Neural Circuitry underlying feeding behavior

BS-MS thesis

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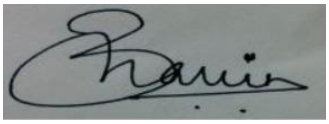
Certificate

This is to certify that this dissertation entitled “Neural mechanisms underlying feeding behavior” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Mr. Bhavin Choksi at IISER, Pune under the supervision of Dr. Aurnab Ghose, Associate Professor, Biology Department during the academic year 2017-2018.



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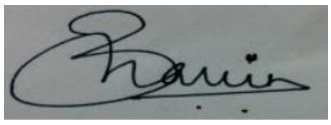
Declaration

I hereby declare that the matter embodied in the report entitled “Neural mechanisms underlying feeding behavior” are the results of the investigations carried out by me at the Department of Biology, IISER Pune under the supervision of Dr. Aurnab Ghose and the same has not been submitted elsewhere for any other degree.



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Abstract

Feeding is a complex behavior having a robust and conserved underlying neural circuitry. Different regions of the brain take part in regulating this behavior along with myriad signaling molecules like orexigenic neuropeptide Y (NPY) and anorexigenic Cocaine and amphetamine-related transcript (CART). Knowing the regions in the brain that express the receptors to such signals allows for identifying regions playing a role in regulating energy homeostasis. Hence, we attempted to map the regions in the zebrafish brain that express Y1 mRNA using *in situ* hybridization to elucidate the neural circuitry underlying the regulation of feeding.

Sensing glucose in the blood is a crucial step in knowing the energy state of the body. It also allows modulation of various processes to ensure energy homeostasis. Various methods for sensing glucose in the blood, apart from the classical glucokinase-dependent methods, have been identified in vertebrates. Glucose sensing via sodium-glucose cotransporters (SGLTs) is one such glucokinase-independent mechanism which is not well studied in zebrafish. Hence, we attempted to look at the role of SGLTs in glucose sensing in the zebrafish brain.

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Chapter 1
Introduction

Living organisms show a wide range of behaviors, varying from simple response towards light to complex problem-solving. These behaviors are controlled by the brain depending upon a large number of factors. Some behaviors are learned over time while others are innate to the species. Some behaviors arise solely due to the internal state of the animals. The maintenance of the circadian rhythms in the absence of light is a popular example. Various other behaviors are elicited depending upon the external clues received, like freezing of rats if they sense any danger. This huge pool of behaviors is under constant evolutionary pressure. The behaviors which positively impact the ability of the organism to find food, water and mating partner have the highest impact on the survival value and are positively selected for during evolution.

A complete understanding of any behavior will beg the understanding of the neural circuitry in the brain which plays a role in its decision making. What were regions in the brain involved in this process? What signals did they sense? What factors made them chose a particular behavior over others? How was the execution command ultimately relayed? How was the real-time feedback incorporated in the decision making? Answers to such questions will provide a complete understanding of the behavior and open the ground for manipulations to cure any pathological conditions.

Feeding is an important behavior which provides energy to the organism to carry out different tasks. It is defined as the ‘combination of foraging, food ingestion and related appetitive behaviors that reflect the motivation to consume food’ (Volkoff et al., 2016). Feeding provides an important substrate for energy metabolism and is a crucial part of energy homeostasis. Neural circuits underlying feeding, thus are evolutionarily conserved and have common component across various species.

1.1 Energy homeostasis

Regulation of the energy, or energy homeostasis, is controlled by the neural circuitry in the brain with the help of the sensory stimulus and neuroendocrine signals from the periphery. Different regions of the brain play a role in this complex phenomenon and try to regulate the metabolism rate, feeding behavior, sensitivity to sensory stimuli to various cues in different organs or tissues in the body.

1.2 Neural circuitry underlying feeding: an insight from mammals

Substantial research has been undertaken to understand the neural circuitry regulating food intake in mammals. Hypothalamus has been recognized as a key player in the regulation of energy homeostasis. Hypothalamus is a conserved brain region and has different nuclei of cells which are responsive to different levels of varied signaling molecules. These nuclei sense different neurotransmitters and neuropeptides, interact with each other, and other regions in the brain to carry out different physiological and behavioral responses depending upon the energy state of the animal.

Different signaling molecules, both from the periphery and within the brain are involved in this process. Hormones like leptin, ghrelin, and cholecystokinin are sensed by the nuclei in the hypothalamus by their receptors. Leptin is a hormone produced by the adipocytes and acts as a satiety signal. It is believed to be an indicator of the body fat stores (Myers et al., 2008). Insulin is produced by pancreatic β-islet cells and increases the glucose uptake and glycogen synthesis. Ghrelin is released by the stomach and acts as an orexic signal, whereas cholecystokinin is released by intestinal endocrine cells and helps in termination of feeding (Barsh and Schwartz, 2002). Neurotransmitters and neuropeptides like CART (Cocaine and Amphetamine related transcript), NPY (Neuropeptide Y), AgRP (Agouti-related protein) also play a role in appetite control.

Genetic or lesion studies have been used to identify the different nuclei in the hypothalamus involved in the regulation of feeding (Kalra et al., 1999). Key players are Arcuate nucleus (ARC), Dorsomedial Hypothalamus (DMH), Lateral Hypothalamus (LH),

Paraventricular nucleus (PVN), and Ventromedial Hypothalamus (VMH). Initial studies led to the formation of a ‘dual center’ hypothesis in which LH acted as a ‘feeding center’ and VMH acted as a ‘satiety’ center (Anand and Brobeck, 1951).

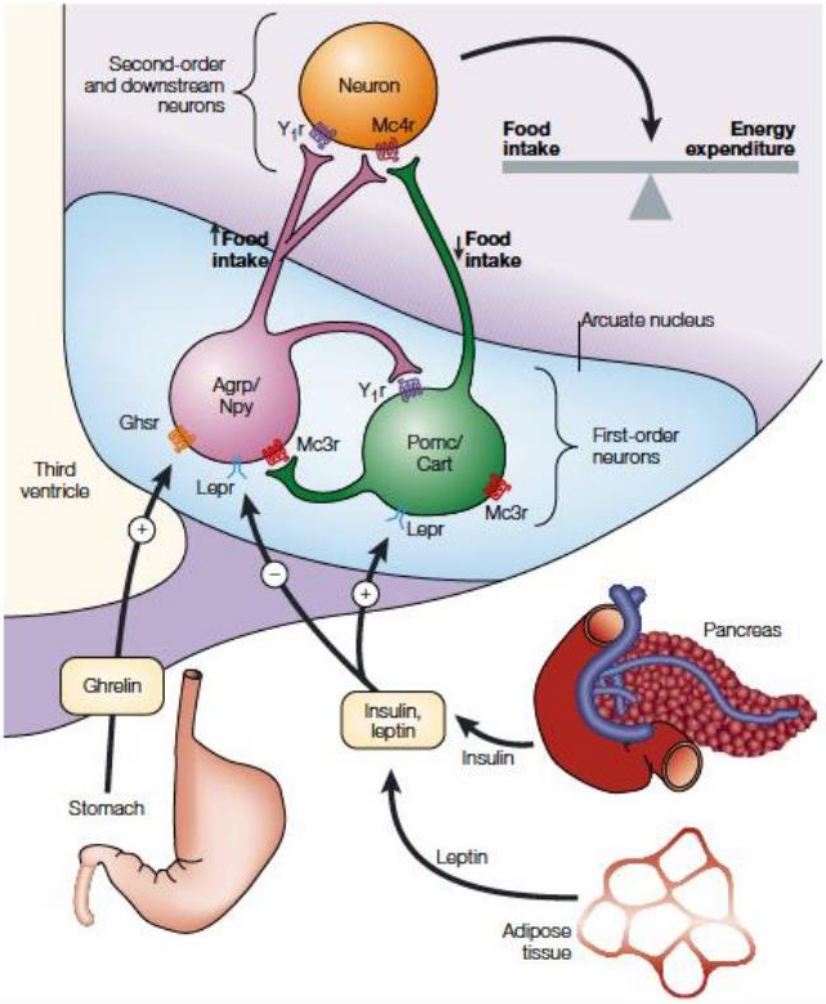


Figure 1: Neural regulation of energy homeostasis: AgRP/NPY and POMC/CART containing neurons in the arcuate nucleus are regulated by the circulating hormones released from the periphery. Leptin and insulin stimulate POC/CART neurons and inhibit AgRP/NPY neurons. Ghrelin activates AgRP/NPY neurons and stimulates food intake. Lepr, leptin receptor; Ghsr: growth hormone secretagogue receptor; Mc3r/Mc4r, melanocortin ¾ receptor; Y1r, one of the receptors of neuropeptide Y. Adopted from (Barsh and Schwartz, 2002).

Arcuate nucleus in the hypothalamus was later identified as an important site for integrating the sensory information regarding the energy state. It contains two distinct groups of cells; cells which co-express AgRP (Agouti-related peptide) and NPY (Neuropeptide Y) and cells that express POMC (Pro-opiomelanocortin). These two groups show opposite effects on the feeding behavior.

AgRP neurons belong to the hunger circuitry. AgRP mRNA increased during fasting in rodents in these neurons (Hahn et al., 1998). Stimulation of these population of neurons also led to increased food intake (Krashes et al., 2011; Aponte et al., 2011) and genetic ablation of these neurons led to rapid starvation (Krashes et al.,2011; Luquet et al., 2005). AgRP/NPY neurons have synaptic connections to areas like POMC neurons in the arcuate nucleus, Paraventricular nucleus (PVN), Bed nucleus of stria terminalis (BNST), Lateral hypothalamus (LH), and Paraventricular Thalamus (PVT). These connections are mostly GABAergic. AgRP neurons can thus actively inhibit the anorexigenic POMC neurons upon activation (Sternson, 2013). AgRP neurons also affect the motivation for seeking food. Activation of AgRP neurons led to frequency and voltage-dependent increase in the motivation to seek food in rodents (Atasoy et al., 2012).

The POMC neurons belong to the satiation circuitry. Neurons expressing POMC and CART are identified to be anorexic in nature, i.e., they reduce the food intake, body weight and heighten the energy expenditure upon activation (Biebermann et al., 2006; Fan et al., 1997; Lee et al., 2006). They are believed to belong to evoke satiety over longer timescales. The PBN (Parabrachial nucleus) is responsible for visceral malaise and inducing satiety on shorter timescales. Activation of PBN region led to decrease in food intake but deactivation of this region did not increase the food intake (Reilly, 1999; Carter et al., 2013).

1.3 Neuropeptide Y system

The neuropeptide Y system consists of three neuropeptides- Neuropeptide Y, Pancreatic Polypeptide (PP), and peptide YY (PYY) and their receptors. In humans, five receptors have been identified, namely Y1, Y2, Y4, Y5, and y6, all of which belong to the family of

G-protein coupled receptors of the rhodopsin family. The neuropeptides have varying distribution and functions. Pancreatic Polypeptide is synthesized by PP pancreatic cells in the islets of Langerhans and stimulates gastric juice secretion (Lonovics et al., 1981). Peptide YY is found in the L cells in the mucosa of the gastrointestinal (GI) tract and in the brainstem. PYY, is anorexic in nature and reduces the speed of gastric mobility to increase the efficiency of digestion and nutrient absorption (Liu et al., 1996).

Neuropeptide Y, only 34 amino acid long, is widely expressed in the brain and is considered to be among the most orexic peptides in the brain (Pedrazzini et al., 2003). Starved rodents depicted an increased NPY mRNA in the arcuate nucleus of the hypothalamus (Sahu et al., 1988). In rats, administration of NPY in the brain increased the food intake three times compared to the increase after human pancreatic polypeptide injection (Clark et al., 1984). NPY injection in the perifornical area of the hypothalamus significantly increased the food intake of the rats to over 12.5 g over baseline at 1 h and 20.0 g at 4 h post injection (Stanley et al., 1993). NPY mRNA was also observed to be significantly increased in the preoptic area of the hypothalamus in chinook and coho salmon upon starvation (Silverstein et al., 1998).

The NPY receptors have varying distribution and different effects on the feeding behavior. Activation of Y1 and Y5 receptors using agonists increased the food intake (Gerald et al., 1996; Hwa et al., 1999; King et al., 1999). Blocking the Y1R using pharmacological antagonist BIBP-3226 ((*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl) methyl] - argininamide) reduced this increase in feeding behavior (Morgan et al., 1998).

In contrast, Y2 or Y4 receptor agonists led to a decrease in the food intake, metabolic rate (Balasubramaniam et al., 2007; Halatchev et al., 2004; McGowan and Bloom, 2004). The Y2 receptor is located in presynaptic membranes in neurons and acts like an autoinhibitor in NPY signaling (Chen et al., 1996)

1.4 Neuropeptide system in zebrafish

Zebrafish as a model organism is gaining more popularity and acceptance due to the ease of genetic amenability, ease of maintenance and high reproduction rate. Furthering

the understanding of the neural circuitry regulating the feeding behavior in zebrafish will allow one to understand the basic and conserved principles involved in regulation of feeding.

The zebrafish NPY system contains three neuropeptides- NPY, PYYa and PYYb along with seven receptors- Y1, Y2, Y4, Y5, Y7, Y8a, and Y8b (Salaneck et al.,2008). Intracerebroventricular administration of NPY in zebrafish led to an increase in the food intake. Pharmacological blocking of Y1 receptor reduced this NPY induced increase in food intake (Yokobori et al., 2012). Starvation increased the NPY mRNA expression in the lateral Hypothalamus in zebrafish (Jeong et al., 2018).

Due to multiple genome duplications in the teleostean lineage, seven NPY receptors in zebrafish have been identified (Salaneck et al., 2008; Fredriksson et al., 2004; Fredriksson et al., 2006; Lundell et al., 1997). Their affinities to the neuropeptides have been studied, but their role in the regulation of appetite control is still not well studied in zebrafish.

1.5 Glucose sensing: current understanding

An important component of energy homeostasis is the regulation of metabolic rate. The body regulates its metabolic rate by sensing the levels of glucose in the blood. Different neural, hormonal and direct nutrient responses are initiated after an exercise or a meal to regulate the glucose concentration in the blood. Areas like hypothalamus, septum, amygdala, striatum, motor cortex, hindbrain in the brain have been shown to get excited after administration of glucose (Levin et al., 2004 a, b; Moran 2010). Pancreatic β-cells also sense the glucose levels in the blood and subsequently produce insulin.

The mechanism of detecting glucose is fairly well understood and involves the uptake of glucose by the low-affinity glucose transporter type-2 (GLUT2) and phosphorylation by glucokinase. Glucose-6-phosphate then undergoes glycolysis and increases in the intracellular ATP:ADP ratio. This leads to the closure of K_{ATP} channels and depolarization of the cell membrane followed by the entry of Ca^{2+} which triggers either neuronal activity

or neurotransmitter release in case of neurons or release of insulin by the pancreatic cells (Polakof, 2011). This mechanism, depended heavily on the availability of glucokinase, is also known as ‘classic glucosensor’ model.

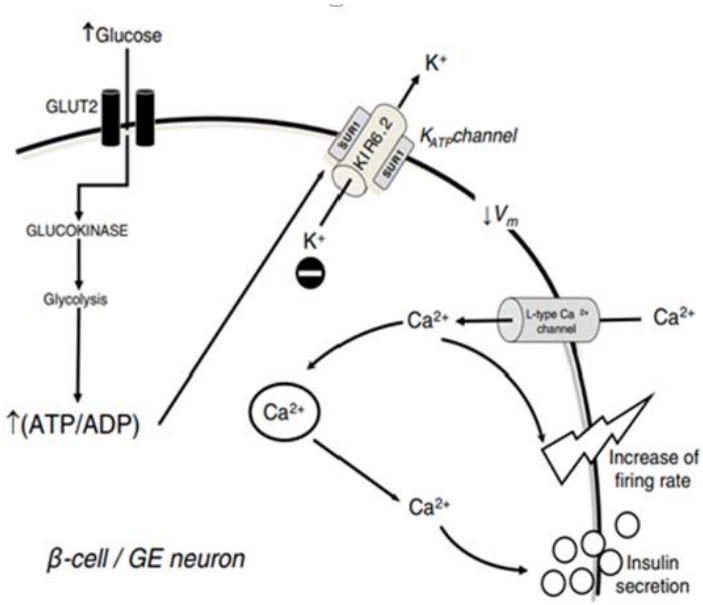


Figure 2: Glucose sensing mechanism in the mammalian pancreatic β-cells and Glucose-excited neurons: Glucose entered in the cell via GLUT2 is phosphorylated by glucokinase. This leads to the production of ATP in the subcellular compartment, which closes K_{ATP} channels in the plasma membrane, causing depolarization. The depolarization leads to Ca^{2+} influx through Ca^{2+} channels, stimulating the release of insulin or the action potential frequency. GLUT2 (glucose facilitative transporter type 2), K_{ATP} (ATP-sensitive inward rectified K^+ channel), Kir6.2 (pore-forming subunit of K_{ATP}), SUR (sulfonylurea receptor). Adapted from Polakof 2011.

Other methods of glucose sensing are also observed in the L-cell of the intestine, hepato-portal vein, carotids, etc. SGLTs were identified to be an important way of glucose sensing in hypothalamic cell cultures (Gonzalez et al., 2009). Sweet taste receptors expressed in the pancreatic B-cells stimulate insulin secretion via cAMP pathway (Nakagawa et al., 2009).

1.6 SGLT:

Sodium-glucose cotransporter was identified to have an important role in renal function. Studies have identified two SGLT isoforms in humans and the hypothesized a mechanism of coupling of sodium and glucose flux for transport of glucose across membranes (Wright, 2001).

In neurons, the sodium-glucose cotransporters (SGLTs) provide an interesting mechanism for sensing glucose. SGLTs transport one or two molecules of glucose along with the sodium influx. This results in overall positive current inwards and thus depolarizes the neurons without the metabolism of glucose (Gribble et al., 2003). Experiments on hypothalamic cultures found that 67% of glucose-excited neurons in the hypothalamus were also excited by α-methylglucopyranoside (α-MDG), a non-metabolizable substrate of SGLTs. This effect was abolished after treatment with phloridzin, an SGLT inhibitor (O'Malley et al., 2006). More studies are necessary to find which SGLT isoform is responsible for this glucose sensing in the brain.

1.7 Objective of the study:

To understand the regions in the brain which can play a role in particular function, one strategy is to find the regions which 'can respond' to the signaling molecules important in that function. Knowing the distribution of receptors allows one to make such a list of regions. Hence, the objective of the study was to find the distribution of NPY Y1 receptor in the zebrafish brain. The study will aid in studies allowing one to look at the role of NPY-responsive region in a particular function in zebrafish. It will also open the ground for evolutionary studies looking at the conservation of the NPY Y1 receptor distribution across species.

Another aim of the project was to look at the role of SGLTs in sensing blood glucose in the adult zebrafish brain. CART is an anorectic peptide and in zebrafish, the number of CART immunoreactive cells in the increase in fed-like condition (Mukherjee et al., 2011). The cells in the Entopeduncular nucleus (EN) show increased CART-ir in fed

state. If SGLTs are involved in the glucose sensing in the zebrafish brain, then blocking SGLTs should impair the capability of neurons to sense glucose levels. Hence, the number of CART-ir cells should not increase even in presence of high glucose. The experiments will give insight about the neural circuitry underlying feeding in zebrafish brain.

Chapter 2
Materials and Methods

2.1 Animal Handling:

Wild-type zebrafish (*Danio rerio*) of the Indian strain were used for all the experiments. They were housed in multiplexed recirculating tanks (Aquatic Habitats, USA) and maintained at 28.5 °C under a 14-10 h light-dark conditions. Optimum water quality ((hardness 100 - 300 mg/L of CaCO3; alkalinity 50 - 300 mg/L of CaCO3; nitrate < 20 mg/ml; pH 6-8; conductivity 180 – 350 µS) was maintained. All the experiments complied with the guidelines of the Institutional Animal Ethics Committee (IAEC) of IISER, Pune, under the Committee for the Purpose of Control and Supervision of Experiments for Animals (CPCSEA), New Delhi, India.

2.2 Synthesis of probes:

The cDNA sequence for NPY Y1 receptor was taken from Salaneck et al., 2008. Using the sequence, primers were designed with T3 and T7 overhangs by Aditi Maduskar in the lab. The amplicons for each primer set were then used to run a BLAST against reference RNA sequence of *Danio rerio.* Sense and antisense probes were synthesized using T3 and T7 polymerases (Promega, Madison, WI, USA) respectively using DIG-labeled dUTPs in the presence of dNPTs.

The plasmid with the exon3 sequence of CART2 inserted between two restriction sites (BamHI and NotI) was previously sythesized in the lab. The plasmid was linearized using restriction enzymes BamHI and NotI (New England Biolabs) and was used for *in vitro* transcription after purification. The probes were synthesized using T7 (for sense probe) and SP6 (for antisense probe) RNA polymerases (Promega, Madison, WI, USA) respectively. DNA templates in all the *in vitro* transcriptions were removed using DNase (Promega, Madison, WI, USA), and the probes were purified using BioRad Micro Biospin columns.

Singh et al. 2017 also did fluorescence *in situ* hybridizations in the zebrafish brain. T3 and T7 overhangs were added to the primers taken from the paper. Sense and antisense probes were synthesized using T3 and T7 RNA polymerases (Promega, Madison, WI, USA).

The probe sequence are as follows:

CART2 exon3 probe:

TGTGATTTGGGCGAGCAGTGCGCGATCAGAAAAGGCTCTCGAATCGGCAAGATGTGCGAC
TGTCCGCGCGGGGCTCTCTGCAACTTTTTCTTGTTAAAGTGTTTGTGATGGAAACACGAAT
TTCGGAGTGGATTGTAACGGGATTGAGGAAAACCATATGCATTATATATCATAAATGACTAC
AGTTTTACTTATATTAATATTTTTTTAAGTGAAGAGACTAAAGATACTGAAGCAAAGTTGTAA
AGAAATTGTTCTTCAAAAATGTATGTGATGTAAAATGTGATATTGTCTTATTCCATGTTTATC
TGCTGTTTTGT

NPY Y1R exon1exon2 probe sequence:

GACACCAACTCATCCTGCACCCTCGTGGATGGAGGCCCAGCCTGAACCACGCTTGCCTGG
GCATCAGCTTGACCTGGGCCCTGGCTGTCCTCACCGCTACACCTTTTCTACTGTTCTCCCG
GGTGACAGACGCCCCGCTCAAGCAGCTGCCTTCAGTGTTTCAGGAGCAGTATCGGGGAAA
AGTGGTGTGTGTGGAGGAGTGGCCCTCCAGAGAAATCAAACTCACCTACACCACCGGCAT
GCTGGTGCTGCAATACATCACGCCTCTCACATTCATCTTCATCTGTTACCTGAAGATATACA
CTCGTCTGCAGCGGCGAAACAACATGATGGAAAGAATCCGTGAAAACAAATACCGTAGCA
GTGAGTCTAAACGGATAAACATCATGCTCTTTTCCATTGTGGTGGCATTTGCAGTTTGCTGG
CTCCCGCTGAACGTGTTTAATGCAGTCATCGACTGGAATCATGAAGTGGCGATGAACTGTA
CCCA

NPY Y1R exon1exon2exon3

CTGACCGACAGCAGTGTGTTACCAGAATTACAAAGACATGGAAAAATCATTACCTAAGAGG
AAGGAGTCTATGATCACCTGATATAAACTAACCCCCTGTGACTACAGAGGAGGCGGAACAA
TGCCAGACTCCGCCTTCTCTCCGCCAGTTCCACCAATCGCTGCTCTGAACTGTTCCCTGGA
CTTATCGAATTGTTCAATCACGAACCTCTCTGCGATTGCATATGGCGACGAGTGCTATGGC
AGCCACTCTTTGTTTGTTATCATGGCTGTTGCCTACAGTGCTGTTGTACTGCTGGGTGTCAT
TGGAAACCTGGCTCTCATCCTTGTGATCGCACGCCAGCGGGAGCTTCACAATGTCACAAAT
GTCTTAATCGCAAACCTCTCGGTTTCAGACTTGCTGATGGCAGTGGTGTGTCTGCCCTTCA
CCTTCATCTACACCTTCATGGACCATTGGGTTTTCGGTGCGGTCATGTGTAAACTCAACAG
CCTGGTTCAGTGCTGCTCCGTCTCAGTGTCGATATTCTCCTTGGTTCTTATCGCCATTGAG
AGACACCAACTCATCCTGCACCCTCGTGGATGGAGGCCCAGCCTGAACCACGCTTGCCTG
GGCATCAGCTTGACCTGGGCCCTGGCTGTCCTCACCGCTACACCTTTTCTACTGTTCTCCC
GGGTGACAGACGCCCCGCTCAAGCAGCTGCCTTCAGTGTTTCAGGAGCAGTATCGGGGAA
AAGTGGTGTGTGTGGAGGAGTGGCCCTCCAGAGAAATCAAACTCACCTACACCACCGG

2.3 In situ hybridization:

The protocol for *in situ* hybridization was adapted from Kuhn and Koster, 2010. Adult zebrafish were anesthetized using 2-phenoxyethanol (1:2000) and were fixed in 4% Paraformaldehyde (PFA) overnight. The brains were dissected out the next day and washed with PTW buffer (PBS with 0.1% Tween20). The brains were further treated with 2% H_2O_2 for 40 minutes at room temperature. This was followed with Proteinase K treatment (10 µg/mL) for 35 minutes without shaking. The brains were later post fixed using 4% PFA for 20 minutes (on shaker) and then washed with PTW buffer (3 washes of 15 minutes each). The brains were later prehybridized for 1 hour in the Hybridization buffer (50% formamide, 5X SSC, 50 µg/ml heparin, 5 mg/ml yeast total RNA, and 0.1% Tween 20). Meanwhile, the probes were heated at 95 °C with the Hybridization buffer (200 µL) for 10 minutes to disrupt the secondary structure and snap frozen in ice for 10 min. The brains were later hybridized with the probes overnight at 60 °C. Next day after hybridization, the brains were washed twice with 50% formaldehyde+2X SSC+0.1% Tween 20 (each wash of 45 minutes), twice with 2X SSC+0.1% Tween 20 (each wash of 15 minutes) and subsequently twice with 0.2X SSC+0.1% Tween 20 (each wash of 45 minutes) at 60°C. The brains were then embedded in 3% agarose blocks and 50 µm sections were taken using a vibratome (VT 1200; Leica, Germany). The sections were washed with PBS and then incubated in Blocking solution (Roche Applied Sciences, Indianapolis, IN) for 1 hour at room temperature. The sections when then incubated overnight with sheep anti-DIG alkaline phosphatase conjugated antibody (1:2000 in Blocking solution) (Roche Applied Sciences, Indianapolis, IN) at 4 °C. Next day, the sections were washed with PTW buffer (3 washes of 20 minutes each) and then twice with coloration buffer (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20)(2 washes of 15 minutes each). For detecting the DIG-labeled probes, chromogen mixture of NBT/BCIP with coloration buffer or BM Purple was used. The reaction was carried out in the dark and was continuously monitored. The incubation time was stopped by giving PBS washes as soon as the desired intensity was reached. The sections were mounted on a slide using glycerol medium (0.5% N-propyl gallate, 70% glycerol, 20 mM Tris pH 8, and 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI)) and were imaged. Photomicrographs of the sections were taken using Apotome (Carl Zeiss, Germany) using

the bright field/epifluorescence modes. Size, contrast and brightness of the images were adjusted using ImageJ software. Neuroanatomical areas in zebrafish brain were identified based on earlier descriptions (Berman et al., 2009; Mukherjee et al., 2012; Wullimann et al., 1996; Yokobori et al., 2012).

2.4 Drug delivery:

Fishes starved for three days were used for all the experiments. The fishes were anesthetized using 1:2000 phenoxyethanol and injected with 2 µL of Glucose or PBS depending upon the experimental group they belonged. They were later allowed to recover for 30 minutes and later fixed overnight in 4% Paraformaldehyde. Next day, the brains were dissected and stored in 30% sucrose solution at 4 °C. The brains were then mounted using Jung Tissue freezing medium (Leica Biosystems, Germany) and 15 µm sections were taken using Cryotome (Leica Biosystems, Germany) on Poly-L-Lysine (Sigma Aldrich, US) coated slides. The sections were later processed for immunohistochemistry.

2.5 Immunohistochemistry

The cryotome sections (as described in 2.4) were rehydrated by giving 3 washes of PBS (phosphate buffered saline) (5 minutes each). They were later incubated in 0.5% PBST (0.5% Triton-X in PBS) for 20 minutes. The sections were later incubated in 5% BSA (bovine serum albumin) to remove non-specific binding for 40 minutes and were later incubated with primary anti-CART antibody (rabbit, 1:2000, Phoenix Pharmaceuticals) overnight at 4 °C. Next day, the primary antibody was washed by giving 3 washes of PBST (10 minutes each) and the sections were again incubated in 30 minutes 5% BSA. They were then incubated in secondary antibody (rabbit, 1:500) for 3 hours at room temperature. The secondary antibody was later washed off by giving 3 washes of PBS (10 minutes each) and the sections were mounted using a glycerol medium (0.5% N-propyl gallate, 20 mM Tris pH 8, 70% glycerol and 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI). The secions were imaged using Apotome (Carl Zeiss, Germany) using the bright

field/epifluorescence modes. Abercrombie's method was used to avoid the over estimation of cell count due to sectioning (Abercrombie, 1946). The equation $N = (nxT)/(T+d)$ was used where N is the corrected cell number, T is the thickness of section, n is the actual cell count and d is the mean diameter of the cells. The graphs were plotted using Graphpad Prism 5.0 statistical software.

Chapter 3
Results and Discussion

3.1 *In situ* hybridization:

Four CART genes have been identified in zebrafish. Akash et al., 2014 had previously described the distribution of all the four CART mRNAs in the adult zebrafish brain. Hence, the CART2 *in situ* hybridizations were used as a positive control. The CART2 mRNA was observed in the Entopenduncular nucleus (EN), NMLF (nucleus of medial longitudinal fascicle), Vs (supracommissural nucleus of the ventral telencephalic area), Vv (ventral nucleus of the ventral telencephalic area), and VM (ventromedial thalamic nucleus) (Figure 3). The observations are in accordance with the results described by Akash et al., 2014.

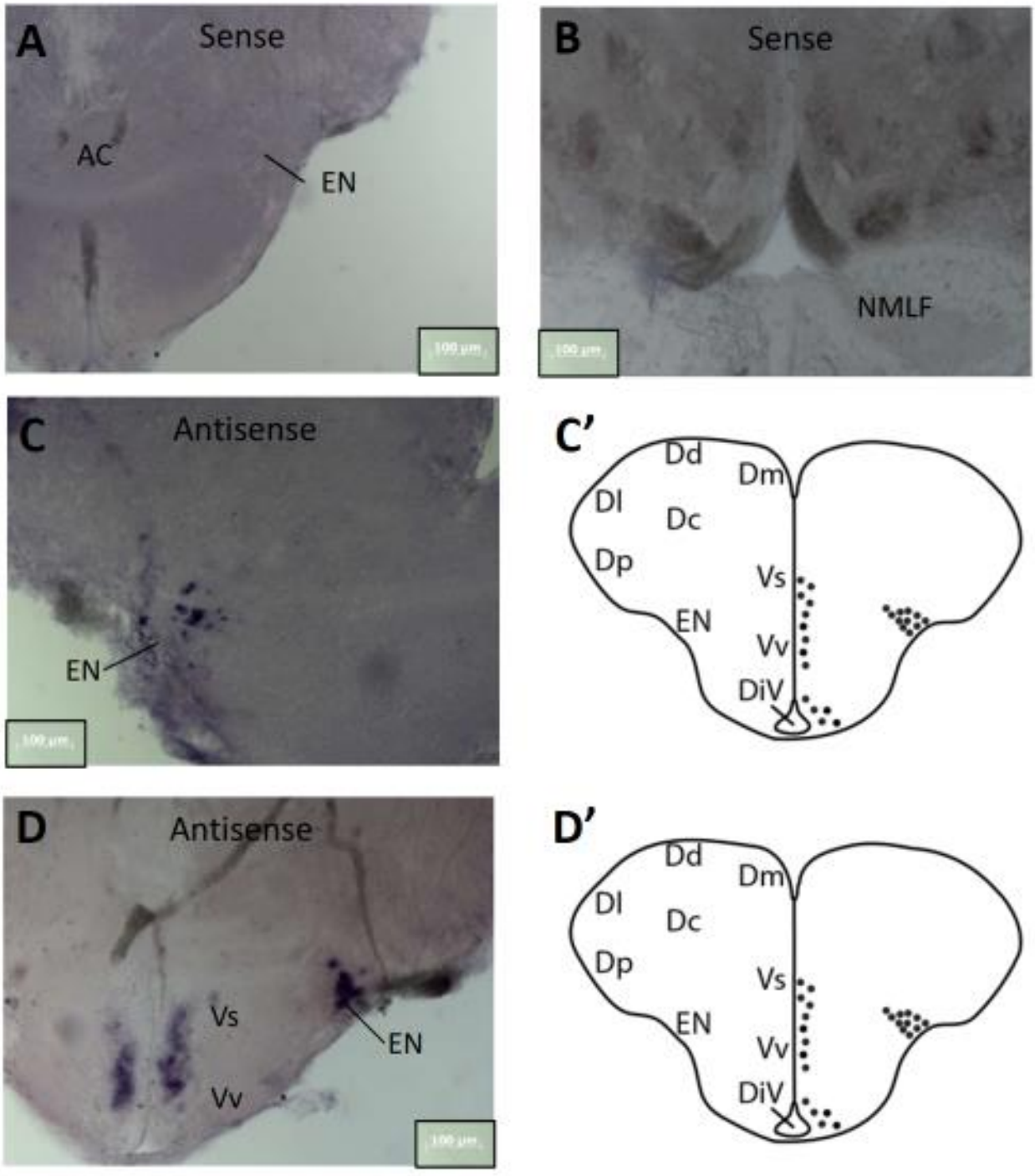
The Y1R *in situ* hybridizations were initially unsuccessful with 300 ng of probe in the Hybridization Buffer. Since there was no hybridization signal observed in the sections, it seemed that it was due to insufficient probe concentration. Hence, in situ hybridizations using 1 µg of probe were done, wherein the sections were developed with high background signal.

Addition of dextran sulphate to the Hybridization buffer (to increase the rate of hybiridizatoin) and using levamisole in the coloration buffer (to reduce the background) with BM Purple instead of NBT/BCIP as a chromogenic enzyme was repeated with 300 ng of probes. However, the experiment conducted with this change did not show any signal in the sections. Thereafter the probe amounts were increased to 600 ng and then to 1 µg using the same setup. Signal was not seen in the experiment with 600 ng of probe but there was diffused color development in the sections hybridized with 1 µg antisense probe after two days incubation in BM Purple. The sections incubated with sense probe showed no such color development (Figure 4A). This was still dismissed as false signal and an anomaly as the cell profile was not clear. The levamisole was removed as there were no signals or any background in the sections. The experiment

with 1 µg of probe and BM Purple showed similar diffused signal without any color development in the negative control (sections incubated with sense probe) (Figure 4). The pattern was diffused, similar to one observed in the experiment with 1 µg of probe. The lower in vivo concentrations of the GPCRs is generally regarded as a factor for low signals in in situ hybridizations.

Singh et al., 2017 described fluorescent in situ hybridizations of Y1R. The group was looking at the role of NPY in sleep modulation in zebrafish. Using their probe to conduct in situ hybridizations might help to either get better quality of signal.

Preliminary experiments done using their probe show no signal development in the antisense sections. This might be due to an unoptimized protocol. Further optimization might be required for this completely new probe. Nevertheless, the authors themselves acknowledge the notoriety of finding the distribution of GPCRs in the tissues as they themselves found less convincing distribution pattern.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Vv
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Vv
DiV

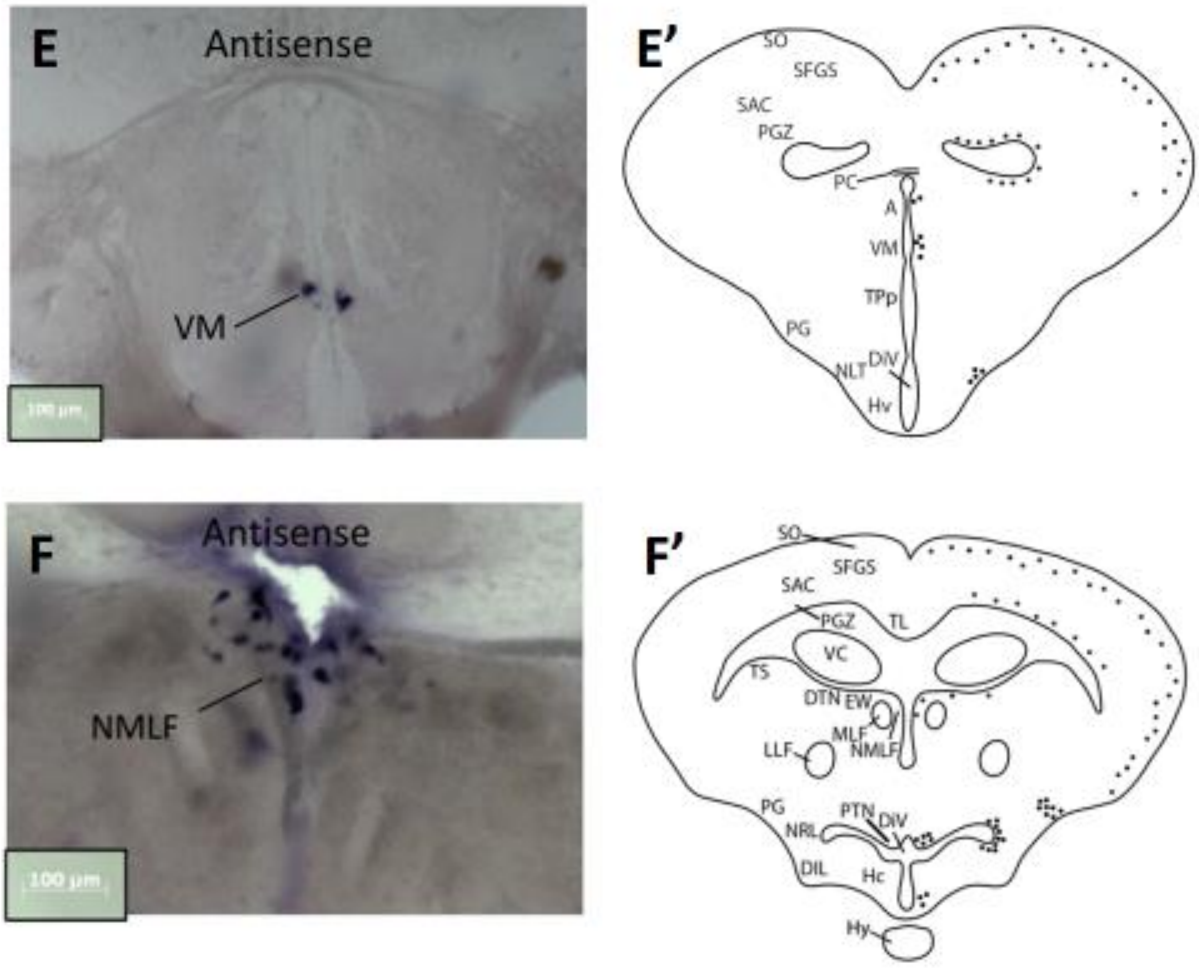
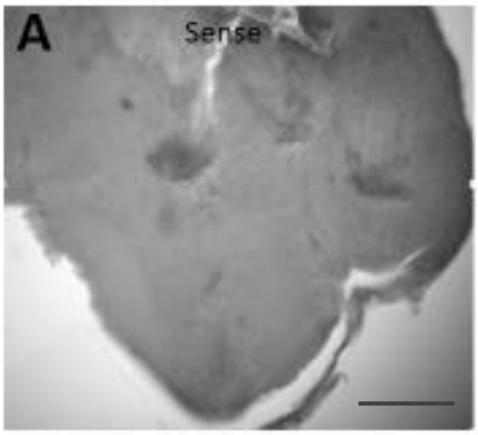
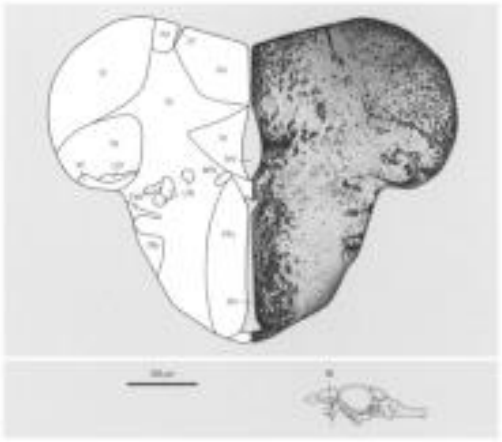
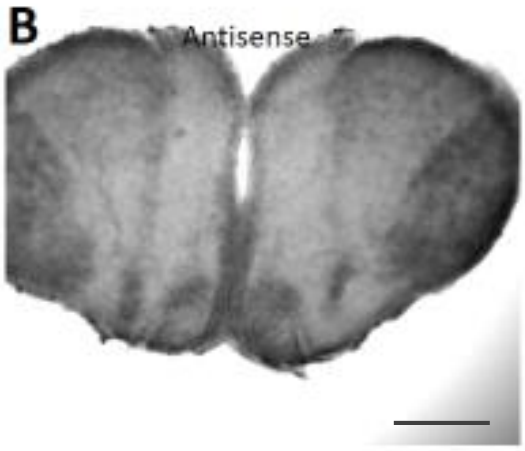
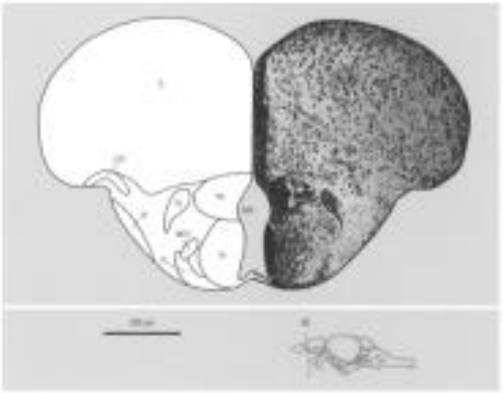


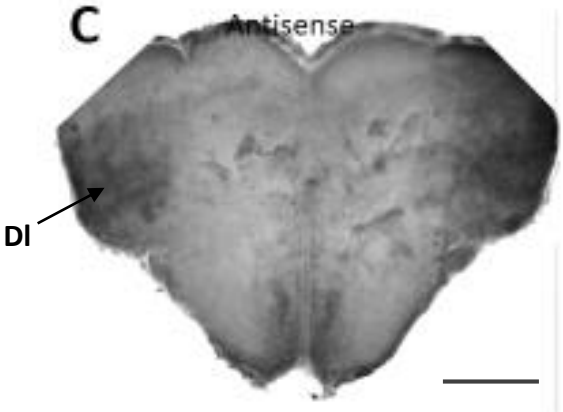
Figure 3: CART2 in situ hybridization: Transverse sections of brain treated with sense probe(A,B) and antisense probe(**C, D, E, F**). (**C', D', E',F'**) are schematics showing the CART2 signal (from Akash et al. 2014). AC (anterior commissure), EN (Entopeduncular nucleus), Vs(supracommissural nucleus of the ventral telencephalic area), Vv(ventral nucleus of the ventral telencephalic area), VM (ventromedial thalamic nucleus), NMLF(nucleus of of medial longitudinal fasciculus). Section thickness: 50 µm. Scale bar = 100 µm.

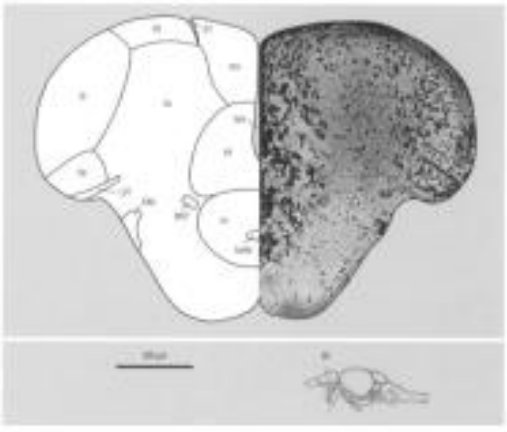

A
Sense




B
Antisense




C
Antisense
Dl



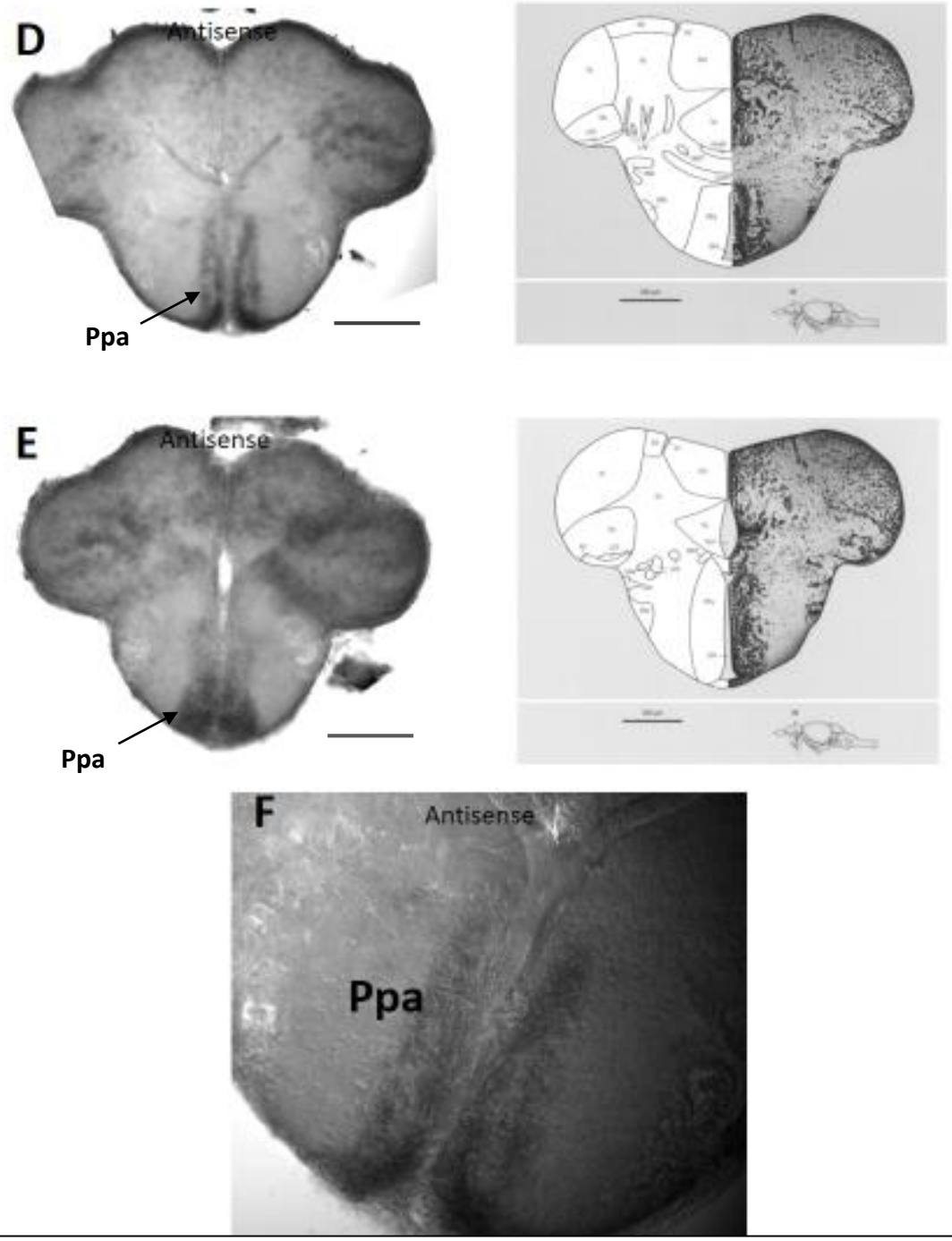


Figure 4: Y1R *in situ* hybridizations: (**A**)Transverse sections of the brain hybridized with sense probe (**B-E**)Transverse sections of the brain hybridized with antisense probe. The images on the right show corresponding regions (adopted from Wulliman et al., 1996). (**F**) Photomicrograph of the Ppa in the section hybridized with antisense probe. EN (Entopeduncular nucleus), Dl (lateral zone of Dorsal telencephalic area), Ppa (Parvocellular preoptic area). Scale bar = 200 µm.

CART immunohistochemistry:

The CART positive cells in the EN are thought to play a part in the regulation of energy homeostasis in the zebrafish brain (Akash et al., 2014; Mukherjee et al., 2012). Glucose treatment in the starved fishes qualitatively increased the CART-ir cells in EN region as compared to those treated with PBS (Figure 5). The observations are in accordance with the previous study conducted by senior lab member (Debia Wakhloo).

The cells in the EN responded to the energy state of the animal. 2-DG (2-Deoxy-D-gluose) is a non-metabolizable homolog of glucose and hence doesn't go through glycolysis which leads to closing of K_{ATP} channels according to the classic 'glucosensor model'. It thus, should mimic starved condition if the glucose sensing is done primarily via glucokinase-dependent pathways. Previous experiments in the lab have shown that 2-DG injections in the zebrafish brain didn't reduce the number of CART-ir cells in the EN. The difference was not seen even in the number of biting attempts towards the food pellets (Devika Bodas, unpublished data). Hence, EN cells might be using different pathways to sense the glucose levels.

SGLT, as discussed above, is used for sensing glucose in various mammalian hypothalamic neurons. To check if they play a role in glucose sensing in zebrafish, one can block the SGLTs using phloridzin and then give glucose injections. Since, the SGLTs are blocked, the increased glucose levels won't be sensed and the number of CART –ir cells in the EN should not increase.

Phlorizin dose was standardized to 5 ng and 12.5 ng after various i.c.v. injections in the zebrafish. 5 ng phlorizin did not show a great reduction in the number of CART-ir cells (Fig.6), though more repetitions are required to conclude this.

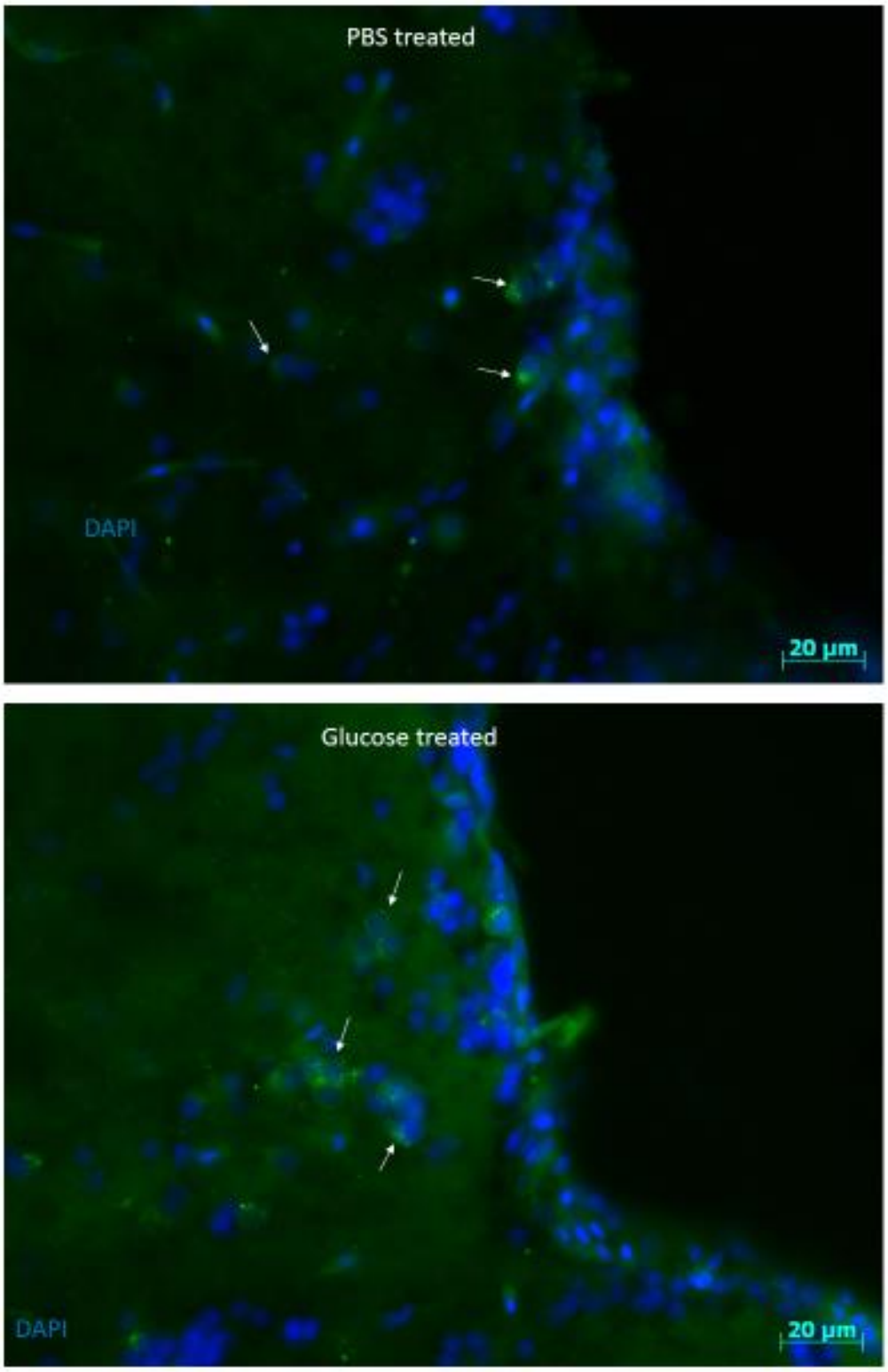


Figure 5: CART immunoreactive cells in the EN: Transverse sections showing cells in the EN (Entopeduncular nucleus) of the zebrafish treated with glucose (**A**) or PBS (**B**). Thickness: 15 µm. Scale bar = 20 µm.

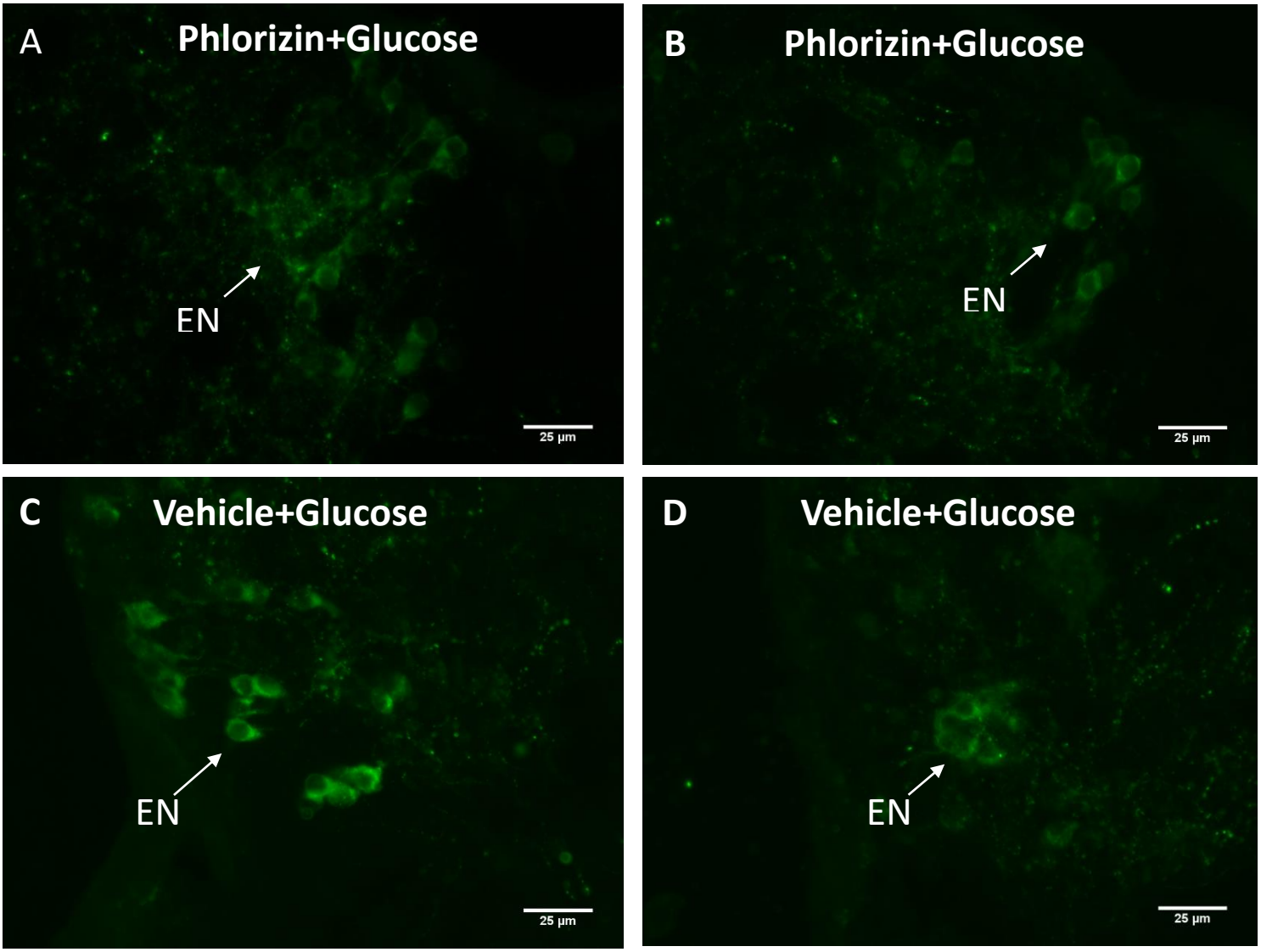


Figure 6: CART immunoreactive cells in the EN after drug treatment: Transverse sections showing cells in the EN (Entopeduncular nucleus) of the zebrafish treated with Phlorizin (5ng)+glucose (**A, B**) or Vehicle+glucose (**C, D**). Thickness: 15 µm. Scale bar = 25 µm.

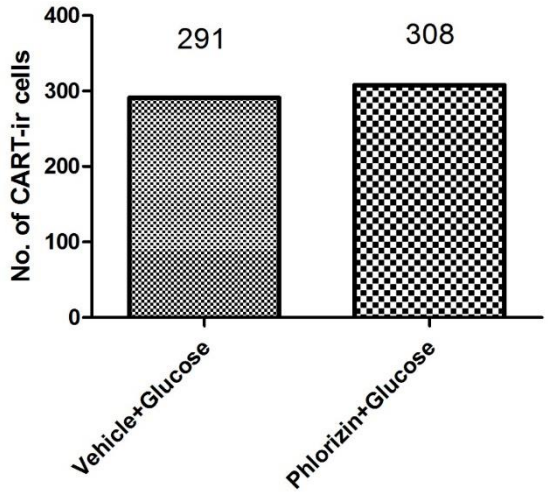


Figure 7: Number of CART immunoreactive cells in the EN. n=1

Conclusions and Future directions

The NPY Y1R *in situ* hybridizations using the designed probe show diffused signal with not so unique cell profile. More optimization of the protocol might increase the quality of signal. Experiments using the probe designed by Singh et al., 2017 might also help to make a better distribution map of NPY Y1R probe in the zebrafish brain.

The map then can be further used to identify regions in the brain that show differential Y1R expression according to the energy state of the animal.

The cells in the Entopeduncular nucleus (EN) in the zebrafish show differential expression of CART-ir depending on the energy state of the animal. This now can be used as a readout for identifying the role of sodium glucose cotransporters in the zebrafish brain.

Preliminary experiments show that 5ng of i.c.v. Phlorizin injection does not affect the number of CART-immunoreactive cells. More repetitions with this dosage are necessary to confirm this observation. Then, one can increase the dosage to see if any significant effect is seen.

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