Calpain-5 Expression in the Retina Localizes to Photoreceptor Synapses

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PURPOSE. We characterize calpain-5 (CAPN5) expression in retinal and neuronal subcellular compartments.

METHODS. CAPN5 gene variants were classified using the exome variant server, and RNA-sequencing was used to compare expression of CAPN5 mRNA in the mouse and human retina and in retinoblastoma cells. Expression of CAPN5 protein was ascertained in humans and mice in silico, in mouse retina by immunohistochemistry, and in neuronal cancer cell lines and fractionated central nervous system tissue extracts by Western analysis with eight antibodies targeting different CAPN5 regions.

RESULTS. Most CAPN5 genetic variation occurs outside its protease core; and searches of cancer and epilepsy/autism genetic databases found no variants similar to hyperactivating retinal disease alleles. The mouse retina expressed one transcript for CAPN5 plus those of nine other calpains, similar to the human retina. In Y79 retinoblastoma cells, the level of CAPN5 transcript was very low. Immunohistochemistry detected CAPN5 expression in the inner and outer nuclear layers and at synapses in the outer plexiform layer. Western analysis of fractionated retinal extracts confirmed CAPN5 synapse localization. Western blots of fractionated brain neuronal extracts revealed distinct subcellular patterns and the potential presence of autoproteolytic CAPN5 domains.

CONCLUSIONS. CAPN5 is moderately expressed in the retina and, despite higher expression in other tissues, hyperactive disease mutants of CAPN5 only manifest as eye disease. At the cellular level, CAPN5 is expressed in several different functional compartments. CAPN5 localization at the photoreceptor synapse and with mitochondria explains the neural circuitry phenotype in human CAPN5 disease alleles.

Keywords: CAPN5, calpain, autosomal dominant neovascular inflammatory vitreoretinopathy, ADNIV

Mutation of the calcium-activated protease calpain-5 (CAPN5) can cause a severe blinding disease, autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV, OMIM #193235).1–3 In their twenties, ADNIV patients begin to display a synaptic signaling defect and intraocular inflammation (uveitis). Over the ensuing five decades, they experience retinal degeneration, retinal neovascularization, and intraocular fibrosis, culminating in phthisis and blindness.1–3 Although CAPN5 is expressed in many tissues, ADNIV patients only manifest disease in the eye.4 Autosomal dominant neovascular inflammatory vitreoretinopathy CAPN5 is hyperactive, since the disease allele reduces the calcium level required for protease activity.7 Thus, the eye-restricted phenotype likely reflects the extraordinarily high calcium concentrations in the retina, where such a hyperactive calcium-dependent protease could be particularly damaging.5,5

Increased calpain activity is a feature of many eye-related pathologies, including retinal degeneration,8–15 retinal hypoxia,10–13 retinitis pigmentosa,14–16 retinal detachment,17 and glaucoma.18–19 Retinal damage from these pathologies can be lessened by administering the calpain inhibitor SJA6017.8,20–22 However, since the human retina expresses several calpains, it
is not known which isoform(s) SJA6017 inhibits. Both CAPN1 and CAPN2 are expressed in the retina and show increased activity in other neurodegenerative conditions and hypoxic cell death.8,24 CAPN10 and calpastatin also are expressed in the retina.8,25 CAPN3 expresses a retina-specific splice variant in rats.8,25 Although CAPN3 is linked to limb-girdle muscular dystrophy type 2A,8,26 it is not associated with any known retinal disease. CAPN5, the most distant calpain family ortholog,7 is the only retinal calpain known directly to trigger retinal disease in humans. Inhibition of CAPN5 might be therapeutic, but a specific inhibitor has never been isolated; and sequence analysis shows CAPN5 does not bind calpastatin, the endogenous calpain inhibitor.7,27

To increase our understanding of CAPN5 in the healthy retina and during ADNIV, we characterized CAPN5 mRNA and protein expression in the normal retina. We also drew from rich compilations of genetic-variation expression databases and performed antibody epitope-structure analysis, immunohistochemistry, and subcellular fractionation.

METHODS

Human ADNIV Electroretinogram (ERG)

The collection of data used in this study was approved by the Institutional Review Board for Human Subjects Research at the University of Iowa, was compliant with the Health Insurance Portability and Accountability Act, and adhered to the tenets of the Declaration of Helsinki. A full-field ERG was performed according to the Declaration of international standards. Briefly, the eyes were dilated and dark adapted for 30 minutes. Electroretinograms were recorded simultaneously from both eyes using Burian-Allen bipolar contact lens electrodes as described previously.20 Evoked waveforms, a 100 μV calibration pulse, and a stimulus artifact were recorded on Polaroid film.

RNA Preparation and Next-Generation Sequencing

The Institutional Animal Care and Use Committee (IACUC) approved all experiments. Rodents were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience. Total RNA was extracted from mouse retinas and cell lines using RNAeasy (Qiagen, Hilden, Germany), and submitted to Oto genetics Corporation (Norcross, GA, USA) for RNA-Seq assays. Libraries were sequenced via Illumina HiSeq2000. Paired-end 90 or 100-nucleotide reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK), and analyzed using DNAnexus (DNAnexus, Inc., Mountain View, CA, USA). Gene expression levels for human retina were collected from GEO Omnibus (accession number, GSE40524).

Variant Annotation and Filtering

Variants in the 1000 Genomes, Epik, and Autism datasets were annotated with minor allele frequencies (MAFs) from EVS and database of single nucleotide polymorphisms (dbSNP) using GATK’s VariantAnnotator29 and SNPSift/SNPEFF.30 Non-coding variants, those not passing quality filtering and those with a MAF >2% were removed. CAPN5 variants were downloaded from the Exome Variant Server (EVS) website (available in the public domain at http://evs.gs.washington.edu/EVS/). Only nonsynonymous variants with a MAF <2% in either the EA or AA populations were analyzed.

We obtained vcf files from the database of genotypes and phenotypes (dbGAP) entry for the ARRA Autism Sequencing Collaboration (phs000298). Only those consented for autism research only (AO) were downloaded. The data set(s) were deposited by the ARRA Autism Sequencing Collaborative, an ARRA funded research initiative. Support for the Autism Sequencing Collaborative was provided by Grants R01-MH089208 awarded to Mark Daly, R01-MH089175 awarded to Richard Gibbs, R01-MH089025 awarded to Joseph Buxbaum, R01-MH089004 awarded to Gerard Schellenberg, and R01-MH089482 awarded to James Sutcliffe.

Exome vcf files from the Epik Epilepsy Phenome/Genome Project (EPPG) were requested and downloaded from dbGAP (dbGAP Study Accession, phs000653.v2.p1). Our appreciation goes to the Epilepsy Epik consortium: Discovery in Epilepsy study (NINDS U01-N077303) and the Epilepsy Genome/Phenome Project (EPPG-NINDS U01-N0859398).31,32

Cell Culture, Reagents, and Antibodies

Cell lines HEK293T, SH-SY5Y, and Y79 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Antibodies used were anti-GAPDH antibody (sc-25778), goat anti-rabbit, and goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-CAPN5 polyclonal antibodies (GTX103264; GeneTex, Irvine, CA, USA; 12573-1-AP; Proteintech Group, Inc., Chicago, IL, USA; sc-50500 and sc-271271; Santa Cruz Biotechnology, Inc.) rabbit anti-myc tag antibody (ab9106) and other anti-CAPN5 antibodies (ab28280, ab38943, and ab97534; Abcam, Cambridge, MA, USA).

HEK293T adherent cells were grown in complete Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine system (FBS). SH-SY5Y adherent cells were cultured in a 1:1 mix of RPMI 1640 and F-12 media containing 10% FBS. Human retinoblastoma Y79 cells were grown in RPMI-1640 medium containing 20% FBS. All cultures contained penicillin (100 U/mL) and streptomycin (100 μg/mL).

Western Blot

Levels of CAPN5 were detected by immunoblot. Cells were lysed 24 hours after transfection, and total cellular protein measured using NanoDrop 2000c (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Samples were electrophoretically resolved by 4% to 12% Bis-Tris PAGE (Life Technologies; Grand Island, NY, USA) and transferred to nitrocellulose (iBlot; Life Technologies). Membranes were blocked with 5.0% nonfat dry milk in 1× TBS 0.1% Tween-20. Primary antibodies were diluted 1:1000; secondary antibodies 1:10,000. Immuno reactive bands were visualized by Supersignal West Dura Extended Duration Substrate and a MYECL Imager (Thermo Fisher Scientific, Inc.).

Subcellular Fractionation

Frozen bovine retinas were thawed and gently vortexed in buffer A (50% sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl2) supplemented with a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). After 13,000g centrifugation (1 hour), rod outer segments (ROS) were collected and the pelleted “rest of retina” (ROR) resuspended in Buffer A without sucrose. A centrifugation step (1 hour) to separate soluble proteins (ROR-S1) from membranes (ROR-P1). The ROR-P1 was loaded on a 1.2 M sucrose cushion and centrifuged (200,000g, 30 minutes). Synaptosomes (Syn-S2) were collected from the top of the sucrose cushion; pelleted material was the “rest of membranes” fraction (ROM-P2). Dilutions for antibodies to retinal markers were: anti-RDS (1:50, Per 2B6, gift from Robert Molday); mouse anti-NKA (1:1000, M7-PB-E9, Santa Cruz Biotechnology, Inc.); rabbit anti-
the 1000 Genomes and EVS databases, which contain hyperactive calpain mutants. Linked to increased calpain activity also are caused by ADNIV-like mutations in normal and disease-associated genomes. No significant enrichment was found in either the epilepsy or autism cohorts. Again, this suggests the increased calpain activity associated with epilepsy is not due to hyperactivating CAPN5 mutations.

Increased calpain activity is associated with cancer, and genetic studies link polycystic ovarian and rectal cancers to CAPN5. We first searched for CAPN5 variants in the Catalogue of Somatic Mutations in Cancer (COSMIC) Database, which holds sequences from 1.1 million sample genomes from 43 types of cancer. Only 39 CAPN5 missense variants occurred in the proteolytic core (and two stop codon variants), 27 of which could potentially alter CAPN5 activity (Supplementary Fig. S1). No variants were found in the CAPN5 gating loop and none matched the previously identified ADNIV alleles. These results suggested CAPN5 variants are rare in cancer, so higher calpain activity in cancer cells is not due to hyperactivating CAPN5 mutations.

CAPN5 is highly expressed in the brain, and increased calpain activity has been associated with epilepsy. To determine if rare CAPN5 coding variants could be contributing to the incidence of neurodevelopmental disorders, we queried an epilepsy and autism dataset for mutations in CAPN5 and compared the variant counts in each cohort to EVS and 1000 genomes. No significant enrichment was found in either the epilepsy or autism cohorts. Again, this suggests the increased calpain activity associated with epilepsy is not due to hyperactivating CAPN5 mutations.

**CAPN5 mRNA Is Widely Expressed**

Prior Northern analysis on adult mouse mRNA showed that CAPN5 mRNA was widely expressed, particularly in the brain, eye, uterus, lung, submaxillary gland, prostate, and epididymis. To further evaluate tissue-specific CAPN5 expression, we used two recent online expression databases (Summarized in the Table). Biogps, which tracked mRNA levels in 79 human and 61 mouse tissues, showed that CAPN5 expression is highest in human colon, heart, liver, prostate, and skeletal muscle. Human retinal tissue expressed CAPN5 at levels slightly above average. Mouse expressed CAPN5 highest in the uterus and digestive system, with only low to moderate expression in the eye. The EMBL-EBI gene expression database reported CAPN5 expression in eight humans and 12 mice, and confirmed high expression in the human digestive system. In contrast to the Biogps, however, these studies found significant expression of CAPN5 in mouse brain tissue. This dataset did not track CAPN5 expression in eyes.
TABLE. Gene and Protein Expression of Calpain Family Members in the Retina

| Gene   | Calpain mRNA in Retina | Calpain Protein in Retina |
|--------|------------------------|---------------------------|
| CAPN1  | Human,* mouse,* rat    | Rat,23,45 pig,23,45 human,* monkey,* rabbit8 |
| CAPN2  | Human,* mouse, rat     | Rat,23,45 pig,23,45 human,* monkey,* rabbit8 |
| CAPN3  | Splice variants rat, human, monkey25 | Splice variants rat57 |
| CAPN5  | Human5, mouse1         | Human1, mouse5 |
| CAPN6  | Human,* mouse*        | N/A |
| CAPN7  | Human,* mouse*        | N/A |
| CAPN8  | –                      | N/A |
| CAPN9  | Human,* mouse*        | N/A |
| CAPN10 | Splice variants human, mouse24 rat49 | Splice variants rat24 |
| CAPN11 | Human,* mouse*        | N/A |
| CAPN12 | Human,* mouse*        | N/A |
| CAPN13 | N/A                    | N/A |
| CAPN14 | N/A                    | N/A |
| CAPN15 | N/A                    | N/A |
| CAPN16 | N/A                    | N/A |

N/A, not applicable. * Current study.

The Retina Expresses Several But Not All Calpain Family Members

We applied RNA sequencing to compare expression of CAPN5 mRNA to that of other calpains in the mouse retina (Fig. 2). Among the 10 calpain genes (CAPN1, 2, 3, 5, 6, 7, 10, 12, CAPN5, and CAST), CAPN5, 7, 10, 2, and CAPN15 were the most highly expressed (Fig. 2A). Interestingly, except for CAPN2, these variants do not contain an EF-hand domain (a calcium binding, helix-turn-helix motif) believed to mediate protein-protein interactions of the most abundant calpains.8 The mouse retina expressed only the full-length CAPN5 transcript, as we reported in humans.1,5 Expression of CAPN5 measured a mean of 0.003259 RKPM, approximately half that of the most abundant calpains, CAPN2 and CAPN7 (Fig. 2A). The relative expression level of CAPN5 RNA was four orders of magnitude less than that of the highly expressed phototransduction genes (Fig. 2). Our transgenic mouse model of ADNIV shows a less severe inflammatory phenotype than humans,5 but here we found that the calpain family mRNA expression pattern in mouse and human retina was similar (Fig. 2B). There were only minor differences between human and mouse. CAPN1, CAPN52, and CAST were higher in human retina (Figs. 2B, 2C).

The human retinoblastoma Y79 cell line offers an in vitro model of photoreceptors. In these cells, CAPN5 was expressed at significantly lower levels (5.580e-005 ± 8.391e-005); otherwise, the pattern matched the human and mouse retinas where CAPN2 and CAPN7 were the most expressed calpains (Fig. 2C).

Calpains mediate inflammatory necrotic cell death and frequently are compared to caspases, which mediate apoptosis.62,63 Eight members of the caspase family were expressed in the retina at levels similar to calpains, which suggests the retina is not biased towards a specific cell-death pathway.

CAPN5 Protein Is Widely Expressed

The Human Protein Atlas describes protein expression across 80 major tissues and organs using antibody-based methods and transcriptomics (summarized in the Table).64,65 CAPN5 protein was detected in 22 distinct normal tissues, mirroring data from the mRNA expression databases. Notably, the Human Protein Atlas data also described equivalently strong expression of CAPN5 in the kidney, bladder, testes, prostate, skin, lungs, and endocrine organs, and moderate expression throughout the digestive tract. CAPN5 also was detected in several brain regions (i.e., cerebral cortex, hippocampus, lateral ventricle, and cerebellum). CAPN5 protein was detected in the cytoplasm, nucleolus, and cell membrane. Limitations of this dataset were the lack of eye tissues and use of only one CAPN5 antibody.

CAPN5 Detection in Neuronal Cells Varies by Antibody

Seven commercial CAPN5 antibodies were tested in Western blots of HEK-293T cells expressing recombinant CAPN5 cDNA. Antibodies had been generated against different CAPN5 regions (Fig. 3B). Antibodies Ab-2, -6, and -7 showed distinct CAPN5 immunoreactive bands. The strongest signal with the least background was detected by Ab-7, which was generated against CAPN5 domains 1, II, and the first 38 amino acids of domain III (Fig. 3C). This antibody did not crossreact with other calpain family members (data not shown). The remaining antibodies detected CAPN5, but gave high background and nonspecific bands (data not shown). Interestingly, two neural cancer lines, SHSY5Y neuroblastoma and Y79 retinoblastoma cells, express endogenous CAPN5 and showed lower molecular weight species not present in transfected HEK-293T cells (Fig. 3D). Since self-cleavage is a known feature of other calpains,51 and calpain autoproteolysis releases a catalytic protease core, the approximately 50 kDa species detected in SHSY5Y and Y79 cells could represent the protease core (domain II) of autoproteolyzed CAPN5. This suggests CAPN5 may undergo a basal level of cell-line specific autoproteolysis, which could impact expression studies.

In Western blots of extracts from mouse and human retina, Ab-7 detected endogenous CAPN5 species that appeared to be the full-length protein1,5 (Fig. 3E); the retinal extracts did not contain low molecular weight species like those found in cancer cells, suggesting calpain autoproteolytic activity is not a feature of the normal healthy retina. Interestingly, Ab-7 also recognized Tra-3, the distant Caenorhabditis elegans paralog of CAPN5 (Fig. 3E) despite that an alignment of Tra-3 with human CAPN5 showed less than 40% identity (Fig. 4A).

Together, these findings suggest that for optimal Western analysis of CAPN5, the best antibodies were polyclonal and likely targeted conformational epitopes in all four domains.66 To test this, a custom antibody was generated using a polypeptide comprised of sequences from all four human CAPN5 domains that were homologous to Tra-3, distinct from other retinal calpain family members, and predicted to be on the CAPN5 surface (according to our published structural model: Figs. 4B, 4C and Supplementary Fig. S2).5 This recombinant antigen was expressed in Escherichia coli, purified, and injected into rabbits to yield polyclonal Ab-8 (Supplementary Fig. S2). Antibody Ab-8 detected CAPN5 via Western blot at levels comparable to Ab-7 (Fig. 4D). Overall, these results suggest CAPN5 antibodies raised against different regions also might show differences in vivo, an important consideration when interpreting CAPN5 expression.

CAPN5 Is Expressed in Several Regions of the Mouse Retina

The retina contains several cell types in layers of highly organized circuits. Previously, we reported CAPN5 was expressed in the inner and outer segments (IS and OS,
respectively) of human and mouse photoreceptors using Ab-2.1,5 Probing the mouse retina with Ab-4, however, revealed that CAPN5 also was modestly expressed in the outer plexiform layer (OPL).5 With these disparities, we used the newly characterized Ab-7. This labeled the photoreceptor inner segments as well as the OPL, where photoreceptors form synaptic connections with bipolar cells and others in the inner nuclear layer (INL; Fig. 5). CAPN5 colocalized with PSD-95, a protein expressed specifically in photoreceptor synapses, where a signaling defect could correlate with the electronegative ERG found in ADNIV patients (Fig. 5). Additionally CAPN5 was detected at a lower level in some, but not all retinal ganglion cells (RGC). Lower levels of CAPN5 were detected in the INL and inner plexiform layer (IPL; Fig. 5). Finding CAPN5 localized to these sites suggests a role in synaptic transmission.

**CAPN5 Localizes to Photoreceptor Synapses**

To explore CAPN5 subcellular localization, retinal extracts were fractionated and analyzed by Western blot (Fig. 6). Rod outer segments were first isolated by sucrose flotation. CAPN5 was detected in the crude ROS fraction. The remaining retinal proteins (ROR) were separated into soluble (ROR-S1) and membrane (ROR-P1) fractions. CAPN5 was detected largely in

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**Figure 2.** RNA sequence analysis of human and mouse retina. (A) Calpain expression in the mouse retina, as compared to levels of caspase expression, phototransduction genes, and housekeeping genes as controls (n = 3). Results show CAPN5 expression is approximately half that of the most abundant calpains, CAPN2 and CAPN7. Calpain expression is comparable to caspase expression in the mouse retina. Expression of CAPN5 is several orders of magnitude less than phototransduction genes and control genes. (B) Slightly different patterns of expression are seen in the human retina (n = 3). (C) Calpain expression observed in Y79 cells as compared to levels of caspase expression, phototransduction genes, and controls (n = 3). Similar to human and mouse retina, CAPN2 and CAPN7 are the most abundant calpains in Y79 cells, but CAPN5 expression is lower.
**FIGURE 3.** CAPN5 Western blot shows specificity of multiple CAPN5 antibodies. (A) Schematic diagram of human CAPN5 protein domain structure. CAPN5 is composed of 640 amino acids that comprise four domains (A). Domain-I spans amino acid residues 1 to 25. Domain-II is the protease core and spans amino acid residues 26 to 343; it consists of two subdomains that together contain the catalytic triad and calcium binding sites. Domain II also contains the three different ADNIIV-causing mutations.3 Domain-III spans amino acid residues 344 to 496 and contains a calcium-binding site leading to speculation that it is a regulatory region. Domain-IV (C2) also is believed to be regulatory; it spans amino acid residues 518 to 619, and contains three calcium-binding sites. (B) A diagram showing the epitopes of the seven different commercially available anti-CAPN5 antibodies tested, as well as the companies they were obtained from. Antibody Ab-1 (ab28280) mainly targeted domain I; Ab-2 (sc-50500) and Ab-3 (sc271271) targeted all of domain I, and part of domain II (catalytic domain); Ab-4 (ab38943) targeted the entire domain II and part of domain III; Ab-5 (12373-1-AP) and Ab-6 (ab97534) targeted domains I, II, and part of III. All antibodies were rabbit polyclonal antibodies, except for Ab-3, which was a mouse monoclonal antibody. (C) Immunoblots of HEK-293 cells transfected with full-length CAPN5 (tagged with c-myc and flag tag) and probed separately with three unique anti-CAPN5 antibodies. Anti-GAPDH antibody was used as a protein loading control. Immunoblot images were captured with an ECL imager. Antibody Ab-7 consistently gave the best bands with the least amount of background, indicating higher specificity for CAPN5. (D) Immunoblots of HEK-293, SY-SH5Y, and Y79 cells transfected with full length CAPN5 and probed with Ab-4. (E) Immunoblots of retinas belonging to human and mice were probed separately with CAPN5 specific Ab-7. Immunoblot of protein obtained from wildtype (N2) *C. elegans*. 10 μg of protein were loaded.
FIGURE 4. Customized antigen raises highly specific CAPN5 antibodies. (A) Alignment of CAPN5 and Tra-3 sequences. Regions of homology are shown in black. Regions corresponding to the customized CAPN5 antigen are underlined with colors corresponding to the different CAPN5 domains. (B) A structural model of CAPN5. The highlighted regions correspond to those recognized by our custom antibody. (C) A diagram showing the epitopes of our custom anti-CAPN5 antibody. (D) Immunoblots of HEK-293 cells transfected with full-length CAPN5 and probed with our custom anti-CAPN5 antibody. We used GAPDH as a protein loading control. Preimmune-serum obtained from the same animal was used as a negative control. The closed arrows indicate CAPN5 bands at approximately 75 kDa, while the open arrowheads indicate GAPDH bands at approximately 37 kDa. Immunoblot images were captured with an ECL imager.
the soluble pool (Fig. 6). Interestingly, CAPN5 also was detected in the membrane fraction that is enriched for synaptosomes (Syn-Soluble, Fig. 6). This analysis was consistent with immunohistochemical localization of CAPN5 to photoreceptor synapses. Expression at these synapses explains a defining feature of ADNIV, loss of the electroretinogram b-wave (see Discussion).

**Subcellular Localization of CAPN5 in the Rat Brain**

The compartmentalization of CAPN5 in the retina suggests that CAPN5 function might be regulated by subcellular localization. To examine localization further, we used rodent brain cells as a source of material, since the brain expresses more CAPN5 than the retina, and brain tissue is far more abundant. Brain extracts were fractionated and probed with CAPN5 Ab-1, -2, and -7. In parallel, fractions also were probed with a panel of antibodies that marked various subcellular compartments (Fig. 7).

As seen in cultured neural cells, the brain expressed multiple species of CAPN5 immunoreactivity, potentially reflecting autoproteolysis. Comparing expression patterns on Western blots, as detected by Ab-1, -2, and -7 (all polyclonal), we noticed each antibody gave a distinct pattern. Antibody Ab-1, which targets the 50 N-terminal amino acids, primarily recognized the smaller, more processed forms of CAPN5, while Ab-1 and Ab-2, which targets Domain I and part of Domain II, appeared to recognize both full-length and proteolytically processed forms, but the electrophoretic mobility of the high molecular weight forms were the same as those detected by Ab-1. Antibody Ab-7, which targets full-length CAPN5, recognized few approximately 50 kD species, preferring the full-length form. Thus, the presence of smaller species could indicate cellular compartments with greater CAPN5 proteolytic activity or the accumulation of the autoproteolyzed CAPN5 proteolytic core (~50 kD).

The patterns on Western blots suggest the different antibodies preferentially recognized different forms of CAPN5. The banding patterns indicate Ab-2 recognized the full-length and proteolytically processed forms of CAPN5, while Ab-1 and Ab-7 preferentially recognized the processed and full-length forms, respectively. The blots show the 50 kD form was enriched for in a crude synaptosomal/mitochondrial fraction (syn-mt). The link between CAPN5 and the synapse and mitochondria has interesting implications, since mitochondria are highly enriched in the synapse due to energy needs associated with neurotransmitter loading and release. Moreover, synapses and mitochondria are rich in calcium stores, which might activate CAPN5 and trigger autoproteolysis.

**DISCUSSION**

The calpains are calcium-dependent, intracellular, cysteine proteases that proteolytically process targets into new functional forms. Calpains are regulatory proteases and have been linked to a broad spectrum of cellular processes, including cell death, cell movement, sex determination, and intracellular signaling. Excess, uncontrolled calpain activity is implicated in cancer pathogenesis, muscular dystrophy, diabetes, and a number of neurologic diseases, such as multiple sclerosis, Alzheimer’s disease, Huntington’s disease, and traumatic brain injury. Compared to the rest of the calpain family, CAPN5 is especially interesting because it is the only known calpain with a human disease mutation that renders the protease intrinsically hyperactive.

Autosomal dominant neovascular inflammatory vitreoretinopathy phenotypically overlaps with several other eye diseases. For example, electronegative ERGs also are observed in retinoschisis, quinine toxicity, and congenital stationary night blindness (CSNB). Interestingly, CSNB and retinal degeneration in the rd1 mouse are associated with excess calpain activity. Additionally, the proliferative retinal neovascularization in ADNIV patients has been confused with diabetic retinopathy, but ADNIV patients are not diabetic and do not show retinal capillary nonperfusion or other signs of retinal or ocular ischemia. Instead, the proliferative changes are likely driven by uveitic inflammatory signals. It is interesting to speculate that calpains might be active in shared downstream pathways.

The expression of CAPN5 varies in abundance and in the appearance of proteolytically processed forms. Since our custom polyclonal CAPN5 antibody was raised to domains unique to CAPN5 that represented surface epitopes across the entire protein, it would be expected to recognize all forms of CAPN5 and all fragments that might be generated by autoproteolysis. Indeed, when interpreting immunohistochemical studies of calpains and their proteolytic targets, it is important to note that the various proteolyzed (or autoproteo-
lyzed) forms would be expected to have different surface epitopes. The different patterns seen with different antibodies might reflect unique autoproteolytic domains of CAPN5, like that seen in the subcellular fractionation experiments.

In the retina, an examination by IHC using different antibodies revealed patterns of CAPN5 subcellular localization. In photoreceptors, for example, CAPN5 is in the outer segment,1,5 where light is converted into cellular signals; the nucleus, where transcription takes place; and the synapse, which transmits signals to the inner retina. Newly available antibodies revealed expression in the inner nuclear layer and ganglion cell layer. In ADNIV patients, however, neither the RGCs nor the optic nerve degenerate. Instead, similar to patients with retinitis pigmentosa, ADNIV patients lose peripheral vision coincident with degeneration of their peripheral photoreceptors. Nevertheless, electrophysiologic testing of the optic nerve with pattern ERG (PERG), for example, may be worthwhile, since PERG can assess macular ganglion cell function. In addition, future immunohistochemistry studies using RGC markers might identify the subtypes of RGC cells expressing CAPN5.

Calpain proteolysis is linked with synaptic function.81 Since the known calpain substrates include cytoskeletal proteins, membrane receptors, and postsynaptic-density proteins, calpain proteolysis of these substrates might be a feature of normal synaptic function.81 Pharmacologic inhibition shows calpains have key roles in many neuronal processes including neurotransmitter release and signal transduction.81 The high expression of CAPN5 we observed in the photoreceptor synaptic layer (OPL) and mitochondria supports a role for CAPN5 at synapses. This is interesting because it may explain the development of early ADNIV electronegative ERG where loss of the b-wave is a sign of defective inner retina neurotransmission.

The genomic survey was designed to help uncover why ADNIV CAPN5 alleles cause retinal disease, and whether such alleles cause other diseases linked to high calpain activity. Our PolyPhen predictions of CAPN5 variants in genome databases suggest damaging alleles arise at a very low frequency, implying they are highly detrimental. More specifically, we found very few protease-core variants, which are likely to be more damaging than variants in the N-terminal or C-terminal regions. Increased calpain activity has been associated with cancer and epilepsy, but we found few CAPN5 mutations, suggesting that in those diseases calpain activity is elevated for reasons other than calpain mutations while the current CAPN5 variants only display retinal disease.
Even though we found calpain mRNA expression patterns were similar between mouse and humans, our previously reported ADNIV mice had a less severe form of the disease; this may happen for several reasons. For example, in one mouse model CAPN5 was expressed from a retroviral vector, which gave a transient and patchy expression pattern in the host retina. In a second, transgenic mouse model, the ADNIV phenotype might be ameliorated by the presence of two wild-type alleles. In both models, expression is restricted to photoreceptors and not other retinal cells where CAPN5 is natively expressed. It also is possible that the short mouse lifespan ends before the disease can develop into full-blown ADNIV, since humans do not typically display severe disease until their fourth decade or later. To distinguish the possibilities from species differences, we will have to generate knock-in mice that replace the normal alleles with disease alleles under the native promoter.

Although ADNIV only affects the eye, CAPN5 mRNA was expressed at only slightly above average levels in the retina and at far higher levels in the colon. Nevertheless, to date no ADNIV patients have gastrointestinal disorders. These data support the idea that CAPN5 is uniquely regulated and/or serving a highly specialized role in the retina. For example, ADNIV CAPN5 mutations might specifically cause a retinal phenotype because photoreceptors use extremely high calcium levels for phototransduction. High calcium could not only activate CAPN5 transiently, but render it permanently hyperactive by autophosphorylytically cleaving out an active fragment that is free of regulatory domains. Alternatively, in the eye, the uniquely slow turnover rate of cells might allow retinal damage to accumulate and become more severe than in a tissue with a faster turnover rate (e.g., the digestive tract). Regardless of the mechanism that restricts the clinical disease to eye tissue, the subcellular localization of CAPN5 indicates it likely targets several different proteins with nonoverlapping functions. This would account for the complex ocular phenotype. Thus, the next important step for understanding ADNIV and devising new therapies will be to identify the proteolytic targets of CAPN5.

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