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Orexin as an input of circadian system in goldfish: Effects on clock gene expression and locomotor activity rhythms

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ABSTRACT

Orexins are neuropeptides mainly known for regulating feeding behavior and sleep–wakefulness cycle in vertebrates. Daily variations of orexin-A expression have been reported in fish, with the highest levels preceding feeding time. However, it is unknown if such variations could be related with daily rhythms of clock genes, which form the molecular core of circadian oscillators. The aim of the present study was to identify the possible role of orexin as an input element of the goldfish circadian system. It was investigated the effects of orexin-A (10 ng/g bw) intracerebroventricular injections on the expression of clock genes, NPY and ghrelin, as well as on daily locomotor activity rhythms. Goldfish held under 12L:12D photoperiod and injected at midday with orexin or saline, were sacrificed at 1 and 3 h post-injection. The analysis of genes expression by qReal Time PCR showed an increment of *Per* genes in hypothalamus and foregut at 3 h post-injection, but not in hindgut and liver. The *gBmal1a* expression remained unaltered in all the studied tissues. Orexin induced NPY in the hypothalamus and ghrelin in the foregut. Locomotor activity was studied in fish daily injected with orexin for several consecutive days under different experimental conditions. Orexin synchronized locomotor activity in goldfish maintained in 24L and fasting conditions. Present results support a cross-talking between orexin-A and other feeding regulators at central and peripheral level, and suggest, for the first time, a role of this peptide as an input of the circadian system in fish.

1. Introduction

The orexins/hipocretins neuropeptides, orexin-A and orexin-B, are generated by the proteolytic cleavage of a precursor, prepro-orexin, mostly produced in mammals in feeding regulatory centers as the perifornical and lateral hypothalamic areas [10,31] with widespread fiber projections through the brain [10]. In non-mammalian vertebrates, orexin neurons are also present in different brain areas, and particularly in the hypothalamus of amphibians [42], reptiles [13] and birds [43]. In fish, conservation of gene sequence, hypothalamic localization of cell bodies, and projection patterns, revealed that the architecture of the orexin system appears well conserved [3,25,55,56].

Accordingly with this broad anatomical distribution, orexins are involved in the regulation of many functions in mammals, as feeding behavior, energy homeostasis, cardiovascular control, sleep–wake cycle and in the reward system [35,44]. The knowledge on the physiological functions of orexins in fish is still limited, but published results suggest conserved functions. The orexigenic action of orexins is a well known function in fish. Centrally administered orexin A, but not orexin B, stimulates food intake with different intensities in different species [30,51,57]. Neuroanatomical and physiological evidences support the interactions among orexin and others appetite-regulating peptides, as NPY [53,56], leptin [52], ghrelin [29], and TRH [1]. Moreover, increases in hypothalamic orexin-like immunoreactivity and orexin mRNA by fasting support this orexigenic action in fish [1,30,54]. Nevertheless, mutant orexin receptor-null zebrafish (*Danio rerio*) displays food intake similar to that of wild-type fish [58].

The role of orexins on activity is still largely unknown in fish. In zebrafish, unlike in mammals, exogenous orexin seems to be a mild sedative not being a strong stimulant [58]. A null mutation in the sole receptor for the orexin causes sleep fragmentation at dark and disrupts the consolidation of sleep/wake behavior in zebrafish adults [3,58], whereas orexin overexpression in larvae inhibits rest and promotes and consolidates wakefulness [38]. Recently, immunohistochemical studies have shown an increased neural activity of hypothalamic orexin-positive neurons correlated

Abbreviations: 24L, constant light; FAA, food anticipatory activity; ICV, intracerebroventricular; LD Cycle, light–dark cycle; ZT, Zeitgeber time.

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Table 1
Primers used for qRT-PCR studies.

Target gene	Accession number		Primer sequences 5′ → 3′	Product (pb)
gPer1a	EF690698	Forward	AGCGCCACTTCCTCCTCTGA	130
		Reverse	TGAAGACCTGCTGTCCGTTGG	
gPer2a	EF690697	Forward	TTTGTCAATCCCTGGAGCCGC	116
		Reverse	AAGGATTTGCCCTCAGCCACG	
gPer3	EF690699	Forward	GGCTATGGCAGTCTGGCTAGTAA	130
		Reverse	CAGCACAAAACCGCTGCAATGTC	
gCry3	EF690702	Forward	GGTGAGACAGAAGCCCTGGAA	102
		Reverse	GCTTGCGAACAGTGATTGAGCG	
Bmal1a	KF840401	Forward	ATCGATGAGTCGTTCCCGTG	161
		Reverse	AGATTCTGTTCGTCTCGGAG	
gGhrl	AF454390	Forward	TTCATGATGAGTGCTCCGTTC	124
		Reverse	GTCAGAATTCAAGTGGCGAATC	
gNpy	M87297	Forward	TTCGTCTGCTTGGGAACTCT	151
		Reverse	TGGACCTTTTGCCATACCTC	
gβ-actin	AB039726	Forward	GGCCTCCCTGTCTATCTTCC	156
		Reverse	TTGAGAGGTTTGGGTTGGTC	

to periods of increased locomotor activity [32]. In goldfish (*Carassius auratus*), an increase in locomotion has been reported 1 h after central injection of mammalian orexin-1, but immunoneutralisation of orexins did not change locomotor activity [30].

Reports in mammals pointed to some interactions between orexin signaling and the circadian system. Orexin neurons are present in brain regions involved in the circadian organization in mammals as the hypothalamic suprachiasmatic nucleus [12,59]. On the other hand, daily variations of hypothalamic orexin with high levels around mealtime and during the daily active period have been shown in rodents [60]. A relationship between orexin and synchronization by food in mice is supported by shifts of the *Fos* expression in orexin neurons induced by restricted feeding [2], and by reductions of food anticipatory activity (FAA) in orexin neuron-ablated mice [2,28]. In fish very few data exist on possible interactions between orexin and the circadian system. Some evidences in zebrafish larvae have shown higher effects of orexin overexpression under constant darkness or constant light than in light–dark conditions [38]. On the other hand, the synaptic structures in orexin cells projecting to the pineal gland and the hindbrain displays a circadian rhythmicity in this teleost [4]. Recently, it is described a peak in hypothalamic prepro-orexin mRNA levels at mealtime in scheduled fed orange-spotted grouper, (*Epinephelus coioides*) [56] and goldfish [21]. Besides, orexin mRNA levels increase during the active phase and decrease during the rest in the Atlantic cod (*Gadus morhua*) [20] and goldfish [21].

In goldfish daily fluctuations of some clock genes and their regulation by light–dark cycle and feeding time have been characterized in some central and peripheral locations [15,33,49], but nothing is known on possible cross regulation between orexin signaling and the circadian system. Deep into this intriguing subject on orexin and circadian system crosstalking in fish, we firstly investigated the possible acute effects of central administration of orexin on central and peripheral clock genes expression in goldfish. Secondly, we studied the possible role of orexin as a zeitgeber in goldfish maintained under different photoperiodic and feeding conditions, analyzing if orexin synchronizes the daily locomotor activity rhythm, a well-known output of circadian system.

2. Materials and methods

2.1. Animals and housing

Goldfish (11.4 ± 0.3 g) were purchased from a commercial local supplier and maintained in the laboratory in 60 l tanks (n = 8/tank). Before the experiment, fish were acclimated at least for two weeks under 12L:12D photoperiod (lights on at 08:00 h) and scheduled feeding at 10:00 h with dry pellets (Sera Pond Bio-flakes, Sera Biogram, Germany) at 1% body weight (bw). The fish handling procedures were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals and were approved by the Animal Experimentation Committee of Complutense University of Madrid.

2.2. Effects of orexin A acute treatment on gene expression

Fish maintained for 2 weeks under 12L:12D photoperiod and scheduled feeding at 10:00 h (2 weeks) received one intracerebroventricular (ICV) injection 4 h after feeding (14:00 h, zeitgeber time ZT6) with saline (1 μl; n = 14) or orexin (10 ng/g bw, American Peptide, Sunnyvale, USA; n = 14). The chosen dose was previously shown to be effective inducing food intake (51) and locomotor activity (30) in goldfish. For injections fish were anesthetized in water containing tricaine methanesulphonate (MS-222, 0.1 g/l, Sigma Chemical, Madrid, Spain). Immediately after loss of equilibrium animals were ICV injected as previously established [11], and after the injections fish were placed in anesthetic-free water tanks where swimming activity and equilibrium was recovered within 1–2 min. At 1 and 3 h post-injection fish where sacrificed (n = 7/group), and central (hypothalamus) and peripheral (liver, foregut and hindgut) tissues were sampled and stored at −80 °C until used.

The expression of clock genes (*gPer1a*, *gPer2a*, *gPer3* and *gBmal1a*) in hypothalamus, foregut, hindgut and liver, and the expression of hypothalamic NPY and ghrelin in foregut were quantified by RT-qPCR in a CFX96TM Real-Time System (Biorad Laboratories, Hercules, USA) as previously described [33]. In brief, the total RNA from goldfish tissues was extracted with Trizol (TRI® Reagent method, Sigma Chemical, Spain) and treated with DNase (Promega, Madison, USA) at 37 °C for 40 min to eliminate genomic DNA. Then, the RNA (1 μg/μl for hypothalamus, hindgut and liver, and 0.5 μg/μl for foregut) was retro-transcribed by using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). The PCR reactions were performed in a 20 μl final volume using 10 μl iTaqTM SYBR® Green Supermix (BIORAD LABORATORIES, Hercules, USA), specific primers (0.4 μM; Sigma Chemical, Madrid, Spain; see Table 1) and 1 μl cDNA for all genes except for *gGhrl* where 3 μl of cDNA were used. The protocol for measuring mRNA expression was as follows for *Per* genes: 1 cycle at 95 °C for 3 min and 40 cycles consisting in 95 °C for 10 s, 58 °C for 30 s and 72 °C for 45 s. The annealing temperature varied for *Bmal1a* (55 °C), *gGhrl* (62 °C), *gNpy* (64 °C), and for β-actin (60 °C) used as reference gene. The specificity of the amplification was checked by the size of the products in agarose gels and by the existence of only one peak at the expected