Morphological Changes in the Hypothalamic Suprachiasmatic Nucleus and Circadian Rhythm of Locomotor Activity in Hereditary Microphthalmic Rats

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Abstract: Analysis of circadian locomotor activity, Golgi-Cox impregnation, and immunohistochemistry were studied on the hereditary microphthalmic rat which congenitally lacked the optic nerve. These blind rats showed free-running circadian rhythms in their locomotor activities. Both the normal and microphthalmic rats had similar ultradian rhythms in addition to circadian rhythms. The neuronal cell population and volume of the hypothalamic suprachiasmatic nucleus (SCN) of the microphthalmic rats were 66% and 71% of those in normal rats, respectively. The number of SCN neurons containing vasoactive intestinal peptide-like immunoreactive substance was dramatically decreased to 35% of that in normal rats. Golgi-Cox impregnation revealed that three types of neurons in the SCN of the microphthalmic rats were consistently distinguished as observed in normal rats. Although there were no changes in the numbers of primary dendrites of the SCN neurons in the microphthalmic and normal rats, the number of secondary and tertiary dendrites in the SCN of the microphthalmic rats was smaller than that of normal rats. These observations suggest that the retinal input may be important for normal morphological formation of the SCN during development, but not for the generation of circadian rhythms and ultradian rhythms.

Key words: blind, circadian rhythm, dendrite, Golgi-Cox, SCN, VIP-neuron

Introduction

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus of mammals is considered to play an important role in the generation of circadian rhythms [13, 26]. It is well known that the SCN receives a direct input from the retina, through the retinohypothalamic tract (RHT) [8, 17]. It is clear that the SCN is a principal component of the mammalian circadian oscillator, and that the oscillator is synchronized with cyclic environmental time cues, such as a light-dark cycle with a period of about 24 hr [13, 26]. The information on the...
light-dark cycle is transmitted to the SCN directly through the RHT and indirectly through the geniculo-hypothalamic tract (GHT) and the environmental day-night cycle drives the rhythm of paro-vasoactive intestinal peptide (VIP) mRNA through the activity of photic SCN afferents [13, 16]. Therefore, the congenital absence of visual input to the SCN might affect the development of the SCN and circadian rhythms. The SCN of rodents is shown to contain neurons immunoactive with antisera to somatostatin [6, 33, 34], vasopressin [3, 16, 33] and VIP [5, 16, 33], and VIP-like immunoreactive (VIP-ir) neurons are suggested to be a recipient of RHT and to be closely related to expressing synchronized behavior with light-dark conditioning, or the circadian rhythm [1, 9].

It has been reported that retino-recipient nuclei such as the lateral geniculate nucleus and superior colliculus of the hereditary microphthalmic rats, the retina of which is seen as a cyst and lacks the optic nerve due to the extreme growth inhibition of the inner layer at the marginal part during the gestation period [10], are 60–70% in volume compared with normal rats [30, 31]. Since the SCN is retino-recipient, the SCN of these blind rats may display abnormalities which might affect the circadian rhythms. We therefore examined the SCN morphologically and the circadian rhythm of locomotor activity in the congenitally blind rats.

**Materials and Methods**

1) Behavioral analysis: Nine hereditary microphthalmic rats (derived from Donryu strain) and the same number of normal Donryu strain rats, weighing 250–310 g (3 month-old), were individually housed in a room maintained at 23 ± 1°C and 55 ± 10% relative humidity, under a 12 hr-12 hr light/dark cycle (lights on 06:00–18:00 hr). The intensity of illumination within the rat cages was approximately 150 lux. Food (MF, Oriental Yeast, Tokyo) and water were freely available. The body weight, consumption of food and water and spontaneous motor activity of the rats were recorded by a monitoring system (W-30-III, Physion-Tech, U.S.A.) [15] after four-days acclimation. The monitoring system was set up in an isolator (KS-06-S, Ishihara, Tokyo) to avoid noise and artifacts. Data of the locomotor activities were collected every 30 min. For graphic illustration, data obtained during 14 days were summed up for every sampling time to visualize the regularity/irregularity of the intrinsic rhythms. Spectral analysis was performed by the maximum entropy method (MEM) [24]. Though the time lag in MEM could not be determined definitely, the stable spectrum would be taken when the time lag was chosen to be 1/3 to 1/2 of the sampling points [14]. Therefore, in this study the time lag was set at 240, half of the sampling points.

2) Cytoarchitectural and Quantitative analyses of the SCN: Nine and eight brains from the mutant and the normal Donryu rats, respectively, weighing 270–300 g (3 month-old), were embedded in celloidin. Serial transverse sections were cut at 25 μm thickness and stained by the Klüver-Barrera method to quantitatively evaluate the volume, cytoarchitecture and the neuronal cell population of the SCN. The volume of the SCN was determined as follows. Contours of the SCN of all sections in each animal were drawn at 40 times magnification and measured by a digitizer system. Then the total volume of the nucleus was estimated by summing the area of the sections. The number of SCN neurons with nucleoli was counted by means of a sampling grid (100 × 100 μm) from a photomicrograph at 400 times magnification. Then the number of cells per μm² was determined and multiplied by the estimated volume. Quantitative data were collected from both sides of the SCN. Statistical differences between the groups were examined by Student’s t-test.

3) Immunohistochemical examination: Ten microphthalmic rats and 12 normal Donryu strain rats, weighing 260–300 g (3 month-old), were used. Twenty-four hours before perfusion, 4 μl of 2% colchicine was injected into the lateral cerebral ventricle to enhance the detectability of the VIP for the cytochemical localization, under sodium pentobarbital (35 mg/kg, ip) anesthesia. Twenty-four hours later, the animals were deeply re-anesthetized with sodium pentobarbital and perfused through the heart with Ringer’s solution, followed by either 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) or Zamboni’s fixative. After the perfusion, the brain was removed from the skull, and soaked overnight in the fixative mentioned above. The brain was cut into serial transverse sections 60 μm thick on a freezing microtome. The immunohistochemical study was performed according to the following protocol: after rinse three times in phosphate-buffered
saline (PBS), the sections were preincubated for 10 min in 1% normal rabbit serum PBS and then incubated for 18 to 24 hr with anti-vasoactive intestinal peptide (VIP) antiserum (polychronal antibody, ZYMED Lab., U.S.A., diluted 1:4,000) at room temperature. After rinsing three times with PBS, the sections were incubated in avidin-biotin complex (ABC) for 1 hr. Finally, these sections were rinsed three times with Tris-HCl buffer, and reacted with H2O2 and 3,3'-diaminobenzidine. Morphometric data for the VIP-ir cells were collected from both sides of the SCN.

4) Golgi-Cox Impregnation: Ten microphthalmic rats and the same number of normal Donryu strain rats, weighing 100–150 g (25 days old), were sacrificed under deep anesthesia with ether. The brains were dissected out and impregnated with the tungstate modification of the Golgi-Cox method. The brains were placed in the fixative for 20 days and immersed twice in 0.05% acetic acid for 24 hr, and then washed with distilled water. After dehydration in ethanol, they were embedded in celloidin. Serial 150 μm-thick sections were cut in the transverse plane. Each impregnated neuron was drawn with the aid of a camera lucida.

Results

1) Behavioral analysis: Sample recordings of spontaneous motor activity are shown in Figs. 1A and 1B. Normal Donryu rats showed daily rhythms of locomotor activity which were synchronized with the environmental 12-hr light, 12-hr dark cycle. From the summation of activity during two weeks (Fig. 1B), it was seen that the normal rats acted mostly during the dark period and only a trace amount of activity could be detected during the light period. In contrast, the hereditary microphthalmic rats showed daily rhythms in the locomotor activity which were not synchronized with the light-dark cycle; these rhythms were free-running (Fig. 1A). These unsynchronized circadian rhythms were also evident from data shown in Fig. 1B. Considerable numbers of activity are seen in the light period as shown in micro-1 and -2 in Figs. 1A and 1B. Seven animals had the same activity patterns as micro-1 and -2. Other animals had the micro-3 pattern. Furthermore, considerable numbers of activities were seen, just before and after the dark period, in the mutants. They represented two large peaks in locomotor activity (Figs. 1A, 1B). A locomotor analysis revealed that the normal and hereditary microphthalmic rats had the circadian rhythms with the periods of 24 ± 0.15 hr and 23.98 ± 0.14 hr, respectively, in an average of nine animals (Table 1). Furthermore, two other ultradian rhythms with periods of about 7 and 12 hr were observed in both normal and hereditary microphthalmic rats (Table 1). Though there were no statistical differences in the frequencies of both circadian and ultradian rhythms between the normal and microphthalmic rats, there was a tendency to be short in the microphthalmia compared with the normal rat (Table 1).

2) Cytarchitectural and quantitative analyses of the SCN: Brains used for histological examinations are shown in Figs. 1C and 1D. The optic nerve and chiasm in the normal rat brain were observed, but none of them in the microphthalmia were observed, and the commissure of Gudsen was visible. The SCN of the normal rat was oval in the transverse section. The cytoarchitecture of the SCN showed that cells in the dorsolateral and ventromedial parts were loosely and tightly packed, respectively. Furthermore, cells in the dorsolateral part were very similar to cells in the adjoining anterior thalamus (Fig. 2A). On the other hand, the microphthalmic SCN was of irregular shape and was unclear in the dorsolateral part. Neurons of the microphthalmic SCN were tightly packed and slightly smaller than those of the normal one (Fig. 2B). As shown in Table 2, the length of the SCN in the rostro-

| Table 1. Cyclic phase of circadian and ultradian rhythmicities of microphthalmic rats |
|---------------------------------|---------------------------------|---------------------------------|
| Animal | Ultradian (shorter phase) | Ultradian (longer phase) | Circadian |
| Normal | 8.21 ± 0.58 hr (n=9) | 12.41 ± 0.65 hr (n=9) | 24.14 ± 0.15 hr (n=9) |
| Mutsnts | 7.29 ± 1.09 hr (n=9) | 11.95 ± 1.61 hr (n=9) | 23.98 ± 0.14 hr (n=9) |

Data are shown as means ± S.D. (standard deviations). No significant difference (P>0.01) in each rhythm between normal and microphthalmia.
caudal direction was estimated as approximately 630 μm and 440 μm in normal and microphthalmic rats, respectively. Thus, the length of the SCN was shorter in the microphthalmic rats than in the normal rats. The middle part of the SCN in the microphthalmic rats was larger in size than that of normal rats (Figs. 2A, 2B),

![Graphs showing spontaneous locomotor activity](image)

Fig. 1. Examples of simultaneous recordings of spontaneous locomotor activity during 5.5 days (A) and averages of these activities for 2 weeks (B), and ventral views of brains of a normal (C) and a hereditary microphthalmic (D) rat. In A and B, L and D show light and dark periods. Micro, microphthalmic rat. Vertical bars in A and B represent the activities per 30 min. Note that circadian activities of these microphthalmic rats were desynchronized. In C and D, the optic chiasma (OC) and commissure of Gudden (GC) are seen. Calibration bars in C and D are 3 mm.
but the volume of the SCN of the microphthalmic rats was smaller (about 71% of the normal). The total number of the SCN neurons was smaller (66%) in the microphthalmic rats than in the normal ones.

3) Immunohistochemical analysis: Immunohistochemical examinations showed that VIP-ir neurons were located in the ventral part of the SCN in normal rats (Figs. 3A, 3B). VIP-ir neurons were also present in the microphthalmic rats (Figs. 3C, 3D), but in the microphthalmic rats the VIP-ir neurons were located not only in the ventral part of the SCN, but they were also scattered throughout the nucleus (Figs. 3C, 3D). Furthermore, many VIP-positive fibers from the SCN were ascending along the wall of the third ventricle (Fig. 3A). The ascending VIP-positive fibers from the SCN were also clearly observed in the microphthalmic rats (Fig. 3C), but this number was smaller than that in normal rats (Figs. 3A, 3C). A quantitative study revealed that the number of VIP-ir neurons in the SCN of microphthalmic rats was reduced to 35% of that of nor-

Fig. 2. Photomicrographs of Klüver-Barrera stained transverse sections. These sections through the middle part of the suprachiasmatic nucleus of normal (A) and hereditary microphthalmic (B) rats. Interrupted lines indicate the boundaries of the suprachiasmatic nucleus. OC, the optic chiasma; SCN, the suprachiasmatic nucleus; V3, the third ventricle. Calibration bars are 100 μm.

<table>
<thead>
<tr>
<th></th>
<th>Normal (a)</th>
<th>Hereditary microphthalmia (b)</th>
<th>(b/a) × 100</th>
<th>Significance (P&lt;0.001)</th>
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<tr>
<td>Length of SCN (μm)</td>
<td>632 ± 58</td>
<td>442 ± 62</td>
<td>70%</td>
<td>a&gt;b</td>
</tr>
<tr>
<td>(n=18)</td>
<td>(n=16)</td>
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<tr>
<td>Total volume (×10⁻³ mm³)</td>
<td>6.9 ± 0.5</td>
<td>4.9 ± 0.5</td>
<td>71%</td>
<td>a&gt;b</td>
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<tr>
<td>(n=18)</td>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>10,981 ± 1,112</td>
<td>7,240 ± 743</td>
<td>66%</td>
<td>a&gt;b</td>
</tr>
<tr>
<td>(n=14)</td>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of VIP-ir neurons</td>
<td>424 ± 232</td>
<td>150 ± 67</td>
<td>35%</td>
<td>a&gt;b</td>
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<tr>
<td>(n=20)</td>
<td>(n=24)</td>
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Data are shown as the means ± S.D. (standard deviations).
4) **Golgi-Cox impregnation**: Successfully impregnated SCN neurons were divided into 3 types according to their pattern of dendritic arborization (Figs. 4, 5). The first type of neurons of the SCN had 5–6 primary dendrites with few secondary and tertiary dendrites, which radiated in all directions away from the cell body (multipolar type). The second type of neurons had 5–6 primary dendrites which extended to the surface of the brain (Figs. 4B, 5B). The third type of neurons had irregularly shaped somata and the directions and arborizing pattern of the dendrites were not constant (Fig. 5C). These dendrites were shorter than the other two types of neurons (Fig. 5C). Moreover, at the ventral border of the SCN we found scarce neurons extending into the superficial portion of the optic chiasm (Fig. 5D).

**Fig. 3.** Photomicrographs of immunostained transverse sections, with the anti-VIP antiserum. These sections through the middle part of the suprachiasmatic nucleus of normal (A and B) and hereditary microphthalmic (C and D) rats. Higher magnifications of A and C are shown in B and D, respectively. Arrowheads in B and D show VIP-ir neurons in the SCN of the normal and hereditary microphthalmic rat. OC, the optic chiasm; SCN, the suprachiasmatic nucleus; V3, the third ventricle. Calibration bars are 200 μm in A and C, and 100 μm in B and D.
4A), but we could not determine their shape because of the irregularity of the staining. Three major cell types identified in the normal animals were also found in the mutant animals (Figs. 4C, 4D, 5A'–C'), but the dendrites of SCN neurons in the microphthalmic rats failed to be fully developed. In the SCN neurons of the microphthalmic rats, 1) dendrites of multipolar neurons were much shorter than those of normal ones, 2) the other 2 types of SCN neurons displayed some dendritic arborization, 3) many lightly impregnated neurons were also found in the ventral part of the SCN of microphthalmic rats, and their dendrites were shorter than the normal ones (Figs. 4C, 4D). In regard to the cell bodies of neurons in the SCN, no apparent differences were observed in the size and shape of the distinguished cell bodies in normal and microphthalmic rats.

Fig. 4. Photomicrographs of Golgi-Cox impregnated transverse brain sections (A–D). A and B show those of a normal rat, and C and D show those of a hereditary microphthalmic rat. Higher magnification of A and C are shown in B and D, respectively. Interrupted lines indicate the boundaries of the suprachiasmatic nucleus. OC, the optic chiasm; SCN, the suprachiasmatic nucleus; V3, the third ventricle. Calibration bars are 200 μm in A and C, and 120 μm in B and D.
Discussion

The present study demonstrated that hereditary microphthalmic rats had a free-running circadian rhythm in locomotor activity and their SCN developed even in the total absence of retinal neural inputs (Fig. 1A and Table 1). Previous reports showed that many animals without optic nerves or visual input have free-running circadian rhythms [13, 26]. Furthermore, ultradian rhythms were also found in the hereditary microphthalmic rats in the present study (Table 1). These results indicated that these microphthalmic rats had a functional circadian oscillator. Van den Pol and Poel [32] showed that circadian rhythms were maintained in rats which more than 50% of SCN neurons were lost. On the other hand, some anophthalmic mice whose SCN contained less than 3,000 neurons lacked the circadian rhythms of wheel-running activity [27]. Interestingly, the rhythmic pattern of the microphthalmic rat showed two peaks just before and after dark period, but the present observations failed to tell us the reason for this phenomenon. Our quantitative data showed that 34% of the number of cells and 29% of the volume of the microphthalmic SCN decreased in comparison with the normal animals; approximately 66% of the SCN remained in these microphthalmic rats. It could, therefore, be considered that microphthalmic rats main-

tained the circadian rhythms and showed spontaneous circadian rhythmicity under the condition in which about 70% of the SCN neurons exist.

It is known that SCN neurons are produced from the 14th to 17th embryonic days in the rat, and that the earlier- and the later-generated cells are concentrated in the ventrolateral and the dorsomedial regions of the SCN, respectively [2]. The retinohypothalamic fibers in albino rats were observed on the 19th embryonic day, or on the first postnatal day [4, 12]. The major increase in synaptic diversity occurred between 3 and 5 weeks of age [12]. Therefore, primary neurogenesis in the SCN seems free from the influence of visual input. These embryological and early postnatal observations suggest that the SCN of hereditary microphthalmic rats develops before completion of the retinohypothalamic projection system. This fact fits the presence of the circadian rhythm in the locomotor activity in this experiment (Fig. 1) and a report that a circadian rhythm in the uptake of 2-deoxy-D-glucose (2DG) into the SCN was detected at the 19th fetal day in rats [25].

In the present experiment, the organization of VIP-ir neurons in the SCN of microphthalmic rats was abnormal; VIP-ir neurons were located in the ventral portion of the SCN and were also confined to the conventional boundaries of the nucleus (Fig. 2). The VIP-ir neurons in the SCN of mutant anophthalmic mice were reported to be sparse [11], though the number of VIP-ir neurons was not calculated. In the present study, the number of VIP-ir neurons in the SCN of the hereditary microphthalmic rats was remarkably smaller (Table 2). On the other hand, VIP-immunoreactivity in the SCN increased 80 days after orbital enucleation [23]. In this respect, it is known that axons of the retinal ganglion cells establish axo-somatic and axo-dendritic synaptic connections with VIP-neurons in the rat SCN [9]. Together with the present findings, these data suggest that the VIP-immunoreactivity and probably the function of VIP-ir neurons in the SCN are influenced by the retinal innervation. Although the number of the VIP-ir neurons was decreased in the SCN, circadian rhythmicity was maintained in hereditary microphthalmic rats. This finding suggests that these VIP-ir neurons were not an essential factor in making the circadian oscillation in rats.

In the rat SCN, several types of neurons have been distinguished; Gündner and Wolff [7] recognized 2 types
of SCN neurons and Van den Pol [33] 5 types. The present Golgi-Cox study indicated that SCN neurons in normal rats are divided into 3 types according to their dendritic patterns as in the mouse SCN [29]. Three types of SCN neurons were also recognized in hereditary microphthalmic rats, but these SCN neurons were morphologically different from those observed in the normal rat; the number of distal dendrites was remarkably smaller and the range of lengths of the primary dendrites was shorter than those of normal ones. Similar phenomena were reported in anophthalmic mice [29]. The present results and previous data suggest that regular formation of the SCN, especially the development of VIP-neurons, may depend upon the presence of retinal input.

Recent evidence has suggested that SCN neurons, probably VIP-neurons, are involved in the regulation of glucose metabolism [19]. Electrical stimulation of the SCN induced hyperglycemia and hyperglucagonemia [18]. Bilateral lesions of the SCN eliminated the hyperglycemia and hyperglucagonemia induced by intracranial injection of 2DG [35, 36]. In surgically blinded rats, hyperglycemia and hyperglucagonemia due to intracranial injection of 2DG were temporarily suppressed [21, 37]. The density of VIP-neurons in the SCN was temporally decreased in the period when the 2DG-induced hyperglycemia was suppressed [22]. The central VIP had a permissive action on the hyperglycemia and hyperglucagonemia due to 2DG [28]. In this connection, it was observed that the hyperglycemia and hyperglucagonemia due to intracranial injection of 2DG were suppressed in hereditary microphthalmic rats [20, 21]. Thus, the reduction in the number of cells and changes in the dendrites of neurons in the SCN, especially the reduction in the number of VIP-neurons, may be related to the suppression of the hyperglycemic and hyperglucagonemic responses to 2DG. Whether these are the case must be investigated in future.

Acknowledgments

The authors wish to thank Miss Ayako Iwaya and Miss Yasuko Kamimura for their excellent technical assistance. This work was supported by Grant-in-Aid for Scientific Research (07306025) from the Ministry of Education, Science and Culture, Japan.

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