Supporting Information (SI) Appendix for

Mosaicism-independent mechanisms contribute to Pcdh19-related epilepsy and repetitive behaviors in *Xenopus*

Jugeon Park[1], Eunee Lee[1], Chul Hoon Kim[2], Jiyeon Ohk[1]*, and Hosung Jung[1]*

[1] Department of Anatomy, [2] Department of Pharmacology, Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul, 03722, Republic of Korea

*Correspondence should be addressed to J.O. (jyeon1703@yonsei.ac.kr) or to H.J. (hosungjung@yonsei.ac.kr).

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LEGEND FOR SUPPLEMENTAL MOVIE S1

EXTENDED MATERIALS AND METHODS

Experimental design and statistical analysis

All the analyzed data were obtained from three independent experiments, each performed with similar numbers of animals per group and per experiment. The numbers of total animals used for each analysis, the type of the statistical test, the outputs of the statistical test are described in the figure legends. The sex of tadpoles is unknown, and it is expected that both males and females are equally represented in the data. Data processing, visualization, and statistical analyses were performed using R (v4.3.0).

Capped RNA and morpholinos

Capped RNAs encoding EGFP were synthesized by using mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Scientific, MA, USA) with pCS2+CEGFP according to the manufacturer's instructions. The following Pcdh19 MO and control MO were designed and supplied by GeneTools (OR, USA): *Xenopus* Pcdh19 MO, 5'-CCCTGCTCAGCCACAACCACATAGT-3'; Control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Xenopus experiments

Xenopus tropicalis were purchased form Xenopus 1 Corp. (MI, USA). Embryos were generated by *in vitro* fertilization and raised in 0.1X Modified Barth's Saline (MBS) (8.8 mM sodium chloride, 100 µM potassium chloride, 100 µM magnesium sulfate, 500 µm 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid, 250 µM sodium bicarbonate, and 1 mM calcium chloride) at 24°C incubator. For the complete knockdown, targeted micro-injections were made to two dorsal animal blastomeres at the 8-cell stage. One ng of morpholino was injected per blastomere. One hundred pg of GFP RNA was injected as a tracer. For mosaic knockdown, injections were made to two dorsal animal blastomeres at the 16-cell stage. Five hundred pg of morpholino was injected per blastomere. Fifty pg of GFP RNA was injected as a tracer. All injected tadpoles were screened under a fluorescence microscope to assess the amount and correct targeting of the MO and GFP.

Western blot analysis

Pcdh19 MO- or control MO-injected embryos were grown to stage 27 (when the brain is mainly composed of neural stem cells), anesthetized in MS222 (Sigma-Aldrich, MO, USA) in 0.1X MBS, and the entire brain was dissected out. Thirty brains per group were lysed in 50 µl RIPA buffer (RC2002, Biosesang, Korea) supplemented with cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche, Germany). The lysate was cleared by centrifugation at 15,000 rpm at 4°C for 20 minutes, and the supernatant was collected. Twenty µg of proteins were resolved 10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on a 0.2 μm polyvinylidene difluoride membrane (Millipore, MA, USA). The Western blot assay was performed using a rabbit polyclonal anti-Pcdh19 (ab191198, Abcam, Cambridge, UK) or a rat monoclonal anti-Tubulin (ab6160, Abcam, Cambridge, UK) antibodies (both at 1:1000), the horseradish peroxidase-conjugated secondary antibodies (ab6271 and ab97057, Abcam), and Supersignal chemiluminescence substrate (Thermo Fisher Scientific). The protein ladder (SM307-250) was purchased from Biofact (Daejeon, Korea). To assess equal loading of samples, the membrane was cut at the 75 kDa molecular marker, and the upper and lower fragments of the same membrane were analyzed for Pcdh19 and Tubulin, respectively. To de-glycosylate Pcdh19 proteins, the lysate was treated PNGase F (P7367, Sigma-Aldrich) according to the manufacturer's instructions. Images were captured by using the ImageQuant™ LAS 4000 (General Electric, MA, USA).

Behavioral assay

For behavioral test, Pcdh19 MO- and control MO-injected embryos were grown to stage 49, and their swimming behavior was recorded in a custom-built dark chamber for 10 minutes following a 10-minute habituation period. Four tadpoles housed individually in a 2.5-cm arena were imaged in a single movie, which was then cropped into four individual movies. Each arena was made by adhering a rubber ring (inner diameter 2.5 cm, outer diameter 3.5, depth 4 cm) on a clean 10-cm petri dish. Four rings were attached per petri dish, and 2 ml of 0.1X MBS was added per arena. To trace the swimming trajectories, the position of the tadpole in each frame was extracted using Deeplabcut (1) trained with resenet50. These frame-by-frame positions were exported as a CSV file. The center of the arena was also calculated for each movie using a custom-written ImageJ macro script (2), which was used to normalize the coordinates of each movie.

To annotate the episodes of seizure-like behaviors, we manually inspected all videos focusing on the previously described categories of behavioral seizures in *Xenopus* (3, 4): intermittent bouts of rapid swimming, repetitive circling, lateral movement of the head with tremors, and/or C-shaped contractions. During the 10-minute video, if any of the four criteria for seizure-like behaviors were observed, the starting and ending times of the behavior were recorded, along with a description of the behavior exhibited. If there was a gap of more than 1 second between seizure-like behaviors, each gap was recorded as a separate episode. In cases where two or more seizure-like behaviors were displayed concurrently, all of them were documented. This was independently performed by two experimenters and two annotations were resolved before finally being documented. The numbers of analyzed animals for Figure 2E(a) are 51, 78, 52, 78, 52, 63, 52, 68, 48, 56, 48, 56, 48, 51, 90, 67, respectively.

For the center-dwell tendency analysis, the tadpoles whose total traveled distance exceeded 5 cm (twice the diameter of the arena) were included. Among these, the most active 1-minute epoch was chosen per movie, and the trajectory during this period was visualized by box and whisker plots, with individual data as points. For visualization, 10 tadpoles per group were randomly selected from each group, the arena was divided into 10x10 areas, and the proportion of the time spent in each area was represented as a density plot. The numbers of analyzed animals for Figure 2E(b) are 34, 43, 21, 20, 32, 23, 53, 65, 29, 24, 13, 21, 28, 36, 62, 48, respectively.

For potassium bromide rescue experiments, we initially recorded the 10-minute swimming behavior of Pcdh19 MO-injected tadpoles at stage 49. Subsequently, the same tadpoles' behavior was recorded in the presence of 8 mM potassium bromide (KBr) in the bath for an additional 10 minutes. Their behaviors were then recorded again in the bath without KBr, also for 10 minutes. We analyzed the duration of seizure-like behaviors and center-dwell time, while tracking individual tadpoles. For pairwise comparisons, we included tadpoles whose total traveled distance exceeded 5 cm in all three imaging epochs in the statistical analysis. As depicted in Figure 2F(a), KBr treatment decreased seizure-like behaviors in 10 out of 11 tadpoles. The one tadpole that did not show an effect had exhibited no seizure-like behavior in the first round of recording. Among the 10 tadpoles that showed the effect, seizure-like behavior rebounded in 7 tadpoles when KBr was withdrawn.

Histology

Anesthetized tadpoles were fixed in 4% paraformaldehyde dissolved in 1X PBS, saturated with 30% sucrose in 1X PBS, embedded in OCT (Tissue-Tek, PA, USA), frozen, sectioned in 16-µm thickness in the coronal plane with a cryostat (Thermo Fisher Scientific), and attached on Superfrost plus microscope slide (Thermo Fisher Scientific). Slides were stained with Hoechst 33342 (1:1000, Sigma-Aldrich), mounted in FluorSave (Sigma-Aldrich), and imaged using a confocal fluorescence microscope (LSM 700, Carl Zeiss).

REFERENCES FOR EXTENDED MATERIALS AND METHODS

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2. J. Schindelin *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
3. K. G. Pratt, A. S. Khakhalin, Modeling human neurodevelopmental disorders in the Xenopus tadpole: from mechanisms to therapeutic targets. *Dis Model Mech* **6**, 1057-1065 (2013).
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SUPPLEMENTAL MOVIE

Movie S1, related to Figure 1E. Examples of seizure-like and normal swimming behaviors in tadpoles. **(A)** Repetitive circling. **(B)** Lateral movement of the head with tremors. **(C)** C-shaped contractions. **(D)** Intermittent bouts of rapid swimming in Pcdh19 knockdown tadpoles. **(E)** Typical swimming behavior of a healthy tadpole. Timestamp, seconds:centiseconds.