SUPPLEMENTARY INFORMATION

An Atypical Role for Collapsin Response Mediator Protein 2 (CRMP-2) in Neurotransmitter Release Via Interaction with Presynaptic Ca^{2+} Channels

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SUPPLEMENTARY METHODS

Isolation of growth cone particles (GCPs). GCPs were prepared by subcellular fractionation of homogenized postnatal day 1 and 8 (PN1/8) rat brain as described previously (1). In brief, the PN1 or PN8 brain homogenate was spun at 1,660 g for 15 min to obtain a lowspeed supernatant (LSS). The discarded pellet contained neural cell bodies, blood cells, and nuclei. The LSS was loaded on a 0.83 M discontinuous sucrose density gradient, and subcellular compartments were separated according to their buoyant density. GCPs were observed just below the soluble proteins of the homogenate and above the 0.83 M sucrose cushion. The GCP fraction was diluted and resuspended in homogenization buffer (0 .32 M sucrose, 1 mM $MgCl_2$, and 1 mM TES, pH 7.3). Sealed, intact GCPs were collected by spinning onto a cushion of Maxidens oil (Nycomed A.S., Oslo, Norway). To prepare a soluble protein extract, pelleted or intact GCPs were resuspended in a lysis buffer consisting of 6 mM Tris (pH 8.1), 0.5 mM EDTA, 0.5 mM EGTA, and 100 U/ml aprotinin. The GCPs were ruptured in a Teflon/glass homogenizer and then stirred for 45 min at 4°C. The lysate was centrifuged for 60 min at 200,000 g. The supernatant and pellet were designated as soluble and membrane fractions, respectively.

Proteomics of GCPs. GCP samples from PN1 and PN8 rats were subjected to LC-MS/MS as described previously (2).

Real time reverse transcriptase PCR (RT-PCR). The mRNA level of genes were quantitatively evaluated by real-time RT-PCR as described (3;4). Single-stranded cDNA was synthesized from hippocampal RNA using Omniscript reverse transcriptase (QIAGEN) using oligo-dT primers. Resultant cDNA samples (~1.5 ng) were amplified with Platinum taq DNA polymerase (Invitrogen) for 30 cycles for non-quantitative RT-PCR. For quantitative RT-PCR, resultant cDNA samples were amplified on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using SYBR Green as a reporter. In most cases, the PCR reaction was run under the following conditions: 1x, 50°C, 2 min; 1x, 95°C, 10 min; 45x, 95°C, 15 sec, 60°C, 1 min; 1x, 72°C, hold. To check if amplification yields PCR products with a single molecular weight, the PCR products were electrophoresed and sequenced. In addition, melting curve analysis was performed to confirm the authenticity of the PCR products. To check for DNA contamination, PCR was run with cDNA samples by using an *L27* (ribosomal housekeeping gene) primer pair, whose PCR product crosses an intron. To check the linearity of detection, a cDNA dilution series (1, 1/10, 1/100, and 1/1,000) was amplified with gene-specific primer pairs, and a correlation coefficient was calculated from the standard curve displaying threshold cycles (C_t) as a function of $\log_{10}$ cDNA concentrations (3;4). The mRNA level for each gene (x) relative to L27 mRNA (internal control) was calculated as follows: $\text{mRNA}(x\%) = 2^{\text{Ct (L27)-Ct}(x)} \times 100$.

Cloning CRMPs and CaVx channel fragments into pGEX-Glu vectors. Using Vector NTI (v. 11; Invitrogen) software, primers were designed to amplify regions corresponding to intracellular loops of rat CaV2.2 cDNA from P3 rat brain cDNA. The primers (see Supplementary Table 1) harbored restriction sites (*Bam HI* or *Bgl II* (5') and *Eco RI* or *Mfe I* (3')) to facilitate cloning into the. In addition to the GST-tag, this vector contains the Glu tag, a sequence of six amino acids (EYMPME). Correctly-amplified PCR products and parent pGex-3x-Glu vector were digested with the indicated restriction

enzymes, and then extracted following electrophoresis on an agarose gel. The extracted DNAs were quantified (Nanodrop 1000, Thermo Scientific), and ligations were performed using 6:1 and 3:1 insert to vector molar ratios. The ligations were transformed into XL-10 *E. Coli* and colonies were screened using colony PCR. Those colonies with the correct-sized inserts were further verified by dideoxy sequencing (Cogenics, Houston, PA).

Supplementary Table 1. Primers used for construction of CaV2.2 fusion proteins.

Construct	***Nucleotides***	***Primer Sequence 5′ → 3′***	***Restriction site***
N-terminus (N-t)	*46-330*	*TAG GAT CCT TAT GGT CCG CTT CGG GGA CGA GCT A*	***BamHI***
		TAT GAA TTC GGC CAT TCG GTG ATG CGC TTA	***EcoRI***
I-II loop (L1)	*1111-1494*	*TAG GAT CCC ATC AGG AGA GTT TGC CAA AGA G*	***BamHI***
		TAT GAA TTC CCG CTC TGT GCT TTC ACC ATA CGA	***EcoRI***
II-III loop proximal region (L2-p)	*2176-3000*	*CTA GAT CTT GGA AGA GGC AGC CAA TCA GAA GC*	***BglII***
		TAT GAA TTC CCT GCA CGG TGC CTG CGT GTG	***EcoRI***
II-III loop distal region (L2-d)	*2914-3471*	*TAC TAG ATC TTG GGC GAG CGT CGC GCA AGA CAT*	***BglII***
		TAT GAA TTC CCG TAA TGG CAG AAG CGA CGG AG	***EcoRI***
III-IV loop (L3)	*4276-4467*	*TGG GAT CCC CTT GAT CAT CAT CAC CTT CCA G*	***BamHI***
		CCG GAA TTC CCA ATG AAG TAC TCA AAG GGT GG	***EcoRI***
C terminus proximal (Ct-p)	*5116-5793*	*TGG GAT CCT CTG TTC CTT TCT GAT GCT GAA*	***BamHI***
		TGA CCG CAA TTG GAA GTT GCA CTC TTT TGT C	***MfeI***
Ct-medial Ct-m)	*5683-6429*	*TAG GAT CCA GAT GGG TCC TGT TTC CCT GTT*	***BamHI***
		CTG GAA TTC CCA AAG CGG TCA CAG GAA TA	***EcoRI***
Ct-distal (Ct-d)	*6400-7044*	*TGG GAT CCA GCG CTT CTA TTC CTG TGA C*	***BamHI***
		TTA TCC CAA TTG CAC CAG TGA TCC TGG TCT	***MfeI***

Purification/Enrichment of Ca^{2+} channels from synaptosomes. Synaptosomes from P1 neonatal rat brains were solubilized with digitonin and enriched by chromatography on WGA-Sepharose as described previously (5-7). Briefly, 30 PN1 neonatal rat brains were homogenized in 180 ml of 320 mM sucrose with a glass-Teflon homogenizer. After a short centrifugation (5000 rpm, 2 min), the supernatant (SN) was centrifuged (42,000 rpm, 60 min). The membranes were solubilized with 1.2% digitonin, 80 mM sodium phosphate buffer, pH 7.4 for 20 min. Unsolubilized material was removed by the centrifugation as before, and the supernatant (S3) was poured over a 40 ml WGA-Sepharose column (50 ml/h). After incubation for 1 hr at 4ºC, the column was washed with 10 column volumes of 0.1% digitonin, 75 mM NaCl, 50 mM sodium phosphate, 10 mM Tris-HCl (pH 7.4) at a flow rate of 50 ml/hr. The glycoproteins bound to the WGA-Sepharose column were eluted with 100 mM N-acetyl-D-glucosamine

(Sigma, St. Louis, MO) in the same buffer at a flow rate of 50 ml/hr. Three milliliter fractions were collected and the protein concentration of each fraction was determined by BCA protein assay kit (Thermo Fisher Scientific, Shelbyville, IN).

To further enrich for Ca^{2+} channels, WGA-column fractions were incubated for 4 hr on ice with 200 µl of heparin-agarose (8). The resin was washed four times with 0.2% CHAPS, 10 mM Tris-HCl, pH 7.4, and once with 10 mM Tris-HCl, pH 7.4. Ca^{2+} channels were gently extracted for 30 min at 50ºC with 100 µl of 5% SDS, 20 mM dithiothreitol, 125 mM Tris-HCl, pH 6.8, 10% sucrose, 20 mM EDTA.

Supplementary Table 2. Antibodies used in this study.

Antibody	Source	Dilutions		
		IF[a]	IP	WB
CaMKIIα (m)	Dr. Andy Hudmon (Indiana University School of Medicine, Indiana, IN)	–	–	1:4000
CaV1.3 (m)	NeuroMab (Davis, CA)	–	–	1:750
CaV2.2 (p)	Calbiochem (La Jolla, CA)	1:150	1:750	1:150
CRMP-1 (p)	Sigma (St. Louis, MO)	–	–	1:750
CRMP-2 (m)	Abcam (Cambridge, MA)	1:500	1:750	1:1000
CRMP-2 (p)	Cell Signaling Technology (Danvers, MA)	1:500	1:1000	1:500
CRMP-2 (p)	Chemicon Int. (Billerica, MA)	1:500	1:1000	1:1000
CRMP-3 (p)	Abcam	–	–	1:500
CRMP-4 (p)	Chemicon Int.	–	–	1:500
CRMP-5 (m)	Chemicon Int.	–	–	1:500
GAP-43 (m)	NeuroMab	–	–	1:1000
GFAP (m)	Sigma	–	–	1:1000
Glu epitope tag (m)	Dr. Clark Wells (Indiana University School of Medicine, Indiana, IN)	–	–	1:2000
MAP2 (m)	Abcam	–	–	1:1000
GluR2	NeuroMab	–	–	1:250
Munc18 (m)	BD Transduction Labs. (San Diego, CA)	–	–	1:500
NaV (p)	NeuroMab	–	–	1:2500
PSD95 (m)	NeuroMab	–	–	1:250
RIM (p)[d]	Synaptic Systems Labs (Göttingen, Germany)	–	–	1:250
Synaptophysin (m)	Chemicon	1:500	–	–
Syntaxin 1A (m)	Sigma	–	–	1:2000
β-III-tubulin (m)	Sigma	1:1500	–	1:2000

[a]Abbreviations used: IF, immunofluorescence; IP, immunoprecipitation; WB, Western blot; m, monoclonal; p, polyclonal; –, not used; CaMKIIα, Ca^{2+}/Calmodulin-dependent protein kinase II alpha subunit; CaV, voltage-gated Ca^{2+} channel; CRMPx, collapsin response mediator protein; GAP-43, growth associated protein of 43 kD; GFAP, glial fibrillary associated protein; Glu epitope, amino acids EYMPME; MAP2, microtubule-associated protein; PSD95; post synaptic density protein of 95 kD; RIM, Rab3a interacting molecule.

[b]RIM2 antibody detects multiple splice variants of the two main RIM genes – RIM1 and RIM2 as well as RIMβ (9-11).

Supplementary References

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Figure S1
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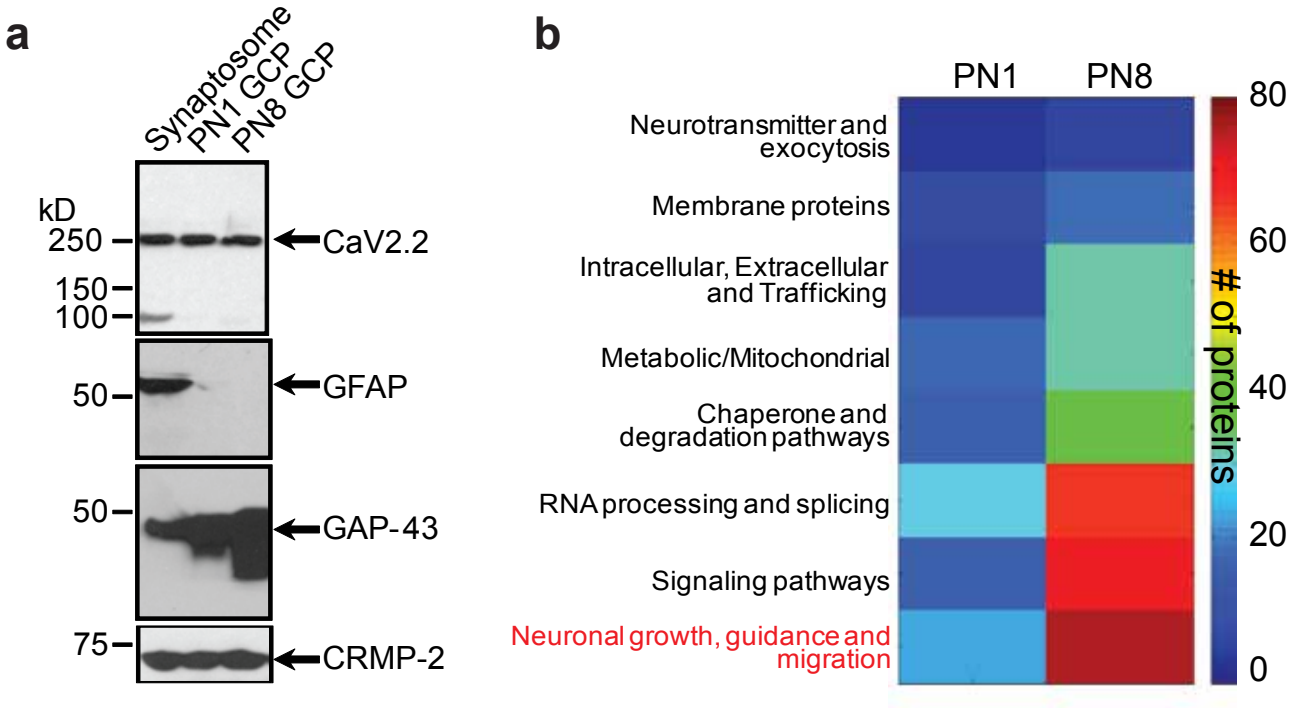

Supplementary Figure 1. Proteomics of growth cone fractions. (**a**) Immunoblot analysis of postnatal day 1 (PN1) rat synaptosomes and growth cone particles (GCP) isolated from PN1 and PN8 rat brains with the indicated antibodies: the N-type Ca^{2+} channel (CaV2.2), the glial fibrillary associated protein (GFAP), the growth cone associated protein of 43 kD (GAP-43) and CRMP-2. Lack of expression of GFAP and abundant expression of GAP-43 in the PN1 and PN8 GCP ensure the purity of our growth cone isolation. (**b**) Summary of the proteomes of PN1 and PN8 GCP identified by tandem mass spectrometry (LC-MS/MS). Classification of the 148 and 464 proteins identified in the PN1 and PN8 GCP fractions, respectively. These 612 proteins were classified within a number of broad functional classes. Particularly enriched in the PN8 GCP fraction were proteins belonging to the neuronal growth, guidance and migration class. Among these proteins were the collapsin response mediator proteins (CRMPs).

Figure S2
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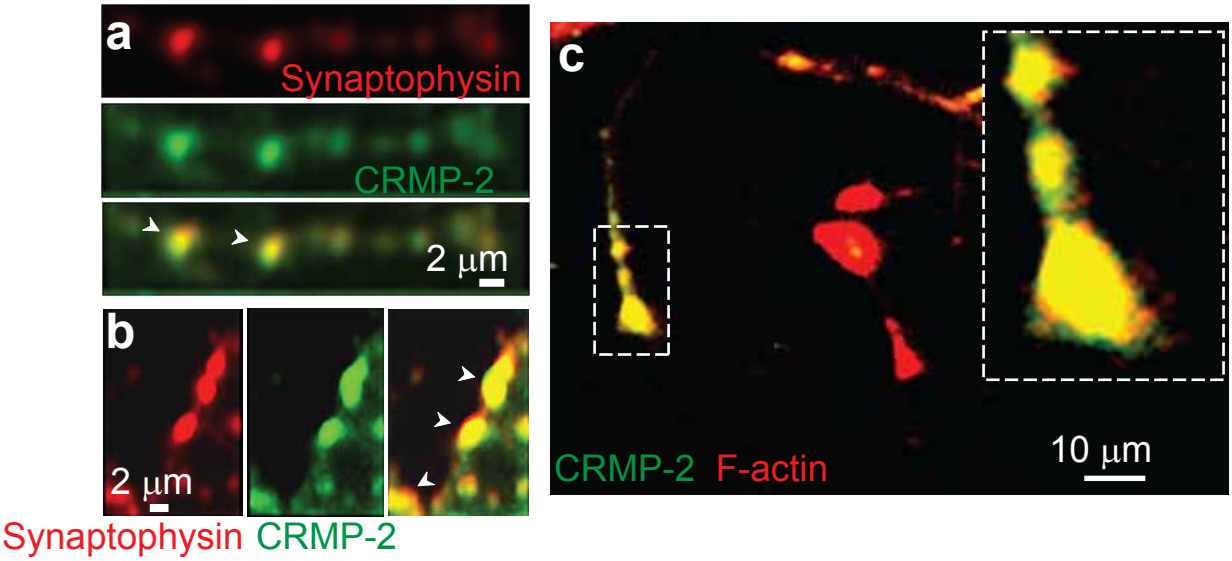

Supplementary Figure 2. Colocalization of CRMP-2 with synaptic and growth cone markers. (**a, b**) Confocal images of hippocampal synaptic boutons along a neurite stained for synaptophysin (*red*; a marker for synapses) and CRMP-2 (*green*). Arrowheads, in the merged panels, indicate areas of strong overlap indicative of CRMP-2 expression in synapses. (**c**) Confocal images of a hippocampal growth cone labeled with the growth cone-enriched protein F-actin (*red*) and CRMP-2 (*green*). Inset shows enlarged region of growth cone (boxed area) with strong colocalization. Scale bar as indicated.

Figure S3
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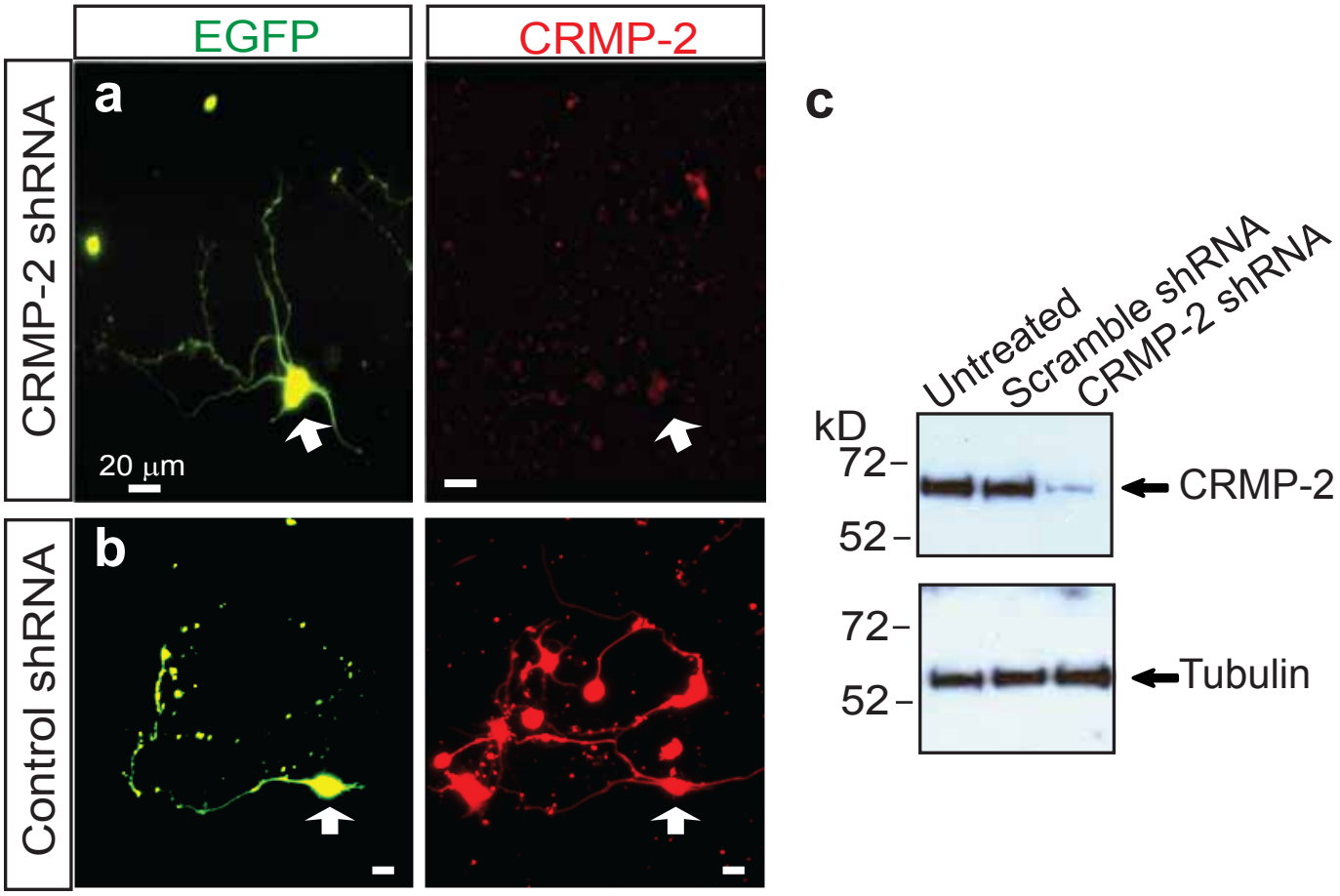

Supplementary Figure 3. CRMP-2 knockdown by lentiviral shRNA. Confocal images of hippocampal neurons infected with CRMP-2 (*top panels*) or scrambled (*bottom panels*) shRNA lentiviruses. Neurons grown for 5 DIV were infected with equal titers of viruses and cultured for a further 7 DIV before immunostaining with CRMP-2 (red). EGFP fluorescence (green) was used to identify infected cells. (**a**) Arrows indicate knockdown of CRMP-2 protein compared to the unchanged EGFP fluorescence. (**b**) Arrow indicates neurons infected with scrambled lentivirus with no corresponding reduction in CRMP-2 protein (*red, panel b right*). (**c**) Western blot analysis with a CRMP-2 monoclonal antibody showing successful (>90%) knockdown of CRMP-2 protein in neurons treated with CRMP-2 shRNA or scramble shRNA lentiviruses for 7 days. Under the same conditions, expression of the neuronal protein β-III-tubulin remained unchanged.

Figure S4
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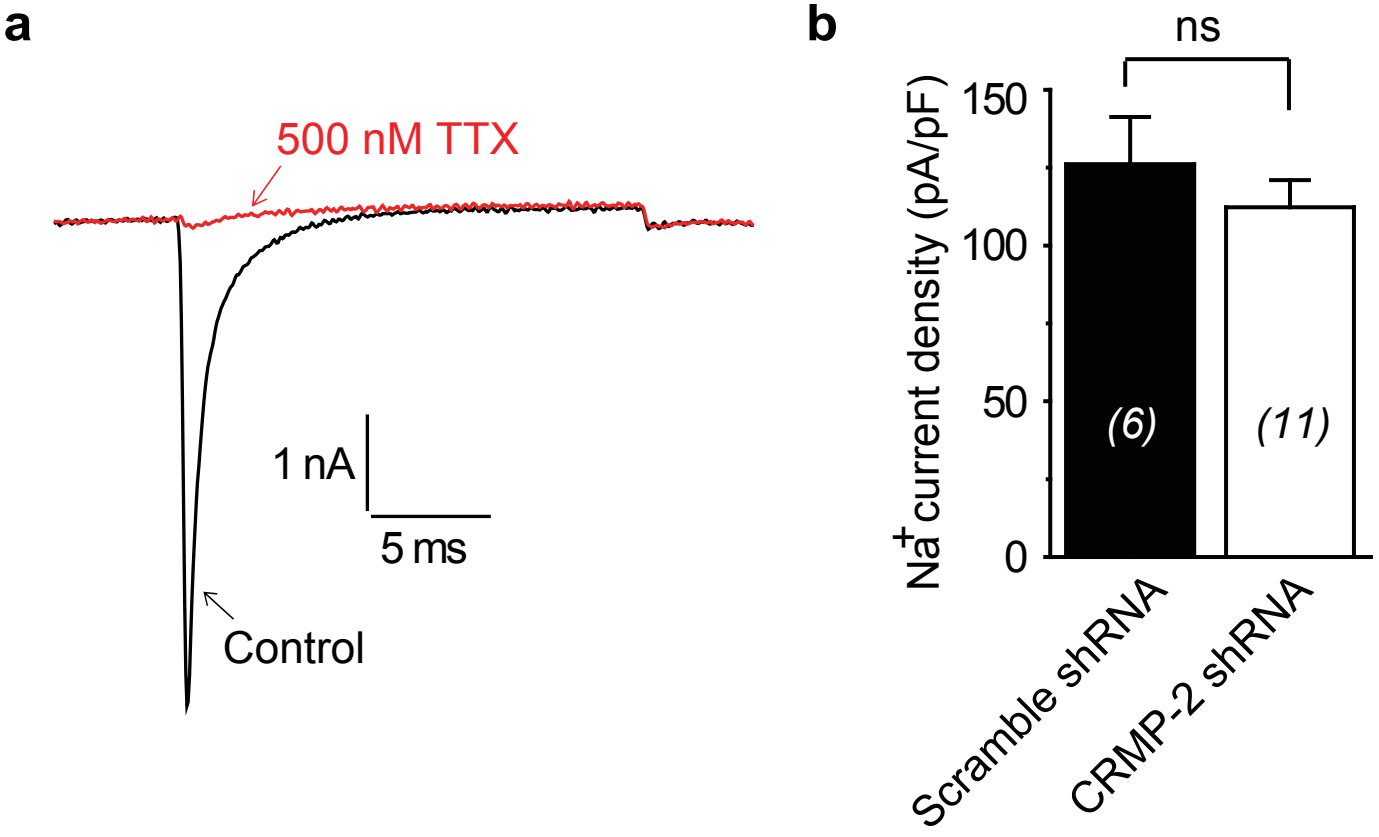
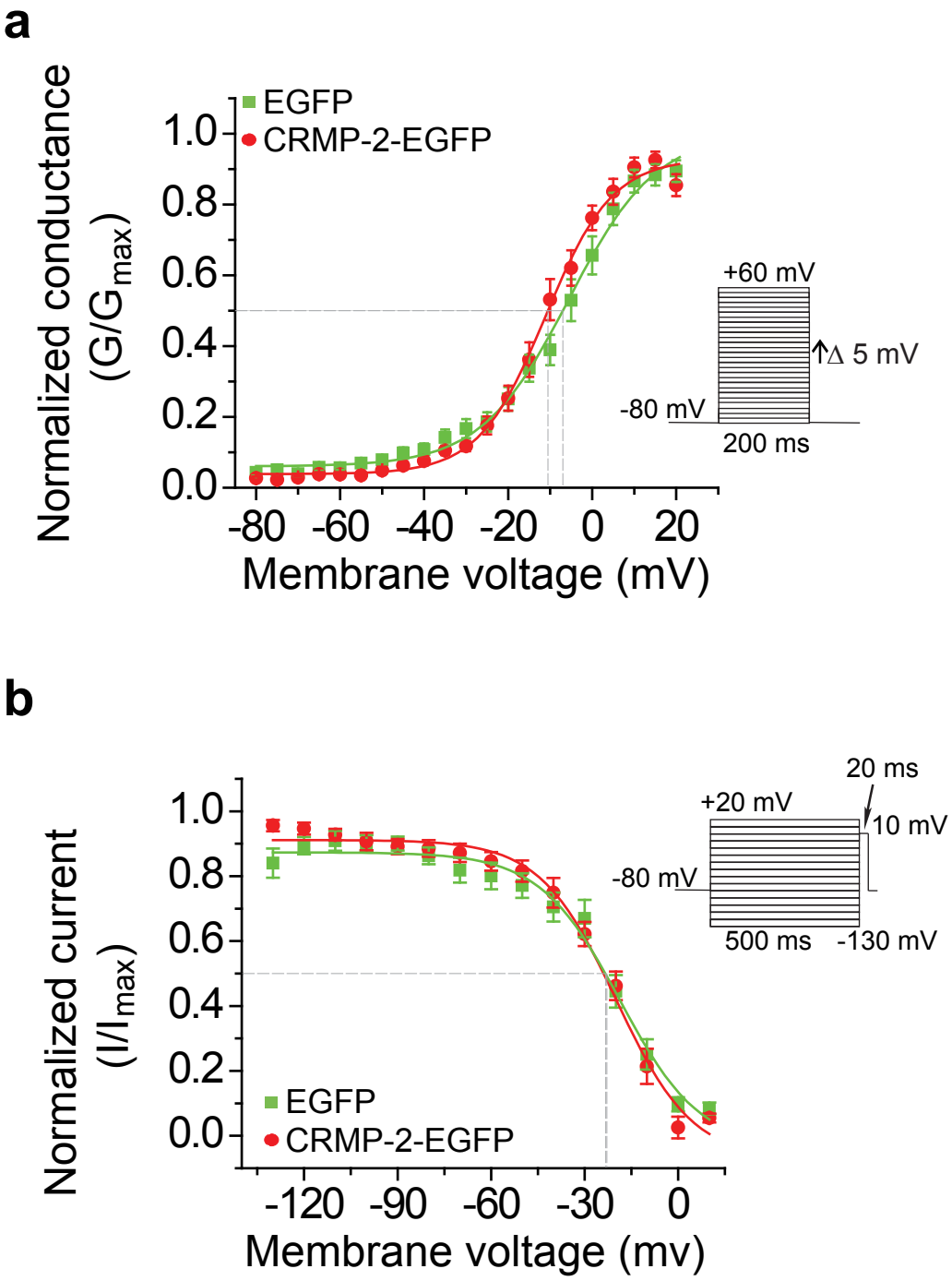
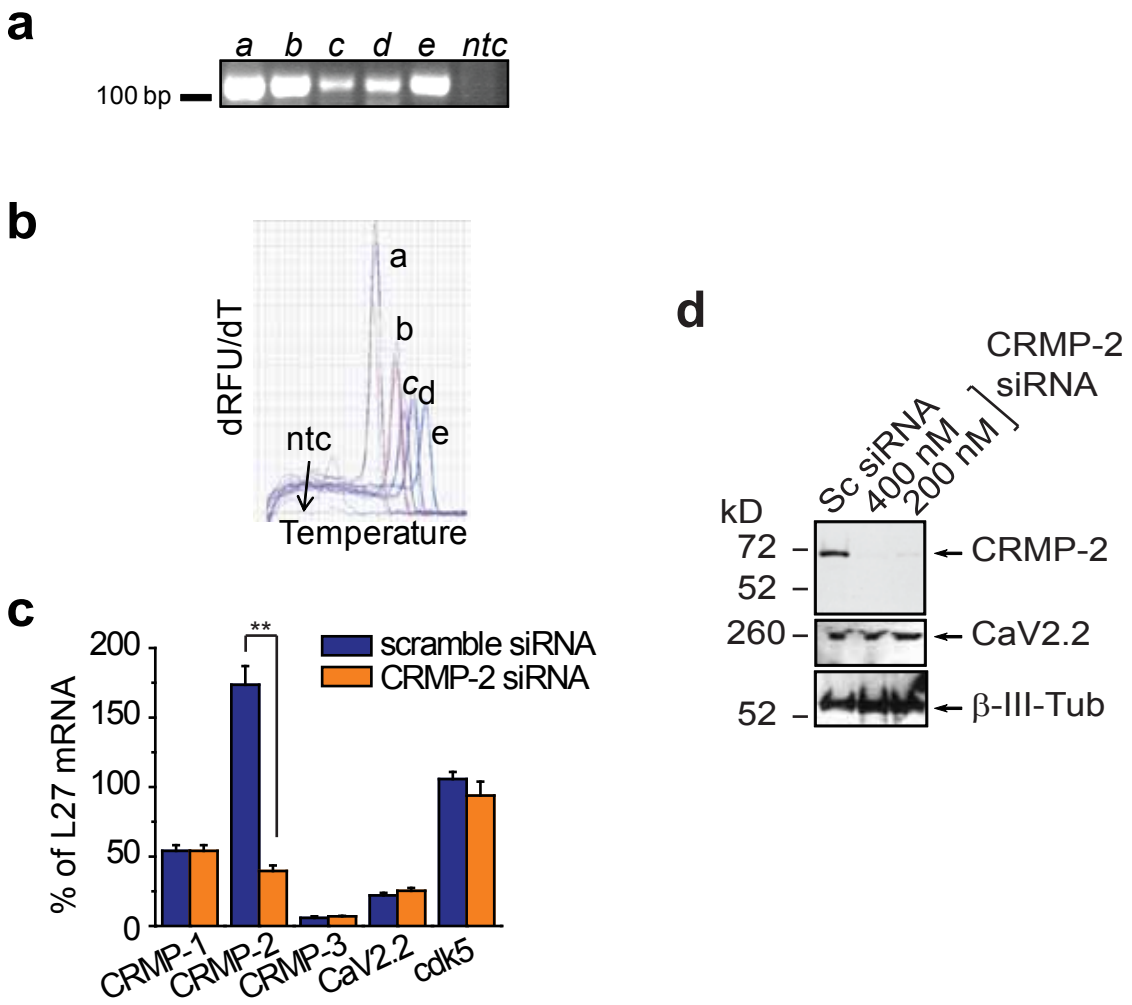


Supplementary Figure 4. CRMP-2 knockdown by lentiviral shRNA does not affect endogenous Na^+ currents in hippocampal neurons. (a) Representative currents from whole-cell recordings of a neuron infected with scramble shRNA lentivirus (identified by EGFP fluorescence) in response to a voltage step to 10 mV from a holding potential of -80 mV for 25 ms. Current before (control, black trace) and 3 seconds after addition of the Na^+-channel selective toxin (500 nM TTX, red trace) showing complete block of Na^+ channels. (b) Summary of peak inward Na^+ current density (pA/pF) measured at +10 mV for CRMP-2 shRNA and scramble shRNA infected neurons. Numbers in parentheses represent numbers of cells tested. There was no statistical difference in peak current densities between the two groups (p>0.5; Student's t-test).

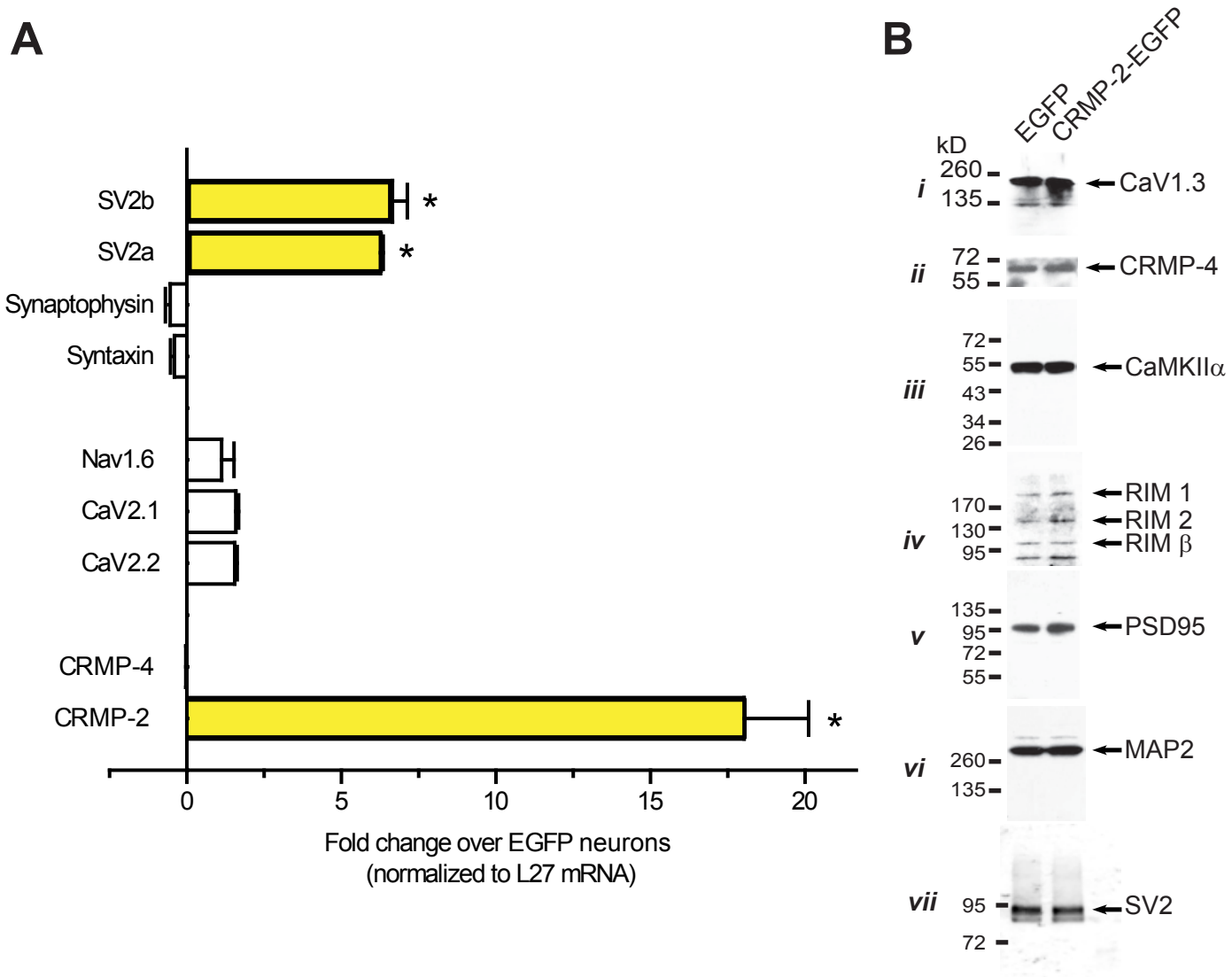


Supplementary Figure 5. Analysis of voltage dependence of conductance and activation relationships of currents from EGFP and CRMP-2–EGFP expressing neurons. (**a**) Summary of the normalized conductance (G) versus voltage relations for EGFP (green squares) and CRMP-2–EGFP neurons (red circles). Currents were elicited from voltage steps from -80 mV to +60 mV in 5 mV increments with a duration of 200 ms from a holding potential of -80 mV. (**b**) Summary of voltage dependent activation. Steady-state voltage dependence of inactivation was assessed by holding the cells at -80 mV and stepping to an inactivating prepulse (ranging from -130 mV to +20 mv) fro 500 ms and then to 10 mV for 20 ms to assess the fraction of current remaining following the prepulse. Data points are average ± SEM of 13–17 cells.



Supplementary Figure 6. Validation of CRMP-2 siRNA knockdown by quantitative PCR and Western blot analysis. (**a**) PCR products from conventional non-quantitative RT-PCR with CRMP-1 (*a*), CRMP-2 (*b*), CRMP-3 (*c*), VGCC (*d*), cdk5 (*e*) primers and no cDNA control (ntc) showing specific amplification of single bands from cDNA isolated from RNA of hippocampal neurons treated with scrambled siRNAs. (**b**) Dissociation curve analysis showing single peaks confirming successful amplification of specific and single PCR (*a* to *e*) products and no peaks in reactions with no template DNA (*ntc*). Dissociation curve analysis was performed after a completed PCR by slowly ramping the temperature of reaction solutions from 60 to 95°C while continuously collecting fluorescence data. (**c**) Quantitative RT-PCR for the indicated genes from neurons treated with scrambled (*purple bars*) or CRMP-2 siRNAs for 72 h (*orange bars*). A specific and robust knockdown of CRMP-2 (**, $p<0.01$, Student's *t*-test vs. control) was observed in CRMP-2 siRNA-treated neurons. Related genes CRMP-1 and -3, the N-type VGCC or the kinase cdk5 were unchanged. Data are expressed as % of L27 mRNA (a ribosomal internal control gene) ±S.E.M. (n=3 for each). (**d**) Western blot analysis with the indicated antibodies showing successful (>95%) knockdown of CRMP-2 protein in neurons treated with 250 nM siRNAs for 3 days. Under the same conditions, expression of the neuronal protein β-III-tubulin and presynaptic CaV2.2 channel remained unchanged. Sc, scrambled siRNA; cdk5, cyclin dependent kinase 5; ntc, no template control.

Figure S7
Khanna



Supplementary Figure 7. Quantitative RT-PCR and immunoblot analysis. (a) Quantitative RT-PCR for the indicated genes from neurons transfected with EGFP or CRMP-2-EGFP for 48 h. The mRNA levels for each gene were normalized to L27 mRNA (a ribosomal internal control gene). The L27-normalized values for CRMP-2-EGFP neurons were divided by the L27-normalized values for EGFP neurons and expressed as fold change over EGFP levels. Data represent mean fold change ±S.E.M. (n=3 for each). A robust upregulation of CRMP-2 mRNA was observed in CRMP-2 overexpressing neurons (*, $p<0.05$, Student's *t*-test vs. control). The mRNAs for the synaptic vesicle genes 2a and 2b (SV2a, SV2b) were also increased in CRMP-2 overexpressing neurons. Importantly, none of the other genes tested – the Ca^{2+} channel genes CaV2.2 and CaV2.1; the Na^{+} channel gene NaV1.6; the presynaptic presynaptic proteins syntaxin, synaptophysin; and CRMP-4 – were significantly altered by CRMP-2 overexpression. (**b**) Equal amounts of total proteins from EGFP and CRMP-2-EGFP transfected hippocampal neurons were immunoblotted with various antibodies as indicated. None of the proteins tested showed any significant changes in expression between the two conditions. *Abbreviations used*: CaV1.3, voltage-gated Ca^{2+} channel 1.3; CaMKIIα, Ca^{2+}/Calmodulin-dependent protein kinase II alpha subunit; RIM, Rab3a interacting molecule; PSD95, post synaptic density protein of 95 kD; MAP2, microtubule-associated protein; SV2, synaptic vesicle protein 2.

Figure S8
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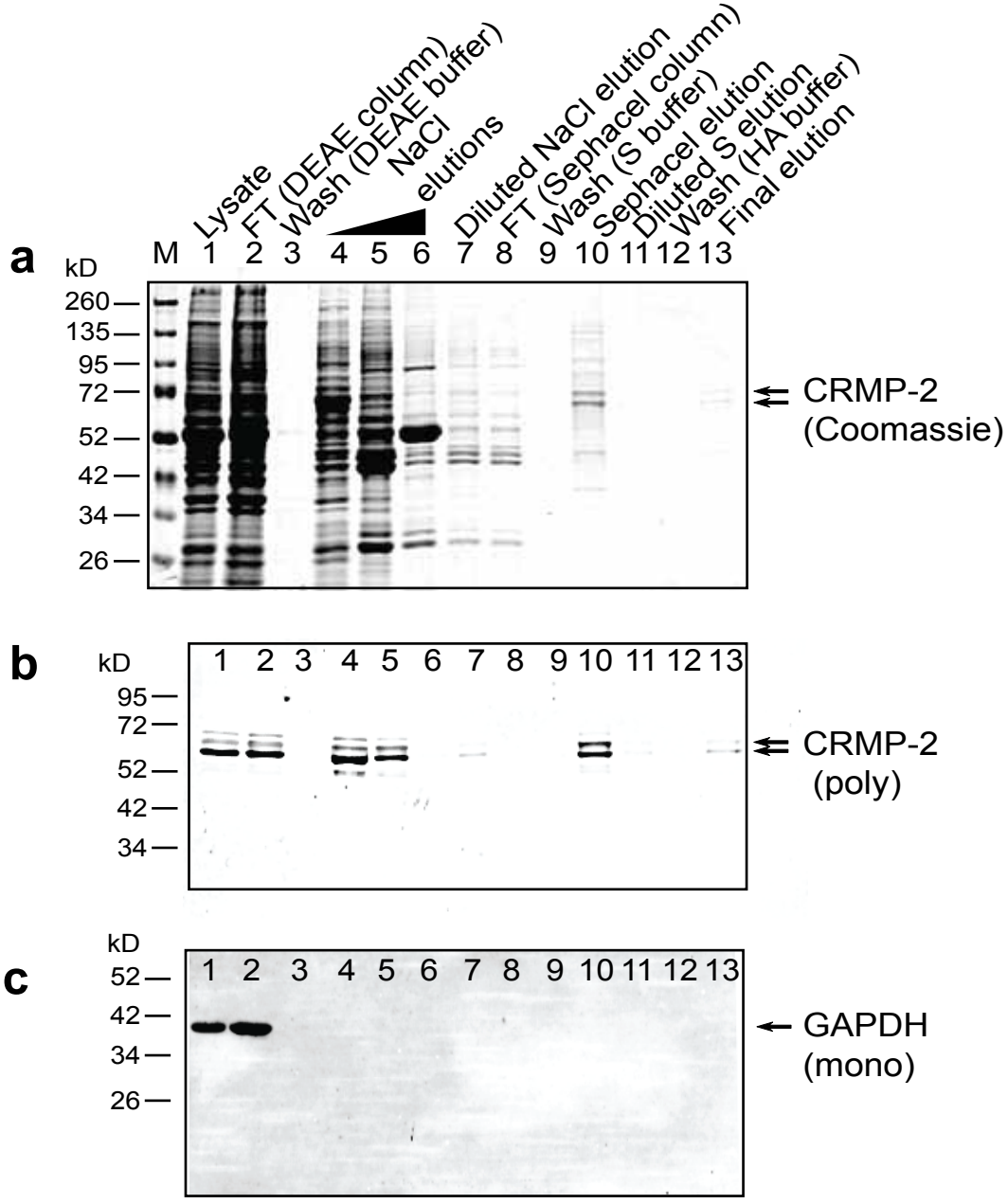

Supplementary Figure 8. CRMP-2 purification. (**a**) Coomassie stained SDS-PAGE gel of CRMP-2 purification from neonatal rat brains. Analysis of the starting lysate, flowthrough (FT), wash and elutions of the various chromatography columns including DEAE-Sephacel column, S-Sepharose and hydroxyapatite (HA). (b) Immunoblot analysis of the above fractions with a polyclonal CRMP-2 antibody (1:2500) showing purification of CRMP-2 protein. Both the nonphosphorylated (*lower band*) and phosphorylated (*upper band*) variants of CRMP-2 are detected in the purified samples in this preparation. (c) Immunoblot with GAPDH shows that only the lysate and flow through of the first DEAE column contain GAPDH. Notably, no contaminating proteins are observed in the final elution.