Intraocular injection of kainic acid does not abolish the circadian rhythm of arylalkylamine N-acetyltransferase mRNA in rat photoreceptors

Katsuhiko Sakamoto, 1 Cuimei Liu, 1 Manami Kasamatsu, 1 P. Michael Iuvone, 2, 3 Gianluca Tosini 1

1 Neuroscience Institute and National Science Foundation Center for Behavioral Neuroscience, Morehouse School of Medicine, Atlanta, GA; Departments of 2 Pharmacology and 3 Ophthalmology, Emory University School of Medicine, Atlanta, GA

Purpose: Melatonin synthesis in mammalian retinal photoreceptors is under photic and circadian control and regulated by changes in the activity of arylalkylamine N-acetyltransferase (AANAT). Recent studies have suggested that retinal dopaminergic neurons contain a circadian pacemaker, and dopamine is the neurotransmitter that drives circadian rhythmicity in the mammalian retina.

Methods: To investigate the role of inner retinal neurons, including dopamine neurons, in generating the rhythm of melatonin synthesis, rat retinas were lesioned with kainic acid (KA), which was shown previously to induce degeneration of neurons in the inner nuclear layer and to eliminate rhythmicity in the dopaminergic system. Aanat, rhodopsin, medium wavelength (mwl) opsin, short wavelength (swl) opsin, and period 1 (Per1), and period 2 (Per2) mRNA levels were measured using real-time quantitative RT-PCR in KA injected and control eyes.

Results: Our data show that intraocular injections of KA did not abolish the daily and circadian rhythms of Aanat mRNA in the photoreceptors, but it did shift the phase of the Aanat transcript rhythm in constant darkness. Surprisingly, KA injections reduced the levels and eliminated daily rhythms of mwl and swl opsin transcripts, but not of rhodopsin mRNA. Per1 and Per2 mRNA levels were rhythmic in saline injected and in KA-treated retinas, and Per2 mRNA levels were significantly reduced (20-50%) in KA-treated retinas.

Conclusions: These findings demonstrate that the circadian clock generating melatonin rhythmicity is largely KA insensitive and likely to be located in the rod photoreceptors, although KA-sensitive neurons do influence its timing. More important, our data demonstrate that dopamine rhythmicity is not necessary for generating the circadian rhythm of Aanat mRNA in the photoreceptors. Our data also indicate that Per1 and Per2 are rhythmically transcribed in the rat retina and KA treatment has a dramatic effect on the overall levels of Per2 mRNA.

In vertebrates, retinal clocks control several circadian rhythms within the retina: from gene transcription to visual sensitivity to photoreceptor damage [1, 2]. In mammals, retinal circadian rhythms persist after lesion of the suprachiasmatic nucleus (SCN) [3,4] and in isolated retinas [5-7], and the retinal clock may contribute to the overall circadian organization of mammals [8, 9].

Melatonin and dopamine (DA) are two of the most studied outputs of the retinal circadian clock, and they act as mutually inhibitory paracrine signals for night and day, respectively [1, 2]. Thus, the melatonin-synthesizing photoreceptors [10] and DA-secreting amacrine and interplexiform cells [11, 12] form a cellular feedback loop that regulates circadian retinal physiology. Although several studies have investigated the mechanisms that regulate the circadian rhythms in retinal melatonin and DA, it is not yet clear if these rhythms are driven by two different circadian pacemakers or if one rhythm is driving the other.

Recent studies have suggested that DA is the key player in the control of retinal circadian rhythmicity, as dopaminergic neurons express several clock genes [13, 14]. An additional investigation in Royal College of Surgeons (RCS) rats reported that circadian rhythms of DA and its metabolites are present in the retina of RCS rats, which virtually lack photoreceptors, thus indicating that the circadian oscillators driving dopamine metabolism are not in photoreceptor cells [15].

These recent findings support the hypothesis that dopaminergic amacrine cells may contain an autonomous circadian clock that drives DA release and metabolism. However, it must be noted that retinal DA content and metabolism are circadian in mice that synthesize melatonin, but not in mice that are genetically incapable of synthesizing melatonin [16, 17]. Daily injections of melatonin induce circadian rhythms of DA in retinas of mice that are unable to synthesize melatonin [17]. These observations suggest that the circadian rhythm of DA synthesis in the mouse retina is controlled by rhythmic release of melatonin.

A few studies have investigated the expression pattern and the cellular localization of putative clock genes in the rat retina. Clock and period 1 (Per1) mRNA levels do not show any rhythmicity [18], whereas Bmal1 and period 2 (Per2) transcript levels are rhythmic [4, 18-20]. The cellular localization of Per1 and Per2 mRNA in the rat retina has also been investigated. Per1 transcripts are expressed in the inner nuclear layer.
(INL) and in the outer nuclear layer (ONL), while Per2 transcripts were primarily expressed in the INL and, to a lesser extent, in the ganglion cell layer (GCL) [20].

Previous investigations have reported that intracocular injection of kainic acid (KA) induces severe degeneration in the inner retina [21,22], eliminates rhythmicity in the dopaminergic system [23,24], but does not affect photoreceptors [25]. KA reduces the b-wave of electroretinogram, which reflects activity of the inner layers of the retina, but has no effect on the a-wave, which is generated by photoreceptors [21].

In the present study, we investigated the effects of KA injection on the regulation of the mRNA encoding Arylalkylamine N-acetyltransferase (Aanat), the key regulatory enzyme in melatonin synthesis, and on the transcripts encoding rhodopsin, medium wavelength (mwl; green cone) opsin, and short wavelength (swl, blue cone) opsin. Finally, in our last experiment we also investigated the pattern of expression of Per1 and Per2 in saline and KA-treated retinas.

**METHODS**

*Animals and tissue collection:* Male rats of the Fisher strain (8-10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Animals were maintained at Morehouse School of Medicine in a 12 h:12 h light-dark (LD) cycle of illumination with light on from Zeitgeber Time (ZT) 0-12. Food and water were available ad libitum.

Animals were maintained in LD for a minimum of two weeks prior to experiments. To investigate the expression of retinal gene expression in LD cycles, animals were sacrificed at ZT 6, 12, 18, and 24. When animals were sacrificed during the night, the procedure was carried out in dim red light (<1 lux). To investigate the pattern of expression in constant conditions, rats were transferred into dark-dark (DD) conditions for two days before sacrifice. Samples were then collected during the second day at circadian time (CT) 6, 12, 18, and 24. Retinas were dissected, immediately frozen on dry ice, and stored at -80 °C.

*Kainic acid injections:* KA (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.4. A volume of 5 µl (200 nmol) was injected intraocularly into the right eye of each rat with a Hamilton microsyringe, while 5 µl of saline was injected in the left eye. Animals were sacrificed 48 h after the injection.

**Real-time quantitative reverse transcriptase polymerase chain reaction:** Total RNA was isolated from each retina using TRIZOL reagent (Life Technologies, Grand Island, NY) following sonication. RNA was treated with DNase I to remove any traces of genomic DNA. First strand cDNA was synthesized from 1 µg of each RNA sample using oligo (dT) and Omniscript reverse transcriptase (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. Each set of samples was simultaneously processed for RNA extraction, DNase I

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### Table 1. Real-time PCR primers

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<th>Name</th>
<th>GenBank accession number</th>
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<td></td>
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<td></td>
<td></td>
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<td>282</td>
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<tr>
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<td>326</td>
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Primers were designed using Primer3 on the indicated GenBank sequences. The length of the PCR product for each primer pair and the cDNA sequence number for each primer are indicated. Each primer pair yielded a single product, as determined by agarose gel electrophoresis and melt-curve analyses.
treatment, cDNA synthesis and PCR reaction. Real-time quantitative RT-PCR was performed with SYBR Green (BioWhittaker Molecular Applications; Walkersville, ME) using an iCycler (BioRad, Hercules, CA). Primers used were as given in Table 1.

To assess the effectiveness of the KA treatment with respect to the dopaminergic system, Tyrosine hydroxylase (Th) mRNA was measured in each of the retina. Only retinas with a >90% reduction in Th mRNA levels with respect to the control were used (see [24]).

Although a recent study has reported that Gapdh mRNA levels may show a significant variation during the 24 h period [18], under our experimental conditions Gapdh mRNA did not show any significant variation in LD or in DD (p>0.1).

In situ hybridization: Eyes were obtained from rats injected with saline and KA at ZT 6 and ZT 18 (that is, at the time of lowest and of highest level of Aanat mRNA). Eyes were punctured and then fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.0) for 6 h at 4 °C. The eyes were transferred to a solution of 30% sucrose for 12-14 h, embedded in Tissue-Tek OCT compound (Miles, Sakura Finetek, Torrance, CA) and cut into 20 µm thick cryosections. The template for transcription was a 1413 bp cDNA fragment of rat Aanat subcloned into a pZLI vector (GenBank U40803) generously donated by Dr. J. Borjigin (John Hopkins School of Medicine, Department of Neuroscience, Baltimore, MD).

Correct orientation of the construct was verified by sequence analysis and restriction enzyme digestion. Antisense and sense cRNA probes were generated using Fluorescein-12-UTP (Perkin Elmer Life Science, Boston, MA) by in vitro transcription. The templates for transcribing RNA probes were made by linearizing recombinant plasmids.

Sections were immersed in prehybridization buffer containing 50% formamide, 5X Denhardt’s solution and 5X SSC for 2 h at room temperature. Then the sections were hybridized with 75 µl of hybridization buffer, coveredslipped, and incubated overnight in a humidified chamber at 67 °C. The best labeling was obtained using a probe concentration of 1:100. Slides were then washed in 5X SSC, 50% formamide at 68 °C for 1 h, in 2X SSC for 1 h at 68 °C before incubation in 20 mg/ml RNase A at 37 °C for 30 min followed by 2X SSC for 1 h and 0.2X SSC for 30 min twice at room temperature. Slides were mounted and viewed with a Zeiss Axioskop microscope equipped with epifluorescence.

Data analysis: Data are expressed as mean with standard error of the mean (SEM). Comparison among treatment groups was carried out using non-parametric analysis of variance (Kruskall-Wallis test) followed, when appropriate, by Dunn’s multiple comparisons test.

RESULTS
KA injection did not abolish the photic and circadian regulation of aanat mRNA: Figure 1A shows the destruction of the retina two days after injection of 200 nmoles of KA. Most of the cells in the INL of the KA-treated retina have degenerated forty-eight h after injection; a few cells were still visible in the GCL. The ONL was almost unaffected. The retinas of KA-treated rats were fragile and fragmented during cryosectioning due to massive disruption of the structural integrity of the tissue. The retinas also appear swollen, which is an initial reaction to KA that has been observed as early as 1 h after intracocular injection [21].

When observed during exposure to LD, Aanat mRNA levels showed robust rhythms that peaked in the middle of the night in both saline-treated and KA-treated retinas (Kruskall-Wallis test, p<0.01), but the peak levels were significantly reduced in retinas from KA test injected eyes (Figure 2A). In animals that were maintained in DD for 48 h, we also ob-

Figure 1. Effect of kainic acid treatment on the retina: histological evaluation of kainic acid treatment. Retinas were dissected 48 h after intraocular injection of 200 nmol kainic acid (KA; A) or saline (control; B), sectioned, and stained with cresyl violet. Micrographs show that in the KA-treated retina, most of the inner nuclear layer (INL) has degenerated. No significant alteration was apparent in the outer nuclear layer (ONL). A few ganglion cells were still visible in the ganglion cell layer (GCL). The scale bars represent 50 µm.
served clear circadian rhythms in Aanat mRNA levels in saline- and in KA-injected eyes (Figure 2B; Kruskall-Wallis test, p<0.01). In DD, no difference was observed in the peak levels of Aanat mRNA between saline- and KA-treated retina (Dunn’s test, p>0.1), but the night/day ratio was reduced in KA-treated animals (2 in the KA group compared to 6 in the saline group). Remarkably, the Aanat mRNA levels of retinas treated with KA peaked earlier than those in control retinas, indicative a shift in phase.

Aanat mRNA expression in KA-treated retinas is restricted to the photoreceptor layer. In the saline-injected eyes, Aanat transcripts were detected in the ONL and to a lesser extent INL and GCL (Figure 3A,C). In the ONL, Aanat mRNA showed a clear day-night difference (Figure 3A,C). In the KA-injected eyes, Aanat mRNA was only observed in the ONL, where a day:night difference in transcript level occurred (Figure 3B,D).

Effects of KA injection on rhodopsin, mwl opsin and swl opsin mRNAs: In the eyes injected with saline, rhodopsin, mwl opsin and swl opsin mRNA levels were rhythmic in LD (Figure 4; Kruskall-Wallis test, p<0.01 in all cases). In the KA-injected eyes, rhodospin mRNA was not significantly different from those measured in the saline-injected eyes (Figure 4A; Dunn’s test, p>0.5, in all cases). In contrast, mwl opsin and swl opsin mRNA levels were dramatically reduced (Figure 4B,C; Dunn’s test, p<0.01 in all cases) and no rhythmic expression was observed (Kruskall-Wallis, p>0.1).

Figure 2. Effects of kainic acid treatment on the daily and circadian rhythm of Aanat mRNA in rat retina. Aanat mRNA levels were rhythmic (Kruskall-Wallis test, p<0.01 in all cases) in saline-injected eyes (white circles) and in KA-injected eyes (black circles) in 12 h:12 h light-dark cycles, LD (A) and in dark-dark cycles, DD (B). The white bar at the top of the graph represents the period of light, while the black bars represent period of darkness. Aanat mRNA levels were measured using real-time quantitative RT-PCR. Mean values are plotted (4 rats/time in LD; 8 rats/time in DD); error bars represent the SEM. All values were normalized with respect to the values obtained in LD at Zeitgeber time 18 in saline injected eyes (100%).

Figure 3. Localization of Aanat mRNA. Aanat mRNA was localized in saline (A,C) or kainic acid (KA; B,D) treated retinas by in situ hybridization. Aanat transcripts were rhythmic in the outer nuclear layer (ONL) of KA-injected eyes. Rats were housed in a 12 h:12 h light-dark (LD) cycle and retinas were obtained at ZT 6 or 18. Aanat transcripts were detected using a fluorescein-labeled probe. A: Saline at ZT 18. B: KA at ZT 18. C: Saline at ZT 6. D: KA at ZT 6. Micrographs are representative of results obtained from at least three animals at each time. The scale bar represents 25 µm.
Effects of KA injection on Per1 and Per2 mRNAs: Per1 mRNA levels were rhythmic in saline- and in KA-injected eyes (Figure 5A; Kruskall-Wallis test, p<0.01). A small reduction in the overall mRNA levels was observed in the KA-treated retinas. As expected, Per2 mRNA levels were rhythmic in the retinae obtained from eyes injected with saline (Figure 5B; Kruskall-Wallis test, p<0.05). In retinas obtained from KA-treated eyes, Per2 mRNA levels were still rhythmic, but significantly (30-50%) reduced (Figure 5B; Kruskall-Wallis test, p<0.01).

DISCUSSION

We have previously demonstrated that Aanat mRNA is rhythmically expressed in photoreceptors cells [7,10], and we have also reported that circadian rhythms in Aanat mRNA in vivo and melatonin synthesis in vitro are still present in the retinas of RCS rats following photoreceptor degeneration [26]. Aanat mRNA rhythmicity in RCS rat retina is generated in the INL, where Aanat transcripts are upregulated compared to controls and show a clear day/night oscillations after, but not before photoreceptor degeneration [26]. It is believed that the circadian pacemakers controlling such rhythms in the RCS rat are probably located in KA-sensitive neurons in the inner retina, as KA injections abolished the rhythmicity [26].

Dopamine amacrine cells in mouse retina express Per1 and other clock genes [13,14], suggesting that these inner retinal neurons may drive circadian rhythmicity in the retina. The data presented in this study demonstrate that the circadian rhythm of Aanat mRNA in the rat photoreceptors does not require the circadian rhythm of dopamine and that it is driven by a circadian pacemaker that is largely insensitive to KA. Therefore, we must conclude that at least two different circadian pacemakers (KA-sensitive and KA-insensitive) are present in the mammalian retina. The KA insensitive pacemaker drives the circadian rhythm of Aanat mRNA (and thus melatonin) and is likely to be located in the photoreceptor cells; while a KA-sensitive pacemaker drives the circadian rhythm of dopamine (or Aanat mRNA in the INL) and is located in neurons of the inner retina.

The reduced amplitude and shifted peak of Aanat mRNA rhythm in KA-lesioned retina suggest that kainate-sensitive neurons modulate the circadian rhythm of Aanat mRNA levels in photoreceptors of the intact rat retina. In the rat retina, DA levels show a clear daily rhythm with high DA levels during the day and low levels during the night [15,24]. Several studies have also shown that DA inhibits melatonin synthesis by acting on D_2/D_4-like receptors present on the photorecep-

Figure 4. Rod and cone opsin transcript levels. Effects of kainic acid (KA) treatment on the daily rhythms of opsin mRNAs in saline-injected eyes (white circles) and in KA injected eyes (black circles). Rhodopsin (A), medium wavelength (mwI) opsin (B), and short wavelength (swI) opsin (C) mRNAs were rhythmical in LD (Kruskall-Wallis, p<0.05) in saline treated retina. KA dramatically affected the transcription of mwI and swI opsins p<0.01, whereas rhodopsin mRNA were not significantly affected. In KA-treated retina rhodopsin mRNA levels were rhythmic (Kruskall-Wallis, p<0.05), whereas mwI and swI opsins mRNA did not show any significant rhythmicity (Kruskall-Wallis test, p>0.1). The white bar at the top of the graph represents the period of light, while the black bars represent period of darkness. mRNA levels were measured using real-time quantitative RT-PCR. Mean values are plotted with 4 animals per time; error bars represent the SEM. Values for rhodopsin and mwI opsin transcript levels were normalized with respect to levels observed at zeitgeber time (ZT) 12 in saline-treated retinas; values for swI opsin mRNA were normalized to levels observed at ZT18 in saline-treated retinas.
Figure 5. *Per1* and *Per2* transcript levels. *Per1* (A) and *Per2* (B) mRNA levels were measured in saline injected eyes (white circles) and in KA injected eyes (black circles) in DD. *Per1* and *Per2* mRNA levels were rhythmic in the retina of eyes injected with saline and KA (A,B; Kruskall-Wallis, p<0.01). mRNA levels were measured using real-time quantitative RT-PCR. Mean values are plotted with 8 animals per time; error bars represent the SEM. All values were normalized with respect to the values obtained at Zeitbeger time (ZT); dark-dark cycle (DD) ZT12 in saline injected eyes.
In conclusion, our results demonstrate that Aanat mRNA levels are rhythmic in the retina of rats treated with KA. Such a rhythm does not require a rhythm in dopamine levels, since abolishing the circadian rhythm in dopamine does not abolish the rhythm in Aanat mRNA. These findings suggest that in rat, as in chicken and *Xenopus*, the photoreceptors cells contain a circadian pacemaker that drives the circadian rhythm in Aanat mRNA or that it is driven by nondopaminergic input from other retinal cells. Our data also suggest that DA plays an important role in the modulation of the Aanat mRNA rhythmicity. Finally, our data indicate that at least two different circadian pacemakers (KA-sensitive and KA-insensitive) are present in the rat retina. The KA insensitive pacemaker drives the circadian rhythm of Aanat mRNA and is likely to be located in the photoreceptor cells; while a KA-sensitive pacemaker drives the circadian rhythm of dopamine and is located in neurons of the inner retina or in the GCL.

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REFERENCES


