Role of the $\beta_2$ Subunit of Voltage-Dependent Calcium Channels in the Retinal Outer Plexiform Layer

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PURPOSE. Mutations in the $\alpha_{1\beta}$ subunit of voltage-dependent calcium channels (VDCCs) have been shown to cause incomplete congenital stationary night blindness (CSNB2). The purpose of this study was to identify which of the four $\beta$ subunits of VDCCs participates in the formation of this channel at the photoreceptor synapse and to determine how its absence affects visual processing.

METHODS. Mice without each of the four known $\beta$ subunits of VDCCs were generated by gene targeting and transgenic rescue (CNS-$\beta_1$, $\beta_2$, $\beta_3$, and $\beta_4$) or by gene targeting alone ($\beta_2$) or were obtained from a commercial provider ($\beta_3$). Retinal function and visual sensitivity were examined by electroretinography and an active avoidance behavioral test, respectively. The structure of the retina and expression of the $\alpha_{1\beta}$ subunit were examined at the light microscopic level and by immunohistochemistry.

RESULTS. Under dark-adapted conditions, CNS-$\beta_2$–null mice had a normal ERG a-wave, but did not have a normal b-wave. In addition, these mice showed decreased sensitivity to light. Both the a- and b-waves appear normal in the CNS-$\beta_1$, $\beta_3$, and $\beta_4$-null mice. Histologic analyses of all four mouse lines indicated that only the CNS-$\beta_2$–null mice had altered retinal morphology. Eyes of these mice had a thinner outer plexiform layer (OPL) than eyes of control animals. In addition, the labeling pattern of the $\alpha_{1\beta}$ subunit in the OPL was altered in CNS-$\beta_2$–null mice.

CONCLUSIONS. The normal distribution of the $\alpha_{1\beta}$ subunit of the VDCCs in the OPL is dependent on the expression of the $\beta_2$ subunit. The expression of both of these subunits is required for normal maintenance and/or formation of the OPL and synaptic transmission. (Invest Ophthamol Vis Sci. 2002;43: 1595–1603)

The visual signal generated in the rod photoreceptors is transmitted to second-order neurons through ribbon synapses in the outer plexiform layer (OPL) of the retina.1,2 These synapses are specialized structures in which glutamate release is mediated by calcium entry through slowly inactivating L-type voltage-dependent calcium channels (VDCCs).3–8 The importance of these channels in visual function was recently highlighted by the discovery that mutations in the $\alpha_{1\beta}$ subunit of VDCCs are responsible for an X-linked disorder, incomplete congenital stationary night blindness (CSNB2) in humans.9,10 More recently, Morgans and colleagues11–15 have shown that the $\alpha_{1\beta}$ subunit is localized to the OPL in rat and chicken.

Neuronal VDCCs are heteromultimers, composed of $\alpha_1$, $\alpha_2/\delta$, and $\beta$ subunits and possibly a neuronal homologue of the skeletal $\gamma$ subunit.14 To date, 10 $\alpha_1$, 4 $\beta$, 3 $\alpha_2/\delta$, and 8 $\gamma$ subunit15 genes have been identified (for review see Ref. 16). In all VDCCs the $\alpha_1$ subunit forms the channel pore, which contains the voltage sensor and the drug-binding site used to define the channel type. The $\alpha_2/\delta$ and $\beta$ subunits are important in determining channel kinetics, and the $\beta$ subunits are necessary for expression of the channel.17 Adult skeletal muscle expresses a single $\alpha_1$ subunit ($\alpha_{1\gamma}$) paired with a $\beta_1$, $\alpha_2/\delta$, and $\gamma_1$ subunit. Inactivation of the $\beta_1$ subunit by gene targeting results in the loss of the $\alpha_{1\gamma}$ subunit and absence of contraction.17 The $\gamma_1$ subunit has been shown to modify channel kinetics of skeletal muscle L-type VDCCs.18,19 but its role in the central nervous system (CNS) is not well defined. In many neurons in the CNS, multiple $\alpha_1$ and $\beta$ subunits are expressed, and the absence of a single $\beta$ subunit appears to be less deleterious than the loss of the $\beta_1$ subunit in skeletal muscle. For example, lethargic mice have no $\beta_4$ subunit20; however, there is no quantitative loss of any specific VDCC type.21,22 Instead, the remaining $\beta$ subunits are reshuffled, resulting in $\alpha_1$ subunit pairings with the remaining $\beta_1$–3.21 Certain aspects of channel function are altered, however, because lethargic mice have an observable phenotype.25

To begin to identify the structural makeup of VDCCs at the photoreceptor ribbon synapse, we examined the role of the $\beta$ subunits in mouse visual function. We describe electrophysiological, anatomic, and behavioral studies in mice with no expression of the $\beta_2$ subunit in the CNS. We show that the absence of the $\beta_2$ subunit in the retina results in an abnormal distribution of the $\alpha_{1\beta}$ subunit in the retina, which has profound effects on visual function and the ERG that are not seen in mice without the other $\beta$ subunits. These results indicate that normal function of the retinal VDCCs at the photoreceptor-to-bipolar cell synapse is highly dependent on the presence of the $\beta_2$ subunit and that mice without this subunit provide an animal model for CSNB2.
METHODS

Experimental Animals

All procedures using animals were approved by the local institutional animal care and use committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice carrying a disrupted version of one of the four β subunits of VDCCs were studied. Mice without the β2 subunit died at birth from asphyxia due to the absence of skeletal muscle contraction.27 The β2-null mutation is rescued by expressing a murine β2 cDNA under the control of the human skeletal actin (HSA) promoter.21 To allow detection of the transgenic β2 protein, we added a T7 tag (MASMTGGGQM; Novagen, Madison, WI) to the β2+ cDNA (Fig. 1Aiv) and produced transgenic mice by standard methods. Three founders expressed the T7/β2+ subunit in skeletal muscle, and two of these were crossed to the β2+ knockout line. After appropriate breeding, mice homozygous for the β2+ knockout allele and carrying the HSA-β2+ transgene were identified. These mice are referred to as CNS-β2−/−. Western blot analysis showed that they do not express the β2+ subunit in the retina (Fig. 1C). They are viable, fertile, and have no obvious phenotypic abnormalities.

Mice without the β2+ subunit were created using gene targeting in embryonic stem (ES) cells derived from the 129/Sv mouse strain. A P1 clone containing the 5′ portion of the β2+ subunit gene was obtained by screening a genomic library (129/Sv strain; Genome Systems, Inc., St. Louis, MO) using a PCR strategy. Bacteriophage clones containing the 5′ portion of the gene were isolated from a mouse 129Sv genomic library (No. 946305; Stratagene, La Jolla, CA) using a cDNA probe encompassing nucleotides 1229 to 1819 (GenBank Accession No. L20543; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/genbank). Mice without the β2+ subunit were produced using homologous recombination in ES cells. The β2− targeting vector was designed to replace exons 4 through 15 of the β2+ gene (Fig. 1Aii). The targeting vector included a TK cassette (pBlue-script; Stratagene; Fig. 1Aii), a 4.4-kb Xho1/BamHI fragment containing the 5′ portion of the β2+ gene including exons 2 and 3, a PGKNeo selection cassette, and a 2.5-kb Stu1/Xho1 fragment containing the 3′ portion of the gene, including part of exon 14 (Fig. 1Aii). A unique Xho1 restriction site was used to linearize the plasmid before its introduction into mouse ES cells by electroporation. Five micrograms of the targeting vector was electroporated into 5 × 106 ABI1 ES cells. G418- and 1-(O-acetyl-2-morpholino)propanesulfonic acid (MOPs) DS running buffer, as recommended by the manufacturer. Transfected ES cells were selected with G418 and MOPs and were suspended in sample buffer and reducing agent and proteins separated by 4% to 12% Bis-Tricine polyacrylamide gels (NuPage; Invitrogen) and were transferred to nitrocellulose membranes. Blots were incubated with the membranes overnight at 4°C. Membranes were washed in TBST (three times for 15 minutes each), the appropriate primary antibody was added, and blots were incubated with an enhanced chemiluminescence kit (PerkinElmer Life Sciences). Western blot analysis with the restriction enzyme EcoRV and probe 1 showed the normal 23-kb band in the parental ES cells and the expected 11-kb band in the targeted ES cell clone, B5 (Fig. 1B). Similarly, Southern blot analysis with the restriction enzyme EcoRI and probe 2 showed the normal 11-kb band in the parental ES cells and the expected 4.5-kb band in targeted ES cell clone B5 (Fig. 1B). Of three clones targeted on the 5′ end, one was also targeted on the 3′ end. This clone was expanded and injected into C57BL/6j blastocysts to produce germine chimeric mice. Mice heterozygous for the targeted allele were viable and appeared grossly normal. We never observed any homozygous (β2−/−) offspring. This was expected because the β2+ subunit is expressed at high levels in heart, and β2−/− mice die during embryogenesis because they have no cardiac contraction (Gregg RG, unpublished observations, 1998).

To rescue the β2−/− null mice, we created a transgenic mouse line expressing the rat β2 subunit in cardiac muscle of FVB mice. This was achieved using a cardiac-specific promoter, the α-mysin heavy chain (αMyHC),25 to express a T7-tagged rat β2 cDNA (Fig. 1Av). Twelve transgenic founder lines were obtained, and three independent lines that showed expression of the transgene in heart tissue were crossed to the β2−/− knockout mice. All three lines were able to rescue the lethal β2−/− null phenotype, and one transgenic line was used to rescue the β2−/− null phenotype in this study. This line showed no detectable expression of the β2+ subunit in the retina (Fig. 1C). Mice homozygous for the targeted β2−/− allele and carrying the T7-tagged rat β2 transgene are fertile, have no obvious abnormalities, and are referred to as CNS-β2−/− mice.

CNS-β2−/− and β2−/− null mice are a mixture of three genetic backgrounds: 129/Sv from the ES cells used for gene targeting, C57BL/6j from the blastocysts into which the ES cells were injected, and FVB used to produce the transgenic lines. To avoid differences due to strain effects, we used littermate control mice whenever possible. Because the FVB strain carries the rd allele, we screened all mice to ensure that none of the mice used in these experiments was heterozygous or homozygous for the rd allele.

Production of the β2−/− null mice (129/Sv background) has been described elsewhere.20 The β2−/− mice (B6EiC3H-Aa-cenhb/K or lethargic) were obtained from Jackson Laboratories (Bar Harbor, ME). Figure 1C confirms that the β2+ and β2− subunits are absent in the β2−/− null mice, as previously reported.20,26 The genotype of the CNS-β2+ and β2− mice was determined using PCR assays specific for the deleted alleles and transgenes. Primers used were: β2− deleted allele: P1 (5′-CAAAATAGCCAGGATGCGGAGT-3′) and P2 (5′-ACACCCCTC- GGGATGIIIAGGAGT-3′); β2+ normal allele, P2 and P3 (ATGGATGTTA- GATGGGGGGATGGGG-3′); T7/β2+ transgene, P4 (5′-AAATTCATGTC- CCGGGGCTTACCCT-3′) and P5 (5′-CTGCCCCATCAACGAGTGCT-3′); β2− deleted allele, P6 (5′-GGTTCGCGGACAGCTCCTACAGCAGC-3′) and P7; β2+ normal allele, P6 and P7; T7/β2+ transgene, P10 (5′-TCTG- CATCTAGGCACTGGCTAG-3′) and P11 (5′-GGTTCGCGCAGCTCCTACAGCAGC-3′). PCR reactions contained 1× Taq polymerase buffer and enhancer, 1 U Taq polymerase (Eppendorf, Hamburg, Germany), 2 μM of each primer, 0.2 mM dNTPs, and 25 to 50 ng DNA in a final volume of 25 μL. Cycling conditions were determined empirically, but in general were: 2 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at the annealing temperature, and 2 minutes at 72°C, followed by a final incubation for 10 minutes at 72°C. Amplified samples were analyzed by agarose gel electrophoresis.

Western Blot Analysis

Mice were killed by anesthetic overdose and the eyes removed. The retinas were dissected and immediately homogenized in 100 μL of solution containing 2 mM HEPES (pH 7.4), 1 mM EGTA, 2 μg/mL leupeptin, and 2 μg/mL aprotinin. Homogenates (12-15 μg protein) were suspended in sample buffer and reducing agent and proteins separated on 4% to 12% Bis-Tricine polyacrylamide gels (NuPage; Novex/Invitrogen, Carlsbad, CA) in 3-(N-morpholino)propanesulfonic acid (MOPS) SDS running buffer, as recommended by the manufacturer. Proteins were transferred to nitrocellulose membranes, and the membranes were blocked using 5% nonfat milk in Tris-buffered saline (TBS: 20 mM Tris-HCl [pH 7.4] and 137 mM NaCl). Primary antibodies were diluted in TBS (TBS and 0.3% Tween 20) including 2% BSA, and incubated with the membranes overnight at 4°C. After membranes were washed in TBS (three times for 15 minutes each), the appropriate horseradish peroxidase (HRP) secondary antibody was diluted in TBS plus 5% nonfat milk and incubated at room temperature for 1 hour. Membranes were washed three times with TBS and developed with an enhanced chemiluminescence kit (Supersignal ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Affinity-purified primary antibodies to β1sub (rabbit 142), β2 (rabbit 143), β3 (sheep 49), and β4 (rabbit 145), were a generous gift from Kevin Campbell. The images were captured on x-ray film and scanned into a computer with image-analysis software (Photoshop; Adobe, San Diego, CA).

Electroretinography

Animals 1 to 6 months of age were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) and placed on a heating pad. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine. Electroretinograms (ERGs) were recorded using a wire-loop recording electrode, which made contact with the corneal surface through a thin layer of 2% methylcellulose. Two platinum needle electrodes used for reference and ground were placed in the cheek and tail, respectively.
Signals were band-pass amplified (1–1000 or 0.01–1000 Hz), digitized at 2500 Hz, and averaged using a signal-averaging system (Compact Four; Nicolet, Madison, WI). To distinguish between rod and cone activity, different stimulus and recording conditions were used. To isolate photoreceptor function, animals were dark adapted for at least 2 hours before testing.
and strobe flashes ranging from \(-3.0\) to \(1.0\) log sec \cdot cd/m\(^2\) were presented in the dark within a ganzfeld (Nicolet). To isolate cone responses, a series of flash intensities was presented on a steady rod-desensitizing adapting field (1.3 log cd/m\(^2\)). Stimulus calibrations were performed with a photometer (Model 550; EG&G, Salem, MA) and are expressed in photopic units.

**Behavioral Analysis**

Light sensitivity was evaluated in 2-month-old CNS-\(\beta_2\)-null and age-matched normal (C57BL/6J) mice, by using a two-way active avoidance paradigm in a shuttle box. Each training period began with a 3-minute acclimation period, and consisted of 100 trials. For each trial, the onset of a light flash on one side of the box preceded by 10 seconds the presentation of a mild foot shock (0.8 mA) on the same side of the cage. The length of time (20–35 seconds) the foot shock was activated was randomized, which also resulted in the randomization of the interflash interval. Animals could either avoid or escape the shock at any point during each trial by moving to the dark, or safe, side of the cage. Each experimental animal was trained for 8 to 12 consecutive days. After the animals had learned the visual task, neutral-density filters (Oriel, Stratford, CT) were used to reduce the intensity of the light cue.

**Histology**

Mice were killed at 3 to 9 months of age by anesthetic overdose, and eyes were removed and immersion fixed (2% glutaraldehyde) for either 1 to 3 hours for morphologic examination or for 10 minutes in 4% paraformaldehyde for immunohistochemical experiments. Eyecups fixed in glutaraldehyde were embedded in epoxy resin (Embed 812; EM Sciences, Fort Washington, PA). Vertical sections were cut at 0.5 mm using a diamond knife on an ultramicrotome (Reichert; Leica, Deerfield, IL) and stained with toluidine blue. Eyecups fixed in paraformaldehyde were cryoprotected by immersion in 10% sucrose in PBS, frozen embedded in optimal cutting temperature compound (OCT), and cut at 10 \(\mu\)m on a cryostat (Ernst Leitz, Rockleigh, NJ) at \(-20^\circ\)C. Frozen sections were blocked by incubation for 30 minutes at room temperature in antibody incubation solution (AIS; 0.5% Triton X-100, 5% horse serum, 0.05% Na\(_2\)S in PBS). Sections were incubated overnight at room temperature in an antibody specific to the human \(\alpha_{1c}\) subunit.\(^{12}\) The sections were washed in PBS, incubated for 1 hour at room temperature in the secondary antibody (donkey anti-sheep IgG conjugated to CY3; carboxymethylindocyanine; Diagram, Hamburg, Germany) diluted 1:50 in AIS, washed in PBS, and coverslipped with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Primary antibodies were omitted from control sections. Photomicrographs were taken with a microscope (Leica) on tungsten film (Ektachrome EPY, 135-56; Eastman Kodak, Rochester, NY) with a fluorescence filter for CY3.

Retinal morphometry was analyzed in plastic-embedded sections that bisected the optic nerve. Measurements were made at an area approximately 1 mm from the optic nerve in three sections from each animal. Results from each animal and then for each group were averaged, and a Student’s \(t\)-test was used to compare averages from the \(\beta_2\)-null mice with each other mutant group. The investigator was masked to the origin of each section analyzed.

**RESULTS**

**Production of Mice without \(\beta_1\) or \(\beta_2\) Subunits in the CNS**

Figure 1Ai–Aiii shows schematic diagrams of the \(\beta_2\)-knockout allele and the transgenes that were used to rescue the \(\beta_2\)- (Fig. 1Aiv) and \(\beta_2\)- (Fig. 1Aiv) knockout mice. Correctly targeted cells had alleles with fragments of predictable size detected by probes 1 and 2. Figure 1B shows the Southern blot analysis of DNA digested with EcoRV and EcoRI and hybridized to probes 1 and 2, respectively. These data show that both probes detected fragments with sizes that were consistent with correct targeting of the cognate \(\beta_2\) allele. No adult mice homozygous for either the \(\beta_1\)- or \(\beta_2\)- knockout alleles were observed. To rescue the lethal phenotype of the homozygous knockout lines, transgenic mice expressing either the \(\beta_2\) subunit in skeletal muscle or the \(\beta_2\) subunit in heart tissue were crossed to the respective knockout background. Figure 1C presents a Western blot analysis of \(\beta\) subunit expression in the retina. Each mouse line showed no expression of the expected \(\beta\) subunit.

**Synaptic Transmission between Photoreceptors and Bipolar Cells**

To eliminate the possibility that any variation in ERG recordings was a function of the varied genetic background of the four lines of mice, littermate control animals were used in all experiments. In addition, the ERG data were compared with a database of responses from control mice collected in other studies.\(^{29}\) Figure 2A shows a series of representative ERGs recorded under dark-adapted conditions to flash stimuli that cover a 4-log-unit range of intensity. Under these stimulus conditions, the responses represent the summed activity of predominantly rod-driven bipolar cells.\(^{30,31}\)

Two major ERG components found in control mice were distinguished in the CNS-\(\beta_2\)-, \(\beta_1\)-, and \(\beta_2\)-null mice. At all intensities, the ERG included a positive-polarity b-wave and high-frequency oscillatory potentials, which represent the summed activity of rod bipolar cells and other inner retinal neurons.\(^{31}\) At the higher flash intensities, the ERG also included a negative-polarity a-wave that is generated primarily by the light-induced closure of cation channels along the rod photoreceptor outer segments.\(^{32}\) There were no apparent differences between either the amplitude or timing of the ERG a- and b-wave responses from CNS-\(\beta_2\)-, \(\beta_1\)-, and \(\beta_2\)-null mice and those recorded from control animals (Figs. 2B, 2C). In comparison, the b-wave of CNS-\(\beta_2\)-null mice was markedly reduced in amplitude at all flash intensities (Fig. 2C), although the a-wave appeared normal (Fig. 2B). These data suggest that CNS-\(\beta_2\)-null mice possess normal photoreceptor function but that postreceptoral activity is greatly reduced, consistent with an abnormality in synaptic transmission between photoreceptors and bipolar cells.

The dark-adapted ERGs from the CNS-\(\beta_2\)-null mice always included a low-amplitude-positive component. To better evaluate this feature, we used an identical protocol to record responses from \(\text{nob}\) (no b-wave) mice, which do not have the postreceptoral components of the dark-adapted ERG.\(^{27}\) The ERGs of \(\text{nob}\) mice are generated by hyperpolarization of rod photoreceptors and a subsequent sustained negativity (slow PII) resulting from the spatial buffering of K\(^+\) by Müller cells.\(^{33}\) Figure 2D compares responses recorded from a CNS-\(\beta_2\)-null and a \(\text{nob}\) mouse at three flash intensities. At each stimulus intensity, it is clear that the CNS-\(\beta_2\)-null response included a late positive component that was missing in the \(\text{nob}\) ERG. This positive ERG component suggests that CNS-\(\beta_2\)-null mice retain a degree of visual transmission from rod photoreceptors to the inner retina.

Figure 3A presents representative light-adapted ERGs from the four groups of mutant mice. At all stimulus intensities, the mouse cone ERG is dominated by a positive-polarity b-wave.\(^{29}\) Distinct b-wave responses that increased in amplitude with increasing stimulus intensity were obtained from CNS-\(\beta_1\)-, \(\beta_3\)-, and \(\beta_2\)-null mice. In comparison, the cone ERGs of CNS-\(\beta_2\)-null mice were of much smaller amplitude and were negative in polarity. Cone ERG intensity–response functions in CNS-\(\beta_1\)-, \(\beta_3\)-, and \(\beta_2\)-null mice fell within the 95% confidence interval for...
control responses (Fig. 3B, dashed lines). In comparison, cone ERGs of CNS-β2-null mice were markedly reduced in amplitude (Figs. 3A, 3B).

Taken together, these ERG data show that in the CNS-β2–, β3–, and β4–null mice, both photoreceptor function and transmission to the inner retina through rods and cones was comparable to that of control mice. In contrast, although photoreceptor function was normal in the CNS-β2–null mice, transmission of the signal to the inner retina was seriously compromised.

Reduced Visual Sensitivity in CNS-β2–Null Mice

Reductions in b-wave amplitude are often associated with marked losses of visual sensitivity.34–35 Although near-normal visual thresholds can be associated with ERG b-wave reductions.36,37 To evaluate light sensitivity in the CNS-β2–null mice, we used a two-way active avoidance paradigm with light as the conditioned stimulus. Figure 4 (left) shows that both control and CNS-β2–null mice quickly learned to avoid the foot shock, each showing an increase in avoidance by the second day of training and an asymptotic level of more than 85% avoidance within 8 days of training. In addition, there was no difference in the time course over which control and CNS-β2–null mice learned the task. To eliminate the possibility that these mice were using nonvisual cues to accomplish the task, mice with eyes enucleated were also trained under these conditions. These mice maintained a low level of performance throughout the eight-day training session. To provide additional confirmation that the performance of the control and CNS-β2–null mice was light dependent, eyes of trained animals were enucleated and then the mice were retested. In these animals, performance fell to the levels observed in untrained animals or those in which eyes had been enucleated (data not shown).

To assess visual sensitivity, performance of trained animals was measured as the intensity of the conditioned stimulus decreased to the scotopic range. Although a reduction in stimulus intensity of 4 log units had no effect on the performance of control mice, the performance of CNS-β2–null mice declined with decreased light intensity, suggesting that overall visual sensitivity is decreased in these animals (Fig. 4, right).

Retinal Structure in CNS-β2–Null Mice

Reductions in the ERG b-wave can be associated with either progressive retinal degeneration,38–40 or a morphologically normal retina.27,41,42 Figure 5 presents representative cross sections from each CNS-β mutant mouse. The overall structure and retinal layer thicknesses of the CNS-β2–, β3–, and β4–null mice were comparable to the same measures made in a group of normal mice in a previous study.29 The CNS-β2–null retina was not substantially different from retinas in other mutant mice, in thickness of the cellular layers or the inner plexiform layer (IPL; Table 1). However, the OPL appeared thinner and disorganized in the CNS-β2 retina and was significantly thinner than in the other mutant mice (P < 0.05).

VDCC α1P Subunit in Photoreceptor Terminals in CNS-β2–Null Mice

In humans, mutations in the VDCC α1P subunit are responsible for CSNB2.9,10 Because the ERG data obtained from the CNS-β2–null mice are similar in many respects to results obtained in patients with CSNB2,35 we examined with immunohistochemistry the distribution of the expression of the α1P subunit in the CNS-β2–null mice. Figure 6 shows that in control mice the α1P subunit was present in both the OPL and IPL, similar to the labeling pattern found in the rat retina.12,13 but was absent in the OPL of CNS-β2–null mice. The distribution of the α1P subunit also appeared to be diminished in the IPL of the CNS-β2–null mice, although quantitative conformation of this conclusion is
In this study, elimination of the \( \beta_2 \) subunit in the retina of mice produced a phenotype that is similar in many respects to that seen in patients with CSNB2.35 The CNS-\( \beta_2 \)-null mice had a normal ERG a-wave, indicating that function of the outer segments was grossly normal. In addition, the amplitude of the b-wave under both light- and dark-adapted conditions was greatly reduced. Further, the CNS-\( \beta_2 \)-null mice were less sensitive to light.

That expression of the \( \alpha_{1F} \) subunit depends on the expression of the \( \beta_2 \) subunit when the \( \beta_1 \) subunit is inactivated.37 In adults, skeletal muscle expresses a single VDCC composed of \( \alpha_{1S} \), \( \alpha_{2/3} \), \( \beta_1 \), and \( \gamma_1 \) subunits. The loss of the \( \alpha_2 \) subunits in both these cases results from a dependence on the \( \beta_2 \) subunit for normal surface expression of the channel (see Ref. 16 for review). The underlying mechanism is not clearly understood, but the \( \alpha_1/\beta \) interaction in the endoplasmic reticulum (ER) appears to mask an ER retention signal present on the \( \alpha_1 \) subunit.44 In contrast to these effects, studies of Schaffer collateral synapses in the hippocampus of \( \beta_2 \)-null (lethargic) mice indicate that loss of the \( \beta_2 \) subunit has little effect on the two main VDCCs at this synapse.72 This indicates that, in contrast to skeletal muscle, other \( \beta \) subunits are expressed in these neurons and consequently are able to form pairings with \( \alpha_1 \) subunits to produce functional channels. This process of alternate \( \alpha_1/\beta \) subunit pairing has been referred to as subunit reshuffling.21 McClenery et al.59 showed that expression of the \( \beta_{1b} \) isoform was increased in several areas of the \( \beta_2 \)-null mouse brain, which could explain the relatively mild effects of deleting this subunit. In \( \beta_2 \)-null mice a significant decrease in the level of \( \alpha_1 \) and \( \alpha_2 \)-type VDCCs was observed in sympathetic neurons in the superior cervical ganglion, indicating that the level of expression of the \( \beta_2 \) subunits may have been limiting.26 In light of these findings, the most likely explanation for the phenotype of the CNS-\( \beta_2 \)-null mice is that the \( \beta_2 \) subunit is the predominant \( \beta \) subunit in photoreceptors, and the expression level of any other \( \beta \) subunit is insufficient to allow normal expression of the \( \alpha_{1F} \) subunit at the ribbon synapse. Alternatively, if other \( \beta \) subunits are present, they are unable to pair with the \( \alpha_{1F} \) subunit. Although possible, the latter explanation is unlikely, because large numbers of in vitro studies have examined \( \alpha_1/\beta \) subunit pairings, and almost all combinations tested to date are able to produce functional channels.16

**DISCUSSION**

Normal transmission between photoreceptors and bipolar cells is dependent on the expression of L-type VDCCs in the OPL, which mediate calcium entry into photoreceptor terminals and subsequent glutamate release. Loss-of-function mutations in the \( \alpha_{1F} \) subunit of VDCCs have been identified in patients with CSNB29,10,43 implicating this subunit in the photoreceptor VDCCs. Patients with CSNB2 show a reduction in the dark-adapted ERG b-wave, although a low-amplitude, late, corneal positive response is retained under all stimulus conditions.35 In addition, the amplitude of the cone ERG is drastically reduced in patients with CSNB2. These electrophysiological changes are associated with a reduction in visual sensitivity.35 The identification of mutations in the \( \alpha_{1F} \) subunit in patients with CSNB2 indicates that this VDCC subunit is required for normal photoreceptor-to-bipolar cell synaptic transmission.

**FIGURE 3.** Light-adapted ERG recordings. (A) Cone ERGs recorded from each mutant mouse line in response to stimulus flashes that ranged from −0.1 to 1.0 log sec · cd/m² presented on a steady rod-desensitizing adapting field (1.3 log cd/m²). Amplifier band-pass:1 to 1000 Hz. (B) Amplitude of the cone ERG plotted as a function of stimulus intensity. The cone ERG was markedly decreased in CNS-\( \beta_2 \)-null mice as the intensity of the light cue was reduced by 2, 3, or 4 log units. Data points indicate the average ± SD of four to five mice. Symbols are as defined in Figure 2.

**FIGURE 4.** Visual sensitivity is reduced in CNS-\( \beta_2 \)-null mice. **Left:** Acquisition of an active-avoidance task with a light cue by control mice (○, \( n = 4 \)). CNS-\( \beta_2 \)-null mice (●, \( n = 4 \)), and mice with eyes enucleated (◆, \( n = 2 \)). **Right:** Performance of control and CNS-\( \beta_2 \)-null mice as the intensity of the light cue was reduced by 2, 3, or 4 log units by addition of neutral-density (ND) filters. Data points indicate the average ± SD.
Although the CNS-β₂-null mice have a dramatically decreased ERG b-wave, they retain some visual function. They exhibit a low-amplitude, late, corneal positive ERG component, and they have the ability to learn a visual task. Therefore, it is likely that some modulation of glutamate release is sensed by second-order neurons. Several explanations are possible. First, another β subunit could form functional channels with α₁F, or α₁F subunits may be inserted in the membrane in the absence of any β subunit. Both of these explanations seem unlikely, because we were unable to detect α₁F in the OPL with immunohistochemistry. Second, there could be other α₄ subunits present that normally form complexes with β₁, β₃, or β₄ subunits. In support of this hypothesis are the observations that α₁C and α₁D are expressed in the OPL and INL of the tree shrew retina, although neither subunit has been localized to the OPL in chicken retina. Perhaps these channels are involved in non-ribbon synapses in the OPL, which are able to support some visual function. The distribution of these subunits in the mouse retina is currently unknown, although α₁D and α₁C have been reported to be present in rat photoreceptors. Regardless of the origin of the signaling responsible for the small positive ERG component and the residual visual ability in the CNS-β₂-null animals, the present results and those obtained from studies of patients with CSNB2 indicate that the primary L-type VDCCs present in the photoreceptor terminal are composed of α₁F and β₂ subunits and presumably one of the three α₁/β subunits.

Unlike retinitis pigmentosa, CSNB1 and CSNB2 are not associated with retinal degeneration. Similarly, in CNS-β₂-null mice there was no evidence of photoreceptor degeneration. However, there was a significant thinning of the OPL in the CNS-β₂-null mice compared with control animals. Subsequent electron microscopic examination of the OPL indicates that CNS-α₁C-β₂-null mice show a nearly complete absence of ribbon synapses. These data suggest that the channels that incorporate this subunit are critical for normal formation and/or maintenance of the OPL. Whether this is because the ribbon synapses fail to form or because they degenerate after formation is currently being examined.

The defect in the nob mouse is now known to involve the nyx gene, which produces the nyctalopin protein. Although the mechanism of action of nyctalopin is unknown at present, electrophysiological studies of nob mice indicate that the defect is most likely postsynaptic to the photoreceptors. In nob mice, the OPL is significantly thinner than in control animals. The distribution of α₁C subunits in the retina of a control and a CNS-β₂-null mutant mouse. Staining is seen throughout the OPL in the control but not in the CNS-β₂-null retina. No background was detected in the no-primary-antibody control section. Left: toluidine blue-stained section from a control mouse. Abbreviations are defined in Figure 5.
mice, the OPL appears normal, as do the ribbon synapses. The OPL is also preserved in two lines of mutant mice in which the bipolar cell response has been inactivated by loss of either the mGluR6 receptor or the Goα protein involved in the postreceptor response to glutamate. These data indicate that activation of the bipolar cell transduction cascade is not required for normal synapse formation and maintenance. The absence of α1F in the photoreceptor terminals of the CNS-β2-null mice indicates that release of glutamate from the photoreceptor terminals is most likely absent, and this release may be critical for normal ribbon synapse formation and/or maintenance. Alternatively, the presence of a specific α1F isoform on the photoreceptor cell bodies may be important in regulation of gene expression of key proteins involved in synapse formation. An example of such a molecule is agrin, whose expression in the motor neurons is required for synaptogenesis at the neuromuscular junction. It is also possible that L-type VDCC-mediated calcium entry into the photoreceptor terminal may be required for synapse formation. Finally, there is evidence that several proteins bind to VDCCs, raising the possibility that the physical presence of the channel may be required for formation and/or maintenance of the ribbon synapse.

In summary, we have shown that expression of the β2 subunit of VDCCs in the retina is required for normal function. The ERG data indicate that studies of the CNS-β2-null mouse may provide insights into the pathophysiologic mechanisms underlying CSNB2. In addition, it is of interest to determine whether mutations in this gene produce an autosomal recessive form of CSNB2. Based on our data, we predict that patients with CSNB2 who carry α1F mutations and α1G-null mice (when they become available) will both have structural abnormalities of the OPL. The availability of the CNS-β2-null mice will provide a valuable resource for understanding the mechanism of formation and/or maintenance of ribbon synapses and will provide a model to evaluate possible therapeutic strategies for CSNB2.

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References


