The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake

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Abstract  
Cannabinoid receptors and the endocannabinoids anandamide and 2-arachidonoylglycerol have been suggested to regulate food intake in several animal phyla. Orthologs of the mammalian cannabinoid CB1 and CB2 receptors have been identified in fish. We investigated the presence of this endocannabinoid system in the brain of the goldfish *Carassius auratus* and its role in food consumption. CB1-like immunoreactivity was distributed throughout the goldfish brain. The prosencephalon showed strong CB1-like immunoreactivity in the telencephalon and the inferior lobes of the posterior hypothalamus. Endocannabinoids were detected in all brain regions of *C. auratus* and an anandamide-hydrolysing enzymatic activity with features similar to those of mammalian fatty acid amide hydrolase was found. Food deprivation for 24 h was accompanied by a significant increase of anandamide, but not 2-arachidonoylglycerol, levels only in the telencephalon. Anandamide caused a dose-dependent effect on food intake within 2 h of intraperitoneal administration to satiated fish and significantly enhanced or reduced food intake at low (1 pg/g body weight) or intermediate (10 pg/g) doses, respectively, the highest dose tested (100 pg/g) being inactive. We suggest that endocannabinoids might variously contribute to adaptive responses to food shortage in fish.  

**Keywords:** anandamide, cannabinoid, CB1 receptor, fish, food intake, receptors.


The cannabinoid CB1 receptor (Matsuda *et al.* 1990) is one of the most abundant G-protein-coupled receptors in the CNS and is believed to be responsible for the majority of the ‘central’ actions of Δ⁹-tetrahydrocannabinol, the major psychoactive component of *Cannabis sativa* and marijuana (Gaoni and Mechoulam 1964). Δ⁹-tetrahydrocannabinol also activates cannabinoid CB2 receptors, which are also coupled to G-proteins of the Gxo family but are absent in the brain under physiological conditions, when they are almost uniquely found in immune tissues and cells (Galiègue *et al.* 1995). CB1 and CB2 receptors share very little homology, thus pointing to a very early separation of the two encoding genes during animal phylogeny. In fact, the only cannabinoid receptor ortholog fully sequenced in an invertebrate species, the sea squirt *Ciona intestinalis* (chordata), exhibits low homology with both mammalian subtypes (Elphick *et al.* 2003). Nevertheless, the orthologs of cannabinoid receptors that have been found so far in non-mammalian vertebrates, including fish, amphibians and birds (McPartland and Glass 2003), are mostly CB1 orthologs, except for the CB2 ortholog identified in the fish *Fugu rubripes* (Elphick 2002) which, contrary to a previous report (Yamaguchi *et al.* 1996), does not express only CB1 orthologs. Endogenous ligands of cannabinoid receptors, or endocannabinoids, have been identified in all phyla studied and they are derived from arachidonic acid. The best studied endocannabinoids are N-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG) (Devane *et al.* 1992; Mechoulam *et al.* 1995; Sugiura *et al.* 1995), which are biosynthesized from...
phospholipid precursors to be immediately released from the cell in order to activate their targets and then rapidly removed from the extracellular space by selective reuptake into the cell followed by enzymatic hydrolysis (Di Marzo et al. 2004). The cannabinoid receptors, the endocannabinoids and the proteins for their biosynthesis and degradation constitute the endocannabinoid system (Di Marzo et al. 2004).

Cannabinoid receptor orthologs have been identified in most vertebrate phyla and in a urochordate species, the sea squirt *C. intestinalis* (Elphick et al. 2003), whereas in invertebrates the action of endocannabinoids seems to be mostly mediated by receptors with little or no homology with the mammalian receptors. In the coelenterate *Hydra vulgaris*, anandamide inhibits a typical feeding response, i.e. the time during which the mouth stays open following exposure to glutathione, a chemical stimulus that mimics exposure to prey. This effect of anandamide is blocked by the CB1 antagonist rimonabant (SR141716A). Importantly, specific binding sites for rimonabant, with high affinity also for anandamide, are found in *H. vulgaris* (De Petrocellis et al. 1999). In *C. intestinalis*, endocannabinoids are found in cerebral ganglia, where specific binding sites for cannabinoid agonists also exist. Accordingly, a CB1/CB2 receptor ortholog was found in this organism, containing all the amino acids necessary for binding to these agonists (Elphick et al. 2003). One such agonist retards the reopening time of the *C. intestinalis* buccal siphon after mechanical stimulation-induced mouth closure (Mattias et al. 2005). Therefore, it is possible that the endocannabinoid system also controls the neuronal mechanisms that regulate the feeding behavior of sea squirts.

In non-mammalian vertebrates there is one example of the involvement of the endocannabinoid system in the control of feeding behavior. In the songbird zebra finch, brain 2-AG levels are increased following food deprivation and concur in inhibiting neural processes involved in new song learning via a CB1 ortholog previously cloned in this bird (Soderstrom et al. 2004). These data suggest that endocannabinoids in birds behave as orexigenic mediators, like in mammals. In fact, in rodents, endocannabinoid levels also increase, after short periods of food deprivation, in brain areas involved in the incentive or appetitive aspects of food intake, i.e. the nucleus accumbens and hypothalamus but not cerebellum (Kirkham et al. 2002), and CB1 receptors are necessary for optimal food intake after a brief starvation (Di Marzo et al. 2001). The present study was aimed at investigating the presence and possible role in food intake of endocannabinoids in the teleost fish *Carassius auratus*. First, we looked at the distribution of CB1-like immunoreactivity (CB1-LI-IR) in the forebrain of this fish. Next, we assessed whether *C. auratus* brain contains endocannabinoids and if their levels vary as observed in birds and rats following food deprivation and refeeding. Finally, we studied the effect of anandamide on food intake.

### Materials and methods

#### Immunohistochemical studies

The experiments were performed under the guidelines established by the Italian law for animal welfare. Adult specimens of *C. auratus* (*n* = 8) of both sexes were deeply anesthetized with tricaine methanesulfonate (1 : 1000, MS222, Sandoz Ltd, Basel, Switzerland). The CNS was removed and fixed for 14 h in paraformaldehyde (4%) in phosphate buffer (0.1 M, pH 7.4), cryoprotected, embedded in Kilik medium (Bio-Optica, Milan, Italy) and frozen in liquid isopentane. Coronal sections (12 mm thick) of the CNS, obtained with a cryostat, were mounted on 3-aminopropyl-triethoxysilane-coated slides and stored at 4°C until use. For molecular biology experiments, the *C. auratus* CNS was rapidly dissected out and immediately frozen at −80°C.

CNS sections were incubated with an affinity-purified primary polyclonal antibody raised against the N-terminus of the rat CB1 receptor (a kind gift of Prof. K. Mackie, University of Washington, Seattle, WA, USA; 1 : 800 dilution in 0.01 M phosphate-buffered saline, 0.1% Triton-X100). The immunoreactivity was revealed by the biotin–avidin complex system and H2O2/3,3′-diaminobenzidine–tetrahydrochloride as substrate/chromogen. The sections were observed under a light microscope (Axioplan, Zeiss, Göttingen, Germany) and then photographed. Images were processed with the software PhotoShop 7.0 (Adobe Systems Incorporated, Mountain View, CA, USA). The specificity of the anti-CB1 antibody was previously assessed in rat (Tsou et al. 1998) and evaluated in *C. auratus* by incubating the sections with the anti-CB1 antibody (1 : 800 dilution) pre-adsorbed for 14 h at 25°C with the immunizing protein (1 µg/ml); moreover, the specificity of the technique was confirmed by omitting the primary antibody. Nomenclature was mainly referred, with modifications, to Meek and Nieuwenhuys (1998).

For western immunoblotting, carried out to further assess the specificity of the antibody used, proteins were extracted from *C. auratus* brain following the procedure described by Tsou et al. (1998). Briefly, brains were homogenized in a fivefold (v/w) excess of a buffer containing 25 mM HEPES, 1 mM EDTA, 6 mM MgCl2 and protease inhibitor cocktail (Sigma, St Louis, MO, USA), pH 7.4. Homogenates were spun for 5 min at 1000 g and the supernatants were then collected. Pellets were rehomogenized in the same buffer and centrifuged as above and the supernatants were then pooled. Alternatively, total proteins were extracted from *C. auratus* and rat CNS by using a boiling buffer containing 2.5% sodium dodecyl sulfate and 125 mM Tris-HCl, pH 6.8. The protein concentration was determined by means of the bichinonic acid technique (Pierce, Rockford, IL, USA); 100 µg of total proteins were loaded on a 10% polyacrylamide gel, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then blotted onto a polyvinylidene difluoride membrane (Amer sham Biosciences, Little Chalfont, UK). Western blotting was performed by using as a primary antibody an affinity-purified polyclonal antiserum raised against the N-terminus of the rat CB1 (1 : 500 in Tris-buffered saline, 5% bovine serum albumin. As a control, the anti-CB1 N-terminus antibody (1 : 500 dilution) was pre-adsorbed for 14 h at 25°C with the immunizing fusion protein (10 µg/mL). After the incubation with an anti-rabbit IgG horseradish peroxidase-linked antiserum, the reaction was revealed with the
ECL Plus Western blotting detection reagent and Hyperfilm-ECL autoradiography film (Amersham Biosciences). The GelDoc gel documentation system and the software Quantity One (Bio-Rad Laboratories, Hercules, CA, USA) were used to determine the molecular weight of the specific CB₁ band on the basis of the migration of the Precision plus protein dual color molecular weight standards (Bio-Rad Laboratories).

**Sequencing of a PCR cDNA fragment from Carassius auratus mRNA encoding a CB₁-like receptor**

Total RNA was extracted from *Ca. auratus* CNS using the TRIzol reagent (Life Technologies, Rockville, MD, USA) and following the manufacturer’s instructions. DNA contaminants were eliminated by digestion with RQ1 RNase-free DNase (Promega, Madison, WI, USA). cDNA was synthesized from total RNA using M-MLV reverse transcriptase (USBiological, Swampscott, MA, USA). cDNA was amplified using RedTag DNA polymerase (Sigma) in the presence of 1.5 mM MgCl₂ at an annealing temperature of 50°C for 30 cycles. *Carassius auratus* CB₁ was amplified using a 5' sense degenerate primer [5'-TACCACTTCAT(T/C/A)GGCAGC(T/C)T-3'] and a 3' antisense degenerate primer [5'-A(G/C/A)GA(G/C/A)AA(G/C/T)GCAAAGC(A/C)A A(T/C)(C/A)GTCT-3'] complementary to the sequences codifying for regions of the second and seventh transmembrane domains of rat CB₁, respectively (see also Cottone et al. 2005). The 690-bp amplification product was cloned into pGEM-T-easy vector using the pGEM-T-easy Vector System (Promega). JM109 high efficiency competent cells were transformed and recombinant colonies were identified by blue/white color screening and restriction digestion; selected recombinant clones (pGEM-T-easy-CB₁Car) were sequenced. The deduced nucleotide sequence of the cloned *C. auratus* CB₁ 650-bp fragment is available at GenBank (Accession no. AY674057). In order to establish the degree of identity of the cannabinoid receptors among the different species, both the nucleotide and the amino acid sequence of *C. auratus* CB₁ were aligned with the CB₁ₐ sequence of *F. rubripes* available in the GenBank database using ClustalW multiple alignment computer programs. The BOX-SHADE alignment graphics program was used to highlight amino acid sequence similarities.

**Determination of endocannabinoid levels**

Goldfish [34.68 ± 1.63 g average body weight (bw)], fed with a daily ration of 1% bw food, were divided into three experimental groups (n = 12 goldfish/group): (1) control group, receiving food in excess 2 h before killing; (2) fasting group, deprived of food for 24 h previously (Matias et al. 2003). Tissues were homogenized in and extracted with chloroform : methanol : Tris-HCl (50 mM, pH 7.5, 2 : 1 : 1, v/v) containing internal standards (10 pmol of [³H]₆ anandamide, [³H]₄ N-palmitylolethanolamine and 100 pmol of [³H]₂-2-arachidonylglycerol, obtained from Cayman Chemicals, Ann Arbor, MI, USA). The lipid-containing organic phase was collected and dried. The mass of dried extract was weighed accurately with an analytical balance in 1.5-M L Eppendorf tubes and pre-purified by open-bed chromatography on silica gel. Fractions eluted with 9 : 1 chloroform : methanol were analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry using an HPLC apparatus coupled to an LCMS-2010 quadrupole mass spectrometer via an atmospheric pressure chemical ionization interface (all Shimadzu, Tokyo, Japan). Mass spectrometry analyses were carried out in the selected ion-monitoring as described previously (Di Marzo et al. 2001). The temperature of the atmospheric pressure chemical ionization source was 400°C and the HPLC column was a Phenomenex (5 mm, 150 × 4.5 mm) reverse-phase column, eluted as previously described (Di Marzo et al. 2001). Anandamide (retention time 13.5 min) and 2-AG (retention time 15.0 min) quasi-molecular ions (m/z = 348.0 and 379.3) were quantified by isotope dilution with the above-mentioned deuterated standards (same retention times and m/z = 356.0 and 384.3) and their amounts in picomoles or nanomoles normalized per milligram of lipid extract. Two liquid chromatography-mass spectrometry peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found at retention times of 15.0 and 16.9 min, respectively, corresponding to 2-AG and 1(3S)-oxygenated-glycerol, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure. Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. Silica column fractions eluted with 1 : 1 chloroform : methanol, which contain the N-acyl-phosphatidylethanolamines, were processed with Streptomyces chromofuscus phospholipase D to yield the corresponding N-acyl ethanolamines in a quantitative manner, as described previously (Di Marzo et al. 2001). Therefore, the anandamide biosynthetic precursor N-arachidonoyl-phosphatidylethanolamine (NArPE) was quantified by measuring the amounts, by means of liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry, of the anandamide produced from its hydrolysis by the phospholipase D.

**Assays for anandamide hydrolysing activity**

These assays were carried out as described previously (De Petrocellis et al. 1999) using [¹⁴C]ethanolamine-labeled anandamide as substrate and membranes from *C. auratus* whole brain prepared as described previously (De Petrocellis et al. 1999). Membranes were incubated at 37°C either with increasing concentrations of substrate (1, 4, 10, 50 and 100 μM) at pH 9.0, with 4 μM substrate in buffers at different pH (De Petrocellis et al. 1999) or in the presence of two distinct inhibitors of mammalian fatty acid amide hydrolase (FAAH), OL-135 and arachidonoylsperotonin (see Di Marzo et al. 2004 for a review). Following the incubation for 30 min, the assay mixture was extracted with chloroform : methanol (2 : 1 by volume) and the amount of [¹⁴C]ethanolamine released into the aqueous phase was used to quantify the anandamide amidease activity.

**Feeding experiments**

Four groups of satiated goldfish (13.9 ± 0.5 g bw, n = 8 goldfish/group) were intraperitoneally injected with 10 μL vehicle/g bw at the following doses of anandamide: 0, 1, 10 or 100 pg/g bw. Fish were anesthetized before the i.p. injection in water containing tricaine methanesulfonate (MS-222, 1/10 000) and the injections were performed when loss of equilibrium was observed. Individual goldfish were transferred to 5-L aquaria. Fish recovered equilibrium and normal swimming activity in anesthetic-free water within 1–2 min.
of the injections. Once recovered, the animals received pre-weighed food in excess (5% bw). Food intake (FI) was measured for 2 h post-injection and calculated as follows: \( FI = W_f - (W_i \times F) \), where \( W_f \) = initial dry food weight and \( W_i \) = remaining dry food weight. 

### Fish cortisol level and activity measurements

Two groups (\( n = 8 \)/group) of satiated goldfish (30.1 ± 0.9 g bw) were injected i.p. with either vehicle (10 μL/g bw) or anandamide (10 pg/g bw). The vehicle composition was (ethanol : cremophor : saline) (1 : 1 : 18), with fish saline being made up of 20 mg Na₂CO₃/100 mL of 0.6% NaCl. The cremophor was from Fluka (Buchs, Switzerland). Blood samples from caudal veins were collected at 2 h post-injection and plasma cortisol levels were measured in duplicate by radioimmunoassay using a commercial kit (DSL 2000, Diagnostic Systems Laboratories, Sinsheim, Germany). The validity of this radioimmunoassay has been previously determined for cortisol titres in goldfish plasma (De Pedro et al. 1997). Locomotor activity was recorded automatically by two infrared sensors located on one side of the aquaria. Every time the fish interrupted light beams it generated an output signal that was transferred via an interface to a computer that recorded and stored the data at 10-min intervals.

### Results

**Carassius auratus brain expresses CB₁-like receptors**

We have analysed the distribution of the CB₁-LI-IR in C. auratus CNS by means of a primary antibody raised against the CB₁ receptor amino-terminus. The specificity of such an antibody has already been demonstrated in rats (Tsou et al. 1998). Specificity controls performed on goldfish brain sections, employing the CB₁ antiserum pre-adsorbed with the immunizing protein [Fig. 1(c)] to be compared with the CB₁-LI-IR shown in Fig. 1(b)] or omitting the primary antibody, resulted in the complete absence of immunostaining. Moreover, western-blotting analysis performed using the same antibody revealed a CB₁-like receptor as a protein with an apparent molecular weight of ~53 kDa, identical to one of the two bands found in the brain of the rat (Fig. 1j); the specific band was not detected when incubating the blot with the antibody pre-adsorbed with the immunogen and it corresponds to the molecular weight of non-modified mammalian CB₁ receptors. The following immunohistochemical observations concern the prosencephalic areas reported as involved in the fish feeding response.

**Telencephalon**

The CB₁-LI-IR was distributed through all of the goldfish prosencephalon, including the olfactory bulbs. In particular, in the telencephalon the immunostaining is observed in cell bodies (15–20 μm diameter) and nerve terminals which are almost dispersed in the central portion of the dorsal area (Figs 1d and e) and through the ventral area. Proceeding caudally, plentiful immunopositive varicosities are observed in the ventral area surrounding negative perikarya, as shown in Fig. 1(f).

### Hypothalamus

A strong CB₁-LI-IR (more intense than that described in any other brain area) is seen in the inferior lobes of the posterior hypothalamus (Fig. 1g). Several labeled cells are neurons seemingly in contact with cerebrospinal fluid and sending their intraventricular processes into the lateral recess of the third ventricle (Fig. 1h), while the cell bodies and nerve terminals more deeply located within the hypothalamic wall possibly belong to the so-called nucleus diffusus of the hypothalamic inferior lobes (Fig. 1g). In the dorsal part of such a nucleus a dense CB₁-LI-IR innervation characterized by thin punctate terminations is shown in Fig. 1(i).

### Partial sequence of a Carassius auratus CB₁ ortholog

Carassius auratus mRNA was found here to amplify by PCR a transcript whose partial sequence yielded an amino acid sequence (GenBank no. AY674057) that was almost identical to that of the previously reported Fugu CB₁A receptor (Yamaguchi et al. 1996) between amino acids 189 and 374 (Fig. 2). In particular, residues necessary for the interaction with cannabinoid receptor ligands (McPartland and Glass 2003), such as the 253–270 CXFXF sequence in Fugu CB₁A important for binding CP55,940 or other residues involved in the binding with anandamide, THC, HU-210 and CP55,940 (e.g. residues F188 and F190) or for CB₁ functional activity (e.g. residues F199, Y275 and W299), were all conserved.

### Endocannabinoid levels in Carassius auratus

Both anandamide and 2-AG were detected in all brain regions of C. auratus. Anandamide was highest in the hypothalamus (\( p < 0.05 \)), whereas 2-AG was highest in the telencephalon (\( p < 0.05 \)) (Figs 3a and b). Food deprivation was accompanied by a significant, more than twofold, increase of anandamide but not 2-AG levels, but only in the telencephalon (Figs 3a and b). However, refeeding did not significantly reduce anandamide levels in this region, although after this treatment the concentration of anandamide in the telencephalon was not significantly different from control fish. As anandamide was the only endocannabinoid whose levels changed during food deprivation, we also analysed the levels of its biosynthetic precursor NArPE. This compound was again detected in all brain regions of control C. auratus (data not shown), thus suggesting that in this fish anandamide is produced through the same pathway as in mammals. As previously observed in mammals, the levels of NArPE were at least one order of magnitude higher than anandamide levels (Fig. 4), as expected from the precursor-product relationship between the two compounds.
Food deprivation was accompanied by a non-statistically significant increase of NArPE in the telencephalon and refeeding resulted in a further increase of this compound (Fig. 4), thus possibly explaining why anandamide levels were not reduced to normal after refeeding.

**Carassius auratus** brain contains a fatty acid amide hydrolase-like enzyme

Membranes prepared from *C. auratus* whole brain were found to actively hydrolyse $[^{14}C]$-anandamide to $[^{14}C]$-ethanolamine in a pH-dependent manner. Maximal activity was found between pH 9 and 10 and a relative peak of activity at pH 6 (Fig. 5a), exactly as in the case of mammalian FAAH. The enzyme followed Michaelis-Menten kinetics, as indicated by the Linewaver-Burk curve shown in Fig. 5(b) and exhibited an apparent $K_m$ of $16.6 \pm 1.9 \mu M$ and apparent $V_{max}$ of $0.3 \pm 0.05 \text{ nmol/mg/min}$ (means $\pm$ SEM, $n = 3$). Finally, two selective FAAH inhibitors, OL-135 (10 nM) and arachidonoylserotonin (10 μM), inhibited the hydrolysis of $[^{14}C]$-anandamide at pH 9 by $35.4 \pm 5.1$ and $55.0 \pm 6.2\%$, respectively (means $\pm$ SEM, $n = 3$).

**Effect of anandamide on food intake, locomotor activity and plasma cortisol levels**

Anandamide, injected intraperitoneally in *C. auratus*, exerted a dual effect on food intake by stimulating it at...
the lowest dose tested (1 pg/g) and reducing it at the intermediate dose (10 pg/g) (Fig. 6a). No effect was observed at the highest dose tested (100 pg/g). As we were not expecting a reduction in food intake, we further studied the nature of the inhibitory dose. We reasoned that the inhibition observed at the intermediate dose might be due to a general inhibition of locomotion or to sedation, as these responses are also observed in mammals. However, anandamide at 10 pg/g exerted no sedation or inhibition of general motor activity, whereas the CB1 receptor antagonist AM251 did (Fig. 6b). Nevertheless, anandamide exhibited a strong trend \( (p = 0.06) \) towards the inhibition of circulating cortisol levels (Fig. 6c), suggesting that at this dose the endocannabinoid might inhibit the stress response in \textit{C. auratus}.

**Discussion**

Studies carried out in rats (Kirkham \textit{et al.} 2002) established that endocannabinoid levels are enhanced following food deprivation in: (1) the hypothalamus, due to the tonic..
inhibitory effect exerted by leptin on endocannabinoid levels in this region (Di Marzo et al. 2001), which is mostly involved in the appetite-inducing behavior that follows food deprivation and (2) the limbic forebrain, which contains the nucleus accumbens and where endocannabinoids appear to play a reinforcing function on the motivational aspects of food intake. Accordingly, injection of endocannabinoids in the nucleus accumbens and hypothalamus enhances food intake (Jamshidi and Taylor 2001; Kirkham et al. 2002). Also in a bird species a brief period of food deprivation causes a significant enhancement of endocannabinoid levels, although only in brain regions involved in the motivational aspects of food consumption and in the learning of songs (Soderstrom et al. 2004). In this study we investigated the presence of the endocannabinoid system in the goldfish *C. auratus* and addressed the possibility that this system plays a role in the physiology of feeding behaviors in fish after a brief period of food deprivation.

We studied cannabinoid CB₁ receptor distribution in *C. auratus* brain. Although both CB₁ and CB₂ orthologs have been found in fish, we focused on CB₁ receptors as these are the only cannabinoid receptor subtype involved in food intake in mammals. Indeed, we amplified and
sequenced a large part of the encoding region of a Fugu CB1A gene ortholog that contained all of the amino acid residues that are most conserved between mammalian CB1 and Fugu CB1A receptors. Our observations largely confirm the description of CB1 immunostaining recently reported in the brain of another bony fish, the cichlid *Pelvicachromis pulcher* (Cottone et al. 2005), although in the present study most attention was focused on the CB1-LI-IR distributed throughout the goldfish prosencephalic areas known to be involved in the fish feeding control. In particular, we found an abundant CB1-LI-IR in the central and ventral areas of the telencephalon as well as in the hypothalamic inferior lobes surrounding the third ventricle lateral recesses. For a relatively long time the interest of comparative neuroscientists has been focused on fish brain areas involved in the feeding response, although neural mechanisms underlying food intake are still under investigation. Electrophysiological studies (Demski and Knigge 1971; Demski 1973) pointed to the bony fish hypothalamus, near the lateral recess of the third ventricle, as an important area responsible for the control of feeding behavior. Furthermore, lesions of the lateral areas of the hypothalamus were found to cause aphagia in *C. auratus* (Roberts and Savage, 1978). However, teleost fish possess an elaborate telencephalon with multiple migrated nuclear groups and a telencephalic involvement in complex behavioral patterns has been demonstrated by electrophysiology. Feeding responses have, in fact, been evoked by electrical stimulation of the medioventral part of the telencephalic hemisphere (Demski and Northcutt 1983). There is evidence indicating that several neuropeptides involved in feeding regulation in fish show a wide distribution in prosencephalic areas (De Pedro and Björnsson 2001). Recently, it has been suggested that neural circuits neurochemically identified within the telencephalon and pre-optic area, such as for example the prosencephalic neuropeptide Y innervation, are deeply implicated in goldfish appetite control (Namaware et al. 2000) which, in fish as in mammals, is under multifactorial regulation (Lin et al. 2000). On the other hand, the present data show that CB1-LI-IR is found in almost the same prosencephalic areas where a well-developed neuropeptide Y neuronal system was described in *C. auratus* by immunohistochemistry (Pontet et al. 1989). In goldfish, using a pharmacological approach, neuropeptide Y was demonstrated to be a physiological brain orexigenic signal as in mammals (Lopez-Patiño et al. 1999) and this stimulatory effect on feeding seems to be dependent on interactions with other neuropeptides (De Pedro et al. 2000; Bernier and Peter 2001; Volkoff and Peter 2001). Moreover, neuropeptide Y gene expression in goldfish prosencephalon is influenced by the composition of the diet (Namaware and Peter 2002). In summary, we have shown here that CB1-like receptors are expressed in all those brain areas of *C. auratus* previously suggested to intervene in the control of food intake.

We have also reported here the capability of a fish species to synthesize brain endocannabinoids. During the preparation of this work, the presence of anandamide and 2-AG was recently reported also in the brain of another fish species, *Pimelophales promelas* (Rademacher et al. 2005). Although these findings were predictable, due to the reported presence of the two major endocannabinoids in all animal phyla investigated so far, we also observed here that the levels of anandamide, but not 2-AG, are augmented by 2.5-fold after food deprivation in the telencephalon but not in the hypothalamus nor in a brain area, the cerebellum, which is not directly involved in the control of food intake. In rats following brief food deprivation, anandamide levels were found to change in the brain region, the limbic forebrain, which, like the telencephalon in fish, is involved more in the motivational than appetitive aspects of food intake (Kirkham et al. 2002), whereas the other endocannabinoid, 2-AG, is also increased in the hypothalamus. However, as outlined above, in fish (and, to some extent, also in mammals), there is no clear distinction in the role played by these two brain areas in the control of energy balance. While in the rat endocannabinoid levels return to basal levels during normal food consumption (Kirkham et al. 2002), anandamide levels in the goldfish telencephalon after refeeding were found here to decrease non-significantly, although they were not significantly different from levels in goldfish fed *ad libitum*. Apart from obvious differences between the two phyla, it is possible that such a discrepancy is due to the different food deprivation/refeeding protocols used in the two studies. Also, the levels of anandamide in the telencephalon of refed goldfish were characterized by marked variability, which may have prevented us from observing a significant difference from the levels in either food-deprived or *ad libitum*-fed fish. We also found that the levels of the anandamide biosynthetic precursor NArPE were increased after refeeding and not, as expected, decreased, thus possibly buffering anandamide levels against a stronger decrease following energy replenishment in *C. auratus*. In turn, the levels of fatty acid precursors necessary for arachidonic acid and *N*-arachidonoylphosphatidylethanolamine biosynthesis are likely to be augmented after refeeding, thus explaining why this condition enhanced, rather than diminished, the tissue concentration of the anandamide precursor.

Having shown that CB1-like receptors, and increased anandamide concentrations to activate them, are present in a *C. auratus* brain region involved in the control of food intake, we needed to demonstrate that anandamide does affect energy intake in this fish. Indeed, we found that, at the lowest dose tested, the endocannabinoid causes more than a twofold stimulation of food consumption over 2 h from administration. The dose of 1 pg/g which exerted this effect is certainly attainable *in vivo* following a food deprivation even shorter than that used in this study (see above). At a
higher dose (10 pg/g), anandamide suppressed food intake. This rather surprising effect is not likely to be due to a sedative effect or to a non-selective impairment of fish motor activity as it could be assessed by simply monitoring fish motor functions. However, it must be emphasized that we did not measure any motor parameter of specific relevance to feeding (e.g. otoromotor control and coordination). In mice, very low doses of systemic anandamide are sufficient to induce appetite (Hao et al. 2000), whereas another putative endocannabinoid, noladin ether, also exhibits a biphasic dose–response curve on food intake and bw (Avraham et al. 2005). It is tempting to speculate that brain endocannabinoid levels are regulated, following food shortage, in a way that they first enhance food intake in order to re-establish energy homeostasis; subsequently, in the presence of persistent food deprivation (which possibly leads to even higher endocannabinoid levels), their role may be to help the organism to adapt to this condition by recovering from the stress caused by the lack of energy and, at the same time, by inhibiting the drive to eat. Some support for this hypothesis comes from the observation that the anorexic dose of anandamide exhibited a strong trend towards inhibiting plasma cortisol levels, which would be in agreement with a similar effect recently described for 2-AG on corticosterone serum levels in rats (Patel et al. 2004). Another endocannabinoid-based adaptive mechanism reported in rodents and their offspring might be represented by the reduction, rather than enhancement, of hypothalamic endocannabinoid levels that follows prolonged (i.e. several days) semistarvation (Hanus et al. 2003; Matias et al. 2003), which again, by reducing the need to consume food, might help the animal to adapt to food shortage. We have no evidence other than that reported here to support a possible dual role of endocannabinoids following food deprivation, as we used only one type of protocol of limited access to food and could not test the effect of anandamide in food-deprived fish. However, it is clear that the endocannabinoid system plays important roles in both the control of energy intake and the recovery from stress, two conditions that may be strictly related or even compete with each other during food shortage (Di et al. 2003). It is likely that one of these two roles of the endocannabinoids may over-ride the other depending on the type and duration of food deprivation.

It should be underlined that, at the highest dose tested, anandamide exerted no overt effect on food intake in *C. auratus*. The only explanation that we can provide for this phenomenon is that, at high doses, anandamide becomes a substrate for a hydrolysing enzyme that we have identified in a fish species for the first time in this study. This enzyme exhibits features similar to those of mammalian FAAH, i.e. an optimal alkaline pH and sensitivity to arachidonoylserotonin and OL-135, two selective FAAH inhibitors, thus suggesting that the *C. auratus* anandamide amidase might be structurally similar to FAAH. However, we provided no molecular data to support this suggestion. Importantly, we found that the apparent \( K_m \) for anandamide of this enzyme is very similar to values found for FAAH enzymes in mammals and is significantly higher than the concentrations probably attained in *C. auratus* following the injection of anandamide. However, it is possible that, at the highest dose tested, anandamide does become a substrate for this enzyme and is subsequently inactivated.

Although we have shown here the presence of CB\(_1\)-like receptors and of endogenous cannabinoid ligands in areas of *C. auratus* deputed to the control of food intake and have described the effect on food consumption of the only endocannabinoid whose levels change during food deprivation, we have not provided any evidence that this latter effect is due to the interaction of anandamide with CB\(_1\)-like receptors. The part of the sequence of the *Ca. auratus* CB\(_{1A}\) ortholog identified here does contain all the highly conserved amino acids known to be necessary for a functional interaction of ligands (including anandamide) with the mammalian CB\(_1\) receptor (McPartland and Glass 2003) and is therefore very likely to be activated by anandamide. We also performed some experiments with the CB\(_1\) receptor antagonists SR141716A and, particularly, AM251 but could not find an inactive dose of these compounds that could also reverse the effects of anandamide on food intake. In fact, AM251 strongly reduced food intake in *C. auratus* (data not shown), which is to be expected if endocannabinoids tonically stimulate appetite in fish and is in agreement with the widely reported effects of CB\(_1\) antagonists in mammals (see Black 2004 for review). However, we found that AM251 also inhibits goldfish general motor activity and we could not separate this effect of the antagonist from its anorexic action (although, again, we did not measure more specific motor parameters that are of direct relevance to feeding behaviors). In summary, also in view of the several molecular targets that have been proposed for anandamide in mammals, we cannot assert conclusively that its dual effects on *C. auratus* food intake, described above, are due to activation of the CB\(_1\)-like receptors detected here in the brain of this fish nor that endocannabinoids tonically stimulate energy intake in this teleost.

In conclusion, we have reported here that the brain of the goldfish *C. auratus* contains CB\(_1\)-like receptors and putative endogenous ligands for these receptors. The distribution of CB\(_1\) immunoreactivity in the brain and the type of changes observed following food deprivation for one of the two major endocannabinoids, anandamide, point to a role of the endocannabinoid system in the control of energy intake in fish, as previously also suggested in birds and as widely demonstrated in mammals. The dual effect of anandamide on food intake suggests that this endogenous compound may not only act to re-establish energy balance but also help goldfish cope with the lack of food, depending on whether or not this becomes available again. Further studies are necessary in order to investigate whether anandamide effects in *C. auratus* are indeed mediated by CB\(_1\)-like receptors.
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