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Journal
Journal of Experimental Biology, 217(15)

ISSN
0022-0949

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Publication Date
2014-08-01

DOI
10.1242/jeb.106161

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RESEARCH ARTICLE

Role of oleoylethanolamide as a feeding regulator in goldfish

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ABSTRACT

Oleoylethanolamide (OEA) is a bioactive lipid mediator, produced in the intestine and other tissues, which is involved in energy balance regulation in mammals, modulating feeding and lipid metabolism. The purpose of the present study was to investigate the presence and possible role of OEA in feeding regulation in goldfish (*Carassius auratus*). We assessed whether goldfish peripheral tissues and brain contain OEA and their regulation by nutritional status. OEA was detected in all studied tissues (liver, intestinal bulb, proximal intestine, muscle, hypothalamus, telencephalon and brainstem). Food deprivation (48 h) reduced intestinal OEA levels and levels increased upon re-feeding, suggesting that this compound may be involved in the short-term regulation of food intake in goldfish, as a satiety factor. Next, the effects of acute intraperitoneal administration of OEA on feeding, swimming and plasma levels of glucose and triglycerides were analysed. Food intake, swimming activity and circulating triglyceride levels were reduced by OEA 2 h post-injection. Finally, the possible interplay among OEA and other feeding regulators (leptin, cholecystokinin, ghrelin, neuropeptide Y, orexin and monoamines) was investigated. OEA actions on energy homeostasis in goldfish could be mediated, at least in part, through interactions with ghrelin and the serotonergic system, as OEA treatment reduced ghrelin expression in the intestinal bulb, and increased serotonergic activity in the telencephalon. In summary, our results indicate for the first time in fish that OEA could be involved in the regulation of feeding, swimming and lipid metabolism, suggesting a high conservation of OEA actions in energy balance throughout vertebrate evolution.

KEY WORDS: Fatty acid ethanolamide, Food intake, Locomotor activity, Monoamines, Triglycerides, *Carassius auratus*

INTRODUCTION

Energy homeostasis in animals is tightly regulated by a complex network of signals adjusting food intake to satisfy metabolic and nutritional requirements. The gastrointestinal tract is involved in feeding regulation in vertebrates through both neuronal and humoral mechanisms. Among these peripheral signals originating in the gastrointestinal tract, lipid-derived messengers such as oleoylethanolamide (OEA) can play a significant role in the regulation of energy balance, as shown by several studies in mammals (Lo Verme et al., 2005; Thabuis et al., 2008; Piomelli, 2013). OEA is a fatty acid ethanolamide (FAE), a structural analogue of the endocannabinoid arachidonoylethanolamide (anandamide) but does not activate the cannabinoid receptors (Rodríguez de Fonseca et al., 2001). This FAE acts as an endogenous ligand for peroxisome proliferator-activated receptor alpha (PPAR-α) (Rodríguez de Fonseca et al., 2001; Fu et al., 2003). In addition to binding to this nuclear receptor, its effects may also be mediated at least in part by the transient receptor potential vanilloid subtype 1 (TRPV1) (Ahern, 2003; Almási et al., 2008) and an orphan G-protein coupled receptor (GPR119) (Overton et al., 2006).

OEA has been detected in different peripheral tissues and brain in mammals (Fu et al., 2007; Izzo et al., 2010). Nutrient status regulates OEA mobilization in a tissue-specific manner. In the small intestine, OEA levels decrease during food deprivation and increase upon re-feeding in rat (Rodríguez de Fonseca et al., 2001; Petersen et al., 2006) and mice (Fu et al., 2007). A feeding-induced OEA mobilization in small intestine of the Burmese python (*Python molurus*) has also been described (Astarita et al., 2006a). By contrast, OEA levels increase in liver, pancreas and fat in response to fasting, and no changes were observed in other peripheral tissues (stomach, colon, lung, heart, muscle and kidney) or in brain structures (brainstem, hypothalamus, cerebellum, cortex, thalamus and striatum) in rats (Fu et al., 2007; Izzo et al., 2010). The periprandial fluctuations of OEA found in small intestine suggest that this lipid amide may contribute to the regulation of feeding behaviour, possibly acting as a satiety signal. Pharmacological studies in rodents support this idea, as systemic administration of OEA causes a dose- and time-dependent suppression of food intake by prolonging the interval between successive meals (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Gaetani et al., 2003; Cani et al., 2004; Nielsen et al., 2004). This response is not due to stress, malaise or aversion, although the anorectic effect of OEA is accompanied by a suppression of locomotor activity in mammals (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005). In rats, OEA injection was followed by reductions in ambulation and in spontaneous activity in the open field, and by an increase in the time that rats pushed their abdomen against the floor with splayed hindlimbs (Proulx et al., 2005). Nevertheless, it has been suggested that OEA modulates feeding and locomotion through distinct mechanisms, because the anorectic action, but not its effect on movement, was abrogated after capsaicin treatment (Rodríguez de Fonseca et al., 2001).

The molecular mechanisms involved in the anorectic effect of OEA have been partially elucidated in mammals. It is known that OEA-induced hypophagia is mediated by the stimulation of vagal sensory nerves that in turn stimulate the brainstem and hypothalamus (Rodríguez de Fonseca et al., 2001; Wang et al., 2005; Fu et al., 2011). Anorectic actions of OEA can be mediated through the modulation of central and peripheral signals involved in feeding regulation. It has been described that this FAE suppresses feeding by activating hypothalamic oxytocin transmission (Gaetani et al., 2010; Romano et al., 2013). Moreover, interactions between OEA and hypothalamic monoamines and cocaine- and amphetamine-regulated transcript (CART) have also been suggested (Serrano et al., 2011). At the peripheral level, some gastrointestinal neuropeptides are modified by OEA administration, although contradictory data have been published in rats. On the one hand, reductions in gut peptides, such as peptide YY and ghrelin, have

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Received 2 April 2014; Accepted 14 May 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.106161

List of symbols and abbreviations

5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine (serotonin)
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
FAE	fatty acid ethanolamide
GHRL	ghrelin
i.p.	intraperitoneal
M_b	body mass
MS-222	tricaine methanesulphonate
NA	noradrenaline
NPY	neuropeptide Y
OEA	oleoylethanolamide
PPAR-α	peroxisome proliferator-activated receptor alpha

been described after OEA administration (Cani et al., 2004; Serrano et al., 2011). On the other hand, Proulx et al. reported that OEA reduces food intake without causing peripheral changes in several gastrointestinal peptides, included peptide YY and ghrelin (Proulx et al., 2005).

In addition to its short-term effects on feeding, OEA has also been implicated in the control of body mass and lipid metabolism. Subchronic (1 week) and chronic (2 or more weeks) administration of this FAE decreased food intake accompanied by a marked inhibition of body mass gain in rodents (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzmán et al., 2004; Fu et al., 2005). It has been proposed that the effect of OEA on body mass is due not only to the feeding decrease but also to a direct effect on lipid metabolism (Lo Verme et al., 2005). Specifically, OEA promotes lipolysis and inhibits lipogenesis in important metabolic tissues such as liver, adipose tissue, muscle and gut (Thabuis et al., 2008; Pavón et al., 2010).

Accumulating evidence indicates that basic mechanisms controlling feeding behaviour are generally conserved among vertebrates. Fish are a valuable experimental model because they show a remarkable diversity that makes them attractive for the study of the evolution of feeding regulation systems in vertebrates (Hoskins and Volkoff, 2012). As in other vertebrates, food intake in fish is regulated by a complex interplay among hormones, neuropeptides and monoaminergic systems, acting at the central and peripheral level. Goldfish, *Carassius auratus* (Linnaeus 1758), is one of the most studied teleost species with regard to feeding regulation (Volkoff et al., 2009). Neuropeptide Y (NPY), orexins and ghrelin are examples of powerful orexigenic factors in this species, whereas cholecystokinin (CCK) and leptin act as anorexic signals (de Pedro and Björnsson, 2001; Volkoff et al., 2009). Dopamine (DA) and serotonin (5-HT) systems have been found to inhibit food intake, while noradrenaline (NA) stimulates it (de Pedro et al., 1998a; de Pedro et al., 1998b). Moreover, interactions between monoaminergic systems and other feeding regulators have been previously reported in goldfish (de Pedro et al., 1998a; de Pedro et al., 2006; de Pedro et al., 2008).

The involvement of FAEs in the control of food intake in fish was reported for the first time by Valenti et al. (Valenti et al., 2005). They demonstrated that the goldfish brain contains the cannabinoid CB_1 receptor, the endocannabinoids anandamide and 2-arachidonoylglycerol, as well as an enzymatic activity similar to the mammalian FAAH (fatty acid amide hydrolase). Intraperitoneal (i.p.) administration of anandamide stimulated food intake at low doses in this species. In agreement with the orexigenic role of anandamide, fasting increased its levels in the telencephalon. Similar results were observed in the sea bream *Sparus aurata* (Piccinetti et al., 2010), with brain anandamide and 2-arachidonoylglycerol raised by 24 h of food deprivation, and a food intake increase induced by anandamide administration. However, to date, nothing is known about whether other FAEs, such as OEA, are involved in food intake regulation in fish. As FAEs, particularly OEA, have been linked to diet and it is known that dietary lipids reduce feeding (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014), this FAE might have an important role in the regulation of feeding and body composition in fish, valuable information for fields such as aquaculture.

The present study was aimed at investigating the presence and possible role of OEA in food intake in fish, using the cyprinid *C. auratus* as an experimental model. First, we assessed whether goldfish peripheral tissues and brain contain OEA and whether this compound is regulated by nutritional status. Thus, OEA levels in liver, intestinal bulb, proximal intestine, muscle, hypothalamus, telencephalon and brainstem of goldfish, fed or following 48 h of the food deprivation, with or without re-feeding, were measured. Next, we analysed the effects of acute OEA administration on food intake, locomotor activity and plasma glucose and triglycerides in this species. Finally, we studied the possible interplay among this FAE and some known feeding regulators in this teleost. With this objective, gene expression of peripheral (leptin, CCK and ghrelin) and central (leptin, NPY and orexin) signals and brain activity of monoaminergic systems were analysed after OEA administration under two feeding conditions: fed and following 24 h food deprivation.

RESULTS

Experiment 1: effects of fasting and feeding on OEA content

Endogenous OEA was detected in all tissues of *C. auratus* studied, both central and peripheral. The OEA content in the intestinal bulb and proximal intestine was almost 5 and 3 times higher than the values observed in muscle and liver, respectively (Fig. 1). In the brain, the highest OEA content was observed in the brainstem, almost 3 and 6 times higher than in the hypothalamus and telencephalon, respectively (Table 1). The OEA levels in the brainstem were comparable to those found in the gastrointestinal tract.

Fig. 1 shows the OEA content in peripheral tissues in fed, fasted (48 h) and fasted (48 h) + re-fed fish 30 and 120 min after feeding. OEA levels at 30 min were markedly decreased ($P<0.05$) after food deprivation for 48 h in intestinal bulb (58%), proximal intestine (45%) and muscle (56%). OEA levels returned to baseline after re-feeding in the three tissues. A similar pattern (decreased OEA content in the fasted group and back to baseline levels with re-feeding) was observed at 120 min in these tissues, though without statistically significant differences (Fig. 1A,B,D). No such changes were observed in liver among the different experimental groups at either of the studied time intervals (Fig. 1C).

The OEA content in the brain (hypothalamus, telencephalon and brainstem) under different feeding conditions is reported in Table 1. Fasting for 48 h significantly ($P<0.05$) increased the OEA content in the telencephalon compared with fed fish 30 min after food intake, and re-feeding did not cause a return to baseline levels. No such differences were observed in the hypothalamus and brainstem at any sampling time analysed (30 and 120 min).

Experiment 2: effects of OEA on food intake and locomotor activity

Fig. 2A–C shows food intake during discrete and cumulative intervals after acute i.p. injection of either vehicle or OEA at doses of 5 μg g^{-1} body mass (M_b) in goldfish. Food intake was

The Journal of Experimental Biology (2014) doi:10.1242/jeb.106161

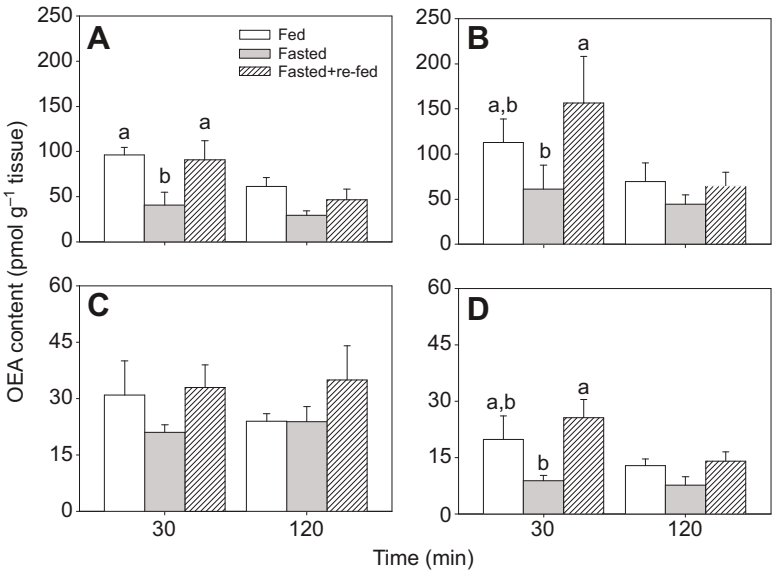

Fig. 1. Effect of feeding conditions on oleoylethanolamide (OEA) content in goldfish peripheral tissues. OEA content in fed, fasted (48 h) and fasted (48 h) + re-fed fish 30 and 120 min after feeding in: (A) intestinal bulb, (B) proximal intestine, (C) liver and (D) muscle. Data are expressed as means + s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups for the same time period.

significantly reduced compared with the control group during the 0–2 h interval (P<0.001; Fig. 2A), but not during the discrete interval 2–8 h (Fig. 2B). Cumulative food intake 8 h after injection was significantly decreased (P<0.05; Fig. 2C) in OEA-treated fish with respect to control fish. These reductions were around 72% at 2 h and 29% at 8 h after the OEA treatment.

The i.p. administration of OEA (5 μg g^{-1} M_b) significantly decreased swimming activity (around 35%) 2 h post-injection (P<0.05; Fig. 2D). A similar trend of decreased swimming was observed during the 2–8 h interval (36%; Fig. 2E) and 0–8 h interval (31%, Fig. 2F), although this reduction in locomotor activity was not statistically significant.

Experiment 3: effects of OEA on plasma metabolites, gene expression of feeding regulators and monoaminergic system

Plasma triglyceride levels were significantly reduced 2 h after OEA i.p. treatment (5 μg g^{-1} M_b) under fasted (24 h) and fed conditions (P<0.005; Fig. 3A). A trend towards higher plasma triglyceride levels was observed in fed fish compared with 24 h food-deprived animals. There were no significant differences in glycaemia in fish treated with OEA relative to the control group (Fig. 3B). Plasma glucose levels were lower in 24 h fasted fish (both control and OEA treated) than in fed fish 2 h post-feeding (P<0.005). There was no interaction between the treatment (vehicle or OEA injection) and feeding conditions (fasted or fed) for both metabolites studied.

Fig. 4 summarizes the results of OEA treatment on gene expression of peripheral feeding regulators. The two-way ANOVA pointed to an interaction between treatment and feeding conditions (P<0.05) in ghrelin (*gGHRL*) gene expression in goldfish intestinal bulb. OEA i.p. treatment reduced *gGHRL* mRNA levels in goldfish intestinal bulb 2 h post-injection in fed fish, but not in 24 h-fasted fish (P<0.05; Fig. 4A). The expression of goldfish CCK (*gCCK*) in the intestinal bulb (Fig. 4B) and goldfish leptin-aI (*gLep-aI*) in the liver (Fig. 4C) was not modified by OEA treatment and/or different feeding conditions in any of the studied groups.

Analysis of the effects of peripheral OEA treatment on central feeding regulators revealed no significant differences in the expression of hypothalamic goldfish leptins (*gLep-aI* and *gLep-aII*), goldfish orexin (*gOrexin*) and goldfish NPY (*gNPY*) 2 h post-injection in both fed and 24 h-fasted fish (Table 2).

Table 3 shows the hypothalamic and telencephalic levels of monoamines (NA, DA and 5-HT) and their metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole acetic acid (5-HIAA)], as well as the monoaminergic turnover (DOPAC/DA and 5-HIAA/5-HT) after i.p. administration of vehicle or OEA (5 μg g^{-1} M_b) in fasted and fed goldfish 2 h post-injection. Feeding conditions modified the hypothalamic NA content regardless of treatment (vehicle or OEA injection), with the highest levels in fed fish compared with 24 h fasted fish (P<0.05; Table 3). No differences by OEA treatment or feeding condition were found in the level of monoamines DA and 5-HT and their main oxidative metabolites (DOPAC and 5-HIAA), and the DOPAC/DA and 5-HIAA/5-HT ratios in goldfish hypothalamus (Table 3). In the telencephalon, a significant (P<0.05) effect of feeding conditions on NA and 5-HIAA content and 5-HIAA/5-HT ratio was observed,

Table 1. OEA content in goldfish brain 30 and 120 min post-feeding

Tissue	Post-feeding time (min)	Fed (pmol g^{-1})	Fasted (48 h) (pmol g^{-1})	Fasted + re-fed (pmol g^{-1})
Hypothalamus	30	47.3±3.0	49.5±1.6	41.4±2.7
	120	43.1±2.3	42.2±2.7	47.0±5.7
Telencephalon	30	20.8±1.8^{a}	28.4±2.1^{b}	26.0±2.2^{a,b}
	120	21.0±1.7^{a,b}	16.6±3.1^{b}	26.0±2.0^{a}
Brainstem	30	130.5±12.1	123.7±9.1	100.5±5.1
	120	96.8±13.3	115.4±9.2	99.4±9.5

Data (pmol g^{-1} tissue) are expressed as means ± s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups for the same time period.
OEA, oleoylethanolamide.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.106161

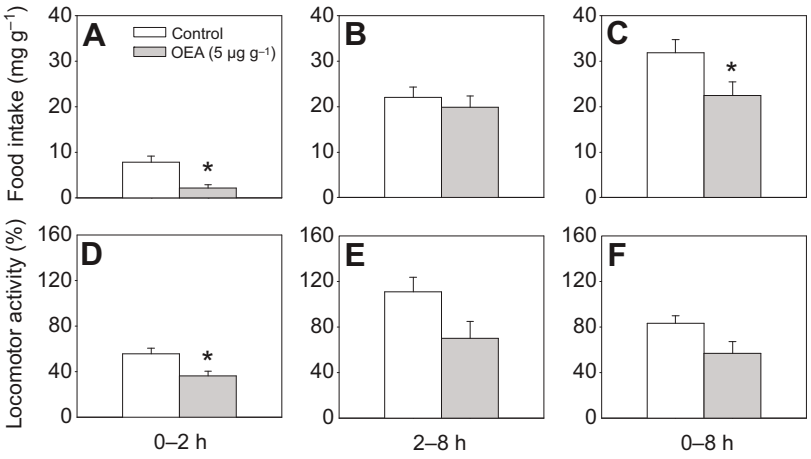

Fig. 2. Effect of OEA on goldfish food intake and locomotor activity. Food intake (A–C) and locomotor activity (D–F) 0–2 h (A,D), 2–8 h (B,E) and 0–8 h (C,F) after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1} M_b). Data are expressed as means + s.e.m. *P<0.05 versus control group.

with lower values in 24 h fasted fish compared with fed fish. The NA content and 5-HIAA/5-HT ratio 2 h post-injection were significantly increased (P<0.05 and P<0.005, respectively) by OEA treatment in both fed and fasted goldfish. The DA and 5-HT telencephalic content was not significantly modified by either treatment or feeding condition in any of the studied experimental groups.

DISCUSSION

The present findings indicate for the first time in fish a potential role of OEA as a lipid-derived satiety factor. The intestinal OEA levels were downregulated during short-term fasting, suggesting that this lipid amide could be involved in the short-term regulation of food intake in goldfish. In support of this hypothesis, i.p. administration of OEA produced a time-dependent inhibition of food intake, accompanied by a decrease of locomotor activity and triglyceride plasma levels. These actions of OEA could be mediated through the modulation of peripheral (ghrelin) and central (monoamines) signals.

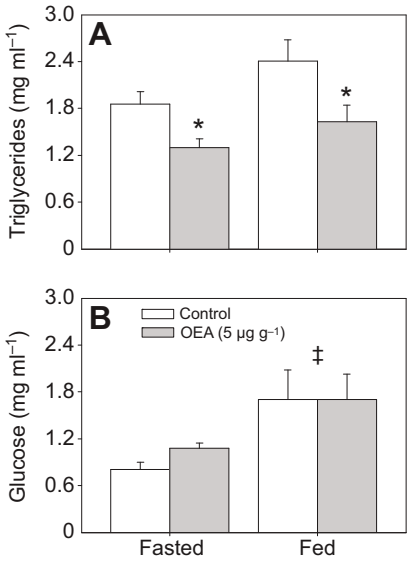

Fig. 3. Effect of OEA on goldfish plasma triglycerides and glucose. Plasma levels of triglycerides (A) and glucose (B) 2 h after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1}) in fed and 24 h food-deprived goldfish. Data are expressed as means + s.e.m. *P<0.05 between control and OEA treatments; ‡P<0.05 between fasted and fed groups.

Regulation of OEA levels by feeding

We have reported the presence of endogenous OEA in both peripheral tissues and brain of goldfish. Gastrointestinal segments (intestinal bulb and proximal intestine) in goldfish fed daily showed similar OEA levels to those previously reported in equivalent regions in fed rats (Fu et al., 2007). OEA was also found in other peripheral tissues (liver and muscle), as well as in brain structures (telencephalon, hypothalamus and brainstem), with lower levels in fish than in rats.

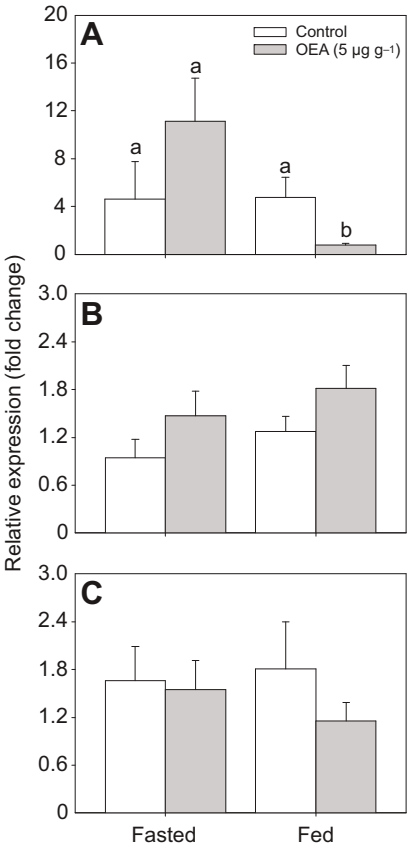

Fig. 4. Effect of OEA on expression of goldfish peripheral feeding regulators. The relative expression of genes encoding (A) ghrelin (*gGHRL*) and (B) cholecystokinin (*gCCK*) in intestinal bulb, and (C) leptin-al (*gLep-al*) in liver 2 h after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1}) in fed and 24 h food-deprived goldfish. Data are expressed as means + s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups.

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Table 2. Relative expression of feeding regulators in goldfish hypothalamus 2 h after i.p. administration of OEA

	Fasted (24 h)		Fed	
Gene	Control	OEA	Control	OEA
gLep-aI	1.04±0.13	1.49±0.29	1.08±0.06	1.24±0.12
gLep-aII	1.07±0.18	1.66±0.33	1.29±0.22	1.23±0.09
gOrexin	1.66±0.49	1.70±0.44	2.28±0.48	3.81±0.54
gNPY	3.31±1.36	5.32±2.51	1.07±0.41	1.67±0.41

OEA was given at 5 μg g^{-1} body mass (M_b). Data are expressed as means ± s.e.m.
gLep-aI, goldfish leptin-aI; *gLep-aII*, goldfish leptin-aII; *gOrexin*, goldfish orexin; *gNPY*, goldfish neuropeptide Y.

Feeding promotes OEA mobilization in the small intestine of studied species, such as rats (Rodríguez de Fonseca et al., 2001; Petersen et al., 2006; Fu et al., 2007), mice (Fu et al., 2007) and Burmese pythons (Astarita et al., 2006a). Our results also support this hypothesis in fish, as intestinal OEA content decreased after 48 h of fasting, and subsequently returned to baseline levels following re-feeding. We cannot confirm intestinal biosynthesis of this FAE in goldfish, as we did not measure the enzymatic activities responsible of OEA synthesis. Similar downregulation of OEA levels has also been observed in goldfish muscle, but not in rats (Fu et al., 2007), and the possible physiological significance of this response in fish remains unknown. The time course of changes in OEA levels in goldfish intestine and muscle indicates higher levels of this lipid amide at 30 min than at 120 min, suggesting that OEA is a rapid satiety signal. In fact, the decrease in OEA content following fasting was rapidly reverted by re-feeding (after 10 min) in rats (Fu et al., 2007). The fact that fasting induces upregulation of OEA content in other peripheral tissues, such as liver, pancreas, spleen and adipose tissue in rats (Fu et al., 2007; Izzo et al., 2010), but not in fish liver (present results) agrees with the downregulation of lipogenesis in liver induced by food deprivation (Pérez-Jiménez et al., 2012) and suggests that nutrient availability regulates OEA mobilization in a tissue-specific manner.

In the brain, the existing evidence in rats does not support a major role for OEA, as there are no fasting/re-feeding-induced changes (Fu et al., 2007; Izzo et al., 2010). Similar results have been found in goldfish hypothalamus and brainstem, but not in the telencephalon, where fasting increased OEA levels, in disagreement with its anorectic role. Fasting also increased anandamide levels in goldfish telecenphalon (Valenti et al., 2005), but this FAE increases food intake (Valenti et al., 2005). Thus, this similar response to fasting of OEA and anandamide does not appear to be in agreement with the opposite effect of these two FAEs. This conflicting result in goldfish suggests there are other roles of OEA in the telencephalon. In accordance with this, other functions of OEA have been described in mammals, such as in memory consolidation, stress, sleep–wake cycle, cellular viability and circadian system (for review, see Sarro-Ramírez et al., 2013).

Effects of OEA on food intake, locomotor activity and plasma metabolites

This is the first report documenting possible actions of OEA in fish. We found that i.p.-administered OEA (5 μg g^{-1} M_b) exerted an inhibitory effect on food intake 2 and 8 h post-injection in goldfish. This result is consistent with previous reports in mammals in which peripheral treatment with OEA was found to reduce food intake at similar dosages (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Cani et al., 2004; Nielsen et al., 2004). The fact that the feeding decrease was observed during the first 2 h after OEA injection, but not during the next discrete interval (2–8 h), suggests that this lipid amide acts for a short time in goldfish. OEA can modify food intake in the first 20 or 30 min post-injection in mammals (Cani et al., 2004; Serrano et al., 2011). Nevertheless, such early changes in feeding intake by FAEs can be extended for some hours, as in the present study. Thus, the OEA-induced decrease of cumulative food intake observed 8 h post-injection in goldfish would reflect the inhibitory action of OEA over a short time (2 h), which is maintained at least 8 h after the treatment. Moreover, the hypophagic actions of OEA appear to depend on the feeding state of the animal. In free-feeding rats, this lipid mediator increased the latency of feeding onset without changes in meal size, while OEA both delayed feeding onset and reduced meal size in food-deprived rats (Gaetani et al., 2003). Our experimental model to study the anorectic effect of OEA utilized 24 h food-deprived goldfish, indicating that OEA reduces feeding induced by fasting, but it is still unknown whether other feeding behaviour parameters, such as latency, post-meal interval or meal frequency, could be modified by OEA in fish. Several lines of evidence in mammals support the idea that OEA decreases food intake by activating PPAR-α receptor. In summary, mice lacking PPAR-α do not respond to OEA (Fu et al., 2003);

Table 3. Brain changes in monoaminergic system in goldfish 2 h after i.p. administration of OEA

	Fasted (24 h)		Fed	
	Control	OEA	Control	OEA
Hypothalamus				
NA (pmol mg^{-1} protein)	50.95±3.37	52.79±2.97	62.66±7.53‡	68.77±6.34‡
DA (pmol mg^{-1} protein)	56.45±3.44	43.40±1.24	52.96±4.53	61.01±10.24
DOPAC (pmol mg^{-1} protein)	1.75±0.19	1.97±0.45	1.95±0.30	1.71±0.20
DOPAC/DA (%)	3.13±0.31	4.46±0.98	3.98±0.76	3.95±1.38
5-HT (pmol mg^{-1} protein)	116.35±8.56	82.45±12.00	109.38±9.09	136.31±17.51
5-HIAA (pmol mg^{-1} protein)	21.76±2.06	21.93±3.28	23.58±1.72	28.34±2.54
5-HIAA/5-HT (%)	18.95±1.75	24.41±2.48	21.85±1.00	22.16±2.26
Telencephalon				
NA (pmol mg^{-1} protein)	46.35±3.68	61.71±5.05*	65.08±6.51‡	74.29±6.10*,‡
DA (pmol mg^{-1} protein)	17.49±5.86	12.24±1.35	12.60±1.30	14.84±1.33
5-HT (pmol mg^{-1} protein)	49.30±11.84	38.69±1.70	43.59±3.17	41.92±2.84
5-HIAA (pmol mg^{-1} protein)	12.42±2.01	13.05±0.72	15.03±1.81‡	18.12±1.25‡
5-HIAA/5-HT (%)	27.10 ±1.78	33.93±2.08*	34.70±3.48‡	43.70±3.48*,‡

OEA was given at 5 μg g^{-1} M_b. Data are expressed as means ± s.e.m. *P<0.05 between control and OEA treatment; ‡P<0.05 between fasted and fed groups.
NA, noradrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid.

PPAR-α agonists have anorectic actions similar to OEA (Astarita et al., 2006b); and OEA stimulates the transcription of various PPAR-α target genes (Fu et al., 2003). The existence of the PPAR subtypes α, β and γ has been demonstrated in fish (Mimeault et al., 2006; Zheng et al., 2013; Carmona-Antoñanzas et al., 2014), but to date it is unknown whether these nuclear receptors could be involved in the effects of OEA in these vertebrates. In addition, TRPV1 and GPR119 receptors have been shown to be involved in the feeding suppression actions of OEA in rodents (Ahern, 2003; Overton et al., 2006; Almási et al., 2008), although genetic removal of either TRPV1 or GPR119 has no effect on OEA-induced hypophagia (Piomelli, 2013). Molecular studies have also demonstrated the expression of TRPV1 and GPR119 receptors in fish species (Fredriksson et al., 2003; Gau et al., 2013), but the physiological roles of these receptors have not yet been elucidated.

Given that the metabolic precursor of OEA is oleic acid, it is important to point out that central or peripheral administration of oleic acid causes satiety effects in fish, probably mediated by fatty acid-sensing systems through different mechanisms related to fatty acid metabolism (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014). Thus, it cannot be ruled out that the OEA mobilization in fish is induced by oleic acid in the intestine, as it has been suggested in mammals (Piomelli, 2013).

Present results suggest that OEA may play a role in the regulation of locomotor activity in fish, as reported in mammals (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005). In both cases, the anorectic effect of OEA was accompanied by a significant reduction of locomotor activity. Rodríguez de Fonseca et al. (Rodríguez de Fonseca et al., 2001) suggested that the two responses are unrelated because the feeding decrease elicited by OEA was eliminated after selective degeneration of sensory fibres by capsaicin treatment, but not the reduction in locomotor activity. The possible interactions of OEA regulation of feeding and swimming activity in fish have not been studied to date. At least two possibilities could be addressed: on the one hand, the anorectic action of OEA might be due to the reduction of locomotor behaviour induced by this lipid amide; on the other hand, a decrease in activity might be related to a decrease in searching behaviour, as a direct consequence of the satiety effect of OEA. We cannot draw conclusions on the independence of these effects, based on the present results, but previous studies in goldfish have suggested that feeding and locomotor activity can be independently regulated by other anorectic hormones, such as leptin (Vivas et al., 2011) and melatonin (Azpeleta et al., 2010).

A significant decrease in triglyceride plasma levels after OEA injection in goldfish is in accordance with the general role of peripheral OEA in increasing fat utilization in mammals (Thabuis et al., 2008; Pavón et al., 2010; Piomelli, 2013). Systemic administration of OEA in rats stimulated lipolysis in adipocytes, decreasing circulating triglycerides and rapidly increasing the circulating non-esterified fatty acids and glycerol (Guzmán et al., 2004; Fu et al., 2005). Similar results were observed after incubation of rat adipocytes in the presence of OEA, suggesting that this lypolitic action of OEA involves the PPAR-α receptor (Guzmán et al., 2004). Moreover, an enhanced fatty acid oxidation was also found in muscle, heart and liver cells of rats and mice (Guzmán et al., 2004). As mentioned above, the effects of OEA might be mediated, at least in part, by oleate and its effects on fatty acid-sensing systems (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014). The reduction in triglycerides does not seem to be due to the reduction in food intake induced by OEA, as this effect was not observed in the pair-fed group in rats (Guzmán et al., 2004). The fact that the decrease in triglycerides in goldfish also occurred in the group that had not received food after OEA injection also supports such a hypothesis in fish. All these findings together suggest that OEA would play an important role in lipid metabolism in mammals and probably in fish.

A 24 h fast reduced glycaemia in goldfish, as expected (Polakof et al., 2012), and this was not affected by OEA treatment. Similar results in rats have shown that OEA administration does not modify blood glucose levels (Guzmán et al., 2004; Fu et al., 2005). However, some experiments *in vitro* have suggested that OEA may be involved in glucose metabolism regulation, as it inhibits insulin-stimulated glucose uptake in isolated rat adipocytes (González-Yanes et al., 2005). This possible inhibitory action of OEA on insulin actions in fish deserves to be investigated.

Interplay between OEA and other feeding regulators

The action of OEA on energy homeostasis in goldfish could be mediated by interactions with ghrelin, as the present results show reductions in ghrelin mRNA levels in the intestinal bulb induced by OEA. Ghrelin is a well-known orexigenic signal in fish that can also increase locomotor activity and lipid deposition in some species (Jönsson, 2013). Thus, OEA might reduce food intake and locomotor activity by decreasing gastrointestinal synthesis of ghrelin. Taking into consideration that OEA inhibits adipogenesis in mammals, and the adipogenic effect of ghrelin in mammals and fish (Thabuis et al., 2008; Jönsson, 2013), it is tempting to speculate that the action of OEA on lipid metabolism could be mediated, at least in part, by a reduction in ghrelin. A decrease in ghrelin expression by OEA was observed only in fed goldfish, but not in 24 h food-deprived fish, suggesting that the OEA–ghrelin interaction could depend on the energy status of the animals. This dependence also seems to occur in mammals, although the results vary. On the one hand, the decrease in circulating ghrelin induced by OEA occurs in fasted rats but not in fed rats (Cani et al., 2004). On the other hand, no changes in plasma ghrelin in fasted rats have been reported (Proulx et al., 2005; Serrano et al., 2011). This apparent discrepancy between the present results in fish and previous results in mammals might arise from species-specific differences, different physiological conditions (such as reproductive stage) and differences in experimental approaches (quantification of mRNA versus plasma levels, duration of fasting imposed on the animals, etc.).

To study whether the anorectic effect of OEA implies modulation of the secretion of other anorectic signals from the gastrointestinal tract in fish, we analysed the expression of CCK in the intestinal bulb of goldfish injected with this lipid amide. In the present study, OEA did not modify CCK expression, supporting previous data in mammals indicating that it is unlikely that CCK mediates the effects of OEA on food intake (Proulx et al., 2005). In fact, the primary contribution of OEA to normal feeding is in the regulation of satiety (delaying feeding onset and prolonging the time between meals), while CCK contributes to the process of satiation or meal termination by reducing meal size (Gaetani et al., 2003).

The unaltered hepatic and hypothalamic leptin expression in OEA-injected fish suggests that the reductions in food intake, locomotor activity and triglycerides induced by this FAE in goldfish cannot be directly attributed to an activation of leptin, an anorectic signal that also induces hypoactivity and lipolytic actions in this teleost (Vivas et al., 2011). The independence of the effects of OEA from leptin agrees with a previous finding in mammals, where OEA reduces both feeding and circulating lipids in obese Zucker rats lacking functional leptin receptors (Fu et al., 2005).

Because the OEA effect is associated with the activation of brain regions involved in feeding regulation, in the present study we

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examined whether peripheral administration of OEA induced changes in the expression of hypothalamic neuropeptides. There were no changes in the expression of NPY and orexin, two important orexigenic peptides in goldfish (Volkoff et al., 2009), following OEA injection. A previous study in rats (Serrano et al., 2011) demonstrated that OEA failed to modulate hypothalamic expression of NPY and AgRP (agouti-related protein) in experimental conditions (fed and 24 h fasted) similar to those of the present study. These data support the hypothesis that these orexigenic peptides in hypothalamus do not play a critical role in the anorectic effect of OEA in fish and mammals, although interactions between OEA and other orexigenic and anorexigenic neuropeptides, such as CART and oxytocin (Serrano et al., 2011; Gaetani et al., 2010), cannot be ruled out.

The central neurotransmitters recruited by peripheral OEA to inhibit food intake in rats have been studied previously (Serrano et al., 2011). The hypothalamic content of NA and DA increased after OEA injection, with a decrease in DOPAC/DA and without modifications to the serotonergic system. These effects were found only with the highest dose (20 mg kg^{-1}) of OEA, but not with 5 mg kg^{-1}. No changes were observed in goldfish hypothalamic monoamines (NA, DA and 5-HT), metabolites (DOPAC and 5-HIAA) and turnover (DOPAC/DA and 5-HT/5-HIAA) following OEA administration. These differences could be the consequence of different experimental approaches such as OEA dose (5 mg kg^{-1} in fish versus 20 mg kg^{-1} in rats) and time post-injection (2 h in fish versus 1 h in rats). Given the telencephalon is involved in the regulation of feeding and swimming in fish (Lin et al., 2000; Wilson and McLaughlin, 2010), the increases in NA, 5-HIAA and 5-HIAA/5-HT ratio induced by OEA in this brain region are potentially very interesting. The fact that these effects of OEA were similar in fed and fasted fish allows us to disregard the possibility that drug-induced feeding changes could be the cause of these monoaminergic neurotransmission alterations. As serotonin reduces feeding and swimming activity in fish (de Pedro et al., 1998b; Kuz'mina and Garina, 2013), the inhibitory effect of OEA on food intake and locomotor activity in goldfish could be mediated by serotonergic activation. The NA increase in goldfish telencephalon could not explain the OEA anorectic action, considering that this monoamine stimulates feeding in fish (de Pedro et al., 1998a; de Pedro et al., 2001). The possible cross-talk between OEA and telencephalic NA could be related to other functions of OEA. In mammals it has been proposed that OEA facilitates memory consolidation through noradrenergic activation of the amygdala (Campolongo et al., 2009). Recent results in rats have suggested that noradrenergic neurons are involved in the circuit responsible for the activation of hypothalamic oxytocin, which mediates the food intake inhibition induced by peripheral OEA administration (Romano et al., 2013). The identification of a functional link between OEA and brain NA is an intriguing question and future studies should examine all these possible interactions.

In conclusion, our results indicate for the first time in fish that OEA may be involved in the regulation of feeding, swimming and lipid metabolism, suggesting a high conservation of OEA actions in energy balance throughout vertebrate evolution.

MATERIALS AND METHODS

Animals

Experiments were performed with goldfish (*C. auratus*). Animals were obtained from a commercial supplier and reared at 21±2°C in aquaria (60 l) with a constant flow of filtered water, under a 12 h light:12 h dark photoperiod (lights on at 08:00 h). The aquaria walls were covered with opaque paper to minimize external interference during the experiments. Fish were fed once daily with 1% M_b commercial dry pellets (32.1% crude protein, 5% crude fat, 1.9% crude fibre, 5.1% humidity and 6.8% crude ash; Sera Biogram, Heinsberg, Germany) at 10:00 h. Animals were maintained under these conditions for at least 15 days prior to experimental use.

All the fish handling procedures comply with international standards for the Care and Use of Laboratory Animals, were approved by the Animal Experiments Committee of the Complutense University of Madrid and were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

OEA administration

OEA (Sigma Chemical, Madrid, Spain) was dissolved in 5% Tween 20, 5% polyethyleneglycol (Sigma Chemical) and 90% teleost saline (20 mg Na_2CO_3/100 ml of 0.6% NaCl). Fish (24 h food deprived) were anaesthetized in water containing tricaine methanesulphonate (MS-222, 0.14 g l^{-1}; Sigma Chemical). Immediately after the loss of equilibrium, fish were weighed and injected at feeding time (10:00 h). Goldfish were not fed for 24 h prior to injections (advisable conditions to test anorexigenic regulators). The i.p. injections were performed using 1 ml syringes and 0.3 mm Microlance needles (Lab-Center, Madrid, Spain), close to the ventral midline posterior to the pelvic fins (de Pedro et al., 2006). Fish were i.p. injected with 10 µl vehicle g^{-1} M_b alone (control group) or containing OEA (5 µg g^{-1} M_b, experimental group). The OEA dose was chosen based on studies previously reported in mammals (Cani et al., 2004; Fu et al., 2003; Nielsen et al., 2004; Rodríguez de Fonseca et al., 2001; Serrano et al., 2011). After the i.p. injections, fish were transferred to the experimental aquaria with anaesthetic-free water, where swimming activity and equilibrium were recovered within 1–2 min.

Experiment 1: effects of fasting and feeding on OEA content

Fish (12.02±0.47 g M_b) were divided into three groups (N=16 fish/group): control (fish were fed 1% M_b at 10:00 h), fasted (animals were food deprived for 48 h) and fasted + re-fed [fish were fasted for 48 h and re-fed (1% M_b) at 10:00 h]. Fish were killed by anaesthetic overdose (MS-222; 0.28 g l^{-1}) followed by spinal section 30 and 120 h after feeding (10:30 h and 12:00 h). Liver, intestinal bulb, proximal intestine (the first centimetre post-intestinal bulb), muscle and brain (hypothalamus, telencephalon and brainstem) were dissected on ice, immersed in liquid nitrogen and immediately stored at −80°C until posterior analysis. These tissues were chosen in accordance with previous studies in mammals and python (Astarita et al., 2006a; Fu et al., 2007), and taking into account the central relevance of the hypothalamus and telencephalon in feeding regulation in fish (Volkoff et al., 2009). Tissues were then weighed and homogenized in a methanol (Thermo Fisher Scientific, Milano, Italy) solution spiked with the deuterated analogue of OEA ([$^2H_4$]-OEA; Cayman Chemical, Ann Arbor, MI, USA), used as internal standard (IS) and mixed with chloroform (Thermo Fisher Scientific) and water (1:2:1). The FAEs in the samples were fractioned by open-bed silica gel column chromatography, as previously described (Cadas et al., 1997). Briefly, the lipid extracts were reconstituted in chloroform and loaded onto small columns packed with silica gel G (60 Å 230–400 Mesh ASTM; Whatman, Clifton, NJ, USA). FAEs were eluted with a chloroform/methanol 9:1 (v/v) solution. Eluates were dried under N_2 and reconstituted in 0.1 ml of acetonitrile with 0.1% of formic acid (Sigma Chemical). Samples were then analysed by LC-MS/MS on a Xevo-TQ triple quadruple mass spectrometer coupled with a UPLC chromatographic system. Standard curves for OEA were prepared in the 1 nmol l^{-1} to 10 µmol l^{-1} range. OEA and its deuterated analogue were loaded on a reversed phase BEH C18 column (50×2.1 mm inner diameter, 1.7 µm particle size) operated at 0.5 ml min^{-1} flow rate. Analytes were eluted from the column using a linear gradient of acetonitrile in water (both added with 0.1% formic acid). The column and the UPLC-MS/MS system were purchased from Waters Inc. (Milford, PA, USA). Quantification of analytes was performed by monitoring the following MRM (multiple reaction monitoring) transitions (parent m/z>daughter m/z, collision energy eV): OEA 326>62, 20; [$^2H_4$]-OEA 330>66, 20. OEA content in the samples was calculated from the analyte to IS peak area ratio and expressed as pmol mg^{-1} tissue.

Experiment 2: effects of OEA on food intake and locomotor activity

Fish (15.67±0.52 g M_b) were divided into two groups (N=16 fish/group): i.p. injected with vehicle or OEA (5 µg g^{-1} M_b). Immediately, individual goldfish were placed alone in 5 l aquaria. Pre-weighed food was supplied in excess (3% M_b) 10 min after fish were injected, and any remaining food was collected after 2 h. New, pre-weighed food (5% M_b) was added to the aquaria and any remaining food was collected at 8 h post-injection. Food intake was measured during the discrete intervals 0–2 and 2–8 h, the sum of which represents the cumulative interval 0–8 h, as previously described (de Pedro et al., 1998b).

Locomotor activity was recorded in groups of six fish (29.51±0.54 g M_b) in tanks of 60 l (N=6 tanks/group), after i.p. injection of vehicle or OEA (5 µg g^{-1} M_b). Swimming was recorded by using infrared photocells (OMRON E3SAD12, Osaka, Japan) fixed on the aquaria wall, as previously described (Azpeleta et al., 2010). The activity values registered in each tank, 2 and 8 h after vehicle or OEA injection, were expressed as a percentage with respect to the locomotor activity recorded at the same time periods in the same tank the day prior to treatment.

Experiment 3: effects of OEA on plasma metabolites, gene expression of feeding regulators and monoaminergic system

Fish (16.98±0.58 g M_b; N=8 fish/group) were i.p. injected with vehicle or OEA (5 µg g^{-1} M_b) at the scheduled feeding time (10:00 h), and maintained under two feeding conditions: fed (1% M_b) or food deprived (24 h). Two hours after injection, fish were anaesthetized and blood was taken by heparinized syringes from the caudal vein. Then, animals were killed by anaesthetic overdose (MS-222; 0.28 g l^{-1}) followed by spinal section, and tissues sampled. Brain (hypothalamus and telencephalon) and peripheral tissues (liver and intestinal bulb) were dissected on ice, immersed in liquid nitrogen and immediately stored at −80°C until posterior analysis. Feeding regulators and tissues studied were chosen considering previous studies in mammals on interactions between OEA and other feeding signals (Serrano et al., 2011; Gaetani et al., 2010), the relevance of these compounds in feeding regulation in fish and their main sites of synthesis and action in fish (Volkoff et al., 2009).

Plasma was obtained after centrifugation (4 min at 6000 rpm) and stored at −80°C until biochemical analysis. Plasma glucose and triglyceride levels were determined using an enzymatic/colorimetric method with commercial kits (GOD-POP and GPO-POD, respectively; Spinreact, Girona, Spain).

The mRNA levels of leptin-aI, leptin-aII, NPY and orexin in hypothalamus; leptin-aI in liver; and CCK and GHRL in intestinal bulb were measured. Feeding regulator gene expression was quantified by quantitative PCR (qPCR) using the goldfish β-actin as a reference gene (no differences between saline- and OEA-injected fish were observed). Total RNA was extracted using Trizol (Sigma Chemical). After DNase treatment (Promega, Madison, WI, USA), total RNA (from 0.25 to 0.8 µg depending on the tissue) was retro-transcribed (SuperScript II Reverse Transcriptase; Invitrogen, Carlsbad, CA, USA). Gene expression analysis was performed in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The qPCR reactions were developed in a 20 µl volume using iTaq SYBR Green Supermix (Bio-Rad). Specific primers (Sigma Chemical; supplementary material Table S1) and qPCR conditions employed for *β-actin*, *gLep-aI* and *gLep-aII* were as previously described (Tinoco et al., 2012). For the other genes, qPCR conditions were similar, but with annealing temperatures of 60°C (*gCCK*) and 65°C (*gNPY*, *gOrexin* and *gGHRL*). All samples were analysed in duplicate. Calibration curves for each gene were generated with serial dilutions of cDNA; all curves exhibited slopes close to −3.32 and efficiencies between 95% and 105%. Negative controls included replacement of cDNA by water and the use of non-retrotranscribed total RNA. The specificity of the amplification reactions was confirmed by the melting temperature of qPCR products (measured at the end of all reactions) and by the size in an agarose gel. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative expression (fold change).

The content of NA, DA, DOPAC (a major DA metabolite), 5-HT and 5-HIAA (a major 5-HT metabolite) in hypothalamus and telencephalon were quantified by HPLC (Agilent 1100, Madrid, Spain) with coulometric detection (ESA Coulochem II, Chelmsford, MA, USA) as previously described (de Pedro et al., 2008). Briefly, the tissues were sonicated in 100 µl of cold perchloric acid (0.3 mol l^{-1}; Scharlab, Sentmenat, Spain) containing 0.4 mmol l^{-1} sodium bisulphate and 0.4 mmol l^{-1} EDTA disodium salt dihydrate (Sigma Chemical). The homogenate was centrifuged (13,000 rpm for 5 min) and the supernatant was injected into the HPLC system. The mobile phase (flow rate 1 ml min^{-1}) consisted in 10 mmol l^{-1} phosphoric acid, 0.1 mmol l^{-1} disodium EDTA, 0.4 mmol l^{-1} sodium octanesulphonic acid (Sigma Chemical) and 3% acetonitrile (Panreac, Barcelona, Spain), pH 3.1. Separation was performed using a reversed phase C18 analytical column, 125×4.6 mm internal diameter, 5 µm particle size (Teknokroma, Barcelona, Spain). The oxidation potential was 200 mV and the signal from the analytical cell was recorded with a sensitivity of 20 nA. Acquisition and integration of chromatograms were performed with Clarity Chromatography Station software (Micronec, Madrid, Spain). Protein content was determined by the method of Lowry et al. (Lowry et al., 1951). The amount of monoamines in the samples was calculated as the area under the peak and expressed as pmol mg^{-1} protein. Metabolite/monoamine ratios are used as an index of monoaminergic activity.

Statistical analyses

Results are expressed as means ± s.e.m. Food intake and swimming activity data were analysed by Student's t-test to ascertain statistical differences between controls and OEA-treated fish in each time period. Plasma glucose and triglyceride levels, feeding regulator mRNA and monoamine content were analysed by two-way ANOVA, using treatment and feeding condition as independent factors. Tukey multiple range test were performed for multi-group comparisons only for significant interactions. One-way ANOVA followed by Tukey test was used to evaluate the effects of fasting and feeding on OEA content. When necessary, values were transformed (logarithmic or square root transformation) to obtain a normal distribution and homogeneity of variances. A Kruskall–Wallis non-parametric test was used to analyse statistical differences in telencephalic content of DA and *gNPY* hypothalamic expression. Analyses were conducted using IBM SPSS Statistics 19 (IBM Corporation, Armonk, NY, USA) and differences were considered statistically significant at P<0.05.

Acknowledgements

The authors thank Dr A. Guijarro for valuable suggestions about the subject of this article; Dr N. Realini and Dr M. Dionisi for technical support at the Italian Institute of Technology; and PhD students A. Sánchez-Bretaño and M. Gómez-Boronat for fish experiment support at the Complutense University of Madrid.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.B.T. and N.D.P. conceived and designed the experiments, and interpreted the findings. E.I. and M.J.D. collected and analysed the data from experiments carried out in Complutense University of Madrid. A.A. and D.P. collected and analysed the data from experiments carried out in Italian Institute of Technology. All authors drafted and revised the article.

Funding

This research was supported by the Spanish Ministry of Economy and Competitiveness project [MINECO; AGL2010-22247-C03-02]. A.B.T. was funded by a PhD grant [BES-2008-008638] from the Spanish Government.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.106161/-/DC1

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Table S1. Primers sequences used in quantitative PCR

Target gene	Accession number	Primer	Primer sequence 5' → 3'	Product (bp)
ß-actin	AB039726	Forward Reverse	GGCCTCCCTGTCTATCTTCC TTGAGAGGTTTGGGTTGGTC	156
gLeptin-aI	FJ534535	Forward Reverse	AGCTCCTCATAGGGGATC TAGATGTCGTTCTTTCCTTA	192
gLeptin-aII	EU095524	Forward Reverse	CTCCAAAAATCTTCATTGATCC AGGATTTCATTCTATCTTTC	195
gNPY	M87297	Forward Reverse	TTCGTCTGCTTGGGAACTCT TGGACCTTTTGCCATACCTC	151
gGHRL	AF454390	Forward Reverse	TTCATGATGAGTGCTCCGTTC GCTAGAATTCAAGTGGCGAATC	124
gOrexin	DQ923590	Forward Reverse	ACTGCACAGCCAAGAGAGTTC GTTATTAAAGCGGCCGATATG	188
gCCK	U70865	Forward Reverse	CTTTCTCTCCCCGCAGTCTC GAGTTGTCCACTGGAGGGTG	102

Goldfish leptin-aI (*gLep-aI*), leptin-aII (*gLep-aII*), neuropeptide Y (*gNPY*), ghrelin (*gGHRL*), orexin (*gOrexin*) and cholecystokinin (*gCCK*).