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Zebrafish ghrelin is expressed in pancreatic endocrine cells and regulated by metabolic state

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ABSTRACT

Mammalian ghrelin is a stomach-derived peptide that stimulates secretion of growth hormone and food intake. Zebrafish is an excellent model system for forward genetic studies, and many aspects of energy homeostasis characterized in mammals appear to be conserved in the zebrafish. In this study, we investigated the expression and regulation of zebrafish ghrelin by metabolic status. Quantitative RT-PCR revealed that zebrafish ghrelin is highly enriched in anterior gut associated tissues. Using *in situ* hybridization with adult zebrafish tissues, we found that zebrafish ghrelin mRNA was not expressed in intestine tissue, but rather in clusters of endocrine pancreas cells distinct from insulin-expressing islets. Fasting specifically upregulated pancreatic ghrelin but not brain ghrelin expression by 3- to 4-fold and refeeding restored ghrelin transcript to control levels seen in the fed group within 5 h.

These results demonstrate that although ghrelin is expressed in a different site in zebrafish, it is responsive to metabolic state in a similar manner as mammalian ghrelin, suggesting a role in the regulation of feeding in teleosts, and thus validate the utility of zebrafish as a genetic model system for the analysis of the ghrelin system and energy homeostasis.

1. Introduction

Vertebrate energy homeostasis is regulated by complex mechanisms involving both central and peripheral tissues. Ghrelin is the first and only peripheral orexigenic hormone identified that sends a hunger signal to CNS and regulates appetite. Ghrelin was identified by reverse pharmacology in an effort to find an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) [1]. In human and rodents, ghrelin is mainly expressed and secreted from oxyntic glands of the stomach [1]. Lower amounts of ghrelin have been observed in the intestine, hypothalamus, pancreas, pituitary, heart, and kidney [1–6]. Mammalian ghrelin is a 28 amino acid peptide and its octanoylation at the third amino acid residue, a serine, is a unique feature of ghrelin and this post-translational modification is essential for the biological activity of the peptide. Ghrelin is mainly involved in growth hormone release and appetite regulation, although other functions, such as gastric acid secretion, gastric motility, and cardiac function have been reported [7].

The orexigenic function of ghrelin appears mainly mediated through AgRP/NPY and POMC neurons. Ghrelin receptor (GHS-R1a) is expressed on NPY/AgRP neurons and intracerebroventricular injection of ghrelin increases c-Fos expression in NPY neurons and upregulates AgRP mRNA [8]. Orexigenic effect of ghrelin is abolished in the AgRP/NPY double null mouse [9]. Plasma levels of ghrelin hit a peak right before onset of a meal and reach their nadir 1 h after a meal [10,11]. In a similar way, Ghrelin mRNA in stomach is regulated [8].

Recently, ghrelin also has been identified in non-mammalian species including, birds [12], reptiles, amphibians [13,14], and fish [15–20]. Like mammalian ghrelin, ghrelin from lower vertebrates has a conserved third serine residue (Threonine in bullfrog) and purified ghrelin peptides have been shown to have the conserved acylation at this residue [16]. Growth hormone releasing activity by ghrelin has been characterized in a couple of fish species. Incubation of tilapia pituitaries with eel ghrelin significantly stimulates GH and prolactin (PRL) release in a culture model, and intravenous (IV) injection of eel ghrelin into rat increases plasma GH levels [16]. In rainbow trout, intraperitoneal (IP) injection of ghrelin significantly increases the plasma GH levels, but not PRL and somatostatin (SL) [15]. Synthetic goldfish ghrelin increases both

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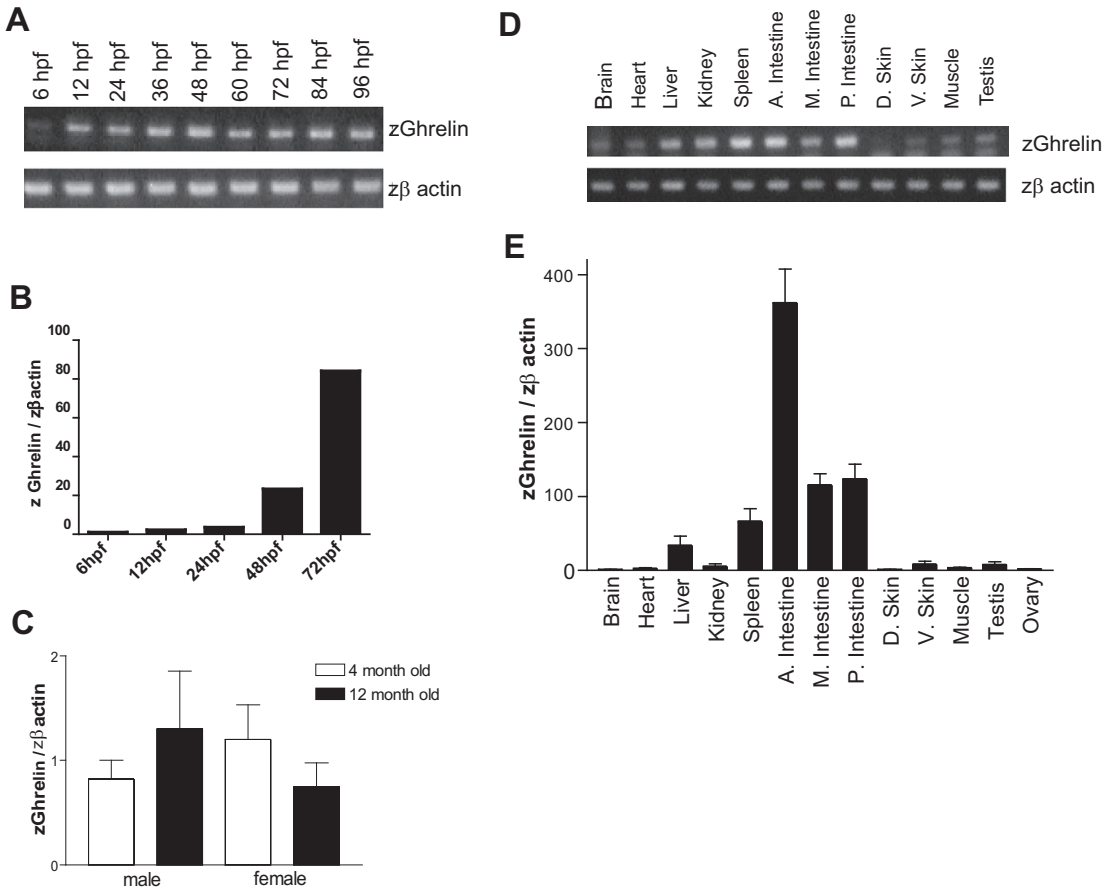

Fig. 1. Temporal and spatial expression of zebrafish ghrelin. (A) Temporal expression of ghrelin was examined by nested RT-PCR analysis. Embryos were collected every six hours from 6 h post fertilization (hpf) to 96 hpf, and subjected to RT-PCR. (B) Real time quantitative RT-PCR analysis of ghrelin expression levels during early development, presented as zGhrelin mRNA level normalized to β-actin expression. (C) zGhrelin mRNA expression was examined from anterior intestine and associated tissues by quantitative RT-PCR between groups of 4 month old and 12 month old (n = 6–9). (D) Zebrafish ghrelin gene expression analysis in various tissues, including brain, heart, liver, kidney, spleen, anterior intestine, middle intestine, posterior intestine, dorsal skin, ventral skin, muscle, and testis, by RT-PCR. (E) Real time quantitative RT-PCR analysis of ghrelin expression level, normalized to β-actin, in tissues from adult specimens. Two male and two female zebrafish were dissected, total RNA was extracted, and ghrelin expression was examined by real time quantitative RT-PCR analysis.

mRNA expression and release of LH and GH when incubated with dispersed pituitary cells and intracerebroventricular (ICV) and IP injection of ghrelin also increases plasma levels of GH and LH [21]. The orexigenic effect of ghrelin has also been examined in goldfish. Goldfish ghrelin is regulated by fasting, and either ICV or IP administration of either goldfish or human ghrelin stimulates feeding in goldfish [17,22].

While the goldfish and trout are well characterized fish models for neuroanatomical and physiological studies, the zebrafish has been developed as a genetic model system for large scale forward genetic analyses [23–25]. Previously we reported that aspects of the control of energy homeostasis by the central melanocortin system are conserved in zebrafish [26,27]. Here, we examine the expression and regulation of the ghrelin system in the zebrafish.

2. Materials and methods

2.1. Zebrafish

Zebrafish were raised and bred as described [28]. 10–15 adult fish were maintained in half-gallon tanks at 26–28 °C, under a 13.5-h light, 10.5-h dark cycle. Animal experimental protocol used in this study is approved by the institution.

2.2. Temporal and tissue specific expression of zebrafish ghrelin

Total RNA was extracted from zebrafish staged between 6 and 96 hpf and from tissues of the adult fish. To quantify the zGhrelin transcripts, RT-PCR was performed with 5′GGCACCAGCTTCCTCAG TCCGACTCAG3′, and 5′CTCCAGAAGATTCTGAAGCAC3′ primers. For real-time quantitative PCR for zGhrelin 5′CAGAAGAGAAGCTGCT-GATCCAG3′, and 5′CTCCAGAAGATTCTGAAGCAC3′, and for β actin with 5′CATCCGTAAGGACCTGTAT3′, and 5′GCAATGATCTTGATCTT-CAT3′ primers were used.

2.3. Fasting and feeding experiments

Adult fish were fed two times a day at 9:00 am and 5:00 pm as previously described in flowing fish system water. For fasting adult fish, 5–19 month old male or female fish were grouped in one tank filled with filter (0.2 μM supor membrane) sterilized fish system water, and manually supplied with fresh filtered system water every other day.

2.4. In situ hybridization

Single or double *in situ* hybridization was performed according to the standard protocol using ^{33}P- or digoxigenin-labeled antisense zGhrelin cRNA. For zebrafish trypsin and insulin *in situ*

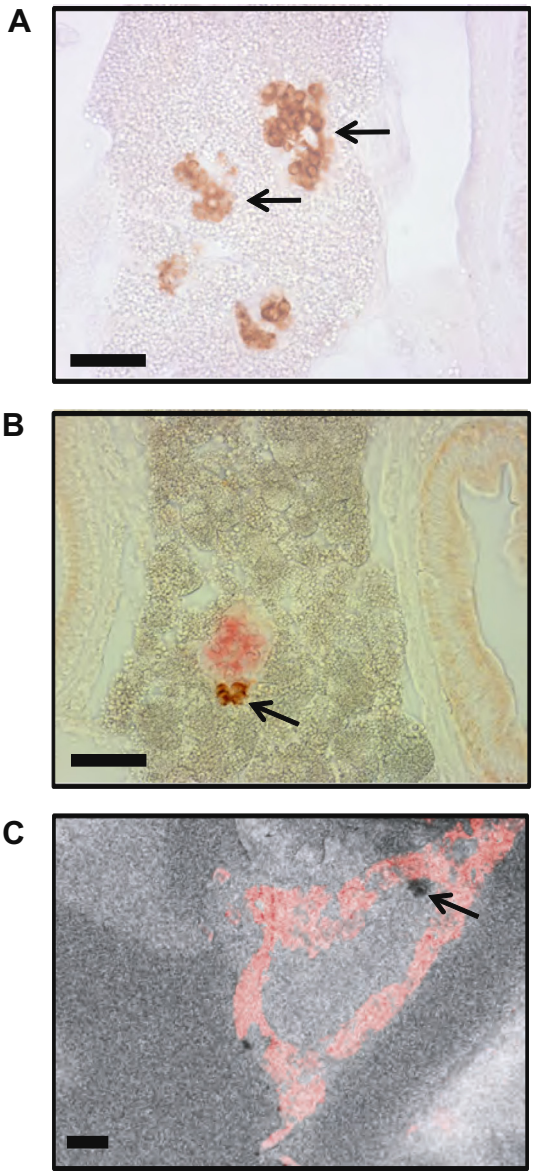

Fig. 2. *In situ* hybridization of adult zebrafish digestive organs with zGhrelin cRNA probes. (A and B) Cross section of double *in situ* hybridization of zGhrelin and zInsulin with Digoxygenin-labeled antisense zGhrelin cRNA probes and Fluorescein-labeled antisense zInsulin cRNA probes in zebrafish gut, and gut associated tissue. Gut and gut-associated tissues were dissected and sectioned in 20 μm slices. zGhrelin signal was developed by DAB staining (brown color) and zInsulin signal was developed by Vector Red staining (red). (C) Double in situ hybridization of ^{33}P-zGhrelin (black color) and zTrypsin-Fluorescein, visualized with Vector Red staining (red color), followed by emulsion dipping, and development. Sense probes did not give any detectable signal. Scale bars represents 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

probes, PCR was performed to amplify the cDNA sequence of zTrypsin according to the published sequence [29,30], and fluorescein labeled cRNA probe was prepared.

2.5. Statistics

Data are presented as means ± s.e.m. Statistical analysis was performed using an unpaired *t* test with Graph Pad Prism software. Statistical significance is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results and discussion

3.1. Temporal expression of ghrelin mRNA in zebrafish

RT PCR using primers spanning intron 1 and 2 showed an expected product of approximately 150 bp using zGhrelin cDNA from 12 hpf embryos. zGhrelin expression appears to increase in early developmental stages, as its expression is minimal at 6 hpf and gradually increased (Fig. 1A and B). Since, zebrafish starts to take food after hatching, earlier expression of ghrelin might have a developmental role, as suggested in the rat [31]. In mouse, circulating ghrelin level decreases with age [32], however there was no difference for anterior intestinal zGhrelin expression between 4 month and 12 month implying zGhrelin mRNA expression reached its maximal basal expression level once development is completed. It is interesting to measure acylated form or secreted form of ghrelin from zebrafish with age in zebrafish. The expression level of zGhrelin between male and female seems also comparable (Fig. 1C).

3.2. Tissue distribution of zebrafish ghrelin

In Tilapia, a fish species containing a defined stomach, ghrelin was detected only in stomach [33]. In rainbow trout, ghrelin expression was detected in the mucosal area of stomach, but not in the intestine [34]. However, in the channel catfish, ghrelin was detected in both stomach and pancreas [35]. Thus, there appear to be significant species-specific differences in the sites of ghrelin expression in fish.

Zebrafish is a stomachless fish, so it was of interest to determine where zebrafish ghrelin was expressed. Goldfish also lacks a stomach and ghrelin expression was detected in brain, spleen, intestine and gill by RT-PCR followed by Southern blot, and Northern blot detected ghrelin mRNA signal only in intestine, suggesting that the goldfish ghrelin is also preferentially expressed in intestinal tissues [17]. First, we examined the tissue expression pattern of zGhrelin by RT-PCR. 40 cycles of RT-PCR detected ghrelin cDNA most strongly from intestinal tissues and spleen but also from a variety of other tissues examined (Fig. 1D). Consistent with RT-PCR data, quantification of zGhrelin mRNA expression by real-time RT-PCR showed that zGhrelin expresses most strongly in anterior intestine. Lesser but significant amounts were detected in mid- and posterior intestine, and lesser amounts in spleen. The expression of zGhrelin in other tissues, such as brain, heart, kidney, skin, muscle, ovary, and testis was minimal (Fig. 1E).

To further characterize the detailed tissue distribution of zGhrelin, we applied *in situ* hybridization techniques. Whole gut associated tissues including intestine, liver, and spleen, were dissected, and subjected to *in situ* hybridization (Fig. 2A–C). zGhrelin mRNA signal was detected in multiple cell clusters from tissues tightly associated with intestinal tissue. Interestingly, the expression of ghrelin was not in gut itself, but rather in a gut-associated tissue scattered along gut and stronger in anterior and decreased in posterior regions (Fig. 2A). In double *in situ* hybridization with zebrafish insulin, we confirmed that zebrafish ghrelin cells are located in the Brockmann body, effectively fish pancreas, in that sometimes zGhrelin-positive cells (brown) are adjacent to insulin-expressing cells (pink) (Fig. 2B). Double *in situ* hybridization with zTrypsin, a pancreatic exocrine marker, shows that zGhrelin expressing cells are surrounded by cells expressing zTrypsin, also indicating that zGhrelin expressing cells are located in the Brockmann body of zebrafish, (Fig. 2C). zGhrelin was never found to coexpress with zTrypsin or zInsulin. The existence of two developmentally independent endocrine populations in the zebrafish pancreas has been suggested [36]. The dorsal posterior pancreatic bud develops into

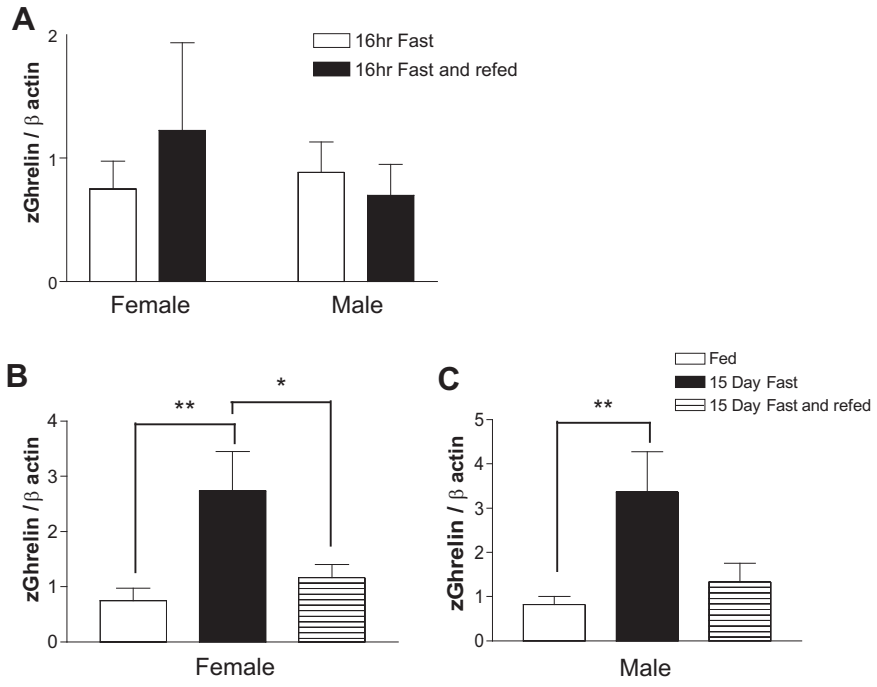

Fig. 3. Pancreatic zebrafish ghrelin mRNA is regulated by metabolic state. zGhrelin mRNA expression level was examined by real time quantitative RT-PCR. (A) Zebrafish were fed as normal at 5 pm previous night and one group was sacrificed about 9 am ($n = 10$). For the other group, fish were allowed free access to food for 1 h then sacrificed ($n = 10$). (B and C) Total RNA was extracted from control fed group, 15 day fast group and a group receiving a 15 day fast followed by 5 h of *ad libitum* refeeding. Relative zGhrelin mRNA expression was normalized to zβ actin mRNA. (B) Female, control group $n = 11$, 15 day fast group; $n = 11$, and 15 day fast and refed group; $n = 6$. (C) Male, control group; $n = 14$, 15 day fast group; $n = 14$, and 15 day fast and refed group; $n = 6$. Results are expressed as mean ± s.e.m., and statistical analyses, comparing fasted values with fed or refed values, were done by unpaired *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

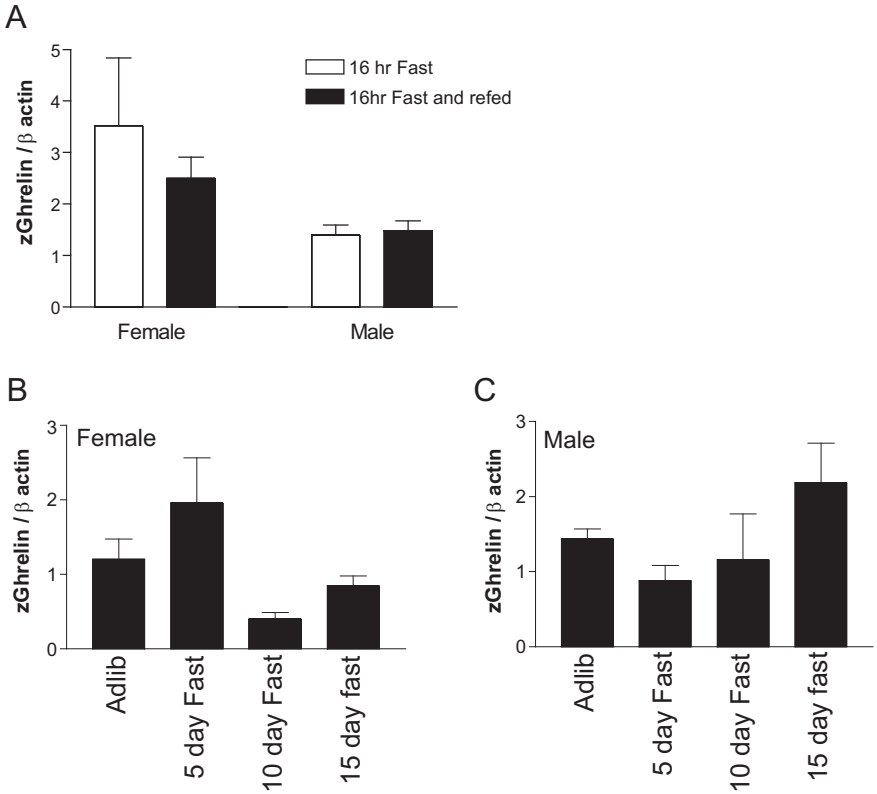

Fig. 4. Brain zebrafish ghrelin mRNA is not regulated by feeding state. zGhrelin mRNA expression level was examined by real time quantitative RT-PCR. (A) Zebrafish were fed as normal at 5 pm previous night and one group was sacrificed about 9 am ($n = 10$). For the other group, fish were allowed free access to food for 1 h then sacrificed ($n = 10$). (B and C) Total RNA was extracted from control fed group, 5 day, 10 day, and 15 day fast group. Relative zGhrelin mRNA expression was normalized to zβ actin mRNA. (B) Female, for each group $n = 6$. (C) Male, for each group $n = 5$. Results are expressed as mean ± s.e.m., and statistical analyses, comparing fasted values with fed or refed values, were done by unpaired *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

the main islet cells expressing insulin, somatostatin, glucagon, and pancreatic polypeptide, whereas the ventral anterior bud gives rise to exocrine cells, pancreatic duct, and small islets. These two buds fuse, and by 76 hpf the main islet is completely surrounded by exocrine cells. Consistent with our results, ghrelin was also detected in smaller islets, suggesting that zebrafish ghrelin originates from ventral anterior bud. Further studies are required to elucidate the origin of ghrelin expressing cells. It may also be interesting to examine the expression of ghrelin from other stomachless fish such as goldfish, to determine in these cases whether the peptide is generally then found in pancreas. The detection of ghrelin by RT-PCR in spleen and liver might be due to the contamination from pancreatic ghrelin tissues.

3.3. Regulation of zGhrelin by metabolic state

Mammalian ghrelin is highly regulated by both short term and long term fasting [11,37–39]. There are discrepancies in gut ghrelin regulation in fish. 7 days fasting did not increase stomach ghrelin expression in Cichlid fish [18,40], but upregulated gut ghrelin in goldfish [22]. Since zGhrelin expresses most strongly in tissues associated with anterior intestine, we decided to look at the metabolic regulation of zGhrelin mRNA expression in anterior intestine associated tissues. We first examined zGhrelin expression before feeding (16 h fast) and 1 h after feeding (16 h fast followed by refed) in the morning. Anterior intestine associated zGhrelin expression level was comparable between the 2 groups (Fig. 3A). However, both in female and male fish, a 15 day fast increased the relative expression of zGhrelin by 3- to 4-fold compared to control fed group. zGhrelin expression returned to normal levels by 5 h after refeeding, following the 15 day fast (Fig. 3B and C). It is possible that, given the ability of zebrafish to consume microorganisms in the water that a true fast does not occur until many days have passed without added food and with regular filtration of the water (see Section 2). Alternately, it is possible that quantification of regulation of ghrelin expression with samples from tissues containing heterogeneous cell types might obscured the alteration of ghrelin expression or acute regulation of ghrelin in fish may be mediated by post-translational modifications rather than transcription or the ghrelin is not regulated acutely by food withdrawal.

Regulated ghrelin expression in the CNS is controversial in mammals. In rat, 24 h and 48 h fasting down regulated hypothalamic ghrelin mRNA expression and n-octanoylated ghrelin expression about 75% [41]. In contrast, goldfish and zebrafish hypothalamic brain ghrelin expression has been reported to be increased by 2- and 200- fold, respectively compared to fed control groups [19,22]. Previously, we showed that zebrafish AgRP, but not zebrafish POMC and AgRP2 expression, is specifically upregulated by fasting similar to mammalian pattern [27,42]. Using the same cDNA samples, we compared zebrafish brain ghrelin expression. However, both in female and male, neither short term fasting (Fig. 4A) nor 15 days of long term fasting upregulated brain zGhrelin expression (Fig. 4B and C). Currently, the divergent results are not understood clearly. Further studies will be needed to better understand hypothalamic ghrelin regulation in the fish.

In conclusion, we have shown for the first time that zGhrelin is expressed in pancreatic endocrine cells rather than in stomach oxyntic cells. Pancreatic but not brain zebrafish ghrelin remains responsive to metabolic state as in mammals, as long term fasting upregulated and refeeding restores its expression. These studies suggest that Ghrelin is likely to be involved in energy homeostasis in zebrafish. Zebrafish is an attractive genetic model system, being one of the few vertebrate systems in which whole genome forward genetic analysis is possible. These together demonstrate fundamental aspects of the ghrelin system that are conserved in the zebrafish, and thus create the possibility of genetic analyses of the ghrelin system in this model.

Acknowledgments

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