

Lesions in the Suprachiasmatic Nuclei Suppress Inflammatory Mediators in Sensitized Rats

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Key Words

Bronchial asthma · Circadian rhythm · Corticosterone · Eosinophil · Histamine

Abstract

Background: Although bronchial asthma is well known as an inflammatory disease that shows obvious circadian rhythms of airway narrowing, it remains to be elucidated whether the suprachiasmatic nucleus (SCN), a major circadian pacemaker, regulates inflammatory mediators relevant to asthma. Thus, we investigated the effects of electrolytic lesioning of the SCN on the antigen-induced immediate allergic response and the late allergic response (LAR) in male Brown Norway rats, i.e. in a model of allergic inflammation. **Methods:** The immediate allergic response, assessed by the histamine levels in plasma and bronchoalveolar lavage fluid (BALF), and the LAR, assessed by the eosinophilic infiltration into BALF and the lamina propria mucosae of the left main bronchus, were examined in three groups of 18 rats each, including (1) an unoperated control group, (2) a sham SCN-lesioned group, and (3) an SCN-lesioned group. **Results:** Both the plasma histamine levels and the number of eosinophils in bronchial tissues in the SCN-lesioned group were significantly lower than those in the other groups. The concentration of histamine and the number of eosinophils in the right BALF showed a similar pattern; however, no significant differences were found. Both

plasma adrenaline and noradrenaline levels were highest during the LAR, whereas the corticosterone level was lowest in the SCN-lesioned group; again, no significant differences were obtained. **Conclusions:** These findings indicate that the SCN has a significant effect on the inflammatory mediators relevant to bronchial asthma.

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Introduction

Bronchial asthma is an inflammatory disease that shows obvious circadian rhythms of airway narrowing [1]. Increasing evidence has demonstrated that asthmatic patients are in a state of bronchoconstrictive circadian periodicity which is more enhanced than in normal subjects, and changes in several kinds of mediators have been considered to be responsible for the pathogenesis of nocturnal asthma [1–3]. Indeed, the blood levels of bronchoconstrictive mediators have been found to increase while the bronchodilator levels decrease from around midnight to early morning [4]. Asthmatic attack occurs most often during sleep, and death from asthma tends to occur at the same time [5, 6].

In relation to changes in working and living situations, the effect of a disturbed sleep-wakefulness cycle, such as night work or shift work, on health has become an important research theme. This disturbance may also have an impact on the pathogenesis of asthma, since nocturnal

asthma has been considered to rely heavily on airway circadian rhythms [1–6]. In comparison with abundant studies on the expression of physiological, endocrinological and immunological circadian rhythms in asthmatics [1, 3, 4], very few studies have so far been carried out regarding the effect of disturbed circadian rhythms on either the physiologic function or biological variables relevant to airway narrowing in asthmatics [2], as well as non-asthmatics [7]. In particular, to the best of our knowledge, no experimental data with animals are available.

It is well known and has been documented that the suprachiasmatic nucleus (SCN) of the hypothalamus is the site of the master pacemaker that controls the circadian rhythms of a variety of behavioral, physiological and endocrinological functions, which are synchronized by the light-dark cycle [8, 9]. Although it is hypothesized that the SCN takes part in the exaggerated circadian rhythms of pulmonary function and several kinds of mediators that affect the functioning of airway caliber, the direct associations are presently unknown. One of the useful methods to assess the role of the SCN in the biological circadian rhythms is to study the electrolytic lesioning of the SCN. A number of studies have shown that the total destruction of the SCN abolishes various behavioral, physiological and endocrinological circadian rhythms [8–11]. However, the effect of the SCN lesions on inflammatory mediators relevant to asthmatic responses has not yet been investigated.

The present study was carried out to examine whether the electrolytic lesioning of the SCN would modify the antigen-induced immediate allergic response (IAR) and late allergic response (LAR) in rats, i.e. in a model of allergic inflammation. To evaluate such responses, histamine levels in plasma and bronchoalveolar lavage fluid (BALF) during the IAR and eosinophilic counts in BALF and bronchial tissues during the LAR were measured. These variables were reported to correlate with the severity of asthma [12, 13], and they are considered to be useful for evaluating the IAR and the LAR, when the assessment of respiratory resistance is unavailable. We focused on eosinophilic, but not neutrophilic, inflammation, because the most frequently used animal model of asthma due to allergen challenge shows that the key pathological feature is airway eosinophilia [14]. This study aims at investigating the effect of the SCN lesioning not on the circadian rhythm of asthmatic responses, but on the antigen-induced allergic inflammations; it is valuable as an initial step of research on the disturbed circadian rhythm-related asthmatic responses.

Materials and Methods

Animals

In this study, all efforts were made to minimize animal suffering and to utilize alternatives to in vivo techniques, if available. A total of 146 male Brown Norway rats (Seiwa Experimental Animal Institute, Fukuoka, Japan), weighing approximately 180 g at the start of the experiment, were used. The rats were housed in standard wire laboratory cages (23 × 33 × 19 cm) in groups of 4 per cage. The cages were kept in a soundproof, air conditioned holding room, with an ambient temperature of $24 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity. A 12-hour light-12-hour dark schedule (lights on at 7.00 h) with ad libitum access to food and water was maintained throughout the experiments.

Sensitization

After more than a 1-week acclimatization to the housing environment, the rats were handled for 5 min on at least 5 randomly scheduled times. The rats were then immunologically sensitized with ovalbumin (OA; Sigma, St. Louis, Mo., USA), based on the method described by Eidelman et al. [15]. Briefly, the rats were actively sensitized by the subcutaneous injection of 1 ml of a sterile suspension of 1 mg OA and 200 mg of aluminium hydroxide in 0.9% (weight/vol) saline. At the same time, 1 ml of *Bordetella pertussis* vaccine containing 6×10^9 heat-killed bacilli was intraperitoneally injected.

Two weeks after the sensitization, a test trial with OA inhalation (10 mg/ml, soluble to physiological saline) was performed using a nebulizer to confirm the presence of asthma-like breathing attacks. A total of 128 rats, which showed respiratory distress such as gasping, was enrolled into the following study. Eighteen rats were removed from this study because of the possibility of unsuccessful sensitization.

Experimental Protocol

The rats were randomly divided into three groups of 18 each – (1) the unoperated control group, (2) the sham SCN-lesioned group, and (3) the SCN-lesioned group – in order to clarify the effect of the SCN lesions on allergic responses. One half of the rats in each group was used to evaluate the IAR, and the second half to examine the LAR. The animals were fixed on a stereotaxic instrument (Model SR-6, Narishige Co, Tokyo, Japan) under anesthesia with sodium pentobarbital (Wako Biochemicals, Osaka, Japan), 50 mg/kg i.p. The electrodes were placed in the mid-portion of the bilateral SCN according to the following coordinates (anteroposterior, interaural, 7.7 mm; lateral, 0.2 mm; ventral, 9.5 mm below the surface of the dura), using the atlas of the rat forebrain [16]. The bilateral electrolytic lesions in the SCN were then produced with the aid of the lesion generator by passing a direct current of 0.6 mA for 20 s through an insulated stainless steel electrode of 0.25 mm with a 1.0-mm bare tip, according to the method of Szafarczyk et al. [17]. A histological examination of the frozen sections of brain, which were stained by hematoxylin-eosin (Wako Biochemicals), was performed later in order to determine the location and the size of the lesions. Only those animals with histologically verified bilateral symmetrical lesions in the SCN were enrolled in this study as the SCN-lesioned group. The sham-operated animals served as the surgical controls which were treated in the same manner without any current applied. The unoperated animals were applied as the sensitized normal controls. Bilateral electrolytic or sham lesions of

the SCN were performed in 110 rats. Among the adequate SCN or sham lesions, we chose 36 rats as the SCN-lesioned and the sham SCN-lesioned groups according to the rank of accuracy of the histologically verified bilateral symmetrical or sham lesions in the SCN.

One week after the operation, the rats for the IAR, three groups of 9 each, inhaled OA (10 mg/ml) for 10 min from 9.00 to 10.00 h in the morning. They were anesthetized with ether (Wako Biochemicals), and venous blood samples were taken about 8–10 min after the exposure. After the animals were sacrificed by cutting the abdominal aorta, right BALF was obtained. The blood and BALF samples were then immediately centrifuged (2,500 and 1,500 rpm for 10 min each). After removing the proteins using perchloric acid (Wako Biochemicals), the supernatants were stored in a freezer at -80°C . Later, the histamine levels in plasma and BALF were measured.

The rats for the LAR, three groups of 9 each, inhaled OA in the same manner as those for the IAR. Seven hours later, venous blood samples were taken, and the levels of corticosterone and catecholamines were measured. Next, we counted the eosinophilic infiltration into the right bronchoalveolar lumen by an analysis of BALF and the number of eosinophils in the lamina propria of the left main bronchus, as previously described [18].

Bronchoalveolar Lavage and Cell Count

After intraperitoneal anesthesia with 50 mg/kg sodium pentobarbital (Wako Biochemicals), a tracheotomy was performed, and the trachea was cannulated with a polyethylene tube. The right lung was then slowly lavaged with 4 ml of sterile saline through the tracheal cannula with a syringe. After the lavage had been repeated 5 times (20 ml total), the BALF was centrifuged at 1,500 rpm for 10 min at 4°C . BALF from 17 to 19 ml was obtained. The pellet obtained was immediately suspended in 5 ml of phosphate-buffered saline, and total cell numbers in the BALF were counted using a hemocytometer. The preparations were stained with Wright-Giemsa (Wako Biochemicals) to determine the cellular composition. The concentration of eosinophils in the right BALF was expressed as the number of cells/cubic milliliter.

Morphologic and Morphometric Studies

The lungs were removed and gently infused with 10% buffered formalin at a pressure of 23 cm H_2O , fixed in the same solution for 2–4 days. The left main bronchus was then sectioned transversely and embedded in paraffin. The fixed specimens were cut into slices measuring 4 μm in thickness and stained with hematoxylin-eosin for microscopic observations. The number of eosinophils were counted in the lamina propria mucosae per 1 mm of basement membrane of the left main bronchus within 5 mm from the carina. All morphometric measurements were made by a single observer who was blind to the group status.

Determination of Histamine, Corticosterone, and Catecholamine Levels

The histamine levels in plasma and BALF during the IAR were measured by a radioimmunoassay according to the method of McBride et al. [19], using a commercial kit (Eiken, Tokyo, Japan). In brief, 100 μl of each sample and standard histamine solutions were placed into designated acylation tubes, and 50 μl of acylation buffer were added. After 1 ml of iodinated (^{125}I) acylated histamine had flown into each tube, 0.5 ml of the same solution was transferred

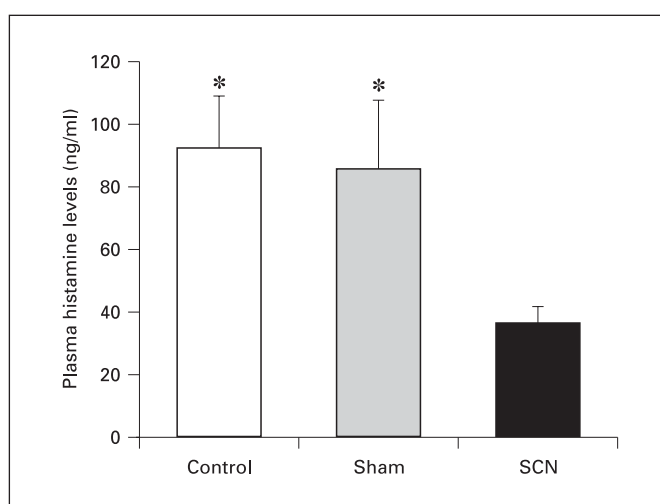


Fig. 1. Comparisons of the plasma histamine levels during the IAR among the control, sham-operated, and SCN-lesioned groups ($n = 9$ rats/group). * $p < 0.05$, significantly different from the SCN-lesioned group (Fisher's PLSD test).

to monoclonal antibody-coated tubes. The solutions were then incubated for 18 h at 4°C and counted in a gamma counter (Aloka RC 2000, Aloka, Tokyo, Japan).

The plasma corticosterone levels during the LAR were measured by the radioimmunoassay method, and the plasma catecholamine levels during the LAR were quantified by high-performance liquid chromatography. Both analyses were performed at the Otsuka Assay Laboratories (Tokushima, Japan).

Statistical Analysis

All the statistical analyses were carried out with SPSS 11.5 for Windows. An analysis of variance was used to compare the histamine, corticosterone and catecholamine levels, as well as the cell counts among the three groups. If significance was obtained, then individual comparisons were subsequently performed with Fisher's protected least squares difference (PLSD) test. Differences were considered to be statistically significant when $p < 0.05$. The data were expressed as mean $\pm$ SEM.

Results

There was a significant difference among the three groups in the plasma histamine levels during the IAR [$F(2, 24) = 3.56$; $p < 0.05$] (fig. 1). The plasma histamine level in the SCN-lesioned group was significantly lower than in the other groups ($p < 0.05$, respectively). The concentration of histamine in the right BALF showed a similar pattern; however, no significant difference was obtained (fig. 2).

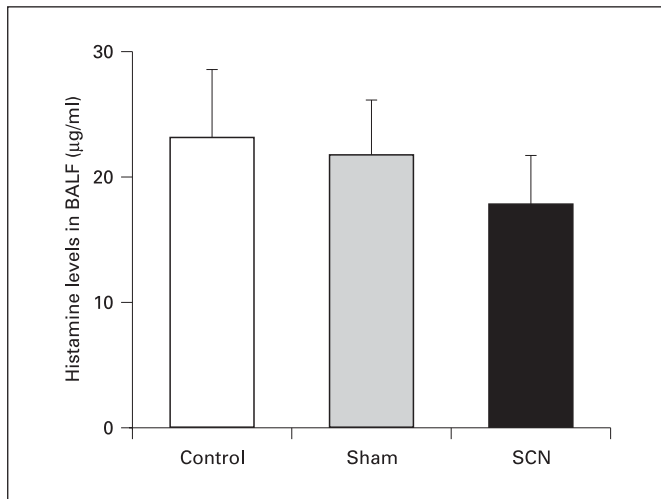


Fig. 2. Comparisons of the histamine levels in the BALF during the IAR among the control, sham-operated, and SCN-lesioned groups (n = 9 rats/group).

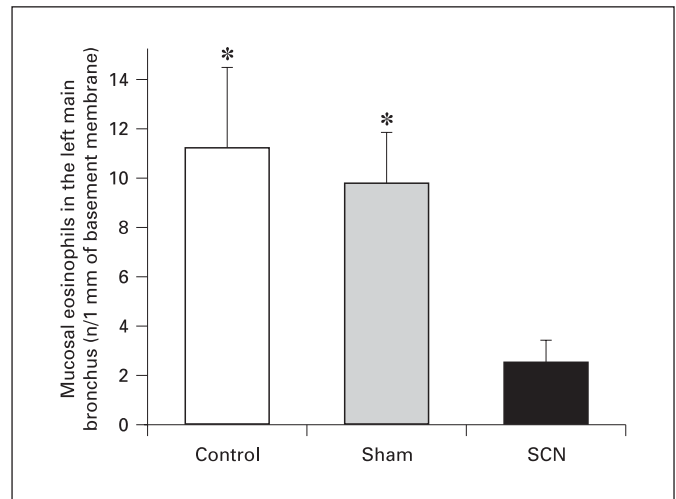


Fig. 3. Comparisons of the number of eosinophils in the lamina propria mucosae of the left main bronchus during the LAR among the control, sham-operated, and SCN-lesioned groups (n = 9 rats/group). * $p < 0.05$, significantly different from the SCN-lesioned group (Fisher's PLSD test).

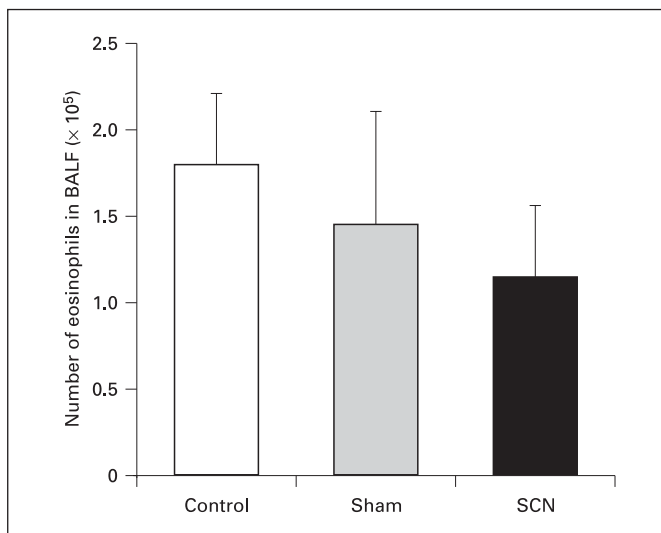


Fig. 4. Comparisons of the number of eosinophils in the BALF during the LAR among the control, sham-operated, and SCN-lesioned groups (n = 9 rats/group).

There was a significant difference among the other three groups in the number of eosinophils in the lamina propria mucosae of the left main bronchus during the LAR [$F(2, 24) = 4.06$; $p < 0.05$] (fig. 3). The number of bronchial wall eosinophils during the LAR in the SCN-

lesioned group was significantly lower than that in the other groups ($p < 0.05$, respectively). The number of eosinophils in the right BALF showed a similar pattern; however, no significant difference was obtained (fig. 4).

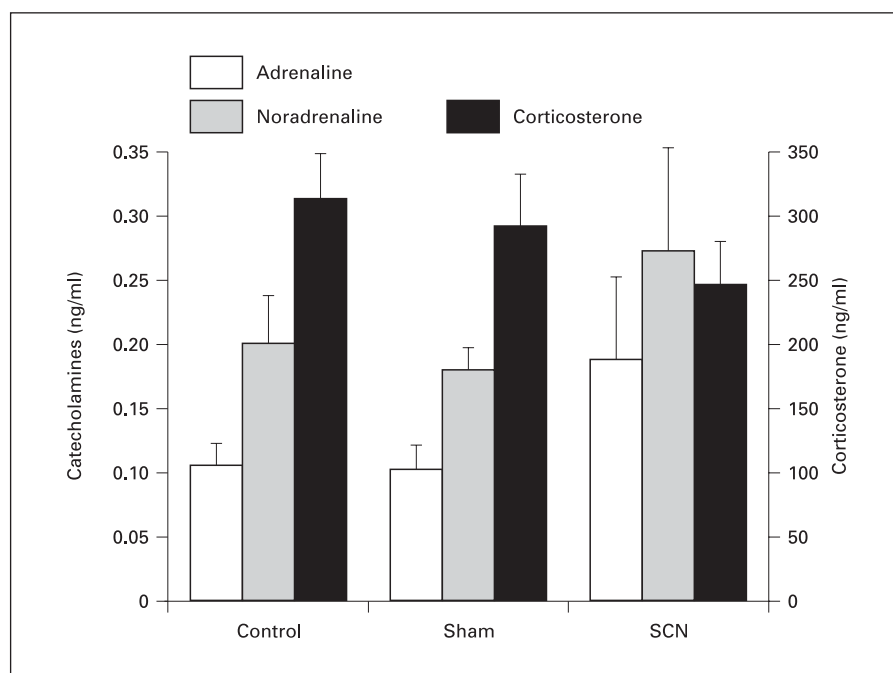
As shown in figure 5, both plasma adrenaline and nor-adrenaline levels during the LAR were highest in the SCN-lesioned group; no significant differences were obtained. In addition, no significant difference was found in the corticosterone level among the three groups, although the corticosterone level was lowest in the SCN-lesioned group.

Discussion

In the present study, we investigated the effects of the electrolytic SCN destruction on both the IAR and the LAR in a rat model of allergic inflammation to speculate the role of the SCN on asthmatic responses. As a result, both responses significantly decreased after the SCN destruction. This study shows that the SCN has a significant effect on the inflammatory mediators relevant to bronchial asthma.

There are several possible reasons why the SCN lesions suppressed the inflammatory mediators. First, the alterations in corticosterone and/or catecholamines due to the SCN lesions might have affected the results, since

Fig. 5. Comparisons of the plasma levels of catecholamines and corticosterone during the LAR among the control, sham-operated, and SCN-lesioned groups (n = 9 rats/group).



these hormones have been reported to suppress both histamine release and airway eosinophilic infiltration [20–23]. In particular, corticosterone has been reported to play a role in the late, but not early, airway response to inhaled allergen [24]. Growing evidence on the SCN seems to support this possibility. For example, a polysynaptic SCN-adrenal cortex pathway has been presented [25]. In addition, the blood levels of adrenocorticotrophic hormone and corticosterone in rats have been reported to increase during lights out and to decrease during lights on [26], and these rhythmicities were lost following the induction of SCN lesions in rats [27, 28]. Buijs et al. [25] also found that a light-induced immediate decrease in the plasma corticosterone level was observed in intact rats, but not in the SCN-lesioned rats; however, no alterations were found in the plasma catecholamine level. As a whole, it is collectively suggested that the SCN plays a strong inhibitory role in corticosterone secretion mediated by vasopressin, particularly in the morning, and a subtle stimulatory role in the evening [29]. Our findings that plasma corticosterone in the evening in the SCN-lesioned rats showed the lowest value among the three groups may correspond with this hypothesis. Nevertheless, the corticosterone level did not significantly differ among the three groups and it was considered to be unrelated to the results, because glucocorticoids have been reported to suppress

both histamine release and airway eosinophilic infiltration [20, 21].

On the other hand, the plasma catecholamines during the LAR in the SCN-lesioned rats showed the highest levels among the three groups, although no significant difference was observed. The reason for the difference between the plasma corticosterone and catecholamine levels remains unclear, but the increase in the catecholamine levels might have contributed to the inhibition of eosinophilic infiltration in the airway caliber during LAR due to the SCN lesioning, because catecholamines have been reported to suppress eosinophilic infiltration [22, 23].

Second, in addition to such hormonal influences, the neuronal influences from the SCN that play a role in the pathogenesis of airway inflammation have been considered. It was thus found that the SCN is involved in the sympathetic outflow from the brain to brown adipose tissue, thyroid gland, kidney, bladder, spleen, adrenal medulla, and perhaps the adrenal cortex, and is linked to the parasympathetic nervous system innervation of the thyroid, liver, pancreas, and submandibular gland [30]. Recently, increasing evidence implied that neural/humoral output signals from the SCN impinge upon peripheral clocks located in other areas of the brain, heart, lung, gastrointestinal tract, liver, kidney, fibroblasts, and most of the cell phenotypes, thus resulting in overt circadian

rhythms in integrated physiological functions [31]. Therefore, we could not rule out the possibility that the SCN innervates bronchial or lung tissues, which might have contributed to these results.

Third, allergic diseases have been shown to be closely related to Th2 immune responses, which are characterized by high levels of interleukin (IL)-4, IL-5 and IL-13. Accumulating evidence demonstrates that a decreased Th1 immune response is also important in the pathogenesis of allergic diseases, and that interferon- γ (IFN- γ) could act as a central regulator in this phenomenon [32]. On the other hand, a light-dependent immunopositivity for the IFN- γ receptor is detectable in the SCN [33]. Accordingly, Th1/Th2 balance-related cytokines, such as IFN- γ , might thus be associated with the outcome.

Another possible explanation for these results may be the change in physical activity. Previous studies have shown that the rats showed an increased locomotor activity during daytime after inducing the SCN lesions [17]. The relationship between asthma, except for exercise-induced asthma, and physical activity shows wide interpatient and inpatient variability [34, 35]. However, well-designed physical training has been considered to improve asthmatic symptoms [34], so that the increase in physical activity might have been associated with the suppression of allergic responses in the present study.

Finally, the potential involvement of other brain tissue lesions might have affected the outcome. The SCN is located in the anterior hypothalamus. Previous electrolytic lesioning studies have shown that the lesions of the anterior hypothalamic area (AHA) provide reliable protection against the lethal effect of anaphylaxis in non-human primates [36, 37]. We also found that the AHA lesions resulted in the suppression of the LAR as assessed by the antigen-induced airway eosinophilic infiltration in rats of allergic inflammation [38]. These findings are consistent with the results obtained in our study. We could not completely neglect the potential involvement of the AHA lesions because of the difficulty of the SCN lesioning procedure; however, the SCN group was strictly selected based on anatomy. Thus, the potential AHA lesions, except for the SCN lesions, were considered to be too small to affect the results. On the contrary, it may be possible to consider that the effects of the previous AHA lesions on either the anaphylactic or late allergic responses partially reflected the involvement of the SCN lesions.

Several potential limitations must also be considered when discussing our findings. First, our findings are limited in terms of the antigen-induced allergic responses partially relevant to asthma in rats, because we did not

measure the physiologic functions (i.e. airway resistance) and we did not study the SCN-related allergic responses or physiologic functions in humans. Whether our findings can be applied to patients with nocturnal asthma is unknown. Nevertheless, only animal experiments allow us to determine the influence of the SCN lesions on allergic inflammation, including asthmatic responses. Second, as mentioned in the introduction, we did not measure the circadian rhythms of either the inflammatory mediators or pulmonary functions, so it remains unclear whether the SCN plays a substantial part in the circadian rhythms of asthmatic responses. We could not completely rule out the possibility that the SCN lesioning might have influenced only peripheral allergic responses, but not the circadian rhythms, because some studies, including a report by us, have demonstrated changes in immunological parameters after brain damage [36–39]. Third, whether the SCN lesioning itself influences airway inflammation is unknown, because we did not investigate it using unsensitized control groups. Fourth, both levels of corticosterone and catecholamines were measured 7 h after the inhalation of OA, so that levels during the 7 h remain unclear.

In conclusion, despite these limitations, our results possibly indicate that the organic destruction of the SCN, a major circadian pacemaker, due to electrolytic lesioning, suppresses both the IAR and the LAR in a rat model of allergic inflammation. This suggests that the SCN centrally regulates the peripheral allergic inflammation which may be responsible for the pathogenesis of nocturnal asthma. We believe that this study highlights the contribution of the circadian rhythm to the allergic responses from a new aspect, such as the involvement of the SCN. Further investigations are required to confirm our suggestions and to develop this research field.

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