Neuropeptide Y: A Novel Olfactory Modulator in Zebrafish, *Danio rerio*

A thesis submitted towards partial fulfillment of

BS-MS Dual Degree Programme

By

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Under the guidance of

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Certificate

This is to certify that this thesis entitled “Neuropeptide Y: A Novel Olfactory Modulator in Zebrafish, *Danio Rerio*" submitted towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research Pune represents original research carried out by "Ajinkya Deogade" at "INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, PUNE", under the supervision of "Professor N.K.Subhedar" during the academic year 2011-2012.

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ABSTRACT

Neuropeptide Y (NPY) has been implicated as a neuromodulator mediating a variety of physiological functions including anxiety, gustatory information and energy homeostasis. We find that a subset of olfactory sensory neurons (OSNs) in zebrafish express NPY from early development through adulthood. Co-localization studies with OSN type markers suggest that NPY is limited to ciliated OSNs. These NPY-positive OSNs innervate in discrete glomeruli in the olfactory bulb. These NPY neurons seem to form synaptic terminals in the olfactory glomeruli since they are colocalized with synaptic vesicle protein SV2.

With a view to interrogate the functional role of NPY in olfaction, we have developed an aversive olfactory assay using zebrafish larvae. The 78 h old larvae showed distinct aversion following treatment with l-cysteine. However, larvae pre-treated with BIBP-3226, a small molecule that serves as NPY receptor antagonist, failed to respond to l-cysteine treatment. For the first time, our data suggest the involvement of endogenous NPY and Y1 receptors in the process of olfaction in zebrafish.

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I. Introduction

The encoding of odorants in the nervous system remains an intriguing and barely understood phenomenon (Korsching et al., 1997). The olfactory sensory system plays a crucial role in acquiring information about food, mate, predator etc. It also plays a crucial role in creating a map of the odorant molecules present in the environment and keeps the organism aware of the natural world. Olfactory system is one of the primitive sensory systems. It has been found in the antennae of the insects, fishes have a well developed olfactory organ to smell in the aquatic environment, the tendrils of plants are especially sensitive to airborne volatile organic compounds. The process of olfaction has been studied in a number of animal models like drosophila, rat and catfish. Zebrafish has also served as a useful model in studies on olfaction because its olfactory circuits are well defined and it offers tremendous opportunities for genetic manipulations.

As in other teleosts, zebrafish have three chemosensory systems: olfaction, taste, and solitary chemosensory cells (Lindsay, 2004). Taste neurons are localized within taste buds distributed over the entire outer body surface including the lips and oropharyngeal cavity. Unlike olfactory and taste neurons, SCCs are distributed over the entire outer body surface (Lindsay, 2004). Most of the odorant molecules like amino acids and bile acids act as both gustatory as well as olfactory stimulants (Lindsay, 2004). While two or more gustatory stimulant can produce same taste sensation, different olfactory senses may be involved in the identification of particular odorants (Korsching, 2001). Very subtle differences in the chemical structure of odorants may lead to a robustly different olfactory perception and hence the task of the olfactory senses proper is not classification but recognition of individual chemosensory stimuli (Korsching, 2001).

1. Olfactory System

Organisms have gained ability to sense light, sound, heat and molecules present in the environment. The ability of an organism to know about its environment depends on these sensory modalities. One of the important sensory modality is olfaction. Olfactory cues produce a perception of the world around us. Odors act as cues invoking both innate and learned behaviors such as attraction and aversion, governing decisions to eat, mate, attack or flee from aggressors and predators (Chédotal et al., 2010). Olfactory sensory epithelium is the first place where odorant molecules come in contact with the olfactory system. These odorant molecules interact with the receptors present on the specialized cell called olfactory sensory neuron (OSN) in olfactory sensory epithelia. These OSNs project onto the olfactory bulb present caudally where they communicate with the second order neurons located in the olfactory glomeruli. OSN expressing same receptor gene converge onto the same spatial location in olfactory bulb called glomeruli. Axons of the mitral cell project onto the higher brain structures.

1.1 Olfactory System in Zebrafish

Zebrafish possess a well developed sense of smell, which is used for the localization of food, detection of predators, mating and other types of inter-individual communications (Fuller et al., 2006). The organization of the olfactory system in zebrafish has the same basic organization as seen in all vertebrates. It is less complex with relatively limited number of receptors and therefore serves as a good model for investigations. Olfactory epithelium of the zebrafish is exposed to environment as a pair of olfactory organs (Fig. 1). The olfactory organ of zebrafish has rosette like structure consisting of several lamellae which project onto a single stalk. These rosettes have separate non-sensory as well as sensory regions. The sensory epithelium is located in the central part of rosettes which is mostly the medial portion of each lamella (Byrd and Brunjes, 1995).

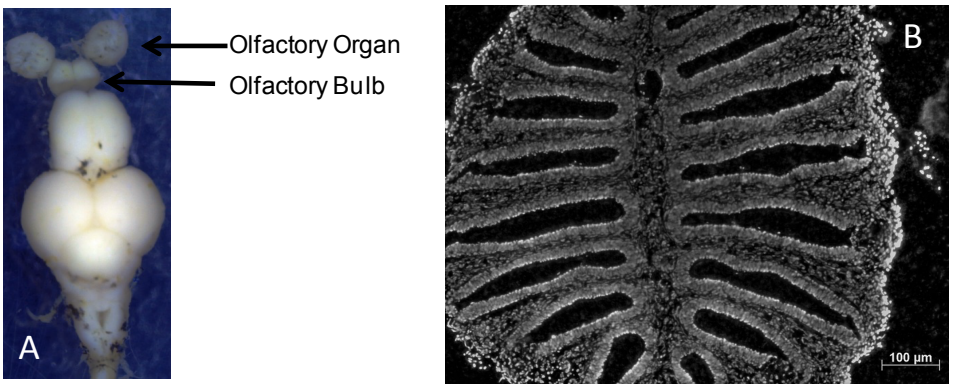

Figure 1: Adult Zebrafish Brain (A) and Section of Olfactory Epithelia (B).

The olfactory bulb of zebrafish is of the sessile type wherein the olfactory organ communicates with the bulb via the olfactory nerve and the bulbs are located in the immediate vicinity of the telencephalic lobes. Though majority of fibers synapse onto the olfactory glomeruli, some noticeable OSNs are reported to terminate in the ventral telencephalon via the olfactory tract forming extrabulbar primary olfactory projections (Castro, 2011). This seems to be a unique feature of the teleost olfactory system. Another interesting aspect of studying zebrafish is the presence of terminal nerve which has also been shown in other fishes and amphibians (Mousley et al., 2006). The terminal nerve (TN) has projections from the olfactory bulb as well as from different parts of the brain involved in processing of information from other sensory modalities such as somatosensory and visual information (Maaswinkel, 2003). The TN fibers have been reported to contain gonadotropin hormone-releasing hormone- like peptide (GnRH) and morphine modulating peptide (FMRFamide) (Maaswinkel, 2003). TN axons also have projections to dopaminergic interplexiform cells in retina apart from projections to forebrain and midbrain (Maaswinkel, 2003). Such centrifugal modulation of visual sensation by olfaction is less understood.

1.2 Olfactory Epithelia and Olfactory Bulb

The olfactory organ of zebrafish is lined with a pseudostartified epithelium consisting of three ultrastructurally distinct cell types namely, the olfactory sensory neurons (OSN), supporting cells and the basal cells (Hansen et al., 1998). OSNs and supporting cells do not form clearly distinguishable layers and can lie above or below the supporting cell (Hansen et al., 1998). The OSNs keep on regenerating throughout life. These OSNs regenerate from the basal cells which act as stem cells. There are 3 types of OSNs found in the zebrafish: ciliated, microvillus, and crypt neurons (Iqbal and Byrd-Jacobs, 2010). There exists no clear arrangement of these neurons around the OE and are present in close proximity with supporting cells (Iqbal and Byrd-Jacobs, 2010). The above arrangement leads to added benefit that the sensation to a particular odorant becomes weaker but does not lead in complete loss when certain areas of olfactory epithelia are damaged (Korsching, 2001).

The olfactory epithelium projects onto the olfactory bulb via olfactory nerve. As in other vertebrates, OSNs expressing same type of receptor gene converges onto the same topographic location called glomeruli in olfactory bulb. In zebrafish, each olfactory bulb is composed of four concentrically arranged layers. These are from outside - the olfactory nerve layer (ONL), the glomerular layer (GL), Mitral/tufts cell layer and the internal cell layer (ICL) (Byrd and Brunjes, 1995). Olfactory nerve layer (ONL) lies in the periphery of the olfactory bulb and is composed of axons of OSN and glial cell bodies. The glomerular cell layer lies next to ONL. Mitral/tuft cell dendrites in olfactory bulb of lower vertebrates make synaptic connections with many glomeruli as compared to mammals (HARA, 1994). The Mitral/Tufts cell axons leave the olfactory bulb and enter the telencephalon by two tracts, the lateral (LOT) and the medial olfactory tract (MOT) (HARA, 1994). The MOT has been associated with transmission of information related to sexual behaviors while the LOT appears to mediate feeding behavior and alerting responses (HARA, 1994). Olfactory bulb neurons project onto the dorsal as well as ventral telencephalon (Rink and Wullimann, 2004).

It has been well established that there exists a chemotopic map of the odorants sensed by an organism at the level of olfactory bulb and even at the higher brain structure. Though the receptors are present diffused at the level of olfactory epithelia they converge onto specific areas of the olfactory bulb and thus acts as the first center for integration of olfactory information. Olfactory bulb of zebrafish is divided into functional map, according to which anterior–lateral subregion responds preferentially to amino acids, the posterior–lateral subregion responds to nucleotides and some amino acids, and a medial subregion responds to bile acids (Friedrich and Korsching, 1998). Recently, a map has been generated of olfactory bulb identifying different glomeruli using cellular markers (Braubach et al., 2012). Such maps are very useful in studying the olfactory system and further will be useful in creating functional maps at the olfactory bulb.

1.3 Development of Olfactory System in Zebrafish

In zebrafish, the olfactory placode first appears at 17 hpf as a thickening of the ectoderm, and this thickening later invaginates to form the naris by 32 hpf (Whitlock and Westerfield, 1998). Pioneer neurons present in the olfactory epithelia form the initial connections between the olfactory placode of the PNS and the telencephalon of the CNS (Whitlock and Westerfield, 1998). The ablation of the pioneer neurons demonstrated that they are required for axon guidance of olfactory sensory neurons to the olfactory bulb (Whitlock and Westerfield, 1998). By 12 hpf olfactory fates are regionalized within the developing neural plate (Whitlock and Westerfield, 2000). Olfactory and visual behaviors appear in the third day with the olfactory avoidance response starting at 72 hpf and visually evoked startle response at 68–79 hpf (Whitlock, 2006).

1.4 Odorants Sensed by Zebrafish

The natural world of the zebrafish is different from that of the land vertebrates i.e. water; the molecules which are relevant with respect to this are also different in their physico-chemical properties. The major odorants sensed by zebrafish are amino acids, bile salts, alarm pheromones (Vitebsky et al., 2005). These molecules act as major cues regarding food, danger and mate. For zebrafish, amino acids act as both olfactory as well as gustatory stimulants (Whitlock, 2006). While some amino acids like L-alanine have been shown to act as attractive odorant (Vitebsky et al., 2005) others amino acids like L-cysteine and L-serine act as aversive odorants (Vitebsky et al., 2005). Zebrafish conditioned to L-Alanine showed more appetitive swimming behavior to L-alanine than any other amino acid (Miklavc and Valentincic, 2012). Zebrafish are able to discriminate similar chemical stimuli because they are represented as dissimilar activity patterns (Miklavc and Valentincic, 2012). Zebrafish larvae have been shown to respond to mixture of amino acids as early as 3 dpf (Lindsay, 2004) at a stage where zebrafish larvae are largely immotile. Bile salts like taurocholic acid act as attractive odorants in zebrafish (Vitebsky et al., 2005). Electro-olfactogram studies have shown that there exists different olfactory receptors for bile salts and amino acids (Michel and Derbidge, 1997). The bile acids may serve as 'signature mixtures'(Wyatt, 2010). 'Signature mixtures' are used to recognize kin, to discriminate sex and age of kin, to discriminate kin from other species, and for shoaling and other non-sexual aggregating behaviors (Braubach, 2011). Physiological evidence suggests that amino acids (and nucleotides) are transduced by microvillous OSNs (Lipschitz and Michel, 2002). The axons of microvillar cells project almost exclusively to the lateral olfactory bulbs of zebrafish (Koide et al., 2009). Thus, suggesting that the lateral portion of the olfactory bulb is involved in processing of amino acid stimuli. Moreover, medial portion of the olfactory bulb has been shown to be innervated almost exclusively by ciliated OSNs and crypt OSNs (Hamdani and Døving, 2007; Sato et al., 2005). Ciliated OSNs have also been shown to be stimulated by bile acids (Døving et al., 2011).

2. Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid, highly conserved neurotransmitter and neuromodulator. It is widely distributed in both the central and peripheral nervous system. It regulates feeding behavior, gastrointestinal activity and central cardiovascular function, and influences seizure threshold and alcohol intake (Hansel et al., 2001). NPY is also shown to act as neuroproliferative factor in rat olfactory epithelia (Hansel et al., 2001). When injected intravenously it causes a vasoconstriction and enhances the effect of noradrenaline (Upsaliensis, 2000). Thus, the overall importance of NPY in the regulation of physiology and metabolism is widely recognized.

2.1 Neuropeptide Y in Olfactory System

Neuropeptide Y has been found in olfactory system of rat (Hansel et al., 2001), cat (Sanides-Kohlrausch and Wahle, 1990), chick (Hilal et al., 1996), xenopus laevis (Tuinhof et al., 1994), goldfish (Peng, 1994), catfish (Gaikwad et al., 2004), killifish (Subhedar et al., 1996), zebrafish (Mathieu et al., 2002). Majority of these studies have found large amount of immunoreactivity for NPY in olfactory bulb. NPY has been reported to occur in the olfactory epithelium of chick, rat, goldfish, and catfish. Some studies aimed at understanding the functional significance of NPY in the olfactory system have been undertaken. NPY was shown to be involved in neuroproliferation in olfactory epithelia of rat (Hansel et al., 2001), mouse (Doyle et al., 2012; Jia and Hegg, 2010), chick (Hilal et al., 1996). NPY knockout study in mouse has shown that NPY acts as a trophic factor for the maturation and survival of olfactory receptor neurons (Doyle et al., 2012). Study in chick (Hilal et al., 1996) has shown that NPY immunoreactive cells in the olfactory epithelia of chick in early developmental stages migrate to caudal brain structures. Gaikwad et al., (2004) have suggested a possible role of NPY as a neurotransmitter in their study on catfish.

2.2 Neuropeptide Y in Zebrafish

Neuropeptide Y has been shown to be present from very early stage of the zebrafish development. Its presence is first reported at 60 hpf (Mathieu et al., 2002). NPY-ir is found in the olfactory pit (72 hpf) as well as during the larval period, between the olfactory pit and the olfactory bulb (Mathieu et al., 2002). The NPY positive cells found in olfactory pit at 72 hpf migrate along the terminal nerve towards the rostral brain (Mathieu et al., 2002). In 1-month/3-month-old animals, new groups of NPY-ir cells appear in the olfactory bulb, the telencephalon and the rhombencephalon (Mathieu et al., 2002). At this stage, NPY-ir is reported in lateral nucleus of the ventral telencephalic area and in the entopeduncular nucleus, and within the rhombencephalon (Mathieu et al., 2002). In zebrafish embryos and in juvenile animals, no NPY reactive cells were found in the mesencephalon (Mathieu et al., 2002).

2.3 Signal Transduction and Expression Pattern of NPY receptors in zebrafish

Neuropeptide Y is closely related to peptide YY (PYY) and pancreatic peptide (PP). These three peptides have a common PP-fold in its tertiary protein structure (Michel et al., 1998). Almost all receptors act via pertusis toxin-sensitive G-proteins (Michel et al., 1998). In some cases pertusis toxin-insensitive receptors have been found but are mostly found in the presynaptic side and act as modulators (Michel et al., 1998). In mammals, six distinct NPY receptors have been found viz. Y1, Y2, Y3, Y4, Y5, Y6 (Michel et al., 1998). Sequence comparisons show Y1, Y4 and Y6 are closer to each other than Y2 and Y5 (Michel et al., 1998). Y1, Y2 and Y5 each bind two distinct endogenous ligands NPY and PYY. Moreover, Y4 has been shown to bind PYY. In mammals signaling via receptors Y1 and Y5 induces feeding, while signaling through receptors Y4 and Y2 inhibits food intake.

In teleosts, Y1-like receptors have been found as Y_a, Y_b and Y_c. While Y2-like subtypes include Y2 and Y7, but lack Y1 and Y5 receptors types from mammals. Expression of zY_b and zY_c has been shown as early as from 24 hpf to 96 hpf in

zebrafish larvae (Mathieu et al., 2005). Whole-mount *in situ* studies by Larhammar et. al. has provided a good insight into the NPY receptor expression in early stages of zebrafish development. They have shown that zY_b mRNA is present in epithalamus at 24 hpf. And new populations of zY_b come up in dorsal portion of anterior telencephalon, ventral hypothalamus and dorsal rhombencephalon. At 96 hpf, zY_b is also present in optic tectum adjacent to mesencephalic ventricle. Similarly, zY_c is also shown to be present in epithalamus at 24 hpf and 96 hpf. zY_c is present in the dorsal region of anterior telencephalon from 48-96 hpf stage. zY_c expression is also found in spinal cord and notochord at 64 hpf.

3. Behavioral Assays in Zebrafish

In order to study behavior of aquatic animals, one must constantly be reminded of their world, a world where water bends light differently, carries sound differently, changes the properties of odor transmission (Whitlock, 2006). Zebrafish is an ideal organism for behavioral studies because of the relative ease in setting up and carrying out the behavioral paradigms. A number of behavioral assays have been used to unlock the mysteries behind the neurocircuitry and to know the role of certain molecules in behaviors. Zebrafish have recently been used to study olfactory behavior, stress and anxiety behavior, spatial recognition, effect of ethanol and nicotine on behavior. Behavioral paradigms looking for the olfactory response to amino acids, bile acids etc have been well established and show robust response.

II. Rationale and Aim of this study

It is clear from the foregoing that NPY is an important signaling agent in the olfactory system. Although there is a possibility that the peptide may also be involved in signal transduction experimental evidence is lacking. The aim has been to (a) study the NPY immunoreactive system in the olfactory component of the adult and larval zebrafish, (b) to test if the NPY neurons synapse in the olfactory glomeruli, (c) to test if the NPY containing OSN neurons are microvillar or ciliated and if they are indeed neuronal in nature and (d) define the role of NPY in transducing olfactory information using an aversive behavioral assay.

In addition, we are interested in finding out if the NPY containing neurons play a role in olfaction. Amino acids have been widely used as odorants in the olfactory assays (Kalueff and Cachat, 2011). Zebrafish responds to amino acids even in very low concentrations in the range of 10^{-6}-10^{-7} M (Vitebsky et al., 2005). While l-alanine produces attraction, l-cysteine and serine are known to produce aversion. L-cysteine aversive assay was employed in the present study. Rationale for employing the aversive assay is as follows. NPY is a well established orexigenic agent. Direct administration of the peptide is known to promote food intake thus suggesting the involvement of a gustatory component. Behavioral assay based on attraction was considered unsuitable because it would not permit distinction between gustatory and olfactory modalities. However, since aversion would be dependent exclusively on the olfactory inputs, we rationalize that this would serve as a useful strategy. L-cysteine aversive assay was used in earlier study by Vitebsky et al. (2005) to screen zebrafish mutant lacking sense of smell. For the current aversive assay, 78 hpf zebrafish were used because at this stage the olfactory system is well established but the taste buds are not. This excludes the involvement of gustatory system in sensing of l-cysteine. Moreover, at this stage the zebrafish larvae are not sensitive to vibration. This excludes the possibility of larvae responding to the vibrations generated during the assay.

III. Materials and Methods

1. Animals

Wild type zebrafish were maintained according to standard zebrafish guidelines (ZFIN). Fish were kept at 28.5 ^{o}C on a 14 hour light / 10 hour dark schedule in 10L and 3L holding tanks (Aquatic Habitats, Apopka, FL, USA) that were continuously supplied with a drip of fresh dechlorinated water, which was filtered through a series of biofilters and a charcoal filter. The fish were fed two times daily with staple fish food (Zeigler Bros. Inc., USA) and live brine shrimp (Salt Creek, Salt Lake City, Utah, USA). Zebrafish embryos were obtained by keeping a cross in breeding tanks (Aquatic Habitats, Apopka, FL, USA) placed in 3 L tanks at night after feeding with males and females separated by a partition. This partition was removed as soon the lights went on in the morning. Eggs were collected around 30-40 min of removal of partition. This point of egg collection was considered as 0 hpf. Eggs were washed thoroughly with E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00001% methylene blue) medium and kept in E3 medium in 100 mm petri dishes at 28.5 ^{o}C. Around 50 embryos were kept in each dish. Dead embryos were removed at regular intervals.

2. Tissue Preparation

The adult zebrafish were anaesthetized using 2-phenoxyethanol (1:2000). The zebrafish brain was exposed from the dorsal side. The head was fixed immediately by immersing in Bouin's fixative and storing overnight at 4^{o}C. The brain along with olfactory organs was dissected out and kept in 70% ethanol overnight at 4^{o}C. The brain was then transferred to 25% sucrose solution for cryoprotection. The brain was then embedded in OCT compound. The olfactory organ and brain were sectioned at 10 µm thickness and mounted on poly-L-lysine coated slides. Similar protocol was used for zebrafish larvae except that they were not anaesthetized before fixing.

3. Immunohistochemistry

The sections were rehydrated and permeabilized using 0.1 % PBST. To reduce the non-specific staining the sections were then pre-incubated in 5 % heat inactivated goat serum (HIGS) for 1 hour and was incubated with the primary antibody in a dark moist chamber at 4 ^{o}C for overnight. On the next day, the sections were washed using 0.1% PBST and pre-incubated with 5 % HIGS for 1 hour and was incubated with desired secondary antibody for 3 hours at room temperature. Then the sections were washed and mounted with glycerol. Primary staining for the sections was carried out using polyclonal anti-NPY antibody (Sigma, 1:2000), monoclonal anti-calretinin antibody (Swant, 1:3000), monoclonal anti-NPY antibody (1:2000, Sigma), polyclonal anti- $G_{\alpha/olf}$ antibody (1:1000, Santa Cruz), anti-Neuron Specific β III tubulin antibody (1:250, Abcam), anti-Hu antibody (1:100, Abcam), anti-SV2 antibody(1:50, DSHB Kathleen M Buckley Harvard Medical School) summarized in table 1. For secondary staining, anti-rabbit Alexa-488 (1:500) and anti-mouse Alexa-568 (1:500) were used. The specificity of antibodies against NPY has been tested (Mukherjee et al., 2012). Sections were observed under the Zeiss Apotome Imager Z1 (Zeiss MicroImaging GmbH, Germany) upright microscope equipped with an Axiovision software 5.1.2600 (AxioVs40v 4.8.0.0.) and LSM710 Confocal microscope (MicroImaging GmbH, Germany, with Axio observer Z1, Zen software).

4. L-cysteine Aversive Assay

In the present study, 78 hours post fertilization zebrafish larvae (n = 10-20) were taken in a 35 mm Petri dish containing 5 ml of E3 medium. Temperature of the room was kept at 27-28.5 ^{o}C. A circle of 1 cm diameter was drawn on the white paper. Petri dish was placed on this paper such that the circle was at the center of the petri dish. The larvae were gently swirled to drive them within the perimeter of the circle below. L-cysteine (10 ul of 3 x 10^{-5} M) was gently introduced over the surface of water, approximately at a point above the center of the circle (making sure that no vibration were generated on the water surface). The larvae were carefully observed for a period

of 30 seconds. The larvae that swam out of the area of the circle were counted as these reflected the aversion to the treatment given (Fig. 2). On the other hand, larvae that remained within the circle were considered as non-responsive. Fresh batch of larvae was taken for each assay. Similarly, the above experimental procedure was followed after NPY Y1 receptor antagonist BIBP-3226(1ug/ml) (Sigma) {(2R)-5-(diaminomethylideneamino)-2-([2,2-diphenylacetyl]amino)-N-[(4-hydroxyphenyl)methyl]pentanamide} treatment for 1 hour. Statistical analysis was carried out using Graphpad Prism 5.0.

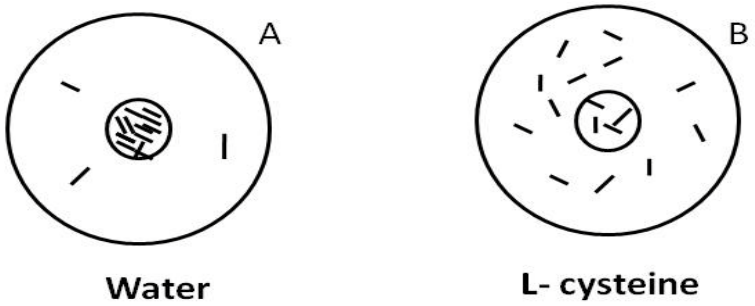

Figure 2: Response by zebrafish larvae (78 hpf) in the aversive assay. Response following control (water) (A) and L-cysteine (B) treatments.

5. *In Situ* Hybridization

In situ hybridization was carried according to the protocol supplied by Dr. Mahendra Sonawne, TIFR, Mumbai. A brief outline of the protocol is given below. Plasmid containing NPY was transfected into competent E. coli DH5α line. DNA was extracted from this E. coli line after overnight incubation using Qiagen MIDI Prep Kit. The extracted DNA was amplified and linearized using T7 and SP6 primers. Amplified DNA was further purified using Qiagen Gel Extraction Kit. DIG-labeled RNA probes for *in situ* were generated by in vitro transcription using the above DNA as template. SP6 polymerase (anti-sense) and T7 polymerase (sense control) were used for in vitro transcription. Similar procedure was used for the probe preparation of Laminin α5 which

was used as positive control for the *in situ* experiments. The mRNA was localized by using antibody against DIG.

Zebrafish larvae of 72 hpf were fixed by incubating overnight in 4% PFA at 4 ^{o}C. On the next day, larvae were washed with PBS and dehydrated using a graded series of methanol/PBST. These larvae were stored in 100% methanol at -20 ^{o}C until the *in situ* hybridization. Before the *in situ* hybridization protocol was carried out the dehydrated larvae were taken through a graded series of PBST/Methanol until the final 100% PBST. They were then permeabilized by incubating in Proteinase K (10 ug/ml) for 45 min at room temperature. They were again washed with PBST and refixed in 4% PFA for 20 min. PFA was removed by washing with PBST (3 times, 5 min). The samples were then incubated in pre-hybridization buffer for 2 hours at 65 ^{o}C. They were then incubated for overnight in the DIG-labeled probe at 65 ^{o}C. On the next day, the larvae were passed through a series of stringency washes to remove the non-specific binding by the probe. These washes were given using (50% Formamide-2x/3x SSCT, 2x SSCT, 0.2 SSCT) 3 times for 30 min each at 65 ^{o}C. After these stringency washes larvae were incubated in blocking buffer for 2 hours at room temperature. Later they were incubated for overnight in Anti-DIG AP-conjugated antibody at 4 ^{o}C. Next day, samples were incubated in maleic buffer followed by PBS washes at room temperature. Then the samples were incubated in staining buffer (100mM Tris-HCl pH 9.5/50mM $MgCl_2$/100mM NaCl/0.1%Tween) at room temperature. The samples were then incubated in p-nitroblue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate NBT-BCIP /staining buffer at room temperature in dark. After 15-30 min a signal starts to develop and it is important to keep observing to stop the reaction at the right amount of signal. The reaction was stopped by washing the samples with PBST. The samples were then fixed by keeping them in 4% PFA overnight. Sometimes, when the staining is too bright, destining was carried out by taking the samples through a graded series of methanol/PBST till 90% methanol. After obtaining the right amount of staining, the samples were again passed through a graded series of increasing PBST concentration. For storage of the samples they were passed through a graded series of glycerol concentration and stored in 100% glycerol at 4 ^{o}C.

Table 1:

Antisera used in present study

Antibody	Antibody	Vendor	Immunogen
polyclonal rabbit anti-NPY	1:5000	Sigma	Neuropeptide Y
monoclonal mouse anti-calretinin	1:2000	Swant	Recombinant human Calretinin
monoclonal mouse anti-NPY	1:500	Sigma	NPY (AAH29497, a.a. 29-98)
polyclonal rabbit anti- $G_{\alpha/olf}$	1:1000	Santa Cruz	Peptide (377-394) mapping at C-terminus of Gα/olf of rat origin
mouse anti-Neuron Specific β III tubulin	1:250	Abcam	
monoclonal mouse anti-Hu	1:100	Abcam	Hu proteins ELAVL2, ELAVL3 and ELAVL4
mouse anti-SV2	1:50	Kathleen M Buckley, Harvard Med. School	Anti-synaptic vesicle protein 2

IV. Results

1. Neuropeptide Y in the Adult Zebrafish Olfactory System

Immunostaining with anti-NPY antibody resulted in immunolabeling of large number of neurons in the olfactory epithelia of adult zebrafish (Fig. 3). NPY containing neurons have an elongated morphology with the cell body lying in layer which is farthest from the receptive surface. Moreover, they have been found in the sensory as well as non-sensory olfactory epithelia.

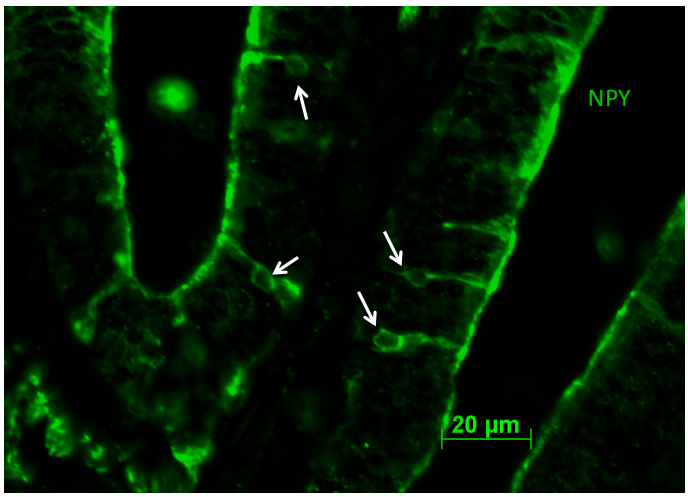

Figure 3: Neuropeptide immunoreactivity in olfactory epithelia of adult zebrafish.
Note the bipolar NPY containing OSNs (arrows) with dendrites extending to the surface.

A large number of neuropeptide Y immunoreactive fibers were seen in the periphery of the olfactory bulb terminating in the olfactory glomeruli (Fig.4A). Double immunostaning using antibody against SV2 (marker for olfactory glomeruli) (Koide et al., 2009) and NPY showed fiber terminals showing both the antigens (Fig. 4B, arrow, yellow) colocalization suggesting that the NPY containing OSNs in the olfactory epithelia are converging onto these colocalizing glomeruli of olfactory bulb.

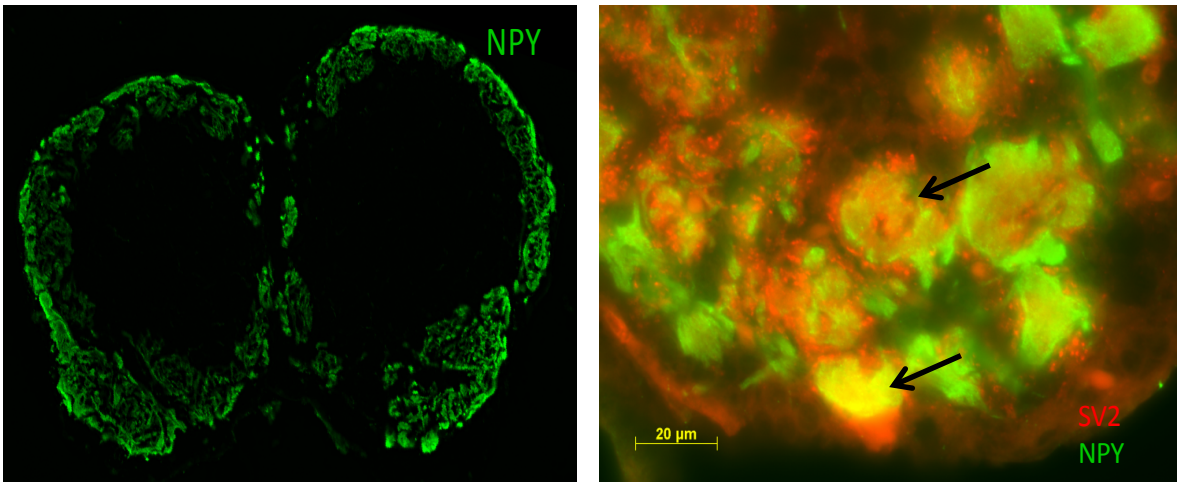

Figure 4: Neuropeptide Y immunoreactivity in olfactory Bulb of adult zebrafish.
(A) NPY (green) containing fiber terminals in the olfactory bulbs; NPY-ir is found in the periphery of olfactory bulb. (B) Co-localization of NPY (green) and SV2 (red) immunoreactivity in the olfactory glomeruli (arrows) of adult zebrafish. This suggests hat NPY containing OSN's converge onto olfactory glomeruli in olfactory bulb. Single SV2 labeled fibers show red immunofluorescence.

2. Neuropeptide Y in the Olfactory System of Zebrafish Larvae

As the NPY is found in adult zebrafish olfactory system in such noticeable amounts it was compelling to check the presence of NPY in developing adult zebrafish olfactory system. Immunostaning for NPY at 78 hpf stage shows the presence of NPY in olfactory epithelia as well olfactory bulb Fig.5 (A, B). NPY was also found in nucleus of terminal nerve near olfactory bulb at this stage (Fig. 5A). A tract showing NPY immunoreactivity from olfactory bulb to more caudal brain regions is also visible (Fig. 5B).

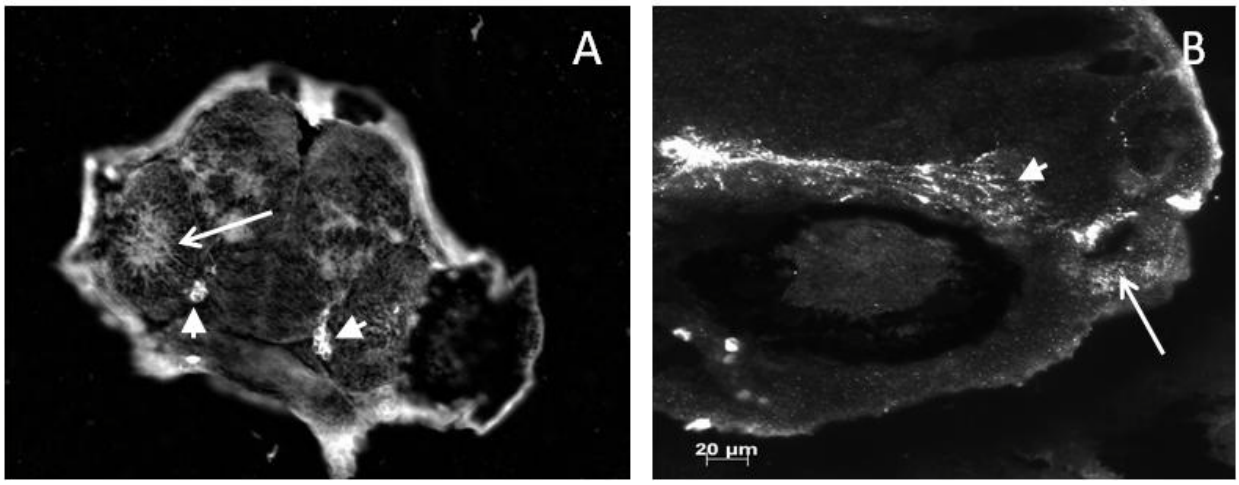

Figure 5: Neuropeptide Y immunoreactivity in 78 hpf zebrafish larvae

(A) Transverse section of 78 hpf zebrafish larvae. Immunoreactivity can be seen in olfactory bulb (arrow). NPY-ir can also be seen nucleus of terminal nerve (arrow head).(B) Sagittal section of 78 hpf zebrafish larvae. NPY-ir can be seen in the olfactory epithelia (arrow) as well as olfactory bulb (arrow head).

3. Neuropeptide Y containing cells in Zebrafish Olfactory epithelia are neurons

To check whether the NPY containing cells in the olfactory epithelia of adult zebrafish are neurons, a double immunostaining using antibody against neuronal marker Hu and NPY was carried out on sections of olfactory epithelia. NPY immunoreactive cells co-localize with Hu, Fig. 6 (A, B), Fig. 7 (A, B, C). Thus, confirming that NPY containing cells in olfactory epithelia of adult zebrafish are indeed neurons.

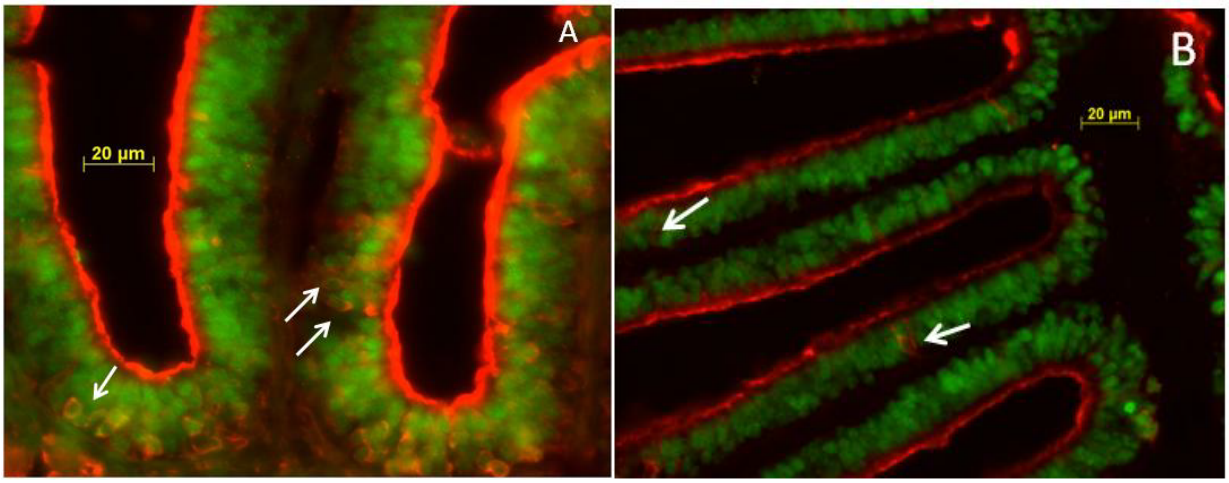

Figure 6: Neuropeptide Y and Hu double immunostaining in adult zebrafish

(A) & (B) NPY and Hu co-localize in the olfactory epithelia of adult zebrafish. This shows that NPY containing cells are indeed neurons.

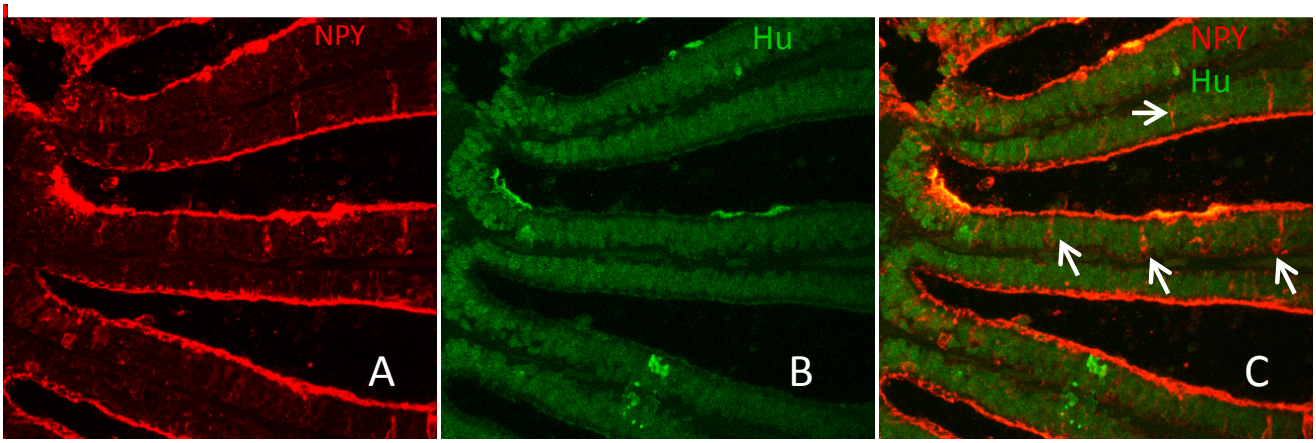

Figure 7: Confocal images of NPY and Hu (neuronal marker) Double immunostaining

(A) NPY – red (B) Hu – green (C) Composite of NPY and Hu (arrows point the co-localized neurons).

4. Characterization of Neuropeptide Y containing Neurons in Olfactory epithelia of Adult Zebrafish

In order to identify the type of OSNs the NPY containing neurons are, antibody co-localization studies were carried out for NPY and calretinin (microvillar OSN marker)/$G_{\alpha olf/s}$(Ciliated OSN marker).

4.1 Neuropeptide Y containing OSNs are not Microvillar

Double immunostaining against NPY and Calretinin showed no co-localization for the two antibodies, Fig.8 (A, B, C). Thus, confirming that NPY containing OSNs are not microvillus. This was suggests further that the NPY containing cells may be ciliated OSNs which is also evident from morphology of NPY OSNs.

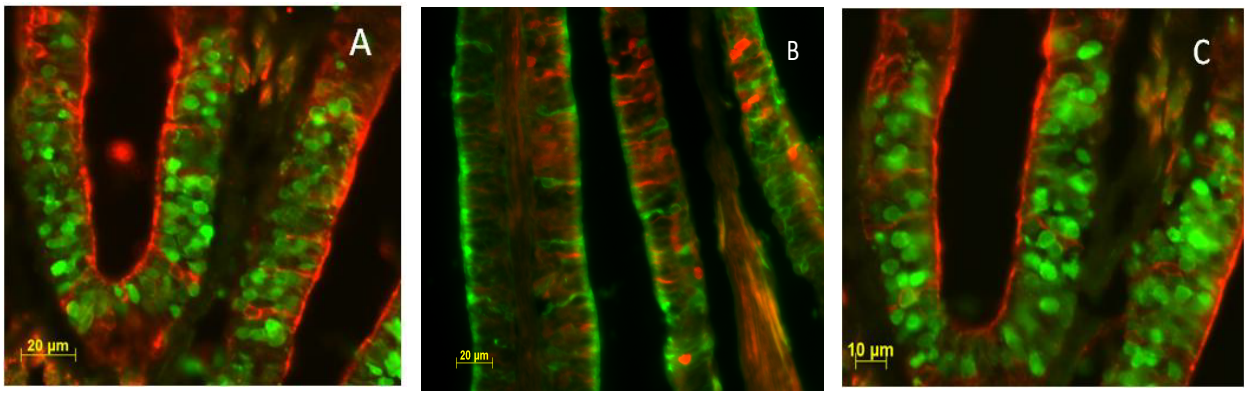

Figure 8: Neuropeptide Y and Calretinin double immunostaning in olfactory epithelia of adult zebrafish.

(A), (B) & (C) Neuropeptide Y (red) and calretinin (green) do not co-localize in the olfactory epithelia of adult zebrafish olfactory epithelia. This shows that NPY containing OSN's are not microvillar in nature.

5. *In Situ* Hybridization for NPY mRNA

In situ hybridization against NPY mRNA showed a huge amount of NPY expression in the brain region. The major regions where NPY expression was found are spinal cord, hind brain and mid brain.

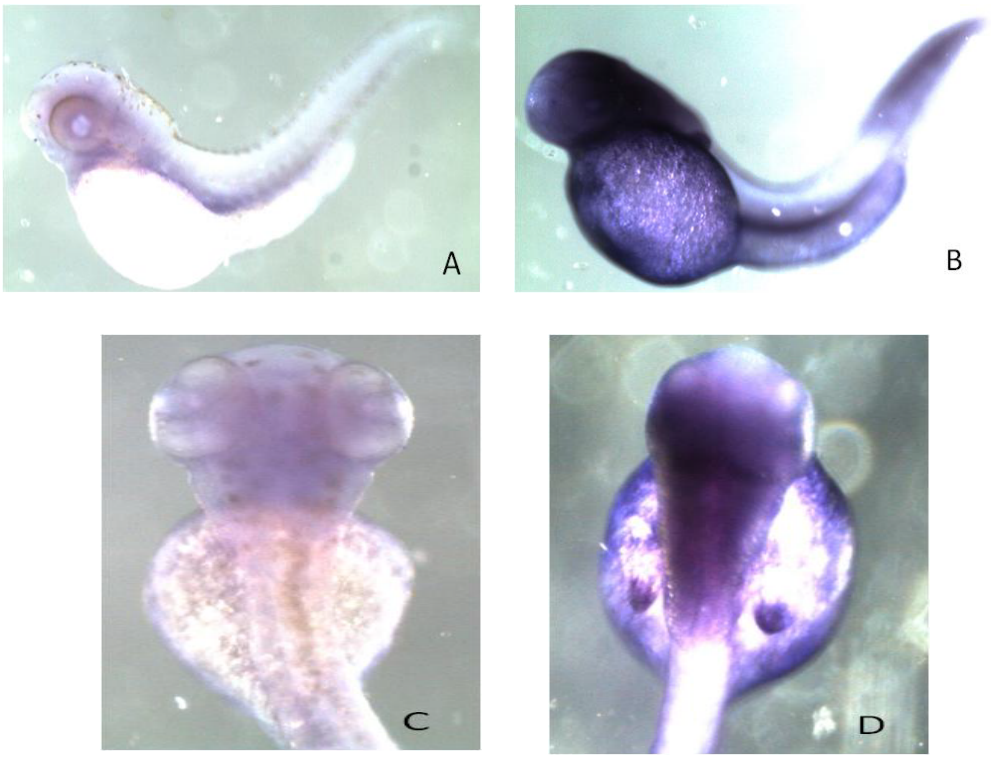

Figure 9: *In situ* hybridisation of NPY mRNA.

(A) Side view of 72 hpf zebrafish whole-mount *in situ* hybridization for NPY sense (negative control) (B) Side view of 72 hpf zebrafish whole-mount *in situ* hybridization for NPY anti-sense. This shows clear localization of NPY mRNA in the brain. (C) Top view of 72 hpf zebrafish whole-mount *in situ* hybridization for NPY sense (negative control). (D) Top view of 72 hpf zebrafish whole-mount *in situ* hybridization for NPY anti-sense. The figure shows clear localization of NPY mRNA as compared to the negative control.

6. Behavioral Assay

Eleven trials of the assay were conducted using 78 hpf zebrafish larvae. Following L-cysteine treatment, a large number of larvae showed aversion as compared to that by the control. A significant ($p<0.015$) difference was found between the response to L-cysteine and response to control (water) after the interval of 30 sec (Fig.10A). No Significant difference was found between the L-cysteine and control when the same assay was carried out on the BIBP-3226 pretreated larvae Fig.10B.

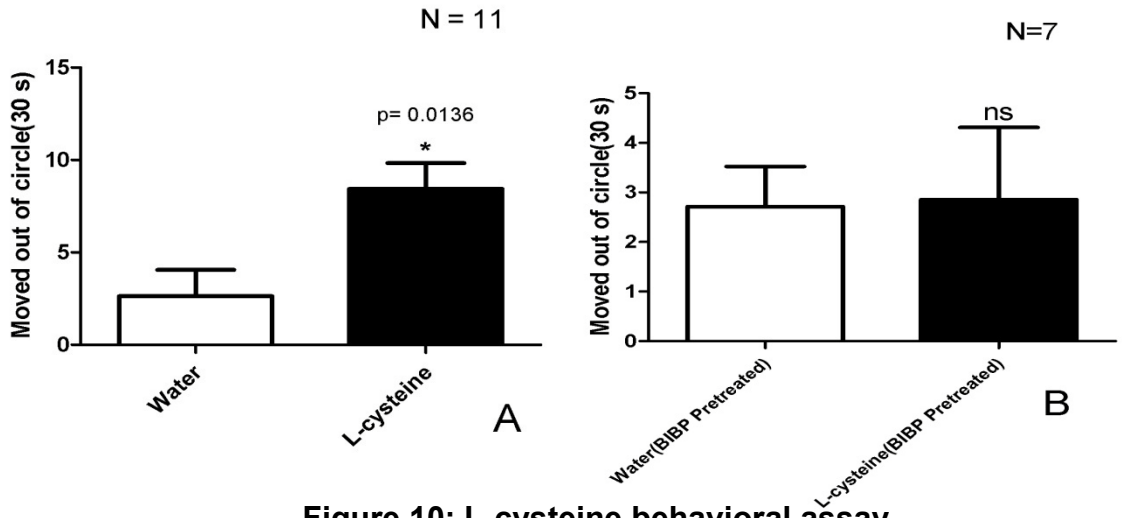

Figure 10: L-cysteine behavioral assay

(A) Response of 78 hpf zebrafish larvae to L-cysteine in the aversion assay. Each bar is a mean of 11 trials p-value =0.0136. (B) Response of 78 hpf larvae to L-cysteine in the aversion assay. Each bar is mean of 7 trials.p-value is not significant. This shows that BIBP was able to block the response to l-cysteine as shown in the samples (B) which were pretreated with BIBP.

V. Discussion

We found NPY-ir in olfactory epithelia and olfactory bulb of adult zebrafish and also in the 78 hpf zebrafish larvae. NPY-ir has been previously shown in the granule cell and mitral cell layer of adult olfactory bulb (Byrd and Brunjes, 1995). NPY-ir has been shown previously in olfactory pit of 72 hpf zebrafish larvae (Mathieu et al., 2002). The presence of NPY at this early stage underscores the importance of the peptide in olfaction and also in development. Previous studies in chick (Hilal et al., 1996), rat (Hansel et al., 2001), mouse (Doyle et al., 2012) suggest that NPY may act as a neuroproliferative factor in the olfactory epithelia. This may be particularly relevant to the regenerative property of the olfactory epithelium seen in all vertebrates including zebrafish. Gaikwad et al. (2004) have reported the presence of NPY in the OSNs of catfish and suggested that the peptide may serve as a neurotransmitter in olfaction. In the present study, presence of NPY in olfactory system of adults, as well as in the 78 hpf larval stage, hints at the possible role of the peptide as a neurotransmitter or neuromodulator in olfaction. Co-localization of olfactory glomeruli marker-SV2 and NPY confirms that the NPY containing OSNs in the olfactory epithelia do indeed synapse onto the olfactory glomeruli in the olfactory bulb. This further suggests role of NPY in olfaction.

NPY is found exclusively in the sustentacular cells (supporting cells) in olfactory epithelia of mouse (Jia and Hegg, 2010). Therefore, before proceeding further to establish NPY's role in olfaction it was important to find out if the NPY containing cells in olfactory epithelia are indeed OSNs. Co-localization of NPY and Hu- neuronal marker in the olfactory epithelia confirmed that NPY containing cells are OSNs.

Present study also looked for the type of NPY containing OSN. Three types of OSNs have been reported in the olfactory epithelium of zebrafish: ciliated, microvillus, and crypt neurons (Iqbal and Byrd-Jacobs, 2010). Previous studies demonstrated that microvillar OSNs, identified as calretinin containing cells, are involved in perception of amino acids. However, possible involvement of ciliated OSNs was not ruled out (Lipschitz and Michel, 2002). We found that the NPY containing neurons in zebrafish do not co-localize with calretinin. It therefore seems that NPY containing OSNs may not be

of microvillus type. Ciliated OSNs have a long dendrites whereas microvillar OSNs are round in shape with a short dendrite (Hansen et al., 1998). Therefore the morphology of the NPY-ir OSNs suggests them to be ciliated OSNs.

We also found NPY-ir in the neurons of terminal nerve at the 78 hpf stage of zebrafish development. This confirms the observations of Mathieu et al. (2002) who reported the cells in zebrafish larvae at 72 hpf. Terminal nerve receives innervations from both the olfactory epithelia and the retina. In addition to NPY, the neurons of the terminal nerve are reported to contain GnRH and FMRFamide (Kawai et al., 2009). While the role of the terminal nerve and the peptide neurotransmitters in the neurons has been debated for sometime recent studies implicate It has been shown that olfactory input of amino acid increases visual sensitivity in zebrafish (Maaswinkel, 2003). This makes our study more important because it suggests NPY may be playing greater role in centrifugal pathways and not just as a neurotransmitter/neuromodulator in olfaction. Mousley et al. (2006) showed that NPY expression changes in the olfactory epithelia with hunger level and is modulated by peptide derived from terminal nerve.

In fishes, amino acids, bile acids act as olfactory cues. NPY-ir fibres were found in the peripheral part of the olfactory bulb. This region has been shown to be responsible for amino acid perception (Koide et al., 2009). So it was evident to first look for whether NPY is responsible for the perception of amino acids. As NPY plays a major role in energy metabolism, the use of an aversive assay was most suitable. A significant difference was shown by 78 hpf larvae in their response to water and l-cysteine. This establishes the fact that there exists an innate aversion for l-cysteine at 78 hpf stage of zebrafish development. The more interesting result of this study is presence of no significant difference between water and l-cysteine when the larvae were pretreated with BIBP-3226 - a small molecule antagonist of NPY Y1 receptor. This result suggests the involvement of NPY in olfactory sensation of l-cysteine. It points that BIBP-3226 was able to antagonize NPY Y1-like receptor in zebrafish present in the olfactory glomeruli and hence disable the perception of l-cysteine. This is for the first time someone has shown the involvement of NPY as a neurotransmitter in olfactory processing. Moreover, this result shows NPY is involved in perception of amino acids. NPY's role as a

neuromodulator in the olfactory process cannot be totally rejected. A possible action of NPY may be to modulate the olfactory signaling. It may thus happen that upon BIBP-3226 treatment the response time for the l-cysteine is increased. This possible aspect of NPY's role is currently been studied in our lab.

The present study put forwards a novel role of neuropeptide Y as a neurotransmitter/ neuromodulator in olfactory processing. Olfaction is an important sensory modality closely related to feeding behavior. Keeping in view the prevalent role of NPY in feeding pathways, currently shown NPY's role in olfaction is interesting. As shown in the present study involvement of NPY in sensing amino acid points toward a unifying role of NPY in feeding and olfaction.

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VII. Supplement

1. Abbreviations

AP-conjugated – Alkaline Phosphatase-conjugated

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

CNS – Central Nervous System

DIG – Deoxygenin

GL – Glomerular Layer

GnRH – Gonadotropin Hormone-releasing Hormone

HIGS – Heat Inactivated Goat Serum

hpf – hours post fertilization

ICL – Internal Cell Layer

LOT – Lateral Olfactory Tract

MOT – Medial Olfactory Tract

NBT – p-nitroblue tetrazolium chloride

NPY – Neuropeptide Y

NPY-ir – Neuropeptide Y immunoreactivity

OCT compound – Optimal Cutting Temperature Compound

ONL – Olfactory Nerve Layer

OSN – Olfactory Sensory Neuron

PBST – Phosphate Buffer Saline Triton-x100

PFA – Paraformaldehyde

PNS – Peripheral Nervous System

PP– Pancreatic peptide

PYY – Peptide YY

SCC – Solitary Chemosensory Cells

SV2 – Synaptic Vesicle Protein 2

TN – Terminal Nerve

2. Vector

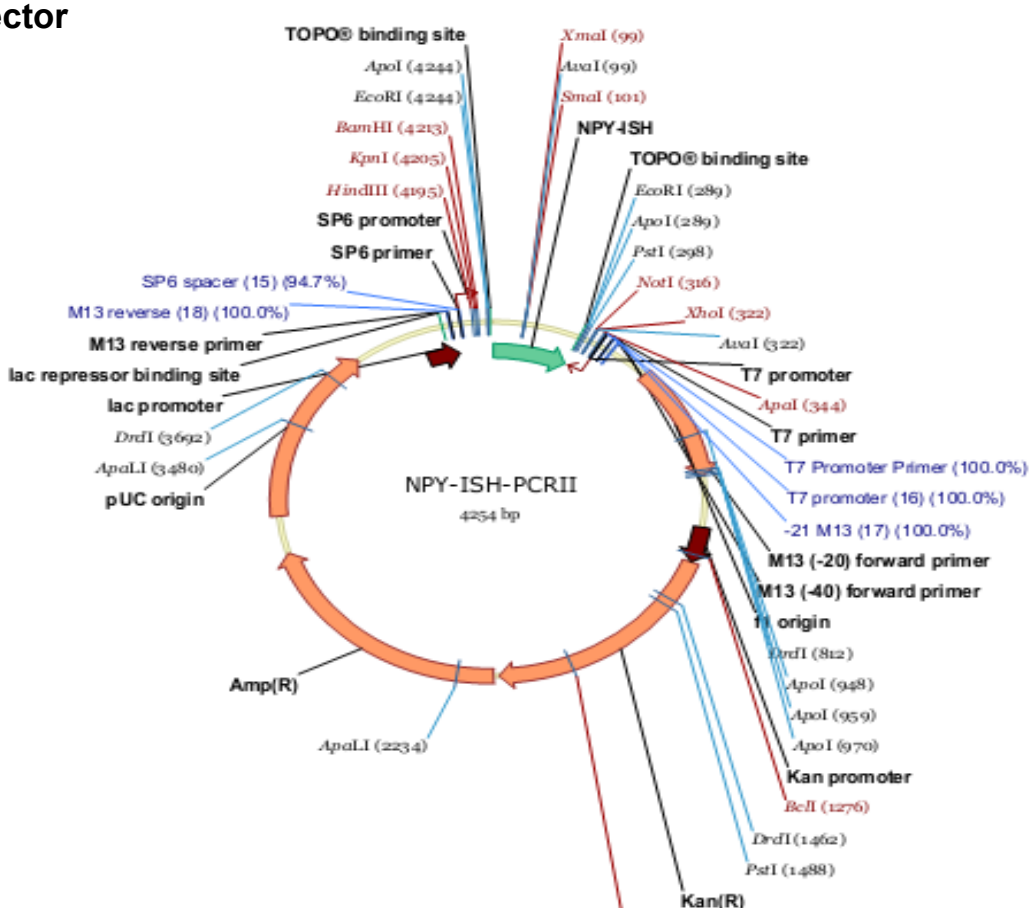

Figure 11: NPY vector