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Circadian pacemaker in the suprachiasmatic nuclei of teleost fish revealed by rhythmic *period2* expression

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ABSTRACT

In mammals, the role of the suprachiasmatic nucleus (SCN) as the primary circadian clock that coordinates the biological rhythms of peripheral oscillators is well known. However, in teleosts, it remains unclear whether the SCN also functions as a circadian pacemaker. We used in situ hybridization (ISH) techniques to demonstrate that the molecular clock gene, *per2*, is expressed in the SCN of flounder (*Paralichthys olivaceus*) larvae during the day and down-regulated at night, demonstrating that a circadian pacemaker exists in the SCN of this teleost. The finding that *per2* expression in the SCN was also observed in the amberjack (*Seriola dumerili*), but not in medaka (*Oryzias latipes*), implies that interspecific variation exists in the extent to which the SCN controls the circadian rhythms of fish species, presumably reflecting their lifestyle. Rhythmic *per2* expression was also detected in the pineal gland and pituitary, and aperiodic *per2* expression was observed in the habenula, which is known to exhibit circadian rhythms in rodents. Since the ontogeny of *per2* expression in the brain of early flounder larvae can be monitored by whole mount ISH, it is possible to investigate the effects of drugs and environmental conditions on the functional development of circadian clocks in the brain of fish larvae. In addition, flounder would be a good model for understanding the rhythmicity of marine fish. Our findings open a new frontier for investigating the role of the SCN in teleost circadian rhythms.

1. Introduction

The rhythms in animal cells are under the control of circadian clocks, which function through the interactions between oscillation feedback loops associated with clock genes [17,30]. These genes include members of the transcriptional activators *Clock* and *Bmal1*, and negative regulatory factors, such as *Periods* (*Per1* and *Per2*) and *Cryptochrome* [17,30]. In mammals, because the rhythm of the circadian clock is not precisely 24 h, it is adjusted to more closely approximate a 24 h-cycle through the actions of the suprachiasmatic nucleus (SCN) in the anterior hypothalamus [3,50,55]. Briefly, expression of the clock genes in the SCN occurs in response to changes in the external environment, which is inferred by processing the photic signals that are transmitted to the SCN from the eyes via the retinohypothalamic tract (RHT) [30]. In this system, *Period 2* (*Per2*), which is involved in light entrainment of the circadian clock, has a well defined transcript expression rhythm that peaks during the day and then decreases at night [17]. As the primary circadian clock, the SCN coordinates the activities of the various peripheral oscillators that control rhythmic physiological processes of organs, such as hormonal secretion and locomotion [30]. In mammals, the pineal gland is considered to be a peripheral oscillator that is under the control of the SCN. In this system, oscillation signals produced by the SCN are relayed to the pineal gland via the multisynaptic efferent pathway [18,47]. This system regulates the production and release of melatonin, an internal time-keeping molecule, by the pineal gland at night. The circadian rhythms of all mammals are considered to be coordinated by this system.

The small teleost fishes, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), are widely used as model organisms in developmental biology, endocrinology, and pharmacology [31,41]. The external development and availability of large numbers of fertilized eggs throughout the year mean that fish embryos are well suited for investigating the ontogeny of the circadian clock and its aberrations due to perturbations in light conditions and exposure to drugs. Several studies have been published on the characteristics of the SCN and *per2* expression in zebrafish embryos to date [8,20,35,42]. The SCN has been identified in zebrafish based on structural

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anatomy and the expression of *tyrosine hydroxylase* (*th*), a key enzyme involved in dopamine production, and vasopressin, a neuropeptide, known to be expressed in the mammalian SCN [8,20,35,42]. However, *per2* expression in the SCN has not yet been observed in zebrafish or other teleosts, and the function of the SCN in the regulation of circadian rhythms is unknown [15,24,53,56,31]. It has been suggested that the presence of a pineal gland containing endogenous, light-sensitive photoreceptors in non-mammalian vertebrates such as fish and birds implies the existence of a photoreceptive circadian oscillator that is responsible for regulating the circadian rhythm of melatonin synthesis [7,9,18,34,36,40]. Unlike the SCN, the pineal gland of zebrafish exhibits rhythmic *per2* expression [52,56]. It is thus possible that the pineal gland of teleosts has an autonomic system that produces melatonin rhythmically, and that this production is governed by an endogenous molecular clock that employs an adjustment system consisting of photoreceptors. As a consequence, the extent to which teleosts are dependent upon the SCN to control circadian rhythms might be lower than that in mammals. It has recently been shown that, although the pineal gland in zebrafish larvae develops circadian rhythms, *per se*, in the absence of an SCN, the maintenance of circadian rhythms under conditions of continuous light requires the presence of the SCN, which suggests that the SCN does play some role in the maintenance of circadian rhythm in the brains of fish larvae [35]. However, the role of the SCN as the circadian rhythm pacemaker in teleosts is currently unclear [35].

The best approach to demonstrate the existence of a circadian pacemaker in the teleost SCN is thus to identify a species that expresses molecular clock genes, such as *per2*, in the SCN. Indeed, such a species would be well suited for use as a model for future investigations on circadian rhythms in vertebrates. In addition to feeding behavior, the reproduction and migration of marine and anadromous fish is frequently synchronized with solar or lunar cycles, with differences in the cyclicity of these behaviors often being observed over the course of seasons or the entire life cycle of the animal [6,51]. We therefore expected that well defined, biological rhythms would be observed in the activity of the RHT and SCN of marine fish when compared with smaller freshwater fish. We recently reported that in flounder (*Paralichthys olivaceus*: Pleuronectiformes) larvae, continuous illumination (LL) during larval development suppresses dopamine synthesis in the SCN, which promotes up-regulation of proopiomelanocortin (*pomc*) mRNA expression in the pituitary [23]. Flounder may therefore have developed the ability to use the RHT and SCN for light entrainment of the circadian clock. In this report on flounder larvae, we examined *per2* expression in the brain using an in situ hybridization (ISH) technique to clarify whether the circadian clock is well developed in the SCN of the flounder. We also sought to demonstrate whether *per2* expression in the SCN could be visualized using whole mounts of early larvae. The obtained data illustrated the role of the SCN as a pacemaker of the circadian rhythms in fish, and also that flounder larvae are well suited for examining various aspects of circadian rhythms and their interaction with the brain via the SCN.

2. Materials and methods

2.1. Experimental fish

Flounder were maintained at the Miyako Station of the Tohoku Research Institute of Fisheries in Iwate, Japan. Fertilized eggs were collected from a spawning tank containing seven females and seven males in June. The obtained embryos were maintained at 20 °C under natural light conditions until metamorphosis; sunrise and sunset times were approximately 5:00 and 18:30, respectively. For whole-mount ISH (WISH) analysis, embryos were anesthetized with MS222 and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 8:00, 16:00 and 24:00 each day between 0–6 days post-fertilization (dpf). Samples were fixed overnight and washed with PBS containing 0.05% Tween20 (PBST), before being stored in 100% methanol at −30 °C. For section ISH (SISH) analysis, larvae were fixed at 11:00 and 23:00 in Bouin's fixative (10% formalin, 5% acetic acid and 85% saturated picric acid) overnight, before being transferred to 100% ethanol and stored at −30 °C.

Amberjack (*Seriola dumerili*: Perciformes) larvae, 22 dpf, cultured at the Shibushi Station of the National Research Institute of Aquaculture in Kagoshima, Japan, were fixed at 11:00 am with Bouin's fixative and stored in 100% ethanol at −30 °C. Medaka (*O. latipes*: Beloniformes) were maintained in the laboratory at 25 °C under 14 L:10 D conditions until they were fixed at 11:00 am at 25 dpf for SISH analysis.

2.2. Cloning of flounder per2 cDNA

By aligning the *per2* coding sequences of zebrafish (NM_182857), *Takifugu* (ENSTRUG00000006817 in Ensembl), stickleback (ENSGACG00000005662), *Xenopus tropicalis* (NM_001127410), chicken (AF246956), mouse (NM_011066) and human (NM_022817), we designed the following set of degenerated PCR primers in a highly conserved region: *per2*_5′ primer, 5′-CCKTCMACCAGYGGCYGCAG; *per2*_3′ primer, 5′-GTKGTGAAGCTGTAGAACACG. The primer pair was expected to amplify a fragment of approximately 450 bp. RT-PCR was performed using cDNA from 30-dph flounder larvae. Thermocycler parameters consisted of 50 cycles of 95 °C for 30 s, 55 °C for 60 s and 72 °C for 120 s. The resulting 450 bp product was gel-purified, ligated into pDrive Cloning Vector (Qiagen, Valencia, CA, USA), and sequenced using an automated sequencer (Applied Biosystems, Foster City, CA, USA). A blastn search of the NCBI database revealed that the cloned sequence was most homologous to zebrafish *per2* cDNA. To obtain longer cDNA fragments for preparing an in situ hybridization (ISH) probe, 3′RACE was performed as described previously [45] using the following gene-specific primer set: RACE 1st PCR primer, 5′-CGTCTCACTCATCAACCCGAAAGATT; RACE 2nd nested primer, 5′-ACTGCAAGCGGGAAGTGTTCAACAA. The resulting 2 kb fragment was ligated to the pDrive Cloning Vector and sequenced. The obtained sequence was deposited in the DDBJ under Accession No. ABxxxx.

2.3. In situ hybridization

To synthesize the antisense RNA probe, the plasmid containing the 2 kb flounder *per2* fragment was linearized by digestion with Not I. A digoxigenin (DIG)-labeled probe was prepared from the digested plasmid using SP6 RNA polymerase with a DIG-RNA labeling kit (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions. Section ISH (SISH) was performed as described in a previous study [28]. Briefly, the larvae were fixed in Bouin's solution and embedded in paraffin before being cut into 8 μm sections. After removing the paraffin by xylene, the sections were hybridized with Dig-labeled probe at 55 °C overnight. The sections were then washed in a formamide and saline-sodium citrate (SSC) buffer series, before being incubated with a 2000-fold dilution of alkaline phosphatase (AP)-conjugated anti-Dig Fab fragments (Roche Diagnostics). NBT/BCIP solution was used as substrate for AP, and treated samples were observed using a conventional microscope (Leica DM2500; Leica, Wetzlar, Germany) and a stereomicroscope (Leica MZ16F) before being digitally photographed (Leica DFC500). Photographs were processed using Adobe Photoshop (ver. 7.0, Adobe Systems, San Jose, CA, USA).

An approximately 1 kb cDNA fragment of medaka *per2* (ENSORLG00000015456) was amplified from embryonic cDNA using