Astroglial Elements in the Suprachiasmatic Nucleus of the Golden Hamster and the Rat —Comparative Morphological Study Using Immunocytochemistry—

Keisuke Inoue¹, Yoshitaka Tamada², Katsuhiko Munekawa¹, Seiji Hayashi¹,
Norio Iijima¹, Akihiko Ishihara³, Teru Ikeda¹ and Masaki Tanaka¹

¹Department of Anatomy & Neurobiology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-0841, ²Department of Anatomy, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121 and ³Laboratory of Neurochemistry, Faculty of Integrated Human Studies, Kyoto University, Sakyo-ku, Kyoto 606-8501

Received March 17, 2000; accepted March 21, 2000

Astroglial elements in the suprachiasmatic nucleus (SCN) in golden hamsters and rats were investigated by GFAP immunocytochemistry and a computer-assisted analyzing system. Serial frontal sections, including the SCN region, were prepared and GFAP immunoreactivity in the following areas were measured as optical density (O. D.) per area (O. D./ μm^2): (1) the whole SCN region, (2) the ventrolateral portion (VLSCN), where vasoactive intestinal peptide (VIP) neurons were mainly distributed, (3) the dorsomedial portion, where arginine vasopressin (AVP) neurons were mainly distributed, and (4) as determined by unilateral intraocular injection of cholera toxin B subunit, the area where

retinohypothalamic tract (RHT) fibers were distributed. In the rat, GFAP immunoreactivity was observed throughout the SCN, particularly in the VLSCN, while in the golden hamster, astroglial elements enclosed the whole region of the SCN. In addition, quantitative analysis revealed that the O. D. of the GFAP immunoreactivity in the VLSCN, where VIP neurons and the RHT fibers were distributed, were significantly higher than those in other regions of the SCN.

These results strongly suggested that the astroglial elements, VIP neurons and RHT fibers have close morphological interactions, although delicate differences were demonstrated in both species.

Key words: GFAP, Suprachiasmatic nucleus, Retinohypothalamic tract, Rat, Golden hamster

I. Introduction

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus contains a circadian oscillator, which affects behavior, sleep-wakefulness and hormonal secretion in mammals [9, 17]. Astrocytes have been identified in the rodent SCN by immunocytochemistry for glial fibrillary acidic protein (GFAP) [4, 14, 29], an astrocytes specific intermediate filament protein, and the SCN shows intense GFAP immunoreactivity compared with other regions of the brain [18]. Although the ratio number of the astrocytic cells to the number of neurons in the SCN is small, compared to that in surrounding areas of the anterior hypothalamus [30], there are a number of extensive astrocytic processes in the SCN [3].

Recently, it has been suggested that the astrocytes in the SCN are involved in the function of the circadian oscillating system. Prosser and his coworkers demonstrated that fluorocitrate, an inhibitor of glial metabolism, disrupted the circadian pattern of neuronal activity in the SCN [21]. In an *in vitro* study, the rhythms of vasoactive intestinal peptide (VIP)- and arginine vasopressin (AVP)-released from cultured SCN slices were not synchronized by the administration of an antimitotic drug, which was used to reduce the number of glial cells [23].

The ventrolateral portion of the SCN (VLSCN) of the rat receives innumerable photic signals from the retinal ganglion cells directly through the retinohypothalamic tract (RHT), or indirectly through the geniculohypothalamic tract (GHT). VIP neurons in the VLSCN receive direct synaptic input from the RHT terminals [8, 27] and VIP immunoreactivity and mRNA expression show diurnal variations according to the presence or absence of light [20, 25]. These findings indicate that photic

Correspondence to: Yoshitaka Tamada, D. D. S., Ph.D., Department of Anatomy, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan.

information entrains the circadian clock through the VLSCN and suggest that GFAP-IR astrocytes in the VLSCN may participate in the circadian entrainment by light.

In our previous study on rat SCN, we have previously demonstrated that astrocytes with GFAP immunoreactive (GFAP-IR) elements were mainly distributed in the VLSCN and that they had a close morphological relationship with VIP neurons, which are the major neuronal components in the VLSCN [26]. In addition, we have also demonstrated that the postnatal development of these astroglial elements, particularly the expansion of GFAP-IR processes in the rat SCN, is regulated by retino-hypothalamic projection (paper submitted for publication). Results obtained in the previous study suggested that retinal input to the SCN may be necessary for the formation and the maintenance of the astroglial network in the SCN. Therefore, astroglial elements have a close relationship with VIP neurons and RHT terminals in the VLSCN. In addition, golden hamsters have also used in the several types of studies on the SCN [18].

The results of this study verified our hypothesis that the distribution of GFAP-IR elements mainly overlapped those of VIP neurons and RHT terminals and we investigated these correlations to determine if they are a common feature in rats and golden hamsters. We employed a GFAP immunocytochemistry and computer-assisted analyzing system in order to investigate the astroglial elements in the SCN of rats and golden hamsters.

GFAP immunoreactivity was calculated as the optical density (O.D.) per area ($\text{O.D.}/\mu\text{m}^2$), as described in several areas of the Materials and Methods section and we also examined the morphological interaction of these elements in the SCN from the view of comparative anatomy.

II. Materials and Methods

Animals

Thirty adult male Sprague-Dawley (SD) rats (body weight: about 250 g) and the same number of adult male golden hamsters (body weight: about 100 g) were housed under a LD cycle (rats: 12 hr light–12 hr dark cycle, golden hamsters: 14 hr light–10 hr dark cycle) in a temperature controlled (22–24°C) environment. All animals were provided with food and water *ad libitum*.

Twenty-four animals of both species were used for immunocytochemistry using antibodies against GFAP, and the other six animals were used for double labeling fluorescence immunocytochemistry. In order to investigate the distribution of RHT terminals in the SCN, twelve animals of both species were injected with cholera toxin B subunit (CTb) (50 $\mu\text{g}/5 \mu\text{l}$ dissolved in water, Sigma), as an anterograde tracer, into the right eyeball under pentobarbital anesthesia (25 mg/kg). After 48 hr, all of the animals were sacrificed at 1400 (Zeitgeber time, ZT7).

The present study was conducted according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The Committee of

Animal Research, Kyoto Prefectural University of Medicine, approved the experiments conducted in the present study.

Immunocytochemistry

All animals were transcardially perfused under deep anesthesia (50 mg/kg, i.p.) with 0.1 M phosphate buffered saline (PBS) and then a fixative solution containing 4% paraformaldehyde, 0.2% picric acid adjusted to pH 7.4 with 0.1 M phosphate buffer. Brains were postfixed in the same fixative solution for 24 hr at 4°C and then soaked in 20% sucrose in 0.1 M PBS for 24 hr at 4°C. Thereafter, serial frontal sections (30 μm in thickness) from the rostral end of the SCN to the retrochiasmatic area were cut with a cryostat. To determine the area of the SCN region, some sections were counterstained with cresyl violet. For the immunocytochemical procedure, free-floating sections of each animal were processed for immunocytochemistry using antibodies against GFAP, AVP, VIP or CTb. The sections were incubated in anti-GFAP, anti-AVP, anti-VIP and anti-CTb sera (GFAP: dilution 1 : 1000, DAKO, AVP: dilution 1 : 5000, Peninsula, VIP: dilution 1 : 3300, code no. 505; courtesy of Dr. N. Yanaihara, Yanaihara Institute, Japan, CTb: 1 : 5000, List Biological Lab.) in PBS for 36 hr at 4°C. The sections were incubated in the secondary antibodies (GFAP, VIP: biotinylated anti-rabbit IgG, AVP: biotinylated anti-guinea pig IgG: dilution 1 : 1000, CTb: biotinylated anti-goat IgG, Vector Lab. Inc.) overnight at 4°C and in avidin-biotin peroxidase complex (ABC) solution (dilution 1 : 1000, Vector Lab. Inc.) overnight at 4°C. The sections were exposed for 10 min at room temperature to 50 mM Tris-HCl buffer containing 0.01% 3,3'-diaminobenzidine 4HCl (DAB) and 0.005% H_2O_2 . All sections were mounted on glass slides and examined by light microscopy.

The specificity of the anti-GFAP, VIP, AVP and CTb sera was confirmed by preabsorption with the respective antigens (1–100 mg), which completely abolished immunostaining.

Quantification of GFAP immunoreactivity in the SCN

In order to quantify the GFAP immunoreactivity in both species, we employed a computer-assisted analyzing system and the GFAP immunoreactivity was calculated as optical density per area ($\text{O.D.}/\mu\text{m}^2$) in the following four areas: (1) the whole SCN region counterstained with cresyl violet (SCN area), (2) the area where AVP neurons were distributed (AVP area), (3) the area where VIP neurons were distributed (VIP area) and (4) the area where RHT terminals were distributed (contralateral side: CT (contra) area, ipsilateral side: CT (ipsi) area).

Serial frontal sections from the rostral pole to the caudal end of the SCN were used for analysis. Every second section was collected and were processed for GFAP immunocytochemistry, the others were counterstained with cresyl violet or immunocytochemistry (VIP, AVP or CTb) simultaneously. In two adjacent sections, the SCN,

VIP, AVP or CT areas were determined on the former section and the O.D. of the GFAP immunoreactivity was measured in the same area of the latter section using NIH image. The background O.D. of each section was also measured in the anterior hypothalamic area (Fig. 1) and this was subtracted from the O.D. of the GFAP immunoreactivity. Values were represented as mean $\pm$ S.D.

One-way analysis of variance (ANOVA) was used to test for statistical significance. Fisher's post hoc tests were used to determine significant group differences ($n=6$, $p<0.001$).

Double labeling fluorescence immunocytochemistry

Two days after the injection of the CTb, six animals of both species were sacrificed and prepared for the light microscopic immunocytochemistry procedures. Coronal sections of the anterior hypothalamus (14 μ m in thickness) including the SCN, were cut with a cryostat and incubated with a mixture of goat anti-CTb (dilution; 1 : 2500, List Biological Lab.) and rabbit anti-GFAP (1 : 3000, DAKO) at 4°C for 3 days. After washing in 0.1 M PBS, the sections were incubated in a solution containing both fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (1 : 500, Bethyl Lab. Inc.) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit

IgG (1 : 2000, Chem. Int. Inc.) at room temperature for 4 hr. After rinsing in 0.1 M PBS, the sections were mounted on glass slides and coverslipped with Vectashield (Vector). They were examined under a fluorescence microscope system (Nikon Y2F-FFD2) equipped with a double filter for both FITC and TRITC fluorescence.

III. Results

Immunocytochemistry

In the sections counterstained with cresyl violet, the SCN of rats and golden hamsters were demonstrated as tiny nuclei located close to the third ventricle above the optic chiasm (Fig. 2a, b). Neurons in the golden hamster SCN appeared to be more tightly packed than those in the rat SCN. The length of the rat SCN along the rostrocaudal plane was greater than that of the golden hamster (rat: approximately 700 μ m, golden hamsters: approximately 500 μ m), as shown by an inspection of the serial frontal sections.

In the rat SCN, GFAP-IR elements were found throughout the region, particularly in the VLSCN (Fig. 2c), however they were less dense than those in the golden hamster SCN. These astroglial elements were localized especially in the VLSCN, and the dorsomedial region of the SCN was less densely labeled than the VLSCN. The distributional pattern of GFAP immunoreactivity in the rat SCN was different from that in the golden hamster SCN. In the golden hamster, GFAP-IR elements covered the whole SCN region and moreover extended a little in the dorsal direction outside the SCN (Fig. 2d). In both species, the distribution of VIP and AVP immunoreactive neurons seemed to show the same pattern: the former were located in the VLSCN and the latter in the dorsomedial portion of the SCN (Fig. 3a-d). However, in the golden hamster, AVP immunoreactive terminals were less dense than those in the rat (Fig. 3a, b). In the golden hamster, VIP immunoreactive terminals formed an extremely dense plexus throughout the SCN, compared to those in the rat (Fig. 3c, d). In the rat SCN, RHT fibers labeled with CTb were distributed mainly in the VLSCN, however in the golden hamster SCN, they were observed throughout the SCN region (Fig. 3e, f). Moreover, there was laterality in respect to the distributional pattern of the RHT between the golden hamster and the rat. Retinohypothalamic projection was dominant on the contralateral side in the rat SCN (Fig. 3e), whereas it was approximately equal on both sides in the golden hamsters (Fig. 3f).

Quantification of GFAP immunoreactivity in the SCN (Fig. 4)

In the SCN area, the GFAP immunoreactivity of the golden hamster was much higher than that of the rat (rat: 4.86 ± 0.22 , golden hamster: 26.16 ± 1.81 , $n=6$). In the rat SCN, the O.D. of the AVP area (2.31 ± 0.69) was very low. The O.D. values shown for the VIP (14.45 ± 1.23) and the CT area (ipsi: 13.61 ± 0.90 , contra: 8.86 ± 0.72)

Fig. 1. Quantification analysis methods in the serial sections. In the former section, the SCN, VIP, AVP and CT areas (1) were determined. The O. D. of GFAP immunoreactivity was measured in the corresponding area (2) of the latter section. (3): background. The O. D. per measured area was calculated using NIH image. OC, optic chiasma; V, third ventricle.

Fig. 2. Sections counterstained with cresyl violet (a, b) and GFAP immunoreactivity in the SCN of the rat (c) and the golden hamster (d). In the sections counterstained with cresyl violet, the size of the SCN was slightly larger in the rat SCN (a) than that in the golden hamsters (b). However, in the golden hamster SCN (b), neurons were appeared to be more tightly packed than those in the rat SCN (a). In the rat SCN, GFAP immunoreactivity was observed throughout the SCN, particularly in the VLSCN (c). In the golden hamster, astroglial elements with GFAP immunoreactivity enclosed the whole SCN region (d). OC, optic chiasma; V, third ventricle. Bar = 100 μ m.

were very high compared to the SCN area. On the other hand, in the golden hamster SCN, the AVP area showed a very low value of GFAP immunoreactivity (13.30 ± 1.22) and the CT area demonstrated a very high value (ipsi: 40.31 ± 1.30 , contra: 41.33 ± 1.60), compared to the SCN area. However, no other remarkable differences were detected between the VIP (25.49 ± 2.34) and the SCN area of the golden hamster. In addition, no significant difference in immunoreactivity was observed between the CT (ipsi) and the CT (contra) area in the golden hamster, while in the rat SCN, the O.D. of the CT (contra) area was about two-thirds that of the CT (ipsi) area, as shown in the morphological observation.

Double labeling immunocytochemistry

RHT terminals were labeled with CTb, which showed green FITC fluorescence. In the rat SCN, GFAP-IR elements were observed mainly in the VLSCN, where they were intermingled and appeared to be in contact with CTb terminals (Fig. 5a). Whereas, in the golden hamster SCN, CTb immunoreactive terminals were observed throughout the whole SCN region. They were intermingled and

appeared to have a close relationship with the GFAP-IR astroglial elements labeled with red TRITC fluorescence (Fig. 5b).

IV. Discussion

The detailed morphological analysis of the SCN in this study demonstrated the prominent morphological similarities of the SCN in the rat and the golden hamster. In both species: (1) the SCN was located close to the third ventricle above the optic chiasm and consisted of tightly packed neurons, (2) the SCN received bilateral retinal inputs, and (3) the SCN was principally divided into two distinct subdivisions; dorsomedial and ventrolateral portions. AVP immunoreactive small neurons were mainly located in the dorsomedial portion and VIP immunoreactive neurons in the ventrolateral portion. A comparative study of the SCN in other mammalian species (opossum, cats, guinea pigs and mice) also indicated similar morphological characteristics [2]. Therefore, these morphological similarities suggest that these nuclei develop characteristic features in mammalian phylogeny. In addition, a three-

Fig. 3. The distribution of AVP (a, b), VIP (c, d) immunoreactivity and RHT terminals labeled with CTb (e, f) in the rats (a, c, e) and golden hamsters (b, d, f) SCN. Although some delicate differences between the rat and the golden hamsters were observed, AVP neurons were mainly distributed in the dorsomedial portion of the SCN and VIP neurons in the VLSCN in both species. The RHT terminals in the rat SCN were mainly distributed in the VLSCN and were dominant in the contralateral side (e). In contrast, in the golden hamster SCN, they were observed throughout the SCN and the contralateral and ipsilateral projections were approximately equal (f). OC, optic chiasma; V, third ventricle. Bar = 100 μ m.

dimensional comparative study demonstrated that the rat SCN has an elongated shape throughout the rostrocaudal plane while the hamster SCN showed a less elongated shape [15]. The functional meanings of these differences in the SCN have not been clarified.

GFAP is the most commonly used astrocytic marker for embryonic development [1, 5] and pathology [11,

12]. We have previously demonstrated that GFAP-IR elements represent the morphological features of astroglial elements in the rat SCN using immunoelectron microscopy [26]. In the present study, GFAP immunoreactivity was measured as O.D. per area and the values were used to represent the astroglial density.

In the present study, prominent differences were

Fig. 4. A graphical demonstration of GFAP immunoreactivity (O. D./μm²) in each area in the SCN of rats (a) and golden hamsters (b). In the SCN area, the GFAP immunoreactivity shown in the golden hamster was much higher than that of the rat. In the rat SCN, the O. D. of the AVP area was very low and those of the VIP and the CT areas were much higher compared to the SCN area. In the golden hamster, the AVP area showed a very low value of GFAP immunoreactivity and the CT areas very high values, compared to the SCN area. Error bars indicate the standard deviation of the mean. *P < 0.001, compared with the value of the SCN area.

Fig. 5. Double immunofluorescence microscopic analyses of the FITC-labeled CTb immunoreactive fibers (green) and TRITC-labeled astroglial elements (red) in the SCN of rats (a) and golden hamsters (b). In both species, CTb immunoreactive terminals were observed to have a close relationship with astroglial elements. OC, optic chiasma; V, third ventricle. Bar = 100 μm.

shown in the distributional pattern and the density of astroglial elements in the SCN between the rats and the golden hamsters. These differences in GFAP immunoreactivity may simply represent the differing amount of GFAP proteins in the respective astrocytes of the two species. In addition, an *in situ* hybridization study suggested that the differences in the amounts of GFAP protein in astrocytes depended primarily on the level of GFAP gene expression [10]. Therefore, we consider that these species dependent differences in GFAP immunoreactivity represent the difference in the level of GFAP gene expression between these two species.

The circadian rhythm of astroglial density in the SCN

in two-month old male Syrian hamster was demonstrated by GFAP immunocytochemistry [13]. In the rat SCN, however, we could not detect any clear rhythmicity of GFAP immunoreactivity under light-dark conditions [26]. Therefore, in the present study, all animals were sacrificed at 1400 to eliminate the effect of possible diurnal variation. These differences may be due to the species differences in the astrocytic function of the SCN between hamsters and rats, since GFAP immunoreactivity was principally observed in the VLSCN in the rat and, in contrast, it was observed in the whole SCN region in the hamster.

In the present study, it was commonly found that the O.D. of the GFAP immunoreactivity was very low in the

AVP area in the dorsomedial portion of the SCN, and very high in the VIP and CT area in the VLSCN in both species. These findings indicate that the amount of astroglial elements is very high in the VLSCN, and its close correlation with VIP neurons and RHT terminals are considered to be a common feature in rats and golden hamsters. In the golden hamster SCN, GFAP-IR elements covered the whole SCN region and the O.D. values shown in the SCN area were very high compared to those shown in the rat. Therefore, in the quantification analysis, we could not detect any significant differences between the SCN and the VIP area in the golden hamster.

It is interesting that in the rat SCN, the O.D. of the GFAP immunoreactivity in the CT areas showed a significant difference between the contralateral and the ipsilateral side. However, in the golden hamster SCN, no significant differences were detected. RHT fibers were more densely distributed in the contralateral portion than in the ipsilateral portion in the rat SCN. In contrast, the contralateral and ipsilateral projections were approximately equal in the golden hamster. Therefore, these differences in GFAP immunoreactivity may be ascribed to the differences in the distribution of the RHT fibers between the rats and the golden hamsters. In the present study, double labeling fluorescence immunocytochemistry revealed that the GFAP-IR elements were mainly overlapped in the CT areas in both species.

We have previously provided morphological evidence that astroglial elements have a close relationship with the VIP neurons in the VLSCN [26] and the postnatal development of these astroglial elements is regulated by retinohypothalamic projection (paper submitted for publication). The axons of the retinal ganglion cells make synapses on VIP neurons in the VLSCN [8, 27] and the main neurotransmitter of the retinal neuronal input on VIP neurons is considered to be glutamate [22, 31].

In the retinal target neurons in the SCN, two types of glutamate receptors, NMDA receptor subunits and AMPA type receptors, were detected using *in situ* hybridization and immunocytochemistry [16]. On the other hand, astrocytes also express various types of glutamate receptors and the proliferation and differentiation of glial cells are regulated by the activation of glutamate receptors [24]. In addition to glutamate and its receptors, it has been demonstrated recently that pituitary adenylate cyclase activating polypeptide (PACAP) exists in RHT and is released from retinal terminals in the SCN as a neurotransmitter [6, 7]. In an *in vitro* culture study, it was demonstrated that PACAP induces the proliferation of astrocytes [28] and that it stimulates mitogen-activated protein kinase (MAPK) in rat astrocytes [19]. These findings strongly suggest that these factors may be necessary to form and maintain the astrocytic network in the SCN.

V. Acknowledgments

This study was supported in part by Grant (No. 11780548) from the Ministry of Education, Science and Culture, Japan. We would like to express our appreciation to Professor Yasuhiko Ibata (Kyoto Prefectural University of Medicine) for his support and encouragement throughout the experiment.

VI. References

1. Botchkina, G. I. and Morin, L. P.: Ontogeny of radial glia, astrocytes and vasoactive intestinal peptide immunoreactive neurons in hamster suprachiasmatic nucleus. *Dev. Brain Res.* 86; 48–56, 1995.
2. Cassone, V. M., Speh, J. C., Card, J. P. and Moore, R. Y.: Comparative anatomy of the mammalian hypothalamic suprachiasmatic nucleus. *J. Biol. Rhythms* 3; 71–91, 1988.
3. Elliott, A. S. and Nunez, A. A.: An ultrastructural study of somal appositions in the suprachiasmatic nucleus and anterior hypothalamus of the rat. *Brain Res.* 662; 278–282, 1994.
4. Eng, L. F., Vanderhaeghen, J. J., Bignami, A. and Grestl, B.: An acidic protein isolated from fibrous astrocytes. *Brain Res.* 28; 351–354, 1971.
5. Hagiwara, Y., Eguchi, M. and Ueda, S.: Vimentin-immunoreactivity in the developing striatum of the rat. *Acta Histochem. Cytochem.* 32; 449–457, 1999.
6. Hannibal, J., Ding, J. M., Chen, D., Fahrenkrug, J., Larsen, P. J., Gillette, M. U. and Mikkelsen, J. D.: Pituitary adenylate cyclase-activating peptide (PACAP) in the retinohypothalamic tract: A potential daytime regulator of the biological clock. *J. Neurosci.* 17; 2637–2644, 1997.
7. Harrington, H. E., Hoque, S., Hall, A., Golombek, D. and Biello, S.: Pituitary adenylate cyclase activating peptide shifts circadian rhythm in a manner similar to light. *J. Neurosci.* 19; 6637–6642, 1999.
8. Ibata, Y., Takahashi, Y., Okamura, H., Kawakami, F., Terubayashi, H., Kubo, T. and Yanaihara, N.: Vasoactive intestinal peptide (VIP)-like immunoreactive neurons located in the rat suprachiasmatic nucleus receive a direct retinal projection. *Neurosci. Lett.* 97; 1–5, 1989.
9. Inouye, S.-I. T. and Kawamura, H.: Characteristics of a circadian pacemaker in the suprachiasmatic nucleus. *J. Comp. Physiol.* 146; 153–160, 1982.
10. Kitamura, T., Nakanishi, K., Watanabe, S., Endo, Y. and Fujita, S.: GFA-protein gene expression of the astroglia in cow and rat brains. *Brain Res.* 423; 189–195, 1987.
11. Kiyozuka, Y., Muraoka, Y., Imamura, K., Senzaki, H., Uemura, Y., Tsubura, A. and Yakushiji, M.: Expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases in gliomatosis peritonei derived from ovarian immature teratoma. *Acta Histochem. Cytochem.* 31; 65–69, 1998.
12. Kubo, S.-i., Tokunaga, I., Yamamoto, A. and Morita, K.: Immunohistochemical studies on paraquat-induced damage to neuronal and glial cells in rat hippocampus. *Acta Histochem. Cytochem.* 32; 373–376, 1999.
13. Lavialle, M. and Serviere, J.: Circadian fluctuations in GFAP distribution in the Syrian hamster suprachiasmatic nucleus. *NeuroReport* 4; 1243–1246, 1993.
14. Lazarides, E.: Intermediate filaments as mechanical integrators of cellular space. *Nature* 283; 249–257, 1980.
15. Lydic, R., Albers, H. E., Tepper, B. and Moore-Ede, M. C.: Three-dimensional structure of the mammalian suprachiasmatic

- nuclei: A comparative study of five species. *J. Comp. Neurol.* 204; 225–237, 1982.
16. Mikkelsen, J. D., Larsen, P. J., Mick, G., Vrang, N., Ebling, F. J. P., Maywood, E. S., Hastings, M. H. and Møller, M.: Gating of retinal inputs through the suprachiasmatic nucleus: Role of excitatory neurotransmission. *Neurochem. Int.* 27; 263–272, 1995.
 17. Moore, R. Y.: Organization and function of a central nervous system circadian oscillator in the suprachiasmatic hypothalamic nucleus. *Fed. Proc.* 42; 2783–2788, 1983.
 18. Morin, L. P., Johnson, R. F. and Moore, R. Y.: Two brain nuclei controlling circadian rhythms are identified by GFAP immunoreactivity in hamsters and rats. *Neurosci. Lett.* 99; 55–60, 1989.
 19. Moroo, I., Tatsuno, I., Uchida, D., Tanaka, T., Saito, J., Saito, Y. and Hirai, A.: Pituitary adenylate cyclase activating polypeptide (PACAP) stimulates mitogen-activated protein kinase (MAPK) in cultured rat astrocytes. *Brain Res.* 795; 191–196, 1998.
 20. Okamoto, S., Okamura, H., Miyake, M., Takahashi, Y., Takagi, S., Akagi, Y., Fukui, K., Okamoto, H. and Ibata, Y.: A diurnal variation of vasoactive intestinal peptide (VIP) mRNA under a light-dark cycle in the rat suprachiasmatic nucleus. *Histochemistry* 95; 525–528, 1991.
 21. Prosser, R. A., Edgar, D. M., Heller, H. C. and Miller, J. D.: A possible glial role in the mammalian circadian clock. *Brain Res.* 643; 296–301, 1994.
 22. Shibata, S., Liou, S. Y. and Ueki, S.: Influence of excitatory amino acid receptor antagonists and of baclofen on synaptic transmission in the optic nerve to the suprachiasmatic nucleus in slices of rat hypothalamus. *Neuropharmacol.* 25; 403–409, 1986.
 23. Shinohara, K., Honma, S., Katsuno, Y., Abe, H. and Honma, K.: Two distinct oscillators in the rat suprachiasmatic nucleus in vitro. *Proc. Natl. Acad. Sci. USA* 92; 7396–7400, 1995.
 24. Steinhauser, C. and Gallo, V.: News on glutamate receptor in glial cells. *TINS* 19; 339–345, 1996.
 25. Takahashi, Y., Okamura, H., Yanaihara, N., Hamada, S., Fujita, S. and Ibata, Y.: Vasoactive intestinal peptide immunoreactive neurons in the rat suprachiasmatic nucleus demonstrate diurnal variation. *Brain Res.* 497; 374–377, 1989.
 26. Tamada, Y., Tanaka, M., Munekawa, K., Hayashi, S., Okamura, H., Kubo, T., Hisa, Y. and Ibata, Y.: Neuron-glia interaction in the suprachiasmatic nucleus: A double labeling light and electron microscopic immunocytochemical study in the rat. *Brain Res. Bull.* 45; 281–287, 1998.
 27. Tanaka, M., Ichitani, Y., Okamura, H., Tanaka, Y. and Ibata, Y.: The direct retinal projection to VIP neuronal elements in the rat SCN. *Brain Res. Bull.* 31; 637–640, 1993.
 28. Tatsuno, I., Morio, H., Tanaka, T., Uchida, D., Hirai, A., Tamura, Y. and Saito, Y.: Pituitary adenylate cyclase-activating polypeptide (PACAP) is a regulator of astrocytes: PACAP stimulates proliferation and production of interleukin 6 (IL-6), but not nerve growth factor (NGF), in cultured astrocytes. *Ann. N.Y. Acad. Sci.* 805; 482–488, 1996.
 29. Turner, J. N., Shain, W., Szarowski, D. H., Lasek, S., Sipple, B., Pace, C., Al-Kafahi, K., Can, A. and Roysam, B.: Confocal light microscopy of brain cells and tissue: Image analysis & quantification. *Acta Histochem. Cytochem.* 32; 5–11, 1999.
 30. Van den pol, A. N.: The hypothalamic suprachiasmatic nucleus of rat: intrinsic anatomy. *J. Comp. Neurol.* 191; 661–702, 1980.
 31. Van den pol, A. N.: Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. *J. Neurosci.* 11; 2087–2101, 1991.