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## Characterization of seizure susceptibility in *Pcdh19* mice

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### Abstract

**Objective**—*PCDH19*-related epilepsy is characterized by a distinctive pattern of X-linked inheritance, where heterozygous females exhibit seizures and hemizygous males are asymptomatic. A cellular interference mechanism resulting from the presence of both wild-type and mutant *PCDH19* neurons in heterozygous patients or mosaic carriers of *PCDH19* variants has been hypothesized. We aim to investigate seizure susceptibility and progression in the *Pcdh19* mouse model.

**Methods**—We assessed seizure susceptibility and progression in the *Pcdh19* mouse model using three acute seizure-induction paradigms. We first induced focal, clonic seizures using the 6-Hz psychomotor test. Mice were stimulated with increasing current intensities and graded according to a modified Racine scale. We next induced generalized seizures using flurothyl or pentylenetetrazol (PTZ), both GABA<sub>A</sub> receptor function inhibitors, and recorded latencies to myoclonic and generalized tonic-clonic seizures.

**Results**—*Pcdh19* knockout and heterozygous females displayed increased seizure susceptibility across all current intensities in the 6-Hz psychomotor test, and increased severity overall. They also exhibited shorter latencies to generalized seizures following flurothyl, but not PTZ, seizure induction. Hemizygous males showed comparable seizure incidence and severity to their wild-type male littermates across all paradigms tested.

**Significance**—The heightened susceptibility observed in *Pcdh19* knockout females suggests additional mechanisms other than cellular interference are at play in *PCDH19*-related epilepsy. Further experiments are needed to understand the variability in seizure susceptibility so that this model can be best utilized toward development of future therapeutic strategies for *PCDH19*-related epilepsy.

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#### Author contributions

AG-G designed the study. JR, NPS, and SM performed the experiments. JR, NPS, and AG-G performed data analysis. JR and AG-G wrote the manuscript. All authors read and approved the final manuscript.

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#### Conflict of interest statement

None of the authors has any conflict of interest to disclose.

#### Ethical Publication Statement

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

## Keywords

Protocadherin 19; Epilepsy; X-linked

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## 1 Introduction

PCDH19 is a cell adhesion molecule belonging to the protocadherin subgroup within the cadherin superfamily. It is predominantly expressed in the nervous system. Heterozygous loss-of-function variants in the human X-linked gene *PCDH19* lead to early infantile epileptic encephalopathy associated with varying degrees of intellectual disability and autistic features. *PCDH19*-related epilepsy shows an atypical pattern of inheritance where heterozygous females present with seizures while hemizygous carrier males are asymptomatic.<sup>1</sup> Seizures occur in brief and reoccurring clusters that are predominantly focal in origin, although generalized seizures (tonic, myoclonic, absence) are also reported.<sup>2</sup> *PCDH19* has been identified as the second most clinically relevant gene in epilepsy.<sup>3</sup> Yet, the pathogenic mechanism behind *PCDH19*-related epilepsy remains largely unknown.

A mechanism of cellular interference has been suggested, wherein the co-existence of neurons expressing wild-type (WT) or mutant PCDH19 disrupts cell-cell interactions.<sup>4</sup> Recent cases of affected males with mosaicism for *PCDH19* pathogenic variants<sup>5-7</sup> and a case of *PCDH19*-related epilepsy in a male with Klinefelter syndrome<sup>8</sup> provide further support for the cellular interference hypothesis. Previous studies investigating PCDH19 function revealed that heterozygous, knockout, and hemizygous mice did not exhibit gross brain abnormalities or spontaneous seizures.<sup>9</sup> Although *Pcdh19* heterozygous females showed increased hyperactivity and a decreased fear response compared to their WT littermates, no differences were observed between *Pcdh19* genotypes when behavioral phenotypes such as anxiety, social interaction behaviors, and spatial/working memory were assessed.<sup>10</sup> No characterization of a seizure/epilepsy phenotype has been reported.

To address this issue, we investigated seizure susceptibility and progression in the *Pcdh19* mouse model. In particular, we characterized susceptibility to focal seizures using the 6-Hz psychomotor model, as well as susceptibility and progression to generalized seizures using flurothyl and PTZ.

## 2 Methods

### 2.1 Mice

All experiments were approved by the Northwestern University Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Animal Welfare Act. Animals were housed on a 14-hour light/10-hour dark schedule, with food and water available *ad libitum*. *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-y</sup> mice (TF2108) on a mixed 129S5.C57BL/6 background were purchased from Taconic Biosciences (Cambridge City, IN) and crossed together to obtain *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-/-</sup> females, as well as *Pcdh19*<sup>+y</sup>, and *Pcdh19*<sup>-y</sup> males. *Pcdh19*<sup>+y</sup> males were crossed with *Pcdh19*<sup>+/-</sup> female mice to also generate WT females. For each experiment, we used age-matched littermates from

these 2 different mating setups. Genotyping was performed following vendor's instructions. The study was done on mice aged 8 to 12 weeks.

## 2.2 Flurothyl seizure induction

Flurothyl seizure induction was performed as previously described.<sup>11</sup> Briefly, mice were individually placed in a sealed induction chamber. Flurothyl (2,2,2-trifluoroethyl ether, Sigma-Aldrich, Milwaukee, WI) was introduced by a syringe pump (KD Scientific, Holliston, MA, U.S.A.) at a constant rate of 20  $\mu$ l/min. Latencies to the first myoclonic jerk (MJ) and generalized tonic-clonic seizure (GTCS) were recorded by reviewers blinded to the genotype following flurothyl induction. At the onset of GTCS, the mouse was immediately placed in a holding cage for recovery. All mice were euthanized at the end of the experimental session.

## 2.3 6-Hz psychomotor seizure test

6-Hz psychomotor seizures were induced via auricular stimulation (0.2 ms pulse width at 6 Hz, 3 s duration). Mice were tested at current intensities of 16, 24, and 30 mA. Prior to stimulation, auricular electrodes were soaked in 0.9% saline solution to ensure complete electrical contact. Animals were manually restrained and delivered a single shock using an ECT unit (Ugo Basile; Comerio, Italy). Following 6-Hz stimulation, mice were observed for 2 minutes and scored by reviewers blinded to the genotype according to a modified Racine scale of seizure severity:

- 0 – No seizure
- 1 – Loss of posture associated with facial clonus and clonus of forelimbs and/or hindlimbs
- 2 – Grade 1 seizure followed by recovery of the righting reflex and low intensity bouncing
- 3 – Grade 1 and/or 2 features with recovery of the righting reflex followed by wild running and popcorning

Animals that did not resume normal exploratory behavior within 10 seconds following 6-Hz stimulation were not tested at higher current intensities.

## 2.4 Video-EEG recording and PTZ seizure induction

Mice were implanted with prefabricated EEG headmounts (Pinnacle Technology, Inc., Lawrence, KS, USA). Briefly, mice were anesthetized with isoflurane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Canada). Headmounts with four stainless steel screws that served as cortical surface electrodes were affixed to the skull with dental cement (GC America, Alsip, IL, USA). Animals were subcutaneously administered 1 mg/kg buprenorphine SR as postoperative analgesic and single-housed for 7 days for recovery. Video EEG started with 1 hour of baseline recording followed by a single dose of PTZ (Sigma-Aldrich, St. Louis, MO, USA) administered intraperitoneally at 40 mg/kg. Video EEG data were collected for one additional 30 minutes post-treatment. Digitized data were acquired and analyzed with Sirenia software (Pinnacle Technology, Inc., Lawrence,

KS, USA.) along with contemporaneous video recordings. Epileptiform activity was scored manually offline by a reviewer blinded to gender and genotype, according to a revised Racine scale<sup>12</sup> as follows: -1, normal baseline; 0, whisker trembling; 1, sudden behavioral arrest; 2 facial jerking; 3, myoclonic jerks; 4, clonic seizure sitting; 5 clonic, tonic-clonic seizure, lying on belly; 6, clonic, tonic-clonic seizure with loss of posture and wild jumping; 7, tonic extension, possibly leading to respiratory arrest and death.

## 2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad, La Jolla, California). Results are presented as mean  $\pm$  standard deviation, unless mentioned otherwise. Outlier detection using the ROUT method (Q=1%) was used whenever the normality criterion was met. One-way analysis of variance (ANOVA) followed by the Tukey's post hoc multiple comparisons test (females), or a two-tailed unpaired *t*-test (males), were used whenever normality and homoscedasticity criteria were met. Otherwise, the nonparametric Kruskal-Wallis test followed by the Dunn's post hoc multiple comparisons test (females), or the Mann-Whitney test (males) were used. A *P* value < 0.05 was considered statistically significant.

The 6-Hz CC<sub>50</sub> and 95% confidence intervals were determined using log-probit analysis.

## 3 Results

### 3.1 *Pcdh19* female mice exhibit lower seizure thresholds and a more severe seizure phenotype following 6-Hz stimulation

We investigated susceptibility to focal seizures by comparing seizure incidence and severity between *Pcdh19* mutant and WT littermates following 6-Hz stimulation (Fig. 1). The percentage of mice exhibiting psychomotor seizure behavior followed a current-dependent increase. *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-/-</sup> females displayed higher seizure incidence at each current intensity when compared with WT female mice. At 16 mA, 55% (11/20) of *Pcdh19*<sup>+/-</sup> and 77% (10/13) of *Pcdh19*<sup>-/-</sup> mice expressed seizures, compared with 22% (4/18) of WT females. At 24 mA, 95% of *Pcdh19*<sup>+/-</sup> and 100% of *Pcdh19*<sup>-/-</sup> mice seized, compared with 44% of WT female mice. At 30 mA, 100% of *Pcdh19*<sup>+/-</sup> mice expressed seizures compared with 67% of their WT female littermates (Fig. 1A). Consequently, the median current level at which half of the mice seized (CC<sub>50</sub>) was significantly lower in heterozygous (CC<sub>50</sub> = 15 mA; CI 95%: 13 to 17) and knockout females (CC<sub>50</sub> = 13 mA; CI 95%: 11 to 16) when compared to WT female mice (CC<sub>50</sub> = 24 mA; CI 95%: 21 to 27) (Fig. 1B). *Pcdh19* female mice exhibited significantly more severe seizures than their WT littermates. Interestingly, at 16 mA, *Pcdh19*<sup>+/-</sup> females expressed a variable phenotype, with behavior ranging from no sign of seizure activity to advanced clonic seizures (wild running), while *Pcdh19*<sup>-/-</sup> females exhibited a significantly more robust seizure phenotype (wild running and popcorning). At 24 mA, both heterozygous and knockout females behaved comparably (Fig. 1C). Hemizygous males showed comparable seizure incidence and severity to their WT male littermates across all current levels (Fig. 1D, E, and F).

### 3.2 *Pcdh19* female mice show shorter latencies to flurothyl-, but not PTZ-induced generalized seizures.

We next assessed susceptibility to induced generalized seizures using the GABA<sub>A</sub> receptor function inhibitors flurothyl and PTZ. Mice exposed to flurothyl express a series of characteristic seizure behaviors, beginning with brief myoclonic jerks (MJ), which eventually progress to generalized tonic-clonic seizures (GTCS). The average latencies to MJ were comparable among all genotypes in both sexes (Fig. 2A, B). However, the average latency to GTCS was significantly reduced in heterozygous females (124 s; CI 95%: 107 to 141) when compared with their WT littermates (177 s; CI 95%: 128 to 226) ( $p = 0.0107$ ) (Fig. 2A). Interestingly, knockout females also exhibited a decreased latency to GTCS (114 s; CI 95%: 105 to 124) when compared to WT mice ( $p = 0.0033$ ) (Fig. 2A). In contrast, no statistically significant differences were observed in latency to GTCS between hemizygous and WT males (latency to GTCS in WT males = 139 s; CI 95%: 99 to 179; latency to GTCS in *Pcdh19*<sup>-y</sup> mice = 123 s; CI 95%: 92 to 154) (Fig. 2B).

It has been previously reported that *Pcdh19*<sup>+/-</sup> mice showed altered network activity with increased number of spike-wave discharge (SWD) events when compared to their WT or *Pcdh19*<sup>-/-</sup> female littermates.<sup>4</sup> SWD incidence on EEG accompanied by behavioral arrest are hallmarks of absence epilepsy. We thus performed video-EEG recordings to analyze spontaneous and induced seizure behavior in *Pcdh19* mice (Fig. 3A). During baseline recording (Fig. 3B), all *Pcdh19*<sup>+/-</sup> mice exhibited SWD activity (Fig. 3D) concomitant with behavioral arrest, with 60% of *Pcdh19*<sup>+/-</sup> (6/10) expressing SWD episodes lasting longer than 3 s (Fig. 4A, F). In contrast, two thirds of WT females (4/6; 2/6 SWD < 3 s, 2/6 SWD > 3 s), and only 40 % of *Pcdh19*<sup>-/-</sup> mice (2/5; 1/5 SWD < 3 s, 1/5 SWD > 3 s) showed SWD. These patterns of SWD-expression were not hugely affected by PTZ injection (Fig. 4A). The 40 mg/kg PTZ dose did not induce generalized seizure in every mouse. 57% of WT females (4/7) reached the GTCS stage (Fig. 3G), which was systematically followed by clonic seizure episodes (Fig. 3E). Half (5/10), and 40% (2/5) of *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-/-</sup> mice, respectively, expressed GTCS. Interestingly, clonic seizure (CS) incidence did not depend on GTCS expression, with 70% (7/10) of *Pcdh19*<sup>+/-</sup> mice and 80% (4/5) of *Pcdh19*<sup>-/-</sup> mice exhibiting CS, compared to 57% of WT (4/7) (Fig. 4B). Average latencies to reach the first MJ (Fig. 3F) and GTCS were similar across all genotypes in both sexes (Fig. 4C, F), as well as seizure severity (Fig. 4E, H). However, *Pcdh19*<sup>+/-</sup> mice expressed significantly less MJs (22 MJs; CI 95%: 10 to 33 MJs) during the 30 minutes post-PTZ injection, compared to WT (75 MJs; CI 95%: 27 to 123 MJs) and *Pcdh19*<sup>-/-</sup> females (82 MJs; CI 95%: 0 to 204 MJs) (Fig. 4D). No statistically significant differences were observed in the total number of MJs in 30 minutes post-PTZ injection between WT and hemizygous males (total number of MJs in WT males = 33; CI 95%: 0 to 68 MJs; total number of MJs in *Pcdh19*<sup>-y</sup> mice = 69; CI 95%: 31 to 108 MJs) (Fig. 4G).

## 4 Discussion

This study is the first to characterize seizure susceptibility in the *Pcdh19* mouse model. We show that *Pcdh19*<sup>+/-</sup> mice have a higher SWD incidence, when compared to their WT and *Pcdh19*<sup>-/-</sup> female littermates, which is consistent with previous data.<sup>4</sup> We also show that

heterozygous female mice exhibit lower seizure thresholds and qualitatively more severe seizure phenotypes while hemizygous males behave comparably to WT males in the 6-Hz psychomotor and flurothyl seizure-induction tests. For these two paradigms, the seizure expression in *Pcdh19*<sup>+/-</sup> females and asymptomatic behavior in *Pcdh19*<sup>-/y</sup> males resembles the atypical pattern of inheritance observed in patients with *PCDH19*-related epilepsy. Interestingly, we also observe lower seizure thresholds and a more severe phenotype in knockout females. This heightened susceptibility somewhat challenges the cellular interference hypothesis, where knockout females are expected to behave comparably to WT females. Taken together, our data suggest that in addition to cellular interference, other mechanisms, possibly gender-related, may also play a role in the pathogenesis of *PCDH19*-related epilepsy. For example, differences in neurosteroid metabolism have been described in female patients with *PCDH19*-related epilepsy.<sup>13</sup> Reduced blood levels of allopregnanolone is particularly relevant,<sup>14</sup> as the neurosteroid is a positive modulator of GABA<sub>A</sub> receptor function, and has also been associated with anticonvulsive properties.<sup>15</sup> Interestingly, besides assisting in homophilic cell binding and sorting,<sup>4</sup> *PCDH19* interacts with synaptic and extra-synaptic GABA<sub>A</sub> receptors, as well as actin cytoskeletal regulators<sup>16, 17</sup>. Moreover, although the majority of mosaic males with pathogenic variants in *PCDH19* develop epilepsy, there have been several reported cases of males with somatic mosaicism who are asymptomatic.<sup>18</sup> Further investigation is needed to understand the broader role for *PCDH19* in epileptogenesis and the cause of the phenotype variability observed in *PCDH19*-related epilepsy.

Contrary to flurothyl, we found that PTZ doesn't affect seizure susceptibility or progression to GTCS in *Pcdh19* mice. However, more *Pcdh19* female mice present with CS. Why does exposing *Pcdh19* mice to two GABA<sub>A</sub> receptor function inhibitors result in such different outcomes? It has been hypothesized that both convulsants inhibit GABA<sub>A</sub> receptor activity through different mechanisms. PTZ interacts with GABA<sub>A</sub> receptors at a domain comparable but distinct from the picrotoxin-binding site,<sup>19</sup> while the flurothyl action site is suggested to be the anesthetic ethers-binding site.<sup>20</sup> The drug delivery method matters too. Indeed, epileptogenic mechanisms following acute PTZ administration or PTZ kindling are different, as repetitive PTZ exposure may modify GABA<sub>A</sub> receptor subunit composition.<sup>21</sup> As *PCDH19* is known to interact with GABA<sub>A</sub> receptor  $\alpha$  subunits,<sup>16</sup> we don't exclude that PTZ-kindled *Pcdh19* mice could show differences in seizure susceptibility compared to the acute PTZ treatment performed in our study.

*PCDH19* patients express seizures ranging from focal clustered seizures, often precipitated by fever,<sup>2</sup> to generalized absence, tonic-clonic, and myoclonic seizures. However, the majority of patients outgrow their seizures over time.<sup>22</sup> The characterization of seizure susceptibility presented in this study was performed on young adult mice. It would be interesting to assess seizure susceptibility in neonatal *Pcdh19* mice using the febrile seizure model or convulsive drug injection during early postnatal development.

In summary, our study demonstrates that *Pcdh19* mice seizure susceptibility varies with the seizure induction model used. Additional experiments are needed to understand the variability in seizure susceptibility seen with heterozygous and homozygous *Pcdh19* female

mice so that this model can be best utilized toward development of future therapeutic strategies for *PCDH19*-related epilepsy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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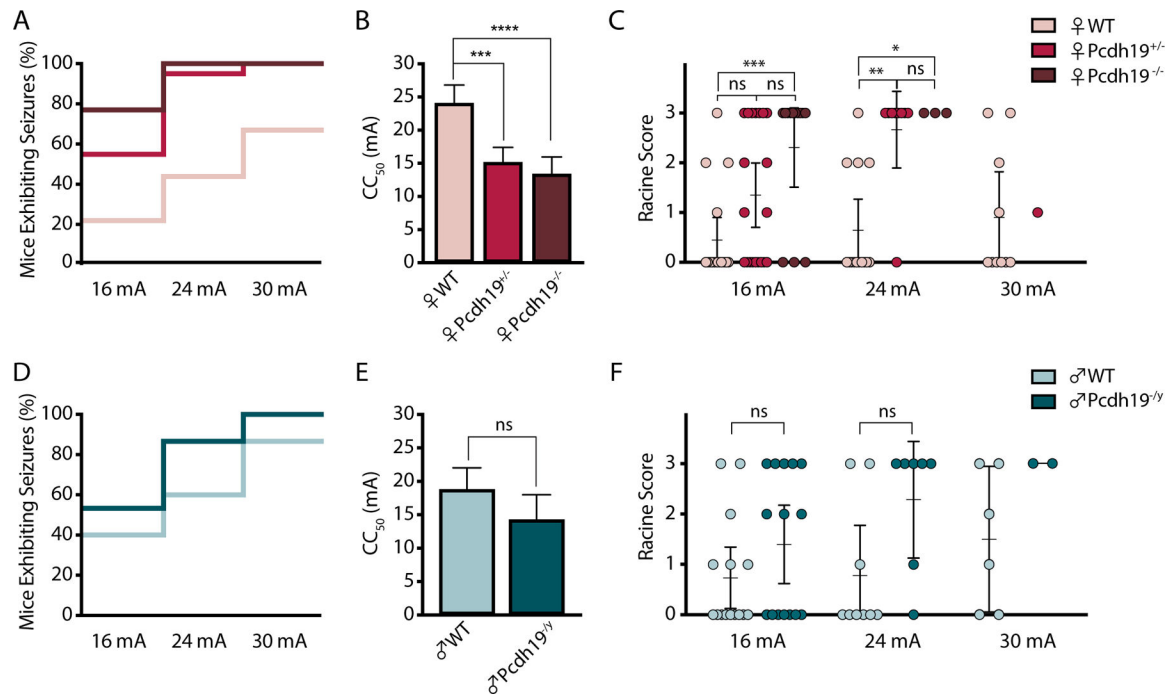
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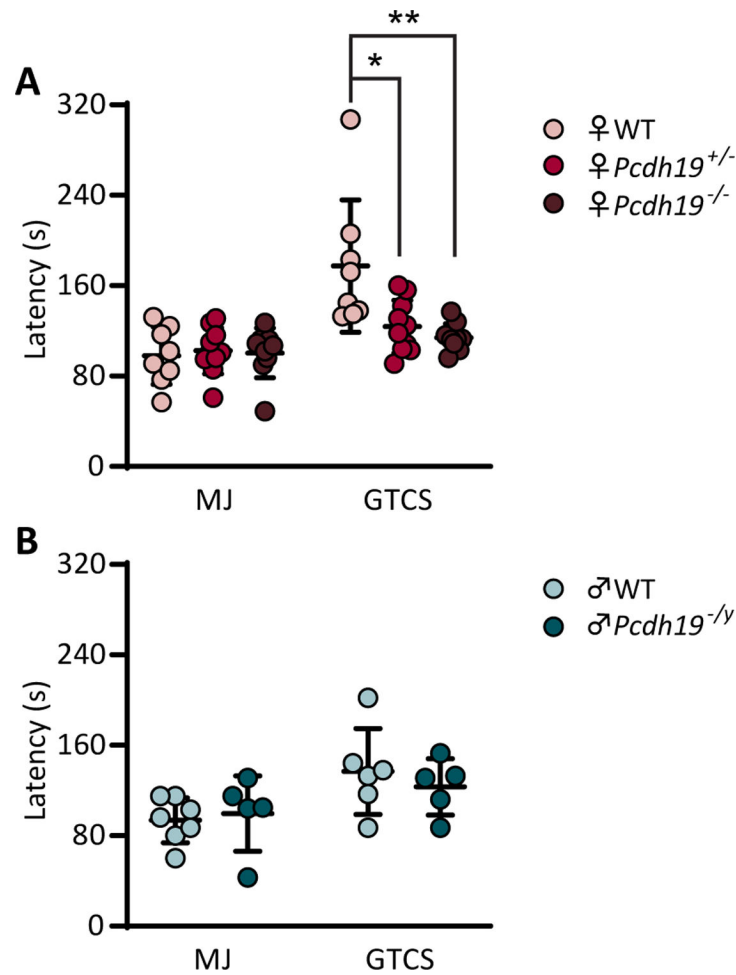
**Key points:**

- *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-/-</sup> female mice show enhanced susceptibility to induced focal seizures.
- Susceptibility to induced generalized seizure in *Pcdh19*<sup>+/-</sup> and *Pcdh19*<sup>-/-</sup> female mice varies with the induction model used.
- Seizure expression in *Pcdh19*<sup>-/-</sup> male mice is comparable to their WT littermates, regardless of the seizure induction model used.
- The heightened seizure susceptibility in *Pcdh19*<sup>+/-</sup> females and asymptomatic behavior in *Pcdh19*<sup>-/-</sup> males resembles the pattern of inheritance observed in *PCDH19*-epilepsy.
- *Pcdh19*<sup>-/-</sup> females expressing heightened susceptibility suggests a broader role for PCDH19 in epileptogenesis.

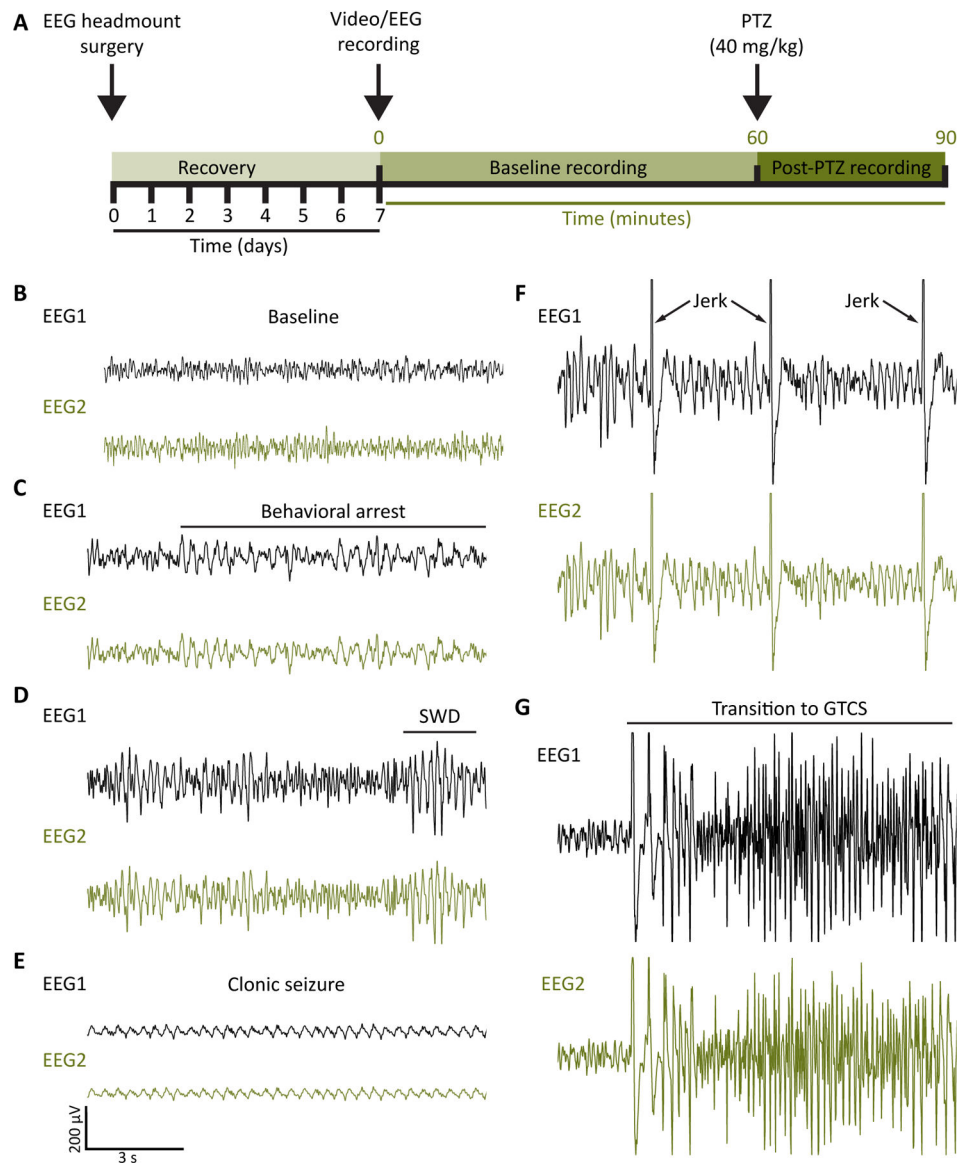


**Figure 1.**

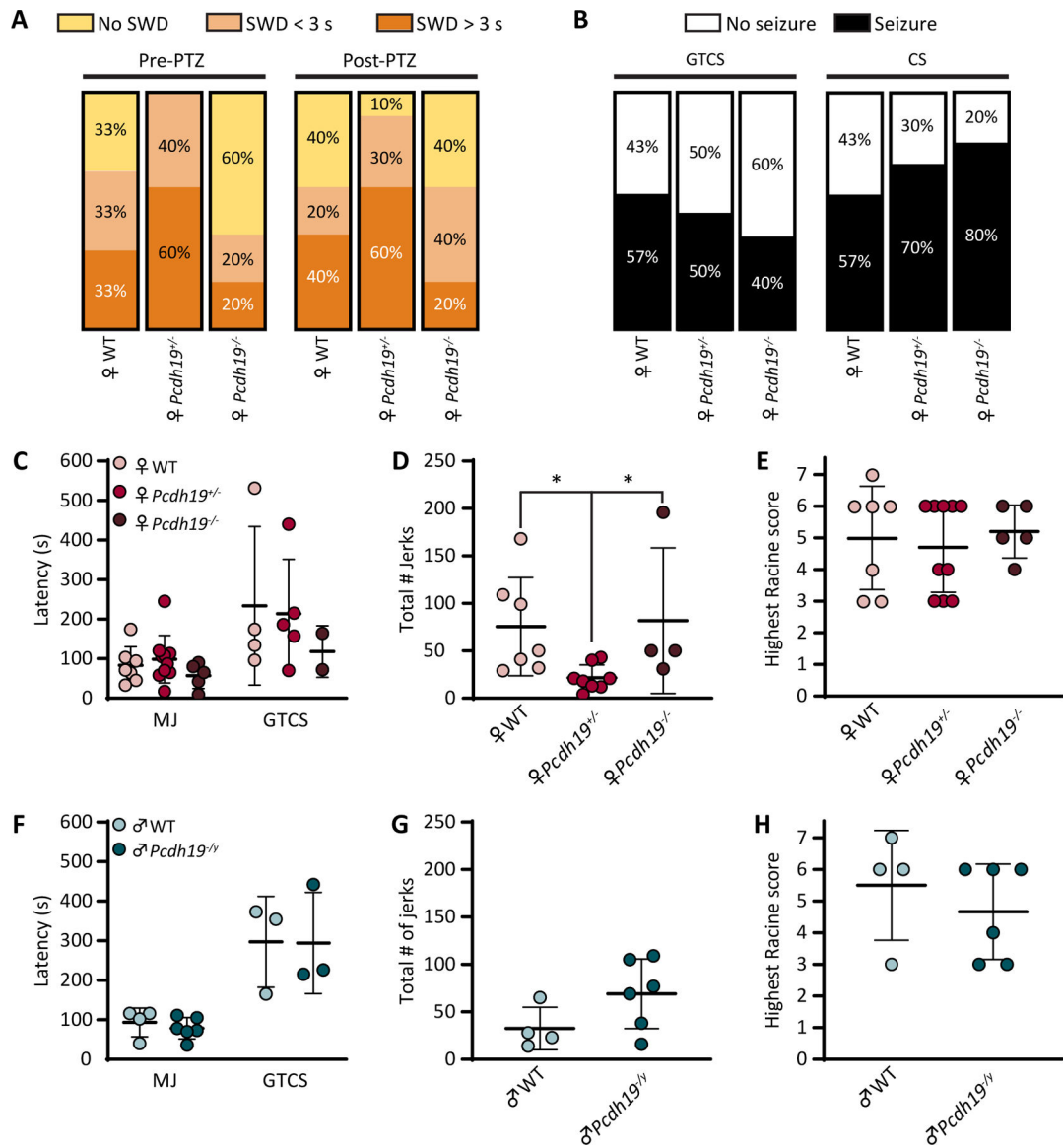
*Pcdh19* female mice show increased susceptibility to 6-Hz seizures. A and D, Percentage of *Pcdh19* female (A) or male (D) mice expressing seizures following stimulation with 16 mA, 24 mA, and 30 mA. At 16 mA, Female n = 18 (WT), 20 (*Pcdh19*<sup>+/-</sup>), 13 (*Pcdh19*<sup>-/-</sup>); Male n = 15 (WT), 15 (*Pcdh19*<sup>-/-</sup>). At 24 mA, Female n = 14 (WT), 9 (*Pcdh19*<sup>+/-</sup>), 3 (*Pcdh19*<sup>-/-</sup>); Male n = 9 (WT), 7 (*Pcdh19*<sup>-/-</sup>). At 30 mA, Female n = 10 (WT), 2 (*Pcdh19*<sup>+/-</sup>), 0 (*Pcdh19*<sup>-/-</sup>); Male n = 6 (WT), 2 (*Pcdh19*<sup>-/-</sup>). B and E, Average  $CC_{50}$  values based on Probit analysis for each genotype in *Pcdh19* females (B) and males (E). \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , ns = not statistically significant. C and F, Racine scores at each current level in *Pcdh19* females (C) and males (F). Female n = 18 (WT), 20 (*Pcdh19*<sup>+/-</sup>), 13 (*Pcdh19*<sup>-/-</sup>) (C). \*  $P = 0.0204$ , \*\*  $P = 0.0015$ , \*\*\*  $P = 0.0008$ ; Kruskal-Wallis ANOVA followed by Dunn's test. Male n = 15 (WT), 15 (*Pcdh19*<sup>-/-</sup>). ns = not statistically significant; Mann-Whitney test. Data are represented as the mean with 95% confidence intervals.



**Figure 2.** *Pcdh19* female mice show lower seizure thresholds following flurothyl exposure. A and B, Latencies to the first myoclonic jerk (MJ) and generalized tonic-clonic seizure (GTCS) in *Pcdh19* females (A) and males (B) Female n = 8 (WT), 10 (*Pcdh19*<sup>+/-</sup>), 9 (*Pcdh19*<sup>-/-</sup>). \*  $P = 0.0107$ , \*\*  $P = 0.0033$ ; Ordinary one-way ANOVA followed by Tukey's test. Male n = 7 (WT), 5 (*Pcdh19*<sup>-/-</sup>). Unpaired  $t$  test (B). Data are represented as the mean  $\pm$  standard deviation.



**Figure 3.** A, Schematic representation of the experimental setup for the video-EEG recording and PTZ administration experiment. B-G, Representative EEG recordings from a *Pcdh19* mouse during baseline (B), and after PTZ injection (C-G). SWD: spike-wave discharge; GTCS: generalized tonic-clonic seizure.



**Figure 4.**

Seizure susceptibility and severity are not affected by loss of *Pcdh19* following acute PTZ treatment. A, Percentages of *Pcdh19* female mice expressing SWD during one-hour baseline recording and 30 minutes post-PTZ injection. B, Percentages of *Pcdh19* female mice expressing generalized tonic-clonic seizures (GTCS), or clonic seizures (CS) following PTZ injection. Female  $n = 6$  (WT), 10 (*Pcdh19<sup>+/-</sup>*), 5 (*Pcdh19<sup>-/-</sup>*). C and F, Latencies to the first MJ and GTCS following PTZ administration in *Pcdh19* females (C) and males (F). D and G, Total number of jerks occurring during the first 30 minutes post-PTZ injection in *Pcdh19* females (D) and males (G). E and H, Highest Racine score reached during the first 30 minutes post-PTZ injection in *Pcdh19* females (E) and males (H). Female  $n = 7$  (WT), 10 (*Pcdh19<sup>+/-</sup>*), 5 (*Pcdh19<sup>-/-</sup>*). \*  $P = 0.0226$  and  $0.0446$  for female WT vs. *Pcdh19<sup>+/-</sup>* and *Pcdh19<sup>+/-</sup>* vs. *Pcdh19<sup>-/-</sup>*, respectively; Kruskal-Wallis ANOVA followed by Dunn's test. Male  $n = 4$  (WT), 6 (*Pcdh19<sup>-/-</sup>*). Mann-Whitney test. Error bars indicate standard deviation.