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Somatodendritic and excitatory postsynaptic distribution of neuron-type dystrophin isoform, Dp40, in hippocampal neurons

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ABSTRACT

The Duchenne muscular dystrophy (DMD) gene produces multiple dystrophin (Dp) products due to the presence of several promoters. We previously reported the existence of a novel short isoform of Dp, Dp40, in adult mouse brain. However, the exact biochemical expression profile and cytological distribution of the Dp40 protein remain unknown. In this study, we generated a polyclonal antibody against the NH_2-terminal region of the Dp40 and identified the expression profile of Dp40 in the mouse brain. Through an analysis using embryonic and postnatal mouse cerebrums, we found that Dp40 emerged from the early neonatal stages until adulthood, whereas Dp71, an another Dp short isoform, was highly detected in both prenatal and postnatal cerebrums. Intriguingly, relative expressions of Dp40 and Dp71 were prominent in cultured dissociated neurons and non-neuronal cells derived from mouse hippocampus, respectively. Furthermore, the immunocytological distribution of Dp40 was analyzed in dissociated cultured neurons, revealing that Dp40 is detected in the soma and its dendrites, but not in the axon. It is worthy to note that Dp40 is localized along the subplasmalemmal region of the dendritic shafts, as well as at excitatory postsynaptic sites. Thus, Dp40 was identified as a neuron-type Dp possibly involving dendritic and synaptic functions.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked hereditary disease characterized by progressive muscular degeneration [1,2]. About one third of all DMD patients manifest variable degrees of cognitive impairment [3,4]. The *DMD* gene encodes dystrophin (also known as Dp427) and its multiple isoforms, including Dp71 and Dp40, which are produced by alternative promoters and/or alternative splicing [5,6]. Mutations in the *DMD* gene cause an absence or dysfunction of Dp427 localized in the plasma membrane of muscle cells, resulting in DMD [7,8]. Although mutations in all parts of the *DMD* gene can be associated with cognitive impairment [9], mutations in the Dp71 coding region is closely associated with the cognitive impairment observed in DMD patients [10–12]. Dp71 is an abundant product of the *DMD* gene that is expressed in the brain and found in both neurons and glia [5]. Most recently, it was found that the shortest dystrophin isoform, Dp40, the NH_2-terminal partial product of Dp71 produced by alternative splicing [13], is also expressed in the brain [14], although little is known about Dp40.

In this study, as the first step toward understanding the role of Dp40, we generated a polyclonal antibody against the NH_2-terminal region common to both Dp40 and Dp71 and investigated the expression profile of Dp40 in mouse brains. We found that that Dp40 emerged from the early neonatal period to adulthood, with preferential expression in neurons. Furthermore, we dissected the subcellular distribution of Dp40 in cultured hippocampal neurons and showed that Dp40 is somatodendritically localized and detected in the excitatory postsynapses. Thus, Dp40 may play critical roles in dendritic and synaptic functions in the brain.

2. Materials and methods

2.1. Animals

All of the animal experiments in this study were approved by the Institutional Review Board for Biomedical Research using Animals at Kyoto Prefectural University of Medicine, and the animals were handled according to the institutional guidelines and

Abbreviations: DMD, duchenne muscular dystrophy; Dp, dystrophin; DAPI, 4,6-Diamidino-2-phenylindole.

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regulations. The experiments were carried out on ICR mice purchased from a breeder (SHIMIZU Laboratory Supplies Co. Ltd., Kyoto, Japan).

2.2. Antibodies

The antibodies used were as follows: anti-Dp71 (ab15277) from Abcam (Massachusetts, USA), anti-hemagglutinin (HA) (3724) from Cell signaling (Massachusetts, USA), anti-MAP2 (ab17838) from Abcam (Massachusetts, USA), anti-PSD95 (P78352) from UC Davis/NIH NeuroMab Facility (California, USA), anti-synaptophysin (MAB5258) from Millipore (Darmstadt, Germany), and anti-gephyrin (147011) from Synaptic Systems (Gottingen, Germany). The recombinant mouse Dp40 fragment (amino acid residues 1–238) was expressed as a GST fusion protein using the pGEX4T-2 vector (GE Healthcare, England)). The fusion protein was soluble in nondenaturing buffer and was purified with glutathione-Sepharose 4B (GE Healthcare, England). Antiserum was obtained by injecting the recombinant Dp40 protein into a Japanese White rabbit followed by booster injection. The antiserum was purified with an affinity column prepared by cross-linking the recombinant protein to CNBr-activated Sepharose 4B (GE Healthcare, England).

2.3. Immunoprecipitations and Western blotting

Protein extracts from mouse tissues or cultured hippocampal cells were prepared by homogenizing in a lysis buffer (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). Two milligrams of protein from each tissue or cell was subjected to immunoprecipitations with anti-Dp40 or negative control antibodies. TrueBlot Anti-Rabbit Ig IP Beads (ROCKLAND antibodies & assays, Pennsylvania, USA) was added to the sample and incubated overnight at 4 °C. Then the samples were washed three times in the lysis buffer. Proteins were eluted by SDS sample buffer and separated on a polyacrylamide gel followed by transfer to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween20), the membranes were incubated with anti-Dp40 antibody (dilution 1:1000) overnight at 4 °C. Blots were incubated for 1 h at room temperature (RT) with Rabbit TrueBlot Anti-Rabbit IgG HRP (ROCKLAND antibodies & assays, Pennsylvania, USA). Chemiluminescence was detected using Western BLoT Chemiluminescence HRP Substrate (Takara Bio, Japan) and Hyperfilm™ ECL (GE Healthcare, England).

2.4. Primary culture and transfection

Cultured hippocampal neurons were prepared from ICR mice. Whole brains were isolated from embryonic day 18 mouse embryos, and the hippocampi were dissected out and treated with a papain dissociation system (Worthington Biochemical Co., New Jersey, USA) according to the manufacturer's instructions. The cells were suspended in DMEM (Sigma, Japan) containing 10% horse serum (Life Technologies, Japan) and plated at a density of 7000 cells/cm^2 on poly-D-lysine (Sigma, Japan)-coated 60 mm dishes or glass coverslips (Fisher Scientific, Massachusetts, USA). Four hours after plating, the medium was changed to serum-free Neurobasal medium containing 2% B27 supplement (Life technologies, Japan) and 0.5 mM L-glutamine (Life technologies, Japan), with incubation at 37 °C in a humidified incubator with 5% CO_2/95% air. Cultured hippocampal neurons growing on glass coverslips were subjected to transfection using Lipofectamine LTX reagent (Life Technologies, Japan) at day 4 *in vitro* according to the manufacturer's instructions. After being washed with Hank's Balanced Salt Solution (Life Technologies, Japan), the neurons were grown in Neurobasal medium containing 2% B27 supplement (Life technologies, Japan), 0.5 mM L-glutamine (Life Technologies, Japan) and 5 μM cytosine arabinoside for a further 3 weeks. Hippocampal non-neuronal cells were prepared from embryonic day 18 mouse hippocampi. Dissociated cells were cultured in DMEM containing 10% horse serum and penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO_2/95% air until reaching 100% confluence.

2.5. Construction of the plasmids

In order to generate COOH-terminal HA-tagged fusion protein, the cDNAs encoding full-length mouse *Dp40* was prepared from adult mouse hippocampus by reverse transcription-PCR using a set of primers (5′-GG GGA ATT CCC GCC ACC ATG AGG GAA CAC CTC AAA GGC CAC G-3′ and 5′-GG GGC GGC CGC TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA CGT TTC CAT GTT GTC CCC CTC TA A CAC-3′) and cloned into the pLVSIN-EF1α-IRES-ZsGreen1 vector (Takara Bio, Japan), which co-expresses ZsGreen (reef coral *Zoanthus* sp. green fluorescent protein) via the internal ribosome entry site.

2.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at RT, blocked with 5% bovine serum, 0.1% Tx-100, 150 mM NaCl, 50 mM Tris–HCl, pH7.5 for 30 min at RT, and then subjected to double-immunostaining with rabbit polyclonal anti-HA and mouse monoclonal anti-PSD95, anti-MAP, anti-synaptophysin, or anti-gephyrin antibodies. The primary antibodies were visualized with the proper combination of secondary antibodies: goat anti-rabbit IgG conjugated to Alexa Fluor 546 and goat anti-mouse IgG conjugated to Alexa Fluor 647. Nuclear DNA staining was processed by using the ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Japan). Fluorescence images were acquired by a confocal fluorescence microscope (LSM510 Ver. 4.0, Carl Zeiss, Wetzlar, Germany). The fluorescence intensity was quantified using ImageJ software for line scan analysis.

3. Results

3.1. Expression profile of Dp40 protein in the mouse brain

In order to reveal the biochemical expression profile of the Dp40 protein, a polyclonal antibody against the NH_2-terminal region of Dp40 was generated by immunizing a recombinant Dp40 protein in a rabbit. The amino acid sequence used for the antigen is totally included in full-length Dp (also known as Dp427) and a short isoform of Dp, Dp71 (Fig. 1A) [6,14]. To validate the reactivity of the anti-Dp40 antibody, anti-Dp40, anti-Dp71, or control IgG immunoprecipitations were performed using adult mouse cerebrum followed by Western blotting with the anti-Dp40 antibody, showing that the anti-Dp40 antibody was able to immunoprecipitate and detect both Dp40 and Dp71 proteins (Fig. 1B), although the anti-Dp71 antibody immunoprecipitated Dp71, but not Dp40 proteins (Fig. 1B). Next, to examine whether Dp40 would be expressed in the muscle, anti-Dp40 immunoprecipitation was carried out using mouse muscle, revealing that neither Dp40 or Dp71 proteins were detected (Fig. 1C, right half), although other Dp isoforms, including Dp427 were detected (Fig. 1C, right half). Consistent with the results shown in Fig. 1B, Dp40 and Dp71 proteins were obviously detected in the cerebrum (Fig. 1C, left half). These results indicated that the anti-Dp40 antibody we generated in this study

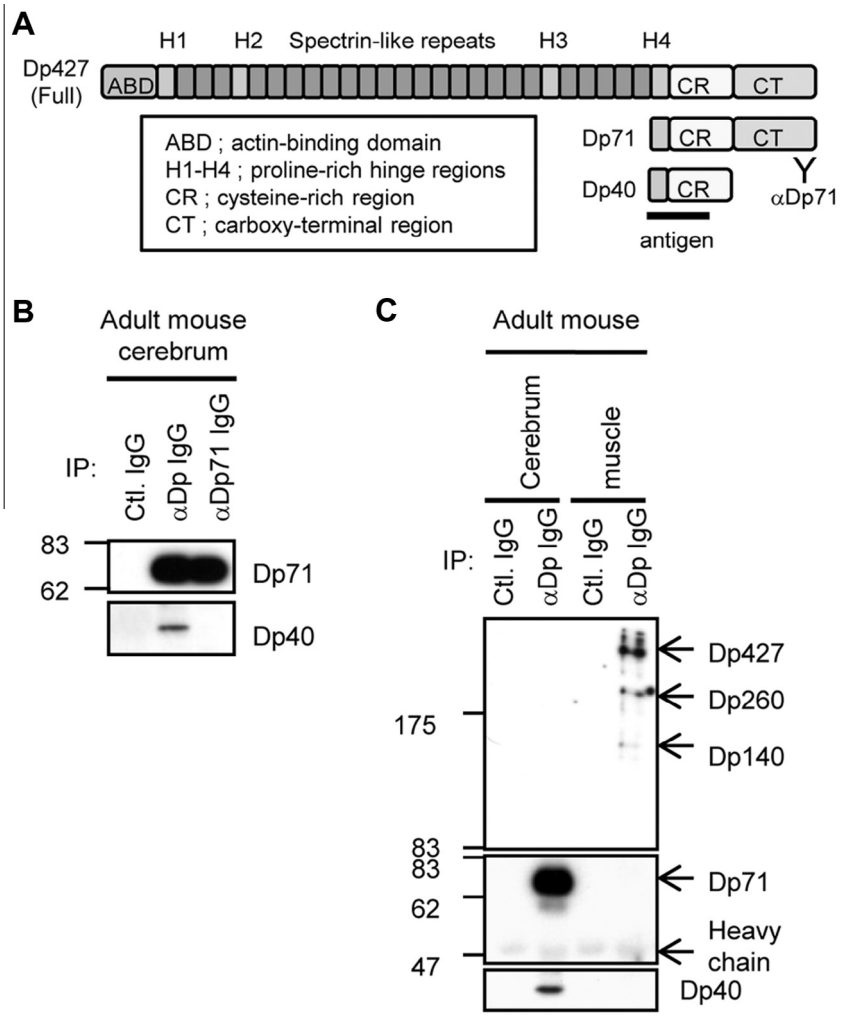

Fig. 1. Generation of the anti-Dp40 antibody. (A) Schematic illustrations of full-length (full) dystrophin (Dp427) and the short isoforms of dystrophin, named Dp71 or Dp40 proteins. The recombinant protein spanning the NH_2-terminal region of the Dp40, designated by the thick bar, was used as an antigen for the production of the anti-Dp40 antibody. The anti-Dp71 antibody (αDp71), purchased from Abcam, recognizes the COOH-terminus of Dp71. (B) Anti-Dp40 (αDp), anti-Dp71 (αDp71), or control (Ctl.) IgG immunoprecipitations were performed using adult mouse cerebrum followed by Western blotting with anti-Dp40 antibody. (C) αDp or Ctl. IgG immunoprecipitations were carried out using adult mouse cerebrum or muscle followed by Western blotting with anti-Dp40 antibody. IP, immunoprecipitation.

was capable of detecting endogenous Dp40 protein expression in the mouse brains.

In order to examine the Dp40 expressions in the distinct brain regions, anti-Dp40 immunoprecipitations using cerebral cortex, hippocampus, and cerebellum from adult mouse brain were performed, showing that similar amounts of Dp40 protein were present in these brain regions (Fig. 2A, lower). In addition, Dp71 protein was also intensely detected in these regions (Fig. 2A, upper). Using cerebrums derived from different developmental stages, we found that the Dp40 protein was expressed from the early neonatal stages, such as postnatal 3, 7, 14, and 21 days, until adulthood (Fig. 2B, lower), although the Dp71 protein expressions were evenly detected through the prenatal and postnatal stages (Fig. 2B, upper), indicating that the Dp40 expression was temporally and differentially regulated in comparison to Dp71 expression in the brain. In order to further clarify the expression profile of Dp40 in the brain, immunoprecipitations using dissociated primary cultured cells were carried out, revealing that Dp40 was abundant in the hippocampal neurons, rather than the hippocampal non-neuronal cells (Fig. 2C, lower). In contrast, Dp71 protein was robustly detected in the non-neuronal cells, compared with the hippocampal neurons (Fig. 2C, upper). These results suggested that Dp40 has a characteristic feature as a neuron-type Dp and functions during neuronal maturation after birth, as well as in the mature neurons.

3.2. Somatodendritic and nuclear localization of Dp40 protein in the cultured hippocampal neurons

Next, we examined the subcellular localization of the Dp40 protein. Because of the lack of antibodies specific for Dp40, we determined the localization of ectopic HA-tagged Dp40 in transfected cultured hippocampal neurons. After three weeks *in vitro*, Dp40-HA was detected in the cell bodies, including the DAPI-labeled nucleus (Fig. 3A). Line scan analysis was used to show the relative intracellular distribution of Dp40-HA, DAPI, or PSD95 within the same field, revealing that Dp40-HA was targeted to the subplasmalemmal region as well as the nucleus (Fig. 3B). Interestingly, when we focused on the neurite region of cultured hippocampal neurons, we found that Dp40-HA was localized only in the MAP2-labeled dendrites, but not in the MAP2-negative axons (Fig. 3C, middle and right), whereas ZsGreen was distributed within both of these neuronal processes (Fig. 3C, left). These observations indicated that

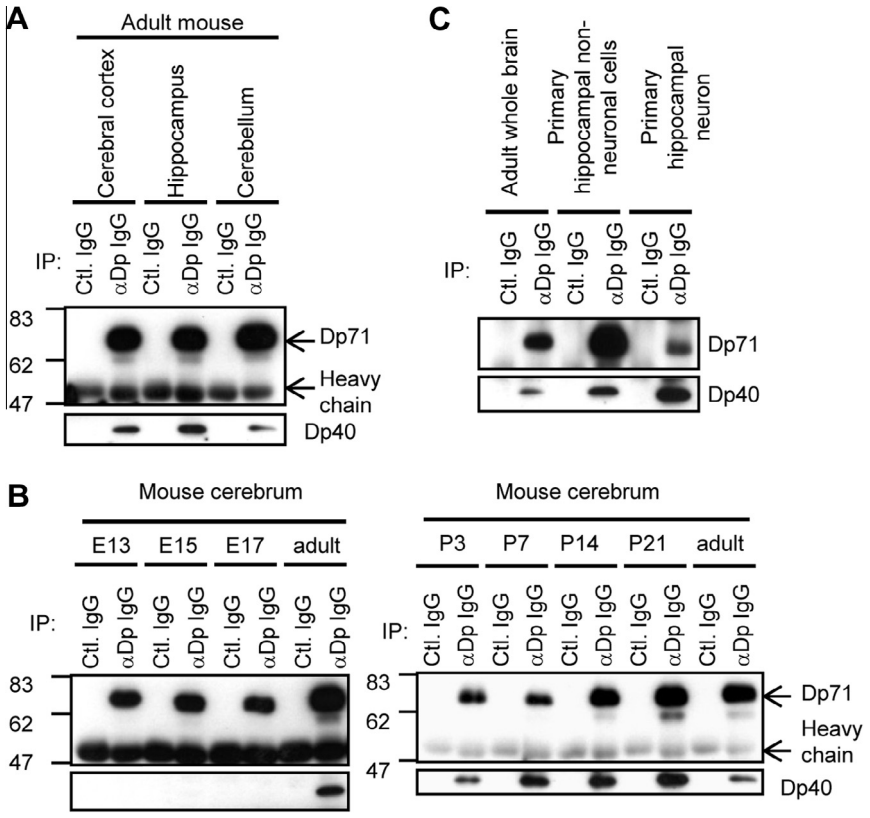

Fig. 2. Dp40 protein expression profile in the mouse brain (A) αDp or Ctl. IgG immunoprecipitations were performed using adult mouse cerebral cortex, hippocampus, or cerebellum followed by Western blotting with anti-Dp40 antibody. (B) αDp or Ctl. IgG immunoprecipitations were carried out using embryonic, neonatal, or adult mouse cerebrum followed by Western blotting with anti-Dp40 antibody. E, embryonic day; P, postnatal day. (C) αDp or Ctl. IgG immunoprecipitations were carried out using adult mouse whole brain, primary cultured hippocampal non-neuronal cells, or primary cultured hippocampal neurons, followed by Western blotting with anti-Dp40 antibody. IP, immunoprecipitation.

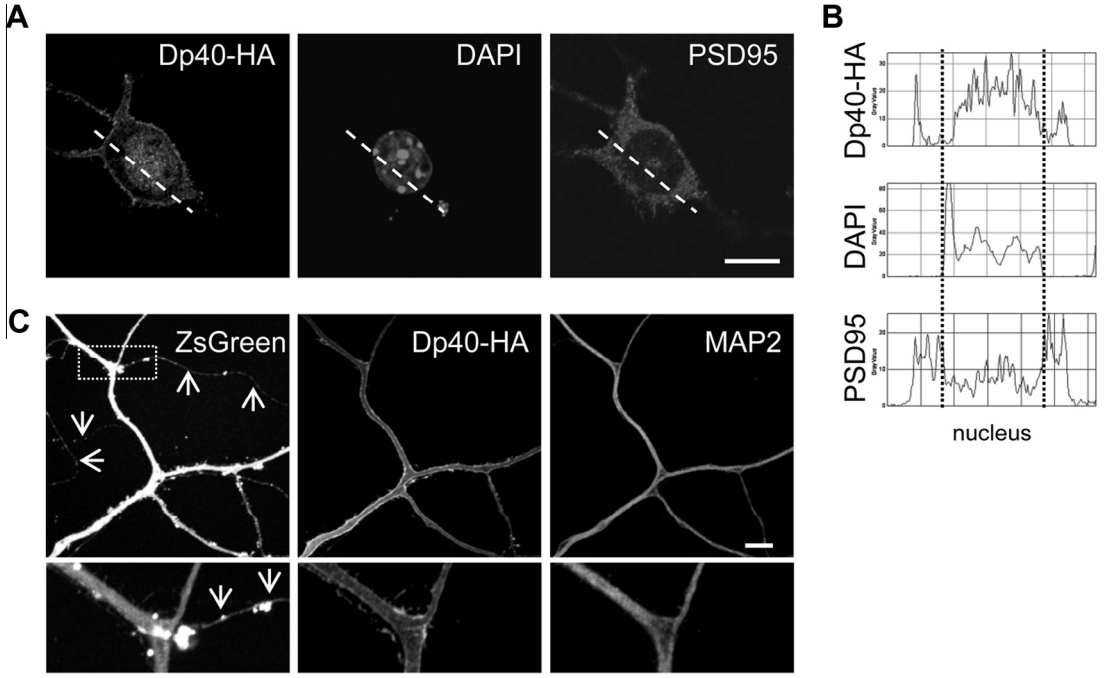

Fig. 3. Somatodendritic and nuclear localization of Dp40 protein in the cultured hippocampal neurons. (A) Fluorescent confocal images of primary hippocampal neurons transfected with HA-tagged Dp40, followed by staining with anti-HA (left), anti-PSD95 (right) antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) (middle). (B) Line scan analysis (region examined indicated by the white dashed line) showing the relative distributions of Dp40-HA, nucleus, or PSD95, as determined by their fluorescence intensities. (C) Fluorescent confocal images of primary hippocampal neurons transfected with HA-tagged Dp40, followed by staining with anti-HA (middle), anti-MAP2 (right) antibodies, or ZsGreen fluorescence (left). Lower panels were magnified images of the region indicated by the dashed rectangle in the upper panel. Arrows show MAP2-negative axon. Scale bar, 10 μm.

the Dp40 protein is a somatodendritic protein that may be involved in dendritic functions.

3.3. Dp40 protein is localized in excitatory postsynapses of the cultured hippocampal neurons

We further addressed the cellular distribution of Dp40-HA in cultured hippocampal neurons by comparing with synapse markers, showing that Dp40-HA was prominently accumulated in the excitatory postsynaptic sites, spines, which were labeled with PSD95 (Fig. 4A–C). It is worthy to note that Dp40-HA was also localized in the subplasmalemmal region of the dendritic shafts (Fig. 4A–C). Conversely, a presynaptic marker, synaptophysin, was not overlapped with Dp40-HA (Fig. 4D), indicating that Dp40 does not exist within the presynapses, which is consistent with the absence of Dp40-HA in axons (Fig. 3D). Additionally, although an inhibitory postsynapse marker, gephyrin, was intensely detected as puncta in the dendritic shaft, Dp40-HA was rarely found colocalized with gephyrin (Fig. 4E). Thus, the Dp40 protein was preferentially targeted toward the excitatory postsynaptic sites in hippocampal neurons.

4. Discussion

The proper expression and function of Dp427, a *DMD* gene product, is essential for the maintenance and regulation of muscle physiology [1,2,7,8]. However, due to complexities in the *DMD* gene expression and in the brain structure and functions, the causative molecules and regulatory mechanisms underlying the cognitive impairment manifested in DMD patients remain to be elucidated. In this study, we demonstrated that the shortest isoform of Dp, Dp40, is a neuron-type Dp. The subcellular localization of Dp40 is distributed somatodendritically and accumulated in the subplasmalemmal region and excitatory postsynaptic sites, suggesting the molecular relevance of Dp40 to the dendritic functions and/or synaptic functions.

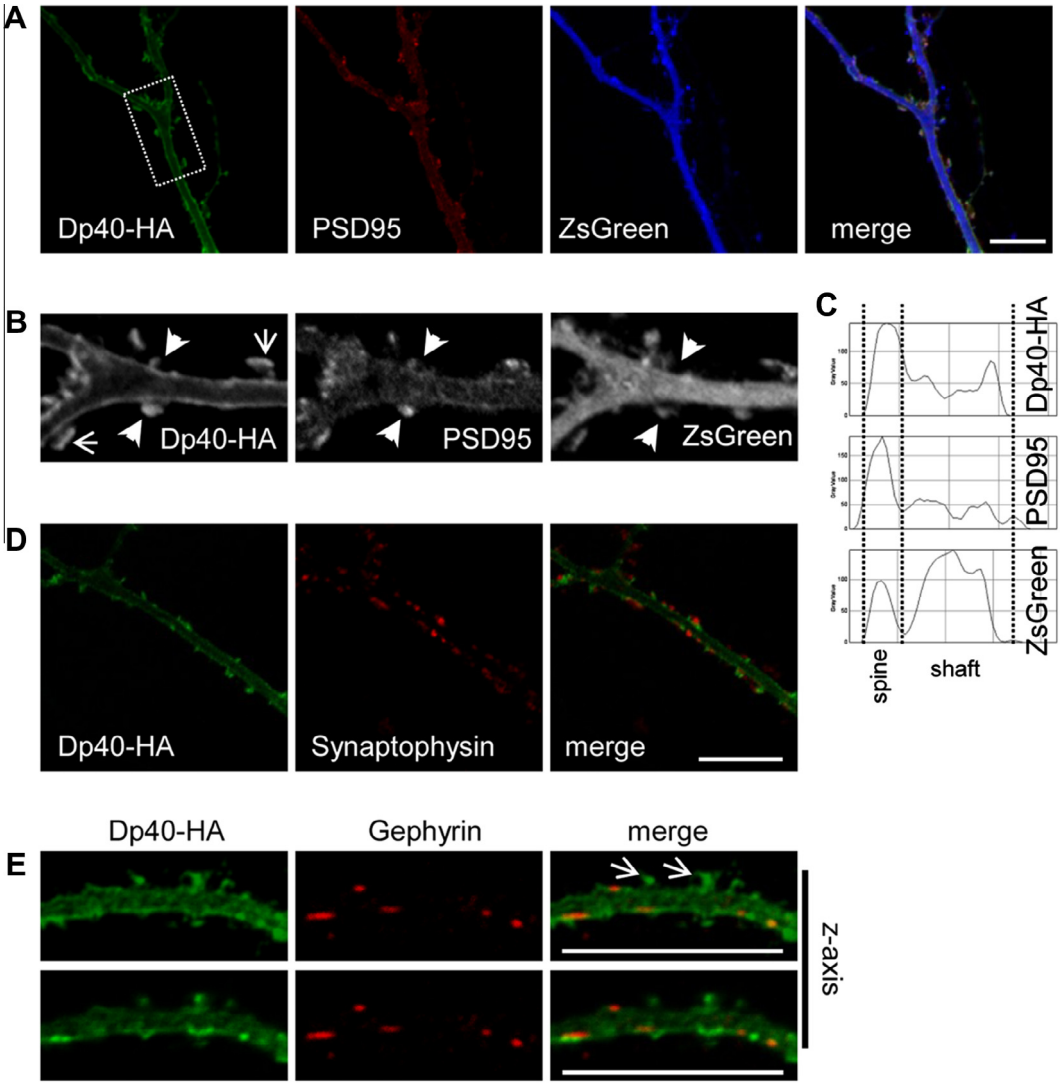

Fig. 4. Dp40 protein was localized in the excitatory postsynapses of the cultured hippocampal neurons. (A) Fluorescent confocal images of primary hippocampal neurons transfected with HA-tagged Dp40, followed by staining with anti-HA (green), anti-PSD95 (red) antibodies, or ZsGreen fluorescence (blue). (B) High magnification images of the region indicated by the dashed rectangle in (A). The arrows show typical PSD95-positive postsynaptic spines on the dendritic shaft. (C) Line scan analyzing the region sandwiched between the two arrowheads in (B). The relative distributions of Dp40-HA, PSD95, or ZsGreen, were determined by their fluorescence intensities. (D) Fluorescent confocal images of primary hippocampal neurons transfected with HA-tagged Dp40, followed by staining with anti-HA (green) and anti-synaptophysin (red) antibodies. (E) Fluorescent confocal images of primary hippocampal neurons transfected with HA-tagged Dp40, followed by staining with anti-HA (green) and anti-gephyrin (red) antibodies. Two images (upper and lower) were collected at different levels perpendicular to the optical axis (the *z*-axis) within a specimen. The arrows indicate postsynaptic spines on the dendrite. Scale bar, 10 μm.

Previous studies have shown that Dp71 is an abundant isoform in the brain, and that Dp71 is expressed in both neurons and the glia [5]. In this study, we showed that Dp40 emerged in cerebrums after birth and that the stage-dependent expression profile of Dp40 was distinct from that of Dp71 (Fig. 2B). Furthermore, Dp40 and Dp71 were abundant in cultured neurons and non-neuronal cells, respectively (Fig. 2C), suggesting that Dp40 and Dp71 have a characteristic feature of neuron-type Dp and glia-type Dp, respectively. This concept is consistent with the fact that Dp71 is highly expressed in perivascular astrocytes [3]. As Dp40 is produced by alternative splicing of the Dp71 transcript [13], it is possible that the regulatory factors involved in the conversion between Dp40 and Dp71 expressions would be activated in cell-type- and stage-dependent manners.

Our observation that Dp40 was localized in the subplasmalemmal region of dendrites in cultured hippocampal neurons is intriguing (Figs. 3A, 3B, 4B and 4C). Considering the fact that Dp427 and the other Dp isoforms, including Dp71, are localized beneath the plasma membranes of muscle and/or neuronal cells by interacting with dystroglycan-related molecules [5–8,15], it is possible that common molecular mechanisms will be involved in the Dp40 localization. However, we previously showed that Dp40 is not likely associated with membrane-spanning glycoproteins [14], suggesting that a particular molecular mechanism different from that related to dystroglycan-related molecules might be responsible for the Dp40 localization. Thus, we must consider the question, "What kinds of molecules are responsible for the Dp40 localization?" As Dp427 and dystroglycan have been reported to be restricted to the inhibitory postsynapses in the hippocampal neurons [6,16], it is notable that Dp40 was rarely colocalized with gephyrin, an inhibitory postsynaptic scaffold (Fig. 4E). Considering that Dp40 was found accumulated in the excitatory postsynaptic sites (Fig. 4A and B) like Dp71, which was previously described as a component of scaffold for structural and signaling molecules in the dendritic spines [5,17], scaffold molecules and cytoskeleton-related molecules orchestrated in the excitatory synaptic structure and their functions may be involved in the Dp40 localization. Most importantly, Dp71-null mice displayed abnormal synaptic organization and maturation *in vivo* and altered synaptic plasticity in the CA1 region of the hippocampus [17,18]. Since Dp40 was also deficient in the Dp-71 null mice, it is plausible that Dp40 was partly responsible for the defects observed in the mice.

Furthermore, it is worthy to note that Dp40 was localized in the nucleus of cultured hippocampal neurons (Fig. 3A). As nuclear localization of the particular Dp71 isoform was also reported [19–22] and the conventional nuclear transporter, importin, seemed to mediate the nuclear import of Dp71 [23], it is pertinent to take into account that Dp40 might have a putative nuclear function. Thus, further investigations concerning the molecular functions of Dp40 in the nucleus, as well as in the dendrites of neurons, will provide a better understanding of various neuronal biological processes.

Although there is no exact correlation between the location of the mutation in the *DMD* gene and the severity of the cognitive impairment observed in DMD patients, mutations in the distal region of the *DMD* gene are closely associated with cognitive impairment [10–12], suggesting that a precise understanding of the full spectrum of the activities of Dp short isoforms, including Dp71 and Dp40, may lead to the development of medicines for patients suffering from cognitive disease.

Conflict of interest statement

The authors hereby declare that there are no conflicts of interest related to this study.

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