Creating a model of PCDH19-related epilepsy syndrome using the *Xenopus tropicalis* and investigating the association of GABA-A signaling

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Directed by Professor Hosung Jung

The Master's Thesis
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Jugeon Park

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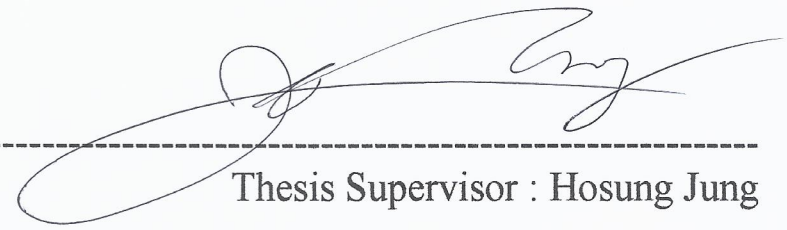
This certifies that the Master's Thesis of
Jugeon Park is approved.



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우선, 2년간 많이 부족했던 저를 열정을 다하여 지도해 주셨던 정호성 교수님께 감사의 말씀을 드립니다. 학위 논문 준비에 있어서 도움과 조언을 아끼지 않으셨던 김철훈 교수님과 이은이 교수님께 감사의 말씀을 드립니다.

아무것도 모르던 저를 하나부터 열까지 가르쳐 주셨던 옥지연 박사님, 정제인 박사님, 김혜영 선배님, 쾌적한 실험실 환경을 위해서 매일 도와주시는 김해리 선생님 그리고 하나밖에 없는 동기 박시현 선생님께 감사의 말씀을 드립니다.

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ABSTRACT

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(Directed by Professor Hosung Jung)

PCDH19-related epilepsy syndrome is a rare disorder characterized primarily by epilepsy onset before the age of 3, developmental delay, intellectual disability, autistic tendencies, and behavioral abnormalities. The causative gene, PCDH19, encodes a calcium-dependent single-pass transmembrane protein known as protocadherin-19. This protein plays a crucial role in neural development by regulating cell-to-cell adhesion through homophilic binding via its extracellular cadherin domains and modulating inter-neuronal signaling. The PCDH19 gene is located on the X chromosome. While males with mutations typically do not manifest the disease, 90% of females with a mutation on one of their two X chromosomes exhibit female-limited epilepsy. This is attributed to random X-chromosome inactivation, leading to a mosaic pattern of normal and PCDH19-mutated cells, causing abnormal cellular interference. Recent findings have also revealed that PCDH19 interacts with the alpha1 subunit of the GABA-A receptor in neurons, and by modulating the expression of PCDH19, the GABAergic neurotransmission signal of the GABA-A receptor can be regulated. In this thesis, using the *Xenopus tropicalis* model, I conducted loss-of-function experiments using PCDH19 morpholinos to create a model of PCDH19-related epilepsy syndrome. I then quantified changes in the epilepsy phenotype of *Xenopus tropicalis* induced by GABA-A agonists and antagonists to determine if the behavioral phenotype caused by PCDH19 deficiency can be alleviated through GABA-A signaling modulation. By microinjecting PCDH19 morpholinos into *Xenopus tropicalis* embryos, I

suppressed PCDH19 gene expression specifically in the central nervous system, induced functional loss, and conducted behavioral tests to quantify the manifestation of PCDH19-related epilepsy. The microinjection was differentiated between 8-cell and 16-cell stages, with the 8-cell stage embryos receiving injections in all cells for complete knockdown and the 16-cell stage embryos receiving injections in only half of the cells to induce a mosaic pattern. The study contrasted the differences between the mosaic pattern and complete knockdown in the central nervous system. While there were no morphological changes in the developing embryos from either group, both exhibited abnormal swimming trajectories and typical epileptic behaviors, including seizures and abnormal behaviors. Detailed analysis and quantification of these behaviors were conducted, and treatment with the antiepileptic drug potassium bromide reduced the frequency and duration of seizures, confirming the behaviors as epileptic. Furthermore, cryosections of *Xenopus tropicalis* brains during the peak of PCDH19 expression revealed that while control group brains had a scattered distribution of morpholino-injected and non-injected cells, PCDH19 knockdown cells clustered together. After inducing the epilepsy phenotype in *Xenopus tropicalis* embryos with PCDH19 morpholino injections, treatment with the GABA-A agonist Muscimol and antagonist Bicuculline during the formation of the central nervous system showed that Bicuculline-treated individuals had relatively reduced seizure behaviors, while Muscimol-treated individuals exhibited increased seizure behaviors. These findings underscore the significance of PCDH19 as a key regulator of GABAergic neurotransmission and provide theoretical evidence for understanding the novel pathogenesis mechanism of PCDH19 epilepsy.

Key words : PCDH19, epilepsy, mosaicism, seizure, GABA-A receptor

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I. INTRODUCTION

PCDH19-Related Epilepsy Syndrome is a rare disorder characterized primarily by seizures that manifest typically before the age of three.[1] Additional symptom of PCDH19 include developmental delays, intellectual disabilities, autistic traits, and behavioral abnormalities.[2-6] The causative gene, PCDH19, encodes a calcium-dependent, single-pass transmembrane cell adhesion protein called protocadherin-19. This protein plays an important role in neurodevelopment by regulating cell-to-cell adhesion through homophilic binding via extracellular cadherin domains and signal transmission between neurons.[7-10]

Located on the X chromosome, the PCDH19 gene presents a unique genetic profile. While males with the mutation usually remain asymptomatic, nearly 90% of females harboring a mutated copy manifest Epilepsy in Females with Mental Retardation (EFMR).[2,11-13] This is thought to arise from cellular interference due to the mosaic presence of both normal and mutated PCDH19 cells, triggered by random X-chromosome inactivation (XCI).[14] In the case of PCDH19 hemizygous males, having only one X chromosome, any potential mutations would affect all cells uniformly, leading to no significant disruption in synaptic function between presynaptic and postsynaptic adhesion. Conversely, for PCDH19 heterozygous mutant females, the coexistence of normal and mutant PCDH19 due to cellular mosaicism results in impaired adhesion between presynaptic and postsynaptic,

ultimately leading to reduced synaptic vesicles, decreased neurotransmitter secretion, diminished long-term potentiation (LTP) plasticity, and impaired cognitive behavior (Figure 1).[15]

PCDH19 is composed of six consecutive extracellular cadherin (EC) domains, a transmembrane (TM) region, and a cytoplasmic domain (CP) with a C-terminal tail.[16] It is evolutionarily conserved in many animals, including *Xenopus tropicalis*. Many mutations, including point mutations and partial or complete gene deletions, have been identified (Figure 2),[17,18] and these mutations are evolutionarily conserved in many animals, including *Xenopus tropicalis* (Figure 3A and 3B). Heterozygous PCDH19 mutations in humans are currently recognized as a cause of the second most common epilepsy, known as PCDH19 GCE (girls clustering epilepsy).

Interestingly, similar patterns of epilepsy have been observed in rodents.[19,20] Initially, symptoms were noted only in heterozygous female mice with PCDH19 mutations.[21,22] However, recent studies have shown that male mice with PCDH19 knockout (KO) also exhibit autistic phenotypes, including repetitive behaviors like self-grooming and rearing, and deficits in sociability.[23]

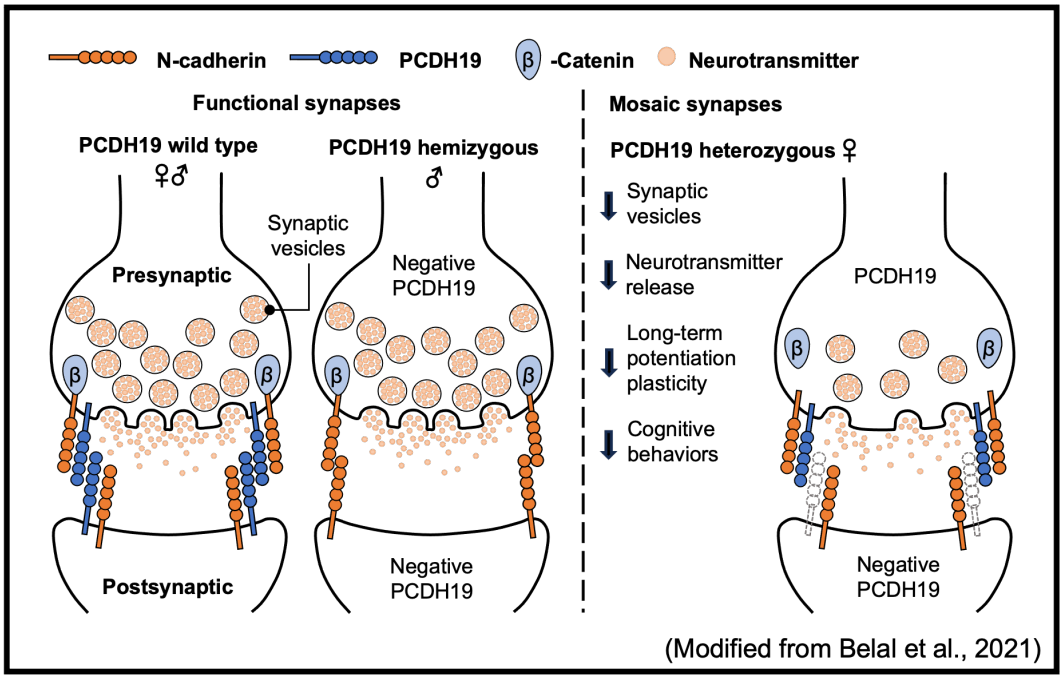


Figure 1. A current view on the pathogenesis of PCDH19-related epilepsy in PCDH19 heterozygous females based on random X-chromosome inactivation. In the case of PCDH19 hemizygous males, having only one X chromosome means that even if a mutation occurs, all cells uniformly possess mutant PCDH19. This does not lead to problems with adhesion between the presynaptic and postsynaptic, and consequently, it does not significantly affect synaptic function. However, in the case of PCDH19 heterozygous mutant females, cellular mosaicism results in the coexistence of normal PCDH19 and mutant PCDH19. This leads to problems in adhesion between the presynaptic and postsynaptic, resulting in reduced synaptic vesicles, decreased neurotransmitter release, reduced long-term potentiation (LTP) plasticity, and impaired cognitive behavior.

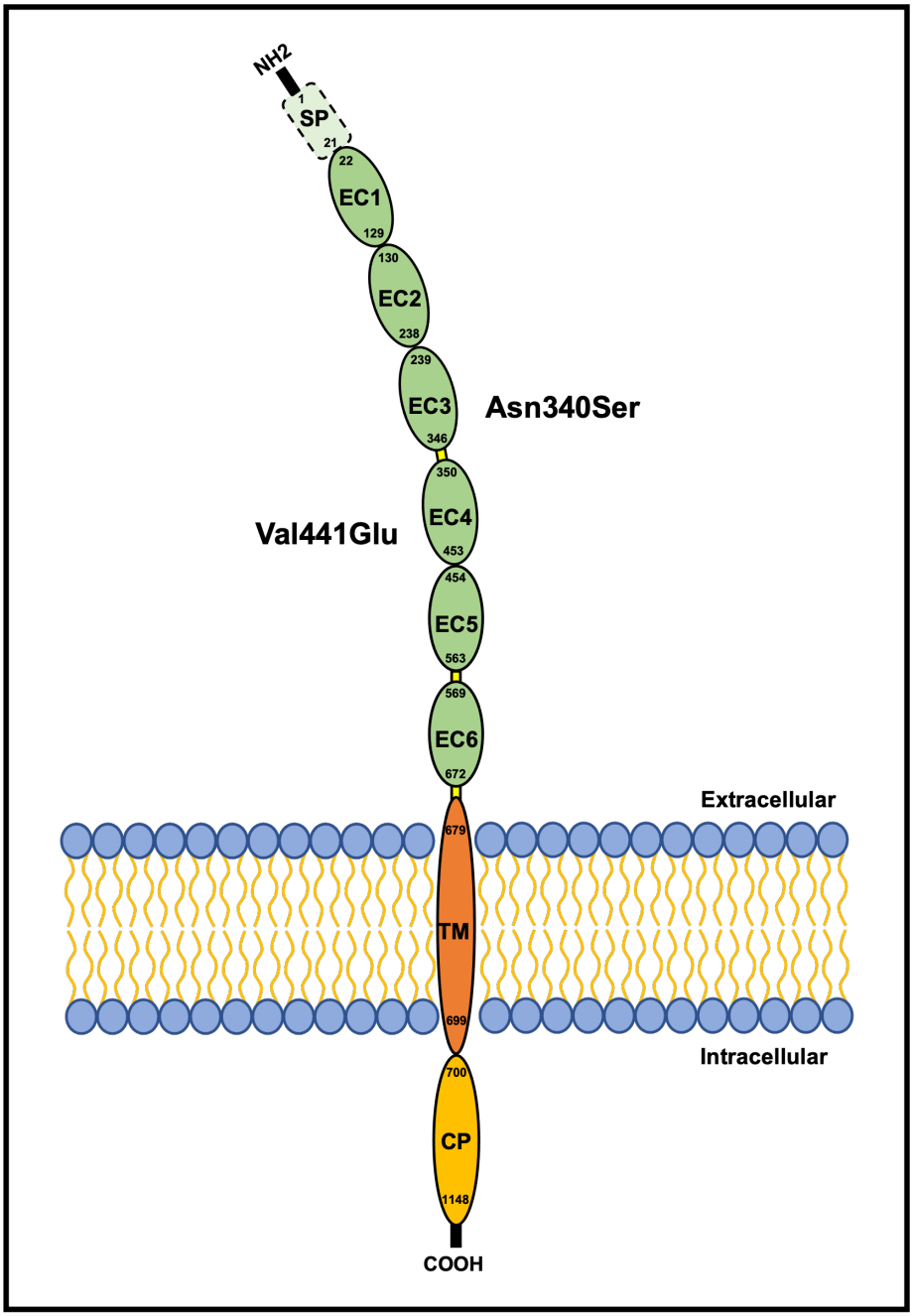


Figure 2. Common pathogenic mutations in the PCDH19 gene. (EC, extracellular cadherin; TM, transmembrane; CP, cytoplasmic)

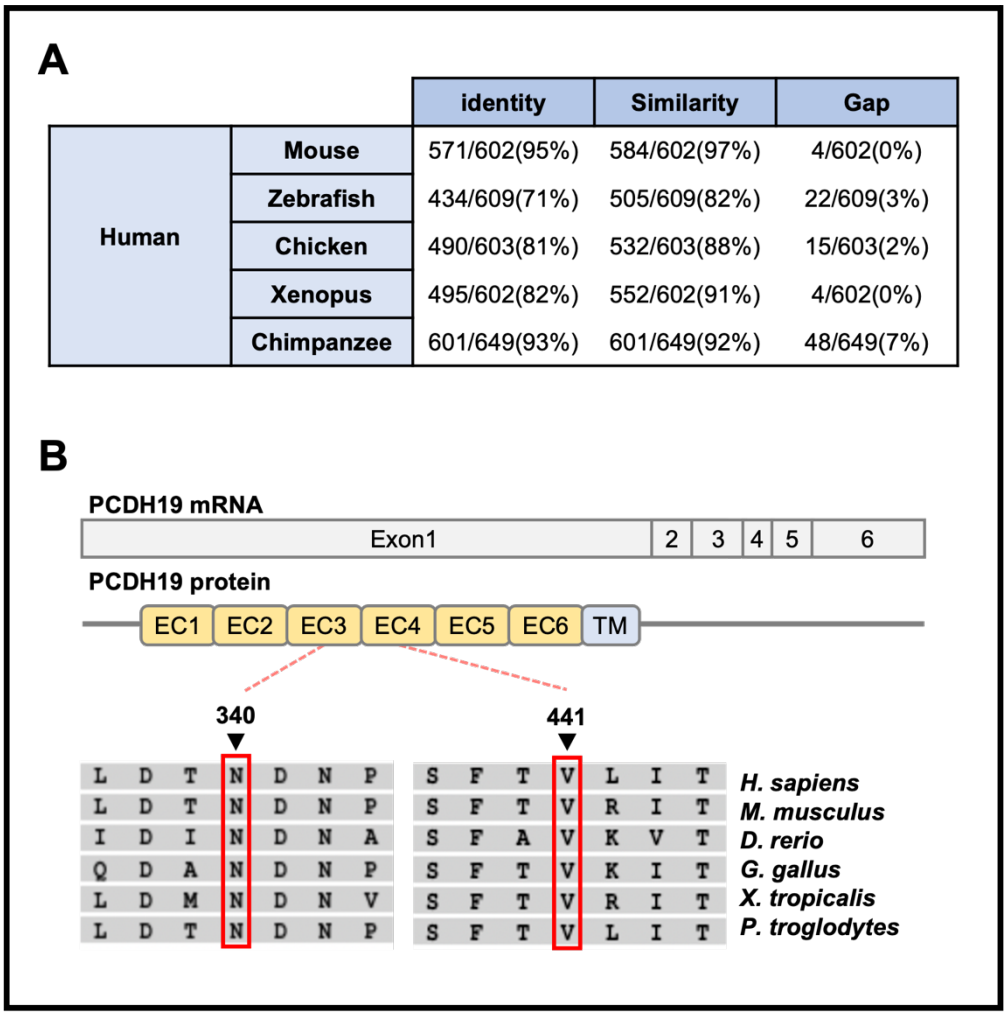


Figure 3. PCDH19 mutations are evolutionarily conserved in vertebrates. (A) Identity, similarity, and the gap between other vertebrates and human. Human PCDH19 shows high sequence identity (571/602, 95%), and similarity (584/602, 97%) with four gaps (4/602, 0%) to mouse; to zebrafish (434/609 (71%) identity, 505/609 (82%) similarity, 22/609 (3%) gaps); chicken (490/603 (81%) identity, 532/603 (88%) similarity, 15/603(2%) gaps); xenopus (495/602 (82%) identity, 552/602 (91%) similarity, 4/602 (0%) gaps); chimpanzee (601/649 (93%) identity, 601/649 (92%) similarity, 48/649 (7%) gaps). (B) Common pathogenic mutations are evolutionarily conserved in vertebrates.

The key symptoms, including seizures and convulsions, observed in disorders induced by PCDH19 mutations seem to be related to the disruption in the excitatory/inhibitory (E/I) balance. Further investigation into the relationship between neurotransmitters like Gamma-aminobutyric acid (GABA), Glutamate and PCDH19 revealed that PCDH19 interacts with the alpha1 subunit of the GABA-A receptor in neurons.[24] Moreover, artificially manipulating PCDH19 expression could modulate the GABAergic neurotransmission signals via the GABA-A receptor.[25,26]

Gamma-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mature central nervous system, and it is associated with two types of receptors: GABA-A receptors and GABA-B receptors. The GABA-A receptor, a critical inhibitory neurotransmitter receptor in the neuronal membrane, is composed of a pentameric structure with two alpha, two beta, and one gamma subunit. This forms a ligand-dependent ion channel that allows chloride (Cl) ions to pass through (Figure 4).[27,28] When two GABA molecules bind to the interface between the alpha and beta subunits, the ion channel opens, allowing chloride (Cl) ions to move into the cell.[29] GABA receptors play a significant role in synaptic neurotransmission in the mature nervous system and are known to be important in various non-synaptic developmental processes, including cell proliferation, neuronal migration, synapse formation, and activity-dependent mechanisms.[30-33]

GABA acts as an inhibitory neurotransmitter in the mature mammalian brain. Activation of GABA-A receptors induces membrane hyperpolarization and inhibition of neuronal activity. However, it has been reported to act as an excitatory neurotransmitter during the early developmental periods, including the embryonic phase and the first week after birth.[34-37]Activation of GABA-A receptors induces depolarization. Similar changes in GABA's role as a neurotransmitter during these developmental periods have been reported in *Xenopus* as well.[38]

Given these findings, PCDH19 emerges as a pivotal regulator in GABAergic neurotransmission and provides insights into the novel mechanisms underlying PCDH19-related epilepsy.

In this thesis, I aim to investigate whether loss-of-function or mosaicism is responsible for PCDH19-related epilepsy using *Xenopus tropicalis* as a model. The *Xenopus tropicalis* model is an experimental animal model that offers the convenience of loss/gain of function experiments, as the fate of each cell is already determined from the early stages of embryonic development. It has genomic proximity to humans, highly resembling the amino acid sequence information of PCDH19. Being a rapidly developing vertebrate with the nervous system fully formed within a week, it has the advantage of a swift developmental timeline. Moreover, it combines embryology and molecular biology techniques, allowing for sophisticated genetic function manipulation. This model permits the manipulation of a single organism, designating one side as the control group and the other as the experimental group. *Xenopus tropicalis* central nervous system development is well-documented by developmental stages and excels in wound healing and neural regeneration capabilities. Additionally, it has been demonstrated to be a valuable model in various studies related to vertebrates, such as epilepsy and autism, making it highly suitable for this research.[39-42]

A Morpholino oligomer (MO) is an oligomer molecule used in molecular biology to regulate specific gene expression. It has a structure consisting of DNA bases attached to a backbone with a Methylene morpholine ring linked through Phosphonodiamidite groups (Figure 5A). MOs function by steric blocking, interacting with specific RNA sequences to inhibit the action of other molecules that interact with RNA. They can attach to the start codon of mRNA to prevent translation via the ribosome or bind to pre-mRNA to regulate splicing (Figure 5B).[43-45]

Using targeted micro-injection of a translation-blocking antisense morpholino oligonucleotide, I will knock-down PCDH19 gene expression in the central nervous system in all or a half of the cells and quantify epilepsy-like behaviors in free-swimming tadpoles. The result of this study will shed light on the molecular basis of PCDH19-like epilepsy.

I aim to elucidate whether the phenotype caused by PCDH19 deficiency can be alleviated through quantitative assessment of phenotypic changes induced by GABA-A agonists and antagonists in *Xenopus tropicalis* expressing PCDH19 MO-induced epilepsy, thereby

exploring the modulation of PCDH19 deficiency-related behavioral phenotypes through GABA-A signaling. Given these findings, PCDH19 emerges as a pivotal regulator in GABAergic neurotransmission and provides insights into the novel mechanisms underlying PCDH19-related epilepsy.

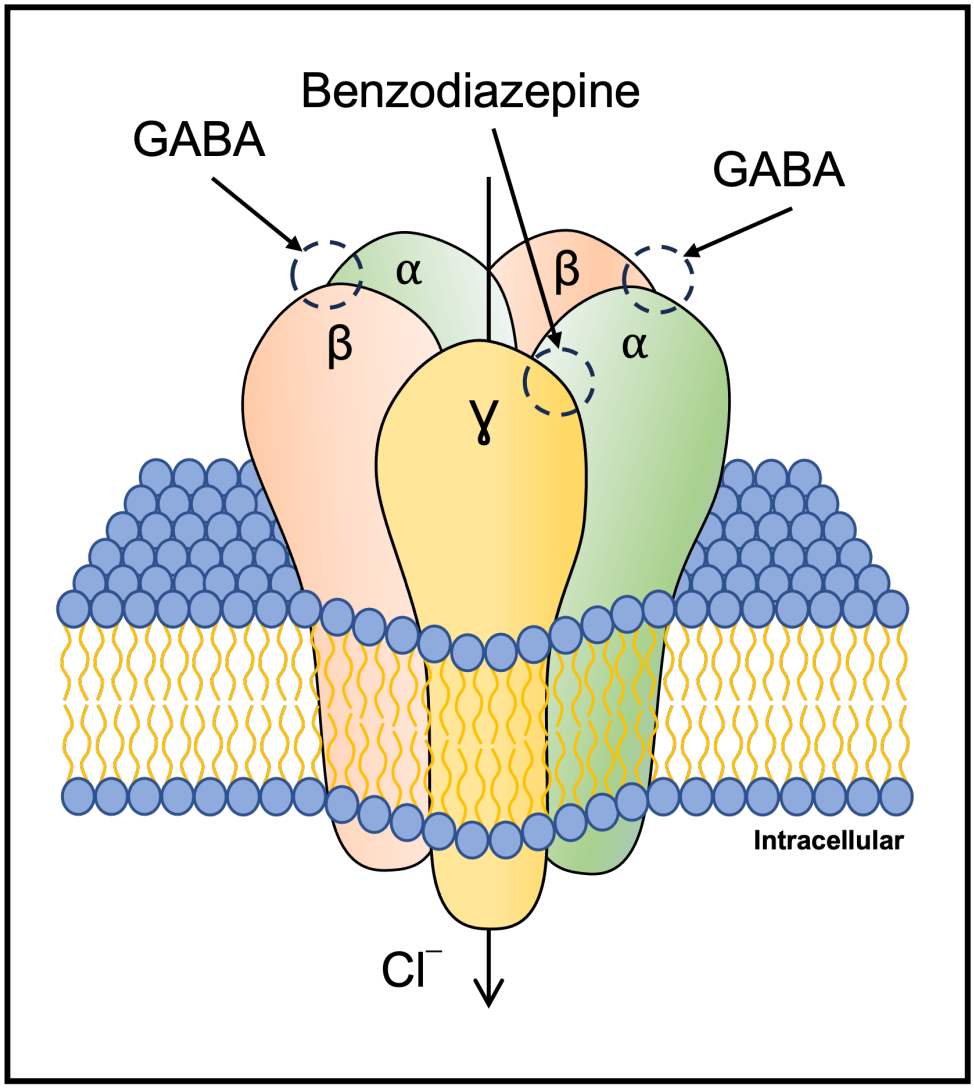


Figure 4. Schematic illustration of the $GABA_A$ receptor. The GABA-A receptor is a pentameric structure consisting of two alpha, two beta, and one gamma subunit, forming a circular arrangement. It serves as an ion channel for chloride (Cl) ions to pass through and is ligand gated. When two GABA molecules bind to the interface between the alpha and beta subunits, the ion channel opens, allowing chloride (Cl) ions to move into the cell. Benzodiazepines, a well-known class of anticonvulsants, act by binding to the interface between GABA and the alpha subunit of the GABA-A receptor.

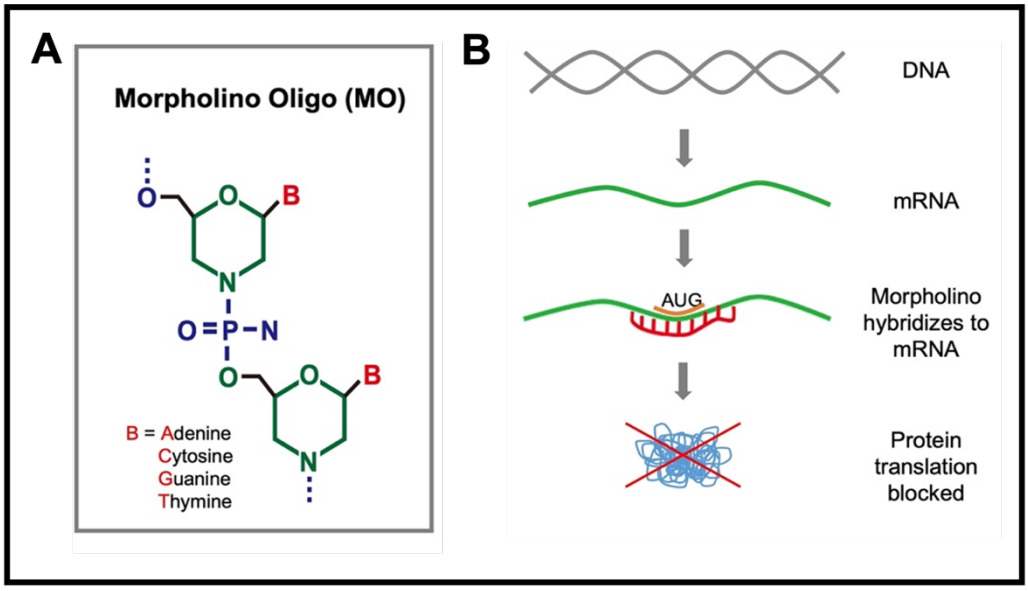


Figure 5. Translation-blocking morpholino oligonucleotide of PCDH19 in *Xenopus tropicalis*. (A) The structure of a Morpholino oligonucleotide. It consists of a backbone with a Methylene-morpholine ring linked through Phosphonodiamidite groups and has attached DNA bases. (B) Schematic illustration of the working principle of a Morpholino oligonucleotide. MO functions by steric blocking, interacting with specific RNA sequences to inhibit the action of other molecules that interact with RNA. It attaches to the mRNA's start codon, thereby preventing translation through the ribosome.

II. MATERIALS AND METHODS

1. *Xenopus tropicalis* embryo

Xenopus tropicalis used in this thesis are out-bred Nigerian frogs from the University of Virginia stock which is from Nasco's Frog Brittle. *Xenopus tropicalis* embryos were generated by in vitro fertilization (IVF) 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℃ incubator.

2. RNA synthesis, and morpholinos (MOs)

Capped RNAs encoding pcs2+CEGFP are synthesized by using mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Science, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Antisense PCDH19 MO and control MO were designed and supplied by GeneTools (Pilomath, Oregon, USA): *xenopus* PCDH19 MO, 5'-CCCTGCTCAGCCACAACCACATAGT-3'; control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

3. Blastomere injection into *Xenopus* embryos

Xenopus embryos were obtained by in vitro fertilization (IVF), raised in 0.1XModified Barth's Saline (MBS) at 24ºC, and staged as described before. For loss-of-function studies, injections were made to two dorsal animal blastomeres at 8-cell stage. One ng of morpholino was injected per blastomere. One hundred pg of GFP RNA was injected per blastomere, used as tracer. For mosaicism studies, injections were made to two dorsal animal blastomeres at 16-cell stage. Five hundred pg of morpholino was injected per blastomere. Fifty pg of GFP RNA was injected per blastomere. All embryos were observed

by light microscope for phenotyping and sectioned for confocal microscopy. GFP RNA or control MO was used as a control to maintain the same amount of total injected RNA or MO in each embryo.

4. Western blot assay

For western blot assay, PCDH19 MO injected embryos were grown to stage 27, then anesthetized in MS222 (Sigma, St. Louis, Missouri, USA) in 0.1XMBS, and the entire brain was dissected with 0.1mm insect pins. 30 whole brains were treated with 50ul of lysis buffer consisting of protease inhibitor cocktail tablet (Roche, Mannheim, Germany), RIPA buffer (Biosesang). Lysates were collected and centrifuged at 15,000 rpm, 4ºC for 20minutes. Using the BCA method (PIERCE, Rockford, USA), protein concentration was determined. 20ug of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and electrotransferred onto 0.2μm polyvinylidene difluoride membrane (Millipore, MA, USA). Membrane was blocked with blocking solution, 5% skim milk in 1X TBST, for 30 minutes at room temperature. Transfer the membrane into 1% skim milk in 1X TBST solution containing anti-PCDH19 antibody (Abcam, Cambridge, UK), anti-Tubulin (Abcam, Cambridge, UK) overnight in 4ºC (1:1000). Membrane was washed with 1X TBST for 10 minutes repeated three times. Then membrane was put into 1% skim milk in 1X TBST solution with horseradish peroxidase-conjugated anti-rabbit antibody (Abcam, Cambridge, UK), horseradish peroxidase-conjugated anti-rat antibody (Abcam, Cambridge, UK) for 1hour at room temperature (1:3000). After washing membrane with 1X TBST for 10 minutes repeated three times, signals were detected using Supersignal chemiluminescence substrate (Thermo Fisher Science, Waltham, Massachusetts, USA). Images were captured by using LAS 4000 program.

5. Behavior test

For behavior test, PCDH19 MO and control MO injected embryos were grown to stage 49 and recorded their behavior 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. To trace swimming trajectories, the position of the tadpole in each frame was extracted using Deeplabcut[46] 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[47], which was used to normalize the coordinates of each movie. Six seizure-like behavior criteria were defined to qualitatively analyze the behavior of a tadpoles.[48] 1. “CSC”, c-shaped contraction, a sudden c-shape contraction in place, 2. “UTB”, uncoordinated tail bend, writhing with slow movement, 3. “CIR”, circling, two or more circling movements without direction, 4. “DAR”, darting, two or more bursts of fast and linear movements, 5. “IMB”, imbalance, swimming sideways, 6. “TRE”, tremor, shaking of the entire body including the head. During the 10-minute video, if any of the six 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.

6. Cryosection and nuclear counterstaining

For nuclear counterstaining, PCDH19 MO and control MO injected embryos were grown to stage 41 and fixed in 4% paraformaldehyde dissolved in 1X PBS overnight in 4ºC and then transferred into 30% sucrose in 1X PBS overnight in 4°C. Embryos were embedded in OCT (Tissue-Tek, Radnor, Pennsylvania, USA) and frozen in dry ice. Embryos were sectioned with a cryostat (Thermo Fisher Science, Waltham, Massachusetts, USA). The 16

μm coronally sectioned embryos were attached to the Superfrost plus microscope slide (Fisher scientific, Pittsburgh, USA). Slides were washed with 1X PBS for 5 minutes repeated three times. Slides were transferred into 1:1000 Hoechst 33342 in 1X PBS for 5 minutes. After washing slides with 1X PBS for 5 minutes repeated three times, slides were mounted using the fluorsave mounting solution and covered with the coverslip. Fluorsave mounting solution is allowed to dry in the dark at room temperature for two days until it solidifies.

7. Drug treatment

For antiseizure drug experiment, potassium bromide (8 mM) was applied in bath to PCDH19 MO injected embryos who exhibited seizure-like behavior based on six criteria at stage 49. After a 10-minute habituation period, all tadpoles were treated with 10 minutes of 0.1X MBS, followed by 10 minutes in 0.1X MBS containing 8mM potassium bromide, and finally, recorded tadpole's behavior for 10 minutes in 0.1X MBS without potassium bromide.

For drug experiments targeting GABA signaling imbalance, embryos injected with control MO and PCDH19 MO were treated with the GABA-A agonist Muscimol (100uM) and GABA-A antagonist Bicuculline (100uM) from stage 27 to stage 41, which is when the central nervous system develops. Subsequently, behavior tests were conducted at stage 49.

8. Imaging

The morphology of all tadpoles was imaged with epifluorescence (Nikon, Eclipse Ti2) at stage 49 while anesthetized with MS222 in 1X MBS. The tadpoles were fixed on a 60mm petri dish filled with 1.5% agarose gel and dish was filled up with MS222 in 1X MBS. Tadpole brain slides with nuclear counterstaining were imaged using a confocal fluorescence microscope (Carl Zeiss, LSM 700).

9. 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).

III. RESULTS

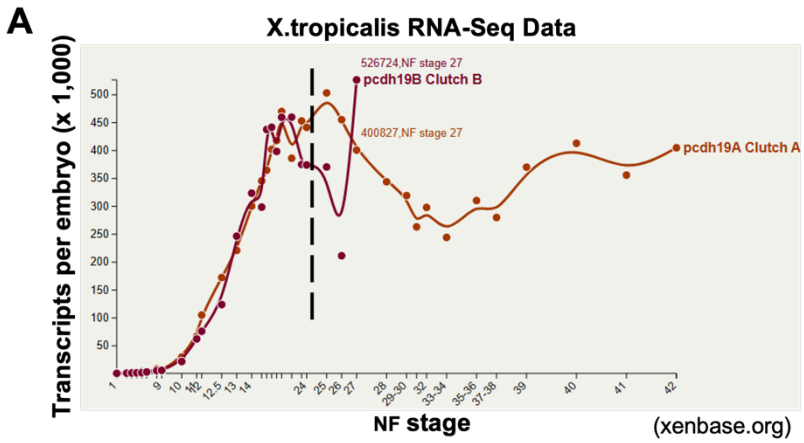
1. Depleting PCDH19 causes abnormal swimming behaviors in *Xenopus tropicalis*

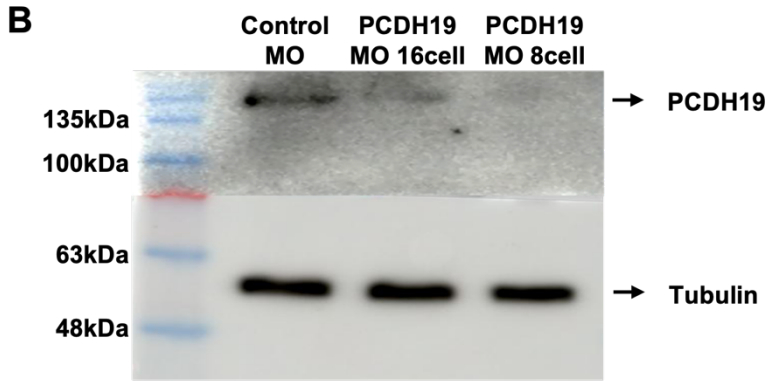
A. PCDH19 is expressed in the developing central nervous system (CNS) and can be specifically knocked down by antisense morpholino oligonucleotides (MO)

To effectively block the expression of the causative gene, PCDH19, I designed a 25-base morpholino oligo. This morpholino oligo was designed to bind to the mRNA sequence containing the start codon of PCDH19, thereby blocking its translation. To verify the efficacy of this morpholino oligo (MO) in blocking PCDH19 gene expression, I quantitatively analyzed the amount of PCDH19 protein using a western blot. During the 8-cell stage of the *Xenopus tropicalis* embryo, I microinjected the MO into the two dorsal blastomeres that differentiate into the central nervous system. At the 16-cell stage, I injected the MO into only half of the four dorsal blastomeres that differentiate into the central nervous system. During the MO injection, I co-injected the fluorescent protein GFP to later select, under a fluorescence microscope, only those specimens where the MO was specifically injected into the central nervous system. To verify the successful suppression of PCDH19 gene expression by the PCDH19 MO in the central nervous system-specifically injected specimens, I dissected the whole brain of the embryo at stages 27-28, when PCDH19 mRNA expression is highest (Figure 6A).[49]

Western blot results showed that the expression of PCDH19 in specimens injected with PCDH19 MO at the 8-cell stage was reduced compared to the control group, and it was relatively less reduced in embryos injected at the 16-cell stage (Figure 6B). Quantification of the band sizes obtained from western blot through densitometry revealed that PCDH19 protein decreased by approximately 17.37% in the PCDH19 depletion group and around 27.39% in the PCDH19 mosaic depletion group compared to the control group (Figure 6C).

These findings confirm that the synthesized morpholino oligo effectively blocks PCDH19 gene expression and is suitable for loss-of-function experiments.





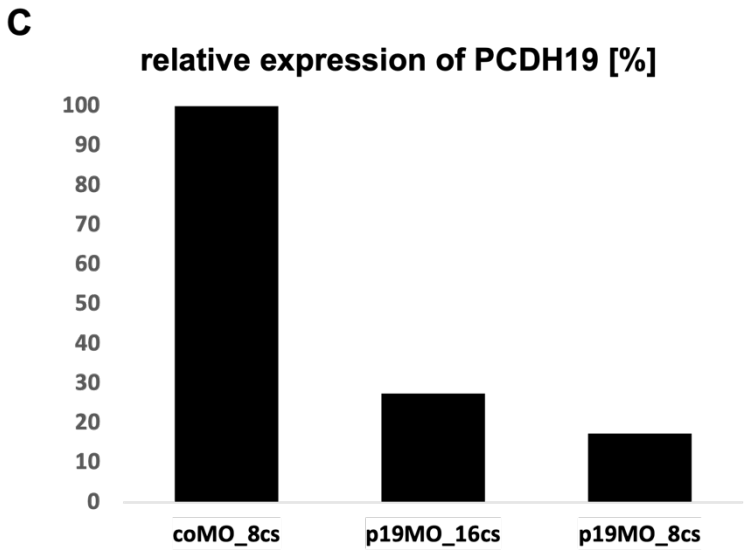


Figure 6. PCDH19 morpholino oligo suppresses PCDH19 gene expression. (A) RNA-seq data of *Xenopus tropicalis* PCDH19. The Y-axis indicated PCDH19 transcripts per embryo (x1,000), and the X-axis indicated the embryonic stages of Nieuwkoop and Faber. The dotted line divided embryonic stage 27. As the development stage progressed, the expression level of PCDH19 increases, with the highest expression occurring at stage 27. (B) Western blot data of PCDH19 and Tubulin in *Xenopus* brain. For the control group, lysates were prepared using the brains of embryos injected with Control MO at stage 27. For the experimental group, lysates were prepared using the brains of embryos injected with PCDH19 MO at stage 27. The size of PCDH19 protein was 135kDa, and the size of Tubulin protein was 50kDa. Tubulin was used as an internal control. (C) Quantification of the effect of PCDH19 MO through densitometry. The Y-axis indicated relative level of PCDH19 protein.

B. Knocking down PCDH19 expression in the entire central nervous system does not cause gross morphology but causes behavioral anomaly, including seizure-like behaviors and repetitive swimming patterns

A cardinal symptom of PCDH19-related epilepsy syndrome is seizures. In humans, seizures typically begin between 3 months and 3 years of age, with the frequency gradually decreasing with age. Seizures can manifest in various forms, including tonic-clonic, myoclonic, absence, atonic, and partial seizures.

To establish a model of PCDH19-related epilepsy syndrome in *Xenopus tropicalis*, I microinjected PCDH19 MO to suppress PCDH19 gene expression and observed whether the embryos exhibited seizure or other abnormal behaviors. I specifically suppressed PCDH19 gene expression in the central nervous system by microinjecting the two dorsal blastomeres that differentiate into the central nervous system at the 8-cell stage.

To observe any abnormal or seizure behaviors, tadpoles were raised to stage 49, habituated in a custom-made dark chamber for 10 minutes, and then video-recorded for 10 minutes (Figure 7). There was no change in tadpole morphology due to PCDH19 knock-down in the central nervous system, but qualitative analysis of videos revealed that while the control MO-injected group exhibited normal swimming patterns around the dish, the experimental group displayed many specimens with abnormal swimming patterns and behaviors indicative of seizures (Figure 8A and 8B).

To further analyze these seizure-like behaviors, I established six criteria for seizure-like behaviors: C-shaped contraction, uncoordinated tail bends, circling, darting, imbalance, and tremor (Table 2). Analysis revealed that embryos in the PCDH19 MO-injected group exhibited a diverse and complex range of these six characteristics. Counting all behaviors fitting these criteria showed a significant increase in seizure-like behaviors in the PCDH19 knock-down group compared to the control MO-injected group (Figure 9A). The total time of seizure-like behavior also increased in the PCDH19 knock-down group (Figure 9B). Comparing the time tadpoles spent at the center of the dish during their most active 1-minute period, it was found that the PCDH19 knock-down group exhibited a longer

duration in the center compared to the control group (Figure 9C). This suggests that the PCDH19 knock-down group displayed abnormal swimming patterns, distinct from normal tadpoles that swim at the dish's periphery. When the total distance covered by tadpoles during their most active 1-minute period was summed, there was no significant difference observed between the two groups (Figure 9D), indicating no significant variance in their motor abilities.

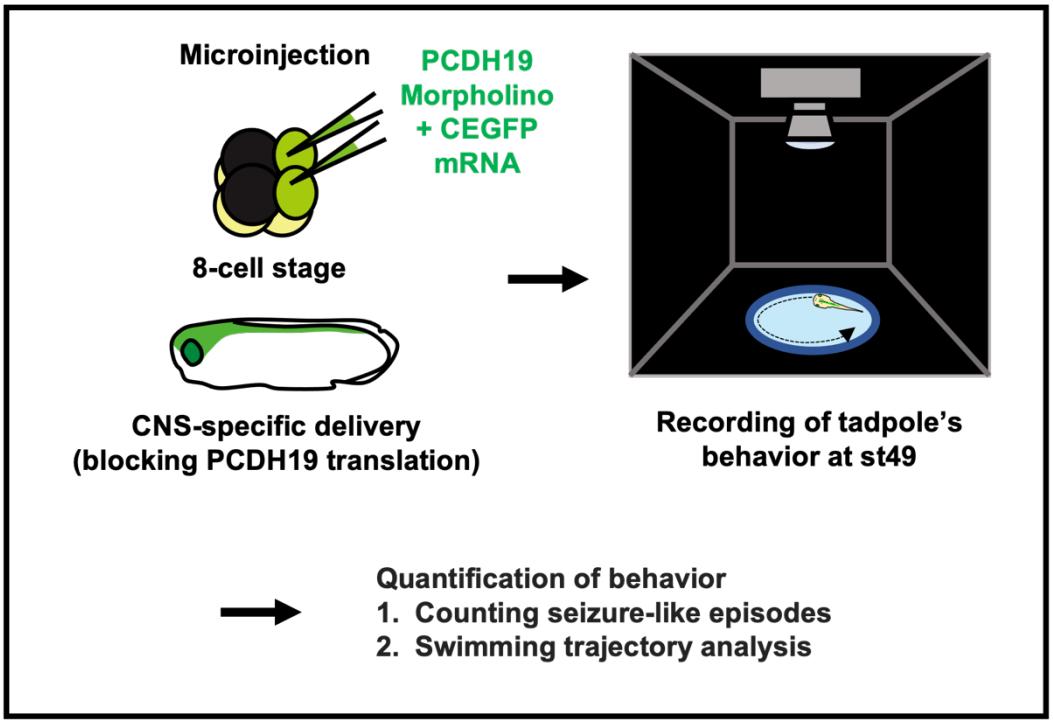


Figure 7. Experimental scheme of behavior test in PCDH19-depleted tadpoles. CNS target microinjected embryos were grown to stage 49 and recorded their behavior in a custom-built dark chamber for 10 minutes. Based on a 10-minute behavioral video, tadpole behavior was quantified by counting seizure-like episodes and analyzing swimming trajectories.

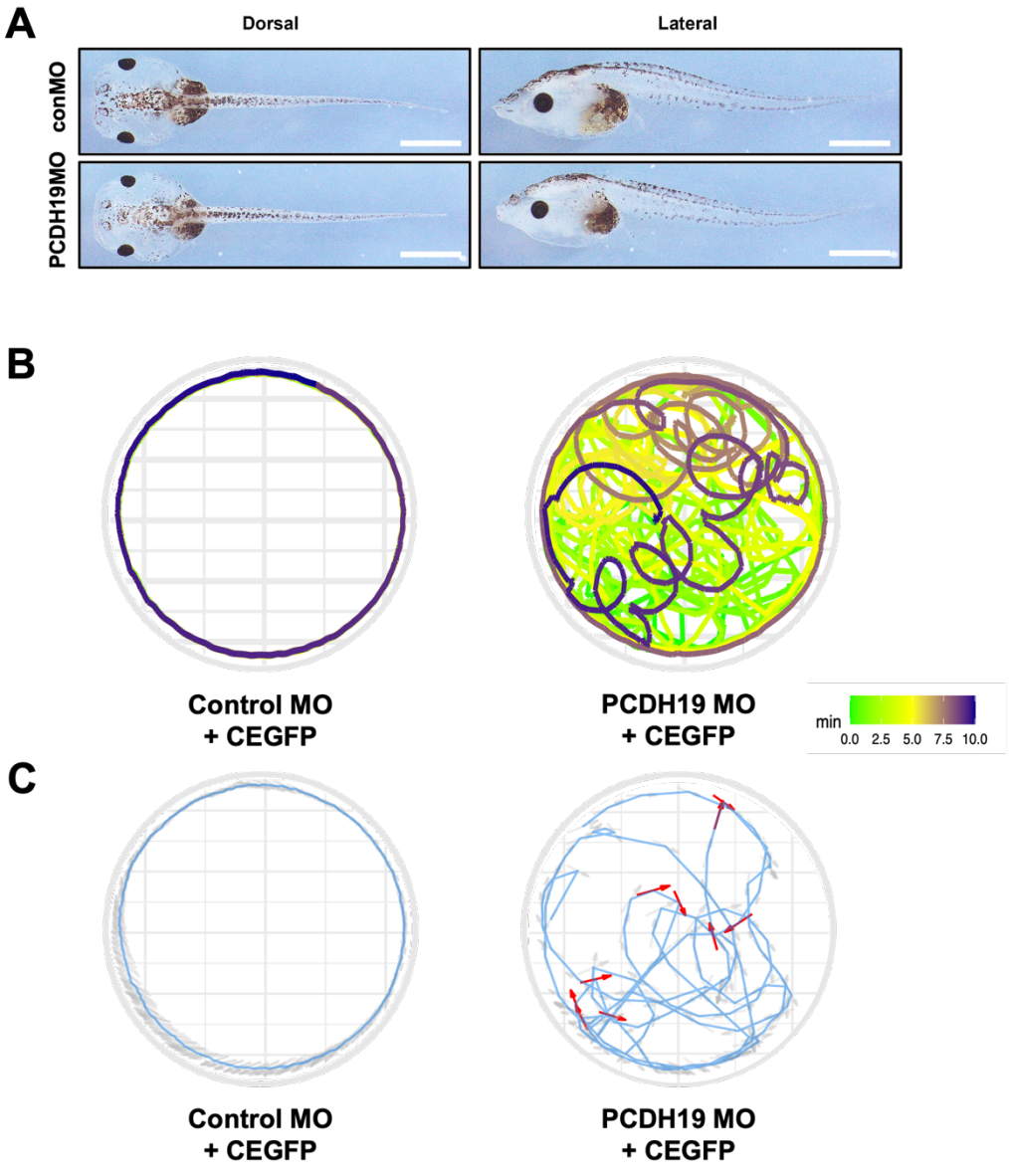


Figure 8. PCDH19 depletion in the CNS causes abnormal swimming behaviors. (A) Morphology of CNS target microinjected embryos at stage 49. PCDH19 knock-down caused no noticeable external morphological changes in developing embryo. Scale bar 1 mm. (B) Representative swimming patterns of tadpoles injected with control MO and PCDH19 MO as CNS targets at the 8cell stage. (C) A video frame representing the most active 1-minute swimming trajectory of the tadpole. The blue line indicates the swimming trajectory, the gray arrows indicate the tadpole's body orientation, and the red arrows indicate the tadpole's body orientation at the moment of a sharp turn.

Table 1. Criteria for seizure-like behaviors in *Xenopus tropicalis*

	Criteria	Feature
CSC	C-shape contraction	A sudden c-shape contraction in place
UTB	Uncoordinated tail bend	Writhing with slow movement
CIR	Circling	Two or more circling movements without direction
DAR	Darting	Two or more bursts of fast and linear movements
IMB	Imbalance	Swimming sideways
TRE	Tremor	Shaking of the entire body including the head

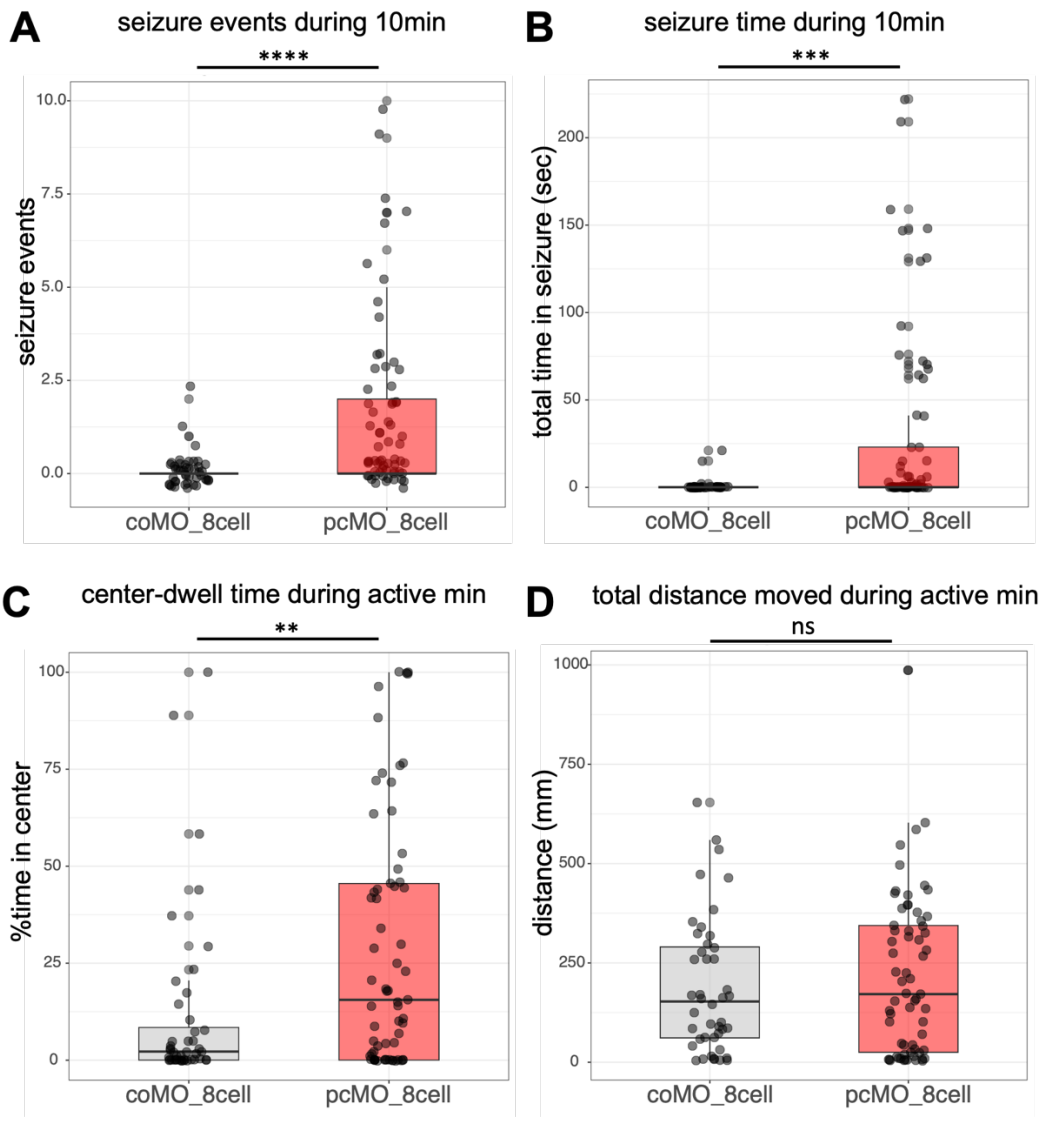


Figure 9. Quantification of seizure-like behaviors and repetitive swimming patterns in PCDH19-depleted tadpoles. (A) Quantification of seizure-like behavior frequency in tadpoles injected with control MO (n = 44) and PCDH19 MO (n = 65) as CNS targets at the 8cell stage. ****p = 0.0000724. (B) Quantification of the total duration of seizure-like behavior in tadpoles injected with control MO and PCDH19 MO as CNS targets at the

8cell stage. ***p = 0.000886. (C) Quantification of the center dwelling time during active time in tadpoles injected with control MO and PCDH19 MO as CNS targets at the 8cell stage. **p = 0.00212. (D) Quantification of the total swimming distance in tadpoles injected with control MO and PCDH19 MO as CNS targets at the 8cell stage. n.s. (not significant) p=0.469. Each point represents an individual tadpole, and the data are summarized by box and whisker plots. Unpaired t-test.

C. Potassium bromide (KBr) ameliorates seizure-like behaviors of PCDH19-depleted embryos

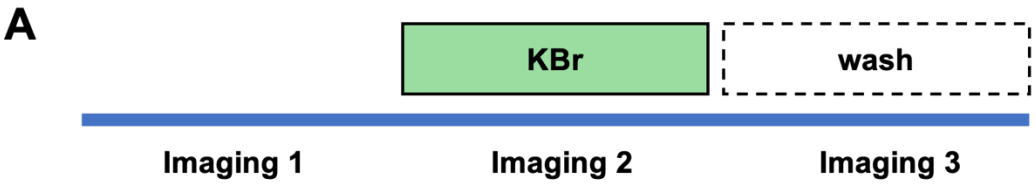
To validate whether the six seizure-like behavior criteria I used for analysis truly represent seizure behaviors, and thus to confirm if I successfully established a PCDH19-related epilepsy syndrome model in *Xenopus tropicalis* using PCDH19 MO for central nervous system-specific knock-down, I conducted experiments using an anti-seizure drug. If the six seizure-like behaviors I identified are eliminated by the anti-seizure drug, then these behaviors can be considered as seizure symptoms, and I can conclude that I have successfully established the model.

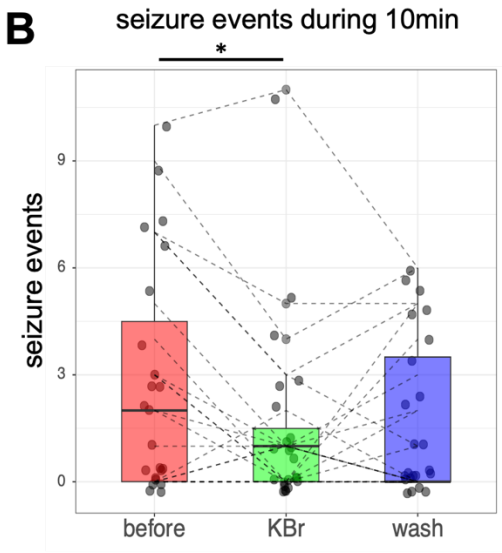
I used potassium bromide, a chemical already used as an anti-convulsant in animals like horses and dogs and has shown effectiveness in patients with PCDH19 mutations, for our drug treatment experiments.[50,51] I first selected embryos displaying seizure-like behaviors from PCDH19 knock-down group, habituated them in a dark chamber for 10 minutes, and then video-recorded their behaviors for 10 minutes. After treating them with potassium bromide, I video-recorded for another 10 minutes and then removed the potassium bromide to see if the seizure-like behaviors reoccurred (Figure 10A). I then analyzed the videos based on our six seizure-like behavior criteria and tracked each embryo individually.

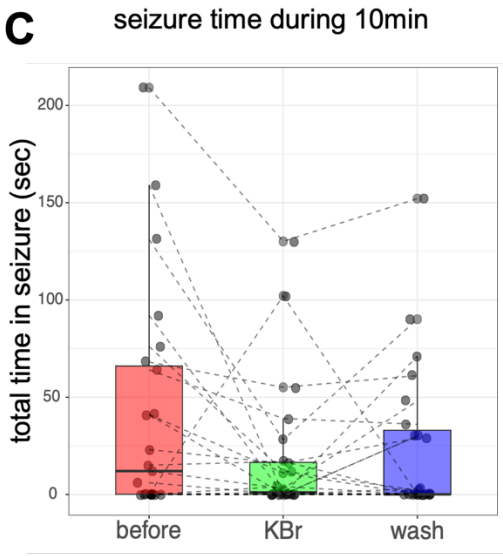
The results showed that the frequency and duration of seizure-like behaviors in tadpoles decreased significantly upon potassium bromide treatment. Once the potassium bromide was removed, the frequency and duration of these behaviors increased again (Figure 10B and 10C), suggesting that this type of behavioral abnormality represents seizure. Comparing the time tadpoles spent at the center of the dish during their most active 1-minute period, it was not affected by potassium bromide treatment (Figure 10D), suggesting that repetitive swimming behaviors in the center of the dish are not related to seizure. As patients with PCDH19-related epilepsy syndrome exhibit repetitive behaviors linked to autism, I hypothesize that this phenotype may represent behaviors commonly associated with autism spectrum disorders. When the total distance covered by tadpoles during their most active 1-minute period was summed, there was no significant difference

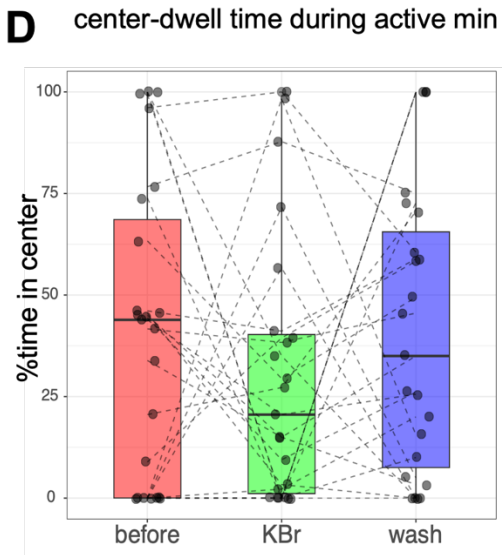
observed between before, during, and after the potassium bromide treatment (Figure 10E).

From these results, I concluded that the behaviors I identified based on my six criteria are indeed seizure symptoms, and thus I have successfully established a *Xenopus tropicalis* model of PCDH19-related epilepsy syndrome using central nervous system-specific knock-down.









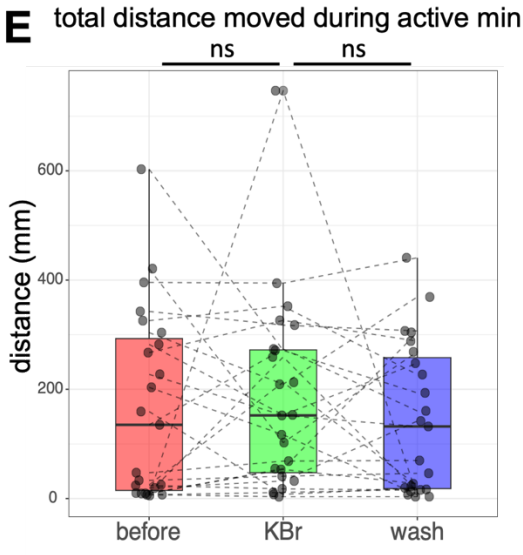


Figure 10. Potassium bromide (KBr) alleviates seizure-like behaviors. (A) Experimental scheme of antiseizure drug treatment. Tadpoles displaying seizure-like behavior among those injected with PCDH19 MO were recorded in video footage for 10 minutes each, three times before, during, and after potassium bromide treatment. (B) Quantification of seizure-like behaviors frequency in tadpoles by individually tracking changes before, during, and after KBr treatment. Before (n = 23) and KBr (n = 23) group. *p = 0.021. Wash (n = 23), n.s. (not significant) p = 0.081. KBr and Wash. n.s. (not significant) p = 1. (C) Quantification of the total duration of seizure-like behaviors of tadpoles by individually tracking changes before, during, and after KBr treatment. Before and KBr group. n.s. (not significant) p = 0.021. Wash, n.s. (not significant) p = 0.102. KBr and Wash. n.s. (not significant) p = 1. (D) Quantification of the center dwelling time during active time in tadpoles by individually tracking changes before, during, and after KBr treatment. Before and KBr group. n.s. (not significant) p = 0.891. Wash, n.s. (not significant) p = 1. KBr and Wash. n.s. (not significant) p = 0.969. (E) Quantification of the total swimming distance in tadpoles by individually tracking changes before, during, and after KBr treatment. Before and KBr group. n.s. (not significant) p = 1. Wash, n.s. (not significant) p = 1. KBr and Wash. n.s. (not significant) p = 1. The data are visualized as points and summarized by box and whisker plots. Repeated-measure one-way ANOVA followed by bonferroni’s post-hoc test.

2. Mosaic loss-of-function of PCDH19 is not required for seizure-like behaviors in *Xenopus tropicalis*

A. PCDH19 expression can be knocked down in all or half of the cells in the central nervous system by injecting MO at 8- or 16-cell stage

The PCDH19 gene, a causative factor in PCDH19-related epilepsy syndrome, is an X-chromosome-linked member. In heterozygous females, the phenomenon of X-chromosome random inactivation leads to a mosaic of both wild type and PCDH19 mutant cells. The aberrant interaction between these two cell types is proposed to induce epilepsy, unlike in hemizygous PCDH19 mutant males where the syndrome is generally not observed.

To test whether similar phenotypes are observable in *Xenopus tropicalis*, PCDH19 was selectively knocked-down in half of the dorsal blastomeres that differentiate into the central nervous system at the 16-cell stage through microinjection of PCDH19 morpholinos (Figure 11). There was no change in tadpole morphology due to PCDH19 mosaic knock-down in the central nervous system. However, like the PCDH19 knock-down group, abnormal and seizure behavior was observed (Figure 12A and 12B). Counting all seizure behaviors showed an increase in the PCDH19 mosaic knock-down group compared to the control MO-injected group (Figure 13A). The total time of seizure-like behavior also increased in the PCDH19 mosaic knock-down group (Figure 13B). Comparing the time tadpoles spent at the center of the dish during their most active 1-minute period, it was found that the PCDH19 mosaic knock-down group exhibited a longer duration in the center compared to the control group (Figure 13C). The total distance covered by tadpoles during their most active 1-minute period was summed, there was no significant difference observed between the two groups (Figure 13D).

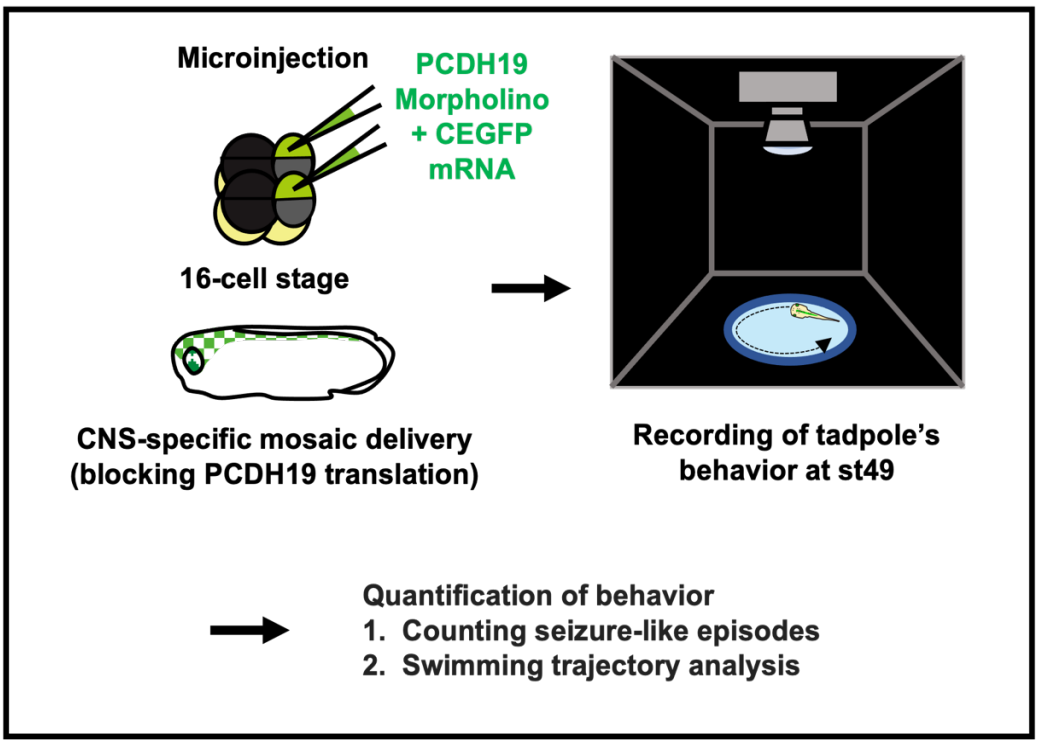


Figure 11. Experimental scheme to induce PCDH19 depletion in the CNS in mosaic pattern. CNS target mosaic microinjected embryos were grown to stage 49 and recorded their behavior in a custom-built dark chamber for 10 minutes. Based on a 10-minute behavioral video, tadpole behavior was quantified by counting seizure-like episodes and analyzing swimming trajectories.

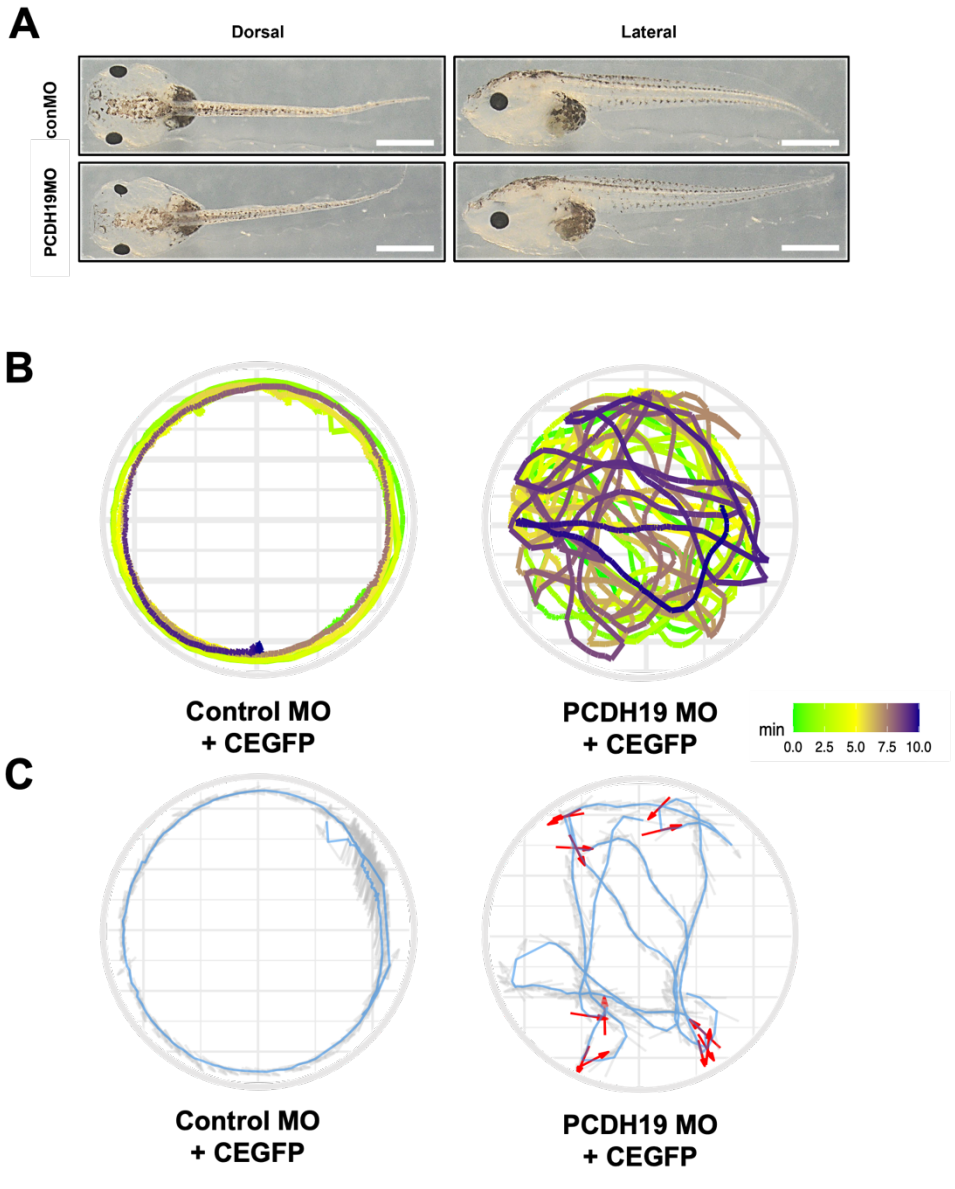


Figure 12. Depleting PCDH19 in half of the cells in the CNS also causes behavioral anomaly. (A) Morphology of CNS target microinjected embryos at stage 49. PCDH19 mosaic knock-down caused no noticeable external morphological changes in developing embryo. Scale bar 1 mm. (B) Representative swimming patterns of tadpoles injected with control MO and PCDH19 MO as CNS mosaic injection at the 16cell stage. (C) A video frame representing the most active 1-minute swimming trajectory of the tadpole. The blue line indicates the swimming trajectory, the gray arrows indicate the tadpole's body orientation, and the red arrows indicate the tadpole's body orientation at the moment of a sharp turn.

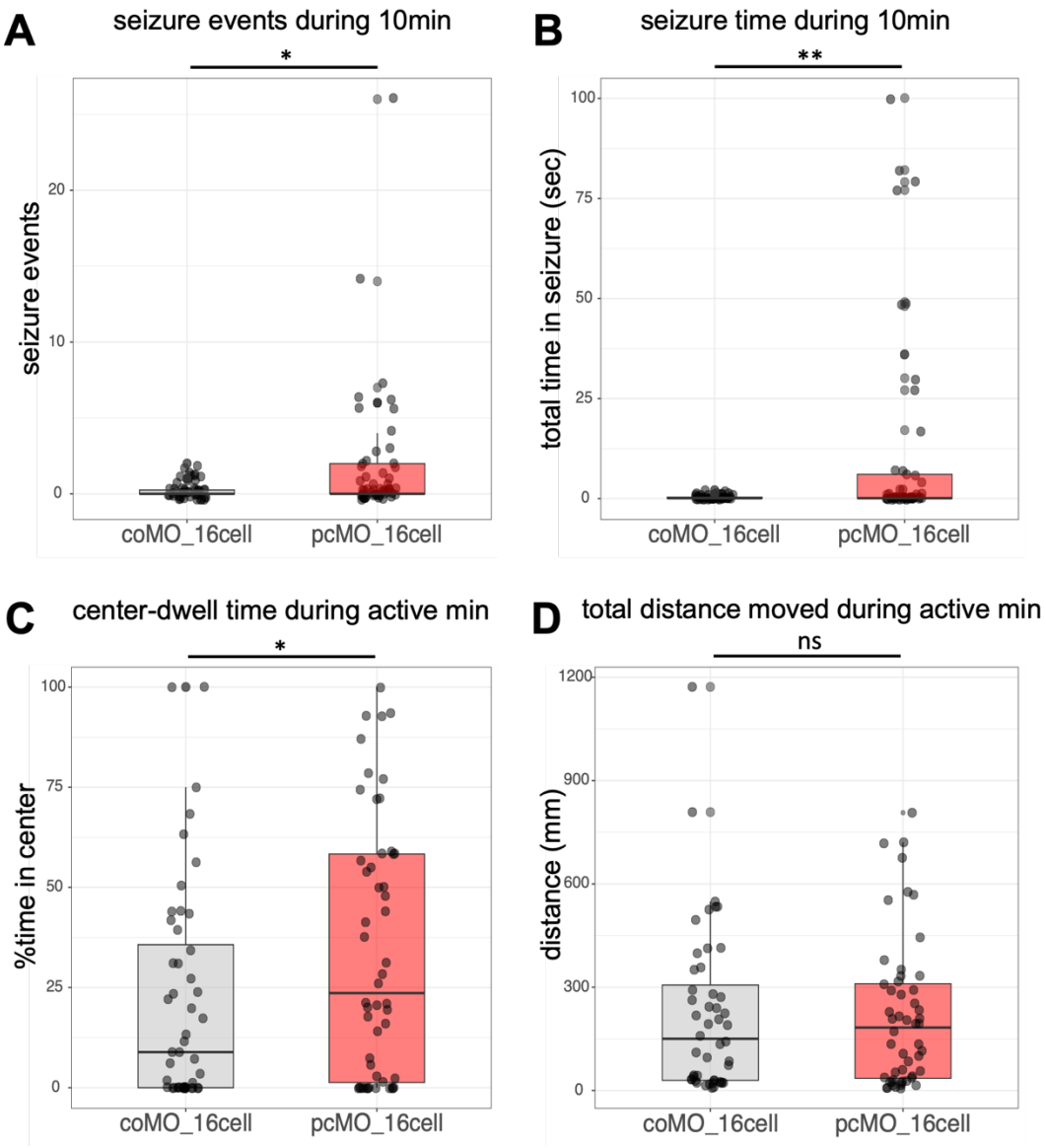


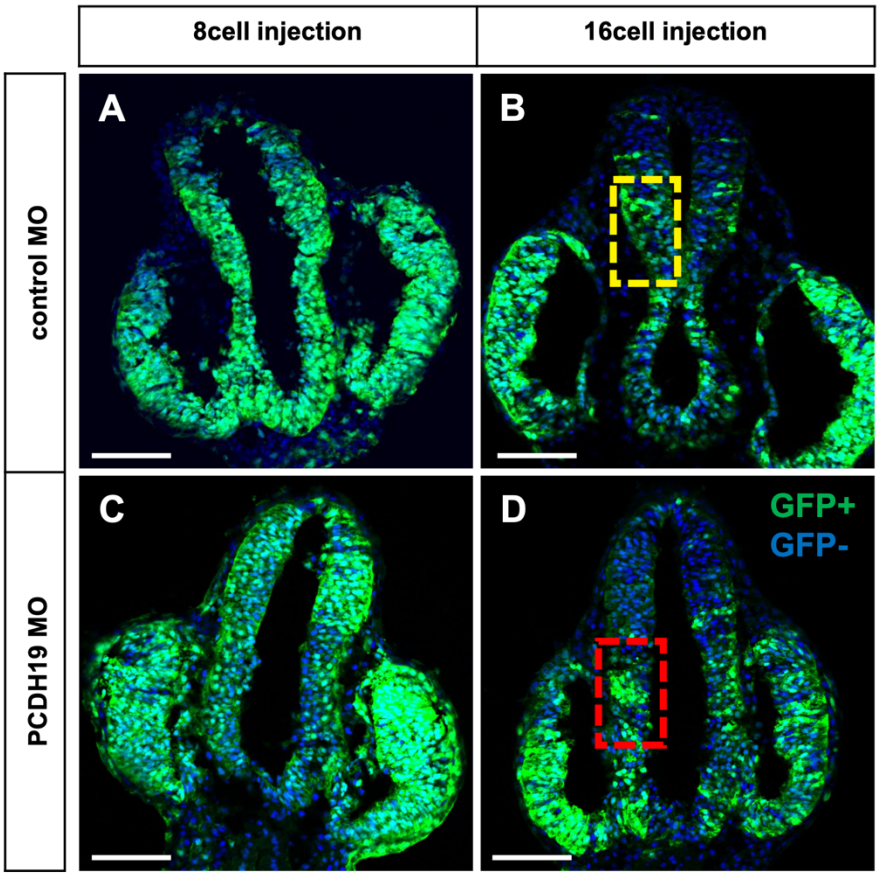
Figure 13. Quantification of seizure-like behaviors and repetitive swimming patterns in PCDH19 depletion in the CNS in mosaic pattern. (A) Quantification of seizure-like behavior frequency in tadpoles injected with control MO (n = 48) and PCDH19 MO (n =

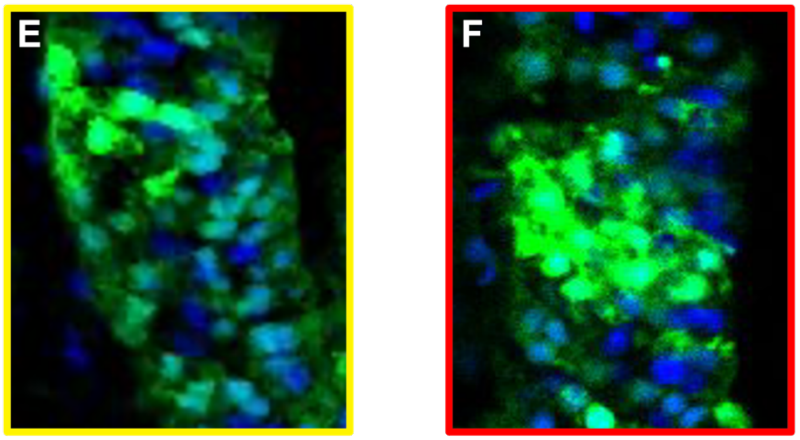
52) as CNS mosaic injection at the 8cell stage. *p = 0.0135. (B) Quantification of the total duration of seizure-like behavior in tadpoles injected with control MO and PCDH19 MO as CNS mosaic injection at the 8cell stage. **p = 0.00258. (C) Quantification of the center dwelling time during active time in tadpoles injected with control MO and PCDH19 MO as CNS mosaic injection at the 8cell stage. *p = 0.0346. (D) Quantification of the total swimming distance in tadpoles injected with control MO and PCDH19 MO as CNS mosaic injection at the 8cell stage. n.s. (not significant) p = 0.981. Each point represents an individual tadpole, and the data are summarized by box and whisker plots. Unpaired t-test.

B. PCDH19-depleted and normal cells segregate in the CNS

PCDH19 is known to be highly expressed in the cortex and hippocampus of the developing mouse brain. Previous studies have reported abnormal "tiger-striped" patterns in the brains of heterozygous PCDH19 knockout female mice.[23,52,53]

To see if the *Xenopus tropicalis* brain with PCDH19 mosaic knock-down also displays the abnormal pattern observed in the mouse brain, I analyzed the location and distribution of cells where pcdh19 was presumed to be deleted due to PCDH19 MO injection. In the case of embryos injected with Control MO and PCDH19 MO at the 8-cell stage, it is estimated that MO was injected into nearly all cells, as indicated by the GFP fluorescence, which suggests a knockdown effect (Figure 14A and 14C). However, when observing the brains of embryos injected at the 16-cell stage, it was observed that MO had been injected into half of the cells, while the other half remained unaffected (Figure 14B and 14D). When calculating the percentage of cells containing MO relative to the total brain cell count, it was found that in the case of injection at the 8-cell stage, the control MO contained approximately 93.57% of cells, while the PCDH19 MO contained approximately 89.92% of the cells. In the case of injections at the 16-cell stage, the control MO contained approximately 46.86% of the cells, and the PCDH19 MO contained about 47.84% of the cells (Figure 15). I found that in the brains of embryos injected with the control MO at the 16-cell stage, the cells injected with MO and those not injected were evenly scattered (Figure 14E, yellow box). However, in the brains of embryos where PCDH19 was knocked down at the 16-cell stage, the cells were clustered in specific areas (Figure 14F, red box).





Control MO
16cell injection

PCDH19 MO
16cell injection

Figure 14. Location and distribution of cells injected with control MO and PCDH19 MO. (A) The brain of control MO group injected in CNS at 8-cell stage. (B) The brain of control MO group injected in CNS at 16-cell stage. The yellow box indicated a MO contained cell. (C) The brain of PCDH19 MO group injected in CNS at 8-cell stage. (D) The brain of PCDH19 MO group injected in CNS at 16-cell stage. The red box indicated a MO contained cell. Scale bars, 100 μm. (E) The enlarged image of the yellow dashed box in B shows that cells containing MO and those not containing MO are evenly distributed. (F) The enlarged image of the red dashed box in D shows that cells containing MO are clustered in the specific area.

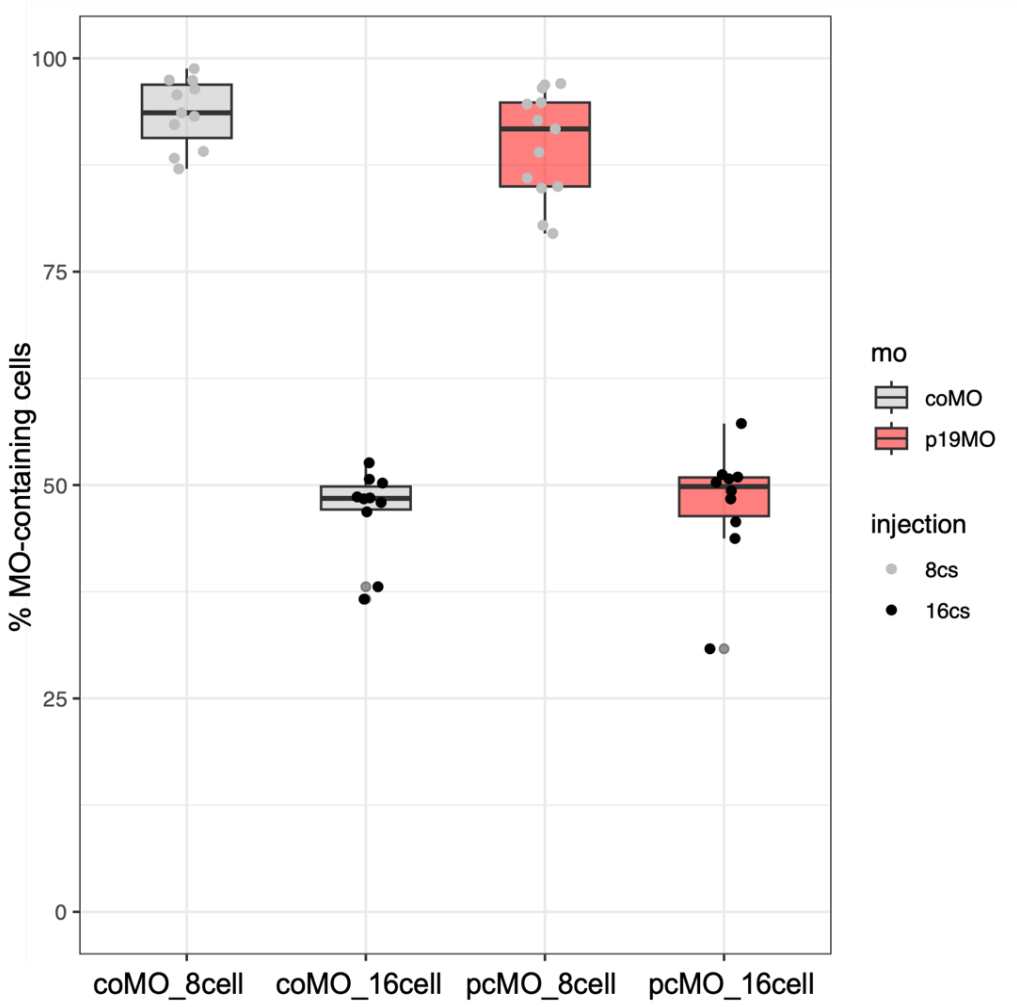


Figure 15. Quantification of proportion of brain cells injected with control MO and PCDH19 MO among the total brain cells. Embryos injected with Control MO and PCDH19 MO at the 8-cell stage, it is estimated that MO was injected into nearly all cells, as indicated by the GFP fluorescence, which suggests a knockdown effect. However, when observing the brains of embryos injected at the 16-cell stage, it was observed that MO had been injected into half of the cells, while the other half remained unaffected. Each point represents an individual tadpole.

C. Depleting PCDH19 expression either in all or half of the cells in the CNS causes behavioral anomaly

In behavioral experiments conducted at stage 49, when comparing seizure behavior between the PCDH19 knock-down group and the PCDH19 mosaic knock-down group with their matching control groups, it was observed that the occurrence of seizure events and the total seizure time increased in both groups in a PCDH19 MO dependent manner (Figure 16A and 16B). Additionally, central–dwelling time increased in both groups compared to the control group (Figure 16C). There was no significant difference in the total distance covered by tadpoles during their most active 1-minute period between the two groups (Figure 16D).

These findings indicate that mosaic knock-down is not required for seizure and repetitive behaviors induced by PCDH19 knock-down in *Xenopus tropicalis*. Furthermore, they suggest that the loss-of-function of PCDH19, other than cellular interference, could play a role in the pathogenesis of PCDH19-related epilepsy syndrome.

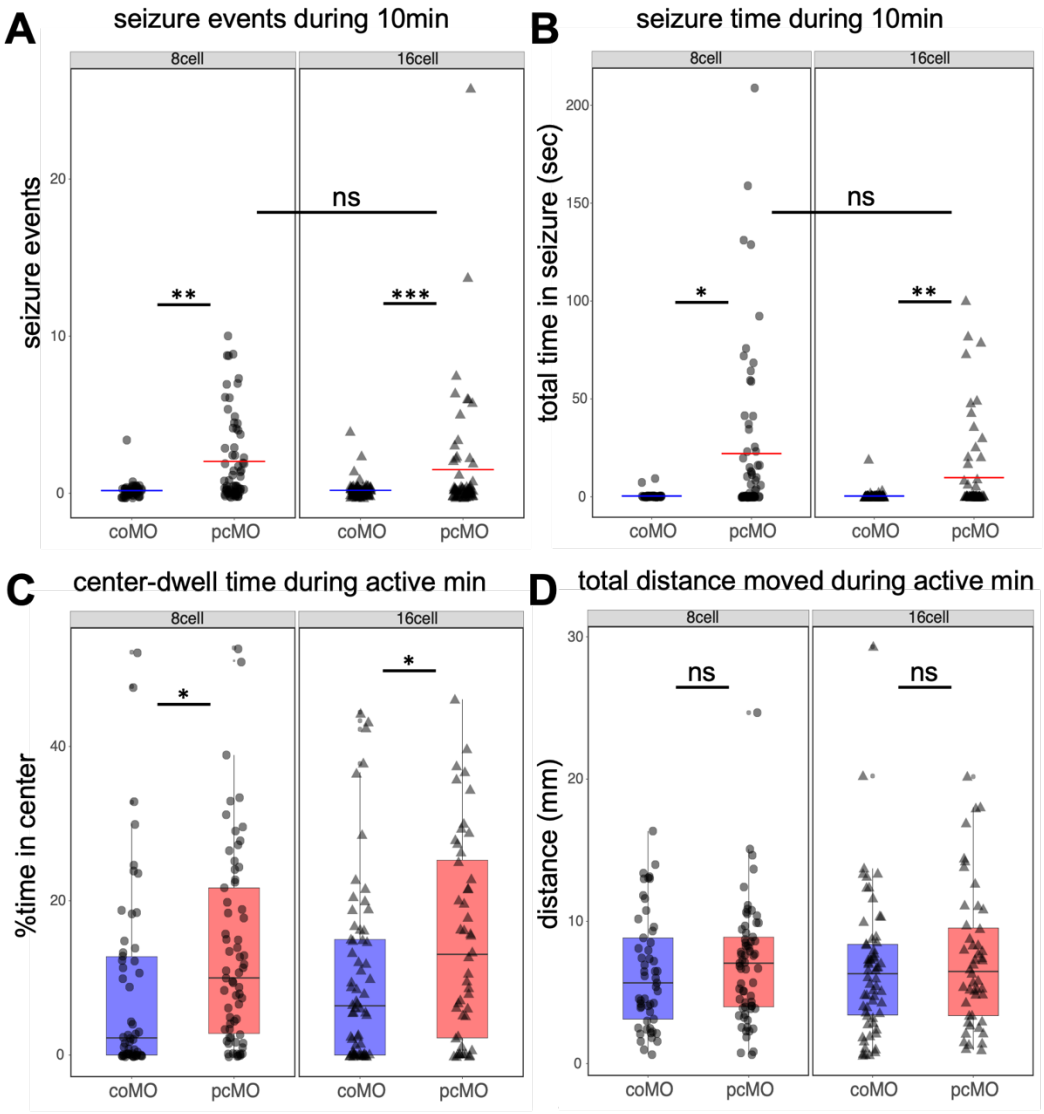


Figure 16. Depleting PCDH19 in all or half of the cells in the CNS causes similar degrees of behavioral anomaly. (A) Quantification of seizure-like behavior frequency and the total duration (B) in tadpoles with PCDH19 knock-down and PCDH19 mosaic knock-down. In both "complete" and "mosaic" knockdown experiments, knockdown tadpoles (pcMO) displayed more episodes of seizure-like behaviors compared to the matching

controls (coMO). Each point represents an individual tadpole (n = 52, 68, 90, and 67 from the leftmost group), and the mean values are represented by solid horizontal lines. *p < 0.05, **p < 0.01, ***p < 0.001, and ns, not significant; post-hoc Tukey test following one-way ANOVA. (C) Quantification of the center dwelling time during active time in tadpoles with PCDH19 knock-down and PCDH19 mosaic knock-down. For this analysis, the tadpoles whose total traveled distance exceeds 5cm (twice the diameter of the arena) were included (n = 53, 48, 62, and 48 from the leftmost group). Among these, the most active 1-minute epoch was chosen per movie, and the trajectory during this period is visualized by box and whisker plots, with individual data as points. (D) Quantification of the total swimming distance in (C). *p < 0.05 in unpaired t-test.

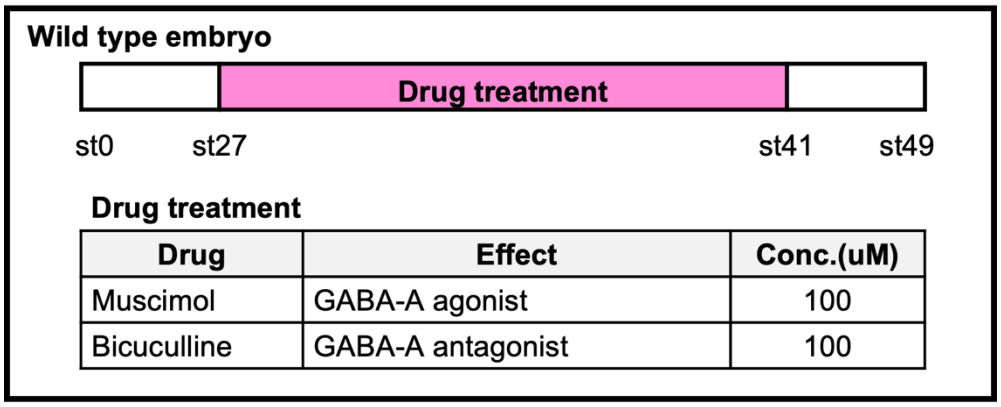
3. Seizure-like behaviors in PCDH19-depleted embryos may involve GABA-A hyperactivation during development

A. Treating GABA-A receptor agonist during CNS development causes seizure-like behaviors, but not repetitive swimming patterns

The cardinal symptom of PCDH19-related epilepsy syndrome, seizure, occurs when the excitatory/inhibitory (E/I) balance is disrupted. While investigating the relationship between PCDH19 and neurotransmitters like glutamate and GABA, I came across reports suggesting that PCDH19 interacts with the GABA-A receptor in neurons. This interaction can regulate the expression of the PCDH19 gene, thereby modulating the gating properties of the GABA-A receptor. I applied this knowledge to our *Xenopus tropicalis* model of PCDH19-related epilepsy syndrome to elucidate the relationship between PCDH19 and the GABA-A receptor.

First, I conducted drug treatment experiments to see how the imbalance of the GABA-A receptor affects the behavior and morphology of *Xenopus tropicalis* tadpoles. When I treated wild-type specimens with the GABA-A receptor agonist muscimol and antagonist bicuculline at a concentration of 100uM during the st27-41 stage, which is when the central nervous system forms (Figure 17), I observed abnormal swimming patterns and seizure behaviors at the st49 stage, even though their morphology remained unaffected (Figure 18A and 18B).

In behavioral experiments conducted at stage 49, it can be observed that in the GABA-A receptor modulating drug treatment group, both seizure events and their duration increased (Figure 19A and 19B). The central-dwelling time and the total moved distance during the tadpole's most active 1-minute period decreased (Figure 19C and 19D).



Drug treatment

Drug	Effect	Conc.(uM)
Muscimol	GABA-A agonist	100
Bicuculline	GABA-A antagonist	100

Figure 17. Experimental scheme of drug treatment for GABA-A signaling imbalance. CNS target microinjected embryos were treated with the GABA-A agonist Muscimol (100uM) and GABA-A antagonist Bicuculline (100uM) from stage 27 to stage 41, which is when the central nervous system develops.

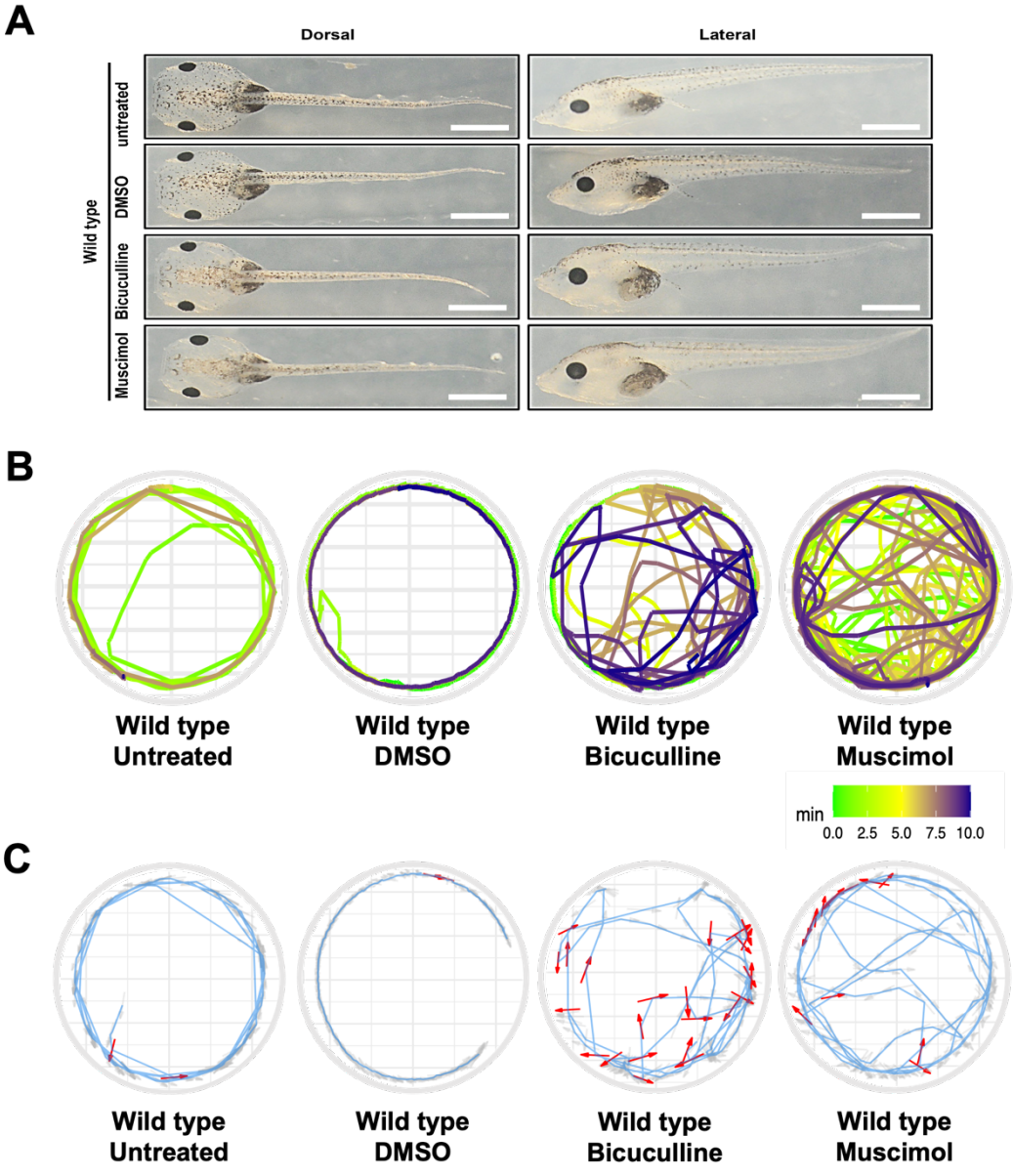


Figure 18. Imbalance in GABA-A signaling during CNS development causes abnormal swimming behaviors. (A) Morphology of drug treated embryos at stage 49. Imbalance in GABA-A signal caused no noticeable external morphological changes in developing embryo. Scale bar 1 mm. (B) Representative swimming patterns of tadpoles treated with GABA-A receptor modulating drugs during CNS development. (C) A video frame representing the most active 1-minute swimming trajectory of the tadpole. The blue line indicates the swimming trajectory, the gray arrows indicate the tadpole's body orientation, and the red arrows indicate the tadpole's body orientation at the moment of a sharp turn.

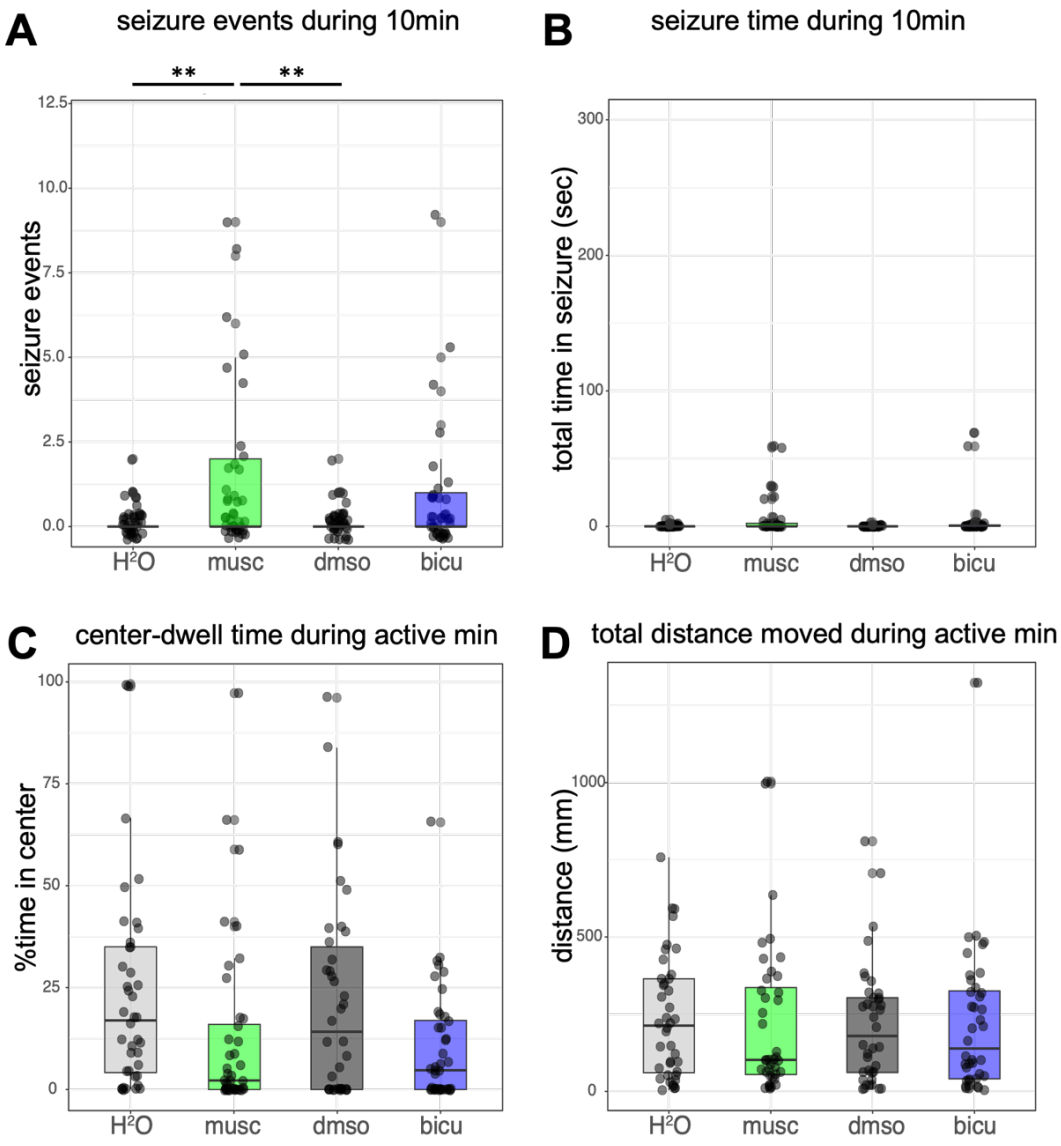


Figure 19. Treating GABA-A receptor agonist or antagonist during development causes seizure-like behaviors, but not repetitive swimming patterns. (A) Quantification of seizure-like behavior frequency in tadpoles treated with GABA-A receptor modulating drugs during CNS development. H^2O (n = 40) and dmso (n = 40) group. n.s. (not significant)

p = 1, musc (n = 40), **p = 0.00363, bicu (n = 40), n.s. (not significant) p = 0.418, dmso and musc group. **p = 0.00278, bicu, n.s. (not significant) p = 0.353, musc and bicu group. n.s. (not significant) p = 0.576. (B) Quantification of the total duration of seizure-like behavior in tadpoles treated with GABA-A receptor modulating drugs during CNS development. H^2O and dmso group. n.s. (not significant) p = 1, musc, n.s. (not significant) p = 0.0624, bicu, n.s. (not significant) p = 0.721, dmso and musc group. n.s. (not significant) p = 0.0517, bicu, n.s. (not significant) p = 0.631, musc and bicu group. n.s. (not significant) p = 1. (C) Quantification of the center dwelling time during active time in tadpoles treated with GABA-A receptor modulating drugs during CNS development. H^2O and dmso group. n.s. (not significant) p = 1, musc, n.s. (not significant) p = 0.255, bicu, n.s. (not significant) p = 0.428, dmso and musc group. n.s. (not significant) p = 0.0674, bicu, n.s. (not significant) p = 0.125, musc and bicu group. n.s. (not significant) p = 1. (D) Quantification of the total swimming distance in tadpoles treated with GABA-A receptor modulating drugs during CNS development. H^2O and dmso group. n.s. (not significant) p = 1, musc, n.s. (not significant) p = 1, bicu, n.s. (not significant) p = 1, dmso and musc group. n.s. (not significant) p = 1, bicu, n.s. (not significant) p = 1, musc and bicu group. n.s. (not significant) p = 1. Each point represents an individual tadpole, and the data are summarized by box and whisker plots. One-way ANOVA followed by bonferroni's post-hoc test.

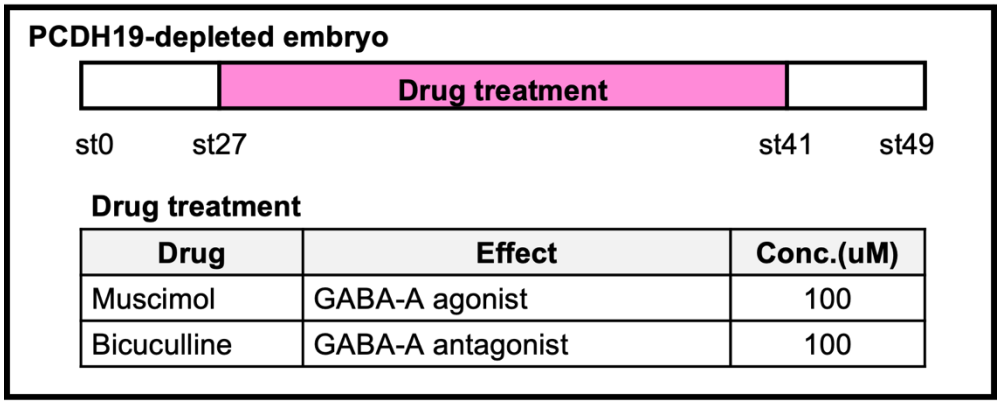
B. Treating GABA-A receptor antagonist to the developing PCDH19-depleted tadpoles ameliorates seizure-like behaviors, but not repetitive swimming patterns

To determine whether regulating the GABA-A receptor can control the abnormal swimming and seizure symptoms observed in our PCDH19-related epilepsy syndrome model in *Xenopus tropicalis*, I conducted further drug treatment experiments to PCDH19 knock-down tadpoles (Figure 20). After treating with muscimol and bicuculline at the st27-41 stage and then analyzing seizure behaviors at the st49 stage, the tadpole's morphology remained unchanged, and abnormal swimming pattern continued to be observed (Figure 21A and 21B).

The results revealed that the group treated with the GABA-A receptor agonist, muscimol, upon visual inspection of the box plot in the graph, both the median and upper quartile increased compared to the group without drug treatment, and bicuculline decreased (Figure 22A). While this suggests a potential influence of GABA-A receptor regulation on the seizure phenotype, statistical analysis did not reveal significant differences in the data. Additionally, there was not a significant difference in the duration of seizure behavior exhibited (Figure 21B). Comparing the time tadpoles spent at the center of the dish during their most active 1-minute period, both the GABA-A receptor antagonist and agonist treatment groups showed an increased in the median and upper quartile of the box plot in the graph compared to the group without drug treatment (Figure 22C). This suggests a potential influence of GABA-A receptor regulation on the repetitive swimming phenotype. However, statistical analysis did not reveal significant differences in the data. When examining the total distance moved during the most active 1-minute period, there was little variation among the four groups, indicating no significant differences in their motor abilities (Figure 21D).

As a result, in the group treated with bicuculline, there was a slight, albeit statistically insignificant, decrease in the frequency of seizure-like behaviors, while in the group treated with muscimol, an increase was observed.

The mechanism of the PCDH19-related epilepsy syndrome model created based on all the results in this study is shown in Figure 23.



Drug treatment

Drug	Effect	Conc.(uM)
Muscimol	GABA-A agonist	100
Bicuculline	GABA-A antagonist	100

Figure 20. Experimental scheme of behavior test of GABA-A receptor modulation in the developing PCDH19-depleted embryos. CNS target microinjected embryos were treated with GABA-A receptor modulating drugs during CNS development then grown to stage 49 and recorded their behavior in a custom-built dark chamber for 10 minutes. Based on a 10-minute behavioral video, tadpole behavior was quantified by counting seizure-like episodes and analyzing swimming trajectories.

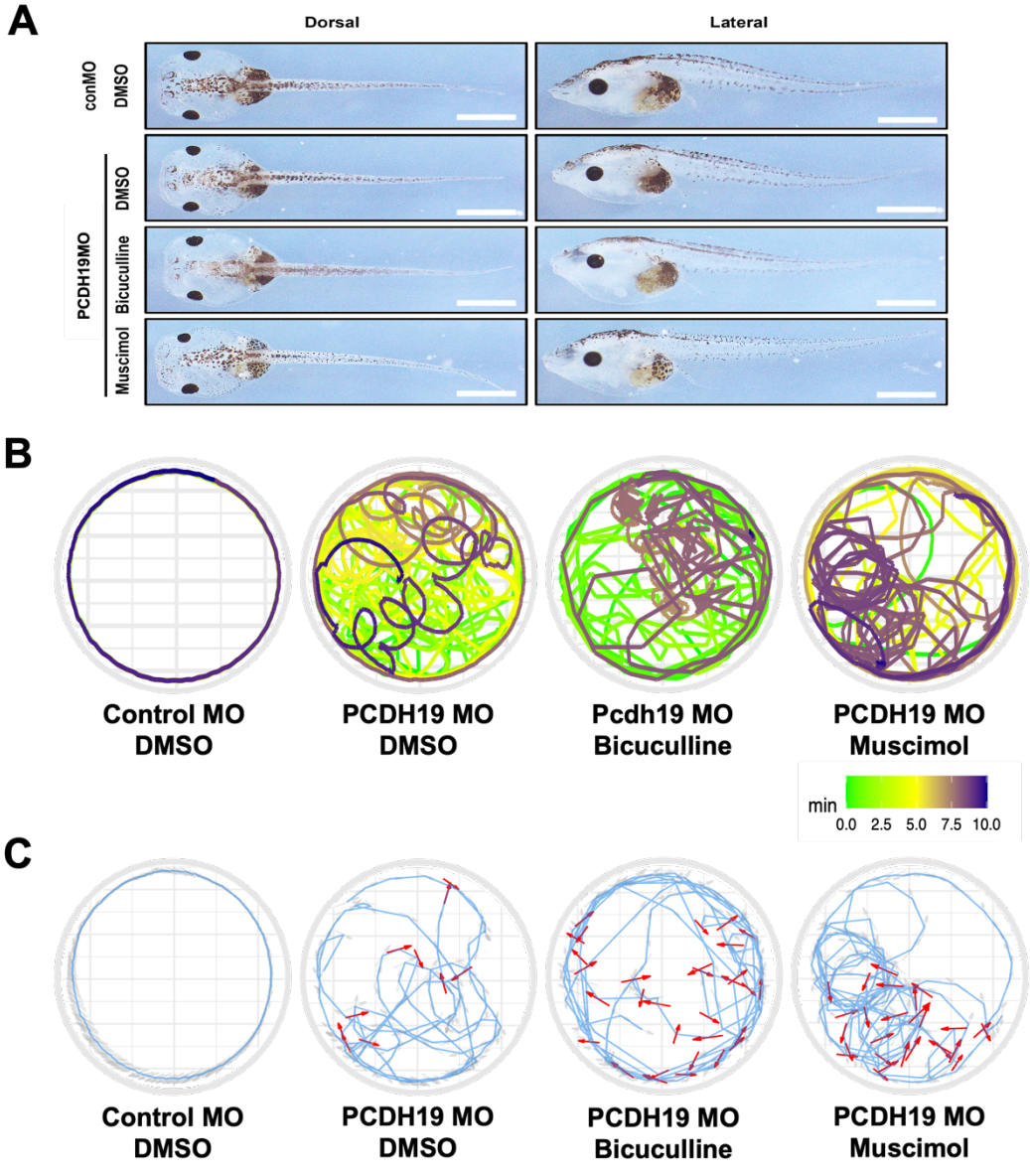


Figure 21. Modulating GABA-A signaling during development affects behavioral anomaly of PCDH19-depleted tadpoles. (A) Morphology of drug treated embryos at stage 49. Imbalance in GABA-A signal caused no noticeable external morphological changes in developing embryo. Scale bar 1 mm. (B) Representative swimming patterns of PCDH19 knock-down tadpoles treated with GABA-A receptor modulating drugs during CNS development. (C) A video frame representing the most active 1-minute swimming trajectory of the tadpole. The blue line indicates the swimming trajectory, the gray arrows indicate the tadpole's body orientation, and the red arrows indicate the tadpole's body orientation at the moment of a sharp turn.

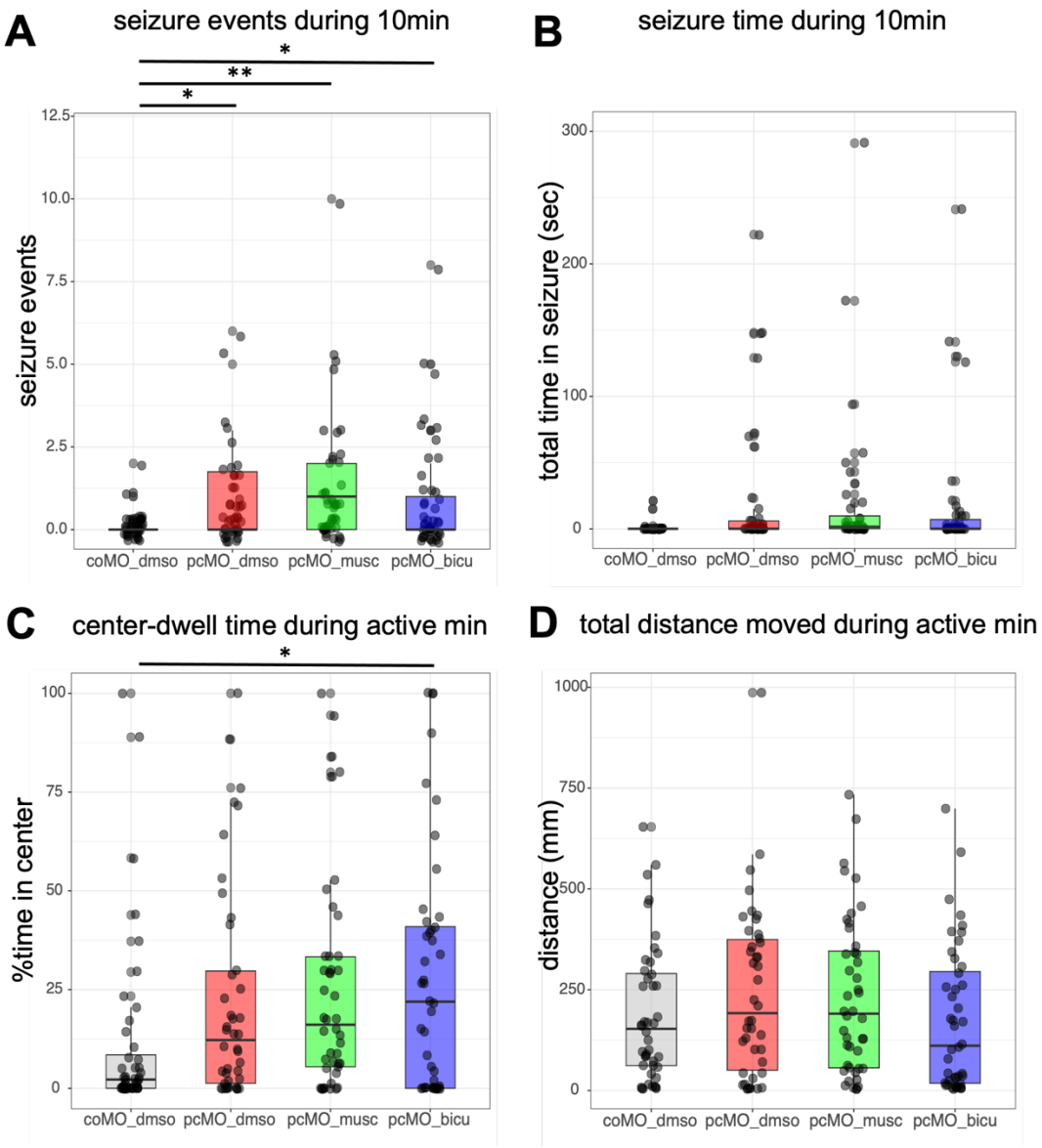


Figure 22. Treating GABA-A receptor antagonist during development ameliorates seizure-like behaviors, but not repetitive swimming patterns, in PCDH19-depleted tadpoles. (A) Quantification of seizure-like behavior frequency in PCDH19 knock-down tadpoles treated with GABA-A receptor modulating drugs during CNS development.

coMO_dmso (n = 44) and pcMO_dmso (n = 42) group. *p = 0.0484, pcMO_musc (n = 44), **p = 0.00162, pcMO_bicu (n = 44), *p = 0.0351, pcMO_dmso and pcMO_musc group. n.s. (not significant) p = 1, pcMO_bicu, n.s. (not significant) p = 1, pcMO_musc and pcMO_bicu group. n.s. (not significant) p = 1. (B) Quantification of the total duration of seizure-like behavior in PCDH19 knock-down tadpoles treated with GABA-A receptor modulating drugs during CNS development. coMO_dmso and pcMO_dmso group. n.s. (not significant) p = 0.153, pcMO_musc, n.s. (not significant) p = 0.261, pcMO_bicu, n.s. (not significant) p = 0.444, pcMO_dmso and pcMO_musc group. n.s. (not significant) p = 1, pcMO_bicu, n.s. (not significant) p = 1, pcMO_musc and pcMO_bicu group. n.s. (not significant) p = 1. (C) Quantification of the center dwelling time during active time in PCDH19 knock-down tadpoles treated with GABA-A receptor modulating drugs during CNS development. coMO_dmso and pcMO_dmso group. n.s. (not significant) p = 0.367, pcMO_musc, n.s. (not significant) p = 0.123, pcMO_bicu, *p = 0.0225, pcMO_dmso and pcMO_musc group. n.s. (not significant) p = 1, pcMO_bicu, n.s. (not significant) p = 1, pcMO_musc and pcMO_bicu group. n.s. (not significant) p = 1. (D) Quantification of the total swimming distance in PCDH19 knock-down tadpoles treated with GABA-A receptor modulating drugs during CNS development. coMO_dmso and pcMO_dmso group. n.s. (not significant) p = 1, pcMO_musc, n.s. (not significant) p = 1, pcMO_bicu, n.s. (not significant) p = 1, pcMO_dmso and pcMO_musc group. n.s. (not significant) p = 1, pcMO_bicu, n.s. (not significant) p = 0.672, pcMO_musc and pcMO_bicu group. n.s. (not significant) p = 0.949. Each point represents an individual tadpole, and the data are summarized by box and whisker plots. One-way ANOVA followed by bonferroni's post-hoc test.

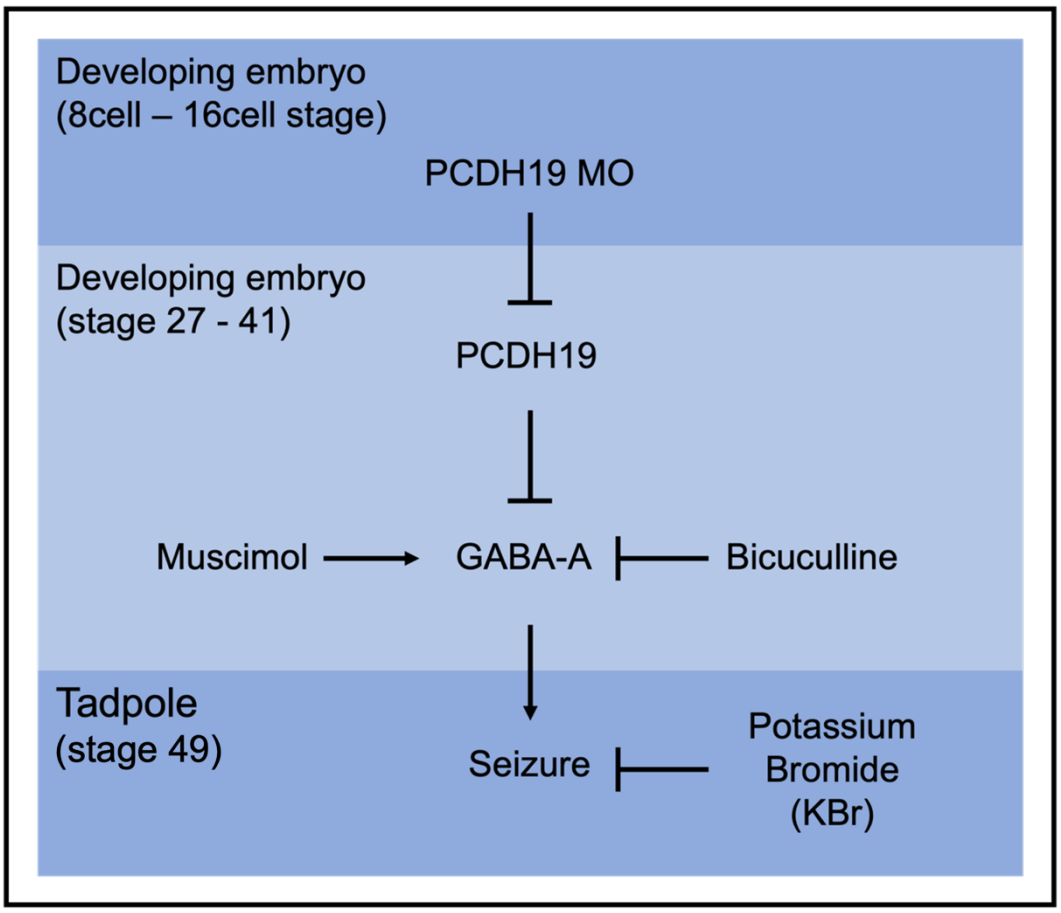


Figure 23. Working mechanism of PCDH19-depleted seizure model.

Table 3. Results of three independent experiments

Experiment number		1st	2nd	3rd	total
Control MO	8cell	20	12	12	44
	16cell	16	24	8	48
PCDH19 MO	8cell	20	11	11	42
	16cell	16	28	8	52

Experiment number	1st	2nd	3rd	total
Wild type	16	16	8	40
Wild type muscimol	16	16	8	40
Wild type DMSO	16	16	8	40
Wildtype bicuculline	16	16	8	40

Experiment number		1st	2nd	3rd	total
Control MO		20	12	12	44
PCDH19 MO	DMSO	20	11	11	42
	muscimol	20	12	12	44
	bicuculline	20	12	12	44

IV. DISCUSSION

To investigate whether complete depletion of PCDH19 in *Xenopus tropicalis* results in the cardinal symptom of PCDH19-related epilepsy syndrome, which is seizures, I first performed microinjections of PCDH19 MO into all cells differentiating into the central nervous system (CNS) at the 8-cell stage. I then observed tadpole behavior through video recording. The results showed that in *Xenopus tropicalis* with PCDH19 depletion in the CNS, abnormal swimming patterns and seizure-like behaviors were observed.

To analyze seizure-like behaviors in more detail, I established criteria for six abnormal behaviors that were expected to represent seizure behavior and quantified all behaviors that met these criteria. As a result, an increased frequency and duration of seizure-like behaviors were observed in individuals with PCDH19 depletion compared to the control group. Quantifying central-dwelling time, measured during the most active 1-minute period of tadpole movement, also showed an increase in this time in the PCDH19 depletion group, while there was no change in total moved distance, indicating that there was no alteration in motor abilities.

In the next experiment, I conducted a test to confirm if the six criteria I had established for seizure behavior indeed represented the cardinal symptom of PCDH19-related epilepsy syndrome. I selectively treated PCDH19 depletion *Xenopus tropicalis*, known to exhibit seizure-like behavior, with the anti-convulsant potassium bromide, commonly used in dogs and horses. During a 10-minute experiment involving drug treatment, I recorded video footage before, during, and after drug administration, and analyzed seizure-like behaviors. The results confirmed that upon treatment with potassium bromide, the frequency and duration of seizures in PCDH19 depletion tadpoles decreased, indicating that potassium bromide rescued the seizure phenotype in PCDH19-depleted tadpoles. This, in turn, allowed me to conclude that the six criteria I had defined indeed represented seizure behavior accurately.

Contrary to what has been previously known, the experimental results as mentioned above revealed that in *Xenopus tropicalis*, the cardinal symptom of PCDH19-related epilepsy

syndrome, seizure behavior, can be observed through the complete depletion of PCDH19 in the central nervous system.

I conducted experiments to investigate whether mosaic depletion of PCDH19 in the central nervous system of *Xenopus tropicalis* resulted in seizure behavior, similar to PCDH19 depletion tadpoles, which are known for exhibiting the cardinal symptom of PCDH19-related epilepsy syndrome. The results showed that, like PCDH19 depletion tadpoles, the frequency and duration of seizure behavior increased, and central-dwelling time also extended. When compared to the PCDH19 depletion group, the frequency of seizure behavior was similar, but the total time was shorter, and central-dwelling time was longer.

I aimed to confirm whether all CNS cells were affected in PCDH19 depletion tadpoles and only half of the cells in mosaic depletion tadpoles. To do so, I examined the amount of PCDH19 protein in the CNS using Western blot at the stage 27-28, the time when PCDH19 gene expression is the highest. I also cryosectioned tadpole brains and performed nuclear counterstaining to observe the distribution of cells injected with MO using a confocal microscope. Quantifying the bands through Western blot revealed a reduction in PCDH19 protein levels in the brain, approximately 17.37% in the PCDH19 depletion group and about 27.39% in the PCDH19 mosaic depletion group compared to the control group. Analyzing the ratio of cells injected with MO relative to the total number of brain cells on brain section slides, at the 8-cell stage, control MO contained approximately 93.57% while PCDH19 MO contained around 89.92% of the cells. At the 16-cell stage, control MO contained about 46.86%, and PCDH19 MO contained approximately 47.84% of the cells. I also observed the distribution of cells containing MO in the brain. As a result, in contrast to the control MO group where cells containing MO and those not containing MO were evenly distributed, in the PCDH19 MO group, I observed that cells containing MO were clustered in specific areas.

Based on the experimental results as mentioned above, it was revealed that by injecting MO into two out of the four cells that differentiate into the central nervous system at the

16-cell stage in *Xenopus tropicalis*, causing half depletion, it is possible to reproduce the behavioral phenotypes of PCDH19-related epilepsy syndrome and the clustering of PCDH19-depleted cells within the brain cortex regardless of gender. Consequently, it was confirmed that a successful PCDH19 epilepsy model using *Xenopus tropicalis* has been established.

Based on the research indicating the interaction between PCDH19 and GABA-A receptors, I conducted experiments to elucidate the relationship between PCDH19 and GABA-A signaling. Firstly, during the neural development stage of wild-type *Xenopus tropicalis*, which occurs from stage 27 to 41, I treated them with a GABA-A receptor modulation drug to observe whether the seizure behaviors and abnormal swimming patterns seen in PCDH19-depleted *Xenopus tropicalis* were present. The results showed an increase in both the frequency and duration of seizure behaviors similar to what was observed in the PCDH19 depletion group. While it was expected that central-dwelling time would also increase, it decreased, unlike in the PCDH19 depletion group. This leads to the consideration that central-dwelling behavior might be categorized as behavior patterns similar to autism seen in PCDH19 knockout mice.

In the next experiment, I treated the PCDH19 depletion group with a GABA-A receptor modulation drug during their neural development stage and compared whether the abnormal behaviors, such as central dwelling and seizure behaviors observed in the previous results, decreased. The results revealed that the group treated with the GABA-A receptor agonist, muscimol, exhibited an increase in the frequency of seizure behavior. Conversely, the group treated with the GABA-A receptor antagonist, bicuculline, showed a decrease in seizure frequency. However, central dwelling time increased in both groups, which suggests that central-dwelling behavior might belong to a category of behavior patterns similar to autism, as seen in PCDH19 knockout mice, and there may be an alternative mechanism at play, similar to the results of the previous experiments conducted on wild-type subjects with drug treatment.

V. CONCLUSION

1. Depletion of PCDH19 in central nervous system (CNS) provokes seizure-like behaviors in *Xenopus tropicalis*.
2. Potassium bromide (KBr) ameliorates seizure-like behaviors of PCDH19-depleted embryos.
3. The loss of PCDH19 function itself, along with cellular interference, may also play a role in the development of PCDH19-related epilepsy syndrome.
4. PCDH19-depleted cells cluster rather than scatter.
5. PCDH19 depletion phenocopies hyperactivation of GABA-A signaling during development.
6. GABA-A antagonist mitigates the seizure-like behaviors of PCDH19-depleted embryos, implicating the antagonistic function of PCDH19 in GABA-A signaling.

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ABSTRACT(IN KOREAN)

제노프스 트로피칼리스 모델을 이용한 PCDH19 관련 뇌전증 증후군 모델 제작 및 GABA-A 신호 연관성 규명

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PCDH19 관련 뇌전증 증후군은 주로 3세 이전에 발병하는 뇌전증과 발달 지연, 지적 장애, 자폐 성향, 행동 이상을 주 증상으로 하는 희소 질환이다. 원인 유전자인 PCDH19는 protocadherin-19이라는 칼슘 의존적 단일 투과막 단백질을 암호화하는데 이는 세포 외 캐드헤린 도메인을 통한 동종친화성 결합에 의한 세포-세포간 접합과 뉴런 간의 신호전달을 조절하여 신경의 발생에 중요한 역할을 한다. PCDH19 유전자는 X염색체에 있으며 남성의 경우 돌연변이가 있어도 보통 발병하지 않으나 두 X염색체 중 하나에서 돌연변이를 가지고 있는 여성의 경우 90%에서 여성 제한적 뇌전증이 발병한다. 이는 X염색체의 무작위적 불활성화로 인한 정상세포와 PCDH19 돌연변이 세포의 모자이크 현상에 의한 세포 간의 비정상적인 간섭을 유발함으로써 발생한다. 또한 최근 PCDH19가 GABA-A 수용체의 alpha1 소단위를 통해 신경세포에서 결합하여 상호작용을 하며 PCDH19의 발현을 인위로 조절해줌으로써 GABA-A receptor의 GABAergic 신경전달 신호를 조절할 수 있음이 밝혀졌다. 이 학위 논문에서는 *Xenopus tropicalis* 모델을 이용하여 PCDH19 모폴리노를 이용한 기능 상실 실험을 진행하여 PCDH19 관련 뇌전증 증후군 모델을 제작하고 뇌전증 표현형을 가지는 *Xenopus tropicalis*의 GABA-A 작용제와 길항제에 의한 표현형의 변화를 정량함으로써 PCDH19 결핍에 의한 행동표현형을 GABA-A 신호 조절을 통해 완화할 수 있는지 규명하고자 하였다. *Xenopus tropicalis*의 배아에 미세주입법을 통해 PCDH19 모폴리노를 주입하여 PCDH19 유전자의 발현을 중추신경계 특이적으로 억제, 기능 손실을 유도하고 행동검사를 실시하여

PCDH19 관련 뇌전증의 행동적 표현형이 나타나는지 뇌전증의 대표적인 증상인 발작의 수를 계산 및 집계하여 정량적인 분석을 진행했다. 미세주입법 시 주입 시기를 8세포기와 16세포기로 나눠 8세포기의 배아는 중추신경계로 분화하는 모든 세포에 주입하여 전체 녹다운을 통한 기능 손실을 유도하고 16세포기의 배아에는 중추신경계로 분화하는 4개의 세포 중 절반인 2개의 세포에만 주입하여 모자이크 현상을 통한 기능 손실을 유도하여 두 실험군 간의 대조를 통하여 중추신경계에서의 모자이크 현상과 전체 녹다운 개체 간의 차이를 분석하였다. PCDH19의 중추신경계 특이적 전체 녹다운과 모자이크 현상 녹다운은 발생 중인 배아의 외형적 형태학상의 변화를 일으키지 않았으나 두 실험군 모두에게서 비정상적인 헤엄 궤도와 뇌전증의 대표적인 증상인 발작행동 및 이상행동이 나타났다. 발작행동 및 이상행동을 세부적으로 분석 및 정량화 하기 위하여 발작행동으로 예측되는 6가지의 기준을 설정하여 행동의 빈도수와 시간을 집계하였고 대표적인 항경련제인 포타슘브로마이드를 약물처리 하였을 때 발작의 빈도수와 시간이 감소하였음을 통해 설정해준 6가지 기준의 행동이 발작행동임을 증명하였다. 또한 PCDH19의 발현이 가장 활발한 시기의 *Xenopus tropicalis* 배아의 뇌를 동결절편하여 대조염색을 통해 PCDH19이 녹다운 된 세포의 위치와 분포를 관찰한 결과 16세포기의 중추신경계에서의 모자이크 현상 녹다운을 해준 개체의 뇌에서 대조군은 모폴리노가 주입된 세포와 주입되지 않은 세포가 고르게 흩어져 산재했지만 PCDH19 녹다운 세포들이 모여서 위치함을 관찰했다. PCDH19 모폴리노의 주입 후 유도된 뇌전증 표현형 *Xenopus tropicalis* 배아의 중추신경계가 형성되는 시기에 대표적인 GABA-A 작용제인 Muscimol과 길항제인 Bicuculline을 처리하고 중추신경계 형성이 완료된 시기의 올챙이의 행동을 행동검사 한 결과 길항제인 Bicuculline을 처리한 개체에서는 발작행동이 상대적으로 감소하였고 작용제인 Muscimol을 처리한 개체에서는 발작행동이 상대적으로 증가하였음을 관찰했다. 위와 같은 사실을 통해 GABA 작동성 신경전달의 핵심 조절자로써 PCDH19의 중요성과 새로운 PCDH19 뇌전증의 발병 메커니즘을 이해하는데 우리에게 이론적 근거를 제시한다.

핵심되는 말 : PCDH19, 뇌전증, 모자이크 현상, 발작, GABAA 수용체