Belg. J. Zool., 138 (1) : 60-69 January 2008

Expression of Collapsin Response Mediator Protein-4 (CRMP-4) in Plastic Brain Areas of Adult Songbird Brain

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ABSTRACT. Collapsin response mediator protein-4 (CRMP-4), a member of CRMP family, is involved in neuronal differentiation and regulation of synaptic rearrangement. Neurite growth and axonal reorganization still continue in the adult songbird brain. It is probable for CRMP-4 to play an important role during these processes. To address this issue, we first obtained CRMP-4 cDNA from the brain of songbird (*Lonchura striata)* by use of RT-PCR and 3' rapid amplification of cDNA ends (3'RACE). We then examined the expression of CRMP-4 mRNA and protein in some plastic brain regions, including the hippocampus, song control nuclei and the cerebellum. Double immunohistochemistry was employed to identify the phenotypes of CRMP-4 expressing cells. The results indicated that: 1) a cloned fragment of CRMP-4 cDNA (798bp) showed high similarity with those comparable cDNA of reported species, confirming the evolutionary conservation of CRMP-4 sequence; 2) both CRMP-4 mRNA and protein were found to be dominantly located in the hippocampus, song control nuclei and the cerebellum among the whole brain; 3) some CRMP-4 positive cells were also labeled by the antibody for HuD (immature neurons), but not for NeuN (mature neurons), and GFAP (astrocytes). The presence of CRMP-4 in the plastic regions suggests that CRMP-4 might be concerned with the neural plasticity of songbird brain.

KEY WORDS : CRMP-4; RT-PCR; *in situ* hybridization; immunohistochemistry; songbird brain

INTRODUCTION

The collapsin response mediator protein-4 (CRMP-4) is a member of the TOAD (turned on after division, 64kDa)/Ulip (Unc-33-like phosphoprotein)/CRMP/TUC/DRP (dihydropyrimidinase-related protein) family of homologous cytosolic phosphoproteins. Unc-33 in *C. elegans* shares sequence homology with CRMP-4 in humans (DRP-3) (HAMAJIMA et al., 1996), rats (TUC-4b) (MINTURN et al., 1995; QUINN et al., 2003), mice (mUlip1) (BYK et al., 1995), chickens (CRMP-4A and CRMP-4B) (YUASA-KAWADA et al., 2003) and *Xenopus* (nsp-1) (BYK et al., 1998), indicating high conservation throughout the evolutionary process. Unc-33 is regarded to play a role in the control of neurite elongation and axonal guidance, according to a finding that neurons have abnormal neuritic outgrowth in the Unc-33 *C. elegans* mutants (LI et al., 1992; MCLNTIRE et al., 1994). Recently, it has been reported that phosphorylation of CRMP-4 can destabilize actin bundles, contributing to the collapse of the actin cytoskeleton (ROSSLENBROICH et al., 2005). CRMP-4 has been studied by immunohistochemistry in the brains of adult rats (NACHER et al., 2000) or lizards (NACHER et al., 2002). The data show that CRMP-4 occurs in newly generated neurons during adulthood, which include those in the dentate gyrus and the subventricular zone migrating through the rostral migratory stream to the olfactory bulb of the rat (MINTURN et al., 1995; NACHER et al., 2000), and those in the medial cortex of the lizard (NACHER et al., 2002). In addition, CRMP-4 has been reported to appear in the cerebral cortex, the piriform cortex and the hypothalamus of the rat (NACHER et al., 2000), or the dorsal and lateral cortices of the lizard (NACHER et al., 2002). It also appears in the other brain regions where no neurogenesis or neuronal migration is detected, but neurite outgrowth, synaptic reorganization or lesion-induced structural plasticity occurs (NACHER et al., 2000; 2002). More recently, CNOPS et al. (2006) revealed spatio-temporal expression profiles of CRMP-4 during visual cortex maturation in the cat, suggesting a potential role of CRMP-4 in the formation of functional network. These data all indicated that CRMPs are involved in neuronal differentiation, neurite growth and regulation of axonal pathfinding in synapse formation (BYK et al., 1998; QUINN et al., 1999, 2003; WANG & STRITTMATTER, 1996).

In songbirds, neuron generation still continues in some adult brain regions such as the hippocampus (ABSIL et al., 2003; BARNEA & NOTTEBOHM, 1994; HOSHOOLEY et al., 2005), some song control nuclei (ABSIL et al., 2003) and the cerebellum (RAO & SHRIVASTAW, 1976). Many studies have further shown that these areas experience significant neural plasticity (hippocampus: CLAYTON & KREBS, 1994; SADANANDA & BISCHOF, 2004; song control nuclei: FOSTER & BOTTER, 1998; BRAINARD & DOUPE, 2000; MOONEY, 2000; the cerebellum: CONSOLE-BRAM et al., 1996; CORVETTI & ROSSI, 2005). For example, the neural plasticity-related genes ZENK (JARVIS et al., 2000; RIBEIRO & MELLO, 2000; BAILEY & WADE, 2005), c-fos (BAILEY & WADE, 2005), c-jun (NASTIUK et al., 1994; RIBEIRO & MELLO, 2000) and BDNF (LI et al., 2000; RIBEIRO & MELLO, 2000) are induced in the hippocampus

and some song control nuclei in the adult songbird brain, following the stimulation of birdsongs.

To our knowledge, no report has been concerned with the distribution of CRMP-4 in the brain of bird. Given the established role for CRMP-4 in neurite outgrowth or synaptic rearrangement, we wanted to know the potential roles of CRMP-4 in the adult songbird brain. To approach this issue, we first obtained CRMP-4 cDNA from the brain of songbird (*Lonchura striata)* by using RT-PCR and 3' rapid amplification of cDNA ends (3'RACE). We then examined the expression of CRMP-4 mRNA and protein in the several plastic brain areas i.e., the hippocampus, song control nuclei and the cerebellum. We also used several immunohistochemistry markers to identify the phenotypes of CRMP-4 positive cells.

MATERIALS AND METHODS

Animals and tissue preparation

The adult male Bengalese finches (*Lonchura striata*) at 12–24 months of age were bred and raised in the breeding colony at Beijing Normal University (Beijing, China). The birds were kept in standard cages (50×62×38cm) under a 14/10hr light/dark cycle at 19-24°C. Seed and water were available ad libitum, supplemented with a mixture of cooked eggs and baby cereal every 2-3days. All experiment procedures were carried out in accordance with the guidelines of Beijing animal protection committee. The birds were deeply anaesthetized by 20% ethyl carbamate (50μL/g body weight). After perfusion with 0.9% saline and then with 4% cold paraformaldehyde in 0.1M phosphate-buffer (pH 7.4), the brains were collected and postfixed 6hr in the same fixative at 4°C. Then the brains were immersed in 30% sucrose at 4°C overnight and cut into 10μm thick sagittal sections on a freezing microtome (CM 1850, Leica).

RNA extraction, reverse transcription–polymerase chain reaction (RT-PCR) and 3' rapid amplification of the cDNA ends (3' RACE)

Since the whole sequence of CRMP-4 cDNA is not yet available in the songbird, we first wanted to get by using RT-PCR and 3'RACE. Total RNA was extracted using the Trizol reagent (GIBCO). 1μg total RNA, 1μL of 50mM poly dT12–18, 4μL of 5 X first strand buffer, 2μL of 0.1M dithiothreitol, 1μL of 50mM dNTPs and 1μL of Moloney-murine leukemia virus reverse transcriptase (GIBCO) were incubated at 37°C for 1hr by using the little genius thermocycler (Japan). PCR was carried out in 25μL reaction mixtures containing 2μL of the first strand cDNA, 1μL 10mM of sense and antisense primers, 1μL 50mM dNTPs and 2U of Taq DNA polymerase (SNBC). Thermocycling was performed using the following protocol: (1) 95°C for 5min, (2) designated cycles of 95°C denaturation for 45sec, 55°C annealing for 45sec and 72°C extension for 45sec, and then (3) 1 cycle at 72°C final extension for 10min before cooling to 4°C. We then employed the 3'-RACE-PCR method using the FirstChoice RACE Kit (Ambion). The RACE procedure was carried out according to the protocol. Primer sets were designed according to the chicken CRMP-4B mRNA sequence (GenBank No: AF301553, the whole length was 2202bp). For 3'-RACE, the outer primer was 5'-gag tac aac atc ttt gaa ggg atg g-3' at positions 1319-1344, and the inter primer was 5'-ggc atg tat gat gga cct gtg ttt ga-3' at positions 1520-1546. The product of 3'-RACE was 798 bp. 3'RACE product shared high sequence similarity (97%) over its entire length with an even longer zebra finch (*Taeniopygia guttata*) clone (GenBank No: CK308729).

In situ hybridization

Digoxigenin-labeled riboprobes were produced by in vitro transcription of specific DNA fragments obtained by PCR amplification of plasmid vectors containing the cDNA for CRMP-4 in a pGEM-T Easy vector (Promega). For PCR amplification, primer pairs were directed against the vector sequences flanking both oppositely located RNA polymerase promoter sites to amplify the insert with both promoters for transcription of both strands using the same DNA preparation (pGEM-s, 5'-TATA GAA TAC TCA AGC TA AAG CAA AGG AAG AAA AAT GG-3'; and pGEM-a, 5'- GGG TGG AGA TGA AGG AGA TG-3'). The labeled probes were approximately 434-base fragments corresponding to the chicken CRMP-4 mRNA (GenBank No: AF301553, nucleotide position 1781–2214, 3' untranslated sequence) and Bengalese finch CRMP-4 cDNA sequence (submitted to NCBI and GenBank No. DQ402134, nucleotide position 262-786, 3' untranslated sequence). Blast NCBI analysis confirmed that none of the cDNA sequence used in the present study showed significant homologues with other CRMP family members or other molecules. Ten-μm-thick sagittal sections were mounted on slides. Digoxigenin-labeled probes were generated by labeling with digoxigenin-11-UTP (Roche Molecular Biochemicals) using SP6 or T7 RNA polymerase (Promega) in a 10μL transcription mixture containing 1μg linearized DNA; 1μL 10 X transcription buffer; 0.5μL 0.2mM dithiothreitol; 1μL of 10mM stocks of ATP, GTP, CTP and digoxigenin-11-UTP; 10U ribonuclease inhibitor; and 10U appropriate RNA polymerase. The transcription mixtures were incubated for 2hr at 37°C. The temperature for hybridization was adjusted according to the GC content of each probe and was selected for high stringency: 52°C for CRMP-4 probe.

Endogenous AP activity was quenched with 0.2M HCl followed by PBS rinses. Proteinase K (GIBCO) digestion was carried out at 37°C, followed by postfixation in 4% freshly depolymerized paraformaldehyde. After PBS rinses the sections were acetylated with 0.25% (v/v) acetic anhydride (Sigma) in a 0.1M triethanolamine (Sigma), pH 8.0 and 0.9% NaCl and then rinsed. After a wash in sterile dH_2O, the sections were incubated with prehybridization solution (50% formamide, 2 X SSC, 0.05g/mL dextran sulfate, 1 X Denhardt's, and 0.1mg/mL salmon sperm DNA). Digoxigenin labeled RNA probe was diluted in prehybridization buffer (1μg/mL). Sections were incubated overnight. After washed with 2 X SSC and 0.05% Tween-20, 0.1M Tris-HCl, and 0.15M NaCl (TTN), the sections were preincubated in 0.5% (w|v) BSA and incubated with a sheep polyclonal anti-digoxigenin antibody $F(ab)_2$ fragment conjugated with alkaline

phosphatase (Roche Molecular Biochemicals, 1: 1000). The sections were washed in TTN and next in Tris, pH 9.4, NaCl: $MgCl_2$ (20: 1), and colour-developed in the presence of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) for 48hr. Negative controls were prepared using the corresponding sense probes.

Protein extraction and Immunoblot analysis

The whole brain of an adult male bird was lysed in 50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100 and 0.1mM phenyl methane sulfonyl fluoride. The samples were separated by 12% SDS-PAGE and electro-transferred onto a polyvinylidene-difluoride (PVDF) membrane (Millipore). Membranes were incubated overnight at 4°C in TBS–Tween (10mM Tris–HCl, pH 7.5, 150mM NaCl, 0.1% Tween 20) containing 5% dry milk. Blots were then incubated with TUC4 performed with peroxidase method with washes after each step.

Immunohistochemistry

The sections were processed for immunohistochemistry as follows: Sections were first incubated in 3% normal goat serum and then incubated with TUC4 (Chemicon, 1: 1000) against the synthetic peptide sequence YDG-PVFDLTTTPK corresponding to amino acids 499–511 from rat CRMP-4 and differing only by one amino acid for the chicken CRMP-4B sequence. This antibody has been shown to recognize exclusively CRMP-4 protein and does not cross-react with other CRMPs. According to the product protocol and previous report (NACHER et al., 2002), the primary anti-sera can cross-react with CRMP-4 into a relatively broad variety of amniotes including *Xenopus*.

The secondary antibody was a biotinylated goat anti-rabbit IgG (Jackson, 1: 400) followed by an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, 1: 150). Colour development was achieved by incubating with 3, 3'-diaminobenzidine 4-HCl (DAB, Sigma). Each step was followed by PBS washing. The specificity of the immunoreactions was checked with reference to the guidelines suggested by SAPER & SAWCHENKI (2003); namely as negative controls, by omitting the primary antisera. For these negative controls, all other immunohistochemical procedures were the same as that described above. In addition, we compared our labeling results with previous demonstrations of which brain areas exhibit or lack CRMP-4 labeling (NACHER et al., 2000; 2002). The findings of all of these control procedures indicated that the labeling reported here was specific.

For double labeling, the sections were treated with 3% normal serum from the same animals as those used in the production of secondary antibodies and reacted with another antibody. The following antibodies were used as the primary antibodies: mouse IgG polyclonal anti-HuD (Molecular Probes, 12.5μg/mL), mouse IgG monoclonal anti-NeuN (Chemicon, 1: 500) and mouse IgG monoclonal anti-GFAP (Chemicon, 1: 200). According to the protocols provided by product companies and our previous work, all these antibodies could be used in songbirds. As the secondary antibodies, the following antibodies were used: FITC-conjugated goat anti-rabbit IgG (Jackson, 1: 200), Rhodamine-conjugated goat anti-mouse IgG (Jackson, 1: 200), FITC-conjugated goat anti-mouse IgG (Jackson, 1: 200) and Cy3-conjugated donkey anti-rabbit IgG (Jackson, 1: 200).

Photography and construction of double coloured fluorescent images

Images were obtained with CoolSNAP colour digital camera (Photometrics) attached to an Olympus microscope (BH-2). Fluorescence signals were detected with Spot digital camera (Diagnostic Instruments) attached to an Olympus fluorescent microscope (1X70) at excitation/emission wavelength of 550/570nm (Rhodamine or Cy3, red) and 492/520nm (FITC, green). The images were corrected for brightness and contrasted by Adobe Photoshop 8.0 (Adobe Systems).

RESULTS

Western blot analysis for CRMP-4 in adult avian brain

Only a band (64kDa) corresponding to TUC4 was shown among total proteins by Western blots (Fig. 1).

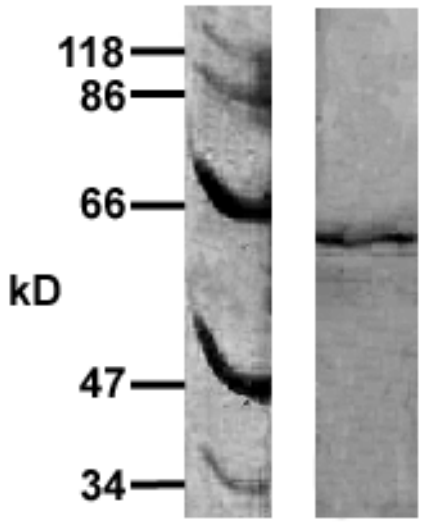

Fig. 1. – Antibody for collapsin response mediator protein-4 (CRMP-4) detects a band of approximately 64kDa on Western blot of adult Bengalese finch brain homogenates.

CRMP-4 labeling *in situ* hybridization and immunohistochemistry

We determined the localization of CRMP-4 through *in situ* hybridization and immunohistochemical staining in the brain of adult male Bengalese finch. For song control nuclei, we examined two nuclei in the motor pathway, i.e., HVC and robust nucleus of arcopallium (RA), several nuclei in the anterior forebrain pathway, i.e., lateral magnocellular nucleus of anterior nidopallium (LMAN), Area X in the basal ganglia, and the nucleus dorsolateralis

anterior, pars medialis (DLM) and an auditory area in the caudal medial nidopallium (NCM) (NOTTEBOHM et al., 1982; VATES et al., 1996). No detectable labeling was observed above background levels by hybridization with the complementary sense probe or in immunohistochemistry controls.

Distributional patterns of CRMP-4 mRNA and protein were similar in the examined plastic regions, as well as in the other brain areas. They were dominantly located in the examined plastic regions among the whole brain. However, this was more obviously reflected from CRMP-4 protein, than that from CRMP-4 mRNA (Figs 2-5). Although positive labeling for CRMP protein was dominantly present in most of studied plastic areas, it was also evident in their surrounding regions for several studied plastic areas such as RA (Fig. 3D) and DLM (Fig. 4B).

Hippocampus

The avian hippocampal complex consists of a medially situated hippocampus (Hp) and parahippocampal area (APH) (COLOMBO & BROADBENT, 2000). A few labeled cells for CRMP-4 mRNA and protein were observed in both subdivisions (Fig. 2). The density of labeled cells in immunohistochemistry was not as high as that in *in situ* hybridization. Although positively labeled cells also appeared in areas underlying the Hp, they were not as robust as in the Hp, especially for those cells positive for CRMP-4 protein (Fig. 2).

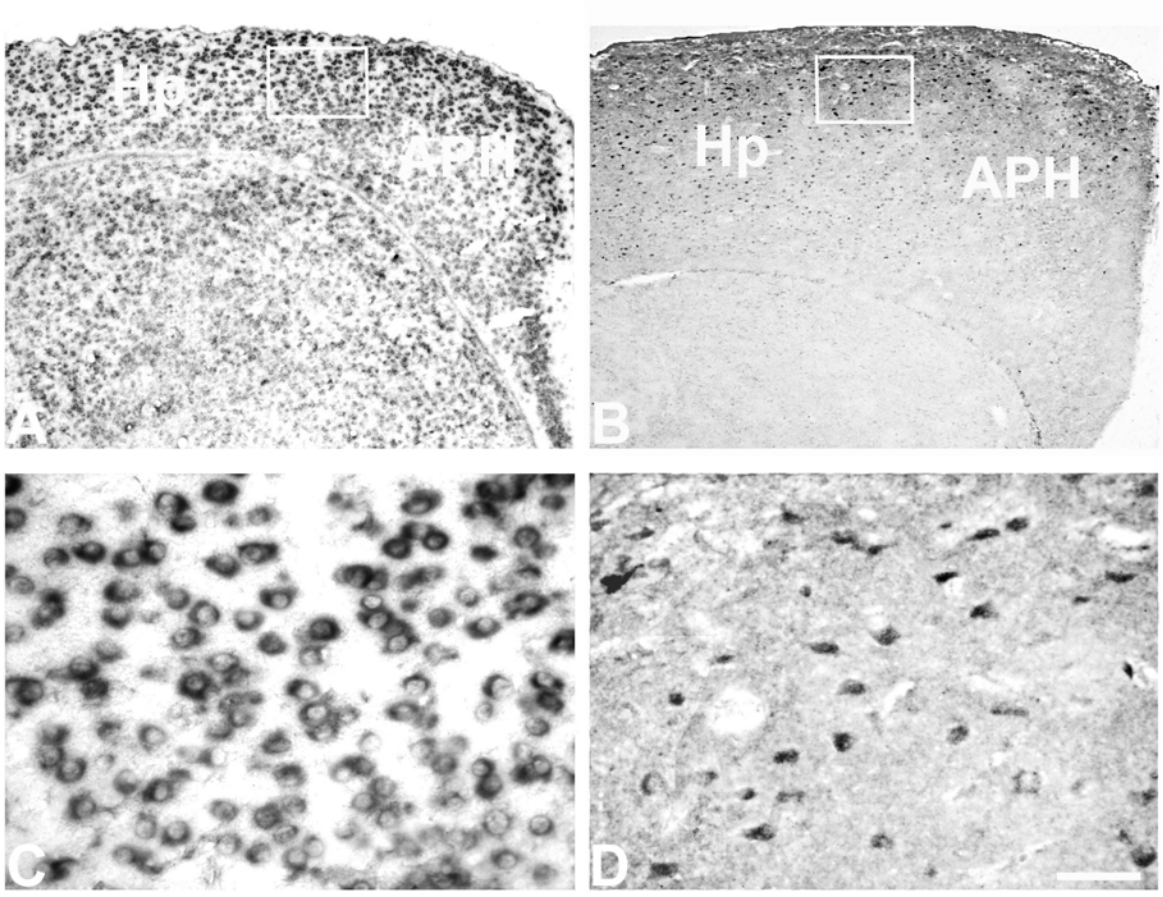

Fig. 2. – The location of CRMP-4 mRNA (A and C) and protein (B and D) in sagittal sections of the hippocampus in the adult Bengalese finch. The boxes in A and B are further illustrated in C and D, respectively. Scale bar: A and B, 150μm; C and D, 30μm.

Song Control Nuclei

CRMP-4 mRNA and protein both appeared in HVC (Figs 3A; 3B), RA (Figs 3C; 3D), LMAN (Figs 3E; 3F), Area X (data not shown), DLM (Figs 4C; 4D) and NCM (Figs 4E; 4F). Some positively labeled cells of HVC and NCM showed two or three basal, thin processes, with two spiny processes emerging from the two poles of the cell bodies (Fig. 4). There were almost no expanding branches around the somata in RA, LMAN, and DLM (Figs 3; 4).

Cerebellum

The cerebellum cortex is a trilayered region, and consists of the molecular layer, the Purkinje layer and the internal granule layer (PALAY & CHAN-PALAY, 1974). The labeling for CRMP-4 mRNA and protein was observed in almost all of the Purkinje cells (Figs 5A; 5B). Labeling of CRMP-4 protein appeared in the somata and dendrites of Purkinje cells. CRMP-4 mRNA labeling could also be seen in the other areas of cerebellar cortex including the

inner granule layer, and to a less extent in the molecular layer, while CRMP-4 protein was both expressed in the inner granule layer and in the molecular layer.

Other Brain Areas

Although positive cells for CRMP-4 mRNA and protein dominantly appeared in the above areas, they also occurred in other brain areas. These areas included some auditory nuclei such as the magnocellularis (NM) (Figs 5C; 5D) in the pons, and the nucleus ovoidalis (OV) in the diencephalon (Figs 5E; 5F), a visual relay nucleus center in the diencephalon (the nucleus rotundus), and some areas in the hypothalamus or the brain stem areas (data not shown).

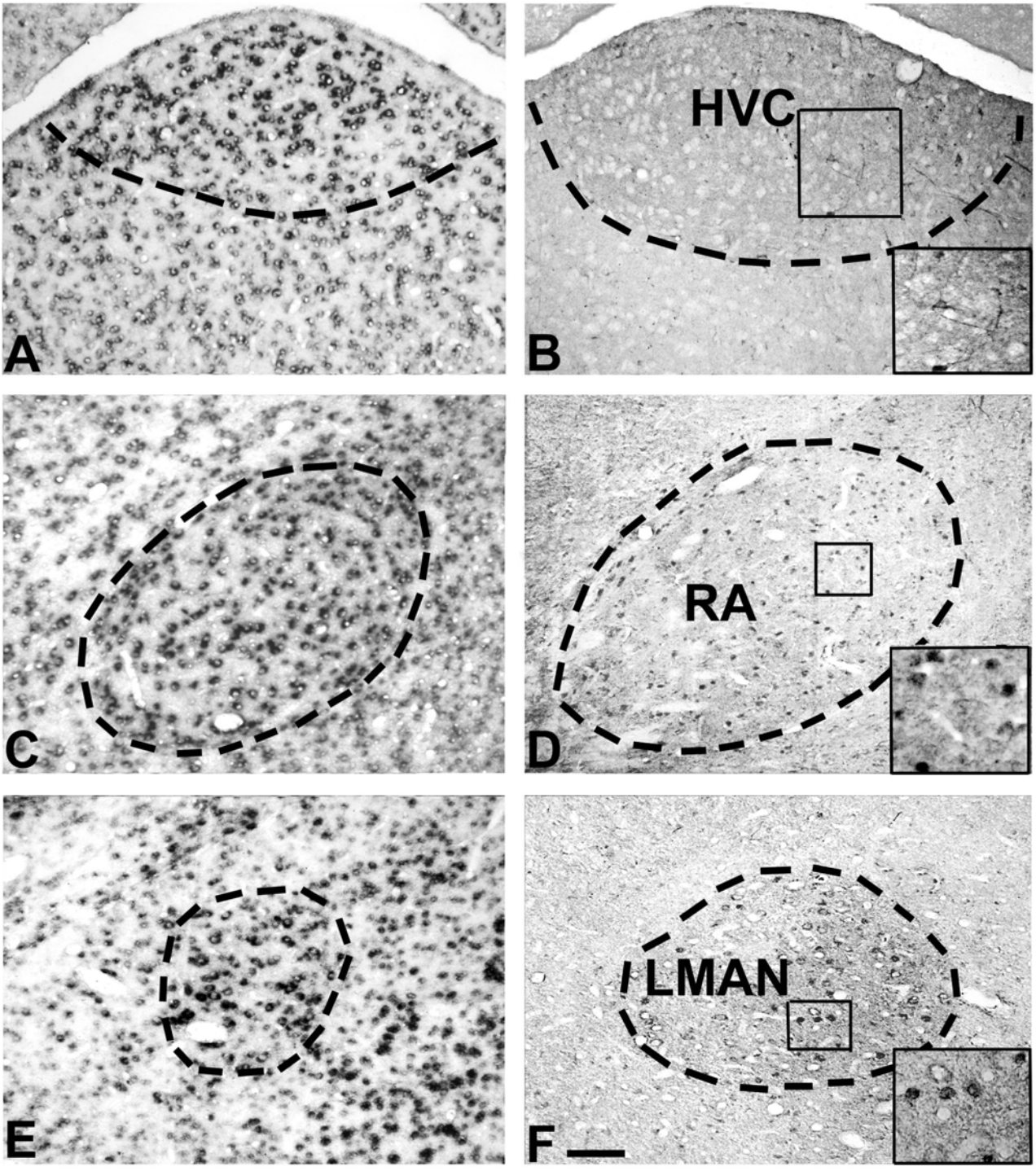

Fig. 3. – The location of CRMP-4 mRNA (A: HVC, C: RA, E: LMAN) and protein (B: HVC, D: RA, F: LMAN) in sagittal sections of song control nuclei of the adult songbird brain. Insets: showing CRMP-4-immunoreactive cells. Scale bar: 75μm; 30μm for insets.

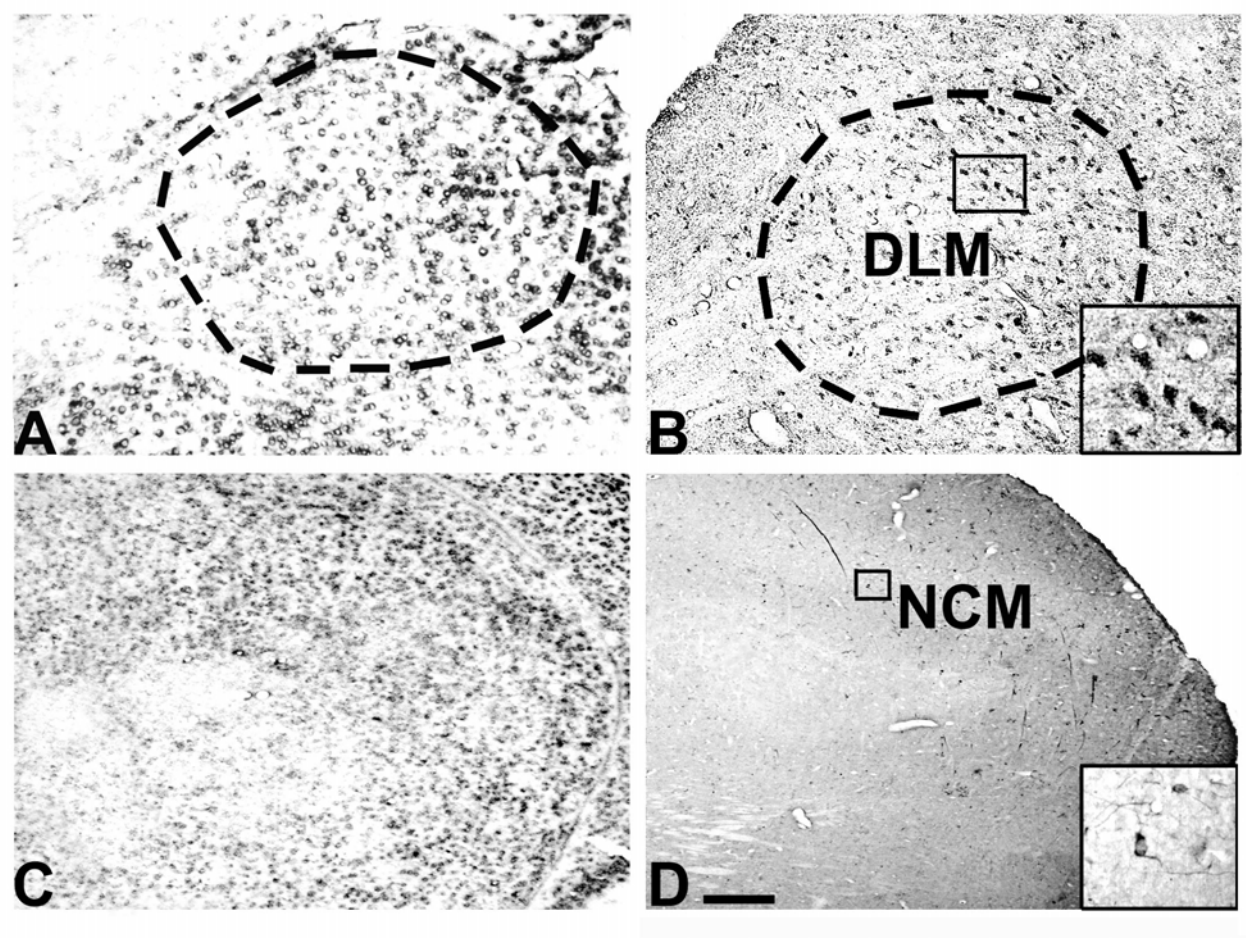

Fig. 4. – The location of CRMP-4 mRNA (A: DLM, C: NCM) and protein (B: DLM, D: NCM) in sagittal sections of song control nuclei of the adult songbird brain. Insets: showing CRMP-4 immunoreactive cells. The hippocampus in D was lost. Scale bar: A and B, 75μm; C and D, 150μm; 30μm for insets.

Double immunohistochemistry

No CRMP-4 immunolabeled cell bodies were found co-expressing with NeuN (Fig. 6A) and GFAP (Fig. 6B) immunoreactivity. A subset of CRMP-4-positive cells was also labeled for neuron marker, HuD. Cells expressing both CRMP-4 and HuD were mainly located in the hippocampus, HVC and NCM (Fig. 7).

DISCUSSION

The present study obtained a fragment of CRMP-4 cDNA with adequate length from the brain of Bengalese finch by employment of RT-PCR and 3'RACE. Both *in situ* hybridization and immunohistochemistry showed that CRMP-4 was located in the hippocampus, song control nuclei and the cerebellum. The double labeling study indicated that some CRMP-4 cells also expressed HuD, but not NeuN and GFAP. In what follows, we will compare the present results with those reported previously, and discuss the physiological implication of CRMP-4 distribution in the brain of songbird.

The sequence of CRMP-4 and its phylogenetic conservation

Although we did not get a complete CRMP-4 cDNA, an adequate length of CRMP-4 cDNA was obtained in the present study. The fragment of CRMP-4 cDNA showed high homology with CRMP-4B cDNA of chicken and other amniotes: human, rat and *Xenopus* (Byk et al., 1995; 1998; Hamajima et al., 1996; Li et al., 1992; Minturn et al., 1995; Nacher et al., 2000; Quinn et al., 2003; Yuasa-Kawada et al., 2003). Our study therefore confirmed again the phylogenetic conservation of CRMPs family (Hamajima et al., 1996).

Location of CRMP-4 in the brain of songbird and its possible physiological function

To our knowledge, the present study first mapped the distribution of CRMP-4 in the adult brain of amniote simultaneously by two approaches i.e., *in situ* hybridization and immunohistochemistry. Since the cDNA sequence corresponding to the CRMP-4 mRNA probe showed no similarities with other CRMPs members or other molecules, we were sure that our results for *in situ* hybridization were specific.

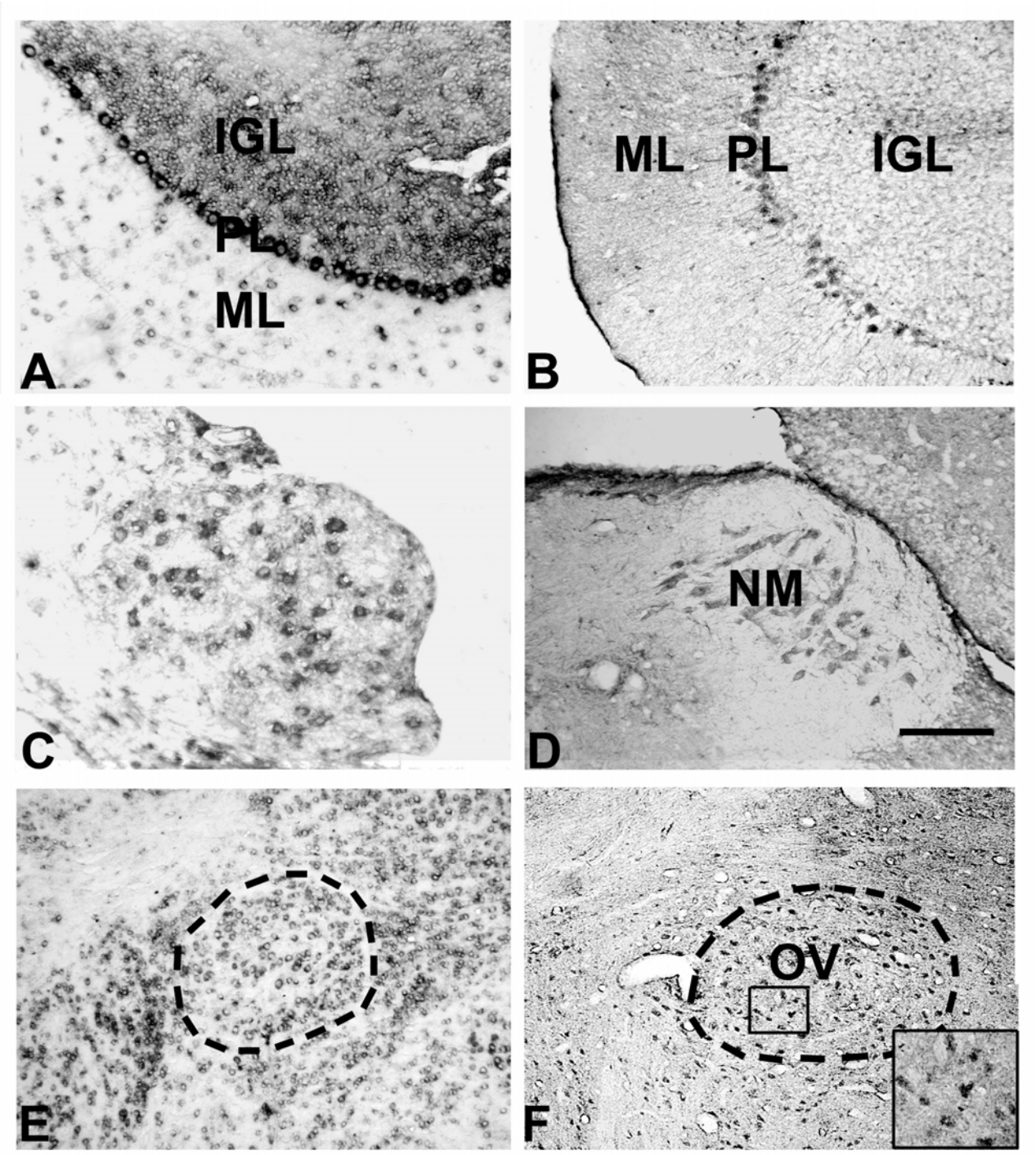

Fig. 5. – A and B: Cells expressing CRMP-4 mRNA (A) or protein (B) in the cerebellum. CRMP-4 mRNA appears in the Purkinje cell layer (PL), the inner granule layer (IGL), and to a less extent in the molecular layer (ML). In contrast, CRMP-4 protein mainly occurs in the PL. C- F: Cells expressing CRMP-4 mRNA (C and E) or protein (D and F) in an auditory nucleus in the brainstem (C and D), and the auditory nucleus in the diencephalon (E and F). Inset in F: showing immunoreactive cells for CRMP-4 protein. Scale bar: 50μm, A-D; 100μm, E and F; 50μm for inset in F.

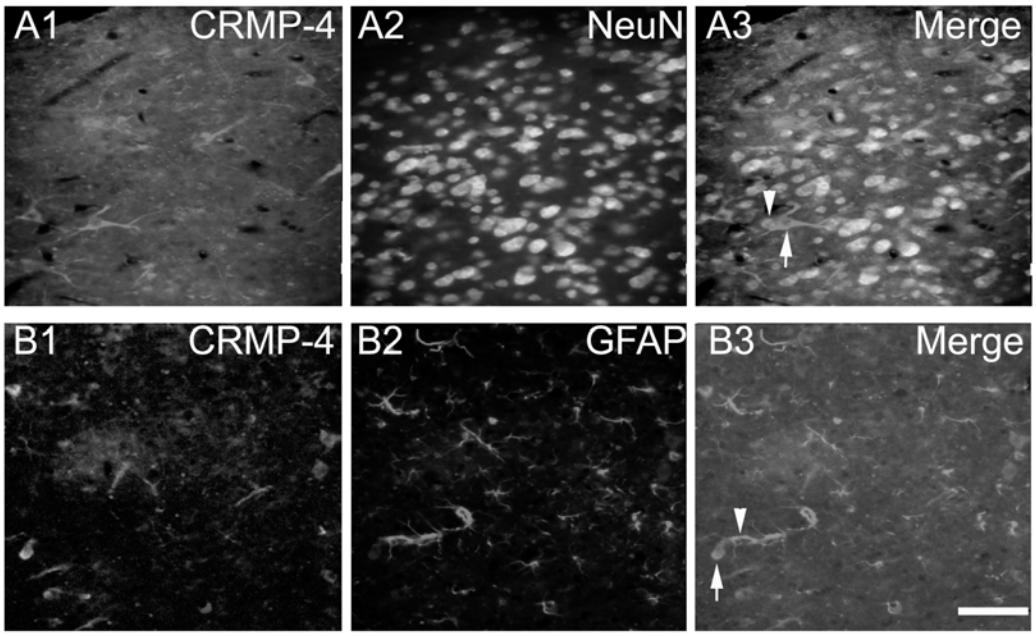

Fig. 6. – Immunofluorescence photomicrographs demonstrating double labeling by anti-CRMP-4 and other antibodies (NeuN and GFAP) in sagittal sections of the caudal medial nidopallium (NCM) in the adult Bengalese finch. Left panels showing cells positive for CRMP-4. Middle panels showing cells positive for another antibody. Right panels showing merged images. A: CRMP-4-positive cell (arrow) devoid of NeuN and NeuN-immunolabeled neuron (arrowhead) negative for CRMP-4. B: labeling cell for CRMP-4 (arrow) not positive for GFAP immunostained astrocyte (arrowhead). For panels in A and B, dorsal is up and caudal is left. Scale bar, 50μm.

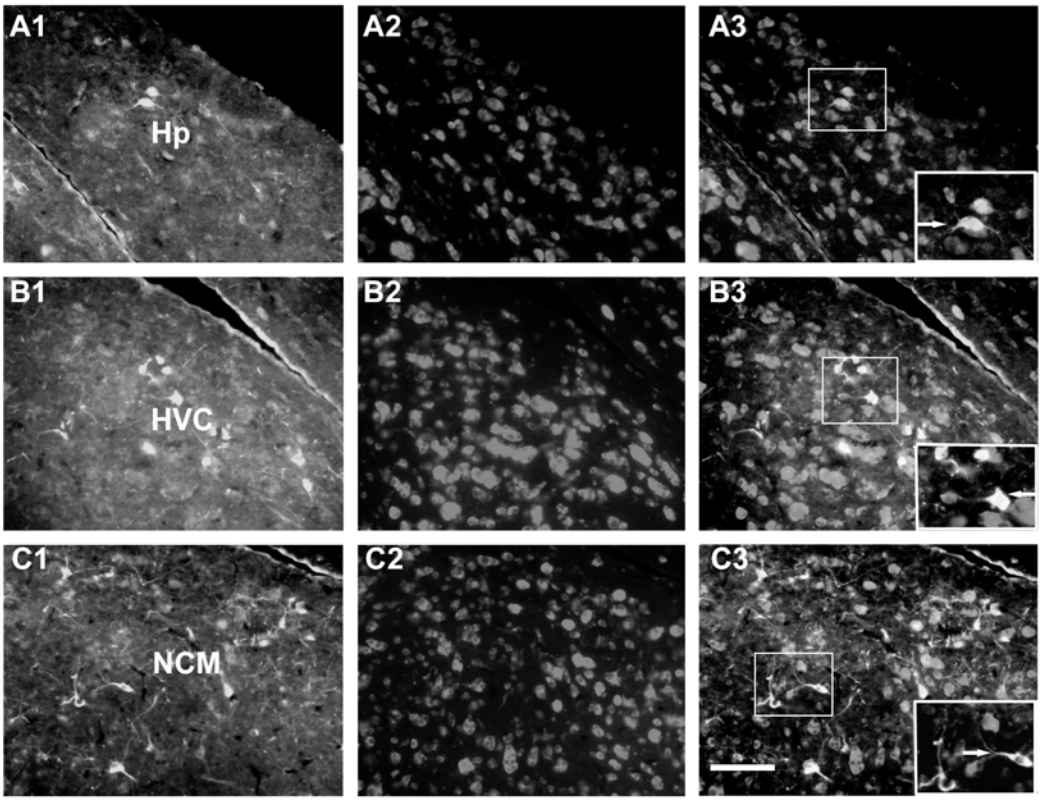

Fig. 7. – Immunofluorescence photomicrographs showing double labeled cells by anti-CRMP-4 and HuD antibodies. Left panels showing cells positive for CRMP-4. Middle panels showing cells positive for HuD. Right panels showing merged images. A: labeling in hippocampus. B: labeling in HVC. C: labeling in the caudal medial nidopallium. Insets: double labeled cells (arrows). For panels in C, dorsal is up and caudal is right. Scale bar, 50μm; 32μm for insets.

We also believed that our immunohistochemistry was specific for CRMP-4. First, the same antibody has been confirmed to be specific in the rat (MINTURN et al., 1995; NACHER et al., 2000), lizard (NACHER et al., 2002) or cat (CNOPS et al., 2004). Second, a single band corresponded to CRMP-4 was identified in our immunoblot analysis as in the rat (MINTURN et al., 1995; NACHER et al., 2000), lizard (NACHER et al., 2002) and cat brain tissue (CNOPS et al., 2004). However, as shown in the result, although the distribution patterns of CRMP-4 mRNA and protein were similar, more cells positively labeled for CRMP-4 mRNA were seen in some of examined areas in the present study (Figs 2-5). This may be due to the reason that CRMP-4 protein was not translated in some cells positive for CRMP-4 mRNA.

It is interesting to find that CRMP-4 is present in the medial cortex of lizard (NACHER et al., 2002), and the hippocampus of rat (NACHER et al., 2000) or Bengalese finch. This supports the view that reptilian medial cortex, and the avian or mammalian hippocampus are evolutionarily homologous from embryological perspectives, neural connection patterns and neurochemistry (see reviews in COLOMBO & BROADBENT, 2000). The presence of CRMP-4 in these homologous areas confirms again the view that the family of CRMP-4 is conserved throughout the phylogenetic scale (BYK et al., 1995; MINTURN et al., 1995; NACHER et al., 2000; 2002; QUINN et al., 2003).

The location of CRMP-4 in the hippocampus, song control nuclei and the cerebellum of Bengalese finch agrees with the previous reports that CRMP-4 expression appears in brain regions where adult neurogenesis and/or neurite outgrowth occur (NACHER et al., 2000; 2002; CNOPS et al., 2006). As mentioned above, many studies have shown that the hippocampus, song control nuclei or the cerebellum in the songbird undergo neuronal generation or synaptic remodeling during development or in adulthood (hippocampus: BARNEA & NOTTEBOHM, 1996; CLAYTON & KREBS, 1994; PATEL et al., 1997; song control nuclei: ABSIL et al., 2003; BRAINARD & DOUPE, 2000; FOSTER & BOTTER, 1998; MOONEY, 2000; cerebellum: CONSOLE-BRAM et al., 1996; CORVETTI & ROSSI, 2005). The presence of CRMP-4 in plastic brain areas suggests that CRMP-4 might be an indicator of continuous synapse growth or sparse fiber replacement, or might be involved in the axon guidance and synapse reorganization in creating a complex pattern of functional neuronal connectivity, to match the responses of the adult brain to physiological or external stimuli. It is also probable in some brain areas that somata and dendrites expressing CRMP-4 protein may do nothing but maintain their presented neural pathways. In other words, CRMP-4 protein is not only involved in axonal outgrowth but also in some other "plastic" events. This may be the reason why CRMP-4 was also detected in other brain areas such as auditory or visual areas and some hypothalamic areas, besides the traditionally regarded plastic brain regions.

Our double labeling study indicates that some CRMP-4 cells also express HuD, but not NeuN and GFAP. HuD is an RNA-binding protein that has been shown to induce neuronal differentiation (FUJIWARA et al., 2006) and plays a role in synaptic plasticity through stabilizing mRNAs associated with ribosomes both in somas and dendrites (BOLOGNANI et al., 2004). It has been reported that HuD expression increases during brain development, nerve regeneration, and learning and memory (SMITH et al., 2004; BOLOGNANI et al., 2004). These data suggest that CRMP-4 may take effect on the synaptic plasticity with the participation of HuD.

Our study showed that only immature neurons (HuD labeled cells), not mature ones (NeuN labeled cells), expressed CRMP-4. This was consistent with previous reports that CRMP-4 protein is located in postmitotic neurons that completed their final mitosis and the transient expression during the first postnatal weeks in the rat (MINTURN et al., 1995). These data support the argument that CRMP-4 can be regarded as immature cell marker, as the reports in the adult rat hippocampus after seizure (PARENT et al., 1997) and ischemia (LIU et al., 2003) or in the cerebral dysplasia (KERFOOT et al., 1999). However, recently reported results in the mature cat visual cortex indicate that CRMP-4 is not a reliable immature neuronal marker, at least not at all developmental stages or in all brain regions (CNOPS et al., 2006).

ACKNOWLEDGMENTS

The work was supported by National Natural Science Foundation of China to SJ ZENG (No: 30370472 and 30470226), MX ZUO (No: 30370196) and XW ZHANG (No: 30460042), and Beijing Natural Science Foundation of China to MX ZUO (No. 5052016)

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Received: January 23, 2007
Accepted: May 25, 2007