Hedgehog-PKA Signaling and gnrh3 Regulate the Development of Zebrafish gnrh3 Neurons

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Abstract

GnRH neurons secrete GnRH that controls the development of the reproduction system. Despite many studies, the signals controlling the development of GnRH neurons from its progenitors have not been fully established. To understand the development of GnRH neurons, we examined the development of gnrh3-expressing cells using a transgenic zebrafish line that expresses green fluorescent protein (GFP) and LacZ driven by the gnrh3 promoter. GFP and LacZ expression recapitulated that of gnrh3 in the olfactory region, olfactory bulb and telencephalon. Depletion of gnrh3 by morpholinos led to a reduction of GFP- and gnrh3-expressing cells, while over-expression of gnrh3 mRNA increased the number of these cells. This result indicates a positive feed-forward regulation of gnrh3 cells by gnrh3. The gnrh3 cells were absent in embryos that lack Hedgehog signaling, but their numbers were increased in embryos overexpressing shhb. We manipulated the amounts of kinase that antagonizes the Hedgehog signaling pathway, protein kinase A (PKA), by treating embryos with PKA activator forskolin or by injecting mRNAs encoding its constitutively active catalytic subunit (PKA*) and dominant negative regulatory subunit (PKI) into zebrafish embryos. PKA* misexpression or forskolin treatment decreased GFP cell numbers, while PKI misexpression led to ectopic production of GFP cells. Our data indicate that the Hedgehog-PKA pathway participates in the development of gnrh3-expressing neurons during embryogenesis.

Introduction

GnRH is a neuropeptide that stimulates the secretion of gonadotropins from the pituitary; it controls reproduction via a neuroendocrine system. Three vertebrate GnRH genes are expressed at different brain regions [1]. GnRH1 (LHRH) is primarily found in the hypothalamus, GnRH2 (cGnRH-II) in the midbrain, while GnRH3 (sGnRH) mainly in the terminal nerve of telecephalon [2]. Zebrafish genome lacks GnRH1, while human genome lacks GnRH3. GnRH2 neurons are expressed in the midbrain from fish to mammals [2]. Deletion of GnRH1 gene or disruption of the migration of GnRH1-expressing cells results in hypogonadal mouse with reproductive dysfunction [3,4], showing the importance of GnRH1 in the development of reproductive organs. GnRH2 and GnRH3 cells appear to modulate sexual behaviors [5–7].

In vertebrates, while GnRH2 cells are originated locally in the midbrain, GnRH1 and GnRH3 neurons migrate from their origins to the final destinations in the hypothalamus and the terminal nerve, respectively [8]. The neuroendocrine cells originated from adenohypophysis form the future gnrh1 cells in the hypothalamus, while zebrafish neuromodulatory cells of the neural crest origin form the future gnrh3 cells in the terminal nerve [8]. Gnrh3-producing cells migrate from the olfactory region via terminal nerve into the olfactory bulb and the preoptic area of the brain in salmon [9–11], medaka [12], barfin flounder [13], European sea bass [14] and zebrafish [15].

It is not clear how gnrh progenitor cells differentiate into GnRH neurons, except it is regulated by cues in the nasal midline [16]. In the mouse nasal placode, fibroblast growth factors (FGFs) stimulate the differentiation and axon targeting of GnRH neurons [17]. Blocking FGF signaling in GnRH neurons leads to reduced numbers of GnRH neurons, although the anatomical distribution of GnRH neurons was unaltered [18]. Other factors like retinoic acid, FGF8, Sonic hedgehog (SHH), bone morphogenetic proteins and transcription factor Lhx2 are also involved in the patterning and differentiation of the olfactory system [19,20].

The Hedgehog (Hh) pathway has been implicated in the development of olfactory neurons. Loss of Shh disrupts the axon trajectory of the olfactory receptor neuron [21]. Loss of Xenopus Xhip, an Hh-specific antagonist in Xenopus, suppresses the formation of olfactory placode, while its overexpression results in a larger olfactory placode [22]. In this report we have examined the development of gnrh3 neurons in vivo by analyzing a gnrh3-GFP/LacZ transgenic fish line that we generated. We showed that the development of zebrafish gnrh3 neurons was regulated by gnrh itself and by the Hedgehog-PKA pathway, and FGF signaling may also affect this process.
Materials and Methods

Animals
Zebrafish of the AB and TL strains were reared at 28.5°C as described [23]. The protocol for the use of zebrafish was approved by the Academia Sinica Institutional Animal Care and Utilization Committee. The transgene construct was injected into fish of the AB background to generate F0 transgenic fish, which was crossed with wildtype AB strain to generate F1 transgenic fish. These F1 fish were crossed with TL to obtain F2 transgenic fish in the AB/TL background. The F2 transgenic fish were intercrossed, and the homozygous F4 transgenic fish were examined for all experiments reported here. The smu577 (slow muscle omitted, Hh signaling component), cycb16 (cyclops, nodal related), and oepm134 (one-eyed pinhead, nodal coreceptor) mutants have been previously described [24–26]. To prevent pigmentation, 0.2 mM phenylthiourea (PTU) was added to the water on day 1 post-fertilization (dpf). PTU treatment did not affect gnrh3 gene expression as GFP and LacZ staining with or without PTU had the same pattern (Fig. S1 in File S1).

In situ Hybridization and Immunofluorescence
Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes followed by detection with anti-digoxigenin alkaline phosphatase-conjugated antibody as described previously [27]. The pGEM-T-gnrh3 plasmid was linearized with NcoI before being used as a template for in vitro transcription with SP6 RNA polymerase. After in situ hybridization, staining and mounting, images were captured with a digital camera (Coolpix 990, Nikon).

For double staining, after in situ hybridization, digoxigenin-labeled gnrh3 antisense RNA probe was first reacted with mouse anti-Digoxin conjugated DyLight 488 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) followed by immunochecmmical reaction with rabbit anti-GFP antibody (sc-8334, Santa Cruz, CA, USA) and detection with Alexa Fluor 647-conjugated to donkey anti-rabbit IgG antibody (Invitrogen Corporation, Carlsbad, CA, USA). The signals were observed using a Leica TCS-SP5-MP-SMD confocal system (Leica Microsystems Wetzlar, Wetzlar, Germany).

Figure 1. Co-localization gnrh3 mRNA and GFP in transgenic fish expressing GFP-LacZ under the control of the gnrh3 promoter. A–D, 3 dpf, E–H, 4 dpf. A and E, bright field (BF) view of the embryos. B and F, green color indicated gnrh3 mRNA detection by in situ hybridization. C and G, GFP immunostaining is shown as red signal. D and H, the merged pictures show co-localization of gnrh3 and GFP signals in the olfactory bulb (OB) at 3 dpf and 4 dpf. The anterior is to the left in all panels, and arrows point to the olfactory bulb.

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Figure 2. Detection of GFP- and LacZ-expressing cells in transgenic fish. A, GFP expression at the olfactory region (OR). B–D, GFP or LacZ cells in the olfactory bulb (OB) and telencephalon (Tel) at 3–8 d (days). E, lacZ cells and axons in the brain at 16 dpf. Hyp, hypothalamus. F–G, At 1 month (m) and 4 months, fluorescent axonal extensions are at OB, Tel and POA (preoptic area). Red arrows indicate cell bodies, yellow arrows indicate axons. A, C, F, G, GFP cells; B, D, E, LacZ-expressing cells. The anterior is towards the left in all panels. A, B, F, G, ventral view; C-E, dorsal view.

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Figure 3. Depletion of gnrh3 expression by morpholinos causes reduction of gnrh3 cells. A–D, in situ hybridization with the gnrh3 probe. E–F, fluorescence detection of GFP cells. G–H, detection of LacZ-expressing cells. After injection of gnrh3-MO1, gnrh3 and GFP expression was reduced at 48 hpf (A, C and E) and LacZ cells in the forebrain was reduced at 7 dpf (G). B, D, F and H, Gnrh3 cells neurons after the injection of control sense MO1 (Ctrl-MO1).

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Gnrh3 Gene Cloning, Reporter Gene Constructs and Microinjection
Genomic clones containing the gnrh3 gene were isolated from a zebrafish BAC genomic DNA library (Incyte Genomic, St. Louis, Missouri) using a gnrh3 cdNA fragment as the probe. Four clones (81d01, 131d09, 135c16 and 148c10) were analyzed, and clones 135c16 and 148c10 were further subcloned. About a 10-kb region
covering the entire *gnrh3* gene and its 5′- and 3′-flanking regions has been completely sequenced several times. The sequence is the same as that reported in GenBank (accession number AJ304429).

To create a zebrafish *gnrh3*-GFP targeting vector, the 2.7-kb fragment containing the *gnrh3* promoter to the ATG and the 2.6-kb fragment containing ATG to *gnrh3* downstream were each ligated into the multiple cloning site of *pChi-GZK* vector (a gift from Dr. Guor-Mour Hez) that contains the genes for LacZ and GFP. The final targeting construct, *pChi-gnrh3-GZK*, was about 15 kb (Fig. S2 in File S1). Transgenic zebrafish were generated by microinjection of 50–100 pg *pChi-gnrh3-GZK* DNA into embryos at the one- or two-cell stage. The reporter gene expression was monitored from 24 hpf to 10 dpf using a fluorescence dissecting microscope (MZ-FLIII with GFP 2 filter, Leica). Fluorescence images were captured with a cooling digital camera (SPOT, DIAGNOSTIC instrument, Inc.).

**Morpholino and mRNA Microinjections**

Morpholinos of *gnrh3* antisense MO1 (5′-cactccagtt-taaactctgtggt-3′), MO2 (5′-gaccagacactctctctactc-3′), MO3 (5′-gaaactctctctctcactatgt-3′), control MO1 (5′-aaacaacaggtttgacaggtct-3′), control MO2 (5′-acctttactctctctcactctc-3′) and *fgf1* (5′-gacgcaggtgctcctctatc-3′) (Gene-Tools, Corvallis, Oregon) were diluted to 10 ng/ml and injected into the yolk of 1-cell embryos at 3–15 ng/injection. Capped RNA was synthesized with mMESSAGE mMACHINE T7/SP6 kit (Ambion, Austin, Texas) from linearized plasmids. Full-length *gnrh3* (100 pg) [1], *gnrh2* (100 pg) [1], constitutively active PKA catalytic subunit *PKA* (50 pg) [28], *PKI* (dominant-negative regulatory subunit of protein kinase A, 100 pg) [28], *shha* (2 ng) [29] and *shhb* (2 ng) [29] mRNA was injected into the yolk of 1-cell embryos, and embryos were allowed to develop at 28.5°C.

**β-galactosidase Histochemistry**

For the detection of LacZ activity, larvae were fixed with 4% paraformaldehyde in PBS for 5–10 min at 4°C, washed with PBS, and then incubated in 1 mg/ml LacZ substrate, Bluo-gal (5-bromo-indolyl-b-O-galactopyranoside), in reaction buffer (3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1.5 mM magnesium sulfate, 0.2% sodium deoxycholate, 0.1% Nonident P-40, and 0.15 mg/ml chloroquine in PBS) at room temperature overnight or two days [30].

**Drug Treatment**

Embryos were soaked in forskolin (200 μM, Sigma), cyclopamine (50 or 100 μM), tomatide (100 μM in EtOH, Calbiochem), or LiCl (200 μM) at 28.5°C from 6 hpf to 10 hpf or indicated otherwise.

**Statistical Analysis**

The total number of GFP expressed as means ± standard error of the mean (S.E.M.). *P*<0.05, **P**<0.01.

**Results**

Transgenic GFP and LacZ Expression can Trace gnrh3 Neurons

We have generated transgenic fish expressing GFP-LacZ under the control of the *gnrh3* promoter. Antibody staining detected GFP in the olfactory bulb of the transgenic fish, and this GFP expression co-localized with *gnrh3* transcripts both at 3 dpf and 4 dpf (Fig. 1). GFP expression started early at around 1 dpf, and was evident at 2 days postfertilization (dpf) at the olfactory region (Fig. 2A) and at the olfactory bulb at 3 dpf (Fig. 2B). The expression then extended towards telencephalon (Fig. 2C, D). The GFP/LacZ-expressing neurons were present in the telencephalon and preoptic area at 16 dpf and becoming stronger at 4 months of age (Fig. 2E–G). This GFP expression pattern is similar to those found in other *gnrh3*-GFP transgenic zebrafish [31,32].

Regulation of gnrh3 Neurons by *gnrh3*

To understand the role of *gnrh3* in these gnrh3 neurons, we knocked down *gnrh3* expression in zebrafish with antisense morpholinos (MOs). This led to a decrease of gnrh3 cells at 48 hpf as detected by in situ hybridization (Fig. 3A–D). The expression of GFP fluorescence in transgenic fish that express *gnrh3*-GFP-LacZ was also decreased (Fig. 3E, F). At day 7, LacZ-expressing gnrh3 cells were detected in control-MO1-injected larvae (Fig. 3H), but were missing after *gnrh3* MO1 injection (Fig. 3G). In addition to *gnrh3*-MO1, a different morpholino, *gnrh3*-MO2, was also injected into zebrafish embryos, and 60% of the embryos had decreased gnrh3-expressing cells at 2 dpf. Similarly,
**Table 1.** Aberrant gnrh3 neuron numbers in embryos with perturbed Hh signaling pathways.

| Mutants or treatment | % embryos with decreased gnrh3 expression after incross | Hh manipulation | % embryos with ectopic GFP expression | % embryos with ectopic gnrh3 expression |
|----------------------|--------------------------------------------------------|----------------|---------------------------------------|----------------------------------------|
| oep                  | 21% (n = 95)                                           | PKI            | 9% (n = 34)                           | 11% (n = 45)                           |
| oep+PKI              | 2% (n = 108)                                           | PKI + gsk3b-MO | 29% (n = 38)                          | 29% (n = 34)                           |
| cyc                  | 24% (n = 127)                                          | gsk3b-MO       | 0% (n = 30)                           | 0%                                     |
| cyc+PKI              | 1% (n = 126)                                           | gsk3b-MO       | 0% (n = 30)                           | 0%                                     |
| smu                  | 27% (n = 59)                                           | gsk3b-MO       | 0% (n = 30)                           | 0%                                     |
|                     |                                                        | gsk3b-MO + csnk1a-MO | 0% (n = 30) | 0%                                     |

Embryos from heterozygote mating were examined for gnrh3 expression and the numbers of embryos were scored. Alternatively embryos from wildtype parents were injected with morpholinos or mRNAs before scoring ectopic GFP and gnrh3 expression at 2 dpf.

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gnrh3-MO3 resulted in a decrease of gnrh3-expressing cells in 30% of the embryos at 2 dpf. Therefore all three independent gnrh3-MO sequences led to reduced presence of gnrh3 cells.

To rule out off-target effects of gnrh3-MOs, we added gnrh transcripts back to zebrafish embryos to see whether this can rescue the defect in gnrh3 morphants. Because the gnrh3-MOs can bind gnrh3 mRNA and destroy gnrh3 mRNA, we rescued morphants with gnrh2 mRNA because gnrh2 mRNA is resistant to gnrh3-MO1 and gnrh2 can also bind to gnrh receptors. After injection of gnrh2 mRNA into fertilized eggs, gnrh2 was misexpressed in all parts of zebrafish embryos and partially rescued the defect of GFP cells caused by gnrh3-MO1 (Fig. 4A). Injection of a control β-gal mRNA together with gnrh3-MO1, however, still led

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**Figure 5.** Hh-PKA pathway is required for the development of gnrh3 neurons. All embryos are at 2 dpf. A–D, gnrh3 neurons were detected by *in situ* hybridization with the gnrh3 probe in the wildtype (wt), but not in oep, cyc or smu mutants. E–F, Ectopic GFP expression near the olfactory region in embryos injected with (F) shhb, but not (E) shha mRNA. G–H, gnrh3 signals were normal in control (G) tomatide-treated embryos, but were lost in embryos treated with (H) Hh inhibitor cyclopamine (CYA, 100 µM) at 6–10 hpf. I–K, More ectopic gnrh3-expressing cells were detected after co-injection of gsk3b-MO or csnk1a-MO with PKI mRNA. L, Gsk3b and csnk1a double MO treatment did not increase the number of ectopic gnrh3-expressing cells. The anterior is to the left in all panels.

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to a reduction of GFP cells. This result indicated that gnrh has a role in the proliferation of gnrh3-expressing cells.

We also investigated the effect of gnrh3 overexpression on the expansion of gnrh3 cells. After gnrh3 mRNA microinjection, GFP cells appeared earlier and their cell numbers increased faster than those in the control fish injected with control β-gal RNA (Fig. 4B). These data indicated that gnrh3 was important for the differentiation and proliferation of gnrh3-expressing neurons.

### Regulation of gnrh3 Neurons by the Hedgehog-PKA Pathway

We further investigated the developmental control of gnrh3 neurons by screening existing mutants for defective gnrh3 expression. Expression of gnrh3 was not detected in about a quarter of offspring from the crosses of oep, cyc, and smu heterozygous parents (Fig. 5A–D). The scoring of these mutants followed a Mendelian ratio (Table 1), indicating that these mutants affect something linked to the appearance of the gnrh3 cells. The oep and cyc mutants do not possess ventral forebrain and thus lose shh expression [24,25], while smu is defective in Hh signaling [26]. We therefore examined the participation of Hh signaling in more detail.

When shha (sonic hedgehog a, syn) mRNA was injected into zebrafish embryos, the pattern of gnrh3 was the same as that found in wildtype embryos (Fig. 5E). However, when shhb (sonic hedgehog b) was misexpressed in zebrafish embryos, increased numbers of ectopic GFP cells were detected (Fig. 5F). Conversely, when fish embryos were incubated with Hh pathway blocker cyclopamine (CYA) from 6–10 hpf, the number of gnrh3 neurons was decreased, while incubation with control chemical tomatide had no effect (Fig. 5G–H, Table 2). These gain- and loss-of-function studies indicate the participation of shhb signaling in the development of gnrh3 neurons.

To dissect the involvement of Hh signaling pathway in gnrh3 cell development, we further examined the participation of kinases that antagonize the Hh pathway. In Drosophila, the Hh signaling is blocked by three kinases, PKA, CK1 and GSK3β [33]. In zebrafish the Hh signaling is also antagonized by the PKA pathway, which can be inhibited by a dominant-negative regulatory subunit of protein kinase A (PKI) [28]. Blocking PKA function by PKI caused ectopic gnrh3 expression in about 10% embryos (Fig. 5I and statistics at Table 1). Co-injection of PKI mRNA and gsk3β-MO also increased the number of cells that express gnrh3 ectopically (Fig. 5J). Similarly co-injection of MO against csk1a (zebrafish CK1 orthologue) with PKI mRNA also increased ectopic gnrh3 expression (Fig. 5K), and the population of embryos with increased gnrh3 expression increased to about 30% (Table 1). Injection of gsk3β-MO and csk1a-MO, alone or together, however, did not cause ectopic gnrh3 expression (Fig. 5L, Table 1). Blocking GSK3β activity by LiCl at 6–8 hpf or 6–10 hpf completely blocked gnrh3 expression, but LiCl treatment at 8–12 hpf or 8–24 hpf caused ectopic gnrh3 expression (Table 2), implying that GSK3β affects the differentiation of gnrh3 neurons differently at different developmental stages. These data suggest the PKA, CK1 and GSK3β pathways regulate the development of gnrh3 neuronal progenitors.

To further examine the effect of PKA signaling on gnrh3 expression, we increased PKA activity by injected into fish embryos mRNA for the constitutively active catalytic subunit of PKA (PKAα). This led to a decrease of GFP- and LacZ-expressing cells at 30 hpf (Fig. 6). PKI mRNA (100 pg /embryo) injection, on the contrary, increased the number of ectopic GFP- and LacZ-expressing cells. High concentrations of PKA activator forskolin (200 μM) decreased the number of GFP/LacZ/gnrh3 cells (Fig. 6). PKI mRNA microinjection also partially rescued gnrh3 cells in embryos treated with forskolin treatment (Fig. 6) or in oep and cyc mutant (Fig. S3 in File S1).

| Treatment          | Treatment Time | % gnrh3 embryos | % embryos with ectopic gnrh3 expression |
|--------------------|----------------|-----------------|---------------------------------------|
| Cyclopamine (100 μM) | 6–8 hpf       | 45% (n = 42)    | 0%                                     |
|                     | 6–10 hpf       | 0% (n = 126)    | 0%                                     |
|                     | 6–24 hpf       | 0% (n = 49)     | 0%                                     |
| Cyclopamine (50 μM) | 6–8 hpf       | 93% (n = 54)    | 0%                                     |
|                     | 6–10 hpf       | 13% (n = 32)    | 0%                                     |
|                     | 6–24 hpf       | 0% (n = 28)     | 0%                                     |
| Tomatidine (100 μM) | 6–10 hpf      | 100% (n = 84)   | 0%                                     |
|                     | 6–24 hpf       | 100% (n = 31)   | 0%                                     |
| LiCl (200 μM)      | 6–7 hpf       | 50% (n = 37)    | 0%                                     |
|                     | 6–8 hpf       | 0% (n = 51)     | 0%                                     |
|                     | 7–8 hpf       | 100% (n = 29)   | 0%                                     |
|                     | 6–10 hpf      | 0% (n = 38)     | 0%                                     |
|                     | 7–9 hpf       | 62% (n = 13)    | 0%                                     |
|                     | 8–10 hpf      | 100% (n = 43)   | 0%                                     |
|                     | 8–12 hpf      | 100% (n = 42)   | 50%                                     |
|                     | 8–24 hpf      | 100% (n = 28)   | 100%                                   |
|                     | 10–24 hpf     | 100% (n = 41)   | 0%                                     |

Embryos were treated with drugs for different time periods, and the numbers of embryos that expressed gnrh3 were scored.
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Effect of Fgf Pathway in the Development of gnrh3 Neurons

We also tested Fgf pathway in gnrh3 neuron development by fgfr1-MO microinjection. The fgfr1-MO treatment caused a decrease in the number of gnrh3 neurons, whereas control-MO2 had no effect (Fig. 7). This result suggests that the Fgf pathway may be involved in gnrh3 neuron development.

Discussion

In this paper, we generated GFP-LacZ transgenic fish that express GFP and LacZ in gnrh3-expressing cells. This fish line was used to analyze the development of gnrh3 neurons. We showed that the differentiation and proliferation of these cells were controlled by gnrh3 itself and by the Hh-PKA signaling pathway.

We showed the effect of the Hedgehog-PKA pathway in the development of gnrh3-expressing cells by inhibitor treatment, analysis of fish mutants, morpholino knockdown, and mRNA overexpression experiments. Hedgehog signaling regulates the patterning of craniofacial neural crest cells [34], which will become gnrh3 progenitors located adjacent to the olfactory placode [8]. The Hedgehog signaling may regulate the patterning and the spatial distribution of GnRH3 neurons by influencing neural crest cell migration and/or differentiation. The effect of hedgehog signaling on gnrh cell fate, however, is not that simple. Zebrafish has two shh genes, shha and shhb. While shhb mRNA misexpression led to ectopic gnrh3 progenitor cells, shha misexpression had no such effect. This data indicates that the functions of these two genes are not identical, and shhb seems to be more instructive than shha in directing gnrh cell expansion. Detailed functions of the Hh pathway in zebrafish still need to be examined in the future.

Multiples signaling molecules appear to be involved in the differentiation of gnrh3 neurons. We found blocking of FGF pathway led to a reduction of gnrh3 cells. Furthermore, misexpression of constitutively active PKA* prevented the expansion of gnrh3-expressing cells from their progenitors. Blocking PKA by PKI or PKI together with gsk3b/cnsk1a morpholinos increased ectopic expression of gnrh3 neurons. GSK3b inhibitor, LiCl, abolished or caused ectopic gnrh3 expression in the zebrafish at different developmental states (Table 2). GSK3b and CK1 also participate in the Wnt pathway [35]; it is possible that WNT pathway may also regulate the development of gnrh3 neurons.

Figure 6. PKA pathway antagonizes the development of gnrh3 neurons at 30 hpf. A, GFP- (top) or gnrh3-expressing (bottom) cells in embryos injected with mRNA for constitutively active subunit of PKA (PKA*), PKI, or treated with forskolin (Fsk) or PKI + forskolin. The anterior is to the left in all panels. B, Quantitation of the numbers of GFP-expressing neurons. Twenty embryos were counted for each data point. *P<0.05. **P<0.01. doi:10.1371/journal.pone.0095545.g006
Regulation of Zebrafish gnrh3 Neurons

Several signaling molecules including BMP, FGF, Wnt, and Hh are expressed in the neural plate close to the preplacodal field or in primordial sensory organs close to the cranial placode [36]. Individually or in combination, these molecules are candidates that may induce the formation of cranial preplacode, as well as of individual cranial placodes [37–39]. Detailed functions of Hh or that may induce the formation of cranial preplacode, as well as of primordial sensory organs close to the cranial placode [36].

Supporting Information

Figure S1 Contains Figures S1, S2, and S3. Figure S1. Similar patterns between LacZ staining and GFP signals at different stages. Figure S2. Structure of the gnrh3 mRNA, its gene, and the design of plasmid to generate transgenic fish expressing GFP-LacZ under the control of the gnrh3 promoter. Figure S3. PKI rescue the gnrh3 neurons in oep or cye mutants at 30 hpf. A, Examination of gnrh3 cell numbers in PKI-injected oep mutant. B, Examination of gnrh3 cell numbers in PKI-injected cye mutant.

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Author Contributions

Conceived and designed the experiments: MWK SWL BC. Performed the experiments: MWK BC. Analyzed the data: MWK BC. Contributed reagents/materials/analysis tools: MWK BC. Wrote the paper: MWK BC.

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