**Title:** Activity-dependent synaptic localization of processing bodies and their role in dendritic structural plasticity.

Running title: P-bodies role in synaptic plasticity

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Abstract

In neurons, transport of a subset of mRNAs to subcellular regions and their translation has a role in synaptic plasticity. Recent studies have suggested a control mechanism of this local translation through mRNA compartmentalization or degradation. Here we report that processing bodies (P-bodies), which are involved in mRNA degradation or storage, are transported to dendrites by conventional kinesin (KIF5A) as a motor protein. Neuronal activation induced by depolarization increased the co-localization of P-bodies with PSD-95 in dendrites. This neuronal activity increased the release of Nd1 and Arp2 mRNA from the P-bodies, and consequently reversed the decrease of F-actin, induced by overexpression of Dcp1a, in the dendrites. Our data suggest that the activity-induced redistribution of P-bodies and mRNA release from P-bodies may have a role in synaptic structural plasticity by altering levels of mRNAs, which are involved in the dynamics of the actin cytoskeleton in dendrites.
Introduction

During the process of local protein synthesis in neurons, specific subsets of mRNAs are transported to local regions, such as dendrites and axons of neurons, and are translated. They have also been reported to play a role in activity-dependent synaptic changes and injury-induced axonal regeneration (Sutton and Schuman, 2006; Twiss and van Minnen, 2006; Bramham and Wells, 2007). The local translation might be regulated by both enhancement and suppression through mRNA degradation and compartmentalization to specific structures in response to external stimuli or synaptic activities (Zeitelhofer et al., 2008a). A specific group of RNA granules known as processing bodies (P-bodies), and also known as GW bodies and Dcp bodies, could mediate these regulations. P-bodies are an aggregate of mRNAs that are translationally repressed and RNA-associated proteins for specialized functions like degradation and compartmentalization (Anderson and Kedersha, 2006; Parker and Sheth, 2007). The major components of P-bodies thus-far identified from studies conducted on yeast and mammalian cells include a variety of proteins associated with mRNA decapping, 5′–3′ mRNA degradation, non-sense mediated mRNA decay (NMD), microRNA (miRNA)- and small interfering RNA (siRNA)-mediated mRNA degradation, and translational repression (Anderson and Kedersha, 2006; Parker and Sheth, 2007; Zeitelhofer et al., 2008a). Some evidence has suggested that mRNAs released from the P-bodies enter the translational machinery or re-enter the P-bodies, indicating a function as a storage site of silenced mRNAs (Brengues et al., 2005; Bhattacharyya et al., 2006; Schratt et al., 2006; Blumenthal and Ginzburg, 2008).
Recent studies have shown that P-bodies are found in neuronal dendrites and cell bodies (Barbee et al., 2006; Vessey et al., 2006; Cougot et al., 2008; Zeitelhofer et al., 2008b). Although Barbee et al. (2006) suggested that transport ribonucleoproteins (RNPs) in the neurons of Drosophila share some components with the P-bodies, only a few of the transport RNPs are found in P-bodies of mammalian neurons, suggesting an independent role as an individual RNP in neurons. Neuronal P-bodies in mammals also contain a decapping enzyme (Dcp1a), an activator of decapping (Dhh1p/RCK/p54), and miRNA-related factors (GW182 and Ago2) (Vessey et al., 2006; Cougot et al., 2008; Zeitelhofer et al., 2008b). Despite the great advancements concerning the functions and structures of neuronal P-bodies, much in the physiological context still remains to be explored.

mRNAs are transported to sub-cellular regions of neurons through specialized modules, transport RNPs, or RNA granules, which are also aggregates of translationally silenced mRNAs, RNA-binding proteins, and ribosomal subunits (Krichevsky and Kosik, 2001; Anderson and Kedersha, 2006; Kiebler and Bassell, 2006). The localization of transport RNPs is modulated by synaptic activities, induced by receptor activations, treatment of neurotrophins, or depolarization (Bramham and Wells, 2007). The conventional kinesin (KIF5, kinesin heavy chain) was isolated as the molecular motor of transport RNPs in neurons (Kanai et al., 2004; Hirokawa, 2006; Jeong et al., 2007). P-bodies are also localized near the synaptic regions and their localization is modulated by neuronal activations (Cougot et al., 2008), suggesting an active transport using molecular motors. However, details involved in transport mechanism and its modulation remain unclear.
In the present study, we isolated KIF5A as the molecular motor involved in dendritic transport of P-bodies. Functional inhibition of KIF5A using a dominant-negative form decreased the amount of P-bodies in dendrites. In addition, neuronal activity induced by depolarization increased the amount of P-bodies localized to synaptic regions, suggesting their possible roles in synaptic functions. Interestingly, overexpression of Dcp1a significantly decreased the level of F-actin in the dendrites. As the actin cytoskeleton in the dendritic spines is dynamically modulated by neuronal activity in developing and matured neurons (Hotulainen and Hoogenraad, 2010), we investigated how P-bodies could regulate synaptic structural changes induced by neuronal activity. Intriguingly, the neuronal activity released P-body-bound Nd1 and Arp2 mRNAs, and consequently reversed the decrease of F-actin, which are induced by expression of Dcp1a, in the dendrites.

Our results indicate that P-bodies could be transported to dendrites by active transport mechanism using the kinesin motor protein and localized to synaptic regions in response to neuronal activities, in turn providing a specific set of mRNAs, involved in dynamics of actin cytoskeleton, to local translational machinery in dendrites. Taken together, these results suggest a role of the P-bodies in synaptic plasticity.
Experimental methods

Hippocampal neuron cultures

Dissociated hippocampal or cortical neuron cultures were prepared from post-natal 1-day-old rat pups, as previously described (Jeong et al., 2007). The cultures were incubated for 12 days before use.

Plasmid constructs

Dcp1a cDNA was isolated from rat brain by RT-PCR (Dcp1a-RT/Dcp1a-RT-S→Dcp1a-RT-A), and cloned into TA vector system (Promega). PCR-amplified coding sequences (Dcp1a-EcoR-S→Dcp1a-Kpn-A) were inserted into the EcoRI/KpnI sites of pCMV-Myc (Clontech), coding sequences amplified by other primer set (Dcp1a-Bgl-S→Dcp1a-EcoR-A) into the BglII/EcoRI sites of pEGFP-C1 (Clontech) or the BamHI/EcoRI sites of pCMV-Tag2b (Stratagene), producing pCMV-Myc-Dcp1a, pEGFP-Dcp1a, or pCMV-Tag2b-Dcp1a. The cDNAs of Dcp1a isolated by the digestion of EcoRI/KpnI were inserted into the site of EcoRI/KpnI sites of pCMV-mRFP, producing pmRFP-Dcp1a. The Myc-tagged Dcp1a and GFP-tagged Dcp1a were amplified by PCR (GFP-Mlu-S→Dcp1a-Sph-A for GFP-Dcp1a, Myc-Mlu-S→Dcp1a-Sph-A for Myc-Dcp1a) and inserted into the MluI/SphI sites of pSinRep5 (Invitrogen) for production of Sindbis virus.

Full-length coding sequences of KIF5A, N-terminal regions (ΔC), C-terminal deleted regions (826 – 1028 amino acids), or C-terminal regions (ΔMD) in which the motor domains (1 – 330 amino acids) are deleted, were amplified by PCR (Kif5a-Bam-S→Kif5a-Apa-A, Kif5a-Bam-S→Kif5a-ΔC-Apa-A, Kif5a-ΔMD-Bam-S→Kif5a-Apa-A) and inserted into the
BamHI/ApaI sites of pCMV-tag2b, producing pCMV-tag2b-KIF5A, pCMV-tag2b-ΔC, or pCMV-tag2b-ΔMD, respectively. The pCMV-tag2b-KIF5A and pCMV-tag2b-ΔMD were digested with BamHI/ApaI and inserted into the BglII/ApaI sites of pEGFP-C1 (Clontech), producing pEGFP-KIF5A and pEGFP-ΔMD. The EGFP-KIF5A or EGFP-ΔMD (KIF5A) were amplified by PCR (GFP-Mlu-S→Kif5a-Apa-A for full length, GFP-Mlu-S→Kif5a-Apa-A for ΔMD) and inserted into the MluI/ApaI sites of pSinRep5, thereby producing pSinRep5-GFP-KIF5A WT (GFP-WT) or pSinRep5-GFP-KIF5A ΔMD (GFP-ΔMD), respectively. For pCMV-mRFP-KIF5A, full-length coding sequences of KIF5A were amplified by PCR (Kif5a-Kpn-S→Kif5a-Apa) and inserted into the KpnI/ApaI sites of pCMV-mRFP. The 3’-untranslated regions (UTR) of rat Nd1 mRNA were isolated by RT-PCR (Nd1-3’UTR-S→Nd1-3’UTR-A) and cloned by pGEM®-T easy TA Cloning system (Promega). The 3’ UTR of Nd1 mRNA were amplified by PCR (Nd1-3’UTR-Xba-S→Nd1-3’UTR-Xba-A) and then inserted into XbaI site of pGL3-promoter vector (Promega), producing a construct that makes mRNAs of firefly luciferase, tailed with 3’-UTR of Nd1 mRNA. All the restriction enzymes employed in the present work were purchased from New England Biolabs.

Transfection, viral infection, drug treatment and depolarization of cultured neurons

Sindbis viruses were constructed in accordance with Invitrogen protocols. For Sindbis viral expression, viruses were directly added to the medium and expression was allowed for 6 – 12 h, according to the experimental design. Transfection into human embryonic kidney (HEK) 293T cells was carried out by Ca²⁺-phosphate method, but
transfection to neurons was performed by following a modified method (Goetze et al., 2004). For drug treatment, green fluorescent protein (GFP)-Dcp1a was expressed by viral infection for 6 h. The cultured hippocampal neurons were treated with 30 μM N-methyl-D-aspartic acid (NMDA; Tocris Bioscience) in HEPES-buffered saline (HBS) for 10 min. For selective blocking of NMDA receptors, 100 μM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris Bioscience) was applied for 15 min before the NMDA treatment. For depolarization of colocalization experiments, the cultures were incubated with high-K+ HBS (60 mM KCl) for 10 min or pretreated with 2 mM EGTA in normal K+ HBS (5.4 mM) for 15 min and then incubated with high-K+ HBS (60 mM) for 10 min. After all the treatments, the cultures were immediately fixed (KCl 10’) or allowed to undergo additional incubation for 50 min (KCl 10’+50’) to examine distribution. For depolarization to induce mRNA release from P-bodies, the cultures were allowed to undergo additional incubation for 20 min, making total time 30 min. For depolarization to examine the level change of F-actin, the cultures were incubated with high-K+ HBS (60 mM KCl) for 10 min and allowed to additional incubation for 50 min.

**Co-immunoprecipitation and Western blotting**

For co-immunoprecipitation (co-IP) analysis, HEK 293T cells were transfected and incubated for 48 h and the cultured rat cortical neurons were infected with Sindbis virus encoding Myc-Dcp1a and incubated for 12 h. Cell lysate was prepared by adding the lysis buffer (150 mM NaCl, 1% IGEPAL® CA-630, 50 mM Tris·Cl [pH 8.0]) and immunoprecipitated with 2 – 3 μg of individual antibody (as indicated in figures), and incubated with 50 μl of protein G-Sepharose (GE Healthcare). The immunoprecipitates were washed thrice with 1 ml of ice-cold lysis buffer and once with 1 ml of 50 mM Tris·Cl
(pH 8.0), and were employed for 8% SDS-PAGE analysis. To examine RNA dependency for the interaction, the cell lysates were incubated with 100 μg/ml RNase A (TypeXII-A; Sigma-Aldrich) in lysis buffer for 30 min at 4°C or with 1 mg/ml RNase A for 30 min at RT. Total RNA was purified by Trizol® reagent (Invitrogen) from one–tenth of the lysate and analyzed by 10% agarose gel electrophoresis. For Western blotting analysis, the blots were incubated with monoclonal anti-Myc antibody (1: 2,000, Clone 9E10; Sigma-Aldrich), monoclonal anti-FLAG antibody (1:2,000, Clone M2; Sigma-Aldrich), or monoclonal anti-Kinesin antibody (1:1,000, Clone H2; Chemicon International). As a control, the blot was washed with stripping buffer (Thermo Scientific) and re-probed.

Immunocytochemistry and Phalloidin staining

Immunocytochemistry was conducted as described in a previous study (Jeong et al., 2007). Monoclonal anti-Kinesin antibody (Chemicon International), monoclonal anti-PSD-95 antibody (Clone: 6G6-1C9; Affinity Bioreagents), or polyclonal anti-Dcp1a antibody (Sigma-Aldrich) were used for staining, each at a dilution of 1:200. A cyanine 3 (Cy3)-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was utilized as the secondary antibody for PSD-95 and KIF5A staining, an Alexa® Fluor 488-conjugated anti-rabbit IgG antibody or Alexa® Fluor 647 goat anti-rabbit IgG antibody (Molecular Probes), a cyanine 3 (Cy3)-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories) for Dcp1a staining. For Phalloidin staining, fixed neurons were treated with 0.5 μM tetramethylrhodamine B isothiocyanate-conjugated Phalloidin (TRITC-Phalloidin, Sigma-Aldrich) for 30 min in phosphate buffered saline (PBS).

In situ hybridization
Probe preparation and *in situ* hybridization were conducted as in a previous study (Jeong et al., 2007). Overnight hybridization was conducted using a hybridization solution containing 1 – 2 μg/ml of digoxigenin (DIG)-labeled antisense or sense firefly luciferase probes at 65°C. The cultures were subjected to a series of stringent washing steps, and then incubated overnight with polyclonal anti-DIG antibody (Roche) and monoclonal anti-Myc antibody (Sigma-Aldrich) at 4°C. *In situ* signals were visualized by staining with fluorescein-conjugated anti-DIG antibody (Roche) and immunostained signals with Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories).

**Image analysis**

Immunostained and/or GFP images were acquired by confocal microscopy using a Zeiss 710 microscope (Carl Zeiss). In order to measure the number of Dcp1a-foci, a major dendrite was selected from a typical hippocampal pyramidal neuron and used in Particle analysis. In order to avoid diffusion of cell body signals in the quantitative analysis of particle number or immunostaining results, dendritic regions (41 – 80 μm) were included in the collection. For co-localization analysis and measurement of F-actin level, typical hippocampal pyramidal neurons with similar morphologies were selected, and a single secondary or tertiary dendrite of similar diameter was scored for each analysis. In order to measure the maximal distance that the Dcp1a-foci could be transported from the cell body, the images were inverted and the background values were subtracted from the acquired images. All the images were processed using NIH image analysis software (ImageJ ver: 1.42q). All the image acquisitions and analyses were conducted through blind experiments. A Student’s *t*-test, paired or unpaired, was used to determine the statistical differences between the two groups. One-way analysis of variance (ANOVA) was used for further
comparison between the groups. *Post hoc* comparisons were conducted by using the Newman-Keuls multiple comparison tests. All the data are represented as mean ± SEM.

*Immunoprecipitation of Dcp1a-bodies and quantitative RT-PCR*

Isolation of Dcp1a-bodies was performed by previously described (Oh et al., 2010). Cultured neurons were infected with Sindbis virus encoding Myc-Dcp1a and supplemented with 1 ml ice-cold lysis buffer (10 mM Hepes [pH 7.4], 200 mM NaCl, 30 mM EDTA, and 0.5% TritonX-100) with 1X complete protease inhibitors (Roche) and 400 U/ml rRNAsin (Promega). The supernatants were pre-cleared and subsequently utilized in immunoprecipitations with 60 μl of protein A sepharose (GE Healthcare) and monoclonal anti-Myc antibody (Clone E910, Sigma-Aldrich) or mouse IgG (Sigma-Aldrich). After IP, each supernatant was saved for RT-PCR. In order to monitor efficiency of IP procedure, 25% of the resulting immunoprecipitates were employed in Western blotting analysis using Myc-antibody, and the other 75% and 10% of the supernatant were used in the RNA preparation using phenol/chloroform extraction method. One–tenth of the purified RNAs were used in RT-PCR using SuperScript™ III (Invitrogen) and Advantage™ II PCR enzyme system (Clontech). After 1.5% agarose gel electrophoresis, band intensities were quantified by the ImageJ imaging analysis program. In order to monitor the specificity of RT-PCR, PCR products were isolated by pGEM®-T easy TA Cloning system (Promega) and sequenced.
Results

*Dcp1a is associated with KIF5 (a conventional kinesin)*

Although recent studies have suggested that P-bodies could be transported to the dendritic regions through a microtubule-based active transport (Cougot et al., 2008; Zeitelhofer et al., 2008b), molecular details including the identification of the motor protein are still undiscovered. As a decapping enzyme, Dcp1a has been isolated from P-bodies of almost all the cell types including neurons and has been widely used as a marker protein of P-bodies (Vessey et al., 2006; Cougot et al., 2008; Swetloff et al., 2009). Therefore we also took advantage of Dcp1a as a marker protein of P-bodies and named P-bodies as Dcp1a-bodies or foci as P-bodies. However, it was necessary to examine whether Dcp1a-bodies produced by overexpression of Myc-Dcp1a or GFP-Dcp1a have identical characteristics of endogenous P-bodies. To this end, Myc-tagged or GFP-tagged Dcp1a bodies were stained with anti-Argonaute 2 (Ago2) antibody, another marker protein of P-bodies. Dcp1a-foci produced by Sindbis viral expression were also stained with Ago2 antibody, thereby suggesting that Dcp1a-bodies could represent endogenous P-bodies (Supplementary Fig. 1).

Transport RNPs use the conventional kinesin (KIF5) as a motor protein (Kanai et al., 2004; Hirokawa, 2006; Jeong et al., 2007). In addition, a part of Dcp1a-bodies was colocalized with Staufen1-RNPs or Staufen2-RNPs in our work (Supplementary Fig. 2). In order to isolate a motor protein involved in dendritic transport of P-bodies, we examined the interaction between Dcp1a and KIF5A, which is a neuron-specific form of KIF5 (Hirokawa and Takemura, 2005), by co-immunoprecipitation assays. The Dcp1a was detected from immunoprecipitates containing KIF5 and, vice versa, KIF5 was found in Dcp1a-precipitates, suggesting their association (Fig. 1A). Subsequently, we defined the interaction regions of
KIF5 with Dcp1a. Dcp1a interacted with KIF5 through the C-terminal region (Fig. 1B), which was consistent with the results of interaction between transport RNP and KIF5 (Kanai et al., 2004). In the following experiment, we examined the interaction between Dcp1a and KIF5 in vivo. Cultured neurons were infected with Sindbis virus encoding Myc-tagged Dcp1a and then neuronal lysates were used in immunoprecipitation assay employing anti-KIF5 antibody. Dcp1a was detected from KIF5 precipitates by Western blot analysis (Fig. 1C). Unfortunately, when the same experiments were performed without any exogenous expression, endogenous Dcp1a was not detected from KIF5 precipitates. We assumed that this might be due to limitation of the sensitivity used in the analysis. As P-bodies contain RNA, we examined the RNA dependency of biochemical interaction between Dcp1a and KIF5. Before the immunoprecipitation, cell lysates were treated with RNase A and then used in the immunoprecipitation assay. The treatments of RNase A with mild (100 μg/ml RNase A for 30 min at 4°C) or higher concentration (1 mg/ml RNase A for 30 min at RT, data not shown) did not block the association, suggesting that this is mediated by protein-protein interaction (Fig. 1D).

In order to further determine whether P-bodies are associated with kinesin in neurons, immunostaining was carried out. Initially, endogenous Dcp1a and KIF5 were immunolabeled with antibodies against each protein and visualized with a secondary antibody, respectively. Next, GFP-tagged Dcp1a was expressed by Sindbis viral infection and then immunostained with anti-KIF5 antibody. As shown in Fig. 2, expression pattern of endogenous Dcp1a as well as overexpressed GFP-Dcp1a showed many foci, particles or granules, both in cell body and dendrites of cultured hippocampal neurons. Overexpression of GFP-Dcp1a by Sindbis viral vector or plasmid transfection made macroscopic P-bodies
which were located on the kinesin motor (Fig. 2). Thus, colocalization between two proteins was examined by the immunostaining of endogenous proteins. The rate at which endogenous Dcp1a-foci colocalized with KIF5 was 61.50 ± 2.89% (N = 18 neurons) and 41.5 ± 2.89% (N = 18 neurons) of KIF5 immunoreactivity was colocalized with endogenous Dcp1a-foci (Fig. 2B). Additionally, the co-localization was examined in the neurons transfected with GFP-Dcp1a and mRFP-KIF5. Interestingly, some particles of each protein were also found to be colocalized in the dendrites (Fig. 2, indicated by arrowhead). In order to further confirm the movement of Dcp1a-bodies by KIF5, a time-lapse imaging using neurons expressing GFP-Dcp1a and mRFP-KIF5 was performed. As expected, a Dap1a-particle moved with an mRFP-particle to distal dendritic regions (Movie S1). In terms of consistency with a previous study (Zietelhofer et al., 2008b), 20 μM nocodazole treatment inhibited the dendritic transport of Dcp1a-foci, whereas 2 μM cytochalasin B (Supplementary Fig. 3) was ineffective, again suggesting their microtubule-based movement.

P-bodies are transported by kinesin motor

As KIF5 has a motor domain in N-terminal regions and function as a dimer (Hirokawa and Takemura, 2005), a mutant (ΔMD) in which motor domains in the N-terminal regions are removed works as a dominant-negative form (Kanai et al., 2004). Thus, we introduced the KIF5 mutant (ΔMD) into cultured neurons and examined Dcp1a-foci in the dendrites by immunostaining. As expected, the expression of WT was significantly increased the number of Dcp1a-foci in distal dendritic regions (41 – 80 μm), but ΔMD reduced it (Fig. 3B; GFP: 36.33 ± 4.50, N = 12; WT: 54.42 ± 5.86, N =12; ΔMD: 28.83 ± 3.23, N =12; ANOVA analysis: ** p < 0.01, *** p < 0.001). In contrast with the results of particle number, the average size of Dcp1a-foci was not significantly increased by the expression of WT and
ΔMD in distal dendritic regions (Fig. 3C; GFP: 180.7 ± 11.77 nm², N = 12; WT: 238.9 ± 25.87 nm², N = 12; ΔMD: 184.2 ± 13.26 nm², N = 12; ANOVA analysis: ns: not significant when compared with GFP controls). Altogether, these results suggest that P-bodies are transported to the dendrites by motor protein, KIF5.

**P-bodies are localized to synaptic regions**

Next, we examined the specific locale of P-bodies in dendrites by comparing with that of PSD-95. Around 42% (±1.98%) of GFP-tagged Dcp1a-foci were colocalized with PSD-95 (Supplementary Fig. 4A,B) and endogenous Dcp1a-foci also showed a comparable co-localization with PSD-95. These results were consistent with previous data indicating synaptic localization of P-bodies (Cougot et al., 2008). Although Dcp1a expression showed a slight reduction of the immunoreactivity of PSD-95 by approximately ~24% in dendrites, it didn’t show a significant difference (Supplementary Fig. 4C).

In order to determine the role of kinesin motor in the synaptic localization of Dcp1a-bodies, we introduced WT or ΔMD into cultured hippocampal neurons and examined the co-localization of Dcp1a-foci with PSD-95. Interestingly, expression of WT itself did not significantly change in the co-localization, but ΔMD reduced level of co-localization (Fig. 4A, B; GFP: 46.03% ± 2.36%, N = 13 dendrites; GFP-WT: 52.19% ± 2.17%, N = 13 dendrites; GFP-ΔMD: 33.16% ± 2.41%, N = 12 dendrites; ANOVA analysis: *** p < 0.001, ns: not significant when compared with GFP controls). It might be due to reduction of PSD-95 caused by the expression of ΔMD (Fig. 4C; GFP: 19.2 ± 1.81, N = 13 dendrites; GFP-WT: 21.18 ± 2.52, N = 13 dendrites; GFP-ΔMD: 4.42 ± 0.57, N = 12 dendrites; AU: arbitrary unit;
ANOVA analysis: *** $p < 0.001$ when compared with GFP controls or GFP-WT). Taken together, these data suggest that kinesin motor protein is necessary for the dendritic localization of P-bodies, but not sufficient for the synaptic localization of P-bodies, indicating a possibility that other factors such as neuronal activity could be required for synaptic localization.

**Neuronal activity regulates synaptic localization of P-bodies**

Although P-bodies are presented in cell bodies and dendrites and near synaptic regions (Cougot et al., 2008; Zeitelhofer et al., 2008b), there are no reports on changes in the localization of P-bodies in synaptic regions in response to neuronal activity. Transport RNPs that are transported by motor proteins to dendrites are modified by neuronal activities (Kiebler and Bassell, 2006). In addition, dendritic transport of P-bodies can also be regulated by various neuronal activations (Cougot et al., 2008). Accordingly, we determined the dendritic distribution, induced by neuronal activities. We induced chemical depression by brief treatment (10 min) with 30 μM NMDA (Lee et al., 1998) or activation by treatment with 60 mM KCl (10 min) to cultured hippocampal neurons. As neuronal activations immediately disassembled P-bodies and number of P-bodies was recovered after 30 min (Zeitelhofer et al., 2008b), drug-treated neurons were additionally incubated for 50 min to permit complete recovery, producing 1 h of experiment time. The brief treatment with NMDA and depolarization did not alter the total amount of Dcp1a-foci in dendrites (Fig. 5C). Interestingly, neuronal activity by depolarization increased the maximal distance at which the Dcp1a-foci can be detected in the cell bodies from $118.8 \pm 4.30$ μm ($N = 52$ dendrites) to $146.7 \pm 5.95$ μm ($N = 42$ dendrites), and NMDA treatment was increased to $160.7 \pm 6.06$ μm ($N = 43$ dendrites) (Fig. 5B). However, these increases were specifically
blocked by D-AP5 treatment (123.5 ± 4.85 μm, N = 58 dendrites) and EGTA treatment (114.3 ± 5.48 μm, N = 41 dendrites) (Fig. 5B), respectively. The results suggested that the observed increases might be mediated by NMDA receptor- or Ca²⁺-dependent mechanism. However, as expected, the average size of Dcp1a-foci was not significantly different from other groups (data not shown). These data suggest that neuronal activity could regulate redistribution of P-bodies in the dendrites.

Thus, in following experiments, we examined whether synaptic localization of P-bodies could be affected by neuronal activity. The neurons infected with GFP-Dcp1a virus were stimulated with high-K⁺ HBS for 10, immediately fixed, and used in co-localization analysis. The co-localization of Dcp1a-foci with PSD-95 was significantly increased by depolarization and blocked by EGTA treatment (Fig. 6B; KCl 10'; CTL: 100% ± 5.95%, N =14 dendrites; KCl: 129.9% ± 8.24%, N = 14 dendrites; KCl + EGTA: 104.5% ± 8.43%, N =13 dendrites; Student’s t-test: ** p < 0.01, * p < 0.05, ns: not significant when compared with controls), suggesting activity-dependent re-localization of P-bodies into synaptic regions.

Nd1 and Arp2 mRNA are released from P-bodies by neuronal activity

We tried to clarify the role of activity-dependent synaptic localization of P-bodies in synaptic plasticity. P-bodies are the sites where mRNA can be stored in repressed state or degraded (Anderson and Kedersha, 2006; Parker and Sheth, 2007). We examined the dendritic spine morphology in cultured hippocampal neurons by overexpression of Dcp1a. Interestingly, Dcp1a expression did not change PSD-95 level (Supplementary Fig. 3C), but significantly reduced level of F-actin by 37% in the dendrites (Supplementary Fig. 5),
suggesting that the P-bodies could be involved in regulation of actin cytoskeleton. As actin cytoskeleton is a major site linking synaptic activity to structural plasticity (Hotulainen and Hoogenraad, 2010), specific mRNAs which are involved in regulation of F-actin were examined in the P-bodies by IP/RT-PCR analysis. Cultured cortical neurons were infected with a Sindbis virus encoding Myc-tagged Dcp1a. The neuronal lysates were immunoprecipitated with anti-Myc antibody, and subsequently RNAs were isolated from the precipitates. The RNAs used for RT-PCR analysis were Arp2/3, ADF/cofilin, Profilin I/II, β-actin, Nd1, FMRP and GluR1. Interestingly, in addition to fragile X mental retardation protein (FMRP) mRNA, Nd1 mRNA, stabilizer of actin filament, and actin-related protein 2 (Arp2) mRNA, nucleator of actin filament was isolated from the immunoprecipitates (Supplementary Fig. 6A,B), suggesting the possibility that they could be candidate proteins, local level of which is regulated by the P-bodies.

There has been a report of the dendritic localization of Nd1 mRNA by translocation in liposarcoma (TLS)-containing RNP and increase of transcripts by activation of mGluR in hippocampal neurons (Fujii and Takumi, 2005). Thus, we examined localization of Nd1 mRNA in the Dcp1a-bodies using in situ hybridization assay. In order to achieve in situ detection of Nd1 mRNA, 3’ untranslated regions (3’ UTR) of Nd1 mRNA was isolated and inserted in the C-terminal region of firefly luciferase (fLuci-3’UTR). As expected, in situ signals of Nd1 mRNA showed a particular pattern and Nd1 mRNAs were strongly co-localized with Dcp1a-bodies in both the cell bodies and the dendrites (Supplementary Fig. 6C). In contrast, those of firefly luciferase mRNA (fLuci), not containing 3’ UTR of Nd1
mRNA, showed a diffused pattern. These data also suggest that the 3’ UTR of Nd1 mRNA might be involved in its dendritic localization.

As the role of P-bodies as a storage site of mRNAs and mRNA release from P-bodies to polyribosomes have been reported (Brengues et al., 2005; Bhattacharyya et al., 2006; Schratt et al., 2006), we examined the level of Nd1 and Arp2 mRNAs in the P-bodies after response to neuronal activity. To do this, we investigated the relative level of each mRNA in the P-bodies by comparing with the ratio of amount of each mRNA isolated from the supernatant and the pellets (Dcp1a-immunoprecipitates) before and after depolarization. Although reduction of Arp2 mRNAs was not significant, depolarization significantly reduced the level of Nd1 mRNAs in the P-bodies by approximately ~20%, thereby indicating an increase of polyribosomal mRNA pool involved in dynamics of actin cytoskeleton (Fig. 7). In following experiment, we tried to examine protein level of Nd1 and Arp2 by depolarization, but did not detect any change. It might be originated from limitation of the sensitivity used in our analysis (data not shown).

Neuronal activity normalizes the level of F-actin reduced by Dcp1 overexpression in dendrites

Our results indicate that overproduction of P-bodies could decrease the level of F-actin in dendrites and P-bodies itself could function as storage sites of mRNAs involved in the dynamics of actin cytoskeleton. In addition, neuronal activity induced the release of the mRNAs from the P-bodies. Accordingly, we investigated whether neuronal activity also rescues the level of F-actin reduced by Dcp1a overexpression. Although there was an increase of F-actin level by depolarization-induced neuronal activity, it was not significant
(Fig. 8; GFP: 100.0 ± 11.07%, N = 18 neurons, 1,595 μm dendrites; GFP + KCl: 107.1 ± 9.27%, N = 18 neurons, 1,576 μm dendrites), but not significantly. However, the level of F-actin reduced by Dcp1a overexpression was normalized by depolarization (Fig. 8; Dcp1a: 73.70 ± 5.93%, N = 18 neurons, 1,588 μm dendrites; Dcp1a + KCl: 96.65 ± 5.65%, N = 18 neurons, 1,674 μm dendrites; Student t-tests: * p < 0.05, ** p < 0.01 when compared with GFP controls), again supporting our hypothesis that P-bodies function as storage sites of releasable mRNAs.
Discussion

*P-bodies are transported by kinesin motor protein to dendrites like transport RNPs*

P-bodies are aggregates of mRNAs and proteins, involved in 5′– 3′ mRNA degradation, nonsense-mediated mRNA decay, miRNA-, siRNA- mediated mRNA degradation, and translational repression (Anderson and Kedersha, 2006; Parker and Sheth, 2007). Thus, a specific subset of mRNAs in the P-bodies might be degraded or stored for subsequent expression. Despite many reports about the components of P-bodies isolated from yeast and HeLa cells, little is known about the core components of P-bodies in neurons. In particular, whether P-bodies contain RNA-binding proteins such as Staufen, fragile X mental retardation protein (FMRP), zip code binding protein 1 (ZBP1), or Brentsz, as like transport RNPs, is still unclear. In Drosophila motor neurons, Staufen and FMRP are present in P-bodies (Barbee et al., 2006), suggesting that the structure of two granules might be interrelated. However, Staufen1 and 2, and Brentsz were not localized to P-bodies in mammalian neurons (Zeitelhofer et al., 2008b). Interestingly, an alternative study showed that ZBP1 and FMRP were localized in the P-bodies and even rRNA in some fractions of the P-bodies in mammalian neurons (Cougot et al., 2008). Considering that there are various types of transport RNPs (Kiebler and Bassell, 2006), these are not surprising. Our data also suggest that some parts of P-bodies might contain Staufen1 and/or 2, and thus share structural properties with some species of transport RNPs (Supplementary Fig. 2). Previous reports have demonstrated that movement of P-bodies in dendrites depend on the active transport, mediated by a motor protein, although the authors did not attempt to isolate a specific isoform of KIF (Cougot et al., 2008; Zeitelhofer et al., 2008b). In the present study, we have isolated KIF5 as a motor protein for the transport of P-bodies in hippocampal neurons.
Consequently, the movement of P-bodies in dendrites showed a transport pattern similar to that of transport RNPs (Rook et al., 2000; Tiruchinapalli et al., 2003). As shown in Fig. 1D, the association of P-bodies with kinesin motor proteins was independent of RNAs. Although these observations suggest that RNAs do not work as a linker or is not required for interaction between P-bodies and kinesin motor, it could not be absolutely excluded a possibility that RNAs which are resistant to RNase A treatment might affect the efficiency of interaction. The previous study that knockdown of KIF5 or kinesin light chain mediated by siRNA delayed disassembly of stress granules and P-bodies suggests that the balance between anterograde and retrograde transport could regulate formation and disassembly of both stress granules and P-bodies (Loschi et al., 2009). Treatment with microtubule-destabilizing drug inhibits the formation of stress granules induced by stress, such as sodium arsenite treatment (Ivanov et al., 2003). Although we did not determine those aspects in the present study, it was proven that motor proteins might also be involved in the formation of P-bodies and stress granules, as well as in the transport mechanism. Interestingly, even not significant, the expression of a dominant-negative form of KIF5A (ΔMD) reduced the level of Dcp1a-foci to by ~ 20% of the level of control groups (Fig. 3), suggesting a possibility that another motor protein might be involved. Nevertheless, our results suggested that P-bodies are transported to the dendrites by motor protein, KIF5.

**Neuronal activity could modulate both transport or localization and mRNA storage in P-bodies**

What is the role of active transport and activity-induced localization of P-bodies to synaptic regions in the dendrites? Activity-induced localization of transport RNPs and subsequent translation of mRNAs from transport RNPs might contribute towards input-
specific synaptic modification (Sutton and Schuman, 2006). Synaptic localization of P-bodies after external stimuli could also contribute towards regulation of local pool of mRNAs. It has been reported that polysomes or translational machineries compete with mRNA decapping machineries or P-bodies (Franks and Lykke-Andersen, 2008). Indeed, the movement of mRNAs from P-bodies to polysomes in response to external stimuli has been reported in yeast (Brengues et al., 2005). It is also recapitulated in human cell line (Bhattacharyya et al., 2006) and cultured neurons (Schratt et al., 2006). Specific miRNAs that regulate target mRNAs at synapses or in development play key roles (Kosik, 2006; Corbin et al., 2009). Major proteins such as Argonaute proteins in miRNP or RISC are enriched in P-bodies (Pillai et al., 2004; Liu et al., 2005). We also could find the conserved target sites for the miRNAs in the 3’ UTR of Nd1 mRNA (data not shown). A study for the miRNA-mediated regulation of local pool of Nd1 mRNA will be an interesting future study. Alternatively, since neuronal P-bodies possess properties of both decapping bodies, for mRNA degradation or storage, and transport RNPs, for mRNA transport, it is plausible that they could supply some mRNAs to local pool for translation from P-bodies itself. Our data also support the view that neuronal activity could regulate both the localization and functional role of P-bodies.

Roles of P-bodies in synaptic structural plasticity (dynamics of actin cytoskeleton)

Actin cytoskeleton is a major regulatory site for structural and functional plasticity of dendritic spines. Many post-synaptic signaling could modulate dendritic spines by changing the activity or level of actin binding proteins (Hotulainen and Hoogenraad, 2010). In the present study, we found mRNAs of Nd1 (a Kelch family protein) and Arp2 mRNA (actin-related protein2) are released from P-bodies in response to neuronal activity (Fig. 7). A
previous study has reported that Nd1-L, an isoform of Nd1, functions as a stabilizer of actin filament and may have a role in actin dynamics (Sasagawa et al., 2002). In addition, Nd1 mRNAs are bound to translocated in liposarcoma (TLS) RNPs, located in hippocampal somatodendritic regions (Fujii and Takumi, 2005). TLS-RNPs are localized to dendritic spines in response to mGluR5 activation and TLS-null mutation induces abnormal spine morphology, thereby consolidating a role of Nd1 in dynamics of actin cytoskeleton (Fujii et al., 2005). Arp2 is a component of Arp2/3 complexes which are enriched in dendritic spines and add a new actin to existing actin filaments, as a nucleator of actin filament. It is the mechanisms required for head formation of dendritic spines (Hotulainen and Hoogenraad, 2010). Although we did not obtain direct evidence that Nd1 and Arp2 mRNAs released from Dcp1a-bodies enter polysomes for translation, neuronal activity-induced release of Nd1 mRNA or/and Arp2 mRNA blocked the reduction of F-actin level by Dcp1a overexpression in dendrites (Fig. 8). These observations suggest that mRNAs involved in dynamics of actin cytoskeleton could be targets of dendritic local protein synthesis through the P-bodies.

In this study, we isolated conventional kinesin (KIF5) as a motor protein for dendritic transport of P-bodies in neurons. The synaptic localization of P-bodies was modulated by neuronal activity. The activity also increased the release of mRNAs involved in dynamics of actin cytoskeleton from the P-bodies and rescued level of F-actin reduced by Dcp1a-bodies overexpression. Taken together, these findings suggest that P-bodies might be involved in synaptic structural plasticity through the activity-induced regulation of mRNA pool in synaptic regions.
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References


Fig. 1. Dcp1a interacts with KIF5A through the C-terminus of KIF5A. (A) Co-immunoprecipitation (IP) of Dcp1a with KIF5A from HEK293T cells transfected with Myc-tagged Dcp1a and FLAG-tagged KIF5A. In the upper panel, IP was carried out using anti-Myc antibody and Western blotting using anti-FLAG antibody, and vice versa in lower panel. The IgG was used as a negative control. (B) Co-immunoprecipiation of Dcp1a with KIF5A mutants (ΔC or ΔMD). HEK 293T cells were transfected with Myc-Dcp1a and KIF5 (full-length, WT), ΔC (the N-terminal regions of KIF5A) or ΔMD (the C-terminal regions in which motor domain was deleted) and used in IP using anti-FLAG antibody. Lower panel shows the expression level of individual proteins. (C) Co-immunoprecipitation of Dcp1a with KIF5A from cultured cortical neurons infected with Sindbis virus encoding Myc-Dcp1a. IP was carried out using anti-KIF5 antibody and Western blotting using anti-Myc antibody. (D) RNA dependency in the association with KIF5. After transfection with Myc-tagged Dcp1a and FLAG-tagged KIF5A, the lysates were treated with 100 μg/ml RNase A for 30 min at 4°C and used in IP and Western blotting analysis. The part of RNAs purified from the lysates was analyzed on an agarose gel electrophoresis (bottom panel) to show the effect of RNase treatment (+, -).
Fig. 2. P-bodies are colocalized with the kinesin motor. (A) The representative images of co-localization. Dcp1a (green) and KIF5A (red) colocalize in neuronal dendrites. Cultured hippocampal neurons were stained with anti-Dcp1a antibody and KIF5 antibody to measure the co-localization of endogenous proteins. And cultured neurons infected with Sindbis virus encoding GFP-tagged Dcp1a (GFP-Dcp1a) were allowed 12 h of incubation for expression and stained with anti-KIF5 antibody. Finally, cultured neurons were transfected with pCMV-GFP-Dcp1a and -mRFP-KIF5A vectors. All the images were acquired by confocal microscopy. The dendrites located within the dot-lined boxes are enlarged for more vivid representation. The yellow-colored dots indicated by the arrow or arrowhead show the colocalized points between Dcp1a and KIF5. Scale bar: 20 μm. (B) The bar graphic representation of the co-localization between endogenous Dcp1a and KIF5A.
**Fig. 3. Dominant-negative form of kinesin (ΔMD) decreases the level of P-bodies in dendrites.**  (A) The representative images used in analysis.  Cultured hippocampal neurons were infected with Sindbis virus encoding GFP, GFP-full-length KIF5A (WT), or GFP-dominant negative form of KIF5A (ΔMD), then incubated for 12 h, and stained with anti-Dcp1a antibody.  Proximal dendritic regions of 1 – 40 μm were excluded to avoid diffusion of cell body signals and regions of 41 – 80 μm were included in the analysis.  Arrowheads indicate the dendrites used in analysis.  Scale bar: 20 μm.  (B) The bar graphic representation of the number of Dcp1a-foci in dendrites.  (C) The bar graphic representation of the size of Dcp1a-foci in dendrites.  Data are presented as mean ± SEM (ANOVA analysis: *** p < 0.001, ** p < 0.01, ns: not significant when compared with GFP controls).
Fig. 4. Expression of full-length kinesin (WT) does not change the synaptic localization of P-bodies. Cultured hippocampal neurons were infected with Sindbis viruses encoding GFP, GFP-tagged full-length KIF5A (WT), or GFP-tagged mutant KIF5A (ΔMD), then incubated for 12 h and fixed. The neurons were stained with anti-Dcp1a antibody and monoclonal anti-PSD-95 antibody, and subsequently stained with Cy3-conjugated goat anti-mouse IgG antibody for PSD-95 and Alexa Fluor 647 goat anti-rabbit IgG antibody for Dcp1a. Immunostained images were visualized by confocal microscopy. For co-localization analysis, typical hippocampal pyramidal neurons with similar morphologies were selected, and a single secondary or tertiary dendrite of similar diameter was scored for each analysis. (A) The representative images of immunostaining results. Scale bar: 20 μm. (B) Change of co-localization by KIF5A or its mutant expression. The colocalization of Dcp1a with PSD-95 is significantly decreased by a dominant-negative form of kinesin (GFP: 46.03 ± 2.36%, N = 13 dendrites; WT: 52.19 ± 2.17%, N = 13 dendrites; ΔMD: 33.16 ± 2.41%, N = 12 dendrites; ANOVA analysis: *** p < 0.001 when compared with GFP controls). (C) Change of PSD-95 intensity by KIF5A or its mutant expression. Expression of ΔMD decreases PSD-95 immunoreactivity in dendrites (GFP: 19.2 ± 1.81, N = 13 dendrites; WT: 21.18 ± 2.52, N = 13 dendrites; ΔMD: 4.42 ± 0.57, N = 12 dendrites; AU: arbitrary unit; ANOVA analysis: *** p < 0.001 when compared with GFP controls or WT). (D) Change of Dcp1a intensity by KIF5A or its mutant expression. Expression of WT increases Dcp1a immunoreactivity in dendrites (GFP: 6.47 ± 0.95, N = 13 dendrites; WT: 15.43 ± 1.67, N = 13 dendrites; ΔMD: 7.48 ± 1.33, N = 12 dendrites; AU: arbitrary unit; ANOVA analysis: *** p < 0.001 when compared with GFP controls).
Fig. 5. Both KCl treatment and NMDA treatment increases the movement of P-bodies. GFP-Dcp1a was expressed by viral infection for 6 h. The cultures were treated with 30 μM NMDA to induce chemical depression or 60 mM KCl to induce chemical activation for 10 min, respectively and incubated for 50 min. For inhibitor treatments, the cultures were treated with individual inhibitors, 100 μM D-AP5 or 2 mM EGTA for 15 min before drug treatments. Maximal distances were scored up to the point at which the GFP signals of Dcp1a-foci were detectable in the subtracted images. (A) The representative images of straightened dendrites. (B) NMDA or KCl treatment transport P-bodies to more distal dendrites. (C) Individual treatment did not significantly alter the amount of P-bodies in dendrites (41-80 μm). Data are presented as mean ± SEM (Student t-test: *** p < 0.001 when compared with not treated controls).
Fig. 6. Neuronal activity localizes P-bodies to synaptic regions. (A) Cultured hippocampal neurons were infected with Sindbis virus encoding GFP-Dcp1a, then incubated for 12 h. The cultures were pretreated with 2 mM EGTA for 15 min and incubated with 60 mM KCl with 2 mM EGTA for 10 min or treated with 60 mM KCl for 10 min without pretreatment, and then immediately fixed (KCl 10'). The cultures were stained with anti-PSD-95 antibody, as a post-synaptic marker. Scale bar: 20 μm. (B) Brief KCl treatment (KCl 10') also increased the co-localization of the P-bodies with PSD-95 in a Ca²⁺-dependent manner. Data are presented as mean ± SEM (Student t-tests: ** p < 0.01, * p < 0.05, ns: not significant when compared with untreated controls).
Fig. 7. Neuronal activity increase release of Nd1 mRNA and Arp2 mRNA from P-bodies.

(A) The change of Nd1 and Arp2 mRNA in Dcp1a-bodies by depolarization. The Myc-Dcp1a expression neurons were treated with 60 mM KCl for 10 min or not treated, and allowed to undergo additional incubation for 20 min, making total time 30 min. After IP procedures, total RNAs were isolated from immunoprecipitates and supernatant, and used in RT-PCR. The relative levels (pellet/supernatant) of each mRNA were measured and compared with those of before treatments. –RT CTL: used total RNA isolated from neuronal lysates before IP as a template without reverse transcriptase, +RT CTL: used total RNA isolated from neuronal lysates before IP with reverse transcriptase, S: used total RNA isolated from the supernatant after IP. P: used total RNA isolated from the pellet after IP. The specificity of RT-PCR was checked by TA cloning and sequencing analysis of RT-PCR products. 

(B) Bar graphic representation of quantitative results. Depolarization reduced the amount of Nd1 mRNA and Arp2 mRNA in Dcp1a-bodies to 83.6% (n=3, Paired t-tests: * p < 0.05) and 81.5% (n=3, ns: not significant when compared with not treated controls), respectively.
Fig. 8. Neuronal activity rescues level of F-actin reduced by Dcp1a expression in dendrites. (A) Cultured hippocampal neurons were infected with Sindbis virus encoding GFP or GFP-Dcp1a and incubated for 6 h. The cultures were incubated with high-K⁺ HBS (60 mM KCl) for 10 min, additionally incubated for 50 min, and used in TRITC-Phalloidin staining. The representative images of Phalloidin staining. The arrows indicates the dendrites used in analysis. Scale bar: 20 μm. (B) Straightened images of dendrites used in analysis. Scale bar: 20 μm. (C) Bar graphic representation of quantitative analysis of F-actin level in dendrites. Data are presented as mean ± SEM (Student t-tests: * p < 0.05, ** p < 0.01; %).
A

GFP  PSD-95  Dcp1a

GFP

GFP-WT

GFP-ΔMD

B

C

D

Co-localization of PSD-95-Dcp1a (%)

PSD-95 intensity in dendrites (AU)

Dcp1a intensity in dendrites (AU)