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Targeted knockdown of *Cerkl*, a retinal dystrophy gene, causes mild affectation of the retinal ganglion cell layer

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ABSTRACT

In order to approach the function of the retinal dystrophy *CERKL* gene we generated a novel knockout mouse model by cre-mediated targeted deletion of the *Cerkl* first exon and proximal promoter. The excised genomic region (2.3 kb) encompassed the first *Cerkl* exon, upstream sequences including the proximal promoter and the initial segment of the first intron. The *Cerkl* −/− mice were viable and fertile. The targeted *Cerkl* deletion resulted in a knockdown more than a knockout model, given that alternative promoters (unreported at that time) directed basal expression of *Cerkl* (35%). *In situ* hybridizations and immunohistochemistry showed that this remnant expression was moderate in the photoreceptors and weak in the ganglion and inner cell layers. Morphological characterization of the *Cerkl* −/− retinas did not show any gross structural changes, even at 12 months of age. However, some clear and consistent signals of gliosis and retinal stress were detected by the statistically significant increase of i) the glial fibrillary antigen protein (GFAP) expression, and ii) apoptosis, as detected by TUNEL. Remarkably, consistent non-progressive perturbation (from birth up to 12 months of age) of ganglion cells was supported by the decrease of the Brn3a marker expression as well as the reduced oscillatory potentials in the electroretinographic recordings. In conclusion, the *Cerkl* −/− knockdown shows a mild retinal phenotype, with increased levels of cellular stress and apoptosis indicators, and clear signs of functional alteration at the ganglion cell layer, but no detectable morphological changes.

1. Introduction

Mutations in the *CERKL* gene are associated to autosomal recessive retinal degeneration. First characterized as a Retinitis Pigmentosa (RP) causing gene [1–6], and later also considered to promote Cone-Rod Dystrophy (CRD) [7,8], its classification as a RP or CRD gene is still under question. In fact, although the association of *CERKL* mutations with retinal pathology is clear, we are still at the dark concerning its physiological function and contribution to the progressive photoreceptor degeneration.

CERKL [6] was named after the amino acid identity conservation with CERK [9], the reported ceramide kinase enzyme, known to phosphorylate the sphingolipid ceramide to ceramide-1-phosphate (C1P) [10], whose balance acts as cellular rheostat between apoptosis *versus* survival signaling pathways [11–13]. Although CERKL has an intact diacylglycerol kinase domain [6,14], no kinase activity neither on lipids nor proteins, could be assigned after intense studies from many groups [14–16]. Remarkably, overexpression of CERKL in cultured cells confers protection against apoptosis caused by oxidative stress injury, providing the first experimental clues on its role in retinal cell protection [16].

Very recently, an accurate assessment of the transcriptional products has unveiled that *CERKL* shows a high repertoire of transcript isoforms due to a combination of extensive alternative splicing plus the use of at least three additional promoters [17]. Adding to the complexity of CERKL functional analysis, the protein subcellular localization is extremely dynamic, shifting from the cytoplasm – where it mainly localizes associated to the endoplasmic reticulum and Golgi membranes – to the nucleus, and seldom at the nucleoli [16].

CERKL shows a wide tissular expression although the tissues where the expression is highest are retina in humans and retina and liver in mouse [17]. *In situ* mRNA hybridization on mouse retinal sections showed strong *Cerkl* specific expression in the ganglion cell layer (GCL), moderate and interspersed staining in the inner nuclear layer (INL), and

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faint detection in the inner segment of photoreceptors, whereas immunohistochemistry with antibodies against specific epitopes revealed strong CERKL colocalization with cones, fainter in rods, and moderate at the INL and GCL.

Our group first identified the R257X mutation in CERKL [6], which it is also the most frequent and accounts for more than half of the reported mutated alleles [17]. This mutation is located on exon 5, one of the alternatively spliced exons, and thus some but not all protein isoforms are compromised. To evaluate the phenotype of this mutation, a mouse model in which exon 5 had been excised was constructed [18]. The homozygous exon 5-deleted mice did not show any apparent phenotype in the retina.

In this context, to shed light on CERKL physiological role and the *in vivo* consequences of its mutations, we aimed to generate a full knockout *Cerkl*−/− mouse model by targeted deletion of the reported promoter and the first exon, according to the knowledge at that time. However, the use of alternative promoters driving residual transcription in the retina turned our knockout into a knockdown model retaining 30–35% of *Cerkl* mRNA isoforms, all of them lacking the first exon and initiating methionine. The homozygous exon 1-deleted *Cerkl*−/− mice show increased levels of retinal stress and apoptosis, and diminished expression of the ganglion cell marker Brn3a, but no gross morphological retinal alterations. Most interestingly, the electroretinographic changes observed in the oscillatory potential waveforms are in accordance with the reduced levels of *Cerkl* mRNA and Brn3a protein levels in the retina of the KO mice.

2. Material and methods

2.1. Animal handling, tissue dissection and preparation of samples

Murine tissue samples were obtained from *Cerkl*+/+ and *Cerkl*−/− C57BL/6J background mice. All procedures were performed according to ARVO statement for the use of animals in ophthalmic and vision research, as well as the regulations of the Animal Care facilities at the University of Barcelona. Animals were euthanized with CO_2 followed by cervical dislocation. Specific tissues and organs were dissected and immediately frozen in liquid nitrogen.

2.2. Generation of the knockout Cerkl −/− mouse model

The strategy, the generation of targeting constructs, initial ES cell screening, breeding, housekeeping and molecular genotyping of all the mice after the G0 generation as well as the crosses to obtain the knockout animal model have been performed in our lab. ES transfection, handling, and the generation of chimeric embryos for the obtention of the founder mouse *Cerkl*+/− were performed by GenOway (Lyon, France). A diagram of the targeted locus is depicted in Fig. 1A. In brief, the genomic sequences corresponding to the 5′ and 3′ flanking regions of the *Cerkl* promoter and exon 1 were amplified from 129/SvPas genomic DNA. These sequences were referred as long (region before exon 1) and short (intron 1) arms of homology (LA and SA). Several sub-cloning steps were performed to obtain the final targeting vector using pPCR-Script SK (+) vector (Stratagene). The first step consisted in the addition of a loxP site into LA. A synthetic exon 1 construct with new restriction sites (GeneArt) was used to clone the amplified SA fragment. A Neomycin resistance gene (NEO) flanked by two FRT and one loxP sites were also introduced into SA. Finally, both homology arms were cloned together and the negative selection cassette, DTA (Diphteria Toxin A), was added. The resulting targeting vector stretched 15384 bp, was fully validated by sequencing, and was electroporated into 129Sv ES cells. Positive selection was performed 24 h after electroporation by adding geneticin (200 μg/ml), and more than 1000 geneticin-resistant clones were isolated and amplified. After PCR screenings for recombination at the 5′ and 3′ flanking regions, a total of 14 clones were obtained. Those were characterized by Southern Blot using two different probes, 5′ probe and 3′ probe (Fig. 1B). Only 5 clones had recombined at the correct sites. ES positive cells were used for C57BL/6J blastocyst injections and led to the generation of 9 male chimeras displaying a chimerism rate ranging from 50 to 80%. Highly chimeric males (80% chimerism) were mated with C57BL/6J Flp-deleter females to allow the excision of the NEO selection cassette. Pups were genotyped, and those that presented the excision of the cassette were breeded with wild-type C57BL/6J. The first generation of floxed mice was obtained by mating 2 heterozygous floxed males with C57BL/6J Cre-deleter females to allow the germline excision of the floxed region. Mice were subsequently bred with C57BL/6J mice for a total of 5 generations and pups were genotyped by PCR from tail or ear samples.

2.3. Genotyping by Southern blot and PCR

Genomic DNA from ES cells growing after double positive–negative selection was obtained by standard protocols, after a mild lysis, overnight proteinase K digestion and nucleic acid precipitation with ethanol. Ten micrograms of DNA were digested with informative restriction enzymes, separated by agarose electrophoresis and transferred to Nylon membranes. Standard protocols for pre-hybridization, hybridization with ^{32}P-labeled probes (whose position is indicated in Fig. 1A) were used. Autoradiographic exposition and image acquisition were performed with BioRad Molecular Imager FX Pro Plus (Bio-Rad, Hercules, CA). Three PCR genotyping primers (Fw, Rv1 and Rv2, sequences in Table 1) were used at the same time to allow genotyping of the WT and the floxed alleles in a single reaction (Fig. 1D). The PCR reaction included a first denaturing step of 120 s at 94 °C, followed by 35 cycles of 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 60 s.

2.4. RNA extraction and RT-PCRs

Twenty-five milligrams of each frozen mouse tissue were homogenized using a Polytron PT 1200 E homogenizer (Kinematica AG, Lucerne, Switzerland). For total RNA extraction, High Pure RNA Tissue Kit (Roche Diagnostics, Indianapolis, IN) was used, following the manufacturer's instructions. Total RNA was quantified using the nanoquant plate in an Infinite 200 microplate reader (Tecan, Männedorf, Switzerland).

The RT-PCR assay was carried out with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN) performed following the manufacturer's protocol, using 200 ng of mouse total RNA. For tissue expression analysis all reaction mixtures (50 μl) contained 10 μM of each primer pair, 2 μM of dNTPs, 1.5 mM $MgCl_2$ and 1 U of Go*Taq* polymerase (Promega, Madison, WI). A pair of primers was used to amplify mouse *Gapdh* (Table 1) to compare and normalize the samples. Two-step PCR conditions were as follows: 120 s at 94 °C and 30 cycles of 94 °C for 20 s and 63 °C for 120 s. Mouse *Cerkl* expression was detected with primers mRT_F and mRT_R (a first denaturing step of 120 s at 94 °C, followed by 35 cycles of 94 °C for 20 s, 60 °C for 30 s, and a final extension step at 72 °C for 20 s). The characterization of the different alternative promoters was performed on cDNAs using a forward primer located in each promoter (NeuroD1_F, UTR_F, 3a_F and 3b_F) and the same reverse primer in exon 12 mRT_R (Table 1). Primers were designed to share the melting temperatures and optimized for the same amplification conditions: 120 s at 94 °C followed by 40 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 90 s. Oligonucleotide sequences are listed in Table 1 and their localization is depicted in Supplementary Fig. 1.

Real-time PCR was performed using the SYBR Green I Master assay in a LightCycler480 (Roche, Indianapolis, IN). Specific primers in different exons were designed to generate an amplicon of approximately 100 bp. Oligonucleotide sequences are listed in Table 1. The expression of *β-2-microglobulin* was used for normalization and comparison to *Brn3a* and *Gfap* target genes. For each real-time PCR reaction, 2 μl of cDNA (diluted 1:5), 1 μM of each primer, 1× master mix and H_2O were mixed in a final volume of 10 μl. The reaction was pre-incubated for