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Summary

Although hippocampal neurons are well-distinguished by the morphological characteristics of their dendrites and their structural plasticity, the mechanisms involved in regulating their neurite initiation, dendrite growth, network formation and remodeling are still largely unknown, in part because the key molecules involved remain elusive. Identifying new dendrite-active cues could uncover unknown molecular mechanisms that would add significant understanding to the field and possibly lead to the development of novel neuroprotective therapy because these neurons are impaired in many neuropsychiatric disorders. In our previous studies, we deleted the gene encoding CRMP3 in mice and identified the protein as a new endogenous signaling molecule that shapes diverse features of the hippocampal pyramidal dendrites without affecting axon morphology. We also found that CRMP3 protects dendrites against dystrophy induced by prion peptide $PrP^{106–126}$. Here, we report that CRMP3 has a profound influence on neurite initiation and dendrite growth of hippocampal neurons *in vitro*. Our deletional mapping revealed that the C-terminus of CRMP3 probably harbors its dendritogenic capacity and supports an active transport mechanism. By contrast, overexpression of the C-terminal truncated CRMP3 phenocopied the effect of CRMP3 gene deletion with inhibition of neurite initiation or decrease in dendrite complexity, depending on the stage of cell development. In addition, this mutant inhibited the activity of CRMP3, in a similar manner to siRNA. Voltage-gated calcium channel inhibitors prevented CRMP3-induced dendritic growth and somatic Ca^{2+} influx in CRMP3-overexpressing neurons was augmented largely via L-type channels. These results support a link between CRMP3-mediated Ca^{2+} influx and CRMP3-mediated dendritic growth in hippocampal neurons.

Key words: CRMP3, Dendrite morphogenesis, Neurite initiation, Ca^{2+} flux, Lamellipodia, Hippocampal neurons

Introduction

Dendritic arbors of neurons play a central role in brain development and plasticity as their specific features provide the structural support for connectivity and information processing. Despite the great diversity between neuronal types, most vertebrate nerve cells exhibit distinctive dendritic morphology depending on their location. These dendrite characteristics influence the electrical and chemical signals that a neuron receives, and consequently impact the neuronal network assembly and function. The dendritic arbor emerges as a convergent product of specific patterns of growth, branching and retraction and is differentially regulated at several points, including control of the number of dendrites, primary branches and their mode and frequency of branching. The balance between growth, retraction and stabilization of dendritic branches persists throughout lifetime (Sakisaka et al., 2004; Huang et al., 2007; Georges et al., 2008; Lin and Koleske, 2010; Kulkarni and Firestein, 2012). As a consequence, studies on dendrite morphogenesis seek, not only to examine their developmental origins such as neurite initiation and dendrite specification and growth (Barnes and Polleux, 2009; Emoto, 2011; Cheng and Poo, 2012), but also to uncover the mechanisms of their inherent remodeling plasticity.

Manipulation of gene expression both *in vivo* and *in vitro* provides excellent paradigms to uncover a regulatory role for a protein in maintaining neuronal polarity and plasticity. The primary hippocampal neuron is one of the most widely used culture systems for investigating neuronal differentiation and regulation. A few hours after plating, neurons extend minor

neurites; then one of them extends rapidly and by morphological transformation generates the axon through stochastic selection. The other neurites mature into dendrites, establishing neuronal polarity (Dotti et al., 1988; Calderon de Anda et al., 2008). Because neurons in culture develop polarity without exogenous guidance or neurotropic cues, it is believed that an internal polarization program exists involving several intra-neuronal organelles and distinct repertoires of signaling molecules intrinsic to the neuron (Goldberg, 2004). Extrinsic signals such as neurotrophins (Song and Poo, 1999), attractant and repellent guidance cues such as semaphorins (Fiore and Püschel, 2003; Schlomann et al., 2009) and guide-post proteins released by guide-post cells (Palka et al., 1992) provide short and long range guidance information. These signals can also stably change the intrinsic ability of neurons to extend or retract neurites during development (Vanderhaeghen and Polleux, 2004) or to engage them into an axono-dendritic differentiation path (Horton and Ehlers, 2003). Extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin also influence neurite development (Lein and Higgins, 1989; Boisseau and Simonneau, 1989; Chamak and Prochiantz, 1989), and together, these various factors provide dendrites with proper routing information to reach appropriate targets or cover appropriate receptive areas (Polleux et al., 2000; Mitsui et al., 2002). Although the mechanisms underlying dendrite development are still largely unknown, recent studies have shown that the Rho family small GTPases are mediators of dendritic morphogenesis through cytoskeleton remodeling (Luo, 2002; Van Aelst and Cline, 2004; Arimura et al., 2000). For instance, cypin increases dendrite morphogenesis by binding directly to tubulin (Akum et al., 2004), and this function is linked to RhoA activity (Chen and Firestein, 2007). However, erbin and δ-catenin, which regulate dendrite branching without affecting number of dendrites, act independently from RhoA and Rac1 (Arikkath et al., 2008).

It has generally been assumed that the axon-dendrite polarity involves distinct repertoires of signaling proteins. Overexpression of LKB1/STRAD (Shelly et al., 2007) or CRMP2 (Arimura et al., 2004) promotes multiple axon formation in cultured hippocampal neurons, whereas cypin (Akum et al., 2004), erbin and δ-catenin (Arikkath et al., 2008), and endogenous Rac1 (Gualdoni et al., 2007), Cdc42 (Chen et al., 2012) and Rab17 (Mori et al., 2012) activities are critical for dendrite development. In contrast, CRMP4 and CRMP5 suppress dendritic branching (Niisato et al., 2012; Brot et al., 2010). Despite these recent observations, the mechanisms involved in early events of neuronal differentiation, such as neurite initiation and maturation into dendrites are still poorly understood (Barnes and Polleux, 2009). Therefore, the identification and characterization of new molecules playing a role in dendrite morphogenesis are critical for the elucidation of the cellular and molecular mechanisms that underlie the generation/maintenance of dendrite arborization. CRMP3 is highly expressed in the brain during embryonic development but is localized mostly in hippocampus post-natally. As there is a spatial and temporal correlation between the pattern of expression of CRMP3 and the timing of neurite outgrowth and the distribution of dendritic plasticity, we investigated the role of CRMP3 by targeted-disruption of the CRMP3 gene and established a line of $CRMP3^{-/-}$ mice. We found that CRMP3 regulates dendritic complexity in vertebrate hippocampal neurons *in vivo*. $CRMP3^{-/-}$ animals displayed alterations in the morphology of dendrites and spines in developing hippocampal neurons that persisted in adult mice. In addition, they exhibited hippocampal-dependent functional impairment with abnormal long-term potentiation (LTP), a deficit in prepulse inhibition (PPI) (Quach et al., 2008a; Quach et al., 2008b) and a significant increase in sensitivity to neurotoxin-induced kindling (T.T.Q., unpublished results). We also showed that overexpression of CRMP3 prevented dendrito-toxicity induced by prion peptide $PrP^{106–126}$ *in vitro*, suggesting that CRMP3 has a role in neuroprotection of hippocampal neurons (Quach et al., 2011). This set of convergent results provides evidence that CRMP3 might be a novel neurite or dendrite outgrowth regulator and because its expression is maintained throughout life in the hippocampus, it could play a critical role in hippocampal function and plasticity, and might be involved in aging and the pathophysiology of neurodegenerative disorders.

Despite these observations, little is known of how dendritic development is regulated by CRMP3. Several observations suggest that Ca^{2+} signaling via Ca^{2+} channels and by release of intracellular stores influence the growth and branching of dendrites and the formation of spines (Lohmann, 2009; Yu and Goda, 2009; Puram et al., 2011). In particular, it has been shown that local Ca^{2+} signaling regulates filopodia dynamics (Lohmann et al., 2005) and influences dendrite branching (Konur and Ghosh, 2005). In line with these observations, our recent studies linked the canonical roles of CRMPs in the specification of neuronal structure and function (Charrier et al., 2003) to the activation of neuronal voltage-gated Ca^{2+} channels (VGCCs) (Chi et al., 2009; Wang et al., 2010; Brittain et al., 2011). Our previous work using whole-cell voltage clamp electrophysiology has also shown that CRMP3 enhances Ca^{2+} current density via VGCCs in transfected hippocampal neurons (Quach et al., 2011). Here, we report, for the first time, that CRMP3 expression affects neurite initiation, primary dendrite formation and branching without affecting axon morphology. We also show that this activity resides in the C-terminus of the protein and that Ca^{2+} influx, largely via L-type Ca^{2+} channels, might be involved in CRMP3-mediated dendritogenic activities in hippocampal neurons.

Results

CRMP3 expression controls neurite initiation and dendrite morphogenesis

CRMP3 is highly expressed in adult hippocampus. To determine its role we created a $CRMP3^{-/-}$ mouse line and found that during early post-natal development, hippocampal pyramidal neurons in these mice displayed dendritic dystrophy that persisted throughout adulthood (Quach et al., 2008a). To complement our *in vivo* observations, in this study we examined the role of CRMP3 on dendrite development *in vitro* using cultured hippocampal cells from $CRMP3^{-/-}$, $CRMP3^{+/-}$ and wild-type (WT) mice. Hippocampal neurons (stained with β-III tubulin, a neuronal marker) prepared from WT mice and grown for 6 days *in vitro* (d.i.v.), displayed a single axon (as assessed by staining with the axonal marker Tau) as well as several dendrites identified by staining with an antibody against microtubule-associated protein (MAP2; supplementary material Fig. S1A,B). In marked contrast, $CRMP3^{-/-}$ hippocampal neurons, identified as LacZ positive by β-galactosidase activity ($LacZ^{+}$, Fig. 1Ai, white arrow) or immunostaining with anti-β-galactosidase antibody (β-gal, Fig. 1Aiii) exhibited a very different morphology with a

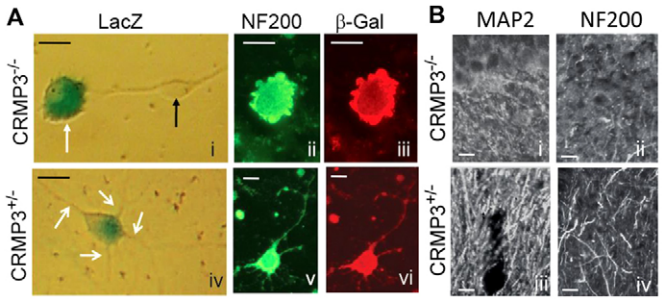

Fig. 1. Deleting CRMP3 curbs neurite initiation and dendrite morphogenesis. (A) Representative LacZ^{+}/CRMP3$^{-/-}$ (blue) cell with no processes (i, white arrow) and LacZ^{-} cell with neurites (i, black arrow) after 10 d.i.v detected using β-galactosidase activity. Colocalization of CRMP3$^{-/-}$ cell with neurofilament 200 (NF200, ii) and anti-β-galactosidase (β-gal) antibodies (iii). Representative LacZ^{+}/CRMP3$^{+/-}$ cell bearing neurites (iv, white arrows) co-stained with NF200 (v) and anti-β-gal antibodies (vi). (B) Representative CA1 hippocampal sections from 10-day-old CRMP3$^{-/-}$ (i,ii) and CRMP3$^{+/-}$ (iii,iv) mice immunostained with MAP2 (i,iii) and NF200 (ii,iv). Scale bars: 15 μm (A); 30 μm (B).

complete absence of neurites. These cells displayed only short and round processes that did not evolve beyond stage 1 of neuronal differentiation (Banker and Cowan, 1979), even after 10 d.i.v. Staining with NF200, a neuronal marker (Fig. 1Aii) and with MAP2 (supplementary material Fig. S2), confirmed that these cells were neurons. Normal neurite initiation and formation were observed in cultures of hippocampal neurons prepared from heterozygous CRMP3$^{+/-}$ mice (Fig. 1Aiv–vi). Altered cellular phenotypes were also evident by immunostaining of hippocampus with a scarcity of neurites in CRMP3$^{-/-}$ (Fig. 1Bi–ii) whereas neuronal processes appeared normal in CRMP3$^{+/-}$ (Fig. 1Biii–iv). In addition, although the main structure of the hippocampus was not grossly altered (supplementary material Fig. S3A–D), there was a small but significantly higher presence of ectopic pyramidal-like cells in stratum oriens that could underlie the differential migratory response of these neurons to CRMP3 (supplementary material Fig. S4A–B).

Our results from gene targeting support the hypothesis that CRMP3 is involved in dendritic development and might be a positive regulator of dendrite formation. To determine whether CRMP3 influences early morphogenetic events in cultured hippocampal neurons, we quantified the effect of increasing CRMP3 expression on the development of neuronal polarity and dendritogenesis. CRMP3 full-length cDNA was tagged with FLAG (Fig. 2A), 1 μg FL-CRMP3 DNA was transfected at 3 d.i.v. and the phenotype of transfected cells analyzed 72 hours later. The dendritic arbor of neurons transfected with control plasmid (Fig. 3C) and untransfected neurons was similar. Remarkably, 70–75% of the FL-CRMP3-expressing neurons developed both abundant short neurites and long highly branching neurites (Fig. 3A). These neurites exhibited the characteristic of dendrites with positive immunoreactivity for MAP2 (Fig. 3A) and no immunoreactivity for the axonal marker, Tau1 (not shown), therefore, these processes are likely dendrites. Quantitative analyses demonstrated that CRMP3-expressing neurons had a large increase in number of primary dendrites (Fig. 3F), total branching points (Fig. 3G) and total dendritic length (Fig. 3H) compared with untransfected or GFP-transfected cells, suggesting that CRMP3 regulates dendritogenesis and

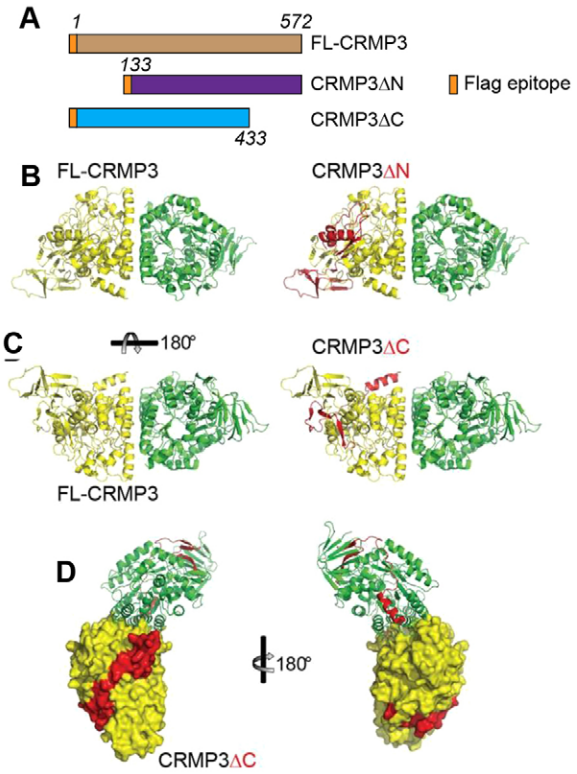

Fig. 2. Homology model of CRMP3 and N- and C-terminus constructs. (A) Linear schematic of CRMP3 constructs. The italicized numbers refer to the amino acids denoting the locations of the N- and C-terminus truncations and the site of the FLAG epitope. A homology model of CRMP-3 [(ribbon representations (B,C); surface representation (D)] was constructed based on the crystal structure of CRMP-1 (PDB: 1KCX) using SWISS-MODEL workspace (Arnold K. et al., 2006). The QMEAN4 global score, a composite scoring function for both the estimation of the global quality of the entire model, as well as for the local per-residue analysis of different regions within a model, was 0.67 with a z-score of −1.67. The structures of full-length CRMP3 (B,C), a construct lacking amino acids 1–132 (CRMP3ΔN; B), and a construct lacking amino acids 434–572 (CRMP3ΔC; C,D) are illustrated. The surface representation of one monomer (D) illustrates the largely surface exposed residues (highlighted in red) that were deleted in the CRMP3ΔC construct. For clarity, only dimers are shown. The rotational axes are as indicated.

dendrite arborization. Interestingly, FL-CRMP3-transfected neurons were often (>75%) surrounded by an extensive prominent thin sheet-like lamellipodium (Fig. 3B, yellow arrows; Fig. 4E, white arrowheads), not present in GFP-transfected or untransfected neurons. Lamellipodium has been shown to strongly influence neurite initiation and filopodia genesis, and to be indispensable for successful dendrite elaboration (Dotti et al., 1988; Yamaguchi et al., 2009). The widespread presence of CRMP3 puncta in the lamellipodium (Fig. 4E) suggests a link between CRMP3 expression and lamellipodial formation.

Dendritogenic activity of CRMP3 resides in its C-terminus

We next investigated which region within CRMP3 is responsible for its dendritogenic activity. To map the domains that possibly impart this activity, we relied on a series of published findings as a guide for the design of CRMP3 constructs. First, a previous

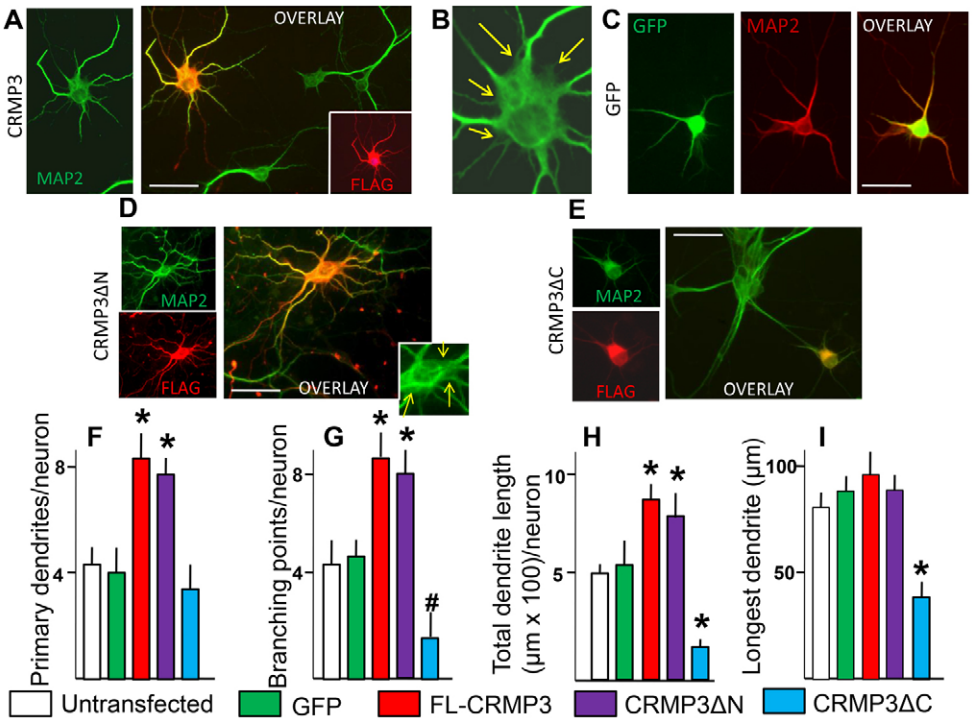

Fig. 3. Dendritogenic activity of CRMP3 resides in its C-terminus. Dissociated 3 d.i.v. hippocampal neurons were transfected with 1 μg FL-CRMP3 (A,B), CRMP3ΔN (D), CRMP3ΔC (E) or GFP control DNA (C) and fixed at 6 d.i.v. Representative transfected neurons immunostained for FLAG (red) and dendritic marker MAP2 (green). Overlay images of transfected neurons are in yellow. Note the morphological features of FL-CRMP3- and CRMP3ΔN-transfected neurons characterized by an increase in lamellipodial formation (yellow arrows in B,D) and dendritic arborization. Quantitative analyses of hippocampal neurons expressing FL-CRMP3 and CRMP3ΔN had significantly more primary dendrites (F), branching points (G) and total dendritic length (H) compared with untransfected and GFP-transfected neurons. Quantitative analyses showed a significant decrease in dendrite number, length, branching and length of longest dendrite in CRMP3ΔC-transfected cells compared with untransfected and GFP-transfected cells (G–I). Data are expressed as means ± s.e.m. of 40–60 neurons per group, and values that differ significantly from GFP-transfected neurons (Student's *t*-test) are indicated as follows: $*P<0.01$, FL-CRMP3 or CRMP3ΔN- or CRMP3ΔC-transfected neurons versus GFP-transfected neurons; $^{\#}P<0.05$, CRMP3ΔC-transfected neurons versus GFP-transfected neurons. Scale bars: 50 μm.

study had implicated the C-terminus of the related CRMP5 protein CRMP5ΔC472 as important for morphogenesis of hippocampal dendrites (Brot et al., 2010). Second, deletion of the C-terminus in CRMP2 resulted in loss of its axon-promoting effect (Inagaki et al., 2001). Finally, it was found that mutating a tyrosine kinase site within the C-terminus of CRMP2, resulted in loss of function of CRMP2 (Varrin-Doyer et al., 2009), and this site is also conserved in CRMP3. Additionally, based on the published crystal structure of CRMP1 (Deo et al., 2004), we developed a homology model of CRMP3 (Fig. 2B–D). This predicted structure revealed that large parts of the C-terminus are surface exposed, not involved in CRMP tetramerization, and therefore could possibly harbor the dendritogenic activity. Reasoning that the CRMP3 C-terminus might harbor its activity, we created FLAG-tagged CRMP3 constructs lacking either the C-terminus (CRMP3ΔC) or the N-terminus (CRMP3ΔN) (Fig. 2A), then transfected them into WT hippocampal neurons. Similar to neurons transfected with FL-CRMP3 (Fig. 3A), the CRMP3ΔN-transfected neurons were surrounded by lamellipodia and exhibited extensive dendrite formation (Fig. 3D), suggesting that the N-terminus domain is not necessary for constitutive CRMP3 dendritogenic activity. In contrast, we found that cultured WT hippocampal neurons transfected at 3 d.i.v. with CRMP3ΔC (Fig. 3E) had significantly shorter dendrites when compared to neurons expressing GFP–control (Fig. 3C), with a significant decrease in branching points (Fig. 3G), total dendritic length (Fig. 3H) and also longest dendrite length (Fig. 3I). Thus, the deletion of 139 amino acids from the C-terminus was sufficient to convert the dendrite-inducing activity to an inhibitory one. Interestingly, when WT neurons were transfected with CRMP3ΔC at 15 hours after plating instead of 3 d.i.v. they did not develop neurites (Fig. 4A1,A2). Their morphology was very similar to the one observed in $CRMP3^{-/-}$ cultured neurons (Fig. 1Ai–iii). Staining with neurofilament, a neuronal marker, confirmed that these cells were neurons (Fig. 4B1–B3), and staining with TUNEL showed that they were not apoptotic (Fig. 4C1–C4). Our current observations support the concept that CRMP3 regulates not only dendrite growth, but also neurite initiation.

To determine whether the CRMP3ΔC truncated protein can directly compete and inhibit the activity of FL-CRMP3 on dendrite arborization, we co-transfected hippocampal neurons with the two plasmids. The C-terminus-deleted CRMP3 exerted a dominant-negative effect on the FL-CRMP3 protein by preventing CRMP3-induced increase in dendritogenesis (Fig. 5B), whereas co-expression of a control GFP protein had no effect (Fig. 5A). Quantitative analyses revealed that, compared with the GFP+FL-CRMP3 co-transfection condition, neurons transfected with CRMP3ΔC+FL-CRMP3 had a significant reduction in the number of primary neurites per

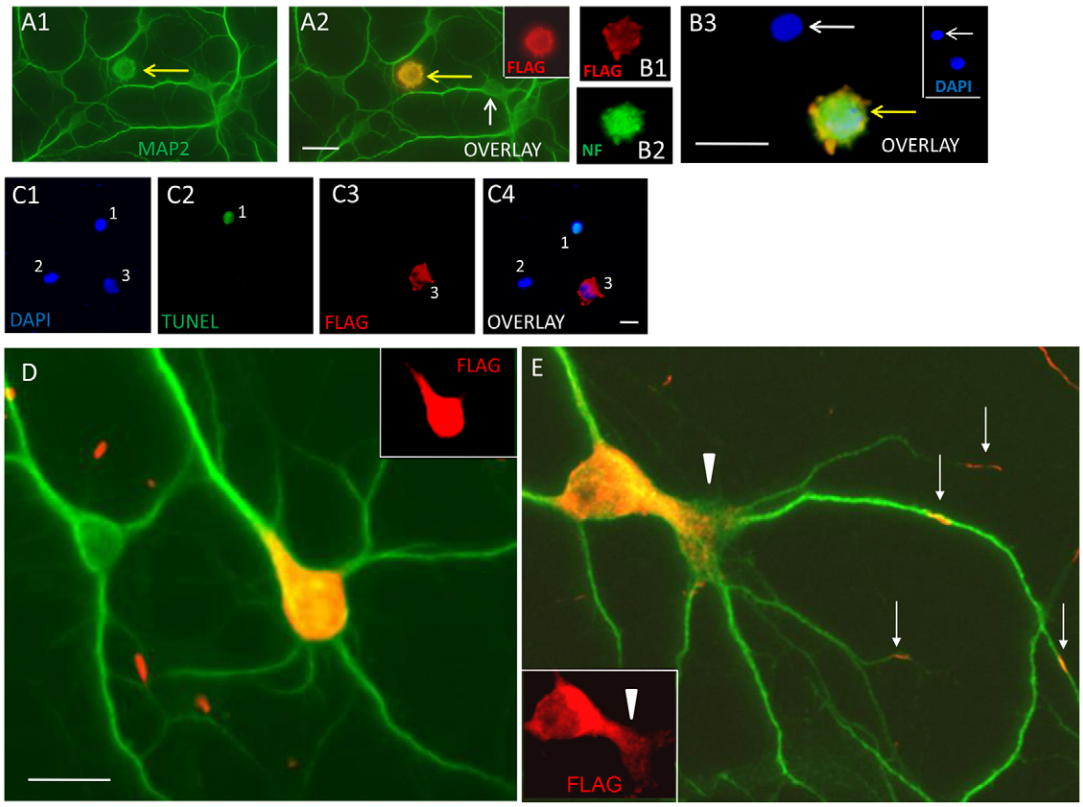

Fig. 4. Early transfection of CRMP3ΔC prevents neurite initiation. Representative neuron transfected with CRMP3ΔC at 15 hours in culture, stained for MAP2 (A1, green) and for FLAG peptide (inset of A2, red). A2 shows merged image. Note the absence of neurites in cells transfected early after plating, similar to cultured CRMP3$^{-/-}$ neurons (Fig. 1Ai–iii). These cells express neurofilament: transfected neurons co-stained with FLAG (red), neurofilament (green) and DAPI (blue) are shown in B1, B2 and inset of B3, respectively. B3 shows merged image (white arrow, untransfected cell; yellow arrow, transfected neuron). (C) TUNEL staining. The transfected cells are not apoptotic: note three cell nuclei stained with DAPI (C1); only the top cell nucleus (C2) is TUNEL^{+}. CRMP3ΔC-transfected cell (C3) is TUNEL^{-} (n=21; all negative). C4 is the merged image. Puncta distribution of CRMP3. The distribution of FL-CRMP3 puncta in dendrites (white arrows) and in lamellipodial formation (white arrowheads) of FL-CRMP3-transfected neurons (0.4 μg/well for 24 hours) shown in E contrasts with the absence of FLAG–(1–150 aa)ΔCRMP3 dendritic distribution in FLAG–(1–150 aa)ΔCRMP3-transfected neurons (0.4 μg or 1.0 μg/well for 24 hours) shown in D. The truncated protein was confined to the cell body. n=20–25 neurons/group. Scale bars: 30 μm.

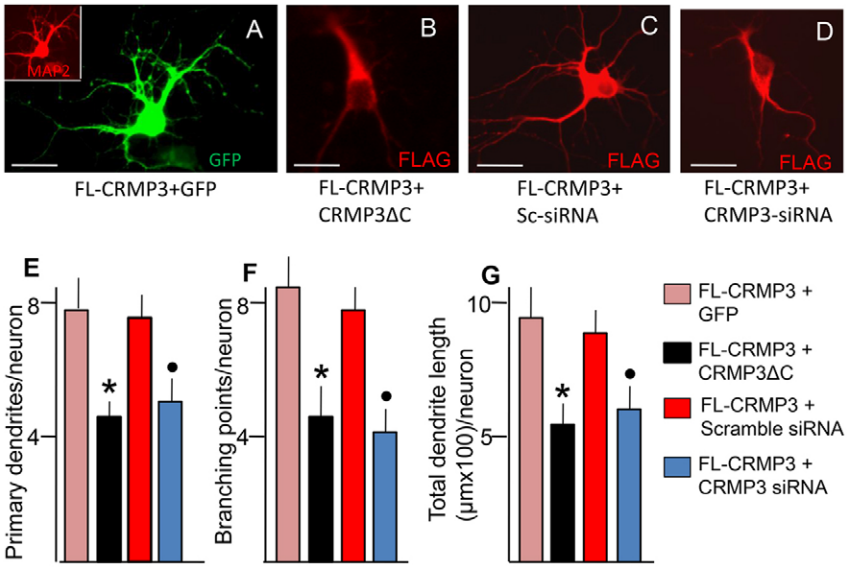

Fig. 5. Co-transfection with CRMP3ΔC or siRNA knockdown of CRMP3 prevents FL-CRMP3-induced increase in dendrite arborization. Representative images of cultured hippocampal cells co-transfected with FL-CRMP3 (1 μg/well) and GFP (1 μg/well) (A; stained for GFP and MAP2, inset) or with FL-CRMP3 (1 μg/well) and CRMP3ΔC (1 μg/well) (B; stained for FLAG). Quantitative analysis of dendrite characteristics is shown in E–G. Representative images of cultured hippocampal neurons co-transfected with FL-CRMP3 (1 μg/well) and scramble siRNA (1 μg/well) (C) or CRMP3 siRNA (1 μg/well) (D) stained with FLAG antibody. Quantitative analysis of dendrite characteristics (Student's t-test) is shown in E–G. *P<0.05, FL-CRMP3+CRMP3ΔC-transfected neurons versus FL-CRMP3+GFP-transfected neurons; n=20–27. $^{\bullet}P$<0.05 FL-CRMP3+CRMP3 siRNA compared with FL-CRMP3+Sc-siRNA; n=17–22). Scale bars: 25 μm.

neuron (Fig. 5E), and in total dendritic length (Fig. 5G). To confirm the role of CRMP3 in dendrite regulation, we used short interfering RNA (siRNA). The dominant-negative phenotype of CRMP3ΔC was phenocopied in neurons co-transfected with CRMP3 and a short interfering RNA (siRNA) against CRMP3 (Fig. 5D) whereas a control scramble siRNA had no effect on dendritic parameters (Fig. 5C). Quantitative analysis revealed a significant decrease in the number of primary dendrites, number of branching points and total dendritic length with CRMP3 siRNA (Fig. 5E–G), with no effect for scrambled siRNA. In all, our findings show that CRMP3ΔC, which lacks the C-terminus of CRMP3, acts in a dominant-negative fashion to suppress dendritic morphogenesis.

Confocal analyses of FLAG immunofluorescence showed that FL-CRMP3 preferentially distributes in the soma and dendrites of transfected hippocampal neurons (data not shown). The proteins probably contain a 'zipcode motif' that is critical for the targeting to dendrites. Whereas the axonal function of CRMP2 has been linked to the formation of the CRMP2–Sra-1–WAVE–Kinesin cargo complex, suggesting the active transport of CRMP2 into the axon (Kawano et al., 2005; Namba et al., 2011), mechanisms underlying the dendritic distribution of CRMP3 are unknown. In a first attempt to determine whether CRMP3 requires similar active transport process, we transfected hippocampal neurons with FL-CRMP3 (0.4 µg/well) for only 24 hours. At this time and dose, rather than a passive lateral diffusion, the protein presented as puncta in the soma and dendrites with some puncta extending in to the dendritic tips (Fig. 4E, white arrows). Comparison with the more intense and homogenous distribution of CRMP3 in dendrites seen at a later time (Fig. 3A) suggests a directional movement of CRMP3 from the soma to the dendrites. As CRMP3ΔN was also found in dendrites and retains CRMP3 activity in dendrite formation, we hypothesized that the cargo binding or transport signal sequence is not present in the 1–150 deleted amino acids of CRMP3ΔN. To control the selectivity and effectiveness of FL-CRMP3 puncta directional drift movement, we constructed and expressed these 150 amino acid tagged with FLAG [FLAG–(1–150)ΔCRMP3]. Consistent with our hypothesis, we found that FLAG–(1–150)ΔCRMP3 was confined in cell bodies and was absent in dendrites (Fig. 4D). None of the FLAG–(1–150)ΔCRMP3-transfected neurons exhibited dendritic FLAG staining, suggesting that the first 150 amino acids from the N-terminus are not associated with active dendritic transport.

CRMP3 does not modify axon morphology

To confirm that CRMP3 affects primary neurites and dendrites but not axons, after axonal specification has occurred, the role of CRMP3 on axonal growth was also investigated. We did not detect any significant changes in the architecture of axons in neurons transfected with FL-CRMP3 or CRMP3ΔN at 3 d.i.v. Neurons had only one axon identified by co-immunostaining of Tau-1 and FLAG without any significant qualitative or quantitative morphological change (Fig. 6). Characteristics of axons of CRMP3ΔC and FL-CRMP3-transfected neurons were not significantly different from those of control GFP-vector transfected neurons. The absence of significant change in axon morphology, including length (Fig. 6C) and number of branching points (Fig. 6D), corroborates the notion that axonal growth is

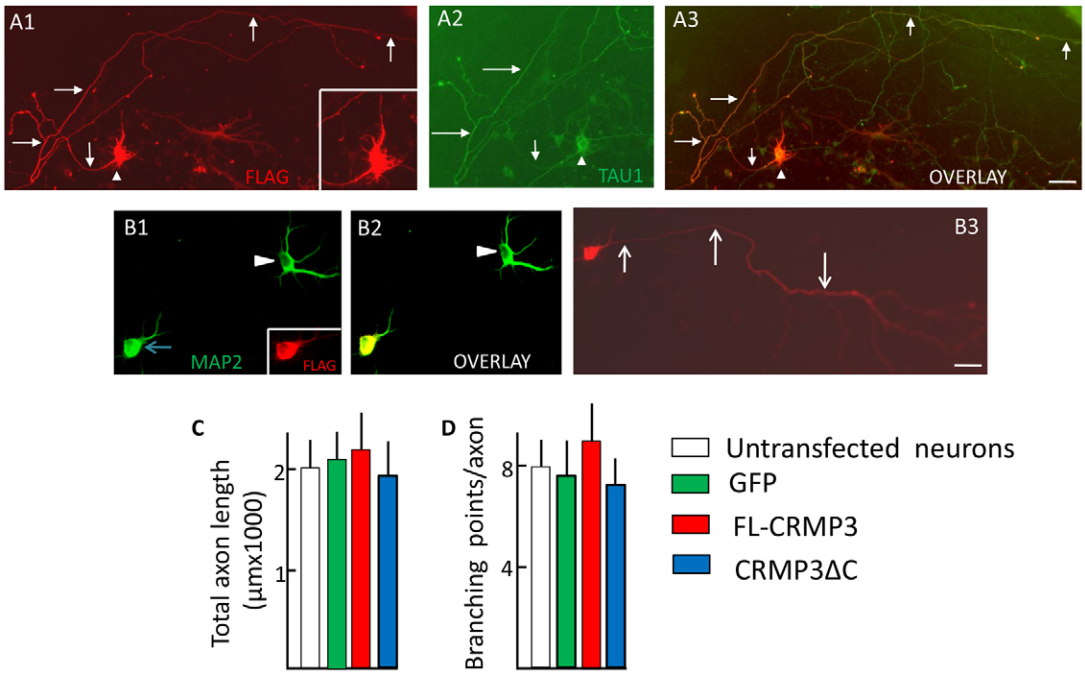

Fig. 6. CRMP3 overexpression and deletion of CRMP3 C-terminus does not affect axon morphology. Representative images of FLAg-antibody-stained (A1,B1 inset) neurons transfected with full-length FL-CRMP3 (A) or CRMP3ΔC (B). Cells were co-stained with the axonal antibody Tau (A2, B3) or MAP2 (B1). The merged images are shown in A3 and B2. White arrows, axon and axonal projection; white arrowheads, untransfected neurons in the field. Quantitative analyses of axon length (C) and number of branch points per axon (D) for untransfected or cells transfected as indicated. Data represent means ± s.e.m., n=20–25 cells (P=0.18, ANOVA). Scale bars: 30 µm.

CRMP3-independent under our experimental condition. Taken together, the present findings confirm our *in vivo* data (Quach et al., 2008a), and indicate that after neurite initiation has occurred, CRMP3 can induce dendritic – but not axonal – genesis in hippocampal neurons. In this aspect, CRMP3 differs from CRMP2, which affects axon specification and growth (Inagaki et al., 2001).

CRMP3-mediated dendritic activity is dependent on Ca^{2+} entry via L- and N-type VGCCs

We have previously shown that overexpression of CRMP3 increases Ca^{2+} influx via somatic VGCCs (Quach et al., 2011). Whether changes in Ca^{2+} flux affect the dendritic promoting activity of CRMP3 is unknown. We co-transfected hippocampal cultures with EGFP or CRMP3 at 3 d.i.v. and assessed dendritic arbor structure in the presence of inhibitors selective for voltage-gated [omega-conotoxin (Ω-CTX) for N-type VGCCs and Nifedipine (Nif) for L-type VGCCs] as well as ligand-gated (APV for NMDARs) Ca^{2+} influx (Fig. 7A). Using the Sholl methodology (Fig. 7B–E), we found that blocking Ca^{2+} influx via N- and L-type VGCCs in CRMP3-overexpressing neurons resulted in decreased dendritic complexity compared with vehicle-treated CRMP3-overexpressing neurons. In contrast, blocking ligand-gated calcium influx did not. The decrease in the number of primary dendrites was not significant.

As the sub-cellular distribution of Ca^{2+} channel types has been shown to change with phases of neuronal differentiation (Pravettoni et al., 2000), stage 4 hippocampal neurons were also analyzed to test for their possible involvement in CRMP3-mediated dendrite maintenance. Neurons were cultured for 10 d.i.v. and transfected with EGFP or with EGFP–CRMP3, and dendritic arbor structure was assessed after incubation with inhibitors selective for voltage and ligand-gated Ca^{2+} influx, as described above. Block of L-type channels with Nifedipine caused a significant decrease in branching complexity in CRMP3-transfected neurons compared with EGFP–transfected neurons [data not shown; $P<0.05$; Student's *t*-test ($n=40–45$ neurons)]. Block of N-type channels or NMDARs did not affect dendritic complexity [data not shown; $P>0.05$; one-way ANOVA with Dunnett's post-hoc *t*-test ($n=40–45$ neurons)]

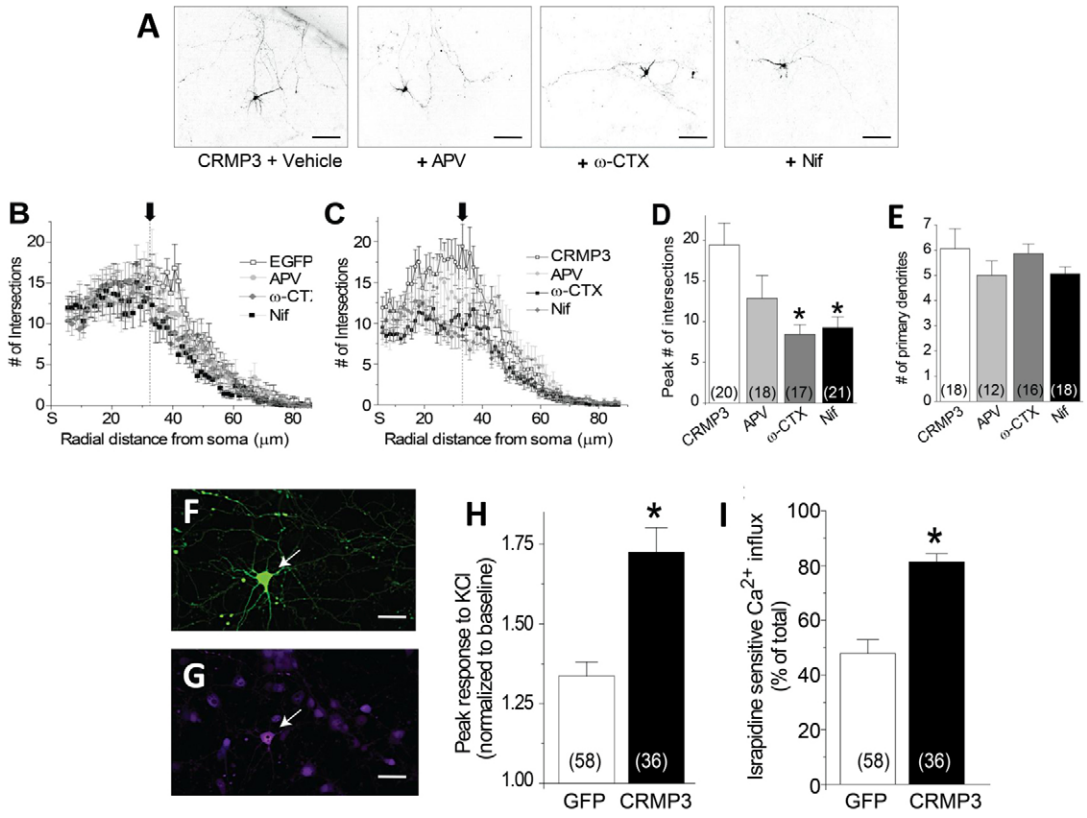

Fig. 7. Involvement of Ca^{2+} channels in CRMP3 activity and CRMP3-mediated facilitation of depolarization-evoked Ca^{2+} responses. Block of L- and N-type Ca^{2+} channels impairs neurite outgrowth in CRMP3-overexpressing neurons. Representative photo-negative images of cultured cortical neurons transfected with CRMP3 and EGFP, and incubated with vehicle or Ca^{2+} channel inhibitors for 24 hours (A). Sholl analysis of neurons overexpressing EGFP (B) or CRMP3 and EGFP (C) incubated with APV (200 μM), nifedipine (20 μM) or ω-conotoxin (1 μM) (B,C). Neurons were transfected at 3 d.i.v.; inhibitors were added 24 hours later and incubated overnight. The black arrows indicate the peak intersections, observed at about 320 μm from the soma. (D) Peak number of neurite intersections from Sholl analysis of CRMP3-overexpressing neurons with and without inhibitors. Overnight application of ω-conotoxin or nifedipine reduced overall neurite outgrowth (D) without affecting the length of the longest neurite or the number of primary dendrites (E). $n=12–21$; $^*P<0.05$ (ANOVA). CRMP3 overexpression increases KCl-stimulated Ca^{2+} influx in hippocampal neurons. Representative images of hippocampal neurons transfected with CRMP3 and EGFP (F) and loaded with the Ca^{2+} indicator Fura-2AM 48 hours post transfection (G). Peak response to a high KCl stimulus (H) normalized to baseline values averaged over several minutes before stimulation. CRMP3 overexpression increased the peak response compared with EGFP alone. Percentage of KCl-induced Ca^{2+} influx sensitive to block by israpidine. Israpidine-sensitive Ca^{2+} influx increased in neurons overexpressing CRMP3 (I). $n=36–58$; $^*P<0.05$, Student's *t*-test. Scale bars: 100 μm (A); 30 μm (F,G).

during this phase of neurite maintenance, suggesting that the type of Ca^{2+} channels involved in CRMP3 dendritic activity varies with stages of neuronal growth.

Ca^{2+} influx is increased in CRMP3-overexpressing neurons

Our results thus far have identified that N- and L-type Ca^{2+} channels regulate dendritic activity of CRMP3, with L-channels maintaining activity at a later stage of neuronal differentiation. So, we tested whether CRMP3 can augment Ca^{2+} influx and whether inhibitors of L-type Ca^{2+} channels can block this effect. Ca^{2+}-imaging experiments performed with Fura-2AM on EGFP-transfected or EGFP–CRMP3-transfected hippocampal neurons (Fig. 7F,G) demonstrated that stimulation with high (45 mM) KCl, a concentration where only CaV1 channels are recruited (Wheeler et al., 2012), produced a transient rise in intracellular calcium ($[Ca^{2+}]_c$). Peak calcium influx in response to the depolarizing stimulus was significantly higher in CRMP3-overexpressing neurons (Fig. 7H). KCl-induced Ca^{2+} influx was reduced by ~80% in the presence of Israpidine, a dihydropyridine blocker of L-type Ca^{2+} channels (Fig. 7I). This analysis was restricted to the soma of transfected neurons where a clear enhancement of dendritic complexity induced by CRMP3 could be observed. Collectively then, our results argue for a link between CRMP3-mediated Ca^{2+} influx and CRMP3-mediated dendritic growth.

Discussion

CRMPs constitute a family of proteins expressed abundantly in the nervous system (Wang and Strittmatter, 1996; Quach et al., 1997; Quach et al., 2000) involved in the control of growth/collapse of neurites (Charrier et al., 2003; Schmidt and Strittmatter, 2007), synaptic activity (Brittain et al., 2009; Wang et al., 2010; Khanna et al., 2012) and nociceptor excitability (Piekarz et al., 2012). Five isoforms of CRMPs have been identified in mammals. Members of this protein family display 75% homology with each other and 30% homology with the Unc-33 gene product involved in axonal guidance and synaptic organization in *C. elegans* (Li et al., 1992). We have shown that in dorsal root ganglia (DRG), CRMP1 is required for the neurite extension induced by neurotrophin-3 (NT3) and CRMP2 participates in the control of neurite extension in NGF-sensitive neurons (Quach et al., 2004). CRMP2 is also necessary for BDNF and NT3-induced axon outgrowth/branching in hippocampal neurons (Yoshimura et al., 2005). Manipulation of CRMPs expression has confirmed their role in neuritogenesis. Overexpression of CRMP2 in hippocampal neurons induced supernumerary axons (Inagaki et al., 2001), axon elongation (Cole et al., 2004) and increased axonal branching (Fukata et al., 2002), whereas its inhibition with a dominant-negative protein suppressed the formation of primary axons. Similarly, CRMP5 overexpression induced supernumerary growth cones and increased collateral axon branching, whereas its inhibition by siRNA led to a decrease in filopodia and growth cone formation (Hotta et al., 2005). CRMP5 also interacts with tubulin to remodel the function of CRMP2 (Brot et al., 2010). However, perhaps the most significant conceptual advance come from our CRMP5 and CRMP1 gene targeting in mice: these two members of the CRMP family, which are distributed in many regions of the CNS, including the cortex, hippocampus and cerebellum, appear to play key integrative roles in neuronal migration, learning and memory (Bretin et al., 2005; Charrier et al., 2006; Yamashita et al., 2006) and in neurological diseases (Camdessanche et al., 2012; Yamashita et al., 2011).

Regarding CRMP3, we first identified the protein as a target antigen of auto-antibodies present in some patients with cancer and autoimmune paraneoplastic neurological syndrome (Honnorat et al., 1999; Knudsen et al., 2007). CRMP3 expression was also found to be altered in Down syndrome (Weitzdoerfer et al., 2001). Recent studies revealed that CRMP3 has histone H4 deacetylase (HDAC) activity (Hou et al., 2013). Dendrites are one of the most plastic structures of the brain. They grow and retract quickly during development as they look for presynaptic counterparts to establish the neuronal circuitries. Although their stability increases in adult brain, dendritic arbors continue to change, albeit at a slower pace (Purves and Hadley, 1985). External stimuli such as stress, deprived environment and depression have been shown to induce reversible retraction of dendrites in the hippocampus whereas exercise and an enriched environment increase dendritic complexity (Conrad, 2006). Consequently, molecules regulating the growth and complexity of dendrites are likely to play a critical role in neuronal function. We are studying the role of CRMP3 and possible mechanism of action on dendrites as a means of understanding the regulation of dendrite growth or arborization during hippocampal development and plasticity. In this study, we addressed the function of CRMP3 in neuronal development by using dissociated hippocampal neurons and found that CRMP3 is essential, not only for dendrite extension, a late-stage event in the differentiation of neurons, but also in the early stage of neurite initiation. Neurons from 1- to 2-day-old hippocampi of control WT mice polarized and grew normally. Strikingly, all $LacZ^{+}$-neurons (identified as neurons by immunopositivity for NF200 and MAP2) from $CRMP3^{-/-}$ littermates mice exhibited no neurite formation even after 10 d.i.v. The neuritogenesis defect was mainly a failure to evolve from stage 1 to stage 2, defined as neurite initiation. This morphological change was replicated by early expression of the dominant-negative CRMP3ΔC. This phenotype seems specific for CRMP3 because knockdown or targeting of other CRMPs did not produce the characteristic rounded shape (Inagaki et al., 2001; Charrier et al., 2006; Yashimata et al., 2011; Niisato et al., 2012) and because neuritogenesis was not defective in $CRMP3^{+/-}$ neurons. As mentioned above, the first step in neuronal polarization requires establishment of lamellipodia often considered as a principal means of initiating primary neurite formation and suggests that its disruption can affect neuritic outgrowth. However, without primary neurites, neurons are not able to form axons and dendrites or to accomplish their ultimate goal of receiving and transmitting information. With such a scenario, which would provide useful guide for studies looking for new intrinsic signals that influence the developmental processes of this structure, one would predict that knockdown or deletion, and overexpression of proteins inducing lamellipodia in cultured neurons might ablate or enhance lamellipodia formation, respectively. Consistent with this idea, we found that in cultured hippocampal neurons, CRMP3 overexpression promoted important lamellipodia formation; in contrast, no $CRMP3^{-/-}$ neurons generated lamellipodia and neurites. Altogether, our studies have established for the first time a link between CRMP3 expression and lamellipodia formation, leading to the notion that CRMP3 is involved in the patterning of this structure as a crucial constitutive protein. Indeed, CRMP3 overexpression in hippocampal neurons significantly increased both dendritic

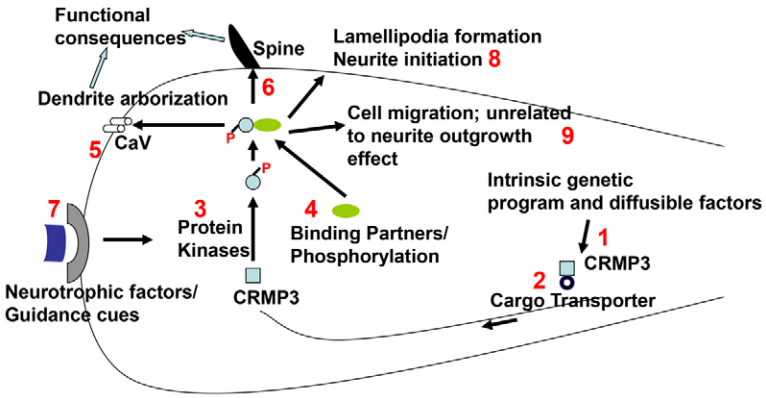

Fig. 8. A proposed model for the regulation of dendrite morphology by CRMP3 activity. We propose a model for CRMP3 roles underlying neurite initiation and dendrite outgrowth throughout Ca^{2+} influx. Combinatorial stimulation of CRMP3 gene expression or activity by intrinsic and extrinsic factors (1). The localization and distribution of FL-CRMP3-positive puncta in dendrites suggests that, similar to CRMP2, CRMP3 could be associated with vesicles or large carrier protein complexes and then actively transported to dendrites (2). Structural and biochemical studies support the idea that CRMP3 activity might be regulated by phosphorylation or other post-translational modifications (3–4) induced by neurotrophic factors or guidance cues (7). Activated CRMP3 binds to different partners and serves as adaptor to a variety of signaling pathways including Ca^{2+} influx (CaV). Our genetic studies showed that CRMP3 contributes to dendrite arborization (5), spine genesis (6) and neurite initiation (8). These effects might involve lamellipodia formation.

number and length per neuron by approximately twofold. The correlation between levels of CRMP3 expression and dendrite extension or spine morphogenesis provides strong support for a role of CRMP3 in neuronal polarization and plasticity (Quach et al., 2008a).

The absence of neurites is a common phenotype of CRMP3$^{-/-}$ cells in culture, whereas dendrites are altered but present *in vivo* in hippocampal neurons of CRMP3$^{-/-}$ mice (Quach et al., 2008a; Quach et al., 2008b). The apparent differences in cellular phenotypes obtained from the identical genotypic background might be related to adaptive mechanisms and functional redundancy or compensation between proteins from the same family *in vivo*. Guidance cues/neurotrophic factors that possibly activate other members of the CRMP family or other pathways leading to neurite formation in CRMP3$^{-/-}$ cells might be present in living animals but absent in cultured cells, and allow partial compensatory mechanisms of repair *in vivo*. *In vitro*, the phenotype depends on the time when CRMP3 was suppressed: CRMP3ΔC transfection of the cells early after plating induced aborted protrusions, similar to the ones seen in CRMP3$^{-/-}$ neurons, whereas CRMP3ΔC transfection at 3 d.i.v. reduced the length and branching of dendrites. These observations suggest that if CRMP3 is absent during neurite initiation *in vitro*, the process is aborted; however, if CRMP3 is absent after neurite initiation has occurred, dendrite complexity is affected. Clearly, both our *in vivo* and *in vitro* studies demonstrate a role of CRMP3 on dendrite formation. To our knowledge, our study is the first to show a direct role for CRMP3 in neuritogenesis. In addition, we have identified CRMP3ΔC as a dominant-negative protein.

Neurite initiation and dendrite branching both proceed by the action of filopodia, which depend on the precisely regulated polymerization, and crosslinking of actin filaments and on the structural network of microtubules (Dent et al., 2007). Several signaling pathways have been proposed for filopodia formation and most of them involve activation of Rho-family GTPases (Mattila and Lappalainen, 2008). In addition, actin-bundling proteins such as fascin and spire proteins, which can crosslink microtubules and F-actin, are necessary for filopodia formation (Renault et al., 2008). It is reasonable to hypothesize that CRMP3 might be associated directly or indirectly to these cytoskeletal-modifying structural proteins to promote changes in neurite morphology through molecular mechanisms that are currently unknown. Alternatively, a potentially weak adhesion site with ECM at the leading edge of lamellipodia of CRMP3$^{-/-}$ neurons might cause a retrograde flow of the lamellipodial cytoskeleton network that moves the membrane sheet upward as a peripheral ruffle and inhibit filopodia formation (Chhabra and Higgs, 2007). In this model, the CRMP3$^{-/-}$ *in vitro* phenotype of hippocampal neurons is linked to the absence of specifically required ECM signaling molecules.

Defining the role of CRMP3 in neurite formation provides clues on the organizing mechanisms of dendrite morphogenesis and plasticity, which in turn could explain some physiopathological aspects of dendritic degeneration. These hypotheses are supported by our results demonstrating an important time delay in hippocampal dendrite development in 10-day-old CRMP3$^{-/-}$ pups. Immunocytochemical experiments (Fig. 1B) revealed a scarcity of neurites, suggesting a developmental defect in the hippocampus of CRMP3$^{-/-}$ mice, which might be overcome progressively, albeit partially, with development over time. Consistent with this argument is the apparently normal dendritic architecture of hippocampi in CRMP3$^{+/-}$ mice, which can be attributed to the selective expression of the CRMP3 gene. Because the levels of expression of CRMP3 in hippocampal neurons were associated with various degrees of dendritogenesis, it is conceivable that CRMP3 is a key element in neurodegenerative diseases affecting hippocampal dendrites. Indeed, our previous data clearly demonstrated that PrP$^{106–126}$ induced dystrophic changes in dendrites, and this effect was prevented by overexpression of CRMP3 (Quach et al., 2011).

In order to gain insights into the mechanism(s) involved in CRMP3-mediated neurite and dendrite genesis, we explored the putative involvement of voltage-gated Ca^{2+} channels. VGCCs comprise a broad class of membrane channels with a wide variety of voltage dependence, activation and inactivation properties, and

are critical contributors to Ca^{2+} signaling within dendrites and spines in both physiological and pathological conditions (McCutchen et al., 2002; Benarroch, 2010). In hippocampal pyramidal neurons, dendritic VGCCs include L-, N-, P/Q- and R-type Ca^{2+} channels, which open following strong synaptically evoked depolarization (Striessnig and Koschak, 2008). Here, we used fluorescent Ca^{2+} imaging in combination with pharmacological assays to define the VGCC subtypes associated with CRMP3 dendritic activity. We observed that Ca^{2+} influx elicited by KCl is significantly increased in EGFP–CRMP3-transfected cells when compared with EGFP-transfected controls (Fig. 7H), highlighting a possible role of CRMP3 as a direct regulator of Ca^{2+} influx through VGCCs. Pharmacological experiments revealed the involvement of L-type Ca^{2+} channels, probably via an association between L-type Ca^{2+} influx and dendrite arborization induced by CRMP3. Additional studies demonstrated that inhibiting L-type Ca^{2+} channels strongly altered dendrite arbor of hippocampal neurons at various stages (Fig. 7A–D), positioning this channel as a regulator of dendrite development during neuronal differentiation. Taken together, our results identify CRMP3 as an important determinant of the development of appropriate dendritic arbor requiring influx of Ca^{2+} via L-type Ca^{2+} channels (see proposed model in Fig. 8) and raise the intriguing possibility of targeting these channels as a way to develop clinically useful therapies for neurodegenerative diseases involving dendritic impairment.

Materials and Methods

Mouse breeding and genotyping

The 129SvE CRMP3$^{-/-}$ line was established in the transgenic mouse facilities of Ohio State University (Quach et al., 2008a). All experiments were conducted in accordance with the guidelines of the National Institutes of Health, USA. Genotyping was performed by PCR on DNA extracted from ear or tail clips after proteinase K digestion. Sequences corresponding to the insertion were amplified using *Taq* polymerase (Promega, WI) with sets of primers for LacZS/LacZA or NeoS/NeoA derived from the LacZ (LacZS:AGCAGAAGCCGTCGATGTCG, LacZA:ACCGTTCATACAGAACTGG) or Neomycin (NeoS:CCGACCTGTCCGGTGCCCTGAATGAA, NeoA:TACCGTAAAGCACGAGGAAGCGGTCA) sequences and primers from the intronic regions surrounding exon 3 (P3:GGCAGATAGGTAACCATTTGG, P4:GCATTTACAGAAACCCAGC).

Cell culture

WT, CRMP3$^{+/-}$ and CRMP3$^{-/-}$ hippocampal cells were prepared following a modification of the methods of Banker and Cowan (Banker and Cowan, 1979). Briefly, 1 and 2 postnatal day old hippocampi were dissected, digested with 1× trypsin-EDTA (pH 7.2; Invitrogen, Carlsbad, CA) for 30 minutes at 37℃, rinsed five times with Neurobasal to remove trypsin, followed by trituration with pipettes. Dissociated neurons were plated at a density of $1–3\times10^4$ cells/ml onto poly-L-lysine (50 µg/ml)/laminin (10 µg/ml, Sigma, St Louis, MO) coated Lab-Tek eight-well chamber slides (Nunc International, NJ). After plating for 20 minutes, medium was replaced with neuronal culture medium (0.5 mM glutamine, 100 U/ml penicillin-streptomycin in neurobasal medium supplemented with 1% B27, 1% N2 and 0.5% glucose) to remove debris. Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37℃, and cells were analyzed at 6 or 10 d.i.v.

Molecular constructs

The C- or N-terminally truncated mutants were generated from full-length FLAG-CRMP3 (FL-CRMP3) by PCR with specific primers (P-ΔNs: 5′-ACGCGGATCCATGTCCTTCCAAGGC; P-ΔNas: 5′-GGACTAGTCTAGATGTTGTACTCTAC; P-ΔCs: 5′-ACGCGGATCCGACTACTCCTTAC; P-ΔCac: 5′-GGACTAGTCTAGGAAAGAGACGTG) introducing *Bam*HI and *Spe*I sites at the 5′ and 3′ end, respectively. PCR products were purified and directionally inserted into the pCMV2Flag vector (Sigma) which generated a protein with a FLAG tag at its N-terminus. The correct DNA sequences of both constructs were verified by sequencing. Plasmid DNAs were purified using Qiagen kit (Gaithersburg, MD).

Transfection assays

The calcium phosphate DNA complex was prepared following the Clontech Calphos mammalian transfection kit manufacturer's protocol (BD Bioscience, Palo Alto, CA, USA). 1 µg of plasmid DNA was mixed with 3.1 µl 2 M $CaCl_2$ and 2× HBS, then adjusted with H_2O to a volume of 50 µl. To avoid the formation of big crystals of Ca^{2+}, 1/5 volume of solution A (plasmid DNA, $CaCl_2$, H_2O) was added at a time into solution B (2× HBS) followed by pipetting several times and gentle vortexing. The mixed solution was kept at room temperature for 20 minutes before being added to each well. After incubation for 3–5 hours, the calcium phosphate precipitate was dissolved with 400 µl transfection medium pre-equilibrated in a 10% CO_2 incubator for 50–60 minutes to reduce cell toxicity. After incubation for 15–20 minutes, medium was replaced with cultured Neurobasal medium (supplemented with B27, 0.5 mM glutamine, and 100 U/ml penicillin-streptomycin; Invitrogen) and cultures maintained in a 5% CO_2 incubator at 37℃. For CRMP3 knockdown by siRNA, hippocampal neurons were transfected with full-length CRMP3 and either siRNA-CRMP3 (5′-UCUUGCUCCAGUAGUGUGAGC-3′) or scrambled siRNA (UUCUCCGAACGUGUCACGU), as previously described (Hou et al., 2006; Brot et al., 2010). The co-transfections were performed with the simultaneous use of Ribojuice and Nanojuice kits (Novagen, Morgantown, WV).

Immunocytochemistry and X-gal staining

Cells were fixed, permeabilized with 0.1% Triton X-100 in PBS containing 1% gelatin and stained with anti-β galactosidase (Promega; 1:500) or anti-FLAG (Sigma; 1:10,000) and anti-MAP2 (Frontier Science, Co, Wisconsin, WI; 1:700), or anti-Tau (Santa Cruz, CA; 1:500) antibodies. Cells were then incubated with one or more of the secondary antibodies (Alexa-Fluor-546-coupled anti-rabbit IgG and Alexa-Fluor-488-coupled anti-mouse IgG; 1:2000, Molecular Probes, OR). Some cultures were incubated with a 0.1 µg/ml solution of DAPI (4,6-diamidino-2-phenylindoldihydrochloride, Sigma) to label cell nuclei. For X-gal staining, cells were permeabilized with 0.1% Triton X-100 in PBS containing 1% gelatin, and stained with X-gal solution (5 mM potassium ferricynide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside in PBS) at 37℃ for 10–12 hours.

Fura-2AM Ca^{2+} imaging

Ca^{2+} imaging experiments were performed on neurons transfected with CRMP3 plus green fluorescent protein (EGFP) or EGFP alone (InVitrogen). Neurons were loaded with 3 µM Fura-2AM (in Tyrode's buffer 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, pH 7.5, 30 mM glucose) for 25 minutes at room temperature in the dark (Wang and Khanna, 2011; Wilson et al., 2012). Neurons were then washed three times with Tyrode's buffer. Experiments were performed on the stage of a Nikon Ti Eclipse inverted microscope equipped with a Lambda DG-4 175 W xenon lamp and a Nikon ×20, 0.4 N.A. epifluorescence objectives. Fura-2 (excitation: 340, 380 nm; emission: 510 nm) ratio images were acquired, every 10 seconds, with an intensified CCD camera Cascade 512B digital camera (Photomometrics, Tucson, AZ) and digitized (256×512 pixels) using Nikon Elements Software (Nikon Instruments, Melville, NY). After a baseline of at least six images was obtained, neurons were stimulated by addition of excitatory Tyrode's buffer (32 mM NaCl, 90 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES pH 7.5, 30 mM glucose) to a final concentration of 45 mM KCl. Background fluorescence from a cell-free region was subtracted from each 340 nm and 380 nm image. Calibrated values were obtained *in situ* by determining F_{min} (10 mM EGTA in Ca^{2+} free-solution) and F_{max} (10 µM ionomycin in 10 mM Ca^{2+} solution; the protonophore p-trifluoromethoxy-phenylhydrazone (FCCP, 2 µM) was given before ionomycin to greatly reduce Ca^{2+} buffering. A K_d of 224 nM was used in the ratio method equation $[Ca^{2+}]_I = K_d*(F_{380max}/F_{380min})*(R-R_{min})/(R-R_{max})$, where $R = F_{340}/F_{380}$ (Piekarz et al., 2012). Cells whose Ca^{2+} responses were smaller than KCl (when challenged with ionomycin) and those whose Ca^{2+} responses were larger than initial baseline (when challenged with EGTA) were not included in the dataset.

Image capture and neurite quantification

Slides were coded before quantitative analysis by a blind-rater. Images of immunofluorescent neurons were taken with Axioplan II fluorescence microscope (Carl Zeiss) using 10, 20, 40 or 100× lens then quantified with image-analyzing software (Metamorph, Universal Imaging or Soft Imaging System, SIS). For fluorescence analysis, three views of the same section were recorded using appropriate filters, as black and white images, which were then color-coded green (SNAT2), red (phenotypic markers) or blue (cell nuclei) by the software and merged together as indicated. Neurites (defined as length ≥ cell body), number of dendrites and branches, total dendrite length per cell and length of longest dendrite were measured. The length of each dendrite was measured by tracing along its length and the lengths subsequently summed for total dendritic length. In some experiments, similar measurements were made for axons.

Sholl analysis of dendritic complexity

Neurite outgrowth of neurons transfected with EGFP or CRMP3 + EGFP and incubated in the presence of various drugs or 0.01% DMSO (control), was assessed as previously described (Brittain et al., 2011; Wang et al., 2010). Transfection of EGFP into neurons allowed optical identification and unequivocal determination

of their arborizations. Images were acquired with a Nikon Eclipse 90i microscope by an experimenter blinded to transfection conditions.

Statistics

A parametric one-way or two-way ANOVA followed by Newman–Keuls or independent Student's *t*-test or a non-parametric one-way or two-way ANOVA followed by a Dunn test or Mann–Whitney test were applied. GB-STAT, and GraphPad-InSTat software were used with significance set at $P<0.05$.

Author contributions

T.T.Q. generated the CRMP3$^{-/-}$ mouse line and did the culture studies. V.R. did the DNA constructs and N.C. participated in the culture experiments. S.M.W. and S.M. performed the calcium experiments. M.K. modeled the CRMP3 structure. T.T.Q., M.F.B., R.K., J.H. and A.M.D. designed the experiments and wrote the manuscript. All authors edited and approved the manuscript.

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