Novel Allele of Crybb2 in the Mouse and Its Expression in the Brain

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PURPOSE. O377 was identified as a new dominant cataract mutation in mice after radiation experiments. The purpose of this study was to genetically characterize the mutation and to analyze its biological consequences.

METHODS. Linkage analysis of the O377 mouse mutant was performed; candidate genes including Crybb2 were sequenced. The authors analyzed eyes and brains of the mutants by histology and the expression domains of Crybb2 by in situ hybridization and immunohistochemistry. RNA was isolated from whole brains of heterozygous and homozygous O377 mutants, and differential expression arrays were performed. All studies were compared with age- and strain-matched wild-type mice.

RESULTS. The mutation was mapped to chromosome 5 and characterized as an A→T substitution at the end of intron 5 of the Crybb2 gene. It led to alternative splicing with a 57-bp insertion in the mRNA and to 19 additional amino acids in the protein. In the brain, βB2-crystallin was expressed in the cerebellum, olfactory bulb, cerebral cortex, and hippocampus. The only morphologic difference in the brain is the increased number of Purkinje cells in the cerebellum of homozygous strain-matched mutants. Differential expression analysis revealed the upregulation of calpain-3 in the brain of homozygous mutants, which was confirmed by quantitative real-time PCR.

CONCLUSIONS. These results confirm the third allele of Crybb2 in the mouse that also affected exon 6 and the fourth Greek key motif. Moreover, expression analysis of Crybb2 identified for the first time distinct regions of expression in the brain, and the differential expression points to the participation of Ca\(^{2+}\) in the corresponding pathologic processes. (Invest Ophthalmol Vis Sci. 2008;49:1533–1541) DOI:10.1167/iovs.07-0788

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the Use of Animals in Ophthalmic and Vision Research and the regulations of the German Law on Animal Protection. The original mutant, expressing progressive cataract, was recovered in the offspring of male mice exposed to 3 Gy x-ray irradiation. The presumed mutation was genetically confirmed and crossed to strain C3H/BL; all further characterizations described here have been performed on this genetic background. For linkage analysis, congenic C3H/BL-O377 mice were outcrossed to wild-type strain C57BL/6J. The mutation was mapped genetically confirmed and crossed to strain C3H/BL; all further characterizations described here have been performed on this genetic background. For linkage analysis, congenic C3H/BL-O377 mice were outcrossed to wild-type strain C57BL/6J. The mutation was mapped to microsatellite markers according to methods described previously.

Segregation data were analyzed with Map Manager (www.mdl.com). The protein has been modeled using the MDL Chime software (http://www.mdl.com). For quantitative real-time PCR (qRT-PCR), 3 μg brain RNA from C3H/BL/FeJ (C3H) and O377 mice (n = 4 mice/strain) was reverse transcribed into first-strand cDNA (Ready-to-Go T-Primed First-Strand Kit; Amersham Biosciences, Freiburg, Germany) in a 35-μL reaction volume, in accordance with the manufacturer’s instruction. cDNA (1 μL) was used in a subsequent PCR reaction using 11.5 μL mix (Absolute qPCR SYBR Green ROX; Abgene, Hamburg, Germany), 1.0 μL each primer, and 10.5 μL RNase-free water. Primers (Table 1) for Capn3, Tmsbix, CR536618, 1700065I16Rik, Sgne1, Stmn1, and Actb were ordered and purchased from metabion International AG (Martinsried, Germany), and analysis was performed with a sequence detection system (ABI PRISM 7000; Applied Biosystems, Foster City, CA). The mRNA sequence was translated using the ExPASy Proteomics server available at http://www.expasy.org/.

The PDB file used to model the proteins was generated from The SwissModel First Approach Mode (http://swissmodel.expasy.org//SWISS-MODEL.html). The protein has been modeled using the MDL Chime software (http://www.mdl.com).

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**TABLE 1. Primer for Quantitative Real-Time PCR**

**Morphologic and Histologic Analysis**

Slit lamp ophthalmologic examination was conducted according to standard procedures (http://www.eumorphia.org/EMPRess). Enucleation of the eyes and histology were performed essentially as described previously.

Brains from adult mice (transcardially perfused with 4% paraformaldehyde in PBS) were paraffin embedded and cut on a microtome in 8-μm-thick sections for immunohistochemistry. The β2-crystallin antibody was kindly provided by Joseph Horwitz (Los Angeles, CA); the ABC kit for polyclonal antibodies was from Vectastain (Vector Laboratories, Burlingame, CA).

Stereoscopic counting of the Purkinje cells of cerebellar lobe V was performed using Cresyl violet-stained, free-floating sagittal cryosections (50 μm thick; HM 560; Microm, Walldorf, Germany) mounted on clean grease-free slides. Nine animals (3 weeks old) each of wild-type and homozygous mutant were analyzed (Stereoinvestigator; Microbrightfield Inc., Williston, VT).

**Protein Analysis**

Water-soluble proteins were extracted from lens and brain tissue using protein extraction buffer (50 mM Tris/HC1 [pH 7.8], 3 mM dithiothreitol, 0.1 mM phenylmethylsulfonil fluoride). Concentration of protein was estimated using a protein assay kit (Protein Quantiﬁcation RC DC; Bio-Rad, Munich, Germany). Samples were stored at −80°C.

**FIGURE 1. Morphology of the O377 mutant eyes.** Gross appearance of the mutant lens at the age of 6 months (left, wild-type; middle, heterozygote; right, homozygous mutant). Isolated lenses of heterozygous and homozygous mutant eyes show severe forms of opacity, as evident from the white patches in dark-field photographs. The eye lenses in heterozygous and homozygous mice are reduced in size.
For Western blotting, 16% SDS PAGE gels were used to separate the proteins. Amounts ranging from 5 to 10 μg protein were sufficient for a reproducible band from the lens, whereas 70 to 80 μg protein was necessary to detect a visible band of B2-crystallin from brain. Before running the gel, the protein samples were denatured by incubation with Laemmli buffer at 100°C for 5 minutes. The gel was run using 1% SDS running buffer at 200 mA for 1 to 1.5 hours. One part of the gel was stained by Coomassie blue, destained, and dried in a gel drier for reference, and the other part was used for blotting. Blotting was performed with the use of a blotter and pure nitrocellulose membrane (0.2 µm; Trans-Blot Transfer Medium; Bio-Rad). After blotting, the membrane was blocked by 5% fat-free milk powder (Roth, Karlsruhe, Germany) solution. It was incubated overnight in the dark at 4°C on a shaker in anti-B2-crystallin antibody (developed in rabbit; kindly provided by Joseph Horwitz, University of California at Los Angeles) dissolved in the blocking solution (1:1000). The secondary antibody used was anti-rabbit IgG (whole molecule) peroxidase conjugate, developed in rabbit; kindly provided by Joseph Horwitz, University of California at Los Angeles) dissolved in the blocking solution (1:1000). The secondary antibody used was anti-rabbit IgG (whole molecule) peroxidase conjugate, developed in goat (Sigma, Taukirchen, Germany). DAB staining was performed to develop the band (3,3′-diaminobenzidine [Sigma]; 5 mL 5× buffer; 20 mL H2O; 100 μL CoCl2 [10 μg/μL]; 10 μL H2O2).

**In Situ Hybridization**

In situ hybridization on paraffin sections of eyes from 1-day-old animals was performed according to standard procedures. Briefly, RNA probes were chosen from the N-terminal extension of Crybb2 and from the entire Capn3 gene and were labeled with digoxigenin-rUTP (Roche, Mannheim, Germany) during synthesis from linearized cDNA templates according to the manufacturer’s instructions. The clones were linearized with EcoRI and XbaI and were synthesized with Sp6 and T7 RNA polymerase for the antisense and sense probes, respectively. Sections were analyzed under a microscope (Axioplan; Zeiss, Göttingen, Germany) and documented with a high-resolution charge-coupled device color camera (AxioCam; Zeiss). Pictures were adjusted for brightness, contrast, and color balance in Adobe Photoshop 6.0 and 7.0.

**Expression Profiling**

Whole brains of wild-type C3HeB/FeJ mice and heterozygous and homozygous O377 mutants were collected at 4 weeks of age. Total RNA was isolated from dissected brains according to the manufacturer’s protocol (RNasey Midi kits; Qiagen, Hilden, Germany). Per DNA
A Genomic sequence of the Crybb2 gene

![Genomic sequence of the Crybb2 gene](image)

<table>
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<th>intron 5</th>
<th>exon 6</th>
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| O377: GTAGGACACTCGAGCCTGCTTCTTTGACCATGCTTTCCGATAGCTGCTCGTGGGATGTTGGGTTACCA | W V G Y Q..

B Predictive modeling of the βB2-crystallin\(^{O377}\) 3-D-structure

![Predictive modeling of the βB2-crystallin\(^{O377}\) 3-D-structure](image)

RESULTS

Eye Morphology, Mapping, and Molecular Analysis of the O377 Mutant

A novel mouse mutant line (O377) with progressive, dominant cataracts and small lenses was established on the C3H genetic background after paternal x-ray irradiation (Fig. 1). Histologic analysis of the eyes at postnatal day (P) 1 indicated no differences between the wild-type lenses and the lenses of the mutants (Figs. 2A–F). At day 4 (Figs. 2G–L), in heterozygous mutants the fiber cell nuclei were not fully degraded and remained present even in the center of the lens. The homozygous mutants exhibited more severe effects on differentiation: the fiber cell nuclei were not fully degraded and remained present even in the center of the lens. The homozygous mutants (Figs. 2A–F). At day 4 (Figs. 2G–L), in heterozygous mutants the fiber cell nuclei were not fully degraded and remained present even in the center of the lens. The homozygous mutants (Fig. 4) Immunohistochemistry (Fig. 5) demonstrated Crybb2 predominantly in neurons of the olfactory bulb (mitral cell layer and glomerular layer), hippocampus (pyramidal cells

Expression Domains of Crybb2 in the Brain

Based on earlier reports, we assayed for and observed Crybb2 and Crybb2\(^{O377}\) transcripts in the entire mouse brain; however, the expression level was much lower (approximately 1:20) than in the lens. Time-course studies suggested that the Crybb2 transcript is best detectable in the brain during postnatal development through adolescence and diminishes thereafter (Fig. 4). Immunohistochemistry (Fig. 5) demonstrated Crybb2 predominantly in neurons of the olfactory bulb (mitral cell layer and glomerular layer), hippocampus (pyramidal cells
of the CAl, CAII, CAIII regions and granule cells of the dentate gyrus, cerebral cortex (pyramidal cells throughout all layers), and cerebellum (Purkinje cells and stellate cells of the molecular layers).

No major morphologic or histologic changes were observed in the brains of 3-week-old mutant animals except for the difference in frequency and size of Purkinje cells in the cerebellum (at vermis) of the homozygous mutants. Using unbiased stereological methods, we counted 12,581 (± 454 SEM) Purkinje cells in the cerebellar lobe V of wild-type mice but 13,993 (± 183 SEM) Purkinje cells in the homozygous mutants, representing a significant increase of 11% (P = 0.011) and indicating subtle morphologic consequences in the brain attributed to the mutation compared with strain-matched controls. The O377 mutants detected at the age of 8 months in mutant or in strain-matched, wild-type mice. m, months.

Expression Profiling

To assess global changes in gene expression levels in the brains of O377 mutants, we performed expression profiling experiments using genomewide DNA microarrays. Only homozygous mutants revealed different RNA expression profiles compared with wild-type mice. We detected six genes upregulated and seven genes downregulated in whole brains of homozygous mutants in all 15 DNA-chip hybridizations. Mean absolute expression ratios of significantly regulated genes ranged from approximately 1.3- to 3.5-fold (Fig. 7A). These 13 regulated genes contained less than one false-positive gene (P > 0.05). The complete expression data set is available from the GEO (Gene Expression Omnibus) database (GSE3761 for the series of experiments and GDS 1216 for the description of the platform).

The upregulated genes coding for calpain-3 (Capn3) and CR536618 showed the strongest difference (3.5- and 2.4-fold) for all probes with reproducible expression in all chip hybridizations. Among the downregulated genes, Sgne1 (coding for a secretory granule neuroendocrine protein) and Stmn1 (coding for stathmin) showed the strongest effects. These differences in the level of gene expression were detected despite the fact that whole brains were used for the assessment of differential expression profiles. The changes of the expression of the top three upregulated or downregulated genes were confirmed by qRT-PCR (Fig. 7B; Capn3, 3.2×; Tmsb15x, 1.8×; CR536618, 2.1×; 1700056J16ktk, 0.5×; Sgne1, 0.6×; Stmn1, 0.8×).

Expression Pattern of Crybb2 and Capn3 in the Eyes of O377 Mutants

The expression pattern of Crybb2 in the lens is shown in Figure 8. It indicates that both the mRNA and the protein are present in the lens cortex and that it is more strongly expressed in the mutants. In contrast, Crybb2 transcripts are also present in the wild-type eye in the anterior part of the retina in the inner limiting membrane. This retinal expression was not observed in the mutants.

Because calpains are frequently discussed in cataractogenesis (see Ref. 25), we also tested Capn3 expression in the developing lens (Fig. 9) because we have seen its overexpression in the O377 mutant brain. It turned out that Capn3 expression in the lens epithelium was stronger in heterozygous and homozygous mutants than in wild-type mice. Moreover, the staining of Capn3 was restricted to the cell nuclei and therefore was different from the staining of crystallin mRNA, as demonstrated for Crybb2 itself (Fig. 8). Further studies will be performed to analyze the different expression patterns of Capn3 in the brains of wild-type and O377 mutant mice.

Discussion

O377 is a new mouse mutant with hereditary, progressive, dominant cataract caused by a mutation in the Crybb2 gene. The A→T base pair substitution in the acceptor splice site of intron 5 of the Crybb2 gene leads to the activation of an alternative splice site resulting in an mRNA with an additional 57 bp. Because of the inclusion of 19 additional amino acids at the beginning of the fourth Greek key motif, the βB2-crystallin protein has a molecular weight 1.9 kDa higher than that in the wild type. The O377 mutants were detected after paternal irradiation (3 Gy x-ray); however, such A→T substitutions are uncommon because of ionizing irradiation.26 Therefore, this particular mutation might be of spontaneous origin coincidentally detected in x-ray irradiation experiments.

In addition to O377, two other mouse mutations in Crybb2—the Philly mouse13 and the Aey2 mutant—have been reported.14 Both have been described as having progressive cataracts. As do the O377 mutants, the Philly lenses develop normally until the first postnatal week, when particles appear in the anterior cortex that extend, by the 10th day, to the anterior subcapsular area. Loss of the normal lens densucation
process (as in the O377 mutants) and swelling of the lens fibers follow; the characteristic bow configuration of the nuclei is replaced by a fan-shaped configuration. In contrast, in the Aey2 mutants, the cataractous changes were observed at eye opening as a diffuse opacity in the cortex and abnormally branched anterior suture. This type of opacity remained stationary until 8 to 11 weeks of age, after which total opacity developed. In addition to the phenotypical similarities, all three mutations affect the start of the fourth Greek key motif. In the lens, the misfolding of the mutated βB2-crystallin might lead to altered aggregation properties, as previously reported for the Philly mouse, and for three different mouse γ-crystallin mutants, including deposition of amyloidlike inclusions. This interpretation is particularly supported by histologic analysis demonstrating, in the early stage of cataractogenesis, the stop

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FIGURE 5. Identification of βB2-crystallin expression in particular areas of the brain. The histology (Cresyl violet staining) of sagittal sections of the olfactory bulb (A), the cerebellum (D), the cortex (G), and the hippocampus (J) of a 5-week-old wild-type brain are given. Immunohistochemistry revealed the expression domains of βB2-crystallin protein to be in the glomerulus and mitral cell layers of the olfactory bulb (B, C). Purkinje cells and stellate cells (molecular layer) of the cerebellum (E, F). Pyramidal cells of the cortex (H, I) and in the CAI, CAII, CAIII (CA = Cornu ammonis), and dentate gyrus of the hippocampus (K, L) in wild-type (B, E, H, K) and homozygous (C, F, I, L) mutant mice. Red arrows: βB2-crystallin-positive regions. Scale bars: (A, G) 50 μm; (B–D, H, I) 20 μm; (E, F) 10 μm; (J–L) 0.1 mm.

FIGURE 6. Stereological study of the Purkinje cells. (A) The index photograph shows the cerebellar lobes of a 3-week-old animal; cerebellar lobe V is marked by a red arrow. (B, C) The same region of cerebellar lobe V of homozygous mutant animals and strain-matched wild-type mice is shown. The size of the Purkinje cells appeared to be slightly smaller in the homozygous mutants (qualitative observation only). (D) Results of the stereological counting are given; the number of Purkinje cells is 11% higher in cerebellar lobe V of the homozygous mutants than in the strain-matched wild-type (wt) mice (9 animals of each genotype were used; 3 weeks of age). *P = 0.011; t-test, two-tailed. Statistically significant difference from the wild type (wt). Scale bars: (A) 0.2 mm; (B, C) 20 μm.
of lens fiber cell denucleation followed by a stop of the elongation process in the equatorial zone and the formation of vacuoles in the center of the lens.

In humans, mutations in **CRYBB2** lead to a remarkable phenotypic heterogeneity. The mutation Q155X is caused by a gene conversion mechanism and affects five geographically and genetically disparate pedigrees. Two more alleles (W151C, D128V) in human are yet to be described. In this study, we were able to demonstrate for the first time the expression of **Crybb2** in distinct areas of the brain, particularly in the olfactory bulb, the cortex, the hippocampus, and the cerebellum. Surprisingly, the only morphologic changes in the brain were found in the cerebellum of homozygous mutant brains (mice 1–4) against a pool of four strain-matched wild-type brains were performed. Expression ratios are depicted in red (up-regulated in mutant) and green (down-regulated in mutant) in the heat plot. The colored scale bar on the left shows the related color code for the ratio. All genes shown in this figure have reproducible upregulation or downregulation in all 15 chip experiments. Genes are ranked according the lowest absolute ratio in 15 experiments (Min abs. on the nonlogarithmic scale). In addition, the absolute mean ratio of expression in 15 experiments is given (Mean abs. on the nonlogarithmic scale). The Lion ID is the unique probe identifier from the Bioscience Array Taq Clone set. Probe sequences were blasted over the mouse genome using MouseBLAST on the MGI interface to determine the current official mouse gene symbol. (B) Transcript levels of calpain 3 (**Capn3**), thymosin-β4-X chromosome (**Tmsb4x**), **CR536618** (AK018879), secretory granule neuroendocrine protein 1 (**Sgne1**), and stathmin 1 (**Stmn1**) in the brains of 4-week-old C3Heb/FeB (C3H) and O377 mice are given. qRT-PCR was performed, and the comparative Ct method (ΔΔCt) was used [where ΔCt = Ct (gene) – Ct (Actb)] for quantitation of the data. The ΔΔCt calculation involved finding the difference between each sample’s ΔCt and the mean ΔCt for the C3H strain. Data are presented as relative C3H expression (mean ± SEM, n = 4 mice/strain). Significant differences in expression levels were found between the C3H and O377 strains for all transcripts (**t**-test, **P** ≤ 0.05), reflecting the microarray results.
...proteins, indicating that in the mutant lens more protein is present in the iris (\textit{m. C}, cornea; \textit{L}, lens; \textit{R}, retina). Scale bars: 100 \textmu\text{m}; (top) 0.2 mm; (middle) 100 \textmu\text{m}; (bottom) 20 \textmu\text{m}.

\textbf{FIGURE 8.} Expression of \textit{Crybb2} in the eye. The expression of \textit{Crybb2} is compared between wild-type eyes (left) and \textit{0377}\textsuperscript{−\textsuperscript{−}} eyes (right) from 1-day-old animals. \textit{Top row:} comparison of the \textit{B2}-crystallin proteins, indicating that in the mutant lens more protein is present in the cortical regions. \textit{Middle row:} an even stronger difference is observed by in situ hybridization for mRNA using digoxigenin-labeled \textit{Crybb2} probes. \textit{Bottom row:} however, magnification of the lens bow region indicates that in the mutants, no \textit{Crybb2} mRNA can be observed in the internal limiting membrane of the retina in the region near the iris (red arrows). Scale bars: (top) 0.2 mm; (middle) 100 \textmu\text{m}; (bottom) 20 \textmu\text{m}.

...in the mutants. In situ hybridization also revealed increased \textit{Capn3} expression in the lens epithelial cells of the mutant embryos suggesting increased proteolytic activity and participation of \textit{Ca}\textsuperscript{2+} in cataractogenesis. Because \textit{B2}-crystallin is discussed as a \textit{Ca}\textsuperscript{2+}-binding protein,\textsuperscript{31} proposing the Greek key crystallin fold as a \textit{Ca}\textsuperscript{2+}-binding motif,\textsuperscript{32} it is tempting to speculate that \textit{Ca}\textsuperscript{2+} signaling and \textit{Capn3} overexpression, with subsequent enhanced calpain activity, are major components contributing to cataract formation in \textit{Crybb2} mutants. Moreover, to achieve an understanding of the function of \textit{Crybb2} in the brain, these findings might be relevant to address the first research questions in this new field.

In summary, our study demonstrated the third independent cataract-causing allele in the mouse \textit{Crybb2} gene. Expression analysis of \textit{Crybb2} pointed for the first time to its expression in particular regions of the brain and to an increased number of smaller Purkinje cells in the cerebellum of the homozygous mutants. In situ hybridization also revealed increased \textit{Capn3} expression in epithelial cells of cataractous lenses, suggesting a participation of \textit{Ca}\textsuperscript{2+} in the pathologic processes initiated by the mutation in the \textit{Crybb2} gene and by the impaired function of the \textit{B2}-crystallin.

\textbf{Acknowledgments}

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References