Seizure Susceptibility in a Genetic Epilepsy Model in Zebrafish

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Introduction

Epilepsy and EAAT2

With over 50 million affected people worldwide, epilepsy is one of the most common neurological disorders (WHO, 2019). Individuals effected by epilepsy have surges of electrical activity in their brains. These surges can cause recurrent and unpredictable seizures, which are the main characteristic of epilepsy. During epileptic seizures the neurons of the brain show excessive and synchronous firing, which results in a prolonged overexcitation of the brain and can cause excitotoxicity. If the main neurotransmitters in the brain are disturbed the result is this excessive neuronal activity, that can be observed during epileptic seizures (Thijs et al., 2019).
The overall excitability of the brain is tightly regulated by balancing the excitatory and inhibitory signals in the brain. In this balancing process the excitatory amino acid transporters (EAATs) play a key role. Their task is to remove glutamate, which is the main excitatory neurotransmitter of the central nervous system (CNS), from the synaptic cleft after a synaptic event. The main and predominant glutamate transporter in the nervous system is EAAT2, which is mainly expressed by astrocytes and only few of these transporters are found in neurons (Bar-Peled et al., 1997). A dysfunction of EAAT2 results in an excessive accumulation of extracellular glutamate. This accumulation has been associated with the development of several neurodegenerative diseases like Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Kim et al., 2011). In humans heterozygous EAAT2 mutations also lead to a very severe form of early infantile encephalopathy, that is associated with epileptic seizures (Epi4K Consortium, 2016) (Guella et al., 2017).

Modelling Epilepsy in Zebrafish

The zebrafish (Danio rerio) is a vertebrate and therefore its nervous system has a lot of similarities in its biochemistry, receptors, channels, transporters and neurotransmitter system compared with the human nervous system. In zebrafish larva all the major components of the brain are already present at the fifth day post fertilisation (dpf) and the brain already contains roughly 100'000 neurons, that form a functional neuronal network (Naumann et al., 2010).
Zebrafish are transparent at larval stages, which makes it an ideal model for in vivo imaging studies. Their transparency can even be further improved by the usage of Propylthiouracil (PTU) which prevents the starting skin pigment formation in the larvae. By genetically altering these animals and introducing a genetically encoded calcium indicator GCaMP, the calcium currents in the brain of the living zebrafish larvae can be observed. GCaMP is a modified green fluorescent protein (GFP). By binding with Ca^{2+} GCaMP undergoes a conformational change, which results in an increased fluorescence (Nakai et al., 2001). GCaMP can be specifically expressed in neurons by setting it under the control of a neuronal specific promotor. This then allows to observe neuronal activity in the living brain (Figure 1). All these factors make the zebrafish an ideal model to study epilepsy.

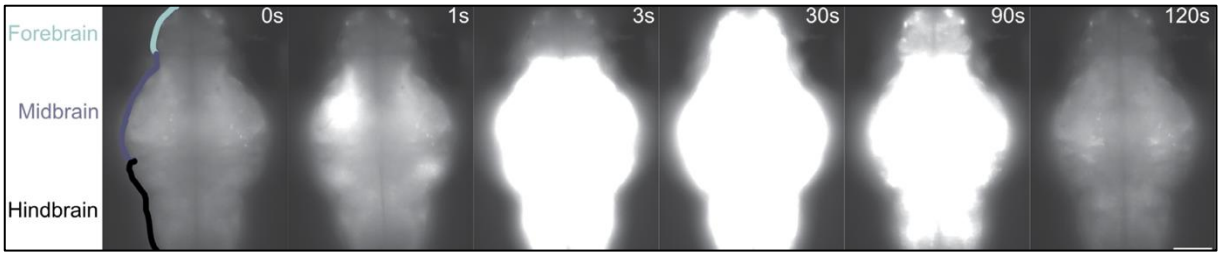

Figure 1 example of calcium imaging (Hotz et al., 2022)

There are different possibilities to replicate and model epilepsy in zebrafish. The most common way is to use pentylenetetrazole (PTZ). PTZ is a gamma-aminobutyric acid (GABA) receptor antagonist and is mostly administered to zebrafish by dissolving it in the water where the animals are currently in. Inhibiting GABA receptors disturbs the neurotransmitter balance in the brain and limits the function of the main inhibitory neurotransmitter of the CNS, which creates an overexcited state of the brain. Acute exposure to PTZ in zebrafish larvae leads to progressive seizures and the prolonged exposure to PTZ leads to epileptic-like seizure activity, which results in death of the larvae (Baraban et al., 2007).

In human patients that suffer from epilepsy these epileptic seizures often arise spontaneously without an obvious trigger event. This means that the acute epileptic seizures due to the exposure to PTZ doesn't model the human condition well enough. There is however, a zebrafish model that shows spontaneous recurrent seizures. As mentioned above humans that have a heterozygous mutation in the EAAT2 gene suffer from a severe form of early infantile encephalopathy associated with epileptic seizures. The zebrafish ortholog of the human EAAT2 gene is the eaat2a gene. Knockout of this gene in zebrafish leads to behavioural and functional impairments in homozygous larvae (Hotz et al., 2022). These homozygous larvae show all three stages of epileptic seizures, that were described by Baraban and colleges in 2007. Compared to their eaat2a$^{+/+}$ (wild type) and eaat2a$^{+/-}$ (heterozygous) siblings, the eaat2a$^{-/-}$ (homozygous knockout) zebrafishes move less, but if they move, they swim longer distances (Figure 2a, b, c) and have a reduced interictal (between seizures) neuronal activity (Figure 3)

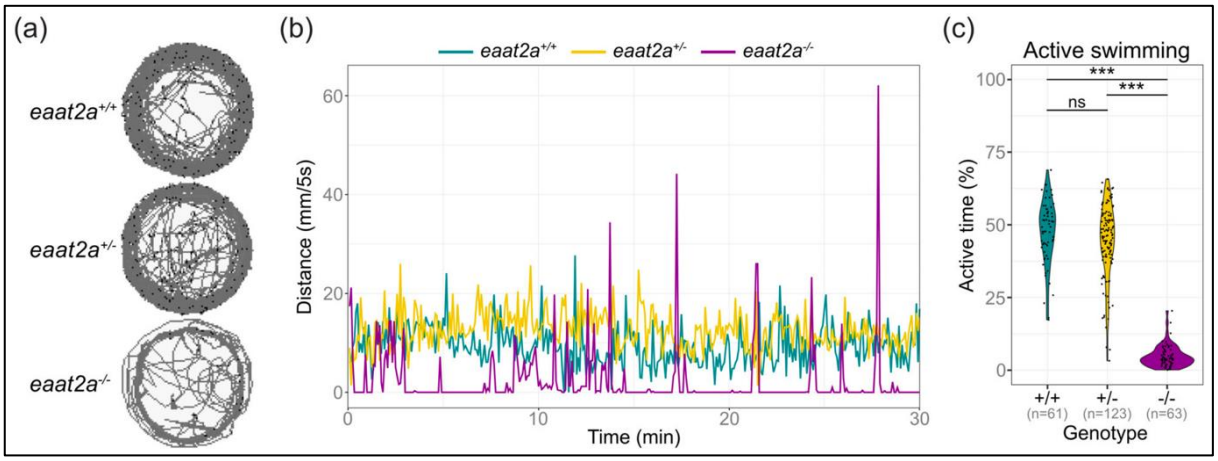

Figure 2, (a) movement of larvae in dish, (b) swimming distance of larvae, (c) time spent actively swimming (Hotz et al., 2022).

The eaat2a$^{+/-}$ mutants were visually indistinguishable to their eaat2a$^{+/+}$ siblings (Hotz et al., 2022). In a different study by Myren-Svelstad and colleagues in 2022, they investigated neuronal excitability and dynamics across multiple zebrafish seizure and epilepsy models. In this study they used photic stimulation to induce and investigate seizures. In figure 5L of this paper they showed the mean neuronal activity across time after photic stimulation. It seems like in some eaat2a$^{+/-}$ the photic stimulation caused seizures or seizure like activity when looking at this data. Although this wasn't significant nor discussed in this study, this led to our idea that eaat2a$^{+/-}$ mutants are more susceptible for seizures, since this activity is not visible in the eaat2a$^{+/+}$ larvae. That's the reason why we investigated the seizure susceptibility of eaat2a$^{+/-}$ mutants.

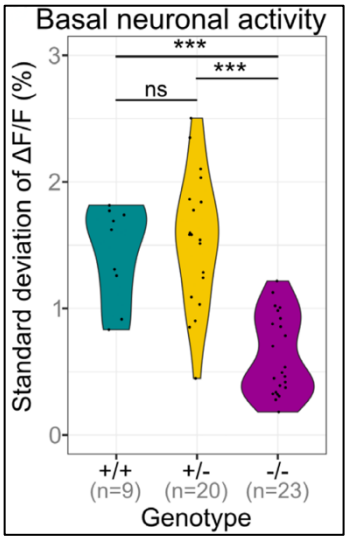

Figure 3, Basal neuronal activity compared between the different larvae (Hotz et al., 2022).

Even though the hypoactivity of eaat2a$^{-/-}$ larvae might seem counterintuitive in an animal that is prone to epilepsy, it is mimicking the slow background brain activity and the reduced muscle tone, which can be observed in human epilepsy patients as well (Epi4K Consortium, 2016) (Guella et al., 2017) (Allen et al., 2013). The mechanism behind the observation of this phenomenon in the eaat2a$^{-/-}$ zebrafish has yet to be fully understood.

A very likely reason behind this hypoactivity could be the attempt of a compensatory mechanism by the neurons to buffer against future seizures. There are multiple possibilities to how the interictal hypoactivity could develop, which might not exclude each other. The transport of glutamate into the astroglia cells via eaat2a is important to recycle glutamate and to restore the glutamate pool in the pre-synapse (Figure 4a, b). Temporarily reduced presynaptic glutamate availability after the occurrence of a seizure therefore might explain the interictal hypoactivity. Another possible mechanism could be the postsynaptic desensitization by internalization of glutamate receptors. Hypoactivity inducing factors could also play a role and would be more long lasting, by the means of an active suppression of glutamate release. This would lead to a decrease of neuronal activity as well and might be caused by a negative feedback of neuropeptide modulators (Figure 4c, d) (Hotz et al., 2022).

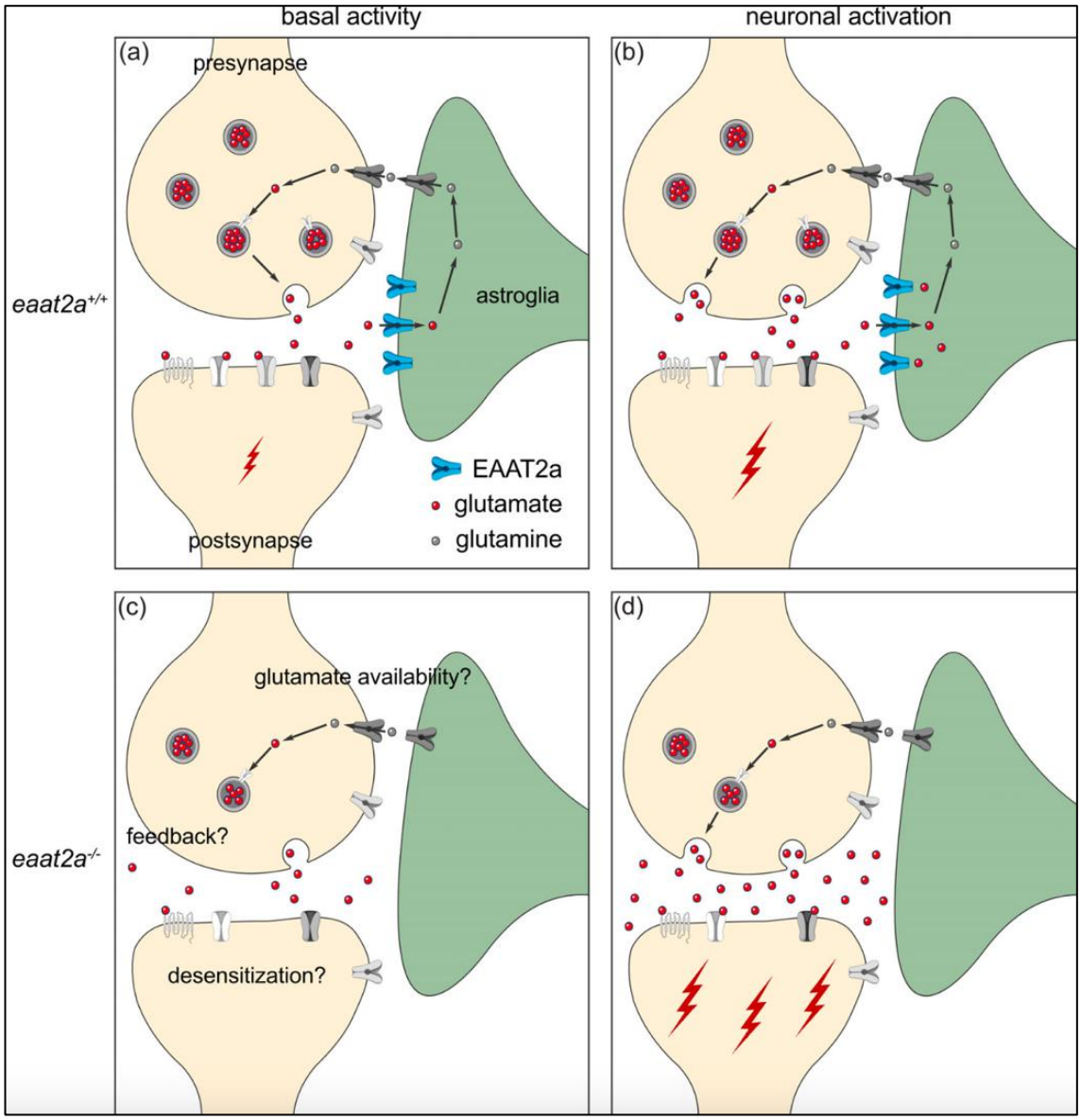

Figure 4, (a, b) clearance and recycling of glutamate, (c) loss of eaat2a leads to hypoactivity during basal periods and possible mechanisms, (d) astroglia cannot sufficiently take up released glutamate leading to hyperactive postsynaptic neurons (Hotz et al., 2022).

Neuropeptides are similar to neurotransmitters. They are chemical messengers, synthesised and released by neurons. The difference between conventional neurotransmitters and neuropeptides is, that neuropeptides are amino acid chains that have a length of 3 to 36 amino acids. Neuropeptides are stored in so called large dense-core vesicles (LDCV), whereas neurotransmitters are stored in synaptic vesicles. Under physiological conditions neuropeptides only get released rarely. This drastically changes upon strong neuronal activation, which leads to a strong increase in neuropeptide release. These neuropeptides mainly bind to G protein coupled receptors. Binding to these receptors activates second messenger pathways, which modulate neuronal activity (Lang et al., 2015).
Galanin is such a neuropeptide, which is known for its sedative effects in zebrafish (Woods et al., 2014) (Reichert et al., 2019) and was upregulated immensely (11.71 fold increase compared to wildtype) in the hypoactive eaat2a$^{-/-}$ mutants. In zebrafish models an overexpression of galanin also leads to behavioural hypoactivity (Woods et al., 2014) (Podlasz et al., 2018), which might explain the hypoactivity in the eaat2a$^{-/-}$ mutants. Galanin signalling reduces the release of glutamate, and by that it reduces the excitability of neurons (Kokaia et al., 2001) (Elliott-Hunt et al., 2004).
Galanin is widely expressed in the brain but has the highest expression in the hypothalamus which is the centre for the homeostatic regulation. In mice there are three different G protein coupled receptors that galanin binds to, GALR-1, GALR-2 and GALR-3. In mice two of these receptors have been shown to play a role in epilepsy, namely GALR-1 and GALR-2. Knockout of GALR-1 lead to spontaneous seizures and reduces the seizure threshold (Jacoby et al., 2002) (Fetissov et al., 2003), while knockout of GALR-2 increases the severity and duration of seizures (Lerner et al., 2008). There are paralogues for GALR-1 and GLAR-2 in zebrafish but there is no gene for GLAR-3 in the zebrafish genome. Also, not much is known about galanin and its role in epileptic seizures in zebrafish. Galanin might have an important neuroprotective role against epileptic seizures, because of a reduction of glutamate release caused by galanin signalling (Kokaia et al., 2001) (Elliott-Hunt et al., 2004). Additionally, it has been observed that galanin is increased after periods of high neuronal activity in zebrafish (Reichert et al., 2019). Therefore, we investigated the possible neuroprotective role of galanin against epileptic seizures in zebrafish.

Aims

1. Investigate the seizure susceptibility of eaat2a heterozygous zebrafish larvae mutants, compared with eaat2a wildtype larvae with drug-induced epileptic seizures.

2. Investigate neuroprotection by galanin with drug-induced epileptic seizures.

Results

Genotyping

The calcium imaging procedure was performed twice with embryos from two different clutches. Therefore, the genotyping procedure was also performed two times separately. The wild type sequence is 204bp long, and the mutated allele has a deletion of -13bp and is 191bp long. In figure 5 lanes 1–10 represent a single larva lysate in each case, and lane 11 is a DNA negative control. A 1kb plus ladder (NEB) is shown in lane 12. The DNA strands of the heterozygous mutants can form a duplex of wild type and mutant alleles, this is visible in the gel as a third upper band (caused by loop structure in the linear DNA). This band combination let us easily distinguish heterozygous and wild types on gel images. In figure 5 larvae 1, 2, 3, 6, 7 and 10 are wild type and larvae 4, 5, 8, 9 are heterozygous.

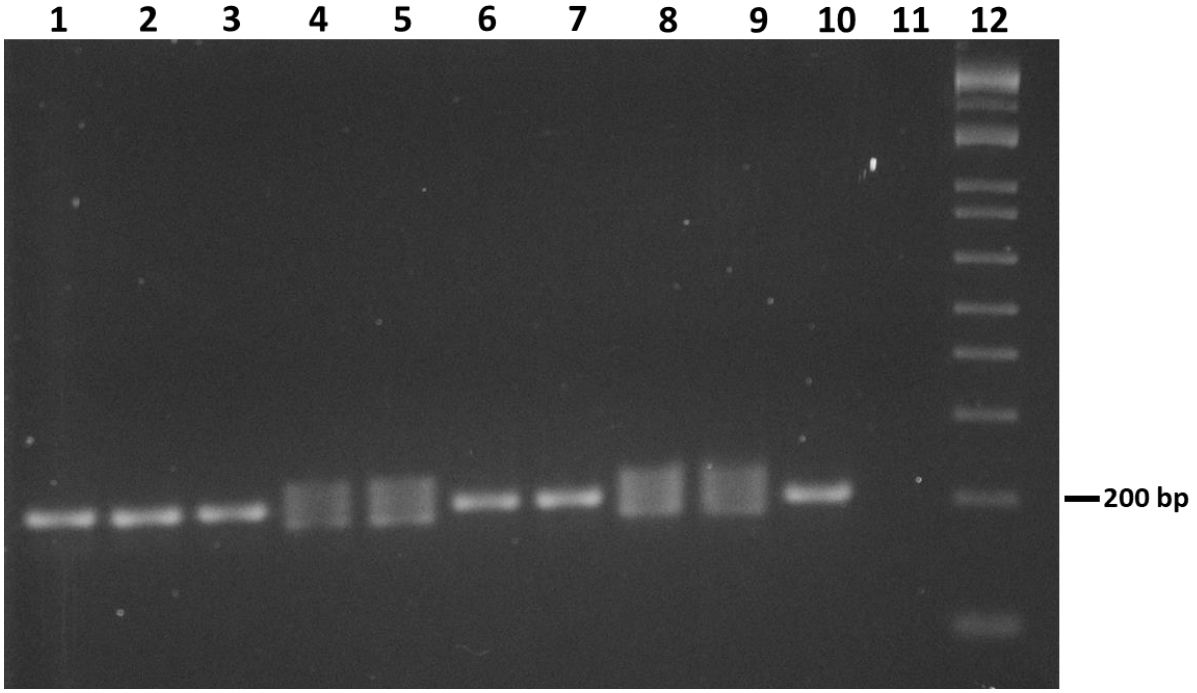

Figure 5: Genotyping for eaat2a of the larvae that were used for the first time of calcium imaging.

In figure 6 lanes 1–14 represent a single larva lysate in each case, and lanes 15,16 are DNA negative control. A 1kb plus ladder (NEB) is shown in lane 17. The DNA strands of the

heterozygous mutants can form a duplex of wild type and mutant alleles, this is visible in the gel as a third upper band (caused by loop structure in the linear DNA). This band combination lets us easily distinguish heterozygous and wild types on gel images. In figure 6 larvae 4, 6, 11 and 13 are wild type and larvae 1, 2, 3, 5, 7, 8, 9, 10, 12 and 14 are heterozygous.

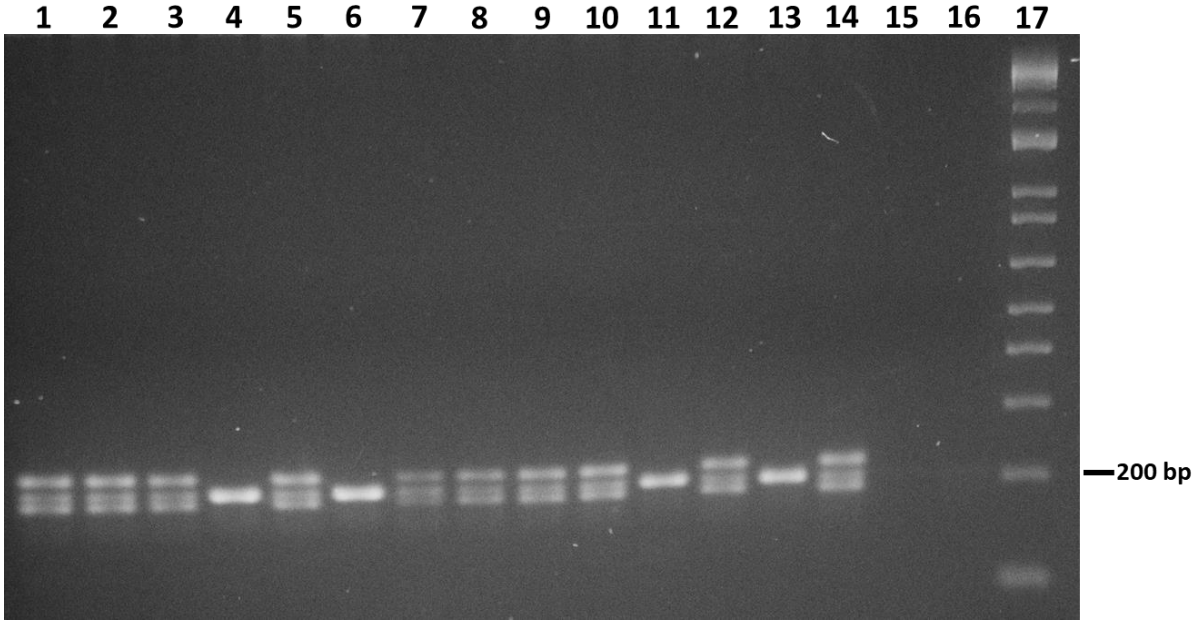

Figure 6: Genotyping for eaat2a of the larvae that were used for the second time of calcium imaging.

Whole Mount Immunofluorescence Staining

Whole-mount immunofluorescence was performed for both galanin wild type and galanin knockout larvae in order to visualize the presence or absence of neurons expressing galanin. The galanin staining is normally detectable in the area between the forebrain and midbrain and has an arced shape (see figure 7).

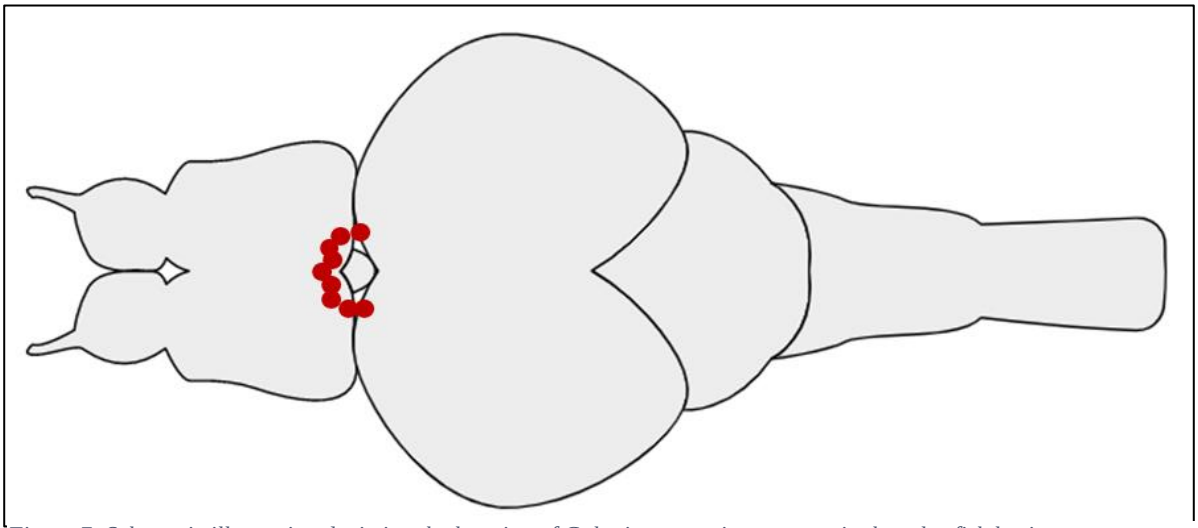

Figure 7: Schematic illustration depicting the location of Galanin expressing neurons in the zebrafish brain.

In figure 8 the GalWT and GalKO conditions are compared, one image shows the shape of the brain and the location where images were acquired. B and C images were expected to be a

comparison of the absence (GalKO) and presence (GalWT) of galanin expression, however, there is no staining for galanin (green) neither in the knockout condition nor in the wild type condition. Besides, there is no staining for acetylated tubulin (pink) present.

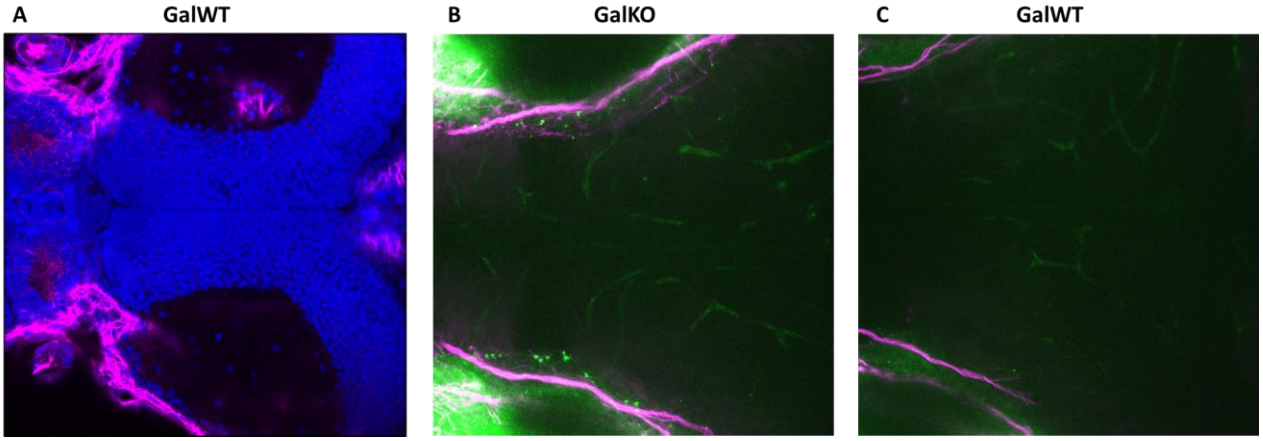

Figure 8: Whole-mount immunofluorescence images for different galanin expression conditions. ***(A)*** *Galanin wild type, blue(DAPI), pink(acetylated tubulin).* ***(B)*** *Galanin knockout, green(galanin), pink(acetylated tubulin).* ***(C)*** *Galanin wild type, green(galanin), pink(acetylated tubulin).*

The staining for these larvae didn't work because the antibodies for both galanin and acetylated tubulin didn't penetrate through the skin of the larvae, that can be observed in figure 9 below. The reasons for such unspecific staining are mentioned in the discussion.

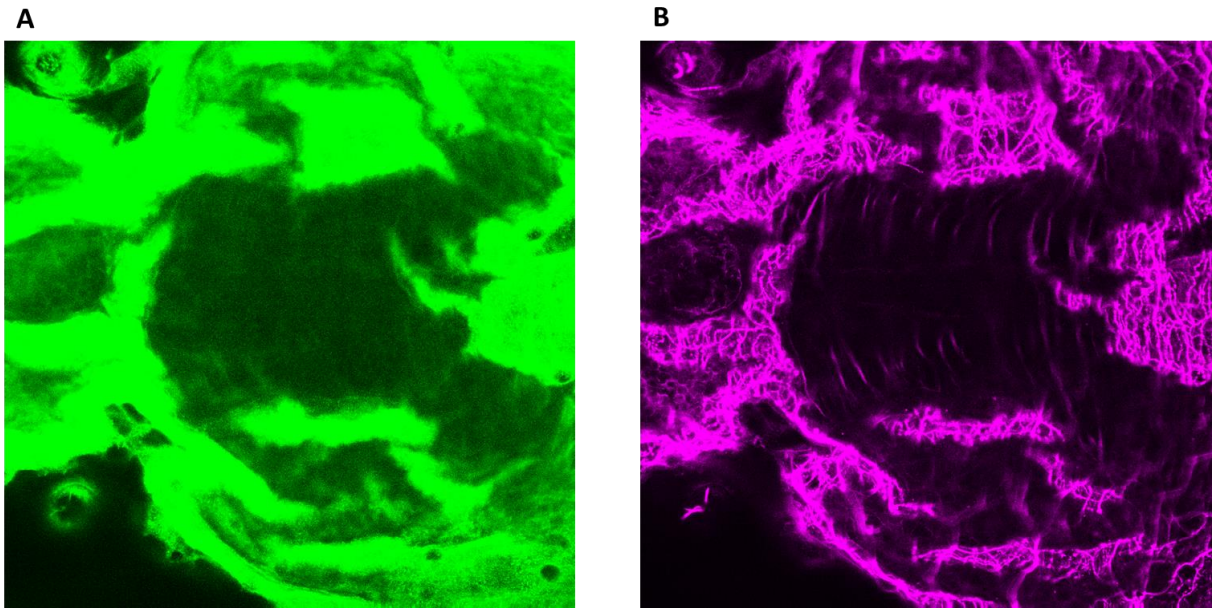

Figure 9: Unspecific staining on the skin of the GalWT larva ***(A)*** *Galanin (green).* ***(B)*** *Acetylated tubulin (pink).*

Seizure Susceptibility in eaat2a Heterozygous Mutant Larvae

Important for the following observations is that the number of larvae per condition were relatively low. Meaning 5 to 7 larvae per condition. This means that the observations are purely qualitative and it would need a higher number of larvae to get to a quantitative conclusion.
Only significant differences between the conditions were: Neuronal activity fluctuation of eaat2a HET galanin WT vs. eaat2a WT galanin KO, p-value = 0.0377, Amplitude of seizure-like events of eaat2a WT galanin KO vs. eaat2a HET vs. galanin WT, p-value = 0.0416

Neuronal Activity Fluctuation

When comparing the neuronal activity fluctuation of both eaat2a heterozygous conditions, galanin KO and galanin WT, it seems like that they have a higher neuronal activity fluctuation. This indicates that the eaat2a heterozygous mutants have more and / or stronger neuronal activity events compared to the eaat2a wild type larvae (Figure 10). These neuronal activity events can either be seizures or seizure like activities. A seizure was defined as an 100% increase of neuronal activity above the baseline. Seizure like events were defined as a 50% increase of neuronal activity above the baseline.

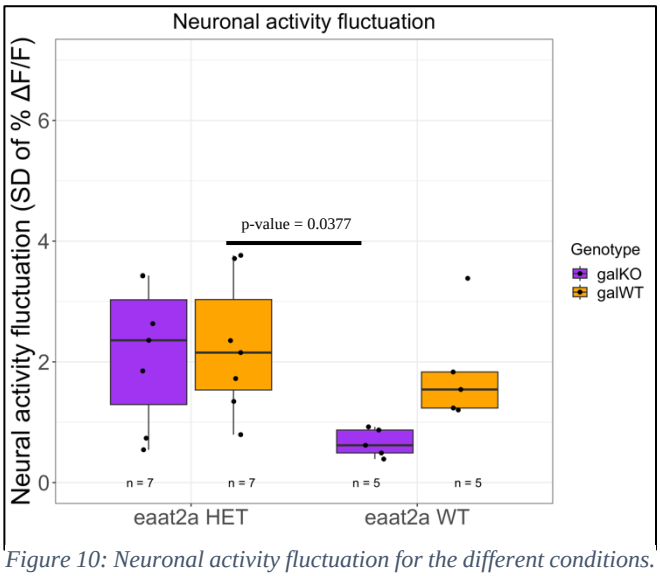

Figure 10: Neuronal activity fluctuation for the different conditions. The Neuronal activity fluctuation is standard deviation of the brain activity across the whole hour of imaging.

Number, Amplitude and Duration of Events

We only looked at the amplitude of seizures (100% threshold above baseline) and seizure like events (50% threshold above baseline). The reason for this is that we were only interested in the seizure susceptibility, therefore the stronger but not seizure like activations (10% threshold above baseline) were not further investigated.
The larvae only had a few seizures (100% threshold) and therefore no meaningful comparison of eaat2a heterozygous mutant vs. eaat2a wild type larvae can be drawn for the number, amplitude and duration of seizures.

In the galanin WT condition the eaat2a heterozygous mutant larvae had fewer seizure like events than the eaat2a wild type larvae. This was the other way around when looking at the galanin KO condition. Here the eaat2a heterozygous mutant larvae had more seizure like events than the eaat2a wild type larvae (Figure 11).

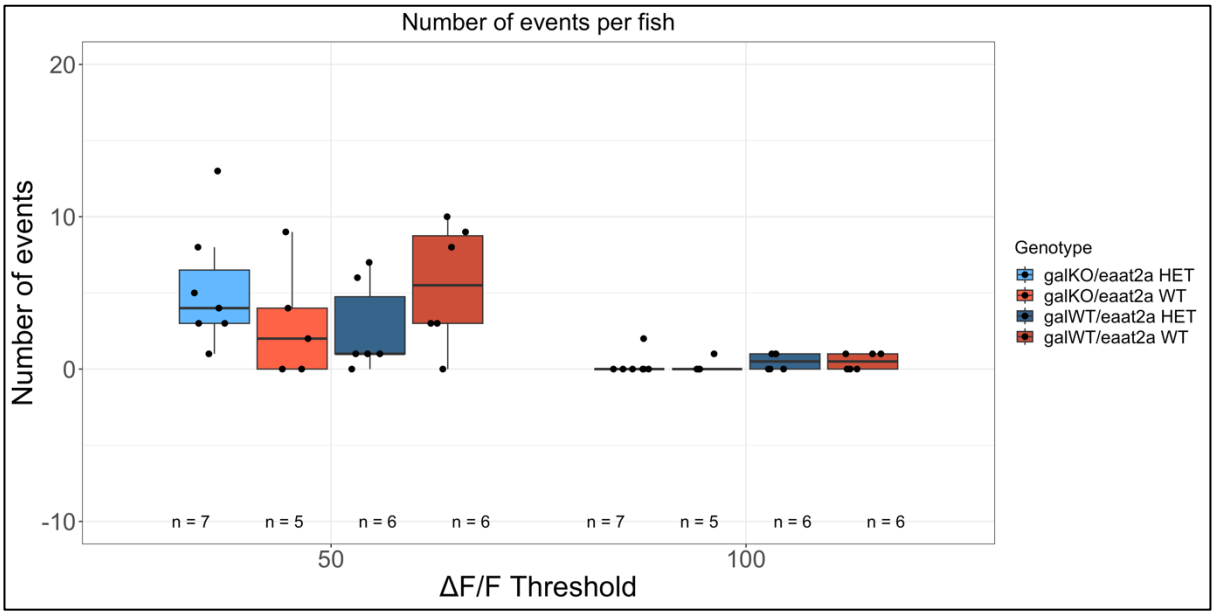

Figure 11: Number of events for both thresholds and for each condition. Seizure like events (50% Threshold) and Seizures (100% threshold).

In comparison with the eaat2a wild type larvae the eaat2a heterozygous mutants had higher amplitudes of seizure like events (50% threshold) in both galanin KO and galanin WT conditions (Figure 12).

The same was true when comparing the duration of seizure like events. In both galanin conditions the eaat2a heterozygous mutants had longer durations of seizure like events (50% threshold) compared to eaat2a wild type larvae (Figure 13).

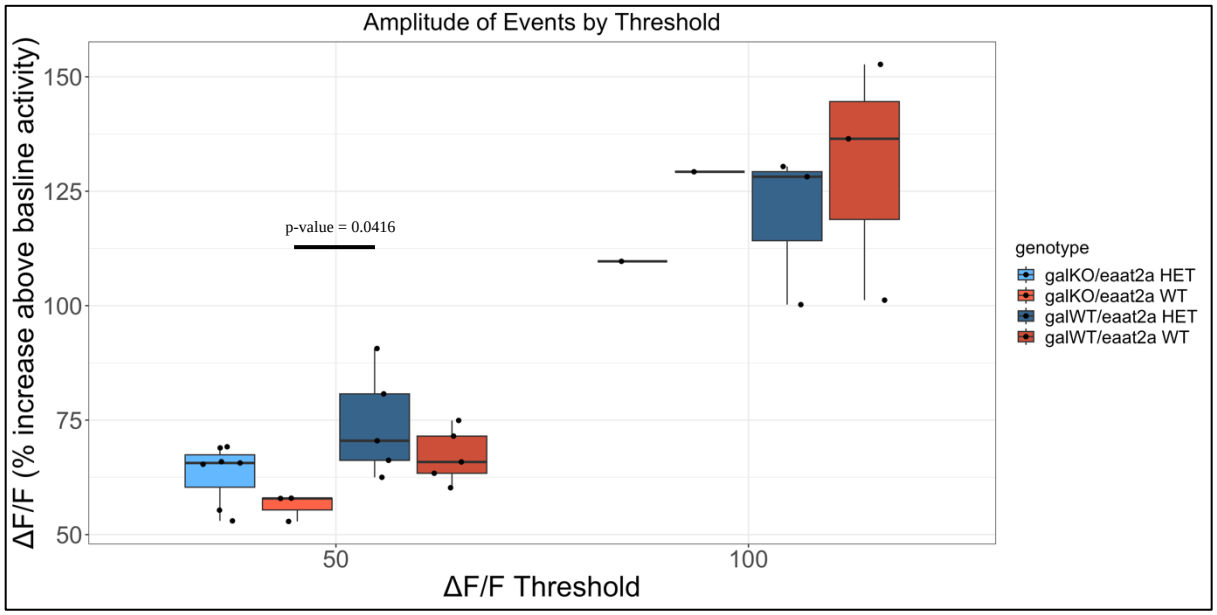

Figure 12: Mean amplitude of events for each larva for both Thresholds. Seizure like events (50% Threshold) and Seizures (100% threshold). Amplitude is the percentual increase of fluorescent signal, representing the brain activity, above the baseline neuronal activity.

Neuroprotection by Galanin

Neuronal Activity Fluctuation

In the eaat2a WT condition the galanin KO larvae have less neuronal activity fluctuation than the galanin WT larvae. Indicating that the galanin KO have less and / or weaker events compared to the galanin WT larvae (Figure 5). For the eaat2a HET condition it was the other way around. Galanin KO larvae had slightly higher neuronal activity fluctuation than the galanin WT larvae (Figure 10). This difference is however extremely small in our sample.

Number, Amplitude and Duration of Events

Again, the definitions of seizures and seizure like events are the same as mentioned above. And the same problem occurs, that there weren't enough seizures to make meaningful comparisons of the number, amplitude and duration of seizures of galanin KO vs. galanin WT larvae.

For the eaat2a WT condition the galanin KO larvae had less seizure like events compared to the galanin WT larvae. Again, this was the other way around when looking at the eaat2a HET condition where galanin KO larvae had more seizure like events compared to the galanin WT larvae (Figure 11).
For both conditions eaat2a WT and HET the galanin KO larvae had lower amplitudes of seizure like activities than the galanin WT larvae of the same eaat2a condition (Figure 12). The same was true for the duration of the seizure like events. In both eaat2a conditions the galanin KO larvae had slightly shorter seizure like events compared to the galanin WT larvae (Figure 13). This effect is however very small.

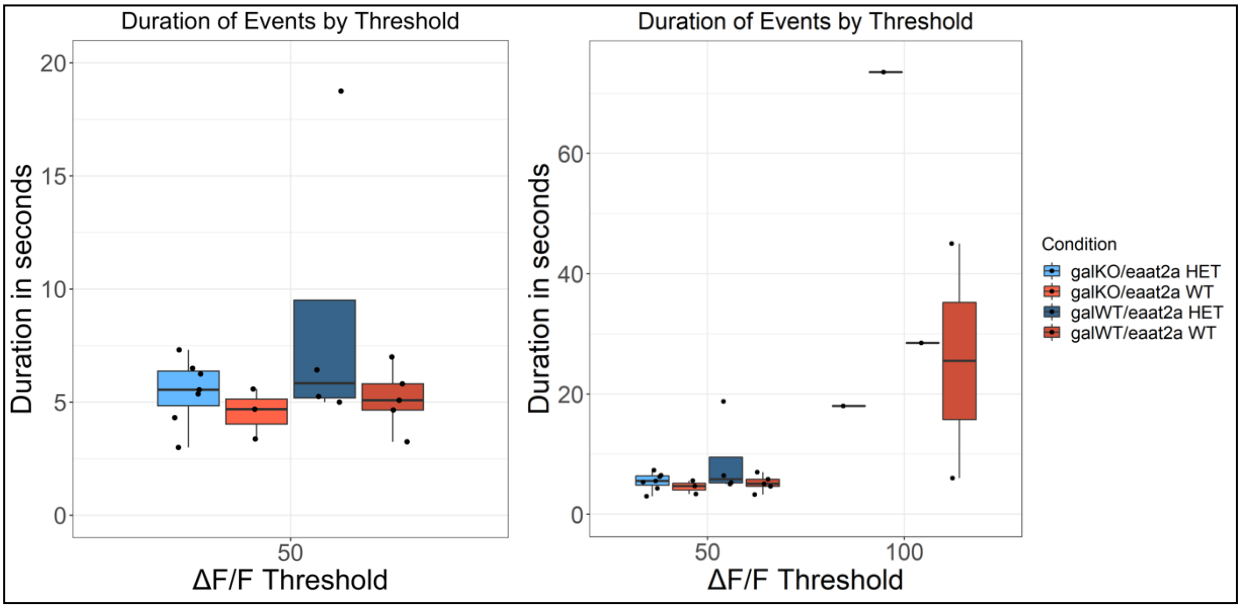

Figure 13: Mean duration of events for each larva for 50% Threshold (left). Mean duration of events for each larva for both Thresholds (right). Seizure like events (50% Threshold) and Seizures (100% threshold).

Discussion

Whole Mount Immunofluorescence Staining

As was mentioned in the Results, almost all antibodies for the different target proteins were unpacifically bound on the skin of the larvae (see Figure 9). The possible reason for this could be the fixation time of the larvae. Normally, larvae at 5 dpf are fixated in 4% paraformaldehyde (PFA) in PBS solution for 2 hours at room temperature, however, due to the timeline of our project, the larvae were fixated at 4 dpf instead of 5 dpf. That possibly made the larvae too impermeable and led to the failure of antibody penetration and to unspecific binding.
However, the same GalKO zebrafish lines were used by Milena Ronchetti to perform the same staining we did, but on the 5 dpf larvae. In figure 14 the GalWT fish image (A) shows the clear signal from neurons expressing galanin. Moreover, as expected, the galanin staining for GalKO fish (B) shows no expression of galanin in neurons. This result let us confirm the absence of galanin expression in GalKO line.

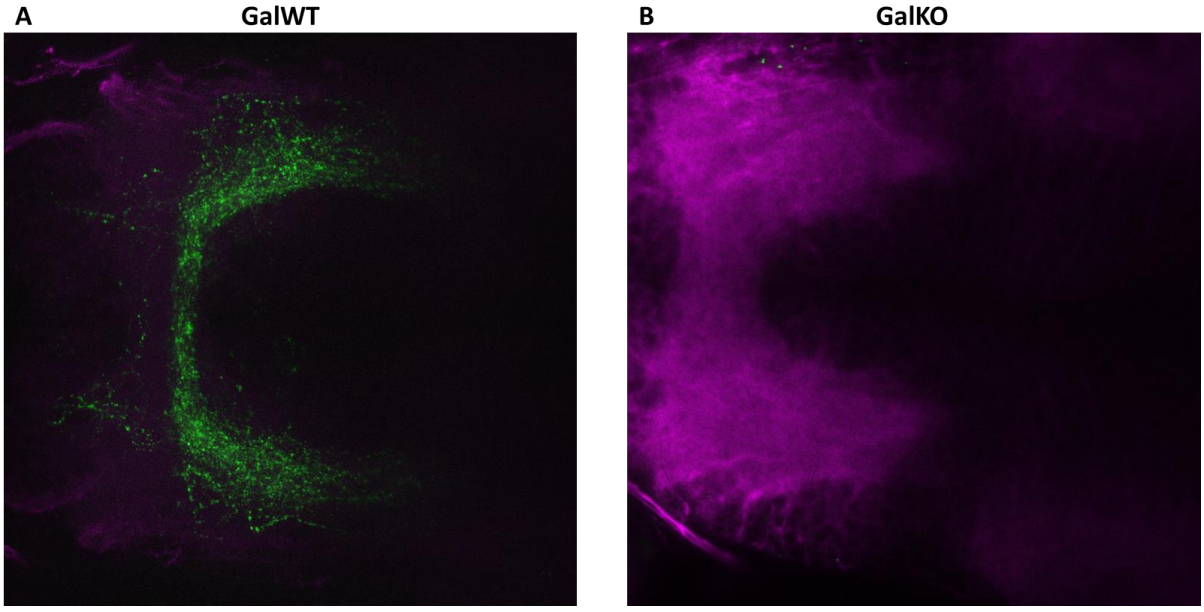

Figure 14: Whole-mount immunofluorescence images for different galanin expression conditions provided by Milena Ronchetti. (A) Galanin wild type, green(galanin), pink(acetylated tubulin). (B) Galanin knockout, green(galanin), pink(acetylated tubulin).

Seizure Susceptibility in eaat2a Heterozygous Mutant Larvae

Important for the discussed possible mechanisms below is that almost no conditions were significantly different from each other and therefore these mechanisms aren't supported by the data. It might be that with a bigger number of larvae these differences would become significant, or they would even out and all the conditions would be similar to each other.

The neuronal activity fluctuation in the galanin WT condition of the eaat2a HET larvae is higher compared with the eaat2a WT larvae. As mentioned in the results section this indicates that the eaat2a HET larvae have stronger, longer or /and more seizure like and seizure events. This however must be investigated more detailed looking at the amplitude, the duration and the number of seizure like events, which will be done below.
The eaat2a HET larvae in the galanin WT condition have less seizure-like events than the eaat2a WT larvae. However, if they have these seizure-like events, they seem to be stronger (higher amplitude) and longer lasting than in the eaat2a WT larvae. As mentioned in the introduction in a previous study it could be qualitatively seen that the eaat2a HET larvae seem to have more seizures when stimulated with light than the eaat2a WT larvae. So this result is partially what we expected to see. We originally hypothesised that the eaat2a HET larvae also would have more seizures than the eaat2a WT larvae.
It might be that, because the eaat2a HET larvae only have one functional copy of the gene, that they are not as effective in removing the glutamate from the synaptic cleft after a synaptic event. This would then lead to a higher baseline glutamate concentrations in the synaptic cleft. If then the activation of neurons is increased by inhibiting GABA, which is the main inhibitory neurotransmitter in the CNS also glutamate levels would increase. This would then lead to the seizure-like events we could observe and because they might be worse in removing glutamate the seizure-like events are stronger. To compensate for the less efficient glutamate removal the larvae might upregulate galanin, because galanin can reduce the amount of glutamate release. The reduced number of seizure-like events then might be caused by this upregulation of galanin and therefore the general downregulation of glutamate. Similar to the hypoactivity observed in the eaat2a homozygous mutants after seizures. So that the number of seizures gets reduced but if the glutamate levels increase enough the seizures are stronger and longer lasting, because of the less effective removal of glutamate from the synaptic cleft.

Neuroprotection by Galanin

Important for the discussed possible mechanisms below is that almost no conditions were significantly different from each other and therefore these mechanisms aren't supported by the data. It might be that with a bigger number of larvae these differences would become significant, or they would even out and all the conditions would be similar to each other.

The neuronal activity fluctuation in the eaat2a WT condition of the galanin WT larvae is higher compared with the galanin KO. As mentioned before this indicates again that they have stronger, longer or /and more seizure like and seizure events.
For the eaat2a HET condition of the galanin WT vs. galanin KO larvae the galanin KO larvae seem to have a slightly higher neuronal activity fluctuation, which would indicate that they have stronger, longer or /and more seizure like and seizure events. This has to be further

investigated in more detail below as it has been done above for the seizure susceptibility of eaat2a HET larvae.

In the eaat2a WT condition the galanin KO caused fewer and less severe seizure-like activities, by the means of shorter duration and weaker amplitude. This might be caused by a overcompensation, due to the loss of galanin in these larvae. Often biological organisms overreact if something is wrong, like when humans get fever we most often can reduce it without impairing the clearance of the pathogen. The theory behind this is that it is most often less harmful for the organism to react to strong than not strong enough. So here the loss of galanin and therefore an impairment in the possibility to decrease glutamate release, the postsynaptic neurons might have desensitized by decreasing the number of AMPA receptors by internalizing these receptors. Desensitization happens if neurons get frequently overactivated. By the loss of galanin the glutamate levels increase, which could then lead to an overactivation. It might also be possible that the neurons of the CNS sensitised for GABA to reduce neuronal activation. Sensitisation for GABA would mean to increase the number of GABA receptors in the postsynaptic part of a inhibitory synapse. Both desensitization for glutamate and sensitization for GABA would explain why the galanin KO didn't have worse seizure-like events but actually decreased their susceptibility to PTZ induced seizure-like events.

For the galanin KO and eaat2a HET larvae the data looks a bit different. When just comparing the galanin KO and eaat2a HET with the galanin WT eaat2a HET larvae we see again a decrease of the severity of the seizure like events. However, the galanin KO eaat2a HET larvae experience more seizure like events than the galanin WT eaat2a HET larvae. These larvae can desensitize their neurons for glutamate and maybe sensitize their neurons for GABA the same way it could be the case in the eaat2a WT galanin KO larvae. This would then explain the reduced severity of seizure like events. However, the eaat2a HET galanin KO are possibly also less effective in removing the glutamate from the synaptic cleft. This could then lead to an increased probability to experience seizure like events, because of the increased glutamate levels in the synaptic cleft. The galanin KO eaat2a HET also cannot downregulate the glutamate levels via a galanin overexpression. Therefore, they additionally cannot reduce the probability of seizure-like events by this possible mechanism.

Comparing the eaat2a WT galanin KO with the eaat2a HET galanin KO larvae we see an increase in the number of seizure-like events and in the severity of the seizure-like event again. This could be explained by the less efficient removal of glutamate from the synaptic cleft again, leading to a more likely accumulation of glutamate and therefore, also to an increased chance of experiencing seizure-like events and simultaneously worsening them.

Outlook

The next steps would be to repeat the experiments to get more larvae and therefore more meaningful results. Then it would become clear if the differences we can see between the different conditions are actual biological differences and not just caused by chance. Having a bigger sample size would potentially also increase the number of seizures the zebrafish have. Therefore, it might then also be possible to compare what the different genetic backgrounds would mean for the seizure susceptibility of the different larvae. Maybe the same differences that could be seen for seizure-like events can be seen for seizures or it might look different, which would then give even more insight into different mechanisms that are involved in seizure development or neuroprotection against seizures.

If this would be the case and it would also turn out, that the differences seen between the conditions is of real biological meaning, the next step would be to perform experiments to confirm the mechanisms that we proposed in the discussion section. This would then give more insight into the mechanisms of seizures and therefore might help to develop new treatments for individuals suffering from a condition that is accompanied by epileptic seizures.

Material and Methods

Whole-Mount Immunofluorescence

At the 4th dpf larvae were anaesthetized on ice for 20 minutes and fixed with 4% paraformaldehyde (PFA) in PBS for 2 hours at room temperature. Then larvae were washed in PBS several times. Before antibody staining the larvae were washed with ddH2O, incubated for 20 minutes in acetone and rewashed in ddH2O, then in PBS, and the preparation of the larvae was finished with two washes with PBDT(PBS + 1% BSA + 1% DMSO + Triton X-100 (1%)). After the preparation steps, larvae were blocked in 10% goat serum in PBDT for 40 minutes at room temperature. Larvae were incubated with primary antibodies (1:400 dilution) in PBDT overnight at 4C.

Antibody	**Host**	**Ratio**	**Supplier**
Anti-Galanin Antibody	Mouse(Ms)	1:400	Sigma-Aldrich
Anti-Acetylated Tubulin antibody, Mouse monoclonal IgG2b	Rabbit (Rb)	1:400	Sigma-Aldrich

Table 1: Primary antibodies (I AB) for the immunofluorescence

The next day the larvae were washed in PBDT four times. Then the secondary antibodies (1:400 dilution) in PBDT were added for 2 hours at room temperature in the dark to prevent the degradation of fluorophores.

Antibody	**Host**	**Ratio**	**Supplier**
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Goat	1:400	Thermo Fisher Scientific
Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	Goat	1:400	Thermo Fisher Scientific

Table 2: Secondary antibodies (II AB) for the immunofluorescence

Then the larvae were washed 4 times with PDT(PBS + 1% DMSO + 0.5% Triton X-100) and incubated in DAPI (diluted 1:1000 in ddH2O) for 15 minutes at room temperature. And afterwards, larvae were washed again 3 times in PDT. Then the clearance through increasing glycerol row (25%, 50%, 70%) was performed. Deyolked embryos were transferred to glass slides and covered with coverslips. The space between the glass slide and the coverslip, where the larvae where pinched was filled with Mowial / DABCO and then sealed with nail polish for confocal imaging using a TCS LSI confocal microscope (Leica Microsystems).

Genotyping for the eaat2a Zygosity

After the calcium imaging procedure, larvae were anaesthetized on ice for 20 minutes and removed from the agarose. Then each larva was placed in a tube with containing lysis buffer (25 mM NaOH, 0.2 mM EDTA). All samples were incubated at 95ºC for 30 minutes. Then the lysates were neutralized in a neutralization solution (40 mM Tris-HCl, pH 5) and kept at 4ºC.

PCR Target Region

Eaat2a mutant fishes can be genotyped by PCR amplification and a subsequent gel-electrophoresis, which allows detection of the 13 base pair deletion. Larvae were genotyped directly after calcium imaging using KAPA2G Fast HotStart PCR Kit (Roche® Life Science Products). To target the eaat2a gene the following primers were used: eaat2a sense/fw (5'-GATGCAGTCGTATGGGAA-3') and eaat2a antisense/rv (5'-CCTTCTCCCAGATTCTCC-3'). Then the cycling protocol was applied (see tableX). After the PCR the samples were loaded in a 3% agarose/TAE gel and run at 120V for 1.5 hours. The gel images were acquired using the following parameters ISO Auto, exp. time 1/100, aperture 5.6.

initial denaturation	95°C	3 min	x 35
denaturation	95°C	15 sec	
annealing	56°C	15 sec	
extension	72°C	1 sec	
final extension	72°C	1 min	

Table 3: Cycling protocol for the PCR reaction

Genotype of Crossed Zebrafish Lines

Genotype Parent I	Genotype Parent II	Genotype Offspring
eaat2a$^{+/+}$ / GCaMP$^{+/-}$	eaat2a$^{+/-}$	25% eaat2a$^{+/+}$ / GCaMP$^{+/-}$ 25% eaat2a$^{+/+}$ / GCaMP$^{-/-}$ 25% eaat2a$^{+/-}$ / GCaMP$^{+/-}$ 25% eaat2a$^{+/-}$ / GCaMP$^{-/-}$
eaat2a$^{+/-}$ / galanin$^{-/-}$ / GCaMP$^{+/-}$	eaat2a$^{+/+}$ / galanin$^{-/-}$	25% eaat2a$^{+/+}$ / galanin$^{-/-}$ / GCaMP$^{+/-}$ 25% eaat2a$^{+/+}$ / galanin$^{-/-}$ / GCaMP$^{-/-}$ 25% eaat2a$^{+/-}$ / galanin$^{-/-}$ / GCaMP$^{+/-}$ 25% eaat2a$^{+/-}$ / galanin$^{-/-}$ / GCaMP$^{-/-}$

Table 4: Genotype of the used adult zebrafish and the resulting offspring.

Raising Zebrafish Larvae

In the evening the zebrafish were put together pair wise into tanks filled with E3 media and the tanks were then covered with a lid. The next day in the morning or directly after noon the eggs were collected as followed. First the zebrafish were put back into their corresponding tanks Then the media of the tanks containing the eggs was poured into a tee strainer. The eggs were then rinsed with tap water to remove the feces. All the eggs of one container were rinsed into a 150mm petri dish filled with E3 media. After collecting of the eggs, it was checked if they were all laid approximately at the same time. If this is the case, they would all be in a similar developing stage. The embryos that were older an therefore were laid already in the evening or during the night were sorted out. In the afternoon the fertilized and living eggs were sorted into new 150mm petri dishes with 30-40 embryos per dish. The new petri dishes were filled with 200µM PTU in E3, so that the fish won't develop pigmentation. Three days later the PTU was refreshed.
On the fourth day the larvae were sorted for GCaMP expression. For this a wide field fluorescent microscope was used. The GCaMP positive larvae were transferred to a new 150mm petri dish. To fetch the larvae, they were anaesthetised with tricaine. After the sorting the GCaMP positive larvae were washed with E3 to remove the tricaine and then put back into 200µM PTU in E3.

Calcium Imaging

Embedding of Larvae

For calcium imaging the larvae where embedded in 2% low melting agarose (LMA) drops in 35mm petri dishes. Per dish two larvae were embedded. One larvae always being a Galanin WT and one being a Galanin Knockout. Once the first larvae was embedded in LMA the residual agarose in front of the head of the larvae was cut away so that only very little agarose was still left in front of it. Then the second larvae was added with as little water as possible. Then LMA was added and the second larvae was aligned to look at the other larvae and to be on the same hight as the first larvae. After this the remaining part of the dish that was not yet covered by LMA was covered with a thin layer of LMA. Once the agarose was no solid the dish was filled with 200µM PTU in E3.

Imaging

The larvae were imaged using a Victron Systems Olympus BX51WI microscope. The intensity of the blue laser was set to 20 for imaging. Each dish was recorded for one hour and every 750ms an image was taken, resulting in 4800 images. A binning of 4 was used to reduce the background noise and to increase the signal intensity.
For imaging the PTU was removed from the 35mm petri dishes and the 20mM PTZ in E3 was added, so that the agarose is completely covered. Then the dishes were put under the microscope focused and the recording was started.

Image and Data Analysis

For the image analysis Fiji was used. For each larvae regions of interest were marked in fiji. These regions of interest were the whole brain, the forebrain, the midbrain, the hindbrain. Then a drift correction was run, because the agarose starts to move during the one-hour imaging period. After the drift correction the regions of interest were readjusted so that the again mark the entire brain and it was checked if the drift correction worked properly by looking if the larvae no longer change their position in the throughout the image sequence. Then the mean fluorescent intensity of each individual frame / image was measured using fiji. The fluorescent intensity in the images is represented by the intensity (brightness) values of the pixels.

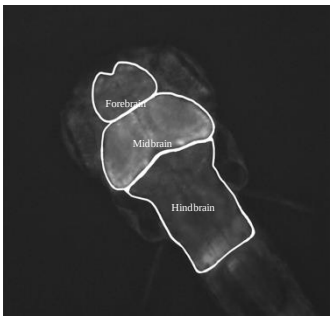

Figure 15: Example of the drawn regions of interest in on of the imaged larva

Then this data was loaded into R for the analysis. First the mean fluorescent intensity values (F) were normalized as followed. Every 400 frames, meaning all 300s, a new F_0 (the value that is considered as zero) was defined. The F_0 in each interval was defined as the first percentile of the F values. These intervals were then shifted by one frame until the end of the image sequence.
Then the three different thresholds were set, 10%, 50% and 100% above the F_0 of each interval. If a mean fluorescent intensity value was above a certain threshold it was accounted to this specific threshold. This value would however not be accounted into the thresholds below. For example, a value above 100% is only accounted into this threshold and not into the threshold of 50% and/or 10%. Seizures were defined as values above 100%, seizure-like events as values above 50% and strong brain activity as values above 10%.

Then for each larvae the brain activity traces were plotted (see Appendix). They represent the ΔF $((F - F_0) / F_0)$ in percent. They were used to check if the data extraction and analysis worked properly. For example, if a larvae would have died during the imaging, a very long and bright fluorescent signal would be visible in the brain activity traces.

Next the data form both days were merchet. From now on only the whole brain was used to for further data analysis. Next the neuronal activity fluctuation was calculated. The neuronal activity fluctuation represents the standard deviation of the brain activity across the whole hour of imaging. Then the number of the different events, the mean amplitudes of the different

events and the mean duration of the different events for each individual larva were calculated. The duration of an event was measured from the time point where half of the intensity of the event was reached to the time point where the half of the intensity of the event was reached again. For example, a the duration of a seizure-like was calculated from 25% intensity before reaching 50% to 25% after the 50% were reached.

Statistical Tests

To analyse if there is a significant difference between the different conditions the kruskal wallis test on each measurement, neuronal fluctuation, number of events, amplitude of events and duration of events. If this test was positive, meaning a p-value of 0.05 it meant that there is a significant difference in the means in at least one of the conditions. To then further analyse between which groups the significant difference is the dunn's test was performed.

Reagents, Mixtures and Antibodies

Reagent	**Mixture Content**
E3	5 mM NaCl 0.17 mM KCl 0.33 mM $CaCl_2$ 0.33 mM $MgSO_4$
Low melting Agarose	-
Universal Agarose	-
Propylthiouracil (PTU)	-
Pentylenetetrazole (PTZ)	-
Double distilled water (ddH_2O)	-
Acetone	-
1x PBS	137 mM NaCl 10 mM PO_4^{3-} 2.7 mM KCl ph 7.4
Mowiol / DABCO	2.4g Mowiol 6g Glycerol 6ml Wasser 12ml TrisHCl 0.2M pH 8.5 2.5% DABCO
Goat serum	-
DAPI	Concentrated Solution
Glycerol	-
PDT	1x PBS 1% DMSO 1% Triton
PBDT	1x PBS 1% DMSO 1% Triton 1% BSA
Triton	-
Bovine Serum Albumin (BSA)	Concentrated Solution
Dimethylsulfoxid (DMSO)	-
Primer forward eaat2a	Concentrated Solution
Primer backward eaat2a	Concentrated Solution
KAPA2G Fast HotStart Ready Mix	KAPA DNA polymerase dNTPs KAPA2G buffer

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Appendix

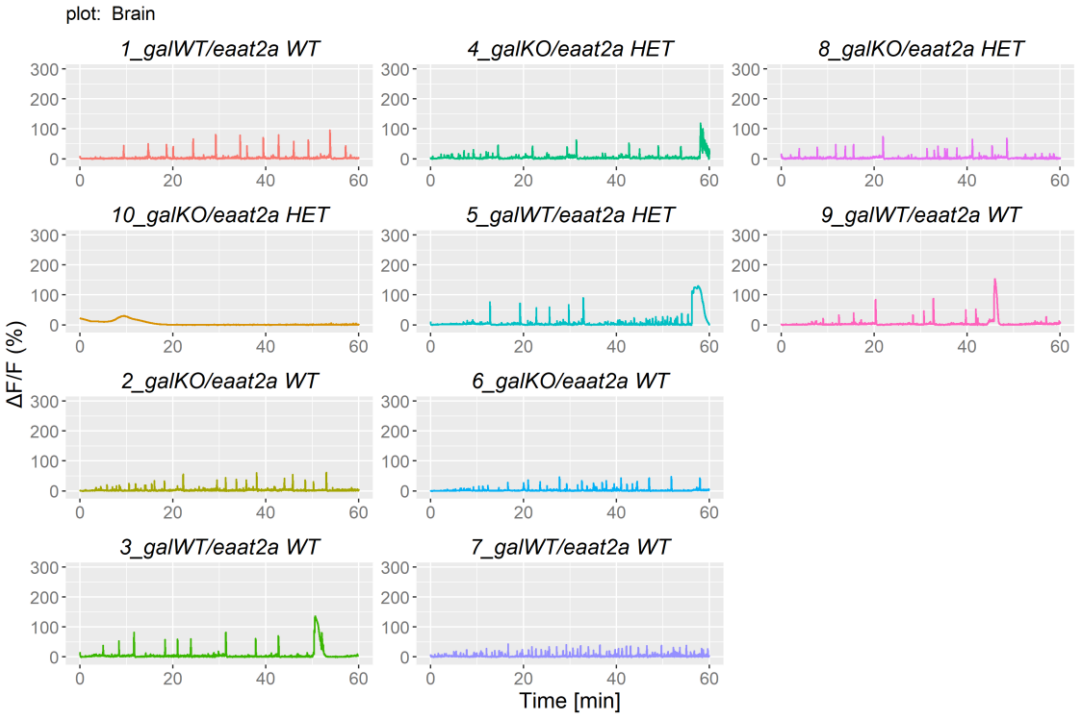

Figure 6: First time of calcium imaging

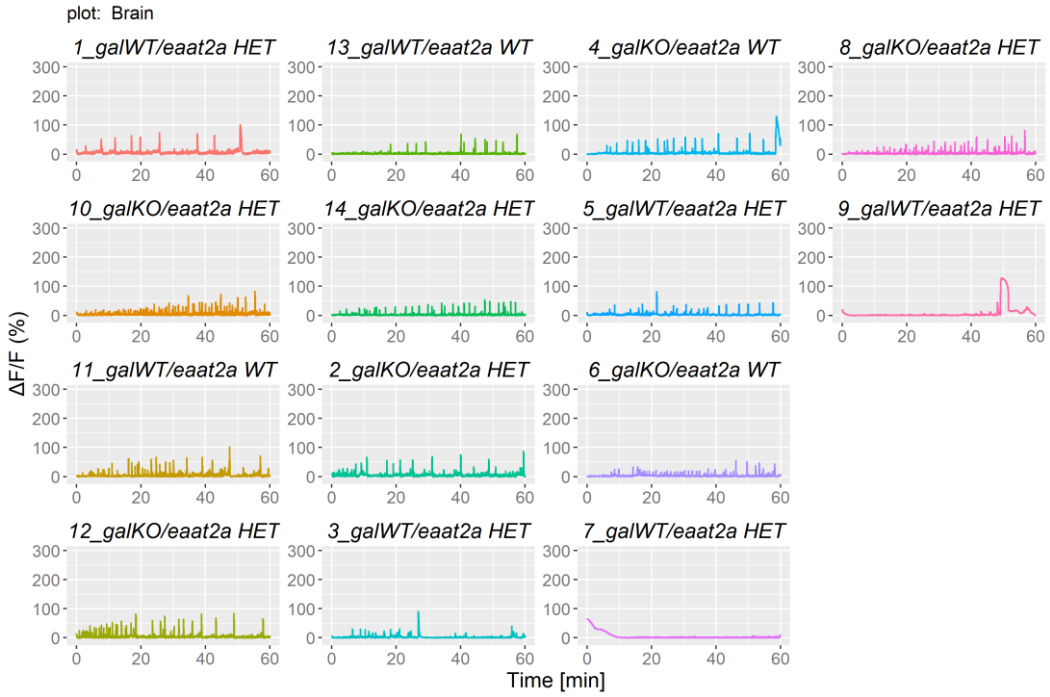

Figure 11: Second time of calcium imaging