Increased food intake in growth hormone-transgenic common carp (Cyprinus carpio L.) may be mediated by upregulating Agouti-related protein (AgRP)

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A B S T R A C T
In fish, food intake and feeding behavior are crucial for survival, competition, growth and reproduction. Growth hormone (GH)-transgenic common carp exhibit an enhanced growth rate, increased food intake and higher feed conversion rate. However, the underlying molecular mechanisms of feeding regulation in GH-transgenic (TG) fish are not clear. In this study, we observed feeding behavior of TG and non-transgenic (NT) common carp, and analyzed the mRNA expression levels of NPY, AgRP I, orexin, POMC, CCK, and CART I in the hypothalamus and telencephalon after behavioral observation. We detected similar gene expression levels in the hypothalamus of TG and NT common carp, which had been cultured in the field at the same age. Furthermore, we tested the effects of GH on hypothalamic fragments in vitro to confirm our findings. We demonstrated that TG common carp displayed increased food intake and reduced food consumption time, which were associated with a marked increase in hypothalamic AgRP I mRNA expression. Our results suggest that elevated GH levels may influence food intake and feeding behavior by upregulating the hypothalamic orexigenic factor AgRP I in GH-transgenic common carp.

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1. Introduction
In aquaculture, enhancing growth is a key objective to improve fish production (Raven et al., 2008). Gene transfer has been employed as a rapid and effective approach to realize this objective. Since the first batch of transgenic fish was produced (Zhu et al., 1997), transgenesis has been used in many fish species (Hu and Zhu, 2010). To date, several stable lines of growth-enhanced transgenic fishes have been generated, including Atlantic salmon (Salmo salar) (Fletcher et al., 2004), coho salmon (Onchorhynchus kisutch) (Devlin et al., 2004), mud loach (Misgurnus anguillicaudatus) (Nam et al., 2002), tilapia (Oreochromis sp.) (Martinez et al., 1999; Rahman et al., 1998) and common carp (Cyprinus carpio L.) (Wang et al., 2001; Zhong et al., 2012). During recent years, it has become clear that elevated growth hormone (GH) levels in transgenic fish induce a wide range of effects apart from growth promotion (Devlin et al., 1999; Du et al., 1992; Martinez et al., 1996; Nam et al., 2001; Rahman et al., 1998; Wang et al., 2001; Zhong et al., 2012), including altered metabolism (Guan et al., 2008; McKenzie et al., 2003; Stevens et al., 1998), swimming performance (Farrell et al., 1997; Lee et al., 2003; Stevens et al., 1998), anti-predator behavior (Abrahams and Sutterlin, 1999; Duan et al., 2010; Dunham et al., 1999) and growth-related neuroendocrine regulation (Raven et al., 2008). In addition, GH treatment resulted in increased food intake and feeding behavior in normal fish (Johnsson and Björnsson, 1994). Similarly, GH-transgenic (TG) fish, with constantly high levels of GH, exhibit accelerated feeding motivation, higher social status, and increased feeding competitive ability (Abrahams and Sutterlin, 1999; Devlin et al., 1999; Duan et al., 2009, 2011; Sundstrom et al., 2003).

Feeding behavior in fish is regulated by numerous environmental factors as well as by complex homeostatic mechanisms, which are mediated by interactions between central and peripheral signals. Peripheral endocrine and metabolic factors convey information about nutritional status to the brain, and then the brain processes this information and induces neurons to secrete neuropeptides to control feeding behavior (Volkoff and Peter, 2006). In the brain, the hypothalamus is the primary center of feeding regulation. A large number of neuropeptides regulate food intake and energy homeostasis, including orexigenic factors, such as neuropeptide Y (NPY), Agouti-related protein (AgRP) and orexins; and anorexigenic factors, such as pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Volkoff et al., 2009). In goldfish (Carassius auratus) and catfish (Ictalurus punctatus), intracerebroventricular (ICV) injections of...
NPY stimulated food intake (Lopez-Patino et al., 1999; Silverstein and Plisetskaya, 2000). Furthermore, the hypothalamic expression of NPY mRNA increased in response to fasting in goldfish and salmon (Narawane and Peter, 2001; Silverstein et al., 1998). AgRP, an antagonist gene of melanocortin receptor 3 and 4 (MC3R and MC4R), participates in the regulation of energy homeostasis and feeding by blocking melanocortin signaling (Cerda-Reverter and Agulleiro, 2011; Stutz et al., 2005). Moreover, fasting led to upregulation of AgRP mRNA in the hypothalamus of goldfish and zebra-fish (Danio rerio) (Cerda-Reverter and Peter, 2003; Song et al., 2003). Two AgRP genes, AgRP I and AgRP II, have been identified in common carp (Wan et al., 2012). Orexins consist of two peptides, orexin A and orexin B encoded by a same precursor, pre-pro-orexin. Orexins play a role in the feeding physiology of fish, as demonstrated by the fact that ICV injections of orexin A and orexin B stimulate feeding behavior and food consumption in goldfish (Volkoff et al., 1999). POMC, the precursor of melanocortin, is mainly processed to alpha-melanocyte-stimulating hormone (α-MSH) and β-endorphin (Cerda-Reverter and Agulleiro, 2011). POMC I and POMC II have already been identified in common carp (Arends et al., 1998). The melanocortin system has been illustrated to play a critical role in the regulation of food intake and body weight in fish (Cerda-Reverter and Agulleiro, 2011). MC4R, a G protein-coupled receptor of α-MSH, possesses seven transmembrane-domain regions and mediates α-MSH to tonic restraint on feeding (Cerda-Reverter et al., 2003; Ringholm et al., 2002). In common carp, two forms of pre-pro-CART derived from separate genes have been identified (Wan et al., 2012). As a well-known appetite inhibiting factor (Volkoff and Peter, 2000, 2001), CART mRNA expression undergoes postprandial increases in common carp (Wan et al., 2012). Cholecystokinin (CCK), a satiety signal mainly produced in the gut and brain, decreases food intake and feeding behavior (Himick and Peter, 1994; Volkoff, 2006). Corticotrophin releasing hormone (CRH) is one of the hypothalamic hormone which controls stress axis and also acts as an anorexigenic factor for satiety regulation. CRH and CRH receptor 1 (CRH-R1) have been cloned and characterized in common carp (Huising et al., 2004).

GH directly or indirectly regulates appetite and food intake through the interaction with hypothalamic neuropeptides. GH and GH receptor (GHR) are present in regions known to participate in the regulation of feeding behavior, energy balance and motivation (Gossard et al., 1987; Hojvat et al., 1982; Lobie et al., 1993). Besides, GH positively regulates AgRP and NPY (Bohlooly et al., 2005; Chan et al., 1996) which are appetite-stimulating neuropeptides. On the other hand, appetite-regulation peptides can influence GH secretion. For instance, NPY negatively regulates GH production through the stimulation of somatostatin (SS)-producing cells (Minami et al., 1998; Peng et al., 1993). In zebrafish, suppression of AgRP I decreased the expression of GH mRNA in pituitary (Zhang et al., 2012). CCK may have direct effects on the pituitary gland and also acts on GH production by reducing SS mRNA (Canosa and Peter, 2004; Himick et al., 1993). Furthermore, changes in GH levels are tightly coupled to nutritional status (Canosa et al., 2007). For example, reduced food intake or starvation causes GH to rise, while GH returns to baseline after resumed feeding (Gabilondo et al., 2006; Pierce et al., 2006; Shimizu et al., 2009).

Feeding behavior influences the ability of fish to compete for food resources which is crucial for survival, competition and growth. Therefore, it is an important fitness parameter for evaluating the ecological effects of TG fish. Besides, the mechanism of GH in feeding regulation is still unknown. In our study, we compared the feeding behavior of TG common carp and non-transgenic (NT) fish, and analyzed the mRNA expression levels of NPY, AgRP, orexin, POMC, CCK, CART, MC4R, CRH and CRH-R1. In addition, we tested the effects of GH on hypothalamic fragments in vitro to confirm our findings. We demonstrated that TG common carp exhibited increased food intake and reduced food consumption time, which were associated with marked increases in hypothalamic and telencephalic AgRP I mRNA expression. We speculate that elevated GH levels may influence food intake and feeding behavior by upregulating the hypothalamic orexigenic factor AgRP I.

2. Materials and methods

2.1. Experimental fish

P0 ‘all-fish’ GH-transgenic common carp were produced by the microinjection of fertilized eggs with the gene construct pCASgGH, which contained the grass carp (Ctenopharyngodon idella) GH gene (gcGH) driven by the β-actin gene promoter of common carp (C. carpio L.). The transmission rates from F1 to F3 followed the expected Mendelian ratio (unpublished data). The GH-transgenic fish used in this study were from the TG2 family as previously described (Zhong et al., 2012).

2.2. Behavioral observations

Experimental fish were maintained indoor in a re-circulating system at the Institute of Hydrobiology, China. Incoming non-chlorinated water was automatically filtered, sterilized and constantly aerated to ensure high water quality. Throughout the experiment, the water temperature, dissolved oxygen and pH were 25–29 °C, 70–85% and 7.4–7.8, respectively. Artificial lighting with a photoperiod set between 07:00 and 19:00 h were provided. Fish were fed with a commercial diet (2 mm diameter pellets; diet containing approximately 42% protein and 3.6% lipids). An approximate 3% of body weight ration of pellets per fish were fed twice a day.

Seven individuals of each genotype, five months post fertilization (mpf), were separately cultured in 14 tempered glass aquaria measuring 50 × 25 × 50 cm with a water depth of 35 cm. The average weight of TG fish was 20.9 ± 0.55 g, ranging from 20.1 g to 23.6 g, and that of NT fish was 21.0 ± 0.56 g, ranging from 20.3 g to 23.3 g. The facility was the same as described in our previous study (Duan et al., 2011).

The experiment consisted of two main parts: (1) a treatment period (days 1–7), and (2) an observation period (days 8–11). Observations began 2 min before the fish were offered pellets in the tank. Each fish was fed with 36 pellets one by one in a 30 s interval time during an 18 min feeding session, twice a day (07:30–12:00 h and 14:00–18:30 h). Two days later, after fasting for a night, all fish were sacrificed for the subsequent experiment.

All experimental procedures employed were approved by the Institute of Hydrobiology Institutional Animal Care and Use Committee (Approval ID: keshuizhuan 08529).

2.3. In vitro experiment

NT common carp (n = 12) were anesthetized with 0.25 ml l−1 Eugenol (purity ≥ 99.99%, Sinopharm Chemical Reagent Co. Ltd., Shanghai, China). The hypothalamus was dissected from each fish; 1 ml cell culture medium was added to each 1.5 ml Eppendorf tube. The tissue samples were aseptically cut, evenly mixed and separated to 24-well plates with common carp GH at final concentrations of 20, 80 and 200 ng ml−1, respectively. The 24-well plates were incubated at 28.6 °C for 2 and 4 h, respectively. After that, the fragments from each well were collected and centrifuged at 4 °C, 4000 rpm for 10 min, the supernatant was removed, 1 ml Triton Reagent (Invitrogen, Carlsbad, CA, USA) was added to each tube, the samples were mixed and stored at −70 °C for subsequent analysis.
2.4. Measurement of serum GH levels by ELISA

TG (n = 12, 5 mpf) and NT common carp (n = 12, 5 mpf) were cultured in the indoor re-circulating system for the measurement of serum GH. Six individuals of each genotype were fasted for two weeks and another six fish of each genotype were fed normally. All 24 fish were anesthetized as described above. Sera from these fish were collected and processed for the measurement of serum GH levels. An enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Wu et al., 2008).

2.5. Expression studies

Total RNA samples were isolated from the hypothalamus and telencephalon of each fish from the behavioral observation experiment. Furthermore, TG (n = 30, 5 mpf) and NT common carp (n = 30, 5 mpf) were divided into four tanks in the indoor re-circulating system. Following the acclimation period, two tanks were food deprived for two weeks and two tanks were maintained on the regular feeding schedule. The hypothalamus of all 60 fish were also dissected for the gene expression analysis. Another 20 individuals of each genotype, 5 mpf, cultured outdoors and fed regularly, were also sacrificed after fasting for overnight.

RNA extractions were performed using TRIzol Reagent (Invitrogen) according to the manufacturers’ protocol. The RNA amount and purity were determined by spectrophotometry and electrophoresis. The quality of RNA samples was assessed by measuring the ratio of sample absorbance at 260 and 280 nm. Only RNA samples with a ratio between 1.8 and 2.1 were used.

After DNase I (Promega, Madison, WI, USA) treatment, 1 µg total RNA was used for the first cDNA transcription. The reverse transcription system contained 1 µg RNA, 4 µl 5· RT buffer, 1 µl ReverTraAce (100 U), 0.5 µl RNase inhibitor (20 U), 1 µl dNTPs (10 mM) and 2 µl random primers 9 (25 pm/µl; TaKaRa, Japan), to a total volume of 20 µl. PCR was conducted under the following conditions: 30 °C for 10 min, 42 °C for 60 min and 95 °C for 5 min. Real-time PCR was performed as previously described (Guan et al., 2011). The primers used in this study are listed in Table 1. The POMC primers used for amplification are specific to both POMC I and POMC II. The primer sequences of CRH, CRH-R1, AgRP II, CART II, MC4R were previously published (Huisings et al., 2004; Wan et al., 2012).

Relative quantification experiments were conducted on 96-well plates under the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 15 s and 72 °C for 45 s. Samples were analyzed in duplicates and experiments were repeated at least twice. In all cases, a no template-negative control (in which cDNA was replaced by water) was included.

Expression levels were compared using the relative Ct (ΔΔCT) method. Briefly, the average CT of the reference gene (β-actin) was subtracted from the average CT of the gene of interest to determine the ΔCT for each sample. The ΔCT of the calibrator (control fish or non-treated group) was then subtracted from the ΔCT of each of the samples to determine the ΔΔCT. This number was then used to determine the amount of mRNA relative to the calibrator and normalized by β-actin. Data were provided as fold changes in expression relative to the reference gene, and compared to a calibrator sample from the control group. The average fold of all control samples were taken and set at 1. The experimental groups were then normalized relative to the control group. These values (1 for the control and other values for the experimental groups) were then statistically compared.

2.6. Data analysis

Student’s t-tests with P < 0.05 were used to detect significant differences in food intake. Gene expression levels between GH-transgenic and NT common carp, in the in vitro experiment, and gene expression levels between GH-treated and non-treated groups were also analyzed by the Student’s t-test. Statistical comparisons between fasted and fed TG and NT carp were carried out with one-way analysis of variance (ANOVA) using GraphPad Prism 5.0 (GraphPad Software, San Diego CA, USA). In the expression studies, real-time PCR data were acquired and analyzed using ABI Prism 7000 SDS 1.1 software (Life Technology, CA, USA). Data were considered reliable when the standard deviation (SD) of three replicate reactions was <15%. All samples are expressed as ratios of the specific target gene to β-actin and normalized as mRNA levels of NT fish or the non-treated group.

3. Results

3.1. Food intake

During the experimental period, we measured the average food intake of TG and NT common carp, which were 0.71 ± 0.024 g and 0.59 ± 0.03 g, respectively (Fig. 1A). TG common carp consumed more food during the experiment. The maximum average food intake in 0.5 h was 0.98 ± 0.05 g and 0.69 ± 0.043 g in TG and NT common carp, respectively (Fig. 1B). The time to consume 0.5 g food was also measured, in TG fish it was 8.65 ± 0.72 min and 14.76 ± 1.8 min in the control group (Fig. 1C). TG common carp exhibited higher and faster food intake.

3.2. Serum GH levels

For control fish, serum growth hormone levels during fasting were 3.10 ± 0.89 ng ml⁻¹. In satiety status when fed normally, the serum growth hormone levels were 0.2 ± 0.12 ng ml⁻¹. In TG fish, serum growth hormone levels were 7.74 ± 2.90 ng ml⁻¹ and 9.81 ± 2.3 ng ml⁻¹, respectively (Fig. 2A).

3.3. Hypothalamic AgRP I mRNA expression in fasted and fed fish

The mRNA expression level of hypothalamic AgRP I in the fed NT common carp is significantly lower (0.52) compared to the fasted NT fish (1). The mRNA expression level of hypothalamic AgRP I in

<table>
<thead>
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<th>Table 1</th>
<th>Primer sequences used in this study.</th>
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<tr>
<td>Primer</td>
<td>F</td>
</tr>
<tr>
<td>GH</td>
<td>5′-TGCTATTTGGCTGGTGT-3′</td>
</tr>
<tr>
<td>GHR</td>
<td>5′-CCACAACCCACACCACGCT-3′</td>
</tr>
<tr>
<td>NPY</td>
<td>5′-TGGCTGGGAACTCTAAGCGGA-3′</td>
</tr>
<tr>
<td>AgRP I</td>
<td>5′-CCCTGCTACCTCCACTACG-3′</td>
</tr>
<tr>
<td>Orexin</td>
<td>5′-ATCTCCCTAGCTAGGGAAGG-3′</td>
</tr>
<tr>
<td>CART I</td>
<td>5′-AGGCTGCGACCAGATGAG-3′</td>
</tr>
<tr>
<td>CART II</td>
<td>5′-ACGATACATCCACTCAAC-3′</td>
</tr>
<tr>
<td>POMC</td>
<td>5′-AACCCCCCTCCACTAAAGG-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GATGATGAAATTGCCGACTG-3′</td>
</tr>
<tr>
<td>R</td>
<td>5′-CTGGTGTTTGGCGACATGC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-CCACTCGGAGCCAGACCT-3′</td>
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<tr>
<td></td>
<td>5′-ACCCCATCGACTCCTCTG-3′</td>
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<td>5′-ACCACCCACCCACCTCCCTT-3′</td>
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fasted and fed TG common carp is 1.74- and 2.90-fold higher when compared to their NT counterparts respectively (Fig. 2B).

3.4. Hypothalamic GH and GHR mRNA expression

TG common carp displayed significantly higher hypothalamic GH mRNA expression, which was about 137.7-fold higher than in control fish. There was no significant difference in hypothalamic GHR mRNA expression levels between TG and NT common carp (Fig. 3).

3.5. Gene expression of behavior-observed fish

We analyzed gene expression levels in TG (n = 7) and NT (n = 7) common carp after observing their feeding behavior. The relative gene expression levels of NPY, AgRP I, orexin, CCK, CART I and POMC in the telencephalon and hypothalamus were detected. Significantly higher expression levels of the orexigenic gene AgRP I were observed in both the telencephalon and hypothalamus in TG common carp, which were 2.97 ± 0.72 and 2.62 ± 0.43-fold higher than in NT control fish, respectively. Other appetite regulation genes (NPY, orexin, CCK, CART I and POMC) showed no significant differences between the two groups (Fig. 4A and B).

3.6. Gene expression of fish cultured outdoors

We analyzed hypothalamic gene expression levels in TG (n = 20) and NT (n = 20) common carp, which had been cultured outdoors. A significantly higher expression level of AgRP I was observed in TG fish, which was increased 2.14 ± 0.39-fold compared to NT fish. The relative gene expression levels of NPY, orexin, CCK, CART I, CART II, POMC, MC4R, CRH and CRH-R1 showed no significant differences between the two groups (Fig. 5).
3.7. In vitro experiment

NT common carp hypothalamic (n = 12) fragments were treated with GH at 20 ng ml⁻¹, 80 ng ml⁻¹, 200 ng ml⁻¹, and cultured for 2 h and 4 h, respectively. Changes in gene expression levels were detected in GH-treated fish at every concentration at each culture time, and also in the non-treated groups. When treated with 20 ng ml⁻¹ of GH for 2 h, the mRNA expression level of NPY increased 2.10 ± 0.31-fold compared to non-treated group. There were no significant changes for the other tested concentrations of GH (80 ng ml⁻¹ and 200 ng ml⁻¹ for 2 h; 20 ng ml⁻¹, 80 ng ml⁻¹ and 200 ng ml⁻¹ for 4 h; Fig. 6A). For AgRP I, when treated with GH at 80 ng ml⁻¹ and 200 ng ml⁻¹ for 2 h, the expression levels of AgRP I were increased 1.85 ± 0.30 and 2.93 ± 0.34-fold compared to the control, respectively. When treated with 20 ng ml⁻¹ GH for 4 h, the expression levels of AgRP I were increased 2.63 ± 0.23-fold compared to the control (Fig. 6B). There was no significant difference in AgRP II mRNA expression levels between the GH-treated and non-treated groups (Fig. 6C). The relative gene expression levels of orexin, CCK, CART I, CART II, POMC and MC4R showed no significant differences between the GH-treated and non-treated groups (Supplemental Fig. 1).

4. Discussion

4.1. GH increased food intake

In fish, it is well established that administration of exogenous GH increases food intake and feeding behavior (Johnsson and Bjornsson, 1994; Markert et al., 1977). Similarly, elevated GH in transgenic salmon induced heightened feeding motivation, increased food intake and feeding behavior (Abrahams and Sutterlin, 1999; Devlin et al., 1999; Sundstrom et al., 2003). In our previous study, we also found that TG common carp consumed more food during competition with normal common carp of the same size, and that this was associated with higher social status and enhanced feeding motivation (Duan et al., 2011). In this study, we demonstrated that under separate culture conditions, the maximum food intake in 0.5 h was significantly higher in TG individuals, and the food consumption time was also significantly shorter than in control fish. These results suggest that overexpressed GH may inherently enhance appetite and feeding motivation in common carp, which result in increased food intake and improve the ability to compete for limited food resources when cultured with control fish.

4.2. GH and hypothalamic feeding regulation

Circulating GH levels are influenced by the nutritional status of fish (Canosa et al., 2007). Our study demonstrated that two weeks
fasting elevated serum GH levels in NT common carp, while serum GH levels were very low in satiated fish. These results are in concordance with the studies in goldfish, flounder, rainbow trout and salmons (Canosa et al., 2005; Fuentes et al., 2012; Cabillard et al., 2006; Pierce et al., 2005; Shimizu et al., 2009). Next, we observed high expression levels of serum GH in both fasted and fed TG common carp, while there is no significant difference between them (Fig. 2A). On the other hand, we observed that the mRNA expression of AgRP I was decreased in fed NT common carp, while there is no significant difference between fasted and fed TG common carp although both are significantly higher than their NT counterparts (Fig. 2B). Interestingly, this mRNA expression pattern is similar to the changes in serum GH levels in NT and TG common carp. In our study, the hypothalamic GH mRNA expression was significantly higher in TG common carp compared with NT individuals. As the GH transgene is driven by the β-actin promoter, the GH mRNA expression was also significantly higher in other tissues, such as muscle, liver, gonad (unpublished data) compared to NT fish. This may provide an explanation for the constant high level of serum GH in TG common carp which is not influenced by feeding condition. Our results suggested that the altered GH secretion pattern may change the mRNA expression levels of hypothalamic orexigenic factor AgRP I in TG common carp. The constant overexpression of GH transgene can result in increased food intake and feeding behavior (Abrahams and Sutterlin, 1999; Devlin et al., 1999; Duan et al., 2009, 2011; Sundstrom et al., 2003), and GH can also regulate food intake by acting on the feeding control center of the central nervous system (CNS) (Bohlooly et al., 2005). Therefore, we speculate that the constant high levels of hypothalamic and serum GH may have a direct or indirect impact on the feeding regulation in the hypothalamus, thereby enhancing appetite and feeding motivation.

4.3. GH upregulated AgRP I

We did not detect any differences in the mRNA expression of CART, orexin and CCK in the hypothalamus and telecephalon of TG and NT common carp. We also found no significant changes in the expression of these genes when hypothalamus fragments were treated with GH in vitro. Similarly, there were no significant differences in the expression of NPY and CCK in TG coho salmon (Raven et al., 2008). However, hypothalamic AgRP I mRNA expression in TG common carp was drastically upregulated in comparison with the control group, independent of whether these fish were bred indoors or outdoors, fasted or fed. Examination of the GH-treated hypothalamic fragments revealed that incubation with GH (80 ng ml⁻¹ or 200 ng ml⁻¹ for 2 h; 20 ng ml⁻¹ for 4 h) could promote the expression of AgRP I. For NPY, no significant difference was found between the transgenic and control fish. In vitro, the mRNA expression levels of NPY only increased when incubated with 20 ng ml⁻¹ of GH for 2 h, while there were no significant changes with other GH concentrations (80 ng ml⁻¹ and 200 ng ml⁻¹ for 2 h; 20 ng ml⁻¹, 80 ng ml⁻¹ and 200 ng ml⁻¹ for 4 h). These results show that AgRP I, instead of NPY, was responsive to the action of GH overexpression in transgenic common carp. A previous study revealed that AgRP and NPY are synthesized in the same neurons, and both are orexigenic neuropeptides in the CNS (Hahn et al., 1998; Volkoff and Peter, 2006), indicating that AGRP and NPY are regulated in parallel. However, in TG common carp, the expression levels of NPY and AgRP were different, which may be the result of NPY and AgRP being involved in two different neuropeptide regulation pathways (Cerda-Reverter and Agulleiro, 2011; Murashita et al., 2009). Some studies also demonstrated that GH could promote the expression of hypothalamic AgRP. Kamegai et al. found that NPY/AgRP neurons expressed GHR mRNA (Kamegai et al., 1996), which was activated following systemic GH administration (Minami et al., 1992). Furthermore, when the GH transgene was overexpressed in the CNS of mice, it could stimulate food intake, which was associated with a marked increase in AGRP (Bohlooly et al., 2005). Moreover, the orexigenic effect of ghrelin, an endogenous GH secretagogue, was partly mediated by AgRP. After injection of ghrelin in GHR-deficient mice, neither the mRNA

Fig. 6. Relative expression levels of NPY, AgRP I and AgRP II in GH-treated hypothalamus fragments. (A) NPY mRNA expression in cultured hypothalamus fragments treated with GH at final concentration of 20 ng ml⁻¹, 80 ng ml⁻¹, 200 ng ml⁻¹, for 2 h and 4 h, respectively. When treated with 20 ng ml⁻¹ of GH for 2 h, the mRNA expression levels increased 2.10 ± 0.31-fold, compared to the non-treated group. (B) AgRP I mRNA expression in cultured hypothalamus fragments treated with GH at final concentration of 20 ng ml⁻¹, 80 ng ml⁻¹, 200 ng ml⁻¹, for 2 h and 4 h, respectively. When treated with GH at 80 ng ml⁻¹ and 200 ng ml⁻¹ for 2 h, the expression level of AgRP I increased 1.85 ± 0.30 and 2.93 ± 0.34-fold, compared to control levels, respectively. When treated with 20 ng ml⁻¹ GH for 4 h, the expression levels of AgRP I increased to 2.63 ± 0.23-fold, compared to the control. (C) AgRP II mRNA expression in cultured hypothalamus fragments treated with GH at final concentration of 20 ng ml⁻¹, 80 ng ml⁻¹, 200 ng ml⁻¹, for 2 h and 4 h, respectively. Expression levels in control for 0 h were normalized to 1. Data are presented as mean ± SEM. Asterisks represent significant differences (Student’s t-test, *P < 0.001).
expression levels of AgRP or food intake changed, while both AgRP and food intake in the control group vastly increased (Egecioglu et al., 2006). It has also been shown that AgRP can compensate for the loss of NPY. GH-releasing peptide-2 (GHRP-2) treated NPY-deficient mice still retained a strong appetite, and there was an increased level of AgRP expression (Tschop et al., 2002). Collectively, elevated GH levels could play a central role in regulating food intake via the hypothalamic AgRP neuropeptide pathway.

It has been revealed that AgRP is an important orexigenic neuropeptide in goldfish and zebrafish (Cerda-Reverter and Peter, 2003; Song et al., 2003). Two AgRP genes, AgRP I and AgRP II have been identified in common carp (Wan et al., 2012). In this study, both in vitro and in vivo showed that only AgRP I was upregulated by GH, while AgRP II was not affected. In phyllogenetic analysis, fish AgRP I and mammalian AgRP were grouped in the same cluster, indicating a conserved function of AgRP I. AgRP II did not share syntenies with AgRP in mammal, which might be a result of whole genome tetraploidization events (Västermark et al., 2012). In zebrafish, AgRP I morpholino oligonucleotides (MOs) injection reduced somatic growth rate which were associated with a marked decrease in pituitary GH mRNA expression. In contrast, suppression of AgRP II expression did not affect linear growth (Zhang et al., 2012). Therefore, we postulate that GH may regulate feeding behavior in common carp through upregulating AgRP I in the hypothalamus. AgRP increases food intake by functioning as an antagonist to the melanocortin 3 and 4 receptors. However, we found no differences in the expression of POMC and MC4R mRNA between TG and NT fish. In GH-treated hypothalamus fragments, we were unable to find any significant changes in the expression levels of these two genes. Some other studies have also demonstrated the orexigenic role of AgRP may act through other pathway besides inhibiting MC4r (Hagan et al., 2000; Kim et al., 2002).

In conclusion, we demonstrated that the expression of hypothalamic AgRP I, but not NPY, was higher in GH-transgenic common carp, suggesting that the constant high levels of GH may influence the AgRP I neuropeptide pathway. Therefore, we speculate that GH-induced hyperphagia in transgenic common carp may be partially due to direct action upon the appetite control centers in the hypothalamus. The mechanisms of GH in regulating appetite are a complex network. Besides its action on the CNS, GH may affect feeding behavior indirectly through a peripheral pathway. It is possible that changes in the feeding behavior of GH-transgenic carp may be the results of both CNS and peripheral regulation. Further research is required to clarify the underlying molecular mechanisms of appetite regulation in GH-transgenic fish.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ygenen.2013.03.024.

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