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Orexin and neuropeptide Y: Tissue specific expression and immunoreactivity in the hypothalamus and preoptic area of the cichlid fish *Cichlasoma dimerus*

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ABSTRACT

Neuropeptide Y (NPY) and orexin are neuropeptides involved in the regulation of feeding in vertebrates. In this study we determined the NPY and orexin mRNA tissue expression and their immunoreactivity distribution in both preoptic area and hypothalamus, regions involved in the regulation of feeding behavior. Both peptides presented a wide expression in all tissues examined. The NPY-immunoreactive (ir) cells were localized in the ventral *nucleus posterioris periventricularis* (NPPv) and numerous ir-NPY fibers were found in the *nucleus lateralis tuberis* (NLT), the *nucleus recess lateralis* (NRL) and the neurohypophysis. Ir-orexin cells were observed in the NPPv, dorsal NLT, ventral NLT, lateral NLT (NLTl) and the lateral NRL. Ir-orexin fibers were widespread distributed along all the hypothalamus, especially in the NLTl. Additionally, we observed the presence of ir-orexin immunostaining in adenohypophyseal cells, especially in somatotroph cells and the presence of a few ir-orexin-A fibers in the neurohypophysis. In conclusion, both peptides have an ubiquitous mRNA tissue expression and are similarly distributed in the hypothalamus and preoptic area of *Cichlasoma dimerus*. The presence of ir-orexin in adenohypohyseal cells and the presence of ir-orexin and NPY fibers in the neurohypophysis suggest that both peptides may play an important neuroendocrine role in anterior pituitary.

1. Introduction

In vertebrates, the regulation of appetite and body weight is a complex phenomenon involving elaborate interactions between the brain and peripheral signals (Volkoff et al., 2005). The hypothalamus, which is the major site in the control of these processes (Demski and Northcutt, 1983; Peter and Crim, 1979), produces key factors that either stimulate (orexigenic) or inhibit (anorexigenic) food intake. Peptides like neuropeptide Y (NPY), α-melanocyte stimulating hormone (αMSH), galanin, ghrelin, agouti related peptides (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), melanin-concentrating hormone (MCH) and orexins (A and B) among others have shown to be involved in feeding regulation (Volkoff et al., 2009).

Neuropeptide Y is a 36-amino acid peptide belonging to the pancreatic polypeptide family (PP) (Tatemoto, 1982; Tatemoto et al., 1982). It is distributed in the brain of cyclostomes (Chiba et al., 1993; Rawitch et al., 1992), elasmobranches (Chiba and Honma, 1992; MacDonald and Volkoff, 2009; Vallarino et al., 1988), dipnoans (Trabucchi et al., 2000; Vallarino et al., 1995), teleosts (Chiba et al., 1996; Cepriano and Schreibman, 1993; Danger et al., 1991; Gaikwad et al., 2004; Pirone et al., 2008; Pontet et al., 1989; Rodríguez-Gómez et al., 2001; Traverso et al., 2003), amphibians (Cailliez et al., 1987; Danger et al., 1985; Perroteau et al., 1988), reptilians (Bennis et al., 2001; Medina et al., 1992), birds (Aste et al., 1991) and mammals (Bons et al., 1990; Smith et al., 1985). NPY is the most potent orexigenic factor described in vertebrates (Eva et al., 2006; Kalra et al., 1999; Morley, 1987). In particular, several studies performed in teleost fish, have demonstrated its orexigenic role. For example, food deprivation promotes the hypothalamic expression of NPY mRNA in goldfish (Narnaware and Peter, 2001) and tiger puffer, *Takifugu rubripes* (Kamijo et al., 2011). Furthermore, intracerebroventricular (ICV) injections of mammalian and fish NPY cause a dose-dependent increase in food intake in goldfish, *Carassius auratus* (de Pedro et al., 2000; López-Patiño et al., 1999; Narnaware et al., 2000; Volkoff and Peter, 2001), rainbow

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trout, *Oncorhynchus mykiss* (Aldegunde and Mancebo, 2006), catfish, *Ictalurus punctatus* (Silverstein and Plisetskaya, 2000) and zebrafish, *Danio rerio* (Yokobori et al., 2012). In fish as in mammals, NPY seems to interact with other appetite regulators, such as CRF, cortisol, CART, leptin, orexins and galanin (Volkoff et al., 2005) and MCH (Matsuda et al., 2009).

Orexin-A and orexin-B (also known as hypocretin 1 and hypocretin 2, respectively) are two peptides produced by proteolytic cleavage of the common precursor prepro-orexin (Sakurai, 2002). The amino acid sequences of fish orexins show regions of low homology with those from other vertebrates and among teleost fish (in Fig. 3 in Xu and Volkoff, 2007). Orexin has been described as an orexigenic factor in fish. In zebrafish (Novak et al., 2005; Yokobori et al., 2011) and barfin flounder, *Verasper moseri* (Amiya et al., 2012), food deprivation induces a significant increase in brain prepro-orexin mRNA levels. Atlantic cod, *Gadus morhua*, fed with a low ration, evidences decreased levels of brain prepro-orexin mRNA levels 2 h post prandial (Xu and Volkoff, 2007). In goldfish, orexin mRNA expression in brain; and particularly the hypothalamic orexin-like immunoreactivity in the *nucleus recess lateralis* (NRL) is induced by a fasting condition (Nakamachi et al., 2006). In addition, ICV injection of orexin stimulates food intake in goldfish (Nakamachi et al., 2006; Volkoff et al., 1999) and zebrafish (Yokobori et al., 2011). The co-administration of orexin and NPY in goldfish brain results in a synergistic orexigenic effect (Volkoff and Peter, 2001; Volkoff et al., 2003).

This study was undertaken in the South American cichlid fish *Cichlasoma dimerus*. *C. dimerus* represents an interesting experimental model due to its high survival, reproductive rates and high growth rates under laboratory conditions. Both NPY and orexin are the most studied orexigenic factors and for that reason we decided to study them in *C. dimerus*. The present work describes the tissue specific distribution of these peptides with particular interest on their distribution in the preoptic and hypothalamic areas. The preoptic area is considered to form a structural and functional continuum with the hypothalamus forming a single complex (Nieuwenhuys et al., 1998).

2. Materials and methods

2.1. Animals

Adult *C. dimerus* of both genders with an average of body weight of 25 g and of total length of 10 cm were collected from "Esteros del Riachuelo", Corrientes, Argentina (27°12′50″ S, 58°11′50″ W). They were transferred to the laboratory and acclimated in fresh water tanks (400 l) under stable condition of temperature (25 ± 2 °C) and photoperiod (14 h light:10 h dark), where they were daily fed with commercial pellets (Tetra Pond Variety Blend) until they were anesthetized with benzocaine 0.1% before sample procedure. For pituitary NPY mRNA expression one week fasted animal were used. Principles of laboratory animal care (guidelines on the care and the use of fish in research, teaching and testing, Canadian Council on Animal Care, 2005) were followed.

2.2. Tissue distribution of the NPY and prepro-orexin mRNA expression in C. dimerus

In order to determine the tissue distribution of NPY and prepro-orexin first we obtained a partial sequence of both mRNA. Briefly, brains from two adults' *C. dimerus* were dissected and total RNA was extracted by TRI Reagent (MRC, Inc., Cincinnati, USA) following the manufacturer's instructions. Total RNA (2 μg) was then treated with DNase I (Sigma, St. Louis, USA) and the first strand cDNA was synthesized by using an AMV enzyme (Promega, Madison, WI, USA). Conditions for the reverse transcription (RT) reaction were: 45 °C for 50 min and 70 °C for 10 min. Degenerate primers (Table 1) were designed according to conserved regions of NPY or prepro-orexin cDNA sequences of phylogenetic related fish available in the GenBank database. PCR amplification for NPY and prepro-orexin were performed by using a GoTaq Flexi DNA polimerase (Promega, Madison, WI, USA). Following initial 3 min of denaturation at 94 °C, the PCR cycle was repeated 40 times with denaturation at 94 °C for 30ʏs, annealing at 52 °C for 30ʏs and elongation at 72 °C for 30ʏs with a last extension step at 72 °C for 10 min. After electrophoresis on 1% agarose gel, an unique expected size band for NPY or prepro-orexin was purified from the agarose gel by using an AccuPrep gel purification kit (Bioneer, Alameda, CA). The purified PCR products were subsequently sequenced (Servicio de secuenciación y genotipificado, EGE, FCEN-UBA, Argentina) and confirmed to be partial NPY or prepro-orexin sequence using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

Table 1
PCR primers for NPY and prepro-orexin sequencing.

Name	Sequence (5′ → 3′)
NPY forward	ACTACTCAGCCCTGAGACAC
NPY reverse	GGTCRTATCTTGACTGTGG
Prepro-orexin forward	TCARCTGRCCTGTGACGC
Prepro-orexin reverse	GTTCTCTTCCCCATTGTCAG

R: G/A.

The tissue distribution of NPY and prepro-orexin was studied by fluorescent-based real-time polymerase chain reaction (qPCR). Two adult *C. dimerus* were anesthetized with benzocaine 0.1% and the telencephalon, hypothalamus, the remaining of the brain (in which the dorsal part of the diencephalon, middle brain and posterior brain without the medulla were pooled together), pituitary, skeletal muscle, liver, spleen, gill, stomach, intestine, ovary, testis, skin, kidney were collected after decapitation. Extraction of RNA from each tissue and synthesis of first strand cDNAs were performed as it was described above. Real time PCRs for NPY, prepro-orexin and acidic ribosomal phosphoprotein P0 (ARP, reference gene) were performed by using FastStart Universal SyBR green Master (ROCHE) with a mixture of forward and reverse specific primers (Table 2) designed on the sequence obtained (for ARP GenBank Accession No. GU244484) and 2 μl of cDNA template per tube. The qPCR protocol was as follows: 10 min of denaturation at 95 °C and 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 20 s. For each amplicon a negative control was performed by template omission. A single melting peak was obtained for each gene amplification and their efficiencies were close to 100%. ARP expression was stable among the replication measures. Raw qPCR data was submitted to LinRegPCR software processing in order to obtain initial fluorescence values per sample for subsequent analysis (Ramakers et al., 2003; Ruijter et al., 2009).

Table 2
Primers used for tissue distribution by real time PCR.

Name	Sequence (5′ → 3′)
NPY forward	GCCCTGAGACACTACATCAAC
NPY reverse	TGTGGAAGCGTGTCTGTG
Prepro-orexin forward	TGTCTGAGTGCTGCAGAGAAC
Prepro-orexin reverse	GCTCGTCCTCTTTTCGTTTG
ARP forward	ACTGTGGGAGCAGACAATG
ARP reverse	TCCAGTGCAGGATTGTTCTC

2.3. NPY and orexin immunohistochemistry in C. dimerus hypothalamus and preoptic area

Six brains with the pituitary attached from adult fish were fixed for 18 h in Bouin's solution, embedded in paraplast and coronally sectioned at 10 μm intervals. Those sections at the preoptic-hypothalamic level were mounted on gelatin-coated slides, deparaffinized in xylene, rehydrated through graded ethanol up to phosphate buffered saline (PBS, pH 7.4) and incubated for 5 min in 0.3% hydrogen peroxide in order to block endogenous peroxidase activity. Then, they were washed in PBS and incubated for 30 min in PBS containing 5% non-fat dry milk at room temperature (RT) to reduce nonspecific staining. Next, they were incubated overnight (ON) at 4 °C with the primary anti-NPY (1:2500 rabbit anti-porcine NPY serum, Peninsula Laboratories Inc., CA) and anti-orexin (1:1000 rabbit anti-human orexin-A serum, code no. 14346-v; Peptide Institute, Osaka, Japan or 1:500 mouse anti-human orexin-B monoclonal, catalog no. MAB734; R&D Systems, Inc., Minneapolis, MN). After that, biotinylated anti-rabbit/goat IgG and peroxidase-conjugated streptavidin were applied for 1 h each. The final reactive products were visualized with 0.3% 3,3′-diaminobenzidine in Tris buffer (pH 7.6) and 0.02% H_2O_2. The sections were then slightly counterstained with hematoxylin, mounted with DPX and examined with a NIKON Microphot FX microscope and digitally photographed. Immunoreactive (ir-) NPY and ir-orexin cell area (in μm^2) were measured at the different levels using an Image Pro Plus 6.1 (Media Cybernetics) software. Only those cells with detectable nuclei were included in the analysis. The anatomical location of the somata and fibers was evaluated following the atlas published for Sea bass (Cerdá-Reverter et al., 2001) due to the anatomical brain similarity between this species and *C. dimerus*.

A great immunostaining of orexin-A was observed in the proximal *pars distalis* of the pituitary, in the same place where the growth hormone (GH) cells were previously found in *C. dimerus* (Pandolfi et al., 2001). In order to determine if ir-orexin was localized in GH cells, serial consecutive sections (10 and 4 μm) were performed in order to compare the immunostaining of ir-orexin-A cells with ir-GH cells. To identify ir-GH cells, anti *Oncorhynchus keta* GH diluted 1:1000 (anti-GH; Bolton et al., 1986) was used. The protocol of immunostaining was the same as described above. No double label immnunohistochemistry was performed due to both antisera were raised in rabbit.

Specificity of the immunoreactions: To check the specificity of NPY and orexin antisera the following controls were performed: sections were incubated with PBS or with primary antisera preadsorbed with porcine NPY (50 μg/ml) (Bachem, Bubendorf, Switzerland) and synthetic human orexin-A (50 μg/ml, Peptide Institute, Osaka, Japan) or orexin-B (50 μg/ml, Peptide Institute) respectively instead of the primary antibody. The specificity of anti chum GH was previously carried out by preadsorption test for *C. dimerus* (Pandolfi et al., 2001).

3. Results

3.1. Tissue distribution of the NPY and prepro-orexin mRNA expression in C. dimerus

Specific products for NPY and prepro-orexin cDNA were amplified from cDNA obtained from brain of adult *C. dimerus* (cd-). The cd-NPY and cd-prepro-orexin fragments consisted of 105 bp and 233 bp respectively, both containing a partial region of the mature peptides. The former presents a high identity (over 90%) with NPY of perciform and pleuronectiform fish such as

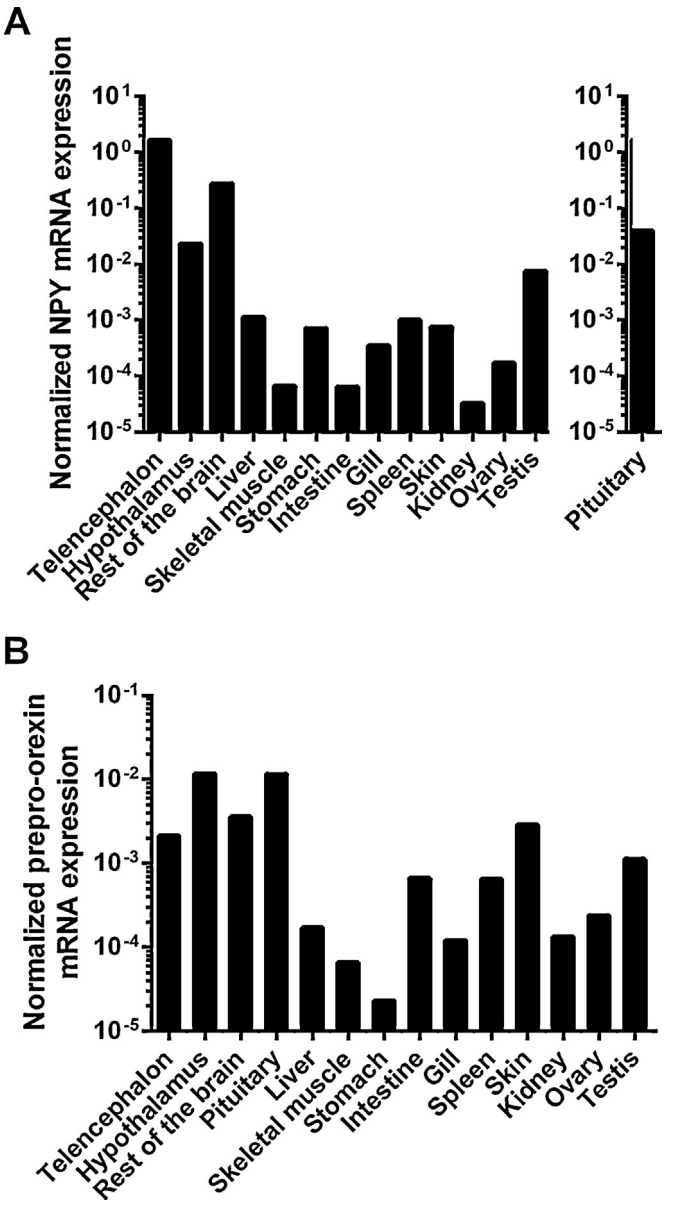

Fig. 1. Real time PCR distribution of NPY (A) and prepro-orexin (B) mRNA in different *C. dimerus* tissues. Samples were analyzed by LinRegPCR software. (A) mRNA levels of pituitaries were measured in one week fasted fish.

Epinephelus coioides (GenBank Accession No. AY626561) and *Paralichthys orbignyanus* (GenBank Accession No. FJ705358) and the latter shows around 90% of identity with prepro-orexin of perciform fish, such as *Oreochromis niloticus* (GenBank Accession No. FJ871159).

The analysis by qPCR showed that NPY and prepro-orexin present a widespread distribution in all the tissue analyzed. The highest levels of NPY expression were found in the brain, particularly in the telencephalon, and also in the testis (Fig. 1A). NPY expression was undetectable in pituitary of *C. dimerus*. However, when we analyzed NPY expression in the pituitaries of one week fasted fish a higher expression was obtained. On the other hand, the highest levels of prepro-orexin were found in the brain, mainly in the hypothalamus and also in the pituitary. In addition, a high expression was observed in skin, intestine, spleen and testis (Fig. 1B).

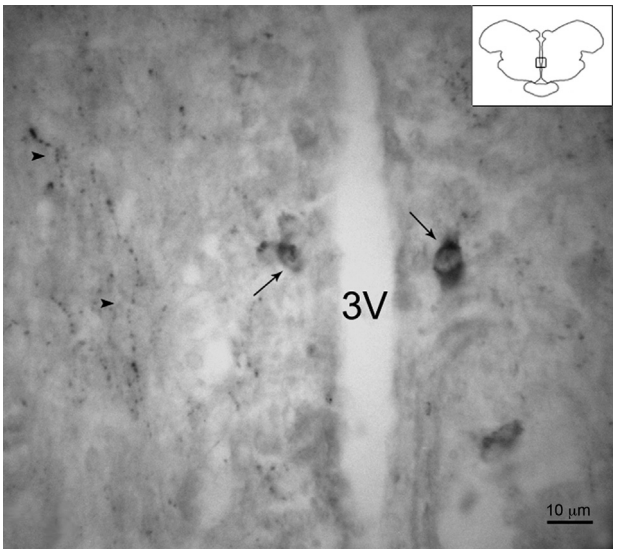

Fig. 2. Microphotography showing ir-NPY cell bodies in the NPPv (arrows) at both sides of the third ventricle (3V) and fibers parallel to 3V (arrowhead). On the right top a camera lucida drawing shows the coronally sections of *C. dimerus* brain and the boxed area indicate the microphotography section. Scale bar: 10 μm.

3.2. NPY and orexin immunohistochemistry in C. dimerus hypothalamus and preoptic area

Immunohistochemistry demonstrated the presence of specific NPY-immunoreactive (ir) material throughout the entire hypothalamus of adult *C. dimerus*. The ir-NPY cells were localized in the preoptic area, specifically, in the ventral *nucleus posterioris periventricularis* (NPPv) at both sides of the diencephalic ventricle (Fig. 2). The somata area, measured on average, was $64.43 \pm 4.15\ \mu m^2$. Particularly, the preoptic area and the hypothalamus displayed numerous positive varicose fibers mainly in the *nucleus lateralis tuberis* (NLT) but also in the NRL (Fig. 3). Numerous ir-NPY fibers were observed in the neurohypophysis at the *pars distalis* and *pars* intermedia level. Preadsorbtion test incubating the primary antiserum with an excess of NPY resulted in complete loss of immunostaining in the hypothalamus, preoptic area (data not shown) and pituitary (Fig. 4).

No immunoreactivity was detected using the orexin-B antiserum, we identified the staining that reacted with the orexin-A antiserum as ir-orexin.

Ir-orexin cells were observed bordering the third ventricle (3V) in the NPPv of the preoptic area and the dorsal NLT (NLTd) and ventral NLT (NLTv) of the hypothalamus (Fig. 5A). Moreover, ir-orexin cells were observed in the lateral NLT (NLTl) (Fig. 5B) and in the lateral NRL (NRLl) (Fig. 5C). Ir-orexin cells were rounded in shape. The average size of the ir-orexin cells bordering the 3V were $27.13 \pm 4.05\ \mu m^2$, the NRL ir-orexin cells were 29.04 and the NLTl ir-orexin cells were $46.97 \pm 6.15\ \mu m^2$. Orexin fibers were widespread distributed along all the hypothalamus, especially in the NLTl (Fig. 6).

Interestingly, in the pituitary we detected ir-orexin in cells bordering the neurohypophysis at proximal *pars distalis* morphological region. Sections incubated with the antiserum preadsorbed with orexin-A completely abolished the immunostaining both in the brain (data not shown) and the pituitary (Fig. 7) whereas preadsorption with orexin-B had no effect on the staining profiles (data not shown). In order to determine if ir-orexin were localized in GH cells, serial sections (10 and 4 μm) were performed and immunostain of both peptides compared. The distribution pattern and comparison of them in both consecutive sections clearly showed that GH cells express orexin (Fig. 8). Moreover, we detected ir-orexin fibers in the neurohypophysis at proximal *pars distalis* level (Fig. 9).

4. Discussion

In this study, we first obtained a partial sequence of NPY and prepro-orexin cDNA and designed specific primers in order to describe the mRNA tissue distribution of both genes in *C. dimerus* by real time PCR. Cd-NPY expression was detected in the brain and in all the analyzed peripheral tissues with different expression levels, as it was observed in other fish (Campos et al., 2010; MacDonald and Volkoff, 2009; Murashita et al., 2009). A similar result showing a different expression profile was found when analysing cd-prepro-orexin by qPCR in brain and peripheral tissues as it was also described in the Atlantic cod (Xu and Volkoff, 2007), in tilapia, *O. niloticus* (Chen et al., 2011) and in *E. coioides* (Yan et al., 2011). Such a widespread distribution for NPY and prepro-orexin

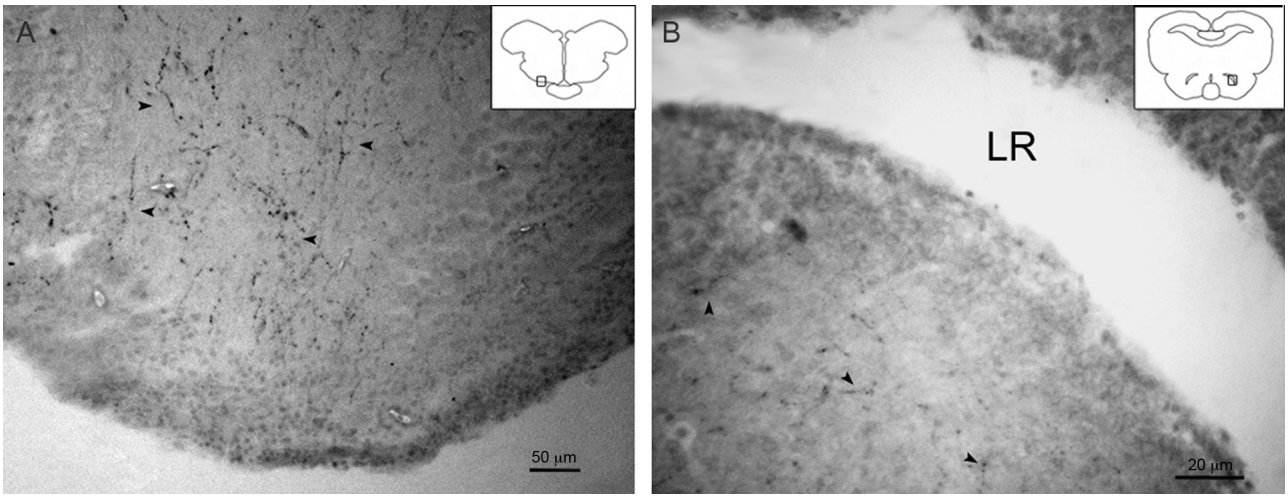

Fig. 3. Ir-NPY fibers in the hypothalamus of *C. dimerus*. (A) Microphotography at NLTl area showing a high density of ir-fibers (arrowheads), scale bar: 50 μm, (B) microphotography at NRL area showing some ir-NPY fibers (arrowhead), LR: lateral recess, scale bar: 20 μm. On the right top a camera lucida drawing shows the coronally sections of *C. dimerus* brain and the boxed area indicate the microphotography section.

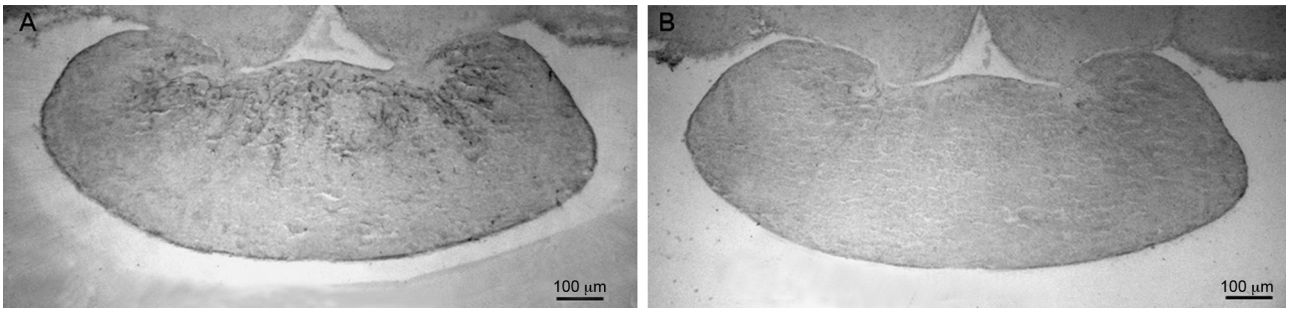

Fig. 4. Pituitary transversal sections showing ir-NPY fibers across the neurohypophysis (A) and the corresponding preadsorbtion in the immediately section (B). Scale bar: 100 μm.

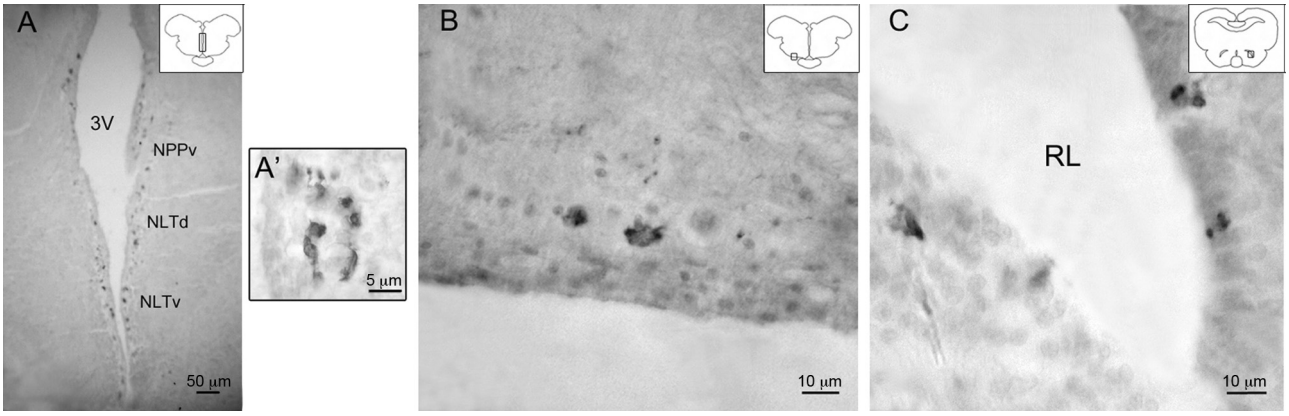

Fig. 5. Microphotography showing ir-orexin cell bodies in the preoptic area and hypothalamus. (A) Ir-orexin cells bordering the 3V, in particular in the NPPv, NLTd, and NLTv. Scale bar: 50 μm. On the right (A′) detail of ir-orexin cells. Scale bar: 5 μm. (B) Ir-orexin cells in the NLTl, scale bar: 10 μm. (C) Ir-orexin cells in the NRLl, scale bar: 10 μm. LR: lateral recess. On the right top a camera lucida drawing shows the coronally sections of *C. dimerus* brain and the boxed area indicates the microphotography section.

suggests other function in peripheral tissues besides orexigenic central function.

The presence and distribution of NPY and ir-orexin cells and fibers in the preoptic area, hypothalamus and pituitary of *C. dimerus* has been described using an immunohistochemical approach. We focused our analysis on brain regions where key factors involved in food intake are located. The absence of immunostaining in the preadsorbtion tests and in the controls conducted by omission of first antibodies indicates the high specificity of the antisera used. Particularly, when we used orexin-B monoclonal antibody no immunoreactivity was detected. Orexin-A antiserum was separately preabsorbed with both orexin-A or orexin-B to evaluate the antiserum specifity. While the preadsortion with orexin-A abolished the immunostaining, orexin-B failed indicating that the antiserum is specifically recognizing orexin-A. In Japanese seaperch, Suzuki et al. (2007a) observed similar results. The results from the present study together with those from the Japanese seaperch, where the same antisera was utilized for immunohistochemistry, suggest the high specificity to the anti orexin-A used in this study.

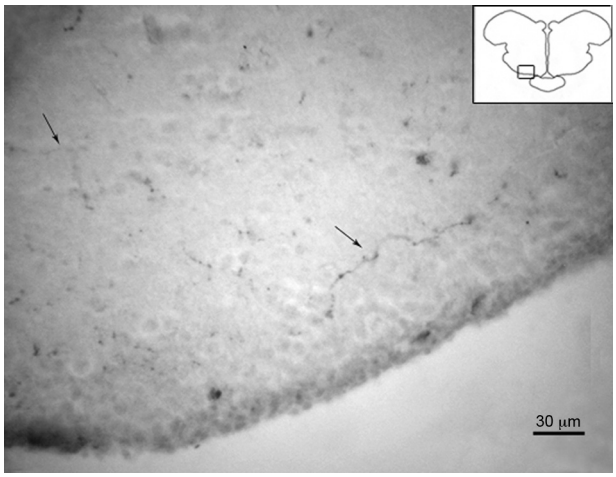

Fig. 6. Microphotography showing ir-orexin (arrows) in the NLTl. On the right top a camera lucida drawing shows the coronally sections of *C. dimerus* hypothalamus and the boxed area indicates the microphotography section. Scale bar: 30 μm.

In this work we detected ir-NPY somata in the NPPv of *C. dimerus* preoptic area. This neuronal distribution was also identified in *Solea senegalensis* (Rodríguez-Gómez et al., 2001), *Polypterus senegalus* (Reiner and Northcutt, 1992), platyfish, *Xiphophorus maculatus* (Cepriano and Schreibman, 1993), *Odontesthes bonariensis* (Traverso et al., 2003) and goldfish (Matsuda et al., 2009; Peng et al., 1994; Pontet et al., 1989) among others. However, in other species such as *Salmo salar* (García-Fernández et al., 1992) and *Sparus aurata* (Pirone et al., 2008), those neuronal populations were not found. This discrepancy in NPY neurons localization could be a species specific characteristic, although we cannot discard differences due to the technique and antibody used. Regarding to the ir-NPY fibers, these were found widely distributed in the *C. dimerus* preoptic area and hypothalamus mainly in the NLTl and NRLl. Also, we found intense ir-NPY fibers in the neurohypophysis intimately associated to the adenohypophysis as it was observed in other fish species (Chiba et al., 1993, 1996; Danger et al., 1991; Gaikwad et al., 2004; Rodríguez-Gómez et al., 2001; Traverso et al., 2003). In goldfish, *C. auratus*, NPY was highly effective in stimulating GH secretion (Peng et al., 1993a,b,c) and directly involved in the release of luteinizing hormone (LH) in pituitary cultures (Cerdá-Reverter et al., 1999). These results suggest that NPY could be involved

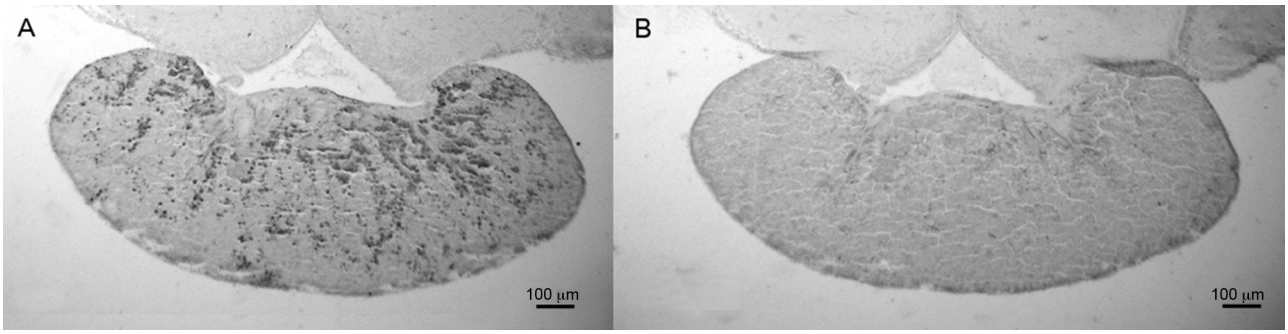

Fig. 7. Transversal sections at pituitary level showing ir-orexin cells in the adenohypophysis (A) and the corresponding preadsorbtion in the immediately section (B). Scale bar: 100 μm.

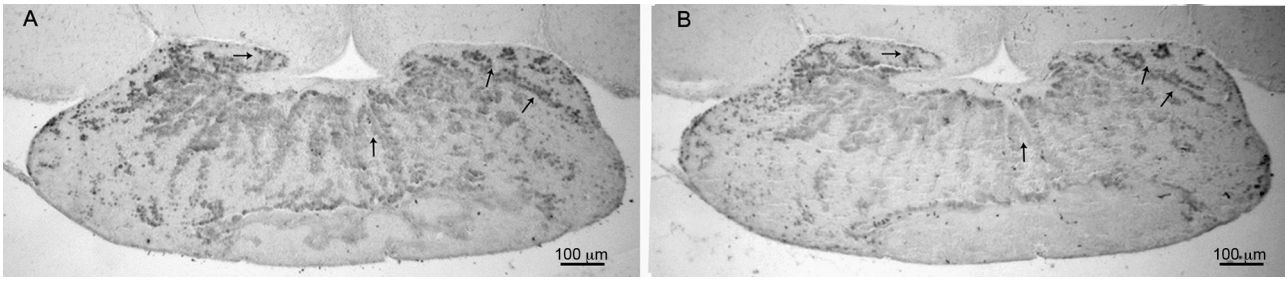

Fig. 8. Transversal sections (10 μm) at pituitary level. (A) Ir-orexin bordering the neurohypophysis, (B) immediately serial section showing ir-GH cell. Arrows show the same pattern of immunoreactivity. Scale bar: 100 μm.

in the control of the synthesis/secretion of adenohypophyseal cells.

In *C. dimerus* ir-orexin cells were detected in the NPPv of the preoptic area, in the NLTd, NLTv, NLTl and in the NRLl of the hypothalamus, whereas orexin fibers were found in all the hypothalamus and preoptic areas particularly in the NLTl and the NPPv. A similar distribution pattern of both orexin fibers and cells was observed in goldfish (Huesa et al., 2005; Nakamachi et al., 2006); whereas in medaka, orexin somata were observed only in the NPPv (Amiya et al., 2007). In addition to the immunoreactivity found in hypothalamic and preoptic areas, a few bundles of ir-orexin fibers were observed in *C. dimerus* neurohypophysis. To our knowledge this is the first report in teleosts fish describing orexin-A fibers in neurohypophysis. Suzuki et al. (2007a,b) observed fiber stains in the Japanese seaperch pituitary but using an antibody raised against orexin-B. On the other hand, orexin receptors have been localized in human, rat, sheep and *Xenopus laevis* pituitary cells (Date et al., 2000; Kaminski and Smolinska, 2012) and central administration of orexin and a posterior analysis of pituitaries for hormone gene expression in rats indicates a possible effect of this neuropeptide as a pituitary hormone modulator (Kohsaka et al., 2001; Pu et al., 1998; Seoane et al., 2004). Moreover, Molik et al. (2008) using sheep pituitary explants demonstrated that orexin modulates GH and prolactin secretion. The presence of orexin-A fibers observed in *C. dimerus* neurohypophysis in addition to the above results cited in other vertebrates suggest that orexin may regulate the synthesis of pituitary hormones and/or hormone release.

Interestingly, in *C. dimerus*, ir-NPY and ir-orexin cells and fibers showed a similar distribution in both preoptic and hypothalamic area. This pattern was also previously observed in other teleost species (Kojima et al., 2009). Although, this anatomical co-localization does not imply a truly physiological relationship, it suggests that these peptides might be interacting and taking part of the neuroendocrine system of feeding behavior in *C. dimerus*. However, in goldfish there are evidences on a co-action between orexin and NPY; even more, the co-administration of orexin and NPY results in a synergistic orexigenic effect (Volkoff and Peter, 2001; Volkoff et al., 2003). In addition, in mammals a large number of orexin axons are in direct synaptic contact with NPY cells (Horvath et al., 1999). The distribution seen in *C. dimerus* and other teleosts fish could support this interaction, but more experiments are necessary to elucidate a possible functional relationship.

Interestingly, in this study we observed the presence of ir-orexin in adenohypophyseal cells bordering the neurohypophysis at the proximal *pars distalis* level where the GH cells are localized in *C. dimerus* (Pandolfi et al., 2001). In other vertebrates species orexin

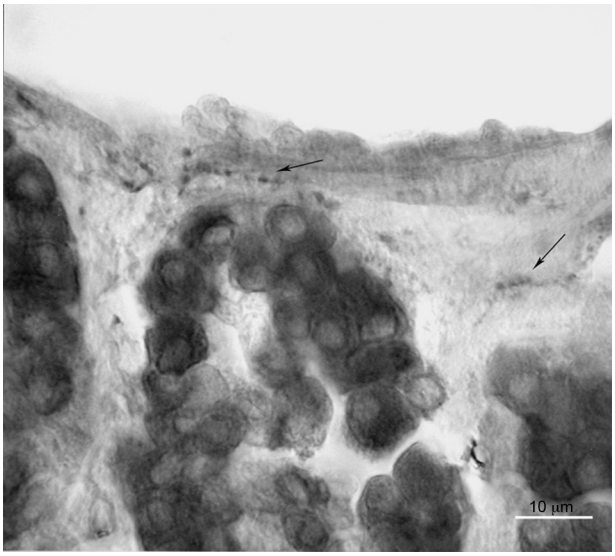

Fig. 9. Detail at proximal *pars distalis* level showing ir-orexin fibers in proximity with ir-orexin cells (arrow). Scale bar: 10 μm.

immunoreactivity was detected in pituitary cells as well. In *Rana catesbeiana*, orexin-A is expressed in prolactin cells (Yamamoto et al., 2004), but in *X. laevis* in thyroid-stimulating hormone (TSH)-containing cells (Suzuki et al., 2007b). Interestingly, in human pituitaries orexin was detected mainly in prolactin cells but also in GH, TSH, LH and FSH cells (Blanco et al., 2001, 2003). As in *C. dimerus* ir-orexin was detected in a region where GH producing cells were previously reported, serial sections and further IHC were performed as it was described above. Ir-orexin was mainly detected in GH cells although we cannot discard immunoreactivity in other pituitary cells. In the Japanese seaperch, Suzuki et al. (2007a) observed that ir-GH cells express orexin. More studies in other fish are necessary in order to elucidate a possible co-secretion and functional cooperativism, e.g. in somatic growth, between both peptides.

In conclusion, both peptides have an ubiquitous mRNA tissue expression and are similarly distributed in the hypothalamus and preoptic area of *C. dimerus*. The presence of ir-orexin cells in the adenohypophysis and the presence of ir-orexin and NPY fibers in the neurohypophysis suggest that both peptides may play an important neuroendocrine role in the anterior pituitary. This study provides basic information necessary to design future researches and also increases the knowledge about the distribution of these neuropeptides among vertebrates.

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