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The Daily Expression Profile of Neuropeptides (*gnih, gnrh3, kiss1* and *kiss2*): A Study of Possible Interaction in the Brain of Zebrafish (*Danio rerio*)

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Abstract

Involvement of neuropeptides in the reproduction of fish (seasonal/regular) is known. The daily rhythmicity and their possible interaction of four major neuropeptides namely *gnih*, gonadotropin-inhibitory hormone; *gnrh*, gonadotropin-releasing hormone; *kiss1/2*, kisspeptin 1/2; is not known to any fish. Our present study on the whole brain of zebrafish (*Danio rerio*) aimed at the daily rhythmicity of the mRNA expression of these four neuropeptides in a 12 h light/12 h dark photoperiod (LD). Only *kiss2* in its expression gives a rhythmicity but other three peptides are not rhythmic. Moreover, the expression of *gnih* is 10-fold lower than *gnrh3*. Our STRING network analysis suggests *kiss2* act as the mediator to communicate with *gnih, gnrh3*, and *kiss1*. Our present finding is indicating the important role of *kiss2* in mediating the reproductive signal and may play a central role in the synchronization of the environmental signal and reproductive periodicity.

Keywords: *gnih, gnrh3, kiss1, kiss2*, Protein-Protein Interaction, Zebrafish

1. Introduction

Reproduction is a complex neuroendocrine and time dependent physiology. Several major pathways have been elucidated while others are hypothesised to explain this process. However, peptides involved in the pathway are alike. In vertebrates, the conserved elements of the reproductive axis, present in the brain are Gonadotropin-Inhibitory Hormone (GnIH) or Neuropeptide VF precursor (NPVF), Gonadotropin-releasing hormone (GnRH), Kisspeptin 1&2 (Kiss1&2), Luteinizing Hormone (LH), Follicular Stimulating Hormone (FSH)[13] and in the ovary, are Transforming Growth Factor-β (Tgf-β), Bone Morphogenetic Protein-15 (Bmp15), etc. However, their functions are not conserved.

Biological clocks, the time-keeping machinery of an organism, anticipate daily and seasonal changes with a neurohormonal mechanism. This anticipation is a crucial process for individual sustainability as it allows the body to cope with the environmental changes. In case of ectothermic animals, such as fish, the importance of clock is more for their sustainability. Apart from day/night cycles, fish have to calibrate its physiology with several parameters like photoperiod and/or water temperature[19,20] at daily basis (circadian), gravitational forces which changes amplitude of tides (circatidal), magnetic waves which play a substantial role in fish migration (circannual) etc. In contrast to its significance in physiology, knowledge is much less about the role of these factors on the reproductive peptides. Moreover, the

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effect of day/night (light/dark), the primary modulator of any rhythm, is not known on the transcript of reproductive genes in any fish.

GnIH was first discovered in quail[31], afterwards its analogues have been reported in other vertebrates including teleost[33,36]. The influence of GnIH varies in respect to species. In mammals, birds and fish it is found to be inhibitory to GnRH neurons and/or pituitary gonadotrops[6,9,15,27,33]. However, GnIH is reported to be stimulatory to GnRH and the downstream gonadotrophs release in Siberian hamster[33]. Our recent finding clearly demonstrated the inhibition of *gnih* by melatonin[35] in the brain-pituitary-reproductive axis of zebrafish.

In zebrafish brain, the Kiss1 and Kiss2 are mainly expressed in the habenular nucleus and ventral hypothalamus, respectively. The Kiss2 neurons are also located at the lateral preoptic area[13,25,37]. It can be concluded from the reports across vertebrate classes that presence of Kiss system (Kiss1&2/GPR54) is cosmopolitan[10,14]. Impact of Kiss1 and 2 on the reproductive axis have been thoroughly studied in several vertebrates including zebrafish. It is now established that the Kiss1/Gpr54 system plays a key role in regulating puberty onset, gonadotropin secretion, and sex steroids feedback by stimulating GnRH release in mammals[17,22]. Similar information has been documented in the complimentary reproductive axis of the teleost[38]. However, *kiss1* and 2 varies in their expression depending on the photic condition in zebrafish[35].

GnRH, a decapeptide was isolated from the mammalian hypothalamus and because of its stimulatory action on the reproductive axis in general and on gonadotrophs in particular, it has been termed as the GnRH[16]. It is now well established from a variety of studies that more than 20 forms of GnRH exist[13,9]. All these forms of GnRH can perform neuroendocrine, neurotransmitter/ neuromodulatory, paracrine and autocrine functions[12].

Despite of ample research on these peptides, the transcriptomic rhythmicity of these peptide was never being studied in any organism. In the current study, we aim to find the daily transcription rhythmicity of these four peptides under normal photic condition in this important animal model. Added to that, we utilized the recent version of protein-protein interaction database "STRING v10" and created individual as well as grouped network maps to understand how they are associated.

2. Material and Methods

2.1 Animals and housing conditions

Wild type zebrafish (*Danio rerio*) of weight 0.4±0.15 g were collected from the North East India and kept in 50 L Glass aquaria (20 fish/ aquaria) in 12L:12D photic condition (300 Lux with florescent tube), maintained by timer (Frontier Digital Timer, Taiwan)[1] and the water temperature was maintained at 28°C using immersion heater (100 W, RS Electrical, India) located in each aquarium. Water was aerated and recirculated through a biological filter (E-Jet, P.R.C). The pH, hardness and other parameters of the water were maintained under standard condition following ZFIN protocol of zebrafish breeding[34]. The fish were fed twice a day with commercial flakes (Taiyo Pet Products Pvt. Ltd., India) in the morning and live *Artemianauplli* (cultured from Artemia Cysts, Ocean Star International, USA) with blood worm (Far East Freeze & Drying Mfg. Co, Ltd, Taiwan) in the evening. Laboratory care of fish and adopted study schedules were in agreement with international standards[18]. The fish were acclimatized in this condition for at least 20 days before performing any experiment.

2.2 Experimental Design

The adult female zebrafish were randomly kept in 12L:12D photic conditions: normal photoperiod (LD; 12 L: 12 D). The light was on at 05.00 hr and off at 17.00 hr with an automated timer for LD group. The sampling time for LD group was every 4 hours interval in 6 time points (04.00 hr, 08.00 hr, 12.00 hr, 16.00 hr, 20.00 hr and 24.00 hr) in a 24 hour daily cycle. The tissue sample during dark phase was carried out under dim red light with utmost care. The tissue samples were collected for these two group after every 24 hr of interval up to three days. During the collection of samples, irrespective of the experimental groups, three adult female zebrafish were taken in 0.1% Tricane (Sigma-Aldrich, USA) solution then kept in ice for anaesthesia before they are euthanized. Whole brain and ovary were removed by dissecting skull and body cavity respectively. The samples were stored at -80°C in TRIzol reagent (Life technologies) until the further studies.

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from the homogenized whole brain and ovary of zebrafish with Trizol reagent (Life technologies, USA) according to manufacturer's instructions. RNA pellets were eluted in RNase-free water (DEPC water, Sigma-Aldrich, USA). The total RNA was quantified using a Nano Spectra (Shimadzu, Japan) then treated with DNase 1 (New England Biolabs, USA) to remove genomic DNA contamination. RNA integrity was checked by staining 28S and 18S RNA bands with GelRed™ (Biotium, USA) nucleic acid stain on 0.8 % agarose gel. The extracted RNA was then reverse transcribed into cDNA using Verso cDNA synthesis kit (Thermo Fisher Scientific, USA) where 0.5 µg of the total RNA was reverse transcribed into cDNA at 42°C for 1.0 h in 20 µl reaction solution according to the given protocol.

2.4 Real-time quantitative PCR

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) was performed using the primers taken from the published data[2,35] listed in (Table 1) in StepOnePlus Real time PCR system (Applied Biosystems, Inc, ABI, USA). Reaction condition comprisesof an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s. Melting curve analysis (Tm) was performed to confirm single gene amplification by the designated primers. Amplification was performed in 10µl reaction volume containing forward and reverse primers, GoTaq qPCR Master Mix (Promega, USA) cDNA. Technical triplicates were used for each sample. Amplification efficiency was tested by standard-curve method generated by serial dilution of primers. The relative expression of all the genes was calculated by 2(-Delta Delta C(T)) method using *Rpl13a* gene as reference[30].

2.5 Statistical analysis

The variation in gene expression at different time points in LD conditions were analysed by one-way ANOVA (SPSS 16.0 software; Macrovision Corporation Santa Carlo, California, USA) followed by Tukey's test to compare the differences between the time points. $P < 0.05$ was considered as statistically significant. The analysis of Rhythm was done using COSINOR PREIODOGRAM 2015 (Boise University, USA) built on cosinor-rhythmometry[21]. Nonlinear regression curve was fitted to the LD data using formula "Y= Mesor + Amplitude Cos (Frequency X + Acrophase)" with Prism software (GraphPad; GRAPHPAD Software Inc., CA, USA).

2.6 In silico network analysis of major regulatory peptides in reproduction

The functional partners of the major genes involved in the regulation of reproduction, namely *gnih* (*npvf*), *gnrh3*, *kiss1*, and *kiss2* were searched individually in STRING database v10 (http://string-db.org/). Further, these four peptides were made as a club to search their relationship within this group. The *Danio rerio* restricted search was automated by the database server with medium interacting confidence score of 0.400. Additionally, to observe the protein-protein interaction map of all 4 peptides with their respective individual networks, a merged and integrated network was derived with the same parameters. The resulted networks with their amino acid sequences, coordinates and annotations were

Table 1. List of primer used for real-time qPCR. Primer sequences for real-time quantitative RT-PCR assays of target and reference genes. F, forward; R, reverse. * Accession Number is provided by the National Centre for Biotechnology Information, Bethesda, MD, USA, references have been given in the text

Gene	Primer Sequence	GenBank Accession No.	Amplicon Size
gnih	F: AGTTACGGCTCTCAGATTGC	NM_001082949.1	282
	R: AGGTTGATGGTAGACTTGGG		
gnrh3	F: TTAGCATGGAGTGGAAAGGAAGGTTG	NM_182887.2	255
	R: CTTTCAGAGGCAAACCTTCAGCAT		
kiss1	F: ACAGACACTCGTCCCACAGATG	NM_001113489.1	201
	R: CAATCGTGTGAGCATGTCCTG		
kiss2	F: ATTCTCTTCATGTCTGCAATGGTCA	NM_001142585.1	344
	R: TGCTTTCTCAGGTAAAGCATCATTG		
rpl13a	F: TCTGGAGGACTGTTAGAGGTATGC	NM_212784.1	148
	R: AGACGGACAATCTTGAGAGCAG		

stored separately in text and tab-delimited file formats respectively. Finally, 6 networks; 4 individual networks, 1 network with group of 4 peptides and 1 network with 32 unique nodes were generated by using Cytoscape[26] Version 3.3.0. It is to mention that this networking was based on both mRNA and peptide data, available from our experiment as well as online repositories.

3. Results

3.1 Expression of gnih and gnrh3 in whole brain in LD

Our study reveals the daily variation in mRNA expression profile of the *gnih* and *gnrh3* in normal photic condition. The cosinor analysis (Table 2) of the qRT-PCR data revealed that both *gnih* and *gnrh3* is arrhythmic in brain. Interestingly, the expression level of the *gnrh3* is 10-fold more that the expression of the *gnih* (Fig. 1).

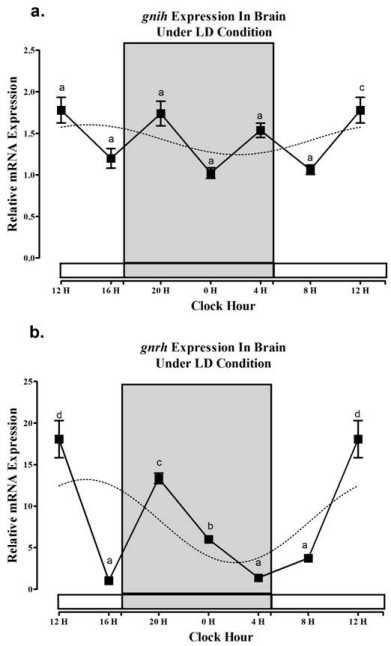

Figure 1. Expression of *gnih* and *gnrh3* in a 12h hour light/dark cycle in the whole brain of zebrafish. **(a)** Expression profile of *gnih* in LD (12h L: 12h D) cycle in whole brain. The dark box resembles the dark phase and open box is light phase. **(b)** Expression pattern of *gnrh3* in LD (12h L: 12h D) cycle in whole brain. The dark box resembles the dark phase and open box is light phase. The relative quantification values in the graph are shown as the mean ± SEM (n = 6). One way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($P < 0.05$). Nonlinear regression curve was fitted to all data.

3.2 Expression of kiss1 and kiss2 genes in whole brain in LD

The Cosinor analysis and one way ANOVA revealed that *kiss1* is arrhythmic while *kiss2* is rhythmic (Table 2). The acrophase of *kiss2* is in the late dark. Their fold change is comparable (Table 2; Fig. 2).

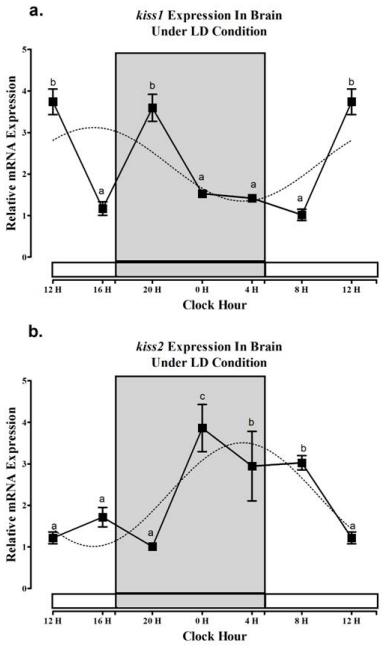

Figure 2. Expression of *kiss1* and *kiss2* in a 12h hour light/dark cycle in the whole brain of zebrafish.**(a)** Expression profile of *kiss1* in LD (12h L: 12h D) cycle in whole brain. The dark box resembles the dark phase and open box is light phase. **(b)** Expression pattern of *kiss2* in LD (12h L: 12h D) cycle in whole brain. The dark box resembles the dark phase and open box is light phase. The relative quantification values in the graph are shown as the mean ± SEM (n = 6). One way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($P < 0.05$). Nonlinear regression curve was fitted to all data.

3.3 STRING network analysis

The results from STRING network analysis are based primarily on the evidence of PubMed text mining and curated databases. A total of 64 separate interactions (GnRH3: 19, Kiss1: 14, Kiss2: 23 and GnIH(npvf): 8) have been observed (Fig. 3). Out of these 64 interacting partners/nodes 32 are found to be unique while remaining are overlapping (Fig. 4; Table 3). The major pathways involved in this network are GnRH signalling and neuroactive ligand-receptor interaction (Table 4). Current network analysis represents that Kiss2 is the

Table 2. Cosinor analysis in LD condition

Genes	F value	P value	Mesor	Amplitude	Acrophase	Robustness	Oscillating
gnih	0.551	0.592	1.3891	0.1283	-230° 15h 18 min	0.00%	No
gnrh	1.336	0.292	7.255	3.567	-229° 15h 14min	00.0%	No
kiss1	1.76	0.204	2.0774	0.7226	-248° 16h 44min	2.8%	No
Kiss2	7.19	0.006	2.2949	1.2005	-51° 3 h 25min	38.70%	Yes

The table is showing the parameters defining the gene expression rhythms in whole brain of zebrafish with oscillation ($P < 0.05$).

central peptide and it co-ordinates between the GnIH (npvf), GnRH3, and Kiss1. The functional network indicates that GnIH and GnRH3 communicates via Kiss2 not Kiss1 (Fig. 4). Additionally, the network map shows associated protein family and domains from PFAM and INTERPRO databases which are automated by STRING search. (Table 4).

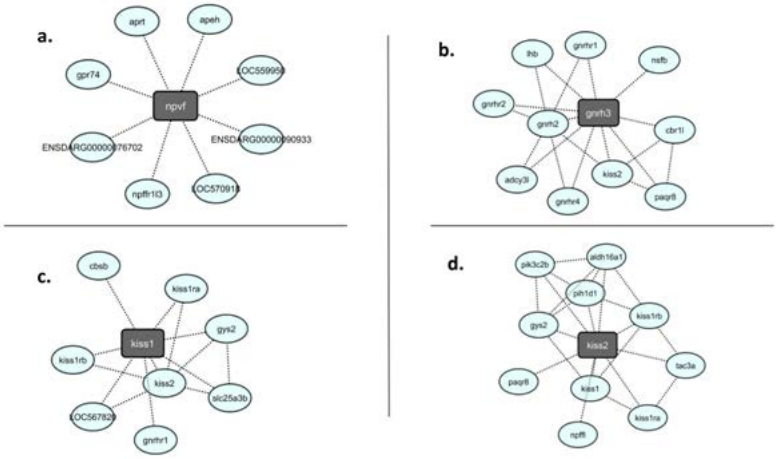

Figure 3. Individual protein-protein interaction network plot. Figure representing individual networks. **(a)** GnIH/npvf with 8 nodes and 8 interactions. **(b)** GnRH3 with 10 nodes and 19 interactions. **(c)** Kiss1 with 8 nodes and 14 interactions. **(d)** Kiss2 with 10 nodes and 23 interactions.

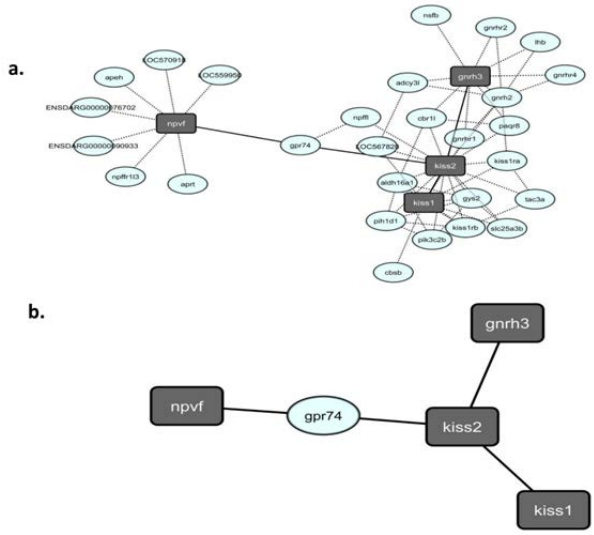

Figure 4. Network plot of protein-protein interactions. Figure representing. **(a)** Merged network of all 4 peptides (GnIH/npvf,GnRH3,Kiss1 and Kiss2) with 32 unique interacting nodes and 61 interactions/edges. Solid line represents the interaction between the studied peptides whereas dotted line shows their respective interacting partners. **(b)** Filtered network of only 4 peptides with gpr 74 as connecter.

Table 3. List of pathways/protein features with their database ID, description, count in gene set, and participating proteins in the network generated by STRING network analysis of GnIH, GnRH, Kiss1 and Kiss2

ID	Description	Count in gene set	participating proteins in the network
KEGG Pathways			
a) 4912	a)GnRH signalling pathway	a)7	a)adcy3l, gnrh2, gnrh3, gnrhr1, gnrhr2, gnrhr4,lhb
b) 4080	b)Neuroactive ligand-receptor interaction	b)9	b)LOC567820, gnrhr1, gnrhr2, gnrhr4, gpr74, kiss1ra, kiss1rb, lhb, npffr1l3
PFAM Protein Domains			
a) PF00446	a) Gonadotropin-releasing hormone	a)2	a)gnrh2, gnrh3
b) PF00001	b)7 transmembrane receptors (rhodopsin family)	b)7	b)LOC567820, gnrhr1, gnrhr2,gnrhr4,gpr74,kiss1r-b,npffr1l3
INTERPRO Protein Domains and Features			
a) IPR001658	a) Gonadotrophin-releasing hormone receptor family	a)3	a)gnrhr1,gnrhr2,gnrhr4
b) IPR005395	b) Neuropeptide FF receptor family	b)3	b)LOC559950,gpr74,npffr1l3
c) IPR000276	c) G protein-coupled receptor, rhodopsin-like	c)8	c)LOC559950,LOC567820,gnrhr1,gnrhr2,gnrhr4,g-pr74,kiss1rb,npffr1l3
d)IPR002012	d) Gonadotropin-releasing hormone	d)2	d)gnrh2,gnrh3
e) IPR017452	e) GPCR, rhodopsin-like, 7TM	e) 8	e)LOC559950,LOC567820,gnrhr1,gnrhr2,gnrhr4,g-pr74,kiss1rb,npf, fr1l3
f) IPR019792	f) Gonadoliberin I	f) 2	f) gnrh2, gnrh3
g) IPR027983	g) Gonadotropin-releasing hormone II receptor	g) 2	g) gnrhr2, gnrhr4

4. Discussion

Studies on the characterization, localization and expression of neuropeptides involved in the reproduction have been studied in great depth in several fish species[38]. The recent study on zebrafish also linked melatonin to the inhibition of *gnih*in the brain-pituitary-reproductive axis of zebrafish in response to photic conditions[35].

However, the daily rhythmicity of these neuropeptide genes had been poorly studied to date. The present communication demonstrated an insignificant daily variation of the mRNA expression of *gnih, gnrh3 and kiss1* with time in normal photic condition while the expression of only *kiss2* showed diurnal rhythmicity in the zebrafish. The interaction of these four neuropeptides has been studied in brain after the manipulation of photic conditions. The same study revealed their interaction depending upon the environmental condition and ultimately to the reproductive physiology[35]. Moreover, all these neuropeptides have already been linked with the reproduction in various animals including fish[15,32,35].

The STRING network analysis is a powerful tool to study functional interactions in proteins. Our present data of STRING analysis includes direct (physical) and indirect (functional) associations of these four neuropeptides through computational prediction, knowledge transfer between organisms, and interactions aggregated from other (primary) databases[29]. It represents the interaction from KEGG pathway, shows PFAM Protein Domains and INTERPRO Protein Domains and Features (Table 3).The 32 unique nodes obtained from this interaction map represent 32 unique ways in which these proteins can crosstalk (Fig. 4).

The daily variation of GnIH was studied in a few mammals and a significant variation was reported in sheep[3,4]. Moreover, in zebrafish the expression of *fsh* and *lh* is reported to be rhythmic and peaks at night time[28] but, the current results revel the arrhythmicity of *gnih* and *gnrh3* gene and rhythmicity of their mediating gene *kiss2*. Taken together the present finding suggests a complex, multi-level environmental regulation in the brain-pituitary-reproductive axis of fish through these peptides. Additionally, these genes might crosstalk for an optimum spawning success.

The present communication reveals three major interpretations. Firstly, the expression of *gnih* is 10-fold lower than the expression of *gnrh3* (Fig. 1), which indicates that the negative loop of the reproduction can be initiated by a comparatively low level of expression than the positive loop. This observation is also supported

Table 4. List of 32 unique nodes in the merged network generated by STRING network analysis of GnIH (npvf), GnRH3, Kiss1 and Kiss2

Sl. No.	Protein	Description
1	gys2	glycogen synthase 2 (701 aa)
2	kiss1ra	KISS1 receptor a (368 aa)
3	gnrhr2	gonadotropin releasing hormone receptor 2 (412 aa)
4	cbr1l	carbonyl reductase 1-like (277 aa)
5	slc25a3b	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3b (356 aa)
6	lhb	luteinizing hormone, beta polypeptide (140 aa)
7	**npvf**	**neuropeptide VF precursor (198 aa)**
8	pih1d1	PIH1 domain containing 1 (287 aa)
9	gnrhr4	gonadotropin releasing hormone receptor 4 (406 aa)
10	nsfb	N-ethylmaleimide-sensitive factor b (747 aa)
11	gnrhr1	gonadotropin releasing hormone receptor 1 (377 aa)
12	gnrh2	gonadotropin-releasing hormone 2; Stimulates the secretion of gonadotropins (By similarity) (86 aa)
13	npffl	neuropeptide FF-amide peptide precursor like (128 aa)
14	LOC559950	neuropeptide FF receptor 1 like 1 (398 aa)
15	**gnrh3**	**gonadotropin-releasing hormone 3; Stimulates the secretion of gonadotropins (By similarity) (94 aa)**
16	gpr74	neuropeptide FF receptor 1 like 2 (484 aa)
17	kiss1rb	KISS1 receptor b (364 aa)
18	apeh	acylpeptide hydrolase (558 aa)
19	LOC570918	pleckstrin and Sec7 domain containing (1603 aa)
20	**kiss2**	**kisspeptin 2 (125 aa)**
21	ENS-DARG00000076702	pleckstrin and Sec7 domain containing (297 aa)
22	**kiss1**	**KiSS-1 metastasis-suppressor (116 aa)**
23	adcy3l	adenylate cyclase 3 (1119 aa)
24	ENS-DARG00000090933	pleckstrin and Sec7 domain containing (1236 aa)
25	npffr1l3	neuropeptide FF receptor 1 like 3 (446 aa)
26	pik3c2b	phosphoinositide-3-kinase, class 2, beta polypeptide (1598 aa)
27	cbsb	cystathionine-beta-synthase b (597 aa)
28	aldh16a1	aldehyde dehydrogenase 16 family, member A1 (795 aa)
29	paqr8	progestin and adipoQ receptor family member VIII (352 aa)
30	tac3a	tachykinin 3a (125 aa)
31	LOC567820	galanin receptor 1 (347 aa)
32	aprt	adenine phosphoribosyl transferase (177 aa)

by our recent findings where melatonin is only controlling the expression of *gnih* not *gnrh*. Secondly, the expression of only *kiss2* is rhythmic in a LD cycle, and finally, the STRING network analysis suggests that the *kiss2* is the central gene for the communication of the *gnih, gnrh3* and *kiss1*. The latter two findings together conclude that the *kiss2* is the most important gene for the reproductive signalling and may play a pivotal role in the synchronization of environmental stimulus and the reproductive peptides in the brain of zebrafish. Recent studies on zebrafish from our group demonstrated cross-talk between the melatonin bio-synthesizing machinery and clock-associated genes in the whole brain and ovary[8], revealing an "orchestrate model" of synchronization[7]. The present finding is also an indicator of the orchestrate synchronization between the environment and the neuropeptides. Although the use of STRING network interaction map utilizes huge data resources, still the scope of this study is limited. The rhythmicity of these genes at translational level should be performed. Future studies withGenome editing tools (CRISPR/TALEN/ Morpholino) should be performed to elucidate the importance of the Kiss2 in zebrafish reproduction.

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