Specification of neuronal polarity regulated by local translation of CRMP2 and tau via the mTOR-p70S6K pathway

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Running head: mTOR controls CRMP2 and tau expression

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Mammalian target of rapamycin (mTOR) is an important regulator for neuronal development and functions. Although it is recently reported that mTOR signaling is critical for neuronal polarity, the underlying mechanism remains unclear. Here, we describe the molecular pathway of mTOR-dependent axon specification, in which the collapsing response mediator protein 2 (CRMP2) and tau are major downstream targets. The activity of mTOR effector 70 kDa ribosomal protein S6 kinase (p70S6K) specifically increases in the axon during neuronal polarity formation. The mTOR inhibitor rapamycin suppresses the translation of some neuronal polarity proteins, including CRMP2 and tau, thereby inhibiting axon formation. In contrast, constitutively active p70S6K up-regulates the translation of these molecules, thus inducing multiple axons. Exogenous CRMP2 and tau facilitate axon formation, even in the presence of rapamycin. In the 5'-UTR of tau and CRMP2 mRNAs, we identified a 5'-terminal oligopyrimidine tract (5'-TOP), which mediates mTOR-governed protein synthesis. The 5'-TOP sequences of CRMP2 and tau mRNAs strongly contribute to the up-regulation of their translation in the axon, in response to the axonal activation of the mTOR-p70S6K pathway. Taken together, we conclude that the local translation of CRMP2 and tau, regulated by mTOR-p70S6K, is critical for the specification of neuronal polarity.

INTRODUCTION

To function, developing neurons must establish axonal-dendritic polarity. The polarization processes have been well studied in cultured hippocampal neurons (1, 2). They first form lamellipodia and small protrusion veils shortly after plating (stage 1), and then extend several immature neurites within 12-24 hours (stage 2). One of the neurites rapidly elongates and becomes the axon, whereas the others become dendrites (stage 3). During these processes, a number of molecules coordinately control the axon specification (3). These molecules spatially regulate cytoskeletal networks of actin filaments and microtubules for specification and maintenance of a single axon.

Mammalian target of rapamycin (mTOR) is a serine-threonin protein kinase and mainly controls protein synthesis via phosphorylation of its downstream targets, such as eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1) and 70 kDa ribosomal S6 protein kinase (p70S6K) (4). In the nervous system, mTOR plays important roles in neuronal survival, dendritic arbor formation, synaptic plasticity, learning, and memory (5). Notably in the regulation of synaptic plasticity, a number of mRNAs are locally translated via activation of the mTOR pathway in response to extracellular signals (5-7). Recently, two research groups reported that the mTOR pathway is also critical for the formation of neuronal polarity (8, 9). They proposed that the mTOR pathway controls the synthesis of polarity proteins, such as SAD kinases (8) and Rap1 (9), and therefore regulates the neuronal polarity. However, the underlying molecular mechanism remains to be fully understood. Here, we demonstrate that p70S6K is necessary and sufficient for the mTOR-controlled axon formation as a downstream effector of mTOR. The mTOR-p70S6K pathway regulates translation of several neuronal polarity genes, including SAD kinases and rap1. In particular, the expressions of collapsing response mediator protein CRMP2
and microtubule-associated protein tau are highly dependent on the mTOR-governed protein synthesis. We further identified a 5'-terminal oligopyrimidine (5'-TOP) tract, which mediates mTOR-governed protein synthesis (10), in the 5'-UTRs of CRMP2 and tau mRNAs. In the axon of developing neurons, the translation of CRMP2 and tau mRNAs is spatially controlled by the mTOR-p70S6K pathway via the 5'-TOP sequences, resulting in accumulation of CRMP2 and tau proteins in the axon. Thus, our study substantially improves understanding of molecular mechanisms underlying the mTOR-dependent development of neuronal polarity.

**EXPERIMENTAL PROCEDURES**

**Materials**- The mTOR inhibitor rapamycin was purchased from Calbiochem. The anti-Tau1 (Chemicon), anti-Tau (Tau46, Abcam), anti-MAP2 (Chemicon), anti-GFP (GF090R, Nakarai Tesque), anti-FLAG (Sigma), anti-HA (3F10, Roche), anti-phospho-p70 S6 kinase (Thr421/Ser424) (Cell Signaling), anti-p70 S6 kinase (C-18, Santa Cruz), anti-E-BP1 (53H11, Cell Signaling), anti-phospho-4E-BP1 (Ser65) (Cell Signaling), anti-CRMP2 (C4G, IBL), anti-Rap1 (121, Santa Cruz), anti-SAD-A (antibody to BRSK2, ProteinTech Group, Inc.), anti-SAD-B (antibody to BRSK1, ProteinTech Group, Inc.), anti-Akt1 (C-20, Santa Cruz), anti-a-tubulin (DM1A, Sigma), anti-β-actin (AC-15, Sigma), anti-GAPDH (FL-335, Santa Cruz), and anti-luciferase (Promega) antibodies were purchased.

**Cell culture and immunostaining**- Hippocampal neurons were prepared from rat hippocampi at embryonic day 18.5. The dispersed neurons were plated on poly-L-lysine-coated coverslips, and cultured in glial-conditioned MEM containing 1 mM pyruvate, 0.6% (W/V) D-Glucose, and 2% B27 supplement (Invitrogen). After 1 week, one-half of the medium was changed to neurobasal medium containing 2% B27 supplement and 0.5 mM L-glutamine. Neurons cultured on coverslips were fixed using 4% paraformaldehyde and then incubated with primary antibody, followed by the appropriate secondary antibody.

**Calcium phosphate-mediated transfection and nucleofection**- Hippocampal neurons were transfected by calcium phosphate precipitation as previously described, with some modifications (11). DNA-calcium phosphate precipitate was prepared using a calcium phosphate transfection kit (Invitrogen). Neurons prepared from rat embryo were plated on a NuncolonΔ surface plate (Nalge Nunc International) and incubated with the precipitate for 3 hours. The transfected neurons were replated on poly-L-lysine-coated coverslips and cultured for 3 days. For higher transfection efficiency, nucleofection was performed using a rat neuron nucleofector kit (Amaxa).

**Expression vectors**- The coding regions for EGFP, rat Rheb, rat p70 S6K, rat eIF4E, rat 4E-BP1, rat CRMP2, rat tau, and human Rap1b were amplified by PCR and cloned into the pCAGGS expression vector. A FLAG-tag sequence was introduced at their 5'-end by PCR. CA-Rheb (Q64L) (12), DN-Rheb (D60K) (13), CA-S6K (ΔCT(T389E)) (14), DN-S6K (T229A) (15), CA-Rap1b (V12) (16), and rapamycin-insensitive 4E-BP1 (5A) (17) were constructed as previously described. For the observation of local translation via the 5'-TOP sequence, the 5'-UTR and promoter region (1,110 bp upstream from the start ATG) of the rat CRMP2 gene were amplified by PCR and cloned into the pGL4.22 vector (Promega). The e-src myristylation motif (MGSSKSKPKDPSQR) was fused to the N-terminus of luciferase by PCR (pGL4.22-CRMP2-myr-Luc) to prevent diffusion of the luciferase proteins.

**Luciferase reporter assay**- The 5'-UTR and promoter region of the rat CRMP2 (1110 bp upstream region from start codon) and the rat tau (857 bp upstream region from start codon) genes were cloned into the pGL4.14 vector (Promega) (pGL4.14-CRMP2 and pGL4.14-tau). The pyrimidine-rich region of the 5'-UTR was deleted by PCR mutagenesis (pGL4.14-CRMP2 (delta 5'-TOP)). 5'-TOP sequence of tau gene was mutated by PCR mutagenesis from ctcctcc to aataaa (pGL4.14-tau (mut 5'-TOP)). These constructs were introduced into hippocampal neurons along with pSV-βGal (Promega), which was used to normalize the transfection efficiency. Twenty-four hours after the transfection, the cells were lysed with Passive
Lysis buffer (Promega), and the luciferase and β-galactosidase activities were measured using the Luciferase Assay System (Promega) and the Luminescent β-galactosidase Detection Kit II (Clontech), respectively.

Quantitative real time RT-PCR- The total RNAs were extracted from neurons using TRIzol reagent (Invitrogen), and reverse-transcribed with PrimeScript Reverse Transcriptase (Takara Bio Inc.). The cDNA was amplified with gene-specific primer pairs using SYBR GreenER qPCR SuperMix Universal reagent (Invitrogen). The quantities measured by real-time PCR were normalized to the GAPDH expression level in each experiment.

RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-5′-RACE)- The total RNAs were extracted from primary hippocampal neurons at 3 DIV, and reverse-transcribed with PrimeScript Reverse Transcriptase (Takara Bio Inc.). RLM-5′-RACE was performed using a GeneRacer Kit (Invitrogen). More than 30 clones were sequenced to determine the multiple transcription start sites of the CRMP2 gene.

RESULTS

Activation of p70S6K is crucial for axon formation. Recently, two research groups reported that the mTOR signaling pathway is critical for the formation of neuronal polarity (8, 9). We also independently found that the mTOR inhibitor rapamycin prevented axon formation by hippocampal neurons in culture (Fig. 1 and supplemental Fig. 1). In contrast, a constitutively active form (CA) of Rheb, an activator of mTOR C1, markedly induced the formation of multiple axons, but a dominant-negative (DN) Rheb mutant inhibited axon formation as strongly as rapamycin (Fig. 1). To examine the molecular mechanism underlying the mTOR-dependent development of neuronal polarity, we analyzed the effects of p70S6K and 4E-BP1, the best-characterized downstream effectors of mTOR, on cultured hippocampal neurons. The mTOR phosphorylates and activates p70S6K. When 4E-BP1 is phosphorylated by mTOR, it releases its binding partner eIF4E, which derepresses eIF4E and initiates cap-dependent translation (4). Consistent with the effect of Rheb, CA-p70S6K (CA-S6K) enhanced the formation of single or multiple axons, but few axons formed in neurons expressing DN-S6K (Fig. 1).

Unexpectedly, overexpression of either an mTOR-insensitive 4E-BP1 (unphosphorylated form, 4E-BP1(5A)) or wild-type eIF4E prevented the specification of neuronal polarity (Fig. 1). Although rapamycin completely inhibited multiple axon formation in CA-Rheb-expressing neurons, CA-S6K-expressing neurons formed single or multiple axons, even in the presence of rapamycin (Fig. 2A and B), indicating that activation of p70S6K is sufficient for the mTOR-dependent axon formation.

It was recently reported that p70S6K and 4E-BP1 are phosphorylated specifically in the axon of hippocampal neurons via the activation of Akt-TSC-mTOR pathway (8, 9). We confirmed that p70S6K phosphorylated on Thr421/Ser424 and 4E-BP1 phosphorylated on Ser65 were specifically localized to the axon, while the total p70S6K and 4E-BP1 proteins were uniformly distributed in the axon and dendrites (Fig. 2C). These data indicate that mTOR pathway is activated specifically in the axon of hippocampal neurons.

Active p70S6K increases the translation of neuronal polarity genes. A number of proteins that control axon specification have recently been reported (3). Because the mTOR-S6K pathway controls the synthesis of certain proteins (10), we investigated the translational regulation of proteins involved in the mTOR-S6K pathway-mediated neuronal polarity. In CA-S6K-expressing neurons, the levels of CRMP2, tau, and Rap1 were significantly increased compared with GFP-expressing control neurons, whereas the levels of the SAD-A and SAD-B kinases were markedly reduced (Fig. 3A and B). Interestingly, however, the mRNA levels of these proteins did not change (Fig. 3C). The expression levels of other proteins, such as Akt, tubulin-α1, and β-actin, were not affected by CA-S6K expression (Fig. 3).

In cultured hippocampal neurons, the phosphorylation level of p70S6K was markedly increased during polarity development from stage 2 occurring at 1 day in vitro (DIV) to stage 3 (3 DIV), and the levels of CRMP2, tau, Rap1,
activity was significantly reduced by CRMP2, 20.0 ± 3.7 %; tau, 21.9 ± 6.6 %; CRMP2/tau, 35.4 ± 3.7 %; CA-Rap1b, 33.8 ± 9.1 %) (Fig. 6). In the presence of rapamycin, 83% of the GFP-expressing neurons were non-polarized, whereas more than 60% of the CRMP2- or tau-expressing neurons formed one or more axons (Fig. 6). Simultaneous expression of CRMP2 and tau more effectively increased the population of axon-forming neurons than their single expression, although the additive effect was rather slight. CA-Rap1b also increased the population of neurons having axons in the presence of rapamycin (36.1 ± 9.4 %), but it was less effective than CRMP2 or tau (Fig. 6).

We further investigated whether CRMP2 and tau can rescue the DN-S6K-induced polarity defect. DN-S6K inhibited axon formation as shown in Fig. 1, and overexpression of CRMP2 and tau overcame the inhibitory effect (Fig. 7). On the other hand, CA-Rap1b had less effect in DN-S6K-expressing neurons (Fig. 7). These data indicate that the mTOR-S6K-governed protein synthesis of CRMP2 and tau is important for the development of neuronal polarity.

**Translational regulation of CRMP2 mRNA via a 5'-TOP sequence.** The mTOR controls protein synthesis by selective up-regulation of the translation of mRNAs that contain a 5'-terminal oligopyrimidine tract (5'-TOP) (10, 18). The 5'-TOP mRNA invariably contains a cytosine at the cap site, followed by an uninterrupted stretch of 4-15 pyrimidine bases (10). We analyzed whether CRMP2 expression, which, among the polarity proteins examined, showed the greatest dependence on mTOR-governed protein synthesis, was also regulated via a 5'-TOP sequence. To determine the transcription start site (TSS) of the CRMP2 gene, we performed RNA ligase-mediated (RLM)-5'-RACE, which is suitable to accurately determine a 5'-terminal sequence of capped mRNA. We found nine TSSs (Fig. 8A, arrows), which were all located in a pyrimidine-rich region, and seven of them were comprised of a cytosine residue followed by an uninterrupted stretch of 5-25 pyrimidine bases (Fig. 8A, red arrows), i.e., a 5'-TOP sequence. This pyrimidine-rich region is highly conserved across species (Supplemental Fig. 3). Therefore, a large proportion of the CRMP2 mRNAs are 5'-TOP mRNAs (10).

To test whether these putative 5'-TOP sequences were functional, the 5'-UTR and promoter region of the CRMP2 gene was inserted into the 5'-end of a luciferase reporter gene, so that the luciferase transcripts contained the CRMP2 5'-UTRs, including the 5'-TOP sequence. In a reporter assay using this construct, luciferase activity was significantly elevated by CA-S6K without increase in luciferase transcription, but deletion of the pyrimidine-rich region extinguished the expression of the luciferase reporter gene.
CA-S6K responsiveness (Fig. 8B), suggesting that the 5'-TOP sequence of the CRMP2 gene is involved in mTOR-S6K-dependent CRMP2 expression.

**Local translation of CRMP2 mRNA via the 5'-TOP sequence.** In polarized neurons, p70S6K was phosphorylated and activated specifically in the axon (Fig. 2C), and endogenous CRMP2 proteins were more concentrated in the axon than in the dendrites (Fig. 8C and 19), raising the possibility that the CRMP2 mRNA is locally translated in the axon in a 5'-TOP-dependent manner. To monitor the 5'-TOP-dependent local translation, the 5'-UTR and promoter region of the CRMP2 gene were inserted upstream of a gene for short-lived luciferase. A myristoylation sequence was fused to the 5'-end of the luciferase gene, to prevent the diffusion of the luciferase proteins. When polarized neurons were co-transfected with GFP and this luciferase expression vector, abundant luciferase was detected in the axon, as was the endogenous CRMP2 protein, but the GFP was distributed diffusely (Fig. 8D). Furthermore, rapamycin dramatically reduced the luciferase expression in both the axon and dendrites (Fig. 8D), strongly suggesting that mTOR controls the local translation of CRMP2 mRNA in the axon via the 5'-TOP sequence.

**tau mRNA is also 5'-TOP mRNA and its translation is locally promoted in the axon.** We found that the reported 5'-terminal sequence of full-length tau mRNA also fits the 5'-TOP mRNA description and this element is highly conserved across species (Fig. 9A). To investigate whether translation of tau mRNA is also regulated via a 5'-TOP sequence, the luciferase reporter gene assay was performed using the 5'-UTR and promoter region of the tau gene. Similarly to the case of CRMP2, luciferase activity was significantly increased by CA-S6K, but mutation of the 5'-TOP sequence extinguished the CA-S6K responsiveness (Fig. 9B). Since tau protein is also known to be concentrated in the axon than in the dendrites as well as CRMP2 protein (Fig. 9C and 20, 21), 5'-TOP-dependent axonal translation of tau mRNA was monitored using the myristoylated short–lived luciferase. The luciferase was abundantly detected in the axon in contrast to the diffuse distribution of GFP, and rapamycin dramatically reduced the luciferase expression in the axon (Fig. 9D). Taken together, these results indicate that the tau mRNA is also 5'-TOP mRNA and its translation is locally promoted in the axon by mTOR signaling.

**DISCUSSION**

In this paper, we reported a novel finding that p70S6K was an important mTOR effector in the development of neuronal polarity. The activity of p70S6K was specifically increased in the axon during neuronal polarity development (Fig. 2C), and CA-S6K-expressing neurons formed multiple axons even in the presence of rapamycin (Fig. 1). In contrast, DN-S6K strongly inhibited axon formation (Fig. 1), indicating that the activation of p70S6K is necessary and sufficient for the formation of mTOR-governed neuronal polarity. In addition, we showed that CRMP2, tau, and Rap1, all are well-known as essential factors for the establishment of neuronal polarity (3), are components of the mTOR-p70S6K pathway. The expression of CRMP2 and tau was particularly dependent on mTOR-p70S6K-governed protein synthesis (Fig. 4). Exogenous overexpression of CRMP2 and tau rescued the rapamycin- and DN-S6K-induced polarity defect (Fig. 6 and Fig. 7), suggesting that they are critical downstream targets of mTOR-S6K pathway in the establishment of neuronal polarity. Both of CRMP2 and tau is known to play roles in regulating microtubule dynamics. Simultaneous expression of CRMP2 and tau more effectively rescued the rapamycin- and DN-S6K-induced polarity defect than their single expression, but the additive effect was rather slight (Fig. 6 and Fig. 7), suggesting that they might have a partially overlapping function in the axon formation. The CRMP2 and tau mRNAs had a typical 5'-TOP motif, and their translation was controlled via the 5'-TOP sequence (Fig. 8 and Fig. 9). Taken together, these results indicate that the activation of p70S6K in the axon of developing neurons leads, at least, to the local translation of CRMP2 and tau, followed by the maintenance and reinforcement of axon specification.

Many mRNAs for proteins whose translation
is controlled by mTOR have a translational cis-regulatory 5'-TOP (10, 18). The result of our RLM-5'-RACE analysis indicated that the CRMP2 mRNA is a typical 5'-TOP mRNA (Fig. 8A). The pyrimidine-rich sequence around multiple TSSs is highly conserved in mammals (Supplemental Fig. 3), suggesting that the 5'-TOP-mediated translation of CRMP2 is important for its function. We also noticed that the tau mRNA also has a conserved polypurinotidene tract at its 5'-terminus (Fig. 9A).

Local translation of CRMP2 and tau mRNAs via 5'-TOP resulted in the axonal accumulation of CRMP2 and tau proteins (Fig. 8C and D). In connection with this, it is well established that the tau mRNA is transported to the proximal portion of the axon via its 3'-UTR axonal targeting element, which may be involved in the accumulation of tau proteins in the axon (22, 23). In addition, Kimura et al. reported that kinesin-1 binds to CRMP2 and transports it to the distal axon (24). These dual mechanisms for the axonal enrichment of CRMP2 and tau proteins could closely regulate the specification and maintenance of a single axon.

The p70S6K was first reported as a downstream effector in the mTOR-governed translation of 5'-TOP mRNAs (15). Recent biochemical and genetic studies, however, revealed that p70S6K is not sufficient for the translational regulation of 5'-TOP mRNAs (25, 26), raising the possibility that p70S6K-dependent and p70S6K-independent pathways transduce the mTOR-regulated translation of 5'-TOP mRNAs. In hippocampal neurons, the activation of p70S6K was required for axon specification and CA-S6K completely rescued the rapamycin-induced polarity defect (Fig. 1 and Fig. 2). CA-S6K increased the translation of endogenous CRMP2 and tau even in the rapamycin-treated cultures (Fig. 3 and Fig. 5), and up-regulated the translation of 5'-TOP-fused reporter genes (Fig. 6B and Fig. 7B). We further confirmed that the translation of canonical 5'-TOP gene rpl32 (27) was also activated by CA-S6K via its 5'-TOP sequence (Supplemental Fig. 4). These results suggest that p70S6K controls the translation of CRMP2 and tau mRNAs via the 5'-TOP sequences in developing hippocampal neurons.

Choi et al. (2008) recently reported that the tuberous sclerosis complex (TSC) proteins, which are negative regulators of the Rheb-mTOR pathway, inhibit axon formation (8). They identified the SAD kinases, whose levels were increased in TSC1/2-depleted neurons. Contrary to their report, we found that the expression levels of SAD kinases were not significantly changed by rapamycin treatment (Fig. 4). Choi et al. (2008) also reported that the basal expression levels of SAD kinases are not changed by rapamycin treatment (8), suggesting that mTOR activity is not required for the basal expression of SAD kinases in developing cultured hippocampal neurons. Unexpectedly, the expression levels of SAD kinases were reduced in the CA-S6K-expressing neurons without transcriptional repression (Fig. 3), raising a possibility that protein levels of SAD kinases may be suppressed by p70S6K through repression of translation efficiency or protein degradation.

Thus, the expressional regulation of SAD kinases must be complex. We observed SAD kinases to be uniformly distributed in the soma, axon, and dendrites, and exogenous SAD kinases showed very little rescue of the rapamycin-induced polarity defect (Supplemental Fig. 5), indicating that SAD kinases are not important for the mTOR-p70S6K-controlled axon formation.

Li et al. (2008) also reported the regulation of neuronal polarity by the Rheb-mTOR pathway (9). In their report, Rap1b was identified as a critical polarity protein regulated by the mTOR-dependent protein synthesis. We also observed the mTOR-dependency of Rap1 expression (Fig. 2). Ectopic CA-Rap1b expression increased the population of polarized cells in the rapamycin-treated and DN-S6K-expressing hippocampal neurons, but it was less effective than CRMP2 or tau (Fig. 6 and 7). They also discussed the importance of 4E-BP1 in axon formation as a downstream effector of mTOR. We confirmed that neurons expressing mTOR-insensitive 4E-BP1 (4E-BP1(5A)) did not form axons, suggesting a possibility that 4E-BP1 is involved in the neuronal polarity formation. However, 4E-BP1(5A) rather severely suppressed neurite outgrowth, compared with DN-Rheb, DN-S6K, and rapamycin (Fig. 1), possibly by a
widespread suppression of translation, including that of polarity-irrelevant proteins. Furthermore, overexpression of eIF4E, which mimics 4E-BP1 that has been phosphorylated by mTOR, did not induce multiple axons. On the other hand, p70S6K was necessary and sufficient for mTOR-controlled axon formation, as described above. Thus, p70S6K is a pivotal effector of the mTOR pathway in the development of neuronal polarity.

In summary, mTOR-p70S6K-governed protein synthesis is crucial for the development of neuronal polarity. The activation of the mTOR pathway in the axon induces the local translation of several polarity genes, most importantly CRMP2 and tau. CRMP2 and tau accumulate specifically in the axon via 5'-TOP-dependent local translation, leading to the specification, formation, and maintenance of a single axon. Thus, this study uncovers the mechanism of mTOR-controlled axon formation, and provides new insights into the establishment of neuronal polarity.

REFERENCES


FOOTNOTES
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The abbreviations used are: mTOR, mammalian target of rapamycin; CRMP2, collapsing response mediator protein 2; p70S6K, 70 kDa ribosomal protein S6 kinase; eIF-4E, eukaryotic translation initiation factor 4E; 4E-BP1, eIF-4E binding protein 1; 5'-TOP, 5'-terminal oligopyrimidine tract; RLM-5'-RACE, RNA ligase-mediated rapid amplification of 5'-cDNA ends; DIV, day in vitro; TSS, transcription start site; CA, constitutively active; DN, dominant-negative.

FIGURE LEGENDS

Fig. 1. The mTOR pathway regulates neuronal polarity. A. Isolated hippocampal neurons were transfected with pCAGGS-EGFP, pCAGGS-FLAG-CA-Rheb, pCAGGS-FLAG-CA-S6K, pCAGGS-FLAG-eIF4E, pCAGGS-FLAG-DN-Rheb, pCAGGS-FLAG-DN-S6K, or pCAGGS-FLAG-4E-BP1. After 3 DIV, the neurons were fixed and stained with anti-GFP (white), anti-FLAG (white), anti-MAP2 (dendrite marker, red), and anti-Tau1 (axon marker, green) antibodies. Bar = 50 µm. B, From the immunostained images shown in (A) the transfected neurons were classified according to their polarity: no axon (white bars); single axon (black bars); multiple axons (gray bars). Statistical analysis was carried out for four independent experiments.

Fig. 2. p70S6K is critical for the mTOR-regulated neuronal polarity. A, Isolated hippocampal neurons were transfected with pCAGGS-EGFP, pCAGGS-FLAG-CA-Rheb, or pCAGGS-FLAG-CA-S6K, and incubated with or without 100 nM rapamycin. After 3 DIV, the neurons were fixed and stained with anti-GFP (white), anti-FLAG (white), anti-MAP2 (red), and anti-Tau1 (green) antibodies. Bar = 50 µm. B, From the immunostained images shown in (A), the transfected neurons were classified according to their polarity: no axon (white bars); single axon (black bars); multiple axons (gray bars). Statistical analysis was carried out for four independent experiments. C, Hippocampal neurons at 3 DIV were fixed and stained with anti-phosphorylated p70S6K (Thr421/Ser424) (p-S6K), anti-p70S6K (S6K), anti-phosphorylated 4E-BP1 (Ser65) (p-4E-BP1), and anti-4E-BP1 (4E-BP1) antibodies. The fluorescent intensities of immunostained images of the dendrites (red) and axon (green) were measured, respectively, and are presented in the graph. Bar = 50 µm.

Fig. 3. Translational regulation of neuronal polarity proteins by the mTOR-S6K pathway. A, Hippocampal neurons were transfected with pCAGGS-EGFP or pCAGGS-FLAG-CA-S6K by nucleofection, and cultured for 2 days. The total proteins were then extracted from the neurons. Neuronal polarity proteins were detected by Western analyses using the indicated antibodies. B, The amounts of proteins were determined by densitometry and statistically analyzed by Student’s t-test. The quantification of each protein was normalized to GAPDH. Statistical analyses were carried out for three independent experiments. C, The total RNAs were extracted from the GFP- or CA-S6K-transfected neurons, and expression of mRNAs for neuronal polarity genes was detected by real-time RT-PCR using gene-specific PCR primers. Statistical analyses were carried out for three independent experiments.
Fig. 4. Effect of rapamycin on the translation of neuronal polarity proteins. A, Hippocampal neurons were cultured with or without 100 nM rapamycin for 1-3 days. The total proteins were then extracted from the neurons. Neuronal polarity proteins were detected by Western analyses using the indicated antibodies. (B) The amounts of proteins were determined by densitometry and statistically analyzed by Student’s t-test. The quantification of phosphorylated p70S6K was normalized to total p70S6K. The quantification of other protein was normalized to GAPDH. Statistical analyses were carried out for three independent experiments. (C) The total RNAs were extracted from the rapamycin-treated neurons, and expression of mRNAs for neuronal polarity genes was detected by real-time RT-PCR using gene-specific PCR primers. Statistical analyses were carried out for three independent experiments.

Fig. 5. CA-S6K rescues the rapamycin-induced reduction in the expression of polarity proteins. A, Hippocampal neurons were transfected with pCAGGS-EGFP or pCAGGS-FLAG-CA-S6K by nucleofection, and cultured for 2.5 days with or without 100 nM rapamycin. The total proteins were then extracted from the neurons. CRMP2, tau, and Rap1 proteins were detected by Western analyses. B, The amounts of proteins were determined by densitometry and statistically analyzed by Student’s t-test. The quantification of each protein was normalized to GAPDH. Statistical analyses were carried out for three independent experiments.

Fig. 6. Overexpression of CRMP2 and tau rescues the rapamycin-induced polarity defect. A, Hippocampal neurons were transfected with pCAGGS-EGFP, pCAGGS-FLAG-CRMP2, pCAGGS-FLAG-Tau, and/or pCAGGS-FLAG-CA-rap1b, and cultured with or without 100 nM rapamycin for 3 days. The cells were fixed and stained with anti-GFP (white), anti-FLAG (white), anti-MAP2 (red), and anti-Tau1 (green) antibodies. Bar = 50 µm. B, From the immunostained images shown in (A), the transfected neurons were classified according to their polarity: no axon (white bars); single axon (black bars); multiple axons (gray bars). Statistical analysis was carried out for four independent experiments.

Fig. 7. Overexpression of CRMP2 and tau rescues the DN-S6K-induced polarity defect. A, Hippocampal neurons were transfected with pCAGGS-HA-DN-S6K, pCAGGS-FLAG-CRMP2, pCAGGS-FLAG-Tau, and/or pCAGGS-FLAG-CA-rap1b, and cultured for 3 days. The cells were fixed and stained with anti-HA (white), anti-MAP2 (red), and anti-Tau1 (green) antibodies. Bar = 50 µm. B, From the immunostained images shown in (A), the transfected neurons were classified according to their polarity: no axon (white bars); single axon (black bars); multiple axons (gray bars). Statistical analysis was carried out for four independent experiments.

Fig. 8. Axonal translation of CRMP2 was regulated by the mTOR-p70S6K pathway through its 5′-terminal oligopyrimidine (5′-TOP) tract. A, The sequences of the 5′-UTR and promoter region of the CRMP2 gene. Transcriptional start sites of the CRMP2 mRNA (black and red arrows) were determined by RLM-5′-RACE. Red arrows indicate the transcriptional start sites conforming to the rule for 5′-TOP mRNAs. Underlined letters indicate the pyrimidine-rich region. Bold letters indicate the translational start codon (ATG). B, A luciferase reporter assay was performed using pGL4.14-CRMP2 or pGL4.14-CRMP2 (delta 5′-TOP) vectors. pCAGGS-FLAG-CA-S6K was co-transferred to activate the 5′-TOP-mediated translation. Total RNAs were extracted from the transfected cells, and expression levels of ectopic luciferase mRNA were determined by RT-PCR. The numbers indicate the relative luciferase expression levels, which were quantitated by densitometry and normalized to β-galactosidase mRNA. C, Localization of endogenous CRMP2 protein. Hippocampal neurons at 3 DIV were fixed and
stained with anti-CRMP2 antibody. The fluorescence intensities of the immunostained images of dendrites (red) and axons (green) were measured, respectively, and are presented in the graph. Bar = 50 µm. D, Hippocampal neurons were co-transfected with pCAGGS-EGFP and pGL4.22-CRMP2-myr-Luc, and cultured with or without 100 nM rapamycin. After 3 DIV, the neurons were fixed and stained with anti-GFP (green) and anti-luciferase (red) antibodies. The fluorescence intensities of the immunostained images of the dendrites and axon were measured, respectively, and are presented in the graph. Bar = 50 µm.

Fig. 9. Axonal translation of tau was also regulated by the mTOR-p70S6K pathway through its 5’-TOP sequence. A, The 5’-UTR sequences of the mouse, rat, and human tau mRNAs were aligned. Red box indicates 5’-TOP sequence. Blue box indicates translational start codon (ATG). Asterisks indicate residues conserved in all three species. B, A luciferase reporter assay was performed using pGL4.14-tau or pGL4.14-tau (mut 5’-TOP) vectors. pCAGGS-FLAG-CA-S6K was co-transferred to activate the 5’-TOP-mediated translation. Total RNAs were extracted from the transfected cells, and expression levels of ectopic luciferase mRNA were determined by RT-PCR. The numbers indicate the relative luciferase expression levels, which were quantitated by densitometry and normalized to β-galactosidase mRNA. C, Localization of endogenous tau protein. Hippocampal neurons at 3 DIV were fixed and stained with anti-tau antibody. The fluorescence intensities of the immunostained images of dendrites (red) and axons (green) were measured, respectively, and are presented in the graph. Bar = 50 µm. D, Hippocampal neurons were co-transfected with pCAGGS-EGFP and pGL4.22-tau-myr-Luc, and cultured with or without 100 nM rapamycin. After 3 DIV, the neurons were fixed and stained with anti-GFP (green) and anti-luciferase (red) antibodies. The fluorescence intensities of the immunostained images of the dendrites and axon were measured, respectively, and are presented in the graph. Bar = 50 µm.
Fig. 1
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Fig. 9
Specification of neuronal polarity regulated by local translation of CRMP2 and tau via the mTOR-p70S6K pathway
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