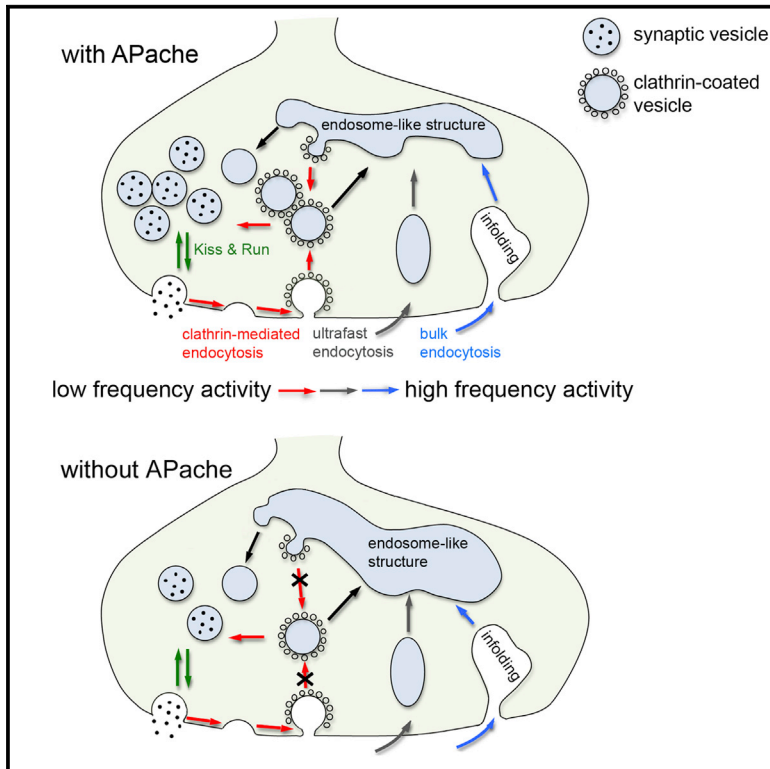


APache Is an AP2-Interacting Protein Involved in Synaptic Vesicle Trafficking and Neuronal Development

Graphical Abstract



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In Brief

Piccini et al. uncovered the AP2-interacting protein APache that acts in the clathrin-mediated endocytic machinery and synaptic vesicle trafficking. They found that silencing APache impairs neuronal development and neurotransmitter release during repetitive stimulation by markedly reducing vesicle recycling.

Highlights

- APache is a presynaptic AP2 interactor on clathrin-coated vesicles
- APache silencing affects the early neuronal development *in vitro* and *in vivo*
- APache-silenced synapses exhibit a marked endocytic phenotype
- APache silencing impairs clathrin-mediated endocytosis and synaptic function



APache Is an AP2-Interacting Protein Involved in Synaptic Vesicle Trafficking and Neuronal Development

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SUMMARY

Synaptic transmission is critically dependent on synaptic vesicle (SV) recycling. Although the precise mechanisms of SV retrieval are still debated, it is widely accepted that a fundamental role is played by clathrin-mediated endocytosis, a form of endocytosis that capitalizes on the clathrin/adaptor protein complex 2 (AP2) coat and several accessory factors. Here, we show that the previously uncharacterized protein KIAA1107, predicted by bioinformatics analysis to be involved in the SV cycle, is an AP2-interacting clathrin-endocytosis protein (APache). We found that APache is highly enriched in the CNS and is associated with clathrin-coated vesicles via interaction with AP2. APache-silenced neurons exhibit a severe impairment of maturation at early developmental stages, reduced SV density, enlarged endosome-like structures, and defects in synaptic transmission, consistent with an impaired clathrin/AP2-mediated SV recycling. Our data implicate APache as an actor in the complex regulation of SV trafficking, neuronal development, and synaptic plasticity.

INTRODUCTION

High-frequency and sustained neurotransmitter release is dependent on the correct reformation of exocytosed synaptic vesicles (SVs) by efficient endocytosis. During physiological activity, clathrin-mediated endocytosis (CME) represents the best-characterized pathway for recycling of fully fused SVs (Heuser and Reese, 1973; Granseth et al., 2006; Dittman and Ryan,

2009; Saheki and De Camilli, 2012), although the precise mechanisms of SV membrane retrieval and functional SV reconstitution remain highly controversial (Soykan et al., 2016; Cousin, 2017).

At the plasma membrane, the most abundant adaptor coordinating coat recruitment and cargo selection into endocytic pits is the heterotetrameric adaptor protein complex 2 (AP2), comprised of two large α and β 2 subunits, a medium-size μ 2 subunit, and a small δ 2 subunit. Among the numerous accessory proteins believed to control the internalization pathway (Slepnev and De Camilli, 2000), clathrin and AP2 constitute the two main protein interaction hubs, around which an extensive and highly dynamic endocytic network is organized to achieve clathrin-coated vesicle (CCV) formation (Schmid and McMahon, 2007). However, other alternate clathrin-associated sorting proteins (CLASP) have recently been identified for the internalization of selected cargo membrane proteins (Traub and Bonifacino, 2013). Thus, synapses have evolved distinct mechanisms to maintain membrane homeostasis and the dominant mode for SV recycling may depend on the type of neuron and its activity pattern (Valtorta et al., 2001; Kononenko and Haucke, 2015; Park et al., 2016). Despite intense research, much remains to be learned about the exact molecular components of the endocytic pathways.

Here, we characterize the highly conserved AP2-interacting clathrin-endocytosis protein APache (NP_001007575.2) and investigate its physiological role in neuronal development and synaptic function. APache is a neuron-specific protein, expressed in axonal processes and presynaptic terminals, that specifically interacts with AP2 on CCVs. Our data indicate that APache plays a role in neuronal development and is required to maintain normal SV recycling in mature neurons. APache can thus be considered an important actor of the clathrin-mediated endocytic machinery at the synapse that is required for normal synaptic transmission.



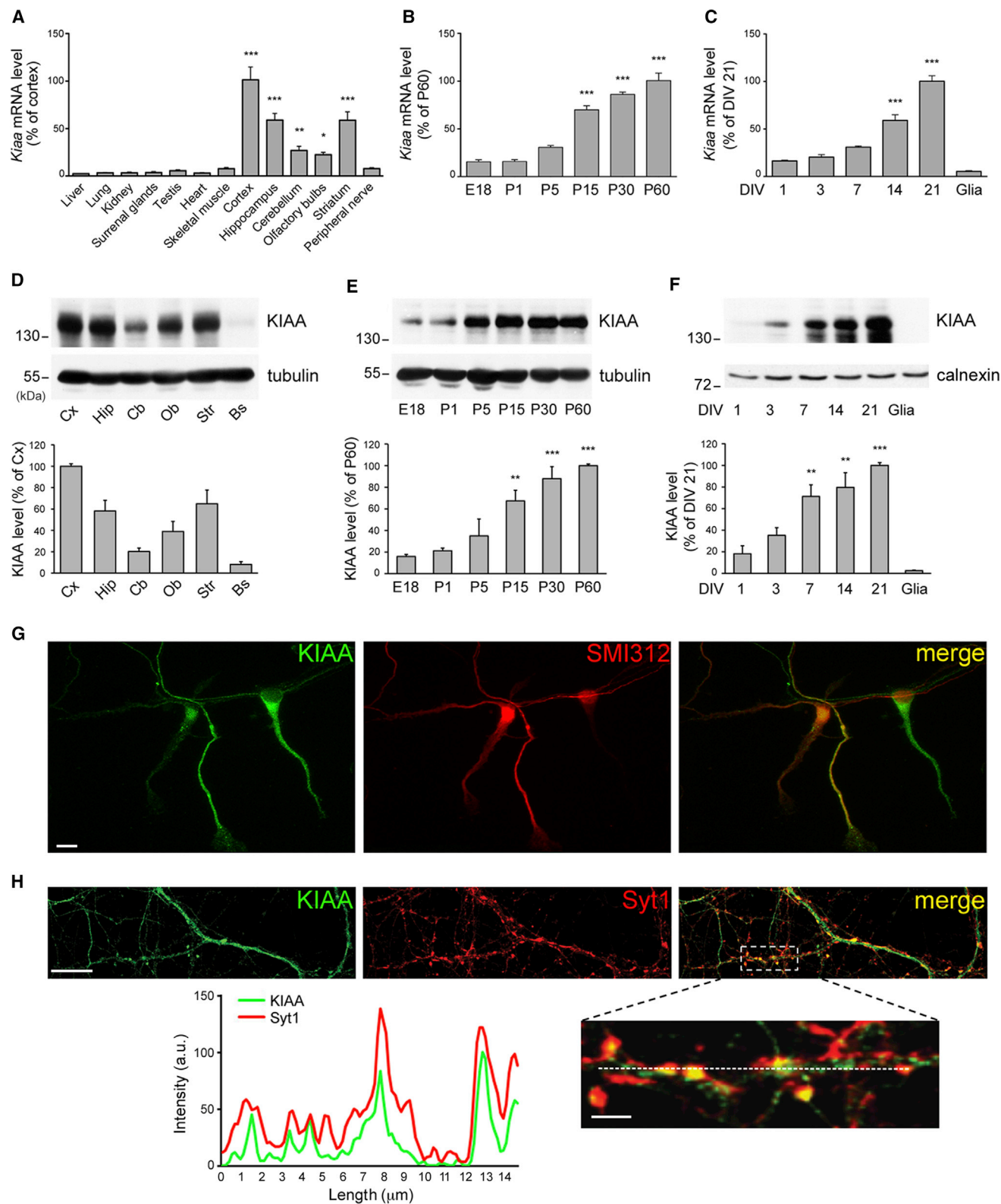


Figure 1. Expression and Localization of Endogenous KIAA1107 in Neurons

(A) Real-time PCR analysis of *Kiaa1107* mRNA levels in various mouse tissues. Means \pm SEM of $n = 3$ animals; one-way ANOVA/Bonferroni's multiple comparison test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus liver.

(legend continued on next page)

RESULTS

Identification of KIAA1107 by GAMMA

Global Microarray Meta-Analysis (GAMMA) is a program previously developed to identify highly correlated transcripts within microarray experiments, which can then be used to infer function, phenotype, genetic network, and disease relevance for uncharacterized genes (Wren, 2009). GAMMA was used to search for uncharacterized genes associated with SV recycling, and KIAA1107 was the highest scoring gene without prior publications. The predicted phenotypes associated with KIAA1107 disruption, predicted disease relevance, and predicted cellular/anatomical structures of relevance to KIAA1107 activity are shown in Figure S1.

KIAA1107 is evolutionarily conserved from zebrafish to human. The murine *Kiaa1107* gene (official symbol: A830010M20Rik) is located on chromosome 5 (forward strand). According to the Ensembl database, the gene gives rise to five differentially spliced transcripts, four of which are predicted to be protein coding. Conversely, the NCBI database includes only two splicing variants (NCBI Refseq NM_001007574.2 and NM_001168557.1, corresponding to ENSMUST00000112671.8 and ENSMUST00000060553.7, respectively, in the Ensembl database). Among these two, one transcript codes for a protein of 1,088 amino acids (aas) and is considered the main isoform, whereas the other codes for a smaller protein of 443 aas. We cloned the major isoform, with an expected molecular mass of 117 kDa, and will refer to it as KIAA1107 throughout. No significant similarity with other proteins could be identified with a BLAST search of the murine KIAA1107 against the *Mus musculus* RefSeq protein database. No conserved protein domains were predicted using bioinformatics tools, such as SMART and InterProScan, but one coiled-coil region was predicted at aa position 820–841 with COILS2 (probability 62.9% with window 21; MTIDK matrix; no weights). Hence, the sequence of KIAA1107 does not reveal any particular information about its possible function or localization, but the GAMMA predictions on its involvement in synaptic function were really strong and persuaded us to investigate it further.

To characterize KIAA1107, we first generated a polyclonal antibody directed against a conserved region comprising aas 732–894 of the mouse ortholog (Figure S2A). The affinity-purified

antibody recognized both overexpressed and endogenous KIAA1107 as a band of ~140 kDa in immunoblotting assays (Figures S2B and S2C). Its specificity was further proved by preadsorbing the primary antibody with a molar excess of the recombinant immunizing peptide (Figure S2D).

To silence KIAA1107 expression, we designed 3 short hairpin RNAs (shRNAs) based on the coding sequence (shRNA#1) and the 3' UTR (shRNA#2 and #3) of the mouse *Kiaa1107* transcript, inserted them into a bicistronic lentiviral vector expressing the fluorescent reporter mCherry, and validated their specificity and efficacy by immunoblotting (Figures S2B and S2C). shRNA#2 was chosen for the subsequent studies, being the most active in knocking down the endogenous KIAA1107 expression. The specificity of the KIAA1107 antibody was subsequently demonstrated by immunocytochemistry of silenced neurons (Figure S2E).

KIAA1107 Is a Neuron-Specific and Developmentally Regulated Protein

We evaluated *Kiaa1107* mRNA and protein levels in various tissues and brain areas of adult mice and determined its developmental expression profile in the intact mouse cortex and primary neuronal cultures (Figures 1A–1F). KIAA1107 was primarily expressed in brain, with the highest mRNA and protein levels in the cerebral cortex, hippocampus, and striatum (Figures 1A and 1D). It was already present in the mouse brain at prenatal and early postnatal stages (embryonic day 18 [E18]–postnatal day 5 [P5]), and its expression increased during postnatal development to reach a plateau at 1 month of age (Figures 1B and 1E). A similar pattern was reproduced in primary cortical neurons, where *Kiaa1107* mRNA and protein levels were discernible at early stages of development (1–3 days in vitro [DIV]) and were greatly enhanced between 7 and 21 DIV (Figures 1C and 1F). Consistent with the strictly neuron-specific expression of the protein, KIAA1107 was not detected in primary astroglial cultures.

To examine the localization of KIAA1107 in neurons during development, 5 and 17 DIV primary cortical neurons were analyzed by immunocytochemistry. In early stages of *in vitro* development, KIAA1107 was expressed in the cell body and growing processes, including the axon, as shown by the colocalization with the pan-axonal neurofilament marker SMI312

(B) Real-time PCR analysis of *Kiaa1107* mRNA expression in the cerebral cortex of developing mice (from embryonic day 18 [E18] to postnatal day 60 [P60]). Means \pm SEM of $n = 3$ animals/developmental stage; one-way ANOVA/Bonferroni's multiple comparison test; *** $p < 0.001$ versus E18.

(C) Real-time PCR analysis of *Kiaa1107* mRNA expression in primary cultures of cortical neurons at various stages of development (from 1 to 21 DIV). Means \pm SEM of $n = 3$ independent cultures/developmental stage; one-way ANOVA/Bonferroni's multiple comparison test; *** $p < 0.001$ versus DIV 1.

(D) Regional expression of KIAA1107 in the adult mouse brain. Representative immunoblot (top) and relative densitometric quantification normalized on β III tubulin levels (bottom) are shown. Means \pm SEM of $n = 4$ animals. Bs, brain stem; Cb, cerebellum; Cx, cortex; Hip, hippocampus; Ob, olfactory bulb; Str, striatum.

(E) Temporal expression profile of KIAA1107 in the developing mouse cerebral cortex (from E18 to P60). Representative immunoblot of KIAA1107 levels (top) and relative densitometric quantification normalized on β III tubulin (bottom) are shown. Means \pm SEM of $n = 3$ animals/developmental stage; one-way ANOVA/Bonferroni's multiple comparison test; ** $p < 0.01$; *** $p < 0.001$ versus E18.

(F) Temporal expression profile of KIAA1107 in primary cortical neurons at various stages of development (from 1 to 21 DIV). Representative immunoblot of KIAA1107 levels (top) and relative densitometric quantification normalized on calnexin (bottom) are shown. Means \pm SEM of $n = 3$ independent neuronal cultures/developmental stage; one-way ANOVA/Bonferroni's multiple comparison test; ** $p < 0.01$; *** $p < 0.001$ versus DIV 1.

(G and H) Localization of KIAA1107 in cultured neurons during development. Representative images of cortical neurons fixed and double stained for KIAA1107 (green) and SMI312 (red) at 5 DIV (G) or for KIAA1107 (green) and synaptotagmin-1 (Syt1) (red) at 17 DIV (H) are shown. The scale bars represent 10 μ m. In the bottom panel, linear intensity profiles of KIAA1107 (green) and Syt1 (red) fluorescence (measured along the dashed lines as indicated in the merge field) illustrate the presynaptic localization of KIAA1107 in mature neurons. The scale bar represents 2 μ m.

See also Figures S1 and S2.

(Figure 1G). In mature neurons, the antibody also revealed a punctate nerve terminal pattern that partially colocalized with the presynaptic marker synaptotagmin-1 (Figure 1H). These data indicate that KIAA1107 is a developmentally regulated, widely expressed neuron-specific protein, mainly present at axonal and presynaptic terminal levels.

KIAA1107 Is an AP2 Interactor

In order to identify KIAA1107 protein-interacting partners, we employed a mass spectrometry (MS) approach using FLAG-KIAA1107, purified from SH-SY5Y human neuroblastoma or COS-7 cells, as a bait to pull-down KIAA1107 interactors from SH-SY5Y cell or subcellular fractions of mouse brain extracts (Figure 2A). The bands of interest were excised from the Coomassie-blue-stained gels, analyzed by liquid chromatography (LC)-MS/MS, and the MS/MS spectra were assigned to peptides with a >95% confidence level. In two independent preparations, a total of 163 proteins were reproducibly identified as specific KIAA1107-binding partners (MSdataSHSY5Y_XTandem.sf3 and MSdataCOS7_XTandem.sf3 in Data S1), seven of which resulted to be in common between the human and murine cellular models (Figure 2B). These included AP2 (α 1 and β subunits) and AP3 (β 2 and δ 1 subunits) found in CCVs that traffic cargoes from the plasma membrane and between the endosomal and lysosomal systems, respectively (Robinson, 2004); Numb-like protein (NUMBL) involved in neural development and clathrin-dependent endocytosis (Sestan et al., 1999; Nishimura et al., 2003; Santolini et al., 2000); Bcl-2-associated transcription factor that interacts with antiapoptotic members of the Bcl-2 family (Kasof et al., 1999); V-type proton ATPase catalytic subunit A, a component of vacuolar ATPase (van Hille et al., 1993); and dynactin subunit1 (DCTN1) involved in organelle transport (Schroer, 2004).

We first focused our attention on AP2, the main adaptor protein responsible for CME (Conner and Schmid, 2003) and proceeded to co-immunoprecipitation assays to validate the potential interaction with KIAA1107. After verifying by MS analysis that the ~140-kDa protein band immunoprecipitated from mouse brain extract with the KIAA1107 polyclonal antibody was indeed the endogenous 1,088-aa KIAA1107 isoform, we found that both AP2 α and β subunits were specifically co-immunoprecipitated with endogenous KIAA1107 from mouse brain extracts (Figure 2C), whereas no interaction of KIAA1107 with clathrin was observed under the same conditions in which the AP2/clathrin binding was evident (Figure 2D). Interestingly, KIAA1107 was also co-immunoprecipitated from mouse brain extracts with anti-AP2 α antibodies (Figure 2D), demonstrating the reciprocity of the interaction between KIAA1107 and AP2.

To restrict the part of the protein that interacts with AP2, we performed additional pull-down assays in mouse brain extracts using FLAG-KIAA1107 full-length, N- and C-terminal fragments. Interestingly, AP2 α and AP2 β were affinity purified only by the N-terminal fragment, whereas no interactions were observed with the C-terminal fragment (Figures S3A and S3B). In addition, the possibility of a nonspecific immunoprecipitation of AP2 was excluded by performing analogous co-immunoprecipitation

assays in mouse liver extracts, a tissue that expresses AP2, but not KIAA1107 (Figure S3C).

We then combined the KIAA1107 binding proteins detected in our study with the results of another large proteomic study (Hein et al., 2015) that also identified KIAA1107 as a potential NUMBL interactor. Thus, we searched for additional shared interactions and overlaps with genes that GAMMA predicted to be relevant to KIAA1107 to infer a potential genetic neighborhood for KIAA1107 (Figure 2E). Such a predictive study revealed connections between KIAA1107 and clusters of genes playing key roles in exocytosis (SNAP25, syntaxin, syntaxin-binding protein, VAMP, NSF, and synaptotagmin-1), endocytosis (dynamin1, AP2, AP3, and Eps15), and neuronal development (Notch1, NUMB, and NUMBL).

KIAA1107 Is Expressed at Nerve Terminals and Associates with CCVs

As AP2 is one of the major coat proteins of CCVs, it was important to determine whether KIAA1107 is associated with CCVs. We isolated a CCV-enriched fraction from cultured rat neurons, successively stripped it and analyzed by immunoblotting the various fractions. The CCV preparation was highly enriched in the coat proteins clathrin and AP2, which were efficiently stripped from the purified vesicles, and in the integral vesicle membrane proteins synaptotagmin-1 and synaptophysin, which were not stripped (Figure 3A). In contrast, an accessory protein of CME, such as dynamin, was neither enriched on CCVs nor stripped, indicating that it does not function as classical clathrin adaptor (Slepnev and De Camilli, 2000). KIAA1107 was significantly enriched in the CCV fraction, although to a lesser extent than clathrin, and could be stripped by treatment with Tris buffer (Figure 3A). These data show that KIAA1107 is a protein associated with the coat component and not with the vesicle fraction.

In subcellular fractions prepared from rat forebrain, KIAA1107 immunoreactivity was mostly associated with the S2 fraction, consistent with the widespread localization of the protein in neurons (Figure 3B). However, it co-enriched with AP2 in the nerve-terminal-derived fractions LS1 and LP2, containing SVs and endosomal membranes, and its distribution roughly paralleled that of AP2 in other fractions (Figure 3B). Consistent with the biochemical data, a close colocalization of endogenous KIAA1107 with the essential components of the endocytic machinery AP2 and dynamin1 was observed in primary cortical neurons (17 DIV; Figure 3C). Notably, dynamin1 was identified as an indirect KIAA1107 interactor in a recent proteomics study (Gorini et al., 2010). These data suggest that KIAA1107 is closely associated with intracellular vesicular structures and binds specifically to AP2 on CCVs.

KIAA1107 Silencing Affects the Early Neuronal Development

CME controls cell surface expression of receptors, including those for axon guidance cues (Tojima et al., 2010), and AP2 plays a key role in directed cell migration (Raman et al., 2014). To interrogate the role of KIAA1107 in neuronal development, we acutely downregulated KIAA1107 expression in primary cortical neurons by RNAi with KIAA1107 shRNA#2

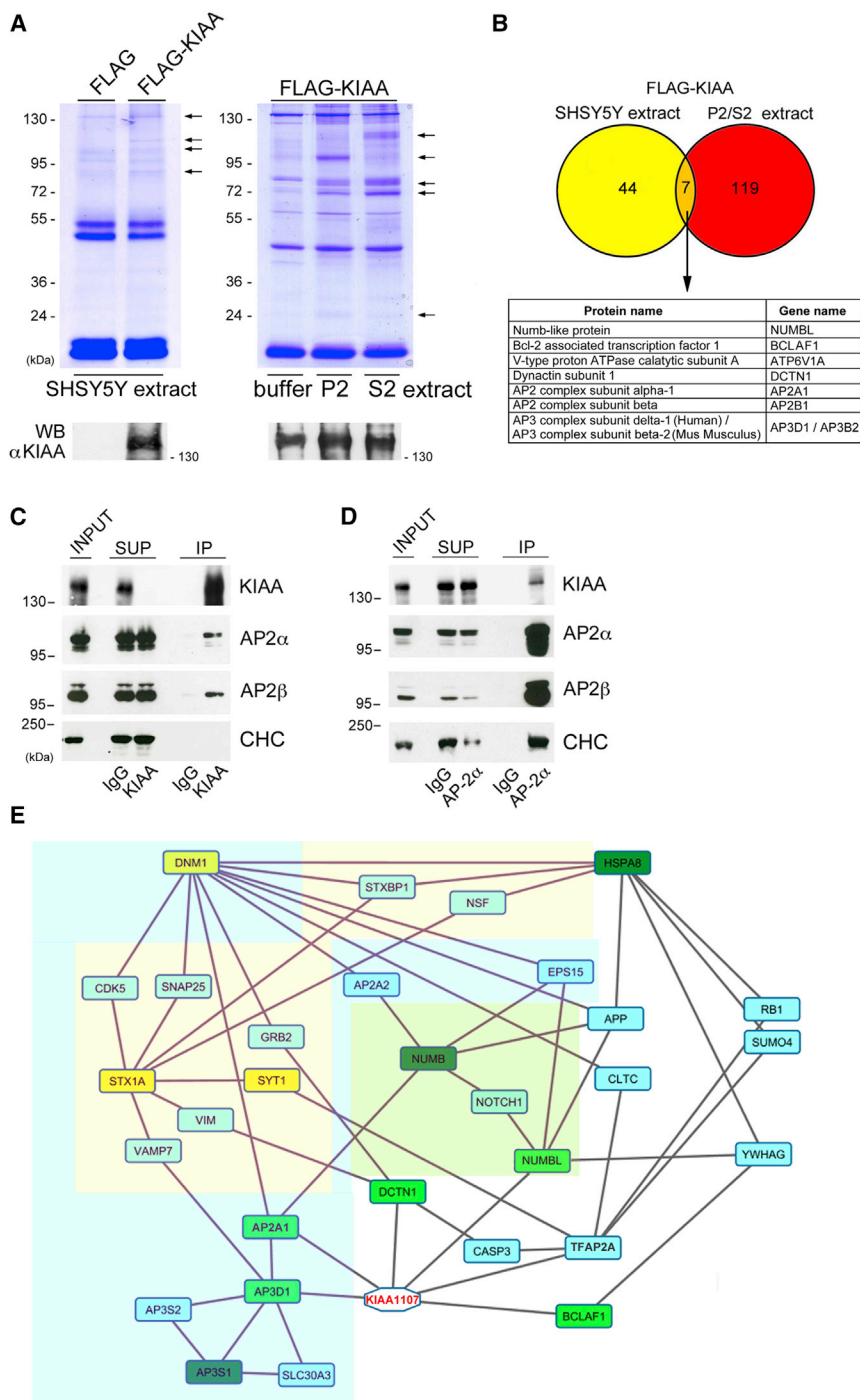


Figure 2. Identification of AP2 as a Specific KIAA1107 Interactor

(A and B) MS analysis of KIAA1107 interactors. (A, top) Coomassie blue stained SDS-PAGE gels for proteins affinity purified by pull-down with overexpressed FLAG-KIAA1107 or FLAG-control in extracts of either SH-SY5Y cells (left panel) or subcellular fractions of mouse brain (cytosolic/microsomal S2 fraction or synaptosomal P2 fraction; right panel) are shown. Selected gel bands (arrows) were excised from the gels and analyzed by LC-MS/MS. (Bottom) KIAA1107 expression and specific precipitation in the samples was confirmed by western blotting (WB). The procedure was repeated twice with independent preparations.

(B) Venn diagram of the number of proteins identified by LC-MS/MS analysis exclusively in FLAG-KIAA1107 samples. The result showed 7 proteins (orange area) shared by both experimental models (protein and gene names are listed in the table) within 44 (yellow area) and 119 (red area) specific proteins for SH-SY5Y cells and mouse brain, respectively.

(C and D) Co-immunoprecipitation of KIAA1107 and AP2. Mouse brain extracts were subjected to immunoprecipitation (IP) with anti-KIAA1107 polyclonal antibodies (C), anti-AP2 α monoclonal antibodies (D), or control immunoglobulin Gs (IgGs). Equal aliquots (2% of total) of the starting material (INPUT) and the supernatants (SUP) together with the IP samples were subjected to immunoblotting with the indicated antibodies (CHC [clathrin heavy chain]). The same membranes were stripped and re-probed for AP2 α and β . The IPs were performed three times with similar results.

(E) Putative KIAA1107 genetic neighborhood based upon protein-protein interactions (PPIs). Our data (light green) were combined with PPIs found in a large proteomics study by Hein et al. (2015; dark green). Then, we searched for their shared PPIs (blue), as documented in Entrez Gene, and looked for overlap with GAMMA-predicted genes that fit into this network (yellow) to infer a potential genetic neighborhood for KIAA1107. Exocytic (yellow area), endocytic (blue area), and developmental (green area) clusters of genes are highlighted. See also Figure S3 and Data S1.

(Figure S4). Cell morphology analysis revealed that silenced neurons displayed a significant impairment in neuronal development in terms of neurite number and length at early stages *in vitro* (3 and 5 DIV) compared to cells treated with control shRNA (shRNA_{actr}) (Figures 4A–4C). Interestingly, silenced neurons also showed a reduced expression level of AP2 (Figure S4), potentially due to partial degradation of the protein in the absence of complex formation with KIAA1107.

The defective neurite outgrowth was completely rescued by the expression of EGFP-KIAA1107, a construct intrinsically resistant to shRNA#2 (Figures 4A–4C), indicating that the developmental impairment was specifically due to the downregulation of endogenous KIAA1107 and not to shRNA-mediated off-target effects. Moreover, KIAA1107 overexpression per se did not affect neuronal maturation, as length and number of processes did not differ between control and EGFP-KIAA1107-overexpressing neurons at both 3 and 5 DIV.

To further investigate the role of KIAA1107 in neocortical development *in vivo*, we downregulated its expression by in

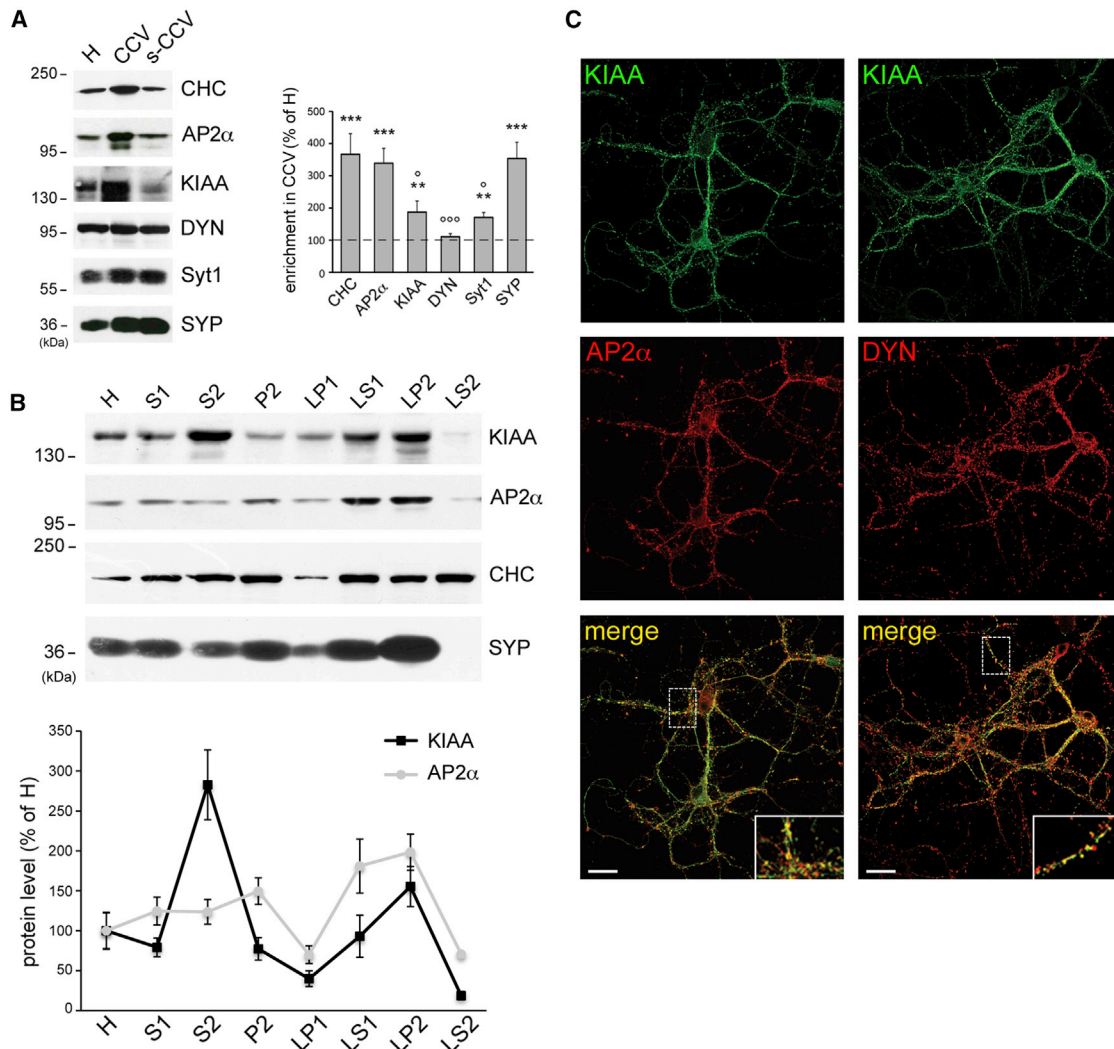


Figure 3. KIAA1107 Is a CCV-Associated Protein

(A) KIAA1107 is enriched in CCVs. (Left) The distribution of KIAA1107 immunoreactivity is compared with that of components of clathrin coats (CHC and AP2 α), SVs (Syt1 and synaptophysin [SYP]), and CCV accessory proteins (dynamitin1 [DYN]). Representative immunoblots are shown. Equal amounts of protein were loaded. H, total homogenate; s-CCV, stripped-CCV. (Right) Densitometric quantification of protein levels in the CCV-enriched fraction expressed as mean (\pm SEM) percentages of H is shown ($n = 3$ independent experiments); ** $p < 0.01$, *** $p < 0.001$ versus H, unpaired Student's *t* test; $^{\circ}p < 0.05$, $^{\circ\circ\circ}p < 0.001$ versus CHC, one-way ANOVA/Bonferroni's multiple comparison test.

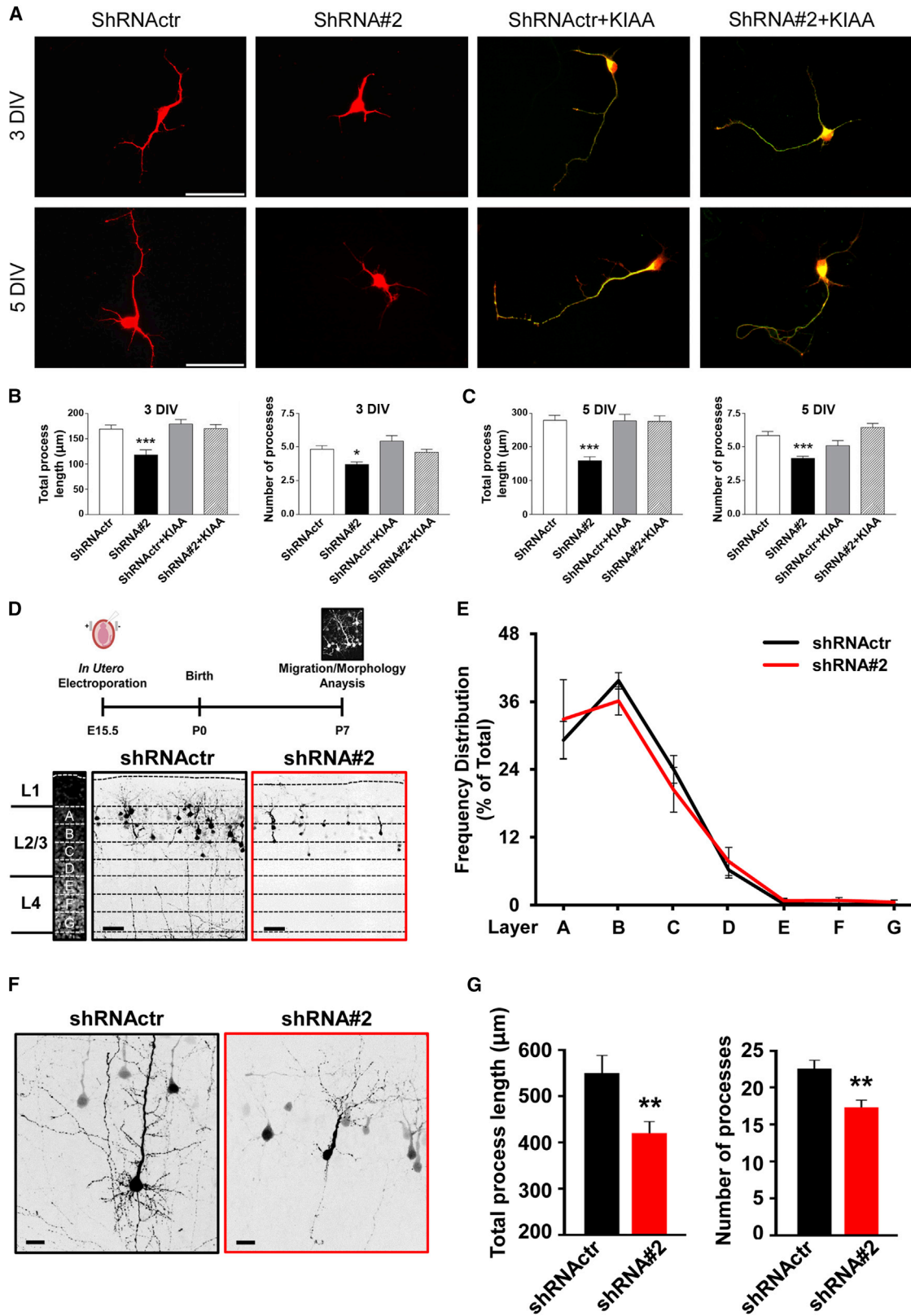
(B) KIAA1107 co-fractionates with AP2. (Top) Subcellular fractions of rat forebrain were analyzed by immunoblotting using KIAA1107, AP2 α , and CHC antibodies. The distribution of the specific SV marker SYP in the same fractions is shown for comparison. LP1, crude synaptic plasma membranes; LP2, crude SVs; LS1, SV-enriched supernatant fraction; LS2, synaptosol; P2, crude synaptosomes; S1, post-nuclear supernatant; S2, cytosolic and microsomal fraction. (Bottom) Densitometric quantification of KIAA1107 and AP2 α immunoreactivities in the various subcellular fractions is shown. Data are expressed in percent of the relative H value (means \pm SEM of $n = 4$ independent experiments).

(C) KIAA1107 colocalizes with the endocytic network. Representative confocal images of mature cortical neurons (17 DIV) double stained for KIAA1107 (green) and AP2 α (red, left panels) or dynamitin1 (DYN) (red, right panels) showing a largely overlapping staining of the proteins (magnified in the insets) are shown. The scale bars represent 10 μ m (4.2 μ m in the insets). Pearson's correlation coefficient is 0.846 ± 0.041 and 0.796 ± 0.039 for KIAA1107/AP2 α and KIAA1107/DYN, respectively ($n = 25$ images obtained from $n = 2$ independent experiments were used for each protein).

utero electroporation (IUE) at E15.5 and analyzed the development of newly generated cortical pyramidal neurons (PNs) derived from shRNA#2-positive progenitors at P7. Whereas the knockdown (KD) of KIAA1107 did not affect radial migration of neural progenitors to layer II/III of the somatosensory cortex (Figures 4D and 4E), it significantly impaired maturation

of PNs that exhibited an aberrant morphology with a significant reduction of total number and length of neurites (Figures 4F and 4G).

These data suggest a crucial role of KIAA1107 in the early stages of *in vitro* and *in vivo* neuronal development, when active SV exo/endocytotic activity at the growth cone is essential for



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process outgrowth (Matteoli et al., 1992; Sabo and McAllister, 2003).

KIAA1107 Markedly Alters the Synaptic Ultrastructure

In view of the potential implication of KIAA1107 in SV endocytosis at mature synapses, we examined the presynaptic ultrastructure of KIAA1107-KD neurons by performing conventional transmission electron microscopy (TEM). Mouse cortical neurons were transduced with a lentiviral vector driving the expression of shRNA#2 or shRNA^{ctr} at 12 DIV. After 5 days, KIAA1107 became undetectable (Figure S5A), whereas no difference in viability was observed between uninfected (ctr) and infected cells (Figure S5B). Notably, KIAA1107-KD synapses were characterized by a markedly reduced density of total SVs (~50% reduction; Figures 5A and 5B), whereas synaptic area, active zone (AZ) length, density of docked SVs, and distribution of SVs with respect to the AZ were comparable to control synapses (Figure 5C). These data were corroborated by a reduced expression level of synaptophysin and AP2 in KIAA1107-KD neurons compared to control (~40% reduction; Figure S5A). Moreover, the density of CCVs was dramatically reduced in silenced synapses (Figure 5B). Finally, KIAA1107-KD synapses displayed enlarged endosome-like structures (~50% increase in size) but with a preserved endosome density (Figure 5B), as confirmed by the increased immunoreactivity of the endosomal marker Rab5 at KIAA1107-KD synapses (Figures S6A and S6B).

To analyze in greater detail the morphology of synaptic terminals, we performed serial sectioning followed by 3D reconstruction of control and KIAA1107-KD synapses (Figure 5D). The morphometric analysis confirmed the severe reduction in the number of SVs and the parallel depletion of CCVs in silenced synapses compared to control (Figure 5E) and revealed that the enlarged endosome-like structures were fully separated from the plasma membrane (Figure 5D). Notably, the ultrastructural effects of KIAA1107 KD were reversible; the silencing phenotype was completely rescued by coinfection of the neurons with EGFP-KIAA1107 (Figures 5D and 5E) resistant to shRNA#2 silencing (Figure S5C). These results show that KIAA1107-silenced synapses, in spite of a normal gross morphology, display severe ultrastructural defects that are consistent with an important role of KIAA1107 in the regulation of SV recycling and in the maintenance of SV pools.

Clathrin/AP2-Mediated Endocytosis Is Impaired in KIAA1107-Silenced Synapses

Mutations in the genes that encode AP2 and other adaptor-like proteins, which are intrinsic components of the clathrin coat and are implicated in the early steps of SV recovery, lead to ultrastructural phenotypes that are similar to that induced by KIAA1107 silencing (González-Gaitán and Jäckle, 1997; Ferges-tad et al., 1999; Zhang et al., 1998).

To uncover whether SV recycling is impaired in KIAA1107-KD synapses, we imaged shRNA^{ctr}- and shRNA#2-infected neurons by electron microscopy upon action potential (AP) firing in the presence of soluble horseradish peroxidase (HRP) to visualize the formation of endocytic intermediates.

We first analyzed synapses under conditions of low-frequency stimulation, when compensatory during-stimulus membrane retrieval largely (but not exclusively) occurs through CME (Granseth et al., 2006; Dittman and Ryan, 2009; Kononenko et al., 2014). Samples were fixed under basal conditions, immediately at the end of the field stimulation (200 APs at 5 Hz) or after 2- and 20-min wash in the absence of HRP. At the end of the stimulus, a significantly decreased density of HRP-positive (HRP⁺) SVs, HRP⁺ CCVs, and HRP⁺ endosome-like structures, all representative of active cycling during stimulation, was observed in KIAA1107-KD terminals compared to control (Figures 6A and 6B). Moreover, the percentage of synapses displaying HRP⁺ CCVs at the end of the stimulus versus total synapses was decreased by about 75% in silenced neurons compared to control (40.9% ± 2.7% and 10.3% ± 6.9% for shRNA^{ctr} and shRNA#2-infected neurons, respectively). After 2-min wash in the absence of HRP, a significant impairment in the formation of HRP⁺ SVs was still evident in KIAA1107-KD terminals, whereas the formation of HRP⁺ endosomal vacuoles recovered to control levels. Finally, after 20-min wash in the absence of HRP, SVs and endosomal structures lost their HRP content in both experimental groups, indicating an active and complete recovery. These results indicate that, in KIAA1107-KD synapses, the recovery of SVs, budding either directly from the plasma membrane or from endosome-like structures, was delayed during mild stimulation. The formation of HRP⁺ endosomal structures derived from either homotypic fusion of CME-derived vesicles or fusion of such vesicles with early endosomes (Heuser and Reese, 1973; Rizzoli et al., 2006; Hoopmann et al., 2010) was

Figure 4. KIAA1107-Silenced Cortical Neurons Display an Impaired Maturation at Early Stages of *In Vitro* and *In Vivo* Development

(A) Representative merged images of 3 and 5 DIV cortical neurons nucleofected before plating with either ShRNA^{ctr} or ShRNA#2 (red) and Sh-resistant EGFP-KIAA1107 (green). The scale bars represent 50 μ m.

(B and C) Quantification of total process length and number of processes at 3 (B) and 5 (C) DIV using ImageJ. Data are means ± SEM (n = 101 and 83 for ShRNA^{ctr} neurons; n = 98 and 105 for ShRNA#2 neurons; n = 74 and 89 for ShRNA^{ctr}+KIAA1107 neurons; n = 66 and 61 for ShRNA#2+KIAA1107 neurons; at 3 and 5 DIV, respectively, from n = 3 independent experiments). *p < 0.05; ***p < 0.001 versus ShRNA^{ctr} neurons; one-way ANOVA/Bonferroni's multiple comparison test.

(D–G) KIAA1107-silencing *in vivo* does not impair radial migration but causes impairment in pyramidal neurons' morphology.

(D, top) Cartoon depicting the experimental design of the *in vivo* experiments is shown. (Bottom) Representative images of GFP fluorescence in neurons transfected with either ShRNA^{ctr} (black) or ShRNA#2 (red) in the somatosensory cortex are shown. The slices were counterstained with DAPI to allow the visualization of cortical layers 2/3/4 (L2/3 and L4), here divided in 8 sub-layers (named from A to G, left). The scale bars represent 50 μ m.

(E) Quantification of the percentage of total transfected cells in each layer is shown. Data are means ± SEM (n = 8 animals per condition, 1 slice per animal).

(F) Representative high-magnification images of GFP fluorescence in neurons transfected with either ShRNA^{ctr} (black) or ShRNA#2 (red) in layer II/III of the somatosensory cortex are shown. The scale bars represent 15 μ m.

(G) Quantification of total process length (left) and number (right) using ImageJ is shown. Data are means ± SEM (n = 21 cells from 8 different animals for ShRNA^{ctr}; n = 23 cells from 8 different animals for ShRNA#2); **p < 0.01; unpaired Student's t test.

See also Figure S4.

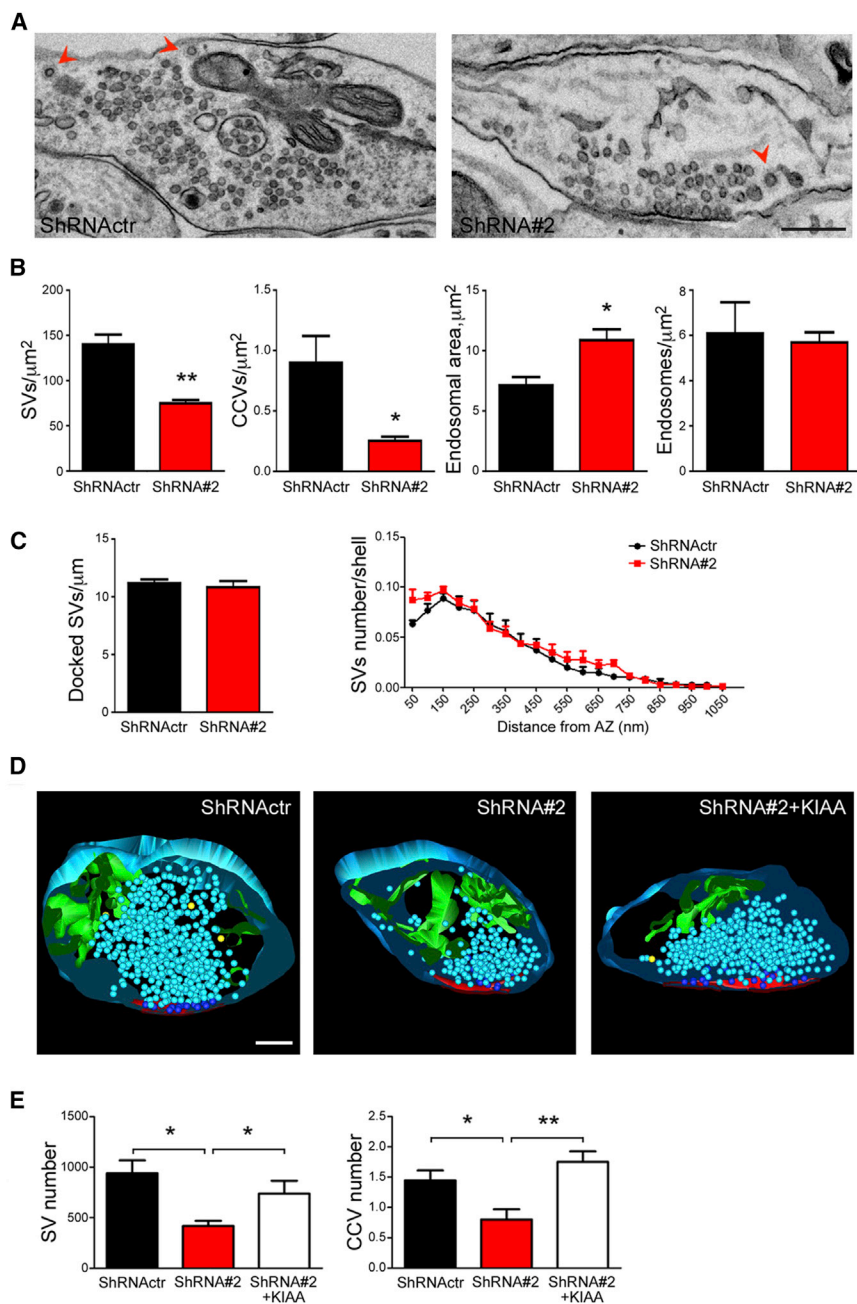


Figure 5. Reduced SV Density and Increased Size of Endosome-like Structures at KIAA1107-Silenced Cortical Synapses

(A) Representative TEM images of nerve terminals from cultured cortical neurons transduced with either ShRNAcr or ShRNA#2 at 12 DIV and processed at 17 DIV. Note the reduced SV density in the KIAA1107-KD synapse compared to control (CCVs, red arrowheads). The scale bar represents 200 nm.

(B) Morphometric analysis from serial ultrathin sections obtained from ShRNAcr- (black bars) and ShRNA#2- (red bars) treated synapses revealed (from left to right) a reduction in the density of total SVs and CCVs and an increase in the area of endosome-like structures in KIAA1107-KD synapses compared to control. * $p < 0.05$; ** $p < 0.01$; unpaired Student's t test.

(C) No changes were observed in the density of AZ-docked SVs and in the spatial distribution of SVs in the nerve terminals of KIAA1107-KD neurons compared to control. The density of SVs located within successive 50-nm shells from the AZ was normalized for the total SV content of each terminal and is given as a function of the distance from the AZ. Nerve terminal areas ($0.716 \pm 0.059 \mu\text{m}^2$ and $0.800 \pm 0.024 \mu\text{m}^2$ for ShRNAcr and ShRNA#2-infected neurons, respectively) and AZ lengths ($0.350 \pm 0.016 \mu\text{m}$ and $0.335 \pm 0.007 \mu\text{m}$ for ShRNAcr and ShRNA#2-infected neurons, respectively) were similar in the two experimental groups. Data are means \pm SEM ($n = 157$ and $n = 160$ synapses for ShRNAcr and ShRNA#2-infected neurons, respectively, from $n = 4$ independent preparations).

(D) Representative 3D reconstructions of synaptic terminals from 60-nm-thick serial sections obtained from cortical neurons confirmed the severe reduction in SV (light blue spheres) and CCV (yellow spheres) number in ShRNA#2-treated neurons, which was completely rescued in ShRNA#2+KIAA1107-treated neurons. Endosomal structures (green), not connected with the plasma membrane, are also visible. The AZ and AZ-docked SVs are shown in red and blue, respectively. The scale bar represents 200 nm.

(E) Morphometric analysis of the number of SVs and CCVs in 3D-reconstructed synapses from neurons transduced with ShRNAcr (black bars), ShRNA#2 (red bars), or ShRNA#2+KIAA1107 (white bars). Docked SVs (19.3 ± 2.38 , 14.57 ± 2.37 , and 20.37 ± 4.15 for ShRNAcr, ShRNA#2, and ShRNA#2+KIAA1107-infected neurons, respectively) were similar in the three experimental groups. Data are means \pm SEM ($n = 10$ synapses per genotype from $n = 3$ independent preparations).

* $p < 0.05$; ** $p < 0.01$; one-way ANOVA/Bonferroni's multiple comparison test. See also Figures S5 and S6.

also delayed in silenced terminals compared to controls. The increased endosomal size observed in silenced synapses both under basal conditions (Figures 5B, S6A, and S6B) and after stimulation (Figure S6C) is compatible with a longer retention of SVs at endosomal level, strongly suggesting an impaired clathrin-mediated SV reformation from endosomal structures.

When neurons were stimulated at higher frequency (200 APs at 40 Hz), i.e., a frequency known to trigger fast membrane

retrieval via endocytic intermediates upstream of clathrin-coat assembly (Clayton et al., 2008; Cheung et al., 2010; Kononenko et al., 2014; Watanabe et al., 2013, 2014), a decreased density of HRP⁺ SVs was observed only at the end of the stimulus in KIAA1107-KD terminals compared to control (Figure 6C), evidence of a moderate impairment of SV reformation at this intense activity level. HRP⁺ CCVs were nearly absent in both genotypes, consistent with the idea that CME is largely dispensable for

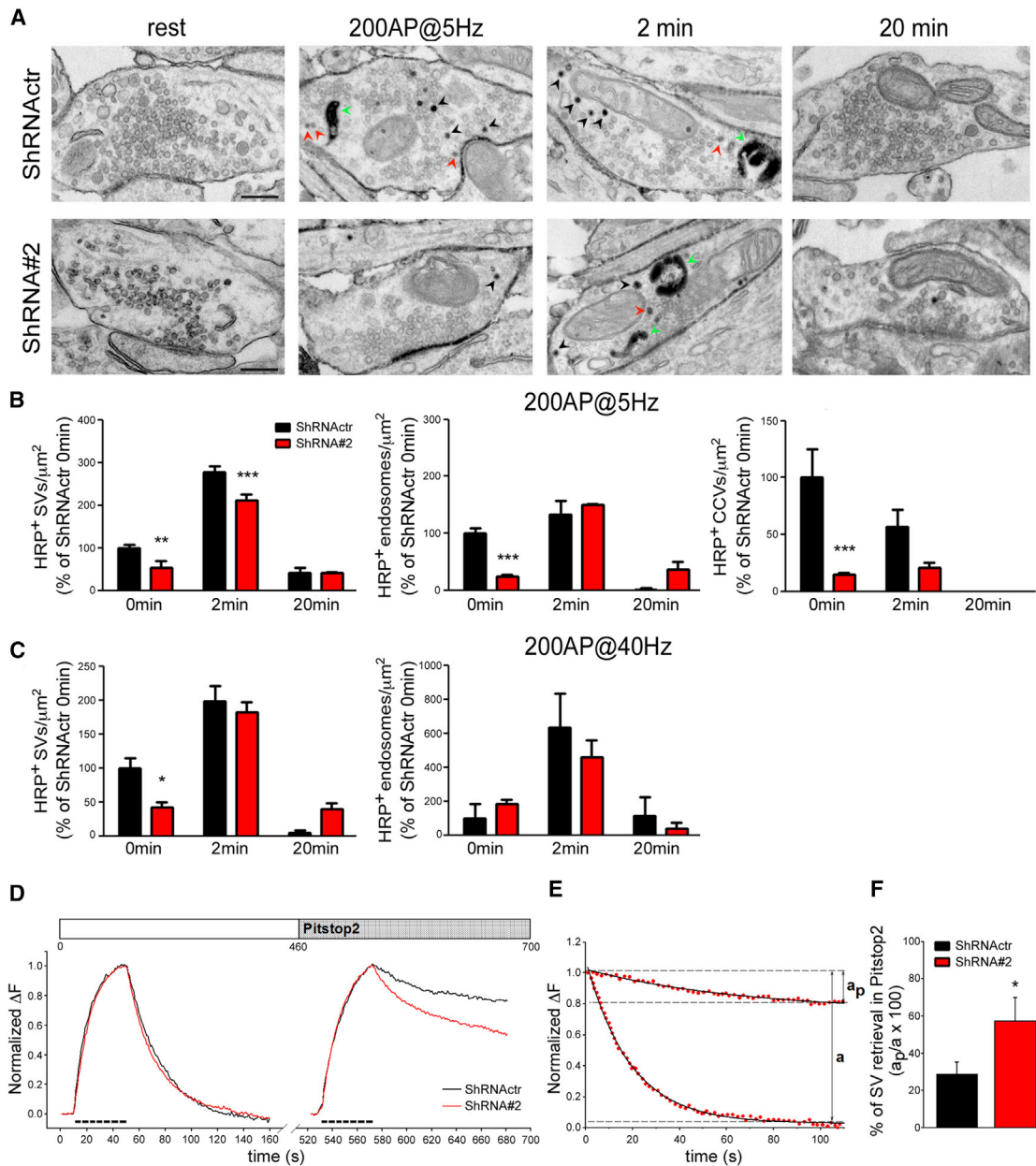


Figure 6. KIAA1107 Knockdown Impairs Clathrin-Mediated Endocytosis in Cortical Synapses

(A) Representative TEM images of presynaptic terminals from control (ShRNActr) and KIAA1107-KD (ShRNA#2) neurons infected at 12 DIV and stimulated at 17 DIV with 200 APs at 5 Hz in the presence of soluble HRP. Synaptic ultrastructure was evaluated by fixing neurons under basal conditions (rest), immediately after the stimulus (0 min), and after 2 or 20 min of recovery in the absence of HRP (HRP⁺ SVs, black arrowheads; HRP⁺ CCVs, red arrowheads; HRP⁺ endosomes, green arrowheads). The scale bars represent 200 nm.

(B and C) Morphometric analysis of HRP-labeled structures after the train stimulation at 5 (B) and 40 (C) Hz (200 APs). The density of HRP-positive (HRP⁺) SVs, HRP⁺ endosome-like structures, and HRP⁺ CCVs in control (black bars) and KIAA1107-KD (red bars) neurons are reported as mean (\pm SEM) percentages of the respective values observed in the control (ShRNActr) group at 0 min (5 Hz: ShRNActr HRP⁺SVs, 3.15 ± 0.25 ; ShRNActr HRP⁺CCVs, 0.90 ± 0.23 ; ShRNActr HRP⁺endosomes, 1.11 ± 0.17 ; 40 Hz: ShRNActr HRP⁺SVs, 1.54 ± 0.23 ; ShRNActr HRP⁺endosomes, 0.1 ± 0.07). $n = 150$ and $n = 120$ images per genotype for the 5- and 40-Hz protocols, respectively, from $n = 4$ independent preparations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ across genotype, two-way ANOVA/Bonferroni's multiple comparison test.

(D) Ensemble average normalized traces of SyHy fluorescence plotted for control (black trace, $n = 8$) and KIAA1107-KD (red trace, $n = 5$) neurons sequentially stimulated with 200 APs at 5 Hz (dotted line) in the absence or presence of $30 \mu\text{M}$ Pitstop-2.

(E) Representative control data points (red dots) and relative fitting (black traces) by a single exponential function ($y = y_0 + a - b^x$). The a values in the absence (a) or presence (a_p) of Pitstop-2 are shown.

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plasma membrane retrieval at this stimulation frequency (Konenko et al., 2014; Park et al., 2016). In addition, the density of HRP⁺ endosomal vacuoles was not altered in silenced synapses (Figure 6C), although their size was increased also at this stimulation frequency (Figure S6D).

We then used Synaptophysin-pHluorin (SypHy) (Tagliatti et al., 2016), co-expressed in hippocampal neurons transduced with either shRNA^{ctr} or shRNA^{#2}, to further assay membrane recycling following low-frequency stimulation (200 APs at 5 Hz). Surprisingly, no differences in the fluorescence increase, indicative of the rate of release, as well as in the fluorescence decay, representative of post-stimulus endocytosis, were observed in KIAA1107-KD synapses compared to control synapses (Figure 6D). The inability of the SypHy assay to detect an endocytic phenotype in silenced terminals could in principle be due either to a compensatory SV membrane retrieval contributed by clathrin-independent endocytosis (CIE) or, alternatively, to a major involvement of KIAA1107 in SV budding from endosomes rather than from the presynaptic membrane. To sort this out, we challenged neurons with the clathrin inhibitor Pitstop-2 (von Kleist et al., 2011; Figure 6D). The inhibitory effect of Pitstop-2 on endocytosis was severe in both control and KIAA1107-KD synapses, despite the presence of a modest compensation mediated by CIE in the latter (Figures 6D–6F). This suggests that KIAA1107 silencing does not primarily impair SV membrane retrieval or vesicle reacidification but rather SV reformation from endosomes.

In summary, these functional data provide strong evidence that KIAA1107 plays an important role both in CME at the plasma membrane and in the reformation of SVs by clathrin coats budding from endosomes. The kinetics of SV reformation, especially under conditions of low-frequency activity when CME is the predominant pathway for SV recycling, is affected by KIAA1107 silencing. Based on these data, we named the KIAA1107 protein APache, for AP2-interacting clathrin-endocytosis protein.

APache-Silenced Autaptic Neurons Exhibit Impaired Presynaptic Function

To determine whether APache plays a role in synaptic transmission, we performed whole-cell patch-clamp recordings in autaptic hippocampal neurons silenced for APache at 6 DIV and analyzed 5 or 6 days after infection. We preliminarily analyzed the effects of APache silencing on the autapse density and found that the density of synaptic contacts was preserved in APache-KD autaptic neurons (Figure S7A).

Then, we proceeded to the analysis of synaptic transmission. Neurons were stimulated with paired stimuli (50-ms interpulse interval) to evaluate evoked excitatory postsynaptic current (eEPSC) amplitude and paired-pulse facilitation, a presynaptic form of short-term plasticity and an indirect measure of the release probability (Pr) (Fioravante and Regehr, 2011). APache-KD neurons displayed a significant reduction of eEPSC ampli-

tude in response to single stimuli but no changes in paired-pulse facilitation (Figures 7A and 7B). To investigate which of the quantal parameters of release was responsible for the decreased synaptic strength in APache-KD synapses, the cumulative eEPSC amplitude analysis was performed by subjecting neurons to high-frequency trains (2 s at 40 Hz) that induce a complete depletion of the readily releasable pool of SVs (RRP) (Figures 7C–7E). Under this condition, the depression during the steady-state phase is limited by the constant recycling of SVs so that an equilibrium is reached between released and recycled SVs (Schneggenburger et al., 1999). The analysis showed that the RRP size was significantly decreased in APache-KD neurons, to the same extent of the reduction in eEPSC amplitude, whereas Pr was not affected (Figure 7F). This suggests that the impairment in evoked release in APache-KD neurons is likely to involve the constant replenishment of the RRP by the recycling SV pool. Despite the change in RRP, the dynamics of facilitation and depression during the 2-s train at 40 Hz were not significantly affected in silenced neurons compared to control (Figure 7D). Because the various endocytic mechanisms are known to be recruited in a frequency-dependent manner, autaptic neurons were challenged with short 2-s trains at frequencies ranging from 5 to 20 Hz and with a long 30-s train at 10 Hz to analyze the expression of facilitation/depression over time (Figures 7G, 7H, S7B, and S7C). APache-silenced neurons exhibited a more pronounced depression that was tightly dependent on the stimulus frequency. Synaptic depression was faster, more intense, and prolonged in APache-KD synapses during 5-Hz stimulation (Figures 7G and 7H) and progressively attenuated with the increase in stimulation frequency (Figure S7B). The strong increase in synaptic depression at 5 Hz, consistent with the RRP depletion and inactivation of release sites (Fioravante and Regehr, 2011), is likely due, similarly to other endocytic mutants (Milosevic et al., 2011), to the impaired clathrin-mediated recycling of SVs. APache silencing was also associated with an accelerated kinetics of depression evoked by long trains (30 s at 10 Hz; Figure S7C). In addition, post-tetanic potentiation (PTP), a form of short-term plasticity evoked after a short high-frequency stimulation and contributed by increases in both Pr and RRP (Valente et al., 2012), was also impaired in APache-KD neurons (~35% reduction; Figure S7D), consistent with the SV depletion observed upon APache silencing.

DISCUSSION

In the present study, using the bioinformatics GAMMA program to search for uncharacterized genes associated with SVs and presynaptic physiology, we identified KIAA1107 with the highest score. The mouse KIAA1107 main isoform is a protein of 1,088 aas lacking both structural data and known function. Our results demonstrate that KIAA1107 is an AP2 interactor that plays a role in early neuronal development and in CME at mature

(F) The a_p/a ratio, representing the percentage of retrieved SVs in the presence of Pitstop-2 versus the retrieved SVs in its absence, is plotted for control (black bar, $n = 8$) and KIAA1107-KD (red bar, $n = 5$) synapses. Data are means \pm SEM from the indicated number of coverslips from $n = 3$ independent preparations. * $p < 0.05$; unpaired Student's t test.

See also Figure S6.

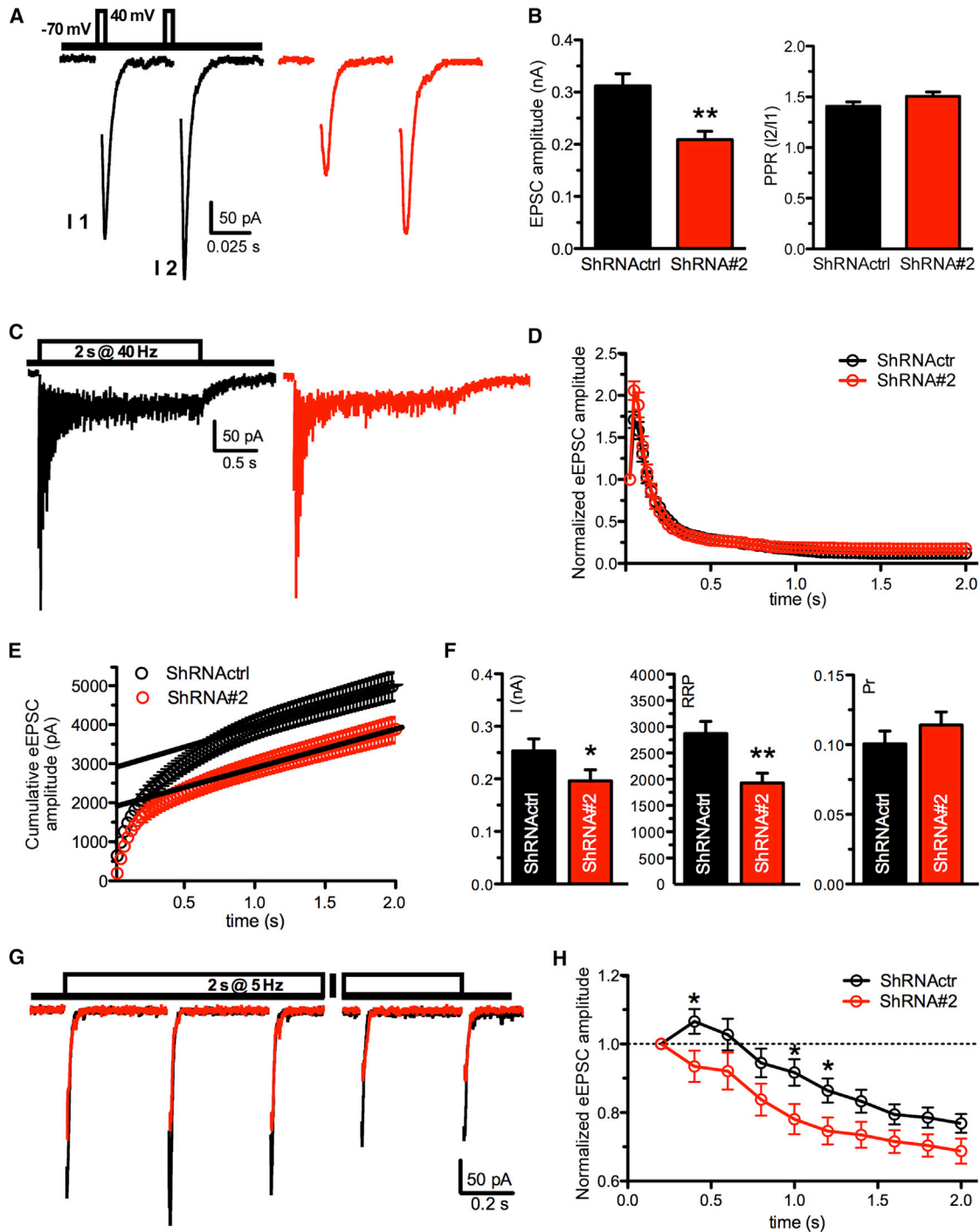


Figure 7. KIAA1107 Silencing Decreases Evoked Excitatory Synaptic Transmission and Enhances Synaptic Depression in Autaptic Hippocampal Neurons

(A) Representative eEPSCs recorded in autaptic neurons transduced with either ShRNActrl (black traces, $n = 67$) or ShRNA#2 (red traces, $n = 63$). eEPSCs were elicited by clamping the cell at -70 mV and stimulating it with two voltage steps to $+40$ mV lasting 0.5 ms at an inter-stimulus interval of 50 ms (inset). (B) eEPSC amplitude evoked by the first pulse (I1, left) and paired-pulse ratio (PPR) (I2/I1, right) recorded under the same conditions of (A). (C) Representative recordings of eEPSC evoked by a 2-s tetanic stimulation at 40 Hz in autaptic neurons transduced with ShRNActrl (black) or ShRNA#2 (red). (D) Normalized values of eEPSC amplitude showing the time course of synaptic facilitation and depression in autaptic neurons stimulated as in (C). (E) Cumulative mean amplitude profiles for eEPSCs during the tetanic stimulation shown in (C) in neurons infected with ShRNActrl (black trace, $n = 40$) or ShRNA#2 (red trace, $n = 37$). Data points in the 1- to 2-s range were fitted by linear regression and backextrapolated to time 0 (solid lines) to estimate the RRP. (F) Bar graphs showing the mean values of I (nA), RRP, and Pr for ShRNActrl (black) and ShRNA#2 (red) neurons. (G) Representative recordings of eEPSC evoked by a paired-pulse stimulation at 5 Hz in autaptic neurons transduced with ShRNActrl (black) or ShRNA#2 (red). (H) Normalized values of eEPSC amplitude showing the time course of synaptic depression in autaptic neurons stimulated as in (G). Data points in the 0.5- to 2-s range were fitted by linear regression and backextrapolated to time 0 (solid lines) to estimate the RRP.

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synapses. We named the protein APache and consider it a molecular component of the clathrin/AP2-dependent endocytic machinery that regulates the fate of endocytosed SVs.

CME has a range of different functions that also include sampling the cell environment for growth and guidance cues and bringing nutrients into cells in developing neurons. An active SV exo/endocytosis in the growth cone has been demonstrated in cultured neurons (Matteoli et al., 1992; Sabo and McAllister, 2003). The present data suggest that, in developing neurons, APache may function in vesicle trafficking events. APache is expressed since the early stages of neuronal development and is present in axonal processes and growth cones, where it promotes neuronal maturation and process outgrowth both *in vitro* and *in vivo*. Moreover, the expression level of AP2 is reduced in developing silenced neurons. It is tempting to speculate that the developmental role of APache is obtained through the clathrin-mediated trafficking pathways that control axon and dendrite outgrowth in developing neurons that are characterized by abundant CCVs (Roos and Kelly, 1999). Intriguingly, AP180 and CALM, two clathrin assembly proteins involved in CME, also play critical roles in controlling the outgrowth of axons and dendrites in embryonic hippocampal neurons (Bushlin et al., 2008), and their silencing elicits morphological phenotypes reminiscent of those of APache-depleted cortical neurons.

Other potential interactors detected in the MS scans can contribute to the effects of APache in neuronal development. NUMBL, an endocytic adaptor binding to the AP2 complex and Eps15 and implicated in CME and neurite outgrowth (Santolini et al., 2000; Sestan et al., 1999; Nishimura et al., 2003) was identified as an APache interactor in our study as well as in a recent proteomic study (Hein et al., 2015). Dynactin, a direct AP2-binding partner that regulates bidirectional transport of vesicles in mammalian neurons, microtubule advance during growth cone remodeling (Kwinter et al., 2009; Grabham et al., 2007), and trafficking of BDNF-TrkB signaling endosomes (Kononenko et al., 2017; Zhou et al., 2012) was also identified as an APache interactor. Thus, in addition to its role in endocytosis at the plasma membrane, APache may also play a role in the internal vesicular/endosomal transport.

Several lines of evidence suggest a potential role for APache in CME at mature synapses. APache is most abundant in brain and, in mature synapses, is concentrated at presynaptic terminals, where it colocalizes with synaptotagmin-1, AP2, and dynamin-1. We have identified AP2 as an APache interactor with the following evidence: (1) APache co-immunoprecipitates with AP2 α and β subunits from brain extracts, suggesting that it may participate in the complex network of interactions regulating

CME and SV recycling; (2) APache is enriched in CCVs to levels comparable to the coat proteins AP2 and clathrin and at a much larger extent than CME accessory proteins, such as dynamin, synaptojanin, amphiphysin, or endophilin; and (3) APache can be stripped from purified CCVs by treatment with Tris buffer, a well-established procedure to remove coat components from SVs. Synapses that lack APache display the typical features of endocytic mutants. The ultrastructural changes include a severe depletion of SVs and CCVs and the presence of enlarged endosome-like structures. The morpho-functional phenotype of APache silencing consists in a global impairment of SV recycling and synaptic strength that peaks under conditions of low-frequency activity.

Mature synapses use multiple activity-dependent SV recycling mechanisms that operate in parallel and influence neurotransmitter release and synaptic plasticity: “kiss and run”; CME; and clathrin-independent mechanisms (i.e., ultrafast or bulk endocytosis). Whereas the retrieval of the majority of fully fused SVs during mild electrical activity occurs through CME, with vesicles reforming directly from the plasma membrane or from endosomal structures (Hoopmann et al., 2010; Uytterhoeven et al., 2011), during intense high-frequency activity, SV membranes are mostly retrieved through CIE.

The experimental evidence indicates that APache is required to maintain normal SV recycling and RRP refilling at the synapse under conditions of mild stimulation by acting in the clathrin/AP2-mediated regeneration of SVs both from the cell surface and from internalized endosomal structures. The latter mechanism seems to be the predominant one, given the lack of effect of APache silencing on SV reacidification in the SypHy assay. Consistent with this, enlarged endosomal vacuoles accumulate at silenced synapses and may contribute to the depression of neurotransmitter release. On the other hand, APache is dispensable for plasma membrane retrieval at high frequencies, although it may be required for the clathrin-mediated SV reformation from endosomal vacuoles generated by CIE. This model is consistent with prior data demonstrating that silencing or conditional knockout of either AP2 (Kim and Ryan, 2009; Kononenko et al., 2014) or AP2-associated endocytic adaptor proteins (Fergestad et al., 1999; Kononenko et al., 2013) significantly slows down, but does not abolish, SV endocytosis and validates the view that alternative molecules or different mechanisms that normally operate in conjunction with AP2 are required to ensure efficient SV and cargo retrieval over a wide range of stimulation frequencies.

Although further structure function studies will be needed to unravel the precise molecular mechanisms that mediate the described APache functions, the identification of an additional molecular component of the complex endocytic pathway is a

(F) Quantal analysis of release in neurons infected with ShRNA^{Actr} (black bars) or ShRNA#2 (red bars). From left to right, amplitude of the first eEPSC, RRP size, and probability of release (Pr) are shown.

(G) Representative recordings of eEPSC evoked by a 2-s tetanic stimulation at 5 Hz in autaptic neurons transduced with either ShRNA^{Actr} (black, n = 31) or ShRNA#2 (red, n = 21).

(H) Normalized values of eEPSC amplitude showing the time course of synaptic facilitation and depression in autaptic neurons stimulated as in (G). In all graphed currents, stimulation artifacts were blanked for clarity. Data are means \pm SEM from the indicated numbers of cells recorded at least from n = 3 independent cell culture preparations. *p < 0.05; **p < 0.01; unpaired Student's t test or Mann-Whitney U test.

See also Figure S7.

step forward for getting insights into fundamental aspects of SV recycling in the healthy and diseased brain.

EXPERIMENTAL PROCEDURES

C57BL/6J mice and Sprague-Dawley rats of either sex were from Charles River Laboratories (Calco, Italy). All experiments, conducted at various stages of development (from E18 to adult mice), were carried out in accordance with the guidelines established by the European Communities Council (directive 2010/63/EU of March 4, 2014) and were approved by the Italian Ministry of Health. The standard procedures for western blotting, CCV purification, pull-down and co-immunoprecipitation assays, immunocytochemistry, real-time PCR, and cultures of low-density and autaptic neurons are reported in detail in the [Supplemental Experimental Procedures](#).

GAMMA

GAMMA relies on identifying gene-gene expression correlations using thousands of publicly available microarray datasets available from the GEO repository. Additional data are reported in the [Supplemental Experimental Procedures](#).

KIAA1107 Antibodies and Constructs

A polyclonal KIAA1107-specific antibody was raised in the rabbit against a conserved region comprising aas 732–894 of mouse KIAA1107. cDNA of *Kiaa1107* was amplified from total mRNA extracted from mouse brain and inserted into the p3XFLAG-CMV-14 or p277.pCCLsin.cPPT.hPGK.eGFP.WPRE vector. shRNAs#1–3 and control shRNA were inserted into the pLKO.1-CMV-mCherry lentiviral vector and used to knockdown the endogenous KIAA1107 in neurons. For detailed description of antibodies, constructs, and neuronal and cellular transfection, see the [Supplemental Experimental Procedures](#).

MS Analysis

Sample preparation, LC-MS/MS analysis, database searching, and criteria for protein identification were conducted as reported in details in the [Supplemental Experimental Procedures](#).

IUE

Standard IUE was performed as previously described ([Szczyrkowska et al., 2016](#)). The images were acquired using a confocal laser-scanning microscope (TCS SP5; Leica Microsystems) or an epifluorescence microscope equipped with NeuroLucida (MicroBrightField) software. For detailed procedures and reagents, see the [Supplemental Experimental Procedures](#).

TEM

Low-density cultures of cortical neurons were infected at 12 DIV with either control shRNA or KIAA1107 shRNA and processed for TEM. For detailed procedures, see the [Supplemental Experimental Procedures](#).

Live Imaging and Patch-Clamp Experiments

Optical recordings with Syphy fluorescent probe were performed at 17 DIV (5 days postinfection). Whole-cell patch-clamp recordings were made from autaptic neurons grown on microislands infected at 6 DIV with either control shRNA or KIAA1107 shRNA. For detailed procedures, see the [Supplemental Experimental Procedures](#).

Statistical Analysis

Data with normal distribution were analyzed by one- or two-way ANOVA followed by the Bonferroni's multiple comparison test or the unpaired Student's *t* test. Non-normally distributed data were analyzed by the Mann-Whitney's *U* test. Statistical analysis was carried out using Prism (GraphPad Software, La Jolla, CA, USA) and OriginPro-8 (OriginLab, Northampton, MA, USA) software. Significance level was preset to $p < 0.05$. Data were expressed as means \pm SEM for number of samples/cells (*n*) as detailed in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one data file and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.11.073>.

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AUTHOR CONTRIBUTIONS

A.P. participated in the design of the experiments and performed research. E.C. performed and analyzed the ultrastructural experiments. F.C.G. and F.C. ran the developmental studies and provided experimental tools. P.V. and G.G. performed the electrophysiological experiments and analyzed the data. D.A. performed the live imaging experiments. M.B. participated in image acquisition. C.M., B.P., and A.S. performed IUE, slice histology, and image acquisition and analysis. A.B. and A.C. performed the MS analysis. A.F. supervised the live imaging experiments. J.D.W. performed the bioinformatic analysis. F.V. contributed to the research design. S.G. performed research, analyzed data, and made the figures. S.G. and F.B. designed and supervised the research and wrote the paper. All authors revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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