Targeted mutation of the gene encoding prion protein in zebrafish reveals a conserved role in neuron excitability

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The function of the cellular prion protein (PrP⁹) in healthy brains remains poorly understood, in part because Prnp knockout mice are viable. On the other hand, transient knockdown of Prnp homologs in zebrafish (including two paralogs, prp1 and prp2) has suggested that PrP⁹ is required for CNS development, cell adhesion, and neuroprotection. It has been argued that zebrafish Prp2 is most similar to mammalian PrP⁹, yet it has remained intransigent to the most thorough confirmations of reagent specificity during knockdown. Thus we investigated the role of prp2 using targeted gene disruption via zinc finger nucleases. Prp2⁻/⁻ zebrafish were viable and did not display overt developmental phenotypes. Back-crossing female prp2⁻/− larvae were found to have increased seizure-like behavior following exposure to the convulsant pentylenetetrazol (PTZ), as compared to wild type fish. In situ recordings from intact hindbrains demonstrated that prp2 regulates closing of N-Methyl-D-aspartate (NMDA) receptors, concomitant with neuroprotection during glutamate excitotoxicity. Overall, the knockout of Prp2 function in zebrafish independently confirmed hypothesized roles for PrP, identifying deeply conserved functions in post-developmental regulation of neuron excitability that are consequential to the etiology of prion and Alzheimer diseases.

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Introduction

Loss-of-function approaches that target disease-related genes have taught us much about the function of the cognate proteins in healthy brains, often leading to basic insights that inspire new therapeutic strategies. Despite much work on disease processes, the function of the prion protein in healthy brains has remained largely enigmatic. This is due in part to the lack of an overt developmental phenotype when Prnp is knocked out in mice (Bueler et al., 1992; Manson et al., 1994). On the other hand, several reports detail a phenotype when PrP homologs are transiently disrupted in zebrafish (Kaiser et al., 2012; Malaga-Trillo et al., 2009; Nouriadbeh-Lillabadi et al., 2010), providing opportunity for insights into PrP’s normal biological function.

Knockout of Prnp in mice produces viable animals with normal development (Bueler et al., 1992). This result suggested that gene redundancy with other prion-family genes such as Shadoo (Sprn) might exist, but recent results showing normal development in Prnp⁻/−; Sprn⁻/− mice refute this (Daude et al., 2012). Phenotypes have been revealed during detailed analysis of adult Prnp knockout mice (Khalife et al., 2011; Steele et al., 2007). These include susceptibility to neuron death during insult with toxins or hypoxia, leading to the suggestion that PrP⁹ has a role as a neuroprotectant factor. Additionally, loss of PrP⁹ in mice leads to a susceptibility to seizures (Carulla et al., 2011; Rangel et al., 2007, 2009; Walz et al., 1999). The mechanisms whereby PrP⁹ can affect neuroprotection and seizures remain largely undefined, but could relate to a role for PrP in metal homeostasis, especially with respect to N-Methyl-D-aspartate (NMDA) channels (Khosravani et al., 2008a; You et al., 2012). Loss of homeostatic control of divalent metals at NMDA channels could accord with both excitotoxicity and increased neuron excitability/seizures (Stys et al., 2012).

In part because mouse Prnp knockouts have been intransigent towards relinquishing functions of PrP, other model systems have been interrogated. Transient knockdown of Prnp homologs in zebrafish has led to intriguing results from several research groups. Zebrafish possess two paralogs of the prion protein gene, prp1 and prp2. Both predict protein products that are substantially larger than mammalian PrP⁹, and thus comparisons on a sequence or amino acid level are difficult to interpret. However the zebrafish homologs share all the identified protein domains and post-translational modifications of mammalian...
PrP (Figs. 1C, S1), are glycosylated and are attached to the membrane with a GPI anchor when expressed in mammalian cell culture (Miesbauer et al., 2006). Further, at least prp1 can be replaced by mammalian homologs to recover normal development (Kaiser et al., 2012; Malaga-Trillo et al., 2009), arguing for a substantial conservation of function.

To date, knockdown of prp1 or prp2 has been accomplished with morpholino oligonucleotide (MO) technology, which uses modified 25mer oligonucleotides expected to transiently block mRNA translation of the target gene. These reagents have been injected into embryos immediately after fertilization and are generally believed to be partially efficacious for approximately 3–4 days of development. This is sufficient time for zebrafish, which hatch after two days, to develop a functioning nervous system, but it remained unclear if more complete or longer-term disruption of prion gene(s) in zebrafish might reveal additional phenotypes. These works repeatedly demonstrated that substantial developmental deficits are induced by transient knockdown of prp1 (Kaiser et al., 2012; Malaga-Trillo et al., 2009) or prp2 (Malaga-Trillo et al., 2009; Nourizadeh-Lilhabadi et al., 2010), including decreased cell adhesion, apoptotic cell death, arrested gastrulation, and impaired CNS development. Our recent work deployed zebrafish genetics and biochemistry of human homologs to link prp1 with amyloid-β precursor protein (APP) in a niche role leading to CNS cell death (Kaiser et al., 2012). Thus data from zebrafish were consistent with an emerging function for PrP in excitotoxic cell death during Alzheimer disease progression as had been suggested by others (Lauren et al., 2009) and implicated a conserved role at synapses (Kaiser et al., 2012).

Intrigued by this collection of results, we sought to gain further insights into prion biology using an independent approach to disrupt the function of prion genes in zebrafish. Zebrafish prp2 was prioritized for analysis because it is akin to mammalian homologs regarding abundant expression in the post-embryonic CNS (results herein and see Cotto et al., 2005; Malaga-Trillo et al., 2009), whereas the expression of its paralog prp1 is primarily limited to early development (Cotto et al., 2005; Malaga-Trillo et al., 2009). Furthermore, it was noted that morpholino knockdown reagents against prp2 failed to meet the most stringent tests of reagent specificity (e.g. rescue of phenotypes with cognate mRNA; see results herein, Malaga-Trillo et al., 2009), whereas MO reagents against prp1 appear to be robust in this regard (Kaiser et al., 2012; Malaga-Trillo et al., 2009). Phenotypes were documented following targeted disruption of prp2 via engineering of a custom zinc finger nuclease (ZF). ZFNs are an emergent technology that, by analogy, represent a custom restriction enzyme technology that, by analogy, represent a custom restriction enzyme designed to cut the target gene in vivo with good specificity; repair of the cut is error-prone and resultant insertions or deletions are heritable, producing an improper translation of the target protein. The mutant fish generated herein demonstrate that prp2 disruption in zebrafish does not induce overt developmental phenotypes and revealed viable adult zebrafish, including larval with increased susceptibility to seizure-inducing drugs and coordinately disrupted NMDA receptor kinetics.

Materials and methods

Zebrafish husbandry and strains

Zebrafish of the AB strain were used as the wild type strain in this study, and this background was used for generation of prp2 mutants. In some instances, Tg(gfp:GFP)mi2001;mitfa w2/w2;roya9/a9 fish were used to examine CNS morphology in transparent ‘Casper’ fish (combining mutant and transgenic lines ZDB-GENO-060623-2 (Bernardos and Raymond, 2006), with ZDB-ALT-990423-22 and ZDB-ALT-980203-444 (White et al., 2008)). Adult fish were maintained at 28 °C under a 14/10 h light/dark cycle and bred as described previously (Westerfield, 2000). Larvae were raised in E3 medium [containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4]. All protocols were approved the University of Alberta's Animal Care and Use Committee: Biosciences under the auspices of the Canadian Council on Animal Care.

ZFN target site selection and cloning of constructs

The zinc finger nuclease (ZFN) targeting methodology described previously (Meng et al., 2008) was adapted to create heritable mutations in zebrafish prp2 (ZFin ID number ZDB-GENE-0412213-3). The zebrafish prp2 target site (5′ cCACCTCCCTacctgTGCTGGAGg 3′, with ZFNs binding to the letters in capitals) was selected from a list of potential sites provided by the online algorithm which can be found on the website http://pgfe.umassmed.edu/ZFPresearch.html, based on the number of guanines, library diversities and other criteria described in the Results. Cloning of single finger oligos and target sites is described in the Supplemental Methods. An overview of the zinc finger nuclease selection is presented in Fig. S2 and described in the Results.

Bacterial one-hybrid selection of ZFNs

Library constructs and their corresponding target site constructs were co-transformed into the bacterial strain USO hisB- pyrF- rpoZ- (Addgene Plasmid 18049) using electroporation. Transformed cells were adapted to NM medium lacking histidine through several washes (Meng et al., 2008). Serial dilutions were spotted onto LBkana/carb plates to determine transformation efficiency and the rest split up onto NMkana/carb plates lacking histidine, one containing a low concentration and the other containing a high concentration of 3-AT (typically in a range between 0.5 and 30 mM) to assay selection. Growth of colonies was assessed after 2–4 days, and if selection occurred (as indicated by observing fewer colonies on more stringent plates), 10–20 colonies were sequenced using the univF1 forward primer (see Supplemental Methods) to assess if selection had occurred.

ZFN combined library assembly and final selections

For each individual finger selection, approximately 100 colonies were picked, cultured for 2–3 h at 37 °C and DNA was prepared using a maxiprep kit (Qiagen # 12163). Library pools were amplified by PCR using phusion DNA polymerase (Thermo Scientific) and the univFx_forward/reverse primers (see Supplemental Methods). The following PCR program was utilized: 1 min initial denaturation at 98 °C followed by 40 cycles of 98 °C for 20 s, 58 °C for 15 s, 72 °C for 15 s plus a final extension at 72 °C for 10 min. Amplification products were run on a 1.5% agarose (TAE) gel, and bands of the appropriate size were excised and purified using a gel extraction kit (Qiagen 28706). In a second step, single finger pools were assembled with overlapping PCR using phusion DNA polymerase (Thermo Scientific) and the univF1_forward plus univF3_reverse primers (see Supplemental Methods and (Meng et al., 2008)). PCR products were again purified from an agarose gel and cloned into the 1352wUV2-kpnI-1bamH1-stop vector (Addgene Plasmid 20704) using BamHI and KpnI. Assembled library diversities were expected to be about 106 (as a result of randomly combining 100 colonies from each of F1, F2 and F3).

Combined library selections were performed as described above, however slightly higher 3-AT concentrations were used. Between 15 and 30 colonies were picked, their DNA extracted and sequenced to determine the optimal ZFN sequence binding to the specific DNA sub-site.
PrP2 transcript localization by in situ hybridization

Antisense riboprobe against the prp2 transcript was generated by amplifying the template using primers that included a transcriptase binding site (5′-CGATTTAATACGACTCACTATAGGGTGAATGTCTAGTTAGAGG GA-3′ and 5′-ATGGTGCTTAAACACTATTTG-3′). This 1688 bp PCR product was used to template the production of DIG-labeled antisense riboprobe. Template for sense riboprobe control amplified the same region (primers 5′-TGAGAATGCATGTAAGAGGA TGTAAGAGGA-3′ and 5′-CATGATTAAATACGACTCACTATAGGGTGAATGTCTAGTTAGAGG TACTATTG-3′). Embryos or larvae were fixed in 4% paraformaldehyde with 5% sucrose in 0.1 M phosphate buffer pH 7.4 and stored at −20 °C in MeOH. Hybridization methods were as previously described (Allison et al., 2010), except that excess riboprobe was incubated on the tissue for 48 h at 65 °C, and signal development utilized 1:5000 anti-DIG-AP (Roche # 11093274910) and NBT and BCIP (4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate, Roche # 11383213001 and 11383221001).

Immunohistochemistry and staining

Zebrafish at the various ages indicated were fixed in 4% paraformaldehyde with 5% sucrose in 0.1 M phosphate buffer pH 7.4. Immunohistochemistry was performed using standard protocols on intact larval fish or on material cryosectioned at 10 μm as reported previously (Fraser et al., 2013). Fixed fish were washed sequentially with water, −20 °C acetone, water and phosphate buffered saline pH 7.4 (PBS). Blocking was in 10% normal goat serum in PBS with 0.1% TritonX-100. Primary antibodies applied were SV2 (previously established to label the synaptic vesicles and motor neurons in zebrafish, (Yazulla and Studholme, 2001), Developmental Studies Hybridoma Bank, ZDB-ATB-081201-1) and 3A10 (previously established to label a neurofilament, Mauthner neurons, and other subsets of neurons in the larval zebrafish (Hatta et al., 1991), Developmental Studies Hybridoma Bank, ZDB-ATB-090108-4). Primary antibodies were diluted 1:100 in PBS with 0.1% TritonX-100 and 2% normal goat serum. Secondary antibody was anti-mouse conjugated to AlexaFluor 555 (1:1000 Invitrogen #A22283) diluted in 1:100 PBS with 0.1% Tween was used to stain nuclei. Phalloidin conjugated to AlexaFluor 546 (1:200 Invitrogen #A12379) and 12.8 megapixel digital camera (DP2T, Olympus). Images were cropped/positioned in Powerpoint 2008 for Mac (Microsoft) and merged or manipulated for brightness and contrast in Zen confocal software (Zeiss), Photoshop CS3 and/or Imaris × 64 7.4.0 (Bitplane).

Knockdown of prp2 by delivery of morpholino oligonucleotide

Transient knockdown of prp2 was performed by injection of morpholino oligonucleotide (MO) as previously described (Kaiser et al., 2012) using an MO directed against the 5′ untranslated region of the prp2 gene (5′-CCT CGG CCT GGT TTA AGG GAG TCC TCC-3′, annotated at ZFin as ZDB-MRPHLNO-100423-7 from Malaga-Trillo et al., 2009). The binding site of this MO is contiguous with the ATG codon, and is thus very similar to the knockdown reagents in another study (shifted by 2 bp compared to “prp2-MO1”, i.e. ZDB-MRPHLNO-101115-1) (Malaga-Trillo et al., 2009; Nourizadeh-Lilabadi et al., 2010). This MO was delivered to 1- or 2-cell stage zebrafish embryos at 4 ng dose, with or without 3 ng of p53 morpholino (5′-CCG CCT GGT TTA AGG GAG TCC TCC-3′, ZDB-MRPHLNO-070126-7 (Langheimrich et al., 2002)) as indicated. In some instances, 225 pg of prp2 mRNA was included in the injection solution, synthesized from a cDNA (accession # JQ994490) as described previously (Kaiser et al., 2012). A standard control MO (5′-CCT CTG ACC TCA GTT ACA ATT TAT A-3′) was delivered in some instances.

Determining relative transcript abundance using RT-qPCR

Experiments were performed in accordance with the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (Bustin et al., 2009).

RNA isolation was performed on samples consisting either of pools of embryos/larvae (n = 15–30 individuals per pool, each pool typically collected from independent clutches of fish) at the age indicated or of individual brains from adult zebrafish. Samples were stored in RNAlater (Ambion AM7021) at 4 °C until RNA extraction. Total RNA was extracted from pools of embryos using the RNAeasy Kit (Qiagen 74104) and manufacturer’s protocols using 600 μL of Buffer RLT containing 1% β-Mercaptoethanol and homogenizing with a rotor stator homogenizer (VWR 47747-370), and on-column DNA digestion was performed using Qiagen DNase I. Total RNA was extracted from adult brain by homogenizing in 1 mL of QIAzol lysis reagent (VWR 47747-370), on-column DNA digestion and using an RNA easy Lipid Tissue Mini Kit (Qiagen 74804). Absence of genomic DNA contamination in each RNA preparation was assessed by examining melt curves and by using primers against APPb exons 3 and 4 (5′-GCCAACACCCGTACCCAT-3′ and 5′-GCACGTGTTAGT GACCTCG-3′); If genomic DNA had been present, a 477 bp band would have been detected in addition to the constitutive 205 bp mRNA band. RNA quantity was determined using a Nanodrop spectrophotometer (GE Healthcare 28 9244-02). All of the samples had similar mRNA profiles and RNA integrity numbers (RIN values of 8.4 or greater when reported) as determined using an Agilent RNA 6000 NanoChip and Agilent 2100 Bioanalyzer.

cDNA was generated using a Long Range 2 Step RT-PCR Kit (Qiagen 205922) using oligo dT primers and Long Range Reverse Transcriptase. The reaction volume was 20 μL, with either 1 μg total RNA or a no template control.

Quantitative PCR was performed using a 7500 Real-Time PCR system (ABI Applied Biosystems). Transcript abundance for prp2 as well as prp1, appa, appb, p53, and cng1, was assessed relative to β-actin and eif1α. The latter two reference genes were chosen based on past literature optimizing RT-qPCR in zebrafish (Casadei et al., 2011; Tang et al., 2007). RT-qPCR primers were designed using Primer Express 3.0 and verified with a standard curve. Primers were designed in the 3′ exons of each gene, and are expected to amplify all splice variants in all genes except p53 (Table S4). Primer sequences and efficiencies are available in Supplementary Tables S4 and S5, respectively. Melting dissociation curves were performed to assess primer specificity, and the presence of a single peak indicated that there was a single product for each primer set and no primer dimers. RT samples were subsequently analyzed at a 24-fold dilution with Ct values ranging from 15 to 30. qPCR reactions were performed with 3 technical replicates of each biological replicate (3–4 biological replicates, each consisting of either separate pools of embryos or tissues dissected from an individual adult fish). Individuals completing these experiments were blinded to the genotype and MO-treatment of the samples prior to beginning the RNA isolation protocol.

Each reaction contained 5 μL Dynamite Master Mix (Molecular Biology Service Unit, University of Alberta, including SYBR Green and platinum Taq hot start enzyme [Invitrogen]), 2.5 μL of pre-mixed primer set, and 2.5 μL cDNA for a total volume of 10 μL. After an initial denaturation step (2 min at 95 °C), cycling consisted of 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. One cycle for melting dissociation curve analysis followed these 40 cycles and consisted of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and finally 60 °C for 1 min. Data analysis was performed using 7500 Software for 7500 and 7500 Fast Real Time PCR Systems version 2.0.1 (AB Applied Biosystems) with auto Cq calling. Transcript abundance was normalized to eif1α and reported with β-actin.
levels normalized to 1. Relative fold change in transcript abundance was analyzed by t-test performed with SYSTAT 12 (Chicago, IL) on the resulting RQ values.

Behavioral analysis of PTZ-induced seizures

Established assays (Baraban et al., 2005, 2007; Bustin et al., 2009) measuring stage I and II seizures were performed. Behavioral tracking software quantified the movement of zebrafish larvae arrayed in a 96-well plate. For trials involving 5 days post fertilization (dpf) *prp2*<sup>−/−</sup> mutants and siblings, larval fish were first acclimatized in E3 medium for 20 min and then subjected to 2.5 mM PTZ (pentylenetetrazol, Sigma-Aldrich #P6500) for 20 min to analyze the response to the convulsant (Baraban et al., 2005, 2007; Bustin et al., 2009). Some trials were performed on embryos from breeding of *prp2*<sup>−/−</sup> fish followed by genotyping the prp2 locus, whereas other trials involved breeding *prp2*<sup>−/−</sup> fish and comparing them to AB fish (the background used to generate the ua5001 allele). For trials involving 3 dpf larvae, fish were acclimatized in their typical growth media (E3 medium) for 30 min and subsequently subjected to 2.5 mM PTZ for 30 min. Swimming behavior during both acclimation and PTZ exposure was monitored. Fish were monitored individually in single wells of a 96 well, flat bottom plate in 100 or 200 μL of fluid. The plate was placed on a light box with an overhead video recording and tracking system. Motion was captured at 30 frames per second using a high resolution camera (SX-920C-HR; Matco Canada, St. Laurent, QC) connected to a video capture card (Picolo H.264; Euresys, San Juan Capistrano, CA) in a PC running EthoVision® XT7 software (Noldus, Wageningen, Netherlands), as described previously (Blinder and Tierney, 2012). Researchers were blinded to fish genotype throughout the behavioral assessment. After tracking the fish movement, raw data (30 data points/second/fish) were exported to Microsoft Excel to calculate swimming speed and distances traveled transformed into 10 s time bins. The average velocity, excluding data points generated when the fish were out of view from the camera, was calculated during two time periods consisting of the final 15 min of the acclimation period, and minutes 5–20 following PTZ addition. Movement following addition of convulsant (2.5 mM PTZ) was normalized to the movement of the larva during an acclimation period prior to addition of convulsant. These values were tested for significance using Kruskal Wallis or Mann–Whitney U tests in Systat 12.

In situ analysis of NMDA receptor kinetics

Recordings from 2 dpf larvae utilized a preparation with intact trunk, spinal cord and hindbrain as previously described (Pattan and Ali, 2009; Pattan et al., 2010). Larvae were anesthetized in MS-222 (Sigma-Aldrich) prior to dissection and subsequently paralyzed in 15 μM α-tubocurarine (Sigma-Aldrich) in an aerated perfusion bath held at 20–24 °C lacking MS-222. Maternal zygotic *prp2*<sup>−/−</sup> larvae, lacking maternal contribution of *prp2* mRNA, were compared to wild type larvae. Miniature excitatory post-synaptic currents (mEPSCs) were recorded from the Mauthner cell, which is readily identified using DIC microscopy. Recording conditions were as reported previously (Pattan and Ali, 2009; Pattan et al., 2010), including use of 1 μM tetrodotoxin to block action potentials. The spontaneous whole cell currents, representing AMPA and NMDA excitatory post-synaptic currents, were recorded with a holding potential of +40 mV with 5 μM strychnine and 100 μM picrotoxin to block γ-aminobutyric acid and glycine receptors. NMDA receptor currents were confirmed with the use of the NMDA receptor antagonist 2-amino-5-phosphonovaleate (APV, 50 μM) which abolished NMDA receptor activity. NMDA mEPSCs returned upon wash out of APV. Currents were amplified with an Axopatch 200B Amplifier, low-pass filtered at 5 kHz (−3 dB) and digitized at 50 kHz to accurately record both NMDA and AMPA mEPSCs (if necessary). Synaptic events were first examined visually, and were then detected and analyzed using AxoGraph X (MDS Analytical Technologies, Sunnyvale CA). Decay kinetics were analyzed by fitting a sum of exponential curves over the first second of the decay phase. Rise time was calculated as the time between reaching 20% and 80% of the current amplitude. In a subset of animals from each genotype, small changes in the recording parameters during the session (drifting access resistance of the pipette) precluded confident analysis of event amplitude or kinetics, though these recordings were used to accurately assess NMDA event frequency. Quantifications were compared between genotypes using t-tests in Sigmastat (Systat Software, San Jose CA).

Results

Targeted disruption of prp2 in zebrafish using zinc finger nucleases

ZFN technology (Meng et al., 2008) was adapted to create heritable mutations in the zebrafish *prp2* gene, ZFin ID number ZDB-GENE-041221-3. A suitable ZFN target site within the gene was identified with assistance of an online algorithm (Meng et al., 2008). The criteria for target site selection included high guanine content, amenable ZFN library diversities (below 10<sup>−3</sup>) and, most importantly, the position of the target site in the gene relative to the functional protein domains. The *prp2* ZFN target site selected based upon these criteria, 5′-CACCT CCTTaccTGCTGGAG-3′, consisted of two regions of 9 base pairs each, separated by 6 base pairs (Fig. 1B). Thus the engineered ZFN enzyme has an 18 bp recognition site and is predicted to specifically bind to a single location within the genome. The selected site is located immediately 5′ of the repeat region of the *prp2* gene (Figs. 1 and S1), and mutations in this sequence (e.g. small deletions caused by the error-prone non-homologous end joining repair process initiated by ZFN cutting) resulting in frame-shifts are predicted to produce a non-functional, truncated prion protein. No alternative start sites were detected in this gene by in silico analysis, and the selected target is present in all splice variants (indeed only one variant is known, with the coding region contained in a single exon).

To engineer a ZFN that would specifically bind this *prp2* target site, two stages of bacterial-one-hybrid selection were performed on libraries of potential zinc fingers (an overview is schematized in Fig. S2, and the resultant fingers were subsequently fused to a nuclease domain). Details of Methods are provided in the Supplementary materials. Zinc fingers that exhibited strong binding to their target site were identified as those that enabled growth of bacteria in the bacterial-one-hybrid selection system (Meng et al., 2008). A library encoding each of the candidate single zinc fingers (three libraries of 5p fingers and three libraries of 3p fingers) was generated by cloning oligonucleotides. These oligonucleotides encoded zinc finger proteins with randomization of amino acid identities at several sites known to effect DNA binding, as suggested by the algorithm (Fig. S2).

Zinc fingers that were good candidates for *prp2* target site binding, as identified by one or two stages of bacterial-one-hybrid selection, were then subjected to functional characterization (two candidate 5p, and five candidate 3p. Supplemental Methods, Table S2, S3). These arrays of zinc fingers were cloned into expression constructs containing the nuclease domain from FokI, and tested for cutting proficiency in concentrated combinations. To preclude time-consuming in vivo testing, screening was performed to ascertain if they successfully cut their target DNA in vitro (Supplemental Methods). As expected, the PrP2 ZFNs cut their respective target sites in a specific fashion (an example is presented in Fig. S3).

To deliver the ZFNs to zebrafish embryos, ZFN mRNA was transcribed from one 5p ZFN and two different 3p ZFNs (Tables S2 and S3), and injected in combinations into single-cell stage zebrafish embryos. Only one of the two tested combinations induced genomic mutations (Tables S2 and S3). The observed frequency of insertion and deletion mutations, primarily small deletions of 1 to 8 base pairs, was
4.6% (6/130 tested clones; Fig. S3). A smaller number of single base pair substitutions were also observed.

To isolate zebrafish carrying prp2 mutations, embryos injected with this ZFN pair were raised until adulthood, and germ line transmission of the mutation was examined by crossing adult zebrafish and genotyping their offspring. As before, genomic DNA of n = 10 larvae at 24 hpf was isolated from each clutch, and an amplicon containing the target site was sub-cloned to assess the occurrence of mutations. One pair of adult zebrafish was found to give rise to embryos carrying a 4 base pair deletion, which was designated as the allele ua5001 (Fig. 1E). Offspring from this pair were raised (producing the F1 generation) until adulthood and tested for heterozygosity by fin-clipping and sequencing. In-breeding of identified heterozygous F1 carriers (n = 4 fish) revealed larvae exhibiting a Mendelian inheritance pattern of the mutation. Larvae from three clutches were sequenced to determine their genotypes (n = 55 larvae: 15 prp2ua5001/+; 27 prp2ua5001/ua5001, and 13 prp2ua5001/ua5001) and the allele inheritance patterns were not different than the expected Mendelian ratios (27%, 49% and 24%, respectively. χ² test = 0.164, p = 0.92).

Disruption of prp2 by ZFN does not produce an overt developmental phenotype

The frameshift in translation caused by the 4 base pair deletion in the prp2ua5001 allele is predicted to result in a null allele, as all protein domains recognizable in the primary sequence of the mature protein are lost (compare Figs. 1D with 1C). Previous efforts aimed at understanding prion protein biology via prp2 loss-of-function in zebrafish utilized morpholin knockdown. These annotated cell death and disrupted head morphology (Malaga-Trillo et al., 2008; Nourizadeh-Lilhadji et al., 2010), which are phenotypes we also observed with these MO reagents (see below). This was in sharp contrast to the fish observed following crosses of prp2ua5001/+ heterozygous fish; these larvae lacked any overt phenotypes throughout development (Fig. 1F). Sequencing confirmed that approximately 25% of these phenotypically normal fish were homozygous prp2ua5001/ua5001 (Fig. 1E).

Maternal contributions of prp2 mRNA do not account for normal development

We were intrigued by the disparity in observed phenotypes when prp2 was disrupted by MO knockdown as compared to ZFN-induced targeted mutation, and set out to examine alternative hypotheses: (i) Maternal contributions of prp2 mRNA into the egg are sufficient for normal development in prp2ua5001/ua5001 fish; (ii) The various prp2 MO knockdown reagents deployed are efficacious but also induce phenotypes that are not specific to prp2 disruption; and (iii) The mutation in prp2ua5001/ua5001 fish fails to disrupt the normal gene product. In subsequent sections we examine the latter, and here we investigate the former. The prp2 MO reagents that produce phenotypes are designed to block translation of both maternal and zygotic mRNA. Robust expression of prp2 mRNA can be detected in recently fertilized zebrafish embryos (Fig. 2A). These observations accord with a role for maternal deposition of wild type prp2 mRNA, and thus masking of phenotypes during breeding of prp2ua5001/+ fish was deemed possible.

It was noted that both male and female prp2ua5001/ua5001 fish were viable into adulthood and had survival rates not different from wild type fish (Figs. 2B, C). Breeding prp2ua5001/ua5001 fish did not suggest any deficits in fecundity. The oldest prp2ua5001/ua5001 fish we had raised at time of writing was 18 months old, and exhibited no apparent deficits, suggesting that at least some prp2ua5001/ua5001 fish can thrive in a manner indistinguishable from wild type fish (adulthood is considered the time of sexual maturity, ~3 months of age, and the normal lifespan of a zebrafish is 3–5 years). prp2ua5001/ua5001 males and females were crossed to generate prp2ua5001/ua5001 fish lacking the hypothesized maternal contribution of wild type prp2 mRNA. Zebrafish lacking both maternal and zygotic contributions of a gene are defined as ‘maternal zygotic’ mutants, as compared to zygotic mutants that do not lack maternal gene function. These maternal zygotic mutant progeny appeared normal (Fig. 2D), and thus maternal mRNA contributions can be excluded as having a causal role in the normal development of prp2ua5001/ua5001 fish, i.e. maternal prp2 mRNA does not account for differences between the mutant and morphant phenotypes. Hereafter prp2ua5001/ua5001 genotypes are denoted as “prp2ua5001/–”. Subsequent back-crosses of homozygous mutant fish to date have supported this conclusion through to the F5 generation.

prp2 expression is disrupted in prp2ua5001/– fish

An alternate interpretation of the normal development observed in prp2ua5001/ua5001 fish is that the mutation does not disrupt the Prp2 protein. This is unlikely, as described above, because the mutation induces a frame-shift early in the prp2 coding region, predicting a truncated molecule lacking all the recognizable protein domains that form the mature molecule (see Figs. 1 and S1). Multiple attempts to generate an antibody against Prp2 protein were not successful, and no cross-reactivity was observed when commercial antibodies against mammalian PrP were investigated. Instead the abundance of prp2 transcripts was assessed in the mutants, because RNA surveillance can reduce transcript levels via nonsense-mediated decay (Culbertson, 1999). This mechanism is especially robust in transcripts harboring premature termination codons in the first coding exon. Nonsense-mediated decay is a conserved mechanism to remove abnormal transcripts encoding truncated proteins, and generally occurs when a premature termination codon is located more than 50–55 nucleotides upstream of an exon–exon boundary (Maquat, 2004). The mechanisms of nonsense-mediated decay are conserved and often observed in embryonic zebrafish mutants (reviewed in Wittkopp et al., 2009).

Transcript abundance was determined by RT-qPCR carried out as per MQE guidelines (Bustin et al., 2009). Integrity of extracted RNA was good regardless of genotype (Figs. 3D, S4), and the RT-qPCR reagents we deployed detected a single product (examples of melt curves in Fig. S4) in a linear range (Figs. 3F, S4). Other aspects ensuring integrity of the RT-qPCR methods are reported in the Methods and associated Supplementary Tables. Multiple biological replicates were compared regarding expression of several genes relative to the reference gene efla. Biological replicates consisted of pools of ~20 larvae, or represented brains of individual adult fish.

The abundance of prp2 mRNA is 6-fold reduced in prp2ua5001/ua5001 larvae compared to prp2ua5001/+ larvae at 48 hpf (Fig. 3G, p < 0.01). An additional reference gene, β-actin, did not change in abundance relative to efla levels, nor did any of the five other genes tested (see below). Similar results for prp2 abundance, including 4- to 6-fold decreases, were observed in 5 day old larvae and adult brains (Fig. S5, p < 0.001).

prp2 expression was also assessed by in situ hybridization. The results confirmed past work showing that prp2 transcripts are abundant in the zebrafish CNS (Malaga-Trillo et al., 2009) (Fig. 3). Consistent with the RT-qPCR results, prp2 transcripts were markedly reduced in prp2ua5001/ua5001 larvae compared to prp2ua5001/– larvae (Figs. 3A–C). Thus, in addition to a predicted disruption of translation that severely truncates the protein, the ua5001 allele leads to downregulation in prp2 transcript abundance consistent with nonsense-mediated decay and a null allele.

Expression of related genes is not perturbed in prp2ua5001/– fish

Alternate explanations for decreases in prp2 transcript abundance in prp2ua5001/– fish could include an idiosyncrasy of the methods, or a general decrement in CNS/synapse transcript abundance might be expected from improper development or degeneration of tissues.
We tested this by assessing genes with similar biological roles and/or distributions of expression compared to prp2. These included RT-qPCR analysis of prp1, appa, and appb (the latter are homologs of the Alzheimer disease gene amyloid-β precursor protein APP). None of these genes were altered when comparing their abundance between genotypes in three developmental stages (Figs. 3, S5). Thus, amongst the eight genes tested (including the reference genes and two others below), decreases in gene transcript abundance were observed only for prp2, supporting the robust specificity of the assays. The results are consistent with the lack of any morphological or neurodegenerative phenotype observed in the CNS.

Previous work analyzing transcript levels in zebrafish injected with either of two prp2 morpholinos reported an increase in abundance of tp53 transcripts, encoding the cell-death-related protein p53 (Nourizadeh-Lillabadi et al., 2010). This is consistent with
cell death observed in the head region of prp2 morphants (Malaga-Trillo et al., 2009; Nourizadeh-Lillabadi et al., 2010). This was replicated herein, in that the use of prp2 MO demonstrated abundant cell death and a three-fold increase in tp53 abundance (Fig. S6C). This contrasts prp2ua5001/ua5001 fish at 48 hpf that demonstrated no such change in tp53 levels compared to wild type fish (Fig. 3G), consistent with the lack of overt cell death in developing mutant embryos. Parallel observations were noted in the cng1 transcript, encoding Cyclin G1 that itself regulates p53 (Jost et al., 1997), in that cng1 was more abundant in 48 hpf prp2 morphants (Nourizadeh-Lillabadi et al., 2010) but was equally abundant in wild type and prp2ua5001/ua5001 48 hpf larvae (Fig. 3G). Increased abundance of tp53 transcript following delivery of a morpholino reagent to zebrafish is typically interpreted as a lack of reagent specificity, often representing the MO reagents causing developmental deficits via off-target effects (Robu et al., 2007). Indeed inclusion of a MO blocking tp53 in the injection solution was able to rescue the cell death phenotypes observed in prp2 morphants (Fig. S6). A lack of specificity in the available prp2 MO reagents is also consistent with results from several groups that fail to rescue prp2 MO-induced phenotypes using cognate prp2 mRNA ((Malaga-Trillo et al., 2009; Nourizadeh-Lillabadi et al., 2010), see also Fig. S6F). Indeed prp2 mRNA is able to partially rescue prp1, but not prp2, MO-induced phenotypes (Malaga-Trillo et al., 2009), and thus the prp2 mRNA delivery is likely a robust test of prp2 MO fidelity (or lack thereof). This contrasts results for the zebrafish paralog prp1, wherein prp1 morpholino induced phenotypes have been demonstrated to be specific to the knockdown reagent because either cognate mRNA or mammalian Prnp mRNAs are able to rescue developmental deficits (Kaiser et al., 2012; Malaga-Trillo et al., 2009). In sum, it appears that previously reported phenotypes resulting from prp2 knockdown are due, in part, to off-target effects of the morpholino reagents; such observations are becoming widespread amongst morpholino papers (Bedell et al., 2011), thus necessitating caution when controls cannot prove reagent specificity (Eisen and Smith, 2008). The disparity of phenotypes between prp2 morphant fish and prp2ua5001/ua5001 mutant fish is also consistent with this interpretation.

It is noteworthy that although the specificity of the prp2 MO reagents is not supported herein, the efficacy of the reagent in partially reducing prp2 abundance is supported by two lines of evidence. Past work shows prp2 MO can reduce both protein and transcript abundance (Nourizadeh-Lillabadi et al., 2010) and data herein at least supports the latter (Fig. S6). Thus it appears that while prp2 can be disrupted effectively by prp2 MO reagents, the previously reported phenotypes are not caused by this disruption. This conclusion is supported by the normal development of prp2 mutants, despite the reduction of prp2 transcript in prp2ua5001/ua5001 mutants being greater in magnitude compared to that observed in the morphants (Fig. S6). Thus discrepancies between prp2 morphants and mutants are most consistent with the interpretation that prp2ua5001/ua5001 mutants represent a null allele, and this is also consistent with a lack
characterization of neuronal and glial markers fails to reveal phenotypes in prp2−/− fish

with an aim of exploring for phenotypes amongst neurons and glia, we examined larvae generated from breeding of prp2−/− fish using immunohistochemistry and/or transgene expression. to better visualize the CNS in fish lacking pigment, we established wild type and prp2ua5001/ua5001 adults expressing GFP under control of the GFAP promoter (Bernardos and Raymond, 2006) on the ‘casper’ background of fish that lack reflective iridophores and melanin pigment throughout their body (excepting the eyes) (White et al., 2008). Thus Tg(gfap:GFP)mi2001;mitfaw2/w2;roya9/a9;prp2ua5001/ua5001 adults were crossed and progeny examined at various ages compared to the same background with wild type prp2, i.e. Tg(gfap:GFP)mi2001;mitfaw2/w2;roya9/a9;prp2+/+. This permitted inspection of organ morphometry in

of phenotypes when Prnp is knocked out of mice (Bueler et al., 1992) or other mammals (Richt et al., 2007; Yu et al., 2009).

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Fig. 3. PrP2 transcript is less abundant in prp2ua5001/ua5001 zebrafish compared to wild type. A, B. In situ hybridization with riboprobe against prp2 transcript in wild type zebrafish 2 days post-fertilization showing prp2 transcript (purple) is abundant in the CNS. Anterior is to the left. Panel A is a dorsal view, with the yolk sac removed. Panel B is a lateral view. sc, spinal cord. C. Prp2 transcript is less abundant in prp2ua5001/ua5001 larvae (top) compared to prp2+/+ larvae (middle); prp2+/+ larvae treated identically except for receiving no riboprobe is presented to assess background staining levels; control for labeling specificity using sense riboprobe also shows low background staining (Fig. S5). D–F. Examples of quality assessment regarding the RT-qPCR analysis, which was performed on all RT-qPCR reagents, including the quality of total RNA assessed by Bioanalyzer being similar regardless of genotype with RNA integrity numbers (RIN) reported above each lane (D); three melting dissociation curves for prp2 transcript at 1:10th dilution to assess qPCR reaction integrity (E), and a plot of Ct values vs. concentration (3 technical replicates presented per dilution) for dilutions of the template demonstrates linearity and high correlation coefficient (R2 = 0.999). See also Fig. S4. G. Quantification of transcript abundance demonstrates prp2 is 6-fold decreased in prp2ua5001/ua5001 compared to prp2+/+ larvae at 2 dpf, whereas other genes analyzed had equal abundance regardless of genotype. Data normalized to reference gene ef1α and presented as fold change of mutant relative to wild type. Samples were independent biological replicates each of which consisted of pools of larvae from different parents, n = 3, **p = 0.002.
a fluorescent stereomicroscope, especially including unprecedented detail being visible with live imaging in the post-larval CNS. No differences in brain morphometry were noted between genotypes (Figs. 4 and S7). Increase in GFAP abundance in the form of gliosis is an early and sensitive outcome of neurodegeneration (Middeldorp and Hol, 2011). No difference in the intensity of GFP expression, driven by the GFAP promoter, was apparent (examples at 7 and 21 dpf in Figs. 4 and S7). Staining of nuclei with To-Pro-3 visualized in whole embryos at 2 dpf or in sectioned material at 7 dpf using confocal microscopy revealed no apparent difference in abundance of pyknotic nuclei (Figs. 5B, E). Markers of synapses and neuron subpopulations that have previously been shown to be sensitive to disrupted neurodevelopment and neurodegeneration (Lemmens et al., 2007) were not different between prp2 genotypes (Figs. 5K–N). We found that the synaptic marker SV2 labeled the same axons as GFAP-GFP fluorescence in the primary motor neurons,

Fig. 4. Prp2−/− zebrafish have grossly normal CNS and organ morphology. prp2−/− fish were crossed to fish with GFP expressed in the CNS, driven by glial acidic fibrillary protein (GFAP) promoter, on the Casper mutant background that enhances transparency. A. Lateral view of 7 days post-fertilization (dpf) larvae, anterior to the left. GFP expression is not different in intensity or distribution based on genotype. Wild type is on top and prp2−/− mutant is on the bottom, and anterior is to the left, throughout the Figure. B. Dorsal view of 21 dpf fish, with GFP revealing normal brain subdivisions and spinal cord in prp2−/− fish. C. Lateral view of 21 dpf fish at higher magnification demonstrating GFP distribution in comparison to brightfield, with no detectable differences between genotypes. Alternate views of these fish are presented in Fig. S7. Scale bar = 1 mm in A, B and 0.5 mm in C.
and that neither was different in its distribution between genotypes (Figs. 5K, L). Labeling with SV2 or 3A10 antibodies in the head region demarcates subpopulations of neurons, and again these were not different between genotypes (Figs. 5M, N). We examined the retina as a tissue that is readily able to reveal CNS phenotypes in zebrafish larvae due to its rapid development and organized structure. Sections of 7 dpf larval zebrafish eyes appeared normal in prp2−/− fish, including normal synaptic and nuclear layers, and unperturbed morphology of

Fig. 5. Histological analysis suggests prp2−/− zebrafish have normal CNS organization, polarity and axon guidance. Structures and markers known to be sensitive to degeneration or abnormal development were not observed to be disrupted in prp2−/− fish. A–F. Transverse sections through the CNS and eye of mutants (A–C) display normal CNS nuclear and plexiform/synaptic layers, compared to wild type (D–F). Actin staining of synapses shows grossly normal plexiform layers (e.g. inner plexiform layer, ipl; inner nuclear layer, inl). Müller glia filled with GFP are normal in their radial projections and overall morphology. Panels C and F are immediately dorsal of the optic nerve (on), similar to the position boxed by dashed lines in panel B. Dorsal is up in all panels of this figure. G–H. Transverse sections through the trunk musculature and spinal cord (sc). Actin staining emphasizes muscle fascicles with normal organization and nuclei. Radial distribution of glia is normal, as revealed by gfap:GFP transgene, with a magnified view in I and J. K, L. Synaptic vesicle (SV2) labeling shows normal motor neuron branching and morphology. Lateral view of trunk, anterior to the left. M, N. A subset of neurons in the head region, labeled with 3A10, have normal axon distribution branching around the eye (outlined with dashed line) from the posterior. The cell bodies of Mauthner neurons (m) are normal, including being em- bedded in rhombomere four of the hindbrain with intense GFP. Mauthner cell axons project posteriorly in the ventral aspect of the spinal cord. A–J are 7, and K–N are 3 days post-fertilization larvae. Scale bars: A–F, K and L are 50 μm; G–J are 30 μm; M, N are 100 μm.
GFP-positive Muller glia that serve various roles including being latent retinal stem cells (Fleisch et al., 2011; Fraser et al., 2013) (Figs. 5A–F).

Disruption of prp2 produces susceptibility to drug-induced seizures

To characterize the role of prp2 with respect to neuron function, we assayed if prp2 loss-of-function leads to increased seizure susceptibility, as has been observed in Prnp−/− mice (Carulla et al., 2011; Rangel et al., 2007, 2009; Walz et al., 1999). Zebrafish have often been used in assays of seizure induction and sensitization, enabling testing of pharmacological and/or genetic interventions. We deployed an assay that has been established as a sensitive proxy of seizures (Baraban et al., 2005; Teng et al., 2010), hyperactivity measured as increased total movement of zebrafish larvae following addition of the convulsant PTZ. Examples of the method and tracking of fish movement are shown (Figs. 6A, B and Supplemental Movie 1).

Our primary analysis focused on assessing fish movement during PTZ application, which was normalized to the levels of fish movement prior to PTZ application. The latter can account for inter-individual variability in baseline activity, which can be substantial in larval zebrafish (Shamchuk and Tierney, 2012). Movement representing Stage I and Stage II seizures was increased by approximately 50% in 5 dpf homozygous mutants compared to wild type fish (p < 0.001, Fig. 6), indicating that prp2 plays a role in modulating neuron excitability and seizures. Prp2ua5001/+ fish were noted to have responses to PTZ that were approximately mid-way between the wild type and prp2ua5001/ua5001 fish, though this difference was not statistically significant from either genotype (Fig. 6C). Similarly, the average velocity after/before the addition of 2.5 mM PTZ was greater for 3 dpf prp2ua5001/ua5001 mutants compared to wild type controls (Fig. 6D, p < 0.05). In sum, prp2ua5001/ua5001 fish are induced to hyperactivity by PTZ to a greater extent compared to wild type controls.

Fig. 6. Prp2 disruption in zebrafish leads to seizure susceptibility. The convulsant pentylenetetrazol (PTZ, 2.5 mM) increases activity to a greater extent in prp2−/− larval fish compared to wild type sibling fish. A. The method involved collecting data traces, examples of which are shown from individual 5 days post-fertilization (dpf) larvae, tracking movement of fish over 20 min, with bath application of PTZ beginning at 1200 s (shaded area). B. Blue lines are example motion paths of 3 dpf prp2−/− and prp2+/+ fish, 5 s in duration, before and during PTZ exposure. Behavior of larvae was monitored in 96-well plates (individual wells shown here, e.g. in 3rd well fish is oriented towards southwest and moved little). Fish of both genotypes exhibited stage I to stage II seizures upon PTZ exposure (defined previously (Baraban et al., 2005, 2007) as a general increase in activity and ‘whirlpool’ swimming patterns, respectively, also presented in the Supplemental Movie 1). C. Quantifying movement as a proxy of seizures shows that prp2−/− fish respond to the PTZ with greater magnitude compared to prp2+/− fish. Data are presented as a ratio of velocity during/before PTZ treatment. **p < 0.001 determined with Kruskal–Wallis one-way ANOVA. Raw data presented in Fig. S8. D. Ratio of the average velocity of 3 dpf prp2−/− and prp2+/− fish during/before treatment with 2.5 mM PTZ. *p = 0.016 with the Mann Whitney U-test.
Further analysis was warranted, however, because changes to this after-PTZ/before-PTZ ratio could represent both increased movement of mutants following PTZ or decreased movement of mutants prior to PTZ exposure. Indeed both effects were observed in 5dpf fish, depending on whether maternal mRNA was present during development. Larvae lacking maternal contribution (denoted as ‘maternal zygotic’ mutants, resulting from breeding prp2<sup>−/−</sup>ωu5001/ωu5001 fish) had substantially lower baseline movement levels compared to wild type fish (Figs. S8A, B), along with the aforementioned increased activity following PTZ (Fig. S8C, p < 0.001, n = 19). This distinctly contrasted larvae with maternal deposition of prp2 mRNA (zygotic mutants, resulting from breeding prp2<sup>−/−</sup>ωu5001/+ fish), where the significant increase in movement with PTZ was observed (Fig. S8C, ratio 1.4, p < 0.001, n = 6) despite mean velocity prior to PTZ movement being comparable to wild type and heterozygous fish (Fig. S8B). Therefore decreased baseline movement cannot by itself account for the increased ratio of PTZ-induced activity. Maternal zygotic mutants at 3dpf also had reduced baseline movement (Figs. S8D and E). Decreased baseline movement is unto itself a phenotype of interest in the maternal zygotic larvae lacking maternal contribution (denoted as ‘maternal zygotic’ mutants, resulting from breeding prp2<sup>−/−</sup>ωu5001/ωu5001 fish), where the significant increase in movement with PTZ was observed (Fig. S8C, ratio 1.4, p < 0.001, n = 6) despite mean velocity prior to PTZ movement being comparable to wild type and heterozygous fish (Fig. S8B). Therefore decreased baseline movement cannot by itself account for the increased ratio of PTZ-induced activity. Maternal zygotic mutants at 3dpf also had reduced baseline movement (Figs. S8D and E). Decreased baseline movement is unto itself a phenotype of interest in the maternal zygotic prp2<sup>−/−</sup>ωu5001 fish.

Overall, prp2<sup>−/−</sup>ωu5001 have greater susceptibility to the convulsant compared to wild type fish. No difference was noted regarding induction of increased movement by PTZ when comparing maternal zygotic and zygotic mutant fish, suggesting that maternal contributions of prp2 mRNA were inert towards the PTZ-induced seizure-like phenotype. Similar results from Prnp<sup>−/−</sup> mice suggest that PrP<sup>+</sup> has a deeply conserved role in modulating neuron excitability.

NMDA receptor kinetics are altered in prp2<sup>−/−</sup>ωu5001 larvae

Having observed an increase in seizure susceptibility, it was of interest to consider if neurophysiological changes at the level of the synapse might correlate with the altered behavior. PrP<sup>+</sup> is known to regulate the activity of various channels, including nicotin acetylcholine receptors, AMPA receptors and voltage gated potassium channels (Alier et al., 2011; Beraldo et al., 2010; Khosravani et al., 2008b). NMDA channels were of particular interest due to their known role in PTZ-mediated behavioral effects in mice (though other channels are also involved), and the potential linkage of PrP to deregulation of NMDA receptors leading to excitotoxicity (Khosravani et al., 2008a; Stys et al., 2012; You et al., 2012).

Spontaneous synaptic NMDA receptor currents were analyzed for a number of properties. Patch-clamp recordings were used to document miniature excitatory post-synaptic currents (mEPSCs) from the Mauthner neuron in the intact hindbrain of wild type and maternal zygotic prp2<sup>−/−</sup>ωu5001 zebrafish. The Mauthner neuron is reliably recognizable by light microscopy, and its activity controls swimming behaviors in the larval fish. Glutamate excitatory currents, representing AMPA and NMDA receptors, were isolated using pharmacology and acquired at holding potentials of −60 mV and +40 mV, but NMDA currents were only apparent at positive potentials that relieve Mg<sup>2+</sup> block of the channel. The accurate identification of NMDA synaptic currents was confirmed, since they were not observable upon addition of the NMDA receptor antagonist APV in either wild type or prp2<sup>−/−</sup>ωu5001 fish (Fig. 7A). Washout of APV resulted in the re-appearance of NMDA mEPSCs, and AMPA currents remained observable throughout. Characterizing NMDA currents in each genotype demonstrated no significant difference in amplitude (Fig. 7D, p = 0.75, n = 4 and n = 7 embryos), but NMDA currents occurred with lower frequency in prp2<sup>−/−</sup>ωu5001 embryos compared to wild type (Fig. 7C, p = 0.026, n = 6 and n = 10 embryos, respectively). It is noteworthy that these currents represent spontaneous events, rather than evoked events, and thus changes in frequency may or may not be due to altered Mauthner neuron excitability. Kinetics of NMDA receptors were considered next, as these are altered in brain slices from Prnp<sup>−/−</sup> mice (Khosravani et al., 2008a; You et al., 2012). Rise time of NMDA mEPSCs was not different between genotypes (p = 0.62) but the decay time was. The increase in decay time is evident in averaged example traces (Fig. 7B). The kinetics of mEPSC decay time were best fit with a tandem of exponential curves consisting of a fast and a slow component, τ1 and τ2 respectively. In particular, τ1 was not different (p = 0.14) but τ2 was doubled in prp2<sup>−/−</sup>ωu5001 larvae (Fig. 7G, p < 0.001, n = 7 wild type, n = 4 prp2<sup>−/−</sup>ωu5001 larvae, with 10 to 68 traces being averaged per recording). Immunohistochemistry and confocal microscopy failed to reveal a difference in Mauthner cell morphology (Figs. 7H, I and 5M, N).

The observations regarding prp2 disruption affecting NMDA receptor kinetics were independently confirmed in wild type zebrafish injected with prp2 morpholino. This included co-injection of p53 morpholino to suppress off-target effects (as discussed above, see Figs. S6E, S6F regarding p53 MO inclusion). The comparator larvae were injected with an equal dose of a standard control MO and p53 MO. The recordings used in the morphant analysis included mixed AMPA and NMDA receptor currents. Thus the very fast decay component in the morphant experiments represents AMPA receptor activity, whereas the slow decay component is dominated by the NMDA receptors comparable to the τ2 component that was altered in the prp2<sup>−/−</sup>ωu5001 larvae above. The slow NMDA component was more evident in the prp2 morphants compared with the controls (Fig. S9 panel A’ vs. A). The slow component was doubled in prp2 MO fish (p < 0.05, n = 3 fish per genotype) with no observed change in amplitude (Fig. S9).

In sum, results using two gene disruption technologies and two sets of recording parameters each revealed that the NMDA channels in prp2<sup>−/−</sup>ωu5001 have altered kinetics: once opened, NMDA receptors remain open longer. This predicts a protective role for PrP2, preventing excitotoxicity during neuronal insult, and accords well with increased neuron excitability that is presumed to be causal of the increased seizure susceptibility reported above (Fig. 6).

Discussion

Knockout of the Prnp gene in mice has been fundamental to understanding of prion disease etiology, allowing confirmation that the gene product is crucial to prion disease progression (Prusiner, 1991). Furthermore, this knockout background has enabled the engineering of transgenic mice that can be infected with prion disease isolates from various species, propelling the study of various impactful prionopathies. Prnp<sup>−/−</sup> mice develop normally, and display few adult phenotypes (Bueller et al., 1992). This has hampered assignment of a function to PrP, a conserved protein that is highly abundant in healthy brains (Aguzzi et al., 2008). Thus we were deeply intrigued by findings that a tractable animal model, the zebrafish, presents with a robust neurodevelopmental phenotype during knockdown of either of two Prnp homologs (Kaiser et al., 2012; Malaga-Trillo et al., 2009; Nourizadeh-Lillabadi et al., 2010). This previous work utilized morpholino technology that permits only transient (multi-day) and partial knockdown of prion protein translation. We sought to confirm these results using an independent method of disrupting the prion gene.

We deployed an emerging technology, zinc finger nucleases, because such approaches have promise to enable targeted mutation of most genes in zebrafish. Use of ZFNs in zebrafish was recently pioneered by three groups (Amacher, Joung, Wolfe and Lawson, see (Doyon et al., 2008; Ekkher, 2008; Foley et al., 2009; McCammon et al., 2011; Meng et al., 2008; Sander et al., 2011; Zhu et al., 2011)) who have been generous in sharing their reagents and experience. The current results represent a rare success deploying ZFN technology in zebrafish beyond the aforementioned groups (see also Ben et al., 2011), thus validating the tractability of the targeted mutagenesis approach for the broader zebrafish community.

We generated zebrafish with a frame-shift mutation early in the prion protein gene 2 (prp2) and find that the fish lacks overt phenotypes. This is consistent with the lack of overt phenotypes when PrP
is disrupted in mice (Bueler et al., 1992) and other mammals (Richt et al., 2007; Yu et al., 2009). Our data suggest the discrepancy between the zebrafish prp2 mutant and morphant can be attributed to a lack of specificity in the morpholino reagents deployed. This is likely to emerge as a common theme in zebrafish biology, as the tractable morpholino technology is slowly supplanted by laborious targeted mutation and gene tilling mutagenesis projects.

The normal development of zebrafish lacking prp2 contrasts the gastrulation and CNS cell death effects observed following knockdown of prp1 in zebrafish using morpholinos (Kaiser et al., 2012; Malaga-Trillo et al., 2009). Future work is needed to decipher if this difference is attributable to the technologies used, the gene products involved (e.g., differences in amino acid sequences) and/or differences in their expression. The latter explanation is probable, in that prp1 is expressed in most cells and very early in development, whereas prp2 expression becomes more robust later in development and is primarily restricted to the CNS.

Characterizing the prp2−/− fish generated herein using behavioral and electrophysiological paradigms confirmed that PrP plays a conserved, and thus important role in regulating neuron excitability. Recent work has suggested that PrP acts to modulate channel excitability in neurons, consistent with its roles in synaptogenesis and neuroprotection (modulating excitotoxicity). The current evidence...
extends these findings to an in vivo paradigm and argues that regulation of ion channels is an ancient function of this protein. This is consistent with this abundant protein having a homeostatic role that comes to the forefront during insults to the CNS.

Alterations to NMDA receptor decay kinetics have been found in brain slices comparing wild type and Prnp−/− mice (Khosravani et al., 2008a; You et al., 2012). The data herein closely parallel this work, including a marked slowing of NMDA receptor deactivation. Other studies indicate Prnp−/− mice have altered abundance of transcripts encoding NMDA receptor (Maglio et al., 2004), and NMDA receptors participate in their increased susceptibility to kainate-induced cell death (Rangel et al., 2007). Here, a role for the prion protein in modulating NMDA receptor kinetics has been confirmed in situ. This deep conservation suggests it as an ancient role for the prion protein in modulating NMDA receptors and neuron excitability.

Decreased baseline movement was observed prior to addition of convulsant in the 5 dpf maternal zygotic prp2−/− zebrafish (lacking maternal contribution of prp2 mRNA into the egg), suggesting that prp2 plays at least two roles with respect to zebrafish movement. First, lack of prp2 affects early development in some fashion that later manifests as decreased basal movement/lethargy in the fish. This is in accord with the decreased frequency of spontaneous NMDA events in maternal zygotic prp2−/− fish. Second, lack of prp2 has an acute effect on zebrafish behavioral susceptibility to the convulsant PTZ, regardless of maternal contributions of prp2 mRNA. This acute effect is in good accordance with altered kinetics of NMDA receptor closure in prp2−/− fish. Future work is required to test whether the observed alterations to NMDA receptor currents cause the observed changes in activity and seizure susceptibility.

The regulation of NMDA receptors by the prion protein PrP has recently been demonstrated to be mechanistically linked, at least in part, to PrP modulating copper availability (Khosravani et al., 2008a; You et al., 2012). PrP is a potent regulator of copper homeostasis (Millhauser, 2008a; You et al., 2012). The data herein closely parallel this work, invertebrate may share fundamental PrP functions at the synapse, while in a hypothesized role of modulating divalent cations (Ehsani et al., 2011). The regulation of NMDA receptor interaction (You et al., 2012). Considering the prion protein homologs in zebrafish, this conclusion is intriguing on at least two levels. First, some of us have recently reported that zebrafish prp1 genetically interacts with the Aβ precursor protein (APP) holoprotein, and extended this by demonstrating biochemical interactions of human PrP−APP in vitro (Kaiser et al., 2012). These observations broaden the landscape of PrP−APP interactions that might prove to be relevant to disease etiology. Second, the regulation of copper abundance at NMDA receptors in mammals is presumed to revolve around PrP’s octarepeat region, yet this region is substantively different in zebrafish. The octarepeats (PHGGGWQ) of mammalian PrP coordinate copper binding at histidine residues in each repeat. The equivalent domain of zebrafish Prp2 protein is homologous, including being unambiguously repetitive (containing multiple PXXXY motifs, Fig. S1), yet none of the repeats in zebrafish Prp2 possess known signatures of copper binding. Thus both mammalian and zebrafish PrP proteins modulate NMDA receptor kinetics in a very similar fashion, but only the former is likely to modulate copper via the repetitive region. This is worthy of further pursuit, as understanding the role of PrP−copper has the potential to inform both prion and Alzheimer disease therapeutics, wherein NMDA receptor antagonists are in clinical trial (Olivares et al., 2012).

In this light, it is intriguing that prion proteins are now understood to have evolved from the transmembrane zip proteins with ancient functions in copper and zinc transport (Ehsani et al., 2011, 2012; Schmitt-Ulms et al., 2009). Mammalian PrP and Zip proteins interact in a hypothesized role of modulating divergent cations (Ehsani et al., 2011, 2012; Schmitt-Ulms et al., 2009). It is striking that PrP in a basal vertebrate may share fundamental PrP functions at the synapse, while apparently lacking core metal homeostatic properties. It will be of interest to understand the role of zebrafish PrP2 with respect to copper homeostasis as this may provide insights into both NMDA receptor-based therapeutics and the evolution of the zip/prion protein family and their myriad mechanisms impinging upon synaptic metal homeostasis.

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