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Two distinct oscillators in the rat suprachiasmatic nucleus *in vitro*

(circadian rhythm/neuropeptide)

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ABSTRACT **In the rat suprachiasmatic nucleus slice culture, circadian rhythms in the release of arginine vasopressin and vasoactive intestinal polypeptide were measured simultaneously and longitudinally. The phase relationship between the two peptide rhythms was relatively constant in the culture without a treatment of antimitotic drugs but became diverse by an introduction of antimitotics, which is generally used to reduce the number of glial cells. By monitoring the two rhythms continuously for 6 days, different periods were detected in culture with the antimitotic treatment. Furthermore, *N*-methyl-D-aspartate shifted the phase of the two peptide rhythms in the same culture differently. These results indicate that the arginine vasopressin and vasoactive intestinal polypeptide release are under control of different circadian oscillators.**

Many lines of evidence indicate that the hypothalamic suprachiasmatic nucleus (SCN) generates mammalian circadian rhythms (1). Arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) are synthesized in neurons within different subdivisions of the SCN (2). *In vivo* studies have shown that on the 3rd day of constant darkness (DD), circadian rhythms are observed in AVP content (3) and mRNA (4, 5) but not in VIP content (6) or mRNA (7). This suggests that AVP and VIP rhythms reflect different aspects of the circadian pacemaking mechanism. In SCN cultures, however, AVP (8–12) and VIP releases (12) undergo circadian changes, which indicates that the VIP is also regulated by the circadian pacemaker as well as the AVP.

Several properties of the circadian pacemaker system that underlie the locomotor activity rhythm in mammals are best interpreted in terms of the two-oscillator system. One such property is the phenomenon known as splitting, in which different circadian periods are simultaneously expressed in constant light (13). The two-oscillator system can account for various other rhythm properties in rodents, such as a compression and decompression of activity time (13), aftereffects by previous entrainment (13), and a difference in the immediate and steady-state phase shifts (14). However, two different circadian oscillations have never been found within the SCN (1).

The present study was conducted to determine whether peptides in different subdivisions of the SCN are regulated by distinct oscillators, using the organotypic slice culture of the SCN. First, we examined 48-hr patterns of AVP and VIP release in the culture with or without antimitotic treatment, which is generally used to reduce the number of glial cells. Second, to measure the respective period in the same culture, we monitored two peptide rhythms simultaneously for 6 days. Finally, we examined the phase-shifting effect of *N*-methyl-D-aspartate (NMDA), whose receptors are suggested to be involved in the visual input to the SCN (15, 16).

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MATERIALS AND METHODS

Preparation of Organotypic SCN Culture. Rats of the Wistar strain were bred and reared in our animal quarters (lights on from 06:00 to 18:00). Organotypic slice culture of rat SCN was performed using a roller tube technique as described (12). Briefly, a hypothalamic slice of 380 μm including a pair of SCN was obtained from a 6-day-old rat. The section was embedded in a plasma clot on a coverslip and placed in a culture tube (Nunc) with 700 μl of culture medium [10% horse serum (GIBCO), 60% Eagle's basal medium (Sigma) with 62 mM D-glucose and 4.16 mM $NaHCO_3$, and 30% Hanks' balanced salt solution (GIBCO) with 4.16 mM $NaHCO_3$]. The SCN slice was cultured at 36°C with rotation (10 revolutions per hr).

Protocols for Sampling and Antimitotic or NMDA Treatment. *Experiment 1.* Forty-eight-hour patterns of the two peptide releases were measured in culture with (Fig. 1*b*, n = 13) or without (Fig. 1*a*, n = 34) a treatment of antimitotic drugs. Antimitotics [arabinonucleoside (cytosine arabinoside), uridine, and fluorodeoxyuridine, 0.01 mM each; Sigma] were applied in the medium for 24 hr. After this, the SCN was cultured without the antimitotics. From the 14th day in culture, the entire medium in a tube was collected and replaced with fresh medium at 2-hr intervals over a period of 48 hr.

Experiment 2. Simultaneous monitoring of the two peptide rhythms was continuously done for 6 days in the culture with the antimitotic treatment (Fig. 1*c*, n = 7). The sampling at 2-hr intervals was started on the 14th day of culture and continued for 128 hr. The medium sampling was performed again (n = 3) for 38 hr on the 28th–29th day and 37th–38th day of culture.

Experiment 3. Effects of NMDA on the phase of the AVP and VIP rhythms in the same SCN culture were examined in the culture with the antimitotic treatment (Fig. 1*d*). After the sampling was carried out for 2 days, the SCN was incubated for 15 min with 20 μM NMDA (n = 14) or without it (n = 12). Afterwards, the medium was replaced by fresh medium, which was exchanged 105 min later. The sampling at 2-hr intervals was started again and continued until 128 hr after the start of sampling. In 8 of 14 cultures, the medium sampling was performed again for 38 hr on the 28th–29th day of culture.

The dose of NMDA was decided after examining glutamate concentration in cerebrospinal fluid (CSF) and horse serum. Glutamate was measured by high-performance liquid chromatography with electrochemical detector after derivation with *o*-phthalaldehyde. The CSF in three rats that was maintained in light–dark (LD) cycles was aspirated via the cisterna magna 2 hr before lights off. Glutamate concentrations were 8.0 ± 2.2 μM (n = 3) in the CSF and 6.1 ± 2.0 μM (n = 4) in the culture medium including 10% horse serum. Accordingly, we used 20 μM NMDA, which is about three times higher

Abbreviations: SCN, suprachiasmatic nucleus; AVP, arginine vasopressin; VIP, vasoactive intestinal polypeptide; NMDA, *N*-methyl-D-aspartate; CSF, cerebrospinal fluid; CT, circadian time; LD, light–dark cycle; DD, constant darkness; Ψ, phase-angle difference.

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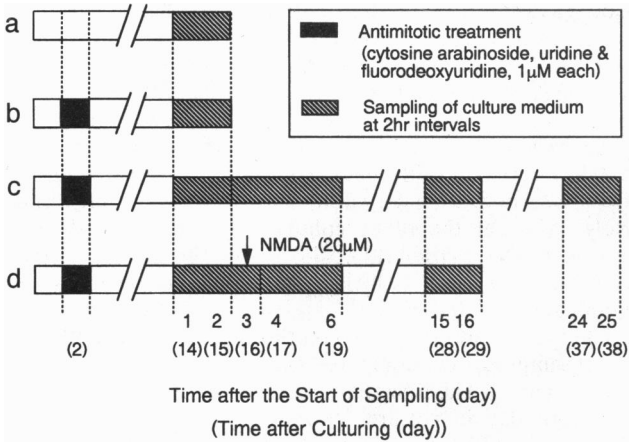

FIG. 1. Protocols for sampling and treatment with antimitotics and NMDA. SCN slice cultures (*b–d*) were incubated in the medium including antimitotics on the 2nd day of culture. Medium changes at 2-hr intervals were started on the 14th day *in vitro* and continued for 48 hr (*a* and *b*) and 128 hr (*c* and *d*). The medium change was performed again for 38 hr after the 28th day (*c* and *d*) and the 37th day (*c*) in culture. In *d*, on the 16th day of culture, the culture was incubated in the medium with 20 μM NMDA or without it in a 15-min pulse, and afterwards, the medium was replaced by a fresh one, which was exchanged 105 min later.

than the glutamate concentration in the CSF and the culture medium.

Peptide Measurement. The collected medium was heated at 100°C for 10 min in 0.1 M HCl, dried up by a speed-Vac concentrator, and stored at −20°C, until assay of AVP and VIP by enzyme immunoassay as described (12).

Data Analysis. The peak phase was determined by a least-squares curve fitting (17) and used as a phase reference point in the AVP and VIP rhythms. The first sampling time was defined as 2 hr. The peak phases in both rhythms were arbitrarily defined as circadian time (CT) 0 since they occurred at almost the same time in the same culture with no antimitotic treatment (see *Results*). Phase-angle difference (Ψ) between the AVP and VIP rhythms was calculated by subtraction of the VIP peak phase from the AVP peak phase. And the difference in the distribution of Ψ was evaluated by their kurtosis (18). The period length of the two peptide rhythms was determined by a χ^2 periodgram (19), using six consecutive cycles. "Measured peak phases" on the 15th–16th day or 24th–25th day of sampling (corresponding to the 28th–29th day or 37th–38th day of culture) (Fig. 1*c*) were determined by the least-squares curve fitting, and "predicted peak phases" were determined by a forward extrapolation of the rhythm of the 1st–6th day with the period calculated by the χ^2 periodgram.

The amount of phase shift was determined by comparing the peak phase predicted by the previous rhythms and the phase observed by the NMDA treatment. A predicted peak phase on the 3rd day was estimated by a forward extrapolation of the rhythms on the 1st and 2nd days. Because the peptide rhythms immediately after the NMDA treatment were distorted, a peak on the 3rd day was determined by backward extrapolation of the rhythms on the 4th–6th day.

RESULTS

Experiment 1. The effect of antimitotics on the AVP and VIP rhythms was examined by measuring their release for 48 hr from the 14th day in culture (Fig. 1 *a* and *b*). As shown in representative cultures (Fig. 2 *a* and *b*), the Ψ between the AVP and VIP rhythms was stable in the SCN slice culture without a treatment of antimitotics. In culture 10 (Fig. 2*a*), the peak phases were 12.0 and 32.2 hr in the AVP rhythm and 12.3 and 32.0 hr in the VIP rhythm, respectively. In culture 12 (Fig. 2*b*), they were 11.0 and 35.8 hr in the AVP rhythm and 11.8 and 36.2 hr in the VIP rhythm. When Ψs between the AVP and VIP rhythms were plotted in a histogram in 2-hr bins (Fig. 3), they proved to be located in a narrow range (0–2 hr). The kurtosis of Ψ distribution was significantly deviated from the normal distribution ($P < 0.01$) (18). This finding indicates that there is a strong coupling between the two peptide rhythms and that the peak phases occur at almost the same phase without the antimitotics (average Ψ = 0.38 ± 0.57 hr; mean ± SD).

In the culture with the antimitotic treatment, however, Ψs were distributed over a wide range. In culture 12G (Fig. 2*c*), the peak phases are 12.8 and 33.2 hr in the AVP rhythm and 22.2 and 42.2 hr in the VIP rhythm. The phase relationship

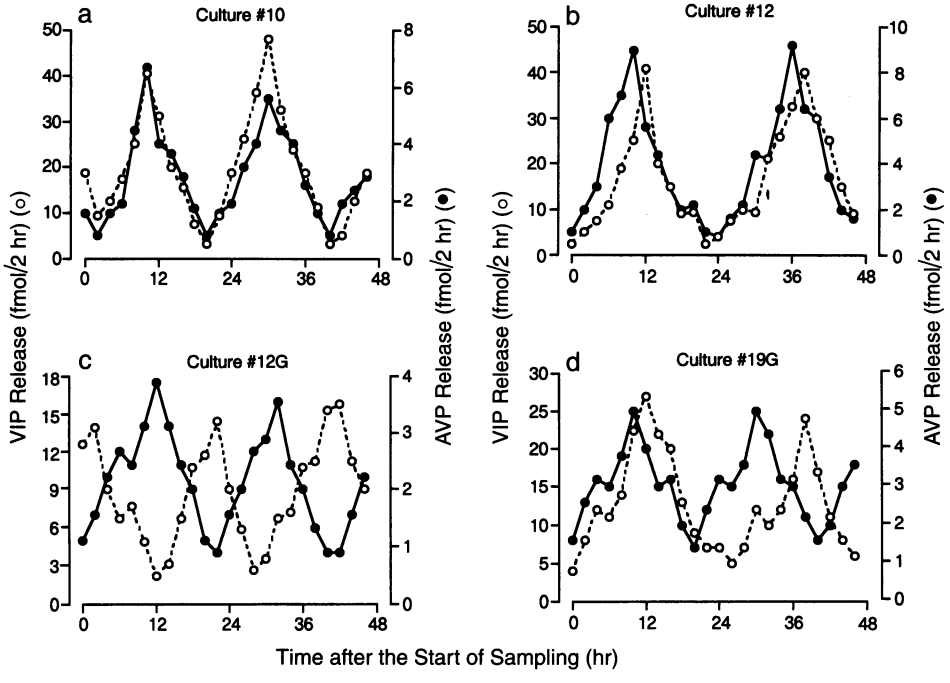

FIG. 2. Forty-eight-hour profiles of the AVP and VIP release in SCN slice cultures without (*a* and *b*) and with (*c* and *d*) the antimitotic treatment.

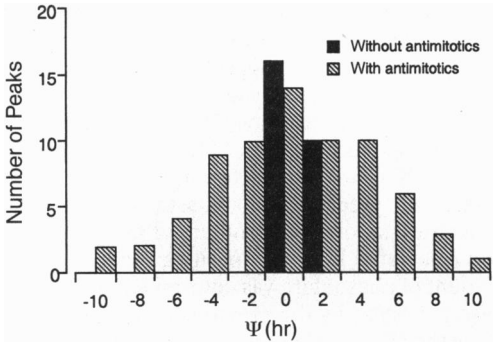

FIG. 3. Distributions of Ψ between the AVP and VIP rhythms in SCN slice cultures with or without the antimitotic treatment. Ψ was determined by subtraction of a VIP peak phase from an AVP peak phase. A positive Ψ indicates that an AVP peak precedes a VIP peak and a negative Ψ indicates that an AVP peak is behind a VIP peak.

between the two peptide rhythms was 180° out of phase, which was never found in the culture without the treatment. In culture 19G (Fig. 2*d*), there was a difference in the peak-to-peak interval between the two rhythms and the first AVP peak preceded the VIP peak by 3.2 hr but the second by 5.8 hr. As shown in Fig. 3, Ψs in the treated culture were widely distributed from −10 hr to 10 hr. The average Ψ in the culture with the treatment was −0.20 ± 4.73 hr (mean ± SD), which was not different from that in the culture without the treatment (two-sample *t* test). The finding indicates that the two peptide rhythms are more loosely coupled in a culture with an antimitotic treatment than without it and suggests that they are regulated by different oscillators.

Experiment 2. To determine the period of the AVP and VIP rhythm, we continuously measured AVP and VIP release simultaneously for 6 days in the culture with the antimitotic treatment. In representative cultures (Fig. 4 *a* and *d*), the Ψ between two peptide rhythms was gradually changed in 6 days. In culture 35 (Fig. 4*a*), Ψs were −1.4, −0.1, 1.1, 1.4, 2.1, and 3.0 hr, which indicates that the VIP peak gradually passed the AVP peak. Also in culture 32 (Fig. 4*d*), the VIP peak was 0.1 hr ahead on the first cycle but 1.7 hr ahead on the 6th cycle.

Analysis of the 6-day data period by the χ^2 periodgram ($P < 0.01$) revealed different periods in the AVP and VIP rhythms—i.e., 20.2 and 19.2 hr and 20.2 and 19.8 hr, respectively. Also, in the other cultures, different periods were observed [AVP rhythm (hr), VIP rhythm (hr): 20.6, 19.2; 21.4, 21.0; 20.2, 19.8; 21.2, 19.4; 19.6, 20.2]. All these periods were significant ($P < 0.01$).

The measured peak phases on the 15th–16th and 24th–25th day of sampling were compared with the predicted peaks (Fig. 4 *b, c, e,* and *f*). The first measured AVP and VIP peaks on the 15th–16th day in culture 35 were 381.9 hr and 366.1 hr, respectively, which represented Ψ greater than 180° (Fig. 4*b*). The measured peaks were almost identical to the predicted ones [AVP rhythm (hr), VIP rhythm (hr): 381.7, 365.7]. On the 24th–25th day, the measured AVP and VIP peaks were 602.3 and 594.1 hr, respectively (Fig. 4*c*), which were consistent with predicted peaks of the AVP rhythm (603.9 hr) and VIP rhythm (596.1 hr). The Ψ on the 24th–25th day seems to be smaller than Ψ on the 15th–16th day. This occurs because the predicted AVP peak phase was the peak of the 30th cycle, when calculated from the cycle on the 1st day (Fig. 4*a*), and the predicted VIP peak was that of the 31st cycle (Fig. 4*c*), assuming that the free-running periods were not changed.

Also, in culture 32, the measured peaks on the 15th–16th day [AVP rhythm (hr), VIP rhythm (hr): 381.4, 377.6] and on the 24th–25th day (604.6, 594.0) almost coincided with the predicted phases on the 15th–16th day (382.4, 375.4) and those on the 24th–25th day (604.6, 593.2). In the other cultures, differences between the measured and predicted phases were within 3 hr in the two rhythms during both periods. These findings indicate that circadian rhythms in the AVP and VIP releases

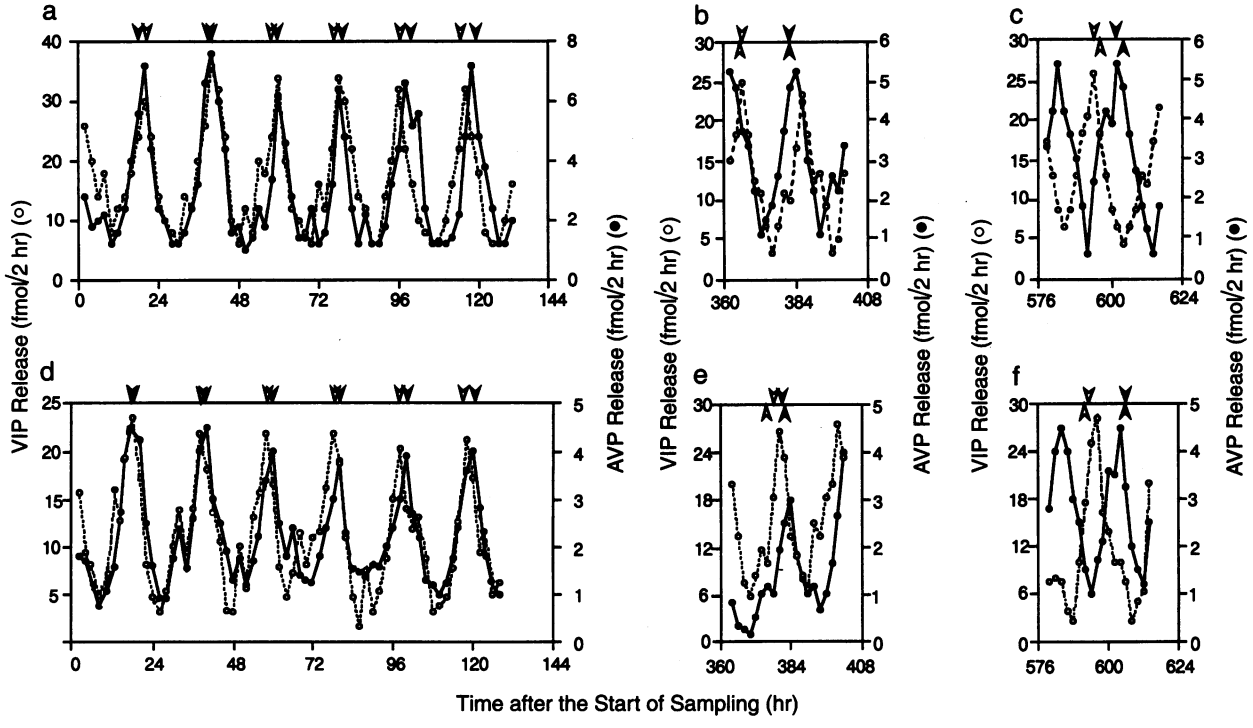

FIG. 4. Circadian rhythms in AVP and VIP release on the 1st–6th day (*a*), 15th–16th day (*b*), and 24th–25th day (*c*) of measurement in SCN slice culture 35 and on the 1st–6th day (*d*), 15th–16th day (*e*), and 24th–25th day (*f*) in SCN slice culture 32. Symbols represent amounts of AVP and VIP in the same medium collected at 2-hr intervals. Black and white arrowheads represent AVP and VIP peaks, respectively. Downward arrowheads indicate measured peak phases that were determined by the least-squares curve fitting. Upward arrowheads represent predicted peak phases that were determined by a forward extrapolation with the period that was calculated by the χ^2 periodgram.

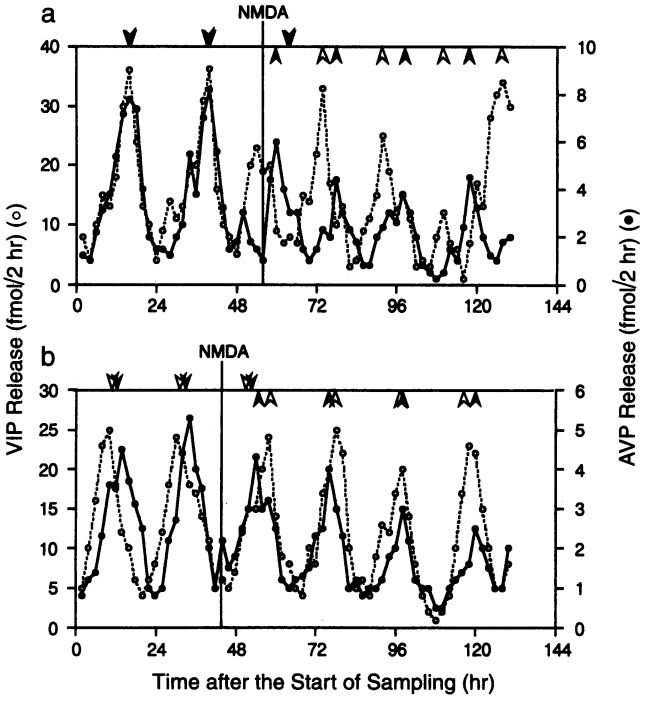

FIG. 5. Effect of a 15-min pulse of NMDA on the AVP and VIP rhythms in SCN slice cultures. Symbols represent amounts of AVP and VIP in the same medium collected at 2-hr intervals. The phase of the NMDA pulse is indicated by the vertical lines. Black and white arrowheads represent AVP and VIP peaks, respectively. The 1st–2nd downward arrowheads indicate measured peak phases before NMDA application and the 3rd indicates a forward extrapolated peak. The 1st upward arrowheads represent a backward extrapolated peak and the 2nd–4th represent measured peaks.

free-run with different periods and that the free-running periods were relatively stable.

Experiment 3. The two-oscillator system was supported by the different responsiveness of the two peptide rhythms to NMDA (20 μM). In SCN culture 17 (Fig. 5*a*), the AVP peak and VIP peak occurred almost at the same time, and the time of NMDA administration was referred to as CT 14.7 in the AVP rhythm and CT 15.2 in the VIP rhythm. When compared with the phase before the treatment, the peak phase in the AVP rhythm was advanced by 7.5 hr, while the VIP phase was delayed by 11.9 hr.

The intervals between the 1st and 2nd peaks, 2nd and 3rd peaks, and 3rd and 4th peaks after the NMDA pulse are 17.6, 19.6, and 20.1 hr for the AVP rhythm and 18.7, 17.8, and 18.1 hr for the VIP rhythm. On the other hand, the peak-to-peak interval on the 15th–16th day was 19.9 hr in the AVP rhythm and 18.2 hr in the VIP rhythm. The finding indicates that the two peptide rhythms go through transients showing slightly different periods. Transient period changes were also reported to occur after administration of glutamate (20). The intervals between the 2nd and 3rd peaks and 3rd and 4th peaks after the treatment (Fig. 5*a*) were averaged and compared with the peak-to-peak interval before the treatment. The interval of the AVP rhythm was altered from 23.8 to 19.9 hr, while that of the VIP rhythm changed from 24.0 to 18.0 hr, which imply that the NMDA treatment also changes the period length differently in the AVP and VIP rhythms in the same culture.

A similar finding was obtained in culture 7 (Fig. 5*b*), where the time of NMDA administration in the AVP rhythm was referred to as 0.6 hr behind that in the VIP rhythm. The pulse produced a 1.5-hr phase-advance shift in the AVP rhythm and 5.3-hr delay shift in the VIP rhythm. As a result, the peak of the VIP rhythm was behind that of the AVP rhythm by 3.2 hr. The peak-to-peak interval of the AVP rhythm was lengthened from 20.0 to 22.1 hr, while that of the VIP rhythm was unchanged. Shifted phases of both rhythms were also kept in the culture on the 15th–16th day.

When the averaged phase shifts were compared with and without the NMDA treatment (Table 1), NMDA application at CT 13 delayed the AVP rhythm but not the VIP rhythm, while that at CT 15 delayed the VIP rhythm but not the AVP rhythm. Significant phase advances in the AVP and VIP rhythms were detected at CT 17 and CT 19, respectively. Phases at which advance and delay shifts occurred were different between the two peptide rhythms, and a significant difference in the amount of phase shift between the two rhythms was detected at CT 15. There was no systematic change in the period length after the NMDA pulse.

DISCUSSION

The present results showed that there were various Ψs between the circadian rhythms in the AVP and VIP release in rat SCN slice cultures with antimitotic treatment, while a relatively constant phase relationship was observed in the culture without the treatment. Long-term and simultaneous monitoring revealed that the two peptide rhythms have different periods. These results indicate that different circadian oscillators are involved in these peptide rhythms. The two-oscillator system was supported by the fact that the two peptide rhythms in the same culture responded to NMDA differently.

To our knowledge, there have been no previous reports of two circadian oscillations in the mammalian SCN that have been simultaneously recorded for >3 days (21). In the same SCN culture, we continuously measured the amount of the two peptides in the same medium for 6 days and the measurement was repeated 9 days and 18 days later. Throughout the experiments, two peptide rhythms were not damped. On the other hand, the average SD of the peak-to-peak interval using six phases from six consecutive records were 0.81 ± 0.31 in the AVP rhythm and 0.61 ± 0.37 in the VIP rhythm (mean ± SD). These values are similar to those of melatonin rhythms in the culture of chicken pineal (22). Accordingly, our *in vitro* SCN system may provide a valuable model for investigating the multi-oscillator system.

Table 1. Phase shifts in the AVP and VIP rhythms after an NMDA pulse

Rhythm	CT 13	CT 15	CT 17	CT 19
AVP				
NMDA	−144.8 ±83.5* (5)	−0.2 ±89.7† (3)	69.7 ± 2.0* (3)	28.1 ±57.2 (5)
Control	17.9 ±35.8 (3)	16.6 ±13.2 (3)	−22.9 ± 39.1 (3)	14.7 ±14.6 (3)
VIP				
NMDA	−62.7 ±93.6 (3)	−115.7 ±86.4*† (3)	17.8 ±120.1 (5)	83.2 ±47.6* (3)
Control	−10.6 ±27.1 (3)	9.6 ±27.7 (3)	4.1 ± 17.4 (3)	16.7 ±15.2 (3)

Values are mean ± SD in degrees. Data are normalized to degrees phase shift (i.e., multiplied by 360/preceding period) and averaged over 2-hr bins of CT. Numbers in the parentheses indicate the numbers of cultures. *, $P < 0.05$ vs. control (Student's t test); †, $P < 0.05$ between the AVP and VIP rhythms (two-sample t test).

Morphological characteristics of the cultured SCN have been well described (11, 23). Two weeks after culturing, a SCN slice is flattened to a few cell layers and two neuronal cell masses surrounded by a peripheral glial cell-dispersed zone in the slice are observed. Until this stage, about 70% of neuronal cells have been lost. However, it is not known whether synaptic connections have developed differently in the SCN culture than those *in vivo*.

The AVP and VIP neurons are different in their location within the SCN and different in the light responsiveness. AVP is contained in neurons within the dorsomedial SCN (2) and displays the circadian rhythms in its content (3) and mRNA (4, 5) in both LD and DD. On the other hand, VIP is contained in neurons within the ventrolateral SCN (2) and shows daily rhythms in its content (6, 24) and mRNA (25, 26) in LD with an antiphase against the AVP rhythms (3–5) but does not oscillate on the 3rd day of DD. These results were interpreted as evidence indicating that the VIP is not regulated by the circadian pacemaker but directly by LD cycles. On the other hand, the AVP is thought to be regulated by the pacemaker. Recently, circadian rhythms of VIP mRNA in the SCN have been reported in pups before eye opening (27) and in adults reared in DD (27). Circadian rhythms of the VIP content in the SCN are observed after a drug treatment (28) with a peak phase similar to that of the AVP. In isolated SCN *in vitro*, the VIP release shows a remarkable circadian rhythm and the average Ψs between the AVP and VIP rhythms were almost 0°. Altogether, an endogenous VIP rhythm with a phase similar to that in the AVP rhythm might be masked by LD cycles. When animals were transferred from LD to DD, an endogenous VIP rhythm might begin to emerge in the presence of an aftereffect of LD, which could result in an apparent arrythmicity. Indeed, there is a similar case in the serotonin content in the SCN (29). In combination of these findings, it could be speculated that the AVP and VIP are controlled by different oscillators with different sensitivities to light.

A 24-hr treatment of cultured cells with antimitotics has been reported to reduce the number of glial cells remarkably in organotypic slice cultures. After treatment, immunoreactive cells for glial fibrillary acidic protein are mainly observed in the peripheral cell-dispersed zone of the SCN (11) and are rarely found in the central neuronal mass of the SCN. On the other hand, slice cultures without the treatment are ensheathed by a thin layer of glial cells (30). Thus, uncoupling of two peptide rhythms after an antimitotic treatment might be due to the loss of the glial cell.

Prosser *et al.* (31) examined the effect of agents that block the glial metabolism on the neural firing rhythm. The drug application produced two peaks per a day in neural activity. Although this observation seems to be inconsistent with the present observation, the discrepancy could be explained, as follows. The neural firing rhythm in the SCN consists of neural activities originating from the ventrolateral and dorsomedial subdivisions of the SCN. If circadian rhythms of the neural firing rate in the two subdivisions are dissociated, it would result in two peaks in neural activities measured in the whole SCN. In turn, the dissociation would cause dissociation of the AVP and VIP rhythms.

Pittendrigh and Daan (13) have advanced a hypothesis that the circadian pacemaker is composed of two mutually coupled oscillators with different light sensitivities. In the present study, the AVP and VIP rhythms showed different periods. Furthermore, the two peptide rhythms responded to NMDA treatment differently. Hence, we safely conclude that at least two independent circadian oscillators are involved in the rat SCN. At present, it cannot be determined whether or not the two oscillators have a relationship of master and slave.

Recently, two oscillators have been found in one cell, a unicellular alga, *Gonyaulax* (32). In a cell population of the *Gonyaulax*, bioluminance and aggregation show circadian rhythms with different periods. The present study demonstrated two circadian oscillations in different peptidergic neurons located in different subdivisions of the SCN. Since other peptidergic neurons are also distributed in each subdivision, it is important to determine the number of oscillations in the two subdivisions—i.e., one each or more. As interactions between two uncoupled rhythms, relative coordination was observed in *Gonyaulax*. In the antimitotic-treated SCN culture, the distribution of Ψ between the two rhythms (Fig. 3) was normal rather than even—i.e., there was observed a preferential Ψ. This might reflect the relative coordination. Based on these results from a unicellular organism and a mammal, it could be assumed that a two-oscillator system might be ubiquitous in the circadian pacemaker organization in most organisms.

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