Characterization of Cav1.4 Complexes (α1, β2, and α2δ4) in HEK293T Cells and in the Retina*

Amy Lee†, Shiyi Wang‡, Brittany Williams‡, Jussara Hagen‡, Todd E. Scheetz‡, and Françoise Haeseleer*†

Departments of Molecular Physiology and Biophysics, Otolaryngology Head-Neck Surgery, and Neurology, University of Iowa, Iowa City, Iowa 52242, the Departments of Ophthalmology and Visual Sciences and Biomedical Engineering, University of Iowa, Iowa City, Iowa 52242, and the Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195

Background: The properties of voltage-gated Ca2+ channels are regulated by auxiliary β and α2δ subunits.

Results: Retinal β2 and α2δ4 subunits interact with Cav1.4 and differentially modulate channel properties compared with other Cav subunits.

Conclusion: β2 and α2δ4 subunits are major determinants of Cav1.4 function.

Significance: Cav1.4 channels in retinal photoreceptors are composed of auxiliary subunits with distinct properties compared with other Cav1 channels.

In photoreceptor synaptic terminals, voltage-gated Cav1.4 channels mediate Ca2+ signals required for transmission of visual stimuli. Like other high voltage-activated Cav channels, Cav1.4 channels are composed of a main pore-forming Cav1.4 α1 subunit and auxiliary β and α2δ subunits. Of the four distinct classes of β and α2δ subunits, β2 and α2δ4 are thought to co-assemble with Cav1.4 α1 subunits in photoreceptors. However, an understanding of the functional properties of this combination of Cav subunits is lacking. Here, we provide evidence that Cav1.4 α1, β2, and α2δ4 contribute to Cav1.4 channel complexes in the retina and describe their properties in electrophysiological recordings. In addition, we identified a variant of β2 named here β2X13† which, along with β2A, is present in photoreceptor terminals. Cav1.4 α1, β2, and α2δ4 were communoprecipitated from lysates of transfected HEK293 cells and mouse retinas and were found to interact in the outer plexiform layer of the retina containing the photoreceptor synaptic terminals, by proximity ligation assays. In whole-cell patch clamp recordings of transfected HEK293T cells, channels (Cav1.4 α1 + β2X13) containing α2δ4 exhibited weaker voltage-dependent activation than those with α2δ4. Moreover, compared with channels (Cav1.4 α1 + α2δ4) with β2α, β2X13-containing channels exhibited greater voltage-dependent inactivation. The latter effect was specific to Cav1.4 because it was not seen for Cav1.2 channels. Our results provide the first detailed functional analysis of the Cav1.4 subunits that form native photoreceptor Cav1.4 channels and indicate potential heterogeneity in these channels conferred by β2A and β2X13 variants.

Ca2+ ions are important for many cellular functions, including neurotransmitter release, muscle contraction, and gene transcription. In neurons, depolarizing stimuli initiate Ca2+ signaling mainly through activation of voltage-gated (CaV) Ca2+ channels. Neuronal Cav channels are multiprotein complexes composed of a major pore-forming α1 subunit, and two auxiliary subunits, β and α2δ, which not only regulate the functional properties of the α1 subunit but also are important for the proper trafficking of the α1 subunit to the plasma membrane and protection of the α1 subunit from proteosomal degradation (reviewed in Refs. 1–4). Ten different genes encode the Cav α1 subunits, whereas the β and α2δ subunits are encoded by four genes each (5, 6). Alternative splicing further adds to the molecular and functional diversity of α1 and β subunits (1, 7).

In photoreceptor terminals, Cav1.1 L-type channels are concentrated near the synaptic “ribbon,” a structure specialized for high throughput and tonic exocytosis (8). At the depolarized photoreceptor membrane potential in darkness, Cav1.1-mediated Ca2+ influx triggers the release of glutamate, which is required for transmission of visual stimuli to second-order neurons (9, 10). Of the different classes of Cav1 channels (Cav1.1–Cav1.4), multiple lines of evidence indicate that Cav1.4 is the major Cav1 channel in rod and cone photoreceptors. Antibodies against Cav1.4 label both rod and cone terminals (11–14). In mice, inactivation of CACNA1F, the gene encoding Cav1.4 (Cav1.4 KO), disrupts photoreceptor synaptic transmission and presynaptic calcium signaling (15) and prevents the maturation of photoreceptor synapses (13, 16). In addition, human mutations in CACNA1F cause vision disorders, including incomplete congenital stationary night blindness 2, which is characterized by impaired rod photoreceptor transmission and low visual acuity in darkness (17–20). Antibody labeling for the Cav1.3 α1 subunit has also been detected in the cones from tree shrew (21, 22) and chick (23). However, in mice lacking Cav1.3, morphological changes in photoreceptor synapses are observed, but visual function is largely normal (24). The auxiliary Cav1.4 subunits in photoreceptors are most likely β2 and α2δ4, because mice lacking functional β2 or α2δ4 subunits...
**Retina Ca$_{1.4}$ Channels**

Exhibit similar morphological defects in the retina and vision impairment as Ca$_{1.4}$ KO mice (25, 26).

However, an understanding of the functional properties of this particular combination of Ca$_{1.4}$ channel is lacking. Previous electrophysiological analyses of Ca$_{1.4}$ channels in heterologous expression systems have employed alternate $\beta$ and $\alpha_\delta$ subunits (27–32) and so may not reflect the properties of native photoreceptor Ca$_{1.4}$ channels. Therefore, the goal of this study was to investigate the association of Ca$_{1.4}$ $\alpha_1$ with $\beta_2$ and $\alpha_\delta$ subunits cloned from human retina and to characterize the electrophysiological properties of the corresponding channels in transfected HEK293T cells. In the course of this work, we identified a splice variant of $\beta_2$ in the retina, which is distinct from the brain $\beta_2a$ subunit and which differentially modulates the functional properties of Ca$_{1.4}$.

**EXPERIMENTAL PROCEDURES**

**Animals**

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Iowa and the University of Washington. These procedures were in accordance with National Institutes of Health guidelines. CaBP4 KO (28) and Ca$_{1.4}$ KO (33) mice were characterized previously. Ca$_{1.4}$ KO mice (B6.Cg-Cacna1f1tm1.15die/J) were obtained from the Jackson Laboratory. Adult mice (WT, 2–4 months old) used in this study were maintained on a 12-h light/dark cycle.

**Antibodies**

Commercially available antibodies were alkaline phosphatase-conjugated anti-rat, anti-rabbit, and anti-mouse (Promega Corp., Madison, WI), mouse anti-FLAG (Sigma-Aldrich), Alexa Fluor 550 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, and Alexa Fluor 550 goat anti-rat (Invitrogen). The antibodies-conjugated anti-rat, anti-rabbit, and anti-mouse (Promega Corp., Madison, WI), mouse anti-FLAG (Sigma-Aldrich), Alexa Fluor 550 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, and Alexa Fluor 550 goat anti-rat (Invitrogen). The antibodies used in this study were maintained on a 12-h light/dark cycle.

**Cloning of Ca$_{1.4}$ $\alpha_1$, $\beta_2$, and $\alpha_\delta$ Subunits and Partial $\beta_{1b}$ and $\alpha_\delta_1$**

Human Ca$_{1.4}$ $\alpha_1$ Subunit—The Ca$_{1.4}$ coding sequence was isolated and cloned from a human retina cDNA library. Five fragments were amplified by PCR with Platinum Pfx DNA polymerase (Invitrogen): nucleotides 1–399 (F1, ATG initiation codon-SnaBl), 393–1306 (F2, SnaBl-SfiI), 1294–3292 (F3, SfiI-ClaI), 3286–3918 (F4, ClaI-HindIII), and 3913–5934 (F5, HindIII-TGA stop codon). A FLAG epitope was added to the first fragment covering the N terminus of Ca$_{1.4}$ (F1, 1–399) by PCR with primers FH736 (5′-CTAGACCATGATGTTACAAAGGTACAGCAGGATAATCGGAAATCTGGACGAGGAGGGAAG-3′) and FH 720 (5′-CCAGGAATGCTACTCCACCCTG-3′). All PCR fragments were subcloned into the pCR-blunt II vector (Invitrogen) and sequenced. Fragments F1 and F2 were first assembled by subcloning into the XbaI and KpnI sites of the pBluescript vector using restriction sites provided by the pCR-blunt II vector for the Ca$_{1.4}$ 1.4 fragments. The F3 fragment was then added by cloning into the SfiI and ClaI sites of pBluescript-F1-F2. The human Cav1.4 full coding sequence was then cloned between the XbaI and NotI sites of the pcDNA3.1 mammalian expression vector by ligating fragments XbaI-ClaI (F1-F3) with ClaI-HindIII (F4) and HindIII-NotI (F5).

$\beta_2$ Subunits—The $\beta_{2x13}$ coding sequence was cloned from human retina cDNA library using PCR with primers FH1168 (5′-CACCATGAGTCTGCGGCTTG-3′) and FH 1169 (5′-TCAATGGCGGATGTTACAAATCCCTG-3′) that hybridizes on the initiation and stop codons, respectively, of $\beta_{2a}$ and $\beta_{2x13}$. After subcloning into the pCR-blunt II vector and confirmation of correct sequence, $\beta_{2x13}$ was transferred into the XbaI and BamHI sites of pcDNA3.1 vector using the XbaI and BamHI restriction sites provided by the pCR-blunt II vector. The $\beta_{2a}$ subunit was cloned from a human retina cDNA library using PCR in two fragments: F1 with primers FH1168 and FH1291 (5′-GGTTTAGGGCGCTGCTG-3′) and FH1291 (5′-TCGTTAGGGCGCTGCTG-3′) that inserts a silent mutation (underlined) at nucleotide 567, creating an Agel site, and F2 with primers FH1290 (5′-GCAAACCCAGGCTTCCAAACC-3′) and FH1169. The human $\beta_{2a}$ full coding sequence was then cloned between the XbaI and BamHI sites of the pcDNA3.1 vector by ligating fragments F1 Xba-Agel (ATG-563) and F2 Agel-BamHI (564-Stop codon). For bacterial expression of partial $\beta_2$ protein fused to a His$_8$ tag, a fragment covering from nucleotide 1225 to the stop codon of human $\beta_2$ was excised with BclI and HindIII from the pcDNA3.1-$\beta_{2x13}$ plasmid and cloned into the BamHI and HindIII sites of the pET30B vector (EMD Millipore, Billerica, MA).

The full-length mouse $\beta_{2x13}$ coding sequence was cloned from mouse retina cDNA using PCR with primers FH1168 (5′-CACCATGAGTCTGCGGCTTG-3′) and FH1278 (5′-TCAATGGCGGATGTTACAAATCCCTG-3′).

Human $\alpha_\delta_2$ Subunit—The $\alpha_\delta_2$ coding sequence was cloned from a human retina cDNA library using PCR in two fragments covering nucleotides 1–988 (F1, ATG-BamHI) using primers FH1227 (5′-CTCATGAGTCTGCGGCTGCTGCGCCCTC-3′) and FH1173 (5′-CTGAGGAGGATCTTTAATCACCAAG-3′) and nucleotides 982–3363 (F2, BamHI-Stop codon) using primers FH1274 (5′-CTGGTTTAAAAGGAGGATCTTCCGTGACG-3′) and FH1272 (5′-CACCCGAGGAGTTGGGGC-GTAG-3′). After subcloning in PCR-blunt II and sequencing, the F1 fragment was then first subcloned into the XbaI and BamHI sites of the pcDNA3.1 vector. Fragment F2 was then added between the BamHI and KpnI sites of pcDNA3.1-F1 in two fragments BamHI-Xhol (nucleotides 983–2778) and Xhol-KpnI (2778-Stop codon). For bacterial expression of a partial $\alpha_\delta_2$ protein fused to a His$_8$ tag, a fragment covering nucleotides 1–1591 of human $\alpha_\delta_2$ cDNA was excised with Ncol and HindIII from the pcDNA3.1-$\alpha_\delta_2$ plasmid and cloned into the pET-SUMO vector (Invitrogen).

$\beta_{1b}$ and $\alpha_\delta_1$ Fragments—For bacterial expression, a fragment of $\beta_{1b}$ corresponding to the sequence used to generate anti-$\beta_{1b}$ antibodies was fused to a His$_8$ tag. A PCR fragment was amplified with primers 5′-GACCGGGCCACTTGAGGAGCAT-3′ and 5′-TACCGGGAGTGTAGACGCCTGTC-3′ and then cloned into the pdest17 vector (Invitrogen). Similarly, a partial fragment of $\alpha_\delta_1$ corresponding to the sequence used to generate the anti-$\alpha_\delta_1$ antibody was amplified by PCR using primers (5′-ATGACTCTGCTGCTGCTGCTG-3′) and (5′-ATACTGCTGCTGCTGCTGCTG-3′).
Retina Ca\textsubscript{v}1.4 Channels

Human retinas were obtained from donors without known eye diseases from the Oregon Lions eye bank as allowed by the Institutional Human Subjects Division of the University of Washington. Total RNAs were isolated from human retina using the Ultraspec RNA isolation system (Biotecx, Houston, TX). A two-step quantitative PCR was then carried out to determine the relative expression of splice variants. Total RNA (1 \mu g) was subjected to first strand cDNA synthesis using Superscript III reverse transcriptase and oligo(dT) in a volume of 20 \mu l according to the manufacturer’s protocol (Invitrogen). For the human \beta\textsubscript{2X13} quantative PCR (qPCR),\textsuperscript{2} primers were designed on exon 7B (FH1280, 5’-GCTAAGCAGAAGCA-GAAATCGAC-3’) and −290 bp downstream of FH1280 on the exon 9-exon 10 junction (FH1284, 5’-TTTACCTCTGAACCTT-TCCGTAAG-3'). For human \beta\textsubscript{2a} primers were designed on exon 7A (FH1279, 5’-GCTATAGACATAGATGCTACT-GGC-3’) and −400 bp downstream of FH1279 on the exon 9-exon 10 junction (FH1284, 5’-TTTACCTCTGAACCTT-TCCGTAAG-3'). For normalization of the qPCR products, primers FH812 (5’-TCAACGGATTGCTGATTTGGGC-3’) and FH813 (5’-AGTGATGCGATGGACTGTGGTCAT-3’) were used to amplify human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Triplicate quantitative PCRs were carried out using 0.5 \mu l of cDNA, a 400 nM concentration of each primer, and 10 \mu l of QuantiTect SYBR Green PCR mix (Qiagen) in a 20-\mu l total reaction volume. After an initial incubation at 95 °C for 15 min, the qPCR was carried out for 40 cycles of denaturation at 95 °C for 15 s, annealing at 68 °C for 30 s, and extension at 72 °C for 1 min on a real-time PCR system (ABI PRISM 7000, Applied Biosystems). Single bands of the predicted size were confirmed at 95 °C for 15 s, annealing at 68 °C for 30 s, and extension for 15 min, the qPCR was carried out for 40 cycles of denaturation. Data were analyzed by comparing \textit{Ct} values. All cDNAs were normalized relative to the \textit{Ct} values of the internal control, GAPDH.

\[
\Delta \text{Ct} = \text{Ct of } \beta_{2a} - \text{Ct of GAPDH} \quad \text{(Eq. 1)}
\]

\[
\text{S.d. of } \Delta \text{Ct} = \sqrt{\text{(S.d. of } \beta_{2a})^2 + \text{(S.d. of GAPDH)}^2} \quad \text{(Eq. 2)}
\]

The normalized \textit{Ct} values of \beta\textsubscript{2X13} and \beta\textsubscript{2a} were then compared by determining $\Delta \Delta \text{Ct} = \Delta \text{Ct of } \beta_{2X13} - \Delta \text{Ct of } \beta_{2a}$. -Fold difference was calculated as 2\textsuperscript{$\Delta \Delta \text{Ct}$} (34).

For mouse RT-PCR, total RNA was isolated from mouse brain or retina using the RNeasy kit (Qiagen, Valencia, CA). After synthesis of cDNA using superscript reverse transcriptase, mouse \beta\textsubscript{2a} was analyzed by PCR using primers FH1279 and FH1284, whereas mouse \beta\textsubscript{2X13} was amplified with primers FH1338 (5’-GCAAAGCAGAAGCAGAAGTCGAC-3’) and FH1284. Primers GAPDH-F (5’-GAAGGGCTAATGACACAGTGTCATCT-3’) and GAPDH-R (5’-TAGCCATTTGTGTTGTCGTACAGG-3’) were used to amplify mouse GAPDH.

RNA-seq

A retinal RNA-seq experiment using samples from donor eyes (35) was used to determine the expression of human retinal \beta\textsubscript{2} splice variants. These sequences were aligned to the human genome (release GRCh37) using the Tuxedo pipeline (36). The resulting alignments were visually evaluated using IGV (37) to determine the specificity of transcriptional inclusion for exons 7A, 7B, and 7C of the CACNB2 gene.

Generation of Anti-\beta\textsubscript{2}, Anti-\alpha\textsubscript{2}\delta\textsubscript{4}, and Anti-CaBP4 Antibodies

Partial \beta\textsubscript{2X13} (amino acids 409–569) and \alpha\textsubscript{2}\delta\textsubscript{4} (amino acids 1–532) were expressed fused to a His\textsubscript{6} tag and purified from bacteria. Anti-\beta\textsubscript{2} and anti-\alpha\textsubscript{2}\delta\textsubscript{4} polyclonal antibodies were raised in rats by subcutaneous immunization with purified \beta\textsubscript{2} or \alpha\textsubscript{2}\delta\textsubscript{4} recombinant proteins mixed with Freund’s adjuvant (Cocalico Biologicals, Inc., Reamstown, PA). Mouse anti-CaBP4 was produced similarly by immunization with purified mouse full-length CaBP4 (28) (Cocalico Biologicals). For affinity purification of rat antibodies, purified \beta\textsubscript{2} or \alpha\textsubscript{2}\delta\textsubscript{4} was coupled to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer’s protocol. After loading of a 10-fold dilution of the sera in PBS, the columns were washed with 20 volumes of PBS. The bound antibodies were eluted with 0.1 M glycine buffer, pH 2.5, and dialyzed overnight against PBS. To test the specificity of the purified anti-\beta\textsubscript{2} by Western blot, the antibody (1 \mu g/ml) was preadsorbed by preincubation with His-\beta\textsubscript{2a} fusion protein (2 \mu g/ml) or His-tagged \beta\textsubscript{1b} (2 \mu g/ml) for 1 h at room temperature before incubation with the membranes. Similarly, to test the specificity of purified anti-\alpha\textsubscript{2}\delta\textsubscript{4}, the antibody (1 \mu g/ml) was preadsorbed by preincubation with His-tagged \alpha\textsubscript{2}\delta\textsubscript{4} (2 \mu g/ml) or \alpha\textsubscript{2}\delta\textsubscript{4} (1 \mu g/ml).

Expression of Ca\textsubscript{v}1.4 \alpha\textsubscript{4} \alpha\textsubscript{2}\beta\textsubscript{2}, \beta\textsubscript{2}, \beta\textsubscript{1b}, \beta\textsubscript{1a}, \alpha\textsubscript{2}\delta\textsubscript{4}, and CaBP4 in HEK293 Cells

HEK293 cells were maintained in DMEM + 10% FBS and 100 units/ml penicillin/streptomycin. For communoprecipitation assays, cells were cotransfected with Ca\textsubscript{v}1.4 \alpha\textsubscript{4}, \beta\textsubscript{2X13}, \alpha\textsubscript{2}\delta\textsubscript{4}, and CaBP4 plasmids using the Ca\textsuperscript{2+}-phosphate method. The transfection medium was replaced 6 h after transfection, and the transfected cells were collected 3 days after transfection. For the analysis of anti-\beta\textsubscript{2} antibody specificity, cells were cotransfected with Ca\textsubscript{v}1.4 \alpha\textsubscript{4}, \alpha\textsubscript{2}\delta\textsubscript{4}, and \beta\textsubscript{2X13} or \beta\textsubscript{1b} (GenBank\textsuperscript{TM} number NM017346), \beta\textsubscript{3} (GenBank\textsuperscript{TM} number NM012838), or \beta\textsubscript{4} (GenBank\textsuperscript{TM} number L02315) (38). For the analysis of anti-\alpha\textsubscript{2}\delta\textsubscript{4} antibody specificity, cells were cotransfected with Ca\textsubscript{v}1.4 \alpha\textsubscript{1}, \beta\textsubscript{2X13}, and \alpha\textsubscript{2}\delta\textsubscript{4}, \alpha\textsubscript{2}\delta\textsubscript{4}, \alpha\textsubscript{2}\delta\textsubscript{4}, or \alpha\textsubscript{2}\delta\textsubscript{4} (plasmids encoding the latter three were a generous gift from Dr. Annette Dolphin (39)).

For electrophysiological experiments, HEK293T cells were grown to 70–80% confluence and cotransfected with human Ca\textsubscript{v}1.4 or Ca\textsubscript{v}1.2 \alpha\textsubscript{1}, \beta\textsubscript{2X13} or \beta\textsubscript{2a}, and \alpha\textsubscript{2}\delta\textsubscript{4} or \alpha\textsubscript{2}\delta\textsubscript{4} cDNAs cloned in pcDNA3.1 and pEGFP cDNA as a transfection marker. Fugene transfection reagent (Promega) was used according to the manufacturer’s protocol. After transfection, cells were maintained at 30 °C. Cells were dissociated 18–24 h.

\textsuperscript{2} The abbreviations used are: qPCR, quantitative PCR; RNA-seq, RNA sequencing; PLA, proximity ligation assay; OPL, outer plexiform layer; ONL, outer nuclear layer; VDL, voltage-dependent inactivation.
Retina Ca\(_{1.4}\) Channels

after transfection and plated at low density for electrophysiological recording of single cells.

**Immunohistochemistry**

C57Bl/6 mouse eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 30 min to 1 h. After fixation, tissues were incubated with increasing concentration of sucrose to 20% sucrose in PB and then embedded in 33% OCT compound (Miles, Elkhart, NY) diluted with 20% sucrose in PB. Eye tissues were cut in 12-μm sections. To block nonspecific labeling, retinal sections were incubated with 3% normal goat serum in PBST buffer (10 mM sodium phosphate, 150 mM NaCl, 0.1% Triton X-100, pH 7.4) for 20 min at room temperature. Sections were incubated overnight at 4 °C in a mix of diluted primary antibodies: mouse anti-CalBP4 (1:100) plus rabbit anti-Ca\(_{1.4}\) (1:1000), rat anti-β\(_2\) (1:25) plus rabbit anti-Ca\(_{1.4}\) (1:1000), or rat anti-α\(_2\)δ\(_2\) (1:25) plus rabbit anti-Cav1.4 (1:1000). A mix of Alexa Fluor 555-conjugated goat anti-mouse IgG or Alexa Fluor 555-conjugated goat anti-rat IgG and Alexa (1:1000). A mix of Alexa Fluor 555-conjugated goat anti-mouse IgG or Alexa Fluor 555-conjugated goat anti-rat IgG and Alexa 488-conjugated goat anti-rabbit IgG was reacted with the sections for 1 h at room temperature. Then the sections were rinsed in PBST and mounted with Prolong antifade reagent (Molecular Probes, Inc., Eugene, OR). Sections were analyzed under a confocal microscope (Zeiss LSM710). Immunofluorescent images were obtained with a Plan-Neofluar ×40/1.3 numerical aperture (Zeiss) objective lens. For immunohistochemistry experiments with rat anti-β\(_2\) and rat anti-α\(_2\)δ\(_2\), antigen retrieval was performed by incubating the sections at 80 °C for 20 min in 10 mM sodium citrate, pH 6.0, 0.05% Tween 20 and washing with PBS before blocking and incubation with the antibodies. Quantification of colocalization was performed using the JACoP plugin for ImageJ (National Institutes of Health), which uses correlation analysis based on Pearson’s coefficient (40). A Pearson’s coefficient near 1 suggests perfect correlation, whereas a number near 0 indicates no correlation.

**Coimmunoprecipitation**

Three days after transfection, whole cell lysates were prepared by incubation of transfected cells at 4 °C for 1 h in 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and inhibitors of proteases (Sigma-Aldrich) with or without 0.1 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\). Lysates were centrifuged at 22,000 × g for 30 min and incubated with mouse IgG or anti-FLAG (5 μg). After a 1-h incubation at 4 °C, 20 μl of protein G-magnetic beads (Invitrogen) were added, and the incubation proceeded for 3 h at 4 °C. After four washes with lysis buffer, proteins were eluted with SDS-sample buffer and analyzed by Western blotting with specific antibodies.

For coimmunoprecipitations from retinas, 12 retinas from WT or Ca\(_{1.4}\) KO mice were lysed in 20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% n-dodecyl-β-D-maltoside and a mix of protease inhibitors (Sigma-Aldrich) at 4 °C for 30 min. Lysates were centrifuged at 10,000 × g for 10 min, and 500 μg of supernatant was incubated with 5 μg of anti-α\(_2\)δ\(_2\) or anti-β\(_2\) at 4 °C. After 1 h of incubation, 30 μl of protein A-Sepharose bead slurry was added to the mix and incubated for 1 h at 4 °C. Beads were washed three times with lysis buffer without detergent, and the proteins were eluted with SDS-sample buffer plus 10 mM DTT before analysis by Western blotting with Ca\(_{1.4}\) antibodies.

**Proximity Ligation Assay**

The proximity ligation assay was performed using the Duolink kit (Sigma-Aldrich). Mouse retina sections were prepared as described above for immunohistochemistry and incubated overnight with rabbit anti-Ca\(_{1.4}\) and mouse anti-CalBP4, rat anti-β\(_2\), or rat anti-α\(_2\)δ\(_2\). For the assay using rat anti-β\(_2\) and rat anti-α\(_2\)δ\(_2\), the sections were then incubated for 1 h at room temperature with mouse anti-rat antibody. Anti-rabbit PLUS probe and anti-mouse MINUS probe were then added to the sections for 1 h at 37 °C. Ligation, amplification, and detection of the probes were carried out according to the manufacturer’s protocol. The sections were mounted with antifade reagent and analyzed under a confocal microscope as described above. To quantify proximity ligation assay (PLA) signals, an arbitrary area of identical size (x = 50 μm × y = 10 μm) was selected in the outer plexiform layer (OPL) of single z plane images as well as in the outer nuclear layer (ONL) to be able to normalize the measurements. Indeed, because there is variability in the background signals from one section to the other, we subtracted the number of PLA signals in the ONL from the number of PLA signals in the OPL. Quantification of PLA spots was performed using the “analyze particles” tools in ImageJ version 1.48.

**Electrophysiological Recordings**

Whole-cell patch clamp recordings of cells transfected with Ca\(_{1.4}\) channel subunits were performed 36–48 h after transfection. Data were acquired with an EPC-9 patch clamp amplifier driven by Pulse software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and analyzed with Igor Pro software (WaveMetrics, Lake Oswego, OR). Extracellular recording solutions contained 140 mM Tris, 1 mM MgCl\(_2\), and 20 mM BaCl\(_2\). Intracellular solutions consisted of 140 mM N-methyl-D-glucamine, 10 mM HEPES, 2 mM MgCl\(_2\), 2 mM Mg-ATP, and 5 mM EGTA. The pH of intracellular and extracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Electrode resistances were typically 1–2 megaohms, and series resistance compensated up to 80%. All averaged data are presented as the mean ± S.E. Statistical significance of differences between groups was determined by Student’s t test as indicated (SigmaPlot, SPSS Science, Chicago, IL.). Normalized tail currents were fit with the Boltzmann equation: \[I/I_0 = \frac{1 + \exp(-(V - V_{1/2})/k)}{b}\] where \(I\) is the maximal current, \(V\) is the test voltage, \(V_{1/2}\) is the voltage of half-activation, \(k\) is the slope factor, and \(b\) is the baseline.

**RESULTS**

**Molecular Identification of Ca\(_{1.4}\) Subunits in Human Retina**—To characterize the molecular constituents of Ca\(_{1.4}\) complexes in the retina, we performed RT-PCR from human retinas using primers specific for Ca\(_{1.4}\) α\(_1\), β\(_2\), and α\(_2\)δ\(_2\). We isolated full-length wild-type Ca\(_{1.4}\) α\(_1\) and α\(_2\)δ\(_2\) cDNAs that were identical to those described previously (GenBank™ accession numbers AF210304 and NM_172364, respectively). The large majority (≥90%; Fig. 1) of PCR products for β\(_2\) corresponded to an alternative splice variant that includes a differ-
ent exon 7. This retinal $\beta_2$ variant includes palmitoylation sites known so far to be specific to $\beta_2a$ and includes exon 7B instead of the exon 7A that is incorporated in $\beta_2a$ (Fig. 1A, $\beta_2a$ GenBank™ number NM_000724; exon numbering according to Buraei et al. (1)). Exon 7B is analogous to the exon 7B included in $\beta_2a$ and $\beta_2c$ mRNAs (1). This new retinal variant has here been named $\beta_2X_{13}$ (Fig. 2). A $\beta_2$ variant with sequence identical to the retinal $\beta_2X_{13}$ was isolated from human jejunum (GenBank™ number AF465485) (41). Although $\beta_2X_{13}$ is expressed at higher levels than $\beta_2a$ in the retina, we were also able to detect the expression of $\beta_2a$ transcripts that include exon 7A (Fig. 1, B and C). By quantitative RT-PCR, the relative
expression of $\beta_{2a}$ and $\beta_{2X13}$ was variable between human retinas, with $\beta_{2X13}$ being 9–77 times more abundant than $\beta_{2a}$ (Fig. 1B and Table 1). $\beta_{2a}$ was undetectable in one human retina (data not shown). By RT-PCR, we also detected $\beta_{2X13}$ expression in mouse retina (Fig. 1C), where its expression was also greater than that of $\beta_{2a}$ (data not shown). $\beta_{2X13}$ was not detected in mouse brain and is thus probably tissue-specific (Fig. 1C). Further evidence for the predominance of $\beta_{2X13}$ in the human retina was obtained in a retinal RNA-seq data set (35), which was specifically evaluated for the inclusion rates of exons 7A, 7B, and 7C. A
TABLE 1

-Fold difference in transcript levels of $\beta_{2X13}$ and $\beta_{2\alpha}$ in human retina

| cDNA | $\beta_{2\alpha} \Delta Ct$ (mean $\beta_{2\alpha} Ct$ - mean GAPDH Ct) | $\beta_{2X13} \Delta Ct$ (mean $\beta_{2X13} Ct$ - mean GAPDH Ct) | $\Delta \Delta Ct$ (mean $\beta_{2X13} \Delta Ct$ - mean $\beta_{2\alpha} \Delta Ct$) | Normalized $\beta_{2X13}$ RNA amount relative to $\beta_{2\alpha}$ RNA or $2^{-\Delta \Delta Ct}$ |
|------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| cDNA1 | 13.36 ± 1.04 | 9.51 ± 1.09 | -3.85 ± 1.09 | 14.45 ± 10.5 |
| cDNA2 | 11.15 ± 0.28 | 9.55 ± 0.55 | -3.15 ± 0.55 | 8.9 ± 3.4 |
| cDNA3 | 11.96 ± 2.00 | 5.69 ± 0.47 | -6.27 ± 0.47 | 77.5 ± 25.1 |

FIGURE 3. Characterization of polyclonal antibodies against $\beta_{2\alpha}$ and $\alpha_2\delta_4$. A, immunolabeling of HEK293 cells cotransfected with $\alpha_{a,1,4}$ $\alpha_2\delta_4$, $\beta_{2X13}$, and $\alpha_2\delta_4$ (left panels) or untransfected cells (right panels) with anti-$\beta_{2\alpha}$ or rat anti-$\alpha_2\delta_4$ B, Western blot of lysates of HEK293 cells untransfected (UC) or cotransfected (TC) with $\alpha_{a,1,4}$ $\alpha_2\delta_4$, and $\alpha_2\delta_4$ or $\alpha_2\delta_4$ (left) or cotransfected with $\alpha_{a,1,4}$ $\alpha_2\delta_4$, and $\beta_{2X13}$, $\beta_{2\alpha}$, or $\beta_{2\alpha}$ (right). Blots were probed with anti-$\alpha_2\delta_4$, or anti-$\beta_{2\alpha}$, or anti-$\beta_{2\alpha}$, respectively. C, Western blot of lysates of HEK293 cells untransfected (UC) or cotransfected (TC) with $\alpha_{a,1,4}$ $\alpha_2\delta_4$, $\beta_{2X13}$, and $\alpha_2\delta_4$ and probed with anti-$\beta_{2\alpha}$, anti-$\beta_{2\alpha}$ preadsorbed with specific $\beta_{2X13}$, or nonspecific $\beta_{2\alpha}$, (left), or anti-$\alpha_2\delta_4$, or anti-$\alpha_2\delta_4$ preadsorbed with specific $\alpha_2\delta_4$ or nonspecific $\alpha_2\delta_4$ (right). Immunoreactivity was blocked by preadsorption with specific antigen only.

$\beta_{2\alpha}$ variant including the 7B exon and concomitantly excluding the 7A and 7C exons was observed to be the dominant retinal isoform in both the macular and peripheral retina (Fig. 1D). In general, exon 7B was found in greater than 95% of sampled transcripts. The mouse $\beta_{2X13}$ sequence was submitted to GenBank™ and assigned accession number KJ789960 (Fig. 2).
Retina Ca\textsubscript{1.4} Channels

Co-localization of Ca\textsubscript{1.4} α\textsubscript{1} with β\textsubscript{2} or α\textsubscript{2}δ\textsubscript{4} at the photoreceptor synaptic ribbon—We next tested whether Ca\textsubscript{1.4} α\textsubscript{1} colocalized with β\textsubscript{2} and α\textsubscript{2}δ\textsubscript{4} in the retina using rabbit polyclonal anti-Ca\textsubscript{1.4} α\textsubscript{1} subunit antibodies that we characterized previously (13). For double labeling, we generated rat polyclonal antibodies against the bodies that we characterized previously (13). For double label-WB analysis, the anti-

FIGURE 4. Colocalization of Ca\textsubscript{1.4} α\textsubscript{1} with β\textsubscript{2} or α\textsubscript{2}δ\textsubscript{4} at the photoreceptor synaptic ribbon. Mouse retina sections were double-labeled with anti-Ca\textsubscript{1.4} α\textsubscript{1} (green) and anti-α\textsubscript{2}δ\textsubscript{4} (red) (A) or anti-β\textsubscript{2} (red) (B). The overlay is shown in the right panel together with Hoechst staining. Higher magnification images of the OPL are shown in the bottom panels. Ca\textsubscript{1.4} shows a high degree of colocalization with β\textsubscript{2} and α\textsubscript{2}δ\textsubscript{4} as indicated by the calculated Pearson's correlation coefficient between the Alexa 488-stained Ca\textsubscript{1.4} and the Alexa 555-stained β\textsubscript{2} (r = 0.710) or α\textsubscript{2}δ\textsubscript{4} (r = 0.608). OS, outer segment; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

In lysates of cells transfected with Cav\textsubscript{1.4} \textsubscript{2X13, but no signal was observed in cells transfected with Cav\textsubscript{1.4} \textsubscript{2X13, and Cav\textsubscript{1.4} \textsubscript{2X13} (Fig. 4). In addition, preadsorption of anti-β\textsubscript{2} with β\textsubscript{2a} but not β\textsubscript{1b} abolished detection of β\textsubscript{2a} (Fig. 3C, left). As well, α\textsubscript{2}δ\textsubscript{4} detection was blocked by preadsorption of anti-α\textsubscript{2}δ\textsubscript{4} with α\textsubscript{2}δ\textsubscript{4} but not α\textsubscript{2}δ\textsubscript{1} (Fig. 3C, right). These results validate the specificity of these antibodies for detection of β\textsubscript{2X13} and α\textsubscript{2}δ\textsubscript{4} by Western blot or immunohistochemistry.

Using these antibodies for double-labeling of mouse retina, we observed selective labeling of the outer plexiform layer containing the photoreceptor terminals; no labeling was observed in other retinal layers (Fig. 4). High magnification images showed anti-Ca\textsubscript{1.4} α\textsubscript{1} labeling of elongated and horseshoe-shaped structures corresponding to the photoreceptor synaptic ribbon that arches around the postsynaptic terminals, as described previously (13, 33). The anti-β\textsubscript{2} or anti-α\textsubscript{2}δ\textsubscript{4} signals colocalized at these structures with anti-Ca\textsubscript{1.4} α\textsubscript{1} staining, as indicated by a Pearson's correlation coefficient of ≥0.710 or
Western blotting with the corresponding antibodies revealed left panels Cav1.4 A control mouse IgG was used instead of FLAG antibodies (Fig. 5, No immunoprecipitated proteins were detected either when CaBP4 with or without (no Cav1.4) FLAG-Cav1.4 both with and without Ca2+/H9252 show input lysates. Coimmunoprecipitations were performed with control mouse IgG or anti-FLAG antibody using lysates of HEK293 cells and mouse retina. Isolation of Cav1.4 and associated proteins. In these experiments, we also tested for the co-immunoprecipitation of CaBP4, a known near photoreceptor synaptic ribbons. Cav1.4 α1, β2, α3δ, δ4 Subunits Interact in HEK293 Cells and in Mouse Retina—We next tested whether Cav1.4 α1 associates with β2X13 δ3 and α3δ in transfected HEK293 cells. The Cav1.4 α1 subunit was tagged with a FLAG epitope, and anti-FLAG antibodies were used to immunoprecipitate FLAG-Cav1.4 and associated proteins. In these experiments, we also tested for the co-immunoprecipitation of CaBP4, a known Cav1.4-interacting protein (28, 42). Because the association of CaBP4 with Cav1.4 can be affected by Ca2+, coimmunoprecipitation experiments were done in the presence and absence of Ca2+. Due to the limited sensitivity of FLAG antibodies, FLAG-Cav1.4 α1 was not detected in cell lysates by Western blot (Fig. 5, left panels). However, FLAG-Cav1.4 α1 was visualized after enrichment using immunoprecipitation (Fig. 5, right panels). Western blotting with the corresponding antibodies revealed the co-immunoprecipitation of β2, α3δ4, and CaBP4 by FLAG-Cav1.4 both with and without Ca2+ (Fig. 5, A and B). This result was not reproduced in cells transfected without FLAG-Cav1.4, which argued against nonspecific immunoprecipitation of β2, α3δ4, and CaBP4 by FLAG antibodies (Fig. 5A). No immunoprecipitated proteins were detected either when control mouse IgG was used instead of FLAG antibodies (Fig. 5, A and B). We also tested whether Cav1.4 α1 associates with β2 and α3δ in mouse retina using immunoprecipitation with anti-β2 and anti-α3δ, Cav1.4 α1 coimmunoprecipitated with β2 and α3δ (Fig. 5C).

To test whether Cav1.4 subunits interact in photoreceptor synapses, we performed in situ PLAs, a technique that allows visualization of protein interactions in fixed tissue. With this method, antibodies against two potentially interacting proteins are coupled to oligonucleotides. If the two proteins interact, the antibodies that recognize these proteins in fixed tissue should be in close proximity (<40 nm) such that their coupled oligonucleotides can be ligated and further visualized with fluorescent probes. As proof of principle, we first tested the interaction of Cav1.4 α1 and CaBP4, which is known to colocalize with Cav1.4 at the photoreceptor terminals in the OPL (Fig. 6A) (28). PLA performed with anti-CaBP4 and anti-Cav1.4 α1 produced strong signals in the OPL, where both proteins are colocalized (Fig. 6, A and B). These signals were specific for the presence of CaBP4 because they were strongly reduced in the retina of mice lacking CaBP4 (CaBP4 KO; Fig. 6C). We have shown previously that immunolabeling for Cav1.4 channels is still present in the OPL of CaBP4 KO retina (13), so the absence of PLA signal is probably due to the lack of CaBP4/Cav1.4 interactions. A few fluorescent puncta were observed in the outer and inner nuclear layers of WT retina, but these were probably nonspecific because they were also seen in CaBP4 KO retina (Fig. 6C). With anti-Cav1.4 α1 and either anti-β2 or anti-α3δ4 antibodies, PLA generated strong signals in the OPL of WT but not Cav1.4 KO mice (Fig. 6, D and K). Quantification showed significantly greater density of PLA signals in WT compared with Cav1.4 KO retina using antibodies for Cav1.4-CaBP4, Cav1.4-β2, or Cav1.4-α3δ4 complexes (Fig. 6, D, H, and L; p < 0.0001). Additional negative controls for PLA were done by omission of one or both antibodies (Fig. 6, M–P) or by preadsorption of anti-β2 antibody with β2 or anti-α3δ4 with α3δ4 (Fig. 6, Q and R). No PLA signals were observed under these conditions. Taken together, our results confirm that Cav1.4 channel complexes at the photoreceptor synapse are composed of Cav1.4 α1, β2, and α3δ4 and CaBP4.

Electrophysiological Analysis of Cav1.4 Channels in HEK293T Cells—Despite the molecular characterization of α3δ1 as a Ca2+ subunit (43), how α3δ4 affects the biophysical properties of Ca2+ channels, particularly Cav1.4, is unknown; previous electrophysiological studies of Cav1.4 utilized the α2δ1 subunit (27, 30–32, 44). To better understand the functional properties of the native photoreceptor Cav1.4 channel complex, we performed whole-cell patch clamp recordings of Cav1.4 channels containing α2δ1 in transfected HEK293T cells. For comparison, we also carried out recordings of Cav1.4 with the α3δ1 subunit. Based on our findings that β2X13 is more highly expressed in the retina than β2a (Fig. 1), we used β2X13 in these experiments. Ba2+ was used as the charge carrier because the greater permeation of Ba2+ compared with Ca2+ increases the resolution of Cav1.4 currents, which are significantly smaller compared with those mediated by other Ca2+ channels in HEK293T cells (31, 32). The properties of Ba2+ currents are largely similar to Ca2+ currents because Cav1.4 channels containing the distal C-terminal domain inhibitory module exhibit little Ca2+-dependent inactivation (31, 32, 44, 45).
Retina Ca\textsubscript{v}1.4 Channels

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Immunohistochemistry (IHC) and proximity ligation assay (PLA) images showing different channels in wild-type (WT) and knockout (KO) mice.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Channel & WT & KO & P-value \\
\hline
Ca\textsubscript{v}1.4/CaBP4 & 60 & 70 & *** \\
Ca\textsubscript{v}1.4/\beta\textsubscript{2} & 40 & 50 & *** \\
Ca\textsubscript{v}1.4/\alpha\textsubscript{2}\delta\textsubscript{4} & 20 & 30 & *** \\
\end{tabular}
\caption{Comparison of PLA spots per 500 \(\mu\text{m}^2\) in WT and KO mice.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Proximity ligation assay (PLA) quantification of different channels in WT and KO mice.}
\end{figure}
In cells cotransfected with Cav1.4, α1·β_{2X13}·α2δ, Ba\(^{2+}\) currents were generally smaller than in cells expressing Cav1.4·β_{2X13}·α2δ, although this difference did not reach statistical significance in plots of current density against test voltage (Fig. 7, A and B). However, analyses of normalized tail current-voltage curves revealed that Cav1.4·β_{2X13}·α2δ channels exhibited weaker voltage dependence of activation compared with channels containing Cav1.4·α1·β_{2X13}·α2δ (Fig. 7C). Boltzmann fits indicated more positive half-maximal activation voltage (V_{\text{h}}) and greater slope (k) for channels containing α2δ compared with those with α2δ (Table 2). In these experiments, tail currents were normalized to those measured upon repolarization from a +80-mV step, at which channel open probability should be maximal. Interestingly, the normalized tail current amplitude exceeded 1 between 0 and +30 mV with α2δ, but not with α2δ (Fig. 7C), similar to that due to Ca\(^{2+}\)-dependent facilitation of Cav2.1 channels (46). Because we used Ba\(^{2+}\) as the charge carrier, this “overshoot” in the normalized tail current...
voltage is probably independent of Ca\(^{2+}\) and may result from a current-dependent mechanism, because facilitation of the tail current was maximal at voltages evoking the greatest inward currents (Fig. 7, B and C). With respect to voltage-dependent inactivation (VDI) during a sustained depolarization, Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) and Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) exhibited similar properties (Fig. 7D). Inactivation was measured as the ratio of the residual current amplitude at the end of the pulse and the peak current amplitude (\(I_{res}/I_{peak}\)). Because Cav1.4 channels inactivate with a very slow time course, inactivation was measured during relatively long (5-s) step depolarizations. There was no significant difference in \(I_{res}/I_{peak}\) for Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) (0.7 ± 0.03, n = 5) and for Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) (0.7 ± 0.1, n = 6; \(p = 0.59\); Fig. 7D). Time constants for VDI were also not different (9.9 ± 1.0 s for Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) versus 9.5 ± 2.1 s for Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\), \(p = 0.88\); Fig. 7D). These results demonstrate that for Cav1.4 channels, \(\alpha_\delta_4\) and \(\alpha_\delta_4\) differ primarily in their effects on voltage-dependent activation.

We next compared the impact of \(\beta_{2a}\) and \(\beta_{2X13}\) on Cav1.4 function. Compared with other Cav subunits, \(\beta_{2a}\) significantly slows VDI of Cav channels (47). Structure/function analyses indicate that this property of \(\beta_{2a}\) is mediated by the HOOK domain (48, 49). Because \(\beta_{2X13}\) and \(\beta_{2a}\) differ in the HOOK domain (Fig. 1A), we predicted that these splice variants may have distinct effects on VDI of Cav1.4. Consistent with previous findings that the HOOK domain did not affect voltage-dependent activation (48), there were no differences in \(V_h\) or \(k\) obtained from normalized tail current-voltage curves in cells transfected with Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) or Cav1.4 \(\alpha_1 - \beta_{2a} - \alpha_\delta_4\) (Fig. 8, A and B, and Table 2). However, VDI during a 5- and 10-s depolarizing pulse was significantly greater for Cav1.4 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) than for Cav1.4 \(\alpha_1 - \beta_{2a} - \alpha_\delta_4\), although the time constants for VDI were not different (see legend to Fig. 8D for values). These results show that the \(\beta_{2X13}\) subunit influences the amount but not the rate of VDI of Cav1.4 channels.

To determine whether \(\beta_{2X13}\) had similar effects on other Cav channels, we compared properties of Ba\(^{2+}\) currents in cells transfected with Cav1.2 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) and those transfected with Cav1.2 \(\alpha_1 - \beta_{2a} - \alpha_\delta_4\). In these experiments, \(\alpha_\delta_4\) was used because native Cav1.2 channels in the brain are not likely to associate with \(\alpha_\delta_4\) based on the near absence of \(\alpha_\delta_4\) in mouse brain (50). Ba\(^{2+}\) current density was generally lower for Cav1.2 \(\alpha_1 - \beta_{2X13} - \alpha_\delta_4\) than for Cav1.2 \(\alpha_1 - \beta_{2a} - \alpha_\delta_4\) but not significantly different. Unlike its lack of effect on Cav1.4 activation, \(\beta_{2X13}\) caused a small but significant (∼+5 mV) shift in \(V_h\) obtained from normalized tail current curves compared with \(\beta_{2a}\) for Cav1.2 channels (Fig. 9, A–C, and Table 2). However, unlike for Cav1.4, \(\beta_{2X13}\) and \(\beta_{2a}\) had similar effects on VDI (Fig. 9D). Therefore, \(\beta_{2X13}\) and \(\beta_{2a}\) differentially modulate the functional properties of Cav1.2 and Cav1.4.

### DISCUSSION

In this paper, we extend previous work indicating the importance of the auxiliary Cav subunits, \(\beta_2\) and \(\alpha_\delta_4\), in the retina. First, we provide the first evidence that Cav1.4 \(\alpha_1\), \(\beta_2\), and \(\alpha_\delta_4\) are physically and functionally interrelated both in transfected HEK293T cells and in photoreceptor synaptic terminals in the retina. Second, we identify the \(\beta_{2X13}\) splice variant, which is distinct from the \(\beta_{2a}\) subunit expressed in the brain, as the major \(\beta_2\) subunit in human retina. Finally, we show that both \(\beta_{2X13}\) and \(\alpha_\delta_4\) have distinct effects on the biophysical properties of Cav1.4 compared with \(\beta_{2a}\) and \(\alpha_\delta_4\). Our results reveal unexpected differences in the modulatory capabilities of Cav1.4 and \(\alpha_\delta_4\) subunits, which may influence the properties of native Cav1.4 channels in photoreceptors.

### Role of \(\beta_2\) and \(\alpha_\delta_4\) as Auxiliary Subunits of Cav1.4 in the Retina

The notion that \(\beta_2\) and \(\alpha_\delta_4\) associate with Cav1.4 channels in the retina is supported by immunochromatic and genetic evidence. First, antibodies against \(\beta_2\) and \(\alpha_\delta_4\) label the OPL of the retina, where photoreceptor synapses are localized (51–53). In contrast, antibodies against \(\beta_{2a}\) label other regions in the retina but not the OPL (51). Second, mice lacking expression of \(\beta_2\) but not mice lacking \(\beta_{2a}\) or \(\alpha_\delta_4\) exhibited strongly reduced b-waves in electroretinograms (25).

The b-wave indicates efficiency of transmission from photoreceptors to bipolar neurons and is highly dependent on proper function of Cav1.4. The b-wave is absent in Cav1.4 KO mice (15) and in mice expressing a mutation in the \(\alpha_\delta_4\) gene, Cacna2d4, in which a truncated \(\alpha_\delta_4\) protein is expressed at very low levels compared with the full-length protein in wild-type mice (26). In the \(\alpha_\delta_4\) mutant mice, there is also delayed degeneration of cones, and human mutations in CACNA2D4 are associated with slowly progressive cone dystrophy (54). Our findings that \(\beta_2\) and \(\alpha_\delta_4\) colocalize with Cav1.4 \(\alpha_1\) at structures resembling photoreceptor synaptic ribbons (Fig. 4) and interact with Cav1.4 \(\alpha_1\) in transfected cells (Fig. 5) and in the OPL (Fig. 6) verify that these auxiliary subunits are primary components of Cav1.4 channel complexes at the photoreceptor synapse.

### Molecular and Functional Characterization of a New Retinal Ca\(^{2+}\) Subunit—\(\beta_{2X13}\) Splice Variant

Cav1.4 \(\beta_{2X13}\) subunits regulate multiple aspects of Cav1.4 channel function, including their gating properties and levels at the cell surface (reviewed in Refs. 1 and 55). Of the four classes of Cav, \(\beta_2\) that have been characterized, \(\beta_2\) is subject to the most significant alternative splicing, with 13 variants thus far identified (1, 41). Despite the evidence in favor of \(\beta_2\) as a Cav1.4 subunit, there has been no characterization of Cav1.4 \(\beta_2\) variants expressed in human retina. In our RT-PCR analyses of human retina, we detected the \(\beta_{2a}\) variant that is present in the brain (56) as well as a \(\beta_2\) splice variant, \(\beta_{2X13}\), which has not

### TABLE 2

Parameters for voltage-dependent activation

| Cav1.4  | \(V_h\) (mV) | \(k\) | \(p\)-value |
|--------|-------------|------|-------------|
| \(+\beta_{2a}; \alpha_\delta_4\) | -16.4 ± 2.0 | 5.3 ± 0.6 | 0.03 |
| \(+\beta_{2X13}; \alpha_\delta_4\) | -9.8 ± 1.9 | 8.2 ± 0.5 | 0.01 |

| Cav1.4  | \(V_h\) (mV) | \(k\) | \(p\)-value |
|--------|-------------|------|-------------|
| \(+\beta_{2a}; \alpha_\delta_4\) | -10.0 ± 2.3 | 8.6 ± 0.9 | 0.97 |
| \(+\beta_{2X13}; \alpha_\delta_4\) | -9.8 ± 1.0 | 8.2 ± 0.7 | 0.03 |

| Cav1.4  | \(V_h\) (mV) | \(k\) | \(p\)-value |
|--------|-------------|------|-------------|
| \(+\beta_{2a}; \alpha_\delta_4\) | -14.8 ± 1.0 | 6.0 ± 0.4 | 0.59 |
| \(+\beta_{2X13}; \alpha_\delta_4\) | -9.6 ± 1.8 | 6.5 ± 0.6 | 0.03 |

Normalized tail current-voltage curves in Figs. 7C, 8C, and 9C were fitted with a single Boltzmann function. The \(p\)-values were determined by student’s t-test. \(V_h\), half-maximal activation voltage; \(k\), slope factor.
been previously reported in the retina. This new $\beta_{2X13}$ variant has the unique combination of containing the two N-terminal cysteines shown to be palmitoylated in $\beta_{2a}$ (57) and containing a shorter HOOK domain partially encoded by exon 7B (Fig. 1A).

Whereas $\beta_{2X13}$ was consistently detected at higher levels by qPCR than $\beta_{2a}$ in RNA isolated from three different individuals, there was large variability in the ratio between $\beta_{2X13}$ and $\beta_{2a}$ (Fig. 1B and Table 1). Previous analyses indicate that $\beta_{2}$ splice variant expression can vary significantly during development (58). Because we did not have information regarding the age of the individuals from which the samples were isolated, it is possible that our results reflect age-related differences in $\beta_{2X13}$ and $\beta_{2a}$ levels. We detected $\beta_{2X13}$ in both mouse and human retina but not in mouse brain (Fig. 1C), which further supports the physiological importance of $\beta_{2X13}$ as a partner for photoreceptor $\alpha_{1.4}$ channels. In addition, RNA-seq analysis of the retinal $\beta_{2}$ transcripts showed that $\beta_{2X13}$ is the major transcript in the cone-rich macula and in the peripheral retina, indicating that $\beta_{2X13}$ is the main isoform in both rods and cones.

The inclusion of exon 7B in $\beta_{2X13}$ creates a shorter HOOK domain (7 amino acids) compared with the exon 7A-containing $\beta_{2a}$ (45 amino acids; Fig. 1A). The HOOK domains of $\beta_{1b}$, $\beta_{1c}$, $\beta_{3}$, and $\beta_{4}$ are the same length as (and contain the same sequence [AKQKQK] that is present in) the $\beta_{2X13}$ exon 7B (1).

Transfer of the HOOK domain from $\beta_{1b}$ to $\beta_{2a}$ causes strong VDI of Cav2.1 channels, typical of $\beta_{1b}$ (48). Moreover, deletion of the HOOK domain from $\beta_{2a}$ enhances VDI of Cav2.2 channels (49). Consistent with these findings, we found that, compared with $\beta_{2a}$, $\beta_{2X13}$ causes stronger VDI of Cav1.4 channels (Fig. 8D). Curiously, this was not the case for Cav1.2 (Fig. 9D). The effect of $\beta_{2a}$ in slowing VDI of Cav1.2 is less profound than observed for other Cav channels (59), so it may be that Cav1.2 VDI is somehow more resistant to modulation by $\beta_{2a}$ subunits than Cav1.4, such that the distinct effects of $\beta_{2a}$ and $\beta_{2X13}$ on VDI are more readily resolved in Cav1.4 than Cav1.2.

The distinct exon 7B present in $\beta_{2X13}$ is orthologous to exon 9 of the zebrafish $\beta_{2.1}$ gene, which is found in a number of $\beta_{2.1}$ variants that are expressed at distinct developmental ages and in different tissues, including the eye (60). Whereas $\beta_{2X13}$ includes the two palmitoylated N-terminal cysteines present in $\beta_{2a}$ (57), these $\beta_{2.1}$ variants do not. Although it is tempting to
speculate on the physiological significance of $\beta_{2X13}$-containing Ca$_{\alpha,1.4}$ channels, it is important to note that VDI is still relatively limited for these channels, with $\sim$35% of the current remaining after 10 s of depolarization (Fig. 9D). Moreover, maximal depolarization of photoreceptors is likely not to exceed $\sim-40$ mV, which should not induce significant VDI of Ca$_{\alpha,1.4}$ channels. Therefore, whether the difference in VDI due to $\beta_{2X13}$ and $\beta_{2a}$ is physiologically relevant for photoreceptor synaptic transmission is debatable. An alternate possibility is that $\beta_{2X13}$ may affect modulation of Ca$_{\alpha,1.4}$ by other factors. For example, Ca$_{\alpha,1.3}$ channels containing $\beta_{2a}$ but not other $\beta$ subunits are relatively resistant to inhibition by arachidonic acid (61). Because synaptic Ca$^{2+}$ currents in salamander photoreceptors are strongly inhibited by arachidonic acid (62), it will be of interest to determine whether $\beta_{2X13}$ alters the sensitivity of Ca$_{\alpha,1.4}$ channels to this arachidonic acid or other neuromodulators.

**Functional Characterization of Ca$_{\alpha,1.4}$ Channels Containing the $\alpha_2\delta_3$ Subunit—**Ca$_{\alpha,\delta}$ subunits are composed of $\alpha_2$ and $\delta$ proteins that are encoded by a single gene; proteolytic cleavage of the $\alpha_2\delta$ preprotein separates $\alpha_2$ and $\delta$ proteins, which remain linked under native conditions by disulfide bonds (reviewed in Ref. 3). Ca$_{\alpha,\delta}$ subunits enhance the cell surface trafficking and decrease the turnover of Ca$_{\alpha,1}$ and Ca$_{\alpha,2}$ channels (63, 64). These effects are due in part to a von Willebrand factor A (VFA) domain and in particular a metal ion adhesion motif (MIDAS), the mutation of which prevents the cell surface trafficking function of $\beta_{2a}$ on Ca$_{\alpha,2.1}$ currents (64). In addition, glycosylation of the extracellular domain is important for the current-enhancing effects of $\alpha_2\delta_3$ (65). $\alpha_2\delta_3$ contains fewer glycosylation sites compared with the three other $\alpha_2\delta$ subunits (43), which may explain why Ca$_{\alpha,1}$ current densities were generally smaller with $\alpha_2\delta_3$ than with $\alpha_2\delta$ (Fig. 7, A and B). Notably, $\alpha_2\delta_3$ does not have the asparagine corresponding to residue
184, which, when mutated to glutamine, inhibits the enhancement of Ca,2.2 channels caused by α,δ1 (66).

Ca, α,δ subunits have been shown to have variable effects on voltage-dependent activation of Ca, channels, which may depend on the particular Ca, α subunit with which they associate (reviewed in Ref. 3). For Ca,1.2 channels, α,δ1 causes a negative shift in the voltage dependence of activation, which depends largely on the δ-encoding sequence (67). Our findings that Ca,1.4 channels containing α,δ subunits exhibit weaker voltage dependence of activation than with α,δ1 may result from relatively weak conservation (~28% sequence identity) in the δ-encoding sequence for α,δ2 and α,δ3. A further distinction between α,δ2 and α,δ3 was in the facilitation of currents evoked by moderate voltages that was unique to α,δ1-containing Ca,1.4 channels (Fig. 7C). Although further analyses are required, this facilitation may result from the binding of permeant Ba2+ ions in the channel protein, similar to what has been proposed for ion-dependent inactivation of Ba2+ currents through Ca,1.2 channels (68). The absence of this facilitation, in addition to relatively weak voltage dependence of activation, in α,δ2-containing Ca,1.4 channels might be considered maladaptive in terms of supporting synaptic Ca2+ signals that drive photoreceptor transmission. However, native Ca,1.4 channels would also be associated with CaBP4, which significantly enhances voltage-dependent activation of Ca,1.4 (28, 42). Thus, native α,δ2-containing Ca,1.4 channels in photoreceptors may be more strongly modulated by CaBP4 than α,δ1-containing Ca,1.4 channels. α,δ subunits also play roles in regulating neurotransmitter release probability (69) as well as synaptic plasticity (70, 71), independent of effects on Ca, function. Understanding the precise roles of α,δ1 in regulating photoreceptor signaling and how its dysregulation leads to vision impairment in humans and mice (26, 54) remains an important challenge for future studies.

Acknowledgments—We thank Dr. Annette Dolfin for the kind gift of plasmids encoding α,δ1, α,δ2, and α,δ3 subunits, Dr. Sharona Gordon and the members of her group for helpful discussions, and the Lions Eye Bank of Oregon for providing human retinas.

REFERENCES
1. Burai, Z., and Yang, J. (2010) The β subunit of voltage-gated Ca2+ channels. Physiol. Rev. 90, 1461–1506
2. Dolphin, A. C. (2012) Calcium channel auxiliary α2β and β subunits: trafficking and one step beyond. Nat. Rev. Neurosci. 13, 542–555
3. Dolphin, A. C. (2013) The α2β subunits of voltage-gated calcium channels. Biochim. Biophys. Acta 1828, 1541–1549
4. Simms, B. A., and Zamponi, G. W. (2014) Neuronal voltage-gated calcium channels: structure, function, and dysfunction. Neuron 82, 24–45
5. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu. Rev. Cell Dev. Biol. 16, 521–555
6. Catterall, W. A., Perez-Reyes, E., Snutch, T. P., and Striessnig, I. (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol. Rev. 57, 411–425
7. lipscombe, D., Andrade, A., and Allen, S. E. (2013) Alternative splicing: functional diversity among voltage-gated calcium channels and behavioral consequences. Biochim. Biophys. Acta 1828, 1522–1529
8. Matthews, G., and Fuchs, P. (2010) The diverse roles of ribbon synapses in sensory neurotransmission. Nat. Rev. Neurosci. 11, 812–822
9. Krizaj, D., and Copenhagan, D. R. (2002) Calcium regulation in photoreceptors. Front. Biosci. 7, d2023–d2044
10. Barnes, S., and Kelly, M. E. M. (2002) Calcium channels at the photoreceptor synapse. Adv. Exp. Med. Biol. 514, 465–476
11. Berntson, A., Taylor, W. R., and Morgans, C. W. (2003) Molecular identity, synaptic localization, and physiology of calcium channels in retinal bipolar cells. J. Neurosci. Res. 71, 146–151
12. Morgans, C. W. (2001) Localization of the α1F calcium channel subunit in the rat retina. Invest. Ophthalmol. Vis. Sci. 42, 2414–2418
13. Liu, X., Kerov, V., Haeseeler, F., Majumder, A., Artemyev, N., Baker, S. A., and Lee, A. (2013) Dysregulation of Ca(V)1.4 channels disrupts the maturation of photoreceptor synaptic ribbons in congenital stationary night blindness type 2. Channels 7, 514–523
14. Knoflach, D., Kerov, V., Sartori, S. B., Obermair, G. J., Schmuckermair, C., Liu, X., Sothillingam, V., Garrido, M. G., Baker, S. A., Glössmann, M., Schicker, K., Seeliger, M., Lee, A., and Koschak, A. (2013) Cav1.4 IT mouse as model for vision impairment in human congenital stationary night blindness type 2. Channels 7, 505–513
15. Mansergh, F., Orton, N. C., Vessey, J. P., Lalonde, M. R., Stell, W. K., Tremblay, F., Barnes, S., Rancourt, D. E., and Bech-Hansen, N. T. (2005) Mutation of the calcium channel gene Cacna1f disrupts calcium signaling, synaptic transmission and cellular organization in mouse retina. Hum. Mol. Genet. 14, 3035–3046
16. Zabouri, N., and Haverkamp, S. (2013) Calcium channel-dependent molecular maturation of photoreceptor synapses. Proc. Natl. Acad. Sci. U. S. A. 110, e6385–e6390
17. Strom, T. M., Nyakatura, G., Apfelstedt-Sylla, E., Hellebrand, H., Lorenz, B., Weber, B. H. F., Wutz, K., Gutwillinger, N., Rüther, K., Drescher, B., Sauer, C., Zrenner, E., Meitinger, T., Rosenthal, A., and Meindl, A. (1998) An L-type calcium-channel gene mutated in incomplete X-linked congenital stationary night blindness. Nat. Genet. 19, 260–263
18. Wutz, K., Sauer, C., Zrenner, E., Lorenz, B., Alitalo, T., Broghammer, M., Hergersberg, M., de la Chapelle, A., Weber, B. H. F., Wissinger, B., Meindl, A., and Pusch, C. M. (2002) Thirty distinct CACNA1F mutations in 33 families with incomplete type of XLCSNB and Cacna1f expression profiling in mouse retina. Eur. J. Hum. Genet. 10, 449–456
19. Bech-Hansen, N. T., Taylor, W. R., and Morgans, C. (1998) Localization and properties of voltage-gated calcium channels in cone photoreceptors of Tupaia belangeri. Vis. Neurosci. 15, 541–552
20. Morgans, C. W. (1999) Calcium channel heterogeneity among cone photoreceptors in the tree shrew retina. Eur. J. Neurosci. 11, 2989–2993
21. Ko, M. L., Liu, Y., Dryer, S. E., and Ko, G. Y. P. (2007) The expression of L-type voltage-gated calcium channels in retinal photoreceptors is under circadian control. J. Neurochem. 103, 784–792
22. Busquet, P., Nguyen, N. K., Schmid, E., Tanimoto, N., Seeliger, M., Ben-Yosef, T., Mizoño, F., Akopian, A., Striessnig, I., and Singewald, N. (2010) CaV1.3 L-type Ca2+ channels modulate depression-like behaviour in mice independent of deaf phenotype. Int. J. Neuroopharmacol. 13, 499–513
23. Ball, S. L., Powers, P. A., Shin, H. S., Morgans, C. W., Peachey, N. S., and Gregg, R. G. (2002) Role of the β2 subunit of voltage-dependent calcium channels in the rat outer plexiform layer. Invest. Ophthalmol. Vis. Sci. 43, 1595–1603
24. Wycisk, K. A., Budde, B., Feil, S., Skoysryk, S., Buzzi, F., Neidhardt, J., Glaus, E., Nürnberg, P., Ruether, K., and Berger, W. (2006) Structural and functional abnormalities of retinal ribbon synapses due to Cacna2d4 mutation. Invest. Ophthalmol. Vis. Sci. 47, 3523–3530
25. Doering, C. J., Hamid, J., Simms, B., McRory, J. E., and Zamponi, G. W. (2005) Cav1.4 encodes a calcium channel with low open probability and
Retina Cav1.4 Channels

unitary conductance. *Biophys. J.** **89**, 3042–3048
28. Haeseeler, F., Imanishi, Y., Maeda, T., Possin, D. E., Maeda, A., Lee, A., Rieke, F., and Palczewski, K. (2004) Essential role of CaV2.1-binding protein 4, a Ca(v)1.4 channel regulator, in photoreceptor synaptic function. *Nat. Neurosci.* 7, 1079–1087
29. Koschak, A., Reimer, D., Walter, D., Hoda, J. C., Heinzel, T., Grabner, M., and Striessnig, J. (2003) Ca(v)1.4 α1 subunits can form slowly inactivating dihydropyridine-sensitive L-type Ca2+-channels lacking Ca2+-dependent inactivation. *J. Neurosci.* 23, 6041–6049
30. Peloquin, J. B., Doering, C. J., Rehak, R., and McRory, J. E. (2008) Temperature dependence of Ca-v 1.4 channel gating. *Neuroscience* 151, 1066–1083
31. Baumann, L., Gerstner, A., Zong, X., Biel, M., and Wahl-Schott, C. (2004) Functional Characterization of the L-type Ca2+- channel Cav1.4α1 from mouse retina. *Invest. Ophthal. Vis. Sci.* 45, 708–713
32. McRory, J. E., Hamid, J., Doering, C. J., Garcia, E., Parker, R., Hamming, K., Chen, L., Hildebrand, M., Beedle, A. M., Feldcamp, L., Zamponi, G. W., and Snutch, T. P. (2004) The CACNA1F gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution. *J. Neurosci.* 24, 1707–1718
33. Specht, D., Wu, S. B., Turner, P., Dearden, P., Koentgen, F., Wolfurum, U., Maw, M., Brandstätter, J. H., and tom Dieck, S. (2009) Effects of presynaptic mutations on a postsynaptic Cacna1s calcium channel colocalized with mGlur6 at mouse photoreceptor ribbon synapses. *Invest. Ophthal. Vis. Sci.* 50, 505–515
34. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔC(T)) method. *Methods* 25, 402–408
35. Whitmore, S. S., Wagner, A. H., DeLuca, A. P., Drack, A. V., Stone, E. M., Tucker, B. A., Zeng, S., Braun, T. A., Mullins, R. F., and Schetz, T. E. (2014) Transcriptomic analysis across nasal, temporal, and macular regions of human neural retina and RPE/choroid by RNA-Seq. *Exp. Eye Res.* 129C, 93–126
36. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7, 562–578
37. Thorvalsdottir, H., Robinson, J. T., and Mesirov, J. P. (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Bioinformatics* 14, 178–192
38. Cui, G., Meyer, A. C., Imanishi, Y., Maeda, A., Lee, A., Rieke, F., and Palczewski, K. (2004) Molecular cloning and characterization of the human voltage-gated calcium channel CACNB2 subunit in current stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1654–1659
39. Bolte, S., and Cordelieres, F. P. (2006) A guided tour into subcellular co-localization analysis in light microscopy. *J. Microsc.* 224, 213–232
40. Wycisk, K. A., Zeitz, C., Feil, S., Wittmer, M., Forster, U., Neidhardt, J., Wissinger, B., Zrenner, E., Wilke, R., Kohl, S., and Berger, W. (2006) Mutation in the auxiliary calcium-channel subunit CACNA2D4 causes autosomal recessive cone dystrophy. *Am. J. Hum. Genet.* 79, 973–977
41. Chaudhuri, D., Issa, J. B., and Yue, D. T. (2007) Elementary mechanisms of the voltage-dependent Ca2+-channel α2δ subunit and effects on channel function. *J. Biol. Chem.* 282, 24645–24648
42. Chu, P. J., Larsen, J. K., Chen, C. C., and Best, P. M. (2004) Distribution and relative expression levels of calcium channel β subunits within the chambers of the rat heart. *J. Mol. Cell Cardiol.* 36, 423–434
43. Catin, C., Nieto-Rostro, M., Foucault, I., Heblich, F., Wrannt, J., Richards, M. W., Hendrich, J., Douglas, L., Page, K. M., Davies, A., and Dolphin, A. C. (2005) The metal-ion-dependent adhesion site in the Von Willebrand factor-A domain of α2δ subunits is key to trafficking voltage-gated Ca2+-channels. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11230–11235
44. Casteel, C., De Waard, M., and Campbell, K. P. (1996) Dual function of the voltage-dependent Ca2+-channel α 2 δ subunit in current stimulation...
and subunit interaction. *Neuron* **16**, 431–440
66. Sandoval, A., Oviedo, N., Andrade, A., and Felix, R. (2004) Glycosylation of asparagines 136 and 184 is necessary for the α2δ subunit-mediated regulation of voltage-gated Ca$^{2+}$ channels. *FEBS Lett.* **576**, 21–26
67. Felix, R., Gurnett, C. A., De Waard, M., and Campbell, K. P. (1997) Dissection of functional domains of the voltage-dependent Ca$^{2+}$ channel α2δ subunit. *J. Neurosci.* **17**, 6884–6891
68. Ferreira, G., Yi, J., Rı́os, E., and Shirokov, R. (1997) Ion-dependent inactivation of barium current through L-type calcium channels. *J. Gen. Physiol.* **109**, 449–461
69. Hoppa, M. B., Lana, B., Margas, W., Dolphin, A. C., and Ryan, T. A. (2012) α2δ expression sets presynaptic calcium channel abundance and release probability. *Nature* **486**, 122–125
70. Eroglu, C., Allen, N. J., Susman, M. W., O’Rourke, N. A., Park, C. Y., Ozkan, E., Chakraborty, C., Mulinyawe, S. B., Annis, D. S., Huberman, A. D., Green, E. M., Lawler, I., Dolmetsch, R., Garcia, K. C., Smith, S. J., Luo, Z. D., Rosenthal, A., Mosher, D. F., and Barres, B. A. (2009) Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell* **139**, 380–392
71. Kurshan, P. T., Oztan, A., and Schwarz, T. L. (2009) Presynaptic α2δ-3 is required for synaptic morphogenesis independent of its Ca$^{2+}$-channel functions. *Nat. Neurosci.* **12**, 1415–1423