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Midkine expression is regulated by the circadian clock in the retina of the zebrafish

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Abstract

Purpose—To investigate the circadian control of the expression of *midkine-a* and *midkine-b* in the retina of the zebrafish.

Methods—Zebrafish were maintained in total darkness for 24 hours, and at four-hour intervals, retinas were collected and the expression of *mdka* during the subjective day and subjective night was evaluated by *in situ* hybridization, quantitative real-time PCR and Western blot analysis. The circadian expression of *mdkb* was evaluated by *in situ* hybridization and real-time PCR.

Results—The expression of *mdka* increases during the subjective day and decreases during the subjective night. In contrast, the expression of *mdkb* increases late in the subjective night and decreases late in the subjective day. Within horizontal cells, the two midkine paralogs show asynchronous circadian regulation. Within the annulus of immature retina adjacent to the proliferative margin, *mdka* shows circadian regulation in Müller glia.

Conclusions—The results of this study show that in the retina of the zebrafish the circadian clock regulates the expression of *mdka* and *mdkb*. These growth factors are components of cyclical signaling events with the vertebrate retina. The asynchronous expression of *mdka* and *mdkb* in horizontal cells suggests these factors may exert different biological activities at distinct times during the circadian cycle. The circadian control of *mdka* expression in immature Müller glia may be related to the persistent neurogenesis mediated by these cells.

Indexing terms

mdka; *mdkb*; *horizontal cells*; *circadian cycle*

Introduction

The circadian clock maintains intrinsic rhythmical changes of biochemical and physiological processes, which provide optimal adaptation to environmental changes, such as light, temperature and access to food. In sighted animals, the retina is the primary tissue that entrains the circadian clock to changes in the dark/light cycle [1]. This occurs through direct projections of retinal ganglion cells to the suprachiasmatic nucleus, the master circadian pacemaker, indirect visual projections to the pineal gland [2,3], and through the synthesis of endocrine/paracrine factors, such as melatonin and dopamine [4,5]. Numerous processes in the retina are dependent on circadian rhythms, such as retinomotor movements [6, 7], disc-

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shedding of photoreceptor outer segments [8], visual sensitivity [9], dopamine release [5], the expression of interphotoreceptor retinoid binding protein [10], and photoreceptor input to cone horizontal cells [11,12,13].

Midkine (MK) is a member of the family of secreted heparin-binding growth/differentiation factors that also includes pleiotrophin [14,15]. MK was first identified in a screen of retinoic acid inducible genes in embryonic carcinoma cells [16,17]; it is highly conserved throughout the animal kingdom and has numerous functions: neurogenic, transforming, neurotrophic, chemotactic, mitogenic, and anti-apoptotic [15,18,19]. In mammals, MK is expressed in numerous tissues during embryonic development, most prominently in the developing neural tube and at epithelial–mesenchymal boundaries [20].

The zebrafish genome encodes two distinct *midkine* genes, *midkine-a* (*mdka*) and *midkine-b* (*mdkb*), which during early development have distinct cellular patterns of expression and different biological functions [19,21,22,23]. In the adult retina, *mdka* is expressed by horizontal cells, whereas *mdkb* is expressed by ganglion and amacrine cells [24].

Midkines are pleiotropic molecules and little is known about their specific receptors or signaling pathways in zebrafish. To study the specific functions of midkines in the *in vivo* retinal environment, we began with a thorough characterization of the cellular patterns of expression during various physiological states. Here we report that in horizontal cells quantitative levels of *mdka* mRNA and protein are regulated by the circadian clock: *mdka* expression increases during subjective day and decreases during the subjective night, and Mdka protein synthesis follows the same time course. Qualitative evaluation of *in situ* hybridization indicates that *mdkb* is also regulated by the circadian clock. The spatial domain of *mdkb* expression within the inner nuclear layer contracts during the subjective day and expands during the subjective night. Within horizontal cells, the two Midkine paralogs show asynchronous circadian regulation. Though the functional significance of the circadian regulation Midkine expression is yet to be determined, this study expands our knowledge of the cellular expression and circadian control of soluble signaling molecules in the vertebrate central nervous system.

Methods

Animals

Wild-type zebrafish (*Danio rerio*), mixed strains and strain AB, 4.5 to 7 months old, were purchased from Aquatica Tropicalis (Plant City, Florida) and acclimated for at least 2 weeks in aquaria at 28.5°C and a 14/10 hour light/dark cycle. For experiments described here, fish were maintained in complete darkness for 24hrs and sacrificed at 4-hour intervals, starting either at 12 AM or at 4 AM. These and the following procedures, except where noted (see below), were repeated in three independent experiments. All animal procedures were approved by the University of Michigan Use and Care of Animals in Research Committee.

Tissue preparation for in situ hybridization and immunohistochemistry

At selected times and using dim red illumination, adult fish were anesthetized in 0.1% 3-aminobenzoic acid-ethyl ester (Sigma-Aldrich, St. Louis, MO) until gill movements stopped and sacrificed by cervical transection. Eyes were enucleated, lenses removed and eyecups fixed by immersion for 4–19h at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer containing 5% sucrose. Eyecups were cryoprotected in 20% sucrose, embedded in 2 parts 20% sucrose 1 part Tissue-Tek® O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA) frozen in Tissue-Tek® OCT and stored at −80°C. Ten-micron thick cryosections through the dorso-ventral axis of the eyecups were mounted on Superfrost Plus microscope slides (Fisher-Scientific, Pittsburgh, PA) and processed for in situ hybridization.

Antisense digoxigenin-labeled riboprobes were synthesized using the DIG RNA Labeling kit (Roche Diagnostics, Indianapolis, IN) from plasmids containing the full-length *mdka* or *mdkb* cDNAs (gift from Dr. Christoph Winkler [19]). In situ hybridization was performed as described previously [25]. Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche Diagnostics, Indianapolis, IN) was used as enzymatic substrate. To compare *mdka* or *mdkb* expression across time points, 4–5 sections from eyes collected at each of the six time points were mounted and processed on the same slide. Therefore, within each replicated experiment, sections were processed identically, and this allowed direct comparison of expression levels based on the intensity and spatial distribution of the color reaction.

Photographic images

Images were taken with a Nikon DMX 1200 digital camera mounted on a Nikon Eclipse E800 epifluorescent microscope equipped with a differential interference contrast filter. Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) was used to construct the figures. The layer tool was used to generate overlays. If needed, brightness and contrast were adjusted identically in all panels of a given figure.

RNA extraction and Quantitative Real-Time RT-PCR (QRT-PCR)

To isolate retinal RNA, eyecups were removed and retinas were dissected and carefully separated from the retinal pigment epithelium. Three to four retinas per sample from different zebrafish were pooled for each sample, homogenized with a sterile pestle (Kontes Pellet Pestle, Fisher Scientific) in 200 μl lysis buffer from the Ambion RNAqueous-Micro RNA isolation kit (Ambion, Austin, TX), and RNA extraction was performed according to the manufacturer's instruction. The amount of RNA was quantified using a spectrophotometer, and the quality of the RNA was assessed on ethydium bromide-stained formalin-agarose gels. 0.5 or 1 μg of total RNA was used to synthesize cDNA using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the manufacturer's protocol with random hexamers. The resulting first-strand reaction was used as a template for the subsequent QRT-PCR using the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) in an iCycler Real-Time PCR detection system (Bio-Rad). The following amplification and melt curve analysis protocol was used: 95°C 3min, 35X(95°C: 20s, 57°C: 20s, 72°C: 30s), 95°C: 1min, 90X55°C: 10s. Gene specific primers (0.4μM) were as follows: for *mdka* (NM_131070) forward: tgaagttttgttactgagctttgtg, and reverse: agccagtgtacataagtgtgtgtgt; for *mdkb* (NM_131716) forward: gctgttgtaatttgtagcaggtttt, and reverse: cattcaatctcgttgtcatttacag. Serial dilutions of the first strand reaction were run for efficiency calculations of each primer using the Pfaffl method [26]. The threshold cycle (Ct) was determined by the iCycler using the maximum curvature approach and then maintained constant for subsequent runs. Relative gene expression values were determined using the calculated primer efficiencies and threshold cycle with the formula: E^{-Ct}. Specificity of the amplification products was verified by agarose gel electrophoresis of sample wells. Values obtained were averaged for 3–6 independent amplification reactions and statistical significance was calculated by one-way ANOVA with Bonferroni correction for multiple comparisons using SPSS software. Samples within each replicate experiment were treated identically. Results were normalized to the 8 PM time point, which allowed comparison between experiments. To verify that equal amounts of RNA were used, QRTPCR was performed with specific primers for *ribosomal protein L-19* (*rpl-19*, accession number: NM_213208, primers forward: gagtatgctcagacttcagaagagg and reverse: atcaaaccatccttcaccaacttac). There were no differences in *rpl-19* expression across time-points.

Midkine-a Antibodies and Western Blot Analysis

Based on antigenicity, hydrophilicity, flexibility and surface probability (Invitrogen, Camarillo, CA), a C-terminal peptide composed of 16 amino acid residues was chosen as the Mdka immunogen (amino acids 131–145: KVKNKPKGKKGKGKGC; accession number NP_571145). Affinity purified polyclonal antibodies were generated in rabbits. In Western blots, the resulting antibodies recognized a single band with the correct size in lysates from both transfected 293T cells expressing zebrafish Mdka-MYC and retinas (data not shown). These antibodies did not recognize Mdkb-MYC (data not shown).

To assay endogenous Mdka, 5 retinas were dissected from 5 different fish at each time-point and pooled. The retinal pigment epithelium was carefully removed, and retinas were homogenized with a Kontes pellet pestle (Fisher Scientific, Pittsburgh, PA) in 75μl of lysis buffer (Phosphate Buffered Saline with 1% Triton X) and protease inhibitors (Complete-mini EDTA free, Roche, Indianapolis, IN). Lysates were centrifuged for 5 minutes at 5000 rpm at 4°C to pellet the nuclei and transferred to fresh tubes. The amount of retinal protein was determined with a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto a 12% SDS-PAGE and processed for immunoblotting.

Proteins were separated by electrophoresis on a 12% Sodium-Dodecyl-Sulphate Polyacrylamide Gel (SDS-PAGE) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were incubated at 4°C for at least 4 hours in Blocking Buffer (Phosphate-Buffered-Saline with 0.5% Tween [PBST] and 5% Non-Fat Dry Milk) followed by overnight incubation in αMdka primary antibodies, diluted 1:500. Membranes were washed for one hour in PBST, then incubated for one hour in peroxidase conjugated anti-rabbit IgG secondary antibodies, diluted 1:1000 (Amersham Biosciences, Arlington Heights, IL). Following 4–5 vigorous washes in PBST, proteins were visualized with the electro-chemiluminescence ECL detection system (ECL- Amersham Biosciences, Arlington Heights, IL) and radiographic film (Kodak, Rochester, NY). Antibodies against Mdka recognize a single band at the appropriate molecular weight for the native protein (see Results). Equal protein loading was verified by reprobing membranes with a monoclonal antibody recognizing α– β– and γ-actin (JLA20, Calbiochem, San Diego, CA).

A flatbed scanner (Epson expression 1600) was used to obtain a digital image of the film from which the intensity of the Midkine and actin bands were measured using the histogram tool in Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). The intensity of each Midkine band was normalized to the intensitiy of the corresponding actin band. To compare results between replicate experiments, the 8pm time-point was chosen as a reference, and the relative protein levels were normalized. The resulting values were then averaged between experiments and plotted as fold-change.

Results

The circadian clock regulates expression of *mdka* and the levels of Mdka protein

To determine if *mdka* expression is regulated by the circadian clock, zebrafish were maintained in their normal aquaria, but in total darkness, for a period of 24 hours, and retinas were collected at four-hour intervals. At each time point, retinas were divided into three groups, and the expression of *mdka* was evaluated by *in situ* hybridization and QRT-PCR, and the synthesis of Mdka was evaluated by Western blot analysis. The *in situ* hybridization confirmed that in the adult retina, *mdka* is selectively expressed in horizontal cells [see 24], and showed that this cellular expression is dynamically modulated during the circadian cycle. Minimum expression was observed in retinas harvested at 12 AM (Fig. 1a), one hour after lights are turned off in our facility. Following this time point, expression increased to reach an apparent maximum at 8 AM (Fig. 1b,c), one hour prior to light onset.

mdka expression then remained high during the first half of the subjective day (Fig. 1d,e) and gradually decreased during the second half of the subjective day (Fig. 1f,a), approaching the minimum at midnight.

To validate these qualitative observations, QRT-PCR was used to measure *mdka* mRNA levels. These quantitative data parallel the *in situ* hybridization data and confirm the circadian rhythm of *mdka* expression. The ANOVA showed that the expression levels *mdka* at 12 AM and 4 AM were statistically significantly different from the 8 AM time point, which, in turn, was significantly different from 8 PM (Table 1).

Finally, Western blot analysis of retinal lysates showed a circadian rhythm for protein levels of Mkda that mirror the *in situ* hybridization and QRT-PCR results (Fig. 3a,b). Minimum levels of protein were present in the retinas at 12 AM, whereas maximum protein levels were present in the retinas at 8 AM. This rhythm was observed in two independent experiments. Taken together, these data show that the expression of Mdka in horizontal cells of the zebrafish retina is regulated by the circadian clock, and both the mRNA and protein levels follow the same dynamic time-related pattern.

mdka is expressed in presumptive Müller glia at the retinal margin in a circadian pattern

In the developing retina, *mdka* is transiently expressed in Müller glia [24]. In central retina, these non-neuronal cells strongly express *mdka* between 3 and 5 days post fertilization (dpf). At 5dpf expression of *mdka* decreases in Müller cells and increases in horizontal cells, where expression is maintained into adulthood. We consistently observed an inverse relationship between the expression of *mdka* in horizontal cells and presumptive Müller glia within immature retina adjacent to the neurogenic retinal margin. When *mdka* expression in horizontal cells was at the minimum (12 AM), its expression increased in cells with large somata located in the inner tier of the inner nuclear layer (Fig. 4). Based on their size and laminar position, we infer these cells are Müller glia. In contrast, when *mdka* expression in horizontal cells was at the maximum (8 AM), there was no visible expression of *mdka* in Müller glia.

The circadian clock modulates the cellular expression of *mdkb*

We next asked whether the expression of *mdkb* is also modulated by the circadian clock. *In situ* hybridization showed that that at 12 PM *mdkb* is expressed by cells within the innermost (amacrine cell) tier of the inner nuclear layer and in the ganglion cell layer (Fig. 1j; see also [24]. In contrast, at the onset of the subjective night – 8 PM - expression levels of *mdkb* increased, and expanded into the outer portion of the inner nuclear layer to include horizontal cells (Fig. 1h,i). This increase in *mdkb* expression was observed in all three replicate experiments. To quantify *mdkb* expression QRT-PCR was performed with *mdkb*-specific primers. In three replicate experiments, a trend that paralleled the *in situ* hybridizations was observed (Fig. 2b), although the level of *mdkb* expression was more variable than for *mdka* and did not reach statistical significance (Table 2).

Discussion

The two zebrafish midkine paralogs are encoded by genes located on different chromosomes; they have different cellular patterns of expression in the central nervous system and in the developing hindbrain subserve different functions [19,21,22,23]. In the zebrafish retina, cellular expression of *mdka* and *mdkb* is actively modulated during two neurogenic events: retinal development and photoreceptor regeneration following light-induced photoreceptor death [24]. Unexpectedly, a time-course analysis of *mdka* expression in the light-lesioned retina, consistently showed a marked decrease at twelve hours after

light onset. Analyzing expression of *mdka* in uninjured retinas from zebrafish maintained in normal lighting conditions revealed the same marked decrease in expression at the end of the day (data not shown), suggesting light-induced or circadian regulated expression.

To test whether *mdka* expression is regulated by the circadian clock we analyzed the expression of *mdka* and *mdkb* over a 24hr-period in fish that were kept in total darkness. This analysis confirmed our hypothesis that in the zebrafish retina expression of midkines is regulated by the circadian clock. For *mdka*, the circadian changes in mRNA expression were paralleled by changes in the levels of Mdka protein, suggesting that mRNA and protein synthesis and degradation are tightly regulated. For *mdkb*, *in situ* hybridization showed that mRNA levels for cells in the inner nuclear layer appear also to be modulated by the circadian clock. The QRT-PCR analysis for *mdkb* expression revealed a trend that mirrored the *in situ* hybridization, but differences were not statistically significant. Unlike *mdka*, which is very distinctly expressed in horizontal cells, *mdkb* is expressed in several types of retinal neurons, so it is possible that the QRT-PCR analysis of whole retina RNA was not able to reflect the circadian changes in expression of *mdkb* in horizontal cells. Nevertheless, *in situ* hybridization showed that in horizontal cells, the expression *mdka* and *mdkb* is out-of-phase, suggesting that in the outer retina these two proteins exert their biological actions at different times during the circadian cycle.

Interestingly, we also observed the circadian-regulated expression of *mdka* in Müller glia, but only in a few immature cells found within the growth zone adjacent to the neurogenic retinal margin (see [27] for a more complete description of neurogenic events in the adult retina of teleosts). This observation is reminiscent of the transient expression of *mdka* in Müller glia in the larval retina and the re-expression of *mdka* in Müller glia during photoreceptor regeneration [24]. Together, these results suggest that expression of *mdka* in Müller glia may be related to the persistent (and injury-induced) neurogenesis mediated by these cells [28].

As for most vertebrates, in teleosts the retina functions in part (though not alone, [29,30,31]) to entrain circadian rhythms. Additionally, within the teleost retina numerous processes are under direct circadian regulation. Among these is the synthesis and release of neurotransmitters and soluble signaling molecules [5,32], retinomotor movements [6,7], and the synthesis of photoreceptor opsins [33,34]. Whereas the function of retinal Midkines are not yet known, our data identify them as components of cyclical signaling events with the vertebrate retina. Further, the demonstration that these molecules are regulated by circadian rhythms points to important avenues of further inquiry.

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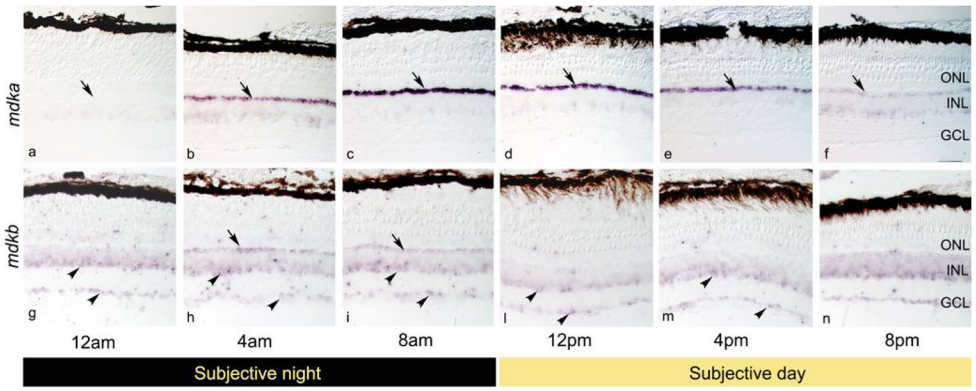

Figure 1. The circadian clock regulates expression of *mdka* and *mdkb* in the zebrafish retina

Panels a–f are in situ hybridization that illustrate retinal expression of *mdka* during subjective day (panels a–c) and subjective night (panels d–f). Panels g–n are in situ hybridizations that illustrate retinal expression of *mdkb* during subjective day (panels g–i) and subjective night (panels l–n). Arrows in panels a–n point to the location of horizontal cells. Arrowheads in panels g–n point to the location of amacrine and ganglion cells respectively. Sections for all time-points for each probe were processed on the same slide. ONL; outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in f equals 50 μm.

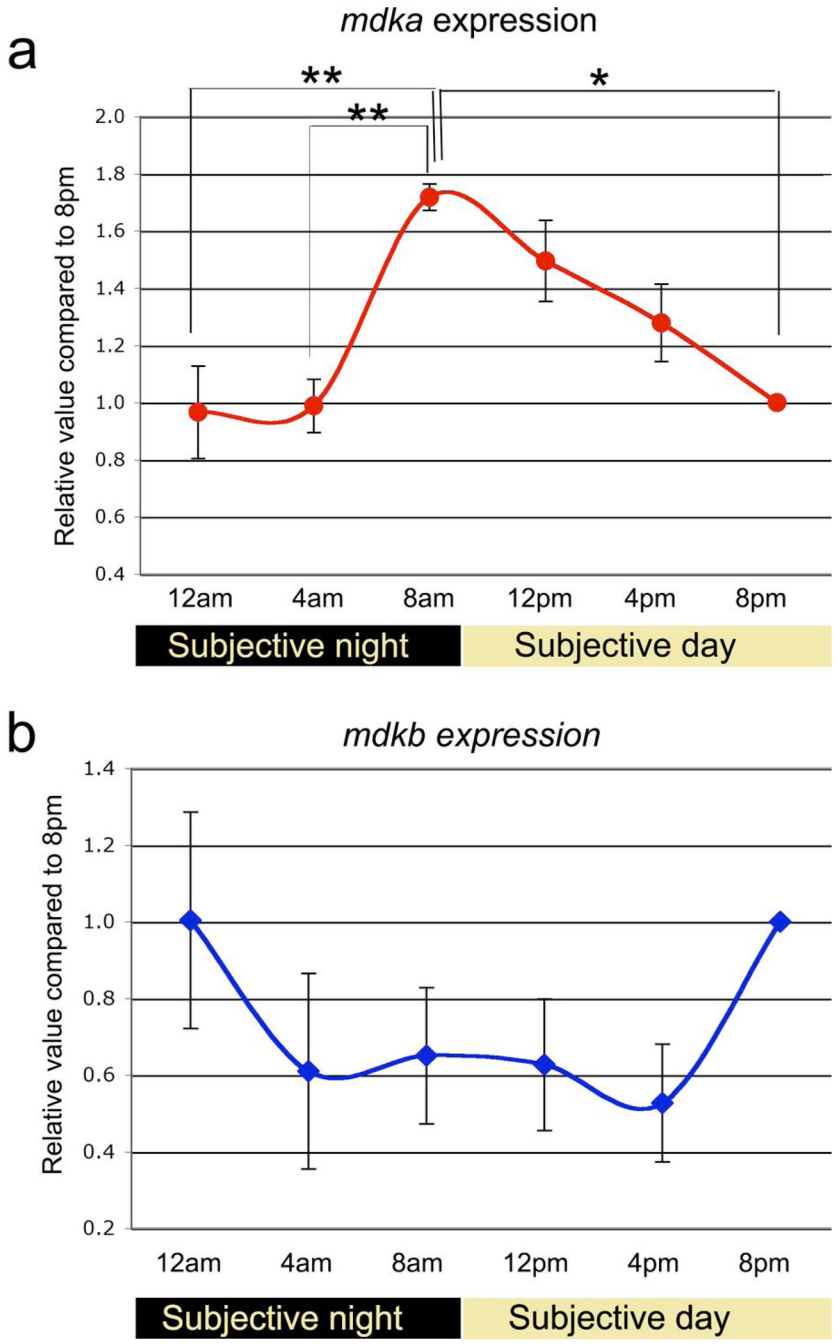

Figure 2. Quantitative analysis of circadian variations of *mdka* and *mdkb*

Panels a and b are graphical representations of the QRTPCR analysis of the expression of *mdka* and *mdkb*, respectively. Gene expression is represented as fold change compared to expression values at the end of the subjective day (8pm). Data represent average values from 3 experiments with standard errors represented for each time-point. Statistical significance was determined through one-way ANOVA with Bonferroni correction for multiple comparisons using SPSS (** $p<0.01$, * $p<0.05$).

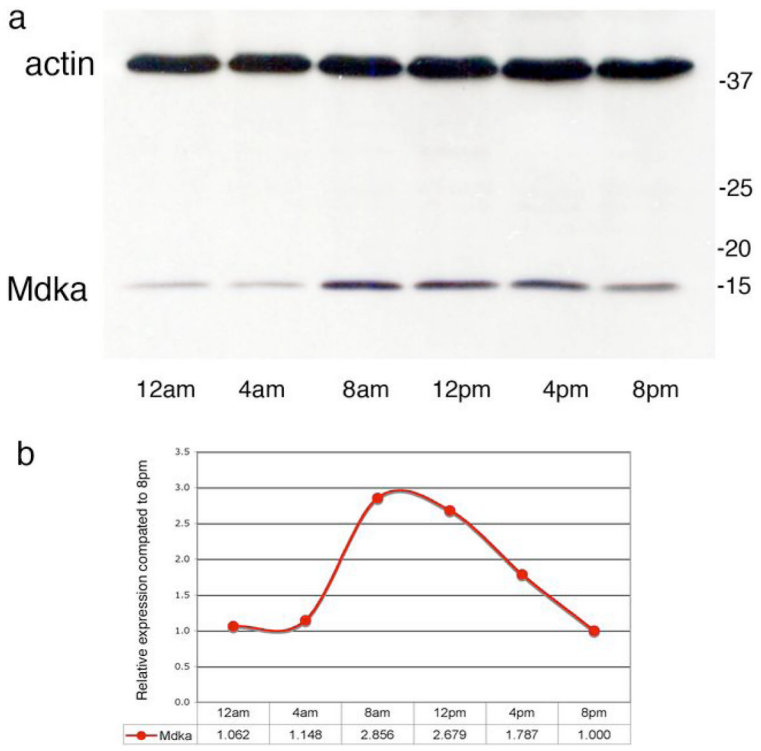

Figure 3. Western blot analysis of Mdka expression

Panel a illustrates an immunoblot of retinal lysates obtained from zebrafish at specified times during the circadian cycle and separated by SDS-PAGE. Lower bands represent the Mdka protein and upper bands represent actin, used as loading control. Panel b illustrates the quantification of circadian changes in retinal Mdka protein expression, normalized to expression of actin, average of 2 experiments. Results are shown as mean fold change compared to the expression at 8pm.

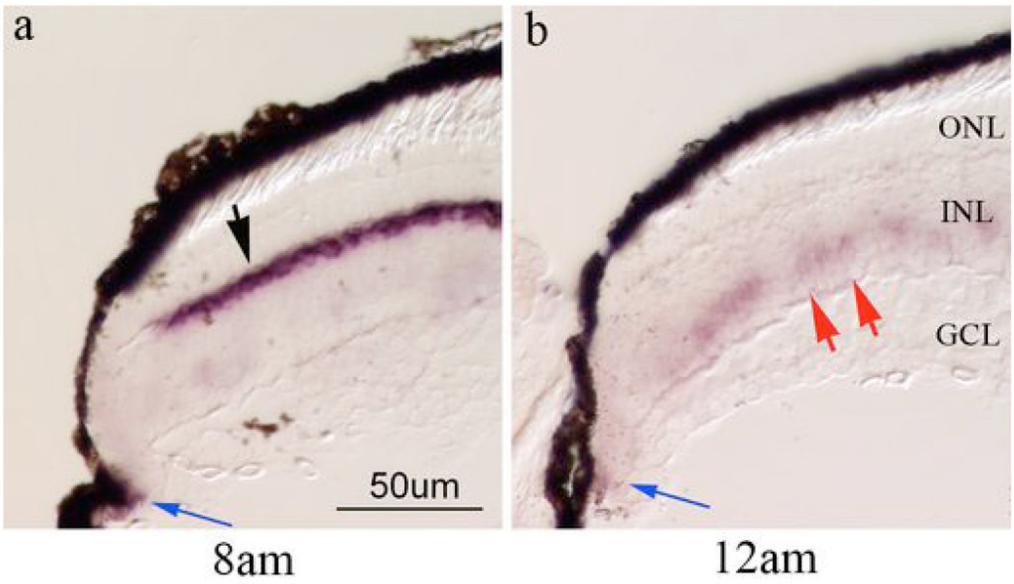

Figure 4. *mdka* expression in immature retina

Panels a and b are in situ hybridization that illustrate epression of *mdka* at the retinal margin at the circadian time of maximum *mdka* expression (8am, panel a) and minimum *mdka* expression (12am, panel b). Black arrow in panel a points to horizontal cells that strongly express *mdka* at this time. Blue arrows in panels a and b point to the location of peripheral-most cells in the CMZ that express *mdka*. Red arrows in panel b point to the presumptive Müller glia that express *mdka* at the time when expression of *mdka* in horizontal cells is minimal. Sections were processed on the same slide. ONL, outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in f equals 50 μm.

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Table 1

Circadian expression of *mdka* in the zebrafish retina

Experiment	12 am	4 am	8 am	12 pm	4 pm	8 pm	Min	Max
1	0.6599	0.8043	1.6980	1.2189	1.1373	1	0.6599 (12am)	1.6980 (8am)
2	1.0282	1.0572	1.6504	1.6875	1.1493	1	1.0282 (12 am)	1.6875 (12pm)
3	1.2099	1.1042	1.8055	1.5799	1.5501	1	1.1042 (4am)	1.8055 (8am)
Mean	0.9660	0.9886	1.7180	1.4955	1.2789	1		
Std. Dev.	0.2802	0.1613	0.0794	0.2455	0.235	0		
Std. Error	0.1618	0.0931	0.0458	0.1417	0.1356	0		
95% CI lower bound	0.2698	0.5879	1.5207	0.8856	0.6952	1		
95% CI upper bound	1.6623	1.3893	1.9153	2.1054	1.8627	1		

One way ANOVA with Bonferroni correction for multiple comparisons								
Pairwise comparison	**Mean difference**	**p value**	**Pairwise comparison**	**Mean difference**	**p value**	**Pairwise comparison**	**Mean difference**	**p value**
12am vs 4am	−0.02253	1.000	8am vs 12am	0.75197	0.007**	4pm vs 12am	0.31290	1.000
12am vs 8am	−.75197	0.007**	8am vs 4am	0.72943	0.009**	4pm vs 4am	0.29037	1.000
12am vs 12pm	−0.52947	0.089	8am vs 12pm	0.2225	1.000	4pm vs 8am	−0.43907	0.255
12am vs 4pm	−0.3129	1.000	8am vs 4pm	0.43907	0.255	4pm vs 12pm	−0.21657	1.000
12am vs 8pm	−0.03397	1.000	8am vs 8pm	0.71800	0.01*	4pm vs 8pm	0.27893	1.000
4am vs 12am	0.02253	1.000	12pm vs 12am	0.52947	0.089	8pm vs 12am	0.03397	1.000
4am vs 8am	−0.72943	0.009**	12pm vs 4am	0.50693	0.115	8pm vs 4am	0.1143	1.000
4am vs 12pm	−0.50693	0.115	12pm vs 8am	−0.22250	1.000	8pm vs 8am	−0.718	0.01*
4am vs 4pm	−0.29037	1.000	12pm vs 4pm	0.21657	1.000	8pm vs 12pm	−0.4955	0.132
4am vs 8pm	−0.01143	1.000	12pm vs 8pm	0.49550	0.132	8pm vs 4pm	−0.27893	1.000

Table 2

Circadian expression of ***mdkb*** in the zebrafish retina

Experiment	12 am	4 am	8 am	12 pm	4 pm	8 pm	Min	Max
1	0.6127	0.1035	0.2938	0.4176	0.2846	1	0.1035 (4 am)	1 (8pm)
2	1.5518	0.9176	0.834	0.9625	0.8120	1	0.8120 (4pm)	1.55 (12 am)
3	0.8471	0.8083	0.8227	0.5001	0.4856	1	0.4846 (4pm)	1 (8pm)
Mean	1.0039	0.6098	0.6502	0.6242	0.5274	1		
Std. Dev.	048878	0.44186	0.30867	0.29642	0.26617	0		
Std. Error	0.2822	0.25511	0.17821	0.17114	0.15368	0		
95% CI lower bound	−0.2103	−0.4878	−0.1166	−0.1121	−0.1338	1		
95% CI upper bound	2.2181	1.7074	1.4170	1.3605	1.1886	1		

One way ANOVA with Bonferroni post-hoc correction for multiple comparisons

Pairwise comparison	Mean difference	p value	Pairwise comparison	Mean difference	p value	Pairwise comparison	Mean difference	p value
12am vs 4am	0.39407	1.000	8am vs 12am	−0.35370	1.000	4pm vs 12am	− 0.47647	1.000
12am vs 8am	0.35370	1.000	8am vs 4am	0.04037	1.000	4pm vs 4am	−0.08240	1.000
12am vs 12pm	0.37967	1.000	8am vs 12pm	0.02597	1.000	4pm vs 8am	−0.12277	1.000
12am vs 4pm	0.47647	1.000	8am vs 4pm	0.12277	1.000	4pm vs 12pm	−0.968	1.000
12am vs 8pm	0.00387	1.000	8am vs 8pm	−0.34983	1.000	4pm vs 8pm	−0.4726	1.000
4am vs 12am	−0.39407	1.000	12pm vs 12am	−0.37967	1.000	8pm vs 12am	−0.00387	1.000
4am vs 8am	−0.4037	1.000	12pm vs 4am	0.0144	1.000	8pm vs 4am	0.39020	1.000
4am vs 12pm	−0.1440	1.000	12pm vs 8am	−0.2597	1.000	8pm vs 8am	0.34983	1.000
4am vs 4pm	0.08240	1.000	12pm vs 4pm	0.0968	1.000	8pm vs 12pm	0.37580	1.000
4am vs 8pm	−0.39020	1.000	12pm vs 8pm	−0.37580	1.000	8pm vs 4pm	0.47260	1.000