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Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) in winter skate (*Raja ocellata*): cDNA cloning, tissue distribution and mRNA expression responses to fasting

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ABSTRACT

cDNAs encoding for neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) were cloned in an elasmobranch fish, the winter skate. mRNA tissue distribution was examined for the three peptides as well as the effects of two weeks of fasting on their expression. Skate NPY, CART and CCK sequences display similarities with sequences for teleost fish but in general the degree of identity is relatively low (50%). All three peptides are present in brain and in several peripheral tissues, including gut and gonads. Within the brain, the three peptides are expressed in the hypothalamus, telencephalon, optic tectum and cerebellum. Two weeks of fasting induced an increase in telencephalon NPY and an increase in CCK in the gut but had no effects on hypothalamic NPY, CART and CCK, or on telencephalon CART. Our results provide basis for further investigation into the regulation of feeding in winter skate.

1. Introduction

In fish as in all vertebrates, appetite is regulated by central and peripheral appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) factors. Neuropeptide Y (NPY) and cocaine- and amphetamine-regulated transcript (CART) are examples of central orexigenic and anorexigenic factors, respectively, whereas cholecystokinin (CCK), although also produced by the brain, is mostly synthesized in the gut and acts as a peripheral satiety factor (Volkoff et al., 2005).

Neuropeptide Y is a 36 amino acid peptide, member of a peptide family that also includes pancreatic polypeptide and peptide YY. In mammals, NPY is one of the most potent orexigenic factors known to date (Chee and Colmers, 2008). NPY has been isolated and characterized in a number of fish species, including goldfish, *Carassius auratus* (Blomqvist et al., 1992), perch, *Siniperca chuatsi* (Liang et al., 2007), trout, *Oncorhynchus mykiss* (Doyon et al., 2003), and cod, *Gadus morhua* (Kehoe and Volkoff, 2007). In fish as in other vertebrates, NPY appears to be involved in the regulation of feeding. Intracerebroventricular (ICV) injections of NPY in channel catfish (*Ictalurus punctatus*) (Silverstein et al., 2001) and goldfish (Lopez-Patino et al., 1999) cause an increase in food intake whereas ICV injections of NPY antagonists decrease feeding in goldfish (Lopez-Patino et al., 1999). In addition, increases in brain NPY mRNA expression levels are seen in food restricted Pacific salmon (*Oncorhyncus* sp.) (Silverstein et al., 1998) and goldfish (Narnaware and Peter, 2002).

Cocaine- and amphetamine-regulated transcript (CART) was first discovered in rats as the transcript of a brain mRNA up-regulated following administration of cocaine and amphetamine and later shown to have a role in the regulation of feeding, as centrally injected CART dose-dependently inhibit food intake in rats (Gorissen et al., 2006). To date, CART has been cloned from mammals (Adams et al., 1999; Douglass and Daoud, 1996; Douglass et al., 1995), amphibians (Lazar et al., 2004) and fish, including goldfish (Volkoff and Peter, 2001), Atlantic cod (Kehoe and Volkoff, 2007) and catfish (Kobayashi et al., 2008). ICV injections of CART decrease food intake in goldfish (Volkoff and Peter, 2000) and fasting induces decreases in CART mRNA in goldfish (Volkoff and Peter, 2001), cod (Kehoe and Volkoff, 2007) and catfish (Kobayashi et al., 2008), suggesting that CART regulates feeding in fish.

Cholecystokinin (CCK) is synthesized by intestinal endocrine cells as a 115 amino acid prepro-CCK polypeptide that is cleaved post-translationally to generate gastrin/CCK-like peptides that share similar carboxy-terminal ends (Chandra and Liddle, 2007;

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Vishnuvardhan and Beinfeld, 2002). In mammals, CCK-8, the most abundant form of CCK (Moran and Kinzig, 2004), acts via vagal afferent pathways to stimulate gallbladder contractions and pancreatic and gastric secretions and to decrease food intake (Chandra and Liddle, 2007; Rehfeld et al., 2007). CCK/gastrin-like immunoreactivity has been shown in the nervous system and gut of several fish species including Atlantic cod (Jonsson et al., 1987), goldfish (Himick and Peter, 1994) and halibut (*Hippoglossus hippoglossus*) (Kamisaka et al., 2001). mRNA sequences have also been determined for a number of fish species including goldfish (Peyon et al., 1998), dogfish (*Squalus acanthias*) (Johnsen et al., 1997), pufferfish (Kurokawa et al., 2003) and Japanese flounder (*Paralichthys olivaceus*) (Kurokawa et al., 2003). Various forms of CCK, including CCK-8, are present in fish and have been shown to influence digestion and appetite. In teleosts, CCK induces contractions of the gall bladder (Aldman and Holmgren, 1995), a decrease in gastric emptying (Olsson et al., 1999) and an increase in gut motility (Forgan and Forster, 2007). Both central and peripheral injections of CCK cause a decrease in food intake in goldfish (Himick and Peter, 1994; Volkoff et al., 2003), oral administration of CCK decreases food intake in sea bass (Rubio et al., 2008) and oral administration of a CCK antagonists causes an increase in food consumption in both trout and sea bass (Gelineau and Boujard, 2001; Rubio et al., 2008), suggesting that CCK also influences appetite regulation in fish. In addition, CCK mRNA levels increase following a meal in goldfish brain (Peyon et al., 1999) and in the pyloric caeca of yellowtail (Murashita et al., 2007).

The winter skate is an oviparous elasmobranch benthic species of the family *Rajidae*, which range extends from the Gulf of St. Lawrence to the south coast of Newfoundland (Scott et al., 1988). Although winter skates have recently become the object of specific fisheries (Frisk and Miller, 2006), little is currently known about their life cycle and physiology, in particular their feeding physiology. To date, most research on winter skates has focused on osmoregulation, as they are well-adapted to exposure to different salinities (Sulikowski et al., 2004; Treberg and Driedzic, 2006).

In order to provide new information on the mechanisms regulating appetite in winter skate, we cloned cDNAs encoding three appetite-regulating hormones (NPY, CART and CCK), examined their mRNA tissue distribution and assessed the effects of fasting on their gene expression.

2. Material and methods

2.1. Animals

Twenty winter skates (average weight of 1.86 ± 0.32 kg) were collected by scubadivers off the shore of St. John's (NL, Canada) in September. Fish were divided into four tanks and acclimated for two weeks in 4 m × 4 m flow through water tanks at an average temperature of 11.4 °C at the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, NL, Canada). Fish consisted of both males and females. Males were recognized by the presence of claspers. All fish except two appeared mature after examination of the gonads (Sulikowski et al., 2005). The sex ratio was approximately 50:50 in all tanks. Skates were fed chopped frozen herring three times a week to satiety at the same time each day (10:00). Skates consumed an average of 59.2 ± 6.1 g of food per fish per feeding (or 31.6 g/kg fish/day). Following the acclimation period, two tanks were food deprived for two weeks and two tanks were maintained on the regular feeding schedule. The experiment ran from the 19th of September 2007 to the 3rd of October 2007. Samples were collected two weeks after the start of the fasting period. Prior to the fasting experiments, three to four acclimated fed fish were sampled for cloning purposes (see below). During all samplings, the weights of fish were measured and the sex and sexual maturity were noted.

2.2. RNA extraction

For cloning and tissue distribution studies, four fed fish were dissected to obtain samples of brain and peripheral tissues (gill, heart, stomach, gut, spleen, liver, kidney, muscle and gonad). For brain tissue distribution, individual brains were further dissected into hypothalamus, telencephalon, optic tectum, and cerebellum according to a previously established brain morphology for elasmobranchs (Northcutt, 2002). For gene expression studies experimental fish were dissected to obtain hypothalamus, telencephalon and gut (adjacent to the pyloric caeca) tissue. Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel Laboratories, Vancouver, BC, Canada) and killed by spinal section. Tissues were dissected and immediately placed on ice in RNA*later* (Qiagen Inc., Mississauga, Ont., Canada) and stored at −20 °C until RNA extractions were performed.

Total RNA was isolated using a trizol/chloroform extraction with Tri-reagent (BioShop, Mississauga, Ont., Canada) following the manufacturers' protocol. Final RNA concentrations were determined by optical density reading at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The quality of RNA samples was assessed by measuring the ratio of sample absorbance at 260 and 280 nm. Only RNA samples with a ratio between 1.8 and 2.1 were used.

2.3. Cloning of cDNA

Two micrograms of total RNA was subjected to reverse transcription into cDNA with a dT-adapter primer (Table 1) using M-MLV Reverse Transcriptase (New England Biolabs, Pickering, Ont., Canada). cDNA (0.5 μg) were then submitted to PCR amplifications using degenerate primers designed in regions of high identity among fish and various vertebrate sequences. The annealing temperature was optimized for each primer set. All PCR reactions were carried out in a volume of 25 μl using JumpStart Taq DNA polymerase (Sigma, St. Louis, MO, USA). PCR products were electrophoresed in a 1% agarose gel, and visualized using an Epichemi Darkroom BioImaging System (UVP, Upland, CA, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP). Bands of predicted size were isolated and purified with the GenElute Gel Extraction Kit (Sigma, Oakville, Ont., Canada), cloned using a pGEM-T easy vector system (Promega, Madison, WI, USA) and sequenced by the MOBIX Lab (McMaster University, Ont., Canada).

In order to isolate winter skate NPY, an initial fragment was obtained using 3′ Rapid Amplification of cDNA Ends (3′RACE) and degenerate primers. Briefly, brain mRNA was subjected to reverse transcription and the cDNA submitted to two rounds of PCRs, using 3′RC-NPY1 and dT-AP, and 3′RC-NPY2 and AP (Table 1). The PCR products were electrophoresed, and the bands of expected size were isolated, purified, cloned, and sequenced as described above. To isolate the 5′ portion of the cDNA, 5′RACE was used. The first strand of cDNA was generated from mRNA with reverse transcription reaction with 5′RC-NPY, purified using a Montage PCR Millipore kit (Bedford, MA, USA) and polyA-tailed using Terminal Deoxynucleotidyl Transferase (Invitrogen, Burlington, Ont., Canada). The product was then amplified using two rounds of nested PCR using 5′RC-NPY2 and dT-AP and 5′RC-NPY3 and AP. PCR products were then purified, cloned and sequenced as described previously. Skate CART was cloned using the same procedures as for NPY but using CART-specific primers (3′RC-CART 1 and 2 and 5′RC-CART 1, 2 and 3, Table 1).

Table 1
Primers used in the cDNA cloning, tissue distribution and qPCR analysis in winter skate (*Raja ocellata*).

Primer	Sequence
NPY	
Primers for 3′ and 5′RACE	
3′R-NPY1	5′ GAGATTTGGCCAAGTATTAYTC 3′
3′R-NPY2	5′ TACAAGGCAGAGGTATGG 3′
5′R-NPY1	5′ TCACATTAAAGAAACTGCAG 3′
5′R-NPY2	5′ATCTCTCAGCATCAGTTCAG 3′
5′R-NPY3	5′ TAGTGCTTCGGGGTTGGATC 3′
Specific primers for RT-PCR	
NPYF	5′ AACATGAAGTCTTGGCTGGG 3′
NPYR	5′ CCACATGGAAGGTTCATCAT 3′
CART	
Primers for 3′ and 5′RACE	
3′R-CART 1	5′ CTCGGGGCTTTACATGANGT 3′
3′R-CART 2	5′ GANGTTCTGGAGAAACTGCA 3′
5′R-CART 1	5′ GGGTCCTTTTCTCACTGCAC 3′
5′R-CART 2	5′ TCCTCCAAATCCTGGGTCCT 3′
5′R-CART 3	5′ TCAGGCAGTTACAGGTCCTC 3′
Specific primers for RT-PCR	
CART qF	5′ GCAGCGAGAAGGAACTGCT 3′
CART qR	5′ GCACACATGTCTCGGATGTT 3′
CCK	
Degenerate primers	
dCCK-F	5′ GTGGGATCTGTGTGTGYGT 3′
dCCK-R	5′ CGTCGGCCRAARTCCATCCA 3′
Primers for 3′ and 5′RACE	
3′RC-CCK1	5′ CAGGCTGAACAGTGAGCAG 3′
3′RC-CCK2	5′ AGCAGGGACCCGGCCTAGTG 3′
5′RC-CCK1	5′ GTAGTAAGGTGCTTCTCTC 3′
5′R-CCK2	5′ GCTGGTGCAGGGGTCCGTGC 3′
5′R-CCK3	5′ TCCCTCTCGGTCCGTCCGTC 3′
Specific primers for RT-PCR	
CCK qF	5′ CACCTACCTGCACAAAGACAA 3′
CCK qR	5′ CCATGTAGTCCCTGTTGGTG 3′
Adaptor primers	
dT-AP	5′ GGCCACGCGTCGACTAGTAC(T17) 3′
AP	5′ GGCCACGCGTCGACTAGTAC 3′
Primers for internal control of RT-PCR	
EF1	5′ AAGGAAGCTGCTGAGATGGG 3′
EF2	5′ CAGCTTCAAACTCACCCACA 3′
Primers for qPCR	
NPY qF	5′ CCCGAAGCACTAATGATGAC 3′
NPY qR	5′ CATGGAAGGTTCATCATACCTAA 3′
CART qF	5′ GCAGCGAGAAGGAACTGCT 3′
CART qR	5′ GCACACATGTCTCGGATGTT 3′
CCK qF	5′ CACCTACCTGCACAAAGACAA 3′
CCK qR	5′ CCATGTAGTCCCTGTTGGTG 3′
EF qF	5′ GAACATGATTACCGGCACCT 3′
EF qR	5′ TTCAAACTCACCCACACCAG 3′

In order to clone winter skate CCK, a small fragment of the unknown sequence was isolated by performing PCR amplifications using degenerate forward and reverse primers (dCCK-F, and -R, Table 1). Following sequencing of this short fragment, 3′RACE (using gene specific primers 3′RC-CCK1 and 2, Table 1) and 5′RACE (using 5′RC-CCK1, 2 and 3, Table 1), were performed.

2.4. Brain and tissue distribution by RT-PCR

Total RNA from brain, gills, heart, gut, liver, spleen, kidney, muscle, skin and gonads and from distinct brain regions (telencephalon, optic tectum-thalamus, hypothalamus, cerebellum) were isolated as described above. Two micrograms of RNA was reverse transcribed with dT-adapter primer using M-MLV Reverse Transcriptase (New England Biolabs). NPY, CART and CCK fragments were then amplified using gene specific primers (Table 1) designed based on our cloned sequences and PCR products were run on a 1% agarose gel. Elongation factor-1 alpha (EF-1α) was used as a control gene. Primers were designed based on little skate (*Raja erinacea*) EF-1α (GenBank Accession No. E988144) (Table 1). Bands amplified with EF-1α were cloned and sequenced in order to verify their nucleotide sequence. A negative control was included for each primer set by omitting cDNA from the PCR reaction.

2.5. Quantitative real-time RT-PCR

Total RNA was reverse transcribed to cDNA, using a QuantiTect Reverse Transcription kit (Qiagen, Mississauga, Ont., Canada), according to the manufacturer's protocol. Briefly, 1ug of template total RNA are submitted to genomic DNA removal and reverse transcribed using an optimized mix of oligo-dT and random primers, Quantiscript RT buffer, and reverse transcriptase Quantiscript RT buffer, and reverse transcriptase. Reverse transcription products were then diluted 1:3 in water and subjected to qPCR using specific primers (Table 1). For all primer pairs, at least one primer was designed to lie across an exon/exon boundary, to avoid risks of amplification of genomic DNA. The primers were designed to have similar melting temperatures and to give similar amplicon sizes. All PCR reactions were prepared using an epMotion® 5070 automated pipetting system (Eppendorf) in a final volume of 10 μl containing 2 μl of cDNA, 1 μM of each sense and antisense primer, and 5 μl of the QuantiFast SYBR Green PCR Kit master mix (Qiagen). SYBR Green real-time quantitative RT-PCR amplifications were performed using the Mastercycler® ep realplex 2S system (Eppendorf). Reactions were conducted in 96-well plates. Samples were analyzed in duplicate. In all cases, a "no template" negative control in which cDNAs were replaced by water was included. In addition, a melting curve was conducted at the end of each qPCR experiment to ensure amplification of only one product. Initial validation experiments were conducted to determine optimal primer annealing temperatures and to ensure that PCRs were reproducible ($0.98 > R^2 > 1.02$) and that all primer pairs had equivalent PCR efficiencies. The genes of interest were normalized to the reference gene (EF-1α) and expression levels were compared using the ΔCt method. Amplification, dissociation curves and gene expression analysis were performed using the Realplex1.5 software (Eppendorf). The reference gene EF-1α was tested to verify that fasting did not affect its expression levels in either hypothalamus or gut, as demonstrated by similar Ct (cycle threshold) values between fed and starved fish.

2.6. Sequence analysis

DNA and deduced protein sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Multiple alignments of amino acid sequences were performed using ClustalW software (www.ebi.ac.uk/clustalw/). Signal peptides were predicted using Signal P 3.0 software (www.cbs.dtu.dk/services/SignalP/).

2.7. Statistics

Gene expression levels between fasted and fed animals were compared using Student's *t* tests. Expression levels were expressed as a percentage relative to a control group, which was set at 100%. Unless specified otherwise, significance was set at $p < 0.05$. All tests were conducted using InStat 3.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Structure of winter flounder NPY, CART and CCK

In all cloning experiments using degenerate primers, multiple bands were generated. All bands within the range of the expected size were cloned and sequenced. For each peptide, only one sequence consisted of the expected peptide sequence.

The winter skate NPY cDNA sequence is a 695 bp sequence (GenBank Accession No. EU684052) that includes a 76 bp 5′UTR and a 325 bp 3′UTR (Fig. 1). The open reading frame (294 bp) contains 98 amino acids which encode for preproNPY, which contains a 28aa signal peptide. The NPY precursor sequence has three putative exons that are divided by two introns located after nucleotides 264 and 348.

The winter skate CART cDNA is 620 bp long (GenBank Accession No. FJ379292) and includes a 50 bp 5′UTR, a 330 bp open reading frame that encodes for a 110aa CART precursor and a 240 bp 3′UTR (Fig. 1B). The CART precursor sequence has a 22aa signal peptide. The CART gene has three putative exons that are divided by two introns located after nucleotides 188 and 279.

Winter skate CCK is a 536 bp sequence (GenBank Accession No. EU684054) with a 78 bp 5′UTR and a 107 bp 3′UTR (Fig. 1C). The open reading frame contains a 20aa signal peptide and pro-CCK, which contains the CCK-8 peptide. The CCK gene has two putative exons that are divided by one intron located after nucleotide 299.

Winter skate proNPY has relatively low sequence similarity to mammalian NPY (55% with mouse) and teleost fish NPY (from 53% with goldfish to 55% with trout) but shared a high degree of similarity with the electric ray NPY (81%) (Fig. 2). Winter skate

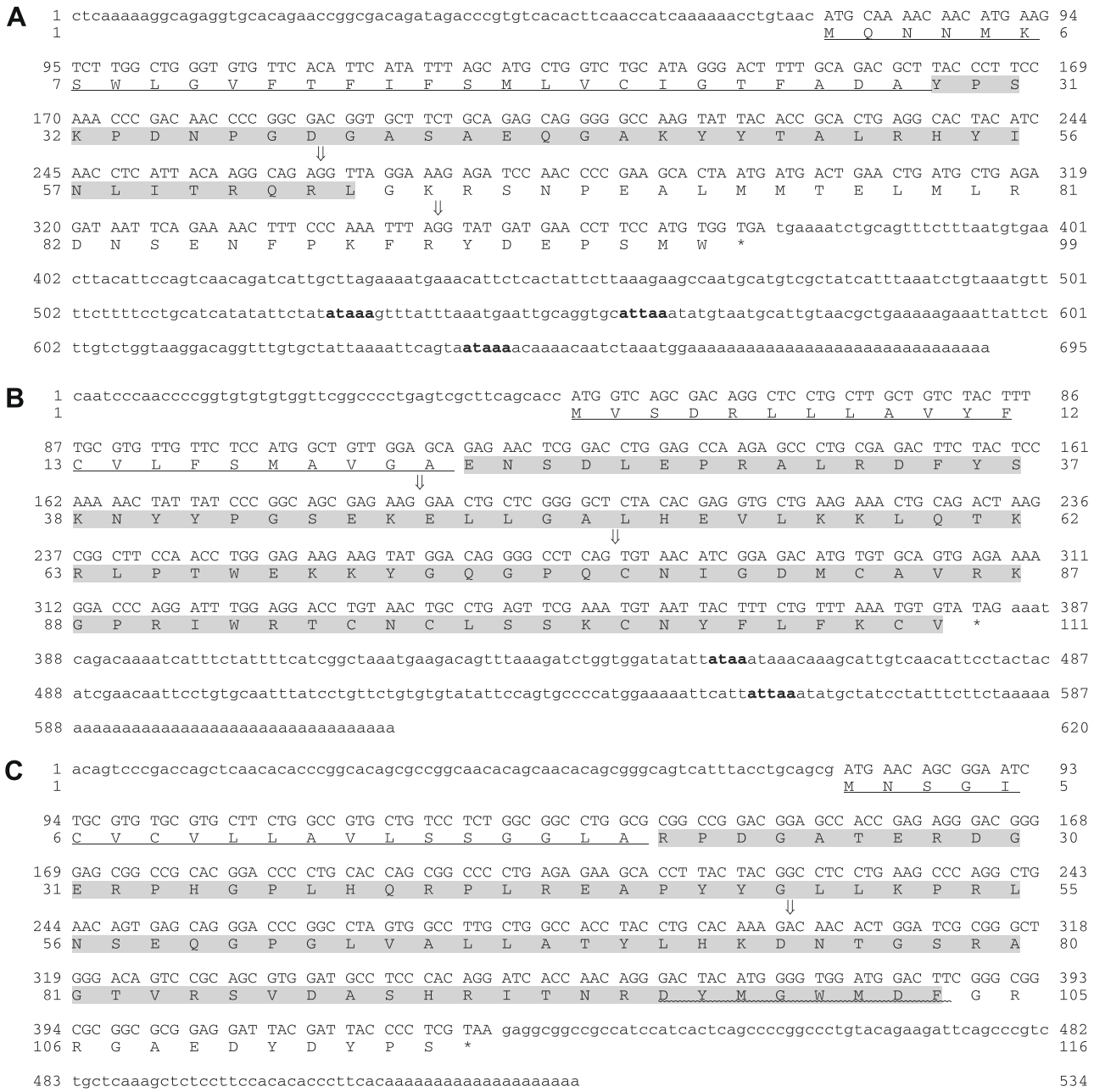

Fig. 1. Predicted amino acid sequence for winter skate NPY (A), CART (B) and CCK (C). Untranslated regions are in small case letters. Putative signal peptides are underlined. Mature peptides are shaded. In the CCK sequence, CCK-8 is wave-underlined. Putative introns are indicated by vertical arrows. Stop codons are indicated by a star (*). Potential polyadenylation sites are in bold letters.

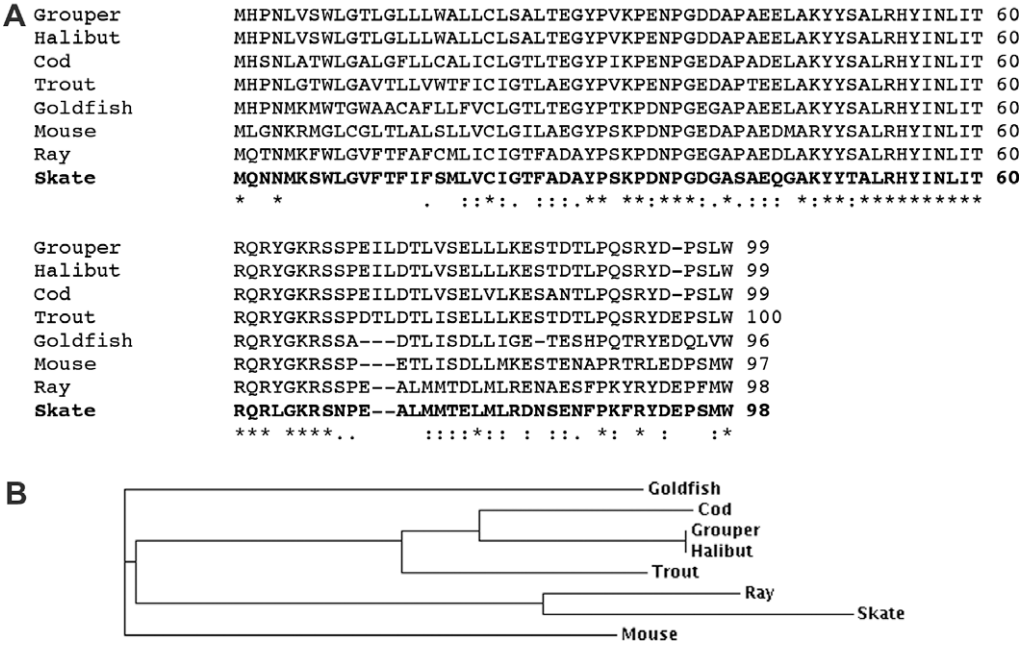

Fig. 2. NPY amino acid sequence alignment (A) and phylogentic tree (B) for winter skate (GenBank Accession No. EU684052), Atlantic cod, *Gadus morhua* (GenBank Accession No. AY822596); orange-spotted grouper, *Epinephelus coioides* (GenBank Accession No. AB055211); goldfish, *Carassius auratus* (GenBank Accession No. M87297); Rainbow trout, *Oncorhynchus mykiss* (GenBank Accession No. AF203902); Bastard halibut, *Paralichthys olivaceus* (GenBank Accession No. AB055211); electric ray, *Torpedo marmorata* (GenBank Accession No. M87296); mouse, *Mus musculus* (GenBank Accession No. BC043012).

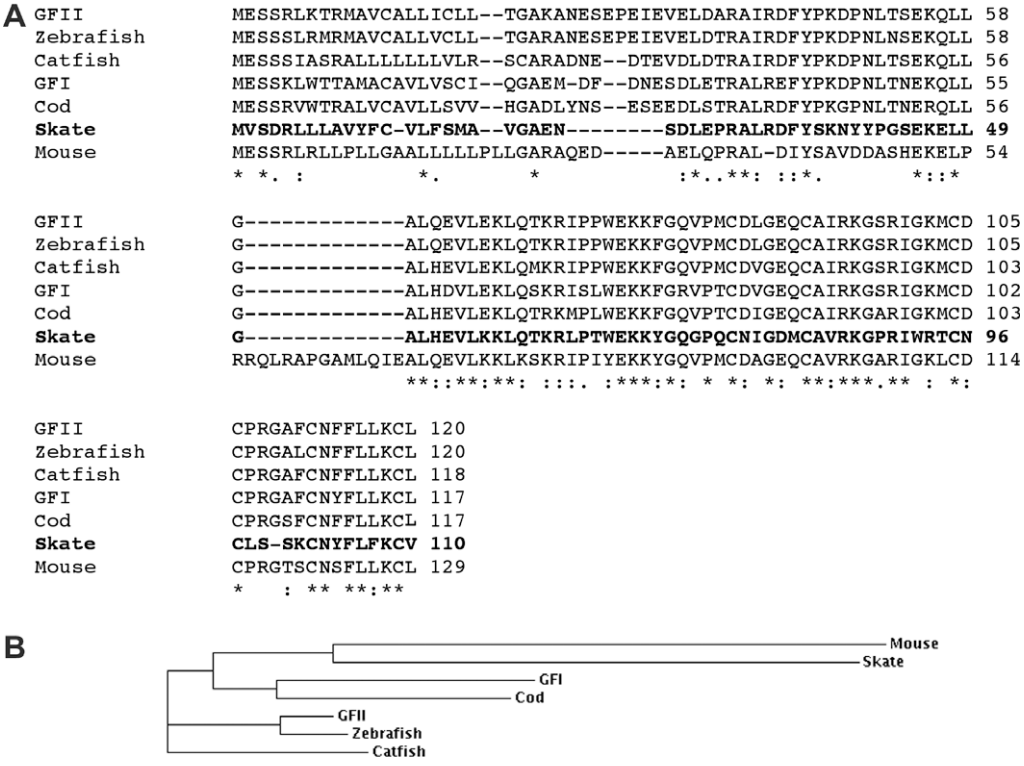

Fig. 3. CART amino acid sequence alignment (A) and phylogentic tree (B) for winter skate (GenBank Accession No. FJ379292), cod, *Gadus morhua* (GenBank Accession No. DQ167210); goldfish CART I and II, *Carrasius auratus* (GenBank Accession Nos. AF288810 and AF288811); mouse, *Mus musculus* (GenBank Accession No. BC056431); zebrafish, *Danio rerio* (GenBank Accession No. XM_680337); catfish, *Ictalurus punctatus* (Kobayashi et al., 2008).

proCART displayed a very low degree of similarity with other vertebrate CART ranging from 40% with mouse to 50% with goldfish catfish and cod (Fig. 3). Winter skate pro-CCK displayed very low amino acid similarity with mammalian CCK (43% with mouse) or teleosts CCKs (ranging from 38% with trout to 44% with goldfish) (Fig. 4). The highest similarity was with dogfish CCK (46%).

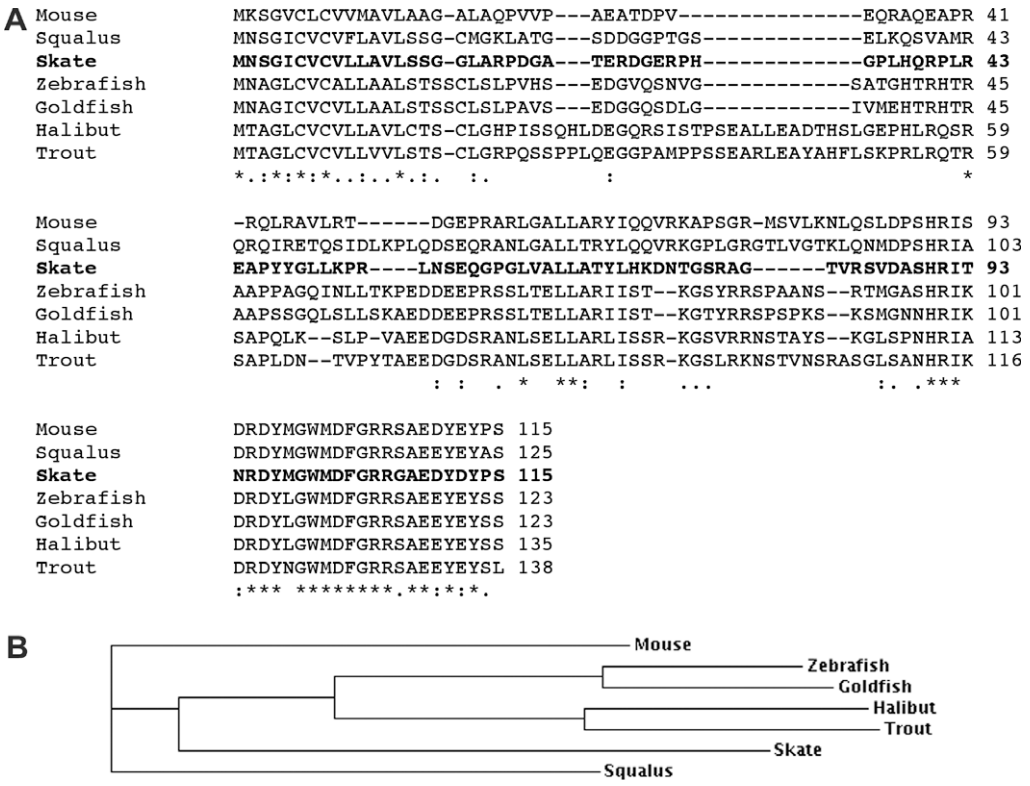

Fig. 4. CCK amino acid sequence alignment (A) and phylogentic tree (B) of winter skate (GenBank Accession No. EU684054), spiny dogfish, *Squalus acanthias* (GenBank Accession No. Z97375); Bastard halibut, *Paralichthys olivaceus* (GenBank Accession No. AB009281); mouse, *Mus musculus* (GenBank Accession No. NM_031161); Rainbow trout, *Oncorhynchus mykiss* (GenBank Accession No. NM_001124345); Zebrafish, *Danio rerio* (GenBank Accession No. XM_001346104).

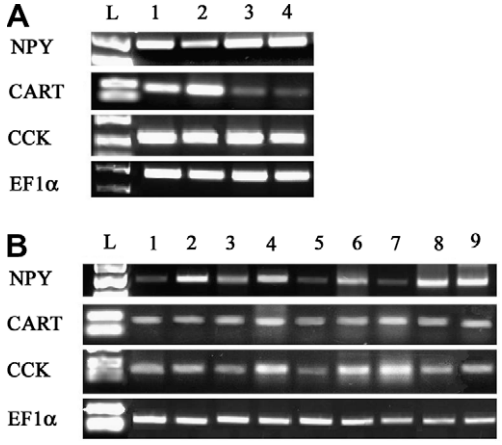

Fig. 5. RT-PCR distribution of NPY (285 bp), CART (92 bp), CCK (120 bp) and EF (247 bp) in different brain regions (A) and different peripheral tissues (B) of the winter skate. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. In (A): L, ladder; 1, hypothalamus; 2, telencephalon; 3, optic tectum; 4, cerebellum. In (B): L, ladder; 1, gill; 2, heart; 3, stomach; 4, gut; 5, spleen; 6, liver; 7, kidney; 8, muscle; 9, gonad.

3.2. Tissue distribution

Reverse transcription PCR (RT-PCR) was used to amplify NPY, CART and CCK in different brain regions as well as several peripheral tissues (Fig. 5). A 285 bp fragment was amplified for NPY, a 92 bp fragment for CART, and a 120 bp fragment for CCK. No expression was detected in any negative control samples. A 247 bp fragment of similar intensity was amplified in all samples using the control gene EF-1α.

Within the brain, NPY, CART and CCK expressions were detected in all regions examined (Fig. 5A). On the basis of visual inspection, NPY and CCK expression appeared similar in all brain regions, whereas CART expression appeared to be highest in the hypothalamus and the telencephalon.

CART, NPY and CCK expression were detected in all peripheral tissues examined (Fig. 5B). NPY expression appeared to be higher in the heart, gut, liver, muscle and gonad. Both CART and CCK displayed relatively constant expression levels, with potentially slightly higher expression levels in the gut, liver and kidney.

3.3. Effects of fasting on gene expression

There were no significant changes in either NPY, CART or CCK expression in the hypothalamus between fed and fasted animals (Fig. 6A and B). There were no significant differences in NPY expressions in the telencephalon between fed and fasted animals (Fig. 6B). NPY expression in the telencephalon and CCK expression in the gut (Fig. 6C) were both significantly higher in fasted fish than in fed fish.

4. Discussion

The structure of winter skate NPY is similar to that of other vertebrates including fish, with potentially three exons divided by two introns (Cerda-Reverter and Larhammar, 2000). As expected, the highest degree of similarity for winter skate NPY occurs with another batoid, the electric ray, with 81% identity. Lower degrees of similarity are found with NPYs from teleost fish, the highest being 55% with rainbow trout NPY. The mature NPY peptide displays higher conservation among species, with a stretch of 13 identical amino acids (ALRHYINLITRQR) at the C-terminus. This high degree

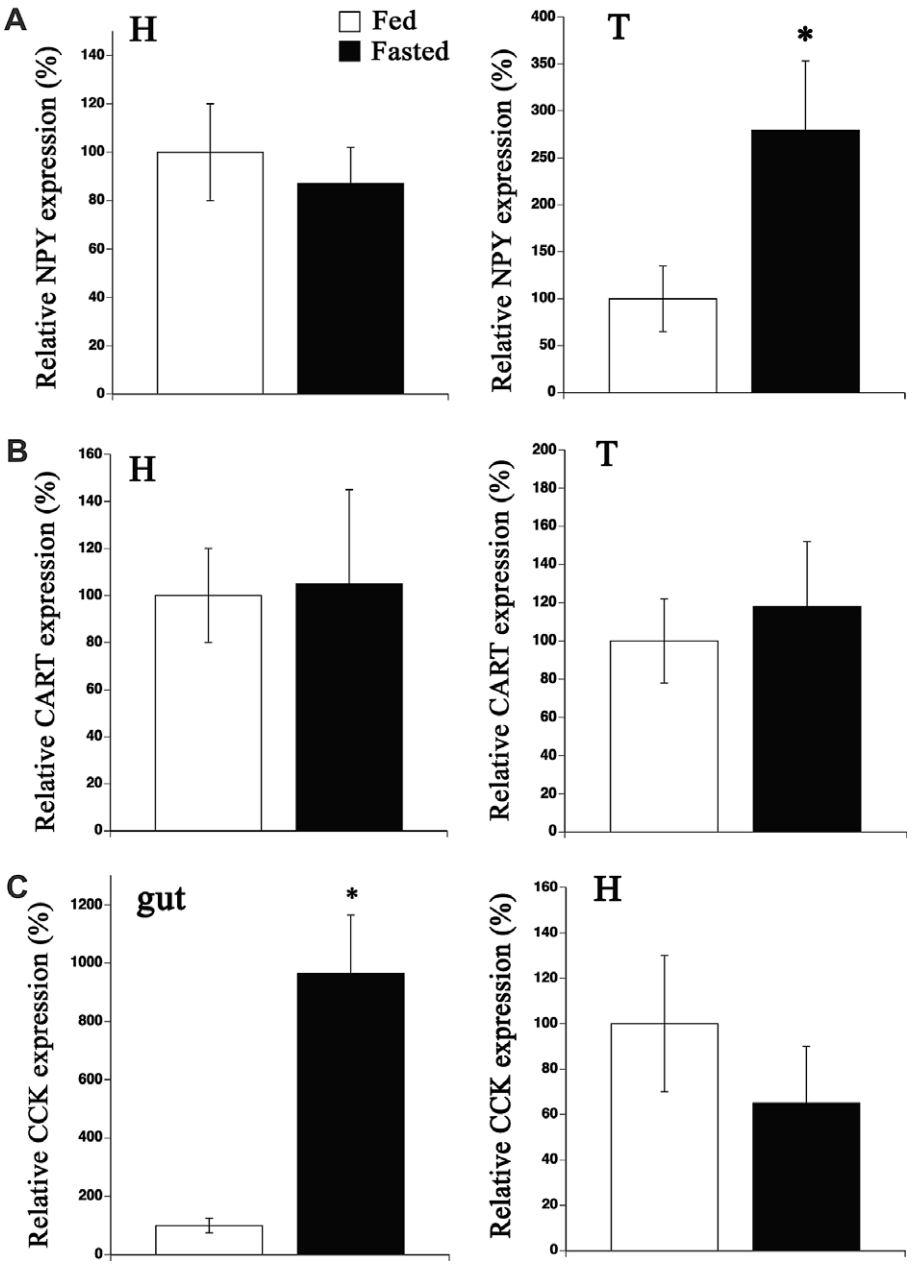

Fig. 6. mRNA expression of hypothalamic (H) and telencephalon (T) NPY (panel A) and CART (panel B) and gut and hypothalamic (H) CCK (panel C) in fed and fasted winter skates (n = 5–8 per group). Expression levels in the fed group were normalized to 100%. Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups.

of conservation suggests that NPY might have similar physiological action among fish, including teleosts and elasmobranchs.

This is the first report of an elasmobranch CART sequence. As in other vertebrates, the winter skate CART gene might have three potential exons. Whereas teleosts CARTs are relatively conserved (70–93% identity), skate CART presents a relatively low degree of homology with CARTs from teleosts, ranging from 45% to 50%. However, the location of cysteine residues is conserved among all fish CARTs, which is not surprising as these cysteines determine the three dimensional structure of CART and are crucial for its biological activity (Couceyro and Fritz, 2003). This conservation suggests that CART might have similar physiological functions across the taxa.

The cloned winter skate CCK sequence contains two putative exons and one intron. Winter skate prepro-CCK shows a very low degree of similarity with CCKs from other fish, the highest sequence similarity being 46% with a CCK of another elasmobranch, the spiny dogfish. CCK-8, the C-terminal sulphated octapeptide fragment of cholecystokinin, appears to be the major product of post-translational processing in fish, although pro-CCK is also cleaved into fragments of different lengths (Jensen et al., 2001). As opposed to the rest of the peptide, the CCK-8 region (DYMGWMDFD) is extremely well conserved among all species, with only one variable amino acid. This conservation suggests that CCK has conserved biological functions among fish species.

Within the winter skate brain, NPY mRNA expression was detected in hypothalamus, telencephalon, optic tectum and cerebellum. Our results are in line with previous studies showing a widespread distribution for NPY mRNA expression within the brain of other fish species, including cod (Kehoe and Volkoff, 2007), sea bass (Cerda-Reverter et al., 2000b), goldfish (Narnaware et al., 2000) and salmon (Silverstein et al., 1998). NPY-immunoreactive

fibers have also been shown throughout the brain of dogfish (*Scyliorhinus canicula*), with the exception of the cerebellum (Vallarino et al., 1988). NPY-immunoreactive cells have also been found in the saccus vasculosus (SV), a circumventricular organ of the hypothalamus, of two elasmobranchs, the dogfish (*S. canicula*) and the brown shy shark (*Haploblepharus fuscus*) (Sueiro et al., 2007) and in the terminal nerve, a supernumerary cranial nerve found in association with the olfactory system, of the cloudy dogfish (*Scyliorhinus torazame*) (Chiba, 2000). In the periphery, NPY mRNA expression was found in every tissue examined, with apparent high expression levels in the heart, stomach, gut, liver, muscle and gonad. NPY-immunoreactive fibers or NPY mRNA expression have also been identified in teleosts pituitary, gut and nerve fibers surrounding blood vessels (Cerda-Reverter et al., 2000a; Marchetti et al., 2000; Rodriguez-Gomez et al., 2001), liver (Liang et al., 2007) and kidneys (Kehoe and Volkoff, 2007). In cloudy dogfish, NPY-immunoreactive fibers are present in the spiral intestine and stomach (Chiba, 1998). These data suggest that NPY might act as a brain gut peptide and have a role in regulating digestive processes in both teleosts and elasmobranchs.

Within the brain, CART mRNA expression was relatively higher in the hypothalamus and telencephalon than in the optic tectum and the cerebellum. These expression patterns are similar to those found in previous studies in cod (Kehoe and Volkoff, 2007) and goldfish (Volkoff and Peter, 2001). In catfish, CART immunoreactivity (Singru et al., 2007) and mRNA (Kobayashi et al., 2008) have a widespread distribution in the brain and pituitary, suggesting that CART peptides may play an important role in the processing of sensory information, motor function and the regulation of pituitary hormone secretion. In skate, CART mRNA expression was present in all peripheral tissues examined including gut, kidney and gonad. Similarly, in both cod (Kehoe and Volkoff, 2007) and goldfish (Volkoff and Peter, 2001), CART mRNA is present gonad, gut and kidney (Volkoff and Peter, 2001). In catfish, however, CART mRNA has only been reported in brain and gonad (testis) (Kobayashi et al., 2008). Interestingly, although CART peptides have been detected in the gut of mammals (Couceyro et al., 1998; Kuhar and Yoho, 1999), CART mRNA has never been detected in the gastrointestinal tract of any vertebrate. The presence of CART mRNA in the gut of skate suggests that CART might have a role in digestive processes in this species.

CCK mRNA expression was found in all brain regions examined, i.e. hypothalamus, telencephalon, optic tectum and cerebellum. CCK expression has been reported in the brain of teleosts, with high levels in hypothalamus and telencephalon (Kurokawa et al., 2003; Murashita et al., 2006; Peyon et al., 1998; Raven et al., 2008). CCK binding sites have also been shown in the brain of elasmobranchs (Oliver and Vigna, 1996). CCK was expressed in all the peripheral tissues tested, including gut, liver and kidney. CCK-like immunoreactivity has been shown in the nervous system and gut of several fish species, including teleosts [trout (Bosi et al., 2004), cod (Jonsson et al., 1987), goldfish (Himick et al., 1993; Himick and Peter, 1994), turbot (Bermudez et al., 2007; Reinecke et al., 1997)] and elasmobranchs [dogfish (Aldman et al., 1989)]. CCK causes gallbladder contraction in the killifish (Honkanen et al., 1988), inhibits gastric secretions in cod (Holstein, 1982), increases gut motility in dogfish (Aldman et al., 1989) and cod (Forgan and Forster, 2007) and slows gastric emptying in trout (Olsson et al., 1999), suggesting that CCK peptides have an important role in the regulation of digestive processes in fish.

A two weeks fasting period induced significant increases in NPY expression levels in the telencephalon, but not the hypothalamus, suggesting that NPY is involved in feeding regulation in winter skate. Our results are consistent with the role of NPY in the regulation of feeding shown in several teleost fish. Central injections of mammalian or fish NPY cause a dose-dependent increase in food intake in goldfish (de Pedro et al., 2000; Lopez-Patino et al., 1999; Narnaware et al., 2000), trout (Aldegunde and Mancebo, 2006) and catfish (Silverstein and Plisetskaya, 2000). In goldfish, both hypothalamic and telencephalon NPY mRNA levels increase after 72 h of food deprivation (Narnaware and Peter, 2001b) and in salmon, NPY hypothalamic mRNA levels increase after three weeks of fasting (Silverstein et al., 1998). Also, forebrain NPY mRNA levels undergo peri-prandial variations in both goldfish (Narnaware and Peter, 2001a) and Atlantic cod (Kehoe and Volkoff, 2007), with highest levels around meal time.

We found no significant differences in the expression of CART in either the hypothalamus or telencephalon of skate following a two weeks fasting period. In goldfish (Volkoff and Peter, 2001), cod (Kehoe and Volkoff, 2007) and catfish (Kobayashi et al., 2008), CART mRNA brain levels decrease following food deprivation. Peri-prandial changes in CART brain mRNA have also been demonstrated in goldfish and cod (Kehoe and Volkoff, 2007; Volkoff and Peter, 2001). The absence of effects of fasting within skate brain might be explained by several factors. First, fasting might induce a response might in CART at a translational or post-translational level, and affect CART protein levels without affecting mRNA levels. It is also possible that another CART form exists in winter skate that is more sensitive to fasting than the form we cloned. Indeed, in goldfish two forms of CART respond differently to fasting, CART I being more sensitive than CART II (Volkoff and Peter, 2001).

Gut CCK mRNA expression was significantly higher in fasted fish compared to the fed fish whereas hypothalamic CCK mRNA expression was not significantly affected by fasting (although a trend in a decrease was observed). Both central and peripheral injections of CCK-8 suppress food intake in goldfish (Himick and Peter, 1994; Thavanathan and Volkoff, 2006; Volkoff et al., 2003) and treatment of trout with CCK antagonists induces an increase in food intake (Gelineau and Boujard, 2001), suggesting that CCK peptides suppress appetite and are involved in the regulation of digestive processes in fish. CCK mRNA levels increase following a meal in both goldfish brain (Peyon et al., 1999) and in yellowtail pyloric caeca (Murashita et al., 2007). In yellowtail, fasting decreases CCK mRNA levels in the anterior intestine (Murashita et al., 2006). In skate, fasting did not significantly affect hypothalamic CCK mRNA expression and induced a significant increase in CCK mRNA levels, which appears to contradict a role for CCK as a satiety factor in fish. In some fish species, such as winter flounder, a long fasting period induces changes in the intestinal mucosa with a reduction in the height and number of the folds (Mcleese and Moon, 1989). In our study, there were no apparent changes in either gut morphology or in the amount of total RNA per gram of tissue between fed and fasted skates, suggesting that changes in RNA expression were not due to morphological change caused by fasting. It is noteworthy that elasmobranchs differ in their digestive physiology from teleosts. For example, elasmobranchs are capable of secreting highly acidic gastric fluids in association with food intake (Holmgren and Nilsson, 1999). Inter-specific differences in the response of gastric acid secretion to fasting exist among elasmobranchs, with some species continuously secreting acid while others periodically cease secretions (Papastamatiou and Lowe, 2005). Also, as opposed to most vertebrates, the pH within the elasmobranch spiral valve (intestine) does not decline progressively from the anterior to the posterior portion (Papastamatiou, 2007). As the presence of acid in the intestine stimulates the synthesis and secretion of CCK—which in turn stimulates bicarbonate secretion in the intestine—(Guilloteau et al., 2006), the increase in CCK expression within winter skate gut could be due to specific patterns of digestive enzymes or acid secretion in this species. Also, metabolic zonation (different cell types and enzymatic profiles) has been observed in the gut of teleosts (Mommsen et al., 2003). As we examined the expression of CCK in the gut region close to

the stomach, it is possible that different expression pattern might have been seen in other gut areas.

In summary cDNAs encoding for NPY, CART and CCK were cloned in winter skate and their mRNA expression shown to have a widespread distribution in peripheral tissues and within the brain. Our results show that all three peptides are expressed in both brain and gut and might have a major role in the regulation of feeding and digestive processes in winter skate.

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References

Adams, L.D., Gong, W., Vechia, S.D., Hunter, R.G., Kuhar, M.J., 1999. CART: from gene to function. Brain Res. 848, 137–140.

Aldegunde, M., Mancebo, M., 2006. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (*Oncorhynchus mykiss*). Peptides 27, 719–727.

Aldman, G., Holmgren, S., 1995. Intraduodenal fat and amino-acids activate gallbladder motility in the rainbow-trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 100, 27–32.

Aldman, G., Jonsson, A.C., Jensen, J., Holmgren, S., 1989. Gastrin/CCK-like peptides in the spiny dogfish, *Squalus acanthias*; concentrations and actions in the gut. Comp. Biochem. Physiol. C 92, 103–108.

Bermudez, R., Vigliano, F., Quiroga, M.I., Nieto, J.M., Bosi, G., Domeneghini, C., 2007. Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa). Fish Shellfish Immunol. 22, 252–263.

Blomqvist, A.G., Soderberg, C., Lundell, I., Milner, R.J., Larhammar, D., 1992. Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones. Proc. Natl. Acad. Sci. USA 89, 2350–2354.

Bosi, G., Di Giancamillo, A., Arrighi, S., Domeneghini, C., 2004. An immunohistochemical study on the neuroendocrine system in the alimentary canal of the brown trout, *Salmo trutta*, L., 1758. Gen. Comp. Endocrinol. 138, 166–181.

Cerda-Reverter, J., Martinez-Rodriguez, G., Zanuy, S., Carrillo, M., Larhammar, D., 2000a. Molecular evolution of the neuropeptide Y (NPY) family of peptides: cloning of three NPY-related peptides from the sea bass (*Dicentrarchus labrax*). Regul. Pept. 95, 25–34.

Cerda-Reverter, J.M., Anglade, I., Martinez-Rodriguez, G., Mazurais, D., Munoz-Cueto, J.A., Carrillo, M., Kah, O., Zanuy, S., 2000b. Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (*Dicentrarchus labrax*). J. Chem. Neuroanat. 19, 197–210.

Cerda-Reverter, J.M., Larhammar, D., 2000. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. Biochem. Cell Biol. 78, 371–392.

Chandra, R., Liddle, R.A., 2007. Cholecystokinin. Curr. Opin. Endocrinol. Diabetes Obes. 14, 63–67.

Chee, M.J., Colmers, W.F., 2008. Y eat? Nutrition 24, 869–877.

Chiba, A., 1998. Ontogeny of serotonin-immunoreactive cells in the gut epithelium of the cloudy dogfish, *Scyliorhinus torazame*, with reference to coexistence of serotonin and neuropeptide Y. Gen. Comp. Endocrinol. 111, 290–298.

Chiba, A., 2000. Immunohistochemical cell types in the terminal nerve ganglion of the cloudy dogfish, *Scyliorhinus torazame*, with special regard to neuropeptide Y/FMRFamide-immunoreactive cells. Neurosci. Lett. 286, 195–198.

Couceyro, P., Paquet, M., Koylu, E., Kuhar, M.J., Smith, Y., 1998. Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in myenteric plexus neurons of the rat ileum and co-localization with choline acetyltransferase. Synapse 30, 1–8.

Couceyro, P.R., Fritz, T., 2003. Production of recombinant CART peptides in Escherichia coli with agonist and antagonist effects on food intake in rats. Protein Expr. Purif. 32, 185–193.

de Pedro, N., Lopez-Patino, M.A., Guijarro, A.I., Pinillos, M.L., Delgado, M.J., Alonso-Bedate, M., 2000. NPY receptors and opioidergic system are involved in NPY-induced feeding in goldfish. Peptides 21, 1495–1502.

Douglass, J., Daoud, S., 1996. Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. Gene 169, 241–245.

Douglass, J., McKinzie, A.A., Couceyro, P., 1995. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J. Neurosci. 15, 2471–2481.

Doyon, C., Gilmour, K.M., Trudeau, V.L., Moon, T.W., 2003. Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. Gen. Comp. Endocrinol. 133, 260–271.

Forgan, L.G., Forster, M.E., 2007. Effects of potential mediators of an intestinal brake mechanism on gut motility in Chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol. C 146, 343–347.

Frisk, M.G., Miller, T.J., 2006. Age, growth, and latitudinal patterns of two *Rajidae* species in the northwestern Atlantic: little skate (*Leucoraja erinacea*) and winter skate (*Leucoraja ocellata*). Can. J. Fish. Aquat. Sci. 63, 1078–1091.

Gelineau, A., Boujard, T., 2001. Oral administration of cholecystokinin receptor antagonists increase feed intake in rainbow trout. J. Fish Biol. 58, 716–724.

Gorissen, M.H.A.G., Flik, G., Huising, M.O., 2006. Peptides and proteins regulating food intake: a comparative view. Anim. Biol. 56, 447–473.

Guilloteau, P., Le Meuth-Metzinger, V., Morisset, J., Zabielski, R., 2006. Gastrin, cholecystokinin and gastrointestinal tract functions in mammals. Nutr. Res. Rev. 19, 254–283.

Himick, B.A., Golosinski, A.A., Jonsson, A.C., Peter, R.E., 1993. CCK/gastrin-like immunoreactivity in the goldfish pituitary: regulation of pituitary hormone secretion by CCK-like peptides in vitro. Gen. Comp. Endocrinol. 92, 88–103.

Himick, B.A., Peter, R.E., 1994. CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. Am. J. Physiol. 267, R841–R851.

Holmgren, S., Nilsson, S., 1999. Digestive system. In: Hamlett, W.C. (Ed.), Sharks, Skates and Rays. The Biology of Elasmobranch Fishes. The Johns Hopkins University Press, Baltimore and London, pp. 144–173.

Holstein, B., 1982. Inhibition of gastric acid secretion in the Atlantic cod, *Gadus morhua*, by sulphated and desulphated gastrin, caerulein, and CCK-octapeptide. Acta Physiol. Scand. 114, 453–459.

Honkanen, R.E., Crim, J.W., Patton, J.S., 1988. Effects of cholecystokinin peptides on digestive enzymes in killifish in vivo. Comp. Biochem. Physiol. A 89, 655–660.

Jensen, H., Rourke, I.J., Moller, M., Jonson, L., Johnsen, A.H., 2001. Identification and distribution of CCK-related peptides and mRNAs in the rainbow trout, *Oncorhynchus mykiss*. Biochim. Biophys. Acta 1517, 190–201.

Johnsen, A.H., Jonson, L., Rourke, I.J., Rehfeld, J.F., 1997. Elasmobranchs express separate cholecystokinin and gastrin genes. Proc. Natl. Acad. Sci. USA 94, 10221–10226.

Jonsson, A.C., Holmgren, S., Holstein, B., 1987. Gastrin cck-like immunoreactivity in endocrine-cells and nerves in the gastrointestinal-tract of the cod, *Gadus morhua*, and the effect of peptides of the gastrin cck family on cod gastrointestinal smooth-muscle. Gen. Comp. Endocrinol. 66, 190–202.

Kamisaka, Y., Totland, G.K., Tagawa, M., Kurokawa, T., Suzuki, T., Tanaka, M., Ronnestad, I., 2001. Ontogeny of cholecystokinin-immunoreactive cells in the digestive tract of Atlantic halibut, *Hippoglossus hippoglossus*, larvae. Gen. Comp. Endocrinol. 123, 31–37.

Kehoe, A.S., Volkoff, H., 2007. Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. A 146, 451–461.

Kobayashi, Y., Peterson, B.C., Waldbieser, G.C., 2008. Association of cocaine- and amphetamine-regulated transcript (CART) messenger RNA level, food intake, and growth in channel catfish. Comp. Biochem. Physiol. 151, 219–225.

Kuhar, M.J., Yoho, L.L., 1999. CART peptide analysis by Western blotting. Synapse 33, 163–171.

Kurokawa, T., Suzuki, T., Hashimoto, H., 2003. Identification of gastrin and multiple cholecystokinin genes in teleost. Peptides 24, 227–235.

Lazar, G., Calle, M., Roubos, E.W., Kozicz, T., 2004. Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the central nervous system of the frog *Rana esculenta*. J. Comp. Neurol. 477, 324–339.

Liang, X.F., Li, G.Z., Yao, W., Cheong, L.W., Liao, W.Q., 2007. Molecular characterization of neuropeptide Y gene in Chinese perch, an acanthomorph fish. Comp. Biochem. Physiol. B 148, 55–64.

Lopez-Patino, M.A., Guijarro, A.I., Isorna, E., Delgado, M.J., Alonso-Bedate, M., de Pedro, N., 1999. Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (*Carassius auratus*). Eur. J. Pharmacol. 377, 147–153.

Marchetti, G., Cozzi, B., Tavanti, M., Russo, V., Pellegrini, S., Fabiani, O., 2000. The distribution of neuropeptide Y-immunoreactive neurons and nerve fibers in the forebrain of the carp *Cyprinus carpio* L.. J. Chem. Neuroanat. 20, 129–139.

Mcleese, J.M., Moon, T.W., 1989. Seasonal-changes in the intestinal-mucosa of winter flounder, Pseudopleuronectes-Americanus (Walbaum), from Passamaquoddy Bay, New-Brunswick. J. Fish Biol. 35, 381–393.

Mommsen, T.P., Osachoff, H.L., Elliott, M.E., 2003. Metabolic zonation in teleost gastrointestinal tract—effects of fasting and cortisol in tilapia. J. Comp. Physiol. B 173, 409–418.

Moran, T.H., Kinzig, K.P., 2004. Gastrointestinal satiety signals II. Cholecystokinin. Am. J. Physiol. Gastrointest. Liver Physiol. 286, G183–G188.

Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T., 2006. Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. Gen. Comp. Endocrinol. 145, 287–297.

Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T., 2007. Changes in cholecystokinin and peptide Y gene expression with feeding in yellowtail (*Seriola quinqueradiata*): relation to pancreatic exocrine regulation. Comp. Biochem. Physiol. B—Biochem. Mol. Biol. 146, 318–325.

Narnaware, Y.K., Peter, R.E., 2001a. Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish. Comp. Biochem. Physiol. B 129, 633–637.

Narnaware, Y.K., Peter, R.E., 2001b. Neuropeptide Y stimulates food consumption through multiple receptors in goldfish. Physiol. Behav. 74, 185–190.
Narnaware, Y.K., Peter, R.E., 2002. Influence of diet composition on food intake and neuropeptide Y (NPY) gene expression in goldfish brain. Regul. Pept. 103, 75–83.
Narnaware, Y.K., Peyon, P.P., Lin, X., Peter, R.E., 2000. Regulation of food intake by neuropeptide Y in goldfish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R1025–R1034.
Northcutt, R.G., 2002. Understanding vertebrate brain evolution. Integr. Comp. Biol. 42, 743–756.
Oliver, A.S., Vigna, S.R., 1996. CCK-X receptors in the endothermic mako shark (*Isurus oxyrinchus*). Gen. Comp. Endocrinol. 102, 61–73.
Olsson, C., Aldman, G., Larsson, A., Holmgren, S., 1999. Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*. J. Exp. Biol. 202, 161–170.
Papastamatiou, Y.P., 2007. The potential influence of gastric acid secretion during fasting on digestion time in leopard sharks (*Triakis semifasciata*). Comp. Biochem. Physiol. A 147, 37–42.
Papastamatiou, Y.P., Lowe, C.G., 2005. Variations in gastric acid secretion during periods of fasting between two species of shark. Comp. Biochem. Physiol. A 141, 210–214.
Peyon, P., Lin, X.W., Himick, B.A., Peter, R.E., 1998. Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. Peptides 19, 199–210.
Peyon, P., Saied, H., Lin, X., Peter, R.E., 1999. Postprandial, seasonal and sexual variations in cholecystokinin gene expression in goldfish brain. Brain Res. Mol. Brain Res. 74, 190–196.
Raven, P.A., Uh, M., Sakhrani, D., Beckman, B.R., Cooper, K., Pinter, J., Leder, E.H., Silverstein, J., Devlin, R.H., 2008. Endocrine effects of growth hormone overexpression in transgenic coho salmon. Gen. Comp. Endocrinol. 159, 26–37.
Rehfeld, J.F., Friis-Hansen, L., Goetze, J.P., Hansen, T.V., 2007. The biology of cholecystokinin and gastrin peptides. Curr. Top. Med. Chem. 7, 1154–1165.
Reinecke, M., Muller, C., Segner, H., 1997. An immunohistochemical analysis of the ontogeny, distribution and coexistence of 12 regulatory peptides and serotonin in endocrine cells and nerve fibers of the digestive tract of the turbot, *Scophthalmus maximus* (Teleostei). Anat. Embryol. (Berl.) 195, 87–101.
Rodriguez-Gomez, F.J., Rendon-Unceta, C., Sarasquete, C., Munoz-Cueto, J.A., 2001. Distribution of neuropeptide Y-like immunoreactivity in the brain of the Senegalese sole (*Solea senegalensis*). Anat. Rec. 262, 227–237.
Rubio, V.C., Sanchez-Vazquez, F.J., Madrid, J.A., 2008. Role of cholecystokinin and its antagonist proglumide on macronutrient selection in European sea bass *Dicentrarchus labrax* L.. Physiol. Behav. 93, 862–869.
Scott, G.K., Davies, P.L., Kao, M.H., Fletcher, G.L., 1988. Differential amplification of antifreeze protein genes in the pleuronectinae. J. Mol. Evol. 27, 29–35.
Silverstein, J.T., Bondareva, V.M., Leonard, J.B., Plisetskaya, E.M., 2001. Neuropeptide regulation of feeding in catfish, *Ictalurus punctatus*: a role for glucagon-like peptide-1 (GLP-1)? Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 623–631.
Silverstein, J.T., Breininger, J., Baskin, D.G., Plisetskaya, E.M., 1998. Neuropeptide Y-like gene expression in the salmon brain increases with fasting. Gen. Comp. Endocrinol. 110, 157–165.
Silverstein, J.T., Plisetskaya, E.M., 2000. The effects of NPY and insulin on food intake regulation in fish. Am. Zool. 40, 296–308.
Singru, P.S., Mazumdar, M., Sakharkar, A.J., Lechan, R.M., Thim, L., Clausen, J.T., Subhedar, N.K., 2007. Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the brain of the catfish, *Clarias batrachus* (Linn.). J. Comp. Neurol. 502, 215–235.
Sueiro, C., Carrera, I., Ferreiro, S., Molist, P., Adrio, F., Anadon, R., Rodriguez-Moldes, I., 2007. New insights on saccus vasculosus evolution: a developmental and immunohistochemical study in elasmobranchs. Brain Behav. Evol. 70, 187–204.
Sulikowski, J.A., Tsang, P.C.W., Howell, W.H., 2004. An annual cycle of steroid hormone concentrations and gonad development in the winter skate, *Leucoraja ocellata*, from the western Gulf of Maine. Mar. Biol. 144, 845–853.
Sulikowski, J.A., Tsang, P.C.W., Howell, W.H., 2005. Age and size at sexual maturity for the winter skate, *Leucoraja ocellata*, in the western Gulf of Maine based on morphological, histological and steroid hormone analyses. Environ. Biol. Fishes 72, 429–441.
Thavanathan, R., Volkoff, H., 2006. Effects of amylin on feeding of goldfish: interactions with CCK. Regul. Pept. 133, 90–96.
Treberg, J.R., Driedzic, W.R., 2006. Maintenance and accumulation of trimethylamine oxide by winter skate (*Leucoraja ocellata*): reliance on low whole animal losses rather than synthesis. Am. J. Physiol. 291, R1790–R1798.
Vallarino, M., Danger, J.M., Fasolo, A., Pelletier, G., Saint-Pierre, S., Vaudry, H., 1988. Distribution and characterization of neuropeptide Y in the brain of an elasmobranch fish. Brain Res. 448, 67–76.
Vishnuvardhan, D., Beinfeld, M.C., 2002. Biosynthesis and post-translational processing of site-directed endoproteolytic cleavage mutants of Pro-CCK in AtT-20 cells. Biochemistry 41, 570–578.
Volkoff, H., Canosa, L.F., Unniappan, S., Cerda-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142, 3–19.
Volkoff, H., Eykelbosh, A., Peter, R., 2003. Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain Res. 972, 90–109.
Volkoff, H., Peter, R.E., 2000. Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, *Carassius auratus*: actions on neuropeptide Y- and orexin A-induced feeding. Brain Res. 887, 125–133.
Volkoff, H., Peter, R.E., 2001. Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. Endocrinology 142, 5076–5088.