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ARTICLE

C9orf10 Protein, a Novel Protein Component of Purα-containing mRNA-protein Particles (Purα-mRNPs): Characterization of Developmental and Regional Expressions in the Mouse Brain

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SUMMARY Purα has been implicated in mRNA transport and translation in neurons. We previously reported that Purα is a component of mRNA/protein complexes (Purα-mRNPs) with several other proteins. Among them, we found the C9orf10 (*Homo sapiens* chromosome 9 open reading frame 10) protein, which was recently characterized as a component of RNA-containing structures. However, C9orf10 itself remains poorly understood. To characterize C9orf10 expression at the protein level, we raised an antibody against C9orf10 and compared the spatial and developmental expressions of this protein and Purα in the mouse brain. C9orf10 was expressed as early as embryo stage 12, whereas Purα was expressed from 5 days after birth. In adults, C9orf10 expression was most prominent in the hippocampus, caudate putamen, cerebral cortex, and cerebellum, unlike the uniform distribution of Purα. C9orf10-positive cells also showed immunoreactivity to Purα. C9orf10 expression was restricted to neurons, judging by the immunoreactivity to neuron-specific nuclear protein or CaM kinase II. These observations suggest an accessory role of C9orf10 for Purα in a limited brain region in addition to other possible functions that have not yet been determined.

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KEY WORDS
Purα
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C9orf10 (*Homo sapiens* chromosome 9 open reading frame 10) was originally found by the human genome sequence project as an annotated protein, and the gene was mapped to chromosome 9q22.31. Thus far, this protein has been detected in some RNA-containing structures, such as mRNA granules (Bannai et al. 2004), polyribosomal poly(A)mRNA–mRNA/protein complexes (mRNPs) (Angenstein et al. 2005), and spliceosomes (Rappsilber et al. 2002). However, there is not yet any functional information about this protein.

In a previous study to determine the binding partners of Purα in the neuronal cytoplasm, we isolated polyribosome-containing structures using anti-Purα antibody (Ohashi et al. 2002). Purα has now been found in a multifunctional protein that binds to single-stranded DNA and RNA; it has also been implicated in DNA replication and transcription, cell proliferation (Gallia et al. 2000), mRNA transport (Ohashi et al. 2000, 2002; Kanai et al. 2004; Johnson et al. 2006), and translation repression (Gallia et al. 2001).

These biological aspects of Purα may be attributable to various interacting proteins (Johnson et al. 1995; Darbinian et al. 1999; Safak et al. 1999; Tretiakova et al. 1999; Ohashi et al. 2002). We have further examined proteins immunoprecipitated using anti-Purα antibody by proteomic analysis and identified 15 additional proteins (unpublished data), of which a mouse ortholog of human C9orf10 protein was detected. C9orf10 is known to belong to a family of putative transmembrane proteins that includes CXorf17 and BC012177 (Holden and Raymond 2003). CXorf17 has been considered a novel candidate protein for one of the many uncharacterized disorders that map to the human chromosome Xp11.22 (Holden and Raymond

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2003), suggesting that C9orf10 may also play a role in neurons. Purα is also known to be indispensable to both postnatal development and the differentiation of certain types of neurons in the mouse brain (Khalili et al. 2003), including synaptogenesis. Thus, it is probable that C9orf10 may, together with Purα, play a crucial role in the development of brain functions. Therefore, it is of great interest to characterize the expression of C9orf10 in the brain in reference to Purα. In this report, we refer to this mouse ortholog as C9orf10 for convenience.

Materials and Methods

Sources of Antibodies

Rabbit anti-Purα antibody was raised by our laboratory and affinity purified as described previously (Ohashi et al. 2002). The affinity purified antibody was used at a concentration of 1:5000 dilutions for Western blot analysis and 1:200 dilutions for immunohistochemistry (IHC).

Rabbit anti-C9orf10 antibody was raised in our laboratory against mixed peptides corresponding to amino acids 413-426 (QNSYSNIPHEGKHT; referred to as JB204) and 1062–1075 (TGDPRVPSHSESAL; referred to as JB205) of C9orf10 (NCBI accession number NP_001028440.2), whose sequences have no meaningful homology with other members of this gene family, such as CXorf17 and BC012177, and was affinity purified using the peptides. The affinity purified antibody was used at a concentration of 1:2000 dilutions for Western blot analysis and 1:200 dilutions for IHC.

The other antibodies used were obtained from the following sources. Rabbit anti-S6 ribosomal protein antibody was obtained from Cell Signaling Technologies (Danvers, MA) and used at 1:2000 dilutions for Western blot analysis. Mouse anti-CaM kinase II (CaMKII) antibody, clone 6G9, which reacts with α-isoform (Erondu and Kennedy 1985), was purchased from Affinity BioReagents (Golden, CO) and used at 1:100 dilutions for IHC. Mouse anti-neuronal nuclei (NeuN) antibody was purchased from Chemicon International (Temecula, CA) and used at 1:300 dilutions for IHC. Alkaline phosphatase–conjugated anti-rabbit IgG was purchased from Promega (Madison, WI) and used at 1:7500 dilutions for Western blot analysis. Biotinylated anti-rabbit IgG was purchased from Vector Laboratories (Burlingame, CA) and used at 1:200 dilutions for IHC. Alexa Fluor 488–conjugated anti-rabbit IgG and Alexa Fluor 555–conjugated anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA) and used at 1:200 dilutions for IHC.

Animals

Pregnant and adult male mice (ddY strain) were purchased from Japan SLC (Hamamatsu, Japan). The use of animals was approved by the Ethics Review Committee for Animal Experimentation of Nihon University.

Preparation of Tissue Extracts

To obtain mouse brains, mice were killed with an overdose of ether. The brains were quickly removed and immediately washed with ice-cold PBS. To obtain embryo brain samples, pregnant mice were killed with an overdose of ether, and the embryos were quickly removed, washed with ice-cold PBS, and placed on ice in PBS. The embryo brains were quickly removed using forceps under microscope and immediately washed with ice-cold PBS. All the following steps were performed at 0–4C unless otherwise stated. Mouse embryos and adult male brains were homogenized with a Teflon tissue grinder in TKM buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 250 mM sucrose) with 1 mM PMSF and 100 μg/ml cycloheximide (CHX), and the homogenate was centrifuged at 10,000 × g for 15 min to yield postmitochondrial supernatant (PMS). The PMS was centrifuged at 130,000 × g for 1 hr, resulting in supernatant (S100) and pellet (P100) fractions. The P100 fraction was resuspended in TKM buffer, and solid KCl was added to a final concentration of 150 mM. The sample was kept on ice for at least 15 min and centrifuged at 10,000 × g for 5 min to remove insoluble materials. The supernatant was used as P100 extracts.

Western Blot Analysis

Western blot analysis was performed as described previously (Ohashi et al. 2000). In brief, samples were electrophoresed and blotted onto a polyvinylidene di-

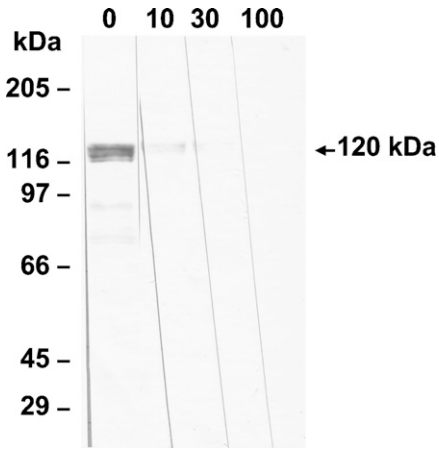

Figure 1 The anti-C9orf10 (*Homo sapiens* chromosome 9 open reading frame 10) antibody specifically recognizes a 120-kDa band in Western blot analysis. P100 extracts from a 4-week-old mouse brain were analyzed, and a 120-kDa band was detected by the anti-C9orf10 antibody (Lane 0). The 120-kDa band signal dose-dependently disappeared by preincubation with C9orf10 peptide JB205 (Lanes 10–100 depict the fold amount of the antibody).

fluoride (PVDF) membrane. Antibody against protein of interest was incubated with the membrane, and the bound antibody was detected by alkaline phosphatase–conjugated secondary antibody followed by visualization with nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate p-toluidine salt. The specificity of the anti-C9orf10 antibody was confirmed by preincubation of the antibody with 10- to 100-fold amounts of JB205 peptide overnight at 4C before processing for Western blot analysis.

Immunoprecipitation Analysis

Immunoprecipitation analysis was performed as described previously (Ohashi et al. 2002). In brief, P100 extracts (100 μg of protein in 250 μl) were incubated with 2.5 μg (in 2.5 μl) of the appropriate antibody for 2 hr at 4C in TKM buffer with 100 μg/ml CHX, 1 mM PMSF, and 0.1% Nonidet P-40 (NP40). For P100 extracts subjected to treatment with EDTA, the antibodies were added after the extracts had been treated with or without 25 mM EDTA on ice for 30 min. Subsequently, 40 μl of Dynabeads M-280 sheep anti-rabbit IgG (Invitrogen) was added, and the reaction mixtures were incubated for an additional 4 hr at 4C. The beads were collected with a magnet and washed five times (5 min each time) in 200 μl of TKM buffer containing 0.1% NP40. The beads were washed for an additional 15 min, after which the immune complex was eluted in 40 μl of SDS-containing sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol). Immunoprecipitated proteins were analyzed by Western blot analysis as described previously (Ohashi et al. 2000).

Fixation of Tissue Samples

To obtain embryo samples, pregnant mice were killed with an overdose of ether, and the embryos were quickly removed. The embryos were fixed with 4% paraformaldehyde (PFA) in PBS. To obtain brain samples, the adult male mice were anesthetized with pentobarbital, and they were perfused transcardially with PBS followed by 4% PFA in PBS. Their brains were dissected and postfixed overnight in 4% PFA in PBS. The embryos and brains were immersed in 30% sucrose in PBS at least overnight. After sinking, they were embedded in Optimal Cutting Temperature Compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), frozen, and sliced at 10 μm by cryostat.

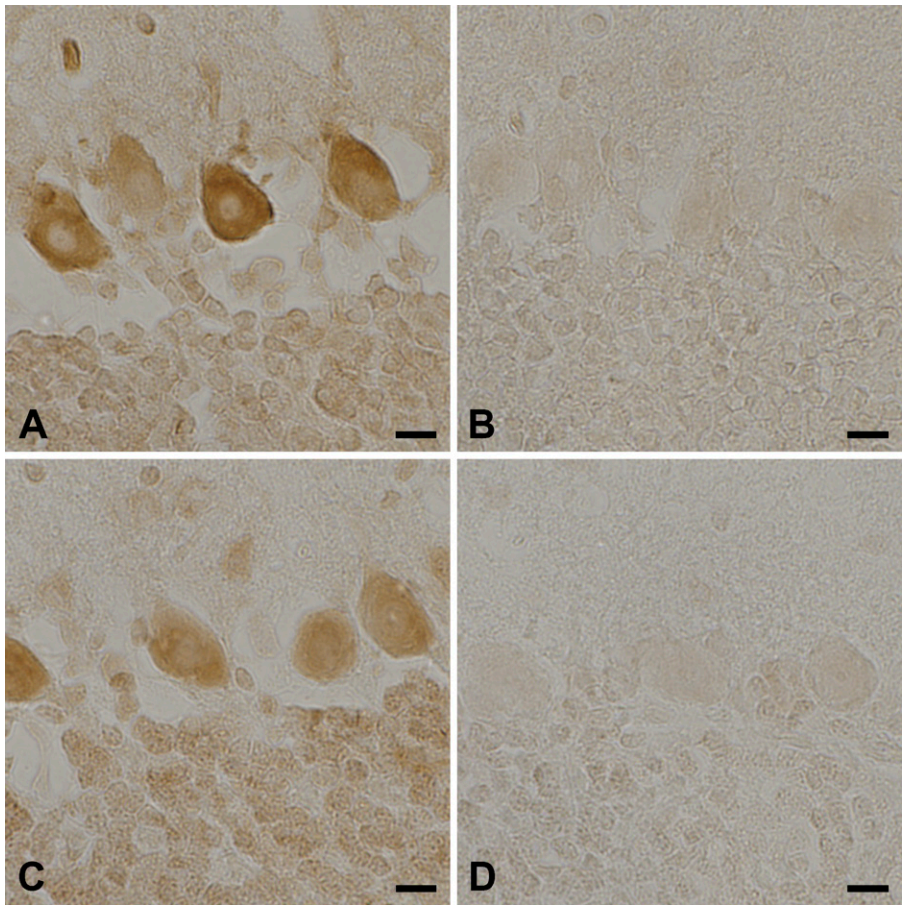

Figure 2 Anti-C9orf10 antibody and anti-Purα antibody specifically stained by IHC. Four-week-old mouse brains were fixed with 4% paraformaldehyde in PBS, and the cerebella were sectioned. Sections were incubated with each antibody, and the bound antibody was detected using the avidin-biotin-peroxidase complex system followed by development with DAB. (**A**) Anti-C9orf10 antibody. (**B**) Anti-C9orf10 antibody preincubated with a 30-fold amount of JB205 peptide. (**C**) Anti-Purα antibody. (**D**) Anti-Purα antibody preincubated with a 30-fold amount of Purα peptide. Bar = 10 μm.

IHC

The sections on glass slides were dried, washed in PBS, and heated at 121C for 3 min in 10 mM sodium citrate buffer (pH 6.0). After the slides were cooled to room temperature, the sections were washed in PBS and incubated in methanol containing 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. The sections were incubated with 2% normal donkey serum in PBS containing 0.3% Tween 20 (blocking solution) at room temperature before processing for IHC. The sections were incubated with the appropriate primary antibody in blocking solution at 4C overnight and were washed with PBS containing 0.1% Tween 20 (PBST). The bound antibodies were detected using the avidin-biotin-peroxidase complex system (Vector Stain Elite ABC Kits; Vector Laboratories) followed by development with DAB (DAB Substrate Kit; Vector Laboratories) in accordance with the manufacturer's instructions.

The specificity of the anti-C9orf10 antibody was confirmed by preincubation of the antibody with a 30-fold amount of JB205 peptide overnight at 4C before processing for IHC. The specificity of the anti- Purα antibody was confirmed by preincubation of the antibody with a 30-fold amount of Purα peptide overnight at 4C before processing for IHC.

Double labeling with anti-C9orf10 and anti-NeuN antibodies or anti-C9orf10 and anti-CaMKII antibodies was accomplished by the simultaneous application of both antibodies. After the sections were incubated with the first antibodies, the sections were incubated with Alexa Fluor 488–conjugated anti-rabbit IgG and Alexa Fluor 555–conjugated anti-mouse IgG for 1 hr at room temperature and washed with PBST. As the negative control, specimens were labeled with the secondary antibodies alone, and none of the control sections showed positive immunoreactivity.

In general, for double labeling performed with antibodies raised against the same species, e.g., rabbit, one-by-one staining with chromogen is well documented (Lan et al. 1995; Xie et al. 2001). However, this method makes it difficult to detect similarities in staining patterns. To study whether or not C9orf10 and Purα are expressed in the same cell, double labeling with anti-C9orf10 and anti-Purα antibodies was conducted under the combination of fluorochrome and chromogen. The first IHC staining for C9orf10 was performed with Alexa Fluor 488–conjugated anti-rabbit IgG and photographed. The slides were soaked in cold PBS overnight to remove the coverslips, followed by washing in PBS and heating at 121C for 3 min in 10 mM sodium citrate buffer (pH 6.0) to inactivate all antibodies bound to the tissue. The second IHC staining for Purα was performed with the avidin-biotin-peroxidase complex system, followed by development with DAB.

Results

Characterization of Antibody Against C9orf10 Peptide

To study C9orf10 expression at the protein level in the mouse brain, we raised an antibody against C9orf10 in rabbit using two peptides (JB204 and JB205). Figure 1 shows that Western blot analysis detected one prominent protein band with an apparent molecular mass of 120 kDa. This band disappeared when the antibody was preabsorbed with peptide JB205 but not with peptide JB204 (data not shown), suggesting that the antibody specifically recognized C9orf10 through peptide JB205. This antibody recognized neurons such as Purkinje cells (Figures 2A and 2B), and immunoreactivity was diminished by preincubation

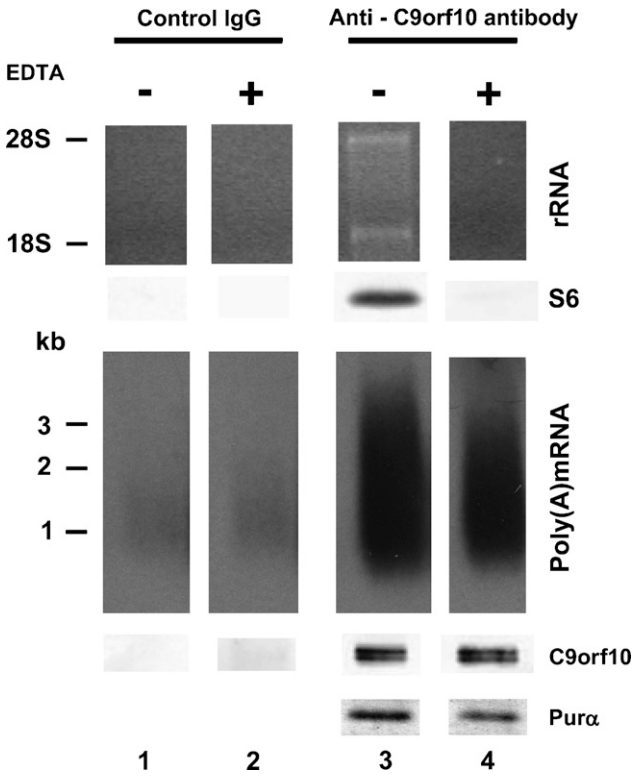

Figure 3 Immunoprecipitation analysis of P100 extracts of the brain using anti-C9orf10 antibody. Immunoisolated proteins using control IgG (Lanes 1 and 2) or anti-C9orf10 antibody (Lanes 3 and 4) from P100 extracts of the brain treated with 0.1% NP40 (Lanes 1 and 3) or 0.1% Nonidet P-40 (NP40) plus 25 mM EDTA (Lanes 2 and 4) were separated by SDS-PAGE, followed by Western blot analysis using antibodies against proteins of interest (panels S6, C9orf10, and Purα). RNA was purified from the immunoprecipitate and analyzed by Northern blotting for the presence of poly(A)mRNA using a ^{32}P-end-labeled oligo(dT) as a probe [panel poly(A)mRNA]. S6, small ribosomal subunit protein S6. Positions of rRNAs are indicated on the left (28S, 18S). Only the eluted fractions are shown.

with peptide JB205. These results indicate that the antibody was specific to C9orf10 in Western blot analysis and IHC.

The specificity of the anti-Purα antibody was also examined for further experiments. The antibody also recognized Purkinje cells, and the immunoreactivity was diminished by preincubation with Purα peptide (Figures 2C and 2D).

Coimmunoprecipitation of C9orf10 With Purα Using Anti-C9orf10 Antibody

First, we confirmed that C9orf10 is a protein component of Purα-mRNPs using the anti-C9orf10 antibody; C9orf10 had previously been detected by mass spectrometry analysis of proteins pulled down by the anti-Purα antibody, which also contained poly(A)mRNAs (unpublished data). Figure 3 shows that the antibody pulled down Purα, rRNAs, and proteins (S6) and poly(A)mRNAs in addition to C9orf10 (Lane 3), suggesting that polyribosomes containing both proteins were immunoprecipitated. Moreover, no significant reduction was observed in the levels of Purα and poly(A)mRNAs after the P100 extracts were treated with EDTA, but ribosomes (rRNA and S6) were no longer detected (compare Lane 4 with Lane 3). Together, these results suggest that C9orf10 is associated with polyribosomal Purα-mRNPs but not with ribosomes.

Developmental Expression of C9orf10 in the Mouse Brain

We determined the changes in C9orf10 expression during mouse development. Figure 4 shows that C9orf10 was expressed in the embryonic brain [at 12 days after gestation (E12)] at a level similar to that of neonatal brain (5 days after birth), whereas no Purα was detected at E12, showing that C9orf10 was expressed at an earlier stage of development than was Purα. C9orf10 expression in the brain increased postnatally and was maintained at an adult level beyond 4 weeks after birth. In contrast to C9orf10, Purα was expressed from 5 days after birth, and the expression was maximal beyond 4 weeks.

Spatial Expression of C9orf10 in the Mouse Brain

The spatial expression of C9orf10 was analyzed by IHC (Figure 5). At 4 weeks after birth, both C9orf10 and Purα became strongly expressed, but some differences in regional expression were also noted (boxed regions in Figure 5, panels M vs N, O vs P, Q vs R, and S vs T). Higher magnifications of the boxed regions are shown in Figures 5C–5T. Essentially, Purα was detected in all of the brain regions examined, whereas the levels of C9orf10 were much lower in particular regions of the brain, including the superior colliculus (Figure 5M), mid-brain (Figure 5O), pons (Figure 5Q), and the granular layer of the cerebellum (gl in Figure 5S). These results suggest that the regional expression in the brain of C9orf10 is differentially regulated, whereas the C9orf10-expressing region was almost always included in Purα-expressing brain regions.

Coexpression of C9orf10 With Purα in Cells in IHC

IHC analyses showed that C9orf10 and Purα proteins were coexpressed in some restricted brain regions (Fig-

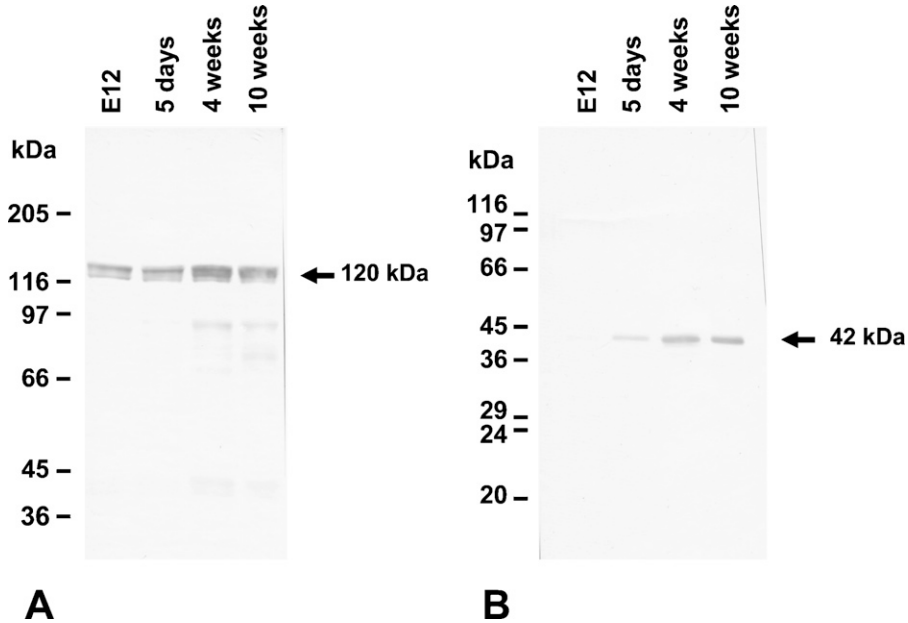

Figure 4 Developmental expression of C9orf10 and Purα in the mouse brain. P100 extracts from developing mouse brains [12 days after gestation (E12) and postnatally at 5 days, 4 weeks, and 10 weeks] were used for Western blot analysis. C9orf10 was detected as a 120-kDa band (**A**), and Purα was detected as a 42-kDa band (**B**).

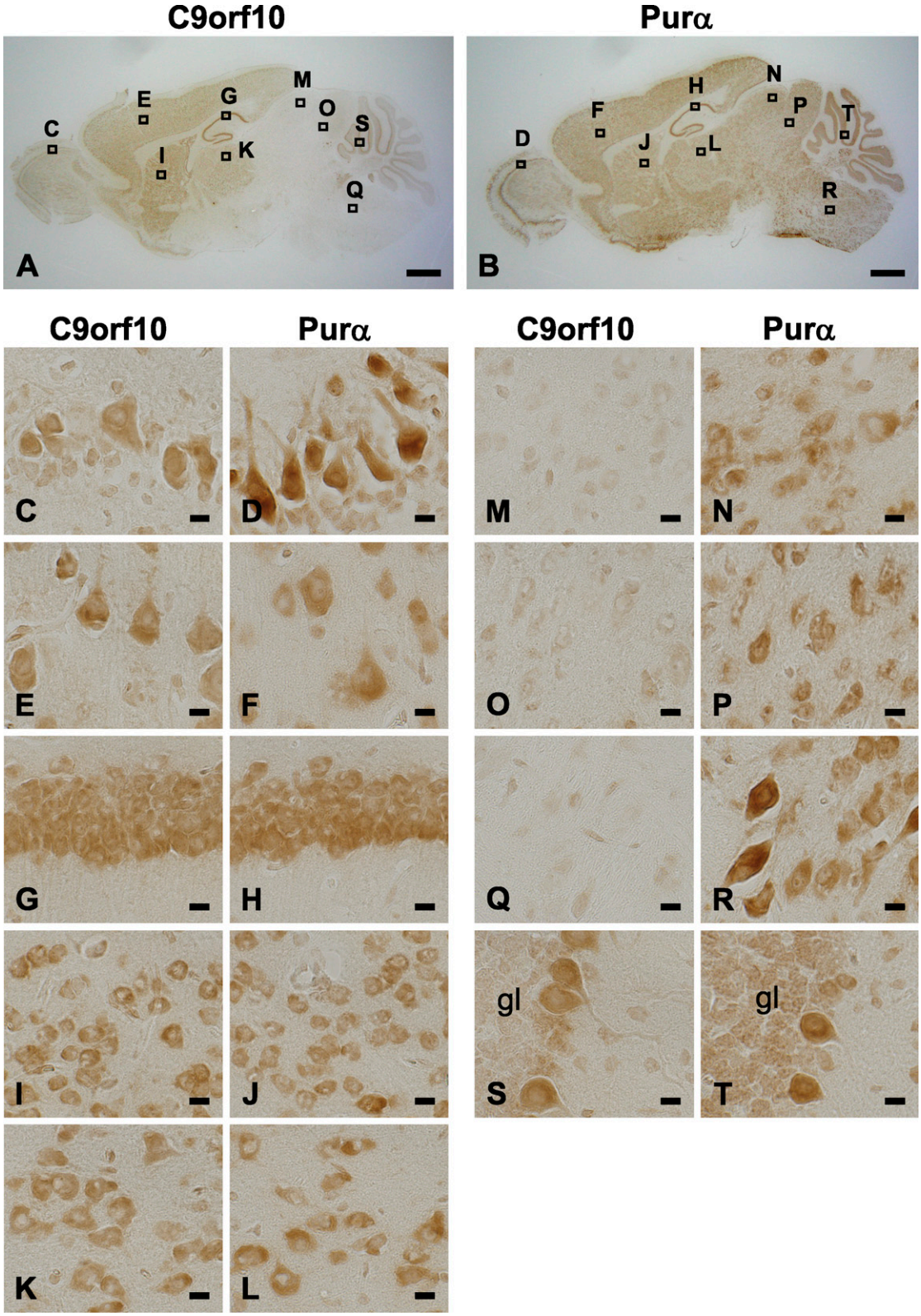

Figure 5 Spatial localization of C9orf10 and Purα. A 4-week-old mouse brain was dissected and DAB stained using anti-C9orf10 antibody (left panel) or anti-Purα antibody (right panel). (**A,B**) Lower magnification. Boxes indicate the locations of higher magnification of **C–T.** (**C,D**) Olfactory bulb. (**E,F**) Cerebral cortex. (**G,H**) Hippocampus. (**I,J**) Caudate putamen. (**K,L**) Thalamus. (**M,N**) Superior colliculus. (**O,P**) Midbrain. (**Q,R**) Pons. (**S,T**) Cerebellum. gl, granular layer. Bars: **A,B** = 1 mm; **C–T** = 10 μm.

ure 5). Furthermore, both proteins were coimmunoprecipitated by anti-Purα antibody (data not shown) or anti-C9orf10 antibody (Figure 3). Nevertheless, this does not necessarily mean that C9orf10 and Purα were coexpressed in the same cells. Hence, to unambiguously show the coexpression of the two proteins in cells, we focused on the cerebral cortex in IHC. For simultaneous staining with antibodies against C9orf10 or Purα, we adopted a unique method using fluorescent labeling followed by DAB staining, because both antibodies to be used were raised in rabbits. First, we incubated cells with anti-C9orf10 antibody using Alexa Fluor 488–labeled secondary antibody, followed by heating in citrate buffer, pH 6.0, to inactivate antibodies, and then incubated them again with anti-Purα antibody with biotinylated secondary antibody using the avidin-biotinylated-peroxidase complex (ABC) and DAB. Judging from the immunoreactivity patterns (Figure 6), anti-C9orf10 antibody (Figures 6A and 6C) and anti-Purα antibody (Figures 6B and 6D) highlighted the same cells. Note also that residual C9orf10 immunoreactivity was abolished by heating, because biotinylated secondary antibody alone did not show immunoreactivity (Figures 6E and 6F). Thus, these results show that C9orf10 and Purα are coexpressed in the same cells in the cerebral cortex.

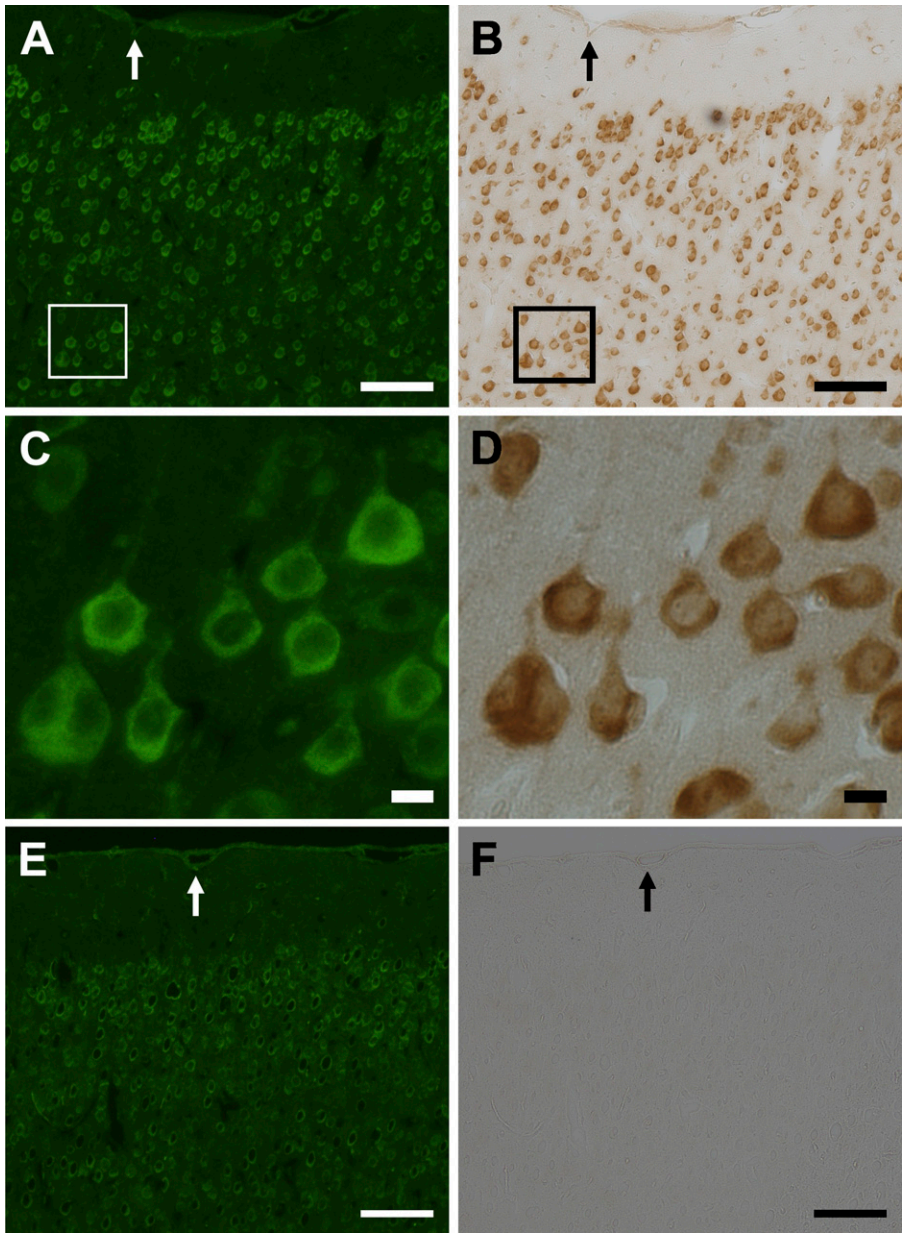

Figure 6 Colocalization of C9orf10 and Purα in cerebral cortex. Cerebral cortex slices were stained with anti-C9orf10 antibody (**A,C**) and subjected to antibody inactivation and then restained with anti-Purα antibody (**B,D**). **C** and **D** indicate higher magnification of boxes in **A** and **B**, respectively. C9orf10 and Purα were localized in the same cells. Complete inactivation of anti-C9orf10 antibody is shown in **F**. C9orf10 staining in **E** was diminished after autoclaving following incubation with the secondary antibody alone (no anti-Purα antibody, **F**). Arrows in **A** and **B** and those in **E** and **F** show the same location. Bars: **A,B,E,F** = 100 μm; **C,D** = 10 μm.

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Neuron-specific Expression of C9orf10 Protein in the Mouse Brain

The immunoreactivity pattern of C9orf10 suggested that expression was restricted to neuronal cells (Figure 5). As shown in Figure 7, this was indeed the case, because all of the cells exhibiting C9orf10 immunoreactivity were also detected by NeuN immunoreactivity in the hippocampus (Figures 7A–7C) and cerebral cortex (Figures 7D–7F). Anti-NeuN antibody detects the neuron-specific nuclear protein, NeuN (Mullen et al. 1992). Furthermore, C9orf10 was also coexpressed with CaMKIIα, the major postsynaptic density protein (Kennedy et al. 1983), in Purkinje cells (Figures 7G–7I), including the proximal portion of the primary dendrite (indicated by an arrow; inset). These results suggest that C9orf10 could play a role in mRNA metabolism in cooperation with Purα, including transport and translation of particular sets of mRNAs in the neuronal dendrites.

Discussion

This study showed that C9orf10 is associated with polyribosomal Purα-poly(A)mRNPs in a reverse immunoprecipitation analysis using anti-C9orf10 antibody (Figure 3), consistent with others' observations that C9orf10 is present in polyribosome-bound (translated) but not in polyribosome-free (non-translated) fractions from the rat cerebral cortex (Angenstein et al. 2005). We also showed that the expression of C9orf10 was detected as early as E12, which was earlier than Purα and maximal at 4 weeks. At 4 weeks, expression in the brain region was restricted and seemed to be included in the Purα-expressing region (Figure 3). These observations may give some hints on its function. One possible function is that C9orf10 cooperates with Purα in neurons coexpressing the two proteins at a translational level. Another possibility is that C9orf10 may escort Purα-containing mRNA subsets to the cytoplasm after processing nascent mRNAs within nuclei, because the protein is reportedly detected in the human spliceosomes (Rappsilber et al. 2002). The other possibility is that, as has been suggested for Purα (Ohashi et al. 2002; Kanai et al. 2004; Johnson et al. 2006), C9orf10 may also participate in mRNA transport in the cytoplasm. In this respect, C9orf10 is known to associate with an mRNA granule containing SYNCRIP, which carries inositol 1,4,5-triphosphate receptor type 1 mRNA in neuronal dendrites (Bannai et al. 2004). We also previously reported that Purα-mRNPs contain two distinct motor proteins (myosin Va and kinesin 5) in addition to one of the rough endoplasmic reticulum (rER) marker proteins, BiP, and we suggested that vesiculated rERs may be involved in the trafficking of Purα-mRNPs (Ohashi et al. 2002). In this context, C9orf10 could tether Purα-mRNPs to such vesicles that are derived from rER, because C9orf10 has amino acid sequences with the structural features of a transmembrane domain (Holden and Raymond 2003).

In conclusion, our findings suggest that C9orf10, in addition to other possible functions that have not yet been determined, plays a cooperative role with Purα

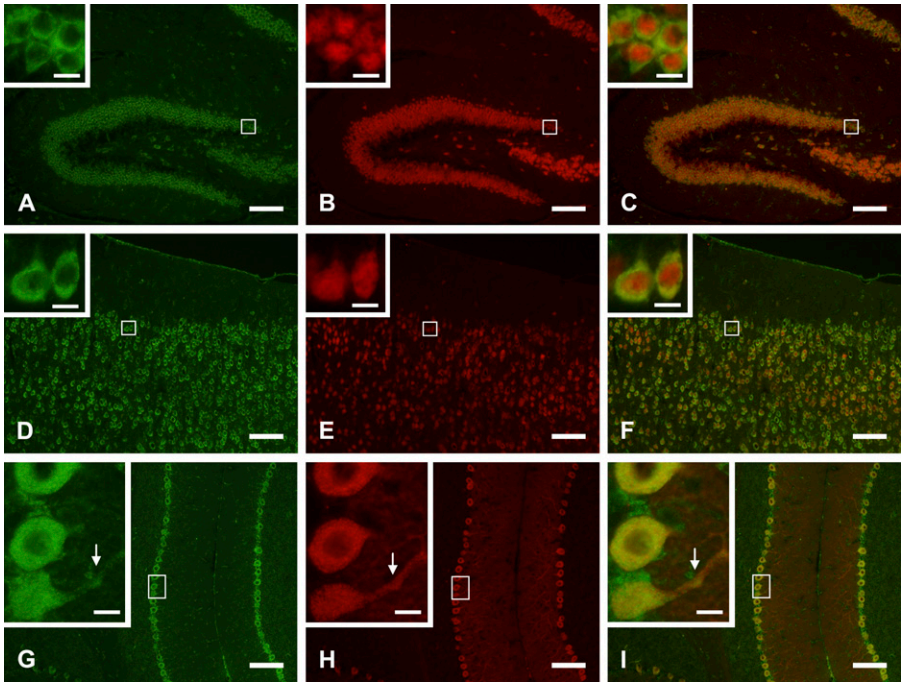

Figure 7 C9orf10 was localized in neurons. Hippocampus slices and cerebral cortex were double-stained using anti-C9orf10 antibody (**A,D**) or anti-neuronal nuclei antibody (**B,E**) and merged (**C,F**), respectively. The cerebellum was double-stained using anti-C9orf10 antibody (**G**) or anti-CaM kinase II antibody (**H**) and merged (**I**). Higher magnifications of cell bodies in boxes are shown in insets. Arrows indicate dendrites. Bars: panels = 100 μm; insets = 10 μm.

in adult mouse brain neurons. This should encourage precise analyses of Purα-mRNP regulation by C9orf10.

Acknowledgments

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