CHAPTER EIGHTEEN

Orexin System in Teleost Fish

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Contents

Abstract

Orexin is a neuropeptide distributed widely among vertebrates. In mammals, orexin and its receptor system are involved in the regulation of food intake, locomotion, and psychomotor activities including the sleep/wakefulness cycle. With regard to nonmammalian vertebrates, there has also been intensive study aimed at the identification and functional characterization of orexin and its receptor, and recent investigations of the role of orexin have revealed that it exerts behavioral effects in teleost fish. Goldfish and zebrafish are excellent teleost fish models, and in these species it has been demonstrated that orexin increases food consumption as an orexigenic factor and enhances locomotor

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activity, as well as being involved in the regulation of active and rest status (circadian rhythmicity and the sleep/wakefulness cycle), as is the case in mammals. This chapter reviews current knowledge of orexin derived from studies of teleost fish, as representative nonmammals, focusing particularly on the role of the orexin system, and examines its significance from a comparative viewpoint.

I. Introduction

Orexin (or hypocretin, as it is sometime known) is a neuropeptide that was first identified as an orphan-receptor ligand, and subsequently as an appetite regulator (Sakurai *et al.*, 1998). Orexin exists as two molecular forms derived from the same precursor: a 33-residue peptide known as orexin A, and a 28-residue peptide known as orexin B. Although it was thought originally that orexin has no functional or structural identity with any other known regulatory peptide, one notable exception is hypocretin, which has been considered to belong to the incretin gene family of peptides, including members of the secretin–glucagon superfamily such as growth hormone releasing hormone, pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal peptide, and glucagon-like peptides (Alvarez and Sutcliffe, 2002; Tam *et al.*, 2011). Subsequently, the orexin receptor was found to have two forms: orexin receptors 1 and 2 (OX1R and OX2R) (Sakurai *et al.*, 1998; Tam *et al.*, 2011; Wong *et al.*, 2011). In mammals, neuronal cell bodies containing orexin-like immunoreactivity are located in the lateral hypothalamus, which is referred to as the "orexigenic center," and nerve fibers containing orexin-like immunoreactivity are widely distributed in various regions including the cerebral cortex, hippocampus, limbic system, and brainstem, suggesting that orexin controls multiple brain functions such as emotional regulation (de Lecea *et al.*, 1998; Sakurai and Mieda, 2011; Thannickal *et al.*, 2000). Consistent with this observation, orexin has been found to regulate the sleep–wakefulness cycle and to suppress the gonadotropin-producing cells in the hypothalamus (Kilduff and Peyron, 2000; Kohsaka *et al.*, 2001; Porkka-Heiskanen *et al.*, 2004; Sakurai, 2005; Sakurai *et al.*, 1998).

The orexin gene or cDNA has also been characterized in nonmammalian species. The structure of fish orexin appears to be less conserved (Kaslin *et al.*, 2004; Tam *et al.*, 2011; Wong *et al.*, 2011). Recent studies indicate that some neuropeptides influence food consumption and related behavior in nonmammalian vertebrates, notably in teleost fish (Kang *et al.*, 2011b; Lin *et al.*, 2000; Matsuda *et al.*, 2011a). Orexin is distributed in the brain of teleost fish and exerts central and neuroendocrine functions (Huesa *et al.*, 2005; Kaslin *et al.*, 2004). In particular, the effect of orexin on feeding

behavior has been studied in teleost fish. In the goldfish, intracerebroventricular (ICV) administration of orexin A stimulates food intake, and the orexin A-induced orexigenic action is mediated via the orexin receptor, subsequently leading to activation of the neuropeptide Y (NPY) Y1 receptor (Matsuda, 2009; Matsuda *et al.*, 2011a; Nakamachi *et al.*, 2006; Volkoff *et al.*, 1999). In this species, the orexigenic response to orexin A is also modulated by cocaine- and amphetamine-regulated transcript peptides and leptin (Abbott and Volkoff, 2011; Volkoff *et al.*, 1999). ICV injection of orexin A also stimulates locomotor activity in the goldfish (Matsuda, 2009; Nakamachi *et al.*, 2006). In the zebrafish, ICV injection of orexin A induces an orexigenic action and hypermotility (Yokobori *et al.*, 2011), and overexpression of orexin promotes wakefulness and inhibits rest (Prober *et al.*, 2006). Taken together, these observations indicate that, in Cypriniformes (including the zebrafish and goldfish), orexin not only regulates energy homeostasis by stimulating food consumption but also involved in the regulation of psychophysiology in teleost fish by enhancing locomotor and psychomotor activities, suggesting that it is involved in multiple brain functions in teleosts (Appelbaum *et al.*, 2009; Rihel *et al.*, 2010). However, except for the case of goldfish and zebrafish, there is no information about the involvement of orexin in feeding and emotional behavior, or other physiological processes, in teleost fish, and the function of the orexin system in fish has not been well studied. Therefore, this chapter focuses on the characterization of orexin and its receptor system, the distribution of the orexin transcript and orexin-like immunoreactivity, and the effect of orexin on food intake, locomotor, and psychomotor activities in teleost fish, especially the goldfish and zebrafish, which have been overlooked hitherto.

II. Orexin and Its Receptor in Teleost Fish

The orexin gene or cDNA has also been characterized in nonmammalian species such as the chicken (*Gallus gallus*), clawed toad (*Xenopus laevis*), zebrafish (*Danio rerio*), tiger puffer (*Takifugu rubripes*), Atlantic cod (*Gadus morhua*), goldfish (*Carassius auratus*), medaka (*Oryzias latipes*), winter flounder (*Pleuronectes americanus*) and winter skate (*Leucoraja ocellata*) (Alvarez and Sutcliffe, 2002; Buckley *et al.*, 2010; Faraco *et al.*, 2006; Kaslin *et al.*, 2004; MacDonald and Volkoff, 2010; Nakamachi *et al.*, 2006; Ohkubo *et al.*, 2002; Shibahara *et al.*, 1999; Xu and Volkoff, 2007). The primary structure of the orexin precursor is conserved among mammals (Tam *et al.*, 2011; Wong *et al.*, 2011). However, as shown in Fig. 18.1A, phylogenetic analysis using the amino acid sequences of the orexin precursor by the neighbor-joining method has revealed that the structures identified in

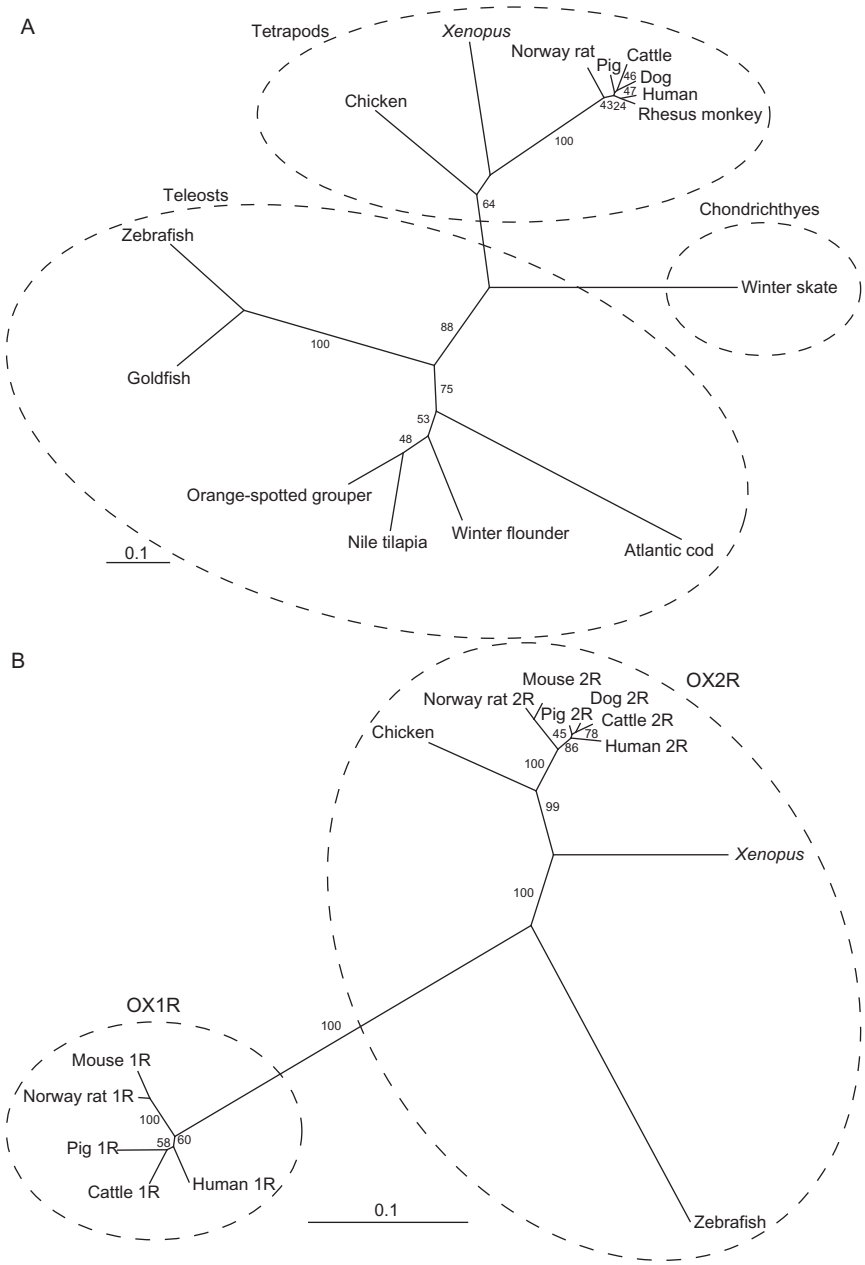

Figure 18.1 (A) A phylogenetic tree of the primary structure of the orexin precursor inferred by the neighbor-joining method using ClustalW. The numbers at the branch points are derived from bootstrap analysis (100 repetitions). The scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank IDs: human

tetrapods are slightly similar to that of the mammalian orexin precursor, whereas the structure of the fish orexin precursor appears to be less conserved (Kaslin *et al.*, 2004; Wong *et al.*, 2011). Two forms of orexin, orexin A and orexin B, are derived from a common precursor encoded by the orexin gene, in which loci have been shown to be conserved throughout vertebrate evolution (Alvarez and Sutcliffe, 2002).

Orexins A and B bind to two orexin receptors, OX1R and OX2R, which belong to members of class B of mammalian G-protein-coupled receptors (Wong *et al.*, 2011). However, information about the orexin receptor in nonmammals, including birds, amphibians, and teleost fish, has been insufficient. To date, one receptor for orexin has been identified in chicken, *Xenopus*, and zebrafish (Ohkubo *et al.*, 2003; Tam *et al.*, 2011; Yokogawa *et al.*, 2007), and this receptor corresponds structurally to mammalian OX2R (Wong *et al.*, 2011). On the other hand, recent studies have failed to identify the gene encoding the OX1R in nonmammals, including zebrafish (Panula, 2010; Wong *et al.*, 2011). Molecular information about the fish OX1R is lacking. From an evolutionary and comparative viewpoint, the OX2R is likely to be a more ancient receptor form in vertebrates. Phylogenetic analyses of the chicken and *Xenopus* orexin receptors by the neighbor-joining method using the amino acid sequences of mammalian OX1R and OX2R have revealed that the nonmammalian orexin receptors may be classified into the mammalian OX2R group. However, in the tree, the zebrafish orexin receptor appears to form a separate cluster (Fig. 18.1B). Further investigations to clarify the relationship between mammalian and nonmammalian orexin receptors, especially the fish receptor, would be warranted.

prepro-orexin, NM_001524; Rhesus monkey prepro-orexin NM_001194432; Norway rat prepro-orexin, AF019565.1; dog prepro-orexin, NM_001033994.1; pig prepro-orexin, MN_214156.2, EF434655.1; bovine prepro-orexin, NM_001166520; chicken prepro-orexin, NM_204185.1, AB056748.1; *Xenopus* prepro-orexin, Shibahara *et al.*, 1999; Atlantic cod prepro-orexin, DQ486137.1, EU096315.1; goldfish prepro-orexin, DQ923590.1; zebrafish prepro-orexin, NM_001077392, DQ831346; Nile tilapia prepro-orexin, FJ871159.1; winter flounder prepro-orexin, GQ397970.1; orange-spotted grouper prepro-orexin, HM992945.1; winter skate prepro-orexin, HM367085.1. (B) A phylogenetic tree of the primary structure of orexin receptors inferred by the neighbor-joining method using ClustalW. The numbers at the branch points are derived from bootstrap analysis (1000 repetitions). The scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank IDs: human OX1R, NM_001525; human OX2R, NM_001526; Norway rat OX1R, NM_013064.1; Norway rat OX2R, NM_013074; mouse OX1R, AY336083.1; mouse OX2R, NM_198962; dog OX2R, NM_001002933; pig OX1R, NM_001043346, DQ321701.1; pig OX2R, NM_001129951, DQ321702.1; cattle OX1R, NM_001048182; cattle OX2R, NM_001192677; chicken orexin receptor, NM_001024584, AB110634.1; *Xenopus* orexin receptor, HQ242647.1; zebrafish orexin receptor, NM_001079868.

III. Distribution of Orexin Precursor Transcript and Orexin-Like Immunoreactivity in Teleost Fish

cDNAs encoding the orexin precursors were isolated from goldfish and zebrafish and cloned as described above (see the Section II). Subsequently, the distribution of the orexin precursor transcript in the central nervous system and peripheral tissues of those species was examined using *in situ* hybridization or RT-PCR. Expression of orexin receptor mRNA was observed in the periventricular regions of the brain (Yokogawa *et al.*, 2007). Figure 18.2 shows the distribution of the orexin precursor transcript in the central and peripheral tissues of the goldfish. Orexin precursor mRNA is strongly expressed in the brain, pituitary, and gonads and is moderately expressed in the other tissues. In the winter flounder, the distribution of the orexin precursor transcript has also been observed (Buckley *et al.*, 2010). The expression of mRNA for the orexin precursor is distributed in the brain, including the telencephalon, mesencephalon, diencephalon, cerebellum, medulla oblongata, and spinal cord. In addition, RT-PCR has indicated that the orexin precursor transcript is present in peripheral tissues such as gill, liver, gut, stomach, spleen, kidney, and gonad.

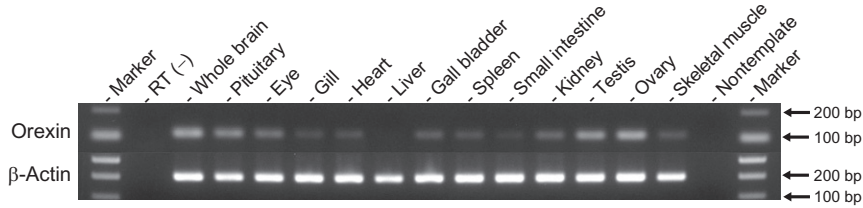

Figure 18.2 Reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis of orexin precursor mRNA in the goldfish tissues. Total RNA was extracted from various tissues including the whole brain, pituitary, eye, gill, heart, liver, gall bladder, spleen, small intestine, kidney, testis, ovary, skeletal muscle. For amplification and quantitation of the cDNA fragments encoding the orexin precursor and β-actin, the two-step RT-PCR method was used. Reverse transcription was carried out at 50 °C for 60 min, and the resulting cDNA was subsequently amplified using 35 cycles for orexin and 23 cycles for β-actin of 94 °C for 40 s and 60 °C for 40 s followed by 72 °C for 30 s. Gene-specific primers for amplification of the orexin cDNA fragment were based on the nucleotide sequence of goldfish orexin precursor (GenBank ID, DQ923590.1). PCR with the sense primer (5′-CGT CAA GGT CCT GCA AAT TAT ACG-3′) and antisense primer (5′-CTG CCG CGT CGT TAT TAA AGC-3′) yielded a 102-bp product encoding part of the goldfish orexin precursor cDNA. Goldfish β-actin-specific primers were used as the internal control for PCR amplification (goldfish, GenBank ID, AB039726.2). Using these primers (sense primer, 5′-CTA GGT ATG GAA TCT TGC GGT A-3′; antisense primer, 5′-TAC TCC TGC TTG CTG ATC CA-3′) a 287-bp product corresponding to a region in the part of the β-actin cDNA for goldfish was obtained.

Orexin-like immunoreactivity was first observed in the brain of the zebrafish (Kaslin *et al.*, 2004), indicating that the major population of neuronal cell bodies possessing orexin-like immunoreactivity is located in the hypothalamic area including the gigantocellular part of the magnocellular preoptic nucleus, the posterior tuberal nucleus, and the lateral recessus in the hypothalamus (Kaslin *et al.*, 2004). Subsequently, it was reported that the goldfish brain contained neuronal cell bodies and fibers showing orexin-like immunoreactivity (Huesa *et al.*, 2005). Schematic drawings of orexin-like immunoreactivity in the brains of the goldfish and zebrafish are illustrated in Figs. 18.3 and 18.4. In these species, the neuronal cell bodies containing orexin-like immunoreactivity are located highly specifically in two regions of the hypothalamus: the preoptic area, including the magnocellular nucleus, and the area bordering the third ventricle, including the nucleus posterioris periventricularis (goldfish) or posterior tuberal nucleus (zebrafish), and the nucleus recessus lateralis. Nerve fibers containing orexin-like immunoreactivity are located mainly in the midbrain and various other regions. The localization of orexin-like immunoreactivity has also been examined in the medaka, and orexin-like immunoreactivity is widely detected throughout the brain (Amiya *et al.*, 2007). In this species, neuronal cells showing orexin-like immunoreactivity are located mainly in the hypothalamic area along the third ventricle, including the nucleus posterioris periventricularis, as is the case in goldfish. In addition, the distribution of orexin-like immunoreactivity has also been examined in the brains of the African and Australian lungfish, *Protopterus dolloi* and *Neoceratodus forsteri* (López *et al.*, 2009). In both the species, the most immunoreactive neurons are present in the suprachiasmatic nucleus and dorsal hypothalamus. Only in *Neoceratodus*, however, important cell populations are located in the preoptic area and infundibular hypothalamus, whereas small numbers of faintly reactive neurons are present in the lateral septum and ventral striatum. Fiber labeling has been shown to be widely distributed in all main brain subdivisions but is more abundant in regions such as the septum, preoptic area, suprachiasmatic nucleus, lateral hypothalamic area, thalamus, pretectum, and tegmentum. Less conspicuous was the innervation is evident in the pallial regions, habenula, optic tectum, rhombencephalic reticular formation, and spinal cord (López *et al.*, 2009).

IV. Effect of Orexin on Food Intake in Teleost Fish

A. Goldfish

Feeding conditions affect the levels of expression of orexin precursor mRNA and orexin synthesis in the hypothalamus of fish. In goldfish that have been fasted for two or more days, the number of neuronal cell bodies

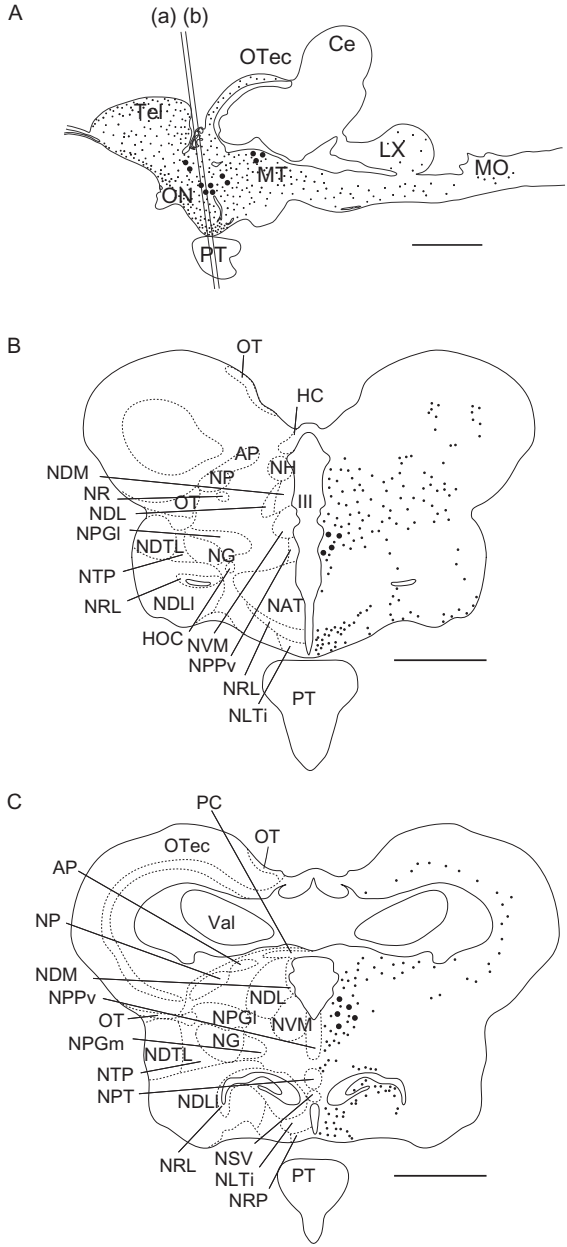

Figure 18.3 Schematic drawings of the distribution of orexin A-like immunoreactivity in the brain of the goldfish (A) is a sagittal section view, and (B) and (C) are cross-sectional views at levels (a) and (b), as indicated by the respective lines in the sagittal section view. Neuronal cell bodies and fibers with orexin-like immunoreactivity are

containing orexin-like immunoreactivity in the hypothalamus is significantly increased in comparison with that in normally fed fish (Nakamachi *et al.*, 2006). Moreover, intraperitoneal (IP) administration of glucose for 12 h induces a significant decrease in the number of neuronal cell bodies showing orexin-like immunoreactivity in the brain. Expression of orexin precursor mRNA in the brain changes according to feeding status: fasting for 7 days induces a significant increase in the levels of expression of orexin precursor mRNA (Nakamachi *et al.*, 2006). These results suggest that fasting enhances orexin-like immunoreactivity and the expression of orexin precursor mRNA in neuronal cells in the hypothalamus, indicating that a relationship exists between the density of orexin-like immunoreactivity and orexin synthesis, or its functional state, in the goldfish brain.

There is a direct evidence that orexin enhances food intake in goldfish. ICV administration of human orexin A at 0.28–2.8 pmol/g body weight (BW) and orexin B at 0.34–3.4 pmol/g BW stimulates food consumption (Volkoff *et al.*, 1999). A subsequent study using goldfish has indicated that ICV administration of orexin A antiserum decreases food intake, that the orexigenic action of human orexin A is more potent than that of human orexin B (Nakamachi *et al.*, 2006), and that the orexigenic action of orexin A at 2.8 pmol/g BW is blocked by treatment with SB334867—an OX1R antagonist in mammals—at 10 pmol/g BW (Miura *et al.*, 2007). However, as described above (see the Section II), there is no evidence that the OX1R is present in teleost fish (Wong *et al.*, 2011). Therefore, SB334867 may antagonize orexin A at the goldfish orexin receptor, which belongs to the OX2R family. Interestingly, the orexigenic action of orexin A at 2.8 pmol/g BW is attenuated by treatment with the growth hormone secretagogue-receptor antagonist, [D-Lys3]-GHRP-6, at 10 pmol/g BW, and the orexigenic action of ghrelin at 1 pmol/g BW is blocked by treatment with SB334867 at 10 pmol/g BW, suggesting that, in goldfish, orexin A and ghrelin have interacting orexigenic effects on the central nervous system

indicated by filled circles and dots, respectively. Scale bar = 1 mm. Abbreviations: AP, area pretectalis; Ce, cerebellum; HC, habenular commissure; HOC, horizontal commissure; LX, vagal nerve; MO, medulla oblongata; MT, midbrain tegmentum; NAT, nucleus anterior tuberis; NDL, nucleus dorsolateralis thalami; NDLI, nucleus diffusus lobi inferior; NDM, nucleus dorsomedialis thalami; NDTL, nucleus diffusus tori inferior; NG, nucleus glomerulosus; NH, nucleus habenularis; NLTi, inferior part of nucleus lateralis tuberis; NP, nucleus pretectalis; NPGl, nucleus preglomerulosus pars lateralis; NPGm, nucleus preglomerulosus pars medialis; NPPv, nucleus posterior periventricularis; NPT, nucleus posterior tuberis; NR, nucleus rotundus; NRL, nucleus recessus lateralis; NRP, nucleus recessus posterioris; NSV, nucleus saccus vasculosus; NTP, nucleus posterioris thalami; NVM, nucleus ventromedialis thalami; ON, optic nerve; OT, optic tract; OTec, optic tectum; PC, posterior commissure; PT, pituitary; Tel, telencephalon; Val, lateral lobe of valvula cerebella; III, third ventricle. The nomenclature used for brain nuclei was based on the brain atlas for goldfish (Peter and Gill, 1975) and for carp (Pirone *et al.*, 2004).

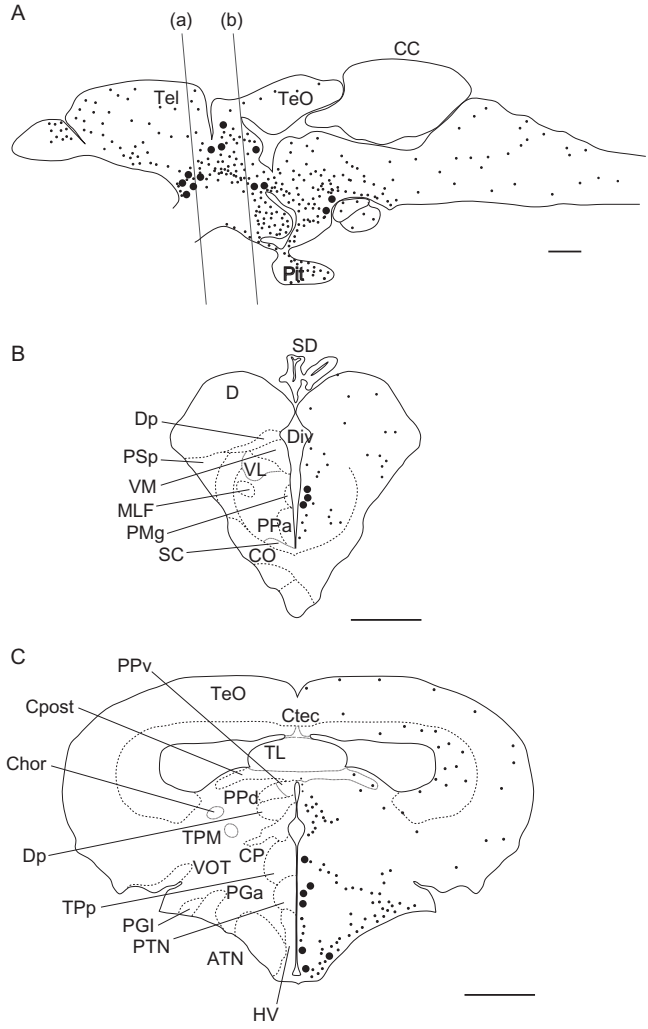

Figure 18.4 Schematic drawings of the distribution of orexin A-like immunoreactivity in the brain of the zebrafish (A) is a sagittal section view, and (B) and (C) are cross-sectional views at levels (a), and (b) as indicated by the respective lines in the sagittal section view. Neuronal cell bodies and fibers with orexin-like immunoreactivity are indicated by filled circles and dots, respectively. Scale bar = 0.2 mm. Abbreviations: ATN, anterior tuberal nucleus; CC, cerebellar crest; Chor, horizontal commissure; CO, optic chiasm; CP, central posterior thalamic nucleus; Cpost, posterior commissure; Ctec, tectal commissure; D, dorsal telencephalic area; Div, diencephalic ventricle; Dp, posterior zone of dorsal telencephalic area; Hv, ventral zone of periventricular hypothalamus; MLF, medial longitudinal fascicle; PGa, anterior preglomerular nucleus; PGI, lateral preglomerular nucleus; Pit, pituitary gland; PMg, gigantocellular part of magnocellular preoptic nucleus; PPa, parvocellular preoptic

(Miura *et al.*, 2007). In goldfish, the orexigenic action of orexin A is also inhibited by treatment with the NPY Y1 receptor antagonist, BIBP-3226, at 100 pmol/g BW, and the action of NPY at 1 pmol/g BW is abolished by treatment with SB334867 at 10 pmol/g BW (Kojima *et al.*, 2009). These data indicate that the orexigenic actions of orexin A and NPY are mediated by mutual signaling pathways in goldfish (Matsuda, 2009). Interestingly, gonadotropin-releasing hormone 2 (GnRH2; chicken GnRH II, another name for GnRH2) mediates the anorexigenic actions of α-melanocyte-stimulating hormone and subsequent corticotropin-releasing hormone, and GnRH2 is known as a potent anorexigenic neuropeptides in goldfish (Kang *et al.*, 2011a; Matsuda *et al.*, 2008). GnRH2 also suppresses expression of orexin precursor mRNA, and orexin inhibits GnRH2-induced reproductive behavior (Hoskins *et al.*, 2008), suggesting the involvement of orexin in the reproduction in fish. In mammals, orexin participates in the regulation of reproduction via the hypothalamo–pituitary–gonadal relationship (Nurmio *et al.*, 2010; Silveyra *et al.*, 2010).

B. Zebrafish

To date, most of the data pertaining to the regulation of food intake by orexin in teleost fish have been obtained using goldfish, as described above (see the Section IV, A) (Kojima *et al.*, 2009; Matsuda, 2009; Miura *et al.*, 2007; Nakamachi *et al.*, 2006; Volkoff, 2006; Volkoff *et al.*, 1999, 2005), and the situation in other teleosts has remained unclear. Recent data have indicated that the number of neuronal cell bodies showing orexin-like immunoreactivity in the posterior tuberal nucleus, but not in the gigantocellular part of the magnocellular preoptic nucleus of the hypothalamus is increased in zebrafish fasted for 7 days (Yokobori *et al.*, 2011), suggesting that orexin-like immunoreactivity in the posterior tuberal nucleus is related to feeding status, and that fasting for 7–14 days induces a significant increase in the orexin precursor mRNA levels in the zebrafish brain (Novak *et al.*, 2005; Yokobori *et al.*, 2011).

So far, in zebrafish, there has been no direct evidence for the involvement of orexin in appetite regulation. However, a method has been developed for measuring the food intake in this species (Piccinetti *et al.*, 2010; Yokobori *et al.*, 2011) by directly observing and recording the number of food pellets eaten by an individual fish, as in the case of the goldfish.

nucleus; PPd, periventricular pretectal nucleus sorsal part; PPv, periventricular pretectal nucleus ventral part; PSp, paravocellular superficial pretectal nucleus; PTN, posterior tuberal nucleus; SC, suprachiasmic nucleus; SD, sorsal sac; Tel, telencephalon; TeO, optic tectum; TL, longitudinal torus; TPM, pretecto-mammillary tract; TPp, periventricular nucleus of posterior tuberculum; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventrolateral optic tract. The nomenclature used for brain nuclei was based on the brain atlas for zebrafish (Wullimann *et al.*, 1996).

A recent study has demonstrated for the first time the stimulatory effect of ICV-administrated human orexin A on food intake in the zebrafish (Yokobori *et al.*, 2011). In that study, ICV administration of orexin A at 0.3 and 3 pmol/g BW induced a significant increase of cumulative food intake during 60 min after treatment. ICV administration of orexin A at 30 pmol/g BW did not stimulate the food intake, being possibly indicative of an overdose effect, receptor downregulation, or receptor desensitization, as is the case in goldfish (Nakamachi *et al.*, 2006; Fig. 18.5). Although the synthetic orexin A employed was of heterologous origin, ICV administration

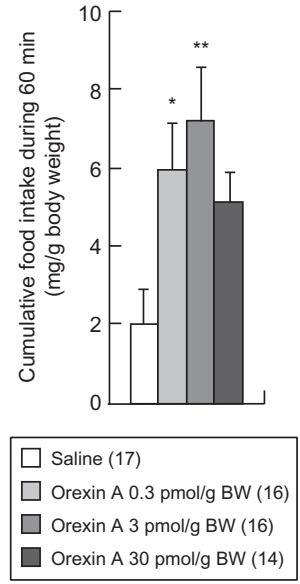

Figure 18.5 Effect of ICV administration of orexin A on food intake in the zebrafish. Zebrafish (0.3–0.6 g body weight, BW) of both sexes were purchased commercially and kept for 2 weeks under controlled light–dark conditions (12 L/12 D) with the room temperature regulated to 20–24 °C. Two hours before the experiments began at 10:00 a.m., each fish was supplied with food at 3% of its BW. In order to examine the effect of orexin A on food intake, ICV administration was carried out as described previously (Yokogawa *et al.*, 2007) with minor modifications for our laboratory (Yokobori *et al.*, 2011). Each fish was placed in wet tissue paper under anesthesia with MS-222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich Co., St. Louis, MO, USA). A small part of the parietal bone was carefully removed using a surgical blade, and 0.5 μL of test solution containing Evans Blue dye was delivered into the third ventricle of the brain of each fish using a microinjecter-holding system designed for small fish and a small Hamilton syringe with 0.1-μL graduations and a disposable fine needle. Correct placement of the injection was confirmed by the presence of Evans Blue dye in the ventricle. Fish were then injected with synthetic orexin A at 0.3, 3, or 30 pmol/g BW. Fish in the control group were given injections of the same volume of saline. After ICV injection, the bone gap was filled with a surgical bonding agent.

of human orexin A enhanced food intake in the zebrafish. The primary structure of orexin precursors has been conserved during evolution among the vertebrates (Wong *et al.*, 2011). However, the amino acid sequence of zebrafish orexin A shows 32% identity with that of human orexin A (Wong *et al.*, 2011). Synthetic human orexin A shows effective physiological activity in zebrafish and goldfish (Nakamachi *et al.*, 2006; Volkoff and Peter, 2000, 2001; Volkoff *et al.*, 1999, 2003; Yokogawa *et al.*, 2007). The orexigenic action of orexin A is blocked by treatment with SB334867 in zebrafish (Yokobori *et al.*, 2011). However, as described above (see the Section II), there is no evidence that the OX1R is present in teleost fish (Wong *et al.*, 2011). Therefore, SB334867 may also antagonize orexin at the zebrafish orexin receptor, which belongs to the OX2R family (Wong *et al.*, 2011).

C. Other fish

In a marine teleost fish, the ornate wrasse (*Thalassoma pavo*), IP injection of orexin A at a high dose of 28 pmol/g BW stimulates the food intake (Facciolo *et al.*, 2009). In the winter flounder and winter skate, fasting stimulates the expression of orexin precursor mRNA in the hypothalamus (Buckley *et al.*, 2010; MacDonald and Volkoff, 2010), suggesting that orexin might play a role in feeding regulation in these species. To date, however, except for the goldfish and zebrafish as described above (see the Section IV), there is no information about the orexin function, and the involvement of orexin in feeding regulation has not been well studied in fish. Further study will be required to elucidate the function of orexin in appetite control in fish.

V. Effect of Orexin on Locomotor and Psychomotor Activities and the Sleep/Wakefulness Cycle in Teleost Fish

A. Goldfish

Orexin exerts locomotor and psychophysiological function in teleost fish. Recent studies have revealed that ICV administration of orexin A at 2–2.8 pmol/g BW stimulates the locomotor activity in goldfish (Fig. 18.6; Matsuda *et al.*, 2011a; Nakamachi *et al.*, 2006). Regarding the mechanism of

Recording of food intake was started 10 min after ICV injection. Each fish received food at 3% per g BW and food intake was measured directly by recording the number of diet pellets eaten by individual fish during the 60 min after treatment. The results are expressed as the mean ± SEM, and the number of fish per group is indicated in parentheses. Significance of differences was evaluated by one-way ANOVA with Bonferroni's method, as compared with a saline-injected group (*$P < 0.05$, **$P < 0.01$).

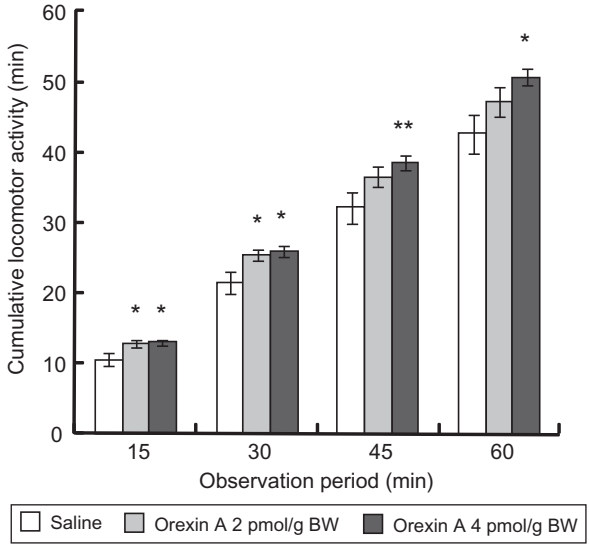

Figure 18.6 Effect of ICV administration of orexin A on locomotor activity in goldfish. Young goldfish (7–10 g body weight, BW) of both sexes were kept under controlled light/dark conditions (12 L/12 D) in a temperature-regulated water tank (20–24 °C) for 2 weeks before use in the experiments. Two hours prior to starting the experiments, at noon, each fish was supplied with food equivalent to at least 2% of its BW. For ICV administration of orexin A, fish were anesthetized in water containing 2 mM MS-222. The animals were then placed in a stereotaxic apparatus and a small part of the parietal bone was carefully removed using a surgical blade. One microliter of test solution was injected into the third ventricle, and the gap was then filled with a surgical bonding agent. Injection into the correct site was verified by the appearance in the ventricle of concomitantly injected Evans blue dye. The fish in the experimental groups were injected with orexin A at 2 and 4 pmol/g BW. The fish in the control group were injected with the same volume of saline. Each fish that had received ICV injection of orexin A or saline was placed in a small white experimental tank filled with tap water. Recording of locomotor activity (swimming time), which was started 15 min after ICV injection and continued for 60 min, was performed with a video-tracking system for automatic recording of goldfish behavior (EthoVision Color Pro, Noldus Information Technology, Wageningen, Netherlands). The results are expressed as the mean ± SEM, and 6–15 fish were used in the experiment. Significance of differences was evaluated by one-way ANOVA with Bonferroni's method, as compared with a saline-injected group ($\star P < 0.05$, $\star\star P < 0.01$).

the increased locomotor activity of teleosts by orexin, there is virtually no report. The plausible mechanistic pathways can be discussed from the studies with rodent models. Dopamine has been importantly implicated in the central nervous control of motor activity (Alachkar *et al.*, 2010; Rizzolatti and Luppino, 2001). The tyrosine hydroxylase-immunoreactive cells as a marker of dopaminergic system in the ventral tegmental area received

innervation from the orexin immunoreactive fibers. In behavioral studies, orexin A-induced hyperlocomotion, stereotypy, and grooming behavior when administered centrally in rats, and these effects were abolished by dopamine antagonists (Nakamura *et al.*, 2000). These results suggest that the involvement of the ventral tegmental area dopaminergic system in orexin-induced locomotor activity.

Recent reports also indicate that the scototaxis protocol (a test to ascertain the preference for light/dark background areas) can be used to evaluate the psychomotor activity of teleost fish, notably anxiety-like behavior (Faganello and Mattioli, 2007; Matsuda *et al.*, 2011b; Maximino *et al.*, 2010). For example, it has been reported that when goldfish are initially kept in a small tank, and then placed in a small rectangular experimental tank with black and white background areas, the locomotor activity is affected (Matsuda *et al.*, 2011a,b). Intact fish transferred to the second tank shows a preference for the black, rather than the white, background area: the average time spent in the black background area is two to three times longer than that spent in the white background area (Matsuda *et al.*, 2011a,b). This scototaxis test appears to be suitable for evaluating the effect of pharmacological compounds on the psychomotor activity in teleost fish. Thus, ICV injection of the mild tranquilizer diazepam, a central-type benzodiazepine receptor agonist, reduces the time taken to move from the black to the white background area, whereas the anxiogenic agent FG-7142, an inverse agonist of the central-type benzodiazepine receptor, prolongs the time spent by the fish in the black background area (Matsuda *et al.*, 2011b). These observations validate the use of this behavioral test for the evaluation of the anxiolytic and anxiogenic properties of pharmacological compounds. In fact, the scototaxis test has recently been employed in fish to investigate the anxiolytic- and anxiogenic-like activities of neuropeptides such as NPY and octadecaneuropeptide in relation to feeding regulation (Matsuda *et al.*, 2011a,b).

B. Zebrafish

The zebrafish has been widely adopted as a model for the studies of sleep and related brain function (Rihel *et al.*, 2010; Zhdanova, 2011), because it has been shown to be a useful laboratory animal in the fields of comparative genomics, developmental biology, endocrinology, neuroscience, and behavioral biology (Faraco *et al.*, 2006). In this species, large populations of γ-aminobutyric acid (GABA)-ergic neurons in the hypothalamus express the orexin receptor (Yokogawa *et al.*, 2007). Overexpression of orexin in this species is reportedly associated with insomnia-like behavior, promoting and consolidating wakefulness, and suppressing a resting status (Prober *et al.*, 2006). On the other hand, zebrafish lacking the orexin receptor exhibits short and fragmented sleep under dark conditions (Yokogawa *et al.*, 2007). In the study of zebrafish hypocretin/orexin receptor mutant (hcrtr−/−),

the level of expression of mRNA for arylalkylamine-*N*-acetyltransferase, an enzyme involved in melatonin synthesis, is reduced in hcrtr −/− pineal gland during the night. Moreover, hypocretin/orexin perfusion of cultured zebrafish pineal glands induces melatonin release, suggesting the regulation of sleep/wakefulness condition by hypocretin–melatonin interaction in zebrafish (Appelbaum *et al.*, 2009). These data suggest that orexin is involved in the regulation of sleep/wakefulness in fish, as is the case in mammals.

C. Other fish

IP injection of orexin A at a high dose of 28 pmol/g BW stimulates locomotor activity in the ornate wrasse (Facciolo *et al.*, 2009), and treatment with a $GABA_A$ receptor antagonist, bicuculline, at 1 μg/g BW reduces the locomotor activity with convulsive behavior in this species, suggesting the involvement of $GABA_A$ and orexin receptor systems in the regulation of motor behaviors (Facciolo *et al.*, 2010).

VI. Conclusions

There is reliable information to indicate that orexin exerts an orexigenic action in goldfish and zebrafish. In other fish, orexin may also be involved in appetite control, since it is expressed mainly in hypothalamic regions, and feeding or fasting conditions affect the expression and synthesis of orexin precursor mRNA.

Recently, behavioral studies have indicated that tests of preference and activity can provide valuable models for evaluating the psychophysiological effects of neuropeptides such as orexin. Such tests in goldfish and zebrafish have shown that orexin induces an increase of locomotor activity. These data indicate that, in fish as in rodents, as well as being implicated in the regulation of food intake, orexin is also involved in the control of locomotor or psychomotor activity in fish. Indeed, orexin regulates the rest and activity (sleep/wakefulness) in zebrafish. It is of considerable interest that the orexin system and its function might be conserved among vertebrates, but that its molecular basis may differ somewhat from the tetrapod system: in teleost fish, there is only one receptor for orexin, and OX1R has not yet evolved. In the zebrafish, the OX1R locus is missing from the chromosome.

It is likely that orexin-induced actions are responsible for energy homeostasis, including food intake, locomotor and psychomotor activities, reproduction, and sleep/wakefulness cycle in teleost fish (Fig. 18.7). However, further study will be required to elucidate the molecular mechanisms underlying the function of orexin in teleosts.

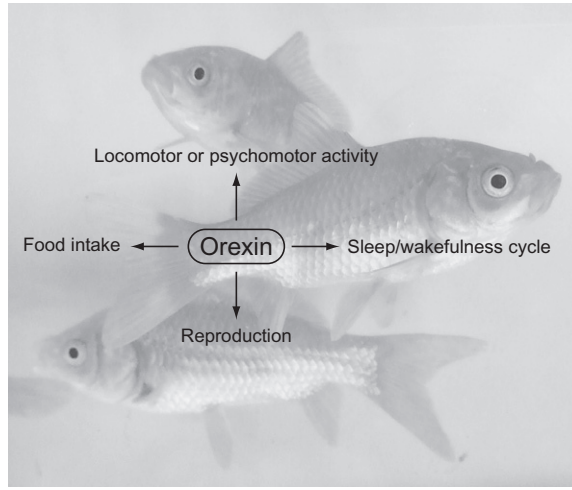

Figure 18.7 Schematic drawing depicting the role of orexin in teleost fish. Orexin-induced actions are responsible for energy homeostasis, including food intake, locomotor and psychomotor activities, reproduction, and the sleep/wakefulness cycle in teleost fish.

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Conflicts of Interest. The authors declare no conflicts of interest.

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