Effect of suprachiasmatic nucleus lesion on circadian dentin increment in rats

MIE OHTSUKA ISOYA, HARUHIDE HAYASHI, AND HISASHI SHINODA

Tohoku University Graduate School of Dentistry, Divisions of 1Pharmacology and 2Physiology, Department of Oral Biology, Sendai, 980–8575, Japan

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Ohtsuka-Isoya, Mie, Haruhide Hayashi, and Hisashi Shinoda. Effect of suprachiasmatic nucleus lesion on circadian dentin increment in rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R1364–R1370, 2001.—Mammalian dentin universally shows circadian increments. However, little is known about the mechanism of this phenomenon. The purpose of the present study was to investigate the role of the suprachiasmatic nucleus (SCN) in the generation of circadian rhythm in dentin increment. Rats underwent lesion of the SCN by electrodes and were maintained under constant light to examine whether the circadian increment free runs. The rats were injected with nitrolotriacetato lead to chronologically label the growing dentin. Two weeks after the operation, maxillary incisors and the locations of lesions in the brain were examined histologically. A harmonic (Fourier) analysis was performed to examine the densitometric pattern of the dentin increments to determine their periodicity. In rats with a completely lesioned SCN, ultradian increments, but no circadian increments, were observed in the dentin. Alternatively, in rats with an intact or only partially lesioned SCN, circadian increments persisted or were only temporarily disturbed. These results suggest that the SCN plays an important role in the generation of the circadian dentin increment in rats.

circadian rhythm; suprachiasmatic nucleus

PERIODIC INCREMENTAL LINES are universally found in the dentin of vertebrates, just as there are annual rings in wood. Previous studies using chronological labeling methods have demonstrated that these incremental lines in dentin are definitely circadian with a period (τ) of ~24 h in rabbits (12, 13), rodents (10, 11, 15), pigs (21), and other mammals (6). It has been shown that incremental lines in human dentin also have a circadian component (4, 9). Elucidation of the cause of such circadian growth increments in dentin is not only important for understanding the mechanism of the growth and development of dental hard tissues, it is also of profound interest from the perspective of chronobiology, because the circadian incremental line in dental hard tissue is a typical circadian structural rhythm, which might be a manifestation of a circadian oscillatory mechanism in the body. Recently, many studies have demonstrated that the suprachiasmatic nucleus (SCN) in the hypothalamus is a neural time-keeper that is responsible for maintaining circadian rhythm in sleeping (3), drinking (14), locomotor activity (17), plasma corticosterone (7), and pineal N-acetyltransferase (8). It has also been shown that destruction of the SCN eliminates such rhythms.

In a previous study, we demonstrated circadian rhythms in the collagen-synthetic and secretory activities of odontoblasts in rats, which might be responsible for the circadian increment in dentin (11). If odontoblast function fluctuates under the control of an endogenous oscillatory mechanism, lesion of the SCN may lead to some disturbance or possibly the disappearance of the dentin increment. On the basis of this hypothesis, the present study was undertaken to investigate the role of the SCN in the generation of circadian rhythm in the dentin increment in rats subjected to SCN lesion.

MATERIALS AND METHODS

Animals. All of the following animal experiments were conducted under the approval of the Animal Care and Use Committee of Tohoku University School of Dentistry, whose guide for the use of experimental animals is based on the Principles of Laboratory Animal Care from the National Institutes of Health.

Twenty-eight male Wistar rats (Japan SLC, Shizuoka, Japan), 12–17 wk old and weighing 250–300 g, were individually housed in identical wire-mesh cages. The room was maintained on a 12:12-h light-dark cycle (lights on at 0800 and off at 2000) at 24 ± 1°C and 55 ± 5% humidity. The rats were allowed free access to food (Laboratory Chow, F-2, Funahashi Farm, Funabashi, Japan) and deionized water. Before the experiment, they were acclimated to the above room conditions for at least 2 wk. The rats were injected subcutaneously with nitrolotriacetato lead (NTA-Pb; 2 mg Pb/kg) on days 0, 5 (when the rats underwent operations), and 12 to chronologically label the growing dentin. The first injection was omitted in some rats.

Locomotor activity. During the experiment, the locomotor activity of the animals was monitored using a Mini Motionlog Laseractigraph (Ambulatory Monitoring) secured to the outside of each cage. The amount of activity was recorded as counts per 30 min.

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Lesion of the SCN. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and then placed in a stereotaxic apparatus. Bilateral electrolytic lesions of the SCN were made by passing anodal and cathodal direct current of 0.3 mA for 3 min through steel wire (0.6–0.8 mm diameter) placed at four loci along the rostrocaudal extent of the SCN. For the control group, four intact rats underwent the same operation without current. After the operation, the rats were housed under constant light. All of the rats were killed within 2 wk postoperatively by perfusion through the left cardiac ventricle with a fixative solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) under excessive pentobarbital sodium anesthesia. Twenty minutes later, the brain and maxillae were dissected out and freed from adherent soft tissue. They were also fixed by immersion in the same fixative solution as above.

Verification of SCN lesion. The brains were further fixed by immersion in the same fixative solution containing 5% potassium hexacyanoferrate (II) trihydrate to stain ferrous ions liberated from heated steel wire in the lesioned brain area. In each brain, the location and extent of SCN lesions were verified by examining 100 \( \mu \text{m} \)-thick serial horizontal frozen sections stained with 1% neutral red solution.

Histological examination and chronological analysis of dentin increments. The maxillae containing the maxillary incisors were demineralized with 10% formic acid solution.
saturated with H₂S gas to fix injected NTA-Pb as insoluble PbS in the dentin matrix. The dentin was embedded in 30% gelatin and sliced transversely into 15 μm-thick sections using a cryostat (OTF/AS, Bright Instrument). The dentin sections were dipped in 0.1% chloroauric acid solution until Pb time-marking lines emerged, rinsed in distilled water, and treated with 5% Na₂SO₄ solution to tone the lines. The sections were stained with Carazzi’s hematoxylin.

Histological observations and chronological analysis of dentin increments were made using a light microscope (Axioptone, Carl Zeiss, Jena, Germany) and an image analyzer (Winroof, Mitani Shoji, Fukui, Japan) attached to the microscope. Densitometry of black and white images of the dentin increment was conducted with the image analyzer in the direction of the dentin tubules at 1-μm intervals (Fig. 1). After densitometry, a Fourier analysis was applied to the densitometric pattern of the dentin increments between the time-marking lines (5- or 7-day interval) to detect the presence of rhythmicity. The period length of dentin increments was determined by periodogram using a harmonic (Fourier) analysis. Finally, the period length was compared with the daily growth rate, which was calculated by dividing the distance between the time-marking lines by the number of days in the interval to estimate whether the period of the dentin increment was circadian. The above time analyses were adopted based on the assumption that the width of a series of growth increments was constant. The adjacent light- and dark-stained increments can be considered to be approximately equidistant, at least for a short period such as 5 or 7 days, although the width of the increments gradually changes from the outer dentin toward the pulp cavity depending on the gradual changes in the matrix-forming activity of odontoblasts with age.

RESULTS

In this experiment, bilateral electrolytic lesions were intended to be made in the SCN using steel wire. However, as has been previously described, it is difficult to place the wire to hit just the SCN, due to the
small size of the SCN. Therefore, in many cases, SCN lesion was not completely achieved, and the SCN remained intact to some extent. In some cases, the entire SCN remained intact. Only 3 of the 28 animals in the present experiment sustained complete SCN lesions. Therefore, to investigate whether or not the SCN plays a key role in the generation of a circadian dentin increment, the relationship between the extent of SCN lesion and the persistence of the circadian dentin increment was investigated in all of the animals.

The number of animals with SCN lesions of various degrees and the corresponding changes in the circadian dentin increment are summarized in Table 1. The details of the examinations, together with the changes in the locomotor activities of the animals, are as follows.

1) In the four rats that underwent a sham operation (Fig. 2A), the circadian dentin increment, consisting of a pair of hematoxylin faintly stained and deeply stained bands, was essentially the same as that before the operation, even under constant light (Fig. 3A). The above observation was further confirmed by a harmonic analysis of the dentin increments before (days 0–5) and after (days 5–12 and 12–19) the operation; in the periodograms for the dentin increments during the three experimental periods, all principal peaks, even those for the after the operation, appeared at period lengths that approximately corresponded to the daily growth rates (Fig. 4A; 25, 30, and 22 μm vs. 24, 29, and 23 μm, respectively). The actogram during the corresponding period is shown in Fig. 5A. Locomotor activity showed irregular fluctuations for several days after the sham operation and then exhibited free-running circadian rhythms through a phase-delay shift.

2) In three rats, the bilateral SCN was completely lesioned (Table 1). The brain section of one of these rats is shown in Fig. 2B. In this animal, the circadian dentin increment was completely abolished after the operation. Instead of the previous circadian increments, ultradian incremental lines, with a periodicity of 14 lines per 7 days (between the line on day 12 and the calcification front on day 19), appeared about 7 days after the SCN lesion (Fig. 3B). In the periodograms of the dentin increment, the principal peaks for the increments disappeared after the operation (days 5–12), and two small peaks appeared on days 12–19. One of the two peaks had a period length of 14 μm, and the other showed 34 μm (Fig. 4B). The daily growth rate of dentin on days 12–19 was 26 μm; therefore, the former is considered to be ultradian (τ < 24 h), whereas the latter is infradian (τ > 24 h).

In the other 2 rats with completely lesioned SCN, circadian increments were also completely abolished after the operation. Ultradian incremental lines with the same periodicity as above or with undefined periodicity appeared immediately after the operation.

Circadian rhythm in locomotor activity disappeared after the operation in all three of these animals with complete SCN lesion. (Fig. 2C)

3) In eight rats in which SCN lesion was restricted to part of the SCN and adjacent dorsolateral (Fig. 2C) or
anterior suprachiasmatic region, circadian dentin increments were only temporarily disturbed (i.e., the circadian dentin increment disappeared or became irregular for several days). In many cases, however, circadian dentin increments reappeared after the temporary disturbance (Fig. 3C). In the periodograms, principal peaks for the increments were observed before (days 0–5) and after (days 12–19) the operation; the period lengths were 22 and 27 μm, respectively, which approximately corresponded to daily growth rates of 21 and 26 μm (Fig. 4C). However, a principal peak was not obvious for the increment just after the operation (days 5–12).

Locomotor activity exhibited free-running circadian rhythms after several days of arrhythmicity or weak circadian fluctuations.

4) In three rats, part of the SCN and adjacent caudal hypothalamic region were lesioned. In one of these animals, both the circadian dentin increment and circadian locomotor activity were abolished (Figs. 3D and 5C), although most of the SCN was still visible (Fig. 2D). Periodograms also indicated that there was no periodicity in dentin increments and no peaks after the operation (Fig. 4D). In the other two rats, the circadian dentin increment was temporarily disturbed and reappeared ~3–7 days after the operation. Circadian locomotor activity reappeared after a few days of arrhythmicity.

5) In 10 rats with an intact SCN (in these cases, outside of the SCN or the unilateral SCN was lesioned), circadian incremental lines were essentially the same as before the operation. Locomotor activity followed the same rhythm as that in rats subjected to sham operations.

DISCUSSION

The present study revealed that the circadian dentin increment is driven by an endogenous oscillatory mechanism, because it free-ran even under constant-light conditions (Fig. 3A). Recently, many studies have shown that the SCN is responsible for maintaining circadian rhythms in various physiological processes (3, 7, 8, 14, 17). The present study demonstrated that the circadian dentin increment was completely abolished in rats in which the bilateral SCN was completely lesioned, whereas in rats with only a partially lesioned SCN or intact SCN, the circadian dentin increment persisted or was only temporarily disturbed. These results strongly suggest that the SCN also plays an important role in the generation of a circadian dentin increment in rats. Because circadian incremental lines are observed in the dentin of various animals (4, 6, 9, 12, 13, 15, 21) in addition to rats, our present findings should be important for understanding the mechanism of circadian increments that are universally found in dental hard tissues of vertebrates.

Complete lesion of the SCN abolished the circadian dentin increment. However, it is still unclear how the circadian dentin increment is associated with the SCN. In several studies, no efferent nerve fibers could be found in the odontoblastic layer or odontoblastic capillary plexus (2). Therefore, the circadian dentin increment might not be directly associated with the nerve fibers in the dental pulp, but may be associated with certain humoral factors. Because odontoblasts are known to be target cells for hormones such as corticosterone (5), growth hormone, thyroxines (1), and parathyroid hormone (22), which are themselves under tight circadian control, it is reasonable to assume that
the response of odontoblasts to such systemic humoral factors would produce the circadian rhythm of dentin increments. In fact, one of the main target hypothalamic areas of the SCN is located in the paraventricular nucleus (PVN), which is closely associated with a wide range of neuroendocrine functions including ACTH secretion (16, 19, 20). In the present study, a rat accidentally received a lesion in the hypothalamic area posterior to the SCN, which is assumed to involve the PVN, and the circadian dentin increment was completely abolished regardless of the remnant SCN (Fig. 3D). It has been reported that the circadian rhythmicity in neither drinking nor feeding could be restored in rats whose hypothalamic area posterior to the SCN was directly destroyed (18).

The rats with complete or partial lesion in the SCN often showed ultradian instead of circadian dentin increments. As was confirmed by harmonic analyses (Fig. 4), the periodicity of the ultradian increment was \(~12\) h, which clearly differs from circadian. A similar ultradian increment has been observed in the dentin of rabbits (13) and humans (4).

Temporary or permanent eradication of the circadian rhythm may lead to disclosure of ultradian rhythm in dentin increments that might usually be masked by circadian increments. According to our previous study on the ontogeny of the circadian dentin increment, ultradian increments emerge before the appearance of circadian increments, and these two rhythms coexist afterward, usually beyond 2–3 wk after birth (10). At this moment, we do not know the physiological meaning of the ultradian increment or how this ultradian increment is related to the circadian increment. Further studies are required on this point.

The circadian dentin increment free-ran even under constant light in rats with an intact SCN and was abolished when the SCN was completely lesioned. In addition, in rats with incomplete SCN lesion, the circadian dentin increment was temporarily disturbed and reappeared under constant-light conditions. These characteristics were essentially the same as those in the circadian rhythm of locomotor activity. Furthermore, the circadian dentin increment persisted even in rats in which most of the SCN was lesioned and locomotor activity became arrhythmic. These results indicate that the circadian dentin increment is a robust and autonomous rhythm that reflects the time course of an endogenous oscillatory mechanism.

**Perspectives**

Lesion of the SCN can lead to obliteration of the circadian dentin increment. Our previous study demonstrated that the formation of circadian incremental lines in dentin reflects circadian rhythm in the collagen-synthetic and secretory activities of odontoblasts and that collagen-rich incremental lines were consistent with incremental layers darkly stained with hematoxylin (11). Therefore, the present observation that lesion of the SCN induced a loss of the circadian dentin increment consequently suggests a loss of circadian rhythm in the collagen-synthetic activity of odontoblasts. On the other hand, lesion of the SCN did not affect the amount of dentin matrix, because after lesion of the SCN, odontoblasts apparently kept secreting nearly the same amount of matrix as those after the sham operation, regardless of the loss of circadian increments (Fig. 3, A vs. B). Therefore, it is possible that the SCN may not directly control the mechanism of dentin formation itself but rather may modulate the function of odontoblasts to generate circadian rhythm. Although the precise mode of the generation of circadian rhythm is not yet known, certain humoral factors might produce pulsing of odontoblast function, because no afferent fibers directly innervate odontoblasts in the rat incisor (2).

Because the rhythm expressed in dental hard tissue is a structural rhythm that leaves a physical record, dentin might be considered a kind of kymograph that faithfully records the individual history of circadian rhythm in the body, and observation of this dentin increment could be a reliable and useful measure in future biorhythmic research.

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**REFERENCES**