SUMMER RESEARCH INTERNSHIP REPORT

NEURAL CIRCUITS AND DEVELOPMENT LAB, NATIONAL CENTRE FOR BIOLOGICAL SCIENCES.

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PREFACE

This report is document of all the activities and experiments performed in Neural Circuits and Development Laboratory, National Centre for Biological Sciences, Bangalore under the guidance of Dr. Vatsala Thirumalai.

During my stay I have worked on 2 parallel projects. The first project was designed to investigate the role of AUTS2 gene on the Dopmainergic system in Zebrafish Larvae by performing the Immunohistochemistry of Tyrosine Hydroxylase. Till now there is no known study on the effect of the knockdown of the AUTS2 gene on the dopmainergic system. This study was done to check the effect on the sizes of dopaminergic neurons in forebrain, pretectum and hindbrain.The second project involved the detection of calcium activity of reticulospinal neurons responsible for descending pathways. The model organism was chosen to be zebrafish and the imaging was performed using an epifluorescence microscope. This was done with help of novel visual stimuli due to which the larva sends motor signals to carry out its swimming activity via reticulospinal pathway.

<u>PREFACE</u>

<u>CONTENTS</u>

EFFECT OF AUTS2 GENE KNOCKDOWN ON DOPAMINERGIC SYSTEM

INTRODUCTION

The discovery of the AUTS2 gene and its effect on the morphological changes post knockdown opened a series of questions about the role of AUTS2 gene in the developmental neurobiology. The functional role of AUTS2 gene is not yet known thoroughly, though it has been established that its knockdown induces symptoms similar to autism spectrum disorder and hence derives its name from *Autism Susceptibility Candidate 2.*

Gene knockdown has been a favourite tool for Developmental Biologists to study the effect of the particular gene. A similar effort has been made in Nadav Ahituv Lab, Univesity of California, San Francisco to study its effect on Zebrafish Larvae. The results of the knockdown resulted in smaller head size, neuronal reduction and decreased mobility. This recent paper was a breakthrough in establishing the repercussions of knocking down the gene and hence suggesting its role in neuronal development and axon guidance. The decreased mobility was measured by calculating the latency in the response after a puff of water was ejected near its aortic vescicle.

This paper opened a door for a pool of questions for developmental scientists regarding the effect of AUTS2 knockdown. The particular question we were interested in was that how exactly is the AUTS2 gene responsible for the development of the Dopminergic system. If it is indeed interfering with the development of the dopmainergic system, then is it affetcting all the neurons or is it specific and say if it is specific, then what are the changes in the behavior .

The model organism for carrying out the experiments to find out the answers of the questions we posed was chosen to be Zebrafish. Zebrafish has turned to be the new labrat in these recent years. The advantages being that it is transparent and hence its easy for non-invasive imaging. It is a vertebrate and has small number of neurons as compared to mice. The developmental period is quite fast compared to mice and hence the relevant experiments can be performed frequently.

IMMUNOHISTOCHEMISTRY OF TYROSINE HYDROXYLASE

Immunohistochemistry is the process of the detecting antigen of interest by introducing the antibodies that bind to it. A secondary antibody containing a flurophore is introduced which binds to the primary antibody which on excitation at a particular wavelength fluoresces. It is widely carried out in basic research to understand the localisation and distribution of the biomarkers in tissue of interest.

The current task proceeded with the immunohistochemistry of tryrosine hydroxylase which is also known as tyrosine-3-monooxygenase. It is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). It does so using molecular oxygen (O_2), as well as iron (Fe^{2+}) and tetrahydrobiopterin as cofactors. L-DOPA is a precursor for dopamine, which, in turn, is a precursor for the important neurotransmitters norepinephrine (noradrenaline) and epinephrine (adrenaline). Tyrosine hydroxylase catalyzes the rate limiting step in this synthesis of catecholamines.
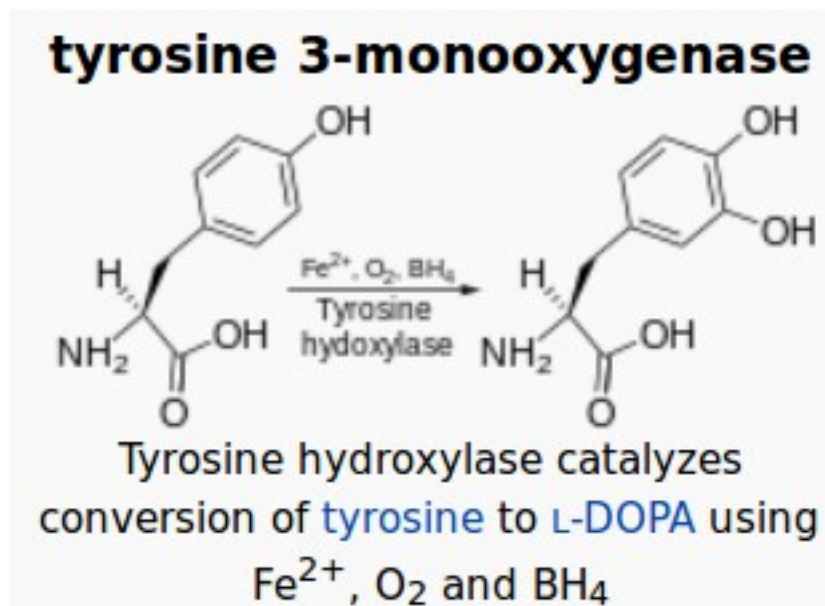

Fig 1: *Conversion of L-Tyrosine to L-Dopa in the presence of Tyrosine hydroxylase*

Since Tyrosine hydroxylase is essential for the conversion of L-Tyrosine to L-DOPA, it is an indirect measure of the dopmaine producing cells. This labeling was done using the primary antibody *Anti Tyrosine Hydroxylase.*

PROCEDURE

The gene knockdown was performed using the morpholino. Morpholino are oligos of nearly 25 nucleotides that hamper the gene expression either by binding to the mRNA so that other molecules does not bind to it or by interfering with the splicing of the pre-mRNA. It is a commonly used research tool for reverse genetics. The larvae were chosen to be IN-WT (Indian Wildtype). The immunohistochemistry was carried out in 3 different sets of zebrafish larvae in each batch. The 3 batches were UNINJ, SCRA and AUTS2. The UNINJ represents the control larvae in which no morpholinom was injected. The SCRA represents the larvae in which the AUTS2 morpholino was injected after chaging few base pairs typically 5. This step was essential to observe if there is an downstream effect on the zebrafish larvae even after changing the sequence of base pairs. The AUTS2 represents the set of larvae injected with AUTS2 morpholino to knockdown the gene function.

1) The morpholino for the microinjection was mixed with 0.01% Fast Green. The fast green was added so that it can be visible during the microinjection. After that the fluorescence of the morpholino mixture was verified in epifluorescence microscope using a green filter.

2) Microinjection was carried out in single to 8 cell stage zebrafish larvae. Each set (UNINJ, SCRA, AUTS2) contained nearly 20-30 larvae. The microinjection was carried out using a P-0 needle programmmed by Sutter instrument. The pressure injection was done using Picospritzer at a pressure of 20 *psi* for 20 *msec* for each puff. A standard of 2 puff of morpholino per egg was injected. The morpholino was injected into the yolk sac.

3) After the microinjection the, the volume of the morpholino injected was calibrated using a micrometer slide. For this a drop of oil was put on the micrometer slide and 2 puffs of morpholino were injected on the surface of the oil. The morpholino would form a sphere. The volume of the drop can be calculated by measuring the radius of the drop from the readings on the micrometerslide using a microscope.

Batch No.	AUTS2	SCRA
1	0.86 nL	0.86 nL
2	3.14 nL	3.14 nL
3	2.144 nL	5.5 nL
4	0.7 nL	0.7 nL

Table 1: *List of volume of morpholino injected*

4) The larvae are stored in petridish in E3 medium. The petridish containing AUTS2 and SCRA were covered in a aluminium foil. It was made sure that the medium is changed daily.

5) Screening was performed the next day. Only those larvae were selected which showed fluorescence throughout and not just in the yolk sac. The screening was done using the epifluorescence microscope in a green filter.

6) At 4 dpf (days post-fertilisation), the larvae were anasthesised in 0.02% MS222 (Tricane) and were then put for fixing in 4% PFA (Paraformaldehyde) in separate eppendorf (each for UNINJ, AUTS2, SCRA). The eppendorfs were stored in 4 degree freezer for overnight.

7) Next day the larvae were taken out and rinsed multiple times using PBST (for permeabalising the cells). This is followed by depigmentation in 3% hydrogen peroxide + 0.5% KOH in PBS. This step is essential for removing the skin pigments and hence useful for imaging non-invasively. After the PFA has been washed thoroughly, the larvae are put in 5% NDS solution with PBST (blocking agent) overnightly.

8) Next, the anti-mouse antibody *Anti-Tyrosine Hydroxylase* with a dilution of 1:1000 in blocking agent is introduced in the all the eppendorfs containing the larvae. The larvae are kept in 4 degree freezer for 48 hours.

9) After 48 hours, the larvae are taken out of the freezer and washed with PBST 5-7 times. This is followed by introduction of secondary anti-mouse antibody either DL649 or DL488 (649 and 488 are the excitation wavelengths of the chromophores of the secondary antibody) to the larvae which was covered with aluminum foil and kept for 24 hours.

10) 24 hours post the introduction of secondary antibody, the larvae are washed with PBS atleast 5 times. This was followed by mounting the larvae in the cover slide with a ProLong Gold Solution to prevent the larvae from bleaching during imaging.

11) The larvae were imaged using Olympus Fluoview confocal microscope with a laser of excitation wavelenght of either 488 nm or 649 nm depending upon the secondary antibody. A 40x objective lens was used with further 2x magnification to focus on the intraregions of the brain.

RESULTS

The AUTS2 deficient larvae had observable morpholical changes. They had abnormally large yolk sac and the tail end was curved. The larvae were in general smaller in size compared to their UNINJ and SCRA counterpart.

The images were grouped into 3 sets : Forebrain, pretectum (rostral to Neuropil, Optic Tectum) and hindbrain. Though there are various types of dopaminergic neurons classified on the basis of size, shape, etc, our observation led us to divide neurons on the basis of morphology into 3 distinct types of neurons : small round shaped(type 1), large pear shaped (type 2) and bipolar (type 3) which is consistent with the results of the work of Rink and Wulliman.
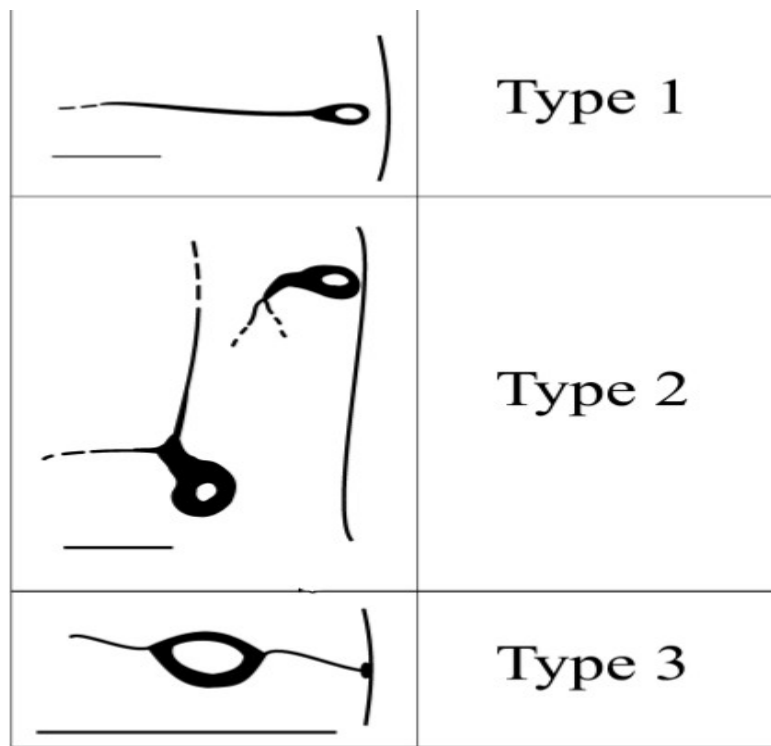

Fig 2 : *Different types of dopaminergic neurons in zebrafish brain*

Ref :"The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum)." Elle Rink, Mario.F.Wullimann, Brain Research Institute, University of Bremen,2000

After the confocal image stacks were obtained, they were carefully analysed. Since we our interested in the effect of AUTS2 knockdown on the size of neurons, we calculated the cross sectional area of individual neurons using the image processing software "FIJI".

Shown below are the confocal images chosen from the stack of different regions in the zebrafish brain:
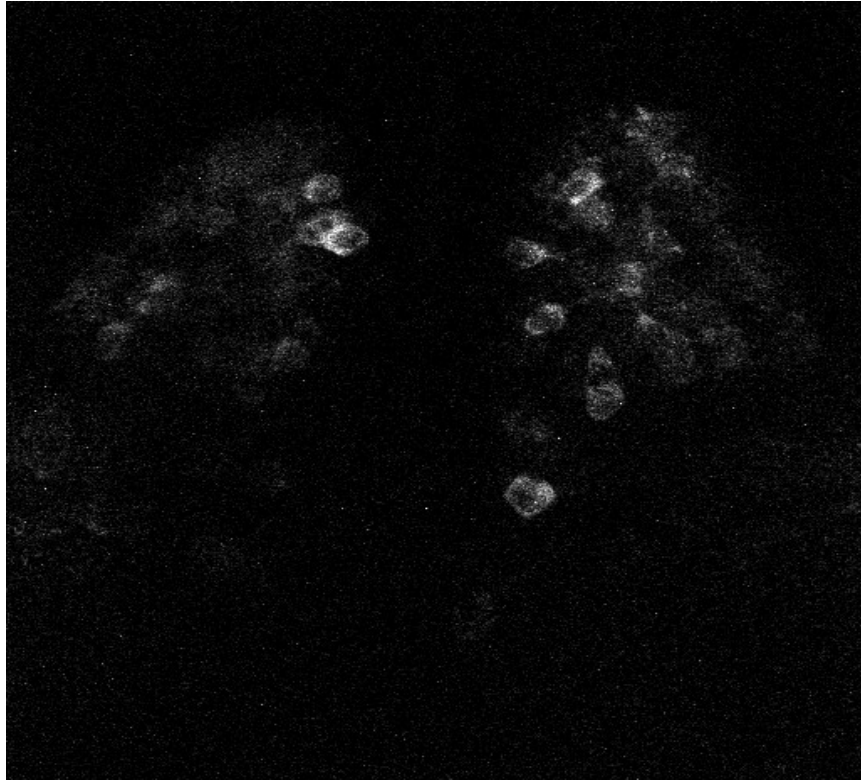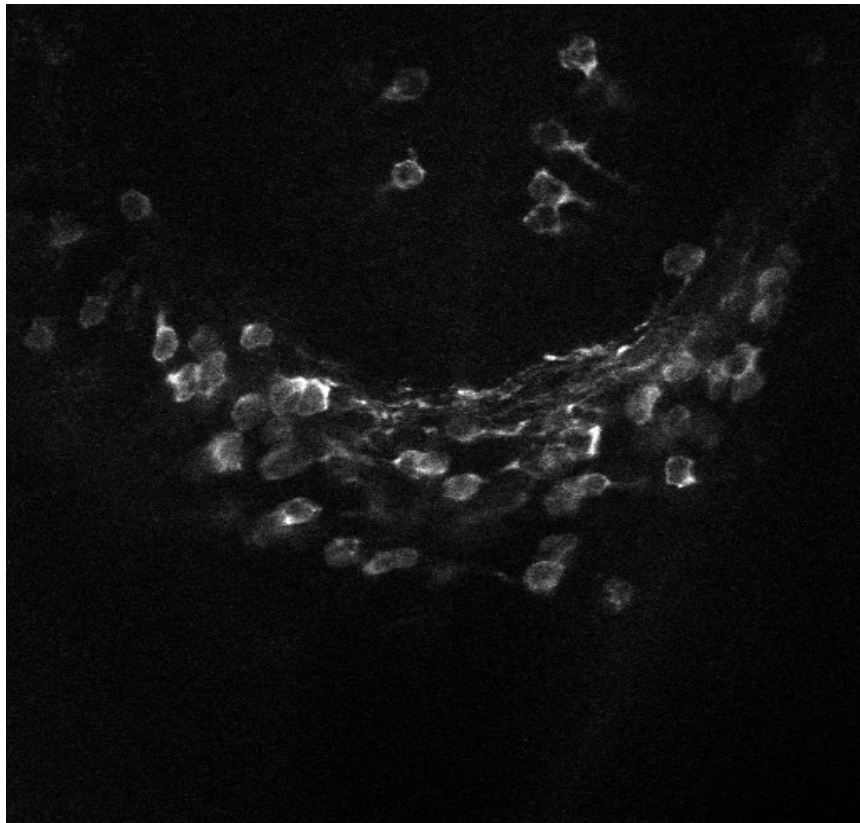
Fig 3: *Forebrain*

Fig 4 :*Hindbrain*

Larva ID	Forebrain Mean Area	Std. Dev	mber of Neurc
AUTS2-I1 (b1)	32.37072	5.305082207	50
AUTS2-I2 (b1)	33.70272727	7.07166341	33
AUTS2 (b2)	35.62479212	6.007029472	34
AUTS2-I1 (b3)	36.58114286	7.147795506	35
AUTS2-I2 (b3)	43.92036111	7.30017376	36
AUTS2-I3 (b3)	38.983	5.040790226	20
AUTS2-I1 (b4)	38.79233333	7.5757123	27
AUTS2-I2 (b4)	34.39575	6.2222243335	36
AUTS2-I3 (b4)	31.371875	8.030091407	40
AUTS2-I4 (b4)	34.33510714	8.503856447	28
AUTS2-I5 (b4)	36.06154167	6.384638079	48
AUTS2-I6(b4)	33.17362222	5.388264809	45
SCRA-I2 (b1)	32.0725	8.411349962	60
SCRA-I3 (b1)	31.224117	6.762736386	60
SCRA-I1 (b2)	27.61103947	4.6497008	76
SCRA-I2 (b2)	30.64812903	5.111375396	62
SCRA-I3(b2)	28.50874286	5.582878418	35
SCRA-I1(b4)	30.62119444	5.131130991	36
SCRA-I2(b4)	32.51697561	5.41812047	41
SCRA-I3(b4)	34.62646341	6.882672715	41
UNINJ-I2 (b1)	37.10377049	8.141542044	61
UNINJ-I3 (b1)	30.15127692	5.484340746	65
UNINJ-I1 (b2)	41.97285714	7.878247167	21
UNINJ-I2 (b2)	28.47233333	4.553356564	54
UNINJ-I3 (b2)	34.84903333	6.587727814	30

Table 2: *Mean size of all the neurons in different larvae in forebrain. The ID in the bracket denotes the batch.*

Larva ID	Pretectum Mean Area	Std. Dev	mber of Neurc
AUTS2-I2 (b1)	30.0286	6.951988696	15
AUTS2-I3 (b1)	33.60590476	8.705027311	21
AUTS2-I4 (b1)	33.65590909	4.373630768	11
AUTS2 (b2)	43.32372727	7.464171712	22
AUTS2-I1 (b3)	40.57471429	7.266242415	14
AUTS2-I2 (b3)	47.470471429	4.785307086	7
AUTS2-I1 (b4)	54.2197	10.27518594	20
AUTS2-I2 (b4)	42.8305625	6.504581944	16
AUTS2-I3 (b4)	43.46125	8.075494125	20
AUTS2-I4 (b4)	59.20970833	9.66617698	24
AUTS2-I5 (b4)	57.9603125	10.39239578	16
AUTS2-I6(b4)	51.8699375	12.24885778	16
SCRA-I1 (b1)	42.06242857	9.167500845	28
SCRA-I2 (b1)	42.75363636	13.35551679	22
SCRA-I3 (b1)	45.34688235	15.05911181	17
SCRA-I1 (b2)	30.74167561	3.652866797	37
SCRA-I2 (b2)	44.59035135	9.7231129	37
SCRA-I3(b2)	29.65006667	5.289539475	30
SCRA-I1(b4)	58.9057037	11.97301845	27
SCRA-I2(b4)	49.60945455	6.733873149	22
SCRA-I3(b4)	49.2224	7.507133273	15
UNINJ-I1 (b1)	38.8585	8.374627213	38
UNINJ-I2 (b1)	38.50625	8.098520418	40
UNINJ-I3 (b1)	45.085	10.20367364	22
UNINJ-I1 (b2)	51.64346429	9.410699023	28
UNINJ-I2 (b2)	47.38078571	9.786650554	28
UNINJ-I3 (b2)	43.76342424	9.108844305	33

Table 3: *Mean size of all the neurons in different larvae in pretectum. The ID in the bracket denotes the batch.*

Larva ID	Hindbrain Mean Area	Std. Dev	mber of Neurc
AUTS2-I1 (b1)	29.80236	6.499517382	50
AUTS2-I2 (b1)	34.95564286	8.249293322	56
AUTS2-I3 (b1)	30.18201149	4.923422251	87
AUTS2-I4 (b1)	33.06	6.58589626	33
AUTS2 (b2)	33.58417647	5.684274352	17
AUTS2-I1 (b3)	26.88458974	5.074401479	78
AUTS2-I2 (b3)	32.48579167	5.769269052	24
AUTS2-I3 (b3)	35.04442857	5.099145395	21
AUTS2-I1 (b4)	33.62935294	8.124852237	17
AUTS2-I2 (b4)	27.64384375	4.332474567	32
AUTS2-I3 (b4)	22.8205333	4.365722242	15
AUTS2-I4 (b4)	31.879	5.684058068	33
AUTS2-I5(b4)	26.63573333	4.507035947	30
AUTS2-I6(b4)	26.14334783	3.871027602	23
AUTS2-I7(b4)	32.70611111	5.527896694	9
SCRA-I1 (b1)	29.21827555	6.55856594	98
SCRA-I2 (b1)	31.95943038	8.496552341	79
SCRA-I3 (b1)	35.60758333	9.461885141	24
SCRA-I1 (b2)	25.28318421	4.872018354	76
SCRA-I2 (b2)	24.41281481	3.720231228	27
SCRA-I2(b4)	32.88553333	6.761963714	15
UNINJ-I1 (b1)	33.3962	8.586970374	5
UNINJ-I3 (b1)	33.38797778	7.220066512	45
UNINJ-I1 (b2)	32.01133333	6.790921629	48
UNINJ-I2 (b2)	29.92638636	5.283500906	44
UNINJ-I3(b2)	29.44531915	5.775732139	47

Table 4: *Mean size of all the neurons in different larvae in hindbrain.*
The ID in the bracket denotes the batch.

From the table we can note that there is no significant change in the size of the neurons present in the pretectum, forbrain and the hindbrain region. Calculating the mean and standard deviation gives little information for a concrete inference and therefore it is necessary to consider the sizes of all the neurons and plot their probability distribution curve.

FUTURE DIRECTIONS

The plotting of the probability distribution curve will give an idea of the kind of statistical analysis needed to extract the information. If the distribution is close to normal distribution, then paired t-test can be performed or pearson's correlation coefficient can be calculated. The correlation coefficient can give us an idea about how different is the distribution of the AUTS2 larvae from the SCRA and UNINJ.

On the other hand, if the distribution is not a normal distribution, then non-parametric statistical analysis can be performed based on it ranking. One of the popular tests is the Wilcoxon rank-sum test which is a paired t-test for non-normal distribution. The statistical analysis will only higlight the morphological changes and not the amount of dopamine produced. This can be verified by performing behavioral tests to correlate the changes in the morphology with that of the behavior deficits.

MEASUREMENT OF CALCIUM ACTIVITY IN ZEBRAFISH LARVA

INTRODUCTION

Non-invasive imaging of the neural networks has emerged out as an useful tool lately for studying network activity pertaining to some stimuli. The information encoded in the spikes can be extracted as to how each neuron's signal is weighted and integrated to perform any motor action. Since we are talking about non-invasive imaging here, zebrafish turns out to be an ideal model organism because of its transparent nature with imaging can be performed at ease. The behavioral experiment performed here was intended to study the calcium activity of the reticulospinal neurons by imaging and correlating the calcium spikes with that of the swimming behavior.

PROCEDURE

1) The larvae were transferred to a medium containing 0.0045% PTU (N-Phenyl Thiourea) solution at 1dpf to inhibit the melanin production. This step was necessary in order to achieve a transaprent larvae. Usually 20-25 larvae were put in a batch. The survival figure was 100%.

2) The backlabeling was performed on 4-7 dpf larvae with OGB-Dextran 1000MW. The dye was injected in spinal cord after puncturing it. Once the dye is injected, the motors in the neurons carry the dye retrogradely to the reticulospinal spinal neurons responsible for motor signaling at the site of injection. The larvae were kept in incubator at room temperature in a 24 well plate in 10% Hank's solution. Each backlabeling batch consisted of 8-12 larvae.

3) After 24 hours, the larvae were taken out and mounted in a 2% low melting agarose. 10% Hank's solution was poured immediately after the agarose cooled down. A small portion of agarose was cut such that the tail was left free but it was made sure that the head was fixed.

4) This was followed by loading the fish in a petridish with a diffuser pasted on the surface of it so that it diffuses the intensity of visual stimuli. This step in essential to make sure that larvae does not go blind.

5) Next, the labeled reticulospinal neurons were excited with a blue light with a wavelength of 488 nm and the activity was observed by measuring the change in the intensity of the light received through the dichroic mirror. A dichroic mirror allows light of higher wavelenght to pass through it and stops a light of low wavelenght. Here the transmitted blue light is of higher wavelength

than the received green light. A 60x objective lens was used to focus on the reticulospinal neurons.
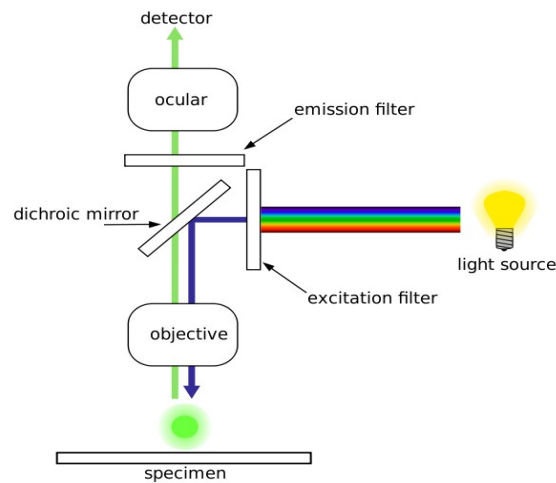

Fig 5 : Epifluorescence Microscope

6) Simultaneously a visual stimuli was provided to the larva. The response was tested against 3 different stimuli : small dots, large dots and gratings. The small dots give the impression of paramocieum (food), the large dots signify predators and hence induce escape responses in the larva. The gratings represent a relative movement of the water stream beneath and hence instructs indirectly to swim so as to keep the pace with stream flow. The grating was designed such that its pace was nearly 1cm/s. All the visual stimuli were designed using the psychopy toolbox, Python. The visual stimuli was projected onto the diffuser by a projector and " ImagePro" was used for capturing the duration within which the visual stimuli was presented.
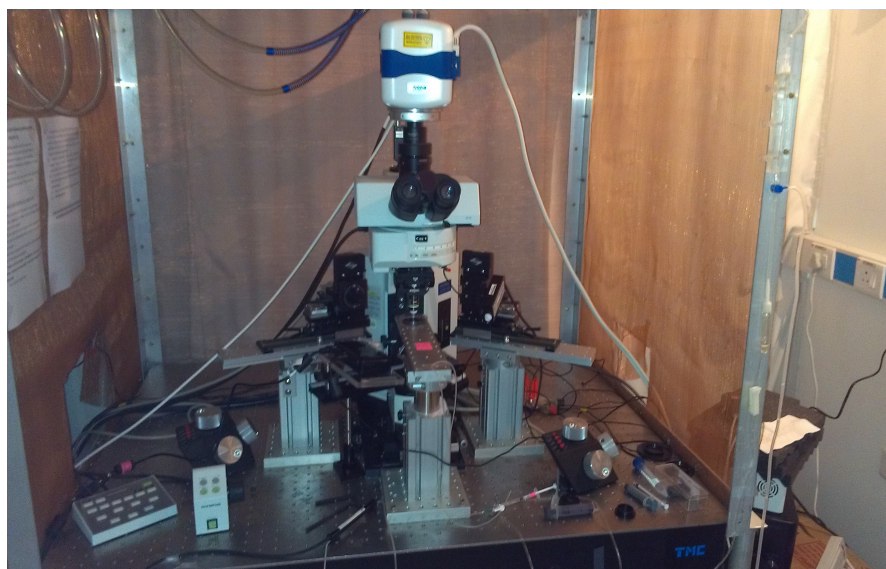

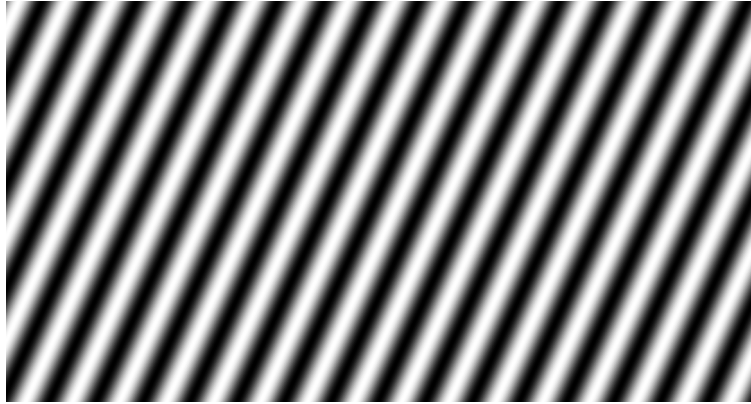*Fig 6: Experiment Setup for the measurement of calcium activity*

STIMULI

Fig 7 : *Grating Stimulus. Different orientations were given to to investigate the changes in the response.*

Fig 8: *Small dots which gives the impression of food to the larva making the larva swim towards the dots.*
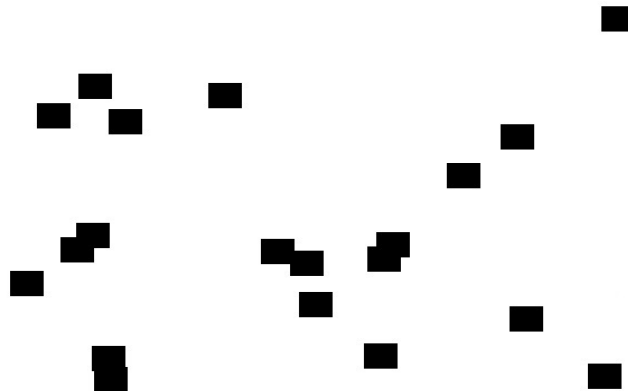

Fig 9: *Large dots which represents predatory attack and elicits escape response making the larvae swim violently.*

RESULTS

The reticulospinal neurons in the hindbrain got succesfully labeled and the calcium activity to the visual stimuli are shown below. As mentioned previously, all the stimui were tried one by one to observe the behavior of the larva.
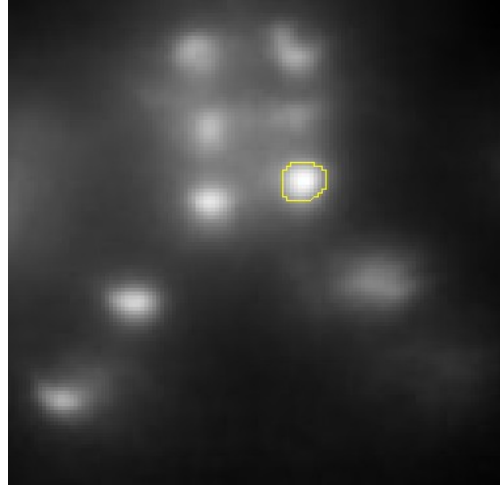

Fig 10*: The labeled reticulospinal neurons present in the hindbrain of the zebrafish larva. Below is the calcium activity of the circled neuron.The movie was captured at 21 fps.*
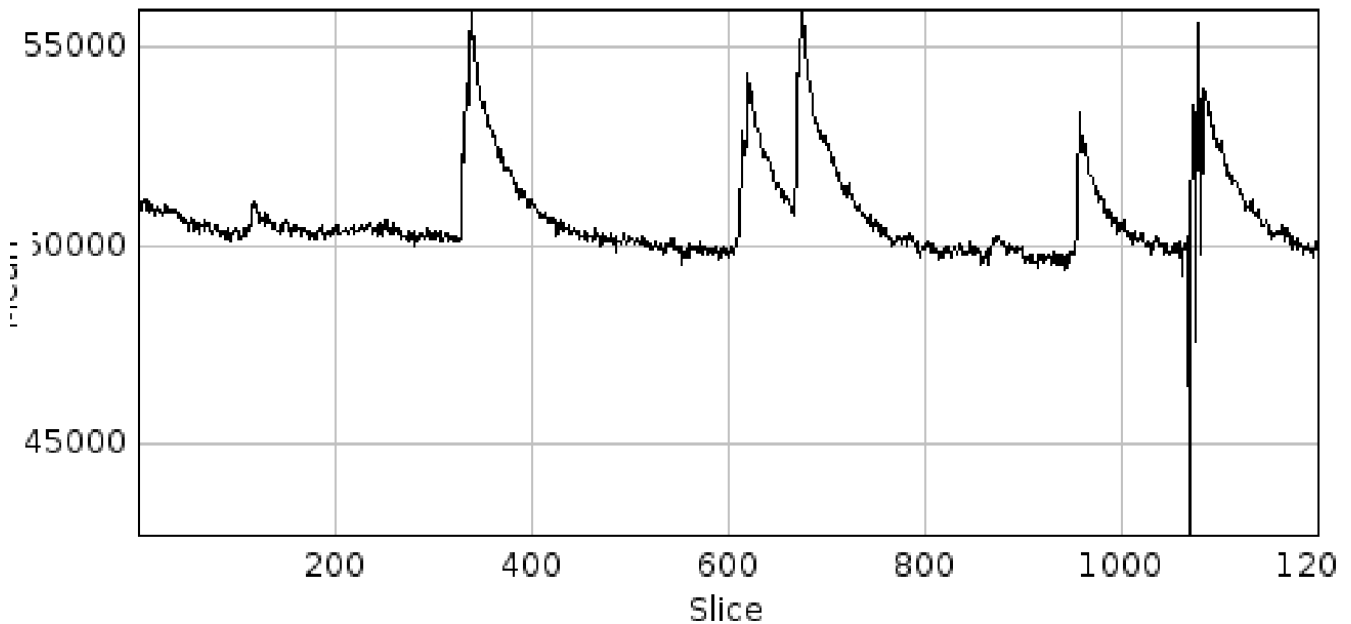

Fig 11: *Below is the calcium spiking activity obtained in response to the small dot stimuli. This was framed at 21fps.*

FUTURE DIRECTIONS

The next step in this experiment would be to paralyse the larvae. Although effort was made to keep the larva's head fixed, the motion artifacts were not completely eradicated. The artifact removal can be done in 2 ways. One way would be to use "Turboreg" plugin offline in FIJI by initialising the first frame as the reference frame. The other way would be to use Bungarotoxin to paralyse the larva. This will require the standardisation of the Bungarotoxin dose that has to be injected into the larva.

Along with this an electrophysiological study could be performed which will correlate how the spiking calcium activity in the reticulospinal neurons to the activity of the muscles at the point of injection. This will require an introduction of a bipolar electrode for muscle potential recording.

An effort can be made to extend this study for different stimuli for example say electrical stimuli. This can be done by injecting a small amount of current near the autolith region near the midbrain of the zebrafish and study the behavior.

In all, a lot can be done to extend this experiment which will surely give an insight into the computations happening within the reticulospinal neurons.

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