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An APPL1/Akt Signaling Complex Regulates Dendritic Spine and Synapse Formation in Hippocampal Neurons

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Abstract

The formation and plasticity of dendritic spines and synapses, which are poorly understood on a molecular level, are critical for cognitive functions, such as learning and memory. The adaptor protein containing PH domain, PTB domain, and leucine zipper motif (APPL1) is emerging as a critical regulator of various cellular processes in non-neuronal cells, but its function in the nervous system is not well understood. Here, we show APPL1 localizes to dendritic spines and synapses and regulates the development of these structures in hippocampal neurons. Knockdown of endogenous APPL1 using siRNA led to a significant decrease in the number of spines as well as synapses and this defect could be rescued by expression of siRNA-resistant APPL1. Expression of exogenous APPL1 increased the spine and synaptic density and the amount of surface GluR1-containing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs). Deletion of the C-terminal phosphotyrosine binding domain of APPL1, which binds the serine/threonine kinase Akt, resulted in a significant decrease in the spine and synaptic density, suggesting a role for Akt in regulating the development of these structures. Consistent with this, knockdown of Akt with siRNA or expression of dominant negative Akt led to a dramatic decrease in spine and synapse formation. In addition, APPL1 increased the amount of active Akt in spines and synapses and the effects of APPL1 on these structures were dependent on Akt, indicating Akt is an effector of APPL1 in the regulation of these processes. Moreover, APPL1 signaling modulates spine and synapse formation through p21-activated kinase (PAK). Thus, our results indicate APPL1 signaling through Akt and PAK is critical for spine and synaptic development and point to a role for APPL1 and its effectors in regulating cognitive function.

Keywords

APPL1; Akt; dendritic spines; synapse formation; PAK; AMPA receptor

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Introduction

Most excitatory synapses in the mammalian brain form between presynaptic axonal terminals and postsynaptic regions, which are comprised of dendritic spines (Hering and Sheng, 2001; Goda and Davis, 2003). Dendritic spines are specialized actin-rich protrusions that generally consist of a thin neck and a bulbous head. The formation and plasticity of spines are essential for cognitive function and abnormalities in their density, size, and morphology are associated with a number of neurological disorders (Fiala et al., 2002; von Bohlen Und Halbach, 2009), underscoring the importance of these structures in the central nervous system (CNS). While the molecular mechanisms that modulate the development and plasticity of dendritic spines and synapses are currently not well understood, emerging data point to an important role for adaptor proteins, which bring together critical signaling pathways, in the regulation of these processes.

APPL1 was originally identified through its interaction with the serine/threonine kinase Akt2 (Mitsuuchi et al., 1999). APPL1 has been subsequently shown to associate with the small GTPase Rab5, the Rab effector Oculocerebrorenal Syndrome of Lowe (OCRL), the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1, as well as several transmembrane receptors, including the tumor suppressor DCC, adiponectin, human follicle-stimulating hormone (FSH), and the neurotrophin receptor TrkA (Liu et al., 2002; Miaczynska et al., 2004; Nechamen et al., 2004; Lin et al., 2006; Mao et al., 2006; Erdmann et al., 2007). Through its multiple protein interactions, APPL1 has been reported to regulate cell survival, proliferation, migration, and vesicular trafficking in non-neuronal cells (Miaczynska et al., 2004; Schenck et al., 2008; Zoncu et al., 2009; Tan et al., 2010). In sympathetic neurons, APPL1 localizes with TrkA in endosomes where it is thought to be critical for TrkA trafficking and signaling (Lin et al., 2006; Varsano et al., 2006), suggesting an important role for this protein in the nervous system.

The function and localization of APPL1 are mediated through several interacting domains, which include an N-terminal Bin–Amphiphysin–Rvs (BAR), a central pleckstrin homology (PH), and a C-terminal phosphotyrosine binding (PTB) domain (Mitsuuchi et al., 1999; Miaczynska et al., 2004). The BAR and PH domains form a functional, symmetrical dimer that is involved in localizing APPL1 to cell membranes (Miaczynska et al., 2004; Li et al., 2007; Zhu et al., 2007). In addition, these domains form a binding interface for Rab5 and are sufficient for targeting this small GTPase to early endosomes (Miaczynska et al., 2004; Zhu et al., 2007). The PTB is a protein-protein interacting domain that mediates APPL1 binding to Akt as well as several other signaling molecules (Mitsuuchi et al., 1999; Liu et al., 2002; Nechamen et al., 2004; Lin et al., 2006).

In this study, we show that APPL1 regulates the formation of dendritic spines and synapses in hippocampal neurons. APPL1 functions in this capacity by increasing the amount of active Akt and PAK in these structures, which is critical for their development. Loss of APPL1 or its effectors severely impairs spine and synapse formation, indicating a critical role for APPL1 signaling in regulating these processes.

Experimental methods

Antibodies and reagents

APPL1 polyclonal antibody was produced by 21st Century Biochemicals (Marlboro, MA) using the following peptides sequences from the C-terminus of human and rat APPL1, respectively, SQSEESDLGEGGKKRESEA and SQSEESDLGEEGKKRESEA. PSD-95 antibody was from Chemicon (Temecula, CA). SV2 antibody was obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA).

LY294002 and antibodies against Akt (C73H10) and phospho-Thr423-PAK were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Phalloidin-TRITC and α-tubulin DM 1A monoclonal antibody were purchased from Sigma (St. Louis, MO). Phospho-Thr308-Akt antibody (sc-16646-R) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). GluR1 antibody was purchased from Calbiochem (EMD Inc., La Jolla, CA). Rabbit polyclonal GAPDH antibody (ab9485) was obtained from Abcam (Cambridge, MA). Alexa Fluor® 488 anti-rabbit, Alexa Fluor® 555 anti-mouse, Alexa Fluor® 647 anti-mouse and anti-rabbit, Alexa Fluor® 680 anti-mouse and anti-rabbit (for Western blotting), and FM4-64 FX were from Molecular Probes (Eugene, OR). IRDye® 800 anti-mouse and anti-rabbit (for Western blotting) were from Rockland Immunochemicals (Gilbertsville, PA). Wortmannin was purchased from VWR International (West Chester, PA). A cell growth determination 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit was obtained from Invitrogen (Carlsbad, CA).

Plasmids and siRNA constructs

Human APPL1 cDNA tagged with EGFP was cloned into a neuronal expression vector, which was a generous gift from Freda Miller, containing a neuronal-specific α1-tubulin promoter (Gloster et al., 1999). GFP-APPL1ΔPTB (aa 1-466) was prepared by amplifying the cDNA using PCR and cloning the PCR product into EGFP-C3 vector (Clontech Laboratories) at *EcoRI* and *KpnI*. Dominant negative Akt (Akt1 T308A/S473A) was generously provided by Brian Hemmings (Friedrich Meischer Institute, Basel, Switzerland). Dominant negative (PAK1 H83L/H86L) and kinase dead PAK (PAK1 K299R) were kindly provided by Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). Small interfering RNA constructs were generated as previously described by inserting sense and antisense 64mer-oligonucleotides into pSUPER vector (Zhang and Macara, 2008). The 19 nucleotide target sequences for APPL1, Akt, and PAK have been previously described (Katome et al., 2003; Lin et al., 2006; Lee et al., 2007; Boda et al., 2008; Yi et al., 2008).

Cell culture and transfection

Low density hippocampal neuronal cultures were prepared and maintained as previously described (Goslin et al., 1998). Neurons were transfected with calcium phosphate as previously described (Zhang et al., 2003). Rat 2 fibroblasts (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. They were transfected with either an Amaxa Nucleofector Kit or Lipofectamine™ 2000 (Invitrogen).

Immunostaining

Neurons were fixed with 4% paraformaldehyde (PFA)/4% sucrose in phosphate-buffered saline (PBS) and then permeabilized with 0.2% Triton X-100 as previously described (Wegner et al., 2008). To stain for PSD-95, neurons were fixed with PFA/sucrose for 3 min and permeabilized with cold methanol for 10 min at -20°C (Wegner et al., 2008). After fixation, coverslips were blocked with 20% goat serum in PBS containing 0.2% Triton X-100 for 1 h. Following blocking, coverslips were incubated with the indicated antibodies diluted in 5% goat serum/PBS. After staining, coverslips were mounted with Aqua Poly/ Mount (Polysciences, Inc., Warrington, PA).

Neurons were immunostained for surface GluR1 as previously described (Lin et al., 2010). After surface staining for GluR1, neurons were permeabilized with 0.2% Triton X-100 and immunostained for SV2 to visualize presynaptic terminals.

Microscopy and image acquisition

Images were acquired with an Olympus IX71 inverted microscope (Melville, NY) using a 60× PlanApo TIRF objective (NA 1.45) and a Retiga EXi CCD camera (QImaging, Surrey, BC). MetaMorph software (Molecular Devices, Sunnyvale, CA) interfaced with a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA) was used for image acquisition. EGFP and Alexa Fluor® 488 were visualized with an Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT). To visualize rhodamine and Alexa Fluor® 555, a TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror) was used. For Alexa Fluor® 647, a Far-red Cy5™ cube (excitation HQ620/60, emission HQ700/75, Q660LP dichroic mirror) was used. Confocal microscopy was carried out on a Quorum WaveFX spinning disk confocal system with a Nikon Eclipse Ti microscope equipped with a PlanApo 60× TIRF objective (NA 1.49). Images were obtained using MetaMorph software and a Hamamatsu ImageEM-CCD camera. For green and red imaging, samples were excited with 491 nm and 561 nm laser lines, respectively.

FM4-64 labeling

Neurons at day 13 in culture were incubated with 6.25 μM FM4-64 FX in high K^+ buffer for 1 min at 37°C as previously described (Wegner et al., 2008). Briefly, the buffer contained 72 mM NaCl, 50 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 11 mM D-glucose, and 20 mM HEPES, pH 7.35. After loading with FM4-64, neurons were washed and fixed with PFA/sucrose.

Results

APPL1 localizes to dendritic spines and synapses and regulates their development

Previous studies showed that APPL1 is expressed in the brain at both the mRNA and protein level (Mitsuuchi et al., 1999; Tan et al., 2010), suggesting a role for this protein in CNS function. To confirm and extend this observation, we assessed the protein distribution of APPL1 in tissue and in hippocampal neurons by western blot analysis. Consistent with previous reports, APPL1 is expressed in the brain (Suppl. Fig. 1A). In addition, we found that APPL1 is highly expressed in hippocampal neurons (Suppl. Fig. 1A). Since APPL1 is highly concentrated in hippocampal neurons, which direct synaptic connections in the CNS via dendritic spines, we hypothesized that this protein modulates spine and synapse formation. To test this, we examined the subcellular localization of endogenous APPL1 with synaptic markers in low density cultures of hippocampal neurons. APPL1 accumulated in puncta that co-localized with synaptic vesicle protein 2 (SV2) and postsynaptic density protein-95 (PSD-95) (Fig. 1A). Almost 90% of the SV2 and PSD-95 clusters contained APPL1 (Fig. 1A), indicating APPL1 is found in a majority of excitatory synapses.

Next, we used a small interfering RNA (siRNA) approach to assess the role of APPL1 in modulating the development of dendritic spines and synapses. Even though we used an APPL1 siRNA target sequence that had been previously shown to knockdown endogenous expression of the protein (Lin et al., 2006), we examined the effectiveness of the siRNA by transfecting it into rat 2 fibroblasts (R2Fs). The APPL1 siRNA knocked down endogenous expression of APPL1 by approximately 75% compared with either pSUPER empty vector or a scrambled siRNA (Fig. 1B). Similarly, when the APPL1 siRNA was transfected into neurons, it decreased endogenous expression of APPL1 by 72.0 ± 1.4% (n = 30 from three independent experiments) compared with control cultures expressing empty pSUPER vector or scrambled siRNA. Transfection of APPL1 siRNA into neurons caused a dramatic decrease in the spine density as determined by GFP fluorescence (Fig. 1C and F). When spine density was determined by staining with phalloidin, which is often used to observe

dendritic spines because it binds to F-actin, a comparable decrease in spine number was seen (Fig. 1E and F). Moreover, the APPL1 siRNA significantly reduced the number of SV2 and PSD-95 clusters, indicating it also impairs the formation of synapses (Fig. 1C, D and F). The rat APPL1 siRNA did not affect expression of human APPL1 due to several nucleotide mismatches within the target sequence, allowing us to perform “rescue” experiments with human GFP-tagged APPL1, which like endogenous APPL1 localized to spines and excitatory synapses (Suppl. Fig. 1B). Expression of human GFP-APPL1 with the APPL1 siRNA completely rescued the siRNA-promoted defect in the development of spines and synapses (Fig. 1C-F), indicating the APPL1 siRNA-mediated defect we observed in spine and synapse formation was due to endogenous loss of the protein. Collectively, these results suggest that APPL1 is an important regulator of spine and synapse formation in hippocampal neurons.

APPL1 promotes the formation of spines and synapses

We further explored the functional role of APPL1 in contributing to the development of spines and synapses by transfecting neurons with GFP-APPL1 at day 5 in culture and examining the spine and synaptic density at day 13. The number of spines was increased 1.6-fold, as determined by both GFP fluorescence and staining with phalloidin, in neurons expressing GFP-APPL1 compared with control GFP expressing cells (Fig. 2A, C and D). Similarly, expression of GFP-APPL1 led to a significant increase in the number of SV2 and PSD-95 clusters compared with GFP expressing controls (Fig. 2A, B and D). In these experiments, we did not observe a significant change in neurite outgrowth with cells transfected with GFP-APPL1 at day 5 in culture compared with GFP controls, suggesting that the effects we observe on spines and synapses are not due to neuritogenesis. However, to confirm this result, we transfected neurons with GFP-APPL1 at day 10 in culture, which represents a time when neurites have attained sufficient length for synapses to form, and examined the effect of APPL1 on spines and synapses. Expression of GFP-APPL1 resulted in a 47.4 ± 1.0% increase in the spine density as well as a 62.1 ± 1.4% and 61.9 ± 1.0% increase in the number of SV2 and PSD-95 clusters, respectively (n = 40 dendrites from three experiments). Taken together, these results indicate that APPL1 regulates the formation of dendritic spines and synapses.

These intriguing results raise the question as to whether the APPL1-containing synapses are functional. FM4-64 is a styryl, fluorescent dye that can be used to visualize functional synapses because it is uptaken into synaptic vesicles in an activity dependent manner (Betz and Bewick, 1992; Betz et al., 1992; Ryan et al., 1993; Betz et al., 1996). When GFP-APPL1 expressing neurons were incubated with FM4-64 in a high K^+ solution, FM4-64 puncta, representing functional synapses, were seen along dendrites. As shown in Fig. 2E, FM4-64 puncta co-localized with GFP-APPL1 clusters. Quantification showed that 81.1 ± 1.1% of APPL1 puncta were positive for FM4-64 (n = 60 dendrites from three separate experiments), indicating the majority of APPL1 containing synapses are functional. In control experiments, when FM4-64 dye was incubated in buffer lacking K^+, FM4-64 puncta were not seen, which indicates FM4-64 uptake is a depolarization-dependent event.

Since expression of AMPAR is important for spine formation and synaptic function, we examined the effect of APPL1 on surface levels of the AMPAR subunit GluR1 (Malinow and Malenka, 2002; Matsuzaki et al., 2004; Nagerl et al., 2004). In these experiments, GFP and GFP-APPL1 expressing neurons were initially immunostained with an antibody against the extracellular epitopes of GluR1 under non-permeabilizing conditions. Neurons were then permeabilized and immunostained for SV2 to show synapses. The amount of synaptic surface GluR1 (sGluR1) in spines was quantified by measuring the background-subtracted integrated fluorescent intensity in individual spines and in the neighboring shafts. The spine to shaft fluorescent intensity ratio was then calculated. The spine/shaft ratio of the

fluorescent intensity for sGluR1 was increased 1.7-fold in GFP-APPL1 expressing neurons compared with GFP controls (Fig. 2F), indicating APPL1 increases sGluR1 levels in spines. These results suggest that APPL1 modulates spine and synaptic function.

APPL1 enhances the amount of active Akt in synapses

Since APPL1 was originally identified through its interaction with the serine/threonine kinase Akt (Mitsuuchi et al., 1999), we hypothesized that Akt is an important effector by which APPL1 modulates spine and synapse formation. To begin to test this hypothesis, we examined the localization of active Akt to spines and synapses. Because Akt is activated by phosphorylation of two conserved amino acids, Thr308 and Ser473 (Alessi et al., 1996), antibodies that recognize the phosphorylated forms of these residues can be used to detect active Akt. When GFP and GFP-APPL expressing neurons were immunostained with a phospho-specific antibody against Thr308, puncta representing active Akt were observed along dendrites (Fig. 3A). These puncta co-localized with SV2 and PSD-95, indicating active Akt accumulates in synapses (Fig. 3A and Suppl. Fig. 2). The phospho-Akt staining appeared to be more intense in synapses of GFP-APPL1 expressing neurons compared with control neurons expressing GFP (Fig. 3A). We confirmed this by measuring the ratio of the fluorescent intensity of phospho-Akt in spines to neighboring shafts from neurons expressing GFP-APPL1 and compared it to that observed in GFP expressing neurons. The ratio was increased 2.4-fold in GFP-APPL1 expressing neurons compared to controls (Fig. 3A and Suppl. Fig. 2). To further show that APPL1 increases the amount of active Akt in spines, neurons were co-transfected with GFP and either FLAG-APPL1 or empty vector at day 5 in culture and immunostained for phospho-Akt at day 13. The spine/shaft ratio of the fluorescent intensity of phospho-Akt in FLAG-APPL1 and control empty vector transfected neurons was 2.4 ± 0.2 and 1.0 ± 0.1 ($n = 200$ spines from three separate experiments; $p<0.0001$), respectively, which is similar to the results obtained with GFP-APPL1. Interestingly, APPL1 did not affect the amount of total Akt in dendritic spines. The spine/shaft ratio of the fluorescent intensity of total Akt was 1.1 ± 0.2 and 1.1 ± 0.1 ($n = 172$ spines from three separate experiments) in GFP and GFP-APPL1 expressing neurons, respectively. Thus, these results show that APPL1 increases the concentration of active Akt in spines and synapses.

Akt signaling is critical for the development of dendritic spines and synapses

To examine the role of Akt in spine and synapse formation, we co-expressed dominant negative Akt (DN-Akt) with GFP or GFP-APPL1 and determined the effect on the density of these structures. Expression of DN-Akt led to an overall decrease in the number of spines and synapses (Fig. 3B-E), suggesting Akt is an important regulator of spine and synapse formation. In GFP-APPL1 expressing neurons, DN-Akt reduced the density of spines and synapses to that observed in control neurons expressing GFP (Fig. 3B-E), suggesting APPL1 modulates the development of spines and synapses through Akt. To further explore this possibility, an APPL1 mutant was generated that lacked the PTB domain (Fig. 4A), which is the Akt binding region of APPL1 (Mitsuuchi et al., 1999; Schenck et al., 2008). As expected, expression of GFP-APPL1 increased the number of spines and synapses while APPL1 lacking the PTB domain (APPL1ΔPTB) failed to enhance the spine and synaptic density (Fig. 4B-E). Indeed, expression of GFP-APPL1ΔPTB caused a decrease in the number of spines and synapses compared with control GFP expressing neurons. This is most likely the result of APPL1ΔPTB forming a dimer with endogenous APPL1 and inhibiting its ability to promote spine and synapse formation. These results suggest that APPL1 modulates spine and synapse formation through Akt.

We next generated Akt siRNA constructs to further show a function for Akt in regulating the development of spines and synapses. Akt siRNA1 and Akt siRNA2 decreased endogenous

expression of the protein by approximately 55% compared with pSUPER empty vector or scrambled siRNA (Fig. 5A). Both Akt siRNAs dramatically decreased the density of dendritic spines and synapses compared with control neurons transfected with pSUPER vector or scrambled siRNA (Fig. 5B-E). The Akt siRNAs did not affect the health of the neurons since greater than 95% of the Akt-siRNA transfected cells were viable at the end of the experiment, as determined by an MTT assay. The effect of the Akt siRNAs on spines and synapses are consistent with those observed with DN-Akt, which further points to a critical role for Akt in spine and synapse formation. In neurons expressing GFP-APPL1, the Akt siRNAs decreased the number of spines and synapses to that observed in GFP control neurons (Fig. 5B-E), which is consistent with our results indicating that APPL1 regulates spine and synapse formation via Akt.

Phosphatidylinositol 3-kinase (PI3K), an upstream activator of Akt, is an important regulator of spine and synapse formation

To further implicate Akt signaling in the modulation of spine and synapse formation, we examined the function of an upstream activator, PI3K, in regulating this process. When GFP and GFP-APPL1 expressing neurons were treated with the PI3K-specific inhibitor LY294002 (20 μM), a significant decrease in the spine and synaptic density was observed (Fig. 6A-D). Indeed, treatment of GFP expressing neurons with LY294002 led to an almost 2-fold reduction in the number of spines and synapses compared with vehicle treated cultures (Fig. 6D), suggesting that PI3K/Akt signaling regulates this process. Moreover, treatment of GFP-APPL1 expressing neurons with LY294002 decreased the spine and synaptic density to that observed in GFP expressing cultures treated with the inhibitor, which suggests APPL1 regulates spine and synapse formation through PI3K/Akt signaling. When GFP and GFP-APPL1 expressing neurons were treated with another PI3K inhibitor, wortmannin (10 nM), similar results were observed (Fig. 6A-D). Wortmannin treatment resulted in an overall decrease in the number of spines and synapses and abolished the effect of APPL1 on these structures (Fig. 6A-D). Collectively, these results indicate an important role for PI3K/Akt signaling in regulating the development of spines and synapses and in mediating the effects of APPL1 on these structures.

APPL1 regulates spine and synapse formation through PAK

Since PAK is a substrate for Akt and has been shown to regulate spine and synapse formation (Penzes et al., 2003; Zhou et al., 2003; Boda et al., 2004; Kumar et al., 2005), we hypothesized that APPL1 mediates its effects on spines and synapses through PAK. To test this, we examined the localization of active PAK to spines and synapses in GFP-APPL1 expressing and control neurons using a phospho-specific antibody against Thr423 because autophosphorylation of this residue strongly correlates with PAK activation (Sells et al., 2000). When GFP and GFP-APPL1 expressing neurons were immunostained with the PAK phospho-specific antibody, puncta representing active PAK co-localized with SV2 and PSD-95 clusters (Fig. 7A and Suppl. Fig. 3), indicating active PAK accumulates in synapses, as previously reported (Zhang et al., 2005). The spine/shaft ratio of the fluorescent intensity of phospho-PAK was increased 3-fold in GFP-APPL1 expressing neurons compared to GFP controls (Fig. 3A and Suppl. Fig. 2). As with Akt, APPL1 did not affect the amount of total PAK in dendritic spines. The spine/shaft ratio of the fluorescent intensity of total PAK was 1.1 ± 0.3 and 1.0 ± 0.3 (n = 150 spines from three separate experiments) in GFP and GFP-APPL1 expressing neurons, respectively. These results indicate that APPL1 significantly increases the amount of active PAK in spines and synapses.

We then co-expressed dominant negative PAK (DN-PAK) with GFP or GFP-APPL1. Expression of DN-PAK resulted in a significant decrease in the number of spines and synapses (Fig. 7B-E), which is consistent with a previous study (Kumar et al., 2005). In

addition, expression of DN-PAK reduced the density of spines and synapses in GFP-APPL1 expressing neurons to that observed in control GFP expressing cells (Fig. 7B-E), supporting our hypothesis that APPL1 regulates spine and synapse formation through PAK. Since the kinase activity of PAK has been previously reported to be essential for spine and synapse formation (Kumar et al., 2005), we co-expressed kinase dead PAK (KD-PAK) with GFP or GFP-APPL1 and examined the effect on the density of these structures. Consistent with the previous study, the kinase activity of PAK is necessary for the formation of spines and synapses, and interestingly is essential for the APPL1-promoted effect on these structures (Fig. 7B-E).

To further show that APPL1 regulates spine and synapse formation via PAK, we used an siRNA approach. PAK siRNA1 and siRNA2 decreased endogenous expression of PAK by approximately 70% compared with pSUPER empty vector or scrambled siRNA (Fig. 8A). The PAK siRNAs significantly reduced the number of spines and synapses compared with control neurons transfected with pSUPER vector or scrambled siRNA (Fig. 8B-E). Moreover, the PAK siRNAs decreased the spine and synaptic density in GFP-APPL1 expressing neurons to that observed in GFP control cells (Fig. 8B-E). Thus, these results suggest that APPL1 promotes the development of dendritic spines and synapses through PAK.

Discussion

Dendritic spines and synapses are known to play a central role in the CNS by forming the complex circuitry underlying cognitive processes, but the molecular mechanisms that modulate the development of these structures are not well understood. Our results reveal a novel function for the signaling adaptor APPL1 in regulating the formation of dendritic spines and synapses. APPL1 is found in a majority of spines and functional excitatory synapses in hippocampal neurons. It promotes spine formation and synaptic activity by increasing the amounts of active Akt and PAK as well as the surface levels of AMPARs in these structures. Akt is critical for their development since a decrease in endogenous Akt or expression of DN-Akt significantly impairs spine and synapse formation. In addition, PAK, which has previously been implicated in spine and synapse formation in hippocampal neurons (Penzes et al., 2003; Boda et al., 2004; Kumar et al., 2005; Hayashi et al., 2007), is an important effector of APPL1 in the regulation of these processes. Thus, our results point to a new role for APPL1/Akt signaling in modulating the formation of dendritic spines and synapses.

APPL1 increases the amount of active Akt in dendritic spines and synapses, which is critical for the development of these structures. APPL1 could function in this capacity by targeting active Akt to spines or by locally enhancing the activation of Akt in these structures. In either case, the increased concentration of active Akt within this region is essential for the formation of spines and synapses. Although the role of Akt in modulating spine and synapse formation is not well understood, this protein had been implicated in the regulation of synaptic plasticity (Peineau et al., 2007). An Akt signaling pathway has been demonstrated to mediate the interplay between two forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Peineau et al., 2007). In addition, Akt signaling has been shown to contribute to filopodia formation and spine morphology (Kumar et al., 2005). Thus, Akt is emerging as an important player in regulating spine and synaptic function, and it will be of great interest to further elucidate the role of this protein in cognitive processes related to learning and memory.

While APPL1 could regulate Akt localization and function through its direct interaction with this protein, our results suggest that PI3K contributes to the APPL1 signaling pathway that

modulates spine and synapse formation. APPL1 has been reported to associate with the p110α catalytic subunit of PI3K and modulate Akt activity in a PI3K-dependent manner (Mitsuuchi et al., 1999; Yang et al., 2003). Perhaps, APPL1 forms signaling complexes with PI3K, Akt, and other Akt associated proteins that regulate the activity and function of Akt in hippocampal neurons. In this context, APPL1 would bring together key components of PI3K/Akt signaling pathways that are critical for regulating spine and synapse development.

PAK is also an effector of APPL1 signaling in the regulation of spine and synapse formation. As with Akt, APPL1 enhances the level of active PAK in dendritic spines and synapses. Interestingly, the phosphorylation and activation of PAK by Akt has been shown to be important for the localization and function of PAK in non-neuronal cells (Zhou et al., 2003). It is possible that APPL1 promotes the development of spines and synapses by increasing the amount of active Akt within this region, which subsequently induces activation of PAK. PAK can function to phosphorylate the regulatory light chain of myosin II, resulting in an augmentation of dendritic spine and synapse formation (Zhang et al., 2005). Alternatively, yet to be defined molecular interactions could be vital for the APPL1-promoted regulation of spines and synapses through PAK. Future studies will be needed to determine if these mechanisms are critical in the pathway by which APPL1 and PAK function in this capacity.

PAK is known to play a key role in the formation and maintenance of dendritic spines and synapses (Penzes et al., 2003; Boda et al., 2004; Zhang et al., 2005; Hayashi et al., 2007). Mutations within PAK have been linked to developmental disorders, such as nonsyndromic mental retardation, which underscores the significance of this protein in contributing to cognitive function (Allen et al., 1998; Bienvenu et al., 2000). Indeed, defects in PAK signaling are implicated in the cognitive decline and memory impairment associated with Alzheimer's disease (Zhao et al., 2006). Moreover, the inhibition of PAK activity in mice leads to a significant deficiency in long-term memory (Hayashi et al., 2004). Thus, our results raise the exciting possibility that APPL1 is an important regulator of cognitive processes through its ability to modulate the activity of proteins, such as PAK. In this context, the contribution of APPL1 to cognitive function represents an exciting avenue for future study.

Our results support a working model in which APPL1 signaling modulates the development of dendritic spines and functional synapses. APPL1 promotes the formation of spines and synapses by increasing the amount of active Akt and PAK at these sites. In addition, APPL1 may contribute to synaptic activity and spine formation by enhancing the surface levels of AMPARs. APPL1 complexes could target active Akt to synaptic sites, or it could locally increase Akt activation at synapses. Akt has been reported to phosphorylate and activate PAK (Zhou et al., 2003), which may contribute to the increased PAK activity observed in APPL1-expressing neurons. Alternatively, other APPL1-dependent mechanisms might be important for this process. Localized PAK activity at synaptic sites, in turn, can regulate processes that underlie the formation of spines and synapses, such as the polymerization and reorganization of the actin cytoskeleton.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. Embo J 1996;15:6541–6551. [PubMed: 8978681]

Allen KM, Gleeson JG, Bagrodia S, Partington MW, MacMillan JC, Cerione RA, Mulley JC, Walsh CA. PAK3 mutation in nonsyndromic X-linked mental retardation. Nat Genet 1998;20:25–30. [PubMed: 9731525]

Betz WJ, Bewick GS. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. Science 1992;255:200–203. [PubMed: 1553547]

Betz WJ, Mao F, Bewick GS. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J Neurosci 1992;12:363–375. [PubMed: 1371312]

Betz WJ, Mao F, Smith CB. Imaging exocytosis and endocytosis. Curr Opin Neurobiol 1996;6:365–371. [PubMed: 8794083]

Bienvenu T, des Portes V, McDonell N, Carrie A, Zemni R, Couvert P, Ropers HH, Moraine C, van Bokhoven H, Fryns JP, Allen K, Walsh CA, Boue J, Kahn A, Chelly J, Beldjord C. Missense mutation in PAK3, R67C, causes X-linked nonspecific mental retardation. Am J Med Genet 2000;93:294–298. [PubMed: 10946356]

Boda B, Jourdain L, Muller D. Distinct, but compensatory roles of PAK1 and PAK3 in spine morphogenesis. Hippocampus 2008;18:857–861. [PubMed: 18481281]

Boda B, Alberi S, Nikonenko I, Node-Langlois R, Jourdain P, Moosmayer M, Parisi-Jourdain L, Muller D. The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. J Neurosci 2004;24:10816–10825. [PubMed: 15574732]

Erdmann KS, Mao Y, McCrea HJ, Zoncu R, Lee S, Paradise S, Modregger J, Biemesderfer D, Toomre D, De Camilli P. A role of the Lowe syndrome protein OCRL in early steps of the endocytic pathway. Dev Cell 2007;13:377–390. [PubMed: 17765681]

Fiala JC, Spacek J, Harris KM. Dendritic spine pathology: cause or consequence of neurological disorders? Brain Res Brain Res Rev 2002;39:29–54. [PubMed: 12086707]

Gloster A, El-Bizri H, Bamji SX, Rogers D, Miller FD. Early induction of Talpha1 alpha-tubulin transcription in neurons of the developing nervous system. J Comp Neurol 1999;405:45–60. [PubMed: 10022195]

Goda Y, Davis GW. Mechanisms of synapse assembly and disassembly. Neuron 2003;40:243–264. [PubMed: 14556707]

Goslin, K.; Asmussen, H.; Banker, G. Rat hippocampal neurons in low-density culture. In: Banker, G.; Goslin, K., editors. Culturing Nerve Cells. MIT Press; Cambridge, MA: 1998. p. 339-370.

Hayashi K, Ohshima T, Hashimoto M, Mikoshiba K. Pak1 regulates dendritic branching and spine formation. Dev Neurobiol 2007;67:655–669. [PubMed: 17443815]

Hayashi ML, Choi SY, Rao BS, Jung HY, Lee HK, Zhang D, Chattarji S, Kirkwood A, Tonegawa S. Altered cortical synaptic morphology and impaired memory consolidation in forebrain- specific dominant-negative PAK transgenic mice. Neuron 2004;42:773–787. [PubMed: 15182717]

Hering H, Sheng M. Dendritic spines: structure, dynamics and regulation. Nat Rev Neurosci 2001;2:880–888. [PubMed: 11733795]

Katome T, Obata T, Matsushima R, Masuyama N, Cantley LC, Gotoh Y, Kishi K, Shiota H, Ebina Y. Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/protein kinase B isoforms in insulin actions. J Biol Chem 2003;278:28312–28323. [PubMed: 12734182]

Kumar V, Zhang MX, Swank MW, Kunz J, Wu GY. Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci 2005;25:11288–11299. [PubMed: 16339024]

Lee IH, Dinudom A, Sanchez-Perez A, Kumar S, Cook DI. Akt mediates the effect of insulin on epithelial sodium channels by inhibiting Nedd4-2. J Biol Chem 2007;282:29866–29873. [PubMed: 17715136]

Li J, Mao X, Dong LQ, Liu F, Tong L. Crystal structures of the BAR-PH and PTB domains of human APPL1. Structure 2007;15:525–533. [PubMed: 17502098]

Lin DC, Quevedo C, Brewer NE, Bell A, Testa JR, Grimes ML, Miller FD, Kaplan DR. APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction. Mol Cell Biol 2006;26:8928–8941. [PubMed: 17000777]

Lin WH, Nebhan CA, Anderson BR, Webb DJ. Vasodilator-stimulated phosphoprotein (VASP) induces actin assembly in dendritic spines to promote their development and potentiate synaptic strength. J Biol Chem 2010;285:36010–36020. [PubMed: 20826790]

Liu J, Yao F, Wu R, Morgan M, Thorburn A, Finley RL Jr, Chen YQ. Mediation of the DCC apoptotic signal by DIP13 alpha. J Biol Chem 2002;277:26281–26285. [PubMed: 12011067]

Malinow R, Malenka RC. AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 2002;25:103–126. [PubMed: 12052905]

Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F, Dong LQ. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol 2006;8:516–523. [PubMed: 16622416]

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H. Structural basis of long-term potentiation in single dendritic spines. Nature 2004;429:761–766. [PubMed: 15190253]

Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, Wilm M, Parton RG, Zerial M. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell 2004;116:445–456. [PubMed: 15016378]

Mitsuuchi Y, Johnson SW, Sonoda G, Tanno S, Golemis EA, Testa JR. Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene 1999;18:4891–4898. [PubMed: 10490823]

Nagerl UV, Eberhorn N, Cambridge SB, Bonhoeffer T. Bidirectional activity-dependent morphological plasticity in hippocampal neurons. Neuron 2004;44:759–767. [PubMed: 15572108]

Nechamen CA, Thomas RM, Cohen BD, Acevedo G, Poulikakos PI, Testa JR, Dias JA. 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 2004;71:629–636. [PubMed: 15070827]

Peineau S, Taghibiglou C, Bradley C, Wong TP, Liu L, Lu J, Lo E, Wu D, Saule E, Bouschet T, Matthews P, Isaac JT, Bortolotto ZA, Wang YT, Collingridge GL. LTP inhibits LTD in the hippocampus via regulation of GSK3beta. Neuron 2007;53:703–717. [PubMed: 17329210]

Penzes P, Beeser A, Chernoff J, Schiller MR, Eipper BA, Mains RE, Huganir RL. Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. Neuron 2003;37:263–274. [PubMed: 12546821]

Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron 1993;11:713–724. [PubMed: 8398156]

Schenck A, Goto-Silva L, Collinet C, Rhinn M, Giner A, Habermann B, Brand M, Zerial M. The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. Cell 2008;133:486–497. [PubMed: 18455989]

Sells MA, Pfaff A, Chernoff J. Temporal and spatial distribution of activated Pak1 in fibroblasts. J Cell Biol 2000;151:1449–1458. [PubMed: 11134074]

Tan Y, You H, Wu C, Altomare DA, Testa JR. 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 2010;285:6377–6389. [PubMed: 20040596]

Varsano T, Dong MQ, Niesman I, Gacula H, Lou X, Ma T, Testa JR, Yates JR 3rd, Farquhar MG. GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling. Mol Cell Biol 2006;26:8942–8952. [PubMed: 17015470]

von Bohlen Und Halbach O. Structure and function of dendritic spines within the hippocampus. Ann Anat 2009;191:518–531. [PubMed: 19783417]

Wegner AM, Nebhan CA, Hu L, Majumdar D, Meier KM, Weaver AM, Webb DJ. N-wasp and the arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses. J Biol Chem 2008;283:15912–15920. [PubMed: 18430734]

Yang L, Lin HK, Altuwaijri S, Xie S, Wang L, Chang C. APPL suppresses androgen receptor transactivation via potentiating Akt activity. J Biol Chem 2003;278:16820–16827. [PubMed: 12621049]

Yi C, Wilker EW, Yaffe MB, Stemmer-Rachamimov A, Kissil JL. Validation of the p21-activated kinases as targets for inhibition in neurofibromatosis type 2. Cancer Res 2008;68:7932–7937. [PubMed: 18829550]

Zhang H, Macara IG. The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. Dev Cell 2008;14:216–226. [PubMed: 18267090]

Zhang H, Webb DJ, Asmussen H, Horwitz AF. Synapse formation is regulated by the signaling adaptor GIT1. J Cell Biol 2003;161:131–142. [PubMed: 12695502]

Zhang H, Webb DJ, Asmussen H, Niu S, Horwitz AF. A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. J Neurosci 2005;25:3379–3388. [PubMed: 15800193]

Zhao L, Ma QL, Calon F, Harris-White ME, Yang F, Lim GP, Morihara T, Ubeda OJ, Ambegaokar S, Hansen JE, Weisbart RH, Teter B, Frautschy SA, Cole GM. Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease. Nat Neurosci 2006;9:234–242. [PubMed: 16415866]

Zhou GL, Zhuo Y, King CC, Fryer BH, Bokoch GM, Field J. Akt phosphorylation of serine 21 on Pak1 modulates Nck binding and cell migration. Mol Cell Biol 2003;23:8058–8069. [PubMed: 14585966]

Zhu G, Chen J, Liu J, Brunzelle JS, Huang B, Wakeham N, Terzyan S, Li X, Rao Z, Li G, Zhang XC. Structure of the APPL1 BAR-PH domain and characterization of its interaction with Rab5. Embo J 2007;26:3484–3493. [PubMed: 17581628]

Zoncu R, Perera RM, Balkin DM, Pirruccello M, Toomre D, De Camilli P. A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 2009;136:1110–1121. [PubMed: 19303853]

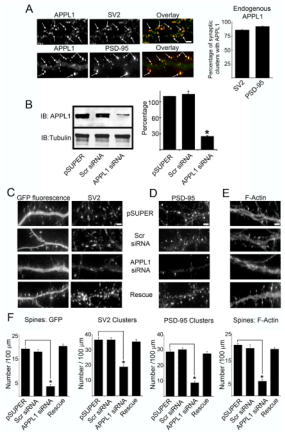

Fig. 1.

APPL1 regulates dendritic spine and synapse formation. (A) Hippocampal neurons at day 13 in culture were co-immunostained for endogenous APPL1 and the synaptic proteins SV2 (upper panels) and PSD-95 (lower panels). APPL1 puncta co-localized with SV2 and PSD-95 clusters (Overlays, arrows). Quantification of the percentage of endogenous APPL1 that co-localized with SV2 and PSD-95 is shown (far right panel). Error bars represent S.E.M. for 50 dendrites from three separate experiments. (B) R2Fs were transfected with APPL1 siRNA, non-silencing scrambled siRNA (Scr siRNA) or pSUPER empty vector. Three days later, cell lysates were immunoblotted for APPL1 and α-tubulin (loading control). Quantification of blots from four independent experiments is shown (right panel). Error bars represent S.E.M. (* $p<0.0001$). Asterisk indicates a statistically significant difference compared with pSUPER transfected cells. (C-E) Neurons at day 5 in culture were co-transfected with GFP and either pSUPER empty vector, scrambled siRNA (Scr siRNA), or APPL1 siRNA and then, stained for SV2 (C), PSD-95 (D), and F-actin (phalloidin) (E) at day 13 in culture. Neurons were transfected with human GFP-APPL1 along with APPL1 siRNA (lower panels, labeled “Rescue”) to show the APPL1 siRNA-mediated spine and synaptic defects were due to loss of the endogenous protein. (F) Quantification of the density of spines, using GFP fluorescence and F-actin staining, as well as synapses (SV2 and PSD-95 clusters) in neurons transfected with the indicated constructs. Error bars represent S.E.M. for 40-50 dendrites from three separate experiments (* $p<0.0001$). Bar, 5 µm (panels A and C-E).

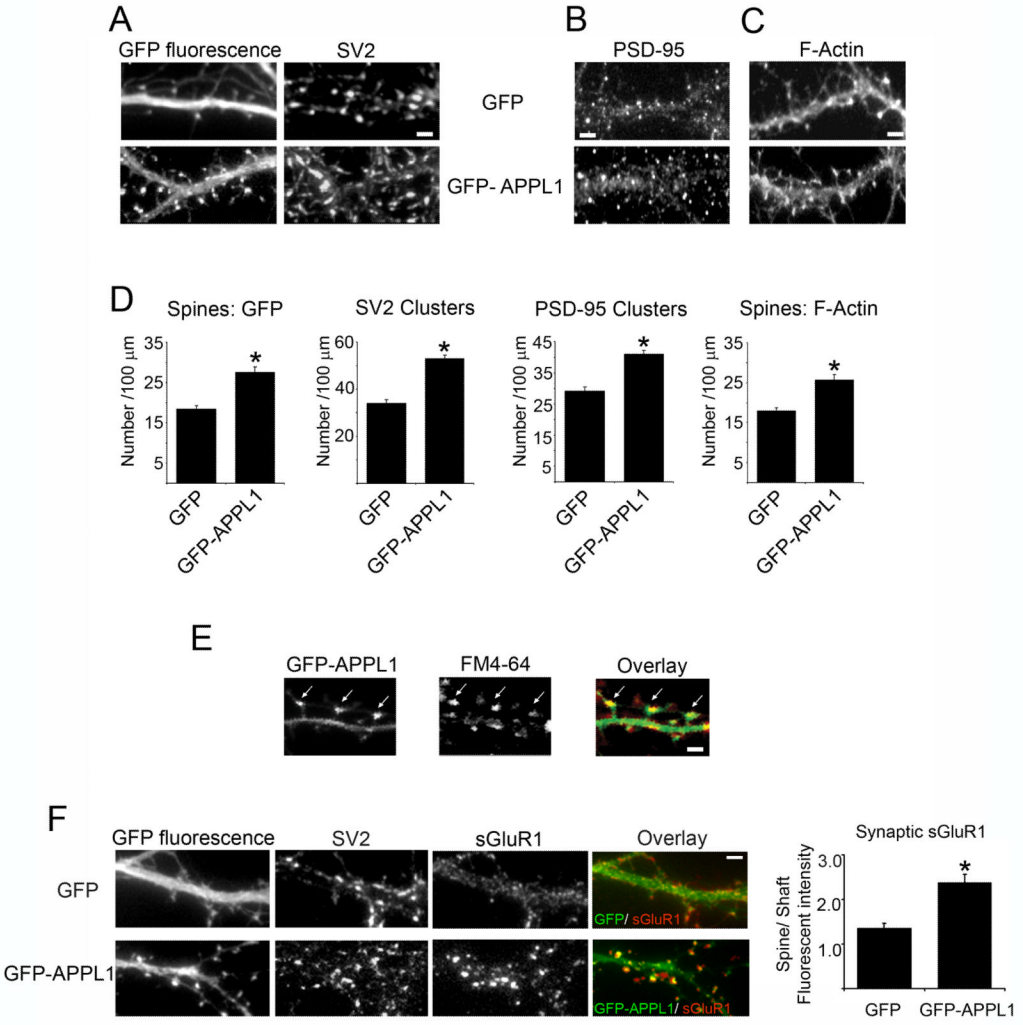

Fig. 2.

APPL1 expression modulates spine and synaptic activity. (A-C) Neurons were transfected with GFP or GFP-APPL1 at day 5 in culture and immunostained for SV2 (A), PSD-95 (B), and actin (C) at day 13 in culture. (D) Quantification of spine density and the number of SV2 and PSD-95 clusters for GFP and GFP-APPL1 expressing neurons is shown. (E) Neurons were transfected with GFP-APPL1 at day 5 in culture and incubated with FM4-64 at day 13 in culture. FM4-64 puncta co-localized with GFP-APPL1 (Overlay, right panel, arrows), indicating APPL1-containing synapses are functional. (F) GFP and GFP-APPL1 expressing neurons were immunostained for sGluR1 under non-permeabilizing conditions. Neurons were subsequently permeabilized and immunostained for SV2 to indicate synapses. Quantification of the spine to shaft ratio of the fluorescent intensity of sGluR1 in spines from GFP and GFP-VASP expressing neurons is shown (far right panel). Error bars represent S.E.M. for 40-50 dendrites (D) or 100 spines (F) from three separate experiments (* $p<0.0001$). Bar, 5 µm (panels A-C, E and F).

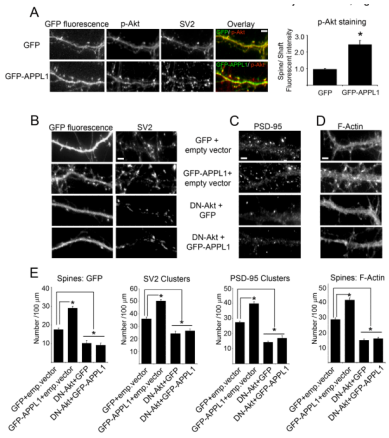

Fig. 3.

APPL1 increases the amount of active Akt in spines and synapses, which is critical for their development. (A) Neurons were transfected with GFP or GFP-APPL1 at day 5 in culture and co-immunostained for active Akt, using a phospho-specific antibody against Thr308, as well as SV2 at day 13 in culture. Phospho-Akt (p-Akt) localized to synapses (overlays). The ratio of the fluorescent intensity in spines to the neighboring shafts for active Akt is shown for GFP and GFP-APPL1 expressing neurons (far right panels). (B-D) At day 5 in culture, neurons were co-transfected with GFP or GFP-APPL1 along with DN-Akt or empty vector and stained for SV2 (B), PSD-95 (C), and F-actin (D) at day 13. (E) Quantification of the spine and synaptic density in neurons transfected with the indicated constructs is shown. Error bars represent S.E.M. for 100 spines (A) or 40-50 dendrites (E) from three separate experiments (*$p<0.0001$). Bar, 5 μm (panels A-D).

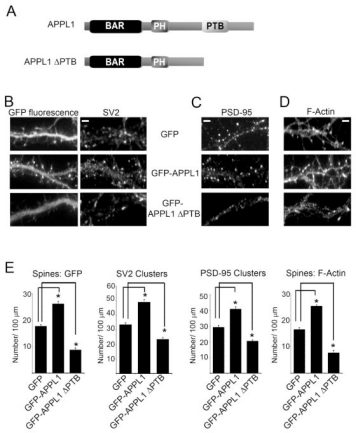

Fig. 4.

The C-terminal PTB domain of APPL1, which binds Akt, is critical for spine and synapse formation. (A) The domain structure of full-length APPL1 is shown (top). A schematic of the APPL1 construct lacking the PTB domain is shown (bottom). (B-D) Neurons were transfected with GFP, GFP-APPL1, or GFP-APPL1ΔPTB at day 5 in culture and immunostained for SV2 (B), PSD-95 (C), and F-actin (D) at day 13. (E) Quantification of synaptic density (SV2 and PSD-95 clusters) and spine number in transfected neurons is shown. Error bars represent S.E.M. for 40-45 dendrites from three independent experiments (*$p<0.0001$). Bar, 5 µm (panels B-D).

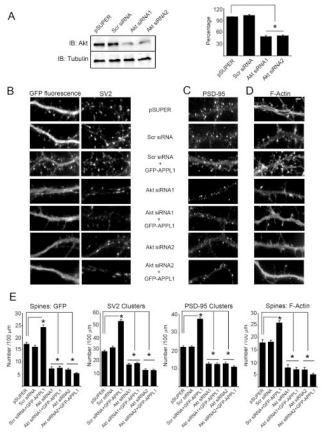

Fig. 5.

Knockdown of Akt affects spines and synapses and abolishes the effects of APPL1 on these structures. (A) Cell lysates from R2Fs transfected with pSUPER empty vector, scrambled siRNA (Scr siRNA), or Akt siRNAs were immunobloted for Akt and α-tubulin (loading control). Quantification of blots from four separate experiments is shown (right panel). Error bars represent S.E.M. (* $p<0.0001$). (B-D) Neurons at day 5 in culture were co-transfected with pSUPER empty vector, scrambled siRNA, or Akt siRNAs and GFP or GFP-APPL1. Cells were immunostained for SV2 (B), PSD-95 (C), and F-actin (D) at day 13 in culture. (E) Quantification of spine and synaptic density for neurons transfected with the indicated constructs. Error bars represent S.E.M. for 40 dendrites from three independent experiments (*$p<0.0001$). Bar, 5 μm.

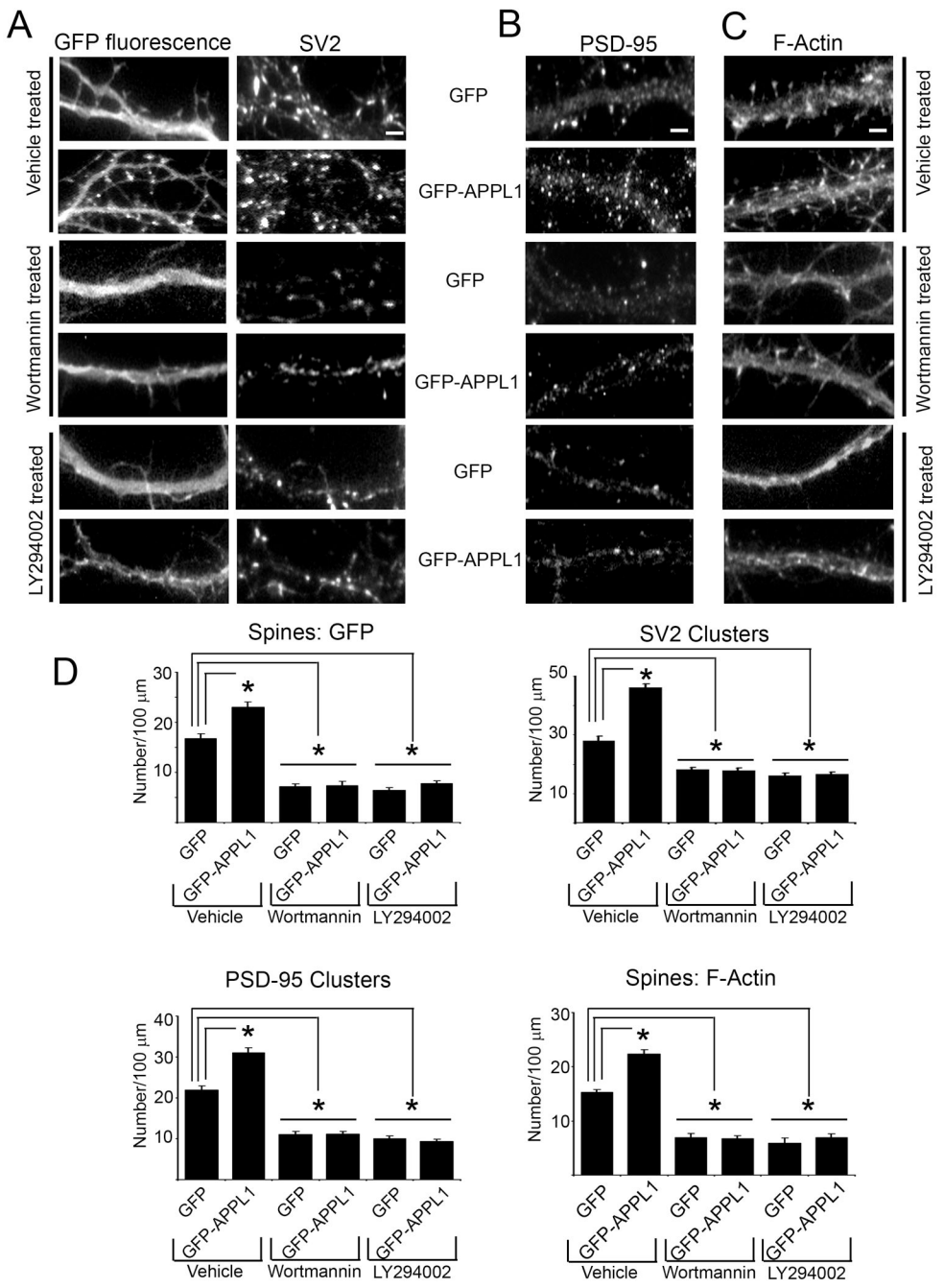

Fig. 6.

The upstream Akt activator, PI3K, regulates spine and synapse formation and mediates the effects of APPL1 on these processes. (A-C) Neurons expressing GFP or GFP-APPL1 were incubated with DMSO vehicle, wortmannin (10 nM), or LY294002 (20 μM) at day 7 in culture and then immunostained for SV2 (A), PSD-95 (B), and F-actin (C) at day 13. (D) Quantification of spine and synapse number from GFP and GFP-APPL1 expressing neurons treated with vehicle, wortmannin, or LY294002 is shown. Error bars represent S.E.M. for 40-60 dendrites from three separate experiments (*p<0.0001). Bar, 5 μm (panels A-C).

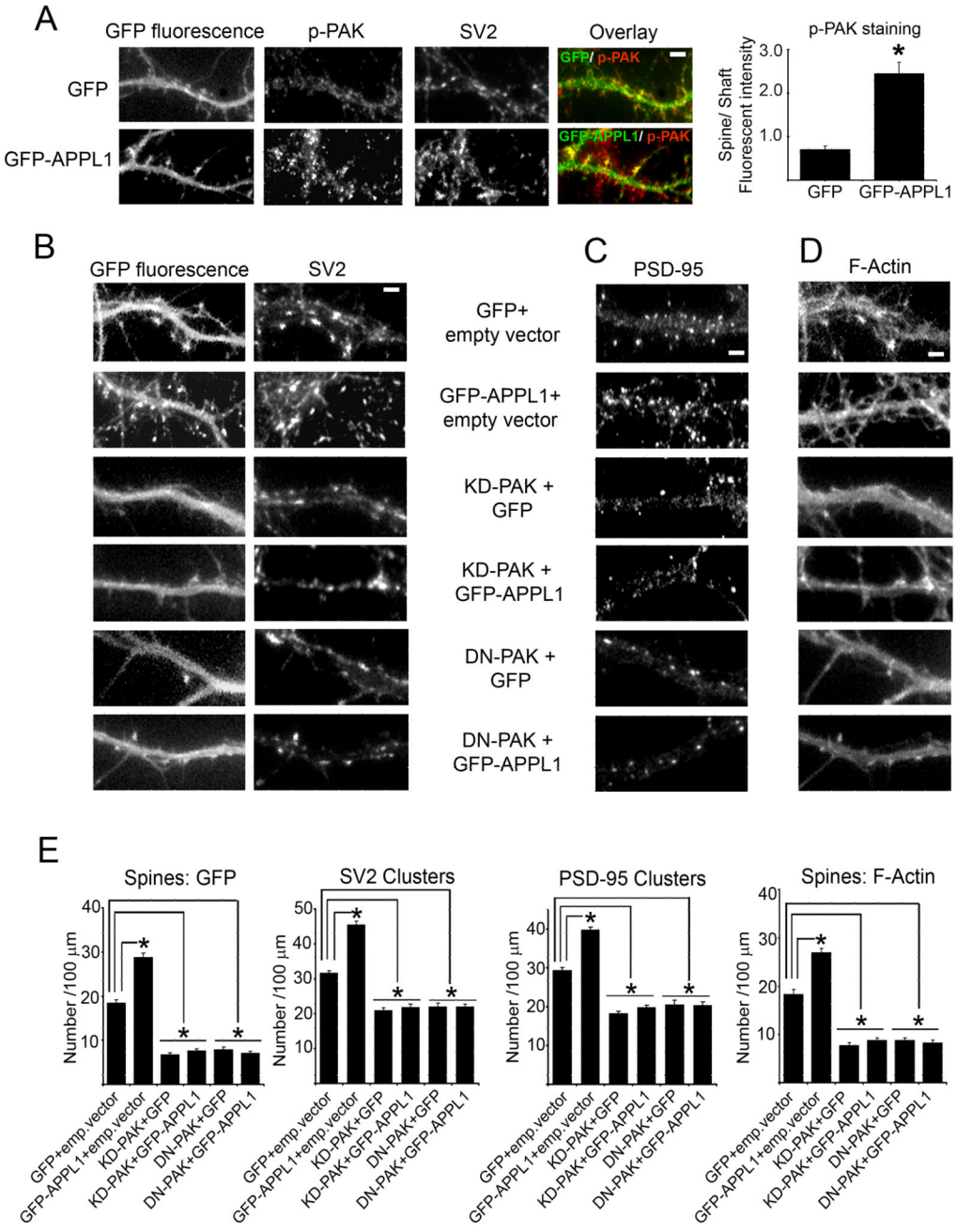

Fig. 7.

APPL1 enhances the amount of active PAK in spines and synapses to modulate their formation. (A) Neurons were transfected with GFP or GFP-APPL1 at day 5 in culture and co-immunostained for active PAK (phospho-Thr423-PAK) as well as SV2 at day 12 in culture. The spine/shaft ratio of the fluorescent intensity of phospho-PAK (p-PAK) is shown for GFP and GFP-APPL1 expressing neurons (far right panels). (B-D) Neurons were co-transfected with GFP or GFP-APPL1 and either empty vector or the indicated PAK constructs at day 5 in culture and then immunostained for SV2 (B), PSD-95 (C), and F-actin (D) at day 12. (E) Quantification of the number of spines and synapses in neurons transfected with the indicated constructs is shown. Error bars represent S.E.M. for 100

spines (A) or 60-80 dendrites (E) from three separate experiments (*p<0.0001). Bar, 5 μm (panels A-D)

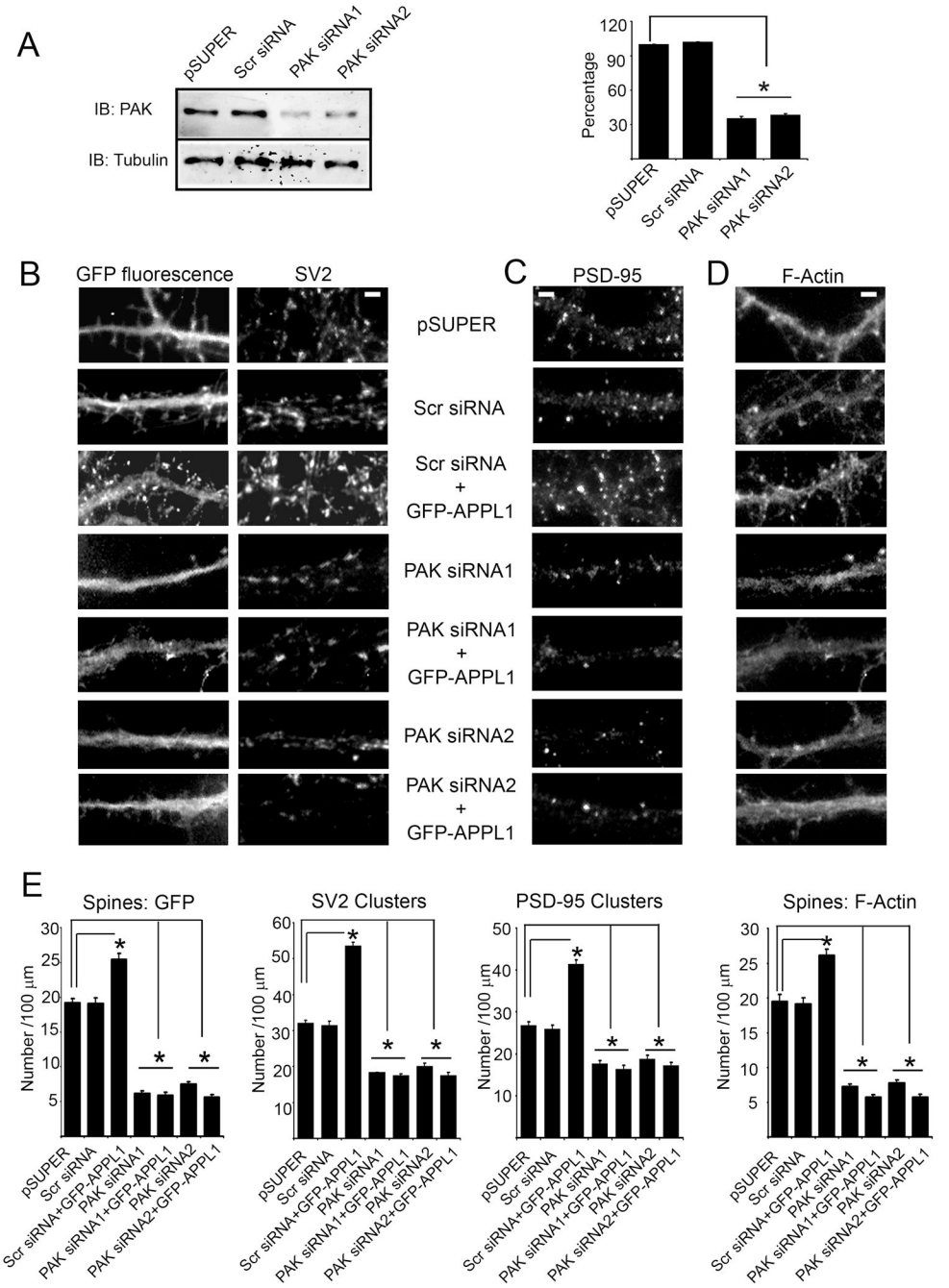

Fig 8.

APPL1 regulates the development of spines and synapses through PAK. (A) R2F cell lysates transfected with pSUPER empty vector, scrambled siRNA (Scr siRNA), or PAK siRNAs were immunobloted for PAK and α-tubulin (loading control). Quantification of blots from four separate experiments is shown (right panel). Error bars represent S.E.M. (* $p<0.0001$). (B-D) Neurons at day 5 in culture were co-transfected with pSUPER empty vector, scrambled siRNA, or PAK siRNAs and GFP or GFP-APPL1 and immunostained for SV2 (B), PSD-95 (C), and F-actin (D) at day 12 in culture. (E) Quantification of the number of spines and synapses for neurons transfected with the indicated constructs is shown. Error

bars represent S.E.M. for 50-60 dendrites from three separate experiments experiments (*p<0.0001). Bar, 5 μm (panels B-D)