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Activity of *etv5a and etv5b* genes in the hypothalamus of fasted zebrafish is influenced by serotonin

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ABSTRACT

Serotonin has been implicated in the inhibition of food intake in vertebrates. However, the mechanisms through which serotonin acts has yet to be elucidated. Recently, *ETV5* (ets variant gene 5) has been associated with obesity and food intake control mechanisms in mammals. We have analyzed a putative physiological function of the two *etv5* paralogous genes (*etv5a* and *etv5b*) in neuronal food intake control in adult zebrafish that have been exposed to different nutritional conditions. A feeding assay was established and fluoxetine, a selective serotonin re-uptake inhibitor (SSRI), was applied. Gene expression changes in the hypothalamus were determined using real-time PCR. Fasting induced an up-regulation of *etv5a* and *etv5b* in the hypothalamus, whereas increased serotonin levels in the fasted fish counteracted the increase in expression. To investigate potential mechanisms the expression of further food intake control genes was determined. The results show that an increase of serotonin in fasting fish causes a reduction in the activity of genes stimulating food intake. This is in line with a previously demonstrated anorexigenic function of serotonin. Our results suggest that obesity-associated *ETV5* has a food intake stimulating function and that this function is modulated through serotonin.

1. Introduction

Ets variant 5 (*ETV5*), also known as ets-related molecule or ERM, belongs to the ETS-family of transcription factors and is a considered transcriptional target of the FGF/MAPK signaling pathway (Krejci et al., 2009). In humans, *ETV5* has been recently related to obesity by genome wide association studies (GWAS) (Thorleifsson et al., 2009) and a single nucleotide polymorphism (SNP), located 6 kb in the upstream region of this gene, appeared in obese people of European, Asian, African-American and Hispanic populations (Elks et al., 2010; Sandholt et al., 2010; Dorajoo et al., 2012; Gong et al., 2013; Graff et al., 2013). In mice, deleted *Etv5* gene function resulted in reduced body weight of the animals suggesting that *Etv5* might counteract weight loss (Schlesser et al., 2008). Mice on a high fat/high sucrose diet had reduced *Etv5* expression levels in the hypothalamic arcuate nucleus, which is involved in food intake control (Boender et al., 2012). In contrast, a diet with limited food resulted in a decrease of *Etv5* expression in the ventral tegmental area and substantia nigra, two nuclei also involved in food intake control that are located in the ventral midbrain. Moreover, a recent study in Drosophila also showed a connection between Ets96B, an ETV5 homologue, with obesity and bipolar disorders (Williams et al., 2016).

In this context, we intended to gain insight to whether the *etv5* homologous genes in zebrafish would correlate to the expression of genes known to be involved in food intake control and metabolism mechanisms. In zebrafish, two paralogous genes, *etv5a* and *etv5b (erm)* have been described (Münchberg et al., 1999). Previous studies have shown that zebrafish *etv5b* gene is expressed in the periventricular hypothalamus (Topp et al., 2008), a region that contains nuclei related to food intake control, and is considered orthologous to the arcuate nucleus (Zohar et al., 2010; Biran et al., 2015). A recent study in zebrafish shows the role of *etv5b* in serotonergic neuron development. This research showed that Fgf signaling acts in the production of serotonin (5-HT) cells through Etv5b, acting on 5-HT progenitors prior to differentiation (Bosco et al., 2013).

In fish, as in mammals, 5-HT is synthesized in the raphe nucleus of the hindbrain from which projections travel to a number of

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higher centres, including the hypothalamus (Hornung, 2003; Lillesaar, 2011). The serotonergic system has been mostly analyzed in relation to mental illnesses, but it shares many common pathways with obesity that may account for the observed co-morbidity of obesity and depression (Stunkard et al., 2003). Studies that addressed the obesity-related role of serotonin found that it has a potent anorexigenic effect following feeding in rodents (Xu et al., 2012), with a significant increase in its release from the superior raphe nucleus (SRa) after a meal (De Fanti et al., 2001) and activating Pomc neurons of the arcuate nucleus (Roepke et al., 2012). Administration of fluoxetine, a common SSRI antidepressant that inhibits re-uptake of serotonin at the synapse and subsequently increases extracellular serotonin levels in the brain, has been shown to reduce feeding in rats (Currie et al., 2004; Myung et al., 2005). Fenfluramine, another drug that activates the central serotonin system causes significant weight loss in humans (Halford et al., 2005). Conversely, manipulations that lower concentrations of serotonin lead to diminished activation of the serotonergic system, hyperphagia and increased body weight (Geyer et al., 1976; Saller and Stricker, 1976). Dysregulation of the serotonergic system is suggested in obesity, with genetically obese rats being shown to have a hyper-excitable raphe nucleus, which leads to increased serotonin levels in the hypothalamus after feeding (Ohliger-Frerking et al., 2003).

Conclusive evidence that serotonin acts as a potential factor to control appetite in teleost fish and that serotonin levels can be experimentally modified using the SSRI fluoxetine has been reported. In goldfish, fluoxetine treatments reduce food intake and body weight (Mennigen et al., 2010) and this anorexigenic effect, can be regulated through corticotropin-releasing factor (CRH) (De Pedro et al., 2008). Similarly, fluoxetine treatments cause decrease in food consumption in fathead minnows (*Pimephales promelas*) (Stanley et al., 2007), evidenced by slow capture of preys (Weinberger and Klaper, 2014). Finally, in rainbow trout, inhibition of food intake has been demonstrated to be induced by serotonin through 5-HT2C and 5-HT1A receptors and to increase the mRNA expression levels of three neuropeptides related to the control of food intake; pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART) and CRH (Pérez-Maceira et al., 2016).

In this study we aimed to determine whether the obesity-association of *ETV5* could relate to the serotonergic system by attempting to establish a functional relationship between *etv5a* and/or *etv5b* and serotonin in adult zebrafish. The utility of zebrafish as a model for the study of obesity has been established in recent years due to the presence of analogous metabolic pathways and structural components of the food intake system (Schlegel and Stainier, 2007). Therefore, we aimed to investigate the effect of fasting on hypothalamic *etv5a/b* expression in adult zebrafish. In this experimental design we have also examined the effect of fluoxetine administration and have included the analysis of food intake and serotonergic marker genes.

2. Experimental procedures

2.1. Animal housing

Adult wildtype (WT) zebrafish were housed in groups of ~30 in 10 L tanks within a circulating water system, providing constant oxygen and removal of waste. A light/dark cycle of 14 h light and 10 h dark was managed by an automated system (AquaSchwarz). Optimal conditions were maintained at 28.5 °C water temperature, pH 7.0, and conductivity 500 μs. All experimental protocols were carried out with the approval of the University of Sydney Animal Ethics Committee.

2.2. Three-week feeding experiment

Adult male WT zebrafish were randomly distributed into three five-liter tanks located within the circulating water system. The nutritional study was conducted at 28.5 °C water temperature for a period of 3-weeks after a 1-week period of acclimation. The first group, (n = 8) was fed ad libitum 3 times daily (Over-fed), the second group (n = 8) was fed ad libitum once daily (Fed) and used as the control group, and the last group (n = 8) was maintained under food deprivation conditions with feeding once weekly per animal ethics committee specifications (Fast). Zebrafish were weighed weekly to determine weight loss during the experiment (Fig. 1A). After 3-weeks of differential feeding all fish were sacrificed and biometric information, standard length (cm), measured from tip to the end of the body, and the body weight (g) were obtained. The lengths of the fish were measured in the beginning and at the end of the experiment while the body weight was defined weekly throughout the study (Fig. 1A). Fish were anesthetized with MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and sacrificed by decapitation. The brains were quickly removed under RNase-free conditions, and the hypothalamus and hindbrain collected to be frozen individually in liquid nitrogen and stored at −80 °C until used.

2.3. Selective serotonin re-uptake inhibition in fasted fish through fluoxetine treatment

Fluoxetine hydrochloride (F132, Sigma-Aldrich) was diluted in nuclease-free water to a concentration of 100 μg/L. Acute treatment was performed in accordance with previous established protocols, with 100 μg/L fluoxetine applied to system water for 3 h before sacrifice (Lynn et al., 2007; Norton et al., 2011). Chronic treatment was also previously established with the application of 100 μg/L fluoxetine for 2 weeks (Egan et al., 2009), but a recent publication demonstrated a shorter effective treatment period of one week (Wong et al., 2013), which was adopted here. Fluoxetine has a half-life of 9 days in fish and 3 days in water containing fish (Brooks et al., 2005; Gaworecki and Klaine, 2008; Paterson and Metcalfe, 2008), therefore water was replaced every 2 days with new system water and fresh fluoxetine in order to maintain a constant concentration.

Adult male WT zebrafish were used for experimentation, with control fish housed in identical conditions to treatment groups. The fish were separated into 4 groups (n = 8) and habituated in 5 L tanks outside the system with constant aeration overnight. Group I (Fed) were fed ad libitum twice daily, with water changed in the morning and night to prevent ammonia build-up due to waste. Group II (Fast) were fasted for 7 days, with water changed every two days. Group III (Fast + Acute Fluoxetine) were fasted for 7 days, with water changed every two days, and an acute dose of 100 μg/L fluoxetine was administered for 3 h before sacrifice. Group IV (Fast + Chronic Fluoxetine) were fasted for 7 days with chronic exposure to 100 μg/L fluoxetine, with water changed and fresh fluoxetine added every two days. At the end of the week all fish were sacrificed, with immediate dissection of hypothalamus to be frozen individually, as explained above (Fig. 1B).

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from each hypothalamus with TRIZOL Reagent (Invitrogen, Carlsbad USA) and all RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to denature any contaminating DNA present in the samples. RNA quantity following DNase I treatment was assessed using a Nanodrop® ND-1000 spectrophotometer (Nanodrop® Technologies Inc, Wilmington, DE, USA). Quality of RNA extraction was determined by analyzing

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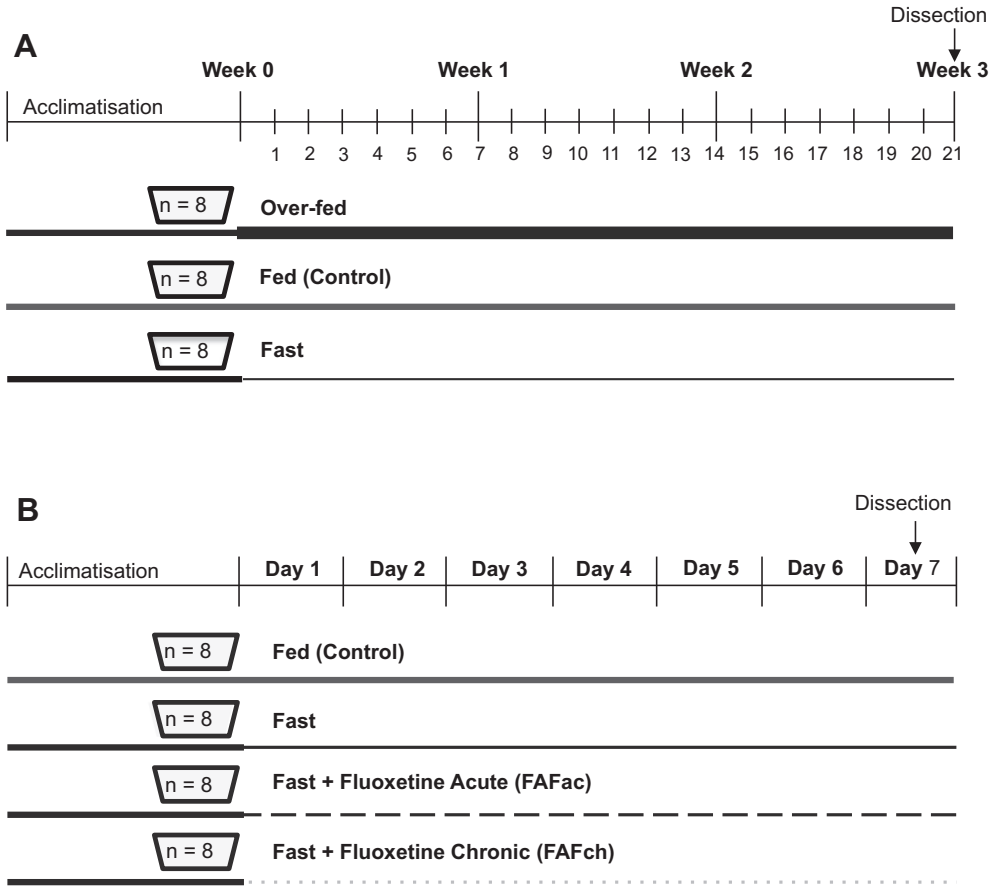

Fig. 1. Experimental Set Up for Physiological Experiments. A) 3 week feeding experiment with over-feeding (Over-fed), normally fed (Fed) and under food deprivation conditions (Fast). Adult male WT zebrafish were acclimatised with normal feeding for 1 week prior to experimentation. 'Over-fed' (n = 8) were fed three times daily, 'Fed' (n = 8) were fed twice daily (control), and 'Fast' (n = 8) were fed once weekly, for three weeks. Weight measures were taken weekly at day 0, day 7, day 14, and day 21. B) 1 week with fasting and with fluoxetine experiment. Adult male WT zebrafish were acclimatised in 5 L tanks with normal feeding for 1 day prior to experimentation. 'Fast' (n = 8) were given no food for one week, 'Fast Fluoxetine acute' (FAFac) were given no food for one week and dosed with 100 μg/L fluoxetine in system water for 3 h prior to sacrifice, and 'Fast Fluoxetine chronic' (FAFch) were given no food and were continuously dosed with 100 μg/L fluoxetine in system water for one week. 'Fed' (n = 8) were fed twice daily and used as control of fasting effects. Both experiments were run in duplicates.

the 260/280 ratio, and considering acceptable values of ~1.9–2.0. RNA integrity of random samples using Agilent 2100 Bioanalyzer (Agilent) showed an RNA integrity numbers (RIN) >8. cDNA synthesis was performed following the manufacturer's instructions using 500 ng of RNA with VILO cDNA synthesis kit (Invitrogen) and first strand cDNA was directly used for PCR into a 10 μl reaction volume.

2.5. Quantitative real-time PCR (qRT-PCR) to establish expression levels in the zebrafish hypothalamus

Expression levels of genes in the hypothalamus of adult male zebrafish were established by quantitative real-time PCR (qRT-PCR) using cDNA preparations as template ($n = 8$). Specific primers were designed to amplify fragments of the genes as shown in Table 1. The qRT-PCR amplification reaction mixture contained 2 μl of diluted cDNA (1:20) (freshly synthesized from 500 ng of RNA), 4 μM of each primer, and 10 l Express SYBR GreenER qPCR Supermix Universal (Invitrogen) in a final volume of 10 μl. Thermal cycling conditions comprised 95 °C for 10 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. At the end of the PCR cycles, the qRT-PCR products were analyzed using a dissociation curve step to confirm that only a single PCR product was amplified. NTC reactions for every primer pair were also included on each reaction plate to check for external DNA contamination. The amplification efficiency (E) of each primer set/target gene was assessed as $E = 10^{(-1/\text{slope})}$ as determined by linear regression of a series of dilutions of the input RNA. The qRT-PCR reactions were loaded with a liquid handler (Tecan robot Freedom EVO; Tecan Group Ltd.) and performed in a LightCycler 480 instrument (Roche).

The data were analyzed using the comparative cycle threshold (Ct) method (Bustin, 2000) (ddCT method) to calculate relative changes in gene expression. Fold change (the relative quantification, RQ) was calculated from the DDCt and normalized to the reference gene *β-actin*. The *β-actin* was tested for its ability to be used as control gene in different hypothalamus and hinbrain cDNA samples. Determinations were carried out in technical triplicates for all the genes analyzed. The RQ values for each sample were averaged and the standard error of the mean (S.E.M.) was calculated, yielding the average fold change of the target gene.

2.6. Statistical analyses

All data reported here are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS v22 software, with comparison of multiple groups determined by one-way analysis of variance (ANOVA) with post-hoc Fisher's least significant difference (LSD) test. Normality and homoscedasticity were tested with Shapiro-Wilk, Kolmogorov-Smirnov test. Data that deviated from normality and homoscedasticity, were analysed

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Table 1
Gene-specific primers used for qPCR.

Gene	GenBank Acc. No.	Primer sequence (5′→3′)	Amplicon size (bp)	Primer name	References
etv5a	NM_001126461	ACGGGACTTCTGCTTCGACT GGAACCACACAGGTGTCATC	144	etv5a-F1 etv5a-R1	This study
etv5b	NM_131205	CAGATGCCTCCTACAGCACA GGGGGATAAGGTGTTTGACA	109	etv5b-F1 etv5b-R1	This study
npy	BC162071	GACTCTCACAGAAGGGTATCC GGTTGATGTAGTGTCTTAGTGCTG	104	npy-F1_qPCR npy-R1_qPCR	Yokobori et al. (2012)
cart1	XM_680337	GCAGAGCAAACGGATCTCAC TCCTCGATCCTTTCCTGATG	95	cart1-F1_qPCR cart1-R1_qPCR	Drew et al. (2008)
pomca	NM_181438	TCTTGGCTCTGGCTGTTC TCGGAGGGAGGCTGTAG	179	pomca_F1_qPCR pomca_R1_qPCR	Zhang et al. (2012)
htr1aa	NM_001123321	ATGAGGATGAGCGGGATGTAG CAATCAGCCAGGACCACG	130	htr1aa_F1_qPCR htr1aa_R1_qPCR	Shimada et al. (2012)
htr1ab	NM_001145766	CTGTGTCGCCTGCACTTTTC TGATCTCCAAAGACTCGCCG	135	htr1ab_F1_qPCR htr1ab_R1_qPCR	Shimada et al. (2012)
βactin	AF057040	AGAGCTATGAGCTGCCTGACG CCGCAAGATTCCATACCCA	106	βactin-F1 βactin-R1	Kitahashi et al. (2009)

using a nonparametric Kruskal-Wallis test. Where there were only two groups to compare students T-test was used. Statistical significance was defined by p-value $\leqslant 0.05$.

3. Results

3.1. etv5a and etv5b gene expression is increased in the hypothalamus during fasting

Fasting over a period of 3 weeks resulted in significantly decreased weight of fasted versus control fish (Fed) ($p < 0.001$), while over-feeding resulted in significant weight gain ($p < 0.001$) (Fig. 2). The weight differences were obvious between the 'Fed' and 'Fast' group after seven days ($p < 0.001$). qRT-PCR analysis of gene expression levels in the hypothalamus of the fish showed a significant up-regulation of both *etv5a* ($p = 0.009$) and *etv5b* ($p = 0.011$) during fasting compared to the groups that were 'Fed' normally and 'Over-fed' (Fig. 3). To analyze a putative effect of *etv5a* and *etv5b* expression in the hindbrain, mRNA expression was also measured in this tissue. Transcripts of *etv5a* were not detected and *etv5b* was expressed only at low levels without differences between the 'Fed' and 'Fast' groups (Table 2). In addition, expression levels of genes related to food intake control, *npy* and *pomca,* were determined. In the hindbrain *pomca* expression was not detected in any condition, whereas *npy* levels were basically the same in normally fed and fasted fish (Table 2).

3.2. Increased etv5a and etv5b gene expression during fasting is lowered by fluoxetine treatment

A link between the serotonergic system that regulates mood and the neuronal circuits that regulate food intake exists, with high serotonin levels corresponding to decreased eating and weight loss. Our aim was to further investigate if up-regulated *etv5a* and *etv5b* expression levels with fasting might be related to low serotonin levels. Fluoxetine is a selective serotonin re-uptake inhibitor (SSRI) that increases extracellular concentrations of serotonin (5HT, hydroxytryptamine) by blocking its re-uptake through its transporter at the synapse. This was used to increase serotonin mediated signaling in the brain. Serotonin receptors were used as markers to ensure that the changes observed were due to alteration of the 5-HT pathway, while *npy, cart1* and *pomca* were included to demonstrate the downstream effects of serotonin on hypothalamic food intake control genes. A one-week fasting assay as established (Fig. 1B) was performed with normal fed fish serving as experimental controls. 100 μg/l fluoxetine was applied to the

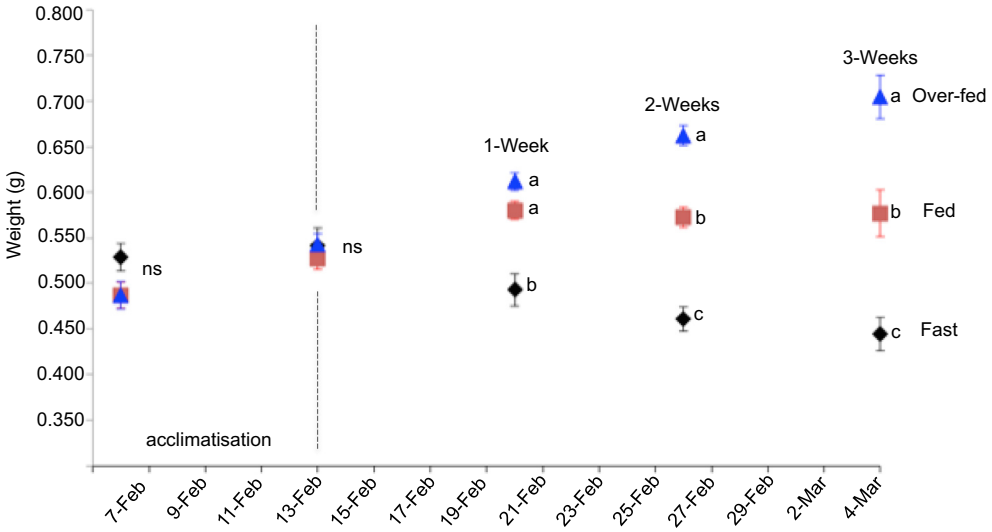

Fig. 2. Mean weights after 3 weeks feeding protocol in adult male zebrafish. Error bars represent SEM. Significance is defined by $p \leqslant 0.05$. Significant differences between letters.

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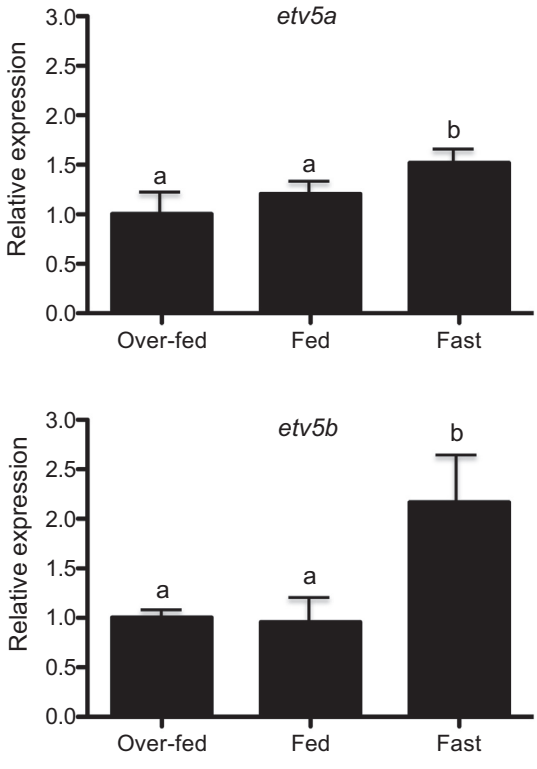

Fig. 3. Physiological analysis of hypothalamic *etv5a* and *etv5b* during fasting. qRT-PCR expression analysis of *etv5a* and *etv5b* revealed an up-regulation in the zebrafish hypothalamus during 3 weeks fasting. Data shown are representative of two independent experiments. Values in the graphs are the mean ± SEM of the fold change over control group (Over-Fed), with controls being set as 1. Significance is defined by $p \leqslant 0.05$. Significant difference between letters.

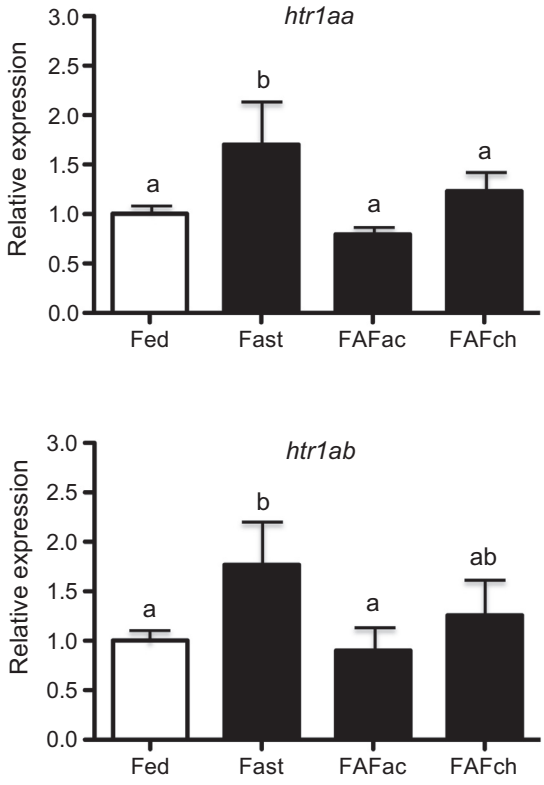

Fig. 4. Expression levels of serotoninergic marker genes following 1 week of fasting with acute (given on last day) and chronic (given for seven days) Fluoxetine treatment. Abbreviations: FAFac, 'Fast Fluoxetine acute'; FAFch, 'Fast Fluoxetine chronic'. Data shown are representative of two independent experiments. Values in the graphs are the mean ± SEM of the fold change over control group (Fed), with controls being set as 1. Significance is defined by $p \leqslant 0.05$. Statistically significance difference between letters.

fasting fish either acutely, for 3 h before sacrifice, or chronically for the whole week, to determine both immediate and long-term effects of increased serotonin levels on gene expression in the hypothalamus (Fig. 1B). Consistent with observations from previous studies, fish chronically exposed to fluoxetine spent more time in the top of the tank than their control counterparts, suggesting that the dosage applied was appropriate to exert an anxiolytic effect (Wong et al., 2013).

Two serotonin receptor genes, *htr1aa* and *htr1ab*, were used to establish the effect of fluoxetine application in fasted fish (Fig. 4), with low serotonin levels resulting in a compensatory increase in the synthesis of these receptors (Diaz et al., 2012). Serotonin receptor gene *htr1aa* was significantly up-regulated in fasted fish ($p = 0.038$) indicating that serotonin levels were lower during fasting. Acute and chronic application of fluoxetine in fasted fish resulted in significantly lowered expression levels compared to fasted fish without fluoxetine treatment normalizing levels comparable to the control group. Similarly, *htr1ab* was significantly up-regulated during fasting ($p = 0.043$) and the acute application of fluoxetine lowered expression levels of the gene compared to fasted fish without treatment ($p < 0.001$). No statistically significant differences were observed in the chronic treatment compared to fasting. In conclusion, as indicated by serotonin receptor expression, the application of fluoxetine increased the serotonin levels in the fasting fish.

Consequently the expression levels of genes involved in the control of food intake were altered (Fig. 5). *Cart1* had significantly increased expression in fasted fish ($p = 0.006$). Increased serotonin levels through acute and chronic application of fluoxetine resulted in significantly lowered expression ($p < 0.001$). The expression of *npy*, did not change in response to fasting, while an acute dose of fluoxetine in the fasted fish caused a significant down-regulation ($p = 0.003$). In contrast to *cart1*, chronic fluoxetine application in the fasted fish did not affect *npy* expression. *Pomca*, was found to have slightly lowered expression in fasted fish, but this did not reach statistical significance. Acute administration of fluoxetine

Table 2
Hindbrain RNA levels of target genes normalized to *β-actin* levels in male zebrafish fasted for 3-weeks. Values reported as mean ± S.E.M (n = 4). Statistically significant differences after the Student's *t*-test were used (p values <0.05). nd: not-detected.

Gene of interest	Fed (Control group)	Fast	Expression	p-value
etv5a	nd	nd		
etv5b	0.0369 ± 0.0154	0.0516 ± 0.0309	No difference	0.6784
Food intake markers				
npy	0.0066 ± 0.0019	0.0086 ± 0.0034	No difference	0.6647
pomca	nd	nd		

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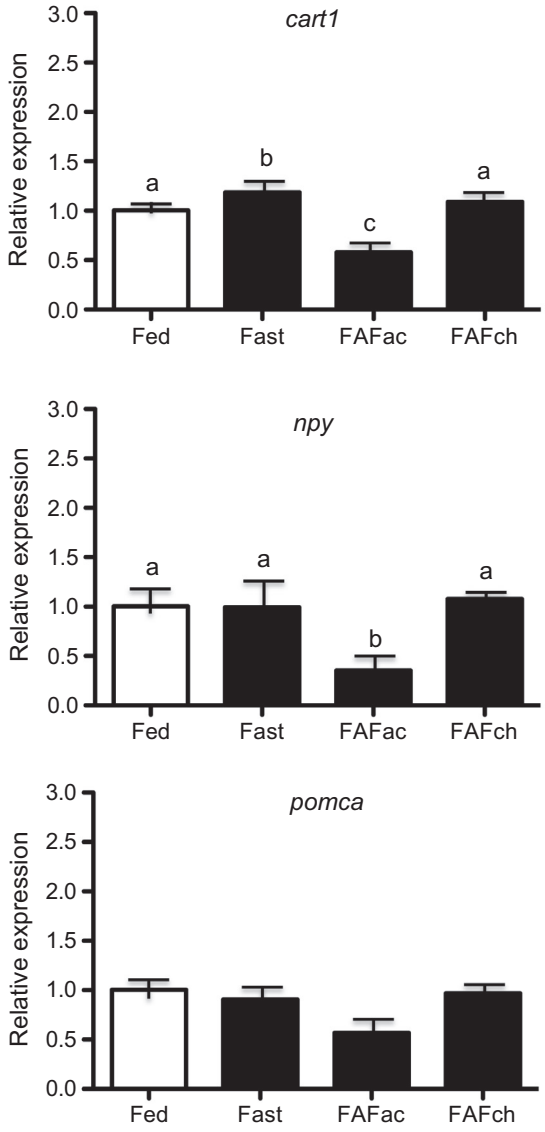

Fig. 5. Expression levels of food intake control markers. Experimental details and abbreviations same as in Fig. 4. Data shown are representative of two independent experiments. Values in the graphs are the mean ± SEM of the fold change over control group (Fed), with controls being set as 1. Significance is defined by $p \leqslant 0.05$. Statistically significance difference between letters.

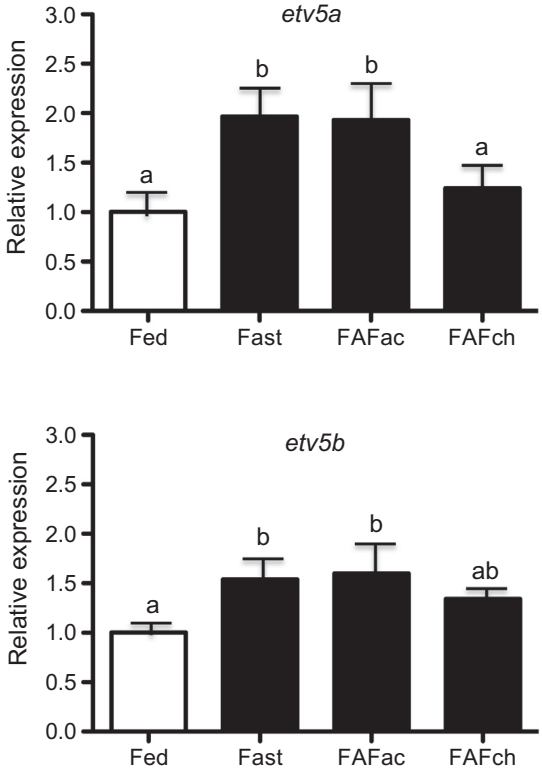

Fig. 6. Expression levels of zebrafish *etv5a* and *etv5b*. Experimental details and abbreviations same as in Fig. 4. Data shown are representative of two independent experiments. Values in the graphs are the mean ± SEM of the fold change over control group (Fed), with controls being set as 1. Significance is defined by $p \leqslant 0.05$. Statistically significance difference between letters.

resulted in a further decrease in *pomca* expression levels, but again statistical significance could not be established (Fig. 5). Overall, *cart1*, *npy and pomca* had decreased expression when serotonin levels were acutely increased in fasted fish. In contrast the application of chronic fluoxetine did not seem to have an effect. Assessment of serotonin receptor expression with chronic application of fluoxetine demonstrated that despite a slight decrease in serotonin levels with repeated exposure, serotonin levels remained at a significantly increased level when compared to non-treated fasting fish. This suggests that the lack of effect of chronic fluoxetine treatment is not due to habituation, but rather another unknown mechanism outside of the scope of this experiment to examine.

Fluoxetine treatment altered the expression of *etv5a* and *etv5b* in fasted fish. A significant up-regulation of *etv5a* ($p = 0.021$) and *etv5b* ($p = 0.042$) compared to the control group was observed in the hypothalamus (consistent with previous results shown in Fig. 3), The increased expression of *etv5a* was maintained with acute application of fluoxetine (Fig. 6). In contrast, chronic application of fluoxetine resulted in significantly lowered expression levels of *etv5a* ($p = 0.045$). Expression levels of *etv5b* responded similarly but no significant expression difference could be established between fast and acute and chronic fluoxetine treatments (Fig. 6). The changes of *etv5a* and *etv5b* expression levels in the hypothalamus with chronic fluoxetine application in fasting fish suggests that the fasting-function of both genes may be regulated through serotonin pathways.

4. Discussion

Results of fasting experiments herein show a previously unreported correlation of *etv5a* and *etv5b* in the hypothalamus during food restriction conditions in zebrafish. *Etv5* has previously been analyzed in overfeeding paradigms, with results demonstrating a down-regulation during high fat diet in rats (Gutierrez-Aguilar et al., 2012) and replication of this down-regulation in the arcuate nucleus and ventromedial nucleus, two specific hypothalamic subpopulations related to food intake control (Boender et al., 2012).

The increase of hypothalamic *etv5a* and *etv5b* in fasting adult fish points to a role of the genes in central pathways of food intake, although it does not identify how these may be involved. High expression levels during fasting, complimented by low levels during satiety, may point to a food intake stimulating function. The attenuation of *etv5a and etv5b* up-regulation during fasting through application of the serotonin re-uptake inhibitor fluoxetine, implicates serotonin as a modulatory partner for *etv5* genes in the hypothalamus. A link between *etv5b* and serotonin during zebrafish development had been established recently, with *etv5b* being

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shown to be crucial for the development of 5-HT neurons in the hypothalamus (Bosco et al., 2013). The responsiveness of *etv5a* and *etv5b* to increased serotonin levels in the adult zebrafish, demonstrates for the first time a physiological connection between *etv5* genes and serotonin.

Our analysis of food intake pathway markers supports the role of serotonin in the inhibition of normal orexigenic responses during fasting. *Npy*, is an appetite stimulating peptide and has previously been shown to be up-regulated during fasting (Wong et al., 2013) and to induce food intake in zebrafish (Yokobori et al., 2012). However, in rainbow trout (*Oncorhynchus mykiss*) *npy* expression did not vary in the hypothalamus under central serotonin administration (Mancebo et al., 2013). Our results did not reveal an up-regulation of the *npy* in fasted fish, however a drastic down-regulation with acute fluoxetine indicates a direct inverse correlation between serotonin and *npy*. Although not statistically significant, *pomca* was found to be slightly down-regulated during fasting, which is in accordance with its appetite inhibiting function. Acute application of fluoxetine lowered *pomca* expression levels further and it may suggest that abundant serotonin made *pomca* functions redundant. However, since *pomca* levels are lowered below levels in normally fed fish it looks like *pomca* would be actively reduced and there might be a negative feedback loop or similar mechanism behind this. Chronic fluoxetine treatment did not have an obvious effect on *npy* and *pomca* levels, and it may be suggested that serotonin levels were not sufficiently elevated to observe an effect on these genes. Increased expression of *cart1* in fasted fish and lowered expression levels with acute fluoxetine administration mimicked the expression profile of *npy*, although this has an opposite, appetite stimulating effect. This may be explained by *cart1* functioning in the hedonic as opposed to homeostatic systems, wherein it acts as in the reward and reinforcement pathways that dictate desire or 'wanting' of food. *Cart1* reinforces 'wanting' during fasting states (Jaworski and Jones, 2006; Vicentic and Jones, 2007), which corresponds to its up-regulation in fasting fish. With high serotonin levels signaling wellbeing and satisfaction, application of fluoxetine would be anticipated to decrease *cart1* dependent activation of the mesolimbic reward system, leading to less 'wanting' of food. Therefore we can interpret the decrease of *cart1* expression as an effect of serotonin on the 'wanting' system. Recently CART has been suggested to be a natural antidepressant associated with serotonin in humans (Job et al., 2011). In this role CART responds to low serotonin levels characteristic of depression and acts directly on the raphe nucleus to stimulate serotonin release. Subsequently pharmacologically increased serotonin levels that lead to down-regulation of *cart1* as shown here, suggest a bi-directional relationship between cart and serotonin. Overall, it is complex to dissect how fluoxetine influences the multiple pathways in which it is implicated in teleost fish. In addition to that already discussed, recent studies have highlighted its ability to influence lipid and amino acid metabolic synthesis (Wong et al., 2013) and this is another potential pathway beyond the scope of this research to examine. Furthermore, while there are several similarities between the food intake control system, brain regions, and serotoninergic mechanisms involved between teleost fish and mammals, conclusions applying to humans must be made with caution.

Changes to the expression levels of *etv5a* and *etv5b* suggest an association with the serotoninergic pathway and its role in both homeostatic and hedonic control of food intake pathways. Serotonin could inhibit the normal induction of appetite stimulating outputs from the hypothalamus, but may also result in less dopaminergic output resulting in less motivation to seek food. However, since serotonin acts in the hypothalamus through specific receptors, we cannot discard modulation of others physiological processes like growth, reproduction, stress and others (Mennigen et al., 2011). Our results suggest that *etv5* may be a mediator in both or either of these pathways during conditions of fasting. SNP's that up-regulate ETV5 may account for its association with obesity via mechanisms seen within this research, whereby increased expression may lead to over-eating (homeostatic) or increased 'wanting' (hedonic) responses in those individuals despite the absence of fasting, giving a predisposition to obesity. Alternatively, genetic pre-disposition to lowered serotonin levels may also be a factor, as there is less inhibition or modulation of ETV5 leading to over-activation of both hypothalamic and mesolimbic pathways leading to increased 'wanting' and decreased signaling of satiety.

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Disclosure statement

The authors have nothing to disclose.

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