ISOLATION OF cDNAs AND GENE EXPRESSION OF THREE FEEDING-RELATED NEUROPEPTIDES, NEUROPEPITDE Y (NPY), COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) AND OREXIN, IN ATLANTIC COD (GADUS MORHUA)

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ISOLATION OF cDNAs AND GENE EXPRESSION OF THREE FEEDING-RELATED NEUROPEPTIDES, NEUROPEPITDE Y (NPY), COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) AND OREXIN, IN ATLANTIC COD (*GADUS MORHUA*)

by

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Abstract

Our understanding of the regulation of food intake in fish has evolved from experiments in the 1970's that identified feeding centres of the fish brain, to recent ones where the molecular cloning of central and peripheral factors regulating appetite is performed. In fish, food intake is regulated by the hypothalamus where neuropeptides controlling feeding are produced. Many neuropeptides have been implicated in the regulation of food intake in fish. In this study, we identified complete cDNAs encoding Neuropeptide Y (NPY) and Cocaine and amphetamine regulated transcript (CART) and a 348 bp partial cDNA of orexin from Atlantic cod (*Gadus morhua*) brain. There is a high degree of identity between the predicted amino acids of Atlantic cod NPY and CART and vertebrate homologs, and we found, using RT-PCR, that NPY and CART mRNA is localized not only in the brain (including the hypothalamus) but also in peripheral tissues. For example, both NPY and CART mRNA are expressed in the ovary, and NPY mRNA is also expressed in the gut, kidney and heart. We examined NPY and CART mRNA expression during a daily feeding period and following food deprivation. Peri-prandial variations were seen in both NPY and CART expression, and CART was affected by fasting. Finally, we examined the effects of environmental temperature on the control of feeding by examining NPY and CART mRNA expression in fish acclimated to 2°C, 5°C, 11°C and 15°C. Our results suggest that both NPY and CART are involved the control of food intake in cod since they are strongly expressed in the hypothalamus, an area of the brain regulating feeding, and they respond to feeding. Further, CART mRNA expression appears to be regulated by temperature whereas NPY does not.

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List of Abbreviations

A	adenine
aa	amino acids
Arc	arcuate nucleus
BBB	blood brain barrier
bp	base pairs
C	cytosine
CART	Cocaine and amphetamine regulated transcript
cDNA	complementary DNA
CPON	carboxy terminal peptide of NPY
dNTP	deoxynucleotides
DMH	dorsomedial hypothalamus
G	guanine
ICV	intracerebroventricular
InsRB	brain insulin receptor
K	thymine or guanine
LHA	lateral hypothalamic area
N	any nucleotide
NPY	Neuropeptide Y
ODNs	oligodeoxynucleotides
OX1R	orexin receptor 1
OX2R	orexin receptor 2

PCR	polymerase chain reaction
PP	pancreatic peptide PP
PVN	paraventricular nucleus
PY	pancreatic peptide Y
PYY	peptide YY
R	purine
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcription polymerase chain reactions
T	thymine
UTR	untranslated region
VHM	ventromedial hypothalamus
W	adenine or thymine
Y	pyrimidine

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1.0 Introduction

1.1 Introduction

Energy homeostasis requires a balance between energy intake and energy expenditure in order to maintain a stable body weight. Energy intake is achieved through feeding, and the hypothalamus, a specialized region of the brain, is the primary site regulating food intake. The Dual Centre Model, established in the 1940's, proposed that specific regions of the hypothalamus controlled food intake. Since the introduction of the Dual Centre Model, other models have been proposed, including the lipostatic model which states that adiposity signals that negatively regulate food intake. The coordinated effort to maintain a constant body weight involves signals creating the motivational drive towards an energy source (hunger signals) and signals terminating these feeding impulses (satiety signals). The identification of hypothalamic neuropeptides controlling food intake has greatly expanded our understanding of the neuroendocrine framework regulating feeding. These neuropeptides consist of orexigenic peptides, which stimulate food intake and anorexigenic peptides, which repress feeding. Neuropeptide Y (NPY) was one of the first discovered orexigenic neuropeptides and has been extensively studied in mammals. In the past ten years, more feeding-related neuropeptides have been discovered, including a group of orexigenic peptides, the orexins, and a potent anorexigenic factor, cocaine and amphetamine regulated transcript (CART). However, due to their recent discovery, orexin and CART have not yet been fully characterized, and information on the cellular and molecular regulation of these peptides is still limited.

Homologues of mammalian neuropeptides have been identified in many species of fish, but their role has not yet been characterized. Studying the neuroendocrine regulation of food intake might provide some important information. Firstly, it could contribute to our overall understanding of feeding behaviour and homeostatic mechanisms. Secondly, comparisons of neuropeptide structure and function between fish and mammals might provide insights into the dynamics of evolution. Thirdly, fish may possibly be developed as models to study human health problems involving weight disorders. Finally, since many fish are commercially important, neuropeptides could be selected as genetic markers to screen for optimal feeding and growth phenotypes.

1.2 Dual Centre Model

The Dual Centre Model, proposed in the 1940's, outlined the anatomical localization of discrete regions within the mammalian hypothalamus that regulate food intake. Lesions and electrical stimulation to specific regions of the hypothalamus showed that the ventromedial hypothalamus (VMH) is the "satiety centre" and the lateral hypothalamic area (LHA) is the "feeding centre" (Brobeck, 1946; Hetherington and Ranson, 1940). This model was supported by studies showing that appetite and food intake are increased by lesions in the VMH (Albert *et al.*, 1971; Bernardis, 1973; Tokunaga *et al.*, 1986), and decreased by lesions in the LHA (Van den Pol, 1982). Later studies, performing bilateral cuts between the VMH and LHA provided evidence that direct contacts are made between these two regions. When contacts between the VMH an LHA are severed, thereby preventing direct communication, food intake increases (Albert

et al., Storlien, 1969; Albert *et al.*, 1971). Based upon these results, it was suggested that the VMH represses the activity of the LHA and consequently inhibits feeding.

The Dual Centre Model, however, is now recognized as an incomplete model as it does not accurately represent the complexity of the neuroendocrine regulation of feeding. The use of bilateral cuts through the hypothalamus by Albert *et al.* (1971) provided evidence that the VMH and the LHA are not the exclusive centres of the hypothalamus regulating food intake. Severing the fibers between the VMH and the LHA does not increase food intake to the same extent as complete lesion of the VMH, and cuts between the VMH and other regions of the hypothalamus also increase food intake. The VHM appears to communicate with other regions of the brain in order to control food intake, and the LHA must receive information beyond the VMH. Subsequently, several other hypothalamic regions involved in the regulation of food intake have been identified, including the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH) and arcuate nucleus (Arc) (Kalra *et al.*, 1999). These specific regions of the hypothalamus synthesize the neuropeptides that control food intake.

In fish, several experiments using electrical stimulation of specific brain regions have suggested that the hypothalamus is the primary feeding centre of the brain (Peter, 1979). Electrical stimulation of the inferior lobes of the hypothalamus from the bluegill sunfish (*Lepomis macrochirus*), cichlid (*Tilapia heudelotti macrocephala*), nurse shark (*Ginglymostoma cirratum*), and goldfish (*Carrasius auratus*) evoke feeding behaviours and increase food intake (Demski, 1973; Demski, 1977; Demski and Knigge, 1971; Savage and Roberts, 1975). In fish, the telencephalon also appears to be involved in the regulation of food intake since electrical stimulation of the telencephalon in cichlids and

goldfish induces feeding behaviours (Demski, 1973; Grimm, 1960). This suggests that in fish, both the hypothalamus and the telencephalon are involved in regulating food intake.

1.3 Lipostatic Model

The lipostatic model, developed in the 1950's, proposed that adiposity factors circulate in the body in proportion to fat stores and act within the brain to decrease food intake (Kennedy, 1953). In mammals, insulin and leptin are the two adiposity signals in the lipostatic model (Schwartz *et al.*, 2000). These factors meet the criteria for true adiposity signals: their levels in the blood are in proportion of body fat, they are able to enter the brain via the blood brain barrier (BBB), their administration decreases food intake and body weight, and deficiency in their signal causes increased food intake and body weight. Within the brain, insulin has been shown to interact with NPY pathways, while leptin appears to interact with NPY, orexin and CART pathways.

There is support for the lipostatic model in fish since there is a negative correlation between adipose stores and food intake. Fish with a higher percent body fat, induced by diet manipulation, consume less food than fish with a lower percent body fat (Johansen *et al.*, 2002; Ogata and Shearer, 2000; Shearer *et al.*, 1997; Yamamoto *et al.*, 2002), and eventually differences in body composition and food intake are abolished. These results suggest that fish adjust food intake in response to fat stores. In Atlantic salmon, diet-induced "fat" fish have a slower growth rate which appears a result of the decrease in food intake (Jobling *et al.*, 2002; Johansen *et al.*, 2002).

Information on the role of insulin and leptin as regulators of food intake in fish is still limited. In coho salmon, circulating insulin levels are regulated by food availability

(Silverstein *et al.*, 1998). There is conflicting information on the anorexigenic effects of insulin in fish. Central injections of insulin decrease food intake in rainbow trout (Soengas *et al.*, Aldegunde, 2004) but have no effect on food intake in channel catfish (Silverstein *et al.*, Plisetskaya, 2000). In fish, there is evidence for the existence of a leptin-like molecule. A cDNA encoding a putative leptin-like molecule has recently been identified from the pufferfish (Kurokawa *et al.*, 2005), but the biological activity of this putative protein product has not yet been investigated. Earlier immunoreactivity and western blot studies have shown that leptin-like peptides are expressed in several species of fish including green sunfish (*Lepomis cyanellus*), largemouth bass (*Micropterus salmoides*), channel catfish and rainbow trout (Johnson *et al.*, 2000). Central injections of murine leptin decrease food intake in goldfish (Volkoff *et al.*, 2003), and increase fat metabolism in green sunfish (Londraville and Duvall, 2002). However, leptin injections do not affect food intake in salmon (Baker *et al.*, 2000), green sunfish (Londraville and Duvall, 2002) or catfish (Silverstein and Plisetskaya, 2000).

1.4 Neuropeptides

The goal of this study was to isolate and characterize the feeding-related neuropeptides NPY, orexin and CART from Atlantic cod (*Gadus morhua*). The following section describes these three neuropeptides as well as interactions between them.

1.4.1 Neuropeptide Y (NPY)

The highly conserved Neuropeptide Y (NPY) is a 36 amino acid neuropeptide belonging to the pancreatic polypeptide family. First isolated from porcine brain

(Tatemoto *et al.*, 1982), NPY has been studied extensively in mammals for its stimulatory effects on food intake. NPY has been identified from mammals, birds, amphibians and fish (Cerda-Reverter *et al.*, Larhammar, 2000). One of the key physiological roles of NPY is the regulation of long-term energy homeostasis during negative energy balance.

In mammals, NPY is widely expressed in peripheral tissues and the CNS, where it is the most abundant peptide (Dumont *et al.*, 1992). The highest levels of NPY mRNA expression occur within the hypothalamus. The primary site of NPY expression within the hypothalamus is the Arc where NPY co-localizes with Agouti-related peptide (AgRP) another feeding-related peptide (Broberger *et al.*, 1998; Hahn *et al.*, 1998). There are extensive NPY/AgRP projections from the Arc to the PVN (Baker and Herkenham, 1995; Broberger *et al.*, 1999), suggesting that the PVN is an important target for NPY neurons.

The first study to localize NPY expression in the brain of a non-mammalian species was performed in goldfish, in which NPY mRNA expression was localized within the forebrain, primarily in the telencephalon (Peng *et al.*, 1994). Subsequently, NPY mRNA expression has been shown to be localized within the forebrain of several fish species including chinook salmon, *Oncorhynchus tshawytscha*, and coho salmon, *Oncorhynchus kisutch* (Silverstein *et al.*, 1998), sea bass, *Dicentrarchus labrax* (Cerda-Reverter *et al.*, 2000), zebrafish, *Danio rerio* (Soderberg *et al.*, 2000) and channel catfish, *Ictalurus punctatus* (Leonard *et al.*, 2001). NPY immunoreactive sites are also localized primarily in the forebrain of fish including rainbow trout, *Oncorhynchus mykiss* (Danger *et al.*, 1991), catfish, *Clarias batrachus* (Gaikwad *et al.*, 2004), killifish, *Fundulus heteroclitus* (Subhedar *et al.*, 1996), and carp, *Cyprinus carpio* (Pirone *et al.*, 2003). In the fish brain, NPY is undetectable in the cerebellum and medulla (Leonard *et al.*, 2001;

Peng *et al.*, 1994; Pirone *et al.*, 2003). Overall, these anatomical studies have shown that NPY is expressed in areas of the brain associated with feeding.

Initial studies that performed intracerebroventricular (ICV) injections of NPY in rats showed that NPY acts within the brain to increase food intake (Clark *et al.*, 1985; Levine *et al.*, Morley, 1984). In rodents, chronic administration of NPY increases daily food intake and body weight (Stanley *et al.*, 1986), and central injections of NPY anti-sense oligodeoxyneucleotides (ODNs) inhibit normal and fasting-induced feeding (Akabayashi *et al.*, 1994; Hulsey *et al.*, 1995; Schaffhauser *et al.*, 1997). Injections of NPY in the PVN increase food intake and *c-fos* expression, an indicator of cellular activity (Stanley and Leibowitz, 1985; Yokosuka *et al.*, 2001), whereas injections of NPY antibodies into the PNV decrease food intake (Shibasaki *et al.*, 1993). Collectively, central injections of NPY in rodents decrease the latency of feeding initiation, increase meal size and increase meal duration (Gehlert, 1999). NPY also has an orexigenic effect in fish since central injections of NPY increase food intake in goldfish (Lopez-Patino *et al.*, 1999; Narnaware *et al.*, 2000) and channel catfish (Silverstein *et al.*, Plisetskaya, 2000). Hence, the orexigenic activity of NPY appears to be conserved between mammals and fish.

In addition to its short-term effects on feeding, NPY appears to also have a central role in long-term energy homeostasis, since its expression is elevated in states of negative energy balance. In rodents, fasting induces hyperphagia, elevates Arc NPY mRNA expression (Brady *et al.*, 1990; Davies and Marks, 1994) and increases NPY protein levels in both the Arc (Brady *et al.*, 1990; Davies and Marks, 1994; Jang and Romsos, 1998) and PVN (Beck *et al.*, 1990; Calza *et al.*, 1989; Sahu *et al.*, 1988).

Refeeding reverses the effects of food deprivation and returns NPY to normal levels within the Arc (Beck *et al.*, 1990). During fasting, the number of neurons expressing NPY mRNA in the Arc increases (Baskin *et al.*, 1999; Hahn *et al.*, 1998). This response to fasting is specific to the hypothalamus, as neurons in other regions of the brain including the hippocampus, thalamic reticular nucleus and cerebral cortex are not affected by fasting (Hahn *et al.*, 1998). In fish, food deprivation increases NPY mRNA in the hypothalamus of goldfish, coho salmon and chinook salmon (Narnaware *et al.*, 2000; Silverstein *et al.*, 1999; Silverstein *et al.*, 1998), and refeeding reverses these effects (Narnaware and Peter, 2001). These results support the role of NPY in the regulation of food intake in a state of negative energy balance in both fish and mammals.

Mammalian NPY is a ligand for members of the NPY-family of receptors which include Y1, Y2, Y4 and Y5 and Y6. The Y1 subfamily is the main subfamily within the NPY receptor family and includes Y1, Y4 and Y6. Further, receptor subtypes Y1 and Y5 appear to be the principal subtypes mediating the orexigenic effects of NPY. There is anatomical evidence for interactions between NPY and Y1 as NPY/AgRP neurons, projecting to the PVN, synapse with Y1-producing cells (Broberger *et al.*, 1999). In rodents, central injections with Y1 antagonists inhibit NPY-induced feeding (Kanatani *et al.*, 2001), but central injections of Y1 anti-sense ODNs do not affect NPY-induced feeding (Schaffhauser *et al.*, 1998), suggesting that Y1 may not be the key receptor responsible for controlling feeding and that other receptors may have a compensatory role in the absence of Y1. This is further supported by the fact that rodents deficient in Y1 do not display any major abnormalities in body weight and food intake but develop late-onset obesity (Kushi *et al.*, 1998; Pedrazzini *et al.*, 1998). The NPY receptor subtype Y5

was initially suggested to be a feeding-related receptor based in its high affinity for NPY (Hu *et al.*, 1996). Subsequent studies have shown that central injections with an NPY analog specific to Y5 increase food intake (Hwa *et al.*, 1999), and central injections with Y5 antagonists or Y5 anti-sense ODNs block NPY-induced feeding (Schaffhauser *et al.*, 1997; Tang-Christensen *et al.*, 1998; Yokosuka *et al.*, 2001), suggesting Y5 is involved in the regulation of food intake. Similar to Y1 knockouts, rodents lacking Y5 do not display any major abnormalities but develop late-onset obesity (Marsh *et al.*, 1998). In mammals, there is evidence that Y1 and Y5 are functionally connected in the regulation of food intake as central injections of Y1 antagonists to Y5 deficient mice block the appetite-stimulating effects of NPY (Marsh *et al.*, 1998). In humans, Y1 and Y5 are also structurally related, as their encoding genes have a common promoter and share common sequence indicating that these genes may be regulated in part by the same transcription factors (Herzog *et al.*, 1997). These data suggest that, in mammals, Y1 and Y5 appear to be feeding-related receptor subtypes.

NPY receptors have been identified in fish, most of which appear to be members of the Y1 subfamily. Three NPY receptor subtypes have been identified in zebrafish: zYa, zYb and zYc (Lundell *et al.*, 1997; Ringvall *et al.*, 1997; Starback *et al.*, 1999). Further, the Yb subform has been cloned from Atlantic cod and rainbow trout (Arvidsson *et al.*, 1998; Larson *et al.*, 2003), and three NPY receptors subtypes, *Squalus* Y1, Y4 and Y6, have been identified from the spiny dogfish (*Squalus acanthias*) (Salaneck *et al.*, 2003). One of the most ancestral fish, the European river lamprey *(Lampetra fluviatilis)* has one NPY receptor which is expressed in the CNS, liver and gonad (Salaneck *et al.*, 2001). Based on sequence analysis and pharmacological studies, fish NPY receptor subtypes

appear to be distinct members of the Y1 subfamily. In goldfish, central injections of non-specific mammalian NPY receptor antagonists block NPY-induced feeding (de Pedro *et al.*, 2000; Lopez-Patino *et al.*, 1999; Narnaware and Peter, 2001), however, it is still unclear which NPY receptor subtypes are involved in regulating food intake in fish.

NPY appears to affect diet choice, and NPY expression in the brain is affected by macronutrients. In rodents, injections of NPY into the PVN increase carbohydrate intake (Stanley and Leibowitz, 1985). Conversely, macronutrients appear to influence NPY expression since high carbohydrate diets increase NPY peptide expression in the PVN (Jhanwar-Uniyal *et al.*, 1993). Fat also influences NPY expression since high fat diets decrease NPY mRNA levels in both the Arc and PVN (Giraudo *et al.*, 1994; Stricker-Krongrad *et al.*, 1998). In goldfish, both carbohydrates and fats appear to influence NPY mRNA expression in the brain (Narnaware *et al.*, Peter, 2002; Preston *et al.*, 1998), suggesting that in fish as in mammals, NPY expression is affected by diet composition.

In addition to its action in the brain, NPY is expressed in peripheral tissues and regulates several physiological processes. In mammals, NPY is a brain-gut peptide that regulates blood flow to the gut and inhibits gastric emptying (Dumont *et al.*, 1992). In fish, NPY is involved in cardiovascular function since NPY induces a vascular response in elasmobranchs and Atlantic cod (Preston *et al.*, 1998; Shahbazi *et al.*, 2002). There is also evidence that NPY is involved in reproduction (Peng *et al.*, 1994) and in the immune response (Volkoff *et al.*, Peter, 2004) in fish.

1.4.2 Orexin

Prepro-orexin, the product of the orexin gene, undergoes proteolytic processing yielding two peptides, orexin-A and orexin-B (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). These two peptides are ligands for the G-protein-coupled receptors orexin receptor 1 (OX1R), which has a higher affinity for orexin-A and orexin receptor 2 (OX2R), which has an equal affinity for both peptides. Orexins have been isolated from mammals (Sakurai *et al.*, 1998), birds (Ohkubo *et al.*, 2002), amphibians (Shibahara *et al.*, 1999) and fish (Alvarez *et al.*, Sutcliffe, 2002; Kaslin *et al.*, 2004). To date, there is limited information on the role of orexins in fish.

Initial studies in rodents showed that orexins are expressed almost exclusively in the brain (Sakurai *et al.*, 1998). Within the brain, prepro-orexin mRNA expression is localized in the LHA, and orexin neurons project widely throughout the brain (Broberger *et al.*, 1998; Cutler *et al.*, 1999; Date *et al.*, 1999; Sakurai *et al.*, 1998). In mammals, central injections of orexin-A induce *c-fos* expression in the PVN, DMH and Arc, suggesting that orexins activate cellular activity in the feeding centres of the hypothalamus (Edwards *et al.*, 1999; Mullett *et al.*, 2000). The distribution of orexin mRNA within the brain has only been examined in a few non-mammalian species. In the Japanese quail (*Coturnix cotunix japonica*), prepro-orexin mRNA is localized in the LHA and PVN (Phillips-Singh *et al.*, 2003), whereas in zebrafish prepro-orexin mRNA is localized in the preoptic area and hypothalamus (Kaslin *et al.*, 2004). In birds, amphibians and fish, orexin neurons have widespread projections throughout the brain including the hypothalamus (Huesa *et al.*, 2005; Kaslin *et al.*, 2004; Phillips-Singh *et al.*, 2003;

Shibahara *et al.*, 1999; Singletary *et al.*, 2005), suggesting that orexins are involved in many physiological processes.

Sakurai *et al.* (1998) were the first to show that orexins act within the brain to stimulate food intake. They demonstrated that, in rodents, injections of orexin-A or orexin-B in the LHA increase food intake, with orexin-A being more potent. Subsequent studies with rodents have confirmed that orexins injected either ICV (Thorpe *et al.*, 2003; Yamanaka *et al.*, 2000), or in the PVN, DMH (Dube *et al.*, 1999; Edwards *et al.*, 1999) or in the Arc (Muroya *et al.*, 2004), increase food intake. In contrast, ICV injections with orexin-A antibodies decrease food intake (Yamada *et al.*, 2000). In rats, chronic administration of orexin-A increases daytime food intake and slightly reduces nighttime food intake, implicating orexin-A in short-term feeding (Yamanaka *et al.*, 1999). Volkoff *et al.* (1999) were the first demonstrate an appetite stimulating role of orexins in fish. ICV injections of either human orexin-A or orexin-B in goldfish increase food intake, and orexin-A is more potent than orexin-B (Volkoff *et al.*, 1999). Overall, these studies support the role of orexins as appetite stimulators in both mammals and fish.

Orexin receptors appear to be expressed exclusively in the brain where they have distinct expression patterns (Sakurai *et al.*, 1998). In rodents, OX1R and OX2R mRNA and immunoreactive sites are widely distributed throughout the brain with high expression levels in the hypothalamus (Backberg *et al.*, 2002; Cluderay *et al.*, 2002; Hervieu *et al.*, 2001; Trivedi *et al.*, 1998). In mammals, injections of an OX1R antagonist decrease food intake and block the appetite-stimulating effects of orexin-A (Haynes *et al.*, 2000; Rodgers *et al.*, 2001). Orexin receptors have also been isolated from birds (Ohkubo *et al.*, 2003), but not from amphibians or fish to date.

Nutritional status influences orexin expression levels in the brain. During fasting, prepro-orexin mRNA levels are elevated in the hypothalamus (Sakurai *et al.*, 1998), and both prepro-orexin mRNA and orexin-A and orexin-B peptide levels are elevated in the LHA (Cai *et al.*, 1999; Mondal *et al.*, 1999). Fasting also increases the number of orexin-producing neurons in the LHA and increases *c-fos* expression within orexin neurons (Diano *et al.*, 2003). Orexin receptors are also influenced by energy status since OX1R and OX2R mRNA and protein levels increase in the hypothalamus during food deprivation (Karteris *et al.*, 2005; Lu *et al.*, 2000). In zebrafish, prepro-orexin mRNA levels increase in the brain during long-term food deprivation (14 days) (Novak *et al.*, 2005), suggesting the response of orexin to fasting is conserved.

Although orexins were initially thought to be expressed exclusively in the brain, they have since been detected in several peripheral tissues (Voisin *et al.*, 2003), suggesting that orexins have many physiological roles other than regulating food intake. In mammals, orexins appear to be involved in regulating feeding behaviours including daily food anticipatory activities (Akiyama *et al.*, 2004), and in the control of gastric acid secretion, reproduction and sleep-wake cycles (Voisin *et al.*, 2003). In zebrafish, orexin neurons innervate aminergic neurons associated with sleep and vigilance (Kaslin *et al.*, 2004) suggesting a role for orexins regulating arousal and wakefulness in fish.

1.4.3 Cocaine and amphetamine regulated transcript peptide (CART)

Douglass *et al.* (1995) originally isolated CART from rats and showed that CART mRNA expression changed with psychostimulant administration. CART is a potent

anorexigenic factor in mammals, and is one of the most highly expressed transcripts in the hypothalamus (Gautvik *et al.*, 1996). CART is spliced into several biologically active fragments (Kuhar and Yoho, 1999), and the expression of these fragments is tissue specific (Thim *et al.*, 1999). In fish, CART has been isolated from goldfish (Volkoff and Peter, 2001), zebrafish (GenBank BQ480503) and pufferfish, *Takifugu rubripes* (Fugu Genome project SINFRUT00000179649). However, to date, a CART receptor has not been identified.

In mammals, CART mRNA is widely expressed throughout the brain including the hypothalamus. Within the hypothalamus, CART mRNA is expressed in several feeding centres including the PVN, LHA and Arc (Vrang *et al.*, 1999), and the highest levels of CART mRNA expression occur in the Arc (Elias *et al.*, 1998; Larsen *et al.*, 2003; Vrang *et al.*, 1999). CART immunoreactive sites are widely distributed throughout the brain and concentrated within the hypothalamus (Koylu *et al.*, 1997). Western blotting has also shown that CART peptides are present in the hypothalamus (Kuhar *et al.*, Yoho, 1999). In mammals, CART appears to target the PVN since CART injections in this hypothalamic region stimulate neuronal activity (Vrang *et al.*, 1999) and block food intake (Stanley *et al.*, 2001). Ablation of CART expression in the Arc, through treatment with a neurotoxin, decreases CART immunoreactivity in the PVN, suggesting that CART peptides localized in the PVN are produced in the Arc (Broberger, 1999). This has been confirmed by immunohistochemical studies showing that CART neurons originating in the Arc innervate neurons in the PVN (Fekete *et al.*, 2000). In amphibians, CART immunoreative sites are localized throughout the brain including the hypothalamus (Lazar

et al., 2004). CART mRNA expression has only been examined in one species of fish, the goldfish, in which CART mRNA is widely distributed throughout the brain (Volkoff and Peter, 2001).

Lambert *et al.* (1998) provided the first behavioural study on the effects of exogenous CART treatments in mammals. They showed that ICV injections of CART decrease food intake in rodents, suggesting that CART acts as an anorexigenic factor. Other studies using central injections have shown that CART decreases food intake in normal and food-deprived rats (Couceyro and Fritz, 2003; Edwards *et al.*, 2000; Kristensen *et al.*, 1998; Zheng *et al.*, 2001), and that central administration of CART antibodies stimulates food intake (Kristensen *et al.*, 1998; Lambert *et al.*, 1998). Chronic administration of CART decreases food intake and body weight in a dose-dependent manner (Larsen *et al.*, 2000; Rohner-Jeanrenaud *et al.*, 2002). CART-deficient mice display increased food intake and increased fat mass when fed a high calorie diet (Asnicar *et al.*, 2001). Little is known about the role of CART in fish but it appears that, as in mammals, it plays a role in the control of food intake as ICV administration of human CART decreases food intake in goldfish (Volkoff and Peter, 2000). In both mammals and fish, CART thus appears to act within the brain to decrease food intake.

In mammals, CART levels appear to be influenced by energy status. Fasting induces a decrease in both CART mRNA levels (Kristensen *et al.*, 1998), and the number of CART-expressing neurons in the Arc (McAlister and Van Vugt, 2004). There is also a decrease in CART peptide levels in several brain regions, including the hypothalamus, during fasting (Vicentic *et al.*, 2005). Decreases in CART mRNA levels during fasting have also been shown in goldfish brain (Volkoff and Peter, 2001).

In addition to its role in food intake, CART has many other physiological roles in mammals, including modulation of gastric function. In rodents, CART peptides are widely expressed throughout the gastrointestinal tract (Ekblad *et al.*, 2003), and central injections of CART decrease both gastric emptying and gastric acid production (Asakawa *et al.*, 2001; Okumura *et al.*, 2000). Since peripheral injections of CART do not affect gastric function, it has been suggested that CART acts via the brain to regulate gastric function (Okumura *et al.*, 2000). CART might also affect pituitary function as it stimulates the release of pituitary hormones (Baranowska *et al.*, 2004).

1.5 Interactions between NPY and orexin

Due to the widespread distribution of orexins fibres within the brain, it has been suggested that orexins might act as mediators between the LHA and other regions of the brain. In particular, data points to a direct action of orexins on NPY. For example, (1) orexin neurons originating in the LHA form synaptic contacts with NPY neurons in the Arc (Horvath *et al.*, 1999; Muroya *et al.*, 2004); (2) in the Arc, NPY/AgRP neurons express OX1R and OX2R (Backberg *et al.*, 2002; van den Top *et al.*, 2004) and orexins alter the neurophysiological properties of NPY/AgRP neurons (van den Top *et al.*, 2004); (3) Y1 and Y5 receptor antagonists block orexin-induced food intake (Dube *et al.*, 2000; Ida *et al.*, 2000); (4) the administration of orexin antibodies attenuates the effects of exogenous NPY (Niimi *et al.*, 2001); and (5) central administration of orexin-A increases both *c-fos* and NPY expression in the Arc (Lopez *et al.*, 2002; Yamanaka *et al.*, 2000).

In fish, there is also evidence that NPY and orexin-A interact in the regulation of food intake. In goldfish, ICV injections with NPY receptor antagonists block the appetite-

stimulating effects of orexin-A, and blocking orexin receptors decreases NPY-induced feeding (Volkoff *et al.*, Peter, 2001). In goldfish, there is evidence that orexins and NPY act synergistically to increase food intake (Volkoff *et al.*, Peter, 2001).

1.6 Interactions between NPY and CART

Anatomical and behavioural studies in mammals have provided evidence of a functional relationship between CART and NPY. In the hypothalamus, NPY and CART immunoreactive sites are in close proximity (Broberger, 1999; Lambert *et al.*, 1998). Both ICV and PVN injections of CART block NPY-induced feeding in rodents (Kristensen *et al.*, 1998; Lambert *et al.*, 1998; Wang *et al.*, 2000). In fish, although there is no anatomical data showing a relationship between CART and NPY, functional studies in goldfish have shown that CART blocks the orexigenic effects of NPY (Volkoff and Peter, 2000).

1.7 Interactions between NPY and Insulin

In mammals, the anorexigenic effects of insulin are achieved in part through interactions with NPY. In rats, central and peripheral injections of insulin block fasting-induced up-regulation of NPY mRNA in the Arc (Sahu *et al.*, 1995; Schwartz *et al.*, 1992), and central injections of insulin brain receptor (InsRB) ODNs increase NPY mRNA in the Arc (Obici *et al.*, 2002), suggesting insulin blocks NPY activity. To date, interactions between insulin and NPY in the regulation of feeding have not been investigated in fish.

1.8 Interactions between NPY, orexin, CART and leptin

The effects of leptin on food intake are mediated in part through interactions with NPY. In mammals, exogenous leptin treatments block NPY-induced feeding (Niimi *et al.*, 2001). In normal and obese mice, central administration of leptin decreases NPY mRNA expression in the Arc (Schwartz *et al.*, 1996; Stephens *et al.*, 1995). NPY neurons in the Arc express Ob-Rb (Baskin *et al.*, 1999), and leptin changes the electrophysiology of NPY/AgRP neurons (van den Top *et al.*, 2004). ICV injections of leptin block the effects of fasting on NPY mRNA expression (Korner *et al.*, 2001). Leptin has also been shown to interact with orexin pathways. During food deprivation, leptin blocks the fasting-induced increase in orexin mRNA expression in the hypothalamus (Lopez *et al.*, 2000). Orexin-induced food intake is only partially inhibited by central injections of leptin, indicating the existence of both orexin-sensitive and insensitive neurons within the brain (Zhu *et al.*, 2002). There is also anatomical evidence of interactions between orexin, NPY and leptin. In the Arc, a subset of NPY neurons co-express the leptin receptor (ObR), OX1R and OX2R mRNA (Funahashi *et al.*, 2003). Leptin has been shown to block orexin-induced activation of NPY neurons in the Arc (Rauch *et al.*, 2000). In rats and monkeys, the leptin receptor protein is expressed by NPY neurons in the Arc and orexin neurons in the LHA (Horvath *et al.*, 1999). To date, only one study has demonstrated an interaction between leptin and NPY and orexin in fish, where central injections of murine leptin inhibit both NPY or orexin-A-induced food intake in goldfish (Volkoff *et al.*, 2003).

Leptin has been shown to regulate the expression of CART within the hypothalamus. In rodents, CART neurons in the hypothalamus express the Ob-R (Elias *et*

al., 2001). In leptin-deficient mice, CART is undetectable in the Arc (Kristensen *et al.*, 1998). ICV injections of leptin block the effects of fasting on CART mRNA expression in the Arc (McAlister and Van Vugt, 2004). In goldfish, leptin treatments increase CART mRNA expression (Volkoff and Peter, 2001).

1.9 Experimental model: Atlantic cod

There is limited information of the neuroendocrine regulation of feeding in fish. As there is a high potential to successfully culture Atlantic cod, these fish are a good model to study feeding-related neuropeptides since acquiring new information on feeding may be used to improve aquaculture conditions. Atlantic cod are mesopelagic fish that feed using visual cues. Fish, crustaceans and krill are the main components of the cod diet, where crustaceans are the major component of the juvenile diet and fish are the major component of the adult diet (Waiwood and Majkowski, 1984). Indeed, there appears to be a diet transition as cod grow since there is a correlation between increasing cod size and increasing fish consumption (Waiwood and Majkowski, 1984). In addition, there are seasonal variations in cod feeding patterns as cod decrease feeding during the winter (Schwalme and Chouinard, 1999).

1.10 Objective of this study

Currently, our understanding of the neuroendocrine regulation of food intake is limited since the majority of studies have been performed using mammalian systems. Little is known about the mechanisms regulating food intake in lower vertebrates including fish. The goal of this study was to isolate and characterize three feeding-related

neuropeptides, NPY, CART and orexin, in Atlantic cod (*Gadus morhua*). We isolated full-length cDNAs encoding NPY and CART and a partial cDNA encoding orexin, and examined the central and peripheral distribution of NPY and CART. To assess if these genes have a role in feeding, we examined the effects of daily feeding and food deprivation on NPY and CART mRNA expression. One important factor motivating this study was the improvement and enhancement of cod aquaculture projects. Since temperature affects food intake in fish, and it is an important abiotic factor in aquaculture projects, we also examined the effects of temperature on NPY and CART mRNA expression to determine if they are involved in mediating temperature-dependent changes in appetite.

2.0 Molecular cloning and tissue distribution of NPY, CART and orexin

2.1 Introduction

NPY is a member of the pancreatic polypeptide family which also includes peptide YY (PYY), pancreatic peptide (PP), and pancreatic peptide Y (PY). NPY and PYY are found in all vertebrates while PY is found exclusively in fish. All members of this family are 36 amino acids (with the exception of chicken PYY), and they form a characteristic hairpin-like tertiary structure, the pancreatic peptide fold. NPY has been isolated from tetrapods and non-tetrapods and is highly conserved across all vertebrates. The members of the pancreatic polypeptide family are ligands for a family of G-protein coupled receptors, the NPY-family receptors, which include Y1, Y2, Y4, Y5 and Y6.

NPY is synthesized as a peptide precursor which includes a hydrophobic signal peptide, a mature 36 amino acid peptide, an amidation-proteolytic site (GKR), and the carboxy terminal peptide of NPY (CPON) (Cerda-Reverter and Larhammar, 2000). In mammals, NPY is the most abundant peptide in the CNS and is involved in the regulation of feeding, cardiovascular function and reproduction (Dumont *et al.*, 1992). Within the mammalian brain, the highest levels of NPY mRNA occur in the hypothalamus whereas in fish, NPY mRNA expression is highest in the telencephalon. In all vertebrates, NPY is expressed in the feeding centres of the brain where it regulates appetite and energy homeostasis.

In fish, NPY cDNA has been isolated from an elasmobranch (electric ray, *Torpedo marmorata*) (Blomqvist *et al.*, 1992), agnathans (river lamprey, *Lampetra fluviatilis*, sea lamprey, *Petromyzon marinus* and Southern brook lamprey, *Ichthyomyzon*

gage) (Montpetit *et al.*, 2005; Soderberg *et al.*, 1994) and teleosts (goldfish, *Carassius auratus*, zebrafish, *Danio rerio*, sea bass, *Dicentrarchus labrax* and channel catfish, *Ictalurus punctatus*) (Blomqvist *et al.*, 1992; Cerda-Reverter *et al.*, 2000; Leonard *et al.*, 2001). NPY peptide sequences have been determined for an elasmobranch (dogfish, *Scyliorhinus canicula*) (Conlon *et al.*, 1992) and teleosts (Atlantic cod, *Gadus morhua* and rainbow trout, *Oncorhynchus mykiss*) (Jensen and Conlon, 1992).

Orexins have been isolated from mammals (Sakurai *et al.*, 1998), birds (Ohkubo *et al.*, 2002), amphibians (Shibahara *et al.*, 1999) and fish including pufferfish (*Takifugu rubripes*) (Alvarez and Sutcliffe, 2002) and zebrafish (Kaslin *et al.*, 2004). In mammals, orexin-A and orexin-B are 33 aa and 28 aa, respectively, and are derived from a 131 aa peptide precursor, prepro-orexin. Orexin-A has four cysteine residues that form two disulfide bridges, and orexin-B is a linear peptide (Lee *et al.*, 1999). Both orexin-A and orexin-B have two helical regions that have been implicated in receptor binding (Miskolzie et al., Kotovych, 2003; Miskolzie *et al.*, 2003). There are two orexin receptors, orexin receptor 1 and orexin receptor 2, which are both G-protein coupled receptors. To date, orexin receptors have not been isolated in fish.

In mammals, orexin mRNA is expressed in discrete nuclei within the hypothalamus, and these neurons project throughout the brain. Orexins are important regulators of feeding and metabolism in mammals since they stimulate both appetite and gastric acid secretion, increase gut motility and increase foraging and vigilance (Rodgers *et al.*, 2002). Orexins appear to have a role in the regulation of feeding in fish, as orexins are expressed in the feeding centres of the brain (Kaslin *et al.*, 2004), and central injections of orexins increase food intake (Volkoff *et al.*, 1999).

CART cDNAs have been isolated from mammals (Douglass and Daoud, 1996; Douglass *et al.*, 1995) and fish including goldfish (Volkoff et al., Peter, 2001), zebrafish (GenBank BQ480503) and pufferfish (Fugu Genome project SINFRUT00000179649). In rodents, two forms of preproCART are expressed, which arise through alternate processing of mRNA. ProCART is processed into several fragments, and the distribution of these fragments is tissue specific (Thim *et al.*, 1999). In goldfish, two forms of preproCART are expressed from separate genes, and both forms have several potential proteolytic sites, suggesting that CART is processed into several fragments. The carboxy terminus of CART peptides is the most conserved region, and the positions of the six cysteine residues that form three disulfide bridges are conserved across all species.

CART mRNA is expressed in the feeding centres of both mammalian (Vrang *et al.*, 1999) and fish (Volkoff and Peter, 2001) brains. CART acts within the brain to decrease food intake (Kristensen *et al.*, 1998; Volkoff and Peter, 2000). In mammals, CART peptides not only decrease appetite, but also increase energy expenditure and thermogenesis and decrease gastric acid secretion (Hunter *et al.*, 2004), suggesting CART is an important regulator of energy homeostasis.

We have isolated complete cDNAs of Atlantic cod NPY, CART and a partial cDNA of orexin. RT-PCR was used to localize NPY and CART mRNA expression in peripheral and central tissues. These sequences were subsequently used to develop molecular tools to examine mRNA expression profiles of these genes and to determine if NPY and CART have an appetite-regulating role in the Atlantic cod.

2.2 Materials and Methods

Animals

Cultured juvenile Atlantic cod (*Gadus morhua*), average starting weight of 100 g, were obtained from the Aquaculture Research and Development Facility, Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Fish were kept under natural photoperiod and temperature conditions. For cloning and tissue distribution studies, 2-3 fed fish were sampled for whole brain, liver, gut, heart, kidney, spleen, ovary, gill and skin. For sampling, fish were anesthetized in 0.05% tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada), killed by spinal section, and tissues were dissected. All tissues were subsequently stored at -20°C in RNA*later* (Qiagen, Mississauga, Ontario, Canada) until RNA isolations were performed.

Preparation of RNA

For cloning and tissue distribution studies, total RNA was isolated from whole brain, peripheral tissues, and specific brain regions including the olfactory tract, telencephalon, optic tectum, hypothalamus, cerebellum and medulla using the RNeasy Mini Kit (Qiagen). Brain regions were dissected following brain morphology determined by Delfini et al., Diagne (1985). Total RNA from the forebrain and hindbrain was isolated using a trizol/chloroform extraction with Tri-Reagent (BioShop, Mississauga, Ontario, Canada). Final RNA concentrations were determined by spectrophotometric readings at 260 nm.

Cloning of cDNA by RT-PCR

First strand cDNA synthesis was performed using 2 μg of RNA from the hypothalamus reverse transcribed with dT-AP (GGCCACGCGTCGACTAGTAC(T)17) and Omniscript Reverse Transcriptase (Qiagen). NPY, CART and orexin were amplified by two rounds of PCR using cDNA as the template in the first round of PCR, and the PCR product from the first reaction as the template for the second round of PCR. The PCR cycling conditions for all reactions were 30 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 45 s. The annealing temperature was optimized for each primer set, and all primer sequences are listed in Table 1.1. A partial sequence of NPY was amplified using degenerate primers NPY 1 and NPY 2 designed from regions of high homology between goldfish (Blomqvist *et al.*, 1992), sea bass (Cerda-Reverter *et al.*, 2000), and channel catfish (Leonard *et al.*, 2001). A partial sequence of CART was amplified using degenerate primers CT 1 and CT 2 designed from regions of high homology between human (Douglass and Daoud, 1996), rat (Douglass *et al.*, 1995), goldfish (Volkoff and Peter, 2001), and zebrafish (GenBank BQ480503). A partial sequence for orexin was amplified using OX 1 and OX 2 designed from regions of high homology between *Xenopus* (Shibahara *et al.*, 1999) , zebrafish (Kaslin *et al.*, 2004) and goldfish (Volkoff, unpublished). The PCR products were electrophoresed on an agarose gel, and bands of expected size were excised from the gel and purified with the 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 at the Core Molecular Biology Facility (York University, Toronto, Canada).

Table 1.1: Degenerate and gene specific primers used for cDNA and genomic DNA amplification.

Primers	Sequence (5′-3′)
NPY primers	
NPY 1	AARCCNGARAAYCCNGGNGA
NPY 2	GTRATNARRTTRATRTARTG
NPY3RC-A	CTGGCCCAAGTATTACTCCGC
NPY3RC-B	TATTACTCCGCGCATTGAGG
NPY5RC-1	AGTAATACTTGGCCAGTTCA
NPY5RC-2	TACTTGGCCAGTTCATCTGC
NPY5RC-3	AGTTCATCTGTTGGGGCGT
gNPY1	AGGCACAGAAGACAACTGAG
gNPY2	CAAGAGTGTCCAGAATCTCAG
gNPY3	CTGAGATTCTGGACACTCTTG
gNPY4	TGCCCTCTGATGACAAATCA
NPY-F	CAACTGAGAAACGGGGAAAA
NPY-R	CGGGTCATATCTGCTCTGTG
CART primers	
CT 1	CAGAACCATGGAGAGCTCC
CT 2	GACAGTCRCACAKCTTWCCGAT
CT3RC-A	CGTGTGATATCGGAGAGCAG
CT3RC-B	GAGAGCAGTGCGCCATCAGG
CT5RC-1	GTCCTCCTCCGACTCCGAGT
CT5RC-2	AAGGCTCTCCTGTCGAGGTC
CT5RC-3	CCATATAGTCTACATTCGAAC
gCART-F	AGAACCATGGAGAGCTCCAG
gCART-R	GTCTACATTCGAACCACGTTCC
CART-F	GTCGTCCATGGAGCTGATCT
CART-R	GAACCACGTTCCCATTTCAC
Orexin primers	
OX 1	GCYGGCATCCTCACKCTGGG
OX 2	ATWGTCAGGCTWCCGGCTGC
OX3RC-A	AGGCGGAGGAGCAGCACTTC
OX3RC-B	AGCAGCACTTCCACAGTCGG
B-actin primers	
β-actin 1	TACAGCTTCACCACCACAGC
β-actin 2	ATGCCACAAGACTCCATTCC

Rapid Amplification of cDNA Ends (RACE)

To isolate the 3' ends of NPY, CART and orexin, mRNA from the hypothalamus was reverse transcribed as described above. The 3' ends were amplified by two rounds of PCR using gene specific nested primers and the PCR cycling conditions described above. The 3' ends of NPY, CART and orexin were amplified using primers pairs NPY3RC-A/dT-AP then NPY3RC- B/AP (GGCCACGCGTCGACTAGTAC), CT3RC-A/dT-AP then CT3RC-B/AP and OX3RC-A/dT-AP then OX3RC-B/AP, respectively.

To isolate the 5' end of NPY, mRNA from the hypothalamus was reverse transcribed with the gene specific primer NPY5RC-1. After reverse transcription, unincorporated primers and dNTPs were removed from the reaction using Montage PCR (Millipore). A polyA tail was then added to the 5' end of the cDNA using Terminal Deoxynucleotidyl Transferase (Invitrogen, Burlington, Ontario, Canada). After the addition of the polyA tail, the reaction was cleaned again using Montage PCR. The 5' end of NPY was amplified using primer pairs NPY5RC-2/dT-AP then NPY5RC-3/AP in two rounds of PCR. To isolate the 5'end of CART, mRNA from the hypothalamus was reverse transcribed with CT5RC-1, then the same protocol was used to clean the reaction and add a polyA tail to the cDNA. To amplify CART cDNA, primers pairs CT5RC-2/dTAP and CT5RC-3/AP were used in two rounds of PCR. All RACE products were gel extracted, ligated, cloned and sequenced as described above.

Cloning of genomic DNA

Genomic DNA was isolated from Atlantic cod liver using the GenElute Genomic DNA Miniprep Kit (Sigma). Due to the large size of the expected NPY introns, three

primers pairs were used to amplify genomic NPY: gNPY1/NPY5RC-2, NPY3RC-A/gNPY2 and gNPY3/gNPY4. The genomic DNA was amplified by PCR with cycling conditions 95°C for 30 s, 55°C for 30 s, 72°C for 2 min for 30 cycles. CART genomic DNA was amplified using gCART-F and gCART-R with cycling conditions 95°C for 30 s, 55°C for 30 s, 72°C for 1 min for 30 cycles. All PCR products were gel extracted, ligated, cloned and sequenced as described above.

Detection of NPY and CART expression in different tissues

One microgram of total RNA isolated from whole brain, peripheral tissues, and specific brain regions was reverse transcribed using dT-AP and SuperScript II reverse transcriptase (Invitrogen). A 30 cycle PCR amplification was performed with the primer sets NPY-F/NPY-R or CART-F/CART-R, and the PCR products were run on a 2% agarose gel. As an internal control, all cDNA samples were amplified with primer set β-actin1/ β-actin2 (see Table1.1), designed from a partial sequence of Atlantic cod β-actin (GenBank CO541508). Negative controls were performed for each primer sets where cDNA was omitted from the PCR reactions. The PCR products obtained for each primer pair were sequenced to confirm the target gene was being amplified. To compare the relative amounts of NPY and CART mRNA between major brain divisions, bands on the agarose gel were detected using the EpiChemi Darkroom (UVP, Upland, California, USA) and quantified using LabWorks (Media Cybernetics, Silver Spring, Maryland, USA). Each sample was expressed as a ratio of NPY/actin or CART/actin.

2.3 Results

Cloning of Atlantic cod NPY and CART

Degenerate primers were used to amplify an 86 bp portion of the coding region of NPY, and the 3' and 5' ends of the cDNA were isolated using RACE. Atlantic cod NPY cDNA (GenBank AY822596) is 491 bp with a 56 bp 5'-UTR and 135 bp 3'-UTR (Fig. 2.1A). The open reading frame has a coding potential for 99 amino acids corresponding to preproNPY. The first 28 amino acids constitute the signal sequence followed by the 36 amino acid mature peptide, the proteolytic/amidation site (GKR), and the 32 amino acid CPON. NPY mRNA is composed of four exons separated by three introns of 388 bp, 926 bp and 546 bp respectively (GenBank DQ256082). Intron I is located between the terminal nucleotide of the 5'-UTR and the start codon, Intron II is located at position 63 and Intron III is located at position 93.

Degenerate primers were used to amplify 321 bp of CART cDNA, and RACE was used to isolate the 3' and 5' ends. The cDNA obtained for Atlantic cod CART is 494 bp with an open reading frame coding for 118 amino acids (GenBank DQ167209). The cDNA is composed of a 30 bp 5'-UTR and a 107 bp 3'-UTR (Fig. 2.1B). There are three potential proteolytic cleavage sites (RK and KK) at positions 69-70, 76-77 and 93-94. We isolated an 860 bp region of genomic CART. Atlantic cod CART is composed of 3 exons, separated by two introns located within the coding region of the gene (GenBank DQ167210). At position 55, intron I is 242 bp, and intron II at position 83 is 182 bp.

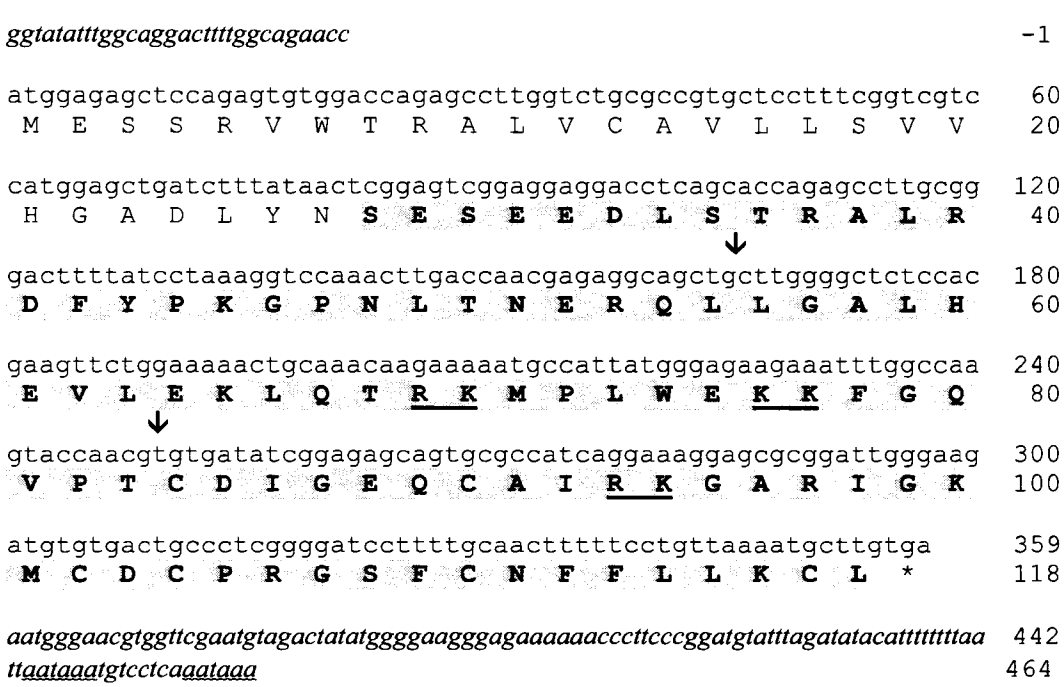
To isolate orexin cDNA, degenerate primers were used to amplify a 113 bp region of the coding region, and RACE was used to amplify the 108 bp 3'UTR (Fig. 2.2). The

FIG. 2.1. Complete cDNA sequences and predicted amino acids of Atlantic cod NPY (A) and CART (B). The 3' and 5' untranslated regions are in italics. Amino acids coding for the mature peptide are shaded in boldface letters. Intron positions are indicated by arrows, and polyadenylation sites are underlined with waves. In CART cDNA potential proteolytic cleavage sites (RK and KK) are underlined.

A


aggcacagaagacaactgagaaacggggaaaaagagcgaccacaaacacgccgata -1
atgcattctaacctggccacctggctcggagctctgggcttcctgctgtgcgcgctgata 60
M H S N L A T W L G A L G F L L C A L I 20
tgtttgggaactctgaccgagggataccccatcaaacccgagaacccaggggaggacgcc 120
C L G T L T E G Y P I K P E N P G E D A 40
ccggcagatgaactggccaagtattactccgcattgaggcactatattaacctcatcaca 180
P A D E L A K Y Y S A L R H Y I N L I T 60
agacaaaggtacgggaagaggtctagtcctgagattctggacactcttgtttcagagctg 240
R Q R Y G K R S S P E I L D T L V S E L 80
gtgctgaaggaaagtgcaaacactcttccacagagcagatatgacccgtcattgtggtaa 300
V L K E S A N T L P Q S R Y D P S L W * 99
tgtctaccctgtcccctcgaccacccacctcaggtgccaggtctgcaactgcccccccccccccgaaccaccccattataaaat 383
attccaacaatgatttgtcatcagagggcaacgcttgtgctacaactgctct 435

B


ggtatatttggcaggacttttggcagaacc -1
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M E S S R V W T R A L V C A V L L S V V 20
catggagctgatctttataactcggagtcggaggaggacctcagcaccagagccttgcgg 120
H G A D L Y N S E S E E D L S T R A L R 40
gacttttatcctaaaggtccaaacttgaccaacgagaggcagctgcttggggctctccac 180
D F Y P K G P N L T N E R Q L L G A L H 60
gaagttctggaaaaactgcaaacaagaaaaatgccattatgggagaagaaatttggccaa 240
E V L E K L Q T R K M P L W E K K F G Q 80
gtaccaacgtgtgatatcggagagcagtgcgccatcaggaaaggagcgcggattgggaag 300
V P T C D I G E Q C A I R K G A R I G K 100
atgtgtgactgccctcggggatccttttgcaacttttcctgttaaaatgcttgtga 359
M C D C P R G S F C N F F L L K C L * 118
aatgggaacgtggttcgaatgtagactatatggggaagggagaaaaaaccttcccggatgtatttagatatacatttttttaa 442
ttaataaatgtcctcaaataaa 464

gggaagcgggaggcggaggagcagcacttccacagtcggctccaccagcttctccgcggt
G K **R E A E E Q H F H S R L H Q L L R G**

ggcgcgcggaatcaggcagccgggatcttgactatgggcaagcggtcagaggaggaagag
G A R N Q A A G I L T M G K R S E E E E

gcggtcgggctgctcatgcaatgggcgcagcaagacttcaccgcttga
A V G L L M Q W A Q Q D F T A *

tggagatggagaggggcggtttcaacaactgcctgttgctggggactttttttttaatgagatggagaggaagaggggtata
actaaaagtccagtgtgttgccgtggtagcaggacatgtgcttagtggatgtgttggatgtaaagtgtttccaaaataaaggc
gcgacctaaagttc

FIG. 2.2. Partial cDNA sequence and predicted amino acids of Atlantic cod orexin. The 3' untranslated region is in italics. Amino acids coding for orexin-B peptide are shaded in boldface letters. The polyadenylation site is underlined with waves.

sequence has a coding potential for 55 amino acids and encodes an orexin-B-like peptide. RACE was not performed to isolate the 5' end of orexin cDNA.

Tissue Distribution of NPY and CART mRNA

RT-PCR of mRNA from central and peripheral tissues of Atlantic cod was used to localize NPY and CART expression. A 332 bp region of NPY and 319 bp region of CART were amplified. NPY was detected at high levels in the brain, proximal intestine, and kidney and detected at lower levels in the heart and ovary (Fig. 2.3), while CART mRNA was detected in the brain and ovary (Fig. 2.3). CART was not detected in the proximal intestine or kidney, and there were no detectable signal of NPY or CART from the liver, spleen, skin or gill.

Within the brain, NPY is expressed primarily in the forebrain (Fig. 2.4A) whereas CART appears to be expressed at similar levels in the forebrain and hindbrain (Fig. 2.4B). NPY was detected at high levels in the telencephalon, optic tectum and hypothalamus, but there were no detectable levels of NPY mRNA in the cerebellum, medulla and olfactory tract (Fig 2.4C). CART mRNA is expressed throughout the brain including the hypothalamus, optic tectum, telencephalon, optic tract, and medulla (Fig. 2.4C), while CART mRNA expression was undetected in the cerebellum.

For all tissues examined, PCR using β-actin gene specific primers was performed as an internal control, and a 230 bp fragment of β-actin was detected in all tissues. As a negative control a reaction with no template was performed using each primer pair, and there was no amplification in these reactions, verifying there was no contamination.

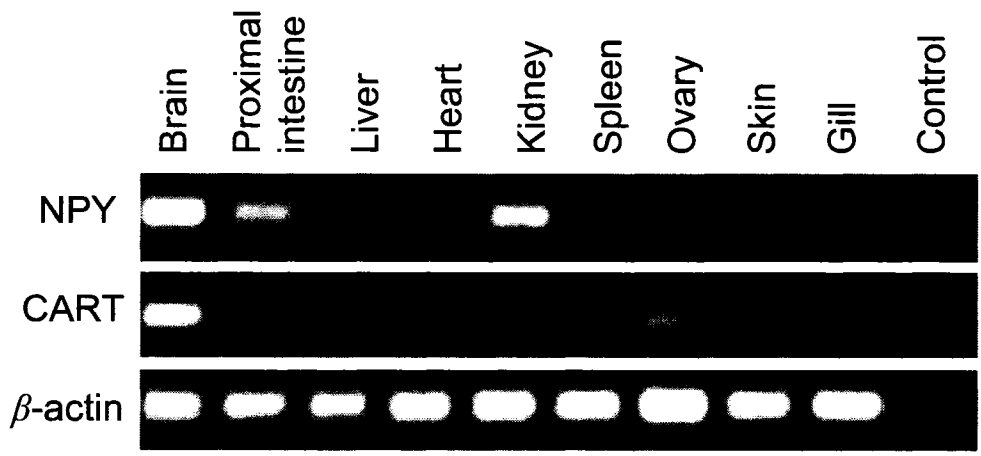
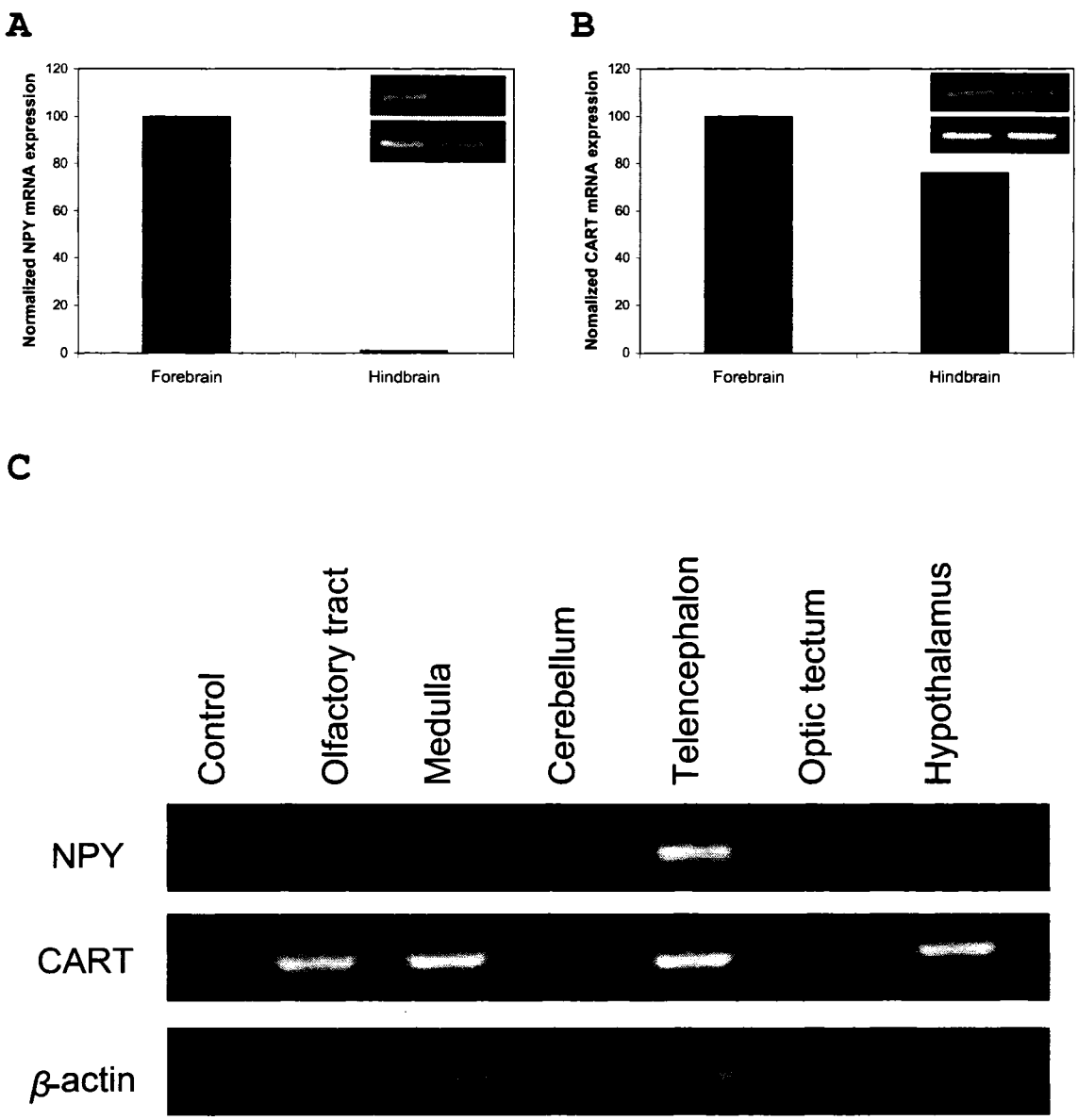


FIG. 2.3. RT-PCR analysis of NPY and CART mRNA expression from central and peripheral tissues. Samples were visualized on a 2% agarose gel stained with ethidium bromide. The cDNA templates were amplified by PCR using primers pairs NPY-F/NPY-R or CART-F/CART-R or β-actin 1/ β-actin 2. The RT-PCR products were of expected size with 332 bp for NPY cDNA, 319 bp for CART cDNA, and 230 bp for β-actin cDNA.

FIG. 2.4. Localization of NPY (A) and CART (B) mRNA from major brain divisions and discrete brain regions (C). RT-PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide. The cDNA templates were amplified by PCR using primers pairs NPY-F/NPY-R, CART-F/CART-R or β-actin 1/ β-actin2. Insets show representative agarose gels of RT-PCR products for forebrain and hindbrain (upper panel NPY (B) or CART (C) and lower panel β-actin).


A
Normalized NPY mRNA expression
120
100
80
60
40
20
0
Forebrain
Hindbrain
B
Nomalized CART mRNA expression
120
100
80
60
40
20
0
Forebrain
Hindbrain
C
Control
Olfactory tract
Medulla
Cerebellum
Telencephalon
Optic tectum
Hypothalamus
NPY
CART
β-actin

2.4 Discussion

Molecular cloning of NPY, CART and orexin

Using RT-PCR with degenerate primers and RACE, complete cDNAs of NPY and CART and a partial cDNA of orexin were isolated from Atlantic cod. The predicted amino acid sequence of mature NPY is in agreement with the peptide sequence determined for NPY peptides obtained from Atlantic cod brain extracts (Jensen and Conlon, 1992). The mRNA organization and the intron-exon boundaries of Atlantic cod NPY are identical to that of NPY from other vertebrates (Cerda-Reverter and Larhammar, 2000). The amino acid sequence of Atlantic cod NPY shares a high degree of homology with NPY from other fish. Atlantic cod preproNPY has 45% to 86% homology with goldfish, zebrafish, European sea bass, channel catfish, and electric ray (Fig. 2.5A), and mature Atlantic cod NPY shares 86% to 94% homology between these species. The high degree of conservation in NPY structure suggests that the physiological role of NPY is also conserved.

The organization of Atlantic cod preproCART, which includes a signal sequence and proCART is similar to CART organization in other vertebrates. The intron-exon organization in Atlantic cod CART with introns at positions 55 and 83 is similar to that of goldfish CART with introns at positions 54 and 82 in form I and positions 57 and 84 in form II. The shift in the intron-exon boundary between Atlantic cod and goldfish form I CART appears to be a result of an additional amino acid in the signal sequence of Atlantic cod CART. Atlantic cod preproCART is 72% homologous to CART peptides from zebrafish and goldfish and 42% homologous to human CART (Fig. 2.5B). Two

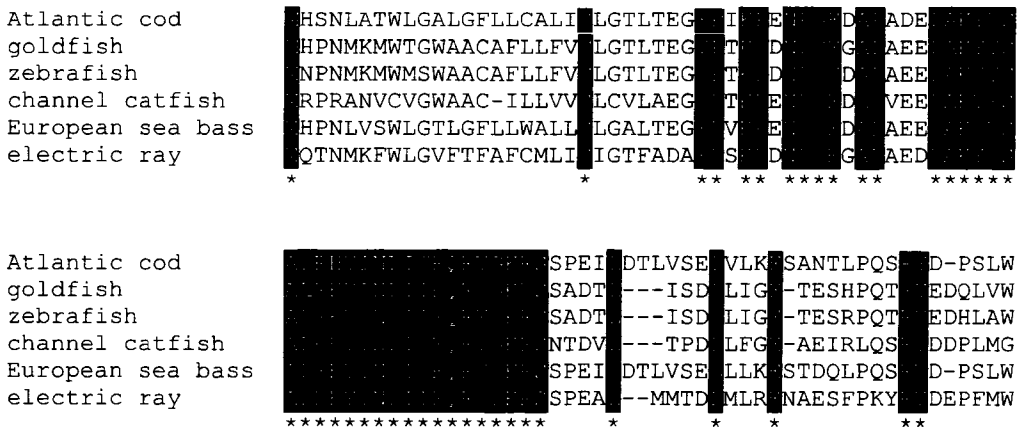
potential polyadenylation sites are present in the polyA tail of CART. Although only one form was isolated in cod, there are two forms of CART in rodents and goldfish suggesting there may be multiple forms of CART mRNA in the Atlantic cod.

There are three potential proteolytic cleavage sites in Atlantic cod CART. Goldfish and zebrafish CART display two potential proteolytic cleavage sites while mammalian CART has three potential proteolytic sites. The positions of two of the three proteolytic sites appear to be conserved across Atlantic cod and mammals (Fig. 2.5B). In the carboxy terminus, the position of the six cysteine residues in the CART is identical to that of other species (Fig. 2.5B). These cysteine residues form disulfide bridges, which maintain the activity of CART. Indeed, unfolded CART peptides do not decrease food intake in mammals when injected centrally (Couceyro and Fritz, 2003). This suggests the carboxy terminal end of CART is the biologically active part of the peptide. CART appears to be a highly conserved neuropeptide across all vertebrates, suggesting that its biological role is also conserved.

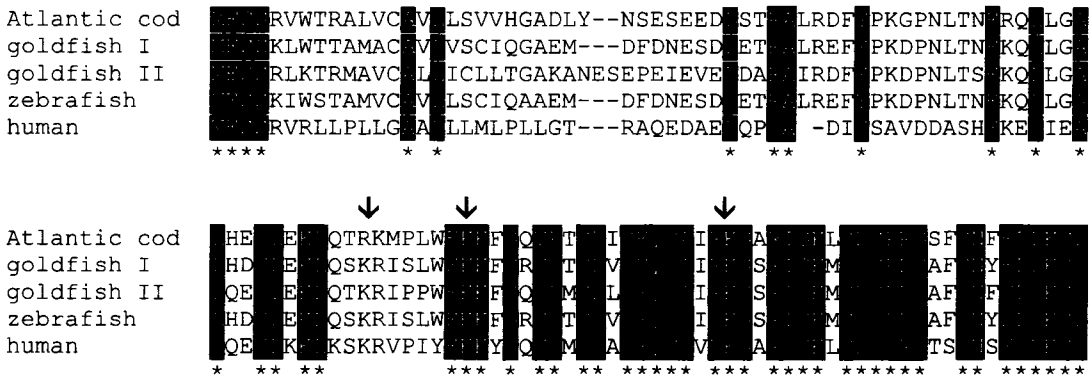
A partial cDNA sequence of orexin was amplified using RT-PCR and RACE. The cDNA encodes the putative amino acids of the orexin-B peptide. The Atlantic cod orexin fragment has 49% and 52% homology with the corresponding zebrafish and pufferfish orexin fragments (Fig. 2.5C). The 3'end of Atlantic cod orexin appears to be homologous to other fish. The 5'end of the peptide has not yet been isolated and must be sequenced in order to develop molecular tools to study orexin expression.

FIG. 2.5. Amino acid sequence alignments with Atlantic cod NPY (A) and CART (B) and orexin (C). Atlantic cod NPY was aligned with preproNPY from goldfish (Blomqvist *et al.*, 1992), zebrafish (Soderberg *et al.*, 2000), channel catfish (Leonard *et al.*, 2001), European sea bass (Cerda-Reverter *et al.*, 2000) and electric ray (Blomqvist *et al.*, 1992). Atlantic cod CART was aligned with preproCART from goldfish (Volkoff and Peter, 2001), zebrafish (GenBank BQ480503), and human (Douglass et al., Daoud, 1996). Atlantic cod partial orexin peptide was aligned with zebrafish (Kaslin *et al.*, 2004) and pufferfish (Alvarez and Sutcliffe, 2002). Conserved amino acids are highlighted in black and indicated with stars. Three potential proteolytic sites in Atlantic cod CART are indicated by arrows.

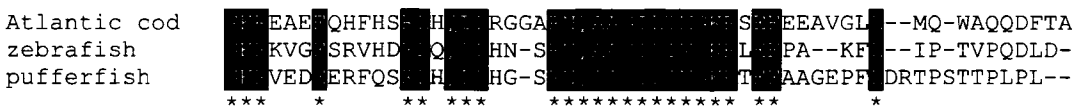
A


Atlantic cod
goldfish
zebrafish
channel catfish
European sea bass
electric ray

B


Atlantic cod
goldfish I
goldfish II
zebrafish
human

C


Atlantic cod
zebrafish
pufferfish

Distribution of NPY mRNA expression

Using RT-PCR we found high levels of NPY mRNA in the brain, proximal intestine, and kidney and lower levels of expression in the heart and ovary. Since the mature NPY peptide has previously been isolated from the Atlantic cod brain (Jensen and Conlon, 1992), our results further support the expression of NPY in the brain. Within the brain, NPY mRNA was detected primarily in the forebrain, in particular in the hypothalamus, the optic tectum and the telencephalon. These results are in agreement with previous studies showing that in other species of fish, the forebrain is the primary site of NPY mRNA expression (Cerda-Reverter *et al.*, 2000; Leonard *et al.*, 2001; Narnaware *et al.*, 2000; Silverstein *et al.*, 1998). The highest levels of NPY mRNA expression within in the Atlantic cod brain appeared to be in the telencephalon, which is also in agreement with previous studies in fish (Leonard *et al.*, 2001; Narnaware *et al.*, 2000; Peng *et al.*, 1994). High expression levels of NPY in the telencephalon suggest that NPY is involved in regulating food intake (Narnaware *et al.*, 2000), innervating the pituitary (Peng *et al.*, 1994) and processing olfactory inputs (Pirone *et al.*, 2003). In goldfish and catfish, the expression of NPY in the telencephalon, optic tectum and hypothalamus is influenced by food availability and displays peri-prandial changes, indicating a possible role of NPY in regulating food intake (Narnaware *et al.*, 2000; Silverstein *et al.*, 1998). The localization of NPY in the telencephalon, optic tectum and hypothalamus of the Atlantic cod suggests that NPY is involved in the regulation of food intake in this species as well.

Using RT-PCR, we examined NPY expression in peripheral tissues and found that NPY mRNA is expressed in the proximal intestine, heart, kidney and ovary. Our results

suggest that, as in mammals and other fish species, Atlantic cod NPY acts as a brain-gut peptide. NPY mRNA and peptides are expressed in the gastrointestinal tract of several fish species, including lamprey, dogfish, skate, rainbow trout and sea bass (Bjenning *et al.*, 1993; Bjenning *et al.*, 1993; Gomez-Visus *et al.*, 1998; Montpetit *et al.*, 2005). In elasmobranchs, NPY inhibits both the contractile activity of the stomach and gastric emptying (Bjenning *et al.*, 1993; Bjenning *et al.*, 1993; Dumont *et al.*, 1992). In Atlantic cod, NPY causes vasorelaxation and induces intestinal contractions (Shahbazi *et al.*, 2002), and NPY immunoreactive neurons project to the gastrointestinal tract (Karila *et al.*, 1997).

NPY mRNA was detected in Atlantic cod ovary. Previous studies have also shown that NPY mRNA is expressed in the fish ovary (Leonard *et al.*, 2001). NPY has been implicated in the regulation of reproduction in goldfish (Peng *et al.*, 1994) and appears to be involved in the release of gonadotropin-releasing hormone in catfish (Gaikwad *et al.*, 2005). The presence of NPY mRNA in the cod ovary suggests that NPY mRNA might play a role in the regulation of reproductive events in Atlantic cod. NPY mRNA was also localized in both the kidney and heart. NPY peptides are expressed in mammalian hearts and kidneys (Dumont *et al.*, 1992), however, in channel catfish, NPY expression was not detected in either kidney or heart (Leonard *et al.*, 2001). NPY appears to be involved in cardiac function of fish since NPY increases heart rate in dogfish (Xiang, 1994). The presence of NPY in the kidneys suggests that NPY might have a role in osmoregulation in cod.

Distribution of CART mRNA expression

Atlantic cod CART mRNA was detected in brain and ovary. CART mRNA was detected throughout the Atlantic cod brain with the exception of the cerebellum. Expression levels appeared to be high in the hypothalamus, telencephalon, medulla and olfactory tract with lower levels in the optic tectum. Our results are in agreement with other studies showing that CART mRNA is expressed throughout the brain of goldfish (Volkoff et al., Peter, 2001) and mammals (Douglass and Daoud, 1996; Douglass *et al.*, 1995). The wide distribution of CART mRNA throughout the brain suggests that CART may have many physiological roles in Atlantic cod, and the localization of CART within the telencephalon and hypothalamus suggests it may regulate feeding. Indeed, in goldfish, CART mRNA expression in the telencephalon, hypothalamus and olfactory bulbs is regulated by food availability (Volkoff and Peter, 2001) as fasting decreases CART expression in these areas.

CART mRNA was also detected in the ovary of Atlantic cod. This is consistent with previous studies that showed CART mRNA is present in the goldfish ovary (Volkoff and Peter, 2001), and that CART mRNA is expressed in bovine ovary where CART inhibits estradiol production (Kobayashi *et al.*, 2004), the latter suggesting an involvement in reproduction and follicle health status. We did not detect CART in the Atlantic cod proximal intestine. In mammals, although CART peptides are present in the gut (Couceyro *et al.*, 1998; Kuhar et al., Yoho, 1999), CART mRNA has never been detected in the gastrointestinal tract, suggesting that CART might not act as a brain-gut peptide. Our results support this hypothesis. The absence of CART mRNA in the heart,

kidney, spleen, skin and gill are in agreement with results from both rat and goldfish (Douglass *et al.*, 1995; Volkoff and Peter, 2001).

Implications

We have isolated NPY, CART and a partial sequence of orexin, three potential feeding-related peptides, from the Atlantic cod. Using this information, we can now develop the molecular tools to study the mechanisms regulating these peptides in the Atlantic cod and determine their role in feeding.

3.0 Effects of food deprivation and daily feeding on NPY and CART mRNA expression

3.1 Introduction

Food intake in vertebrates is regulated by the brain, and in particular the hypothalamus, which responds to peripheral signals and mediates changes in the expression of central effectors that regulate appetite. Several feeding-related neuropeptides have been isolated from fish, including NPY, orexins, CART, cholecystokinin, galanin, bombesin and ghrelin (Volkoff *et al.*, 2005). These central effectors are neuropeptides that act as either orexigenic or anorexigenic signaling peptides.

NPY, one of the first orexigenic peptides discovered, is a potent appetite stimulator expressed in the feeding centres of the brain. One of the principal physiological roles of NPY is the regulation of energy homoeostasis. NPY acts within the brain to increase food intake in rodents (Levine and Morley, 1984) and fish (Lopez-Patino *et al.*, 1999; Silverstein and Plisetskaya, 2000). In both fish and mammals, NPY is associated with hyperphagia after fasting, and NPY levels in the brain respond to changes in energy status (Jang and Romsos, 1998; Narnaware and Peter, 2001; Silverstein *et al.*, 1998).

CART is a neuropeptide that is widely expressed throughout the brain, and has potent anorexigenic effects. This has been demonstrated in studies in which ICV injections of CART decrease food intake in both mammals and fish (Kristensen *et al.*, 1998; Volkoff and Peter, 2000), and blocking CART signaling by injecting CART antibodies increases food intake in mammals (Kristensen *et al.*, 1998; Lambert *et al.*, 1998). In addition, the expression of CART within the brain decreases during fasting in

both mammals (Kristensen *et al.*, 1998; Vicentic *et al.*, 2005) and fish (Volkoff et al., Peter, 2001).

Previous studies have provided anatomical and behavioural evidence of a functional relationship between CART and NPY. In rodents, hypothalamic NPY and CART immunoreactive sites are in close proximity, suggesting these neurons interact (Broberger, 1999; Lambert *et al.*, 1998). Further, ICV injections of CART block NPY-induced feeding in both rodents and fish (Kristensen *et al.*, 1998; Lambert *et al.*, 1998; Volkoff and Peter, 2000), suggesting that CART blocks the orexigenic effects of NPY.

The Atlantic cod, like other marine species, experiences seasonal variations in energy stores and is able to survive for long periods with very little food intake. In the wild, these periods of declined food intake occur during the winter months, when Atlantic cod have the highest percent of empty stomachs and lowest stomach content weights (Dutil *et al.*, 2003), and they can lose up to 27% of their body weight (Schwalme and Chouinard, 1999). However, nothing is known about the endocrine mechanisms regulating feeding and these fasting episodes in cod.

In mammals, NPY and CART appear to regulate feeding during a state of negative energy balance. Further, recent studies have implicated these peptides in the regulation of daily feeding of fish (Narnaware *et al.*, 2000; Volkoff and Peter, 2001), making them good candidates as appetite regulators in cod. In order to determine if NPY and CART have a role in the regulation of food intake in Atlantic cod, we used slot blots to examine changes in NPY and CART mRNA expression at several times during a scheduled feeding regimen (peri-prandial changes). In addition, we examined the effects of food deprivation on NPY and CART mRNA expression.

3.2 Materials and Methods

Animals

Cultured juvenile Atlantic cod (*Gadus morhua*) were obtained from the Aquaculture Research and Development Facility, Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Four groups of fish with an average starting weight of 100 g were acclimated for 5 weeks in 1000 L tanks (60 fish per tank) with flow-through water at 7°C under a 16:8-h light-dark cycle. The fish were fed EWOS Marine Diet, composed of 58% protein and 12% fat, once daily at the same time (12:00) with a total ration of 1% body weight. Fish were weighed weekly so that the average body mass of each tank could be estimated, and feed rations were adjusted in proportion to increasing body mass. To evaluate peri-prandial changes in NPY and CART mRNA expression, groups of fish (n = 15) were sampled 2 hours before feeding time (10:00), at mealtime (12:00), and 2 hours after feeding time (14:00). To examine the effects of fasting, one group (n =15) was food deprived for one week before sampling and sampled at mealtime (12:00). The remaining 45 fish from each tank were sampled and will be used for other experiments, not described here. For sampling, fish were anesthetized in 0.05% tricaine methanesulfonate, killed by spinal section and whole brains were dissected. All tissues were stored at -20°C in RNA*later* (Qiagen) until RNA isolations were performed.

Preparation of RNA

Total RNA was isolated from the forebrain (cerebellum and medulla removed) using a Trizol/chloroform extraction method with Tri-Reagent (BioShop). Final RNA

concentrations were determined by spectrophotometric readings at 260 nm. The forebrain was chosen based on previous results showing that this brain region displays the highest NPY and CART mRNA expression levels (Chapter 2, Fig. 2.4). Due to time constraints, we examined changes in the whole forebrain instead of examining changes in discrete brain regions.

Slot Blots

Slot blots were used to examine peri-prandial changes in mRNA expression, and to evaluate the effects of food deprivation. Biotin-labeled DNA probes were synthesized for slot blot detection of mRNA. NPY and CART were amplified from cDNA by PCR using primer pairs Biotin-NPY-1 (AGGCACAGAAGACAACTGAG) and Biotin-NPY-2 (AGAGCAGTTGTAGCACAAGC) or CART-F/CART-R (Chapter 2, Table 1.1). Before probe synthesis, the PCR products were cloned and sequenced (as per Chapter 2, section 2.2) to verify that the PCR products were either NPY or CART. Clones with NPY or CART inserts were used as templates for PCR amplification. The PCR products were quantified and used as templates for the synthesis of biotin-labeled DNA probes using the BioPrime DNA Labeling System (Invitrogen). A β-actin probe was generated as described above using primer set β-actin 1/β-actin 2 (Chapter 2, Table 1.1). Before performing slot blots with experimental samples, dilutions of RNA were blotted onto a membrane and detected to determine the optimal quantity of RNA to use. For slot blots, ten micrograms of total RNA from individual fish was denatured at 65°C for 10 min. Samples were blotted onto a Biodyne Membrane (Pierce, Rockford, Illinois, USA) by vacuum suction using a slot blot apparatus (Bio-Rad Laboratories, Mississauga, Ontario,

Canada). The membranes were then fixed by baking at 80°C for 1 h. Membranes were hybridized overnight at 55°C with either the NPY or CART biotin labeled probe. After hybridization, membranes were washed and the probe detected using the North2South Chemiluminescent Hybridization and Detection Kit (Pierce). After detection, the membranes were stripped for 1 hour at 60°C in 0.5% SDS and re-probed with the β-actin probe.

Data analysis and statistics

In gene expression studies, the hybridization signals were detected and quantified using the EpiChemi Darkroom and LabWorks. Each sample was expressed as a ratio of NPY/actin or CART/actin. All samples were then expressed as a percentage relative to the control group (Time +2H) which was set at 100%. Before any statistical tests, the data set was tested for normality and homogenous variances. To compare between different feeding groups, a one-way ANOVA followed by Tukey's multiple comparison was performed. Significance was set at $p<0.05$. All data are expressed as mean ± SEM.

3.3 Results

Peri-prandial changes in NPY mRNA expression

NPY mRNA expression levels were 50% lower 2 hours before feeding (-2H) compared with NPY mRNA levels at the scheduled feeding time (0H), and 30% lower compared to NPY mRNA levels 2 hours after mealtime (+2H). In contrast, NPY expression levels 2 hours after feeding time (+2H) were not significantly different from NPY expression levels at mealtime (0H) (Fig. 3.1A).

Peri-prandial changes in CART mRNA expression

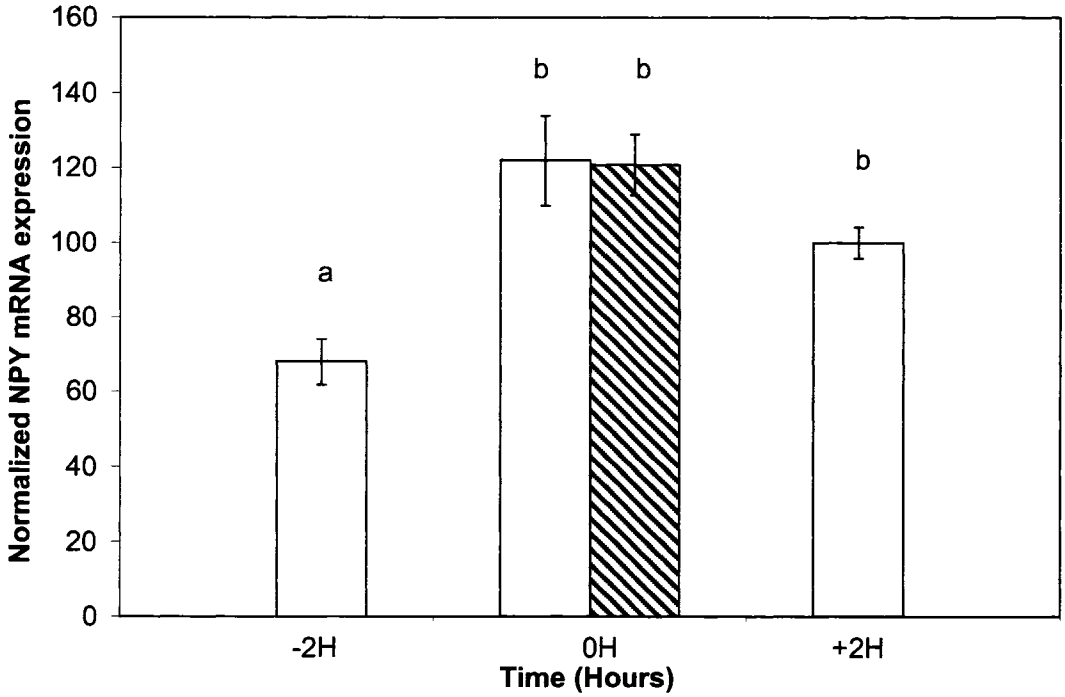
There were no significant differences between CART mRNA levels 2 hours before feeding time (-2H) and at feeding time (0H), however, CART mRNA levels 2 hours after mealtime (+2H) were 30% lower than CART levels at mealtime (0H) (Fig 3.1B).

Effects of food deprivation on NPY and CART expression

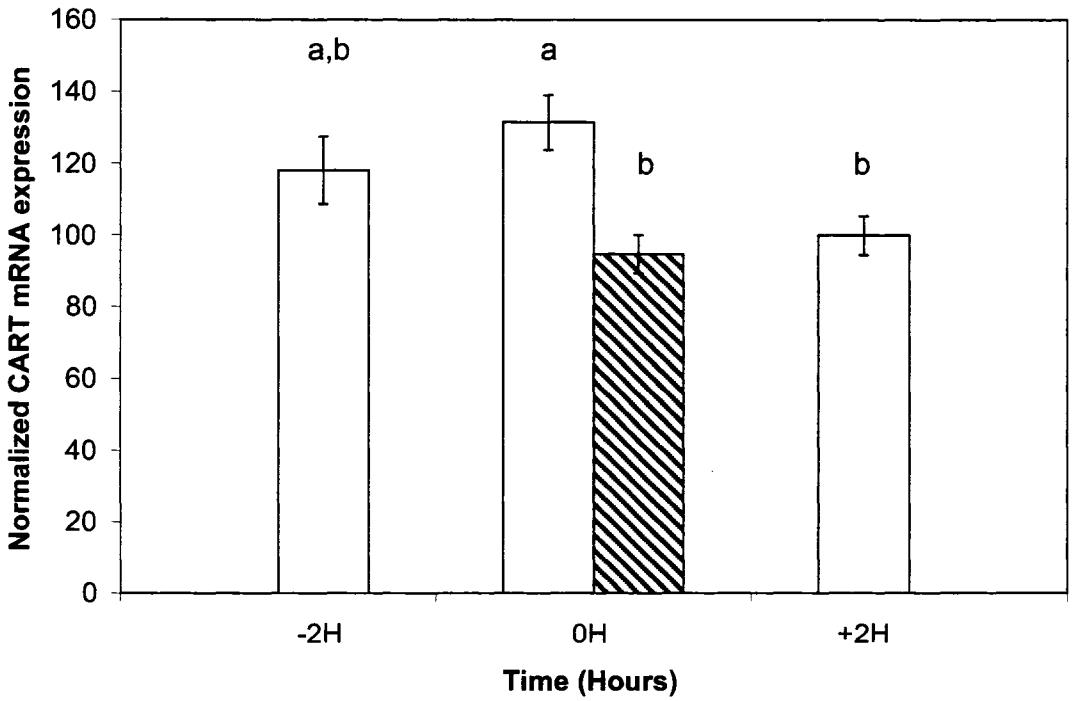
After seven days of food deprivation, there were no significant differences in NPY mRNA expression between fed and unfed fish (Fig 1A). Food deprivation resulted in a significant 35% decrease in CART mRNA expression in the forebrain of unfed fish compared to fed fish at mealtime (0H) (Fig 3.1B).

FIG. 3.1. Peri-prandial changes in NPY (A) and CART (B) mRNA expression within the forebrain and differences in NPY and CART mRNA expression in fed (open bars) and unfed fish (hatched bars) at scheduled feeding time (0H). Total RNA was isolated and detected using slot blots and biotin-labeled probes. NPY, CART and β-actin levels were quantified. NPY and CART expression levels were expressed as a ratio between NPY or CART to β-actin. All values were normalized to time +2 hours (+2H). Data is expressed as means ± SEM for both NPY (n = 8) and CART (n = 6). Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA. Groups that differ significantly are indicated with different letters.

A


160
140
120
100
80
60
40
20
0
Normalized NPY mRNA expression
a
b
b
b
-2H
0H
+2H
Time (Hours)

B


160
140
120
100
80
60
40
20
0
Normalized CART mRNA expression
a,b
a
b
b
-2H
0H
+2H
Time (Hours)

3.4 Discussion

Peri-prandial changes in NPY mRNA expression

Our results show that there are changes in NPY mRNA expression related to the daily feeding patterns of Atlantic cod. NPY mRNA expression increased 50% from 2 hours before the meal (-2H) to mealtime (0H). These results are in agreement with previous studies in goldfish that show that NPY mRNA expression in the brain increases dramatically 1-3 hours before feeding (Narnaware *et al.*, 2000). Peri-prandial changes in NPY expression have also been shown in rats where NPY peptide levels in the brain are elevated at meal time (Kalra *et al.*, 1991). The rapid increase in NPY expression at mealtime suggests that NPY is involved in the regulation of food intake in Atlantic cod, i.e. it might generate a hunger signal prior to meals.

No significant post-prandial changes in NPY mRNA expression were seen in the cod brain. These results differ from a previous study in goldfish that showed a rapid decrease in NPY mRNA expression 1 hour after mealtime (Narnaware *et al.*, 2000). Similarly, in rats, NPY peptide levels decrease progressively during a four hour feeding episode (Kalra *et al.*, 1991). The lack of significant decrease in NPY mRNA expression in this study could be explained by the fact that the fish used in the present experiment were fed a food ration of 1% body mass whereas in the study using goldfish, a 2% ration of total body mass was fed to the animals. Animals used in the current experiment were thus not fed to satiation and might not have received a sufficient amount of food to trigger a rapid decrease in NPY expression levels after a meal. Previous studies in goldfish have demonstrated that ration affects NPY expression levels. For example, a ration of 2% body

mass significantly decreased NPY mRNA expression in the brain as compared to fish fed 1% body mass (Narnaware *et al.*, 2000). Although we did not detect any post-prandial variations in NPY expression, NPY mRNA levels increased dramatically at mealtime suggesting that NPY is involved in daily feeding.

Peri-prandial changes in CART mRNA expression

Our results show that in cod, CART mRNA expression levels did not change before the scheduled feeding time, but decreased 30% after mealtime (+2H). To our knowledge, this is the first study to examine CART mRNA expression before feeding in fish. In rodents fed *ad libitum*, CART mRNA increases in the brain before the onset of the dark phase, when rodents increase food intake (Vicentic *et al.*, 2005). It is difficult to compare the results of our study with these results since both the species examined and the feeding regimens used in these studies were different.

The post-prandial decrease in CART mRNA expression seen in this study contrasts with a previous study in goldfish showing that CART mRNA expression increases in the brain after mealtime (Volkoff and Peter, 2001). However, in goldfish, post-prandial variations only occurred in the hypothalamus and olfactory bulbs (Volkoff and Peter, 2001). Since we examined changes using the whole forebrain, it is possible that small post-prandial increases in CART expression within discrete brain regions could not be detected. Since there is limited information on the appetite-regulating role of CART in fish, it is difficult to explain the decrease in CART mRNA levels after mealtime. As for NPY, CART mRNA levels may be influenced by food ration. We used a food ration of 1% body mass whereas Volkoff and Peter (2001) used a food ration of 2%

body mass. However, we can only speculate on the effects of ration on CART expression since this has not been investigated.

Effects of food deprivation on NPY and CART mRNA expression

Food deprivation did not affect NPY expression in cod, as NPY mRNA expression levels were the same for fed and unfed fish at their scheduled feeding time. This is contrary to other studies in fish that have shown that NPY mRNA is up-regulated in the brain during fasting (Narnaware *et al.*, 2000; Silverstein *et al.*, 1998). In previous studies examining the effects of fasting, NPY mRNA expression was measured in discrete brain regions whereas we used the whole forebrain. The effects of fasting on NPY appear to vary according to specific brain regions and differ between species. For example, during fasting, NPY mRNA levels in the optic tectum increase in goldfish (Narnaware *et al.*, 2000) but do not change in salmon (Silverstein *et al.*, 1998). It is possible that by using the whole forebrain we were not able to detect small changes in NPY expression within specific brain regions. In addition, sampling both the fed and unfed fish at the scheduled mealtime may have affected our ability to detect the effects of food deprivation on NPY mRNA levels. Since NPY mRNA levels appear to be highest at the scheduled mealtime as shown by our peri-prandial studies, it is possible that the basal NPY mRNA levels of unfed fish were elevated and small differences in expression between fed and unfed fish could not be detected. Now that we have evaluated peri-prandial changes in NPY mRNA expression in cod, optimal sampling times can be determined for future studies in order to compare fed and unfed fish. We must also consider that the duration of food deprivation may have not been sufficient to cause any

significant changes in NPY mRNA expression. In goldfish, NPY expression levels increased after 72 hours of food deprivation (Narnaware and Peter, 2001), while in catfish, three weeks of food deprivation were required to reveal any detectable changes in NPY expression in the brain (Silverstein and Plisetskaya, 2000). However, cod have a different physiology and life history than that of these two freshwater, warm water fish. Previous studies have shown that, in adult cod, five weeks of fasting does not induce any significant changes in total mass, length or condition factor (Belanger *et al.*, 2002). Cod submitted to longer periods of food deprivation either display high mortality rates (100 days starvation) (Dutil and Lambert, 2000) or have significantly lower body weights and lower growth rates compared to fed fish (10 weeks starvation) (Guderley *et al.*, 1996). It is possible that the one week food deprivation period used in the present study was not sufficient to cause significant changes in NPY expression.

Fasting caused a 30% decrease in CART mRNA expression in the forebrain of Atlantic cod as compared to fed fish just prior to mealtime (0H). This result suggests that CART mRNA expression levels are affected by food deprivation. Similar studies in mammals and goldfish have shown that CART mRNA decreases in the brain during fasting (Kristensen *et al.*, 1998; Vicentic *et al.*, 2005; Volkoff and Peter, 2001).

In mammals, leptin is an important peripheral signal of energy stores, and NPY and CART neurons in the brain express leptin receptors (Baskin *et al.*, 1999; Elias *et al.*, 2001). The reduction of circulating leptin levels during fasting is thought to be the primary feedback signal to the brain (Schwartz *et al.*, 2000). Since Atlantic cod CART mRNA expression is affected by nutritional status, CART neurons probably receive

information on energy stores. However, our data do not allow us to conclude which peripheral signals might relay this information.

Implications

The present study provides evidence that NPY and CART are feeding-related peptides in the Atlantic cod. Both NPY and CART appear to be involved in daily feeding. We demonstrated that CART is regulated by food deprivation, however, we were unable to show that NPY is regulated by food deprivation. Given the complexity of the endocrine regulation of feeding in vertebrates, it is likely that other neuropeptides are involved in the regulation of food intake in Atlantic cod. Future studies will help elucidate if this is the case.

4.0 Effects of temperature on NPY and CART mRNA expression

4.1 Introduction

Temperature is one of the most important abiotic factors regulating physiological processes. Atlantic cod (*Gadus morhua*) are eurythermal and can survive in temperatures ranging from below zero to 24°C (Jobling, 1988). Temperature influences many feeding-related factors in Atlantic cod including appetite (Brown *et al.*, 1989), feeding frequency (Waiwood *et al.*, 1991), feed conversion (Bjornsson *et al.*, 2001), food consumption rates (Peck *et al.*, 2003), and gastric evacuation rates (Singh-Renton and Bromley, 1996). Atlantic cod increase food consumption at higher temperatures (Brown *et al.*, 1989; Singh-Renton and Bromley, 1996; Waiwood *et al.*, 1991). A positive correlation between temperature and food intake has also been demonstrated in several other species including Atlantic halibut, *Hippoglossus hippoglossus* (Jonassen *et al.*, 2000), Atlantic salmon, *Salmo salar* (Bendiksen *et al.*, 2002), turbot, *Scophthalmus maximus* (Burel *et al.*, 1996) and channel catfish, *Ictalurus punctatus* (Buentello *et al.*, 2000).

Food intake affects growth rate, and increased food intake at higher temperatures is associated with higher growth rates in Atlantic cod (Peck *et al.*, 2003). Further, North Atlantic cod stocks from geographic areas with a higher mean temperature (11°C) have higher body masses compared to stocks from waters with lower temperatures (2°C) (Brander, 1995). In addition, temperature also affects food acceptance since Atlantic cod display an increase in feeding latency, food rejection rate and food handling at 3°C compared to 4.7°C or 9.1°C (Clark *et al.*, 1995). Feeding strategies are also affected by temperature as juvenile Atlantic salmon switch from diurnal to nocturnal foraging at low

temperatures (Fraser *et al.*, 1993). Cod display seasonal changes in activity, being nocturnal during the summer and diurnal in the autumn (Clark and Green, 1990), but it is not clear how these changes in activity relate to feeding.

In mammals, there is limited information on the endocrine regulation of food intake at different temperatures. Since mammals are endotherms, they respond to cold exposure by increasing their metabolic rate in order to maintain a stable core body temperature. Consequently, mammals must increase food intake during cold exposure to sustain the increased metabolic demand. NPY and CART expression in mammals is affected by cold exposure (Kong *et al.*, 2003; McCarthy *et al.*, 1993; Mercer *et al.*, 1997), suggesting that these peptides are involved in mediating temperature-dependent changes to food intake. In contrast, fish are ectotherms that decrease their metabolic rate and food intake at lower temperatures. The effect of temperature on feeding-related peptides has never been examined in fish, but it is likely that feeding-related peptides respond differently to temperature in fish and mammals.

In this study, we performed a pilot study to assess the role of feeding-related neuropeptides in mediating changes in food intake at different temperatures in the Atlantic cod by examining NPY and CART mRNA expression in fish acclimated at 2°C, 5°C, 11°C and 15°C.

4.2 Materials and Methods

Animals

Cultured juvenile Atlantic cod (*Gadus morhua*) were obtained from the Aquaculture Research and Development Facility, Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Fish with an average starting weight of 100g were acclimated in 5000 L tanks (n = 30) under natural photoperiod. To evaluate the effect of temperature, four groups (n = 30) were acclimated for 5 weeks to different temperatures, 2°C, 5°C, 11°C and 15°C. The fish were fed EWOS Marine Diet, composed of 58% protein and 12% fat, once daily at the same time (12:00) with a ration of 1% body mass. Cod held at 11°C were considered as the control group since this is the normal temperature at which cultured fish are maintained and because cod feed well at this temperature (J. Brown personal communication). To examine the effects of cold temperature, 2°C and 5°C were chosen, and 15°C was chosen to examine the effects of a high temperature. At the end of the acclimation period, 20 fish from each group were sampled for brain tissue 30 minutes after the scheduled feeding time. Fish were anesthetized in 0.05% tricaine methanesulfonate and killed by spinal section. Whole brains were dissected and stored at -20°C in RNA*later* (Qiagen) until RNA isolations were performed. After the brain was dissected, the gastrointestinal tract of each fish was removed and qualitative observations on the gut contents were made. The remaining fish were used for other experiments that are not described here.

RNA Isolations

Total RNA from the forebrain was isolated using a trizol/chloroform extraction as per Chapter 3, section 3.2.

Slot Blots

Total RNA from individual fish brains was applied to a membrane and hybridized with either an NPY or CART biotin-labeled probe. Probe synthesis, blotting and detection were performed as per Chapter 3, section 3.2.

Data analysis and statistics

All data was normalized to the control group (11°C) which was set at 100%. To compare between different groups, a 1-way ANOVA was performed as per Chapter 3, section 3.2.

4.3 Results

Observations on gut contents at different temperatures

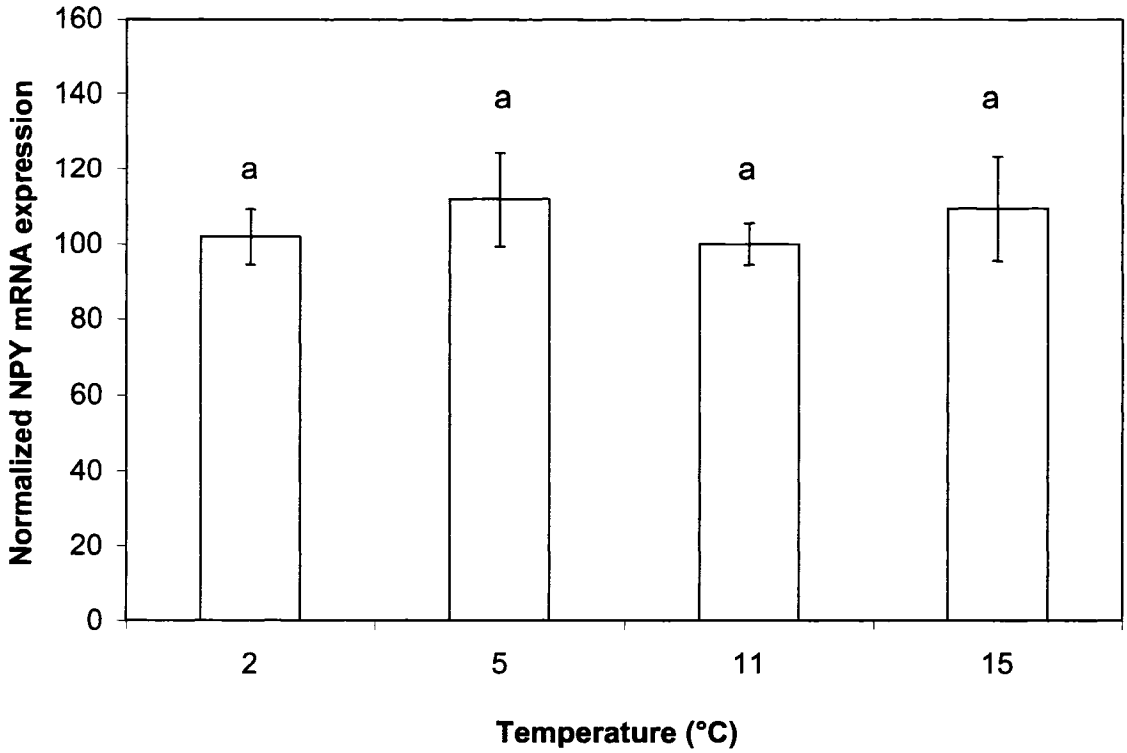
We observed that the stomachs from the fish acclimated to 2°C contained less food than the fish acclimated to the higher temperatures, as seen by a lower number of pellets. Several of the fish acclimated to 2°C had empty stomachs, which was not observed in any fish acclimated to the 3 other temperatures. Furthermore, the appearance of the gastrointestinal tract in 2°C fish differed from that of the other fish in that their gastrointestinal tracts appeared watery and distended.

Effects of temperature on NPY and CART mRNA expression

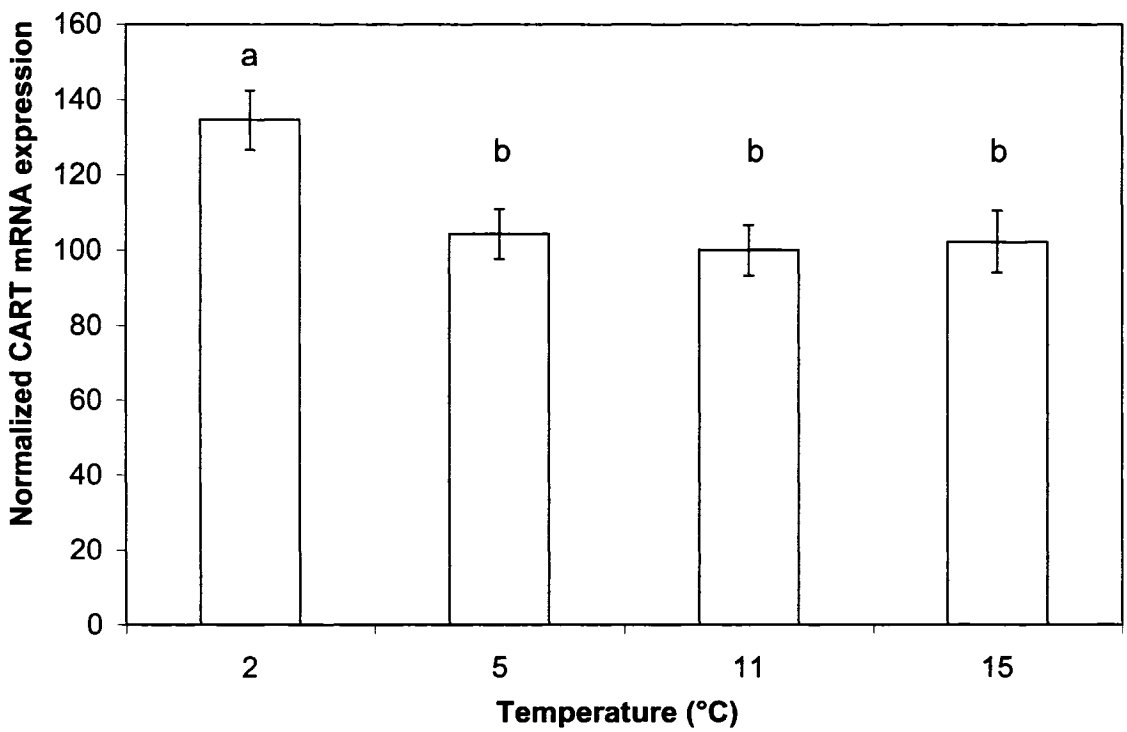
There were no significant differences in NPY mRNA expression at the different temperatures (Fig 4.2A). In contrast, CART mRNA expression was 35% higher in fish acclimated at 2°C compared to fish acclimated at 5°C, 11°C or 15°C. There were no significant differences in CART mRNA expression between fish acclimated to 5°C, 11°C and 15°C (Fig 4.2B).

FIG 4.2. NPY (A) and CART (B) mRNA expression levels in the forebrain of Atlantic cod acclimated to different temperatures. Slot blots and biotin-labeled probes were used to measure mRNA expression levels. NPY and CART expression levels are expressed as a ratio between NPY or CART to β-actin. All values were normalized to the control group (11°C). Data are presented as mean ± SEM for both NPY (n = 11-12) and CART (n = 10). Significant differences ($p<0.05$) between groups were detected using a 1-way ANOVA. Groups that differ significantly are indicated with different letters.

A


Normalized NPY mRNA expression
160
140
120
100
80
60
40
20
0
a
a
a
a
2
5
11
15
Temperature (°C)

B


Normalized CART mRNA expression
160
140
120
100
80
60
40
20
0
a
b
b
b
2
5
11
15
Temperature (°C)

4.4 Discussion

Gut contents at different temperatures

Fish acclimated to 2°C appeared to have less food in their guts, suggesting food intake was lower at this temperature. However, we were only able to make qualitative observations on gut contents from fish acclimated to different temperatures, and further studies using a quantitative method of evaluating food intake (e.g. behavioural observations or pellet counts in the stomach) should be used for a more accurate assessment of the effects of temperature on food intake.

NPY mRNA expression at different temperatures

Our results suggest that changes in temperature from 2°C to 15°C do not affect NPY mRNA expression. Although NPY does not appear to be regulated by temperature at the gene level, it is possible that temperature regulates NPY activity at the peptide level. Further studies using western blots may clarify the effect of temperature on NPY peptide levels.

In fish as in mammals, NPY is an important regulator of energy homeostasis. Previous studies have shown that Atlantic cod have a lower metabolic rate at lower temperatures (Schurmann and Steffensen, 1997). After a five week acclimation period, fish may alter their metabolic rates in response to temperature, adjusting their food intake and NPY levels accordingly. Previous studies in mammals have shown that NPY mRNA expression levels increase during acute cold exposure (Mercer *et al.*, 1997), but not during chronic cold exposure (Bing *et al.*, 1998), suggesting that NPY mRNA expression

is normalized during prolonged temperature changes. Although it is difficult to compare between mammals and fish, it is possible that a five weeks acclimation period induced a "normalization" of NPY levels. Acute exposures to low and high temperatures might be necessary to induce more dramatic changes in NPY expression in cod.

CART mRNA expression at different temperatures

Our results show that CART mRNA expression levels were 30% higher at the lowest temperature (2°C). Previous studies in cod have shown that food intake decreases as the temperature is decreased from 8°C to 1°C (Brown *et al.*, 1989; Waiwood *et al.*, 1991), and from 12°C to 6°C (Singh-Renton and Bromley, 1996). Our results suggest that the anorexigenic peptide CART may be involved in mediating this decrease in food intake. There were no differences in CART mRNA expression between fish acclimated at 5°C, 11°C and 15°C suggesting CART peptides do not regulate changes in appetite, if any, across these temperatures.

Conclusion

In our study, NPY mRNA expression did not appear to be regulated by temperature whereas CART mRNA expression was only up-regulated at the lowest temperature but not affected by higher temperatures. The absence of changes in NPY expression might reflect a true physiological phenomenon. It is also possible that our experimental conditions were not appropriate. Temperatures used might not have been extreme enough since cultured cod live at 11°C and their preferend zone ranges from 9-17°C (Jobling, 1988). It is also possible that gradual acclimation affected the results, and

acute exposures might assist in determining the effects temperature has on these neuropeptides. In Atlantic cod, the physiological mechanisms regulating changes in appetite at different temperatures is still unclear. This study was a pioneering study examining the effects of temperature on feeding-related neuropeptides in fish. Our results show for the first time that CART may be involved in mediating changes in appetite at lower temperatures. However, more studies are needed in order to determine which temperature-sensitive signals induce these changes in neuropeptide expression.

5.0 Summary

We have cloned complete cDNAs encoding for Atlantic cod NPY and CART and a partial cDNA for orexin. NPY mRNA is localized within the forebrain whereas CART mRNA is localized throughout the brain. Both NPY and CART mRNAs are expressed in the hypothalamus, suggesting they are involved in the regulation of food intake. During a daily feeding period, there are peri-prandial changes in NPY and CART mRNA expression. CART mRNA is down-regulated by a negative energy balance produced by fasting. In contrast, NPY mRNA does not appear to be affected by food deprivation. Temperature does not appear to regulate NPY mRNA expression whereas CART mRNA is up-regulated at very low temperatures (2°C). Overall, our results suggest that NPY and CART are involved in regulating food intake in cod.

The regulation of food intake is an important factor for the commercial success of aquaculture projects. The application of genetic and molecular tools for the improvement of aquaculture production is currently being investigated for many commercially important fish. In channel catfish, different feeding phenotypes can be accounted for by genetic variability since different strains and families display differences in food intake (Silverstein *et al.*, 2001; Silverstein *et al.*, 1999). Strain differences in feeding efficiency have also been observed in rainbow trout (Silverstein *et al.*, 2005). Although there is limited information on the impact feeding-related neuropeptides have in determining feeding phenotypes, some of these neuropeptides might represent good candidates as genetic markers to select for optimal feeding phenotypes. For example, some strains of

channel catfish are more responsive to NPY (Silverstein, 2002), and NPY is currently being evaluated as a possible biomarker for food intake.

We have isolated three potential feeding-related peptides from the Atlantic cod and have shown that two of them are affected by nutritional status and environmental condition. In this pioneering study in cod, we examined changes in mRNA expression in a major brain division (the forebrain), and future studies examining NPY and CART expression within discrete brain regions might provide more detailed information on the regulation of these peptides. Certainly, more central and peripheral factors regulating food intake are present in cod. Future studies using behavioural and molecular tools will help elucidate their structure and role in the neuroendocrine regulation of feeding in cod.

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