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PDZ-scaffold protein, Tamalin promotes dendritic outgrowth and arborization in rat hippocampal neuron

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ABSTRACT

Tamalin is a scaffold protein known to regulate membrane trafficking through its interaction with cytohesin-2/ARNO, guanine nucleotide exchange factor (GEF) on ADP-ribosylation factor (Arf) 1/6, and induces actin reorganization. However, the neuronal function of Tamalin is not well understood. Here, we report that Tamalin participates in neurite development through the association with exchange factor for Arf6 (EFA6A)/Arf6 signaling. In immature hippocampal neuron, Tamalin knockdown markedly reduced the dendritic outgrowth, the number of dendritic tips and the levels of filamentous actin (F-actin) and microtubule-associated protein 2 (MAP2) in dendrites. In addition, Tamalin colocalized with EFA6A and Arf6 in the dendritic shaft. Tamalin knockdown reduced the number, size, and intensity of endogenous EFA6A cluster, whereas overexpression of Tamalin showed opposite effects compared with those of knockdown. These results suggest that Tamalin is responsible for neuronal dendritic development via regulation of EFA6A/Arf6-mediated cytoskeleton dynamics.

1. Introduction

Tamalin (also termed *GR*P1-*a*ssociated *s*caffold *p*rotein, GRASP) is a scaffold protein that contains multiple protein-interacting domains, including alanine-rich, PDZ (PSD-95/discs-large/ZO-1), leucine-zipper, proline-rich domains and C-terminal PDZ binding motif [1,2]. The PDZ domain of Tamalin binds to C-terminal PDZ binding motifs of metabotrophic glutamate receptors (mGluRs), gamma-aminobutyric acid B receptor 2 ($GABA_{B2}$), SAPAP3, and Tamalin itself, whereas the leucine-zipper region interacts with the coiled coil domain of cytohesin-2/ARNO. In addition, Tamalin forms a direct complex with PDZ domains of S-SCAM, Mint2, and PSD-95 via its C-terminal PDZ binding motif [3].

In the rat brain membrane fractions, Tamalin forms multimolecular protein complex comprising not only mGluR1 but also c-Src, Fyn, and a protein phosphatase, SHP-2, suggesting that Tamalin is a novel signaling molecule mediating Syk signaling [4]. In addition, neurotrophin-3 (NT3) binding to TrkCT-1 causes the recruitment of Tamalin and cytohesin-2/ARNO to the TrkCT-1 cytoplasmic domain, which in turn produces the active Arf6-GTP form. Arf6-GTP activates Rac1 GTPase and induces membrane ruffling and the formation of cellular protrusions via actin reorganization [5]. Knockdown of Tamalin in Madin-Darby Canine Kidney (MDCK) cells prevents the association of ARNO and Dock180, and blocks ARNO-mediated Rac activation [6]. Especially, PSD-95, Tamalin-interacting synaptic scaffold protein, regulates negatively dendritic outgrowth and branching in an activity-dependent manner. In addition, PSD-95-mediated inhibition of dendritic branching is antagonized by cypin [7] which binds directly to tubulin heterodimers and promotes microtubule polymerization [8]. Recent study showed that Tamalin deficiency blocks electroconvulsive shock (ECS)-induced proliferation and neurite development of adult hippocampal progenitors in dentate gyrus [9].

Here, we report the involvement of Tamalin and EFA6A in neuronal dendritic development. Results from immunocytochemistry, overexpression, and knockdown suggest that cellular function of Tamalin in early neuronal development is associated with EFA6A/Arf6 pathway.

2. Materials and methods

2.1. Expression and short hairpin RNA constructs

Rat Tamalin (full length, GenBank Accession No. AF374272.1) was subcloned into pRK5-myc (Clontech). For short hairpin RNA (shRNA) knockdown construct, oligonucleotide targeting the following region of Tamalin was subcloned into pSUPER.gfp/neo (Oligoengine); nt 1320–1338 of rat Tamalin, 5′-GTCCCAGCACAA-AGAAGAA-3′.

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2.2. Primary neuron culture, transfection, and immunocytochemistry

All experiments were approved by Korea University Institutional Animal Care & Use Committee. Cultured hippocampal neurons were prepared from embryonic (E18) Sprague Dawley rats (of either sex). Dissociated neurons on poly-L-lysine-coated (1 mg/ml) coverslips were placed in neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM L-glutamine, 12.5 μM glutamate, and penicillin–streptomycin (Invitrogen) for 3 h and were grown in fresh medium without glutamate. Neurons at DIV5–7 were transfected by calcium phosphate precipitation (Calphos™ transfection kit; Clontech). For immunostaining, neurons were permeabilized by phosphate buffered saline containing 0.2% Triton X-100, and then incubated with primary antibodies raised against GFP (1:500), Tamalin (1:500), PSD-95 (1:250), EFA6A (1:250), Arf6 (1:250), and MAP2 (1:500), followed by Cy3-, Cy-5 or FITC-conjugated secondary antibodies (Jackson Immunoresearch). F-actin in cultured neurons was visualized using FITC- or rhodamine-conjugated phalloidin (1:500; Invitrogen).

2.3. Antibodies

Antibodies were purchased: Tamalin, β-tubulin, and MAP2 from Chemicon; PSD-95 from Affinity BioReagents; GFP and myc from Santa Cruz; Guinea pig anti-EFA6A and rabbit anti-Arf6 were obtained from Dr. Eunjoon Kim of KAIST [10].

2.4. Coimmunoprecipitation

Transfected HEK293T cells were solubilized with phosphate-buffered saline containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche). After centrifugation at 22,000*g* at 4 °C for 30 min, the supernatant was incubated with anti-FLAG M2-agarose (Sigma) for 2 h at 4 °C. Precipitated proteins were immunoblotted with Tamalin (Chemicon) and FLAG (Sigma) antibodies.

2.5. Preparation of rat brain lysates

Rat brains (~postnatal 0, 7, 12, 18, 26 days and 6 weeks) were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with 2 mM PMSF and protease inhibitor cocktail (Roche). After centrifuging the homogenates at 16,000*g* for 10 min, the supernatant was saved as whole lysates. For the analysis of brain regional expression, four brain regions – the cerebral cortex, hippocampus, olfactory bulb, and cerebellum were dissected out from adult rat brain (6 weeks).

2.6. Image acquisition and statistical analysis

Fluorescent images were acquired using a confocal microscope (LSM510). The same parameter settings were used for all scans. Each experiment was repeated two to three times, and images were blindly analyzed using MetaMorph (Universal imaging). Quantification of the total length of dendrites, the number of dendritic segments and tips were performed using images acquired with a 20× objective. To measure the length of dendrites, all dendrites in a single neuron were manually traced, followed by automatic length calculation in MetaMorph. Dendrites of GFP expressing neurons were identified by immunostaining for dendritic marker MAP2. Total dendritic length represents the total length of all dendrites per neuron, and total dendritic tips show

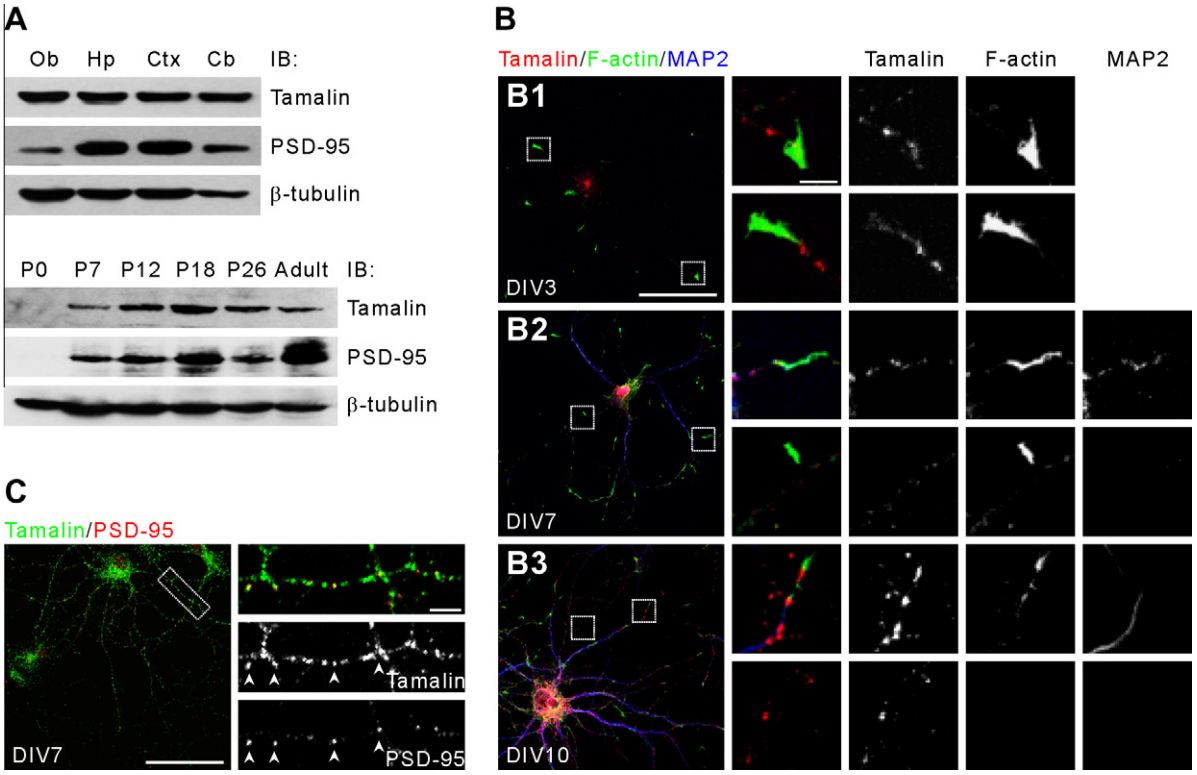

Fig. 1. Expression patterns of Tamalin. (A) Tamalin protein showed widespread distribution in adult rat brain regions (upper panel; Ob, olfactory bulb; Hp, hippocampus; Ctx, cortex; Cb, cerebellum). Tamalin expression increases gradually during rat brain development (bottom panel). (B) Tamalin is localized mainly in dendrites and rarely in axons in cultured hippocampal neurons. Cultured hippocampal neurons (B1, DIV3; B2, DIV7; B3, DIV10) were immunostained using anti-Tamalin and subcellular markers (anti-MAP2 for dendrites and FITC-conjugated phalloidin for F-actin). Tamalin distributes to MAP2-positive dendrite as well as MAP2-negative axons. Tamalin colocalizes partially with F-actin in dendritic growth cones. (C) Tamalin also colocalizes partially with PSD-95. Sites of colocalization are indicated by arrowheads. Enlargements of the insets are shown right the original images. Scale bar 50 μm; inset, 5 μm.

the total number of all dendritic processes and branches per neuron. Total length and branching number of dendrites from single neurons were averaged to obtain a population mean and SEM. To measure the changes of endogenous F-actin, MAP2, and EFA6A, their immunofluorescence intensities in knockdown and overexpression were compared with that of control. The Student's unpaired *t*-test was used in experiments with two groups. $p < 0.05$ was considered significant. Values are expressed as mean ± SEM.

3. Results

3.1. Expression patterns of Tamalin

Because Tamalin is a functionally novel protein, we investigated its expression pattern in the adult rat brain (6 weeks), using immunoblot analysis. Tamalin protein is widely expressed in various rat brain regions, including cortex, cerebellum, hippocampus, and olfactory bulb (Fig. 1A, upper panel). Expression levels of Tamalin protein gradually increased during postnatal rat brain development, similar to PSD-95 (Fig. 1A, bottom panel), but decreased in adult stage. We determined the subcellular localization of Tamalin. In cultured hippocampal neurons (days *in vitro* or DIV3), Tamalin was detected in discrete, but often diffuse, structures in MAP2 (a dendritic marker)-positive dendrites, although relative weak signals were also detected in MAP2-negative axons (Fig. 1B2 and B3). In dendrites, Tamalin showed a widespread subcellular distribution, with some of the Tamalin signals being colocalized with PSD-95 (an excitatory postsynaptic marker) in dendritic shafts and F-actin in dendritic growth cones (Fig. 1B and C).

3.2. Tamalin knockdown caused a suppression of dendritic development

To study the role of Tamalin in neuronal development, we next examined the effects of Tamalin knockdown on dendritic outgrowth, using an shRNA construct that significantly reduced both

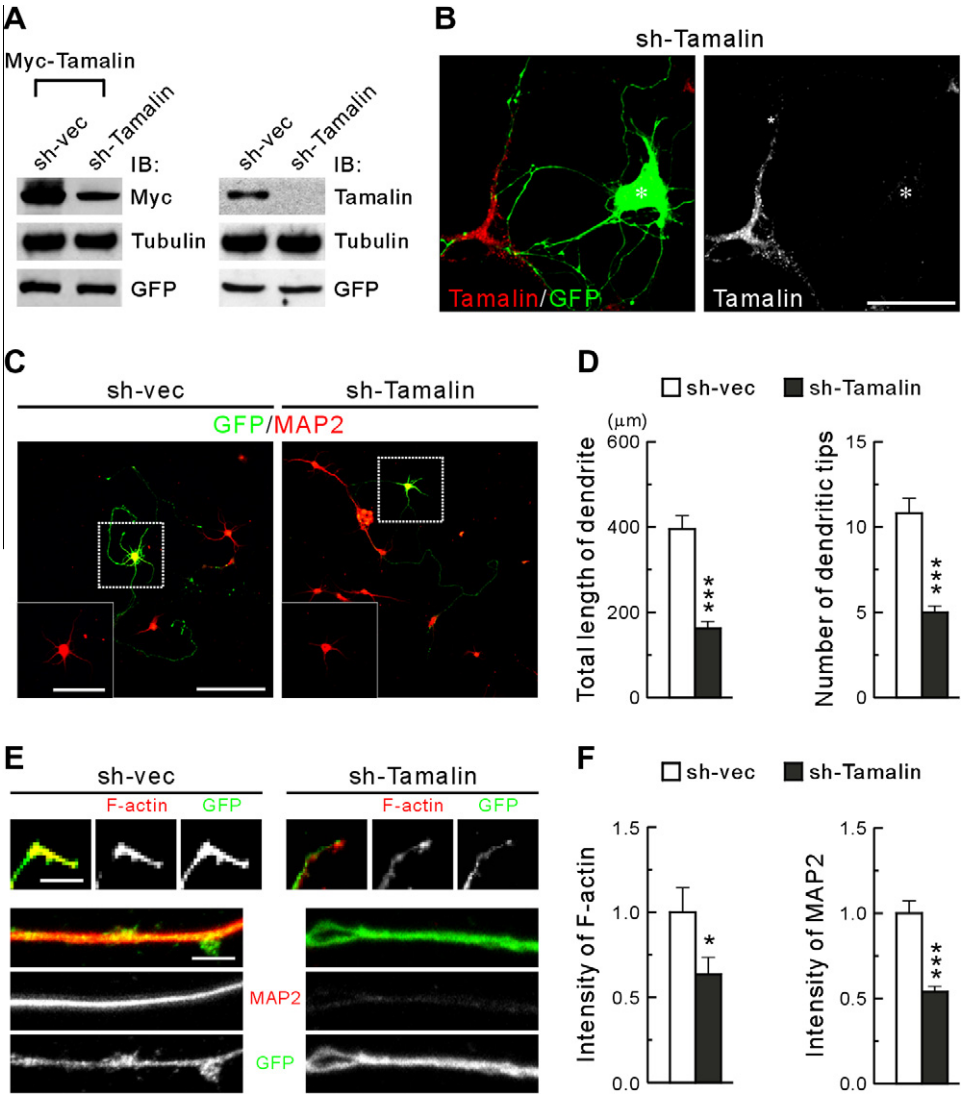

Fig. 2. Knockdown of Tamalin suppresses dendritic development and reduces dendritic cytoskeletons. (A) Knockdown construct of Tamalin reduces exogenously expressed Myc-Tamalin (left panel) and endogenous Tamalin (right panel) in PC12 cells. The cell lysates transfected with sh-vec or sh-Tamalin were analyzed with Western blotting. (B) Knockdown construct also reduces the expression of endogenous Tamalin in cultured hippocampal neurons, which were transfected at DIV5 with sh-vec or sh-Tamalin, immunostained at DIV7 with anti-Tamalin, and subsequently stained with Cy3-conjugated goat anti-rabbit IgG antibody. Scale bar 50 μm. (C) Tamalin knockdown decreases the dendritic outgrowth and branching. Dendrites were identified by both MAP2 and GFP fluorescence. Scale bar 100 μm; inset, 50 μm. (D) Quantification of (C). Histogram represents mean ± SEM (sh-vec, $n = 23$ cells; sh-Tamalin, $n = 23$ cells; $^{*}p < 0.05$; $^{***}p < 0.001$, Student's *t*-test). (E) Tamalin knockdown leads to significant decreases in F-actin and MAP2 immunofluorescence signals in dendritic tips and shaft, respectively. (F) Quantification of (E). Histogram represents mean ± SEM based on ratio of arbitrary unit values, normalized to that in sh-vec (sh-vec, $n = 15$ cells; sh-Tamalin, $n = 25$ cells; $^{***}p < 0.001$, Student's *t*-test).

exogenous and endogenous Tamalin expressions in PC12 cells (Fig. 2A), and endogenous Tamalin expression in cultured hippocampal neurons by ~75% (Fig. 2B, DIV5–7).

Cultured hippocampal neurons with Tamalin knocked down by shRNA (DIV5–7) displayed a marked reduction in the total length of dendrites and the number of dendritic tips, compared with neurons expressing empty shRNA vector (sh-vec) (Fig. 2C and D). This result strongly suggests that Tamalin is required for dendritic development including dendrite outgrowth and arborization. During neuronal development, the organization of cytoskeleton, including microtubules and F-actin, plays a critical role in neurite outgrowth [11]. Therefore, it seemed highly likely that the inhibitory effect of Tamalin knockdown on the neurite outgrowth was associated with cytoskeletal disorganization. We thus investigated whether reduced expression of Tamalin affects the levels of F-actin and MAP2 in the dendritic growth cones and shafts, respectively. To this end, neurons were labeled with rhodamine-conjugated phalloidin for visualization of F-actin or MAP2 antibody. Tamalin knockdown led to a significant reduction in the levels of F-actin and MAP2 (Fig. 2E and F). Although degradation and reduction of MAP2 induce the destabilization of microtubule [12,13], intensity of microtubule was not changed (data not shown) in dendritic shafts. These results suggest that Tamalin is a positive regulator in dendritic development in immature neurons.

3.3. Tamalin overexpression enhanced dendritic development

Because Tamalin knockdown decreased dendritic outgrowth and branching (Fig. 2), we reasoned that an increased expression of Tamalin may positively regulate dendritic development. Indeed, Tamalin overexpression in cultured hippocampal neurons (DIV5–7) significantly increased the total length and branching tips of dendrites, compared with control (GFP alone) (Fig. 3A and B). In addition, our finding that Tamalin knockdown decreases the contents of F-actin and MAP2 (Fig. 2E and F) suggests the possibility that Tamalin might directly regulate the organization of F-actin and microtubule cytoskeletons. Therefore, we investigated whether Tamalin overexpression affects the levels of F-actin and MAP2. Tamalin overexpression significantly enhanced the levels of F-actin in dendritic shafts as well as in growth cones (Fig. 3C, upper panel), and also increased the intensity of MAP2 (Fig. 3C bottom panel, and D). These results suggest that Tamalin contributes to dendritic development including outgrowth and branching through mechanisms that depend on regulations of dendritic cytoskeletons.

3.4. Tamalin regulates the EFA6A–Arf6 complex

Tamalin forms molecular complex directly with cytohesin-2/ARNO [1], which is a member of GEFs for the Arf1 and Arf6 GTP-binding proteins. The EFA6A is known as an Arf6-specific GEF and has been suggested to have a role in neurite outgrowth [14] and dendritic spine dynamics [10]. Therefore, to determine whether Tamalin-dependent regulation of dendritic development accompanies change of EFA6A, we observed the association of Tamalin with EFA6A by immunocytochemistry. As we expected, Arf6 and EFA6A well colocalized (Fig. 4A) and Tamalin also colocalized with EFA6A (Fig. 4B) in cultured hippocampal neuron. In order to determine that Tamalin contributes to regulation of EFA6A signaling via direct interaction between Tamalin and EFA6A, HEK293T cells co-transfected with Tamalin and EFA6A were used in *in vitro* coimmunoprecipitation. Full length Tamalin

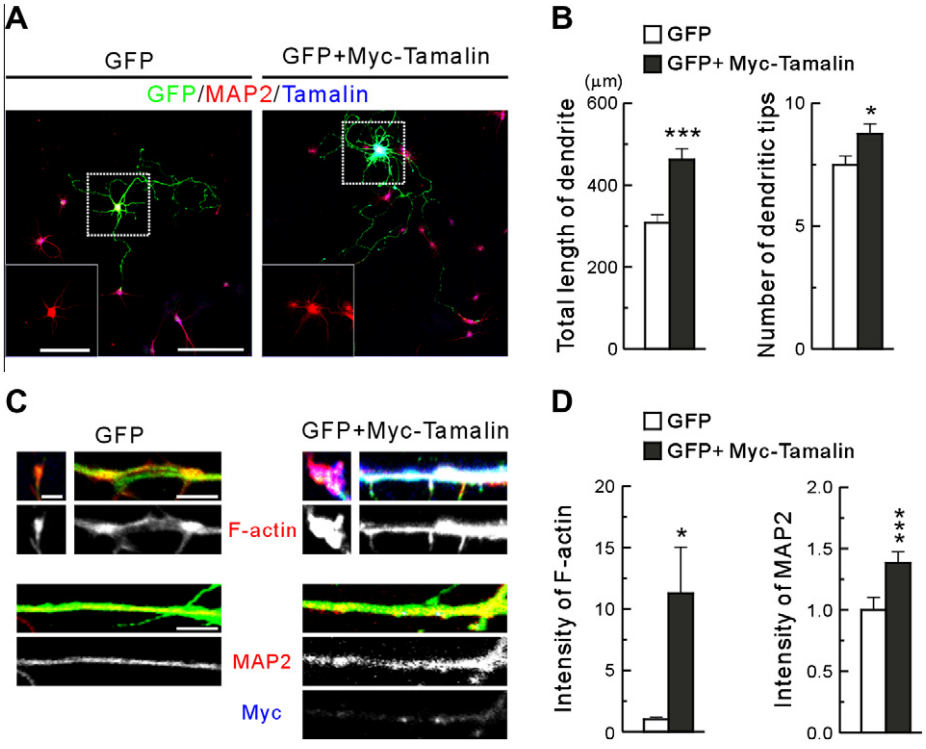

Fig. 3. Tamalin overexpression augments dendritic development and increases dendritic cytoskeleton. (A) Dendritic outgrowth and branching are increased by Tamalin overexpression. Cultured hippocampal neurons were singly or doubly transfected at DIV5 with GFP alone or GFP plus Myc-Tamalin, and labeled at DIV7 for anti-GFP, anti-Tamalin, and anti-MAP2. Scale bar 100 μm; inset, 50 μm. (B) Quantification of (A). Histogram represents mean ± SEM (GFP, $n = 43$ cells; GFP + Myc-Tamalin, $n = 25$ cells; $^{*}p < 0.05$, $^{***}p < 0.001$, Student's t-test). (C) Tamalin overexpression in cultured hippocampal neurons at DIV5–7 leads to significant enhancement of F-actin and MAP2 immunofluorescence signals in dendrites. (D) Quantification of (C). Histogram represents mean ± SEM based on ratio of arbitrary unit values, normalized to that in GFP alone (Left graph; GFP, $n = 10$ cells; GFP + Myc-Tamalin, $n = 11$ cells; $^{*}p < 0.05$, Student's t-test. Right graph; GFP, $n = 17$ cells; GFP + Myc-Tamalin, $n = 23$ cells; $^{***}p < 0.001$, Student's t-test). Scale bar, 2.5 μm in dendritic tips; 5 μm in dendritic shaft.

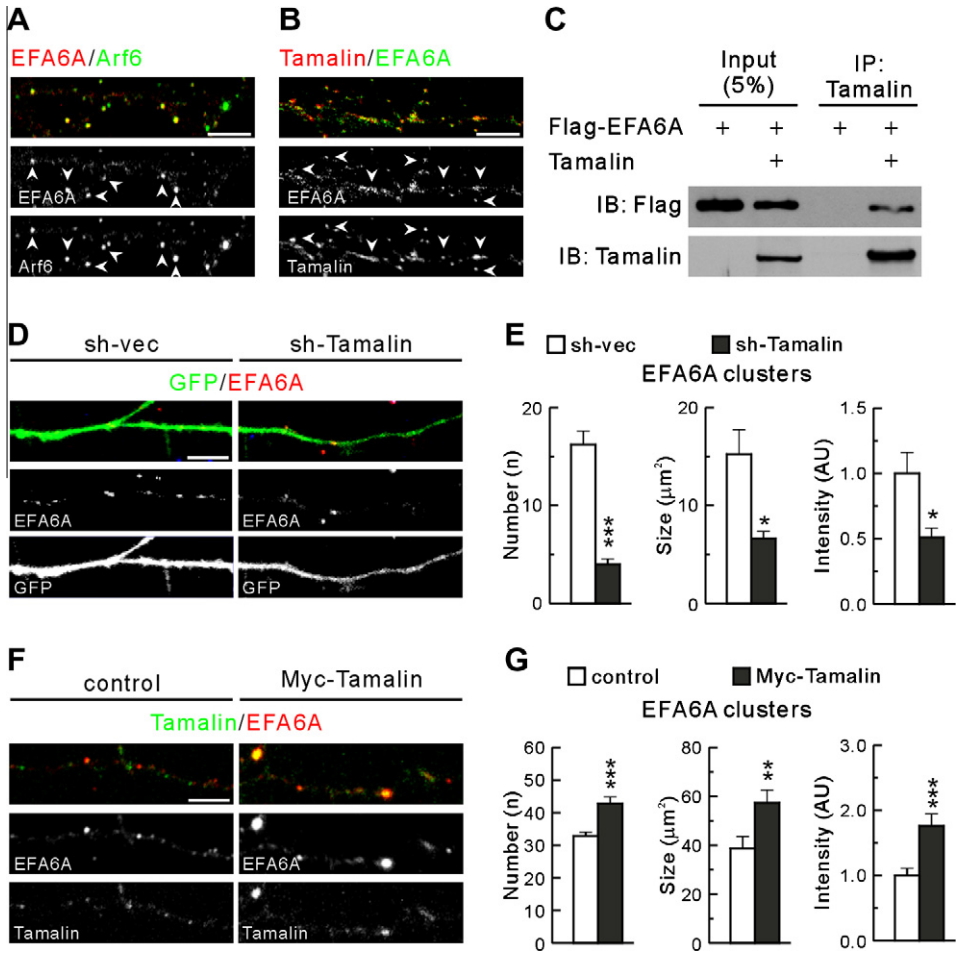

Fig. 4. Tamalin regulates the intensity of EFA6A clusters in dendrites via direct interaction. (A) EFA6A colocalizes with Arf6 protein in cultured hippocampal neurons (DIV7) labeled with anti-EFA6A and anti-Arf6. (B) EFA6A also colocalizes with Tamalin in hippocampal neurons (DIV7) labeled with anti-EFA6A and anti-Tamalin. Scale bar 5 μm. (C) Tamalin forms a complex with EFA6A in HEK293T cells. HEK293T cells doubly transfected with untagged-Tamalin and Flag-tagged EFA6A were immunoprecipitated with anti-Tamalin antibodies and immunoblotted with Flag and Tamalin antibodies, compared with HEK293T cells singly transfected with Flag-EFA6A. (D) Tamalin knockdown in cultured hippocampal neurons (DIV5–7) induces a marked decrease in the number, size, and intensity of EFA6A clusters. Scale bar, 5 μm. (E) Quantification of (D). Histogram represents mean ± SEM (sh-vec, $n = 44$ cells; sh-Tamalin, $n = 26$; $^{*}p < 0.05$; $^{***}p < 0.001$, Student's t-test). (F) Tamalin overexpression in cultured hippocampal neurons (DIV5–7) leads to an increase in the number, size, and intensity of EFA6A clusters. Scale bar 5 μm. (G) Quantification of (F). Histogram represents mean ± SEM (control, $n = 70$ cells; Myc-Tamalin, $n = 66$; $^{**}p < 0.01$; $^{***}p < 0.001$, Student's t-test).

formed a complex with EFA6A (Fig. 4C), suggesting that Tamalin and EFA6A may form a complex and this pathway affect neuronal development and morphology. These results led us to further explore the effects of Tamlain on subcellular localizations of EFA6A.

The reduction of dendritic outgrowth in Tamalin knockdown may be attributed to decrease in EFA6A clusters. As shown in Fig. 4D and E, the elimination of Tamalin significantly altered the clustering and distribution of EFA6A in cultured hippocampal neurons. Tamalin knockdown reduced the number, size, and intensity of EFA6A immunofluorescence signals in dendrites. To further characterize the effects of Tamalin on EFA6A, we overexpressed Tamalin in cultured neuron. As we expected, Tamalin overexpression increased the number, size and intensity of EFA6A cluster in dendrites (Fig. 4F and G). These results suggest that Tamalin has a role in dendritic development by regulation of EFA6A/Arf6 pathway in a manner requiring direct interaction with EFA6A.

4. Discussion

Our results presented herein indicate that Tamalin promotes dendritic outgrowth and arborization via regulation of EFA6A/Arf6 pathway. Endogenous Tamalin is highly expressed during postnatal rat brain development. Knockdown of Tamalin suppressed dendritic outgrowth and reduced dendritic branching tips. In contrast, its overexpression increased the outgrowth and branching of dendrites. A notable feature of Tamalin is that it colocalizes with EFA6A via direct interaction in dendrites of developing neurons, suggesting that Tamalin is associated with EFA6A-dependent signaling. This notion was further supported by the facts that Tamalin knockdown showed the reduced number, size, and intensity of immunofluorescence signals of EFA6A compared with those of control, and its overexpression induced reverse effect.

The cytoskeleton is an essential element for neuronal morphology, including neurite formation or outgrowth and synapse structure. We found in the present study that Tamalin affected the contents of F-actin and MAP2 in dendritic growth cones and dendritic shaft, respectively (Figs. 2E and F, 3C and D). Thus, the effects of Tamalin on neurite development appeared to be associated with regulation of neuronal cytoskeletons. In neurons, Rho family small GTPases play roles in structural changes of neuronal dendrites and axons [15], and their activities are up-regulated by Rho-guanine nucleotide exchange factors (Rho-GEFs). Arfs function as GDP/GTP-regulated switches in actin reorganization and membrane ruffling [14], and the Arf6 signaling pathway has a role played in

endosomal–plasma membrane recycling and cortical actin cytoskeleton remodeling [16]. In addition, a recent report suggested that the activity of EFA6A and Arf6 signaling enhances the recycling of endosomal compartment containing BACE1 (β-site amyloid precursor protein (APP) cleaving enzyme 1) and reduces amyloid-β (Aβ) production in neurons [17]. Dominant mutant of Arf6 (T27N) blocks recycling from the endosomal recycling compartment to the cell surface, which allows APP to be processed efficiently by BACE1 and enhances production of Aβ. Aβ is responsible for degradation of MAP2 and neuronal apoptosis [18]. Consistent with these notions, the reduction of EFA6A by Tamalin knockdown may enhance Aβ, and cause destabilization of MAP2, resulting in decreased microtubule stability.

Neurite outgrowth requires transport of proteins such as receptors for extracellular signaling molecules, delivery of cell adhesion molecules, and supply of membrane lipids [19–21]. In the regulation of membrane trafficking, the roles of several PDZ domain-containing scaffold proteins have been proposed [22]. These proteins are often involved in the assembly of multimolecular complexes that perform signaling functions at particular subcellular locations [23]. Glutamate receptors, including NMDA and AMPA receptors, regulate dendrite branching [19], and PSD-95 is involved in targeting these receptors to the dendritic surface and synapse [24]. An earlier study reported that Tamalin forms a complex with the guanine nucleotide exchange factor cytohesin-2, and its interaction is mediated by leucine zipper domain of Tamalin and coiled-coil region of cytohesin-2. Moreover, N-terminal PDZ domain of Tamalin binds directly to the class I PDZ-binding motif of mGluR1a and regulates the dendrite-targeted surface expression of mGluR1a via cytohesin-2-interaction-dependent manner [1]. Group I mGluR plays a critical role in dendritic outgrowth [25,26]. In the present study, we found that Tamalin colocalized with EFA6A, and overexpression of Tamain induced the increase of endogenous EFA6A clusters in cultured hippocampal neurons (Fig. 4). Interestingly, EFA6A is a member of the ARNO/cytohesin and also has two coiled-coil motifs at C-terminus [27]. It is, therefore, possible that the leucine zipper domain of Tamalin binds to the coiled-coil regions of EFA6A, similar to its interaction with cytohesin-2. Although we could not rule out the influence of cytohesin-2, the above described results indicate that Tamalin plays a key role in the association of EFA6A-downstream molecules and contributes to intracellular signaling affecting neuronal development.

In mature neuron, EFA6A/Arf6 signaling is involved in the regulation of spine development and maintenance [10], and most proteins interacting with Tamalin are synaptic molecules [1,3]. Avenues for future studies include delineating additional molecular details of the underlying mechanisms and *in vivo* studies with Tamalin-deficient mice in synapse plasticity.

Acknowledgments

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