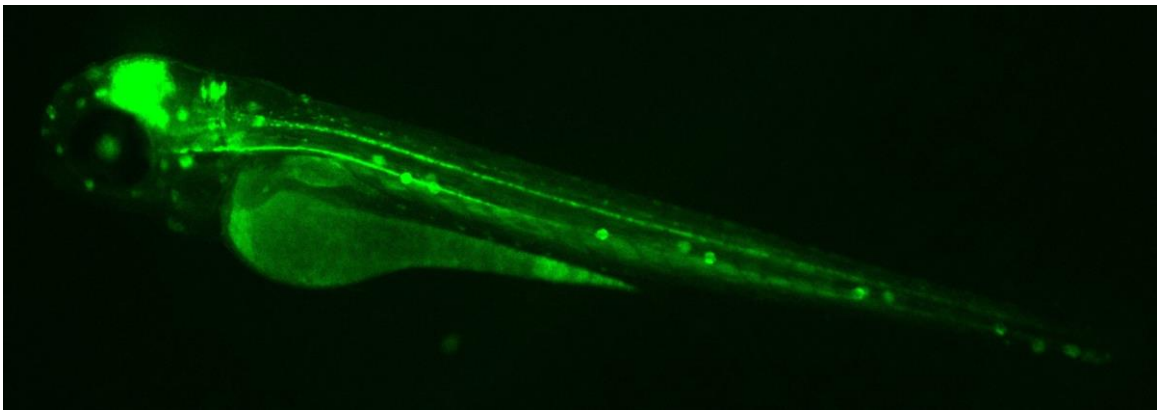
UPPSALA
UNIVERSITET

Visualizing neuronal cell sub-populations using novel transgenic zebrafish lines.



Aikaterini Zafeiriou

Master's Degree Project in Medical Research, 30 credits, Spring 2021
Department: Neurosciences
Supervisors: Henrik Boije, Judith Habicher

When life gets you down, do you know what you've got to do?
Just keep swimming.

Contents

Abstract

Zebrafish is a frequently used model organism with an array of transgenic lines that have been used in developmental and physiological studies.

We aim to generate novel transgenic zebrafish reporter lines to study subpopulations of spinal neurons *in vivo*.

The gene editing system called CRISPR/Cas9 system was used to knock in reporter genes such as green fluorescent protein (GFP) or Gal4 transcription factor, to generate transgenic fish lines. Zebrafish embryos were injected with gRNAs targeting *gabrb1* or *nr4a2a* and GFP or Gal4 plasmid, respectively. F0 larvae were screened, positive fish were raised until sexual maturity, and founders characterized to verify germline insertion. Three founders were found for *gabrb1* and the location and the direction of the insert verified. The GFP expression was studied during development and differential expression patterns were identified whereas all founders had expression in brain and spinal cord. In parallel, positive fish from the Gal4 injections were raised and will be screened. Immunohistochemistry was performed to check if *nr4a2a* is expressed in the same cells as known neuronal markers. However, no co-localization was detected.

The three *gabrb1* founders identified in this study highlight the challenges into creating stable transgenic lines recapitulating true expression of the gene of interest. Sequencing, in-situ hybridization and immunohistochemistry should be performed to verify the line. A possible reason for the varying expression may be that through the knock-in we may interfere with regions regulating gene. The *nr4a2a*-Gal4 line will be used to perform functional studies. Those experiments will be performed using reporter genes, such as opsins or GCaMP, controlled by Upstream Activation Sequence (UAS). These transgenic lines will provide important insights regarding neuronal subpopulations that express *gabrb1* and *nr4a2a* to unravel how the locomotor network is formed.

Popular Scientific Summary

Can a fluorescent fish unravel the mystery of spinal cord neurons?

Zebrafish is a small fish that is found in freshwater streams in India and Burma. This fish is widely used in developmental and physiological studies. Among its many advantages, the fish eggs and larvae are transparent which makes it possible to monitor the changes in development in real time. Zebrafish are also frequently used as model organism to study the nervous system. The reduced complexity of the fish makes it easier to study the complex structures and functions of the nervous system. The main goal in this thesis was to use zebrafish to mark and study specific groups of cells in the nervous system, called neurons. By investigating sets of neurons in the spinal cord, we hope to gain insights into how a relatively simple circuit coordinates its activity to generate a coherent output in the form of movements.

In order to mark these neurons, we used a gene-editing tool called CRISPR. With this technique we can cut the DNA of the fish in a specific place of choice and insert a reporter gene to mimic the expression of the gene of interest. Our target place in the DNA was close to *gabrb1*, a gene that produces a protein present in neurons and is mainly responsible for de-activating cells. The goal was to be able to distinguish the cells that are functionally different but look morphologically alike. In order to visualize the cells, we cut the DNA close to the *gabrb1* gene and inserted another gene that creates a protein that emits in green color. With this technique, when we see green, it means that the *gabrb1* gene is expressed in that cell.

This procedure was performed in fish eggs just after fertilization and fish that expressed the green protein were raised until they were sexually mature. After 2 months, the fish were mated, and the eggs were screened using a fluorescent microscope. If we found green cells in the offspring, it meant that the parent fish carried the modified genome in their germ cells. From our experiments, we found three parent fish that gave positive offspring. However, these three fish gave offspring that expressed the green protein in different cells; therefore, we observed three different patterns. One possible explanation why we observed different patterns could be that the insert was probably introduced in different places in the fish DNA or that the insertion modified DNA sequences with a regulatory function differently in these three fish.

In parallel with this method, we also used an already established line of fish that had the same inserted gene (green protein) close to the *nr4a2a* gene. In these fish, we wanted to check if the gene is expressed in the same cell types as some other known marker genes. To achieve that, we used specific antibodies that target those genes in larvae producing the green protein in nr4a2a expressing cells and then imaged the fish with a confocal microscope. By using different colors for the antibodies, we could monitor if one cell expressed many different target genes. Our results showed that we did not have co-expression of those markers in the neurons which in some cases verifies the results from previous studies.

By performing these experiments, the main goal is to use the new transgenic fish in other experiments to study the role of these cells in generating the motor output. If we can specifically label different populations of cells, we can use optogenetics to turn them on and off in a living animal. This will give us the opportunity to study the activity of those cells and see their effect on locomotion. By knowing the basic structures and functions of the neurons we can make conclusions of their role in different circuits in the brain and spinal cord. Thereafter, we can use this knowledge to understand the mechanisms underlying of many motor and neurological diseases in neurologically more complex organisms, such as humans.

Introduction

The nervous system is considered one of the most complex systems in the body. The human brain contains more than 100 billion cells and the connections between them control every function and every decision that we make. Our focus is to study how this complex system is formed and more specifically how the different cells and cell types interact with each other to generate motion. One specific type of motor activity, called locomotion, is studied intensively by researchers and is of interest even for this thesis. Locomotion is an umbrella term that includes the different ways an animal uses to move from one place to another. This process is controlled by many neuronal networks that include different types of neurons that interact with each other and the muscles to create a specific behavior.

One way to study how the locomotor network translates into behavior is by using model organisms. In model organisms, we can study the behavioral output of a locomotor circuit, but we can also generate transgenic lines to study specific populations of neurons *in vivo*.

Zebrafish as a model organism

One of the most common model organisms used in science is zebrafish. Zebrafish (*Danio rerio*) is a small fish about 2,5-4cm long that is native to rice paddies in India and Burma (Engeszer et al., 2007). It inhabits freshwater streams; it survives about 2 years in nature, and it reaches sexual maturity after 2 months. It is widely used as an experimental animal for studying developmental and physiological processes due to its many advantages.

The development of zebrafish is fast, and the fertilization takes place in the water. The female lays transparent eggs in clutches of about 200 and the male is fertilizing them. The embryo remains transparent for all the larval stages. At about 2 days post fertilization (dpf), pigmentation starts appearing on the skin and at the adult phase the fish is covered with horizontal stripes. The transparency at the early stages of life gives the opportunity to monitor the changes that occur during development in real time. This advantage makes zebrafish widely used for developmental studies (Bao et al., 2019).

Husbandry of zebrafish is relatively easy since they can be housed in a manageable space. Due to that fact, many different lines of fish can be kept and used in many experiments.

Along with the other major advantages, zebrafish has established itself as a model organism because of the plethora of molecular tools available. The full zebrafish genome was sequenced and annotated in 2013 and it contains about 26,000 protein coding genes. From those genes, 70% have similar genetic structure with the human genes and 84% of genes related to human disease have homologous counterparts in zebrafish (Howe et al., 2013). This has made zebrafish a very promising model of human disease but also for unraveling the structures and functions of many different biological systems.

One of the most complex systems that is studied in zebrafish is the nervous system. The basic organization of the nervous system is similar among higher vertebrates and zebrafish. Neural circuits, cell types, structure and basic functions seem to be conserved (Burton, 2015). However, the reduced complexity in the fish makes it easier to study basic functions as well as the fine-tuning between the different cells and cell types. Zebrafish can also be used in experiments that cannot take place in other vertebrates. Larvae can be stored in 96-well plates and be treated with a variety of drugs; in vivo screenings can take place as well as electrophysiological recordings (Burton, 2015). The most important tool though is the ability to

genetically manipulate the fish and create many different transgenic lines in relatively short periods of time (Costantini, 2001; Ma and Liu, 2015; Sertori et al., 2016).

Zebrafish genetic tools

In order to interfere with the genome, three ways are very popular among groups that use zebrafish. The Cre-LoxP system, the Gal4-UAS and CRISPR/Cas9 are widely used to study gene expression, gene interactions and disease traits either by introducing reporter genes in the genome or by inducing mutations. Cre-LoxP is a recombination process where structural mutations like deletions, insertions, translocations and inversions can be induced. The system contains a recombinase, Cre, that can target specific sequences in the genome called Lox. The Cre cuts in the Lox sites and according to the number of Lox sites the DNA can be cut in one, two or more places.

Introduction of specific reporter genes can indicate the place and time where a gene is expressed. Green and red fluorescent protein (GFP and RFP respectively), as well as other fluorescent proteins can be very useful since their expression can be visualized with a simple fluorescent microscope. Also, reporter genes are or can be involved in the next two methods, Gal4-UAS and CRISPR/Cas9.

The Gal4-UAS system was first developed in 1988 (Kakidani and Ptashne, 1988), adapted in 1993 (Brand and Perrimon, 1993) and is currently considered one of the most powerful techniques -along with the in-situ immunohistochemistry- to study gene expression. It was used widely in drosophila, but it has been used in zebrafish studies as well (Davison et al., 2007). The system consists of two parts, the gene that encodes the Gal4 protein and the Upstream Activation Sequence (UAS) which functions as an enhancer activated by Gal4. The way it is used in fish is that one line is created which carries the *Gal4* gene and another one with the UAS and a reporter gene (*e.g., GFP*). In the first fish line the *Gal4* gene is expressed under the promoter of the gene of interest, but the enhancer is not present, whereas in the second fish line the enhancer is inactive and GFP is not expressed because Gal4 is not present in the cell. However, if those two fish are crossed then the offspring will carry both the Gal4 and the UAS, enabling an active enhancer and GFP expression. With this system, lines that express Gal4 only in specific tissues can be created and different types of information can be obtained based on the reporter gene. For example, when a fluorescent protein is used then information on which cells express Gal4 can be obtained. Additionally, opsins like channelrhodopsin and halorhodopsin can also be used as reporter genes (Arrenberg et al., 2009; Zhu et al., 2009). These can be used to study the function of certain neurons, since neurons can be activated or suppressed by the light-sensitive channels. The Gal4-UAS system allows also for calcium imaging where proteins of the GCaMP family are used as reporter genes. Those proteins are fluorescent when calcium is released and since this happens when neurons are firing (transmission of the electrical signal between neurons) it can give insights of how the nervous system operates in real time (Kettunen, 2020, 2012).

CRISPR (Clustered Regulatory Interspaced short palindromic repeats) was first discovered in 1987 in bacteria, and it served as an immune response to bacteriophages (Barrangou, 2015). It is a cluster of DNA sequences in the bacterial genome derived from the phages. Those sequences are used to identify viral DNA that enters the bacterium in a subsequent infection (Barrangou et al., 2007). There are three types of those immune responses in bacteria and each of them is associated with a different enzyme. The method used in the present work is based on type II (Chylinski et al., 2014). Type II CRISPR is associated with an enzyme called Cas9 (CRISPR-associated protein 9) which is an endonuclease. The Cas9 has two active sites, one is for binding the RNA from the CRISPR site and one that creates a double strand break in the DNA that is complementary with the RNA sequence. This bacterial system was adopted to be used for

editing genomes of organisms. The CRISPR/Cas9 was introduced as a genetic tool in 2014 and can be used to target specific sequences in the genome (Doudna and Charpentier, 2014). The CRISPR RNA can be designed, and it can guide the Cas9 to an exact location in the genome where the enzyme introduces the double-strand break. The CRISPR/Cas9 system has been used to modify crops, to de-activate genes in human cells, to treat genetic disorders and in other applications (Jiang and Doudna, 2017; Zhang et al., 2014). In the present thesis, CRISPR/Cas9 was used to create a double strand break upstream of the 5' UTR (Un-translated region) of *gabrb1*. Instead of knocking out the gene, a sequence of a reporter gene was introduced upstream of the gene we are interested in. In that way, the reporter gene -*GFP*- is expressed in cells and the developmental stages where *gabrb1* is expressed. This method was published in 2014 (Kimura et al., 2014).

gabrb1

gabrb1 is the gene encoding beta1 subunit of the GABA receptor. γ-aminobutyric acid (GABA) is one of the main neurotransmitters in the central nervous system. Its effect is mainly inhibitory, and it controls neural circuits through the $GABA_A$ receptors. There are two types of GABA receptor; $GABA_A$ and $GABA_B$. The difference between them is that $GABA_A$ is mediating rapid hyperpolarization, while $GABA_B$ produces slow and prolonged signals. In this thesis we focus on the $GABA_A$ receptor. This receptor was first discovered in crustacean muscles, since it was easier to test neuroactive substances from mammals in crustacea (Florey, 1954; Florey and McLennan, 1959; Kuffler, 1954). The $GABA_A$ receptors play numerous roles in the central nervous system. At the beginning of development, they are involved in the generation of neurons and the synapses and promote neuronal excitation (Ben-Ari, 2002; Ben-Ari et al., 2007). However, their role in generation of neurons and neuronal excitation was challenged but their involvement remains very crucial (Bregestovski and Bernard, 2012; Valeeva et al., 2016). In general, the $GABA_A$ receptors are involved in two areas, the inhibitory synapses and the extrasynaptic sites. In inhibitory synapses, GABA is released quickly and briefly but causes acute inhibition of the neuron. On the other hand, in extrasynaptic sites it initiates tonic inhibition which is less intense but lasts for longer periods of time (Farrant and Nusser, 2005; Mody, 2001; Sadamitsu et al., 2021).

Despite their different types on inhibition, all $GABA_A$ receptors constitute of five subunits which form a Cl^- dependent channel. Each of the subunits penetrates the membrane four times and contains a ligand binding site in the N-terminal domain. Mammals have 19 subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ, and ρ1–3) that comprise the $GABA_A$ receptors and despite the amount of different possible combinations, most receptors are composed by two α, two β and one γ subunit. $GABA_A$ receptors are also studied in zebrafish, where 23 subunits have been found (eight α, four β, three γ, one δ, one π, one ζ, and five ρ) (Cocco et al., 2017; Sadamitsu et al., 2021). Cocco at al. also studied the expression levels of the $GABA_A$ receptors in the adult zebrafish brain. $GABA_A$ receptors are also highly expressed, but also tissue specific, in larval stages as indicated by in situ hybridization (Monesson-Olson et al., 2018). Another in situ hybridization in 5dpf larvae showed that some are widely expressed in forebrain, midbrain, hindbrain and eye, like α1 subunit. Others had more constrained expression like in the olfactory bulb. β subunit, which is the focus of this study, were broadly expressed in the whole brain region (Sadamitsu et al., 2021).

Zebrafish is used as a model organism to study the effect of those receptors in human disease since the receptor subunits are very conserved among mammals and teleosts (Sadamitsu et al., 2021). Loss of function studies with morpholinos or CRISPR/Cas9 have revealed disease phenotypes in larvae and juvenile fish (Reyes-Nava et al., 2020; Samarut et al., 2018). Those results are consistent with studies of $GABA_A$ receptors and *gabrb1* specifically, in mice and humans. Many variants in the $GABA_A$ subunits

and b1 among them (*gabrb1*) are linked with epilepsy and alcohol consumption (Anstee et al., 2013; Hernandez and Macdonald, 2019; Hirose, 2014).

nurr1 (*nr4a2a*)

nr4a2 (nuclear receptor subfamily 4 group A member 2), also called *nurr1* (nuclear receptor related 1 protein) was first identified by Law et al. in 1992 (Law et al., 1992). This nuclear receptor is a key protein for the maintenance of the dopaminergic system of the brain as it is involved in the development of the dopaminergic neurons in the central nervous system (Sacchetti et al., 2006; Zetterström et al., 1997). Mutations and loss of function studies in this gene have shown that it is involved in differentiation and survival of the dopaminergic neurons. It is also linked with disorders related to dopaminergic dysfunction such as Parkinson's disorder and schizophrenia (Decressac et al., 2013; Lei et al., 2011; Montarolo et al., 2019). *nr4a2a* is a very important factor that is involved in the regulation of dopamine transporter and tyrosine hydroxylase along with other genes such as *Pitx3* and *Foxa2* (Arenas, 2005; Yi et al., 2014). Wnt pathway and retinoid signalling are regulating the *nr4a2a* gene (Smidt et al., 2009; Volakakis et al., 2009). In zebrafish this gene is present in two paralogs, a and b, with overlapping expression patterns. From Blin et al. 2008, it is known that *nr4a2a* is homologous to *Nr4a2* in Amniota (Blin et al., 2008). This fact is supported by loss of function studies, where knock-down of *nr4a2a* results in loss of dopaminergic phenotype in different neurons in the central nervous system. This outcome is the same when knocking down both *nr4a2a* and *nr4a2b,* but the phenotype is less severe when only *nr4a2b* is lost (Filippi et al., 2007).

Aim of the study

The main goal of this thesis was to generate a transgenic zebrafish line that expresses GFP in cells where the *gabrb1* gene is expressed in order to mark and study those neurons. Moreover, we wanted to insert Gal4 upstream of the *nr4a2a* gene with the aspiration to create a transgenic line and use it in optogenetic or calcium imaging experiments to study the neurons that express this transcription factor. Those two genes were selected after an extensive screening in mice where many possible candidate genes were assessed for their ability to mark only few neurons. In the mouse spinal cord, the genes were expressed in limited number of interneurons, and they have homologous zebrafish genes.

In summary, we followed the timeline of generating a transgenic line from the injections until the verification using two gene targets. For *gabrb1*, we injected one cell stage embryos with gRNA, Cas9 and the GFP plasmid, we screened the fish that had cells expressing GFP in the spinal cord and raised them until sexual maturity. After that we screened the offspring of these fish trying to find a fish with germline insertion and identified three founders. These founders showed different GFP expression patterns and we performed PCR to explain the differences in the patterns. In parallel, we injected a Gal4 plasmid for the *nr4a2a* gene in order to generate a *nr4a2a*:Gal4 driver line to combine with UAS-opsins or UAS-GCaMP fish in order to perform functional experiments. Also, immunohistochemistry was performed in the already established *nr4a2a*-GFP line by using antibodies targeting known neuronal markers like Wt1, Calb2 and Isl1/2. With this experiment we wanted to characterize the cell types where GFP and therefore *nr4a2a* was expressed by examining co-localization with other known transcription factors. The next step in the process would be to use the lines to study the sub-populations of neurons, that express the specific markers, and use them in functional studies in order to see how the neural networks are formed and how these neurons are involved in locomotion.

Materials & Methods

Ethical statement

All zebrafish lines were housed at Genome Engineering Zebrafish National Facility (SciLife Lab, 342 Uppsala, Sweden) under the local welfare standards as well as the European Union legislation (EU-Directive 201_63). The fish were kept under standard conditions of 14 hours light/ 10 hours dark cycles at 28^{o} C and in densities of max 5 fish/L tanks for adult fish. All embryos used were anesthetized and euthanized using Tricaine and rapid cooling according to up-to-date publications (Aleström et al., 2020; Köhler et al., 2017). The ethics permit was obtained from the local ethical board in Uppsala and carries the following number: C164/14 and 14088/2019.

Embryo treatment

All eggs used for further experiments were collected from zebrafish natural crosses. In order to collect fish for injections, the fish were kept in tanks with separators the previous day. The separators were removed the following morning and the eggs were used at the one cell stage. For the eggs used in other experiments the fish were left to mate freely. All the collected eggs were kept in an incubator protected from light and at 28^{o}C. Unfertilized eggs and dead embryos were removed from the petri dishes and the rest of the eggs were kept in water with methylene blue. Embryos used for immunohistochemistry were treated with 1-Phenyl-2-thiourea (PTU, 0.003% final concentration) at 24 hours post fertilization (hpf) to avoid pigmentation.

Injections

Preparation

Injection needles were made from glass capillaries (Narishige, GD-1) using a needle puller (Narishige, model PC-10). The needle was backfilled with 5µL mix that contained gRNA targeting a region upstream the 5' UTR of the *gabrb1* gene, a GFP carrying plasmid, mbait gRNA to cut open the plasmid, Cas9 mRNA and protein and phenol red dye (50ng/µL target gRNA, 15ng/µL plasmid, 50ng/µL mbait gRNA, 4.55ng/µL Cas9 protein, 112.5ng/µL Cas9 mRNA, 0.075% phenol red (SIGMA Life Science, P0290)) (Kimura et al., 2014). The needle was then placed in the microinjection manipulator (Narishige, IM-31) that was connected to a microinjection pump (Narishige, IM-31) and the tip was opened using a pair of forceps. Balance and pressure in the pump were adjusted in order to obtain the covetable injection volume. The volume of the injection droplet was calculated at using a micrometer slide (ZEISS) and the whole process was carried out under a stereomicroscope (Nikon SMZ1500). The droplet volume was optimized by administering the mix into an oil droplet until the diameter of 0.5mm was achieved. This droplet diameter corresponds to injection volume of 0.06µL using the following formula, $V=1/6\pi d3$ (1bar). This process was repeated whenever the needle was changed.

Procedure

Fish (AB or NACRE lines) were set up using separators in the breeding tanks. In the morning, the separator was removed, and the eggs were collected after 20-30 minutes. The one-cell staged embryos were lined up against a microscope slide that is placed inside a petri dish. The excess water is removed so that the eggs are immobilized and then they are injected in the one cell while avoiding injection to the yolk. In every clutch of eggs collected, about 10-20 were kept as non-injected controls. After the injection, the embryos

were kept in a dark incubator at 28° C and after 3h were cleaned from dead or unfertilized eggs. The embryos were kept in the incubator and screened every 24h until 5 dpf for GFP positive cells in a fluorescent stereomicroscope (Leica, MZ10F). At 5dpf the healthy, GFP positive fish were put in the system to grow and were screened after 2 months.

gRNA construction

For the gRNA construction, the protocol from burgess lab was adjusted (Varshney et al., 2015). The method is using oligos and amplification during a Polymerase Chain Reaction (PCR) and RNA synthesis to make the sgRNA followed by RNA clean-up with an affinity column process.

Oligo assembly & preparation
At first, the lyophilized primers (Forward: tgtaaaacgacggccagttgaaaattcagaccaaattgttt, Reverse: gtgtcttGCCGACTCAACACCTCTTTC) were diluted to a final concentration of 10μM via 100μM stock solution. Then a PCR mix containing 5XPhusion buffer (1X, 5μL), dNTPs (10mM, 0.5μL), gene specific oligo 1 (taatacgactcactataGGGACCGTGGATGAGAGGAAgttttagagctagaaatagcaag) (10μM, 0.5μL), oligo2(AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC) (10μM, 0.5μL), Taq Phusion polymerase (2U/μL), RNase free water (18.3μL) run at the following conditions: denaturation 98°C for 2 minutes, annealing 50°C for 10 minutes, extension 72°C for 10 minutes. The PCR product was imaged on a 2.5% agarose gel where a 120bp single band was visible (3-5μL of PCR product and 3μL of ladder (GeneRuler DNA Ladder, ThermoFisher Scientific)).

RNA synthesis and clean up.
For RNA synthesis the T7 quick high yield RNA synthesis kit (New England Biolabs, E2050S) was used. The whole process was performed on ice and a tube with dNTP mix (10μL), ddH_2O (5μL), assembled oligos for sgRNA mbait (mbait is a specific sequence in the plasmid) (3μL) and T7 polymerase (2μL) was then incubated at 37°C for 2-16h in a PCR machine (Biorad T100 thermal cycler). After the incubation 1μL of DNase (ThermoFisher Scientific) was added and the sample was incubated at 37°C for 15 minutes. For the clean-up of the product, Gene JET RNA Cleanup and concentration micro kit (ThermoFisher Scientific, K0841) was used, and the final sample was eluted in 10μL RNase-free H_2O. The final RNA was kept on ice and its concentration was verified via nanodrop measurements. A 2% agarose gel was also used as an extra check point. As a final step the sgRNA was diluted to 250ng/μL and aliquots of 3μL were kept in a -80 °C freezer.

Immunohistochemistry

Immunohistochemistry was performed in *nr4a2a*-GFP transgenic fish at 3dpf. The fish were set-up without separators and the eggs were collected one hour after the light was turned on. The dishes were cleaned from dead or unfertilized eggs and the healthy embryos were kept in a dark incubator at 28°C. After about 24h from fertilization, the eggs were treated with 1-Phenyl-2-thiourea (PTU, 0.003% final concentration) and at 3dpf they were screened for GFP positive cells. From the positive fish, 10-15 were selected for the experiment.

Larvae were fixed in 4% PFA (ThermoFisher Scientific, Pierce™ 16% Formaldehyde (w/v), Methanol-free) in Protein Bovine Serum (PBS) for 15 minutes at room temperature, equilibrated three times

10minutes in PBS-T (1xPBS, pH7.3, 0.1% Triton X-100). After equilibration, cryoprotection with 30% sucrose solution in PBS for 2 hours at room temperature, was performed followed by three 10-minute washes with PBS-T. The permeabilization was achieved with pre-chilled acetone (100%) for 20 minutes at -20^{o}C. The preceding step was a series of washes; first with PBS-T, then with ddH_2O and then again with PBS-T, each for 2 rounds of 5 minutes. The preparation for the primary antibody was initiated with a one-hour incubation with 1% BSA in PBS-T (blocking buffer) at room temperature. Thereafter, the fish were incubated with the primary antibody against GFP (chicken, 1:1000), Il1/2 (mouse, 1:200), Wt1 (mouse, 1:100) and Calb2 (rabbit, 1:1000) for the *nr4a2a*-GFP fish diluted in blocking buffer at 4^{o}C overnight.

On the second day, the fish were washed twice with PBS-T for one hour at room temperature and then incubated with the secondary antibody -diluted in blocking buffer- at 4^{o}C, in the dark (488nm, goat, anti-chicken, 1:500 for GFP, 594nm, donkey, anti-mouse, 1:400 for Isl1/2, 594nm, donkey, anti-mouse, 1:400 for Wt1, 647nm, donkey, anti-rabbit, 1:400 for Calb2).

On the final day, two washes with PBS-T were performed for one hour each at room temperature and the fish were kept in PBS, in the dark at 4^{o}C. It is noted that every incubation always took place with gentle agitation and that the tubes were kept in the dark.

Imaging with fluorescent stereomicroscope and confocal microscope

For imaging of the samples and the injections we used 3 different microscopes. For the injections, the Nikon SMZ 1500 was used. The screening of the injected fish as well as the potential founders took place in the Leixa MZ10F fluorescent stereomicroscope. The characterization of the founders and the immunohistochemistry imaging was conducted in SP8 Leica confocal microscope, and the image analysis was done in Fiji, LasX software, GIMP, InkScape and Adobe Illustrator.

PCR & Sequencing

For verification of the location and direction of the insert, PCR was used to amplify the region where the plasmid was induced. Combination of primers were used that were specific to the genomic area upstream of the *gabrb1* gene as well as primers inside the GFP gene and the heat-shock promoter (hsP) (Table 1).

***Table 1**: List of primers used for the PCRs to verify the position and direction of the insert.*

Primer name	Sequence (5'- 3')
gabrb1 Forward	TGAAAATTCAGACCAAATTGTTT
gabrb1 Reverse	GCCGACTCAACACCTCTTTC
hsP Forward	GACCGCAGAGAAACTCAACC
hsP Reverse	GCCCGTCTGTTCATTGTTTT
EGFP Forward	ATCATGGCCGACAAGCAGAA
EGFP Reverse	TCTCGTTGGGGTCTTTGCTC

Results

Knock-in of GFP resulted in three founders

From the CRISPR/Cas9 injections many positive fish were identified from screening but not all of them were suitable for further in the experiments. A large number of fish developed an unhealthy phenotype and others had unspecific expression of the GFP, mainly in the muscles. About 50 larvae that expressed the GFP only in the spinal cord were continued to grow until sexual maturity. These F0 fish were set up and screened and three of the fish gave GFP-positive offspring (F1). These F1 fish (Fig. 1), which are named A, B and C based on the chronological order they were found, expressed GFP in three different patterns. All the F1 fish had expression in the brain and spinal cord; but expression in the blood vessels and neuromasts was present in some of them (Fig.1).

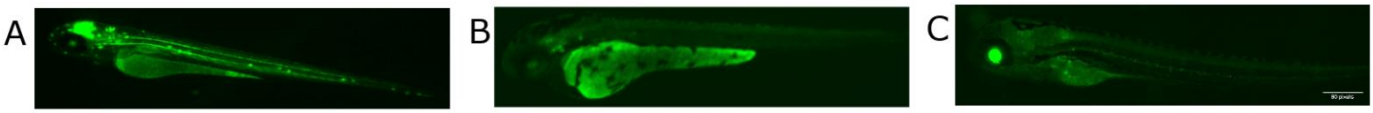


***Figure 1**: Lateral views on zebrafish larvae at 3dpf. Brain and spinal cord expression were found in offsprings from founder A(A), founder B(B) and founder C(C)*

Neurons in the spinal cord express GFP in all three founders

The first area we characterized for GFP expression was the spinal cord. In F1 embryos from founder A and B, the GFP started to be expressed from day 1 but this was not the case for the offspring of founder C where the expression was not detected before day 2. Offspring from founder A (hereafter referred to as founder A) displayed very strong expression in relatively few cells of the spinal cord on day 1 but also some expression was seen in the muscles (Fig. 2, A1). On the contrary, offspring from founder B (hereafter referred to as founder B) had weaker expression in the spinal cord on day 1 and it was also in few cells (Fig. 2, B1).
On day 2, the expression patterns diverged even more. In founder A we found expression in few cells, mainly on the dorsal and ventral side of the spinal cord, while Rohon-Beard cells (white arrows, Fig. 1, A2) were also fluorescent on the dorsal side. On founder B the expression was stronger, different types of neurons were highlighted and many of the neuronal axons were also expressing GFP (Fig. 2, B2). Offspring from founder C (hereafter referred to as founder C) developed high levels of GFP expression in day 2 (Fig. 2, C2). The majority of the spinal neurons were expressing GFP, and expression was also present in the blood vessels (white arrow, Fig. 1, C2). The muscles did not display strong GFP expression.

Founder A continued to express GFP in a few cells on the dorsal and ventral side of the spinal cord at day 3 and 4, and the Rohon-Beard cells were not present or not labelled anymore (Fig. 2, A3, A4). For founders B and C at day 3 and 4, the expression pattern remained the same with strong cells in the spinal cord and in the blood vessels (Fig. 1, white arrows, C3) in founder C.

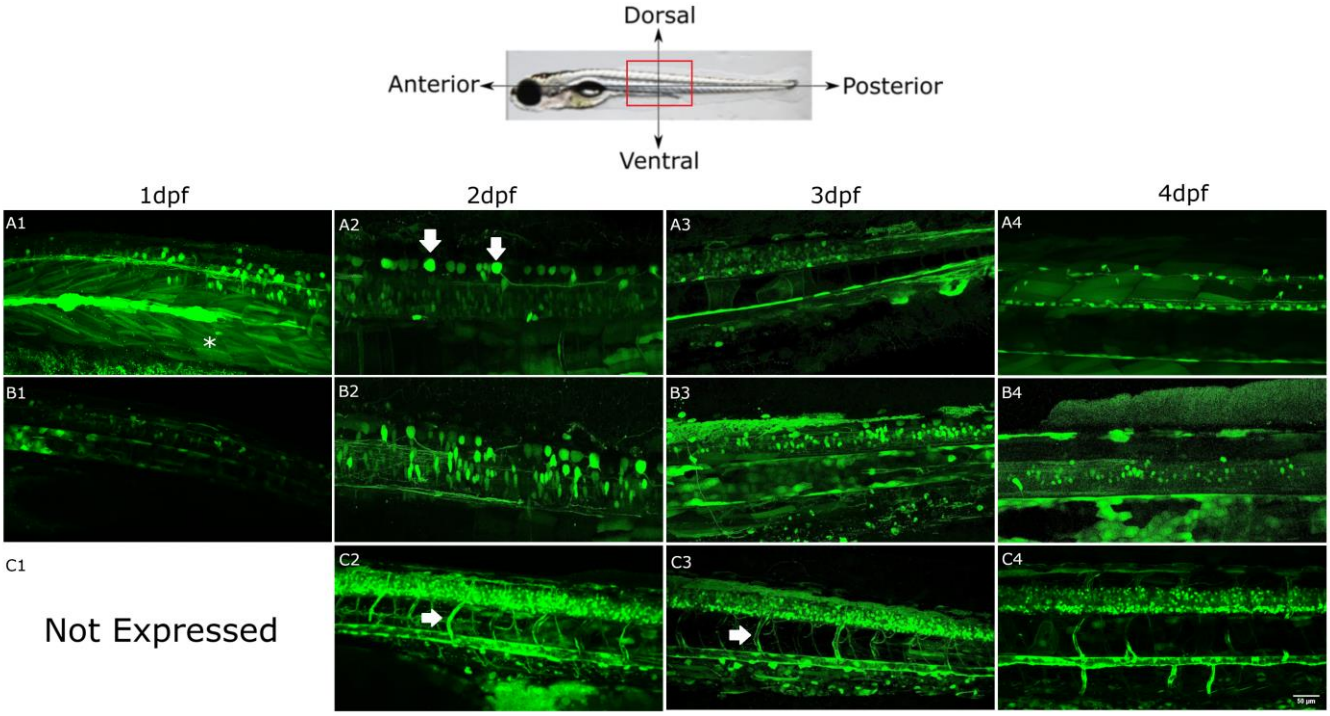


***Figure 2**: Confocal images from larvae spinal cords from 1dpf to 4dpf for offspring from the three founders. On day 1, we observed GFP expression in founders A and B but not in founder C. On day 2, all the founders expressed GFP but in different patterns. Founder A expressed GFP in few neurons and the Rohon-Beard cells (white arrows A2), founder B had expression in more cells both dorsally and ventrally and founder C had very strong expression throughout the spinal cord and the blood vessels. On days 3 and 4, in founder A the expression in Rohon-Beard cells was not present and the GFP expression was limited in neurons mainly in the dorsal and ventral side. In founders B and C, in the same period, the expression was in all of the spinal neurons and in founder C in the blood vessels as well (white arrows, C2, C3). GFP expression in the muscles was also noticed mainly in founder A (white star in A1). Scale bar at 50µm.*

Expression patterns in the brain of the three founder fish

The brain of the larvae of all founders showed cells expressing GFP. In day 1, founders A and B exhibited strong expression of the fluorescent protein but GFP expression in founder C is not present (Fig. 3, A1, B1, C1). Founder A showed at 2-4dpf very strong expression in the forebrain and hindbrain where some bouquet-like structures of cells are very distinguishable (Fig. 3, red box, A3). Founder B had strong expression in the posterior lateral line ganglion and some cells on the surface of the head, probably some skin cells at day 2 (Fig. 3, white arrow, B2). Clear axonal projections to the neuromasts and hair cells in the ear were detected at day 3-4 (Fig. 3, B3, B4). Founder C expressed GFP mainly in the midbrain and retina, but some fluorescence could also be detected in the heart (Fig. 3, C2).

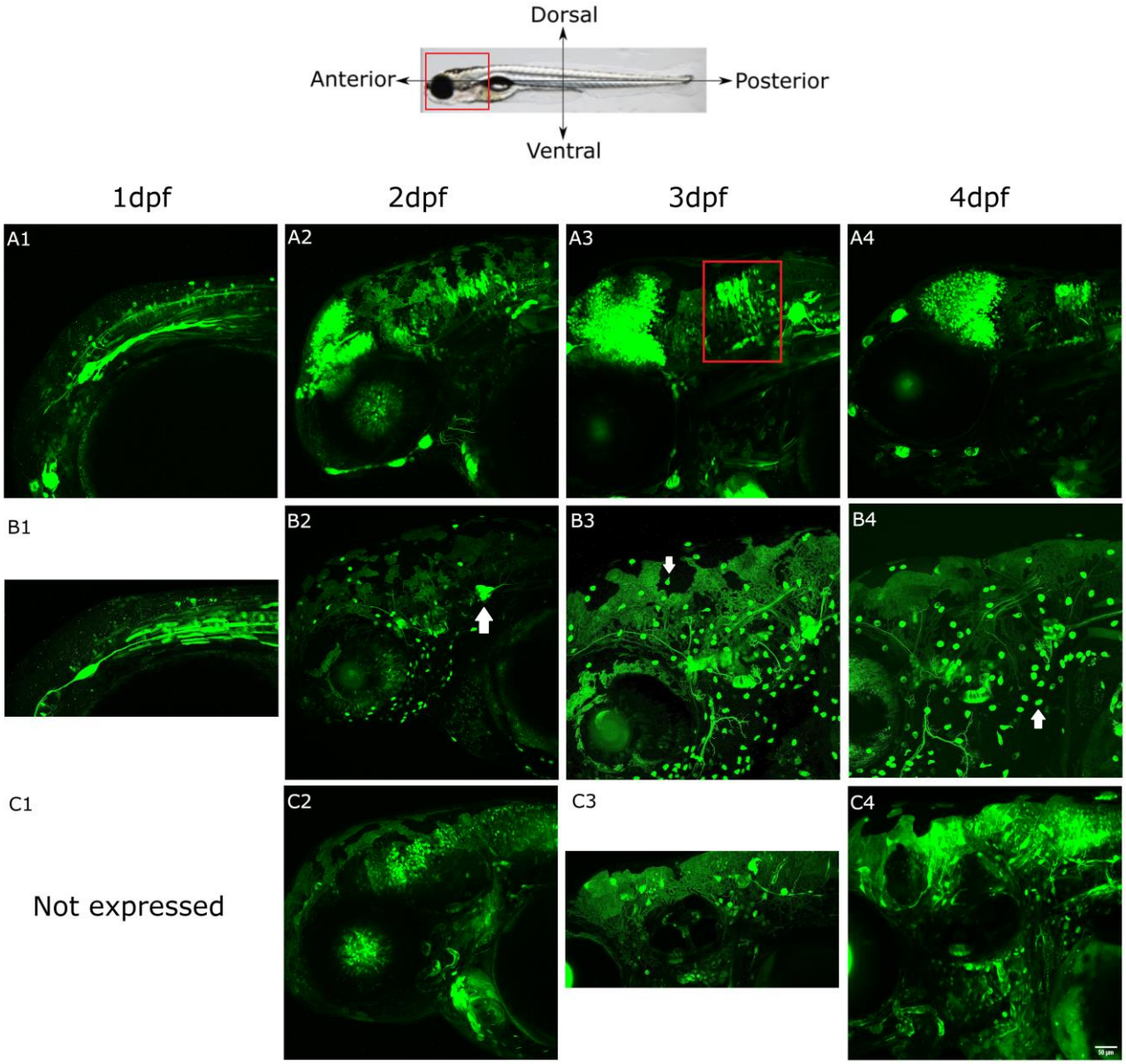


***Figure 3**: Confocal images of the head and brain of the offsping of the three different founders. On day 1, founders A and B strongly express GFP in the head area while in founder C expression was not detected. In founder A, strong GFP expression remained until day 4 and it was detected in the forebrain, midbrain and hindbrain. Some bouquet-like structures were obvious as well (red box, A3). In founder B, on day 2 some superficial cells expressed GFP, and the expression remained until day 4 (white arrows, B3, B4). The posterior lateral line ganglion was also fluorescent in founder B. In founder C, axons towards the neuromasts were also visible and the retina, midbrain and heart cells expressed GFP. Scale bar at 50μm.*

Neuromasts express GFP in two of the founders

Neuromasts are sensory patches that are present in the surface of the fish throughout the body. They are stimulated by water flow, and they help the fish move through the water, avoid obstacles and catch pray. This group of cells consists of a rosette of hair cells and the supporting cells (Wada and Kawakami, 2015). In the confocal images of the founders, we identified that neuromasts were also GFP positive (Fig. 4). Founder A had GFP positive neuromasts in the head and along the spinal cord (Fig. 4, A). From the confocal images, we can observe that the cells expressing the insert are mainly the supporting cells.

Founder C also displayed fluorescence in the neuromasts but not the ones in the head but only the ones along the side of the body (Fig. 4, C). Founder B's neuromasts were not labelled by GFP.

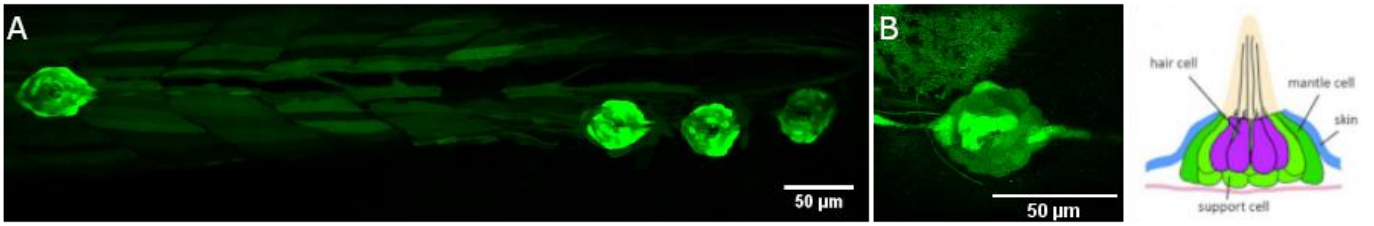


***Figure 4**: Offspring of the founders A and C had expression of GFP in the neuromasts. From a schematic picture of a neuromast (modified from (Wada and Kawakami, 2015)), we observe that the supporting cells are the ones that expressed GFP. The confocal image for founder A (A) was taken at 4dpf and the one from founder C (B) at 3dpf but the expression was visible from day 2 until day 4. Scale bar at 50µm.*

Verification of the line via PCR

In order to examine the location of the insert and its direction in the genome we performed a series of PCRs. This was also a way to explain the differences observed in the expression patterns of the three founders. For the PCRs, we used primers that target the genomic area upstream of the 5' UTR of the *gabrb1* gene as well as two sets of primers for the GFP gene and the heat-shock promoter (Fig. 5). For the PCRs all the different combinations were used but the amplification was not successful (Fig. 6). When the genomic primer and the EGFP primers were used, the expected product was 1600bp but the bands that were visible in the gels were not corresponding to the expected molecular weight of the desired products (Fig. 6).

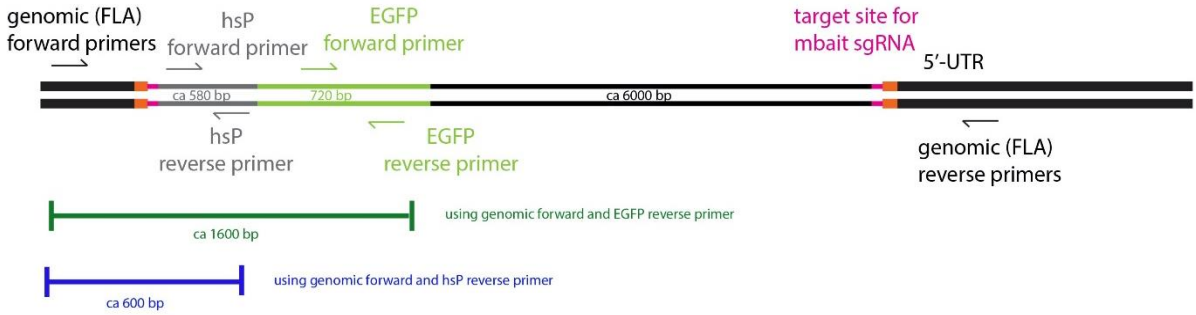


***Figure 5**: Schematic approach of the genomic areas recognized by the different primers. The genomic primers were specific for gabrb1 while the hsP primer and the EGFP primer were specific to bind to sequences in the insert. The fish used for the PCR were heterozygous so when the genomic forward and reverse primers were used, we were getting an amplification from the wild type copy of the genome that did not include the insert. When the EGFP or hsP primers were used and we got a band it indicated that the insert is present in the genome. What we were not able to amplify was a band when using genomic forward primer and one of the hsP or EGFP primers. If the insert was in a forward direction, the genomic forward primer with the EGFP reverse one would have given a 1600bp band. In the same concept, if we used the genomic forward primer and the hsP reverse we would have amplified a 600bp piece. (Modified from J. Habicher)*

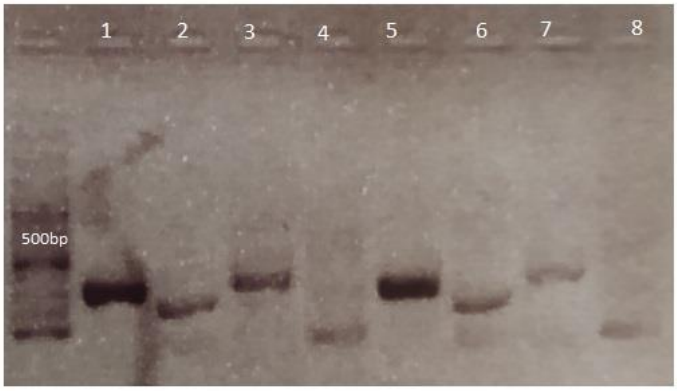


***Figure 6**: Example of a gel from the PCR using combinations of primers. The wells are from left to right: ladder (Gene Ruler 100bp, ThermoFisher Scientific), genomic forward and reverse primers (1,5), EGFP forward and reverse primers (2,6), genomic forward and EGFP reverse primers (3,7) and genomic reverse and EGFP reverse primers (4,8). The PCR was performed in 2 fish samples of founder B. Only the controls (wells 1,2 for fish 1 and 5,6 for fish 2) gave the expected bands while the combinations of primers gave bands of lower molecular weight than the expected.*

Gal4 injections resulted in ca. 50 positive fish

We performed injections with a gRNA targeting upstream of the 5' UTR of the *nr4a2a* gene. We used UAS-GFP and UAS-RFP lines to inject the CRISPR/Cas9 mix. If the injections are successful and the *Gal4* is inserted upstream of the *nr4a2a*, then the UAS will be activated, and we could screen for GFP or RFP cells. From our injections with gRNA targeting *nr4a2a* we had about 50 fish growing. After 2 months, when they are sexually mature, they will be screened for positive cells in their offspring.

Characterization of the *nr4a2a*-GFP cells in the spinal cord

Two different immunohistochemistry (IHC) processes were performed in the *nr4a2a*-GFP line. We used three known neuronal markers Wt1, Calb2 and Isl1/2. In the first one, we used an antibody against GFP, one for Wt1 and one for Calb2 (Fig. 7, B). For the second one, GFP and Isl1/2 antibodies were used (Fig. 7, A, 7, C). While in the maximum projection we can observe some yellow cells, searching in the z-stack images we concluded that in both IHC there was no co-localization of the known markers with the GFP. This indicates that the cells expressing GFP, and therefore also the *nr4a2a* gene, are not the same cells that express Wt1, Calb2 and Isl1/2.

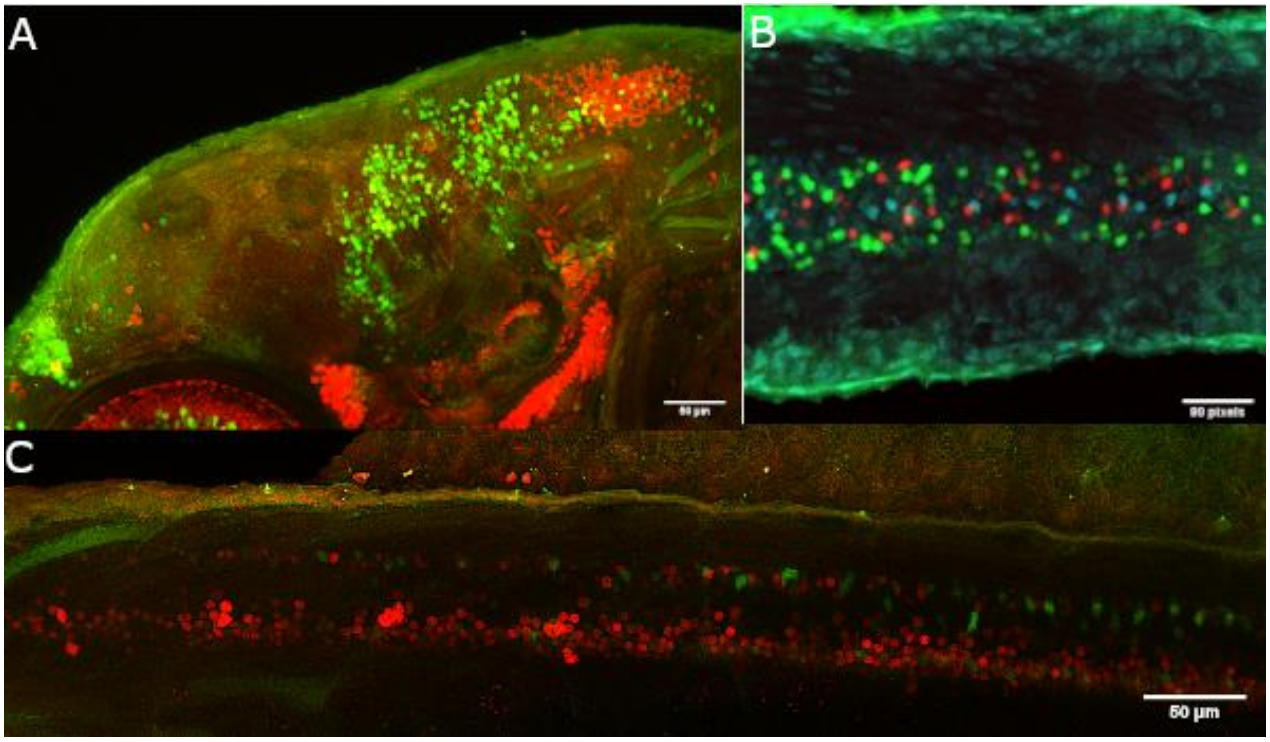


Figure 7*: Confocal images of the performed immunohistochemistries. A and C are lateral views of the head and spinal cord, of 3dpf fish, respectively. In green is the GFP positive cells and in red is the Isl1/2 expressing cells. Attention should be paid to the fact that the images are depicting the maximum projections. Some yellow cells are visible but by examining the z-stack images, overlapping was not found. Image B is a dorsal view of the spinal cord of 3dpf fish where in green are the GFP expressing cells, in red the Wt1 positive cells and in blue the Calb2 expressing cells. No overlapping is present in this immunohistochemistry as well. Scale bar at A and C at 50µm and at 80pixels at B.*

Discussion

Studying the nervous system is difficult but very interesting and zebrafish is an important model organism for these types of studies. This model system gives the opportunity to study neuronal development from the first hours after fertilization due to the transparency of the larvae. Despite its reduced complexity, compared to mammals, zebrafish has more than 20 different classes of embryonic spinal neurons (Goulding, 2009). These neurons are distinguished by the combinations of transcription factors they express. In this study we wanted to assess whether the two candidate genes, *gabrb1* and *nr4a2a* can be used as markers for a subpopulation of neurons in the spinal cord. In order to achieve that, we created a transgenic line that expressed GFP in the cells that *gabrb1* is expressed. Moreover, we used an already established line to perform immunohistochemistry to check if the *nr4a2a* is co-expressed with other known markers.

From the first part of the thesis, we managed to create a transgenic line that expressed GFP in some of the cells where *gabrb1* is expressed. However, we generated three different founders with three different expression patterns. This inconsistency is possibly a result of the insert that includes the GFP gene, the heat-shock promoter (hsP) and the rest of the plasmid. With the CRISPR/Cas9 method, the gRNA that cuts the genome creates a double-strand break and by Non-Homologous End Joining (NHEJ) repair the plasmid can be inserted in the genome. With this process, nucleotides can be inserted or deleted in the site of the break and create different types of mutations. The mutations can interfere with the regulatory sequences that are upstream of the gene of interest which can lead to differential expression of the controlled gene. The nucleotides that are added to the normal genomic sequence can alter the function of enhancer sequences and as a result the gene is not expressed in the same way in all the different founders.

Moreover, the genomic gRNA that cuts the genome is tested to check if it cuts in the correct area upstream of the 5'UTR of the gene of interest. However, the gRNA may have some unspecific targets in other genomic sites. If the gRNA cuts in other places as well, then the insert can be introduced in the wrong place in the fish genome. As a result, the GFP is not expressed in the cells where *gabrb1* is expressed but either randomly or in cells where some other gene target is expressed. This explanation is very possible since we have many different subunits of the GABA receptors that may have high sequence similarity. As a result, our gRNA may have off targets due to high complementarity with other subunit sequences. This reasoning is also in line with our PCR results. Our GFP controls gave a band and GFP is also visible in the fish via stereomicroscope and confocal microscope. However, we could not amplify the estimated products from the combinations of primers. This can be an indication that the insert is not in the correct place in the genome but is inserted in a different place. If the GFP is introduced in three different places in the fish genome then this also explains why we observe three different expression patterns but also expression in places where GABA receptors are not expected to be expressed.

In order to verify the location of the insert, at first, we can try to perform a PCR in homozygous fish. All the PCRs we performed were in heterozygous fish in order to have two internal controls. We had a positive band from the genomic primers from the wild type allele without the insert and a band from the EGFP primers from the allele with the insert. If we would test homozygous fish, then both alleles should have the insert. This means that the genomic primers would not be able to amplify the part with the insert, since it is longer than 7kb, therefore we would only have a band from the EGFP primers. However, if the insert is not in the correct place in the genome, then the genomic primers would give us a band. This is an indirect way to get a primary conclusion of the location of the insert.

Another method to check in which cells GFP is expressed is immunohistochemistry. We can use an antibody for the *gabrb1* protein and investigate if the gene is co-expressed in the same cells with other known neuronal markers. With this method, we will be able to monitor the expression of the protein in the cells and check if it is the same among the three different founders. Other more time-consuming and difficult methods such as whole genome sequencing can also give an answer, but it might be hampered by the sequence similarity of the receptors.

The other part of the thesis focused on some later steps of the process of generating a transgenic line. Generating a transgenic line is a time-consuming task. It starts with inserting the *GFP* in the specific place in the genome, then characterizing it over development and verifying that it is expressed in the cells where the target gene is expressed. It is also important to check which cells are marked either by setting up crosses with other known transgenic lines or performing immunohistochemistry. If the marked cells are from a specific population of neurons that means that the targeted gene is a promising marker for that cell population. Then, the next step is to generate a Gal4 line and use it for functional analysis. Since the present study was limited by time, we used an already established transgenic line to perform the Gal4 injections. This line was generated with the same method as the *gabrb1* line, but the gene target was *nr4a2a*. For the *nr4a2a* line we performed two different immunohistochemical labellings. The first one targeted Wt1 and Calb2 and the other Isl1/2.

From the RNA sequencing data, we know that nr4a2a expression is overlapping with Doublesex And Mab-3 Related Transcription Factor 3 (dmrt3) (Andersson et al., 2012). Wt1 and Calb2 are two markers of the dmrt3 lineage and since nr4a2a is expressed in dmrt3 neurons we expected that it might be co-expressed with Wt1 and Calb2 as well. However, this was not confirmed by the immunohistochemistry performed in this thesis. In our experiments, we did not observe any expression overlapping between the markers which verifies, only in one case, the RNA sequencing data. We need to keep in mind that immunohistochemistry directly targets the protein of interest in the expressing cell which means that the protein is present in this cell. RNA sequencing is targeting the mRNA in a pool of many cellular mRNAs. Therefore, the fact that the RNA is present is not an implication that it is also translated into a protein.

From the experiments performed, it is obvious that generating transgenic lines is easier than in the past, but it remains a challenging task. The main goal when generating these lines is to be able to use them for functional studies. The Gal4 injections performed aim to create a line that has Gal4 upstream of the *nr4a2a*. When we cross this line with other UAS:opsin or UAS:GCaMP lines we can perform optogenetics and calcium imaging and be able to study the activity of the neurons expressing *nr4a2a* in the spinal cord. These experiments in the *nr4a2a*:Gal4 line but in other lines as well, can give us insights of the structure and function of different neuronal sub-populations. These types of experiments can subsequently help us unravel the complexity and the connections between the different neurons in the neural circuits. Last but not least, knowledge on the structure and function of the nervous system can contribute to understanding neuronal disease processes and help in the development of treatment.

Acknowledgements

First of all, I would like to express my very great appreciation to my supervisors Henrik Boije and Judith Habicher for their advice, encouragement and critiques of this research work. I am grateful for the opportunity to work with you and learn from you. A special thanks goes to Judith for being with me at the lab guiding and helping me whenever I needed it. Thank you for pushing me to give my very best self every day.

I am particularly grateful for all the members of the lab. Ana, Remy, David, Harmen and Akram thank you for all the advice and your help.

I would also like to express my appreciation to the people working at the SciLife zebrafish facility for taking care of the fish and to Manolis for the valuable conversations.

On a more personal note, I want to heartfully thank my parents and my sister for sending love, good thoughts and Greek treats to keep me going. Thank you for always being next to me, even if you are far away, and for supporting me to every stage of my life!

Finally, a very big thank you to my other family, my boyfriend Kostas and all my friends, for tolerating me and being there for me when I found everyday life harder than it was. Kosta thank you for being practically involved in the experiments troubleshooting, for reading the drafts and helping with the figures. But most of all thank you for your love and support. Nothing would have been the same without you! Ahmad, my dear friend, thanks for our chats, for the BBQ in the snow and for being my baking companion.

References

1. Aleström, P., D'Angelo, L., Midtlyng, P.J., Schorderet, D.F., Schulte-Merker, S., Sohm, F., Warner, S., 2020. Zebrafish: Housing and husbandry recommendations. Lab Anim 54, 213–224. https://doi.org/10.1177/0023677219869037
2. Andersson, L.S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C.-J., Patra, K., Arnason, T., Wellbring, L., Hjälm, G., Imsland, F., Petersen, J.L., McCue, M.E., Mickelson, J.R., Cothran, G., Ahituv, N., Roepstorff, L., Mikko, S., Vallstedt, A., Lindgren, G., Andersson, L., Kullander, K., 2012. Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. Nature 488, 642–646. https://doi.org/10.1038/nature11399
3. Anstee, Q.M., Knapp, S., Maguire, E.P., Hosie, A.M., Thomas, P., Mortensen, M., Bhome, R., Martinez, A., Walker, S.E., Dixon, C.I., Ruparelia, K., Montagnese, S., Kuo, Y.-T., Herlihy, A., Bell, J.D., Robinson, I., Guerrini, I., McQuillin, A., Fisher, E.M.C., Ungless, M.A., Gurling, H.M.D., Morgan, M.Y., Brown, S.D.M., Stephens, D.N., Belelli, D., Lambert, J.J., Smart, T.G., Thomas, H.C., 2013. Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. Nature Communications 4, 2816. https://doi.org/10.1038/ncomms3816
4. Arenas, E., 2005. Engineering a dopaminergic phenotype in stem/precursor cells: role of Nurr1, glia-derived signals, and Wnts. Ann N Y Acad Sci 1049, 51–66. https://doi.org/10.1196/annals.1334.007
5. Arrenberg, A.B., Del Bene, F., Baier, H., 2009. Optical control of zebrafish behavior with halorhodopsin. PNAS 106, 17968–17973.
6. Bao, W., Volgin, A.D., Alpyshov, E.T., Friend, A.J., Strekalova, T.V., de Abreu, M.S., Collins, C., Amstislavskaya, T.G., Demin, K.A., Kalueff, A.V., 2019. Opioid Neurobiology, Neurogenetics and Neuropharmacology in Zebrafish. Neuroscience 404, 218–232. https://doi.org/10.1016/j.neuroscience.2019.01.045
7. Barrangou, R., 2015. The roles of CRISPR-Cas systems in adaptive immunity and beyond. Curr Opin Immunol 32, 36–41. https://doi.org/10.1016/j.coi.2014.12.008
8. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712. https://doi.org/10.1126/science.1138140
9. Ben-Ari, Y., 2002. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3, 728–739. https://doi.org/10.1038/nrn920
10. Ben-Ari, Y., Gaiarsa, J.-L., Tyzio, R., Khazipov, R., 2007. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87, 1215–1284. https://doi.org/10.1152/physrev.00017.2006
11. Blin, M., Norton, W., Bally-Cuif, L., Vernier, P., 2008. NR4A2 controls the differentiation of selective dopaminergic nuclei in the zebrafish brain. Mol Cell Neurosci 39, 592–604. https://doi.org/10.1016/j.mcn.2008.08.006
12. Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.
13. Bregestovski, P., Bernard, C., 2012. Excitatory GABA: How a Correct Observation May Turn Out to be an Experimental Artifact. Front. Pharmacol. 3. https://doi.org/10.3389/fphar.2012.00065
14. Burton, E.A., 2015. Chapter 7 - Zebrafish, in: LeDoux, M.S. (Ed.), Movement Disorders (Second Edition). Academic Press, Boston, pp. 117–138. https://doi.org/10.1016/B978-0-12-405195-9.00007-X
15. Chylinski, K., Makarova, K.S., Charpentier, E., Koonin, E.V., 2014. Classification and evolution of type II CRISPR-Cas systems. Nucleic Acids Res 42, 6091–6105. https://doi.org/10.1093/nar/gku241

16. Cocco, A., Rönnberg, A.M.C., Jin, Z., André, G.I., Vossen, L.E., Bhandage, A.K., Thörnqvist, P.-O., Birnir, B., Winberg, S., 2017. Characterization of the γ-aminobutyric acid signaling system in the zebrafish (Danio rerio Hamilton) central nervous system by reverse transcription-quantitative polymerase chain reaction. Neuroscience 343, 300–321. https://doi.org/10.1016/j.neuroscience.2016.07.018
17. Costantini, F., 2001. Transgenic Animals, in: Brenner, S., Miller, J.H. (Eds.), Encyclopedia of Genetics. Academic Press, New York, pp. 1990–1998. https://doi.org/10.1006/rwgn.2001.1305
18. Davison, J.M., Akitake, C.M., Goll, M.G., Rhee, J.M., Gosse, N., Baier, H., Halpern, M.E., Leach, S.D., Parsons, M.J., 2007. Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. Dev Biol 304, 811–824. https://doi.org/10.1016/j.ydbio.2007.01.033
19. Decressac, M., Volakakis, N., Björklund, A., Perlmann, T., 2013. NURR1 in Parkinson disease—from pathogenesis to therapeutic potential. Nature Reviews Neurology 9, 629–636. https://doi.org/10.1038/nrneurol.2013.209
20. Doudna, J.A., Charpentier, E., 2014a. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346, 1258096. https://doi.org/10.1126/science.1258096
21. Doudna, J.A., Charpentier, E., 2014b. The new frontier of genome engineering with CRISPR-Cas9. Science 346. https://doi.org/10.1126/science.1258096
22. Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish in The Wild: A Review of Natural History And New Notes from The Field. Zebrafish 4, 21–40. https://doi.org/10.1089/zeb.2006.9997
23. Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6, 215–229. https://doi.org/10.1038/nrn1625
24. Filippi, A., Dürr, K., Ryu, S., Willaredt, M., Holzschuh, J., Driever, W., 2007. Expression and function of nr4a2, lmx1b, and pitx3 in zebrafish dopaminergic and noradrenergic neuronal development. BMC Dev Biol 7, 135. https://doi.org/10.1186/1471-213X-7-135
25. Florey, E., 1954. An Inhibitory and an Excitatory Factor of Mammalian Central Nervous System, and Their Action on A Single Sensory Neuron. Archives Internationales de Physiologie 62, 33–53. https://doi.org/10.3109/13813455409145367
26. Florey, E., McLENNAN, H., 1959. The effects of factor I and of gamma-aminobutyric acid on smooth muscle preparations. J Physiol 145, 66–76. https://doi.org/10.1113/jphysiol.1959.sp006127
27. Goodings, L., He, J., Wood, A.J., Harris, W.A., Currie, P.D., Jusuf, P.R., 2017. In vivo expression of Nurr1/Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics. J Comp Neurol 525, 1962–1979. https://doi.org/10.1002/cne.24185
28. Goulding, M., 2009. Circuits controlling vertebrate locomotion: moving in a new direction. Nat Rev Neurosci 10, 507–518. https://doi.org/10.1038/nrn2608
29. Hernandez, C.C., Macdonald, R.L., 2019. A structural look at GABAA receptor mutations linked to epilepsy syndromes. Brain Res 1714, 234–247. https://doi.org/10.1016/j.brainres.2019.03.004
30. Higashijima, S., 2008. Transgenic zebrafish expressing fluorescent proteins in central nervous system neurons. Development, Growth & Differentiation 50, 407–413. https://doi.org/10.1111/j.1440-169X.2008.01023.x
31. Hirose, S., 2014. Chapter 3 - Mutant GABAA receptor subunits in genetic (idiopathic) epilepsy, in: Steinlein, O.K. (Ed.), Progress in Brain Research, Genetics of Epilepsy. Elsevier, pp. 55–85. https://doi.org/10.1016/B978-0-444-63326-2.00003-X
32. Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones,

M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Crollius, H.R., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. https://doi.org/10.1038/nature12111

33. Jiang, F., Doudna, J.A., 2017. CRISPR-Cas9 Structures and Mechanisms. Annu Rev Biophys 46, 505–529. https://doi.org/10.1146/annurev-biophys-062215-010822
34. Kakidani, H., Ptashne, M., 1988. GAL4 activates gene expression in mammalian cells. Cell 52, 161–167. https://doi.org/10.1016/0092-8674(88)90504-1
35. Kertes, D.A., Kalsi, G., Prescott, C.A., Kuo, P.-H., Patterson, D.G., Walsh, D., Kendler, K.S., Riley, B.P., 2011. Neurotransmitter and neuromodulator genes associated with a history of depressive symptoms in individuals with alcohol dependence. Alcohol Clin Exp Res 35, 496–505. https://doi.org/10.1111/j.1530-0277.2010.01366.x
36. Kettunen, P., 2020. Calcium Imaging in the Zebrafish. Adv Exp Med Biol 1131, 901–942. https://doi.org/10.1007/978-3-030-12457-1_36
37. Kettunen, P., 2012. Calcium imaging in the zebrafish. Adv Exp Med Biol 740, 1039–1071. https://doi.org/10.1007/978-94-007-2888-2_48
38. Kimura, Y., Hisano, Y., Kawahara, A., Higashijima, S., 2014. Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. Scientific Reports 4, 6545. https://doi.org/10.1038/srep06545
39. Kindt, K.S., Sheets, L., 2018. Transmission Disrupted: Modeling Auditory Synaptopathy in Zebrafish. Front. Cell Dev. Biol. 6. https://doi.org/10.3389/fcell.2018.00114
40. Köhler, A., Collymore, C., Finger-Baier, K., Geisler, R., Kaufmann, L., Pounder, K.C., Schulte-Merker, S., Valentim, A., Varga, Z.M., Weiss, J., Strähle, U., 2017. Report of Workshop on Euthanasia for Zebrafish—A Matter of Welfare and Science. Zebrafish 14, 547–551. https://doi.org/10.1089/zeb.2017.1508
41. Kuffler, S.W., 1954. Mechanisms of activation and motor control of stretch receptors in lobster and crayfish. J Neurophysiol 17, 558–574. https://doi.org/10.1152/jn.1954.17.6.558
42. Law, S.W., Conneely, O.M., DeMayo, F.J., O'Malley, B.W., 1992. Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol 6, 2129–2135. https://doi.org/10.1210/mend.6.12.1491694
43. Lei, Z., Jiang, Y., Li, T., Zhu, J., Zeng, S., 2011. Signaling of glial cell line-derived neurotrophic factor and its receptor GFRα1 induce Nurr1 and Pitx3 to promote survival of grafted midbrain-derived neural stem cells in a rat model of Parkinson disease. J Neuropathol Exp Neurol 70, 736–747. https://doi.org/10.1097/NEN.0b013e31822830e5

44. Ma, D., Liu, F., 2015. Genome Editing and Its Applications in Model Organisms. Genomics, Proteomics & Bioinformatics 13, 336–344. https://doi.org/10.1016/j.gpb.2015.12.001
45. Mody, I., 2001. Distinguishing Between GABAA Receptors Responsible for Tonic and Phasic Conductances. Neurochem Res 26, 907–913. https://doi.org/10.1023/A:1012376215967
46. Monesson-Olson, B., McClain, J.J., Case, A.E., Dorman, H.E., Turkewitz, D.R., Steiner, A.B., Downes, G.B., 2018. Expression of the eight GABAA receptor α subunits in the developing zebrafish central nervous system. PLOS ONE 13, e0196083. https://doi.org/10.1371/journal.pone.0196083
47. Montarolo, F., Martire, S., Perga, S., Spadaro, M., Brescia, I., Allegra, S., De Francia, S., Bertolotto, A., 2019. NURR1 deficiency is associated to ADHD-like phenotypes in mice. Transl Psychiatry 9, 207. https://doi.org/10.1038/s41398-019-0544-0
48. Mullins, JonathanG.L., Chung, S.-K., Rees, M.I., 2010. Chapter 4 - Fine architecture and mutation mapping of human brain inhibitory system ligand gated ion channels by high-throughput homology modeling, in: Donev, R. (Ed.), Advances in Protein Chemistry and Structural Biology. Academic Press, pp. 117–152. https://doi.org/10.1016/B978-0-12-381264-3.00004-7
49. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F., 2013. Genome engineering using the CRISPR-Cas9 system. Nature Protocols 8, 2281–2308. https://doi.org/10.1038/nprot.2013.143
50. Reyes-Nava, N.G., Yu, H.-C., Coughlin, C.R., Shaikh, T.H., Quintana, A.M., 2020. Abnormal expression of GABAA receptor subunits and hypomotility upon loss of gabra1 in zebrafish. Biol Open 9. https://doi.org/10.1242/bio.051367
51. Sacchetti, P., Carpentier, R., Ségard, P., Olivé-Cren, C., Lefebvre, P., 2006. Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1. Nucleic Acids Res 34, 5515–5527. https://doi.org/10.1093/nar/gkl712
52. Sadamitsu, K., Shigemitsu, L., Suzuki, M., Ito, D., Kashima, M., Hirata, H., 2021. Characterization of zebrafish GABA A receptor subunits. Scientific Reports 11, 6242. https://doi.org/10.1038/s41598-021-84646-3
53. Samarut, É., Swaminathan, A., Riché, R., Liao, M., Hassan-Abdi, R., Renault, S., Allard, M., Dufour, L., Cossette, P., Soussi-Yanicostas, N., Drapeau, P., 2018. γ-Aminobutyric acid receptor alpha 1 subunit loss of function causes genetic generalized epilepsy by impairing inhibitory network neurodevelopment. Epilepsia 59, 2061–2074. https://doi.org/10.1111/epi.14576
54. Sertori, R., Trengove, M., Basheer, F., Ward, A.C., Liongue, C., 2016. Genome editing in zebrafish: a practical overview. Briefings in Functional Genomics 15, 322–330. https://doi.org/10.1093/bfgp/elv051
55. Smidt, M.P., Peter, J., Burbach, H., 2009. Terminal Differentiation of Mesodiencephalic Dopaminergic Neurons:, in: Pasterkamp, R.J., Smidt, M.P., Burbach, J.P.H. (Eds.), Development and Engineering of Dopamine Neurons, Advances in Experimental Medicine and Biology. Springer, New York, NY, pp. 47–57. https://doi.org/10.1007/978-1-4419-0322-8_4
56. Thiele, T.R., Donovan, J.C., Baier, H., 2014. Descending control of swim posture by a midbrain nucleus in zebrafish. Neuron 83, 679–691. https://doi.org/10.1016/j.neuron.2014.04.018
57. Valeeva, G., Tressard, T., Mukhtarov, M., Baude, A., Khazipov, R., 2016. An Optogenetic Approach for Investigation of Excitatory and Inhibitory Network GABA Actions in Mice Expressing Channelrhodopsin-2 in GABAergic Neurons. J. Neurosci. 36, 5961–5973. https://doi.org/10.1523/JNEUROSCI.3482-15.2016
58. Varshney, G.K., Pei, W., LaFave, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M., Harper, U., Huang, S.C., Prakash, A., Chen, W., Sood, R., Ledin, J., Burgess, S.M., 2015. High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. Genome Res 25, 1030–1042. https://doi.org/10.1101/gr.186379.114

59. Volakakis, N., Joodmardi, E., Perlmann, T., 2009. NR4A orphan nuclear receptors influence retinoic acid and docosahexaenoic acid signaling via up-regulation of fatty acid binding protein 5. Biochem Biophys Res Commun 390, 1186–1191. https://doi.org/10.1016/j.bbrc.2009.10.116
60. Wada, H., Kawakami, K., 2015. Size control during organogenesis: Development of the lateral line organs in zebrafish. Development, Growth & Differentiation 57, 169–178. https://doi.org/10.1111/dgd.12196
61. Yi, S.-H., He, X.-B., Rhee, Y.-H., Park, C.-H., Takizawa, T., Nakashima, K., Lee, S.-H., 2014. Foxa2 acts as a co-activator potentiating expression of the Nurr1-induced DA phenotype via epigenetic regulation. Development 141, 761–772. https://doi.org/10.1242/dev.095802
62. Zetterström, R.H., Solomin, L., Jansson, L., Hoffer, B.J., Olson, L., Perlmann, T., 1997. Dopamine neuron agenesis in Nurr1-deficient mice. Science 276, 248–250. https://doi.org/10.1126/science.276.5310.248
63. Zhang, F., Wen, Y., Guo, X., 2014. CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet 23, R40-46. https://doi.org/10.1093/hmg/ddu125
64. Zhu, P., Narita, Y., Bundschuh, S.T., Fajardo, O., Schärer, Y.-P.Z., Chattopadhyaya, B., Bouldoires, E.A., Stepien, A.E., Deisseroth, K., Arber, S., Sprengel, R., Rijli, F.M., Friedrich, R.W., 2009. Optogenetic Dissection of Neuronal Circuits in Zebrafish using Viral Gene Transfer and the Tet System. Front Neural Circuits 3, 21. https://doi.org/10.3389/neuro.04.021.2009