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Localization of CRMP5 mRNA by in situ hybridisation during development of the mouse forebrain

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Abstract

The expression of the collapse response mediator protein CRMP5 in the prenatal mouse is largely unknown. Evidence suggests that CRMP family members play important roles in neurite outgrowth, and CRMP5 is known to modulate outgrowth of processes in oligodendrocytes through signalling via neuropilin-1 and SemaA. Furthermore, CRMP family members function in axon regeneration after injury and are implicated in the early stages of Alzheimer's disease. Despite these findings relatively little is known about the specific roles these proteins play. The aim of the present study was to evaluate CRMP5 expression in the developing mouse forebrain using in situ hybridisation. Serial coronal sections of brain from E12.5 to E18.5 were analysed. We found highly specific patterns of expression which were restricted to the post-mitotic layers of both the ganglionic eminence and neocortex, and an additional domain of strong expression in the pyramidal layers of the hippocampus in all prenatal ages. Our results are therefore consistent with a role for CRMP5 in process extension. Interestingly, our results also revealed a temporal switch in high-expression levels from the ganglionic eminence to the cortex at a critical time during tangential cell migration. However, the pattern of expression appeared more representative of a general permissiveness for neurite outgrowth rather than one which is restricted to a particular cell subset or cell class. Additionally, expression was also found during periods predominated by neurogenesis and not neurite extension. We conclude that expression of CRMP5 is consistent with a dynamic implicit role in forebrain development.
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The collapsing response mediator protein/Unc-33-like phosphoprotein (CRMP/Ulip) was identified as a signalling molecule of the repulsive axon guidance cue Sema3A [5]. The CRMP family is now known to be comprised of five homologous cytosolic proteins, all of which are expressed in the adult nervous system, and in immature neurons of the developing forebrain and hippocampus [10,13]. CRMP2, which is enriched in the growth cone, is the most widely studied family member and shares homology with the product of the Unc-33 gene of *C. elegans*, the mutation of which leads to aberrant axon guidance and outgrowth [7]. CRMP-2 localizes on microtubules, clathrin-coated pits, and actin filaments in dorsal root ganglion neuron growth cones and can promote microtubule assembly, which can be disrupted by phosphorylation by Rho-kinase to promote axon growth cone collapse [1]. A second member of the family, CRMP1 has recently been implicated in the Reelin signalling pathway [17]. CRMP1 was found to colocalize with disabled-1 (Dab1), an adaptor protein in Reelin signalling, and loss of CRMP1 in a Dab1 heterozygous background led to the disruption of hippocampal lamination, a Reeler-like phenotype.

CRMP genes are reportedly expressed with increasing intensity in the CNS towards the end of embryonic life during the period of maximal axonal growth [16,9]. Consequently, these proteins are implicated in developing processes, particularly axonal pathfinding and connection refinement during periods of differentiation and plasticity, supported by data showing coexpression with other developmentally regulated factors such as PSA-NCAM [13].

In addition, CRMP proteins may play important roles in neuronal disease and repair, and have been implicated in recovery after brain injury, in motor axon regeneration, and in the early stages of Alzheimer's disease [18,14,15]. Sema3A is associated with degeneration of neurons in vulnerable fields of the hippocampus during Alzheimer's disease, and recent findings

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suggest that accumulation of the beta-amyloid protein which causes alterations in neurite outgrowth and is the hallmark of Alzheimer's disease pathology, phosphorylates CRMP-2 to interfere with tubulin assembly in neuritis [4].

Surprisingly, considering the potential importance of these proteins in development and disease, the role of CRMP proteins remains poorly understood and detailed expression data incomplete or unavailable. Expression of CRMP5, the last member of CRMP family to be identified, has not been described in the mouse at embryonic stages before E19. In the post-natal brain CRMP5 has been found to be expressed together with CRMP2 in Sema3A-sensitive oligodendrocytes, playing a role in process extension mediated by neuropilin-1, the major component of the Sema3A receptor complex [11,12]. Previous studies in Rat have found mRNA expression after E19 in the hippocampus and post-mitotic layers of the telencephalon, and by antibody detection at E16.5 in the thalamus and cortex [12].

The aim of the present study was to map the expression of the CRMP5 gene from E12.5 to E18.5, the period encompassing the main growth, expansion and morphogenesis of the mouse forebrain, including the peak periods of genesis and migration of projection and interneurons, as well as neurite outgrowth, pathfinding and initial stages of differentiation and process refinement. To do this we used in situ hybridisation with a digoxygenin labeled oligonucleotide probe.

In situ hybridisation was performed as previously described [8]. Time mated C57BL6 mice were killed at E12.5, E14.5, E16.5, E18.5 (vaginal plug was considered as day 0); perfused with PBS and finally, buffered 4% paraformaldehyde. Brains were removed, cryoprotected in 30% sucrose and sectioned at 18 μm using a cryostat. Sections were then treated with proteinase (10 μg Proteinase K/ml PBS) for 10 min at room temperature followed by fixation in cold 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 20 min. Sections were then washed in PBS and incubated in 0.1M PBS + 0.1% Tween 20 for 30 min. Prehybridisation was performed for 2 h at 65 °C in 50% formamide/50% 5 × SSC buffer. Hybridisation was performed in humidified conditions for 16 h at 65 °C in the same buffer as for prehybridisation with 0.4 μg/ml DIG-labeled sense or anti-sense probe added. The plasmid used in these experiments was previously described [12], and was a generous Gift from Dr. Veronique Rogemond, Lyon, Laboratoire de Neuropathologie-U 433 Hospital Neurologique, Lyon, France. Sense or anti-sense digoxigenin-labeled riboprobes were generated by transcription of the human Ulip6/CRMP5 cDNA (GenBank accession number AF264015) in pBluescriptSK, containing a 1.9 kb cDNA coding for 564 amino acids; following linearization with EcoRV (sense) and SpeI (anti-sense), transcription using the T3 or T7 promoters respectively, and labeling with digoxigenin-UTP (Roche, Meylan, France), following the manufacturer's instructions. Lack of cross reactivity was previously verified by alignment of the sequence of the Ulip6/CRMP5 protein with those for the four known human Ulip/CRMP proteins which showed 48–50% identity, and by Western blot analysis using a rabbit polyclonal anti-serum that recognized the Ulip6/CRMP5 recombinant protein but not the other four Ulip/CRMPs [12]. The human Ulip6/CRMP5 cDNA-derived riboprobe was suitable for hybridization with mouse tissue sections because the sequence of this human riboprobe displays >90% homology with the corresponding mouse sequence. Sections were sequentially washed in 2 × SSC (30 min, room temperature), 2 × SSC (1 h, 65 °C), 0.2 × SSC (1 h, 65 °C), PBS/0.1% Tween 20 (10 min, 65 °C) and PBS/Tween 20 (10 min, room temperature) before being treated with blocking reagent (0.1% Tween 20, 20% fetal calf serum, 2% fetal calf serum) for 2 h at room temperature. Antibody reaction was performed by incubating the slides for 16 h at 4 °C in a 1:5000 dilution of anti-DIG alkaline phosphatase-coupled FAB fragment (Roche, Germany) in blocking solution. Sections were washed thoroughly in PBS/0.1% Tween 20 and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl pH9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 min. Alkaline phosphatase activity was detected with 45 μl/ml 4-nitrobluetetrazolium chloride (NBT, Promega, USA) and 35 μl/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Promega, USA) in alkaline phosphatase buffer for ≥2 h at room temperature. The reaction was stopped with PBS. Alternate sections were hybridized with anti-sense and sense probes to ensure the specificity of the hybridisation signals. Experiments were repeated three times at each age. All procedures involving animals in these experiments conformed to international guidelines on the ethical use of experimental animals.

At E12.5 we observed CRMP5 expression in the post-mitotic region of the ganglionic eminence, extending slightly into the cortical pre-plate which begins to form at this stage (Fig. 1). By E14.5 expression remained in the ganglionic eminence and prominent expression was clearly evident throughout the cortical pre-plate, including the medial telencephalon in the region of the future hippocampus (see high-magnification view of boxed area at E14.5). Interestingly, from E16.5 we observed a gradual switch in expression between the ganglionic eminence and neocortex, such that from E16.5 to E18.5, expression in the ganglionic eminence was greatly reduced, whilst neocortical expression was maintained at E16.5 and was most strongly expressed by E18.5. Comparison of micrographs showing the caudal most aspect of the forebrain to those at mid-hippocampal levels at E12.5, E16.5 and E18.5, show staining is present throughout the post-mitotic cell layers in each case. Consistent with previous experiments in the rat after E19, expression was restricted to the post-mitotic layers of the telencephalon [12]. Additionally, we observed a sharp boundary of expression at the cortico-striatal border indicating that CRMP5 could play an inhibitory or permissive role in restricting cell movement between these two regions and may also form a useful marker for the neocortex at later developmental stages.

Overall, the pattern of expression is reflective of the temporal sequence of neuronal migration and axon outgrowth, first in the ganglionic eminence and a short time later, in the cortical plate and would therefore be consistent with other members of the CRMP family in playing a role in these processes. Interneuron generation occurs from E12.5 in the mouse, reaching a peak at E14.5 after which time generation diminishes [3]. Our expression analysis reveals likelihood that newly generated interneurons are positive for CRMP5 and that the switch in expression intensity between the ganglionic eminence and cor-

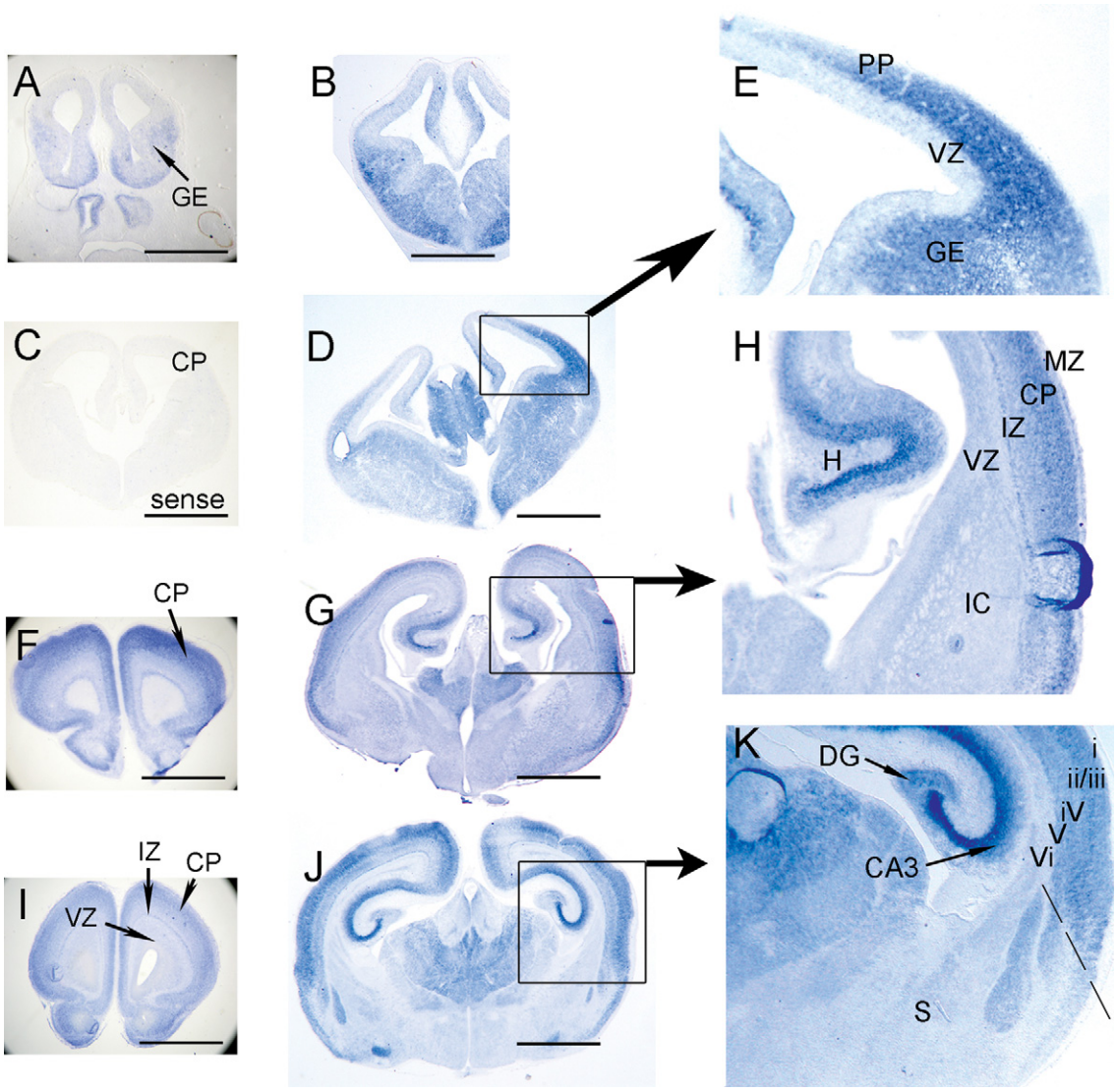

Fig. 1. Developmental regulation of CRMP5 expression in the forebrain from E12.5 to E18.5 Coronal sections hybridized with a digoxygenin labeled oligonucleotide anti-sense probe. At the height of neurogenesis in the cortex and ganglionic eminence, expression is localized to the non germinal layers at E12.5 (A and B), E14.5 (C–E) and E16.5 (F–H) (more clearly seen in the higher magnification images denoted by the boxed regions: E, H and K). At E18.5 (I–K) expression is prominent throughout the post-mitotic layers of the cortical plate and is absent in layer 1 of the cortex. Comparison of the rostral most aspect of the cortex (A,F, and I) to mid-hippocampal levels (B, G, and J) reveals a consistent and restricted staining pattern. From a position approximating to the cortico-striatal boundary (indicated by a dashed line in the boxed region at higher magnification) expression appears down regulated in the striatum. At all ages of hippocampal development, strong expression is present in the dentate gyrus and pyramidal layers of the CA regions. Expression in the dorsal and ventral thalamic nuclei can also be observed after E12. A comparative section at E14.5 hybridized with the sense probe shows no specific staining (C). Abbreviations: Mz, marginal zone; PP, cortical pre-plate; Vz, ventricular zone; GE, ganglionic eminence; H, hippocampus; DG, dentate gyrus; IC, internal capsule; S, striatum; I–VI, cortical layers 1–6, respectively. Scale bars: 400 μm.

tex may indicate an involvement in their tangential migration and pathfinding into the cortex. However, during the process of tangential migration interneurons are restricted largely to the sub ventricular zone or marginal zones of the telencephalon, whereas we found CRMP5 expression throughout the cortical plate from an early stage. This may rather reflect a general regional permissiveness and may also suggest that the role of CRMP5 is not restricted to, or involves interneurons. Alternatively, previous work has implicated CRMP5 in oligodendrocyte development in other areas of the nervous system [11], and a subset of oligodendrocytes are known to be generated at this time from the ganglionic eminence which subsequently migrate into the cortex [6], suggestive of a potential role for CRMP5 in the migration and pathfinding of this cell class. Alternatively, CRMP5 may play an important developmental role in myelination of neurons by oligodendrocytes which also contain microtubules and may therefore undergo cytoskeletal remodelling through signalling mediated by CRMP family members. This would be supported by data indicating increased expression from early to late postnatal stages [13,7], and may also indicate a role in conditions of demyelination additional to neurodegenerative disorders such as Alzheimer's disease.

Although the functional role of CRMP5 remains to be elucidated, the results presented in this study, together with evidence

of CRMP expression at sites of adult neurogenesis [10], and similar patterns of expression with other CRMP family members such as CRMP4, indicates that CRMP5 may serve a general role in the outgrowth and plasticity of new processes which is not cell type specific, and that some level of functional redundancy among family members may exist. Consistent with a wider role in process outgrowth, our expression analysis revealed high-mRNA levels in the pyramidal layer of the hippocampus at a time of active neuronal elaboration in this layer, whilst lower levels were found in the granular layers of the dentate gyrus. Conversely, it has been shown that the protein products of different members of the CRMP family may be targeted to specific subcellular compartments within the cell such as the axon, growth cone or dendrite, and therefore raises the possibility that different cell types present at the same locations during early development may respond differently to CRMP signalling in a refined manner [2]. Finally, our results also highlight expression at ages prior to these processes where neurogenesis is the predominant process and indicate that a role in this process or in neuronal migration may also be likely.

Conflict of interest

The authors declare no conflict of interest.

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