CRMP-2 induces axons in cultured hippocampal neurons

Supplementary information

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Supplementary discussion

As described in the text, we observed variations in morphology of CRMP-2-transfected neurons. One may expect that all the neurites will become axons in the case that the level of myc-CRMP-2 expression is very high. In our system, the amount of overexpressed myc-CRMP-2 was limited and about 4 times that of endogenous CRMP-2 (estimated by the intensity of CRMP-2 immunoreactivity as described in Methods). Hippocampal neurons prepared from E18 rat brain are heterogeneous in terms of developmental stage. In addition, the levels of expressed myc-CRMP-2 vary in individual cells. Such heterogeneity of the neurons may account for the variety of changes in morphologies induced by CRMP-2.

We did not observe remarkable accumulation of CRMP-2 in a neurite of stage 2 neurons before it acquired an axonal identity (Fig. 1a). This raises a possibility that there are other upstream molecules that govern more initial events of axon determination. Alternatively, the initial period of axonogenesis may not be so clear-cut. There is competition between neurites to become an axon. The neurites of this period repeat elongation and retraction, and the longest one becomes an axon1. In these stages, there may be competition for the CRMP-2 concentration between neurites, and a small difference in CRMP-2 levels not detected by immunocytochemistry may trigger a stabilized elongation of neurites to become an axon.

In stage 3 neurons, one neurite, to become an axon, may win in the scramble for high level of CRMP-2 and accumulate further CRMP-2. Then, the overwhelming levels of CRMP-2 in an axon (Fig. 1b) may maintain the neuronal polarity. In either case, we conclude here that CRMP-2 is involved in the
regulatory pathway for axonogenesis because overexpression of CRMP-2-induced axon formation and the deletion mutants inhibited it. CRMP-2 provides an important clue to study upstream and downstream molecules for axonogenesis.

**Supplementary methods**

**Gene construction.** The cDNA of human CRMP-2 was amplified by PCR from a human fetal brain cDNA library with primers 5'-AGATCTATGCTTTATCAGGGGAAGAAAAA-3' and 5'-AGATCTCTAGCCCAGGCTGGTGATGT-3', and subcloned into pCAGGS-myc expression vector or pEGFP-C1 (Clontech, Palo Alto, California). The cDNA fragments of CRMP-2 deletion mutants were amplified by PCR and subcloned into pCAGGS-myc expression vector. Immunoblot analysis confirmed that the deletion mutants were expressed comparably to the wild-type myc-CRMP-2 in COS7 cells without degradation.

**Protein preparation.** Recombinant CRMP-2 was expressed in *E. coli* as glutathione S-transferase (GST) fusion protein and purified on a glutathione-Sepharose column (Amersham Pharmacia Biotech, Backinghamshire, England). The rabbit polyclonal anti-CRMP-2 antibody r1 was raised against the GST-CRMP-2. Monoclonal anti-CRMP-2 antibody C4G3 was provided by Y. Gu and Y. Ihara (Tokyo University).

**Cell culture, transfection and axon identification.** Hippocampal neurons prepared from E18 rat embryos by use of papain as described were seeded on polylysine-coated coverslips, polylysine- and laminin-coated coverslips, or astrocytes obtained from newborn rat hippocampus, and cultured in Neurobasal Medium (Invitrogen-Gibco BRL, Carlsbad, California) supplemented with B-27 supplement (Invitrogen-Gibco BRL), 1 mM glutamine, and 2.5 µM cytosine β-d-arabinofuranoside. In the case of culturing on astrocytes, cytosine β-d-arabinofuranoside was removed from the medium. All experiments were done by using neurons plated at a high density (2 × 10⁵ cell/well in a 24-well plate) except for the case in which pictures of single isolated cells were to be taken. Neurons were transfected with plasmids by the calcium phosphate method before plating. Briefly, dissociated neurons in the transfection buffer (Neurobasal medium/B-27 supplement/1 mM glutamine/10 mM HEPES) with plasmid DNA were plated
out on 10 cm hydrophobic culture dishes and incubated for 90 min in an atmosphere of 5% CO₂. During this transfection procedure, most of the cells adhered to the dishes. The neurons were gently washed with DMEM 3 times and detached from the dishes by vigorous pipetting. The collected cells were then suspended in the culture medium and plated out on the coverslips. The transfection efficiency was reduced for better observation of single neurons. Because less than 10 neurons per well were transfected and visualized by myc immunoreactivity, it was possible to observe the morphology of single neurons expressing myc-CRMP-2 constructs. We also confirmed the morphology of myc-positive neurons by co-transfection with myc-CRMP-2 constructs and GST, and by double immunostaining with anti-myc and anti-GST antibodies. In the case of statistical studies, cells were counterstained with an antibody against a dendritic marker MAP-2. Only the neurites that showed the typical morphology of axons (long, thin and branching at right angles), and were MAP-2-negative were counted as axons. We also observed long neurites that had MAP-2-positive proximal regions. When we could follow the processes to MAP-2 negative regions, we counted them as axons. The quantitative data at 10 d.i.v. with axonal markers tau-1, synaptophysin, GAP43 and synapsin I were essentially identical to those obtained for MAP-2. We sometimes encountered neurons with complicated axons that looped back to the cell bodies or neurons that made contact by processes with other myc-CRMP-2 positive cells and were difficult to analyze even by careful examination with a 63× oil immersion objective lens (Plan-Apochromat; NA, 1.4; Zeiss, Oberkochen, Germany). Such cells (10–15%) were excluded from the data. The health of the CRMP-2 transfected neurons was good. The transfection efficiency of CRMP-2 and that of a control gene, GST, were similar in primary hippocampal neurons. The number of CRMP-2-expressing cells did not decrease, and their morphology appeared healthy at least up to two weeks during culture as in the case of the control neurons expressing GST. However, because a small fraction of the cells were transfected, we cannot rule out the possibility that the present data may be applied to a subset of hippocampal neurons that is more susceptible to transfection.

**Immunocytochemistry and time-lapse fluorescence microscopy.** Cells were fixed with 3.7% formaldehyde in PBS for 10 min, followed by treatment with −20°C methanol for 10 min. They were then immunostained using the following antibodies: polyclonal anti-CRMP-2 antibody r1, monoclonal anti-CRMP-2 antibody C4G, rabbit anti-myc antibody (Santa Cruz, Santa Cruz, California), monoclonal
anti-MAP-2 antibody (HM-2, Sigma, St. Louis, Missouri), or anti-tau-1 antibody (Roche Molecular Biochemicals, Mannheim, Germany). For anti-tau-1 staining, cells were fixed with 3.7% formaldehyde at 37°C for 30 min. To estimate the amount of myc-CRMP-2 expression, we triple-stained hippocampal neurons with anti-myc antibody, anti-CRMP-2 antibody C4G and CMFDA (Molecular Probes, Eugene, Oregon). Anti-myc antibody detects neurons overexpressing CRMP-2, whereas CMFDA is a green cytoplasmic marker and diffusely stains the cytoplasm. Fluorescent images of C4G immunoreactivity and CMFDA staining in myc-positive and myc-negative cells were obtained by DeltaVision microscopy (Applied Precision, Issaquah, Washington) within the dynamic range of 256 brightness units (8 bits), then the background fluorescent intensity was subtracted from the data. Fluorescent intensities were calculated by using the Image pro system (Media Cybernetics, Silver Spring, Maryland). The relative amount of CRMP-2 expression in myc-positive cells (n = 30) and myc-negative cells (n = 30) was calculated by using CMFDA staining as an internal standard (C4G immunoreactivity/CMFDA staining). The cells expressing EGFP-CRMP-2 were observed by using DeltaVision microscopy.