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## Interaction and Colocalization of CaBP4 and Unc119 (MRG4) in Photoreceptors

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### Abstract

**Purpose**—To characterize the interaction of the neuron-specific protein CaBP4 with the synaptic photoreceptor protein Unc119 homolog (MRG4).

**Methods**—The interaction of CaBP4 and Unc119 was studied using affinity chromatography, yeast two-hybrid system, coimmunoprecipitation, and gel overlay assay. The colocalization of CaBP4 and Unc119 was analyzed using immunohistochemistry. Unc119, CaBP4, and synaptic proteins were examined in photoreceptors using immunohistochemistry and in synaptic tangential sections of flatmounted frozen retinas using Western blot analysis.

**Results**—Biochemical evidence supported the interaction of CaBP4 with Unc119. CaBP4 and Unc119 colocalized in the photoreceptor synapse of adult retina and during postnatal retinal development. A reduction in Unc119 levels was observed in the photoreceptor terminals of CaBP4-knockout mice compared with wild-type mice and was higher than the reduction of other synaptic proteins.

**Conclusions**—This study provides evidence for the interaction of CaBP4 with Unc119 at the photoreceptor synapse. This interaction suggests a functional relationship between CaBP4 and Unc119, further supporting a role for these proteins in neurotransmitter release and in the maintenance of the photo-receptor synapse.

The neuron-specific protein CaBP4 is a member of a sub-family of neuronal Ca<sup>2+</sup>-binding proteins that are highly similar to calmodulin.<sup>1-5</sup> CaBP4 is localized in photoreceptor synaptic terminals and is required for normal neurotransmission between photoreceptors and bipolar cells. CaBP4-knockout mice (*Cabp4*<sup>-/-</sup>) have morphologically and functionally deficient synapses. Electroretinograms from these *Cabp4*<sup>-/-</sup> mice indicate impaired cone and rod synaptic function. Phenotypically, *Cabp4*<sup>-/-</sup> mice resemble mice deficient in the  $\alpha_1$  1.4 or  $\beta_2$  subunit of the Ca<sub>v</sub>1.4 channel<sup>6-8</sup> and patients with incomplete congenital stationary night blindness (CSNB) who carry *CACNA1F* (encoding  $\alpha_1$  1.4) gene mutations.<sup>9,10</sup> In fact, CaBP4 interacts with the  $\alpha_1$  1.4 subunit of Ca<sub>v</sub>1.4 L-type voltage-dependent calcium channels and modulates the functional properties of these channels.<sup>4</sup> More recently, patients with autosomal recessive incomplete CSNB with no mutation in *CACNA1F* have been shown to carry mutations instead in the *Cabp4* gene.<sup>11</sup> CaBP4 has also been observed in auditory inner hair cells, and it modulates the Ca<sub>v</sub>1.3 L-type voltage-dependent calcium channels expressed in those cells.<sup>12,13</sup>

In addition to modulating voltage-gated calcium channel activity,<sup>12,14,15</sup> other members of the CaBP subfamily also modulate TRP channels and inositol 1,4,5-trisphosphate (IP3)

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receptors.<sup>16-19</sup> It is possible that the role of CaBP4 is not restricted to voltage-gated Ca<sup>2+</sup> channel modulation and that CaBP4 can also interact with other synaptic proteins.

To gain further insight into the biological function of CaBP4, the interaction of CaBP4 with the Unc119 synaptic protein was analyzed. Unc119 homolog, also called mouse retinal gene 4 or MRG4, was identified as a putative target for CaBP4 using an affinity chromatography/mass spectrometry approach. Unc119 was selected because, similar to CaBP4, it is a synaptic photoreceptor protein.<sup>20</sup> This study demonstrated that CaBP4 interacts with Unc119 and colocalizes with Unc119 throughout development. Although the precise role of Unc119 is not yet known, Unc119 has been reported to play a role in neurotransmitter release and to be required for nervous system maintenance.<sup>21-23</sup> Its interaction with CaBP4 further corroborates the importance of the function of both proteins for neurotransmitter release and maintenance of the photoreceptor synapse.

## METHODS

### Animals

The mice were housed in the Department of Comparative Medicine at the University of Washington and were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures for the maintenance and use of animals were approved by the Institutional Animal Care and Use Committee of the University of Washington. The mice used in this study were approximately 10 weeks old unless specified (see postnatal development studies).

### Antibodies

Commercially available antibodies were alkaline phosphatase-conjugated anti-mouse and anti-rabbit (Promega, Madison, WI); mouse anti-6His-tag (EMD Biosciences Novagen, Madison, WI); anti-PKC alpha (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PSD-95 (Upstate Biotechnology, Lake Placid, NY); anti-syntaxin 3 (Synaptic Systems, Göttingen, Germany); anti-rhodopsin (Millipore, Bedford, MA); Cy3 goat anti-rabbit and Cy3 goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA); Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit and Alexa 488-labeled peanut agglutinin (PNA) lectin (Molecular Probes, Eugene, OR). The mouse anti-SV2, developed by Kathleen Buckley, was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA). The development and characterization of rabbit anti-CaBP4 (UW145) was described in Haeseleer et al.<sup>4</sup> Rafeul Alam has generously provided a sample of rabbit anti-Unc119. To generate anti-Unc119 monoclonal antibody, mice were injected with 50 µg purified His-tagged Unc119 protein in a RIBI adjuvant system. After two boosts at 2-week intervals, the mouse sera were analyzed using Western blot and immunohistochemistry. One mouse was used for fusion with myeloma. Ninety-six clones were screened for Unc119 immunoreactivity using Western blot and immunohistochemistry. One clone, A2, which produced antibodies that gave positive signals on retina tissues using Western blot and immunohistochemistry, was selected.

### Cloning of Recombinant Unc119 and CaBP4

Mouse Unc119 cDNA was amplified by PCR from mouse retina cDNA (clone MMM1013-62981; Open Biosystems, Huntsville, AL) with primers K218 (5'-CACCGAGGCCATGAAGGTGAAGAAAGG-3') and K219 (5'-TCAGGGTGTCCCCTGTAGGAATAG-3') and was subcloned into the pENTR-topo vector (Invitrogen, Carlsbad, CA). Mouse CaBP4 cDNA was subcloned into the pENTR-topo vector and into the pCRII vector (Invitrogen) after PCR amplification with primers K198 (5'-

CACCATGGCAACAGAGCACAAT-3') and K160 (5'-TCAGCCTGTAGATAGCATCAT-3') from a cloning vector.<sup>4</sup> The sequence of all constructs was confirmed by DNA sequencing. The cDNA encoding the mouse CaBP4 was then subcloned as a fragment *NcoI-BamHI* into the pGBKT7-BD vector (Matchmaker; Clontech, Palo Alto, CA) opened *NcoI-BamHI*. Unc119 cDNA was subcloned, using the Gateway Technology System (Invitrogen), into pGADT7-AD that was converted to a Gateway Destination vector after ligation of a blunt-end cassette containing *attR* sites flanking the *ccdB* gene and the chloramphenicol resistance gene into the multiple cloning site of the pGADT7-AD. For expression in bacteria, the cDNA sequences encoding CaBP4 and Unc119 were subcloned, using the Gateway Technology System (Invitrogen), into the Gateway expression vector pDest17 in fusion to a 6His-tag and were purified using Ni-NTA agarose (Qiagen, Valencia, CA) or into the pDest15 vector for fusion to a GST-tag and were purified on glutathione resin (Promega) as recommended by the manufacturer.

Deletion mutants of mouse CaBP4 were generated by PCR with primers confined at the 5'- and 3'- ends of the truncated segments. The resultant sequences were subcloned into the pENTR-topo vector and were further subcloned, using the Gateway Technology System (Invitrogen), into the pDest17 vector for fusion to a GST-tag.

### CaBP4 Affinity Chromatography

The 6His-tagged CaBP4 was coupled to CNBr-activated beads of 4% agarose (Sephacrose 4B; Pharmacia/GE Health Care, Piscataway, NJ) according to the manufacturer's protocol. Bovine retinas were homogenized in 5 mM bis-tris-propane (BTP), pH 8.0, 1 mM CaCl<sub>2</sub>, 10 mM dodecyl- $\beta$ -maltoside (DM), 0.5 mM dithiothreitol, and 1 mM benzamide and was then centrifuged at 40,000g for 10 minutes at 4°C. The supernatant was loaded on CaBP4 – bead-form agarose, and the column was washed with the homogenization buffer containing 150 mM NaCl followed by 2 mM EGTA in 5 mM BTP, pH 8.0, 10 mM DM, and 1 mM benzamide. Elution was performed using 0.1 M glycine (Gly), pH 2.5.

### Isolation of Proteins for Mass Spectrometry Analysis

The identification of interacting partners for CaBP4 was carried out by liquid chromatography–tandem mass spectrometry. Proteins were prepared and analyzed by mass spectrometry using a method similar to that described in Zhu et al.<sup>24</sup> Briefly, the CaBP4 interacting proteins were separated by electrophoresis on SDS-PAGE and visualized by Coomassie staining. Excised bands were destained and dehydrated and then digested with trypsin at 37°C overnight. The supernatant was collected and analyzed by liquid chromatography–tandem mass spectrometry. Peptide sequences were compared with databases using the BLAST program.

### Unc119 Affinity Chromatography

The 6His-tagged Unc119 was coupled to CNBr-activated beads of 4% agarose (Sephacrose 4B; Pharmacia/GE Health Care) according to the manufacturer's protocol. For binding in the presence of Ca<sup>2+</sup>, the Unc119 – bead-form agarose (approximately 300  $\mu$ g Unc119/300  $\mu$ L) was equilibrated with 10 mM BTP, pH 8.0, 2 mM benzamide, and 0.1 mM CaCl<sub>2</sub>. Purified 6His-tagged CaBP4 (300  $\mu$ g) was incubated with the bead-form agarose at 4°C for 1 hour. Unc119 – bead-form agarose was then washed with the equilibration buffer (40 $\times$  the volume of the column) followed by the same buffer containing 150 mM NaCl. Elution was performed with 3 mM EGTA followed by 0.1 M Gly, pH 2.5. Fractions were collected, and aliquots were analyzed by Western blot with an anti-CaBP4 antibody. The loaded fraction and flow-through fraction were analyzed by Coomassie staining. For binding in the presence of EGTA, Unc119 – bead-form agarose was equilibrated with 10 mM BTP, pH 8.0, 2 mM benzamide, and 0.1 mM EGTA and was washed with this buffer with or without 150 mM NaCl. Elution was performed with 5 mM CaCl<sub>2</sub> followed by 0.1 M Gly, pH 2.5.

## Coimmunoprecipitation Assays

Mouse retinas were lysed by homogenization in 10 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM dodecyl- $\beta$ -maltoside, 1 mM CaCl<sub>2</sub>, and 5 mM benzamidine. Lysates were cleared by centrifugation at 13,000g for 10 minutes at 4°C, and the supernatants were incubated with 50  $\mu$ L of a 50% slurry of protein G agarose (Roche, Indianapolis, IN) for 3 hours at 4°C. After centrifugation, the supernatants were incubated with 15  $\mu$ g immunoprecipitating anti-CaBP4 (UW145) for 1 hour at 4°C. Incubation was prolonged overnight at 4°C after the addition of 50  $\mu$ L of a 50% slurry of protein G agarose. Protein G agarose-bound immune complexes were recovered by centrifugation and washed three times in immunoprecipitation buffer. Proteins were eluted by boiling for 3 minutes in SDS-PAGE sample buffer and were subjected to electrophoresis and Western blot analysis.

## Gel Overlay Assay

Recombinant GST-tagged purified proteins (2  $\mu$ g) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) membranes. After overnight saturation at 4°C in PBS, 0.1% Tween-20, and 3% nonfat milk, the membranes were incubated in PBS, 0.1% Tween-20, and 2% nonfat milk (blotting buffer) containing 2  $\mu$ g/mL of 6His-tagged proteins for 1 hour at room temperature. The blots were washed three times for 5 minutes with 10 mL blotting buffer and then were incubated for 1 hour with anti-6His antibody in blotting buffer at room temperature. After three washes of 5 minutes each, the blots were incubated with an anti-mouse antibody conjugated to alkaline phosphatase for 1 hour at room temperature. Bound recombinant proteins were visualized by incubation with NBT/BCIP (Promega). When indicated, the membranes were incubated with a retinal extract prepared in PBS and 0.1% Tween-20 and containing 3 mg total protein/1 mL blotting buffer. Bound proteins were detected by incubation with the mouse anti-Unc119.

## Yeast Two-Hybrid System

The coding sequence for the mouse CaBP4 was cloned in fusion to the DNA-binding domain into the pGBKT7-BD vector (carrying the gene for tryptophan; Clontech). The cDNA encoding Unc119 was cloned in fusion to the Gal4-activation domain into the pGADT7 vector (carrying the gene for leucine; Clontech). AH109 yeast was cotransformed with both plasmids (0.2  $\mu$ g each) using the lithium acetate method according to a standard transformation protocol described by the manufacturer (yeast protocols handbook; Clontech). To determine the cotransformation efficiency, yeast cells (20% of the transformed cells) were allowed to grow for 4 days at 30°C on plates containing selective synthetic dropout (SD) medium without tryptophan and leucine. To test reported gene expression, a fraction of the cotransformed yeasts was also plated on selective medium without tryptophan (Trp), leucine (Leu), histidine (His), or adenine (Ade) and with X- $\alpha$ -galactosidase (X- $\alpha$ -gal; Biosynth, St. Gallen, Switzerland) because a high-affinity interaction of the recombinant proteins would result in the transcription of reporter genes that code for nutritional markers (Ade, His) and X- $\alpha$ -gal. In addition, 10 mM 3-amino-1,2,4-triazole (3-AT; Sigma, St. Louis, MO) was added to inhibit leaky expression of His3 proteins. To test whether low-affinity interaction can occur between the recombinant proteins, an equal amount of yeast was also plated on SD medium without Trp, Leu, or His containing 10 mM 3-AT. These colonies were then further streaked on selective SD medium without Trp, Leu, His, or Ade and with X- $\alpha$ -gal and 10 mM 3-AT.

## Immunohistochemistry

Mouse eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, (PB) for 1 hour. After fixation, tissues were incubated with a sucrose series to 20% sucrose in PB and then were embedded in 33% OCT compound (Miles, Elkhart, NY) diluted with 20% sucrose in PB. Eye tissues were cut in 12- $\mu$ m sections. To block nonspecific labeling, retinal

sections were incubated with 3% normal goat serum in PBST buffer (136 mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) for 20 minutes at room temperature. Sections were incubated overnight at 4°C in a mix of diluted primary antibodies (1:500 for rabbit anti-CaBP4 with 1:200 for mouse anti-Unc119; 1:100 for rabbit anti-syntaxin 3 with 1:200 for mouse anti-Unc119; 1:200 for mouse anti-SV2 with 1:2000 for rabbit anti-Unc119; 1:500 for mouse anti-PSD95 with 1:2000 for rabbit anti-Unc119). Control experiments were carried out with antibodies preabsorbed for 2 hours at 37°C with the purified proteins that were used as antigens. A mixture of Cy3-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG or Cy3-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-rabbit was reacted with sections for 1 hour at room temperature. Then the sections were rinsed in PBST and mounted with antifade reagent (Prolong; Molecular Probes) to slow photobleaching. Sections were analyzed under a confocal microscope (Zeiss LSM510; Carl Zeiss, Thornwood, NY). Immunofluorescent images were obtained with a 40×/1.3 NA objective lens (Plan-Neofluar; Carl Zeiss). For the experiments studying the protein levels, the double-labeled retinas were analyzed simultaneously with the confocal microscope configured with the same settings for the wild-type and CaBP4-knockout mice. For the analysis of wholemount retinas, the retinas of 2-month-old mice were dissected after fixation for 4 hours in 4% paraformaldehyde in PB. The retinas were incubated with 5% normal goat serum in PBST buffer (136 mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) overnight at 4°C. Retinas were then incubated overnight at 4°C with anti-Unc119 (1:200), anti-CaBP4 (1:500), or both. After three washes for 15 minutes in PBST, a mixture of Cy3-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse (1:100) was added to the retina and incubated overnight at 4°C. For double staining with PNA, the retinas were incubated overnight at 4°C with Cy3-conjugated goat anti-rabbit or anti-mouse IgG (1:100) and Alexa 488-PNA (1:50). Retinal wholemounts were mounted photoreceptor side up and were analyzed under a confocal microscope.

### Analysis of Tangential Sections Using Western Blotting

The procedure used to prepare serial tangential sections was similar to that described by Arshavsky and colleagues.<sup>25</sup> Briefly, the lens and vitreous were dissected out of the mouse eye immersed in Ringer solution (10 mM HEPES, pH 7.4, 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 0.02 mM EDTA, 313 mM glucose). A central piece of the retina was dissected out with a 1.5-mm trephine and was transferred to a PVDF membrane placed on a small glass coverslip with the photoreceptor facing up. The retina was then gently flattened between two glass slides separated by 0.5-mm-thick spacers and frozen on dry ice. The retina attached to the PVDF membrane and coverslip was then mounted with freezing compound onto the cryostat microtome holder parallel to the cutting blade and was cut in 15- $\mu$ m serial sections. Each section was collected in 50  $\mu$ L SDS-PAGE sample buffer, and 10- $\mu$ L aliquots were loaded on SDS-PAGE and analyzed by Western blot with selected antibodies after transfer onto a transfer membrane (Immobilon; Millipore).

## RESULTS

### Binding of Unc119 to CaBP4 in Retina

Retinal proteins eluted from a CaBP4 affinity column were analyzed to identify novel CaBP4-interacting proteins. Among the major bands identified by mass spectrometry, one band was identified as Unc119 homolog or mouse retinal gene 4/MRG4 (Fig. 1A). Because of its specific localization in the photoreceptor synapse, Unc119 was selected as a physiologically relevant interacting partner for CaBP4. To investigate whether the interaction between CaBP4 and Unc119 is direct, this interaction was analyzed using affinity chromatography with purified proteins. Unc119 was coupled to bead-form aga-rose and incubated with 6His-tagged mouse CaBP4 in the presence of Ca<sup>2+</sup>. Western blot analysis of the eluted proteins revealed that no

proteins eluted with EGTA but that CaBP4 eluted with an acidic buffer (Fig. 1B). This result indicates that CaBP4 can bind to Unc119 in the absence of  $\text{Ca}^{2+}$ . To further verify this observation, Unc119 – bead-form agarose was incubated with 6His-tagged mouse CaBP4 in the absence of  $\text{Ca}^{2+}$ . Although the binding of CaBP4 to Unc119 was observed in the absence of  $\text{Ca}^{2+}$ , it was partially disrupted after the addition of  $\text{CaCl}_2$ , suggesting a stronger binding of CaBP4 to Unc119 in the absence of  $\text{Ca}^{2+}$  (Fig. 1C).

To further investigate the physiological interaction of CaBP4 and Unc119, we analyzed their interaction by coimmunoprecipitation from lysates of mouse retina using an anti-CaBP4 antibody. A protein of approximately 35 kDa, corresponding to CaBP4, was immunoprecipitated from the wild-type but not from the CaBP4-knockout mouse retina lysates (Fig. 1D). As shown in Figure 1D, Western blot analysis of CaBP4 immunoprecipitates from wild-type mice also shows the presence of Unc119. In control experiments, no Unc119 is detected in immunoprecipitates using retina lysates from CaBP4-knockout mice, confirming the specific interaction of CaBP4 and Unc119.

### CaBP4 Interacts with Unc119 in Yeast

To confirm whether CaBP4 directly binds to Unc119, the ability of CaBP4 to interact with Unc119 was studied in the yeast 2-hybrid assay. Mouse CaBP4 cDNA was fused to the DNA-binding domain (BD) and mouse Unc119 was fused to the Gal4-activation domain (AD) by subcloning into yeast expression vectors (i.e., pGBKT7-CaBP4 and pGADT7-Unc119). In the reporter yeast strain AH109, expression of the *His-3*, *Ade-2*, and *Mel-1/LacZ* reporter genes required the colocalization of the binding domain with the activation domain mediated by the interaction of the fused proteins. Yeasts were cotransformed, and the interaction was assayed on selective synthetic dropout media. Coexpression of CaBP4-BD with Unc119-AD resulted in growth on SD-Leu-Trp and SD-Leu-Trp-His+3AT plates but not on SD-Leu-Trp-His-Ade+3-AT+ X- $\alpha$ -gal plates (Fig. 2A). Growth on SD-Leu-Trp plates indicates that both expression vectors are present in yeast. Growth on SD-Leu-Trp-His+3-AT but not on SD-Leu-Trp-His-Ade+3-AT+X- $\alpha$ -gal plates suggests a low-affinity protein interaction. In the case of lowaffinity interactions, restreaking of colonies that grow on SDLeu-Trp-His+3-AT plates can result in growth on SD-Leu-Trp-His-Ade+3-AT+ X- $\alpha$ -gal plates. Therefore, four individual yeast colonies isolated on SD-Leu-Trp plates were further retested for their ability to grow on SD-Leu-Trp-His+3-AT and SD-Leu-Trp-His-Ade+3-AT+ X- $\alpha$ -gal plates. Yeast growth was observed on both media 2 days after inoculation (Fig. 2B), whereas 4 days were needed for the colonies to turn blue. Control experiments in which yeast was cotransformed with pGBKT7-CaBP4 and the pGADT7 vector (Fig. 2) or with pGBKT7-calmodulin and pGADT7-Unc119 (data not shown) did not show growth on SD-Leu-Trp-His+3-AT and SD-Leu-Trp-His-Ade+3-AT+ X- $\alpha$ -gal selective media. This result confirms that the expression of the three reporter genes in yeast cotransformed with CaBP4 and Unc119 resulted from the specific interaction between those proteins.

### Interaction of the CaBP4 N-Terminus with Unc119 in a Gel Overlay Assay

To determine whether the interaction of mouse CaBP4 with Unc119 involved a linear domain of CaBP4, their interaction was analyzed using a gel overlay assay. GST-tagged CaBP4 or GST was separated on SDS-PAGE and transferred to PVDF membranes. The blots were incubated with 6His-tagged Unc119. The 6His-tagged Unc119 bound to GST-tagged CaBP4 but not to control GST (Fig. 3A, left). We further investigated the binding of Unc119 to CaBP4 using gel overlay assay with a retinal extract. Native Unc119 directly bound to GST-tagged CaBP4 but not to GST (Fig. 3A, right). This finding confirmed the specificity of this interaction and also suggested that a linear domain of CaBP4 is involved in the interaction with Unc119 because this assay is performed after denaturation of the protein in the presence of SDS. To define the region in CaBP4 that interacts with Unc119, GST-tagged, CaBP4-truncated

segments of varying lengths (amino acids residues 1–61, 1–82, 1–118, 119–271, 82–271, and 1–271; Fig. 3B) were constructed, and potential interactions with 6His-tagged Unc119 were analyzed using an overlay assay. Unc119 bound CaBP4 fragments that included the amino acid 82–118 domain (Fig. 3C). This result was confirmed by showing that Unc119 binds as well to the internal amino acid 82–118 domain as to the full-length CaBP4 (Fig. 3D). In CaBP4 orthologs, this is also the only well-conserved segment of the CaBP4 N-terminus upstream of EF-hand domains.

### Colocalization of Unc119 with CaBP4 in Retina Sections

To be a physiologically relevant interacting partner for CaBP4, Unc119 must colocalize with CaBP4 in the retina. To study whether Unc119 colocalizes with CaBP4 in the retina, a monoclonal antibody specific for mouse Unc119 was generated after immunization of mice with bacterially expressed Unc119. Figure 4A shows that one major band of approximately 34 kDa immunoreacted with the anti-Unc119 antibody in mouse retina extract, as seen by Western blot analysis.

To analyze whether CaBP4 is coexpressed with Unc119, mouse retina sections were double labeled with mouse anti-Unc119 and rabbit anti-CaBP4 antibodies. A strong staining signal for both CaBP4 and Unc119 was observed at the photo-receptor synapse (Fig. 4B). Some staining was also observed in the photoreceptor inner segment. After preabsorption of the anti-CaBP4 and anti-Unc119 antibodies with the purified proteins, staining at the synapse and inner segment was abolished, indicating their specificity. The next step was to determine whether CaBP4 and Unc119 were coexpressed in both types of photoreceptor. PNA labeling was used to demarcate the cone pedicles. However, because CaBP4 and Unc119 localize in the cone synaptic pedicles alongside the PNA-labeled synaptic membranes, no yellow signal was observed in double-labeling experiments with PNA.<sup>5</sup> PNA labeling of cone pedicles in wholemouted retinas revealed that Unc119 and CaBP4 are expressed throughout the cone pedicles (Fig. 4C). The remaining staining signals observed between the PNA-labeled pedicles reflected the labeling of CaBP4 and Unc119 in rod terminals. CaBP4 immunoreactivity colocalized with Unc119 labeling throughout cone and rod terminals (Fig. 4C).

### Colocalization of CaBP4 and Unc119 during Mouse Postnatal Development

To determine whether CaBP4 and Unc119 interact during retinal development, their expression was analyzed by immunohistochemistry during postnatal development. Both CaBP4 and Unc119 are detectable by immunohistochemistry beginning at postnatal day 6 (Fig. 5), though RNA is detectable at postnatal day 4 using RT-PCR (data not shown). Throughout development, CaBP4 and Unc119 are largely colocalized in the outer plexiform layer (OPL) and throughout the outer nuclear layer (Fig. 5). In the adult retina, both Unc119 and CaBP4 staining were restricted to the photoreceptor synapse and inner segment.

### Unc119 Less Abundant in CaBP4-Knockout Mice

To determine whether the absence of CaBP4 can affect the level of Unc119 protein, Unc119 was analyzed in the retinas of CaBP4-knockout mice (*Cabp4*<sup>-/-</sup>). To isolate and enrich in proteins localized to the outer plexiform layer, the method described by the Arshavsky group,<sup>25</sup> which combines serial tangential sectioning of flatmounted frozen retinas with Western blot analysis, was used. Serial sectioning of retinas was performed on frozen retinas with the photoreceptor side up. The collected fractions included approximately two thirds of the retina from the photoreceptor side. Serial sections were obtained from either 3 *Cabp4*<sup>+/+</sup> or 3 *Cabp4*<sup>-/-</sup> mouse retinas to compare the protein content of multiple synaptic markers. The distribution of Unc119 in all retinas was analyzed using PKC $\alpha$  as a marker protein. PKC $\alpha$  is expressed throughout the rod bipolar cells, from the axon terminals in the inner nuclear layer to their dendrites that form a dense network in the OPL, where they contact photoreceptor

pedicles<sup>26</sup> (Fig. 6A). The distribution of PKC $\alpha$  was analyzed, together with the distribution of Unc119 or other synaptic proteins, to monitor the reliability of the section series and the relative amounts of the studied synaptic proteins to PKC $\alpha$  in the OPL. The distribution of CaBP4 was found to overlap the distribution of Unc119 (Fig. 6A). The distributions of rhodopsin, syntaxin 3, and PSD95 were also analyzed in all fractions. Because there is always some variation inherent to the Western blot analysis, samples can only be compared if loaded on the same Western blot. Therefore, and because of the limited amount of material obtained using this method, fractions 3 to 5, which contained the higher amounts of Unc119 in all retinas (Fig. 6A), were combined and used to compare the level of Unc119 with other synaptic proteins. Figure 6B shows that the amount of Unc119 protein in CaBP4-knockout mice was lower than that in wild-type mice, though no differences were observed for PKC $\alpha$  marker proteins in the same fraction. As expected, CaBP4 was not detected in fractions from *Cabp4*<sup>-/-</sup> mice. The amount of Unc119 protein was also compared with the amount of other photoreceptor synaptic proteins (i.e., PSD-95 and syntaxin 3).<sup>27</sup> Similar amounts of syntaxin 3 were detected in synaptic fractions of *Cabp4*<sup>+/+</sup> and *Cabp4*<sup>-/-</sup> mice, in contrast to Unc119 (Fig. 6B). PSD-95 was also reduced in *Cabp4*<sup>-/-</sup> mice, but Unc119 was still more severely decreased (Fig. 6B).

To confirm the reductions in the levels of Unc119 at the photoreceptor synapse in *Cabp4*<sup>-/-</sup> mice, an analysis of photoreceptor proteins was performed using immunohistochemistry. Mouse retina sections from *Cabp4*<sup>-/-</sup> and *Cabp4*<sup>+/+</sup> mice were double labeled with antibodies for Unc119 and photoreceptor synaptic proteins and were analyzed simultaneously using confocal microscopy. Although the photoreceptor terminals of *Cabp4*<sup>-/-</sup> are disorganized and flat, the synaptic proteins—syntaxin 3, SV2, and PSD95—were detected at similar levels in *Cabp4*<sup>-/-</sup> and *Cabp4*<sup>+/+</sup> retinas, though synapses were slightly more weakly labeled in *Cabp4*<sup>-/-</sup> (Fig. 7). In contrast, almost no fluorescence was observed for Unc119 in *Cabp4*<sup>-/-</sup> mice, whereas Unc119 labeling in *Cabp4*<sup>+/+</sup> retinas revealed a strong signal similar to that of other synaptic proteins. This result confirmed that Unc119 levels are strongly reduced in *Cabp4*<sup>-/-</sup> mice compared with other synaptic proteins.

## DISCUSSION

Our study provides the first evidence that CaBP4 interacts with Unc119, a synaptic photoreceptor protein. The interaction between these proteins is compelling because CaBP4 and Unc119 have both been reportedly involved in neuronal development. Unc119 is localized in the synaptic terminals of rod and cone photoreceptors.<sup>20</sup> Unc-119/HRG4/MRG4 (human retinal gene 4/mouse retinal gene 4) has been observed in many organisms and has been well conserved throughout evolution.<sup>23,28-31</sup> Although the function of Unc119 has not yet been clearly established, it has been suggested that it plays a role in the mechanism of neurotransmitter release through the synaptic vesicle cycle.<sup>20,22</sup> A truncation mutation in HRG4, the human ortholog, was discovered in a patient with late-onset retinal degeneration.<sup>22</sup> The phenotype of this patient was similar to that of transgenic mice that express mutant HRG4.<sup>22</sup>

Given our biochemical data that indicate CaBP4 and Unc119 interact in vitro and in the retina, the interaction of these proteins in vivo was investigated by studying their localization using immunohistochemistry. Not only did both proteins colocalize in the adult retina, they began to be expressed at the same time at postnatal day 4 and then colocalized throughout postnatal development. This observation further corroborated the physiological interaction between these proteins in vivo. To test this conclusion further, the effects of the absence of CaBP4 on the levels of Unc119 at the photoreceptor synapse were analyzed. Western blot analysis of isolated synaptic photoreceptor fractions showed a reduction in Unc119 protein in the photoreceptor terminals of CaBP4-knockout mice compared with wild-type mice. The analysis of Unc119 using immunohistochemistry showed strongly reduced immunoreactivity in



CaBP4-knockout photoreceptor synapses compared with wild-type retinas. The fact that the levels of the other synaptic proteins (e.g., PSD-95, SV2, syntaxin 3) decreased only slightly in the same retina sections that showed severely reduced levels of Unc119 suggests that this strong decrease was not only a consequence of a decrease in the number of photoreceptor terminals.<sup>4</sup> Rather, the absence of CaBP4 may trigger a loss of Unc119 or may affect a common pathway that involves CaBP4 and Unc119 and, consequently, results in the instability and reduction of Unc119 protein. In any case, this effect of CaBP4 deletion on Unc119 protein further supports the possibility that the functions of these proteins are related. A decrease in the levels of functionally related proteins was previously observed in photoreceptors of other mouse models, such as in SV2B-knockout mice and transgenic Unc119 mutant mice.<sup>32,33</sup>

CaBP4 and Unc119 have been shown to colocalize with synaptic vesicle proteins and to be involved in neurotransmitter release.<sup>4,20</sup> Neuronal activity is important for the maintenance of synapses. Unc-119 and CaBP4 appear to be important for the stability of the synapse because abnormal axon branching, retinal degeneration, or both are observed when these proteins are absent.<sup>4,21,34</sup> Their coexpression and interaction suggest that Unc119 and CaBP4 participate in the same pathway and are important for the maintenance and correct localization of the synapse after neuronal outgrowth rather than during development.

Although Unc119 is prominent in the retina, it is also expressed in myeloid and lymphoid cells,<sup>35</sup> where it activates Src-type tyrosine kinases important for eosinophil survival and T-cell function.<sup>35,36</sup> However, the interaction of Unc119 with Src kinases might not be involved in the normal function of mouse adult retina. Unc119, with or without CaBP4, did not stimulate the phosphorylation of retinal proteins in a phosphorylation assay using a mouse retinal extract (data not shown).

CaBP4 and Unc119 are expressed specifically at the specialized ribbon synapse of photoreceptor cells. These synapses are composed of a particular set of proteins, including a specific subtype of voltage-gated calcium channel.<sup>37-40</sup> CaBP4 was previously shown to modulate neurotransmitter release through the regulation of presynaptic Ca<sub>v</sub>1 L-type Ca<sup>2+</sup> channels. In photoreceptors, the neurotransmitter release is linearly proportional to the Ca<sup>2+</sup> influx through Ca<sub>v</sub>1. This linearity requires proximity of the Ca<sub>v</sub>1 channels with the fusion site of synaptic vesicles. In fact, there is a tight association between voltage-dependent calcium channels and the core complex of proteins involved in synaptic vesicle docking/fusion.<sup>41</sup> The present study suggests that CaBP4 regulates neurotransmitter release by interacting not only with Ca<sub>v</sub>1 but also with Unc119. CaBP4 binding to Unc119 is a Ca<sup>2+</sup>-sensitive process. In the affinity chromatography experiments, CaBP4 is partially eluted from Unc119 on the addition of CaCl<sub>2</sub>. One could speculate, therefore, that the interaction of Unc119 and CaBP4 is dependent on the Ca<sup>2+</sup> concentration. One possible scenario is that the entry of Ca<sup>2+</sup> through the Ca<sub>v</sub>1 activated by CaBP4 results in a change of affinity of CaBP4 for Unc119. The CaBP4/Unc119 complex is disrupted, and Unc119 is free to play its role in the mechanism of synaptic vesicle release in close proximity. Although beyond the scope of this article, it is tempting to speculate that CaBP4 might thus play a role in linking Ca<sup>2+</sup> influx and subsequent neurotransmitter release.

Our initial studies demonstrate that CaBP4 is crucial for morphologically and functionally normal synapses, likely through the modulation of L-type Ca<sup>2+</sup> channels and transmitter release. This study raises the possibility that CaBP4 interacts with multiple synaptic proteins that play roles in mechanisms important for normal neurotransmitter release, including Ca<sub>v</sub>1.4 and Unc119. The identification of CaBP4-interacting partners is important for determining the synaptic mechanisms that involve CaBP4 and will shed light on future roles of CaBP4.

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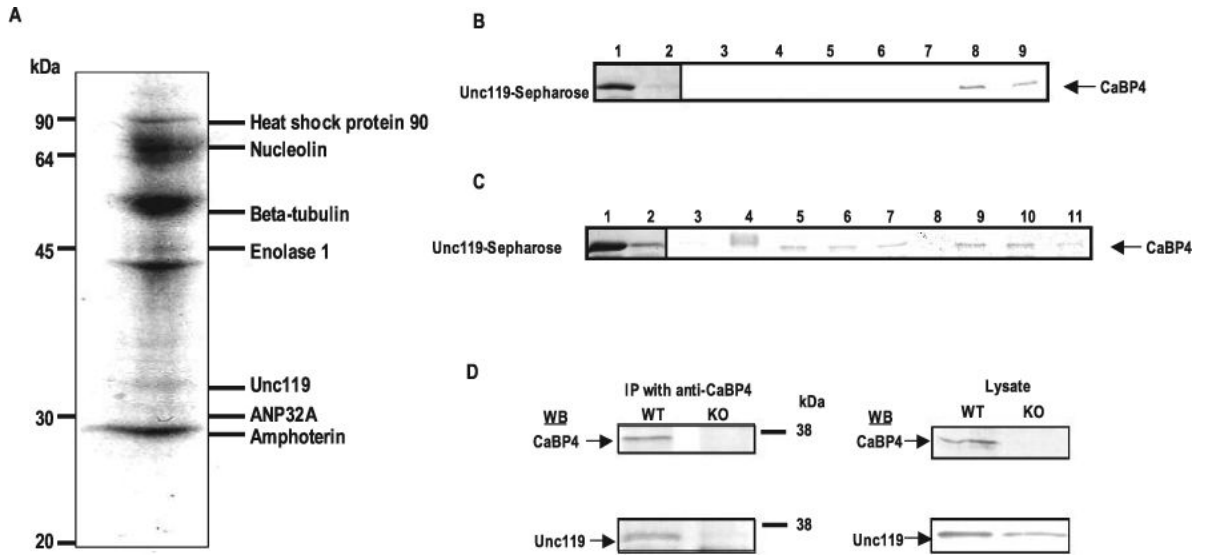
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## References

1. Haeseleer F, Sokal I, Verlinde C, et al. Five members of a novel Ca<sup>2+</sup>-binding protein (CABP) subfamily with similarity to calmodulin. *J Biol Chem* 2000;275:1247–1260. [PubMed: 10625670]
2. Haeseleer F, Imanishi Y, Sokal I, Filipek S, Palczewski K. Calcium-binding proteins: intracellular sensors from the calmodulin superfamily. *Biochem Biophys Res Comm* 2002;290:615–623. [PubMed: 11785943]
3. Haeseleer, F.; Palczewski, K. Calmodulin and Ca<sup>2+</sup>-binding proteins (CaBPs): variations on a theme.. In: Baehr, W.; Palczewski, K., editors. *Photoreceptors and Calcium*. Landes Bioscience; Georgetown, TX: 2002.
4. Haeseleer F, Imanishi Y, Maeda T, et al. Essential role of Ca<sup>2+</sup>-binding protein 4, a Ca(v)1.4 channel regulator, in photoreceptor synaptic function. *Nat Neurosci* 2004;7:1079–1087. [PubMed: 15452577]
5. Maeda T, Lem J, Palczewski K, Haeseleer F. A critical role of CaBP4 in the cone synapse. *Invest Ophthalmol Vis Sci* 2005;46:4320–4327. [PubMed: 16249514]
6. Ball SL, Powers PA, Shin HS, Morgans CW, Peachey NS, Gregg RG. Role of the beta(2) subunit of voltage-dependent calcium channels in the retinal outer plexiform layer. *Invest Ophthalmol Vis Sci* 2002;43:1595–1603. [PubMed: 11980879]
7. Mansergh F, Orton NC, Lalonde MR, et al. Mutation of the calcium channel gene *Cacna1f* disrupts calcium signaling, synaptic transmission and cellular organization in mouse retina. *Hum Mol Genet* 2005;14:3035–3046. [PubMed: 16155113]
8. Chang B, Heckenlively JR, Bayley PR, et al. The nob2 mouse, a null mutation in *Cacna1f*: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses. *Vis Neurosci* 2006;23:11–24. [PubMed: 16597347]
9. Strom TM, Nyakatura G, Apfelstedt-Sylla E, et al. An L-type calcium-channel gene mutated in incomplete X-linked congenital stationary night blindness. *Nat Genet* 1998;19:260–263. [PubMed: 9662399]
10. Bech-Hansen NT, Naylor MJ, Maybaum TA, et al. Loss-of-function mutations in a calcium-channel alpha(1)-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness. *Nat Genet* 1998;19:264–267. [PubMed: 9662400]
11. Zeitz C, Kloeckener-Gruissem B, Forster U, et al. Mutations in CABP4, the gene encoding the Ca<sup>2+</sup>-binding protein 4, cause autosomal recessive night blindness. *Am J Hum Genet* 2006;79:657–667. [PubMed: 16960802]
12. Yang PS, Alseikhan BA, Hiel H, et al. Switching of Ca<sup>2+</sup>-dependent inactivation of Ca(V)1.3 channels by calcium binding proteins of auditory hair cells. *J Neurosci* 2006;26:10677–10689. [PubMed: 17050707]
13. Cui, G.; Haeseleer, F.; Lee, A. Ca<sup>2+</sup>-binding protein 4 is expressed in cochlear hair cells and modulates Cav1.3 L-type Ca<sup>2+</sup> channels.. Presented at: Society for Neuroscience 36th Annual Meeting; Atlanta, GA. October 14–18, 2006;
14. Zhou H, Kim SA, Kirk EA, et al. Ca<sup>2+</sup>-binding protein-1 facilitates and forms a postsynaptic complex with Ca(v)1.2 (L-Type) Ca<sup>2+</sup> channels. *J Neurosci* 2004;24:4698–4708. [PubMed: 15140941]
15. Lee A, Westenbroek RE, Haeseleer F, Palczewski K, Scheuer T, Catterall WA. Differential modulation of Ca (v) 2.1 channels by calmodulin and Ca<sup>2+</sup>-binding protein 1. *Nat Neurosci* 2002;5:210–217. [PubMed: 11865310]
16. Kinoshita-Kawada M, Tang JS, Xiao R, Kaneko S, Foskett JK, Zhu MX. Inhibition of TRPC5 channels by Ca<sup>2+</sup>-binding protein 1 in *Xenopus* oocytes. *Pflugers Arch* 2005;450:345–354. [PubMed: 15895247]

17. Kasri NN, Holmes AM, Bultynck G, et al. Regulation of InsP(3) receptor activity by neuronal Ca<sup>2+</sup>-binding proteins. *EMBO J* 2004;23:312–321. [PubMed: 14685260]
18. Yang J, McBride S, Mak DOD, et al. Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca<sup>2+</sup> release channels. *Proc Natl Acad Sci U S A* 2002;99:7711–7716. [PubMed: 12032348]
19. Haynes LP, Tepikin AV, Burgoyne RD. Calcium-binding protein 1 is an inhibitor of agonist-evoked, inositol 1,4,5-trisphosphate-mediated calcium signaling. *J Biol Chem* 2004;279:547–555. [PubMed: 14570872]
20. Higashide T, McLaren MJ, Inana G. Localization of HRG4, a photoreceptor protein homologous to Unc-119, in ribbon synapse. *Invest Ophthalmol Vis Sci* 1998;39:690–698. [PubMed: 9538874]
21. Knobel KM, Davis WS, Jorgensen EM, Bastiani MJ. UNC-119 suppresses axon branching in *C. elegans*. *Development* 2001;128:4079–4092. [PubMed: 11641230]
22. Kobayashi A, Higashide T, Hamasaki D, et al. HRG4 (UNC119) mutation found in cone-rod dystrophy causes retinal degeneration in a transgenic model. *Invest Ophthalmol Vis Sci* 2000;41:3268–3277. [PubMed: 11006213]
23. Manning AG, Crawford BD, Waskiewicz AJ, Pilgrim DB. Unc-119 homolog required for normal development of the zebrafish nervous system. *Genesis* 2004;40:223–230. [PubMed: 15593328]
24. Zhu L, Jang GF, Jastrzebska B, et al. A naturally occurring mutation of the opsin gene (T4R) in dogs affects glycosylation and stability of the G protein-coupled receptor. *J Biol Chem* 2004;279:53828–53839. [PubMed: 15459196]
25. Strissel KJ, Lishko PV, Trieu LH, Kennedy MJ, Hurley JB, Arshavsky VY. Recoverin undergoes light-dependent intracellular translocation in rod photoreceptors. *J Biol Chem* 2005;280:29250–29255. [PubMed: 15961391]
26. Greferath U, Grunert U, Wassle H. Rod bipolar cells in the mammalian retina show protein kinase-C-like immunoreactivity. *J Comp Neurol* 1990;301:433–442. [PubMed: 2262600]
27. Morgans CW. Presynaptic proteins of ribbon synapses in the retina. *Microsc Res and Tech* 2000;50:141–150. [PubMed: 10891878]
28. Maduro MF, Gordon M, Jacobs R, Pilgrim DB. The UNC-119 family of neural proteins is functionally conserved between humans, *Drosophila* and *C. elegans*. *J Neurogenet* 2000;13:191–212. [PubMed: 10858820]
29. Maduro M, Pilgrim D. Identification and Cloning of Unc-119, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics* 1995;141:977–988. [PubMed: 8582641]
30. Higashide T, Murakami A, McLaren MJ, Inana G. Cloning of the cDNA for a novel photoreceptor protein. *J Biol Chem* 1996;271:1797–1804. [PubMed: 8576185]
31. Chung S, Kang S, Paik S, Lee J. NgUNC-119, *Naegleria* homologue of UNC-119, localizes to the flagellar rootlet. *Gene* 2007;389:45–51. [PubMed: 17123749]
32. Lazzell DR, Belizaire R, Thakur P, Sherry DM, Janz R. SV2B regulates synaptotagmin 1 by direct interaction. *J Biol Chem* 2004;279:52124–52131. [PubMed: 15466855]
33. Kubota S, Kobayashi A, Mori N, Higashide T, McLaren MJ, Inana G. Changes in retinal synaptic proteins in the transgenic model expressing a mutant HRG4 (UNC119). *Invest Ophthalmol Vis Sci* 2002;43:308–313. [PubMed: 11818371]
34. Ishiba Y, Higashide T, Mori N, et al. Targeted inactivation of synaptic HRG4 (UNC119) causes dysfunction in the distal photo-receptor and slow retinal degeneration, revealing a new function. *Exp Eye Res* 2007;84:473–485. [PubMed: 17174953]
35. Cen O, Gorska MM, Stafford SJ, Sur S, Alam R. Identification of UNC119 as a novel activator of SRC-type tyrosine kinases. *J Biol Chem* 2003;278:8837–8845. [PubMed: 12496276]
36. Gorska MM, Stafford SJ, Cen O, Sur S, Alam R. Unc119, a novel activator of Lck/Fyn, is essential for T cell activation. *J Exp Med* 2004;199:369–379. [PubMed: 14757743]
37. Heidelberger R, Thoreson WB, Witkovsky P. Synaptic transmission at retinal ribbon synapses. *Prog Retinal Eye Res* 2005;24:682–720.
38. Dieck ST, Brandstatter JH. Ribbon synapses of the retina. *Cell Tissue Res* 2006;326:339–346. [PubMed: 16775698]

39. Morgans CW. Localization of the alpha(1F) calcium channel subunit in the rat retina. *Invest Ophthalmol Vis Sci* 2001;42:2414–2418. [PubMed: 11527958]
40. Morgans CW. Calcium channel heterogeneity among cone photo-receptors in the tree shrew retina. *Eur J Neurosci* 1999;11:2989–2993. [PubMed: 10457194]
41. Sheng ZH, Westenbroek RE, Catterall WA. Physical link and functional coupling of presynaptic calcium channels and the synaptic vesicle docking/fusion machinery. *J Bioenerg Biomembr* 1998;30:335–345. [PubMed: 9758330]



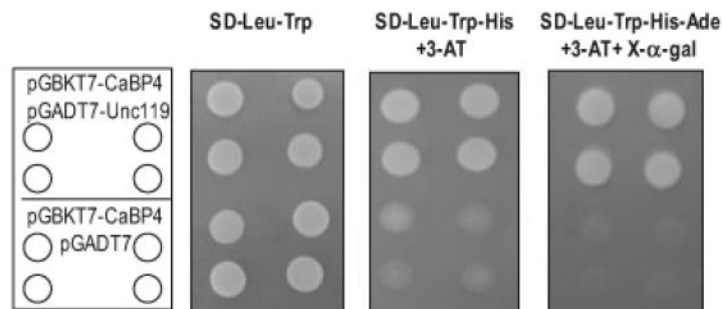
**Figure 1.**

Interaction of CaBP4 with Unc119 in the retina. **(A)** Identification of CaBP4-binding proteins by affinity chromatography and mass spectrometry. Affinity chromatography on CaBP4 – bead-form agarose was performed using extracts of bovine retina. The eluted proteins were separated by electrophoresis on SDS-PAGE. Excised bands were then analyzed by mass spectrometry. Several proteins were identified: amphotericin (database accession number: XP\_001072913), acidic leucine-rich nuclear phosphoprotein 32 family member A, ANP32A (AAI25144), Unc119 homolog B (AAI15530), enolase 1, alpha (AAP36132),  $\beta$ -tubulin 4Q (AAL32434), nucleolin (EDL40223), heat shock protein HSP 90- $\alpha$  (P07900). **(B, C)** Direct interaction of Unc119 with CaBP4 analyzed by affinity chromatography. **(B)** Purified 6His-tagged Unc119 was coupled to bead-form agarose and incubated with 6His-tagged CaBP4 in the presence of  $\text{Ca}^{2+}$ . The column was washed with 150 mM NaCl, and the proteins were eluted with 3 mM EGTA followed by 0.2 M Gly buffer, pH 2.1. The loaded and flow-through fractions (lanes 1, 2) were analyzed using Coomassie staining. Elution fractions (lanes 3–9) were analyzed by Western blot with an anti-CaBP4 antibody. Lane 1, CaBP4 loaded on the column; lane 2, flow-through fraction; lane 3, last wash; lanes 4–6, elution with 3 mM EGTA; lane 7, final elution with EGTA; lanes 8 and 9, elution with 0.1 M Gly. **(C)** After incubation of 6His-tagged Unc119 – bead-form agarose with 6His-tagged CaBP4 in the absence of  $\text{Ca}^{2+}$ , the column was washed with 150 mM NaCl and the proteins were eluted with 5 mM  $\text{CaCl}_2$  followed by 0.1 M Gly buffer, pH 2.1. The elution fractions were then analyzed by Western blot with an anti-CaBP4 antibody. Lane 1, CaBP4 loaded on the column (Coomassie staining); lane 2, flow-through fraction (Coomassie staining); lane 3, last wash; lanes 4–7, elution with 5 mM  $\text{CaCl}_2$ ; lane 8, final elution with  $\text{CaCl}_2$ ; lanes 9–11, elution with 0.1 M Gly buffer. **(D)** Unc119 coimmunoprecipitates with CaBP4 in retina extract. CaBP4 was immunoprecipitated from mouse retina lysates using the anti-CaBP4 antibody. A retina lysate from *Cabp4*<sup>-/-</sup> retina (KO) was used as a negative control for the immunoprecipitation with the anti-CaBP4. Immunoprecipitated proteins were analyzed by Western blot using anti-CaBP4 (upper) or anti-Unc119 (lower) antibodies. Right: CaBP4 and Unc119 proteins in a sample of the initial lysates.

A

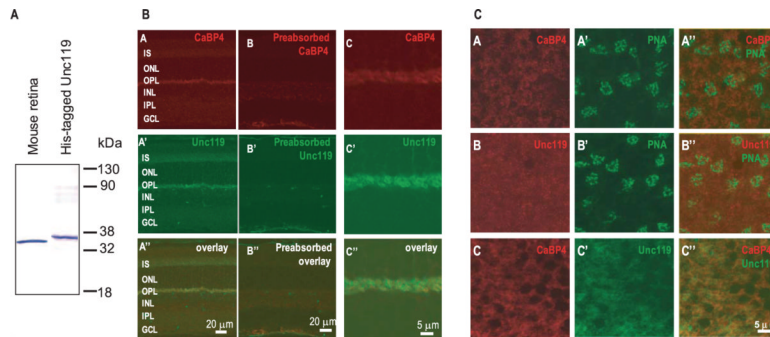
	pGBKT7-CaBP4 + pGADT7-Unc119	pGBKT7-CaBP4 + pGADT7
SD-Leu-Trp	227±39	280±6
SD-Leu-Trp-His+3-AT	41±13	0
SD-Leu-Trp-His-Ade+3-AT+X- $\alpha$ -gal	0	0

B

**Figure 2.**

Interaction of Unc119 with CaBP4 in yeast. **(A)** Analysis of yeast-transformant growth on selective synthetic dropout media. Number of primary yeast colonies (mean  $\pm$  average absolute deviation;  $n = 3$ ) cotransformed with pGBKT7-CaBP4 and pGADT7-Unc119 or pGBKT7-CaBP4 and pGADT7 (control) obtained on selective synthetic dropout media (SD). Growth on SD-Leu-Trp indicates that both constructs are present in yeast. Growth on SD-Leu-Trp-His+3-AT and SD-Leu-Trp-His-Ade+3-AT indicates weak and strong protein-protein interaction, respectively. **(B)** Replicates of primary yeast colonies on selective media. Four individual yeast colonies isolated on SD-Leu-Trp were further retested for their ability to grow on SD-Leu-Trp or SDLeu-Trp-His+3-AT or SD-Leu-Trp-His-Ade+3AT +X- $\alpha$ -gal. Plates were photographed after 2-day incubation at 30°C.

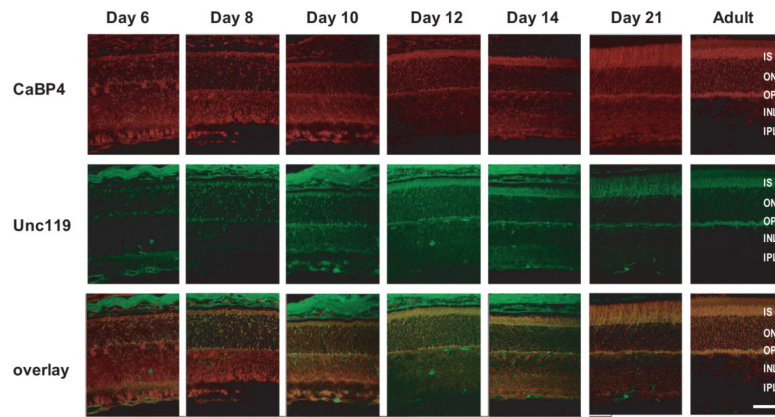




**Figure 4.**

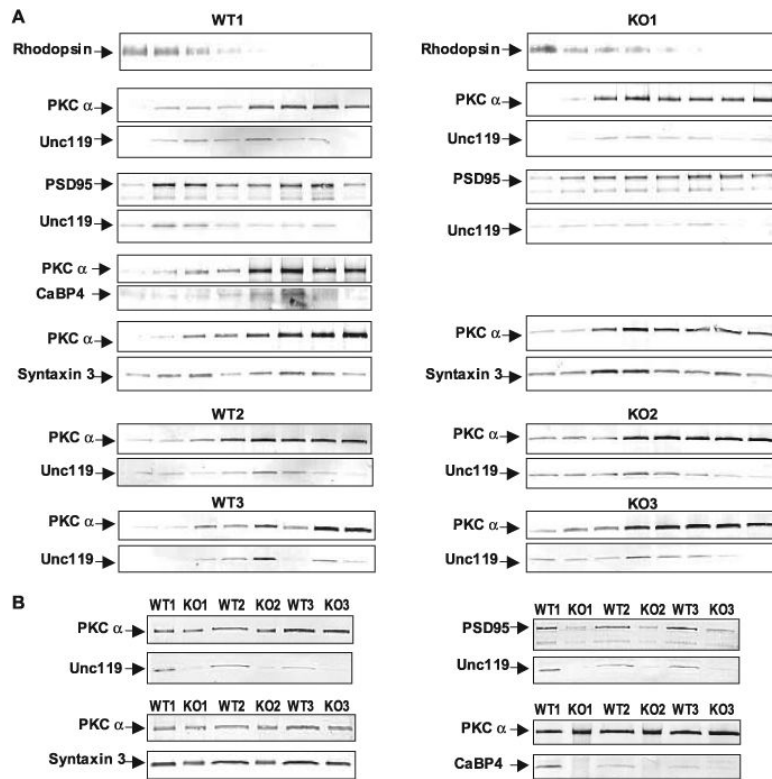
Colocalization of CaBP4 and Unc119 at the photoreceptor synapse. (A) Characterization of the mouse monoclonal anti-Unc119. Western blot analysis of recombinant 6His-tagged Unc119 and mouse retina extract with anti-Unc119 antibody. A protein of approximately 34 kDa was detected in mouse retina extract. (B) CaBP4 and Unc119 labeling in the retina (A, A', A'', B, B', B'') and in the outer plexiform layer (C, C', C''). Mouse retina sections were immunostained with anti-CaBP4 (red) or anti-Unc119 (green) antibodies. The overlay of CaBP4 and Unc119 is shown at the bottom. Yellow: colocalization of CaBP4 and Unc119 at the photoreceptor synapse. Control experiments were carried out with antibodies preabsorbed with the respective purified proteins (B–B''). IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (C) CaBP4 and Unc119 labeling in mouse retinal wholemounts. Wholemount view of CaBP4 labeling (red) at cone pedicles labeled with PNA (green)(A, A', A''). Unc119 labeling (red) at cone pedicles labeled with PNA (green) (B, B', B''). Flatmounted retina double stained with CaBP4 (red) and Unc119 (green). Scale bar, 5  $\mu$ m.



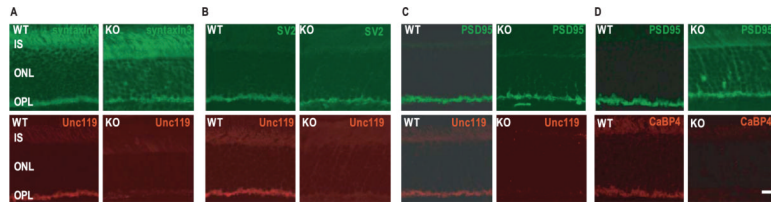


**Figure 5.**

CaBP4 colocalized with Unc119 during postnatal retina development. Mouse retina double labeled at different time points of postnatal development with anti-CaBP4 (*red*) and anti-Unc119 (*green*). Overlay of CaBP4 and Unc119 (*bottom*). Postnatal ages of the mouse retina are indicated above the columns. IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 40  $\mu\text{m}$ .



**Figure 6.** Western blot analysis of synaptic proteins in 15- $\mu$ m retina serial sections from *Cabp4*<sup>+/+</sup> and *Cabp4*<sup>-/-</sup> mice. **(A)** Distribution of synaptic proteins in OPL fractions. Fifteen-micrometer serial sections from 3 *Cabp4*<sup>+/+</sup> (WT1, WT2, WT3) and 3 *Cabp4*<sup>-/-</sup> (KO1, KO2, KO3) retinas were analyzed by Western blot with anti-Unc119. The distribution of PKC $\alpha$  proteins used as a marker protein was analyzed in parallel. The distribution of rhodopsin, syntaxin 3, PSD95, and CaBP4 was analyzed in sections from 1 *Cabp4*<sup>+/+</sup> and 1 *Cabp4*<sup>-/-</sup> retina. **(B)** Unc119 proteins are less abundant in *Cabp4*<sup>-/-</sup> retina than in *Cabp4*<sup>+/+</sup> mice. Levels of various synaptic proteins in fractions 3 to 5 of three series of fractions, as shown in **(A)**, from *Cabp4*<sup>+/+</sup> retina (WT) or *Cabp4*<sup>-/-</sup> retina (KO) were compared using Western blotting. PKC $\alpha$  and PSD95 were used as marker proteins.



**Figure 7.** Analysis of Unc119 proteins in photoreceptors from *Cabp4*<sup>+/+</sup> and *Cabp4*<sup>-/-</sup> mice using immunohistochemistry. *Cabp4*<sup>+/+</sup> (left, WT) and *Cabp4*<sup>-/-</sup> (right, KO) retina were double labeled using antibodies for Unc119 (A–C, bottom, red) and syntaxin 3 (A, upper, green), SV2 (B, upper, green), or PSD95 (C, upper, green). (D) Retinas were double labeled with anti-CaBP4 (bottom, red) and anti-PSD95 (upper, green). The inner segment (IS), outer nuclear layer (ONL) and OPL are identified. Scale bar, 10  $\mu$ m.