

ORIGINAL ARTICLE

Molecular cloning of medaka orexin and orexin receptor and decreased spontaneous movement in response to an orexin inhibitor

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Abstract: Orexin (hypocretin) is a neuropeptide secreted by hypothalamic neurons that is activated during motivating behaviors and active waking. Our knowledge of orexin is mainly limited to mammalian species, such as humans, mice, and rats. In the present study, we cloned orexin and its receptor from the medaka brain. Additionally, we analyzed the effect of an orexin inhibitor (SB-649868) on spontaneous behavior. The orexin inhibitor decreased spontaneous movement of the fish, suggesting the importance of the orexin system in spontaneous movement. Our results indicate that medaka might be a suitable animal model for neurological behavioral studies.

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Key words: medaka; orexin; cDNA; neuropeptide; fish.

Introduction

Orexin, also known as hypocretin, is a neuropeptide that regulates arousal, wakefulness, and appetite^{1,3)}. The orexin system regulates feeding, energy homeostasis, sleep, and wakefulness^{1,3)}. Symptoms of a narcolepsy-like sleep disorder are observed in mice with deficiencies in orexin and the orexin-2 receptor^{4,5)}. The most common form of narcolepsy (> 90%) is caused by a lack of orexin in the brain⁶⁾. There are two orexin peptides in the mammalian central nervous system (CNS), orexin-A (33 amino acids) and orexin-B (28 amino acids), which are derived from post-translational cleavage of a common precursor prepro-orexin^{1,2)}. Orexin A and B have 46% similarity in their amino acid sequences. Neurons expressing orexin are located within the lateral and posterior hypothalamus, and project throughout the hypothalamus, brain

stem, and thalamus. In mammalian species, orexins orchestrate diverse central effects following binding of two G-protein coupled receptors, orexin receptor-1 (OX₁; 425 amino acids) and orexin receptor-2 (OX₂; 444 amino acids). OX₁ is linked to Gq proteins and OX₂ is associated with Gq or Gi proteins³⁾. The importance of the orexin receptor in narcolepsy is known, but only one receptor (similar to type-2) has been reported in zebrafish⁷⁾. Although the functions of orexin and orexin receptors have primarily been characterized in mammalian species, recent zebrafish studies have demonstrated the importance of orexin and its receptor in small fish⁷⁾. Recent zebrafish work has shown considerable progress, but in other small fishes, such as medaka, the importance of orexin is still unknown⁸⁾.

The Japanese rice fish (*Oryzias latipes*), also known as the medaka or Japanese killifish⁹⁾, has

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been a small and popular aquarium species since the 17th century. The medaka is found in East and Southeast Asia¹⁰⁾ and has been used for biological experiments since 1921¹¹⁾. Similar to the zebrafish (*Danio rerio*), which has been widely used since the 1980s¹²⁾, the medaka has become a prominent vertebrate model for genetic and embryological studies.

To fully realize the potential of medaka as a cost-effective animal model for drug screens affecting the CNS, we analyzed the orexin system of the medaka. We cloned orexin and its receptor in the medaka brain. In addition, we established a stereotaxic apparatus for injecting drugs and a videotracking system to assess movement.

Materials and methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Hirosaki University School of Medicine. We used 6-month-old HO5 medaka, which is a standard inbred line from Southern Japan.

RNA isolation and reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

Total RNA was isolated from the brain using the RNeasy Kit (Qiagen, Valencia, CA, USA). The reverse transcription reaction was performed in a solution of 10 pmol oligo-dT primer, 1 µg RNA, 1 × first strand cDNA buffer (Life Technologies, Rockville, MD, USA), 10 mM dithiothreitol, 0.4 mM dNTPs, and 200 U Superscript III (Thermo Fisher Scientific, Waltham, MA, USA), in a volume of 20 µl at 42°C for 45 min, as described previously¹³⁾. The sequences of orexin and its receptor were identified using human orthologous sequences. We mapped the sequences of these transcripts using BLASTN (version 2.2.31) and the whole genome shotgun

sequence of medaka from the National Center for Biotechnology Information (NCBI). Specific oligo DNAs were synthesized according to the predicted sequences. The medaka orexin cDNA sequence (420 bp) was amplified with medakaORXfor (atgtgggaccctcaaactctca) and medakaORXrev (gaaagggaaagggtgtcacgatagtgg). The medaka orexin receptor cDNA sequence (1275 bp) was amplified with medakaORXRfor (atgtctggaatctctgggaatttggga) and medakaORXRrev (tcaagacaaaacgatctgctctgac). The amplified PCR products were subcloned into pGMAX and sequenced¹⁴⁾.

Western blot analysis

Lysates from brain were prepared using TNE buffer in the presence of a protease inhibitor cocktail (Roche Pharma, Basel, Switzerland). Lysates (100 µg) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with the following antibodies: anti-orexin-A (N-18; Santa Cruz Biotechnology, Dallas, TX, USA), anti-orexin-A (C-19; Santa Cruz Biotechnology), and anti-orexin R-2 (Santa Cruz Biotechnology), followed by incubation with alkaline phosphatase-conjugated secondary antibodies¹⁵⁾. Anti-orexin antibodies (N-18 and C-19) were raised against human orexin, while they detect orexin of mouse and rat. Anti-Orexin R-2 antibody was raised against human orexin receptor-2, while it also recognizes orexin receptor-2 of mouse, rat, bovine, canine and porcine. The results were visualized by a colorimetric reaction using the Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, WI, USA).

Cerebroventricular microinjection of an ORXR antagonist

Cerebroventricular microinjection consists of generating an opening through the skull into the cerebroventricular fluid (CVF) of the adult medaka and injecting into the CVF. The fish

were anesthetized in 0.04% tricaine in a Petri dish, fixed on a hand-made stage, and the cranial bone close to the midline above the optic tectum was incised using the sharp edge of a fine needle with a short bevel (35 G needle; Saitouryoukikai, Tokyo, Japan) under monocular vision (multi monocular 4 × 12; Vixen Co., Ltd., Higashi-Tokorozawa, Saitama, Japan). This incision was made through a slit in the skull with a diameter of approximately 200 µm without damaging the underlying optic tectum. Approximately 1000 nl of solution dissolved in artificial cerebrospinal fluid (127 mM NaCl, 1.0 mM KCl, 1.2 mM KH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 2.4 mM CaCl₂, and 1.3 mM MgCl₂) was injected using thin glass capillaries. The injection apparatus contained a microinjection holder and a needle. The stereotaxic device was made from a Micro Miller MF70 apparatus (Proxxon GmbH, Föhren, Germany).

Medaka movement tracking system

Behavioral assays

Spontaneous activity was monitored using a videotracking system. To acclimate the medaka to the new environment, the fish were placed in a round dish (water bath; diameter 15 cm) filled with 40 ml of water at 25°C for 30 min. The water bath was placed in a white color box (25 x 15 cm), and the fish were illuminated with a 900 lux white light.

Data collection and analysis

Movement was captured by a web camera (HD webcam pro9000, Logicool Co. Ltd., Lausanne, Switzerland) with an acquisition sampling frequency of 1 fps and resolution of 1280 × 720 pixels^{16, 17}. Video data was converted to time-lapse video data (H.264; MP4) for further analysis with Panolapse 1.15 software (<http://www.panolapse360.com/jp/>). The developed time-lapse sequences were analyzed using

Kinovea 0.8.15 (<https://www.kinovea.org/>) motion analysis software. Once the medaka head was identified on the video, it was automatically tracked by the software, which extracted the displacement in two dimensions. Then, data of the two displacement signals (*x*- and *y*-axes at each second) were collected, and the distance of each frame was calculated (Δx and Δy).

Statistics

Data are expressed as mean ± standard error. Prior to statistical analyses, data were analyzed with the Shapiro-Wilk test. Following confirmation of a normal distribution, statistical differences were determined using Student's *t*-test. A *p*-value < 0.05 was considered significant.

Results

Molecular cloning of orexin (hypocretin) and the orexin receptor

We screened databases containing the medaka genome (eEnsembl; http://asia.ensembl.org/Oryzias_latipes/Info/Index and NCBI; <https://www.ncbi.nlm.nih.gov/>) using the mouse pre-pro-orexin cDNA sequence [NM_010410]. We identified the medaka pre-pro-orexin cDNA (XM_011477947). We also identified a partial cDNA (384 bp, KT202308.1). The medaka genomic sequences revealed that this gene was located from 6191387 to 6191753 (exon2) and from 6193375 to 6193428 (exon1) bp, at a contig of chromosome 8 in its local position (CP020672.1), and consisted of two exons. We constructed specific oligonucleotide primers based on these exons. RT-PCR with these primers amplified a single band in the medaka brain (Figure 1A, ORX).

We also searched the databases containing the medaka genome using the mouse orexin receptor type-2 cDNA sequence [NM_198962]. We identified a cDNA of the orexin type-2 receptor like-sequence [XM_004077422]. We

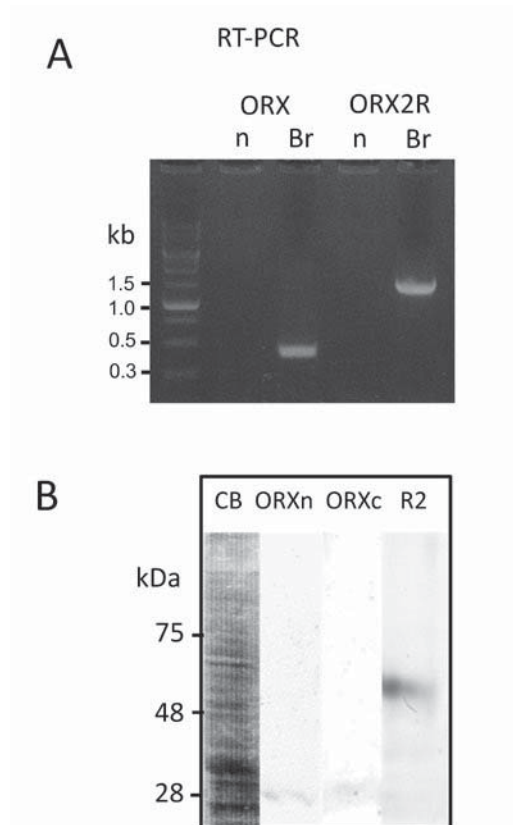


Figure 1 (A) Reverse-transcription polymerase chain reaction (RT-PCR) analysis to determine prepro-orexin and the orexin receptor. DNA ladder is indicated (left). n, negative control and Br, medaka brain. PCR primer sets for orexin (ORX) and the orexin receptor (ORX2R) are indicated. (B) Western blot analysis using anti-orexin and anti-orexin receptor type-2 antibodies. CB, Coomassie Brilliant Blue staining of total protein; ORXn, antibody against the N-terminal sequence of orexin-A; ORXc, antibody against the C-terminal sequence of orexin-A; and R2, antibody against the type-2 orexin receptor. Protein molecular-weight size markers are indicated (28, 48, and 75 kDa).

identified several candidates with the mouse orexin type-1 receptor sequence (NM_198959), [DK017798.1, DK003185, and DK061979]. However, they proved to be GPCR orthologs (DK017798.1, delta-type opioid receptor; DK003185, delta-type opioid receptor; and DK061979, alpha-1A adrenergic receptor). We constructed specific oligonucleotide primers based on the medaka orexin type-2 receptor-like sequence. RT-PCR with these primers amplified a single band in the medaka brain (Figure 1A, ORX2R).

Protein expression of medaka orexin and its receptor

Following cloning of ORX and its receptor cDNA, we expected to detect expression of

orexin and its receptor in the brain. To confirm this, we performed immunoblotting with anti-orexin-A and anti-orexin-2 receptor antibodies. The anti-orexin-A antibody, which recognizes the N-terminal sequence of orexin-A, revealed a faint band (Figure 1B; 30 kDa, ORXn). Anti-orexin-A antibody revealed a single band (30 kDa) with low expression in the medaka brain (ORXc), while the anti-orexin-2 receptor antibody revealed a single band (53 kDa) with low expression (R2). It has been reported that orexin localizes predominantly in the perifornical area and lateral hypothalamus, although it projects whole brain except cerebellum^{1, 2)}. Therefore, low expression of orexin was expected.

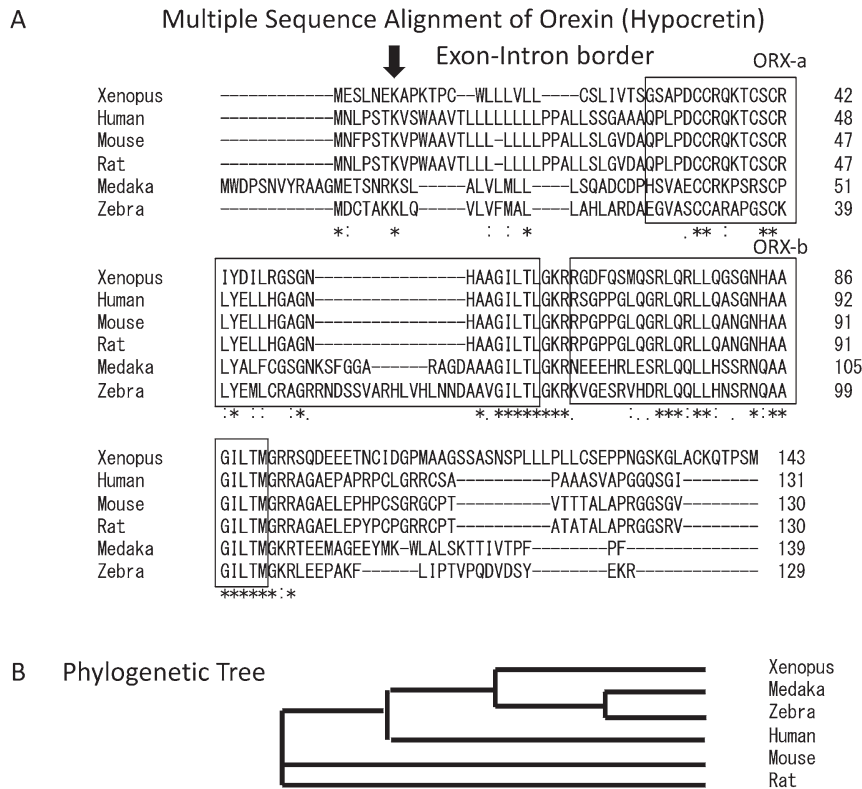


Figure 2 Alignment of the orexin homologs of various species (A). Exon-intron border of the medaka orexin gene is indicated (arrow). Amino acid sequences of orexin-a and orexin-b are indicated. Phylogenies of the orexin genes of *Xenopus*, medaka, zebrafish, human, mouse, and rat (B). The amino acid sequences were analyzed using CLUSTALW (<http://www.genome.jp/tools/clustalw/>).

Molecular cloning of prepro-orexin

Database analyses of mouse orexin cDNA revealed the amino acid sequences of medaka orexin (Figure 2A). Using the NCBI database, we compared *Xenopus*, human, rat, and zebrafish orexin homologs. Figure 2A shows the amino acid sequence alignment. These sequences contain two conserved domains, orexin-A and orexin-B. The overall homologies of the orexins were relatively low. The homology between medaka and *Xenopus* orexin was 28.8% while the homology between medaka and zebrafish orexin was 40.3%. The homology between *Xenopus* and zebrafish orexin was the lowest (27.9%). Medaka orexin showed a tendency to be more similar to mammalian sequences than to that of zebrafish.

Molecular cloning of the orexin receptor

Database analyses with mouse orexin-1 and -2 receptor cDNA revealed the amino acid sequences of the medaka orexin-2 receptor (Figure 3). Using the NCBI database, we compared *Xenopus*, human, rat, and zebrafish orexin homologs. Figure 3 shows the amino acid sequence alignment. The overall homologies of the orexin-2 receptors were relatively high. The homology between the medaka and zebrafish orexin-2 receptor was 76.7%. The homology between the medaka and *Xenopus* orexin-2 receptor was 67.0%. The medaka orexin-2 receptor showed a higher similarity (68.4%) to the human orexin-2 receptor than to the human orexin-1 receptor (64%). During the database search, we tried to find another orexin receptor (like type-1) in medaka and found several

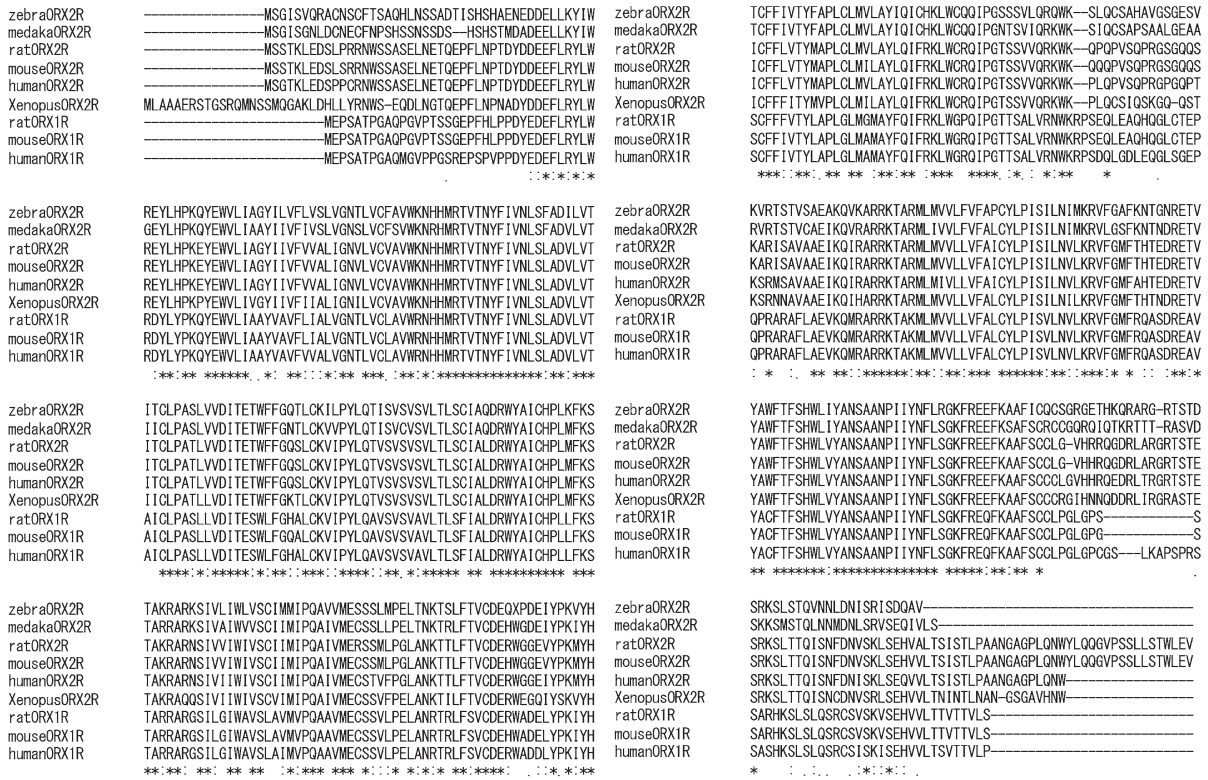


Figure 3 Alignment of the orexin receptor homologs of various species. The amino acid sequences were analyzed using CLUSTALW (<http://www.genome.jp/tools/clustalw/>).

candidate genes (medakaNPFFR2L, neuropeptide FF receptor 2-like; olbr52c23, delta-type opioid receptor; olbr10c17, delta-type opioid receptor; and olea50e18, alpha-1A adrenergic receptor), though their homologies were low (< 29% of the medaka orexin-2 receptor). Taken together, our database analysis revealed only one orexin receptor in Medaka fish.

Behavioral analysis

To further analyze the importance of orexin in medaka, we established a videotracking system to detect spontaneous movement of the fish. A stereotactic apparatus was prepared with a micro Miller apparatus and sponge surgical-bed (Figure 4A and B). The hand-made sponge surgical bed was mounted on a ball (3 cm diameter) located on a ring, which enabled fine movement of the surgical bed. The hand-made sponge surgical bed was mounted on a ball (3 cm diameter) located on a ring, which enabled fine movement of the surgical bed. The hand-made sponge surgical bed was mounted on a ball (3 cm diameter) located on a ring, which enabled fine movement of the surgical bed. The hand-made sponge surgical bed was mounted on a ball (3 cm diameter) located on a ring, which enabled fine movement of the surgical bed.

a water bath and its behavior was monitored with the web camera-based videotracking system (Figure 4C). The video data were analyzed with a semi-automated videotracking program (Kinovea 0.8.15). The adult medaka fish were active following cerebroventricular microinjection of the artificial CSF (Figure 5Ai). By contrast, the medaka showed significantly decreased spontaneous movement following microinjection of the orexin inhibitor (SB-649868, 10 mg/kg; Figure 5Aii, right panel). Statistical analysis revealed significantly decreased spontaneous movement due to SB-649868 (Figure 5B), indicating the significance of orexin in spontaneous activity.

Discussion

In the present study, we cloned orexin and its receptor for the medaka brain. mRNA and

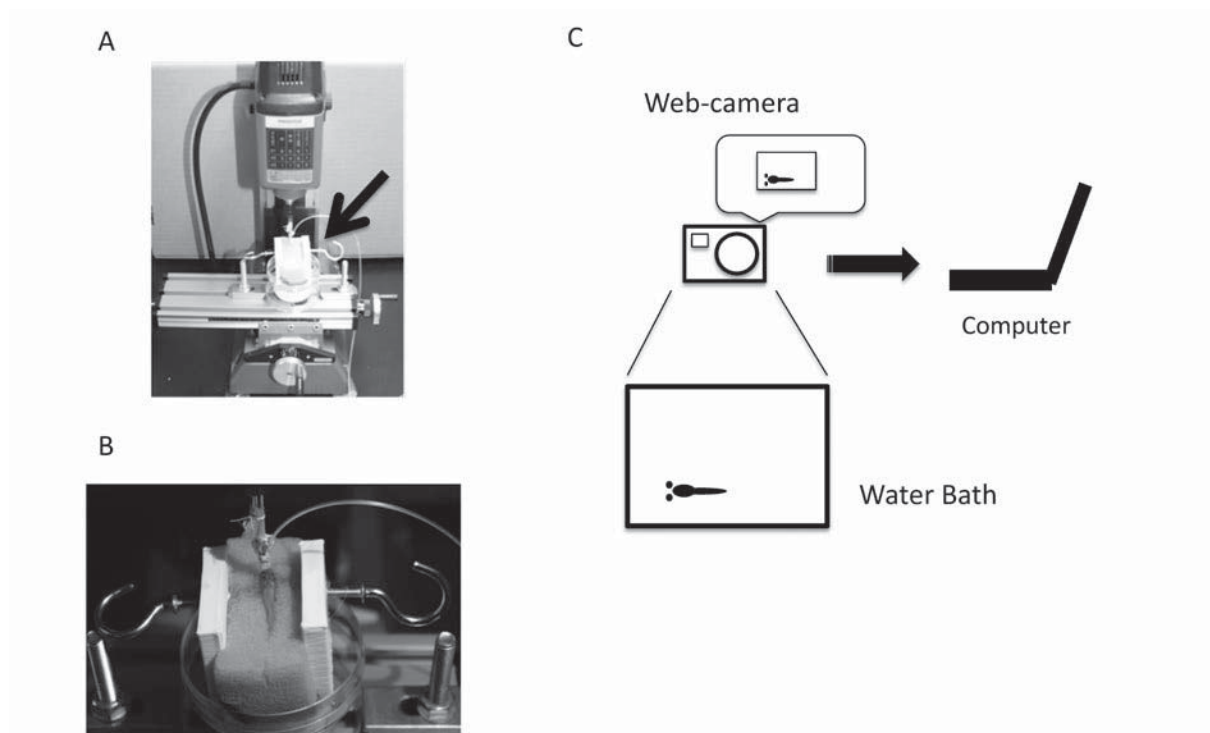


Figure 4 Overview of stereotactic apparatus (A). Surgical bed for medaka (arrow). An incision needle was inserted in the medaka skull for microinjection (B). Diagram of videotracking system (C). The spontaneous movement of medaka fish was recorded with video-system, digitized and saved in a computer. Data were analyzed with Kinovea program.

protein expression of orexin and its receptor was confirmed in the medaka brain. We also analyzed the effect of an orexin inhibitor on spontaneous behavior. The orexin inhibitor (SB-649868) decreased spontaneous movement of medaka, suggesting involvement of the orexin system in spontaneous movement. Our results indicate the existence of an orexin system in medaka, suggesting that medaka might be a suitable animal model for neurological behavioral studies.

We report the complete two-exon structure of the medaka orexin gene. In a previous study, Kaslin et al. reported that ORX has a single exon¹⁸⁾. Faraco et al. reported an additional first exon in zebrafish¹⁹⁾. Combined with our results, it is apparent that the orexin gene has a two-exon structure.

Faraco et al. predicted the medaka orexin amino acid sequence, which was 10 amino acids

shorter than our sequence at the N-terminus¹⁹⁾. As Faraco et al. only performed *in silico* analysis and the computer database is still being developed, medaka may have a longer pre-pro-orexin sequence; further analysis is necessary to determine the first start codon. ATG is typically used as the start codon, although the first in-frame ATG is not always the start codon. Therefore, we tried to find the Kozak sequence and analyzed the 5'-terminal sequence with the online program ATGpr (<http://atgpr.dbcls.jp>), which predicted that the first ATG had 0.07 reliability, while the second ATG had lower reliability (0.04). The first ATG contains only a single base that conforms to the Kozak rule (AXXATGt), while the second ATG contains two (GXXATGG). Taken together, the first ATG might be the start codon. Nevertheless, a future study will be needed to determine the first codon of the medaka orexin gene.

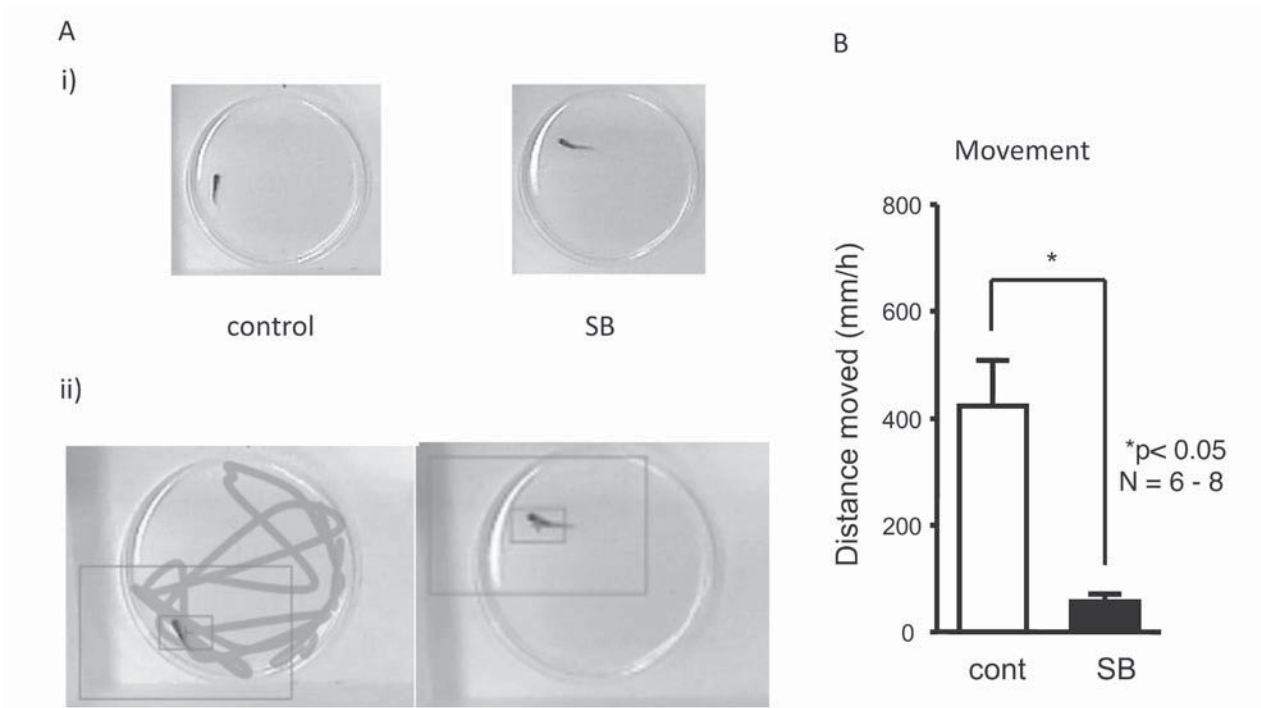


Figure 5 Behavioral analysis. Medaka fish following microinjection of the negative control (artificial cerebrospinal fluid [Ai]) and SB-649868 (Aii). The water bath containing medaka was mounted in a white box for videotracking. Spontaneous movements of the control (left panel) and SB-649868-injected medaka (right panel) are shown (Aii). Control medaka exhibited active movements (blue lines), while the SB-649868-injected medaka showed marginal movement. Statistical analysis of spontaneous movement (B). $n = 6-8$. * $p < 0.05$ vs. control.

In the present study, we used medaka fish (Japanese rice fish) as an animal model. Medaka fish has several advantages over zebrafish. One advantage is the low cost for animal care. Medaka fish live in a wide range of temperatures (from 4 to 40 °C in nature), while zebra fish needs more rigorous condition. Medaka fish spawn 100 to 200 eggs every week and become adults only two to three months after hatching. Another advantage is trunk transparency, comparing with zebrafish. This advantage is useful for the observation of internal organs.

In conjunction with a previous study on the orexin receptor in zebrafish⁷, we identified a single orexin receptor gene in medaka by *in silico* database analysis. As noted, the medaka orexin receptor (ORX2R) has higher homology to mammalian type-2 ORX receptor than to the type-1 receptor⁷. Considering the importance of

the type-2 receptor, as its mutation causes the narcolepsy phenotype in mammalian species^{4, 20}, a defect in the type-1 receptor in medaka, as well as zebrafish, is interesting. As the type-1 receptor is widely expressed in the brain of mammalian species, the type-1 receptor might have neurological importance.

Since the discovery of the orexin system and its importance in sleep and narcolepsy has been clarified^{4, 20}, we examined the effect of an orexin antagonist by cerebroventricular microinjection²¹. For this purpose, we established an approximate 1000 nl microinjection system, which was originally used for DNA microinjection to produce transgenic mice. As the medaka is smaller than a rodent, such as a rat or mouse, our behavioral analysis following cerebroventricular microinjection suggests that medaka might be a useful animal model for behavioral analyses.

Conclusion

We identified orexin and orexin receptor cDNA from the medaka brain. Cerebroventricular microinjection of the orexin inhibitor (SB-649868) decreased spontaneous movement of medaka. Our results indicate that medaka might be a suitable animal model for neurological behavioral studies.

Acknowledgments

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Conflict of interest (COI)

We declare no conflict of interest.

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