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Cloning, distribution and effects of season and nutritional status on the expression of neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) in winter flounder (*Pseudopleuronectes americanus*)

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ABSTRACT

cDNAs encoding for neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) were cloned in winter flounder, a species that undergoes a period of natural fasting during the winter. Tissue distribution studies show that these peptides are present in several peripheral tissues, including gut and gonads, as well as within the brain. We assessed the effects of season and fasting on the expression of these peptides. Our results show that NPY and CCK, but not CART, show seasonal differences in expression with higher hypothalamic NPY and lower gut CCK expression levels in the winter. In the summer, fasting induced an increase in hypothalamic NPY expression levels and a decrease in gut CCK levels, but did not affect hypothalamic CART expression levels. None of the peptides examined was affected by fasting in the winter. Our results suggest that NPY and CCK, but maybe not CART, might have a major role in the regulation of feeding in winter flounder and might contribute to the seasonal fluctuations in appetite in this species.

Introduction

Winter flounder (*Pseudopleuronectes americanus*) are bottom-dwelling right-handed flatfish of the family *Pleuronectidae* that undergo pronounced seasonal changes in behavior. In Newfoundland, these fish move inshore during the winter (Burton and Idler, 1984) and enter a dormant-like state during which feeding and locomotor activity are reduced (He, 2003; Martell and McClelland, 1994; Meise et al., 2003; Stoner et al., 1999). Interestingly, winter flounder spawn in late winter or early spring (Scott et al., 1988), following a period of fast and weight loss that paradoxically also coincides with gonadal development (Burton and Idler, 1984). It has been suggested that in winter flounder (Mcleese and Moon, 1989) and plaice, *Pleuronectes platessa* (Dawson and Grimm, 1980), which both undergo a similar period of winter fasting and spring spawning, lipid reserves are mobilized to provide energy during the fast, whereas protein is mobilized to provide energy for reproduction. Owing to its high quality meat (Cho, 2005; Mercier et al., 2004), and its resistance to adapt to harsh and changing environments (de Montgolfier et al., 2005; Plante et al., 2003) and to chronic stress in captivity (Plante et al., 2003), this species might represent a good candidate as a cold water aquaculture fish. However, to date, the mechanisms controlling appetite and the obligatory fasting period of winter flounder have never been examined.

In fish as in all vertebrates, appetite is regulated through endocrine pathways involving key appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) factors. The regulation of feeding involves the central nervous system as well as peripheral organs such as the gastrointestinal (GI) tract, which are engaged in complex interactions (Volkoff et al., 2005). 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. Environmental factors such as temperature and photoperiod also influence feeding. Seasonal changes in feeding behavior have been shown in a number of fish species for which increased day length and warm water temperatures are usually associated with increased feeding behavior (Brown et al., 1989).

Neuropeptide Y is a 36 amino acid peptide originally discovered in the porcine brain (Tatemoto, 1982) and is a member of the 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 identified in a number of fish species, including perch, *Siniperca chuatsi* (Liang et al., 2007), trout, *Oncorhynchus mykiss* (Doyon et al., 2003), and cod, *Gadus morhua* (Kehoe and Volkoff, 2007). NPY appears to be involved in the regulation of feeding in fish as intracerebroventricular (ICV) injections

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of NPY in both channel catfish (*Ictalurus punctatus*) (Silverstein et al., 2001) and goldfish (*Carassius auratus*) (Lopez-Patino et al., 1999) cause an increase in food intake and 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 fasted Pacific salmon, *Oncorhyncus* sp. (Silverstein et al., 1998) and in goldfish fed low nutrient diets (Narnaware and Peter, 2002).

Cocaine- and amphetamine regulated transcript (CART) was first discovered in rats (Douglass et al., 1995) as the transcript of a brain mRNA up-regulated following administration of cocaine and amphetamine (Douglass et al., 1995). In mammals, central injections of CART dose-dependently inhibit food intake (Vrang et al., 1999; Zheng et al., 2001). To date, CART has been isolated 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 food intake in fish.

Cholecystokinin (CCK) is synthesized as a preprohormone which is later proteolytically cleaved to produce gastrin/CCK-like peptides that share the carboxy-terminal ends (Chandra and Liddle, 2007; Vishnuvardhan and Beinfeld, 2002). Several biologically active forms exist, with CCK-8 being the most abundant form in mammals (Moran and Kinzig, 2004). In mammals, CCK is released from intestinal endocrine cells during a meal and decreases gastric emptying, stimulates pancreatic and gastric secretions and reduces food intake via vagal afferent pathways (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), yellowtail (*Seriola quinqueradiata*) (Murashita et al., 2006), rainbow trout (Jensen et al., 2001), pufferfish (Kurokawa et al., 2003) and Japanese flounder (*Paralichthys olivaceus*) (Kurokawa et al., 2003). In fish as in mammals, CCK influences digestion and appetite. In salmonids, CCK induces contractions of the gall bladder (Aldman and Holmgren, 1995), decreases gastric emptying (Olsson et al., 1999) and increases gut motility (Forgan and Forster, 2007). CCK also influences appetite in fish. 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 decrease in 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). In addition, CCK mRNA levels increase following a meal in goldfish brain (Peyon et al., 1999) and in pyloric caeca of yellowtail (Murashita et al., 2007).

In the present study, we have cloned cDNAs encoding NPY, CART and CCK in winter flounder, and examined their tissue and brain distributions. We then examined the effects of fasting on the gene expression of these appetite-regulating hormones. Experiments were conducted both in summer and winter to assess the effects of season on the expression levels of these hormones and on the response of the fish to fasting.

Materials and methods

Animals

Wild winter flounder were collected by scubadivers off the shore of St. John's (NL, Canada) and kept in 2 m × 2 m flow through tanks at the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, NL, Canada). Fish were kept under natural photoperiod and temperature conditions (see below). Fish consisted of both males and females and the sex ratio was approximately 50:50 in all tanks. Fish were fed frozen herring twice or three times a week at the same time of the day (10:00). Prior to the starvation 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 for all fish. GSIs (gonadosomatic index = ovary weight/somatic weight) were calculated for each animal. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide.

Experimental design

Winter experiment

Sixty flounder (average weight of 365 ± 16 g) were divided into four tanks (15 fish per tank), and acclimated for two weeks in flow through water tanks at an average temperature of 0 °C. The experiment was conducted from of March 21st to May 2nd 2007. Fish were fed twice a week as described above. Following the acclimation period, two tanks were food deprived for six weeks and two tanks continued to be fed at the above-described conditions. Five flounder were sampled from each tank two, four and six weeks after the start of the experiment (for a total of 20 animals per sampling day).

Summer experiment

Thirty-six flounder (average weight of 446.9 ± 12.8 g) were divided among four tanks (eight fish per tank). The experiment ran from August 1st to August 29th 2007. The average water temperature was 11.9°C. As fish were more active than in the winter, they were fed three times a week as opposed to twice a week for the winter experiment. After a two-week acclimation period, two tanks were food deprived for four weeks and two tanks continued to be fed as described above. Two to five flounder were sampled from each tank two and four weeks after the start of the experiment, for a total of 24 animals. As winter flounder move off shore during the summer, thus reducing the number of animals available for collection, only 2 samplings could be performed in the summer experiment (as opposed to 3 samplings in the winter).

RNA extraction

For cloning and tissue distribution studies 4 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 flatfish (Evans, 1937). For gene expression studies experimental fish were dissected to obtain hypothalamus and gut (adjacent to the pyloric caeca) tissue. Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada) and killed by spinal section. Tissues were dissected and immediately placed on ice in RNA*later* (Qiagen Inc., Mississauga, Ontario, 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, Ontario, 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.

Cloning of cDNA

Two micrograms of total RNA were subjected to reverse transcription into cDNA with a dT-adapter primer (see Supplementary Table 1) using M-MLV Reverse Transcriptase (New England Biolabs, Pickering, Ontario, Canada). Fragments of the unknown sequences were initially obtained by performing PCR amplifications using degenerate primers designed in regions of high identity among fish and various vertebrate sequences and the above cDNA. 0.5 μg of cDNA was used for each PCR. The annealing temperature was optimized for each primer set. The PCR reactions were carried out in a volume of 25 μl consisting of 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, and 1 U of Taq polymerase (Sigma, St Louis, Missouri, USA). A negative control was included for each primer set by omitting cDNA from the PCR reaction. The PCR products were electrophoresed in a 1% agarose gel and bands of predicted size were isolated and purified with a GenElute Gel Extraction Kit (Sigma, Oakville, Ontario, Canada), ligated into the pGEM easy vector using the pGEMeasy vector system (Promega, Madison, Wisconsin, USA) and sequenced by the MOBIX Lab (McMaster University, Ontario, Canada).

In order to clone winter flounder NPY, a small fragment of the unknown sequence was isolated by performing PCR amplifications using degenerate forward and reverse primers (dNPY-F, and -R, see Supplementary Table 1). Following sequencing of this short fragment, 3′ Rapid Amplification of CDNA Ends (3′RACE) using gene specific primers was conducted. Briefly, brain mRNA was subjected to reverse transcription and the cDNA submitted to two rounds of nested PCRs, using 3′RC-NPY1 and dT-AP, and 3′RC-NPY2 and AP (see Supplementary Table 1). The PCR products were 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 by 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, Ontario, 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. The PCR product was purified, cloned and sequenced as described previously. In order to isolate flounder CART, an initial fragment was obtained using 3′RACE and degenerate primers (dT-AP and 3′R-CART1 and AP and 3′R-CART2, see Supplementary Table 1) in two rounds of nested PCR. 5′RACE was then conducted as described above with primers 5′RC-CART1, 5′RC-CART2 and 5′ RC-CART3. Flounder CCK was isolated as described for winter flounder NPY (with CCK specific primers, see Supplementary Table 1).

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. 2 μg of RNA was reverse transcribed with dT-adapter primer using M-MLV Reverse Transcriptase (New England Biolabs). NPY, CART and CCK were amplified using gene specific primers (see Supplementary Table 1) designed based on our cloned sequences. PCR products were run on a 1% agarose gel and visualized using the 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). Elongation factor-1 alpha (EF-1α) was used as a control gene. Primers were designed based on winter flounder EF-1α (GenBank accession number AW013637, see Supplementary Table 1). Bands amplified with EF-1α were cloned and sequenced in order to verify their nucleotide sequence.

Quantitative real-time RT-PCR

Total RNA was reverse transcribed to cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Mississauga, Ontario, Canada), according to the manufacturer's protocol. Reverse transcription products were then diluted 1:3 in water and subjected qPCR using specific primers (see Supplementary 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 and samples were run in duplicate. In all cases, a "no template" negative control in which cDNAs were replaced by water was included. Initial validation experiments were conducted to determine optimal primer annealing temperatures and to ensure that PCRs were highly specific and reproducible ($0.98 > R^2 > 1.02$) and that all primer pairs had equivalent PCR efficiencies. Amplification, dissociation curves and gene expression analyses were performed using the Realplex1.5 software (Eppendorf). The relative Ct (ΔΔCt) method was used to quantify expression. Briefly, the fold change of each target gene was normalized to the housekeeping gene (*EF1-α*), and expressed relative to a calibrator sample from the control group (fed fish at the 2 week sampling). The average fold expression of sample from the control group was set at 100% and the expression levels of all the other groups were expressed as a percentage relative to a control group. The reference gene *EF1-α* was tested to verify that starvation did not affect its expression levels in either hypothalamus or gut, as demonstrated by similar Ct (cycle threshold) values between fed and starved fish.

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/).

Statistics

Feeding levels and gene expression levels between summer and winter animals were compared using Student's *t*-tests. For starvation experiments, gene expression levels were compared using one-way ANOVAs followed by Student–Newman–Keuls multiple comparison tests. Expression levels were expressed as a percentage relative to a control group, which was set at 100%. Significance was set at $p<0.05$. All tests were conducted using InStat 3.0 (GraphPad Software, San Diego, CA).

Results

Structure of winter flounder NPY, CART and CCK

The winter flounder NPY sequence is 398 bp long (GenBank Accession number EU684053) with a 338 base pairs (bp) 5′ untranslated (UTR) region and a 60 bp 3′UTR (see Supplementary Fig. 1). The open reading frame encodes for a 99 amino acids preproNPY. Based on comparisons with other fish NPY sequences, winter flounder NPY likely has four putative exons that are separated by three introns located after nucleotides 60, 251, and 337.

The sequence for winter flounder CART has a 396 bp (GenBank Accession number FJ379291) coding sequence with an 82 bp 5′ UTR and a 40 bp 3′UTR (see Supplementary Fig. 2). The open reading frame encodes a 91 amino acids preproCART. Based on

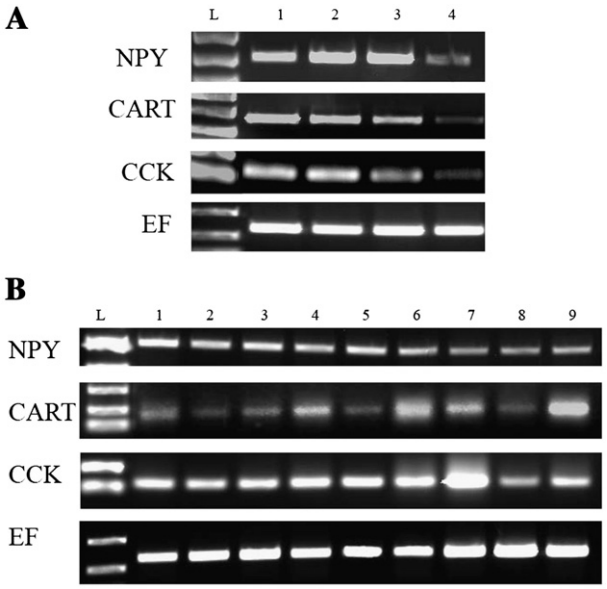

Fig. 1. A. RT-PCR distribution of NPY (300 bp), CART (123 bp), CCK (87 bp) and EF (201 bp) in different brain regions of the winter flounder. L, PCR maker; 1, hypothalamus; 2, telencephalon; 3, optic tectum/thalamus; 4, cerebellum. B. RT-PCR distribution of NPY (300 bp), CART (123 bp), CCK (87 bp) and EF (201 bp) in different peripheral tissues of the winter flounder. L, PCR maker; 1, gill; 2, heart; 3, stomach; 4, gut; 5, spleen; 6, liver; 7, kidney; 8, muscle; 9, gonad. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide.

comparisons with other CART sequences, flounder CART likely has three exons that are separated by two introns located after nucleotides 121 and 200.

The sequence for winter flounder CCK is 469 bp (GenBank Accession number EU684055) and includes a 56 bp 5′UTR (see Supplementary Fig. 3). The open reading frame encodes for a 130 amino acids preproCCK. Based on comparisons with other CCK sequences, flounder CCK has two putative exons that are divided by one intron located after nucleotide 323.

The amino acid sequences were aligned with sequences from other fish species and with one mammalian sequence. Winter flounder NPY has 53 to 96% amino acid similarity to NPY from other fish species, with highest similarity (96%) to the bastard halibut and orange spotted grouper (see Supplementary Fig. 4). Winter flounder CART has 65 to 84% amino acid similarity to other fish species, with highest similarities to Atlantic cod and goldfish II (see Supplementary Fig. 5). Winter flounder CCK shows an amino acid similarity ranging from 38% to 91%, with the highest similarity to halibut CCK (see Supplementary Fig. 6).

Tissue distribution

Reverse transcription PCR (RT-PCR) was used to amplify NPY, CART and CCK in different brain regions as well as in several peripheral tissues of winter flounder (Fig. 1). Fragments of 300 bp, 123 bp and 87 bp were amplified for NPY, CART and CCK, respectively. No expression was detected in any control amplification (without DNA template), verifying the absence of contamination. All samples were amplified with EF-1α and produced a band of expected size (201 bp).

Within the brain, NPY, CART and CCK expressions were detected in all regions examined (Fig. 1A). Expression levels appeared to be lower in the cerebellum compared to the other brain regions on the basis of visual intensity (Fig. 1A). NPY, CART and CCK expressions were detected in all peripheral tissues examined (Fig. 1B). CART expression appeared to be higher in the gill, gut, liver, kidney and gonad compared to the other tissues. No apparent differences in expression between tissues could be detected for either CCK or NPY.

As our tissue distribution studies show that NPY and CART are highly expressed in the hypothalamus and that CCK is highly expressed in the gut and as hypothalamic peptides and gut CCK has previously been shown to have a major role in the regulation of feeding of both mammals (Shioda et al., 2008) and fish (Nelson and Sheridan, 2006; Volkoff and Peter, 2006), we chose these tissues to perform our subsequent gene expression studies. As the distribution of CCK-immunoreactive (CCK-IR) cells has been shown to be mostly on the anterior intestine and pyloric caeca in Japanese flounder and halibut (Rønnestad, 2002), the portion of the intestine adjacent to the pyloric caeca was used for CCK expression studies.

Effects of season on food intake, size and GSI

Flounder consumed an average of 2.12 ± 0.2 g of food per fish per feeding (or 5.8 mg/g ± 0.5 g of fish per day) during the winter (0 °C) and an average of 12.7 ± 0.89 g of food per fish per feeding (or 28.4 mg/g ± 1.1 g of fish per day) during the summer (11 °C), which was significantly higher compared to the winter experiment.

The average weight of fish was similar in winter (365 ± 16 g) and in summer (446.9 ± 12.8 g). The average GSI for males was significantly lower in the summer (0.47 ± 0.02%) than in the winter (4.8 ± 0.4%). The average GSI for females was significantly lower in the summer (1.08 ± 0.2%) than in the winter (8.7 ± 0.8).

Effects of season on gene expression

NPY expression levels in the winter flounder hypothalamus were significantly higher in the summer experiment compared to the winter experiment (Fig. 2A). There were no differences in

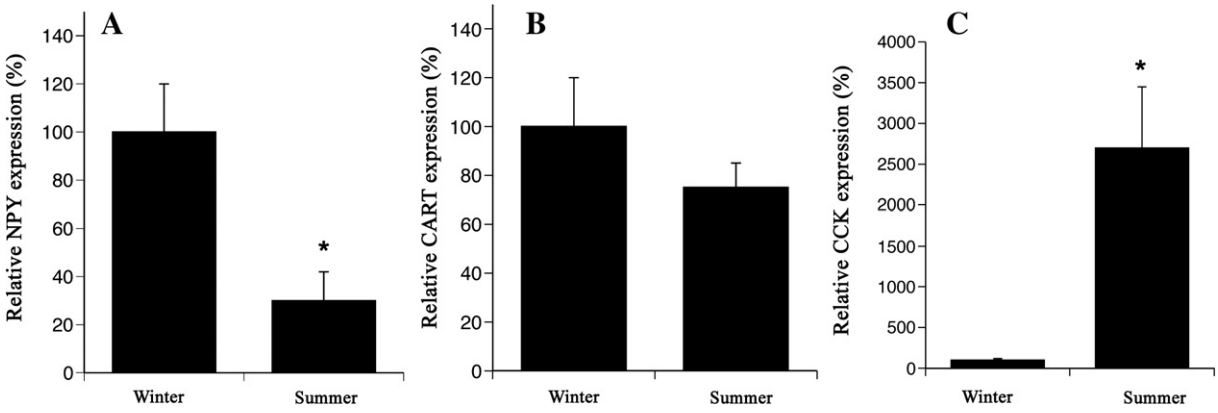

Fig. 2. Hypothalamic NPY, hypothalamic CART and gut CCK mRNA expressions in fed winter flounder collected at four weeks in the summer ($n = 10$ fish per group) and in the winter ($n = 5$–6 fish per group) experiments. Expression levels are expressed as a percentage normalized to the control (fed) group. Data is presented as mean ± SEM. A star (*) indicates significant differences between groups ($p < 0.05$).

hypothalamic CART expression between summer and winter (Fig. 2B). CCK gut mRNA expression levels were higher in summer than in winter (Fig. 2C).

Effects of fasting on gene expression during the winter

There were no significant differences in NPY expression in the hypothalamus of flounder between fed fish and fasted at either two, four or six weeks of fasting (Fig. 3A). There were no significant differences between NPY levels at 2, 4 or 6 weeks for either fed or fasted fish although levels tended to decrease in fed fish. There were

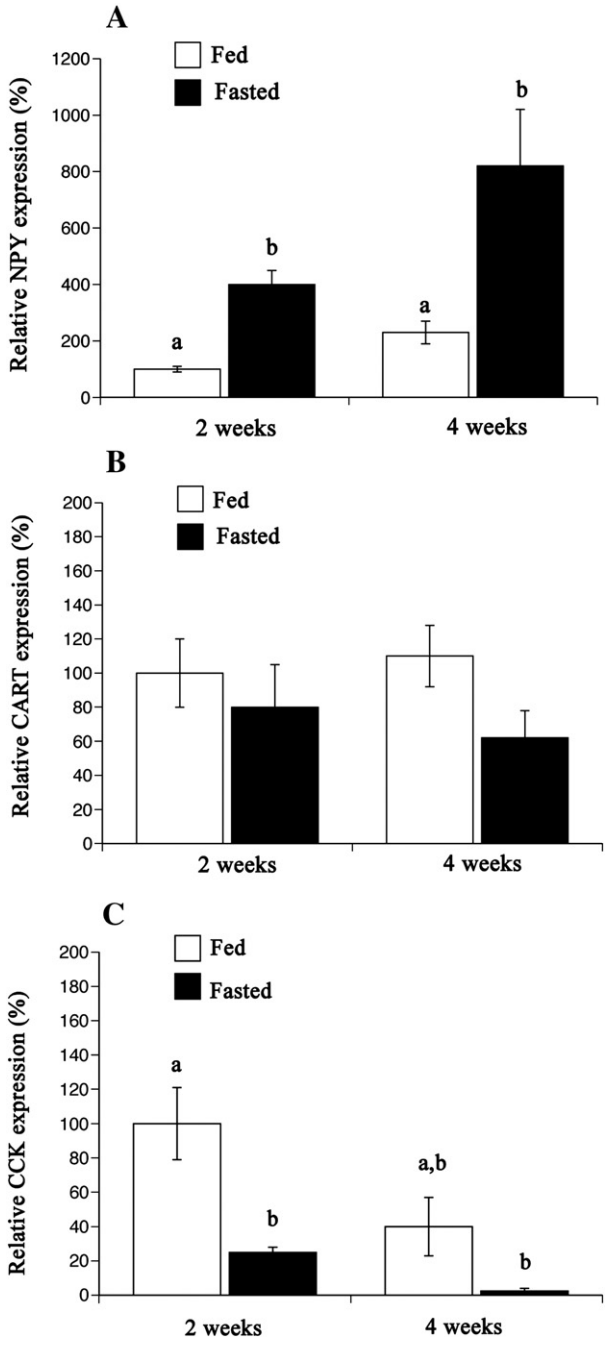

Fig. 3. Effects of fasting on hypothalamic NPY (A), hypothalamic CART (B) and gut CCK (C) mRNA expression levels during the summer experiment. 5–6 fish were sampled from both fed and starved groups at each collection. Expression levels are expressed as a percentage normalized to the control group (fed fish at the 2 week sampling), which was set at 100%. Data is presented as mean ± SEM. Different superscript letters indicate groups that are significantly different ($p<0.05$).

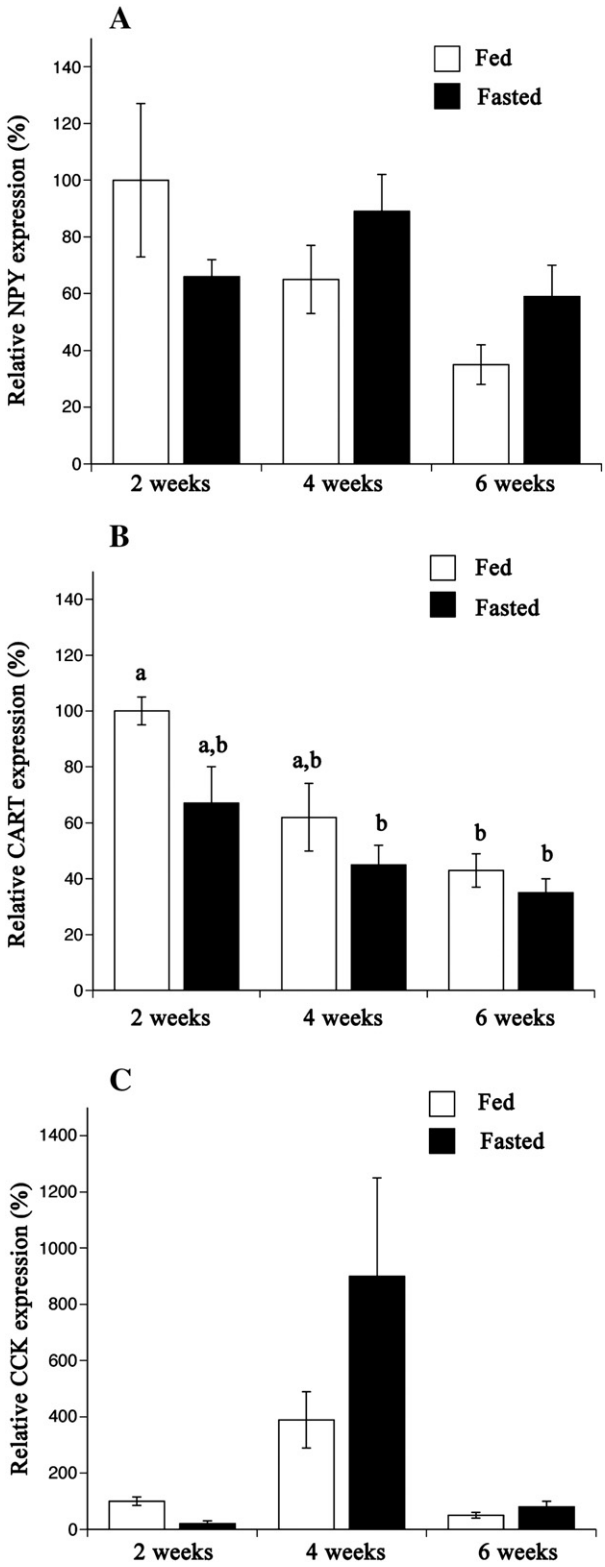

Fig. 4. Effects of fasting on hypothalamic NPY (A), hypothalamic CART (B) and gut CCK (C) mRNA expression levels during the winter experiment. Ten fish were sampled from both fed and starved groups at each collection. Expression levels are expressed as a percentage normalized to the control group (fed fish at the 2 week sampling), which was set at 100%. Data is presented as mean ± SEM. Stars indicate groups that are significantly different ($p<0.05$).

no significant differences in CART expression in the hypothalamus of flounder between fed fish and starved at either two, four or six weeks of starvation (Fig. 3B). Within the fed group, CART expression levels

were significantly higher at two weeks compared to fed fish at 6 weeks. There were no significant differences in CCK expression levels in the gut of flounder both between fed and starved fish, or between collection dates (Fig. 3C).

Effects of fasting on gene expression in the summer

At both two and four weeks of fasting, NPY expression in the hypothalamus was significantly higher in fasted fish compared to fed fish (Fig. 4A). In both fed and fasted fish, NPY expression levels were similar at two and four weeks. There were no significant changes in CART mRNA expression in the hypothalamus between the fed and starved groups at either two or four weeks of starvation or between both collections (Fig. 4B). CCK gut mRNA levels were lower in fasted fish than in fed fish at two weeks but were similar in fed and fasted fish at four weeks. For both fed and fasted fish, there were no significant differences in CCK expression between two and four weeks (Fig. 4C).

Discussion

The main objective of our study was to assess whether NPY, CART and CCK mRNA expressions were influenced by fasting and by season in winter flounder. As sequences were not available for this species, we initially cloned cDNAs encoding these three peptides and examined their tissue distribution. The amino acid sequences of winter flounder NPY, CART and CCK all show a relatively high degree of homology with sequences from other fish (from 38 to 96%), with highest sequence similarity with another flatfish, the bastard halibut (96% for NPY and 91% for CCK). Overall, the relatively well conserved amino acid structure of the precursors and the mature peptides between fish species suggests that the physiological function of these hormones may also be conserved.

In order to assess where these peptides were expressed, mRNA expression levels were examined in four brain regions and in eight peripheral tissues. Within the brain, NPY, CART and CCK mRNAs all appear to be highly expressed in forebrain (hypothalamus, telencephalon) and midbrain (optic tectum/thalamus) with lower levels in the cerebellum. High NPY expression levels in the forebrain have previously been reported in other fish species including salmon (Silverstein et al., 1998), sea bass (Cerda-Reverter et al., 2000) and cod (Kehoe and Volkoff, 2007) and are consistent with a role of NPY in the regulation of feeding (Narnaware and Peter, 2002). CART (Kehoe and Volkoff, 2007; Volkoff and Peter, 2001) and CCK (Kurokawa et al., 2003; Murashita et al., 2006; Peyon et al., 1998) mRNA expressions have all also been shown in forebrain and midbrain of other fish species. NPY, CART and CCK mRNA expressions were detected in several peripheral tissues with relatively high levels in gut and gonad. High NPY mRNA expression levels in the gastrointestinal tract have been reported for several fish including cod (Kehoe and Volkoff, 2007) and goldfish (Peng et al., 1994) and CCK mRNA is found in stomach, pyloric caeca and intestine of yellowtail (Murashita et al., 2006) and in trout stomach and intestine (Jensen et al., 2001). However, none of the fish species examined to date express CART in their gut (Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Volkoff and Peter, 2001). Similarly, CART mRNA has never been detected in the gastrointestinal tract of mammals, although CART peptides have been detected in rat gut (Couceyro et al., 1998; Kuhar and Yoho, 1999). This study is the first to report the presence of CART mRNA in the gut of any vertebrate and suggests that CART might act as a brain–gut peptide in flounder. The expression of NPY (Gaikwad et al., 2005; Leonard et al., 2001; Peng et al., 1994), CART (Kehoe and Volkoff, 2007; Kobayashi et al., 2008) and CCK (Peyon et al., 1998) has previously been reported in fish gonads and suggests a role for these peptides in the regulation of reproductive processes in fish.

Significant differences in food consumption and GSI were found between fish in summer and in winter. Our results showing lower food intake and higher GSIs in the winter are consistent with previous reports on captive and wild winter flounder (Burton and Idler, 1984; Kennedy and Steele, 1971; Stoner et al., 1999). A number of fish species have also been shown to display both decreased growth rates and food consumption in colder water compared to warmer water (Kehoe and Volkoff, 2007; Martell and McClelland, 1994; Meise et al., 2003; Stoner et al., 1999). Winter flounder in the winter also showed very little swimming activity compared to animals held in the summer (MacDonald, personal observation). It is noteworthy that during the winter, winter flounder produce antifreeze proteins to protect themselves from freezing (Fletcher, 1981; Fletcher et al., 2001; Gauthier et al., 2005). As metabolite levels have been shown to affect feeding in fish (Banos et al., 1998), it is possible that high antifreeze protein levels affect feeding and appetite-related hormonal systems in flounder.

In order to examine the effects of season on gene expression, we compared the expressions of NPY, CART and CCK in fed animals in winter and summer. NPY mRNA expression was significantly lower in the summer compared to the winter. As food consumption is higher in the summer compared to the winter and NPY has been shown to be an orexigenic peptide in fish, one would have expected higher NPY expression levels in summer animals. High NPY expression levels in the winter might be indicative of a stimulation of appetite-related NPY pathways in the brain by an empty gut and a down-regulation on NPY receptors within the brain. In contrast to NPY, CART mRNA expression levels in the hypothalamus were similar in winter and summer. As CART mRNA is also detected in the telencephalon of flounder (this study) as in other fish including cod, catfish and goldfish (Kehoe and Volkoff, 2007; Leonard et al., 2001; Narnaware et al., 2000), it is possible that variations in CART expression occur in this region rather than the hypothalamus. It is also possible that another CART form exists in winter flounder that is more sensitive to seasonal changes. Indeed, in goldfish two forms of CART respond differently to starvation, CART I being more sensitive than CART II (Volkoff and Peter, 2001). Finally, it is also possible that CART may not be affected by seasonal changes or have a major role in the regulation of feeding of winter flounder. CCK gut mRNA expression levels were higher in summer than in winter. Given a higher food consumption in the summer and the putative anorexigenic role of CCK in fish, higher levels of CCK in the fed fish in the summer were expected. Interestingly, in wild coho salmon, in which appetite is also reduced during winter, higher levels of CCK were detected in the gut and in the telencephalon in winter compared to summer (Lohmus et al., 2008).

Given the seasonal differences in expression in some of these peptides, we examined the effects of fasting on the expression of NPY, CART and CCK in both summer and winter. NPY hypothalamic expression levels responded differently to starvation in the winter and in the summer. Whereas in the winter, no significant differences in NPY expression could be detected between starved and fed fish, in the summer, NPY expression was higher in starved animals compared to the fed animals at both two and four weeks of starvation. Previous studies in salmon and goldfish report an increase in NPY expression in the brain of starved fish compared to fed fish (Narnaware and Peter, 2001; Narnaware et al., 2000; Silverstein et al., 1998), which supports a role for NPY as an appetite-regulating peptide in fish. In addition, in goldfish, mRNA expression levels display periprandial variations, with high expression levels in the forebrain before feeding (Narnaware et al., 2000) and are regulated by diet composition (Narnaware and Peter, 2002). In cod, however, forebrain NPY mRNA expression does not appear to change in response to starvation (Kehoe and Volkoff, 2007). Similar to cod, winter flounder is capable of withstanding long periods of fasting in the wild. The lack of changes in NPY expression during the winter months could be indicative of this feeding adaptation. A longer period of fasting might be necessary to induce changes in NPY expression. As winter flounder undergo a dormancy-like phase in the colder winter months where their movement and

food consumption decrease (Martell and McClelland, 1994; Meise et al., 2003; Stoner et al., 1999), the lack of effects of fasting on NPY expression might be indicative of a general "shutdown" of the NPY system during dormancy.

Hypothalamic CART mRNA expression was not affected by starvation in either summer or winter. In the summer, CART mRNA tended to be lower in the starved group compared to the fed group after four weeks of starvation, but this decrease was not significant. In goldfish, cod and catfish (Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Volkoff and Peter, 2001), brain CART mRNA expression levels decrease following starvation. In our study, CART mRNA expression in the hypothalamus was relatively low and it is possible that differences in expression levels were too small to be detected. As stated previously, it is also possible that CART in other brain regions might be more sensitive to starvation or that another CART form exists in flounder that is more sensitive to starvation. Interestingly, CART mRNA levels in fed winter fish decreased from week 2 to week 6. The reasons for this decline are unclear. As CART mRNA is present in the ovary and CART has been implicated in reproduction (Kehoe and Volkoff, 2007; Kobayashi et al., 2004; Volkoff and Peter, 2001), and as flounder spawn in late winter or early spring (Scott et al., 1988), a decrease in CART levels might be related to reproductive cycles, in particular to spawning.

Similar to NPY, CCK expression levels in the gut displayed seasonal differences in responses to fasting. Whereas there were no significant changes in expression in the winter, CCK levels in the summer were lower in fasted fish than in fed fish after 2 weeks of starvation and tended to be lower after 4 weeks. CCK has previously been shown to regulate digestive processes (Aldman et al., 1989; Forgan and Forster, 2007; Honkanen et al., 1988; Olsson et al., 1999) and to act as a satiety factor (Himick and Peter, 1994; Volkoff et al., 2003) in fish. Our results for the summer experiment are in line with previous studies showing that gut CCK expression levels decrease following fasting in several fish species, including yellowtail, dogfish and rainbow trout (Aldman et al., 1989; Murashita et al., 2006; Olsson et al., 1999) and that during the fasting period, the intestinal mucosa of winter flounder exhibits a reduction in the height and number of the folds and a change in their shape (Mcleese and Moon, 1989). This data suggests that CCK is involved in the regulation of digestive processes and feeding in winter flounder. The very low expression levels of CCK during the winter months made quantification difficult and might have masked any effects of fasting on CCK expression levels.

In summary cDNAs encoding for NPY, CART and CCK were cloned in winter flounder and their mRNA expression shown to have a widespread distribution in peripheral tissues and within the brain. Our results show that NPY and CCK, but not CART, show seasonal differences in expression with higher hypothalamic and lower gut CCK expression levels in the winter, which corresponds to a natural fasting period for winter flounder. None of the peptides examined was affected by fasting in the winter. In the summer, fasting did not affect hypothalamic CART expression levels but induced an increase in hypothalamic NPY expression levels and a decrease in gut CCK levels, which is consistent with the orexigenic and anorexigenic roles for NPY and CCK, respectively. Our results suggest that NPY and CCK, but maybe not CART, have a major role in the regulation of feeding in winter flounder and might contribute to the seasonal fluctuations in appetite in this species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2009.03.002.

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