Adaptor protein APPL1 links neuronal activity to chromatin remodeling in cultured hippocampal neurons

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Local signaling events at synapses or axon terminals are communicated to the nucleus to elicit transcriptional responses, and thereby translate information about the external environment into internal neuronal representations. This retrograde signaling is critical to dendritic growth, synapse development, and neuronal plasticity. Here, we demonstrate that neuronal activity induces retrograde translocation and nuclear accumulation of endosomal adaptor APPL1. Disrupting the interaction of APPL1 with Importin α1 abolishes nuclear accumulation of APPL1, which in turn decreases the levels of histone acetylation. We further demonstrate that retrograde translocation of APPL1 is required for the regulation of gene transcription and then maintenance of hippocampal late-phase long-term potentiation. Thus, these results illustrate an APPL1-mediated pathway that contributes to the modulation of synaptic plasticity via coupling neuronal activity with chromatin remodeling.

Keywords: APPL1, excitation–transcription coupling, synaptic plasticity, chromatin remodeling, gene transcription

Introduction

Activity-dependent regulation of gene expression (excitation–transcription coupling) is a powerful means by which neurons build up stable changes of neuronal properties, a process that is essential for long-term synaptic plasticity and memory (Impey et al., 1996; West et al., 2002; West and Greenberg, 2011). A great number of studies in these years has been focused on identifying pathways that couple synapse to the nucleus to elicit transcriptional responses (Ch’ng et al., 2012; Bading, 2013; Karpova et al., 2013; Ma et al., 2014).

Calcium signals are the major route for communication of synaptic activity to the nucleus (Adams and Dudek, 2005; Bading, 2013). Synaptic activity induces a rapid and transient rise in calcium levels within the postsynaptic specialization, which then triggers the release of internal calcium stores from the endoplasmic reticulum, creating a regenerative calcium wave that propagates toward the soma (Dolmetsch et al., 2001; Thiagarajan et al., 2005; Zhao et al., 2005; Adams et al., 2009). The influx of calcium at the soma or the nucleus acts as a second messenger to initiate a cascade of signaling events and results in the activation of a program of gene expression within minutes. The active transport of signaling molecules is another well-known mechanism for synapse-to-nucleus signal coupling. In recent years, a burgeoning list of signaling molecules has been identified to be implicated in synapse to nucleus communication (Wellmann et al., 2001; Proepper et al., 2007; Dieterich et al., 2008; Lai et al., 2008; Jordan and Kreutz, 2009; Schmeisser et al., 2009; Marcara and Kennedy, 2010; Dubielecka et al., 2011; Ch’ng et al., 2012; VanLeeuwen et al., 2014). Most of these proteins directly associate with NMDA receptor (NMDAR) complex, making them in a privileged position to sense local synaptic events. Synaptic activity can drive the disassociation of

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these proteins from synapse, and then long-distance retrograde trafficking along microtubule via an association with motor proteins such as dynein (Liu et al., 2005; Ben-Yaakov et al., 2012; Karpova et al., 2013; Dent and Baas, 2014).

APPL1 and APPL2 are multifunctional adaptor proteins that contain an N-terminal bin1/amphiphysin/Rvs 167 domain, a pleckstrin homology domain, and a C-terminal phosphotyrosin binding (PTB) domain (Diggins and Webb, 2017). APPL1, as a Rab5 effector, can be recruited to a subset of Rab5-positive early endosomes and participate in vesicle trafficking (Miaczynska et al., 2004; Erdmann et al., 2007; Zoncu et al., 2009). Moreover, APPL1 facilitates cross-talk between different signaling pathways via its interaction with many receptors and signaling proteins through its PTB domain (Mao et al., 2009). APPL1 plays additional role in the modification of gene expression in non-neuronal cells, mainly through directly shuttling into the nucleus to stimulates changes in chromatin remodeling (Miaczynska et al., 2004; Banach-Orlowska et al., 2009) or indirectly regulating the activity and nuclear location of other partners to promote transcription (Rashid et al., 2009; Banach-Orlowska et al., 2015).

APPL1 is also highly expressed in the central nervous system, although its function is far from well-known. Our previous work has shown that APPL1 couples synaptic NMDARs with downstream PI3K/AKT signaling pathway and participates in neuroprotective effect (Wang et al., 2012). In this study, we demonstrate that neuronal activity induces retrograde transport of APPL1 into the nucleus via the interaction of APPL1 with Importin α1. This dendritic APPL1-mediated pathway induces chromatin remodeling and thus regulates gene transcription and plays key roles in long-term synaptic plasticity.

Results

**Neuronal activity induces nuclear accumulation of APPL1 in the cultured hippocampal neurons**

To examine whether APPL1 undergoes nuclear translocation in the cultured hippocampal neurons, we first used immunostaining with antibodies that specifically recognize APPL1 (Supplementary Figure S1A and B) to test the intracellular localization of endogenous APPL1 under basal conditions. APPL1 was present in several subcellular compartments, such as Golgi fractions (indicated by GM130), early endosomal membrane fractions (indicated by EEA1), and late endosomal membrane fractions (indicated by Rab7) (Supplementary Figure S1C). Moreover, APPL1 was wildly distributed in the soma and along the dendrites under basal conditions but was excluded from the nucleus of hippocampal neurons (Figure 1A).

Next, we incubated cultures with GABA<sub>A</sub> receptor antagonist Bicuculline and K+ channel antagonist 4-aminopyridine (BMI/4-AP) together to drive excitatory synaptic transmission. As shown in Supplementary Figure S1D and E, the total amount of APPL1 was unchanged after incubation with BMI/4-AP for 1 h. Interestingly, the distribution of APPL1 underwent a ∼2.5-fold increase in the nuclear/cytoplasmic intensity ratio (Figure 1A and B). Similar changes were evoked by incubation with another GABAA receptor antagonist Picrotoxin (PTX) or depolarization with KCl (Figure 1A and B). In contrast, treatment with Na<sup>+</sup> channels blocker Tetrodotoxin (TTX) to block action potentials or activation of extrasynaptic NMDARs showed no nuclear accumulation of APPL1 (Figure 1A and B). Furthermore, pretreatment with NMDAR antagonist APV or L-type voltage-gated calcium channels blocker Nimodipine (Nimo) or removal of calcium from the extracellular media completely abolished activity-induced nuclear accumulation of APPL1 (Figure 1A and B), indicating that the influx of extracellular calcium is essential for activity-induced nuclear APPL1 accumulation. To exclude the possibility that nuclear accumulation of APPL1 depends on protein synthesis, we pretreated cultures with Anisomycin, an inhibitor of protein synthesis and observed that treatment with Anisomycin did not affect activity-dependent nuclear accumulation of APPL1 (Figure 1A and B).

We also analysed the time course of nuclear APPL1 accumulation and observed that the nuclear/cytoplasmic intensity ratio of APPL1 was significantly increased when neurons were incubated with BMI/4-AP for 15 min or longer, but not for 5 or 10 min (Figure 1C and D). Similarly, we detected the nuclear and cytosolic levels of APPL1 using subcellular fractionation and observed that APPL1 was accumulated in the nucleus when neurons were incubated with BMI/4-AP for 40 min (Supplementary Figure S1F and G). Furthermore, when cultures were incubated with BMI/4-AP for 15 min followed by a quick washout, the immunoreactivity of APPL1 in the nucleus was gradually decreased (Figure 1E and F), indicating that nuclear accumulation of APPL1 is dynamically regulated by synaptic activity.

In contrast, we observed that APPL2, another isoform of APPL (Miaczynska et al., 2004), existed in the nucleus under basal conditions and the abundance of APPL2 in the nucleus showed no significant change after stimulation with BMI/4-AP for 1 h (Supplementary Figure S1H and I).

**APPL1 undergoes activity-dependent translocation along the dendrites**

Next, we transfected GFP-tagged APPL1 (APPL1-GFP) into the cultured hippocampal neurons at DIV 12 (12 days in vitro) and assayed the movement velocity of APPL1-GFP in the distal dendrites at DIV 14 using fluorescent recovery after photobleaching (FRAP) (Figure 2A and B). Our results revealed that the recovery rate of APPL1-GFP in the distal dendrites was much faster when the neuron was stimulated with BMI/4-AP compared to the unstimulated neurons. These data indicate that the movement velocity of APPL1 in the dendrites is increased following enhanced synaptic activity.

We also monitored the transport of APPL1 using a photoconvertible fluorescent protein Dendra2-labelled APPL1 (APPL1-
Dendrites (Figure 2C and D). A brief UV illumination of distal dendrites converted the Dendra2 signal from green to red. Using time-lapse imaging, we followed the transport of photoconverted signals over a period of 30 min postconversion. Our results revealed that the photoconverted (red) dendritic APPL underwent stimulus-induced trafficking toward the nucleus.

Furthermore, to test whether endogenous APPL undergoes nuclear accumulation following stimulation of distal dendrites, we infected the neurons with a lentivirus expressing EGFP at DIV 6 to visualize the entire dendritic arbor of individual neurons. At DIV 13, neurons were applied with MNI-caged glutamate or vehicle as control, which was then uncaged at the distal dendrites of GFP-expressing neurons by UV illumination, and 30 min later, fixed and immunostained with anti-APPL antibodies. We observed that a brief UV pulse at the distal site of the dendrites significantly increased the immunoreactivity of APPL in the nucleus as compared to the control (Figure 2E and F), indicating that activation of a subset of synapses in the distal dendrites promotes nuclear APPL accumulation.

Finally, pretreatment with Ciliobrevin D to inhibit dynein ATPase or with Nocodazole to induce microtubule depolymerization completely blocked nuclear accumulation of APPL induced by BMI/4-AP (Figure 2G and H), indicating that retrograde trafficking of APPL is dependent on both dynein and microtubule.

**APPL interacts with Importin α1 via its nuclear localization signal**

Bigger proteins can be actively transported in the nucleus by dedicated importins, which recognizes the nuclear localization signal (NLS) located in the protein (Gama-Carvalho and Carmo-Fonseca, 2001; Hanz et al., 2003; Harel and Forbes, 2004). Through bioinformatics analysis, we identified a predicted NLS in APPL (Figure 3A). Consistently, full-length APPL (GST-APPL), but not APPL with this sequence deleted (GST-APPLΔNLS), interacted with Importin α1, while both GST-APPL1 and GST-APPL1ΔNLS interacted with Rab5 (Figure 3B). Furthermore, BMI/4-AP treatment significantly enhanced the interaction between APPL1 and Importin α1, indicating that neuronal activity promotes the recruitment of Importin α1 to APPL1 (Figure 3C and D). Next, we examined whether the interaction...
between APPL1 and Importin α1 is necessary for nuclear APPL1 translocation. We designed a cell membrane penetrating peptide according to the NLS of APPL1 which was fused with cell membrane transduction domain of trans-activating transcriptional activator from human immunodeficiency virus 1 (Tat-APPL1). Co-immunoprecipitation (co-IP) assay confirmed that pretreatment with Tat-APPL1 blocked the interaction between APPL1 and Importin α1 (Figure 3E and F). Additionally, pretreatment with Tat-APPL1 completely blocked nuclear APPL1 accumulation induced by synaptic activity, whereas pretreatment with scramble peptide (Tat-APPL1-SCR) had no such effect (Figure 3G and H).

CREB-regulated transcription coactivator 1 (CRTC1) is another protein undergoing nuclear translocation in hippocampal neurons and an NLS is located at its N terminus (Ch'ng et al., 2012; Nonaka et al., 2014). Incubation with Tat-APPL1NLS had no effect on the nuclear accumulation of CRTC1 induced by BMI/4-AP stimulation (Supplementary Figure S2A and B), suggesting that Tat-APPL1NLS specifically blocked the interaction between Importin α1 and APPL1.

**Nuclear APPL1 influences the association of HDAC2 with chromatin**

To examine the possible function of nuclear APPL1, we first transfected an NLS-tagged APPL1 into the cultured hippocampal neurons and observed that overexpression of APPL1 in the nucleus had no effect on the abundance of nuclear pERK (Supplementary Figure S3A and B) or pCREB (Supplementary Figure S3C and D), indicating that nuclear APPL1 alone does not induce phosphorylation of nuclear ERK or CREB.
Next, we focused on histone deacetylases (HDAC1/2) which have been identified as binding partners of nuclear APPL1 via proteomic analysis (Miaczynska et al., 2004). We transfected GFP-NLS-APPL1 into the PC12 cells and the interaction of nuclear APPL1 with HDAC2 was observed via co-IP (Figure 4A). Moreover, the interaction between endogenous APPL1 with HDAC2 was detected in the cultured neurons treated with BMI/4-AP for 30 min, which was completely blocked by pretreatment with Tat-APPL13, but not with scramble peptide (Figure 4B). However, no interaction was observed between GST-APPL1 and HDAC2 (Supplementary Figure S4), indicating that APPL1 has no direct interaction with HDAC2.

To test whether nuclear APPL1 affects HDAC2 activity, PC12 cells were transfected with NLS-APPL1 or NLS only, cell lysates were immunoprecipitated with an HDAC2 antibody, and HDAC2 activity was assessed using a fluorimetric assay. As shown in Figure 4C, no difference in HDAC2 activity was detected between cells transfected with NLS-APPL1 or NLS, indicating that APPL1 has no effect on HDAC2 activity.

To test the effect of nuclear APPL1 on histone acetylation, PC12 cells were transfected with NLS-APPL1 and acetylation of histone H4 was detected. As shown in Figure 4D and E, the acetylation levels of H4 at the H4K5 and H4K12 sites were significantly increased after overexpression of APPL1 in the nucleus. Furthermore, treatment with BMI/4-AP induced rapid acetylation of histone H4 in the cultured hippocampal neurons, and this effect was completely blocked by pretreatment with Tat-APPL13, but not with scramble peptide (Figure 4F and G). In the following, we performed chromatin immunoprecipitation (ChIP) assays using PC12 cells to test whether nuclear APPL1 affects the binding ability of HDAC2 to chromatin. We surveyed a total of five genes, which are regulated by neuronal activity and contain a CREB/HDAC2 binding site in their promoters. As shown in Figure 4H, the association of HDAC2 with the promoters for these genes...
was significantly attenuated in PC12 cells transfected with NLS-APPL1, compared to the control group expressing empty vector. Taken together, these results indicate that nuclear APPL1 participates in chromatin remodeling via interrupting the interaction between HDAC2 and chromatin.

**Nuclear translocation of APPL1 regulates gene transcription**

Next, to examine whether nuclear translocation of APPL1 is necessary for the regulation of gene expression, we analysed several genes, including *bdnf, c-fos, homer1, camk2a,* and *arc,* which were reported to be transcribed at early phase of synaptic plasticity (Zhang et al., 2009). Here, RNA isolated from the hippocampal neurons was used for quantitative reverse transcriptase (RT–qPCR) analysis. Incubation with BMI/4-AP significantly increased the transcription levels of most of the above-mentioned genes, and pretreatment with Tat-APPL113 (Figure 5A), but not with Tat-APPL1Scr (Figure 5B), completely blocked these effects.

**Nuclear translocation of APPL1 is critical for the maintenance of late-phase long-term potentiation**

Among the genes analysed in Figure 5, *Bdnf* and *camk2a* are genes tightly related to synaptic plasticity. Next, we examined whether nuclear translocation of APPL1 plays a role in synaptic plasticity. Immunostaining assay revealed that nuclear accumulation of APPL1 occurred in parts of hippocampal pyramidal neurons of the brain slices induced by four trains of tetanic stimulation, but not in that of the brain slices induced by a single train of stimulation (Figure 6A). We then measured synaptic transmission at hippocampal Schaffer collateral-CA1 pyramidal (SC-CA1) synapses. Basal excitatory transmission, such as input–output (Figure 6B) and paired-pulse ratio (Figure 6C) was...
lated genes were measured by qRT–PCR. In this study, we demonstrated that adaptor protein APPL1 shuttles into the nucleus under stimulation and promotes histone acetylation, and this process is required for the activity-induced gene transcription and the maintenance of LTP. Therefore, APPL1 acts as a linker coupling neuronal activity with gene transcription, and this pathway contributes to the modification of synaptic plasticity.

Previous researches have observed the nuclear translocation of APPL1 in non-neuronal cells (Miaczynska et al., 2004). Here, we confirmed the activity-induced nucleocytoplasmic shuttling of APPL1 in both cultured hippocampal neurons (Figure 1) and in acute brain slice (Figure 6). To analyse the causal role of nuclear APPL1 in gene transcription, we utilized a transmembrane peptide to block the interaction between APPL1 and Importin α1, instead of changing the expression level of APPL1 (Banach-Orlowska et al., 2009, 2015; Rashid et al., 2009). It should be kept in mind that APPL1 is a multifunctional protein and synaptic APPL1 has been identified to couple NMDA receptors with AKT signaling (Wang et al., 2012) and gate LTP (Fernandez-Monreal et al., 2016). It is better to specifically interrupt the nuclear translocation of APPL1, while leaving its other functions unaffected.

In our study, we observed that enhanced neuronal activity triggers retrograde translocation of APPL1 along the dendrites (Figure 2), indicating that nuclear-accumulated APPL1 is mainly from the dendrites. However, we cannot completely exclude the possibility that retrograde translocation of APPL1 along the axon may also contribute to nuclear APPL1 accumulation. Especially, Zerial’s group has shown data indicating that APPL1 undergoes both retrograde and anterograde trafficking along the axon under basal conditions (Goto-Silva et al., 2019). It is interesting to differentiate the functional roles of dendritic transport of APPL1 from that of axonal transport in future.

Similar to APPL1, several endocytic proteins involved in vesicle trafficking and sorting, such as clathrin (Borlido et al., 2009), β-arrestin1 (Beaulieu and Caron, 2005), Huntingtin interacting protein 1 (Mills et al., 2005), and intersectin 1-short (Alvisi et al., 2018), are capable of shuttling between the nucleus and the cytoplasm and being involved in nuclear signaling in response to extracellular stimuli (Pilecka et al., 2007; Borlido et al., 2009). It is still unclear to what extent these diverse functions are interconnected to coordinate various cellular processes, or whether they are largely independent. At least, these findings suggest that nuclear translocation of endocytic proteins may represent a powerful channel for communicating information from the extracellular environment to the nucleus. Moreover, endocytosis
mediated by APPL/Rab5 constitutes a novel APP-dependent pathogenic pathway in Alzheimer’s disease (AD) (Kim et al., 2016) and APPL1 has also been found to accumulate as granules around neurons in postmortem human brain of AD (Ogawa et al., 2013). Thereafter, endocytic protein-mediated pathway should be a potentiated target to unveil the pathophysiology of these neurodegenerative diseases in future studies.

**Materials and methods**

**Animals**

Mice were housed under a 12-h light and dark cycle with food and water provided ad libitum. All experiments were performed in accordance with the guidelines of Zhejiang University Animal Experimentation Committee. Male C57BL/6 mice (6–8 months of age) were used in related experiment.
Plasmids
GST-APPL1 and GST-APPL1ΔNLS were constructed by cloning the corresponding cDNA of mouse APPL1 into pGEX-4T-1 between XhoI and EcoRI. FLAG-NLS-APPL1 was constructed by cloning the cDNA of human APPL1 into pCMV-Tag4A vector between EcoRV and XhoI. NLS-GFP, GFP-NLS-APPL1, or Importin α1 was constructed by cloning the cDNA into pEGFP or pERFP vector between XhoI and EcoRI. APPL1-Dendra2 was constructed by cloning the human APPL1 into pCMV-Dendra2 between EcoRI and XhoI.

Reagents, peptides, and antibodies
4-Aminopyridine, PTX, poly-L-lysine, D(-)-2-amino-5-phosphonopentanoic acid, Trolox, DAPI nuclear dye, Nocodazole, and Ciliobrevin D were purchased from Sigma. Bicuculline methiodide, MNI-caged-L-glutamate, Anisomycin were purchased from Tocris Bioscience. Leptomycin B (10 nM) was purchased from Beyotime. Peptides used are Tat-APPL131-137 (YGRKKRRQRRRRRA SEKQKEIERVKEK) and Tat-APPL131-137ΔC (YGRKKRRQRRRRASEKQKEIEAA AAA). The following antibodies were used: anti-APPL1 (sc-67402) and anti-Rab5 (sc-46692) from Santa Cruz Biotechnology, anti-pERK (4370S) and anti-GAPDH (2118) from Cell Signaling Technology, anti-HDAC2 (ab32117), anti-Importin α1 (ab84440), anti-Histone H4K5 (ab51997), anti-Histone H4K12 (ab77793), anti-APPL2 (ab95196), and anti-Histone H4 (ab10158) from Abcam, anti-MAP2 (M9942, M3696), anti-FLAG (F1804), and anti-APPL1 (1409089) from Sigma–Aldrich, and anti-pCREB (06-519 and 04-218) from Millipore. Glutathione sepharose beads and protein A sepharose beads were purchased from GE Healthcare. Phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitor cocktails 2 and 3 were purchased from Sigma. Horseradish peroxidase (HRP)-linked goat anti-mouse immunoglobulin G (IgG), goat anti-rabbit IgG, and donkey anti-goat IgG, secondary antibodies conjugated to Dylight (488 or 555), and chemiluminescence kit were purchased from Pierce.

Cell cultures and transfection
PC12 cell line was grown on Dulbecco’s modified Eagle media (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco) at 37°C under 5% CO2. Hippocampal or cortical neurons were cultured as described previously ([Luo et al., 2002; Wang et al., 2012]). Hippocampal neurons were transfected with the plasmids using Lipofectamine 3000 reagent (Life Technologies) at DIV 10–13 or Calcium Phosphate Transfection Kit (Clontech) at DIV 6–9 according to the manufacturer’s instructions.

Pharmacological treatment
For most of the experiments, neurons were incubated with various pharmacological reagents in cultured medium in a 37°C, 5% CO2 incubator for the appropriate amount of time before cells were either fixed for immunostaining or lysates were collected for western blotting. For extrasynaptic NMDAR activation, cultures were first stimulated by BMI/4-AP in the presence of the non-competitive NMDAR antagonist MK801 (10 μM) for 5 min to inactivate synaptic NMDARs. After washout, extrasynaptic NMDAR were then selectively activated by NMDA (10 μM) for 1 h. KCl depolarization was induced in solutions containing 140 mM NaCl, 1.3 mM CaCl2, 50 mM KCl, 35 mM HEPES, and 33 mM glucose (pH 7.4) for 30 min. When peptides or antagonists were used to pretreat the cultured neurons, these reagents were applied 30 min before stimulation unless otherwise indicated. When peptides or antagonists were used to pretreat the cultured neurons, these reagents were applied 30 min before stimulation unless otherwise indicated.

Immunostaining
Hippocampal neurons were fixed at room temperature with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min, washed three times in PBS, permeabilized with 0.2% Triton X-100 (Amresco) in PBS, and then blocked for 30 min in blocking solution containing 2.5% BSA fraction V (Amresco) in PBS. Next, neurons were incubated in primary antibodies for 1 h at room temperature or overnight at 4°C, washed three times with PBS, and then incubated with secondary antibody in blocking solution for another 1 h at room temperature. After being washed, neurons were mounted with ProLong Gold or ProLong Gold with DAPI (Molecular Probes).

Acute brain slices (50 μm) or frozen sections (25 μm) were fixed with 4% PFA overnight at 4°C, permeabilized with 0.4% Triton X-100 in PBS for 30 min at room temperature, and then blocked in 2.5% BSA for 2 h at room temperature. Slices were incubated with primary antibodies for 48 h at 4°C. After being washed with PBS, slices were then incubated with an appropriate secondary antibody and nucleus dye DAPI for 4 h at room temperature. Slices were mounted with ProLong Gold.

Recombinant protein purification and pull-down assay
GST-APPL1 or GST-APPL1ΔNLS was transformed in Escherichia coli BL21 and induced with 0.1 mM isopropyl β-D-thiogalactopyranosid (IPTG) for 6 h at 30°C. The recombinant APPL1 and APPL1ΔNLS were purified using glutathione-sepharose beads. To pull down proteins from brain lysates, fresh mouse brain was homogenized in lysis buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.6) with protease and phosphatase inhibitors. After homogenization, the mixture was incubated for 30 min at 4°C, and then centrifuged at 16000× g for 15 min at 4°C. Subsequently, 500 μl supernatant was incubated with 10 μg purified recombinant proteins for 3 h at 4°C. The complex was washed four times with lysis buffer and subjected to western blotting analysis. For binding assay, 10 μg purified GST-tagged protein was incubated with approximately the same amount of His-tagged recombinant protein in lysis buffer. Proteins were pulled down with GST beads, washed four times with lysis buffer, and then detected with His antibody.
Quantitative real-time PCR

Total RNA was extracted from cortical neurons using Trizol (TaKaRa) and reversely transcribed into cDNA with PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer’s instructions. qRT–PCR was carried out using CFX96 Real-Time PCR Detection System (Bio-Rad). Gene expression levels were calculated according to the 2−ΔΔCt method. The relative amounts of mRNA were normalized to β-actin as an internal control, and hprt, an activity-independent gene, was set as a negative control. Primer sequences were as follows (Sequence hprt

\begin{align*}
405 & \text{AGAAGA;} \\
100 & \text{b} \\
30 & \text{actin} \\
\end{align*}

were acquired using 488 nm line of laser before photoswitch and 561 nm line of laser after switch. Subsequently, image analysis was carried out with the ImageJ and FV10-ASW2.0 software (Olympus).

Slice preparation and electrophysiology

C57Bl/6 mice at 8–12 weeks of age were anesthetized with diethyl ether, and the brains were rapidly removed and placed in ice-cold, high sucrose cutting solution containing 194 mM succrose, 30 mM NaCl, 26 mM NaHCO3, 10 mM glucose, 4.5 mM KCl, 1.2 mM NaH2PO4, 7 mM MgSO4, 0.2 mM CaCl2, and 2 mM MgCl2. Slices were cut on a Leica vibratome in the high sucrose cutting solution and immediately transferred to an incubation chamber with artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 26.2 mM NaHCO3, 11 mM glucose, 2.5 mM KCl, 1 mM NaH2PO4, 1.3 mM MgCl2, 11 mM glucose, and 2.5 mM CaCl2. The slices were allowed to recover at 34°C for 30 min before being allowed to equilibrate at room temperature for another 1 h. During recordings, the slices were placed in a recording chamber constantly perfused with heated ACSF (28°C–32°C) and gassed continuously with 95% O2 and 5% CO2. Extracellular field EPSPs (fEPSPs) were recorded in stratum radiatum of CA1 using a glass pipette filled with ACSF (1.5–3 mM). The SC pathway was stimulated every 20 sec using a concentric bipolar stimulation electrode. For LTP, the stimulation intensity was adjusted to give fEPSP slopes of 30%–50% of maximum, and three successive responses were averaged and expressed relative to the normalized baseline. After a stable baseline was recorded, one train of 100 Hz (100 Hz, 1 sec) or four trains of 100 Hz (3 min apart) stimulation were applied to induce E-LTP or L-LTP. Peptides (20 μM) were bath-applied for >30 min before the LTP protocol application. For immunohistochemistry, brain slices induced by high-frequency stimulation were kept in ACSF for another 30 min and then immunostained accordingly.

Statistical analysis

No statistical methods were used to pre-determine sample sizes, but our sample sizes were similar to those reported in previous studies. Samples were assigned randomly to the experimental and control groups. Experimental treatments were also randomized. All data are shown as the mean ± the standard error of the mean (SEM). Differences between two groups were tested by unpaired, two-tailed Student’s t-test or Mann–Whitney rank sum test, based on a normality test (Shapiro–Wilk). For comparison of more than two groups, one-way analysis of variance (ANOVA) and Bonferroni’s test for multiple comparison post hoc tests were used. We used two-way ANOVA and Tukey test for post hoc test if there were two independent variables. Statistical analysis was carried out using Prism 5 software (GraphPad). Significance was indicated as ***P < 0.005, **P < 0.01, and *P < 0.05.
Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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References

Adams, J.P., and Dudek, S.M. (2005). Late-phase long-term potentiation: getting to the nucleus. Nat. Rev. Neurosci. 6, 737–743.

Adams, J.P., Robinson, R.A., Hudgings, E.D., et al. (2009). NMDA receptor-independent control of transcription factors and gene expression. Neuron 60, 1429–1433.

Alvigi, G., Paolini, L., Contarini, A., et al. (2018). Intersectin goes nuclear: secret life of an endocytic protein. Biochem. J. 475, 1465–1472.

Bading, H. (2013). Nuclear calcium signalling in the regulation of brain function. Nat. Rev. Neurosci. 14, 593–608.

Banach-Orlowska, M., Pilecka, I., Torun, A., et al. (2009). Functional characterization of the interactions between endosomal adaptor protein APPL1 and the NuRD co-repressor complex. Biochem. J. 423, 389–400.

Banach-Orlowska, M., Szymanska, E., and Miczynska, M. (2015). APPL1 endocytic adaptor as a fine tuner of Dvl2-induced transcription. FEBS Lett. 589, 532–539.

Beaulieu, J.M., and Caron, M.G. (2005). β-arrestin goes nuclear. Cell 123, 755–757.

Ben-Yaakov, K., Dagan, S.Y., Segal-Ruder, Y., et al. (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J. 31, 1350–1363.

Borlido, J., Zecchini, V., and Mills, I.G. (2009). Nuclear trafficking and functions of endocytic proteins implicated in oncogenesis. Traffic 10, 1209–1220.

Ch’ng, T.H., Üzgil, B., Lin, P., et al. (2012). Activity-dependent transport of the translational coactivator CRTC1 from synapse to nucleus. Cell 150, 207–221.

Cheng, K.K., Lam, K.S., Wu, D., et al. (2012). APPL1 potentiates insulin secretion in pancreatic β cells by enhancing protein kinase Akt-dependent expression of SNARE proteins in mice. Proc. Natl Acad. Sci. USA 109, 8919–8924.

Dent, E.W., and Baas, P.W. (2014). Microtubules in neurons as information carriers. J. Neurochem. 129, 235–239.

Dieterich, D.C., Karpova, A., Mikhaylova, M., et al. (2008). Caldendrin-Jacob: a protein liaison that couples NMDA receptor signalling to the nucleus. PLoS Biol. 6, e34.

Diggins, N.L., and Webb, D.J. (2017). APPL1 is a multifunctional endosomal signaling adaptor protein. Biochim. Bioph. Trans. 45, 771–779.

Dolmetsch, R.E., Pajvani, U., Fife, K., et al. (2001). Signaling to the nucleus by an L-type calcium channel-calcmodulin complex through the MAP kinase pathway. Science 294, 333–339.

Dubielecka, P.M., Ladwein, K.I., Xiong, X., et al. (2011). Essential role for Ab1 in embryonic survival and WAVE2 complex integrity. Proc. Natl Acad. Sci. USA 108, 7022–7027.

Erdmann, K.S., Mao, Y., Mc Cra, H.J., et al. (2007). A role of the Lowe syndrome protein OCR1 in early steps of the endocytic pathway. Dev. Cell 13, 377–390.

Fernandez-Monreal, M., Sanchez-Castillo, C., and Esteban, J.A. (2016). APPL1 gates long-term potentiation through its plektin homology domain. J. Cell Sci. 129, 2793–2803.

Galan-Davila, A.K., Ryu, J., Dong, K., et al. (2018). Alternative splicing variant of the scaffold protein APPL1 suppresses hepatic adiponectin signaling and function. J. Biol. Chem. 293, 6066–6074.

Gama-Carvalho, M., and Carmo-Fonseca, M. (2001). The rules and roles of nucleocytoplasmic shuttling proteins. FEBS Lett. 498, 157–163.

Goto-Silva, L., McShane, M.P., Salinas, S., et al. (2019). Retrograde transport of Akt by a neuronal Rab5-APPL1 endosome. Sci. Rep. 9, 2433.

Hanz, S., Perlson, E., Willis, D., et al. (2003). Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40, 1093–1104.

Harel, A., and Forbes, D.J. (2004). Importin β: conducting a much larger cellular Symphony. Mol. Cell, 16, 339–350.

Impex, S., Mark, M., Villacres, E.C., et al. (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 6, 973–982.

Jordan, B.A., and Kreutz, M.R. (2009). Nucleocytoplasmic protein shuttling: the direct route in synapse-to-nucleus signaling. Trends Neurosci. 32, 392–401.

Karpova, A., Mikhaylova, M., Bera, S., et al. (2013). Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus. Cell 152, 1119–1133.

Kim, S., Sato, Y., Mohan, P.S., et al. (2016). Evidence that the rab5 effector APPL1 mediates APP–JCTF1-induced dysfunction of endosomes in Down syndrome and Alzheimer’s disease. Mol. Psychiatry 21, 707–716.

Lai, K.O., Zhao, Y., Ch’ng, T.H., et al. (2008). Importin-mediated retrograde transport of CREB2 from distal processes to the nucleus in neurons. Proc. Natl Acad. Sci. USA 105, 17175–17180.

Liu, S., Hennessey, T., Rankin, S., et al. (2005). Mutations in genes encoding inner arm dynein heavy chains in Tetrahymena thermophila lead to axonemal hypersensitivity to Ca2+. Cell Motil. Cytoskeleton 62, 133–140.

Luo, J.H., Fu, Z.Y., Losi, G., et al. (2002). Functional expression of distinct NMDA channel subunits tagged with green fluorescent protein in hippocampal neurons in culture. Neuropharmacology 42, 306–318.

Ma, H., Groth, R.D., Cohen, S.M., et al. (2014): γCaMkII shuttles Ca2+/CaM to the nucleus to trigger CREB phosphorylation and gene expression. Cell 159, 281–294.

Mao, X., Kikan, C.K., Riojas, R.A., et al. (2006). APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat. Cell Biol. 8, 516–523.

Marcera, E., and Kennedy, M.B. (2010). The Huntington’s disease mutation impairs Huntington’s role in the transport of Nf-kB from the synapse to the nucleus. Hum. Mol. Genet. 19, 4373–4384.

Miczynska, M., Christoforidis, S., Giner, A., et al. (2004). APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell 116, 445–456.

Mills, I.G., Gaughan, L., Robson, C., et al. (2005). Huntingtin interacting protein 1 modulates the transcriptional activity of nuclear hormone receptors. J. Cell Biol. 170, 191–200.
Nonaka, M., Kim, R., Fukushima, H., et al. (2014). Region-specific activation of CRTC1–CREB signaling mediates long-term fear memory. Neuron 84, 92–106.

Ogawa, A., Yamazaki, Y., Nakamori, M., et al. (2013). Characterization and distribution of adaptor protein containing a PH domain, PTB domain and leucine zipper motif (APPL1) in Alzheimer’s disease hippocampus: an immunohistochemical study. Brain Res. 1494, 118–124.

Pilecka, I., Banach-Orlowska, M., and Miaczynska, M. (2007). Nuclear functions of endocytic proteins. Eur. J. Cell Biol. 86, 533–547.

Proepper, C., Johannsen, S., Liebau, S., et al. (2007). Abelson interacting protein 1 (Abi-1) is essential for dendrite morphogenesis and synapse formation. EMBO J. 26, 1397–1409.

Rashid, S., Pilecka, I., Torun, A., et al. (2009). Endosomal adaptor proteins APPL1 and APPL2 are novel activators of β-catenin/TCF-mediated transcription. J. Biol. Chem. 284, 18115–18128.

Ryu, J., Galan, A.K., Xin, X., et al. (2014). APPL1 potentiates insulin sensitivity by facilitating the binding of IRS1/2 to the insulin receptor. Cell Rep. 7, 1227–1238.

Schenck, A., Goto-Silva, L., Collinet, C., et al. (2008). The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. Cell 133, 486–497.

Schmeisser, M.J., Grabrucker, A.M., Bockmann, J., et al. (2009). Synaptic cross-talk between N-methyl-D-aspartate receptors and LAPSER1–β-catenin at excitatory synapses. J. Biol. Chem. 284, 29146–29157.

Thiagarajan, T.C., Lindskog, M., and Tsien, R.W. (2005). Adaptation to synaptic inactivity in hippocampal neurons. Neuron 47, 725–737.

VanLeeuwen, J.E., Rafalovich, I., Sellers, K., et al. (2014). Coordinated nuclear and synaptic shuttling of afadin promotes spine plasticity and histone modifications. J. Biol. Chem. 289, 10831–10842.

Wang, Y.B., Wang, J.J., Wang, S.H., et al. (2012). Adaptor protein APPL1 couples synaptic NMDA receptor with neuronal prosurvival phosphatidylinositol 3-kinase/Akt pathway. J. Neurosci. 32, 11919–11929.

Wellmann, H., Kaltschmidt, B., and Kaltschmidt, C. (2001). Retrograde transport of transcription factor NF-κB in living neurons. J. Biol. Chem. 276, 11821–11829.

West, A.E., and Greenberg, M.E. (2011). Neuronal activity-regulated gene transcription in synapse development and cognitive function. Cold Spring Harb. Perspect. Biol. 3, a005744.

West, A.E., Griffith, E.C., and Greenberg, M.E. (2002). Regulation of transcription factors by neuronal activity. Nat. Rev. Neurosci. 3, 921–931.

Zhang, S.J., Zou, M., Lu, L., et al. (2009). Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. PLoS Genet. 5, e1000604.

Zhao, M., Adams, J.P., and Dudek, S.M. (2005). Pattern-dependent role of NMDA receptors in action potential generation: consequences on extracellular signal-regulated kinase activation. J. Neurosci. 25, 7032–7039.

Zoncu, R., Perera, R.M., Balkin, D.M., et al. (2009). A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 136, 1110–1121.