Special Issue Article

Electron microscopic analysis of a fusion protein of postsynaptic density–95 and metallothionein in cultured hippocampal neurons

Yuko Fukunaga1,2, Ai Hirase1,2, Hyeji Kim1, Natsuko Wada1, Yuri Nishino1,2 and Atsuo Miyazawa1,2,*

1Bio-multisome Research Team, RIKEN SPring8 Center, Harima Institute, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan 2CREST, JST
*To whom correspondence should be addressed. E-mail: atsuo@spring8.or.jp

Abstract

The subcellular localization of biomolecules at high resolution has traditionally been investigated by combining transmission electron microscopy (TEM) and chemical staining with heavy metals or immuno-based labeling with gold-conjugated antibodies. Here, we employ genetically encoded tags to examine the localization of proteins in transfected cultured cells by TEM. We purified a fusion protein of postsynaptic density-95 (PSD-95) coupled to three tandem repeats of metallothionein (MT) (PDS-95–3MT) from COS7 cells grown in the presence of Cd2++. PSD-95–3MT was detected as black particles by TEM. To visualize the subcellular localization of PSD-95–3MT, expression constructs encoding this fusion protein were transfected into primary hippocampal neurons cultured in medium containing Cd2+. The subcellular accumulation of PSD-95–3MT and Cd2+ provided excellent contrast in TEM micrographs. To address if genetically encoded tags affect the function of the target proteins, we found that the conjugation of 3MT to PSD-95 did not alter its association with known binding partners. These results demonstrate that 3MT coordinating Cd2+ is a valuable genetically encoded tag to study the localization of proteins by TEM.

Keywords
transmission electron microscopy, metallothionein, genetically encoded tag, PSD-95

Introduction

Light microscopy (LM) and transmission electron microscopy (TEM) are commonly employed to examine protein localization at high resolution. Detection with immuno-based labeling is a routine approach for LM and TEM. Ultrastructural localization of specific proteins in tissues has also been studied with immuno-gold antibody conjugates. Recently, Quantum dots, made of CdSe and related materials, have been applied to cell biology [1]. These dots are bright fluorophores and electron dense. Covalently coupling of Quantum dots to biomolecules, such as antibodies, provides an excellent tool for studying intracellular processes at the single-molecule level [2] and for labeling endogenous proteins for LM and at high resolution for TEM [3].

Previously, we demonstrated that metallothionein (MT), a heavy metal-binding protein, is a useful genetically encoded tag [4]. The high electron density of this protein complex, arising from heavy metal clusters, is visible by TEM. MT fusion proteins provide several advantages for studying the distribution of proteins at high resolution. First, proteins for which high specificity and high affinity antibodies are not available are amenable to labeling with genetically encoded tags. Second, the stoichiometry of target protein and tag protein approaches a 1:1 ratio. Third, as the electron-dense heavy metal is incorporated during culturing conditions, the degree of labeling is not susceptible to variations in specificity, contrast, and distribution that arise as consequence of fixation and antibody penetration. However, while individual proteins are not sufficiently electron dense for detection by TEM, the multimer of tagged proteins is discernable.
Thus, proteins that multimerize or are enriched at subcellular locations are the most tractable.

Postsynaptic density protein, postsynaptic density-95 (PSD-95), is a founding member of a large family of scaffolding proteins that contain several protein interaction domains. PSD-95 has been proposed to cluster and anchor numerous proteins at the postsynaptic density, including N-methyl-D-aspartate (NMDA) receptors [5]. It has been shown that mice harboring a genetic disruption of PSD-95 retain functional NMDA receptors, but exhibit aberrant long-term potentiation and learning deficits [6]. Thus, one function of PSD-95 is to couple NMDA receptors to intracellular signaling proteins that contribute to mechanisms of synaptic plasticity [7]. An improved characterization of the subcellular distribution of PSD-95 would aid our understanding of the mechanisms of synaptic plasticity.

In this study, we examined the subcellular localization of PSD-95 with an MT fusion protein. In addition, we determined that the three tandem repeats of MT (3MT) conjugation did not disrupt the localization and the intrinsic function of PSD-95.

**Materials and methods**

**Cell culture**

COS7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. One day before transfection, cells were plated onto 100 mm Petri dishes. At the time of transfection, cells were 80–90% confluent.

Hippocampal neurons were prepared from embryonic day 19 Sprague-Dawley rats (Japan SLC, Japan). Dissected hippocampi were dissociated by trypsin treatment and triturated. The cell suspension was seeded on poly-L-lysine and laminin-coated 15 mm round glass coverslips at 2.5 × 10⁴ cells cm⁻² for adenovirus transduction or 6 × 10⁴ cells cm⁻² for lipofection. Neurons were maintained in Neurobasal medium (Invitrogen) supplemented with glutamate, B-27 (Invitrogen), Glutamax (Invitrogen), penicillin, and streptomycin, using a humidified atmosphere containing 10% CO₂. Two days after plating, arabinoside C was added to the culture medium to inhibit glial proliferation. Three days after plating, the culture medium was exchanged with Neurobasal medium supplemented with B-27, Glutamax, penicillin, and streptomycin. Thereafter, the growth medium was changed once a week.

**Cloning and plasmid construction**

A full-length murine PSD-95 in pcDNA3.1(−) as well as Kv1.4, and c-Myc-tagged NR2B in pcDNA3.1(+) was a kind gift of Mr. F. Imamura and Professor Y. Fujiyoshi (Kyoto University). The c-Myc tag (EQKLISEEDL) was inserted between amino acid residues 27 and 28 of NR2B. A full-length murine neuronal nitric oxide synthase (nNOS) clone in pCR2.1 was kindly provided by Dr. T. Ogura (National Cancer Center Research Institute, Japan).

To construct PSD-95-Flag-pET21b, the MT fragment was excised with BlnI and SpeI and the vector ligated PSD-95 was deleted from PSD-95–3MT-Flag-pET21b previously prepared [4] by digesting the plasmid with NheI and BlnI, excising the PSD-95 fragments and religating the vector to yield MT-Flag-pET21b. PSD-95–3MT-Flag-pET21b, PSD-95–Flag-pET21b, and MT-Flag-pET21b (Fig. 1) were digested with NheI and Xhol and the insert sequences were ligated into the corresponding restriction sites of pcDNA3.1(−) (Invitrogen). Cloning and plasmid construction were prepared in *Escherichia coli* Top10F⁺ (Invitrogen). For brevity, PSD-95–3MT-Flag is abbreviated as PSD-95–3MT throughout the manuscript.

**Transfection**

COS7 cells were transfected with expression vectors using Lipofectamine 2000 according to the manufacturer’s instructions. PSD-95 and Kv1.4, NR2B or nNOS were transfected at a ratio of 1:1. Hippocampal neurons were transfected with PSD-95–3MT, PSD-95 or MT clone in pcDNA3.1(−) at 13–14 days in vitro (DIV13–14). CdCl₂ was added to the culture medium at a concentration of 20 µM (COS7 cells) or 5 µM (hippocampal neurons) 24 h after transfection. COS7 cells or hippocampal neurons were cultured for additional 19 h prior to coimmunoprecipitation or immunocytochemistry, respectively.

**Coimmunoprecipitation**

COS7 cells were washed with phosphate-buffered saline (PBS) resuspended in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40) and sonicated for 5 s on ice. Extracts were centrifuged at 40 000 × g for 30 min at 4°C. The resulting supernatant was incubated with anti-Flag M2 affinity gel (Sigma) overnight at 4°C. The beads were subsequently washed five times with ice-cold immunoprecipitation buffer. The bound proteins were eluted with sodium dodecyl sulfate (SDS) sample...
buffer and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, protein samples were transferred to nitrocellulose by semidry electoblotting. Immunoblots were probed with the following primary antibodies: rabbit anti-Flag antibodies (1:500, Affinity BioReagents); rabbit anti-Kv1.4 antibodies (1:2000, Chemicon); rabbit anti-nNOS antibodies (1:2000, BD Transduction Laboratory); mouse anti-Myc antibodies (1:200, Santa Cruz Biotechnology), and visualized with alkaline phosphatase-conjugated secondary antibodies and a chromogenic substrate.

**Purification of PSD-95–3MT proteins**

Cells were suspended in Tris buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 10 µg ml⁻¹ DNase, 10 µg ml⁻¹ RNase and 1 tablet per 100 ml complete proteinase inhibitor mix (Roche, Germany) and disrupted by sonication. The lysate was incubated at 4°C for 1 h and centrifuged at 10,000 × g for 1 h at 4°C. The soluble fraction was applied to an anti-Flag M2 affinity column (Sigma) equilibrated with Tris buffer. The column was washed with 10 column volumes of Tris buffer and the protein was eluted with 100 mM glycine, 150 mM NaCl (pH 3.5). The eluent was neutralized immediately with the addition of 500 mM Tris-HCl (pH 8.0). Subsequently, protein was concentrated to 0.05 µg ml⁻¹ with a vivaspin100 (VIVASCIENCE, Germany).

**Analysis of the metal content**

The CdCl₂ concentration in the purified protein solution was determined using inductively coupled plasma analysis by Oki Engineering (Japan). The protein concentration was determined by comparing the density of the band stained with Coomassie brilliant blue on a SDS-PAGE gel using BSA as a standard.

**Adenovirus construction and transduction**

Adenovirus containing an expression construct for PSD-95–3MT (pAdPSD-95–3MT) was prepared with the ViraPower Adenovirus Expression System (Invitrogen) according to the manufacturer’s instructions. Briefly, PSD-95–3MT was subcloned into the pENTER-D/TOPO vector using the pENTER Directional TOPO cloning kit (Invitrogen). Next, the cDNA inserts were transferred into the pAd/CMV/V5-DEST vector by the Gateway system using LR Clonase. The plasmids were purified and digested with PaCl (New England Biolabs). The linearized plasmids were then mixed with Lipofectamine 2000 in Opti-MEM medium and transfected into subconfluent 293A cells maintained in Opti-MEM. The 293A cells were subsequently cultured for 1–2 weeks in DMEM containing 10% fetal calf serum, with the medium being replaced every 2 days. When most cells detached from the plates, the cells and the culture medium were harvested together, freeze-thawed twice, and centrifuged to obtain supernatant enriched in adenovirus. Aliquots of this supernatant were added to fresh 293A cells and cultured for 2–3 days to amplify the adenovirus. After second round of amplification, the resulting adenovirus-containing media were used as virus stocks. The viral titer was determined by calculating the 50% tissue culture infectious dose (TCID₅₀ = 4.4 × 10⁵).

Aliquots of the adenovirus-containing medium were added to hippocampal primary cultures after DIV9. Three days after application of the viral vector, CdCl₂ was added to the culture medium at 5 µM. The cells were then incubated for 19 h until fixation for immunocytochemistry or TEM.

**Immunocytochemistry**

The neurons were fixed with 4% paraformaldehyde in PBS for 20 min and quenched by 10 mM glycine in PBS, and then permeabilized using 0.5% NP-40 in PBS for 5 min at room temperature. For detection of synaptophysin, neurons were treated with methanol at −20°C for 10 min, instead of paraformaldehyde. Coverslips were blocked for 1 h in PBS containing 1% BSA, 10% goat serum, and 0.5% Triton X-100, and then incubated over night at 4°C with one of the several primary antibodies: rabbit anti-Flag antibody (Affinity BioReagents) diluted 1:2000; mouse monoclonal anti-synaptophysin antibody (Sigma) diluted 1:1000; mouse monoclonal anti-MT antibody (Transgenic, Japan) diluted 1:1000; or rabbit polyclonal anti-MAP2 antibody (Chemicon) diluted 1:1000. After washing the cells three times with PBS, the cells were incubated with the Alexa 488-conjugated (1:500, Molecular Probes) or Alexa 543-conjugated secondary antibodies (1:500, Molecular Probes) for 1 h. The cells were then washed three times with PBS and mounted in Vectashield (Vector). Neurons were imaged with a LSM510 confocal laser-scanning microscope (Carl Zeiss, Germany). The fluorescence intensity of MT staining on neuronal cell bodies was measured as the mean intensity of manually selected areas from neurons stained with anti-MAP2 antibodies. The confocal settings, including laser power, aperture size, gain, and offset, were held constant for image acquisition from different coverslips. The cells exhibiting more than three times the intensity of the background were counted as MT-positive cells in a fixed area (460 × 460 µm). The number of MT-positive cells was normalized to the total number of MAP2-immunoreactive neurons.

**The assessment of viability**

The cell viability rate was assessed with a calcein and propidium iodide (PI) double staining kit, Cellstain (Dojindo, Japan). Following supplementation of the media with Cd²⁺, COS7 cells and the primary hippocampal neurons were stained with the Cellstain Double Staining Kit according to the manufacturer’s instructions, except that the concentration of calcein (0.25 µM) and PI (0.5 µM) were optimized. The number of living cells, stained with calcein, and the
number of dead cells, stained with PI, were counted with the aid of a confocal microscope.

Transmission electron microscopy
Purified proteins were applied onto glow-discharged carbon-coated copper grids and washed in water. The grids were either stained with 2% uranyl acetate or washed in water. At each step, excess solution was removed with filter paper. Dried grids were examined at ×15 000 with a JEM-2010 electron microscope (JEOL, Japan) operating at an accelerating voltage of 100 kV. Images were acquired using a CCD camera (TVIPS, Germany) and recorded as 8-bit images.

The neurons were fixed for 2 h in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 30 mM HEPES buffer (pH 7.2) for the neurons without postfixing with osmium tetroxide and stained with uranyl acetate and lead citrate, or in PBS for the neurons with the postfixing and electron staining. Following fixation, they were washed for 10 min then dehydrated through a series of graded solvents (50% ethanol for 10 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 10 min, 100% anhydrous ethanol twice, each time for 10 min). The neurons were soaked in propyrenoxide for 10 min and then in Epon 812/propyrenoxide (volume ratio = 1:1) for 18 h. They were subsequently transferred to 100% Epon 812, and after 6 h, the Epon was polymerized in a programmed oven. Ultrathin sections, ∼80 nm thick, were cut on an Ultramicrotome EM UC-6 (Leica Microsystems, Germany) and collected on copper grids. Sections that were postfixed with osmium tetroxide were double-stained with uranyl acetate and lead citrate. Dried grids were examined at ×20 000 using an electron microscope operating at an accelerating voltage of 200 kV for the neurons without postfixing with osmium tetroxide and stained with uranyl acetate and lead citrate, or of 100 kV for the neurons with the postfixing and electron staining. Images were acquired using a CCD camera (TVIPS) and recorded as 8-bit images.

Results
Cytotoxicity of Cd\(^{2+}\) and expression of PSD-95–3MT in COS7 cells
To detect MT-fused target proteins by TEM, cells were incubated with heavy metal ions. MT binds several heavy metal ions including Ag\(^+\), Cd\(^{2+}\), Cu\(^{2+}\), Hg\(^{2+}\), and Zn\(^+\) [8]. Following addition of 50 μM Ag\(_{2}\)O\(_{3}\) or 80 μM Hg\(_{2}\)Cl\(_{2}\) to the culture medium, Ag\(^+\) or Hg\(^{2+}\) did not bind to MT (data not shown). However, following the addition of 20 μM Cd\(_{2}\)Cl\(_{2}\), Cd\(^{2+}\) did bind to MT specifically.

Cd\(^{2+}\) is a potent cell poison and may promote both necrotic and apoptotic cell death [8,9]. To estimate cell viability after exposure to Cd\(^{2+}\), COS7 cells were treated with 20 or 40 μM Cd\(_{2}\)Cl\(_{2}\), Cd\(^{2+}\) did bind to MT specifically.

Electron micrographs of purified PSD-95–3MT
To detect purified PSD-95–3MT by TEM, COS7 cells were transfected with expression plasmids encoding PSD-95–3MT and treated with 20 μM Cd\(_{2}\)Cl\(_{2}\), PSD-95–3MT was immunostained with anti-Flag antibodies and Alexa488-conjugated secondary antibodies.

30 h of exposure to 20 or 40 μM Cd\(_{2}\)Cl\(_{2}\) and after 20 h of exposure to 40 μM Cd\(_{2}\)Cl\(_{2}\), but did not decrease after 20 h of exposure to 20 μM. An analysis of metal content indicated that PSD-95–3MT purified from COS7 cells binds Cd\(^{2+}\) after 20 h of exposure to this concentration of CdCl\(_{2}\). COS7 cells, which were transfected with plasmids encoding PSD-95–3MT cDNA and were treated with 20 μM Cd\(_{2}\)Cl\(_{2}\), expressed PSD-95–3MT in 2 days after transfection (Fig. 2b).
but not without CdCl$_2$ (Fig. 3f). Thus, purified PSD-95–3MT coordinating Cd$^{2+}$ is visible by TEM.

**Cytotoxicity of Cd$^{2+}$ and expression of PSD-95–3MT in primary hippocampal neurons**

After DIV13 or 14, 5 or 10 µM of CdCl$_2$ were added to the culture medium of hippocampal neurons and the percentage of living cells was calculated 10, 20, or 30 h after Cd$^{2+}$ exposure. The percentage of living cells decreased after 30 h of exposure to 10 µM CdCl$_2$, but did not decrease significantly after 30 h of exposure to 5 µM CdCl$_2$ (Fig. 4a). An analysis of metal content at 20 h indicated that Cd$^{2+}$ was present in lysate from cells treated with 5 µM CdCl$_2$, and approximately 7.5–10 µM in the cells (unpublished data). Therefore, we treated primary cultures of hippocampal neurons with 5 µM CdCl$_2$ for less than 20 h to obtain specimens suitable for TEM.

To examine the distribution of PSD-95–3MT in neuronal cells by TEM, we transduced neurons with adenovirus harboring an expression construct for PSD-95–3MT (AdPSD-95–3MT) (Fig. 4b). The highest infection efficiency (approximately 65%) was obtained when primary culture was infected with $1.8 \times 10^7$ pfu ml$^{-1}$ of AdPSD-95–3MT, and...
Cytotoxicity of Cd$^{2+}$ and expression of PSD-95–3MT in primary hippocampal neurons. (a) The percentage of viable cells was calculated for DIV13 or 14 hippocampal neurons at 10, 20, and 30 h after the addition of 5 or 10 µM CdCl$_2$. The results from three independent experiment sets were averaged. (b) The primary hippocampal neurons were transduced with adenovirus containing an expression construct for PSD-95–3MT and treated with 5 µM CdCl$_2$. PSD-95–3MT was immunostained with anti-Flag antibodies and Alexa543-conjugated secondary antibodies. Another optimal condition for infection of AdPSD-95–3MT was to start infection at DIV9, seed the dissociated cells with $2.5 \times 10^4$ cell cm$^{-2}$, and fix cells at DIV13 (data not shown).

Observation of PSD-95–3MT
The primary hippocampal neurons were transduced with AdPSD-95–3MT and treated with 5 µM CdCl$_2$ for 19 h prior to preparation for TEM. To limit the loss of signal for Cd-bound PSD-95–3MT (Cd-PSD-95–3MT), while retaining features of cell architecture, these samples were prepared for plastic embedding without osmium tetroxide and electron staining (Fig. 5a–d). Images of the sectioned material provided reasonable contrast, despite the absence of osmium tetroxide and electron staining. Many large electron-dense deposits were present in neurons exposed to AdPSD-95–3MT and treated with Cd$^{2+}$ (Fig. 5a), but not in untreated controls (Fig. 5b) or in neurons treated with CdCl$_2$ alone (Fig. 5c). In addition, no signal was detected in neurons transduced with AdPSD-95–3MT, but not incubated with CdCl$_2$ (Fig. 5d). Small electron-dense dots were observed in not only neurons incubated with CdCl$_2$ (Fig. 5c), but also neurons transduced with AdPSD-95–3MT and incubated with CdCl$_2$, controls, and neurons only transduced with AdPSD-95–3MT (data not shown). As electron-dense deposits are distinguishable from these small electron-dense dots, we conclude that the electron-dense deposits are Cd-PSD-95–3MT.

To examine the cellular localization of electron-dense deposits, next we prepared the samples that were postfixed with osmium tetroxide and stained with uranyl acetate and lead citrate (Fig. 6a–d). Electron-dense clusters were present in neurons exposed to AdPSD-95–3MT and treated with Cd$^{2+}$ simultaneously (Fig. 6a), but not in untreated controls (Fig. 6b), neurons treated with CdCl$_2$ alone (Fig. 6c) or neurons transduced with AdPSD-95–3MT only (Fig. 6d).

Localization and binding ability of PSD-95–3MT
We examined whether appending MT to PSD-95 disrupted the functions of this scaffolding protein. PSD-95 is enriched in the postsynaptic density and PSD-95 overexpressed in transfected neurons localizes to synapses (Fig. 7). PSD-95–3MT colocalized with the presynaptic marker synaptophysin on dendritic spines (Fig. 7a). The synaptic localization was also observed when neurons were transduced with an adenovirus rather than transfected (Fig. 7b). This distribution was indistinguishable from that of an epitope-tagged PSD-95 (Fig. 7c). In comparison, MT was distributed throughout transfected neurons (Fig. 7d). Thus, we conclude that fusion of PSD-95 to MT does not disrupt the synaptic targeting of PSD-95.

PSD-95 interacts with a number of proteins also present at the synapse, including NMDA receptors, the shaker potassium channel Kv1.4, and nNOS. To examine whether the PSD-95-MT fusion protein altered the ability of the PSD-95 fragment to interact with these proteins, we performed pull-down assays (Fig. 8). Left and right columns of Fig. 8 represented the input sample from COS7 cells (input) and immunoprecipitation (IP), respectively. PSD-95 and PSD-95–3MT, but not MT, bound to the NMDA receptor NR2B subunit (upper panel; Fig. 8), Kv1.4 (middle panel) and nNOS (lower panel). Therefore, we conclude from these results that the binding ability of PSD-95 is not changed by the fusion with MT.

Endogenous metallothionein
Cd$^{2+}$ induces MT expression in various tissues [10,11]. In our experiments, induction of endogenous MT by 5 µM CdCl$_2$ was examined after 19 h, using cells stained with anti-MT. Endogenous MT was not observed in untreated neurons (Fig. 9a) and appeared in neurons after 19 h exposure...
Fig. 5. Electron micrographs of PSD-95–3MT in primary hippocampal neurons without osmium postfixation and electron staining. Primary hippocampal neurons were fixed with paraformaldehyde and glutaraldehyde without osmium postfixation and electron staining. Electron-dense deposits (arrowheads) were observed in neurons transduced with AdPSD-95–3MT and incubated with CdCl₂ (a), but not controls (b) or neurons only incubated with CdCl₂ (c) or only transduced with AdPSD-95–3MT (d).

of 5 µM CdCl₂ (Fig. 9b). Endogenous MT was distributed throughout the dendrite (Fig. 9c). There were fewer MT-positive cells present after 24 h of 5 µM CdCl₂ than after 48 h of 5 µM CdCl₂ (Fig. 9d). In spite of the induced expression of endogenous MT, electron micrographs of primary hippocampal neurons incubated with 5 µM CdCl₂ for 19 h did not produce visible grains by TEM (Figs. 5c and 6c). We conclude that endogenous MT, though it may bind Cd²⁺, is not electron dense enough to yield a signal by TEM.

Discussion
Here, we demonstrate a useful method for examining the localization of proteins at high resolution. This method relies on expressing a fusion construct of the protein of interest with MT and then providing a heavy metal ion to bind MT. This method overcomes several limitations associated with immuno-gold labeling techniques as it does not require high specificity and affinity antibodies to the protein of interest. MT binds Au⁺ [12] and electron micrographs of purified Au-bound MT complexes reveal black spots of various sizes [13]. These reports suggest the possibility that Au-bound MT may be ideal genetically encoded tag for TEM. However, in contrast to Cd²⁺, which binds specifically to MT, Au⁺ binds to a number of cytosolic proteins [12]. Therefore, we used Cd²⁺ in this study. Although Cd²⁺ is a potent cell poison [9,14], cellular dysfunctions can be avoided by optimizing the concentration of Cd²⁺ and incubation times required to promote adequate contrast by TEM, while not decreasing cell viability.

In this study, we appended 3MT to the target protein. As up to seven atoms of cadmium can be bound by a single molecule of MT [15,16], this fusion protein could potentially bind as many as 21 atoms of cadmium. However, in a previous study, we were unable to detect individual 3MT proteins by TEM [4]. Here, we investigated whether or not we might be able to visualize fusion proteins to 3MT that were enriched at subcellular locations or were components of a larger supramacromolecular structure. We focused our efforts on PSD-95, a scaffolding protein that is enriched in the postsynaptic density of neurons. Cd-PSD-95–3MT localizes to excitatory synapses (Fig. 7) in a manner similar to endogenous PSD-95 [17]. These sites of enrichment for the fusion protein were visible by TEM. Further work, including enhancement of the labeling intensity or efficiency of metal binding, will be required to extend this approach to include individual proteins that are less concentrated at subcellular locations.

The electron-dense deposits associated with the Cd-PSD-95–3MT were larger than typical areas of postsynaptic
Fig. 6. Electron micrographs of PSD-95–3MT in synapses of primary hippocampal neurons with osmium postfixation and electron staining. Primary hippocampal neurons were fixed with paraformaldehyde and glutaraldehyde, and with osmium postfixation and electron staining. Electron-dense deposits (arrowheads) were observed in postsynaptic site of neurons transduced with AdPSD-95–3MT and incubated with CdCl₂ (a), but not controls (b) or neurons only incubated with CdCl₂ (c) or only transduced with AdPSD-95–3MT (d). S, spine; Sh, dendritic shaft; T, presynaptic terminal.

Fig. 7. Synapse targeting of PSD-95–3MT to synapses of hippocampal neurons. PSD-95–3MT (green) colocalized with synaptophysin (red). PSD-95–3MT (a, b), PSD-95 (c) and MT (d). cDNA was introduced into the primary hippocampal neurons by lipofection (a, c, d) and viral transduction (b) and CdCl₂ was treated for 19 h before fixation. Upper panel: immunostaining with an anti-Flag antibody, middle panel: immunostaining with an anti-synaptophysin (syn) antibody, bottom panel: merged image.
Fig. 8. Binding of PSD-95–3MT to NR2B, Kv1.4, and nNOS COS7 cells were transfected with PSD-95–3MT, PSD-95 or MT (tagged with Flag) in the presence or absence of NR2B (upper panel), Kv1.4 (middle panel), or nNOS (lower panel) and were treated with 20 µM CdCl2 for 19 h. NR2B, Kv1.4, or nNOS were immunoprecipitated (IP). Whereas MT did not bind to NR2B, Kv1.4, and nNOS, both PSD-95–3MT and PSD-95 were coimmunoprecipitated with these proteins.

density characterized by TEM [18]. As PSD-95 is enriched on the postsynaptic membrane under physiological conditions, overexpression of PSD-95–3MT may induce larger areas occupied by PSD-95 at postsynaptic sites of these primary neurons, and this might result in that the electron densities were observed not only at the PSD, but also beneath the PSD in postsynaptic sites, as shown in Fig. 6a. In neurons transduced with AdPSD-95–3MT only, these extra electron densities were not observed at the PSD, but also beneath the PSD in postsynaptic sites, as shown in Fig. 6a. In neurons transduced with AdPSD-95–3MT only, these extra electron densities were not observed at the postsynaptic sites, indicating that Cd-3MT could permit the detection of the accumulated proteins, which were not detected by staining with uranium acetate and lead citrate in TEM. Our approach may elucidate where PSD-95 is preferentially enriched within a population of excitatory synapses. However, further experiments using the double-stained specimens by immuno-gold labeling of PSD-95 with Cd-PSD-95–3MT will be required to ensure this approach for TEM of genetically encoded fusion proteins.

For genetically encoded tags suitable for TEM to be informative, the appending of the fusion protein ‘tag’ must not interfere with the functions of the protein of interest. In many cases, GFP has overcome this problem with fluorescence microscopy [19,20]. GFP has been employed as a tag for not only PSD-95 [19,20], but also for a wide variety of proteins with disparate localization and function. In this study, we confirmed that 3MT did not affect the synaptic targeting or association with known interacting proteins. Thus, this 3MT tag is useful for studying the localization of PSD-95 with TEM. Nevertheless, the possibility remains that 3MT might impair the activity and conformation of other target proteins and each fusion protein will require individual analysis.

In the specimens without postfixation and/or electron staining, small electron-dense dots that were distinct from large electron-dense deposits were observed in neurons transduced with AdPSD-95–3MT and incubated with CdCl2, controls, and neurons transduced with AdPSD-95–3MT only. As few cells expressed endogenous MT at 24 h following CdCl2 treatment, and MT does not form clusters, a feature that would increase the visibility of Cd-MT molecules by TEM, we consider it unlikely that these small electron-dense dots are endogenous MT coordinating Cd2+. These small electron-dense dots might be contaminants arising during fixation, embedding, or ultrathin sectioning. In addition, these small electron-dense dots might be masked in samples stained with heavy metals, as these dots were not evident in cells stained with uranyl acetate and lead citrate.

The domains of PSD-95 that mediate the synaptic localization of this scaffolding protein have been determined predominantly by LM. Higher-resolution information on subcellular localization of PSD-95 has been provided with immuno-based methods of TEM [21,22]. However, these immuno-based TEM methods require extensive optimization with regard to the fixation protocol, strength of fixative, membrane permeabilization, concentration of antibody, and incubation time of antibody, according to the properties of each cell or tissue. This fusion protein approach permits the detection of target proteins in cells without any other labeling for TEM. Therefore, Cd-3MT may become a valuable tool for characterizing the localization of proteins by TEM.

### Concluding Remarks

A fusion protein of PSD-95 coupled to three tandem repeats of metallothionein (PDS-95–3MT) was synthesized in COS7 cells in the presence of Cd2+, and was subsequently purified and visualized by TEM. We identified conditions of CdCl2 treatment (5µM, 19 h-treatment) which did not induce cytotoxicity in cultured hippocampal neurons. This fusion protein localized to synaptic sites in transfected or transduced primary hippocampal neurons. The accumulation of Cd-PSD-95–3MT at synapses permitted the detection of this fusion protein by TEM. In addition, 3MT did not affect its association with known binding partners. Cd-3MT provides an alternative approach to immuno-based labeling that may in the future be a general tool for TEM.
Fig. 9. Endogenous metallothionein DIV14 hippocampal neurons in primary culture were treated with 5 \( \mu \)M CdCl\(_2\) and fixed 19 h later. Cultures were stained for MT. (a) Control cells. (b) CdCl\(_2\)-treated cells. (c) High magnification of dendrite 19 h postCdCl\(_2\). (d) DIV14 primary hippocampal neurons were treated with 5 \( \mu \)M CdCl\(_2\) and fixed either 24 or 48 h later. Neurons were stained for MT. The graph indicates the mean percentage of MT positive cells ±SD. The data represent the results from at least 100 cells from 4–6 different areas for each treatment.

Funding

Ministry of Education, Culture, Sports, Science and Technology of Japan (proposal No. 17657053).

Acknowledgements

The authors are very grateful to Dr. N. Unwin (Medical Research Council) and Professor Y. Fujiyoshi (Kyoto University) for their helpful suggestions. The authors thank Mr. S. Aida of JEOL DATUM LTD (Japan) for supporting the experiments relating to TEM.

References