CHARACTERIZATION OF GENETICALLY LABELED DOPAMINE NEURONS AND CIRCADIAN STUDIES OF THE ZEBRAFISH RETINA

By

Shi Meng

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Approved:

Douglas G. McMahon, Ph.D.

Bruce H. Appel, Ph.D.

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CHAPTER I

CHARACTERIZATION OF GENETICALLY LABELED DOPAMINE NEURONS IN THE ZEBRAFISH RETINA

Introduction

In the central nervous system, dopamine (DA) plays important roles in modulating a variety of physiological events such as movement, reward, emotion and memory processing. In the vertebrate retina, dopamine is involved in mediating neuronal adaptation to light (Witkovsky and Dearry 1991, Djamgoz and Wagner, 1992), circadian rhythmicity (Mangel, 2001, Ribelayga *et al*., 2002, Sakamoto *et al*., 2005), as well as cell survival and eye growth (Yamauchi *et al*., 2003, Linden *et al*., 2004). In teleost retinas, dopamine is released by dopaminergic interplexiform cells (DA-IPCs), which contact horizontal and bipolar cell dendrites in the outer plexiform layer (OPL), and amacrine cells and bipolar cell terminals in the inner retina (Dowling and Ehinger, 1975, 1978; Witkovsky and Dearry, 1991; Dowling, 1991). DA-IPCs have been proposed to be a centrifugal pathway for information flow from the inner to the outer retina (Dowling and Ehinger, 1978, Zucker and Dowling, 1987), as well as shown to mediate the modulatory effect of olfactory input on retinal ganglion cell activity (Huang *et al*., 2005).

Despite the diverse roles of DA cells in retinal functions, the understanding of DA cell function has been limited due to the fact that they have a low density in the retina and cannot be identified in living retina by morphological characteristics (Negishi *et al*., 1981, Versaux-Botteri *et al*., 1984). In the mouse, transgenic lines have been created in which reporter genes are driven by the promoter for the *tyrosine hydroxylase* (*TH*) gene, the rate-limiting enzyme for dopamine biosynthesis. These transgenic lines provide strategies to identify dopaminergic neurons in the mouse *in vitro* (Gustincich *et al*., 1997) and *in situ* in living retina (Zhang *et al*., 2004). Here we report marking dopaminergic neurons *in vivo* in zebrafish retina using a similar strategy.

Zebrafish (Danio rerio) has become of interest for neurogenesis and dopaminergic system development due to fast embryonic development and the availability of mutagenesis and transgenesis. For example, tyrosine hydroxylase immunoreactivity studies have revealed the presence of dopaminergic neurons in the ventral diencephalon as early as 18 hours post fertilization (hpf) (Holzschuh *et al*., 2001), and in the retina at three days post fertilization (dpf) (Arenzanna *et al*., 2006). In addition, using a large-scale mutagenesis screen, mutant zebrafish lines with disrupted catecholaminergic or dopaminergic system development have been isolated (Guo *et al*., 1999, 2000, Ettl *et al*., 2006). Studies of these mutants can enhance our understanding of the dopaminergic system development and function. In order to investigate the normal function and morphology of living DA cells in the zebrafish retinas, we established a transgenic zebrafish model in which GFP is driven by the zebrafish *TH* promoter. Here, we report morphological, molecular and physiological characterization of genetically labeled dopamine neurons in this transgenic zebrafish line.

Results

Generation of *TH*::GFP transgenic fish

The *TH*::GFP transgenic fish was generated by W. Driever (University of Freiburg). Briefly, a 12-kb PAC fragment containing the zebrafish *TH* promoter was isolated and ligated with cDNA encoding enhanced green fluorescence protein (EGFP) followed by a SV40 polyadenylation tail (pA) (Fig.1A). The recombinant DNA was injected into ~ 1-2 cell zebrafish embryos which were allowed to grow to adulthood. The founder fish were then crossed with wild-type fish and screened for germ-line transmission of the transgene. Larval fish with GFP expression were raised as transgenic F1 fish.

TH-driven GFP expression during development

To determine the GFP expression pattern in these TH::GFP fish, we crossed hemizygous transgenic F1 fish with wild type fish and examined GFP expression in their progeny using

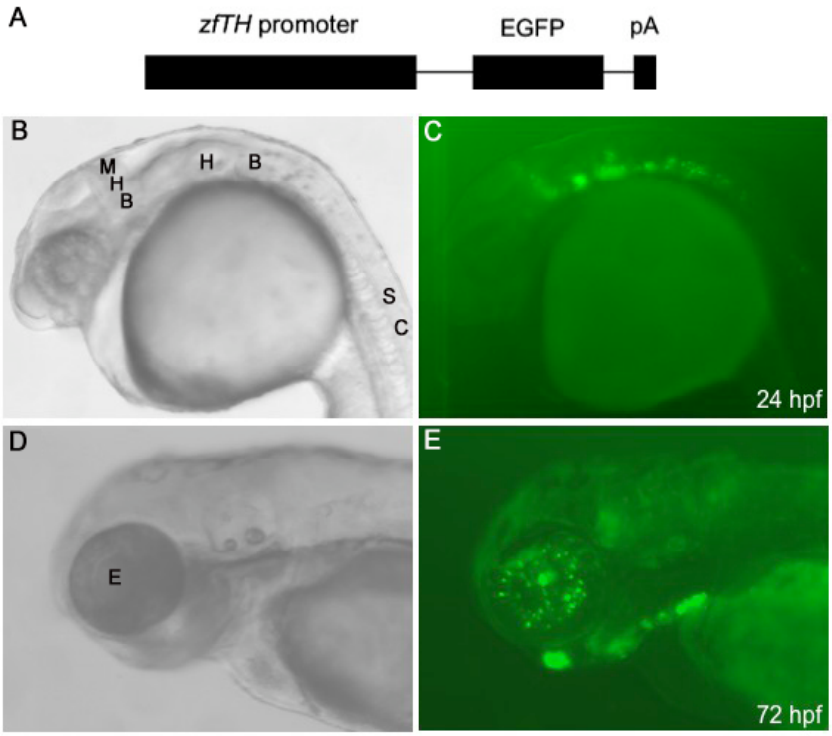

Figure 1. ***TH* promoter-driven GFP expression during zebrafish development.**
A, Schematic map of the *TH*::GFP construct. The 12 kb sequence containing the zebrafish *TH* (*zfTH)* promoter was ligated with EGFP followed by a SV40 polyadenylation tail (pA). **B**, bright field image, and **C**, fluorescence image of GFP expression in the embryonic neural system at 24 hpf. MHB: midbrain and hindbrain boundary. HB: hindbrain. SC: spinal cord. **D,** bright field, and **E**, fluorescence images of GFP expression in the embryonic retina (72 hpf). E: eye. For B – E, Dorsal is on the top and anterior is on the left.

fluorescence microscopy. The transgenic embryos showed robust GFP expression in the embryonic brain region, including the midbrain and hindbrain boundary (MHB), as well as the hindbrain and spinal cord at 24 hpf (Fig.1B). At 72 hpf, individual neurons exhibiting GFP fluorescence were identified within the retina (Fig.1C). Confocal Z-stack imaging, which reconstituted a 3-D fluorescent image of the retina, showed that at 72 hpf, individual GFP-expressing neurons were observed and all GFP expression was contained in cells in the inner retina (data not shown).

The morphology of GFP-expressing cells in the retina

To describe the morphology of GFP-expressing cells in the retina, vertical sections of the retina from one-month-old zebrafish were processed for immunocytochemistry (ICC) and GFP immunoreactivity (IR) was visualized with confocal microscopy. GFP-expressing cells were observed at the proximal cellular row of the inner nuclear layer (INL) (Fig. 2A). There was no obvious difference in the shape and size among individual GFP-expressing cells. To further investigate their morphology, z-stack images of individual cells were made in the living whole mount retina (Fig. 2B). Two primary processes arose from the cell body and formed dense branches in sublaminas b of the IPL. Fig. 2C shows the fiber network within the focal plane of the IPL. GFP-expressing cells exhibited extensive processes in proximal position of the IPL, consistent with DA cell morphology.

Co-localization of the GFP-expression cells with TH antibody staining

In order to determine the overlap of *TH::*GFP with native TH expression, we performed double-labeled immunocytochemistry using antibodies for TH and GFP on vertical sections and whole mount adult retinas. Figure.3A shows a vertical section in which GFP was expressed within two cells in the INL. The cell on the right was co-labeled with TH antibody (arrow), while the one on the left was not (arrowhead). No difference in their size and shape was observed. In addition, two distinct narrow bands of GFP-expressing processes were observed descending from the somata to the proximal IPL. Immunocytochemistry of whole mount retinas was performed to

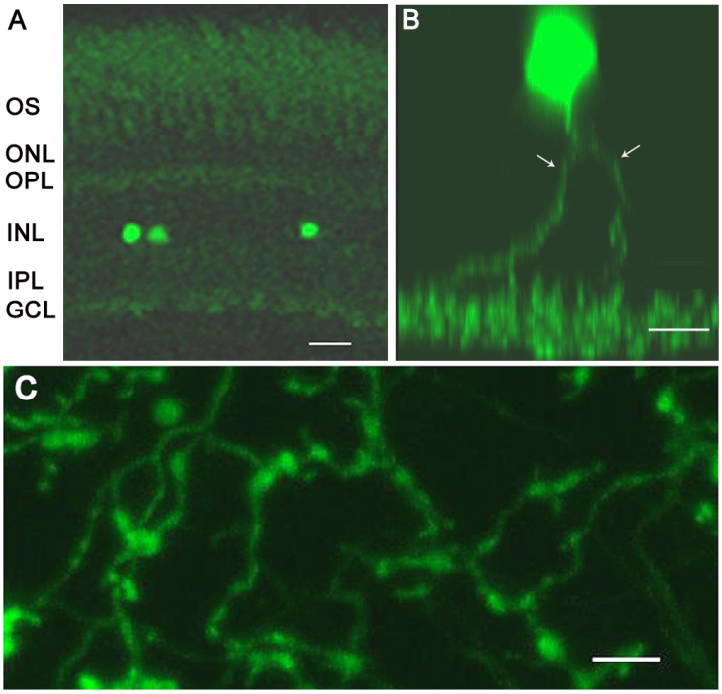

Figure 2. The morphology of GFP-expressing cells in the retina.
A, A vertical retinal section showing the localization of GFP-positive cells. Most of the GFP-expressing cells were found at the proximal cellular row of the inner nuclear layer. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer. **B**, A z-stack image showing the somata and processes of a single cell. **C,** GFP fluorescent fiber network in the inner plexiform layer. Scale Bar = 20 μm for A and C, and 10 μm for B.

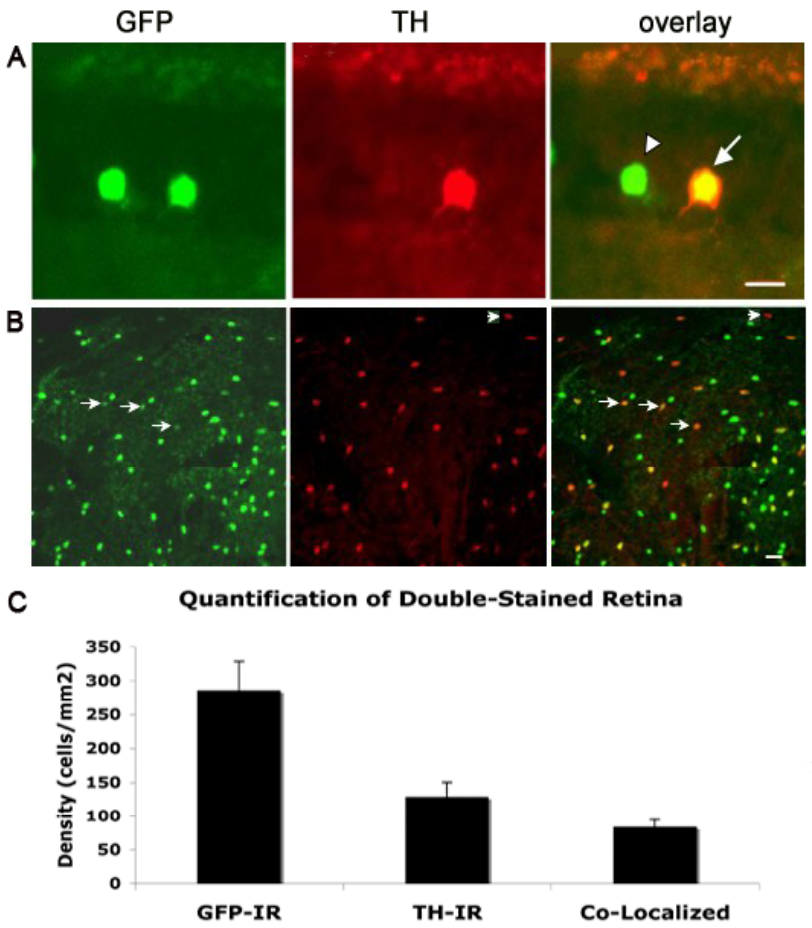

Figure 3. Double immunostaining using anti-GFP and anti-TH antibodies.
A, an image shows the vertical retinal sections and **B,** whole mount retina**.** Green: GFP-IR; Red: TH-IR. **C**, Quantification of double-staining in the whole mount retina. Overall, 29 ± 2% (mean ± SD) of GFP-labeled cells co-expressed TH. Scale bar = 10 μm for A and 20 μm for B.

examine the co-localization of GFP-IR and TH-IR (Figure 3B). The arrows indicate the cells which expressed both GFP and TH. Note the fact that while the majority of TH-IR cells also expressed GFP, there was one single-labeled by TH antibody (pointed by arrow head). In order to quantify the co-localization rate of TH and GFP in the whole retina, four whole mount retinas were processed for ICC, and images of the entire retina were analyzed. On average, 29.2 ± 1.7% (mean ± SD, n =4) of GFP-expressing neurons also expressed TH in entire zebrafish retinas, and GFP-expressing neurons exhibited a density of 286 ± 43 cells/mm^2. Meanwhile, TH-expressing cells had a density of 129 ± 21 cells/mm^2 throughout the retina and 65.0 ± 3.4% TH-expressing cells were labeled by GFP. Images of entire retinas that had been double-labeled for GFP-IR and TH-IR showed that both GFP-IR neurons and TH-IR neurons had a relatively even distribution throughout the retinas (data not shown).

An interesting fact was that there was a trend toward a greater incidence of co-localization with TH for dimmer cells, whereas the neighboring bright GFP-stained cells were single labeled. In fact, the average gray value analysis using MetaMorph on confocal images of GFP-immunostained whole mount retinas indicated a 2-fold difference in fluorescent intensity between bright GFP- or dim GFP-expressing cells (Figure 4).

Expression of *th* and *dopamine transporter* mRNA in GFP-labeled cells

To further asses what proportion of GFP-expressing cells are dopaminergic and to test the possibility that molecular components of GFP cells could be analyzed *in vitro*, we sought to detect *th* and *dopamine transporter* (*dat*) expression in isolated living cells. We acutely dissociated the retinas and plated retinal cells into culture dishes. Living retinal tissue revealed two subpopulations of GFP-positive cells: one with bright fluorescence and another with a much lower intensity. While these two subpopulations were distinguishable in fluorescence intensity (Fig. 5A), they exhibited similar cell size and shape. In order to profile the molecular characteristics of the two types of neurons, performed single-cell RT-PCR, using primers specific for *th* and *dat*, markers for DA neurons. To increase reaction specificity, semi-nested PCR primers spanning different exons of the genes were used (Fig. 5B). Bright or dim GFP-expressing

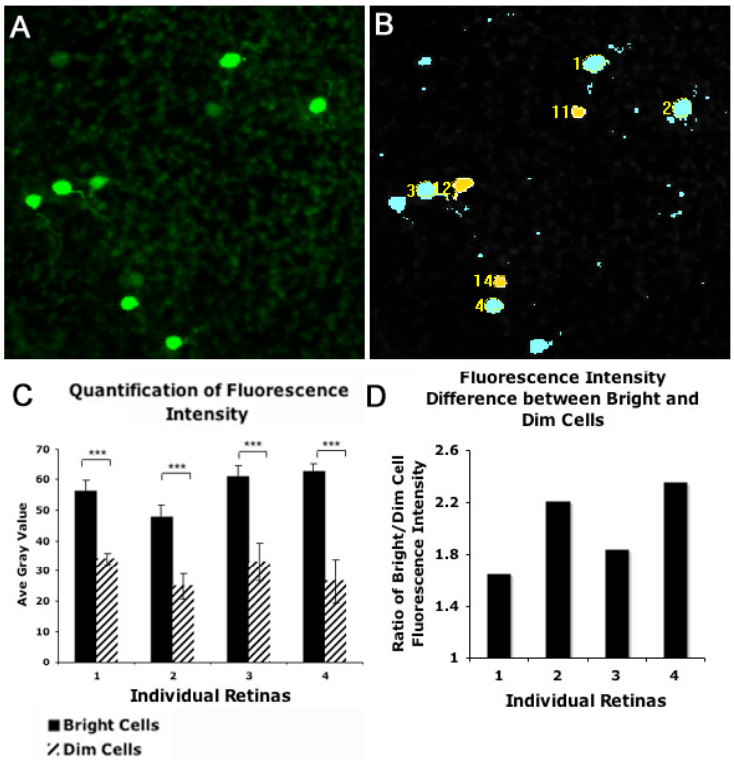

Figure 4. Quantification of fluorescence intensity among GFP-labeled cells.
A, A representative confocal image (partial) of GFP-immunostained whole mount retina. **B**, the same image area as A, as analyzed in MetaMorph. Bright cells were color coded as blue (cell 1, 2, 3 and 4), and dim cells were yellow (cell 11, 12, and 14). **C**, quantification of fluorescence intensity by analyzing bright cells and dim cells in four individual retinas. For each retina, 10 bright cells and 10 dim cells were chosen using the same threshold. Value represents mean ± SD (n = 10 for each cell subpopulation). *** indicates $p < 0.001$. **D,** the ratio of bright cell fluorescence intensity to dim cell fluorescence intensity. For all four retinas, bright cells are 2.0 ± 0.3 (mean ± SD) fold of dim cells in fluorescence intensity.

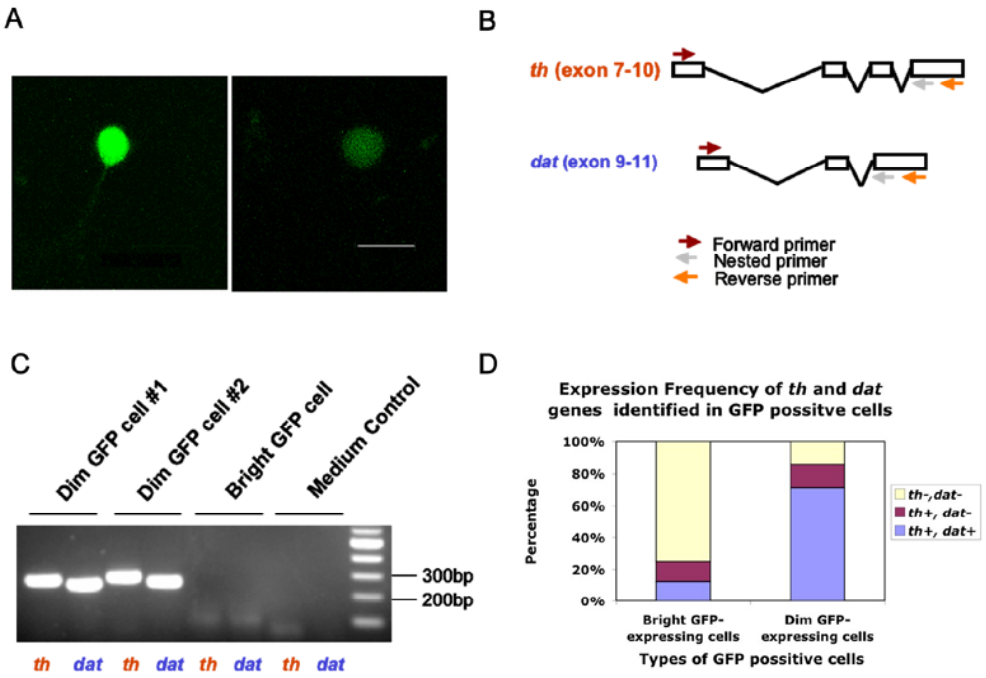

Figure 5. Analysis of two subpopulations of GFP-positive cells using single-cell RT-PCR. **A,** Two representative GFP-positive cells in the dispersed retinal cells. The pictures were taken under the same excitation intensity. Note the left cell was much brighter than the right cell. Scale Bar = 10 μm. **B,** Schematic diagram showing the primers used for semi-nested PCR. For *th*, the size of the 2nd round PCR product was 277 bp. For *dat*, the size was 250 bp. **C,** A representative gel image showing the 2nd round PCR product from dim GFP cells, bright GFP cells or medium control sample. Primer sets used were indicated at the bottom. **D,** Expression frequency of *th* and *dat* genes identified in GFP-positive cells using single-cell RT-PCR. For bright GFP-expressing cells, 8 cells were examined. For dim GFP-expressing cells, 7 cells were examined.

cells were collected and analyzed by reverse transcription followed by semi-nested PCR. Amplicon from the second round PCR was visualized on agarose gel stained with ethidium bromide (Fig. 5C). Among the seven dim GFP-expressing cells collected, five cells expressed both *th* and *dat*, while only one out of eight bright GFP-expressing cells did (Fig. 5D). These results showed that most dim GFP-expressing cells co-expressed both dopamine-cell genes, suggesting that labeled cells of this subpopulation are dopaminergic neurons.

Spontaneous spike activity of GFP-expressing neurons *in situ*

As described previously, dopaminergic amacrine cells generate spontaneous action potentials in dissociated or whole mount mouse retinas (Gustincich *et al*., 1997, Feigenspan *et al*., 1998 and Zhang *et al*., 2007). To examine the neuronal activity of dopaminergic cells in the *TH*::GFP zebrafish retina, we performed extracellular loose-patch recordings on GFP-expressing cells. Based on the initial observation that most dim-GFP-expressing cells are dopaminergic neurons, for recording we prioritized cells with relatively low GFP expression (see Figure 6A for overlaid fluorescent and DIC images of an example cell). Among the 20 GFP+ cells, 11 exhibited spontaneous spikes. Figure 6B shows a typical recording from a GFP+ cell. The cell fired in a single irregular pattern with a spiking rate of 0.75 Hz. For the 11 cells exhibiting spontaneous spikes, the firing rate ranged from 0.47 - 4.70 Hz with an average of 1.48 ± 1.17 Hz (mean ± SD).

Discussion

We report here, the characterization of GFP-expressing retinal neurons in a transgenic zebrafish line where the fluorescent reporter is driven by the zebrafish TH promoter. In this transgenic line, TH-driven GFP exhibited a robust expression in the brain and retina. In juvenile and adult zebrafish retinas, about 30% of GFP-expressing cells are dopaminergic cells. Single-cell RT-PCR results further indicated that most dim GFP-expressing cells are dopaminergic neurons. In addition, spontaneous action potentials were observed in labeled cells in darkness, suggesting these cells are functionally active. These results indicate that this transgenic line

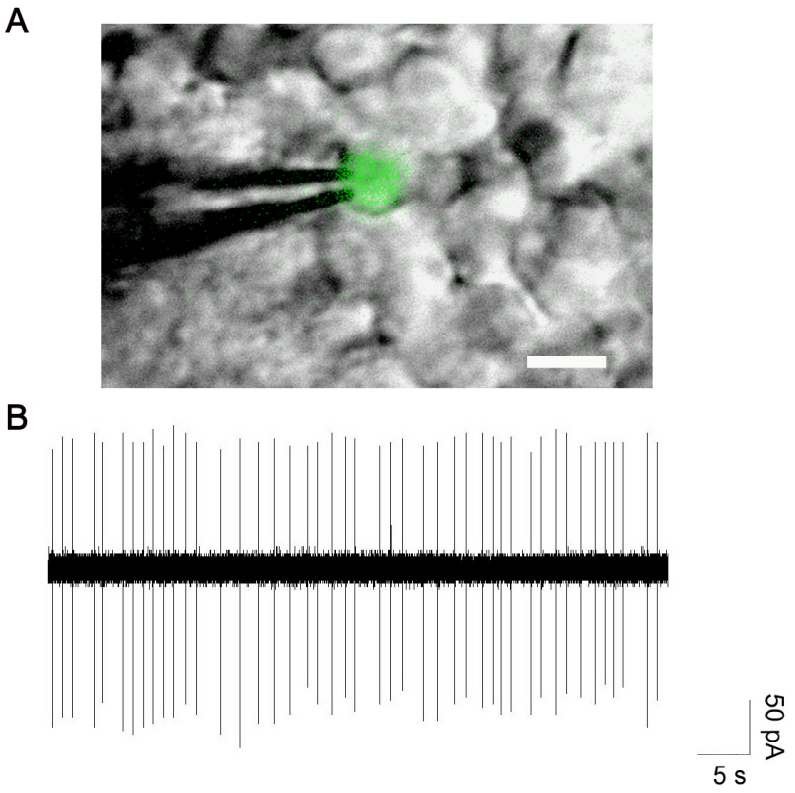

Figure 6. In isolated whole mount retina, GFP-labeled neurons exhibit spontaneous spikes. **A**. An overlap image of fluorescence with infrared images of *TH*::GFP neurons in whole mount zebrafish retina. Scale bar = 10 μm. **B,** an example of a recording demonstrates spontaneous spike of a GFP-labeled cell. Loose-patch recording were made using a voltage-clamp mode with the electrode holding a potential of 0 mV.

provides means to target zebrafish retinal dopaminergic neurons in the living retina and in primary cell culture.

A similar strategy for labeling zebrafish dopaminergic neurons has been reported by Gao, *et al*. (2005). In their transgenic line, GFP expression was driven by the rat *TH* promoter and was expressed in many more neurons throughout one to two cell layers in the INL, as well as in the ganglion cell layer (GCL). In our line, GFP driven by the zebrafish *TH* promoter was restricted to a limited number (~1000) of neurons in the INL. The zebrafish *TH* promoter used in the present study may more faithfully drive reporter expression compared to the mammalian promoter. It is possible that presence of different transcriptional regulators in teleost and mammalian organisms is responsible for transcriptional regulation of the transgene.

In our *TH*::GFP transgenic line, about one third of the GFP-expressing retinal neurons are TH-IR, while the rest remain to be identified. One possible explanation for this is that the ~12 kb zebrafish *TH* promoter may not include all regulatory elements, so that GFP was ectopically expressed in some non-DA cells. A silencer or negative regulatory element that would normally suppress TH expression might be missing from the relatively small constructs, as has been proposed to be a potential reason for ectopic transgene expression in mouse and zebrafish (Matsushita *et al*., 2002 and Jessen *et al*., 1998). Another possibility is that GFP-expressing, TH-negative cells could be non-DA catecholaminergic (CA) cells, as referred to as Type 2 CA cells in the mouse and other mammalian species (Versaux-Botteri et.al.,1986 and Gustincich et.al., 1997). These studies indicate distinct differences between DA cells and Type 2 cells in soma size, processes morphology and distribution pattern throughout the retina. However, our results indicate no significant differences in cell size or shape between GFP-TH co-localized- and GFP single-labeled cells. Instead, we found a fluorescent intensity difference between individual labeled neurons. Although the identity of the non-DA cells is unknown to us, it is important to note that the GFP-fluorescent intensity can be used to reliably identify DA cells for molecular and physiological analysis.

Our single-cell RT-PCR results of two sub-populations of GFP-labeled neurons revealed a useful strategy for identifying dopaminergic neurons for future *in vivo* and *in situ* studies. Our

results also indicate the possibility of analyzing the molecular components of GFP+ neurons at the single-cell level. For example, DA neurons in the mouse have been reported to express circadian clock genes, as well as multiple subunits of $GABA_A$ receptors in the mouse (Gustincich *et al*., 2004, Ruan *et al*., 2006, Gustincich *et al*., 1999). Investigation of these genes in the current *TH::*GFP fish will provide the information about teleosts from a comparative aspect. In the present study, it is interesting that there were cells that only express *th* but not *dat* in both bright- and dim-GFP-expressing cells, suggesting the possibility that they could be adrenergic/noradrenergic neurons, similar to the type 2 catecholaminergic neurons described in mammalian retinas (Versaux-Botteri *et al*., 1986 and Gustincich *et al*., 1997). Previously, Holzschuh *et al*., (2001) reported the co-localization of TH and *dat* in four-day-old zebrafish retinas. However, their finding does not exclude the possible existence of adrenergic/noradrenergic neurons in the adult fish retina. In addition, the sensitivity of different methods (immunocytochemistry and *in situ* hybridization in their study, and single-cell RT-PCR in our case) could be different. It is possible that in adrenergic/noradrenergic neurons, low levels of the *th* transcript were amplified by RT-PCR, but could not be detected by immunocytochemistry assay in whole mount embryos.

Electrophysiology recordings of GFP-expressing cells demonstrated that in the dark, these cells generate spontaneous action potentials. Spontaneous activity of dopaminergic retinal neurons has been implicated in the maintenance of the basal level of dopamine release from fish IPCs in a calcium-dependent manner (Djamgoz and Wagner, 1992). After prolonged darkness, fish cone horizontal cell responsiveness is suppressed, as is receptive field size of cone horizontal cells, both due to dopamine release (Tornqvist et al., 1988, Mangel and Dowling 1987). These facts suggest that dopamine, released by spontaneous oscillatory spiking of DA-IPCs, modulates the dark-adaptation process of cone horizontal cells. Due to the fact that horizontal cells mediate lateral inhibitory effects in the OPL and form the antagonistic surround responses of cones, bipolar cells, and certain ganglion cells, dopamine may have a broader effect on dark-adaptation in the retina via cone horizontal cells. In addition, Li and Dowling (2000) studied effects of dopamine depletion in zebrafish and found that in DA-cell depleted fish, rod signals were blocked in the inner plexiform layer during dark adaptation. They suggested this rod

signaling defect was due to the lack of dopamine release, which is required for the rod signal to be transmitted from the rod to cone bipolar cells in darkness. Our results provide direct evidence of dopaminergic cell activity in darkness in the zebrafish retina.

In summary, our results indicate that by using this *TH*::GFP transgenic line, we can identify and target dopaminergic neurons *in vitro* and *in vivo*. *TH*::GFP targeting of DA retinal neurons can be used as a valuable approach for developmental and functional studies of dopaminergic cells in the zebrafish retina.

Materials and methods

Screen of transgenic offspring

TH::GFP transgenic zebrafish hemizygous for the *TH*::GFP transgene were crossed with wild type AB* fish to produce the hemizygous *TH*::GFP fish used in this study. Eggs were collected in the morning, 1 hr after mating, and treated with 0.003% (w/v) phenyl-2-thiourea (PTU; Sigma) after 24 hpf to prevent the development of melanin pigment. The transgenic offspring were screened by visualizing GFP expression at 24 ~ 30 hpf with a fluorescent microscope (Leica MZFLIII). The fish were treated in accordance with NIH and Vanderbilt University Division of Animal Care guidelines.

Imaging

TH::GFP expression in the zebrafish embryos was visualized using a fluorescent dissecting microscope (Leica MZFLIII) connected to a QImaging Retiga 1300 CCD digital camera. The bright field or fluorescent images were captured using QCapture v 1.2.0 software. All other images, including whole mount retina and immunostained vertical sections of adult zebrafish retina, were visualized with a laser scanning confocal microscope (Zeiss LSM5 PASCAL) at excitation wavelengths of 488 nm or 543 nm. The z-stack images were scanned at an optical slice of 4 μm for whole-retina expression in 3 dpf embryos and1 μm for individual cells

in living retina. For cell counting on complete retinas, tile-scans of sixteen 512 x 512 μm^2 images were collected from double immuno-stained whole mount retinas.

Immunocytochemistry

Transgenic fish (~ 1-3 month-old) were kept in darkness overnight and then killed on ice. Both eyes were removed from the fish and then hemisected to remove the cornea and lens. The eyecups were fixed for 2 hr and then rinsed three times for 5 min each in Phosphate buffered saline (PBS) (pH 7.4), cryoprotected in 30% sucrose overnight and cryosectioned at 15 µm. Cryostat sections were then blocked with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.1 M PBS for 2 hr, and stained with rabbit anti-GFP (1:20000; Molecular probes, Carlsbad, CA), and sheep anti-TH (1:2000; Chemicon, Temecula, CA) primary antibodies overnight at room temperature. After rinsing, a secondary incubation was performed for 2 hr in a mixture containing cy3-conjugated donkey anti-rabbit IgG(H+L) and cy5-conjugated donkey anti-sheep IgG(H+L) antibodies (1:500 for both; Molecular Probes). Whole mount retina ICC was performed similarly, except that isolated retinas, instead of whole eyecups, were processed for staining, and the retinas were incubated in primary antibodies for 48 hr.

Single-cell RT-PCR

To make dispersed cell cultures, the retinas were isolated in Leibovitz's L-15 medium (GIBCO), digested with papain (20 units/ml) for 20 min, gently triturated in L-15 medium, and distributed into 35 mm culture dishes. The culture dishes were kept at room temperature for 30 min. Cells were observed with an inverted microscope (Nikon, Lewisville, TX). A perfusion system was used to avoid contamination. The inlet tube perfused the target cell with extracellular solution for zebrafish (containing 137 mM NaCl, 2.5 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, and 10 mM Glucose, pH 7.4). Individual cells were aspirated into the pipette and after washing with extracellular solution, the tip of the pipette harboring the target cell was broken into a 0.5 ml tube with 14 µl of Master Mix 1 and maintained on dry ice. Reverse transcription (RT) was performed using SuperScript II Reverse Transcriptase (Invitrogen) according to

manufacturer's recommendations. For semi-nested polymerase chain reaction (PCR), the following primers were used: *th* forward primer: 5'-CAGGATTTCAGTTGAGGCCAGTG-3'; *th* reverse primer: 5'-ATGCACCAGCTCTCCATAGGATG-3'; *th* nested primer: 5'-TCCACAGTGAACCAGTACATTGTCG-3'; *dopamine transporter* (*dat*) forward primer: 5'-AACTCTCTGACCAGCTTCTTCTCCG-3'; *dat* reverse primer: 5'-ATTCCACCGTTGGTGACGCAG-3'; *dat* nested primer: 5'-GTCGATGAGTCCCGTGATCAC-3'. Semi-nested PCR was performed using an Eppendorf Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). The first round reaction was first incubated at 94°C for 5 min, then at 94°C for 30 s, 50°C for 30 s, 72°C for 30 s for15 cycles, followed by 72°C 10 min. Nested-PCR reactions were performed using 1 µl of the first round PCR product in a 100 µl sample. The second round reaction was performed at 94°C for 5 min, then at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 30 cycles, followed by 72°C 10 min.

Electrophysiological recordings

TH::GFP zebrafish were dark-adapted overnight before the day of recording. The separation of retina was performed in oxygenated extracellular solution under dim red light. The extracellular solution contained the following (in mM): 116 NaCl, 2.4 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1 NaH_2PO_4, 28 $NaHCO_3$, 10 Glucose, bubbled with a gaseous mixture of 95% O_2/5% CO_2. The retina was placed photoreceptor-side down in the recording chamber and maintained in the dark for at least 1 hr before recording. The chamber was then mounted on the stage of an upright conventional fluorescent microscope (Leica DM LFSA, Leica Microsystems, Wetzlar, Germany). Oxygenated medium was continuously perfused into the recording chamber at a rate of ~2-3 ml/min, and the recording was performed at ~ 21°C by a Dual Channel Heater Controller TC-344B (Warner Instrument Corporation).

During recording, *TH*::GFP cells were identified by fluorescence microscopy using a brief illumination of fluorescent excitation light. GFP-labeled cells and glass electrodes were visualized using infrared differential interference contrast (DIC) optics for recording. The glass electrodes were prepared using DMZ universal puller (Zeitz-Instrumente, Munich, Germany) and filled with a

solution containing 150 mM NaCl and 10 mM HEPES (pH 7.5). The resistance of pipettes filled with this solution was ~ 9-10 MΩ. The recording was made from the soma of GFP-labeled cells using a pipette with holding potential of 0 mV. Experiments were executed and data were recorded using Clampex 8.0 software connected to an AxoClamp-2B amplifier (Molecular Devices, Palo Alto, CA), via a digitizer 1322A (Molecular Devices). Data were analyzed with Clampfit 9.2 (Molecular Devices). Action potential frequency was calculated from 40 s recordings from each cell. The values are given as the mean ± SD.

CHAPTER II

PRELIMINARY STUDY OF ZEBRAFISH RETINAL CIRCADIAN RHYTHMS

Introduction

Circadian rhythms are daily rhythms of physiological and behavioral processes driven by endogenous and autonomous circadian clocks. These rhythms provide organisms the selective advantage of adapting to and anticipating day/night changes of environment (Pittendrigh, 1993). In vertebrates, the circadian clocks are located within both central (Moore 1983; Klein *et al*., 1991; Zimmerman and Menaker, 1979) and peripheral tissues (Yamazaki *et al*., 2000), including the retina (Besharse and Iuvone, 1983; Cahill and Besharse, 1993). In the vertebrate retina, circadian clocks regulate a variety of cellular, biochemical, and physiological processes, including gene expression (Ivanova and Iuvone, 2003 a and b, Pierce *et al*., 1993), release of the neurohormone melatonin (Cahill and Besharse 1993; Tosini and Menaker 1996), rod outer segment disc shedding (La Vail, 1976; Grace *et al*., 1996), electroretinogram (ERG) responses (McGoogan *et al*., 2000 and Brandenburg *et al*., 1983), and visual sensitivity (Barlow 2001). Despite the profound influence of circadian rhythmicity on retinal physiology, many questions about vertebrate retinal circadian organization remain to be answered.

Recently, the zebrafish has become of interest for circadian clock studies as a diurnal vertebrate species (Li and Dowling, 1998; Hurd *et al*., 1998), providing information from a comparative aspect in circadian studies. Zebrafish retina exhibits daily rhythmicity in gene expression, including that of *Per3* (Delaunay *et al*., 2000) and the interphotoreceptor retinoid binding protein (IRBP; Rajendran *et al*., 1996). In addition, the absolute visual sensitivity is high in the subjective late afternoon and low in the subjective early morning in constant darkness (DD) or constant light (LL) (Li and Dowling, 1998); and long wavelength opsin mRNA expression correlates to this fluctuation of rhythmic cone sensitivity (Li *et al*., 2005). *In vitro* cultured zebrafish retina displays rhythmic *Per3*-driven luciferase expression in constant light or dark conditions and

temperature compensation of circadian rhythms (Kaneko *et al*., 2006). While ample evidence indicates that the zebrafish retina is a rhythmic tissue, several basic questions remain to be answered. For example, what are the retinal cell types potentially critical for circadian rhythms, and how individual retinal oscillators are modulated to exhibit an overt rhythm? Our long-term goal is to generate transgenic zebrafish, in which the promoter sequence for *Per3* drives dynamic green fluorescent protein (d2GFP) expression, and examine the circadian rhythm expression with single-cell resolution. Meanwhile, *Per3::*LUC transgenic fish (Kaneko *et al*., 2006) have been used to study the effect of light on zebrafish retinal circadian rhythm.

Results

2.1 Generation of transgenic zebrafish model for circadian study

Modification of Bacteria Artificial Chromosome that Contains *Per3*

To generate transgenic zebrafish in which the circadian clock gene (*Per3*) is labeled by dynamic green fluorescent protein (d2GFP), we started with a BLAST search for BAC clones that contain at least 10 kb sequence upstream from the start codon and the entire coding region of the *Per3* gene. We ordered these BAC clones from CHORI (http://bacpacchori.org) and checked the BAC DNA by PCR using primers specific for *Per3*.

To modify the BAC DNA with a fluorescence marker, we adapted an *E.coli*-based bacteria artificial chromosome (BAC) engineering method (Lee *et al*., 2001 and Shin, *et al*., 2003). As shown in Fig. 7, we used a relatively long homologous sequence (~1 kb) on both sides of the inserts in order to increase the recombination efficiency. Next, we introduced the targeting construct into the BAC-transformed host cells, which contain the defective lambda prophage and an arabinose- inducible *flpe* gene to allow homologous recombination and flip recombination. Restriction endonuclease digestion analysis confirmed the clones, which did not undergo major recombination after the modification. Further PCR analysis and DNA sequencing around the targeting region showed correct modified *Per3*::d2GFP rBAC DNA.

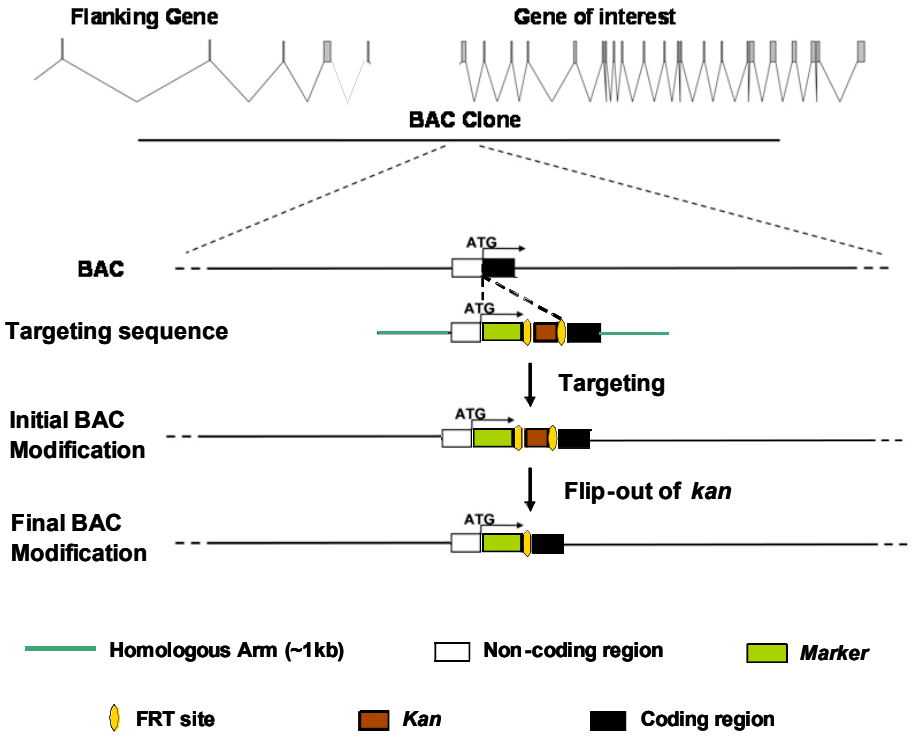

Figure 7. Schematic map of the BAC modification strategy.
Adapted from Lee *et al.,* (2001) with modification. A relatively long homologous sequence (~1 kb) on both sides of the inserts in order to increase the recombination efficiency. The targeting construct was introduced into the BAC-transformed EL250 cells, which contain the defective lambda prophage and an arabinose- inducible *flpe* gene to allow homologous recombination and flip recombination.

Transient expression of *Per3*::d2GFP in injected fish

After the final modification, we linearized the *Per3* BAC with Not I and injected the purified DNA (~100 ng/ul) into the boundary of between cell and yolk of 1-cell stage embryos (Figure 8A). Transient expression of *Per3*::d2GFP was observed in the retina and other brain regions from injected fish at 1 dpf (Figure 8B). ~ 400 injected fish were identified as GFP+ fish and were allowed to grow to adulthood for further screening.

Screen for germ line-transformed founders

To screen for germ-line transformed founders, we raised the fish expressing GFP to adulthood, intercrossed them and screened their progeny for GFP expression. Among ~ 150 fish we have examined, 2 pairs produced GFP+ embryos. Figure 9 shows two representative images of the embryos that expressed GFP in the brain and the retina at 1 dpf (Figure 9A) and 2 dpf (Figure 9B). However, these embryos showed abnormal morphology (Figure 9C) and did not survive to adulthood. This could be due to random insertion of the transgene disrupting a critical gene for development. Currently more injected fish are set to be crossed and examined for germ-line transmission of the transgene.

2.2 Bioluminescence rhythm recording from *Per3*::LUC transgenic fish retina

Long-term culture of the zebrafish retina

As we are developing the *Per3*::d2GFP system for rhythm studies for individual retinal neurons, we are cognizant of the potential concern that the repeated exposure to fluorescence excitation light could affect the properties of retinal rhythms by the light-induction of clock genes. An alternative real-time reporter, luciferase, can allay this potential problem since it does not required excitation light. In order to study the zebrafish retinal circadian rhythm with long-term recording, we obtained the *Per3*::LUC transgenic fish (Kaneko *et al*., 2006), and adapted the method for mammalian retinal explant culture developed in our lab (Ruan *et al*., manuscript in preparation) for zebrafish retina culture. Vertical sections of the retina, which had been cultured

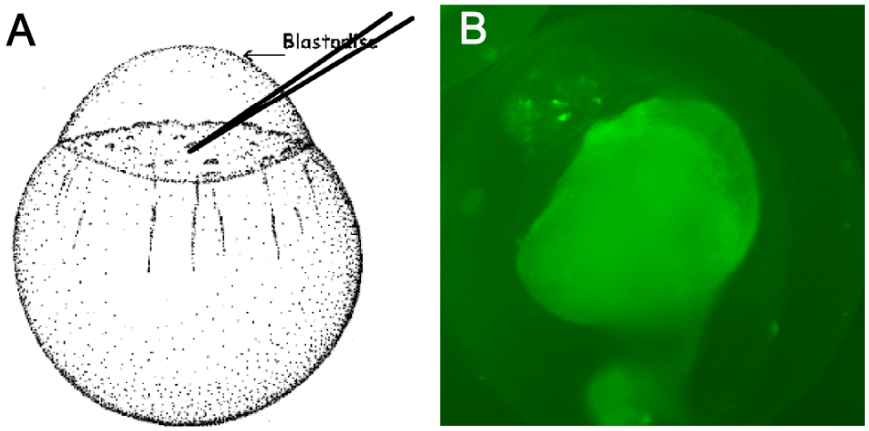

Figure 8. Transient expression of *Per3*::d2GFP in injected fish.
A. Microinjection of final modified BAC (*Per3*::d2GFP) into zebrafish embryo. The modified BAC DNA (~100 ng/ul) was injected to the boundary of between cell and yolk of 1-cell stage embryos.
B. Transient expression of *Per3*::d2GFP in BAC-injected fish. Short half-time GFP expression was observed in the retina and other brain regions at 1 dpf.

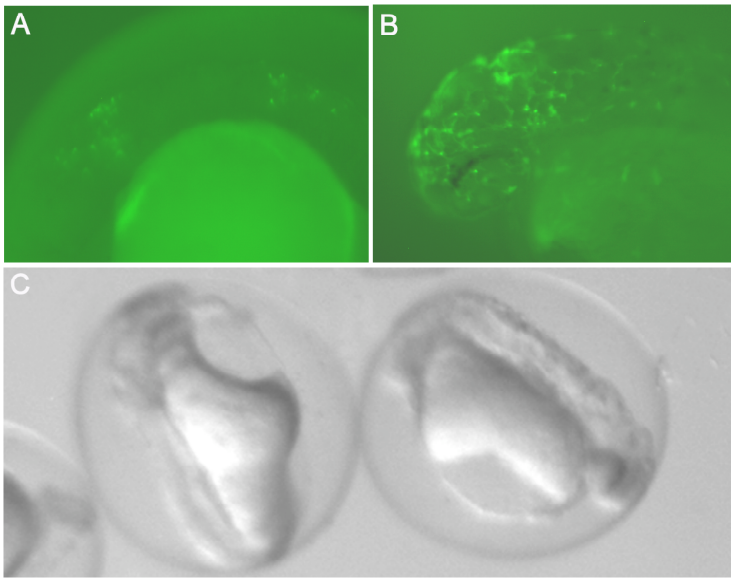

Figure 9. *Per3*::d2GFP expression in progeny of injected fish.
A. d2GFP expression was observed in the midbrain and hindbrain of embryos from intercross of injected fish at 1 dpf. **B.** d2GFP was more broadly expressed in the brain and eye at 2 dpf, **C.** Bright field image of typical embryos at 2 dpf. These embryos developed abnormal morphology, and did not survive to adulthood. For **A** and **B**, dorsal is on the top and anterior is on the left.
for 15 days, were examined for histological morphology.

for 15 days, were examined for histological morphology. After 15 days in culture, the retina maintained proper tissue morphology with stratified layers, except a significant number of outer segments were detached from the retina when the retina was removed from the culture membrane for morphological analysis (Fig. 10).

With the *Per3*::LUC transgenic line, we are able to record luminescence rhythms driven by the z*fPer3* promoter for at least 10 days (Figure 11A) and up to 24 days with medium change (Figure 11B). The bioluminescence rhythm from all retinal cultures persisted in constant darkness at least 10 day in culture with period of 27.54 ± 0.49 hr (mean ± SD, n=30, as determined by Lumicycle Analysis) for the first two cycles.

Resetting *Per3::*LUC rhythm by short light pulses

One of the most important functions of the circadian clock is to ensure the behaviors and internal metabolic adjustments are appropriately timed with daily environmental changes. Light is usually the most important Zeitgeber (or, 'time-giver') for entraining circadian oscillators. Therefore, light phase response curve (PRC), a plot of phase-shifts as a function of circadian phase of light stimulus, has been valuable in understanding how circadian pacemakers are entrained to the daily environmental light/dark cycle. Considering the free-running period of the zebrafish retina, as measured by bioluminescence reporter, is remarkably longer than 24 hr in constant darkness, we speculate that the regular 24-hour light/dark cycle must impose an important role in entraining the internal clock of the zebrafish retina to adapt to a 24-hour period. It's of interest to examine the light-induced phase response properties of the zebrafish retina.

To determine the PRC for the zebrafish retina, 15-min light pulses at ~ 500 lux were administered to cultured retinas every three hours of their circadian time (CT), and phase changes were measured as phase difference between expected peak time before and after the stimulus. The zebrafish retina PRC (Figure 12A) represents a typical light PRC of circadian oscillators, exhibiting delay phase shifts in the early subjective night and advance phase shifts in the late subjective night, with relatively little phase shifting occurring during the subjective day. In addition, there are the two types of PRCs: type 1 displays relatively small phase- shifts and has a

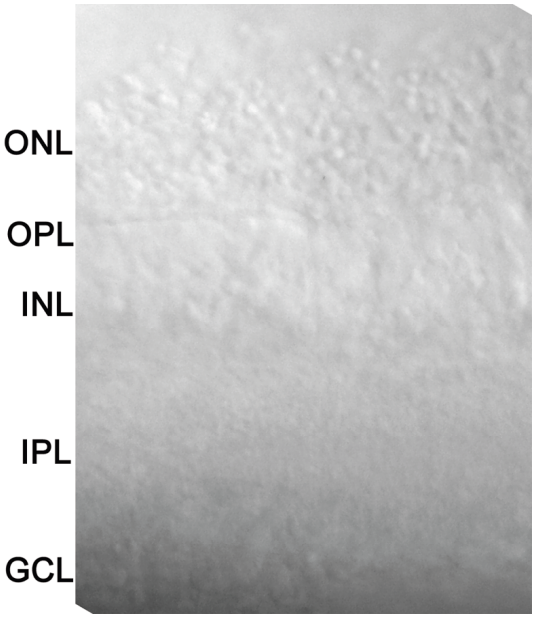

Figure 10. Zebrafish retina maintained proper morphology in culture.
An image of a vertical section of a zebrafish retina, which had been cultured 15 days *in vitro.* The retina maintained proper tissue morphology with stratified layers. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer

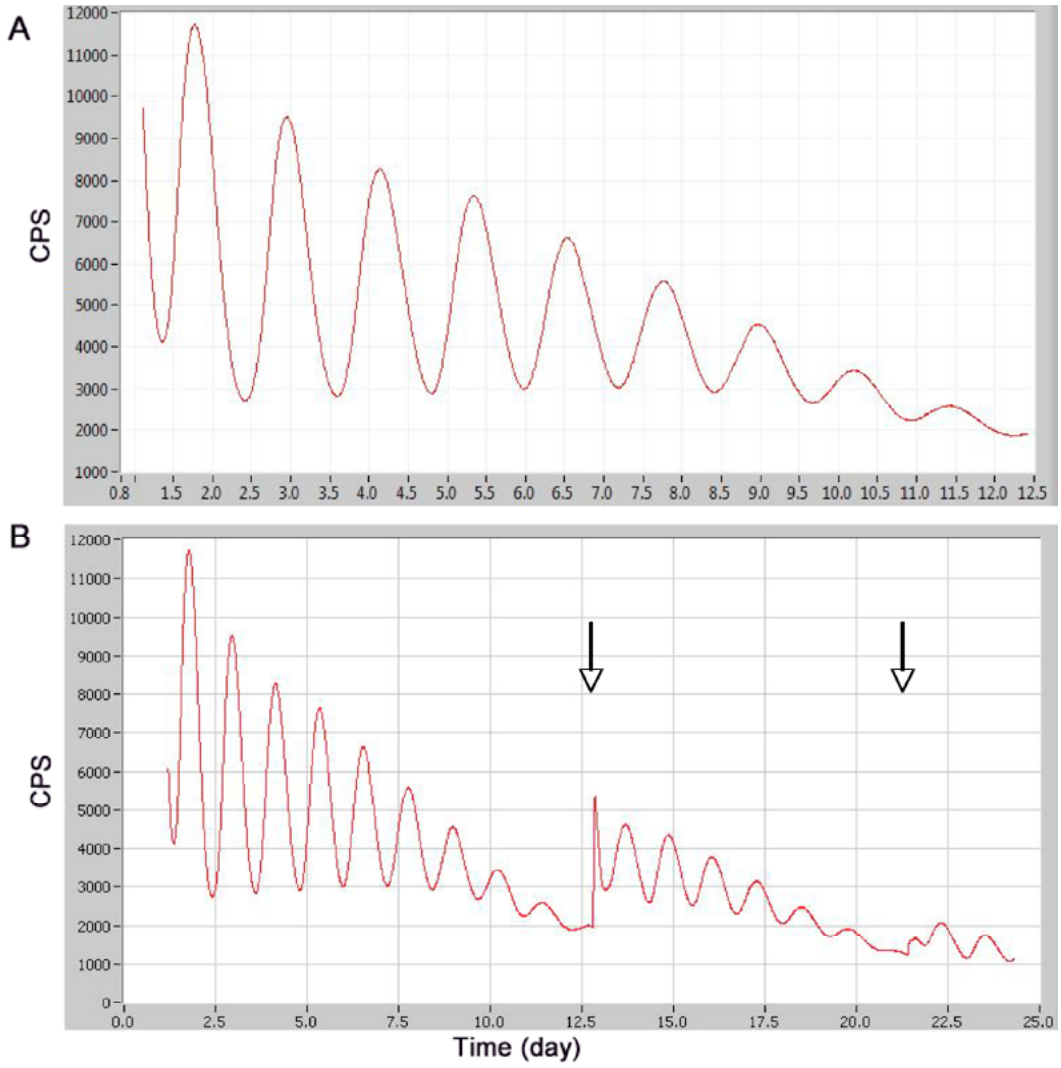

Figure 11. Retinal bioluminescence rhythm from *Per3*::LUC transgenic fish.
A. A representative example of the bioluminescence recording from an piece of isolated *Per3*::LUC zebrafish retina. Vertical axis is photon counts; horizontal axis is time *in vitro* in days. 9 successive cycles were recorded. **B.** Bioluminescence rhythm form long-term cultured retina. The rhythm persisted up to 24 days in cultured retina with medium change. Arrows indicate medium changes. CPS: counts per second.

continuous transition between delays and advances, whereas type 0 PRCs show large phase-shifts. Our results of the zebrafish retina PRC showed large phase shifts of up to 6 hours and was close to type 0 resetting, which suggests that a 15-min light pulse (~500 lux) was a strong stimulus for resetting the zebrafish retinal oscillators. It is an interesting question for us whether every zebrafish retinal neuron was responsive to the light, or the phase shift was due to the responses of photoreceptors and melanopsin containing ganglion cells only (Hattar *et al*., 2002). A notable fact is that besides shifting the phase of retinal clocks, light also induced different amplitude changes depending on the time of the light pulse. Figure 12B shows the representative normalized *Per3*::LUC rhythm with a light stimulus at CT 7, CT 16 and CT 19. At CT 19, when phase advances are maximal, light severely damped rhythm amplitude; whereas at CT 7, when no phase shift was induced by light, the amplitude was increased substantially. A light pulse at CT 16 led to a large phase delay with moderate amplitude increase. These results were similar to the study in effect of light using a photoentrainable mammalian fibroblasts system (Pulivarthy *et al* 2007). Their results suggest an intriguing reciprocity between phase shifts and amplitude changes in the mammalian circadian clock. Our results suggest such a reciprocal relation between circadian phase shifts and amplitude changes may also exist in cultured the zebrafish retina.

Discussion

We have successfully constructed a recombinant BAC in which short-half life GFP is under the control of the *zfPer3* gene promoter and demonstrated expression of the modified BAC clone in zebrafish embryos. If we eventually identify a stable transgenic line, it could be useful to address several interesting questions. First, it will help us to understand the cellular location of the autonomous circadian clock within the zebrafish retina. Photoreceptors have been demonstrated to contain intrinsic circadian clocks that drive rhythmic melatonin secretion or biosynthesis in *Xenopus* (Cahill and Besharse, 1993 and Hayasaka *et al.,* 2002) and chickens (Piece *et al.,* 1993; Ivanova and Iuvone, 2003 b). However, in mammalian retinas, core circadian clock genes are concentrated in the inner layers (Gekakis *et al.,* 1998; Namihira *et al.,* 2001;

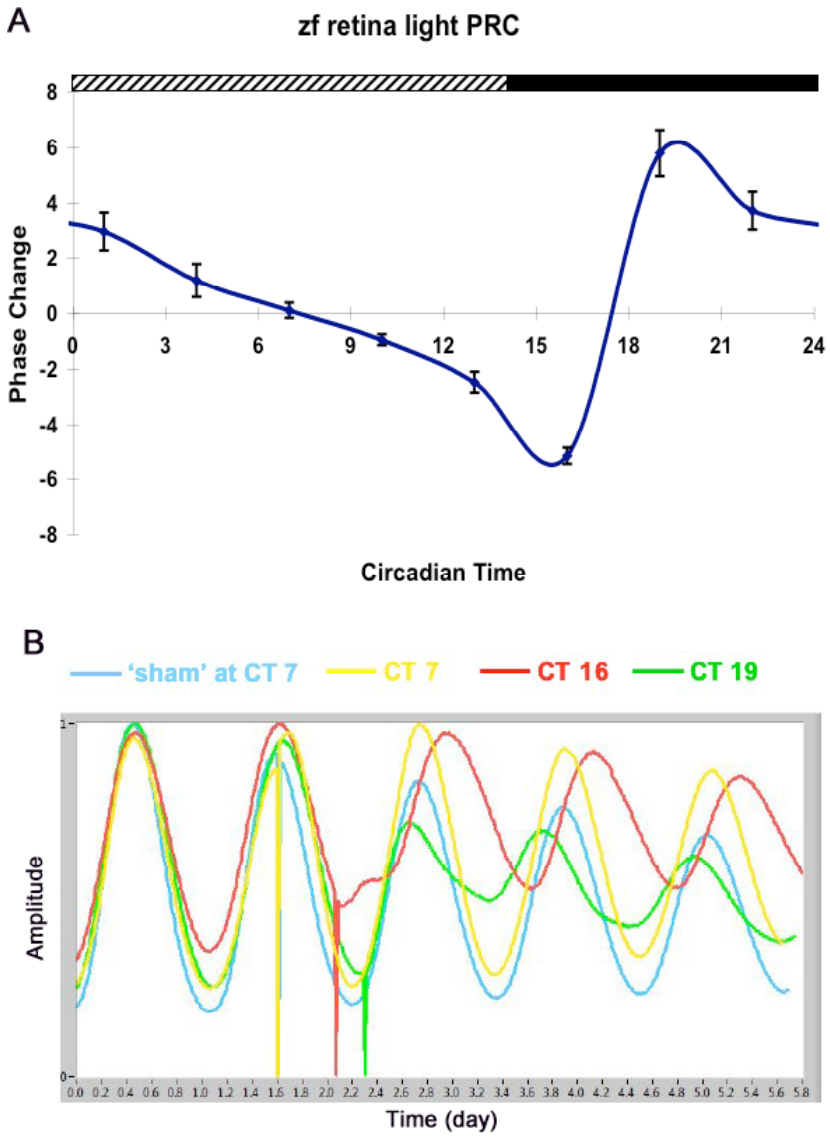

Figure 12. Resetting *Per3*::LUC rhythm by short light pulses.
A. Zebrafish retina phase response curve. Phase responses to 15-min light pulses were determined in CT for the cultured retina against the time of light pulse. For each time point, average phase changes of four to five samples were plotted. Error bars represent SD. The stripped bar on the top represents subjective day and the black bar represents subjective night.
B. Representative examples of the effects of light on *Per3*::LUC phase and amplitude. The traces were normalized by amplitude and aligned by the time of the first peak.

Ruan *et al.,* 2006),and circadian rhythmicity in dopamine content still persists in Royal College of Surgeons (RCS) rat retina lacking photoreceptors (Doyle *et al.,* 2002), suggesting that the location of functional circadian clocks lies outside of the photoreceptors. The expression of *Per3*-driven GFP may provide us the information of zebrafish retinal circadian organization from a comparative aspect. In addition, we could also examine the co-localization of GFP and cellular markers for specific neuronal types of the retina. For example, we could address the question of whether dopaminergic neurons co-express GFP, and therefore understand whether DA neurons are important for retinal circadian functions.

A more attractive aspect of the GFP reporter is that it can be measured in real-time manner with single-cell resolution. As revealed by *Per3*::LUC bioluminescence reporter, the phase and amplitude of the zebrafish retinal circadian rhythm are largely affected by light pluses. It is tempting for us to explore whether light resets the circadian rhythm of cultured zebrafish retina by altering the property of a few 'master clock' cells (or a specific type of cells) and shifts the overt rhythm through the coupling of between these cells and the rest of the retinal neurons. Or, otherwise, it's possible that every single zebrafish retinal neuron is responsive to light. Considering the fact that peripheral tissues and zebrafish cell lines can be reset by light (Whitmore *et al*., 1998 and 2000; Pando *et al*., 2001), it's possible that in the zebrafish retina, circadian origination is more 'generalized' so that every single neuron independently responds to light through some unknown receptors. These two possibilities could be teased out by using a GFP-reporter system. Our lab has established a time-lapse recording system for *Per1*::GFP mouse SCN (suprachiasmatic nucleus) culture. Once we obtain a stable transgenic zebrafish line for *Per3*::d2GFP, the method could be transferred to zebrafish retinal rhythm recording. With the high resolution of confocal microscopy, the expression of d2GFP within a single neuron will be monitored in real-time manner. We could further examine whether there is potential coupling between different oscillators by studying single-cell rhythmicity from dissociated retinal culture.

Using the *Per3*::LUC transgenic fish, we are able to examine the bioluminescence rhythm from long-term retinal cultures. Our recordings showed robust rhythms, which exhibited stable phase and period for up to 10 days without medium change. However, for some samples we

observed a lengthened period, a fact also observed in mouse retinal culture (unpublished result from our lab). The reason is mysterious to us now, but we assume it could result from changes in circadian properties during long-term culture. A notable fact is that there is a variation among different retinal preparations in terms of circadian period and damping rate. As suggested by the Cahill group, the zebrafish retina might be susceptible to different batches of medium (Kaneko *et al.*, 2006), and we speculate some other subtle differences during the sample preparation may also lead to these variations. Even so, the *Per3*::LUC line provides an attractive system for zebrafish retina circadian study, because it makes long-term bioluminescence rhythm recording possible. Our future direction using this fish line is to study the mechanisms critical for circadian organization of the zebrafish retinal function. For example, we could use this system to test the effect of dopamine, melatonin or other neuromodulators on zebrafish retinal circadian oscillators by administration of agonists or antagonists to the retina culture. We could also chemically lesion the dopaminergic cells, or use morpholino technology to knockdown a specific gene of interest and examine their effects on the zebrafish retinal circadian rhythm.

The preliminary study of the effects of short light pulses using *Per3*::LUC fish important properties of zebrafish retinal circadian rhythms. In contrast with zebrafish pineal gland, which displays small phase changes (~ 3 hr maximum phase advance and 2.3 hr maximum delay), the large phase shifts obtained during the subjective night suggest that a 15-min light pulse is strong enough for cultured zebrafish retina entrainment. Instead, the PRC for the retina is similar to that of the zebrafish heart (Kaneko *et al*., 2006). These results raise an interesting question of whether the zebrafish retina shares similarity in photosensitivity, phototransduction pathways or some pacemaking properties with the heart, which is usually not considered as a photosensitive organ in other vertebrates. This may support the possibility that in the zebrafish retina, besides photoreceptors, other types of neurons are also responsive to light by one or multiple photopigment(s) ubiquitously expressed in all zebrafish tissues, probably the teleost multiple tissue (tmt) opsin (Moutsaki *et al*., 2003). Further study may reveal important information about the zebrafish retinal circadian organization.

In summary, we have constructed a recombinant BAC in which short-half life GFP is under the control of the *zfPer3* gene promoter and demonstrated expression of the modified BAC clone in zebrafish embryos. Further intercrosses of injected fish and screening of their progeny may identify transgenic fish lines that express the dynamic GFP reporter. In combination with using *Per3*::LUC transgenice fish, we may elucidate the retinal cell types and mechanisms critical for circadian organization of the zebrafish retina. These studies may provide important knowledge about vertebrate retinal circadian rhythms from a comparative aspect.

Materials and methods

Modification of the *Per3* BAC clone

Nucleotide sequence of 10 kb upstream of the *Per3* coding sequence was used for blast search. A ~ 158 kb BAC clone, CH211-138E4 was used as it contains ~ 50 kb upstream sequence of *Per3* and its complete coding region. The clone was confirmed by PCR using two sets of *Per3* specific primers: per3 ef: 5'-AGTGCTAGAACCGTATGAGC-3' and per3 er: 5'-CCTTGTGCTTCGAGACAATC-3'; per3 if: 5'-ATGTCCGCAGAGTTTAACTGG-3' and per3ir: 5'-CGACAGAATCGTGATCGCTG-3'. For PCR, *Per3* BAC DNA was prepared using a QIAGEN Plasmid midi kit (QIAGEN ltd.), following the purification protocol for low copy number plasmids and dissolved in nuclease-free water.

To produce a targeting sequence, we first amplified a 1.3 kb fragment near the *Per3* start codon using primers per3 if and per3 ir. Before we cloned the targeting sequence into pPCR-Script (Stratagene), a Kpn I site within the pPCR-GFP plasmid was destroyed by digesting the clone with Kpn I and Xho I, followed by treatment with the Klenow fragment, and ligation of these blunted ends together. Then the targeting sequence was inserted into the above de-Kpn-pPCR-GFP plasmid by blunt end ligation. Spe I and Kpn I sites were introduced to per3-pPCR-Script plasmid by using primers SpePer3: 5'-GCACTAGTGACGGCTTTCCTGAT-3' and KpnPer3: 5'-GCGGTACCTCCCCCTGGCATTCCC-3' followed by phosphorylation and ligation of the PCR

product. The new plasmid, named as k/s-per3/pPCR-Script was confirmed by Kpn I and Spe I restriction digest.

We then made a construct containing *d2gfp* and kanamycin resistance gene (*kan*^{r}). To do that, we amplified the sequence contains SV40 poly A and FRT sites flanking *kan*^{r} from pPCR-GFP plasmid (kindly gifted by the Appel lab) using following primers: SVpA5: 5'-TAAGATACATTGATGAGTTTGG-3' and SpeKan: 5'-GGACTAGTCTATTCCAGAAGTAGTGAGGAG-3' , and inserted the sequence into pPCR-Script plasmid by blunt end ligation. Then we cloned the *d2gfp* gene from P1PG plasmid (a construct from the lab) using primers containing Kpn I and Xho I site: KpnGFP: 5'-TCGGTACCATGGTGAGCAAGGGCGAG-3' and XhoDGFP: 5'-CCCTCGAGCTACACATTGATCCTAGCA-3'. Next, the *d2gfp* gene was inserted into the *kan*^{r}/pPCR-GFP between Kpn I and Xho I sites and the new construct was named as d2gfp-*kan*^{r}/pPCR-GFP.

Next, the d2gfp-*kan*^{r} gene cassette was ligated into k/s-per3/pPCR-Script plasmid between Kpn I and Spe I sites and the transformed DH5α competent cells was selected by 50 μg/ml kanamycin for successful ligation. Therefore, we obtained the construct containing *Per3* targeting sequence with *d2gfp-kan*^{r} gene, which we named as *Per3- d2gfp-kan*^{r}/pPCR-Script.

To modify the *Per3* BAC, we followed the method of Lee *et al.,* (2001) and Shin *et al*., (2003), which employs EL250, a modified DH10B strain carrying a heat-inducible recombination genes expression and an arabinose-inducible *flpe* gene. Briefly, EL250 cells were first transformed with the original *Per3* BAC by electroporation (1 mm cuvettes, using a Bio Rad Gene Pulser II set at 2.5 Kv, 25 μF and 200 Ω) and selected by 12.5 μg/ml chloramphenicol at 32 ^{o}C. Colonies were screened by PCR using *Per3* specific primers and compared by restriction digestion and gel electrophoresis of the BAC DNA prepared from these cultures. A confirmed *per3*+ EL250 single colony was selected and grown to OD_{600} = 0.6, then shifted to 42 ^{o}C for 15 min with shaking followed by chilling in an ice-water bath for 30 min. The heat-induced culture was then centrifuged and prepared for electroporation together with 500 – 1000 ng purified Kpn I and Spe I fragment of the targeting DNA. Cells were incubated at 32 ^{o}C for 24 -28 hr on LB-agar

plates containing 25 μg/ml kanamycin and 12.5 μg/ml chloramphenicol. Colonies were screened by whole-cell PCR, using two sets of primers: primers specific for upstream of the per3 homologous region (per3f2 5'-GGGTCTGTGCAATCCTAGC-3') and d2gfp (GFPr2 5'-TAGGTCAGGGTGGTCACGAG-3'); primers specific for *kan*^{r} (Kanf2 5'-GACCGCTTCCTCGTGCTTTAC-3') and downstream of per3 homologous region (Per3r2 5'-CCACCCTTGTGCTTCGAGAC-3'). After identification of modified BAC clones, an overnight culture was then treated with 0.1% L-arabinose (Sigma) to remove the *kan*^{r} gene. Colonies were tested by PCR to confirm the removal of *kan*^{r} gene.

Modified *Per3* BAC microinjection and transgenesis

Modified BAC was linearized with Not I restriction enzyme, phenol/chloroform extracted, ethanol precipitated and dissolved in nuclease-free water. For injection, 100 ng/μl DNA was prepared with 0.1 M KCl and 0.1% phenol red. Microinjections were performed using thin wall borosilicate glass pipettes (OD = 1.0 mm, WPI) pulled to a fine tip using a micropipette puller (P-97, Sutter Instrument Co.), with the tip broken with a blade. The micropipettes were backfilled with the injection solution and connected to a pressure injector (MPPI-2, Applied Scientific Instrumentation) driven by compressed air. Eggs were collected immediately after mating of adult pairs and placed into furrows molded into 1.2% agarose injection ramps and carefully manipulated with end-blunted micropipettes so that the animal cap was on the top. About 1 nl DNA solution was injected to the boundary of between cell and yolk of 1-cell stage embryos. After injection, the embryos were removed from the ramps and screened at about 24 hpf using a fluorescence microscope. The embryos expressing GFP in the brain and eye region were raised to adulthood. To identify germ line-transformed founders, we intercrossed the injected fish and screened their progeny for GFP expression.

Retinal Cultures

Per3::LUC zebrafish (originally purchased from the Zebrafish International Resource Center) between 2 – 7 months old were dark-adapted for about 2 hr then killed between ZT 7 – 9

(3:00- 5:00 pm of local time). Eyes were removed from the fish and hemisected in Hank's balanced salt solution (Invitrogen) to remove the cornea and lens. Each retina was isolated and cut into three pieces. Each piece of retina was placed on Millicell culture membrane (Millipore) with ganglion cell layer on the top. The membrane was first transferred to 1.0 ml neurobasal medium (Invitrogen) supplemented with 2 mM L-glutamine (Sigma), 2% B-27 (Invitrogen), 25 units/ml penicillin and 25 μg/ml streptomycin (Invitrogen), incubated in 5% CO_2 incubator at 28 ^{o}C for 24 hr, then transferred to medium 199 (Sigma) supplemented with 0.7 mM L-glutamine, 4 mM sodium bicarbonate (Sigma), 10 mM HEPES (Sigma), 20 mM D-glucose (Sigma), 2% B-27, 0.1 mM beetle luciferin (Promega), 25 units/ml penicillin and 25 μg/ml streptomycin (Invitrogen). Bioluminescence emitted from cultured retina was monitored with a LumiCycle (Actimetrics, Wilmette, IL). Data was then exported to Lumicycle Analysis (Actimetrics, Wilmette, IL) to determine the period and phase of *Per3*::LUC rhythm.

Phase Response Curve

For each light treatment, 4 – 5 samples were given a 15 min light pulse at specified times between the third and the forth cycle in culture. The retinas were illuminated by LED lamps with the light intensity of about 500 lux. Two samples were kept in DD as a control. To calculate the time of light pulses in CT, free-running periods and peak phases were estimated by Lumicycle Analysis. Then the time of the light pulses administration was converted to CT, assuming that the peak of *Per3*::LUC expression corresponds to CT 7 (7 hr after lights on, the time of CT 0 for zebrafish) as deduced from recording of retina cultures which were put in constant darkness immediately after dissection with stable free-running period. The phase shifts were calculated as differences of the expected peak time before and after the stimulus.

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