Mutations in PROSC and vitamin B6 dependent epilepsy – a functional study in zebrafish

Degree project in Medicine

Rasmus Selin

Supervisors: Alexandra Abramsson

Rakesh Banote

Institute of neuroscience and physiology, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Program in medicine

Gothenburg, Sweden 2018

Table of contents

Abstract

Background: Vitamin B6 dependent epilepsies are a group of rare, recessive genetic diseases that causes seizures refractory to standard epilepsy treatment but that respond rapidly to vitamin B6. Several disease-causing mutations have already been described in detail, but the mechanism behind one of them, PROSC (proline synthetase co-transcribed homolog), remains poorly understood. Although the seizures can be treated effectively, the syndrome is accompanied by features such as brain defects and developmental delay that are not improved by todays treatment regime, which motivates further research.

Aim: To investigate whether absence of PROSC causes vitamin B6 deficiency and epilepsy in a zebrafish model.

Methods: Morphological characteristics, survival rate, and behavior were assessed in mutant fish with and without vitamin B6 treatment. Epileptic zebrafish typically show a hyperactive swimming pattern that can be quantified objectively using automated video-tracking equipment and software.

Results: Mutant fish cannot be distinguished from wildtype based on physical appearance. No signs of epileptic activity have been observed, instead mutant fish are significantly less active than their wildtype siblings. Activity levels were normalized in mutant fish treated with vitamin B6. All mutants died between day ten and fourteen, however a significant reduction in mortality rate was observed in B6 treated fish.

Discussion: This study has shown that homozygous *prosc* mutant fish are vitamin B6 deficient. Although no signs of seizure-like epileptic activity were found in the present study this does not contradict the findings in humans. It is quite possible that the fish were epileptic only for a short time and not observed frequently enough to detect it or that the hypo activity observed may be due to an abnormal electroencephalograph (EEG) response. Vitamin B6 deficiency causes both brain malformations and a wide range of systemic disturbances that might have affected the behavior of the fish to such an extent that the hyperactivity that otherwise would have arisen due to a lowered seizure threshold, was masked.

Introduction

Vitamin B6 dependent epilepsies are a rare group of recessive genetic diseases that by definition causes seizures refractory to standard epilepsy treatment but that respond to vitamin B6. Brain malformations and developmental delay of varying degrees are seen in most cases as well. There are six different forms of vitamin B6 and the one most commonly used as medication is pyridoxine. Vitamin B6 dependent epilepsies are therefore often referred to in the literature as "pyridoxine-dependent epilepsies", abbreviated PDE.

PDE has been linked to several mutations in different pathways, and though the clinical characteristics are similar, the etiology is not. Despite recent advances in diagnosing PDE, there are some patients in which the cause of the disease cannot be determined.

Researchers investigating one such case, a Syrian family with three children with PDE negative for all previously known mutations, found that they shared a homozygous mutation in the protein PROSC (proline synthetase co-transcribed homolog) (1). Examining the apparent link further, 29 children with PDE of unknown origin were screened for homozygous mutations in PROSC and it was detected in four of them (1). No individuals homozygous for the mutations has yet been found in healthy controls. After the first study was published, a second study has confirmed the effects PROSC deficiency, finding it in four additional patients with PDE (2).

How PROSC deficiency causes epilepsy is unknown but the protein seems capable of binding PLP (3) and is likely involved in intercellular PLP homeostasis (1).

Even though the seizures can be treated effectively, accompanying features such as brain defects and developmental delay are not improved by todays treatment regimens which motivates further research.

In recent years zebrafish has emerged as a popular animal model for studying human disease in general and epilepsy in particular. In an attempt to ascertain if the mutation does indeed

cause an epileptic phenotype in zebrafish we aimed to investigate mutant fish morphology, mortality rate and behavior with and without B6 treatment. If the fish displayed typical epileptic behavior that normalized after B6 supplementation, this would indicate that they are a good model organism for the disease that can be used for further research.

Pyridoxine dependent epilepsy

1.1 Prevalence

The PROSC mutations have so far only been found in 11 patients and no large-scale studies have been performed. It is therefore not possible to say how many actually suffer from the disease. However, many patients with PDE have previously been labeled idiopathic or successfully treated with pyridoxine without a specific diagnosis, so it is quite likely that at least some of them have PROSC deficiency. How common PDE is, is still debated. According to a one survey conducted in the United Kingdom the prevalence is around 1:100000 (4) while a study in the Netherlands reported a prevalence of 1:396000 births (5). In a study done in a German center where pyridoxine is routinely administered to neonates with intractable seizures, the number of cases was estimated to be around one in every 20000 births (6).

1.2 Clinical presentation

The diagnosis should be considered in children younger than three years with intractable seizures and encephalopathy when no evidence of other metabolic conditions or ischemic encephalopathy exists (7). In most cases, the seizures commence in the first weeks after birth and show no or only partial response to standard epilepsy treatment but rapid response to vitamin B6. In general, the seizures present themselves quite dramatically with recurring episodes of status epilepticus but more benign types, such as partial and self-limiting seizures, have also been reported (7).

However, patient symptoms vary to a considerable degree both in severity and presentation. Seizures may start several months or even years (up to three have been reported) after birth or, in some cases, possibly even before birth since mothers have reported fetal movements that might indicate a seizure (8). The response to pyridoxine is also highly variable. Normally, intravenous administration of 50-100 mg of pyridoxine is enough to reverse the seizures within minutes but some patients require much higher doses or even addition of standard epileptic drugs to become seizure free (9). If treatment is stopped, seizures tend to recur quickly, often within days and always within two months (10). High dose pyridoxine treatment may lead to respiratory depression when first administered and patients with PDE must be monitored closely during the initial treatment attempt (11).

This effect is seen only in patients with the disease and occurs only if the treatment is used while the patient is having a seizure.

Brain abnormalities accompanies the syndrome in most cases. Microcephaly and reduction in size of the corpus callosum are common findings as well as a general underdevelopment of the brain (12). Periventricular cysts and hydrocephalus are also frequently observed (13).

The degree of intellectual impairment patients exhibits range from non-existent (or very mild) to very severe. Cognitive impairment is seen despite successful seizure control in most patients and the time of diagnosis and initiation of treatment seem to have little impact on final outcome (14). Vitamin B6 participates in more than a hundred different reactions and deficiency causes more symptoms than just epilepsy.

Disturbances in electrolyte count, hypoxia and metabolic acidosis are frequent findings as well as large fluctuations in body temperature hypothyroidism and diabetes insipidus (7).

Before onset of clinical seizures, patients regularly display symptoms indicating encephalopathy, for example, irritability, poor feeding and sensitivity to sound and touch. Pyridoxine administration usually relives these symptoms (6).

1.3 Vitamin B6

There are six different vitamers of vitamin B6, three of them pyridoxine, pyridoxal and pyridoxamine are distinguished from each other based on the group bond to the 4'-carbon atom. Each vitamer may carry a phosphate group instead of a hydroxymetyl group on the 5'-carbon atoms, yielding a total of six different molecules.

The biologically most active of these molecules, pyridoxal 5' phosphate (PLP) can either be created by phosphorylation of pyridoxal by pyridoxal kinase or from pyridoxamine 5'-phosphate and pyridoxine 5'-phosphate by the actions of the enzyme PNPO.

Dietary vitamin B6 enters the circulation as PLP bound to albumin. In order to enter cells, PLP is dephosphorylated into pyridoxal by an alkaline phosphatase (TNSALP).

Once inside the cell, pyridoxal is rephosphorylated by pyridoxal kinase to produce active PLP. Since vitamin B6 is abundant in most animal and plant derived food (especially meat, nuts and grain), dietary deficiencies are rare (15, 16). When they do occur, the classic symptoms include angular chelitis, atrophic glossitis, eruptions similar to those seen in seborrheic dermatitis and neurological symptoms such as neuropathy and extreme sleepiness. Epilepsy due to dietary deficiency has also been seen but only a handful of cases have been reported (17).

1.4 Pathophysiology of vitamin B6 dependent epilepsy

Since vitamin B6 acts as cofactor in about 140 different reactions, pinpointing exactly how deficiency causes epilepsy is difficult. PLP is especially prevalent in reactions involving neurotransmitter metabolism. An imbalance between GABA and glutamate might explain why B6 deficiency leads to seizures and encephalopathy (7). The higher levels of glutamate and lower levels of GABA seen in PLP deficient subjects might in part be caused by dysfunction of the PLP dependent enzyme glutamate decarboxylase (18). Glutamate concentrations could also be increased by the higher levels of a-ketoglutarate and lysine seen in PDE (7). However, cases of PDE have been seen with normal levels of GABA and glutamate suggesting a complex cause of the seizures (19).

Vitamin B6 seems to be especially important during the embryonic development of the brain. Rats subjected to a B6 restricted diet while pregnant produce offspring with impaired neuronal development (20) and motor function (22). There is also evidence indicating that B6 levels are higher in the fetus than later in life. Neonates have higher plasma concentration of B6 than their mothers (22) and babies born prematurely have even higher levels (23).

It is likely that B6 depletion causes epilepsy in fish in a manner similar to mammals.

Gingotoxin (4-O-methylpyridoxine), an inhibitor of pyridoxal kinase (the enzyme converting pyridoxal to PLP), has been shown to produce seizure-like behavior in zebrafish that can be reversed by PLP supplementation (24).

1.5 Etiology

The different mutations causing PDE cannot be distinguished from each other based on patient's symptoms and response to treatment alone. Analysis of biomarkers or genetic

screening is needed to determine subcategory. The mutations causing PDE act in different pathways but all have in common that they reduce the amount of PLP available in the brain. The most common cause of PDE is due to mutations in the gene Aldh7a1 which results in antiquitin deficiency (25, 26). Antiquitin (ATQ) catalyzes the conversion of α-aminoadipic semialdehyde (a-AASA) to a-aminoadipic acid (a-AAA). When ATQ no longer functions, the buildup of a-AASA causes an increase in L-Δ1-piperideine 6-carboxylate (P6C), which in turn reacts with and inactivates PLP. Diagnosis can be made either by genetic screening or by measurement of a-AASA in plasma (CSF or urine), elevated levels is highly specific for antiquitin deficiency (7). Pipecolic acid levels can also be measured, but elevated levels are less specific and is seen in liver disease as well. PLP plasma levels are not affected, but measurement of PLP in the CNS shows reduced concentrations (27).

Since the P6C is a breakdown product of lysine and because some scientists speculate that the brain damage observed is due to accumulation of toxic metabolites as well as B6 depletion, attempts to treat antiquitin deficiency with lysine restricted diet have been made. Some studies seem to indicate more favorable outcomes with lysine treatment, but due to the small number of patients tested, it is still uncertain if it yields better results in the end (28).

Among other known but more rare causes of PDE, TNSALP deficiency (tissue non-specific alkaline phosphatase), PNPO deficiencies and hyperpolinemia type 2 should be mentioned. The most prominent symptom of TNSALP deficiency, also known as hypophosphatasia, is demineralization of bone tissue resulting in frequent fractures and a deformed skeleton, but seizures are also seen (22). Since PLP is unable to enter cells or cross the blood brain barrier, it must first be converted to pyridoxal by TNSALP. Once inside the brain cells (and other tissue) pyridoxal is converted back to PLP by pyridoxal kinase. When TNSALP no longer functions, PLP concentrations in the neurons drop and epilepsy develops.

PNPO converts pyridoxine to PLP in the liver and PLP bound to albumin enter the circulation. Hence a dysfunctional PNPO protein results in low levels of PLP and high levels of pyridoxine. PNPO deficiency was initially thought to only respond to PLP treatment, but some individuals have been found who respond to pyridoxine as well (29).
Hyperprolinemia type 2 causes epilepsy in a manner similar to antiquitin deficiency.
It is caused by accumulation of L-D1-pyrroline-5-carboxylic acid (P5C) which like P6C reacts with and inactivates PLP (30). The clinical phenotype is similar to antiquitin deficiency.

1.6 PROSC

The gene encoding PROSC was first discovered in 1999 (31). It is 2530 base pairs long and located on chromosome 8p11.2 (31). PROSC is a protein highly conserved between species and is expressed in all human tissues indicating an important biological function. The protein is located in the cytoplasm and it is capable of binding PLP but seems to lack enzymatic activity (32). It has been suggested that its action might be to facilitate intracellular PLP transport, supplying it to enzymes while preventing it from reacting with other molecules (1). Support for this hypothesis comes from the fact that PROSC deficient individuals seem to accumulate PLP inside cells and that this excess PLP binds to small molecules and proteins indiscriminately (1). However, that the protein is involved in intracellular PLP binding is not a precise statement and its exact function is still unknown.

1.7 The zebrafish

In recent years the zebrafish (*Danio rerio*) has emerged as a popular animal model for studying human disease in general and epilepsy in particular.

They spawn quickly, their embryonic development is fast and they are easy to maintain, making it possible to generate and screen large numbers of larvae in a relatively short time. They reach their adult stage in about three months and are fertile for at least two years. An additional advantage is that the larvae are transparent in their first ten days of life making it possible to view the development of organs to observe abnormalities or use stains to show protein expression without the need to dissect the fish. Furthermore, equipment designed for analyzing zebrafish behavior and methods specifically for evaluating epileptic behavior based on movement patterns already exist.

1.8 Epileptic behavior in zebrafish

It has been shown that certain behavioral patterns in zebrafish strongly correspond to epileptic activity in their brain (33). Normal larvae have an infrequent and darting swimming pattern whereas epileptic fish behave in a more predictable (constant/uniform) manner.
Three distinct behavioral patterns exist, graded as stage 1-3 depending on severity, i.e. degree of epileptic activity in the brain as measured by an EEG equivalent. Bursts of very fast swimming and or hyperactivity is classified as stage 1, rapid "whirlpool like" swimming as stage 2 and whole-body convulsions followed by loss of posture as stage 3 (34). Movement tracking equipment can be used to detect stage 1 and 2, by analyzing swimming speed and path. Stage 1 is detected as dramatic increase in swimming, particularly at speeds exceeding 20mm/s since healthy fish do not swim this fast for longer periods unless provoked (36). Stage 3 presents itself as a sharp decrease in locomotor activity on movement plots, and whether this is due to a seizure or simply regular inactivity is impossible to determine based on movement data alone. Only stage 1 activity will be considered in this paper since it is the first stage that appears and the easiest to quantify.

Methods

2.1 Fish maintenance and breeding

Adult fish and larvae older that six days were kept in room with a temperature of 28°C and a natural light cycle, that is, the room was lit between 8 a.m. and 10p.m. Young fish were kept in a rotifier bath during their first days of life and later given artemia in addition to the dry food juvenile and adult fish were fed. Fish were breed by placing one or two males in smaller tanks together with two females.

2.2 Maintenance of larvae

During their first six days of life, larvae were kept in embryonic media (consisting of etc.) in petri dishes and put in an incubator maintaining a constant temperature of 28°C. Larvae were continuously observed from the day they were fertilized to the termination of the experiment. Fifty or less larvae were kept in each dish, dead larvae were counted and removed each day when the embryonic media was changed.

2.3 The mutations

Two mutant lines were created to confirm that the observed phenotype is due to the mutation in *prosc* and not to other mutations that might accidentally be introduced in the genome while the mutants are created. The two mutations, labeled $prosc^{27\_1}$ and $prosc^{27\_3}$, were selected as they give rise to a premature stop codon by means of a deletion of one base pair (27_1) and four bases (27_3) that will prevent protein translation.

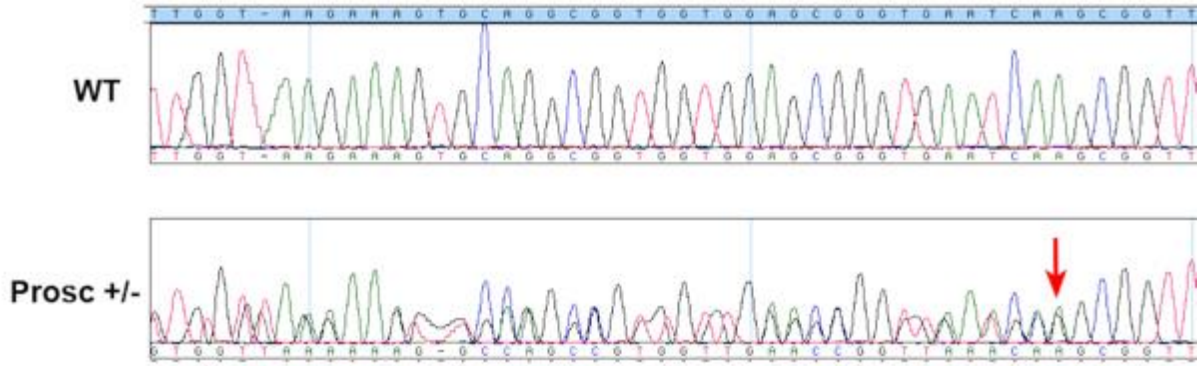

Fig 1 DNA sequence from wildtype and heterozygous fish obtained by PCR and Sanger sequencing. The introduced mutation causes a frameshift (red arrow), seen in the heterozygous fish (Prosc +/-) as double peaks in the sequence.

2.4 Survival analysis and B6 treatment

For the survival analysis, as many eggs as possible were obtained from both mutant lines. Each day, the number of dead larvae were counted and removed. At day six, they were transferred to tanks and were fed rotifers. Since heterozygous fish were bred to create mutants this will result in 25 % of the fish being mutants, 25 % being wildtype and 50% heterozygous if survival rates are not affected by the mutation. Since the larvae are very sensitive during their first days of life, even slight differences in conditions between tanks will have a substantial impact on survival. Therefore, the best control group against which to compare the survival of the mutants were their wildtype siblings kept in the same tank. However, to confirm that nothing went wrong during their upbringing and allow comparison of mortality (wildtype fish generally have very low mortality rates after six days) before genotyping, a tank with only wildtypes was used as a control as well.

For survival and behavior experiments, half of all available mutants were exposed to pyridoxine. Larvae six days or older, was exposed to 10mM of pyridoxine for 30 minutes each day, since this treatment regime has been shown to successfully alleviate the epileptic phenotype seen in zebrafish with a mutated antiquitin gene (35). Larvae older than six days

are kept in tanks and had to be manually transferred (by pouring all larvae into a net) to a small bowl for each treatment session.

2.5 mRNA extraction

The fish bodies were put in 1.5ml Eppendorf tubes, between three and ten fish in each tube depending on supply, and suspended in 200µl Tri Reagent. To dissolve the bodies properly, the sample (fluid and fish) were repeatedly "drawn up and ejected" using a syringe.
Once properly dissolved, an additional 200µl of Tri Reagent was added to the tubes.
In order to get rid of the excess fat and protein, the samples were centrifuged at 12000xg for 10 minutes in 4C. This yielded at thin surface layer containing fat and a pellet of protein at the bottom of the tubes. The liquid in between containing RNA and DNA was removed and placed in new tubes. 40 ml of 1-bromo-3-chloropropane was then added to each tube, the tubes vortexed and allowed to stand in room-temperature for 10 minutes.
The resulting mixture was then centrifuged at 12000 x g for 15 minutes at 4 C.
At this stage the tube contains three different layers, a red layer at the bottom containing protein, a thin middle layer containing DNA and a clear upper layer containing RNA.
The upper layer was transferred to a new tube, 200µl of 2-propanol was added and the tubes put in room temperature for 10 minutes. Once again, the tube is centrifuged for 10 minutes at 12000 x g at 4 C which creates at pellet of RNA at the bottom of the tube.
Now, the supernatant was removed and 200µl of 75% ethanol added.
This was followed by vortexing and another round of centrifugation at 12000 x g for 5 minutes. As much ethanol as possible was removed by pipetting and what still remains in the tubes evaporated by placing them in 37 C with the lid open for 10 minutes. If everything is done correctly, a small RNA pellet is left at the bottom of the tube. About 25µl RNase free water was added (depending on the pellet size) and the sample heated to 55C for 10 minutes.

RNA concentration and purity was measured after this step using a Nanodrop 2000 (spectrophotometer) before treating the sample with DNase to remove any DNA left. For the DNase treatment 8µl of RNA and water was transferred to a new tube, the amount of RNA was not allowed to exceed 1 µg hence the varying amounts of sample added (poorly written, change). To this new tube, 1µl of DNase and 1µl of DNase buffer were added and the sample incubated at 37 C for 30 minutes. After incubation 1ul of stop solution was added and the sample heated to 65C for 10 minutes to inactivate the DNase. The samples were put on ice to prevent degradation of RNA before cDNA synthesis started.

2.6 cDNA synthesis

To be able to compare the relative expression of the gene of interest at different developmental stages or between mutant and wildtype fish, the total amount of RNA used for cDNA synthesis needed to be the same in all samples. For this reason, 300 ng of the extracted DNases treated RNA was transferred to a new tube and water added until the total volume reached 8µl.

For each sample 10µl of 2x reaction mix, 2µl RT (reverse transcriptase) enzyme mix was added (yielding a total volume of 20ul). In order to start the cDNA synthesis, the samples were put in a thermal cycler (same type as used for regular PCR) which kept them at 25 °C for 10 min, 50 °C for 30 min followed by 85 °C for 5 min. Excess RNA was removed by adding 1ul of (2U) Ecoli RNAse H and incubating for 20 min. The samples were then diluted with 579 µl water, creating a stock of cDNA (600 µl per sample) used for running the PCR.

2.7 Optimization of primers

Primers amplifying exon 1 were used to genotype the fish. For looking at protein expression by converting RNA to cDNA, different primers were used. The primers in question amplified 1: exon 1-6 536 bp 2: exon 1-5 438 bp, 3: exon 1-8 (full length) 1159 bp, 4: exon 1-8 (full length) 1152 bp. In order to determine at which temperatures the primers achieve the highest specificity for the region of interest a PCR reaction was run for each primer at eight different temperatures ranging from 58 C to 68 C during (step 3 of the reaction) amplification.
The samples were placed on an 2 % agarose ethyl bromide gel and subjected to electrophoresis and then placed under an UV light source for visualization of the DNA. When DNA is amplified successfully, it appears as luminous bands on the gel. An optimal temperature is one in which a single band of the expected length appears, indicating that a single region of the genome has been amplified and not others with sequences almost matching the ones of the primer.

2.8 Excluding alternative splicing and confirming knockout of PROSC

cDNA corresponding to all proteins expressed in zebrafish during every developmental stage is readily available as Kalibrator cDNA. This cDNA was amplified using three of the primers mentioned previously and then subjected to electrophoresis on an agarose gel. All clearly visible bands were cut out, lysed and sequenced to determine which bands amplified sections of the PROSC protein. If alternative splice sites exist, sequencing of the bands would reveal that the exons are not put together in the same way as in the full -length protein. Expression of PROSC in mutants were compared to wildtype fish by amplifying cDNA synthesized from mutant and wildtype fish obtained at 3 dpf, with PCR. The DNA samples were then run on an ethidium bromide gel for visualization. Absence of PROSC expression would result in the

absence of a band. To confirm that the cDNA synthesis was successful, primers amplifying the gene *aldh7a1* was used on the PROSC mutants.

2.9 PCR and Sanger Sequencing

Due to the lethal phenotype of homozygous mutants and the lack of visible and early phenotypes all fish used were obtained from heterozygous inbreeds and genotyped using Sanger sequencing.

PCR

The fishes were anesthetized and a part of their tail fin was cut and placed in a PCR tube. To dissolve the fin, 75 µl alkaline lysis reagent was added to each tube and the tubes were heated to 95 C for 30 min and then cooled to 4 C. Afterwards, 75 µl neutralization buffer was added to the tubes. A solution containing 7.3ul MQ 1 1µl 10xadv2PCR buffer 0.2 µl dNTP 0.2 µl revers and forward primer 0.1 µl 50x Adv 2 taq polymerase mix per DNA sample was prepared. This mixture was then added to the reaction plates along with 1ul of the sample DNA and the PCR reaction was started. The protocol used was 95°C 2 minutes, followed by 39 repetitions of the following series: 95 °C for 20 seconds, 63 °C for 40 seconds and 72 °C for 30 seconds. The samples were then cooled down to 4°C for storage.

The PCR product was cleaned by adding l µl ExoI and 0.25 µl Fasp to each sample and then incubating them at 37 °C for 15 min and 80 °C for 15 min. After cleaning, DNA concentrations and purity were measured using a spectrophotometer.
A new solution (mastermix) containing the primers, BigDye Term v1.1 cycle (2µl/sample), BigDye Term 5xseq buffer (1µl/sample) the revers or forward primer (0.5µl/sample) and MQ 0-7.5 µl/sample, depending on DNA concentration was prepared. Between 1 and 7.5 µl of the

cleaned PCR product was added (depending on DNA concentration) and together with the master-mix yielded a total volume of 10 µl to be added to a 96 well plate. Now the second round of PCR used to prepare the samples for Sanger sequencing was performed. The protocol used was the following: 95°C for 1 minute followed by 25 repetitions of: 96° for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes.

Cleaning of samples

The DNA sample amplified by the PCR was cleaned prior to sequencing using a standard protocol. The samples were placed in a 96 well plate (if this had not been done previously), 2ul 125 mMol EDTA, 2ul 3Mol NaAc (pH 5.2) and 50ul 95% ethanol added to each sample. The plate was then incubated in room temperature for 15 minutes followed by centrifugation in 4C at 1650*g (what does that stand for exactly, the centrifugal force = 1650 times the gravitational?) for 45 minutes. To remove the excess liquid the plate was centrifuged again upside down for 1 minute (190*g). To the dried samples 60 µl 70% ethanol was added, the plate centrifuged again (15 min 190 190*g) followed by removal of the excess liquid as described previously. In the last step of the process, 15 ul of Foramid (HiDi) was added to each sample and allowed to incubate in room temperature for 20 min. After this step, the samples were cleaned and ready for Sanger sequencing.

2.10 Morphological analysis of mutants and mutant mortality

Fish were breed in the same way as for the behavioral analysis.

Differences in the larvae morphology was assessed without prior knowledge of the genotype and later confirmed with PCR and Sanger sequencing. Pictures of fish at different developmental stages were taken.

2.11 Behavioral analysis

Fish heterozygous for the two PROSC mutations were breed producing offspring heterozygous or homozygous for the mutation as well as wildtype larvae. Fish were not genotyped until after the experiment had been performed.

Chosen time-points for the recordings were five and nine days post fertilization.

Before the larvae are five days old, they generally move very little and was therefore only observed in their petri-dishes for signs of obviously abnormal behavior.

For recording of behavior, a total of four plates were used, each containing 48 fish separated into 48 individual wells filled with 1 ml of embryonic medium.

To avoid that we unconsciously selected fish with a certain trait, each plate was filled with fish from a single petri dish. The plate was then placed in the ZebraBox, an equipment especially designed for monitoring zebrafish. The recording was performed under light conditions between 10 am to 5pm and lasted one hour. Room temperature was 28 C and the fish were allowed to acclimatize for at least one hour before we started recording their behavior. The fish were allowed to acclimatize to the new environment inside the ZebraBox for 20 min before the filming started and great care was taken to ensure that all fish were tracked correctly. Background color was set to transparent and detection threshold to 20. Movements faster than 20 mm/s were classified as fast, between 6 and 20 as medium speed, and below 6 as slow. Total distance at each speed, total duration of activity and number of times any activity was detected was obtained by letting the software analyze the film.

Since epileptic fish display a behavior pattern that is dramatically different from wildtype fish, a more than 50% increase in total activity of mutant fish when compared to wildtype would support the hypothesis that they are epileptic (type 1 activity). Type 1 activity (hyperactivity or increased speed) is used as screening and to confirm the presence of epilepsy

type 2 (rapid “whirlpool-like” swimming) and type 3 (loss of posture, twitching) is used.

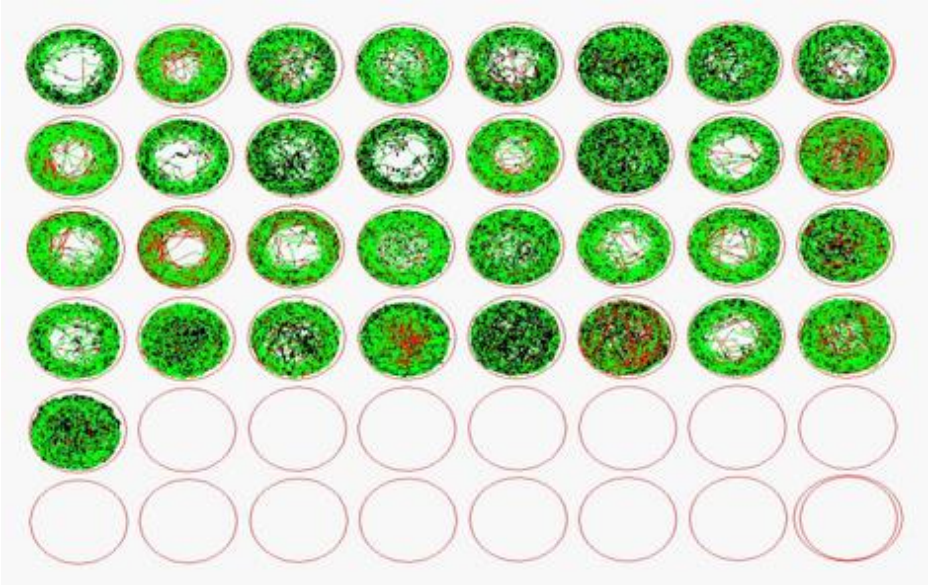

Fig 2 Total movement plots obtained after recording 33 fish for one hour. Each circle represents a well and the movement of fish inside it is seen as red lines for high speed movements, green for medium speed, and black for low speed. Similar plots exist for each ten second period, making it possible to track the path each fish has taken.

2.12 Statistical methods

No power analysis was performed for the survival study. Since the fish are fragile and mortality rates increase rapidly if conditions deteriorate, fish were kept at low densities and as many fish as possible were used. This meant that about 800 larvae were kept in Petri dishes the first six days and slightly less than 200 were kept in tanks for long term survival, which was the maximum number the that could be kept in the fish room at the time of the experiment.

Since I had no data available before recording the behavior, no power analysis could be carried out prior to the experiment. The first trails were run using 196 fish which afterwards proved to be sufficient.

For the fish analyzed at five days post fertilization (abbreviated dpf), logarithmic data for total distance traveled appeared normally distributed (fig 3 and 4) and hence a t-test could be used to determine the statistical significance of different means between groups.

The total distance traveled for each fish had a standard deviation after logaritmation of 1.1. Since 32 mutants and 33 wildtype fish were used in each group, the power of the t-test to detect a significant difference in activity if the true difference between groups was 50%, is over 95%. Hence a failure to detect a, for epilepsy, significant difference in activity is likely not due to a small sample size. Results were similar for the fish monitored at 9 dpf (fig 5 and 6), that is data still looked log-normally distributed for all genotypes.

For survival analysis, Fisher's exact test was used since some of the groups tested had very few (or no) fish after the experiment had been performed.

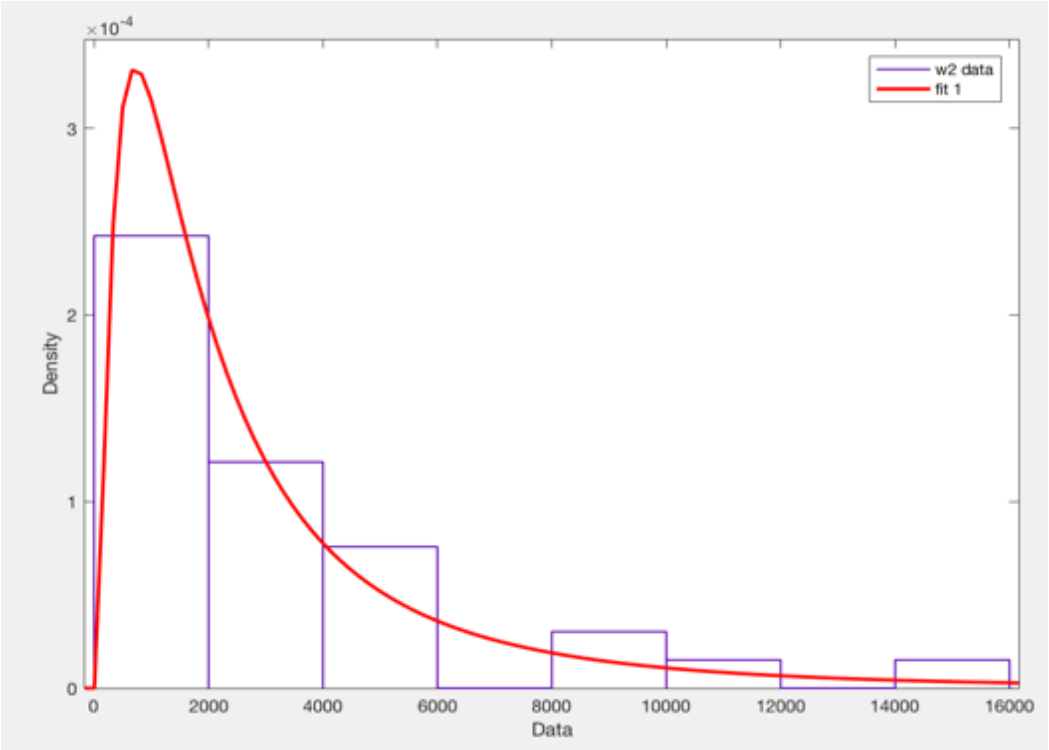

Fig 3a Total distance traveled appear to follow a log-normal distribution

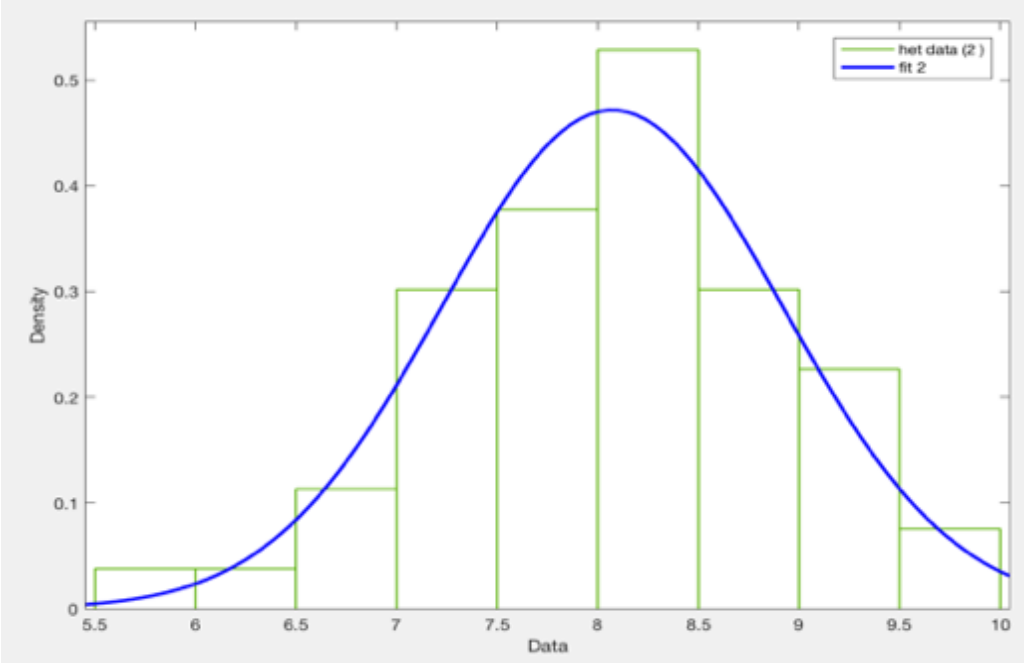

Fig 3b Probability density of the logarithm of the data for total distance traveled and a fitted normal distribution.

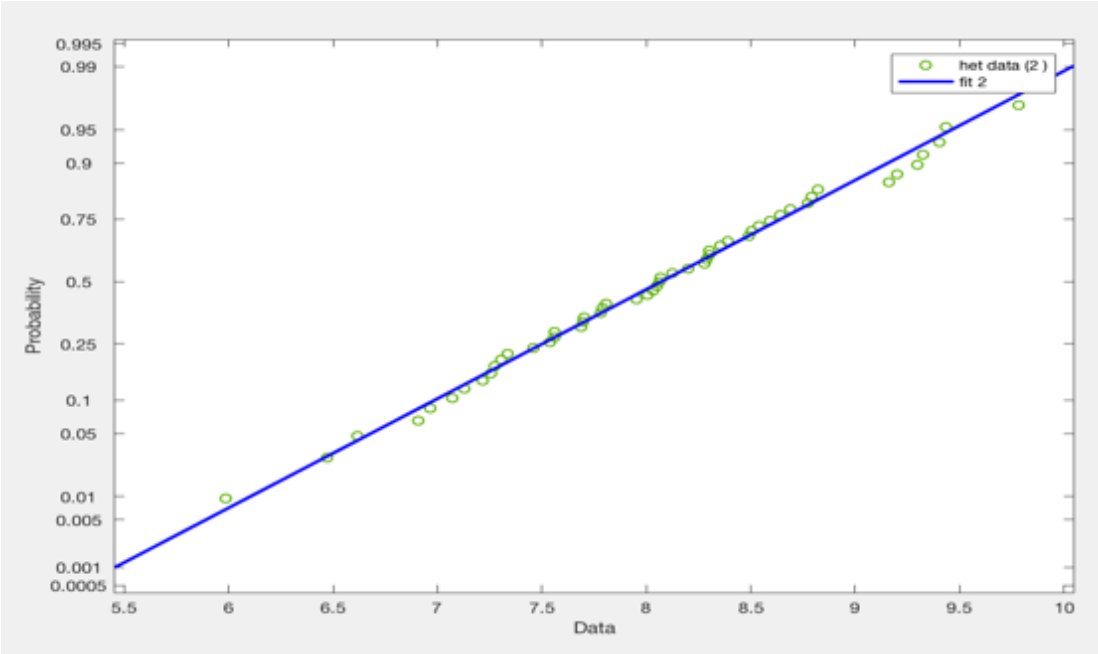

Fig 4. The probability plot shows data from heterozygous fish represented as green rings and the fitted log-normal distribution as a straight line. Data points lie close to the line indicating that the underlying distribution is log-normal.

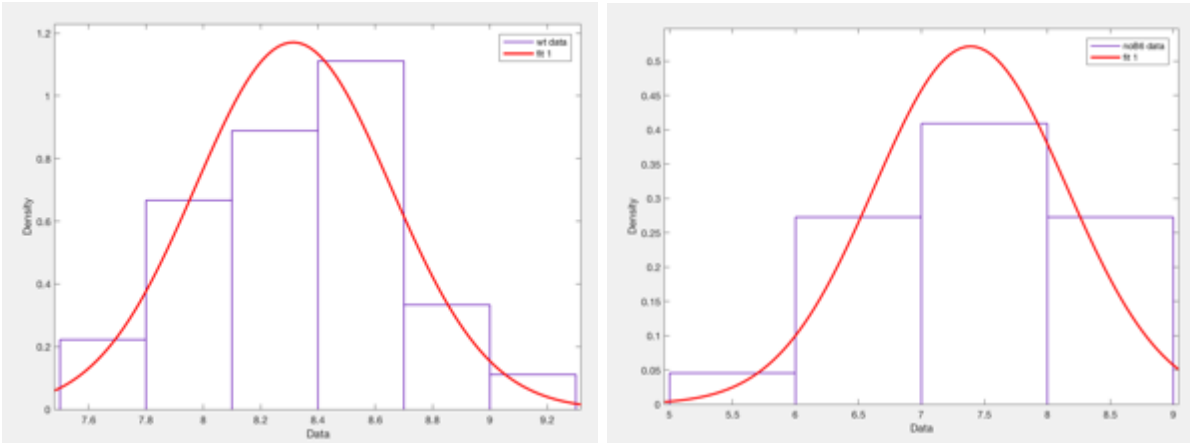

Fig 5 Probability density of the logarithm of the data for total distance traveled of wildtype (left) fish and untreated mutants (right) at 9 dpf.

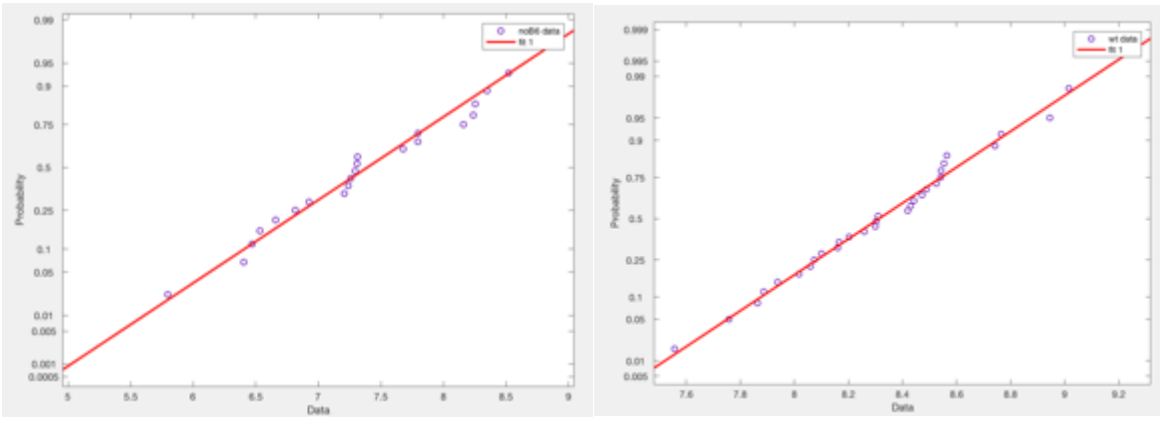

Fig 6 The probability plot shows data from mutant (left) and wildtype (right) represented as purple rings and the fitted log-normal distribution as a straight line. Data points lie close to the line indicating that the underlying distribution is log-normal.

Results

3.1 Morphology

Mutant fish cannot be distinguished from wildtype and heterozygous based on any distinctive morphological trait.

Mutant 3 dpf

Wildtype 3 dpf

3.2 Optimization of primers and excluding alternative splice sites

Of the four primers ordered for amplifying PROSC cDNA, successful amplification was achieved with three of them namely number 1 (exon 1-6), 2 (exon 1-5), and 4 (full length exon 1-8). Successful amplification was seen as one clearly visible band on an agarose gel (fig7).

Primer 1 (top) 2 (bottom) Primer 3 (top) 4 (bottom)

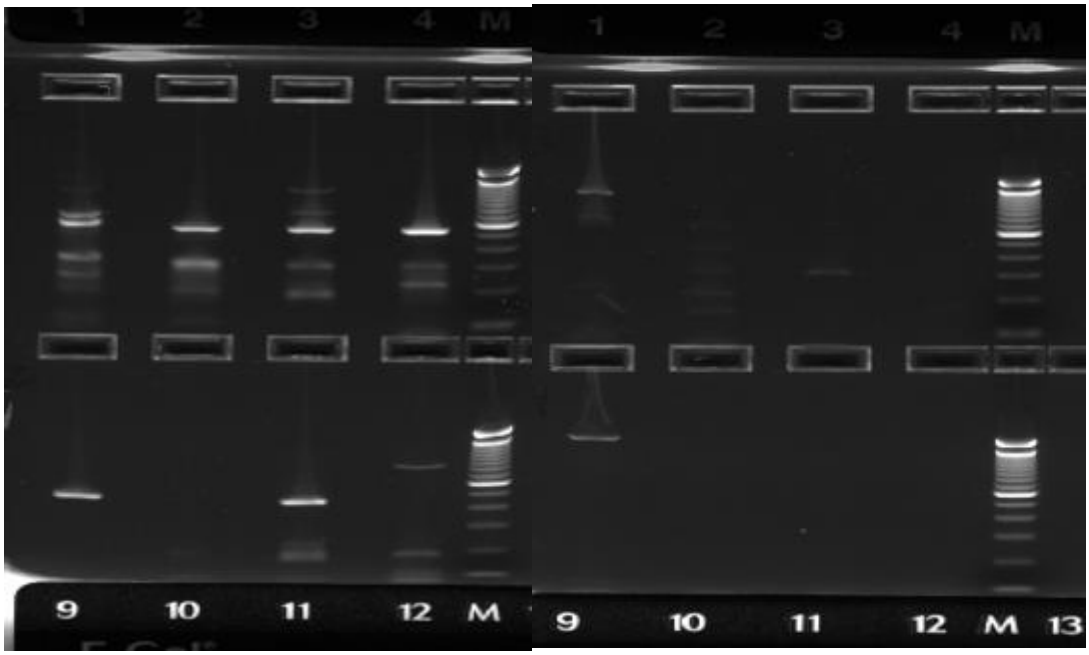

Figure 7. Gradient pcr for primer optimization. Temperatures range from 63 (left) to 68 °C (right), with successful amplification of primer 1, 2 and 4 as seen by a single, clearly visible band. Optimal temperatures for primer 1: 64-68°C, primer 2: 63 and 66°C, primer 4: 63 °C

Optimal temperatures were found to be 66°C for primer 1 and 63°C for 2 and 4. Since several bands are seen with primer one, the pcr product were run on an agarose gel and all visible bands were purified and sequenced (fig 8). To analyze for alternative splice variants we usedcDNA corresponding to all protein expressed in zebrafish and primers that amplifies different fragments of the PROSC gene. Thus, if alternative splice variants exist, they will be visible as extra bands on the gel. Although a few faintly visible bands are seen on the gel (fig 4), sequencing of these bands showed that none of them corresponded to a section of the PROSC protein while all the clearly visible bands did.

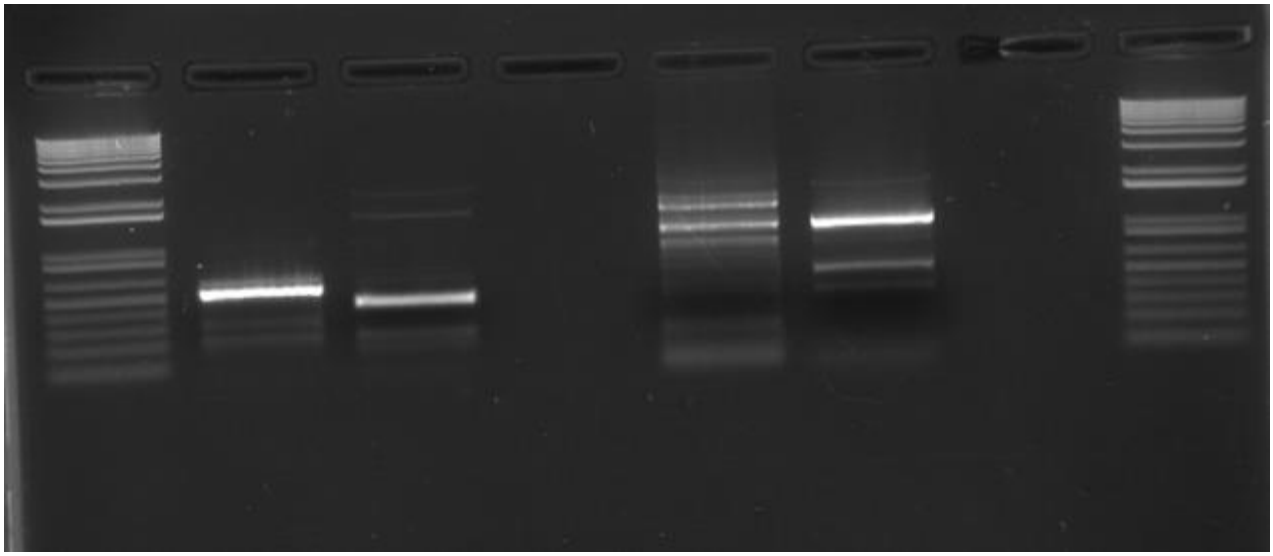

fig 8 Primer (from the left) 1, 2, 4 display clear bands, a 100 bp ladder was put on their left-hand side for visual comparison of fragment size. The largest fragments appear at the top of the image and the smallest at the bottom.

Confirming knockout of PROSC

Successful knockout of the *prosc* gene was confirmed by amplifying cDNA extracted from wildtype and mutant larvae at 3 dpf with PCR using primer no 2. Wildtype fish show PROSC expression, visualized as a band on the ethidium bromide gel, whereas no band is in the PROSC mutants (fig 9).

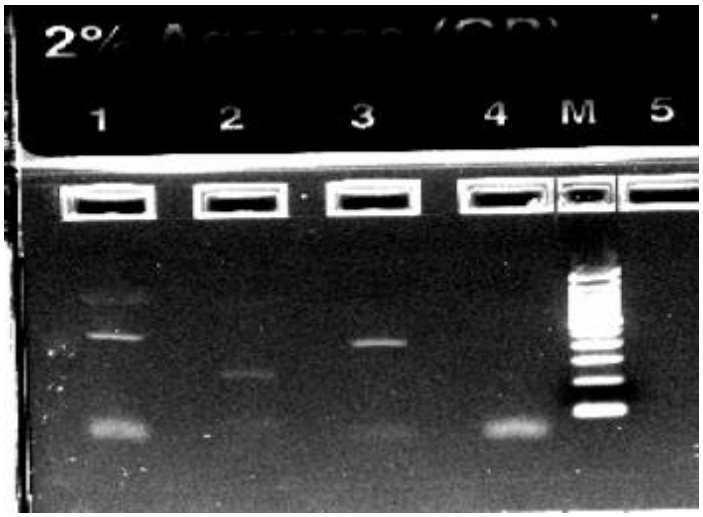

Figure 9. cDNA extracted from wildtype and mutants amplified by PCR
Wildtype in well 1,2 and 3, mutant in well 4. cDNA synthesis failed in lane 2, likely due to RNA degradation.

That the absence of the band in the mutants was not due to failed cDNA synthesis was confirmed by running the sample of mutant cDNA with primers amplifying a protein that is known to be expressed (antiquitin was used), which resulted in clear bands.

3.3 Survival of mutants and wildtype fish

9 dpf

Mortality rate of mutant fish is identical to that of wildtype for at least the first nine days. B6 treatment had no effect on survival before 9 dpf.

Fish were genotyped when 5, 9, 14, 17 days old. Results for genotyping at 5 dpf were similar to 9 dpf and will not be presented here.

	Wildtype	Heterozygous	Mutant
B6 treatment	17	55	24
No B6 treatment	32	42	22
Total	49	97	46

Table 1 Genotypes of fish 9 days post fertilization, these fish were used for behavioral analysis.

For the total number of fish, approximately 25 % are mutants, 25% wildtype and 50% heterozygous which indicates that survival of mutant fish is identical to that of wildtype at nine days post fertilization.

Survival data for fish genotyped at 14 and 17 dpf

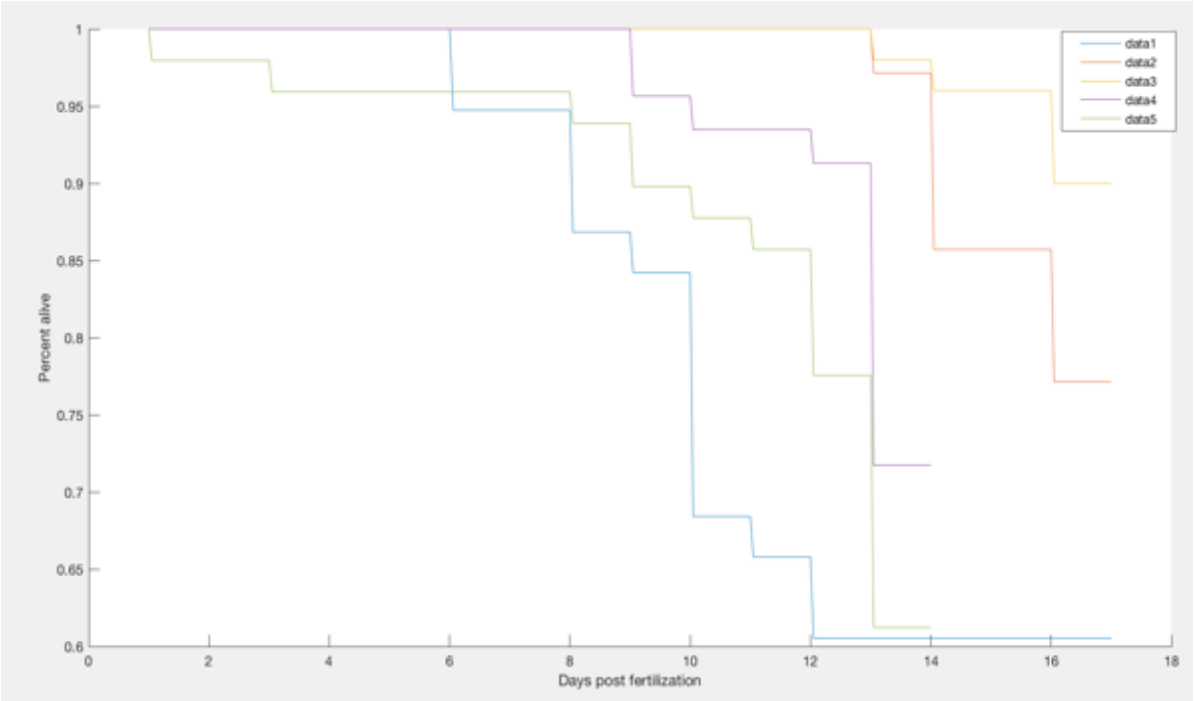

Fig 10. All groups, except 3 which contained only wildtype fish as a control, were created by breeding heterozygous fish. Every line represents the percentage of fish alive in each tank at each time-point. Data: 1 no B6 treatment, 2 treated with B6, 3 wildtype, 4 treated with B6, 5 no B6 treatment.

All tanks have a low mortality rate until about 9 dpf, when the number of fish first start to decline gradually in all tanks except wildtype (fig 10). The gradual decline is followed by one or two sharp spikes in mortality that occurred between 10 and 14 dpf. B6 treatment was discontinued at day 16 for the tanks genotyped at 17 dpf. This is unfortunate since the previously B6 treated group showed an immediate increase in mortality the next day. Groups treated with B6 show both a lower mortality rate overall and a later spike in mortality. Eight mutants were left in the B6 treated groups and no mutants were still alive in untreated groups.

	Wildtype	Heterozygous	Mutant
B6 treated	24	22	8
No B6 treatment	25	31	0

Table 2 Genotypes of fish 17 days post fertilization

The difference in mortality between treated and untreated tanks was statistically significant, p =0.025 according to Fisher's exact test. Heterozygous fish seemed to have an increased mortality as well. The expected number of heterozygous fish in each group is around 50 and the low numbers are statistically significant ($p < 0.001$ Fishers test), but no improvement is seen in the B6 treated group. The experiment was terminated at 14 dpf for two of the tanks, and at 17 dpf for the other three. The reason for this was that behavior of the 14 dpf fish was monitored and this required the fish to be transferred to individual wells. Since no mutants were alive in the B6 untreated group at this time, the data has not been analyzed.

3.4 Behavior

5 days post fertilization

To ascertain that the fish were acclimatized properly total distance traveled for all the fish were plotted at an interval of 100 s (fig 11). No dramatic decline or increase in activity is visible during each run (or no visible trend), indicating successful acclimatization.
One of the plates, 27_3 p2, had to be removed due to an error in the data file obtained after the experiment. Very few fish traveled at speeds exceeding 20 mm/s so only total distance traveled at all speeds will be presented here.

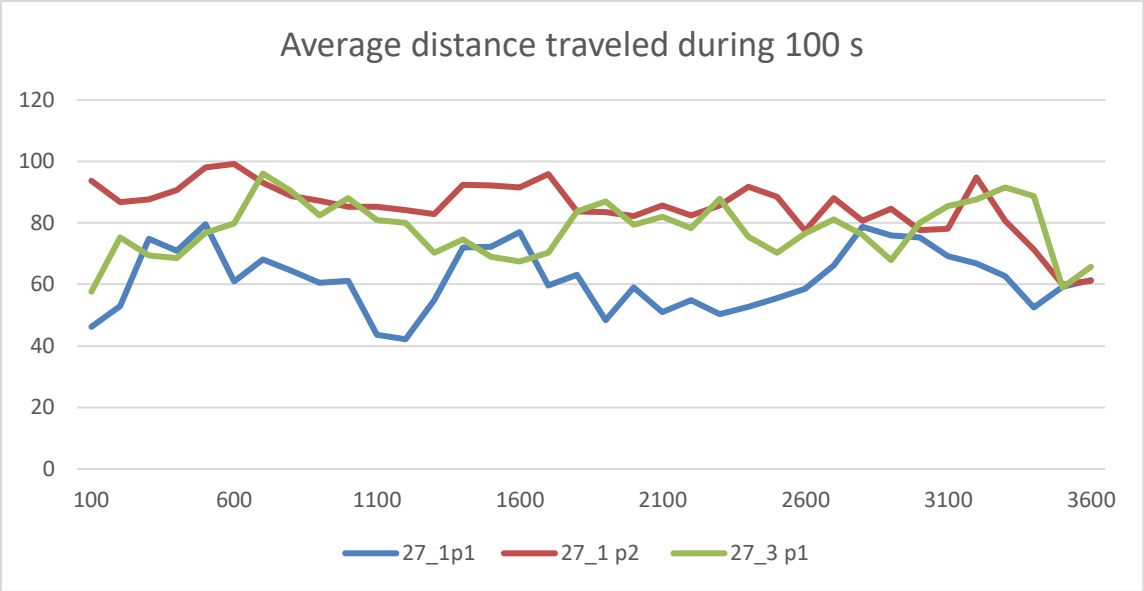

Fig 11. Average total distance traveled in mm by all fish integrated over a 100 second period. On the x-axis, time in seconds, y-axis, average distance all fish last 100 seconds in mm.

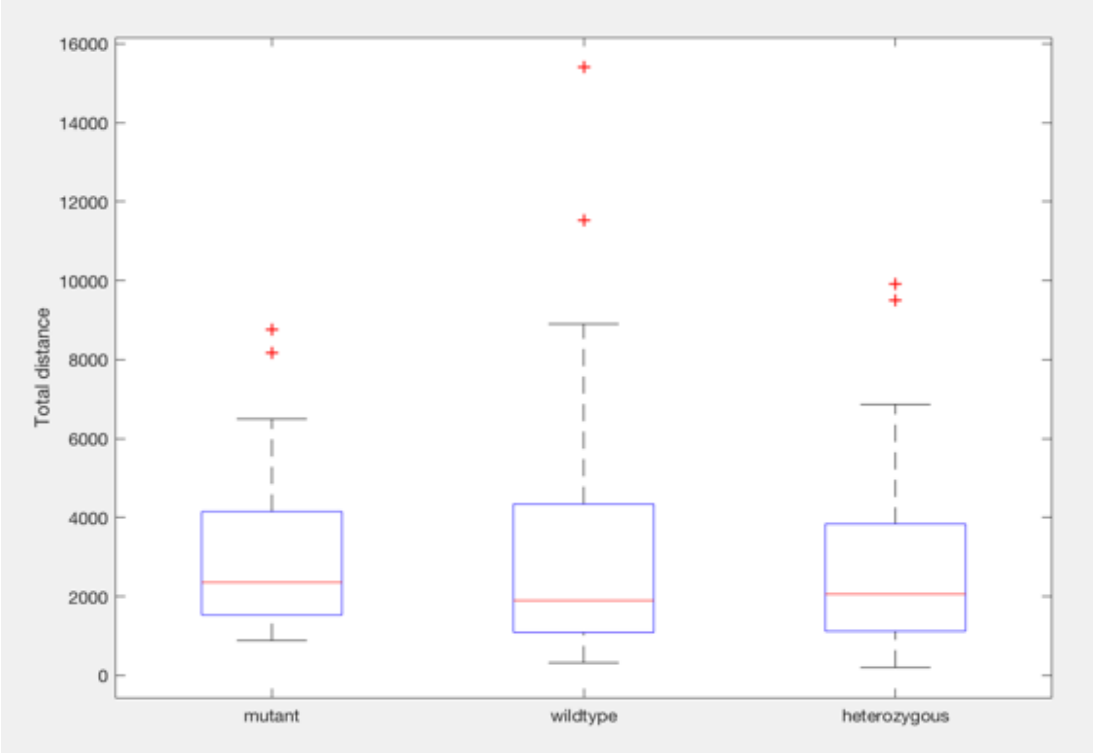

Fig 12 Summary of total distance traveled by all fish according to genotype

Red lines = median value for total distance traveled by all fish, blue lines represent the 25th and 75th percentiles, black lines = highest and lowest values, red crosses = outliers, representing very active fish.

Average distance traveled for wildtypes (n = 33) were 3190 mm and for mutants (n= 32) 3030mm. This difference is not statistically significant according to a t-test (p= 0.77).
Some fish were much more active than the others (fig 12), but this was seen in all genotypes and is therefore not caused by the mutation.

9 days post fertilization

All plates were recorded one hour except plate 4 which was filmed 30 minutes. Activity levels were generally a bit higher in the morning. A problem that be arose was that the fish in the first plate recorded (plate 2) was not sufficiently acclimatized which is evident by their very high average distance in the beginning followed by a sharp decline (fig 13).
For this reason, only the last 20 minutes of the recording was used which showed approximately the same activity levels as the plate analyzed shortly after (p1).
The average distance traveled in the other plates show only a slight decline over time indicating successful acclimatization (fig13).

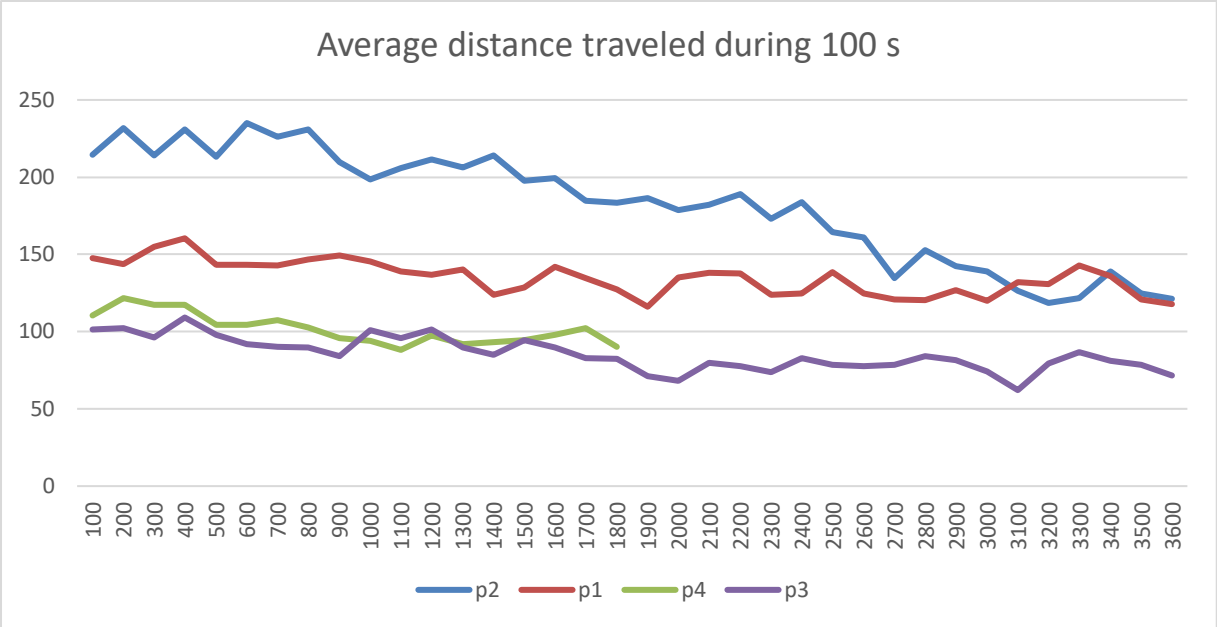

Fig 13 Average total distance traveled in mm by all fish integrated over a 100 second period. On the x-axis, time in seconds, y-axis, average distance all fish last 100 seconds in mm. plate 2 fish are twice as active in the beginning compared to the end of the recording indicating unsuccessful acclimatization.

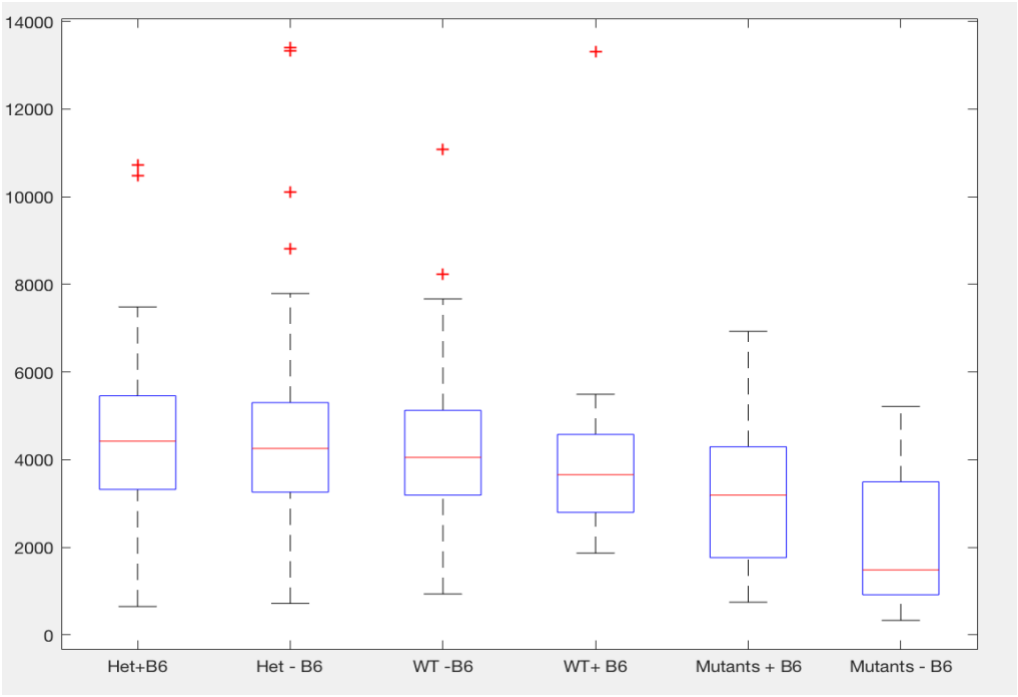

Fig14 Summary of total distance traveled by all fish according to genotype

Red lines = median values for total distance traveled by all fish, blue lines represent the 25th and 75th percentiles, black lines = highest and lowest values, red crosses = outliers, representing very active fish.

The mutant fish were in general much less active than their wildtype siblings. Average distance traveled by untreated mutant fish was 2100 mm and 4471 for wildtype, which is statistically significant according to a t-test $p < 0.001$. The difference between mutant treated and not treated with B6 was significant ($p=0.013$) as well as the difference between treated mutants and wildtype ($p < 0.001$). No mutant fish showed signs of hyperactivity (fig14). Difference detected in average distance traveled between wildtype (4471 mm) and heterozygous fish (4610mm) was not statistically significant ($p= 0.98$). B6 treatment did not affect the behavior of heterozygous and wildtype fish. For heterozygous fish, no difference in average distance was detected (4609mm and 4611mm) while a small but insignificant difference between groups was detected in wildtype (4417 and 4196 respectively, $p = 0.6$). None of the fish traveled much by high speed movements, average values reflect the results found when analyzing total movement and are not presented here.

	B6 treatment	No 6 treatment
Mutant	3206	2100
Heterozygous	4609	4611
Wildtype	4196	4417

Table 3 Average total distance traveled for fish recorded at 9 dpf.

Discussion

The introduced mutations successfully eliminated PROSC synthesis in mutant fish. Mutant and wildtype fish cannot be visually distinguished from one another. This is consistent with findings in antiquitin deficient zebrafish (35). However, the fish were neither dissected nor measured, thus it is not possible to exclude that they suffered from brain defects similar to those seen in humans. All untreated mutants die between day ten and fourteen. A similar

result was found in fish with antiquitin deficiency (35). An improvement in overall survival rate is seen in mutants treated with vitamin B6. The heterozygous fish show an increase in mortality that is hard to explain. That it should be due to B6 deficiency appears unlikely since the B6 treated group had slightly higher mortality rates among heterozygous than the untreated group did. It is evident that the B6 treatment protocol used is not optimal since the majority of mutants died. In humans, the required does of pyridoxine needed to achieve seizure control varies widely (9), so it is very likely that treatment outcomes in zebrafish could be substantially improved by a different treatment regimen.

No signs of epileptic behavior were observed. The markedly lower activity levels seen among mutant fish is an unspecific finding that only proves that they are not healthy.

The more normal behavior and increased survival rates of B6 treated mutants strongly suggest that the untreated group was vitamin B6 deficient.

That no epileptic activity has been observed might be due to several factors.

B6 deficiency causes electrolyte disturbances and brain malformations that might affect the behavior of the fish to such an extent that the hyperactivity that otherwise would arise due to a lowered seizure threshold by the B6 deficiency, disappears. The fact that mutant fish move much less than wildtype and that improvement is seen upon B6 administration supports this explanation. It is also possible that the fish were not monitored regularly enough to detect the seizures. In a study performed on zebrafish with antiquitin deficiency, seizure like activity was observed only shortly before the fish died (35). Electro-graphical measurements of brain activity can be performed on zebrafish and is a more sensitive and specific method than behavioral analysis. It is very time consuming and technically difficult to perform and was therefore not undertaken. However, in this case were the fish likely suffer from systemic disease that affected their behavior, it could have yielded additional information.

In the antiquitin model, epileptic activity could be elicited using a light stimulus before spontaneous seizures occurred (35). Another possibility is to use chemicals such as pentylentetrazol to provoke seizures and measure the concentration needed in wildtype and mutant fish. Visual characterization of stage 2 and 3 seizure-like activity would have been good to do if any of the fish had shown signs of hyperactivity but would probably not have added anything to this paper since no seizure like activity was observed.
It would also be good to have a measurement of vitamin B6 metabolites to shed light upon pathogenesis and the function of PROSC.
It would be especially interesting to measure levels of homocarnosine in the CSF since it was undetectable in the one patient with PROSC deficiency in whom it was measured (1) and could potentially be used as a biomarker.

Despite the fact that I failed to detect any signs of epilepsy in the fish, this study provides support for the role of PROSC in B6 homeostasis and has shown that it is essential for survival. In fact, one could argue that since the fish clearly display signs of B6 deficiency and that since B6 deficiency in humans is known to cause epilepsy, proving that knockout of PROSC in zebrafish causes B6 deficiency is enough to support the hypothesis that it also causes epilepsy in humans. After my work on the project had started, a second article confirming the effects of the PROSC mutation in four additional patients was published (2). Given the weight of all the evidence, it appears very likely that the mutation causes epilepsy while the mechanism behind it remains obscure.

Populärvetenskaplig sammanfattning

Vitamin B6 beroende epilepsi (förkortat PDE) är en sällsynt grupp av ärftliga sjukdomar som alla har det gemensamt att nivåerna av vitamin B6 i hjärnan är för låga.

De epileptiska anfallen startar oftast inom några dagar till veckor efter födseln och svarar inte på vanlig epilepsibehandling men blir snabbt bättre av vitamin B6. Livslång substitution med vitamin B6 krävs, annars drabbas patienterna av nya anfall strax efter att behandlingen avbrutits. Förutom epilepsin drabbas barnen av mer eller mindre allvarliga missbildningar i hjärnan med medföljande utvecklingsstörningar av varierande svårighetsgrad som kvarstår trots B6 behandling. En mutation som nyligen hittas och som tros kunna vara sjukdomsorsakande är i proteinet PROSC. Hittills har mutationen hittats i elva patienter och misstanken att den orsakar epilepsi är ganska stark, men vet vi inte exakt vad proteinet gör annat än att det verkar kunna binda till vitamin B6.

För att få svar på om mutationen verkligen orsakar epilepsi och vitamin B6 brist har vi använt oss av genmodifierade zebrafiskar som saknar ett fungerande PROSC protein.

Om dessa fiskar visade upp ett typiskt epileptiskt beteende (epileptiska fiskar simmar väldigt mycket mer och fortare än friska fiskar) som dessutom förbättrades genom att medicinera dem med vitamin B6 skulle det ge en stark indikation på att mutationen i PROSC ger upphov till epilepsi, både hos människor och i zebrafisk.

När detta är bekräftat kan man gå vidare med annan forskning på fiskarna för att lära sig både om hur PROSC fungerar och hur epilepsin bäst kan upptäckas och behandlas.

I min studie fann jag inga direkta tecken på att fiskarna hade epilepsi, däremot visade de tydliga tecken på att ha vitamin B6 brist. Samtliga fiskar som helt saknade PROSC dog nämligen i ung ålder medan flera av fiskarna utan PROSC som dessutom fick vitamin B6 tillskott överlevde tills studien avslutades. De behandlade fiskarna betedde sig dessutom mer normalt än de obehandlade (som rörde sig mindre, inte mer, än friska fiskar) vilket också

tyder på att de obehandlade har B6 brist.

Att vi inte hittade tecken på epilepsi hos fiskarna behöver inte betyda att mutationen inte orsakar epilepsi hos människor. Epilepsi i fisk orsakar inte alltid en konstant hyperaktivitet och det är mycket möjligt att man kan behöva provocera fram anfallen genom att använda t.ex. blinkande ljus eller kemikalier. Dessutom ger vitamin B6 brist upphov till en komplex blandning av symtom som skulle kunna göra fiskarna så sjuka att hyperaktiviteten som epilepsin annars skulle orsaka, döljs.

Sammantaget ger alltså det faktum att vi kunnat påvisa B6 brist hos zebrafisk med PROSC mutationen det mycket troligt att mutationen även orsakar B6 brist hos människor och därmed även epilepsi.

Ethical aspects

Permission for breeding and using zebrafish for scientific studies was obtained from Jordbruksverket.

References

1 Darin N, Reid E, Prunetti L, Samuelsson L, Husain RA, Wilson M, El Yacoubi B, Footitt E, Chong WK, Wilson LC, Prunty H, Pope S, Heales S, Lascelles K, Champion M, Wassmer E, Veggiotti P, de Crécy-Lagard V, Mills PB, Clayton PT.
Mutations in PROSC disrupt cellular pyridoxal phosphate homeostasis and cause Vitamin-B6-Dependent epilepsy.
American journal of human genetics 2016; 99:1325–1337.

2 Plecko B, Zweier M, Begemann A, Mathis D, Schmitt B, Striano P, Baethmann M, Vari MS, Beccaria F, Zara F, Crowther LM, Joset P, Sticht H, Papuc SM, Rauch A.
Confirmation of mutations in PROSC as a novel cause of vitamin B 6 -dependent epilepsy.
Journal of medical genetics. 2017; 54:809–814.

3 Tremiño L, Forcada-Nadal A, Contreras A, Rubio V.
Studies on cyanobacterial protein PipY shed light on structure, potential functions, and vitamin B6 -dependent epilepsy.
FEBS Letters 2017; 591:3431-3442

4 Baxter P, Griffiths P, Kelly T, Gardner-Medwin D.
Pyridoxine-dependent seizures: demographic, clinical, MRI and psychometric features, and effect of dose on intelligence quotient.
Developmental medicine and child neurology. 1996; 38:998–1006.

5 Been JV, Bok JA, Andriessen P, Renier WO.
Epidemiology of pyridoxine dependent seizures in The Netherlands.
Archives of disease in childhood. 2005; 90:1293–1296

6 Ebinger M, Schutze C, Konig S.
Demographics and diagnosis of pyridoxine-dependent seizures.
The journal of Pediatrics. 1999; 134:795–796

7 Stockler S, Plecko B, Gospe SM Jr, Coulter-Mackie M, Connolly M, van Karnebeek C, Mercimek-Mahmutoglu S, Hartmann H, Scharer G, Struijs E, Tein I, Jakobs C, Clayton P, Van Hove JL.
Pyridoxine dependent epilepsy and antiquitin deficiency: clinical and molecular characteristics and recommendations for diagnosis, treatment and follow-up.
Molecular genetics and metabolism. 2011; 104:48–60

8 Haenggeli CA, Girardin E, Paunier L.
Pyridoxine-dependent seizures, clinical and therapeutic aspects.
European journal of pediatrics. 1991; 150:452-455.

9 Baxter P. Pyridoxine-dependent and pyridoxine-responsive seizures.
Developmental medicine and child neurology. 2001; 43:416-20.

10 Mills P.B., Footitt E.J., Mills K.A., Tuschl K., Aylett S., Varadkar S., et al.
Genotypic and phenotypic spectrum of pyridoxine-dependent epilepsy (ALDH7A1 deficiency),
Brain 2010; 133: 2148–2159

11 Kroll J.S.
Pyridoxine for neonatal seizures: an unexpected danger.
Developmental medicine and child neurology. 1985; 27:377–379.

12 Friedman SD, Ishak GE, Poliachik S, Poliakov A, Otto RK, Shaw DWW, Willemsen MA, Bok LA, Gospe SM.
Callosal alterations in pyridoxine-dependent epilepsy
Developmental medicine and child neurology. 2014; 56:1106–1110.

13 Gospe SM, Jr.
Pyridoxine-dependent epilepsy and pyridoxine phosphate oxidase deficiency: unique clinical symptoms and non-specific EEG characteristics.
Developmental medicine and child neurology. 2010; 52:602-603.

14 Plecko B, Hikel C, Korenke GC, Schmitt B, Baumgartner M, Baumeister F, Jakobs C, Struys E, Erwa W, Stöckler-Ipsiroglu S
Pipecolic acid as a diagnostic marker of pyridoxine-dependent epilepsy.
Neuropediatrics. 2005; 36:200–205.

15 Gospe SM Jr.
Pyridoxine-dependent seizures: New genetic and biochemical clues to help with diagnosis and treatment.
Current opinion in neurology. 2006; 19:148-153.

16 Wang HS, Kuo MF.
Vitamin B6 related epilepsy during childhood.
Chang Gung Medical journal. 2007; 30:396-401.

17 Yisha Tong
Seizures caused by pyridoxine (vitamin B6) deficiency in adults: A case report and literature review.
Intractable & Rare Diseases Research. 2014; 3:52-56.

18 G. Kurlemann, R. Ziegler, M. Gruneberg, T. Bomelburg, K. Ullrich, D.G. Palm,
Disturbance of GABA metabolism in pyridoxine-dependent seizures
Neuropediatrics. 1992; 23: 257–259.

19 T. Goto, N. Matsuo, T. Takahashi
CSF glutamate/GABA concentrations in pyridoxine-dependent seizures: etiology of pyridoxine-dependent seizures and the mechanisms of pyridoxine action in seizure control.
Brain Development. 2001; 23: 24–29.

20 Groziak SM, Kirksey A.
Effects of maternal restriction in vitamin B-6 on neocortex development in rats: neuron differentiation and synaptogenesis.
The Journal of nutrition. 1990; 120:485-492.

21 Alton-Mackey MG, Walker BL.
Graded levels of pyridoxine in the rat diet during gestation and the physical and neuromotor development of offspring.
The American journal of clinical nutrition. 1973; 26:420-428.

22 Link G, Zempleni J.
Intrauterine elimination of pyridoxal 5'-phosphate in full-term and preterm infants.
The American journal of clinical nutrition. 1996; 64:184-189.

23 Albersen M, Groenendaal F, van der Ham M, de Koning TJ, Bosma M, Visser WF, et al.
Vitamin B6 vitamer concentrations in cerebrospinal fluid differ between preterm and term newborn infants.
Pediatrics. 2012; 130:191-198.

24 Lee GH, Sung SY, Chang WN, Kao TT, Du HC, Hsiao TH, et al.
Zebrafish larvae exposed to ginkgotoxin exhibit seizure-like behavior that is relieved by pyridoxal-5'-phosphate, GABA and anti-epileptic drugs.
Disease models & mechanisms. 2012; 5:785-795.

25 Bennett CL, Chen Y, Hahn S, Glass IA, Gospe SM, Jr.
Prevalence of ALDH7A1 mutations in 18 North American pyridoxine-dependent seizure (PDS) patients.
Epilepsia 2009; 50:1167-1175.

26 Mills P.B., Struys E., Jakob, C., Plecko B., Baxter P., Baumgartner M., Willemsen M.A., Omran H., Tacke U., Uhlenberg B., et al.
Mutations in antiquitin in individuals with pyridoxine-dependent seizures.
Nature Medicine. 2006; 12: 307–309.

27 Shin Y.S., Rasshofer R., Endres W.,
Pyridoxal 5′-phosphate concentration as marker for vitamin-B6-dependent seizures in the newborn.
The Lancet 1984; 324: 870–871.

28 Yuzyuk T, Thomas A, Viau K, Liu A, De Biase I, Botto LD, Pasquali M, Longo N.
Effect of dietary lysine restriction and arginine supplementation in two patients with pyridoxine-dependent epilepsy.
Molecular genetics and Metabolism. 2016; 118:167–172

29 Mills, P.B., Camuzeaux, S.S., Footitt, E.J., Mills, K.A., Gissen, P., Fisher, L., Das, K.B. Varadkar, S.M., Zuberi, S., McWilliam, R., et al.
Epilepsy due to PNPO mutations: genotype, environment and treatment affect presentation and outcome.
Brain 2014; 137:1350–1360.

30 Farrant R.D. Walker, V., Mills, G.A., Mellor, J.M., and Langley, G.J
Pyridoxal phosphate de-activation by pyrroline-5- carboxylic acid. Increased risk of vitamin B6 deficiency and seizures in hyperprolinemia type II.
The journal of biological chemistry 2001; 276:15107–15116

31 Ikegawa S, Isomura M, Koshizuka Y, Nakamura Y
Cloning and characterization of human and mouse PROSC (proline synthetase co-transcribed) genes.
Journal of human genetics. 1999;44 :337-342.

32 Ito T., Iimori J., Takayama S., Moriyama A., Yamauchi A., Hemmi H., and Yoshimura T.
Conserved pyridoxal protein that regulates Ile and Val metabolism.
J. Bacteriol. 2013:195; 5439–5449.

33 Tatiana Afrikanova1., Ann-Sophie K. Serruys1., Olivia E. M. Buenafe, Ralph Clinckers, Ilse Smolders, Peter A. M. de Witte1, Alexander D. Crawford1, Camila V. Esguerra
Validation of the Zebrafish Pentylenetetrazol Seizure Model: Locomotor versus Electrographic Responses to Antiepileptic Drugs
Plos One 2013; 8: (online publication)

34 Baraban S. C., Taylor M. R., Castro, P. A., Baier H.
Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression.
Neuroscience 2005; 131: 759−768.

35 Pena IA, Roussel Y, Daniel K, Mongeon K, Johnstone D, Weinschutz Mendes H, Bosma M, Saxena V, Lepage N, Chakraborty P, Dyment DA, van Karnebeek CDM, Verhoeven-Duif N, Bui TV, Boycott KM, Ekker M, MacKenzie A.
Pyridoxine-Dependent Epilepsy in Zebrafish Caused by Aldh7a1 Deficiency
Genetics 2017; 207:1501-1518.

36 Matthew J. Winter, William S. Redfern, Amanda J. Hayfield, Stewart F. Owen, Jean-Pierre Valentin, Thomas H. Hutchinson
Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs.
Journal of Pharmacological and Toxicological Methods. 2008; 57:176–187