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Abbreviations

suprachiasmatic nucleus	SCN
2-deoxy-*D*-glucose	2DG
vasoactive intestinal peptide	VIP
peptide histidine isoleucine	PHI
arginine vasopressin	AVP
retinohypothalamic tract	RHT
artificial cerebrospinal fluid	aCSF
lateral cerebral ventricle	LCV

Summary

It is known that in mammals the SCN includes a circadian oscillator. It was shown in our laboratory that the SCN had an important role in the regulation of plasma glucose concentration; bilateral lesions of the SCN eliminated the hyperglycemia due to intracranial injection of 2DG. Furthermore, from the evidence that the 2DG-hyperglycemia was temporarily and permanently suppressed in surgically and congenitally blind rats with abnormal SCN, respectively, we speculated that the neurons receiving the retinal neural input in the SCN might be involved in the hyperglycemic response to intracranial injection of 2DG. On the other hand, it is known that the neurons containing VIP-like immunoreactive substance have synaptic contact with the RHT from the retinal ganglion cells. Accordingly, I examined whether VIP is involved in the central regulation of the SCN in the plasma glucose concentrations. Consequently, I found that in surgically blind rats VIP-neurons in the SCN were dispersed and invaded to neighbors during the period when the hyperglycemia due to 2DG was temporarily suppressed, but that the neurons became tightly packed again when the hyperglycemic response was restored. From these results, it was considered that VIP-neurons in the SCN might has an essential role in the hyperglycemic response to 2DG. Furthermore, I also found that VIP enhanced synergistically 2DG-hyperglucagonemia as well as 2DG-hyperglycemia, and that a VIP-antagonist inhibited both the 2DG-hyperglycemia and 2DG-hyperglucagonemia. Furthermore, It was found in our laboratory that intracranial injection of 2DG elicited increase in the neural activities of the sympathetic efferents to the pancreas, liver and adrenal , but that, the increase of sympathetic nerve activity of adrenal efferents in response to 2DG was eliminated by bilateral lesion of the SCN. In this connection, I found that in normal rats the increase of sympathetic nerve activity by

2DG was enhanced by intracranial injection of VIP. These findings suggest that VIP included in the SCN acts as an endogenous modulator that enhances 2DG-hyperglycemia through regulation of sympathetic nerve activities.

On the other hand, it was found that the SCN might have a role in the regulation of body water balance; in congenitally blind rats with abnormal SCN the increase of the plasma AVP concentration induced by 24 hours water-deprivation was eliminated. In this study, I found that bilateral lesions of the SCN lowered the increase in the plasma AVP concentration induced by 24 hours water-deprivation and intraperitoneal injection of hypertonic saline. Furthermore, I also found that intracranial injection of VIP enhanced the increase in the plasma AVP concentration after intraperitoneal injection of hypertonic saline, but that injection of VIP-antagonist suppressed it. These results also suggest that VIP-neurons of the SCN have a role to facilitate the increase of the plasma AVP concentration by hypertonic challenge.

Finally, I observed that central administration of AVP suppressed 2DG-hyperglycemia, but that administration of an AVP-antagonist enhanced it. These findings suggest that in the brain AVP has a role in suppressing the plasma glucose concentration, that is counteraction to VIP. Thus it can be concluded that AVP-neurons existed in the SCN is involved in the regulation of glucose metabolism.

General Introduction

In mammals, the SCN works as a master oscillator of the circadian rhythms, and that they are synchronized by a light-dark cycle with a period of about 24 hours through direct and indirect projections from the retina. It was shown in our laboratory that the hyperglycemic response and increase of sympathetic nerve activity to 2DG was eliminated by bilateral lesions of the SCN. Furthermore, it was also shown that the hyperglycemic response to 2DG was temporarily suppressed during 4 to 6 weeks after bilateral orbital enucleation, but that 2DG-hyperglycemia was not observed in congenitally blind rats with abnormal SCN. In mammals, glucose is usually used as a sole energy source in the brain. 2DG is a derivative of glucose that inhibit glucose metabolism through inhibition of glucose uptake into cells by glucose transporters and phosphoglucoisomerase activity after its conversion to 2DG-6-phosphate. Therefore, 2DG causes glucopenia after injection into the brain and thus leads to energy deficiency in the brain. To overcome this crisis, animal increases the blood glucose level (hyperglycemic response) through the elevations of the blood glucagon and adrenaline levels and the suppression of the increase in the blood insulin level depending on the increase of sympathetic nerve activity. Thus, our findings suggested that the SCN neurons receiving retinal inputs might be responsible for this hyperglycemic response through the sympathetic nervous system. Based on histological studies, it was reported that the neurons containing VIP-like immunoreactive substance (VIP-neurons) in the SCN had synaptic contacts with the axons of retinal ganglion cells. This prompted me to examine the role of VIP-neurons in the SCN in the hyperglycemia and increase of sympathetic nerve activity to 2DG. In this study, I compared in surgically blind rats the change of the localization of the VIP-neurons of the SCN with the intensity of the hyperglycemic response to 2DG, and

studied the effects of intracerebroventricular injection of VIP on the hyperglycemia and increase of sympathetic nerve activity induced by 2DG. Furthermore, from the results that the increase of AVP level by 24 hours water-deprivation was eliminated in congenital blind rats with abnormal SCN, it was suggested that the VIP-neurons in the SCN also had a role on regulation of plasma AVP level. AVP acts as an antidiuretic hormone in peripheral organs so that its plasma concentration increases by such osmotic stimulations as water-deprivation and administration of hypertonic saline. Therefore, I examined the effects of bilateral lesions of the SCN and intracranial injection of VIP in the increase of the plasma AVP level by osmotic stress. On the other hand, it is also known that the neurons containing AVP-like immunoreactive substance (AVP-neurons) exist in the SCN and that these neurons project so far as outside areas of the SCN. Thus, I examined the role of AVP in the central nervous system on the 2DG-hyperglycemia. These results are also included in this paper.

Chapter 1

Effect of orbital enucleation on glucose homeostasis and morphology of the SCN

SUMMARY

It was shown in our laboratory that in SCN-lesioned rats and blind hereditary microphthalmic rats with abnormal SCN, the hyperglycemic response to intracranial injection of 2DG was not observed. Furthermore, it was also shown that in surgical blind rats the hyperglycemic response to 2DG was temporarily suppressed from week 4 to 6 after blinding. In rats there are direct and indirect neural connections from retinal ganglion cells to the ventrolateral part of the SCN, in which the neurons containing VIP and PHI are included. From these findings, I supposed that the neurons responsible for the hyperglycemic response to 2DG were present in the SCN, that after blinding these neurons temporarily lost such activity, and that this functional change was reflected in the morphology of the SCN, especially the neurons containing VIP and PHI. To investigate this possibility, I examined the morphological changes of the SCN by Nissl staining and immunohistochemical studies with anti-VIP and anti-PHI antibodies in surgically blinded rats, and analyzed the relationship between these morphological changes and the hyperglycemic response to 2DG. After surgical blinding, the following changes was observed. (1) The optic chiasm became thinner. (2) The SCN became displaced rostrally. (3) The density of neurons in the middle to caudal part of the SCN, where the retinal ganglion cells projected, decreased markedly without change in cell number during the period when the hyperglycemic response to 2DG was temporarily suppressed after blinding. (4) the neurons containing VIP and PHI were distributed dispersively when the hyperglycemia to

2DG was temporarily suppressed, but these neurons are tightly packed when the hyperglycemia was observed. The first two changes seemed to reflect reduction of fibers and axon terminals from retinal ganglion cells and their innervation, respectively. The last two changes were parallel with suppression of the hyperglycemic response to 2DG. The latter findings may reflect functional change of the neurons in the SCN that are responsible for the hyperglycemia due to 2DG, especially the containing VIP- and PHI-neurons .

INTRODUCTION

In mammals the SCN of the hypothalamus is established as a location of a circadian oscillator of behaviors, hormonal levels and enzyme activities and this oscillator is synchronized with a light-dark cycle with a period of about 24 hours [12, 15, 19, 25]. There is a direct neural input from retinal ganglion cells to the ventrolateral part of the middle to caudal part of the SCN [6, 11-13]. This neural pathway is called the retinohypothalamic tract (RHT). The neurons containing VIP- or PHI-like immunoreactive substance are present in the ventrolateral part of the SCN where the axons of the RHT terminate [1, 3, 4, 22]. In rats, intracranial injection of 2DG causes a time-dependent hyperglycemic response [17] and this response is eliminated by bilateral lesions of the SCN [28]. Furthermore, surgical blinding by bilateral orbital enucleation results in a temporary suppression of the hyperglycemia induced by intracranial injection of 2DG from week 4 to 6 after blinding [31]. These temporary changes after blinding are similar to those observed after bilateral lesions of the SCN [28]. These findings suggest that the SCN contains the neurons that are responsible for the hyperglycemia caused by 2DG-injection into the lateral cerebral ventricle and that the function of these neurons is temporarily suppressed by orbital enucleation which causes loss of innervation by the retinal ganglion cells. These made me suppose that this functional suppression might be reflected in morphological changes of neurons in the SCN. Thus, Nissl-staining and immunocytochemical staining with antisera against VIP and PHI were used to examine the changes in morphology in relation to the hyperglycemic response to 2DG.

MATERIALS AND METHODS

Animals

Four-weeks-old and new born male Wistar strain rats were used. Rats of both ages were divided into 2 groups, bilateral orbital enucleation and control groups. Bilateral orbital enucleation was performed under ether anesthesia. After enucleation, the animals were housed in plastic cages in a room maintained at 24 ±1 °C and illuminated for 12 hours a day (0700h-1900h) by fluorescent light bulbs (80 lux). Food (type MF, Oriental Yeast Co., Osaka) and water were given freely. New born rats were kept with their own mothers until the age of 4 weeks, and then weaned. Technical errors due to differences in staining conditions in the morphological study of the SCN were avoided by examining the effect of unilateral blinding. Unilateral orbital enucleations were also done in rats at the age of 4 weeks and 1 day.

Administration of 2DG and blood sampling

In weeks 5 and 10 after bilateral orbital enucleation of 4 -weeks-old rats and in week 5 after blinding of 1-day-old rats, a polyethylene cannula (PE-10) was inserted into the right lateral cerebral ventricle (LCV) by the method of Altaffer *et al.* [2] under pentobarbital anesthesia. Another catheter made of silastic and polyethylene tubing (PE-50) was inserted into the right atrium of the heart by the method of Steffens [24]. Three days after cannulation, 60 μmol of 2DG or saline, each in a volume of 30 μl, was injected through the brain catheter into the LCV of rats deprived of food for 2-h. The injection was started 5 hours after the beginning of the subjective light period, which was determined for each blind rat by monitoring the free-running circadian drinking rhythm. Blood samples (0.2 ml each) were collected from the right atrium through the heart cannula 0, 30, 60, 90 min after the injection. Food was withdrawn until the end of the experiment.

Measurement of plasma glucose concentration

The plasma glucose concentration was measured by the glucose oxidase method with a Blood Sugar GOD Period Test Kit (Boehringer, Mannheim).

Data and statistical analysis

Data are expressed as means ± standard errors of means. Statistical analysis was performed by Student's t-test.

Histological examination

Brain sectioning --- After the experiment on the effect of 2DG, rats were anesthetized with sodium pentobarbital (0.5 mg/kg), and their brains were perfused first with 0.85 % NaCl solution containing 0.1 % sodium nitrite and heparin (1 U/ml) and then with Zamboni's fixative (2 % paraformaldehyde, 0.13 M phosphate buffer pH 7.4, 0.21 % picric acid). Colchicine (30 μg/ 20 μl saline) was injected into the LCV of rats, and 2 days later their brains were perfused with the above fixative. These brains were postfixed in the same fixative overnight and then immersed in phosphate buffered saline containing 30 % sucrose for 2 days at 4 °C. Then, brain sections with the thickness of 25 μm thick mass were cut with a cryostat. These brain sections were used in successive order from the anterior to posterior for Nissl-staining and immunostaining with anti-VIP and anti-PHI antisera, respectively. In other words, these stainings were done on every 3 sections. Rats suffered unilateral orbital enucleation were killed by an overdose of sodium pentobarbital and their brains were perfused with the above fixative without injecting 2DG at the time corresponding to that when rats with bilateral orbital enucleation were killed.

Nissl-staining --- Sections were stained with cresyl violet, and examined under a light microscope.

Immunostainings with anti-VIP and anti-PHI antisera --- Sections were stained by an immunocytochemical method [10]. For this, they were treated with phosphate buffered saline (PBS) containing 0.3 % Triton X-100 at room temperature and incubated in PBS containing 1 % normal goat serum, 1% bovine serum albumin and 0.3 % Triton X-100 (solution A) for 3 hours at 4 °C. Then they were incubated with anti-VIP antiserum (Peptide Institute Inc., Osaka) or anti-PHI antiserum (a gift from Dr. L. A. Frohman [7]) each diluted 1:1000 with solution A for 2 days at 4 °C. After washing with PBS, they were transferred to solution A, incubated in goat anti-rabbit IgG diluted 1:1000 with solution A overnight at 4 °C, washed with PBS, treated with solution A and incubated with rabbit PAP complex (ZYMED, California) diluted 1:1000 with solution A overnight at 4 °C. Next, they were washed with PBS and 50 mM Tris-HCl (pH 7.4), and immersed in substrate solution containing 0.02 % 3,3'-diaminobenzidine, 0.0045 % H_2O_2 and 0.45 % $(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$ in 50 mM Tris-HCl (pH 7.4). The reaction was terminated by transferring them to 50 mM Tris-HCl (pH 7.4). Finally the sections were mounted on slides with 0.75 % gelatin solution, dried and examined under a coverslip by light microscopy.

RESULTS

Effect of blinding on the hyperglycemic response to 2DG

Figure 1 shows the changes in the plasma glucose concentration after intracerebroventricular injection of 2DG in the subjective light periods of control and blind rats in week 5 and 10 after a sham operation or bilateral orbital enucleation at the age of 4 weeks and in week 5 after blinding at the age of 1 day. As reported previously [31], the hyperglycemic response to 2DG injection was significantly reduced in week 5 after blinding at the age of 4 weeks, but not in week 10 after

blinding at the age of 4 weeks or in week 5 after blinding at the age of 1 day. These findings confirm the existence of temporary suppression of the hyperglycemic response to 2DG in these surgically blinded rats.

Effect of blinding on the morphology of the SCN

The morphological changes of the SCN of blinded rats were examined by Nissl staining and immunocytochemical stainings. Figure 2 shows photomicrographs of immunocytochemical staining with anti-VIP antiserum of brain sections of normal control and rats in week 10 after blinding at the age of 4 weeks. In normal rats (Figs. 2a and 2b), the neurons and nerve terminals with immunoreactivity to anti-VIP antiserum were not observed at the level of the anterior commisure (AC), which is usually about 200 μm anterior to the rostral tip of the SCN, and neurons containing VIP-like immunoreactive substance remain in the SCN. However, after bilateral orbital enucleation (Figs 2c and 2d) the neurons containing VIP-like immunoreactive substance were observed at the level of the AC. The neurons containing VIP-like immunoreactive substance were also seen at the level of middle to caudal part of the SCN. These findings indicate that orbital enucleation caused rostral displacement of the SCN. To confirm these findings, the effect of unilateral orbital enucleation on the morphology of the SCN was examined. Figure 3 shows photomicrographs of cresyl violet staining of horizontal sections of brains of a normal rat, a rat with unilateral orbital enucleation (half blind) and a rat with bilateral orbital enucleation (blind) in week 5 after enucleation at the age of 4 weeks. The frontal edge of the bilateral SCN of normal rats and that on the ipsilateral side of the SCN to the enucleated eye of half blind rats were located within the arch formed by extensions of the rostral edges of the bilateral supraoptic nuclei (Figs. 3a and 3b). However, the frontal edges of the bilateral SCN of the blind rat and of the contralateral SCN of the half blind rat extended over this

arch (Figs. 3b and 3c). Since the unilateral retinohypothalamic projection to the SCN preferentially (about 70 %) terminates in the ventrolateral part of the contralateral SCN in rats [5, 6, 8, 11], it was possible to examine the effect of the exclusion of the unilateral retina by comparing the morphologies of the ipsilateral and contralateral SCN. This comparison using unilateral orbital enucleation has the advantage that it minimizes the error due to differences in staining conditions. Morphological changes in brain sections of rats with unilateral enucleation were found to be similar to those in rats with bilateral orbital enucleation on the contralateral side, whereas their morphology on the ipsilateral side was similar to that of normal control rats. Thus, I examined the morphological changes in the SCN of rats with unilateral orbital enucleation. In this series of experiment I examined 2 groups of rats in week 5 and 10 after unilateral orbital enucleation (blinding) at the age of 4 weeks (these 2 groups are referred to as groups 4W-5W and 4W-10W, respectively). Figure 4 shows brain sections stained with cresyl violet and anti-VIP and anti-PHI antisera of rats of group 4W-5W. On comparison with the control (ipsilateral) side of the SCN, the following marked changes can be seen in morphology of the contralateral side of the optic chiasm and the SCN to the enucleated eye: 1) The optic chiasm became thin. 2) SCN neurons became located far more rostrally. 3) In the middle to caudal part of the normal SCN, the area occupied by SCN-neurons and the cell numbers became reduced, but SCN-neurons seemed to extend to the same level in the caudal tip as in the ipsilateral SCN.

Similar changes were observed in rats of group 4W-10W and in rats in week 5 after unilateral orbital enucleation at the age of 1 day (this group is referred to as group of 1D-5W) (Figs. 5 and 6). Tracings of sequential coronal section at 75 μm-intervals of the SCN of rats of groups

4W-5W, 4W-10W and 1D-5W are shown in Figs. 7, 8 and 9, respectively. In these rats, the contralateral SCN was displaced rostrally, and the area occupied by the SCN neurons became smaller in the middle to caudal part than on the ipsilateral side. Gross observation of the SCN structure by Nissl-staining showed no significant difference in these 3 groups of rats, except that the area occupied by the SCN cells in the middle to caudal part of the SCN was slightly wider in group 4W-5W than in the other 2 groups. Therefore, I counted cell numbers and calculated cell densities in the SCN in these 3 groups of rats. Table I shows numbers of cells in the ipsilateral and contralateral SCN. As these cell numbers were counted in one third of the brain sections, the actual cell numbers in the SCN must be roughly 3 times more, and are thus consistent with reports that the unilateral SCN contains about 10,000 cells [11, 14, 19]. I calculated the densities of SCN cells in each brain section and showed the results as ratios of densities in the contralateral SCN to those in the ipsilateral SCN in Fig. 10. In the rostral part, the contralateral SCN had higher density than the ipsilateral SCN in all three groups of rats. However, in the middle to caudal part, the contralateral SCN had a much lower density than the ipsilateral SCN in rats of group 4W-5W, whereas in rats of groups 4W-10W and 1D-5W it had a lower density than the latter in some regions of the middle to caudal part of the SCN, but a higher density in other regions. In other words, in the middle to caudal part of the SCN contralateral to orbital enucleation, the relative density of SCN neurons was lower in group 4W-5W than in group 4W-10W and 1D-5W.

As mentioned previously, the RHT innervates the ventrolateral portion of the posterior three-quarters of the SCN [5, 6, 8, 11] and the ventrolateral SCN has the neurons containing VIP- and PHI-like immunoreactive substance [1, 3, 4, 7, 22]. Thus, I examined the SCN in

these rats with unilateral orbital enucleation immunohistochemically with antibodies against VIP and PHI. The distribution of the neurons containing VIP- and PHI-immunoreactive substances in the SCN confirmed the dramatic changes in morphology of the SCN observed in brain sections stained with cresyl violet (Figs. 4-6). In addition, the results showed that the SCN neurons containing VIP- and PHI-like substances in the middle to caudal part of the SCN were more loosely packed in rats of group 4W-5W than in those of groups 4W-10W and 1D-5W (Fig. 11).

DISCUSSION

Bilateral lesions of the SCN were found to eliminate the circadian rhythms of plasma corticosterone concentration [12], locomotor activity and drinking behavior [25] in rats. It was also found in our laboratory that in rats the circadian rhythm of feeding disappears after bilateral lesions of the SCN [15]. Subsequent studies showed that, the SCN is a master circadian oscillator which synchronizes to light-dark cycle [14, 19]. In studies on whether time-dependency (photoperiodicity) in the hyperglycemic response due to intracranial injection of 2DG [17] disappears after lesions of the SCN, it was found that bilateral lesions of the SCN not only abolish this time-dependency, but also completely eliminated the hyperglycemic response itself [28]. It was also found that bilateral lesions of the SCN induced hyperinsulinemia under fasting conditions [26, 27, 28] and hypoglucagonemia [29, 30], and that it eliminated the hyperglucagonemic response to 2DG [29] and enhanced glucose tolerance [27]. These findings indicate that neurons or neural fibers in the SCN or its vicinity are essential for the hyperglycemic response to 2DG and are involved in the mechanism of glucose homeostasis.

The circadian rhythms of blind rats are not synchronized with an environmental light-dark cycle, but are free-running, and these rats show a subjective time-dependent (photoperiodic) hyperglycemic response to intracranial injection of 2DG [31]: hyperglycemia is higher in their subjective light period than in their subjective dark period. However, from week 4 to 6 after bilateral orbital enucleation (blinding) the hyperglycemic response is temporarily suppressed and remarkable hyperinsulinemia is observed before 2DG injection [31]. These findings in blinded rats are similar to those in rats with bilateral lesions of the SCN. The hyperglycemic response to 2DG was also shown to have disappeared or to be largely suppressed in congenital blind rats (hereditary microphthalmic rats) which have no optic nerve and have an abnormal SCN [16]; in these rats, the SCN contains fewer neuronal cells and occupies more space than that in control rats. As there is a direct neural connection between the retinal ganglion cells and the ventrolateral part of the SCN by the RHT [5, 6, 8, 11, 13], these findings suggest that the SCN neurons are essential for the hyperglycemic response to intracranial injection of 2DG, and that their neural activity is temporarily suppressed during week 4 to 6 after blinding. Therefore, I wonder whether the functional changes of these SCN neurons involved in this hyperglycemic response might be reflected in changes in morphology of the SCN. Accordingly, I compared the effects of blinding on the hyperglycemic response to 2DG and the morphological structure of the SCN.

In this study the following results were obtained: 1) In rats blinded by bilateral orbital enucleation at the age of 4 weeks, the hyperglycemic response to intracranial injection of 2DG was significantly suppressed after 5 weeks (Fig. 1a), but not after 10 weeks (Fig. 1b). 2) In rats blinded at birth, however, temporal suppression of the hyperglycemic

response to 2DG was not observed 5 weeks after blinding (Fig. 1c). 3) In all groups of rats blinded unilaterally corresponding at times to those mentioned above, the structure of the SCN contralateral to the enucleated eye showed dramatic changes; that is, the SCN was markedly displaced rostrally, and the area occupied by Nissl-positive SCN cells and cell numbers in the middle to caudal part of the SCN were reduced (Figs. 2-9). 4) However, the total cell numbers in the SCN did not seem to be changed by orbital enucleation (Table I). 5) The cell density in the middle to caudal part of the SCN was significantly lower in rats of group 4W-5W than in those of groups 4W-10W and 1D-5W (Fig. 10), and cells containing VIP- and PHI-like immunoreactive substances in the middle to caudal part of the SCN were more loosely packed in the rats in group 4W-5W than in those in the other two groups (4W-10W and 1D-5W) (Figs. 4-6,11).

Considering the previous findings mentioned above, the parallelism that were observed between the temporary suppression of the hyperglycemic response to 2DG and the reduction in cell density in the middle to caudal part of the SCN (especially that of the SCN neurons containing VIP- and PHI-immunoreactive substances) in the rats of group 4W-5W suggests that the neurons in the middle to caudal part of the SCN are responsible for the hyperglycemic response to 2DG, and thus for glucose homeostasis. Furthermore, our findings suggest that these SCN neurons temporarily lose their neural activity during a certain period after the blinding. Moreover, the looseness of the packing of VIP- and PHI-positive cells found in the middle to caudal part of the SCN of the rats in group 4W-5W (Fig. 4) raises the possibility that the lack of innervation of these neurons from the retinal ganglion cells reduces their activity, and that these neurons containing VIP- or PHI-immunoreactive substances might be responsible for the hyperglycemic response to 2DG. These

possibilities are consistent with the following reported findings: 1) The RHT projects to the ventrolateral part of the middle to caudal part of the SCN [6, 11, 13, 14] and its neural fibers are in close contact with VIP-immunoreactive dendrites in the SCN [18]. 2) Immunoreactivity to anti-VIP antibody in fibers and terminals in the SCN but not in perikarya increases 7 days after blinding [18]. (The authors suggested that this was due to inhibition of the release of VIP). 3) The neurons located in the ventrolateral SCN receive only the excitatory input from the retinal axon [21]. 4) Electrical activity of neurons in slice preparations of the ventrolateral part but not the dorsomedial part of the SCN decreases 10 days after bilateral orbital enucleation [20].

In this experiment, neonatal blinding did not suppress the hyperglycemic response to 2DG (Fig. 1c). This finding was consistent with the morphological observation that the cell density of the middle to caudal part of the SCN of these rats in week 5 (group 1D-5W) was not less in the SCN contralateral to the enucleated eye than in the ipsilateral SCN (Fig. 10) and that the SCN cells were tightly packed in the contralateral SCN (Figs. 6 and 11). However, it is unknown why blinding at the age of 4 weeks temporarily suppresses the hyperglycemia due to 2DG, whereas blinding at birth does not. Young rats are generally thought to have more plasticity and the RHT has not yet formed synapses with the SCN neurons on the day of birth [9, 23], so at this stage the SCN neurons involved in the hyperglycemic response may be innervated by other neurons whose dysfunction was not detected. Therefore, this difference in changes in the hyperglycemic response might be explained by differences in plasticity of new born and 4-week-old rats. Tightness of neuron packing is considered to depend on innervation, so the morphological differences 5 and 10 weeks after blinding at the age of 4 weeks, could be due to loss of neural activity of the neurons responsible

for the hyperglycemic response in week 5 because of denervation from the retinal ganglion cells, and this reinnervation by neurons other than retinal ganglion cells, which restored their neural activity, and the hyperglycemic response in week 10.

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Table 1. Summations of cell numbers of the ipsilateral and contralateral SCN to the enucleated eye in brain sections (1 of every 3 sections of 25 μm thickness).

	4W-5W	4W-10W	1D-5W
Ipsilateral	3260	3310	3080
Contralateral	3190	3460	3060

Fig. 1. Effect of 2-deoxy-D-glucose injection into the lateral cerebral ventricle (LCV) on the plasma glucose concentration in blind rats in week 5 (a) and week 10 (b) after bilateral orbital enucleation at the age of 4 weeks and in week 5 after bilateral orbital enucleation at the age of 1 day (c) and of corresponding control rats. Statistical analyse were done by the t-test (*, $p<0.05$; **, $p<0.01$).

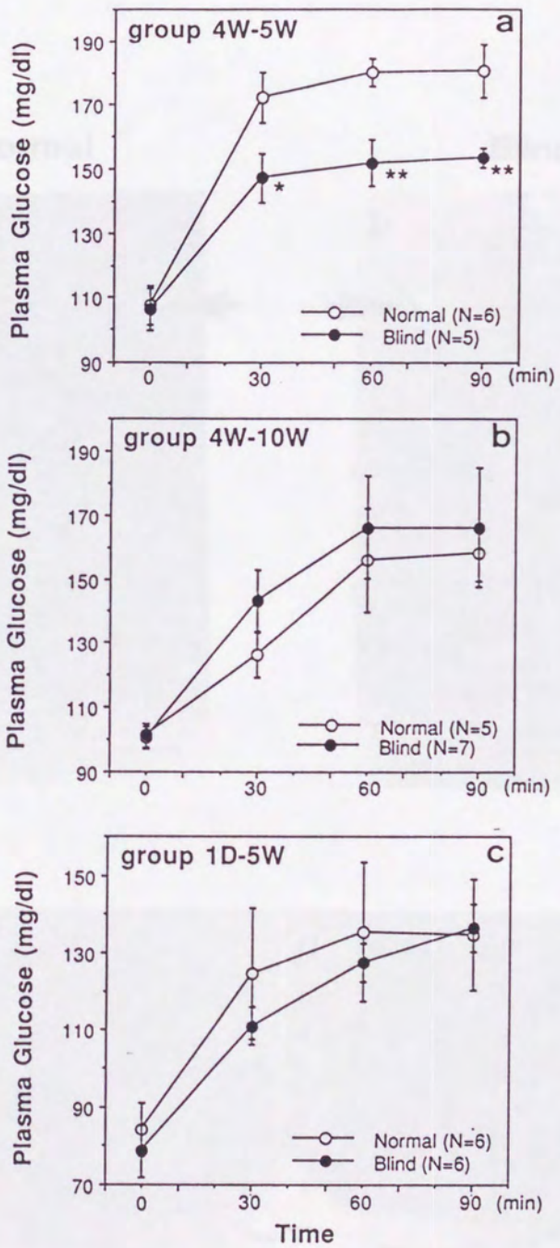


Fig. 2. Photomicrographs of coronal brain sections of control and blind rats immunohistochemically stained with anti-VIP antiserum.

The rat was blinded by bilateral orbital enucleation at the age of 4 weeks and sacrificed at 10 weeks later. Arrows indicate the anterior commisure (AC) (a and b). The SCN neurons containing VIP-like immunoreactive substance were observed at the level of the AC in the blind rat (b), but not in the control rat (a). The SCN neurons containing VIP-like immunoreactive substance were observed in more caudal parts in both normal and blind rats (c and d). (Bar = 500 μm)

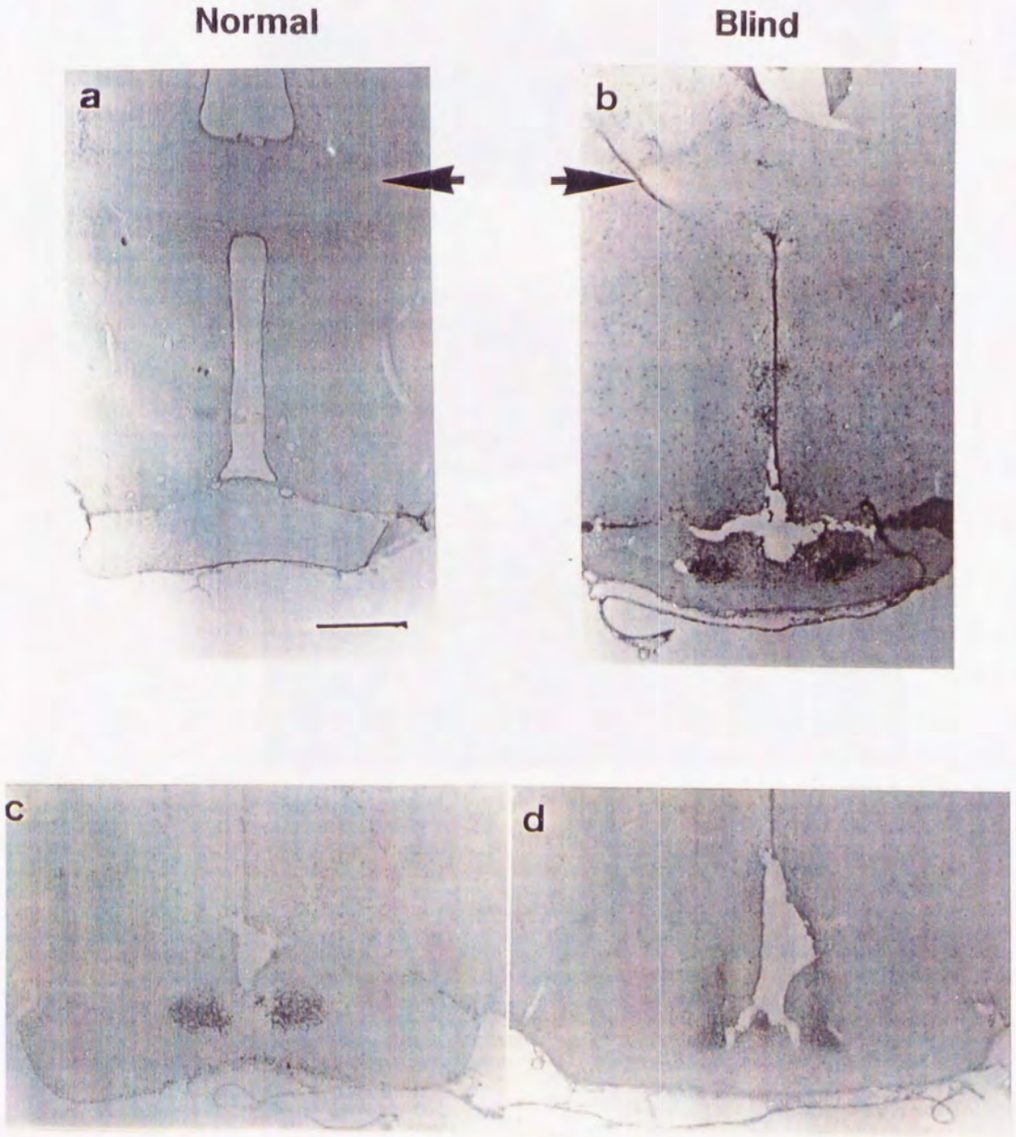
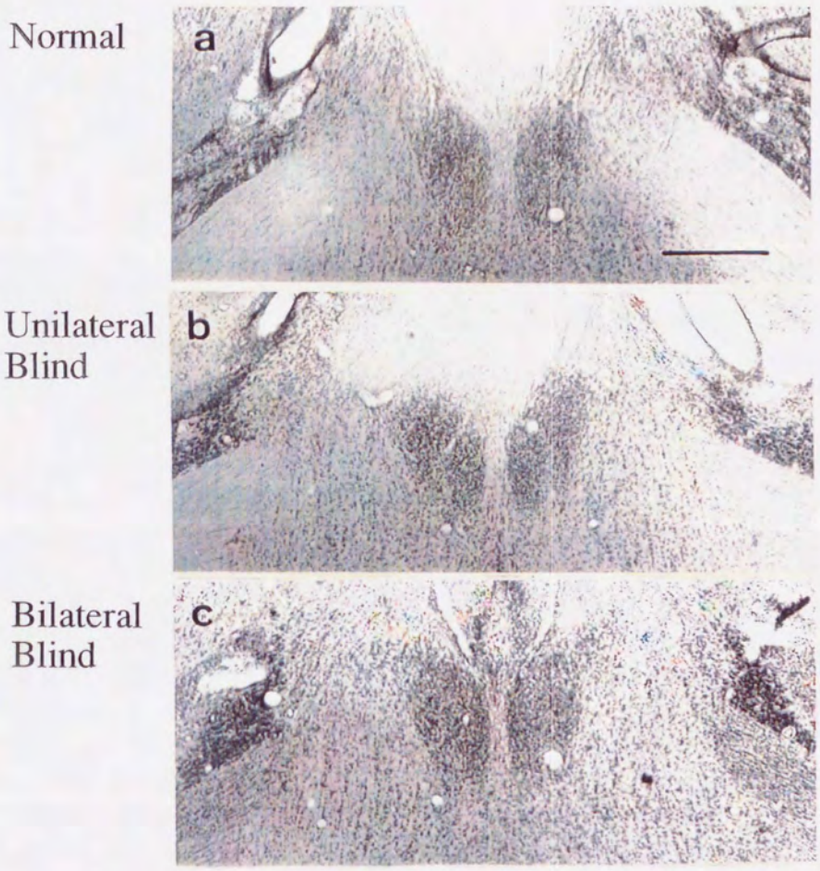


Fig. 3. Photomicrographs of horizontal brain sections stained with cresyl violet of a control rat (a), a rat with unilateral orbital enucleation (b) and a rat with bilateral orbital enucleation (c) 5 week after blinding at the age of 4 weeks.
In section b, the SCN contralateral to the enucleated eye is shown on the right side. (Bar = 500 μm)



Bar = 500 μm

Fig. 4. Photomicrographs of coronal brain sections of a rat with unilateral orbital enucleation (group 4W-5W).

Brain sections were stained with cresyl violet (a-d), anti-VIP serum (e-h) and anti-PHI serum (i-l). Sections of the SCN contralateral to the enucleated eye are shown on the right side. (Bar = 200 μm)

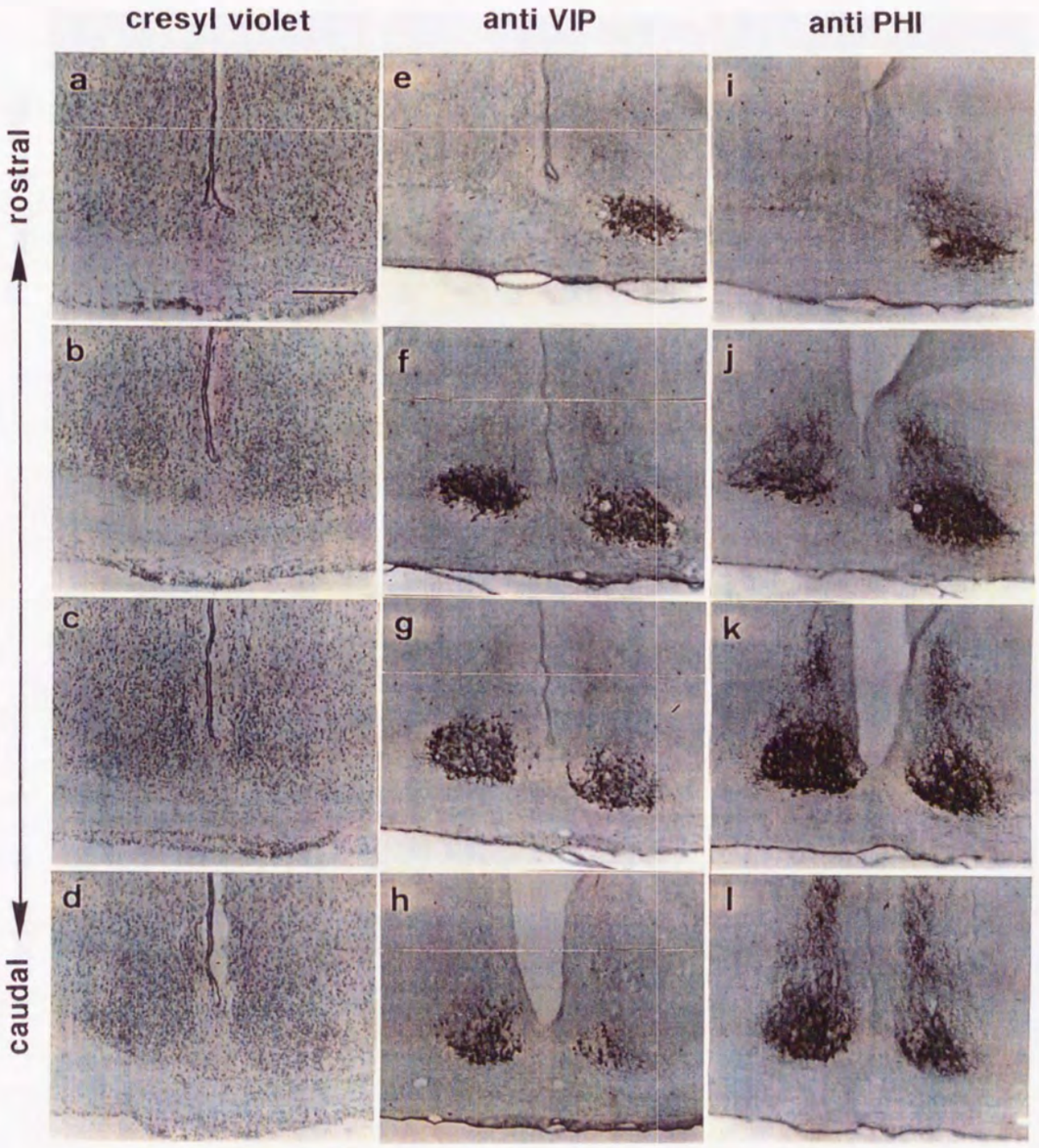


Fig. 5. Photomicrographs of coronal brain sections of a rat after unilateral orbital enucleation (group 4W-10W).
Explanations are the same as for Fig. 4. (Bar = 200 μm)

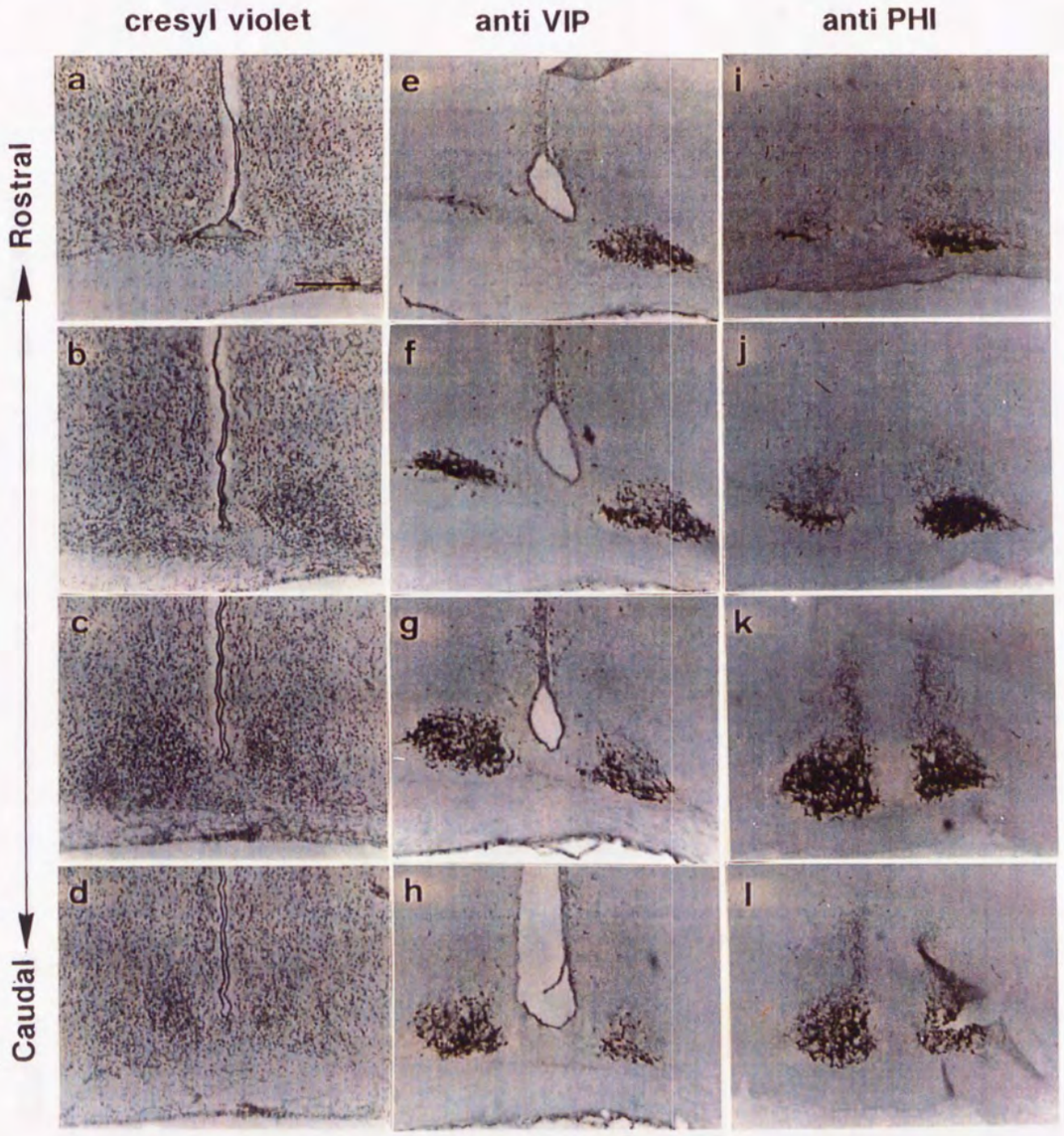


Fig. 6. Photomicrographs of coronal brain sections of a rat after unilateral orbital enucleation (group 1D-5W).
Explanations are the same as for Fig. 4. (Bar = 200 μm)

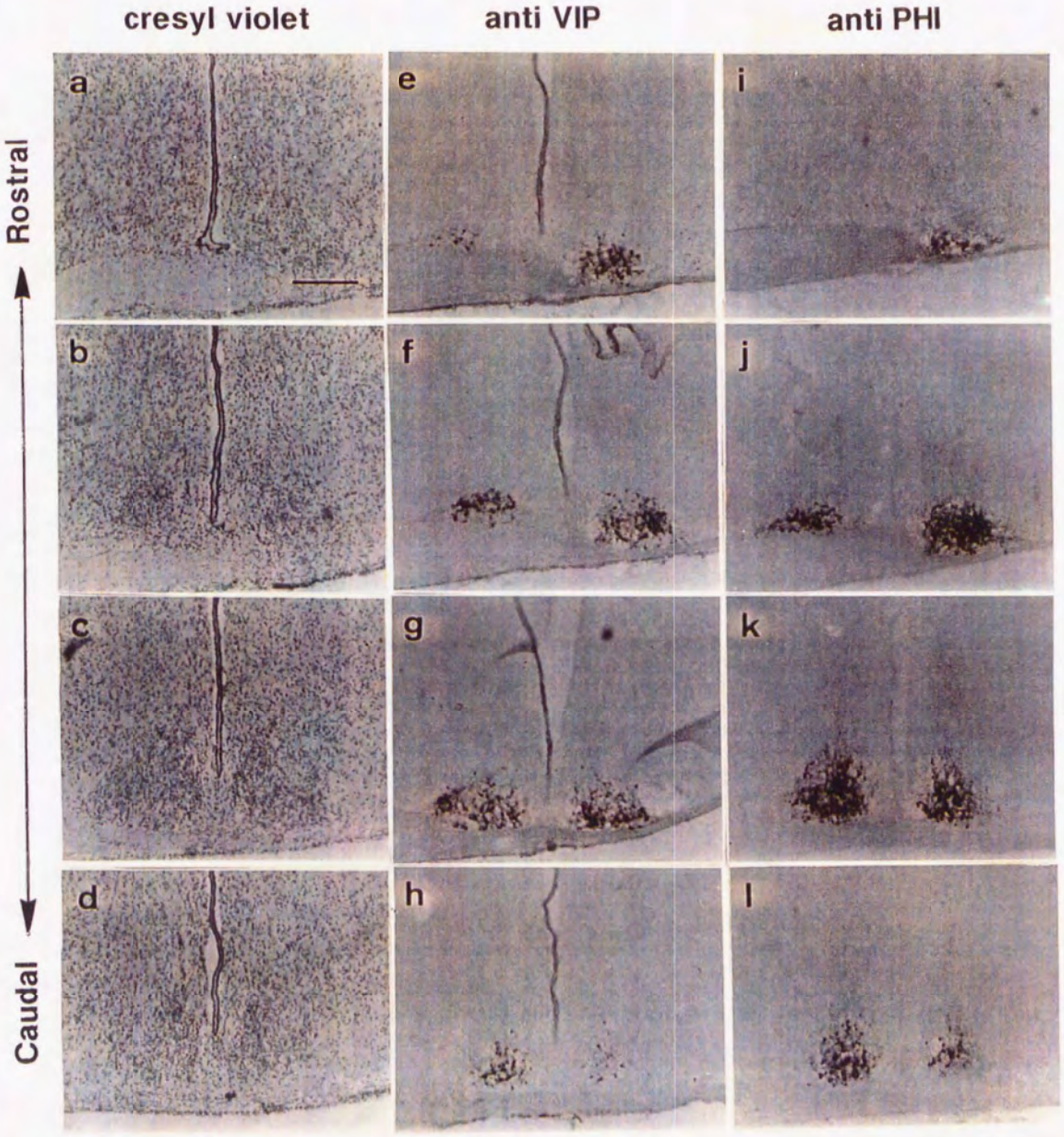


Fig. 7. Tracings of sequential coronal sections at 75 μm intervals of the SCN in a rat with unilateral orbital enucleation (group 4W-5W).

The SCN contralateral to the enucleated eye is displaced rostrally.

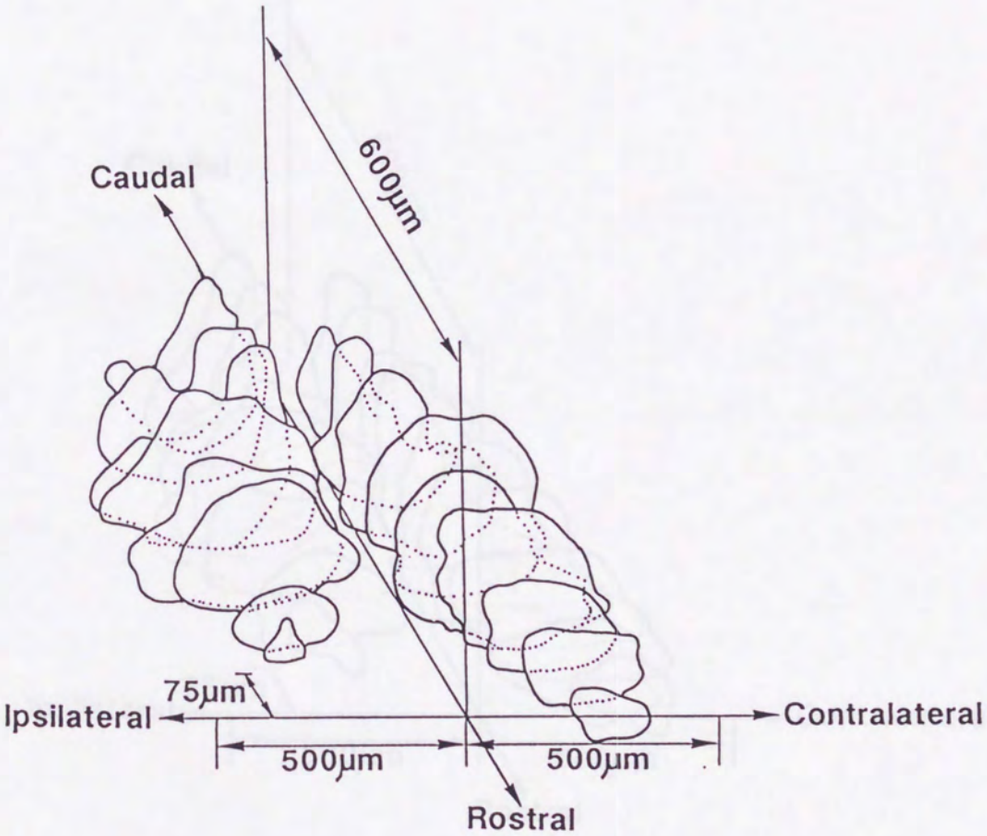


Fig. 8. Tracings of sequential coronal sections at 75 μm intervals of the SCN in a rat after unilateral orbital enucleation (group 4W-10W).

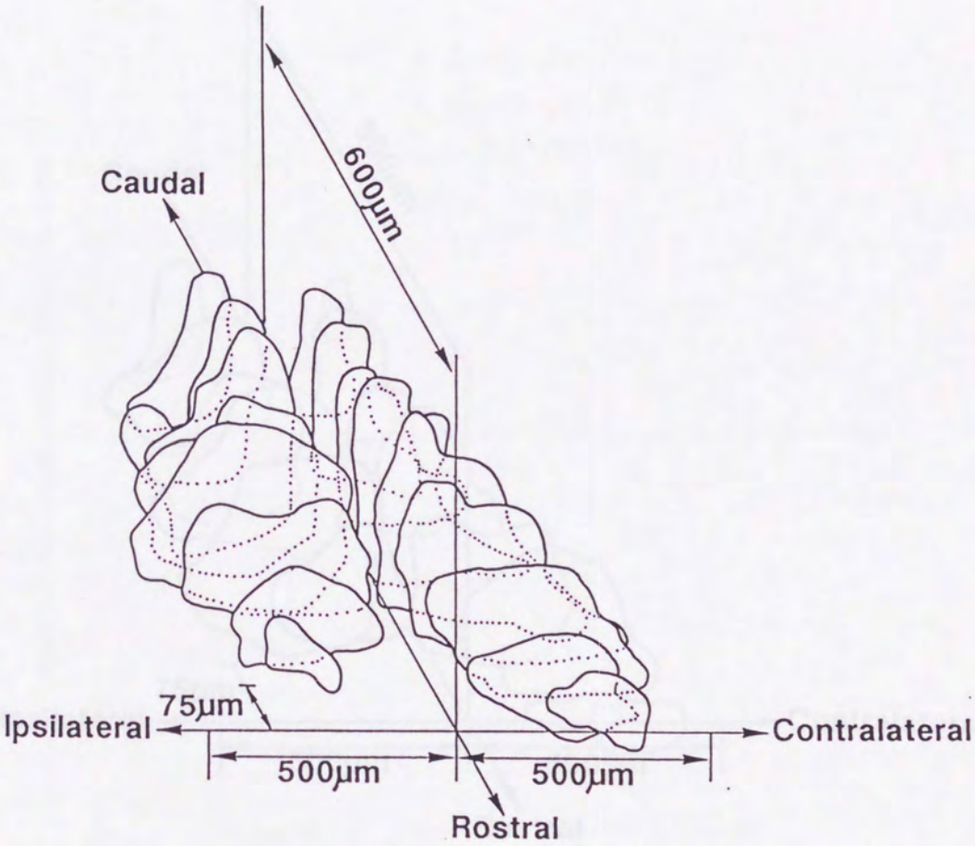


Fig. 9. Tracings of sequential coronal sections at 75 μm intervals of the SCN in a rat after unilateral orbital enucleation (group 1D-4W).

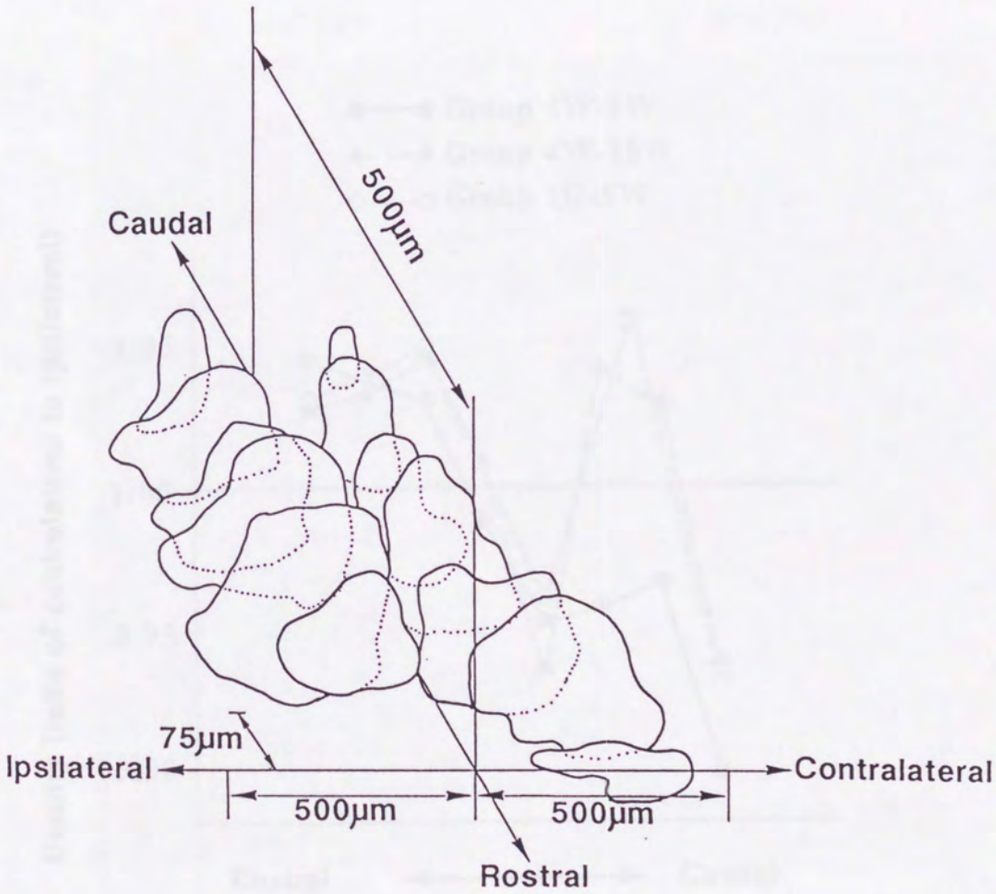


Fig. 10. Cell densities of the SCN of rats with unilateral orbital enucleation (groups 4W-5W, 4W-10W and 1D-5W).
Cell densities are shown as ratios of the cell density (cell numbers/SCN occupied area) of the SCN contralateral to the enucleated eye to that of the ipsilateral SCN along the rostrocaudal axis.

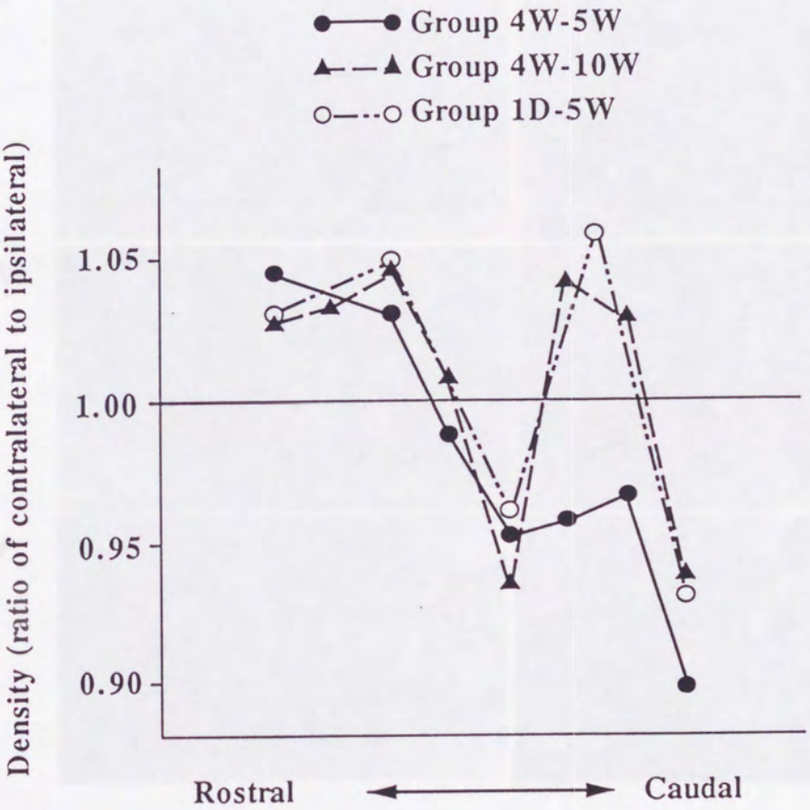
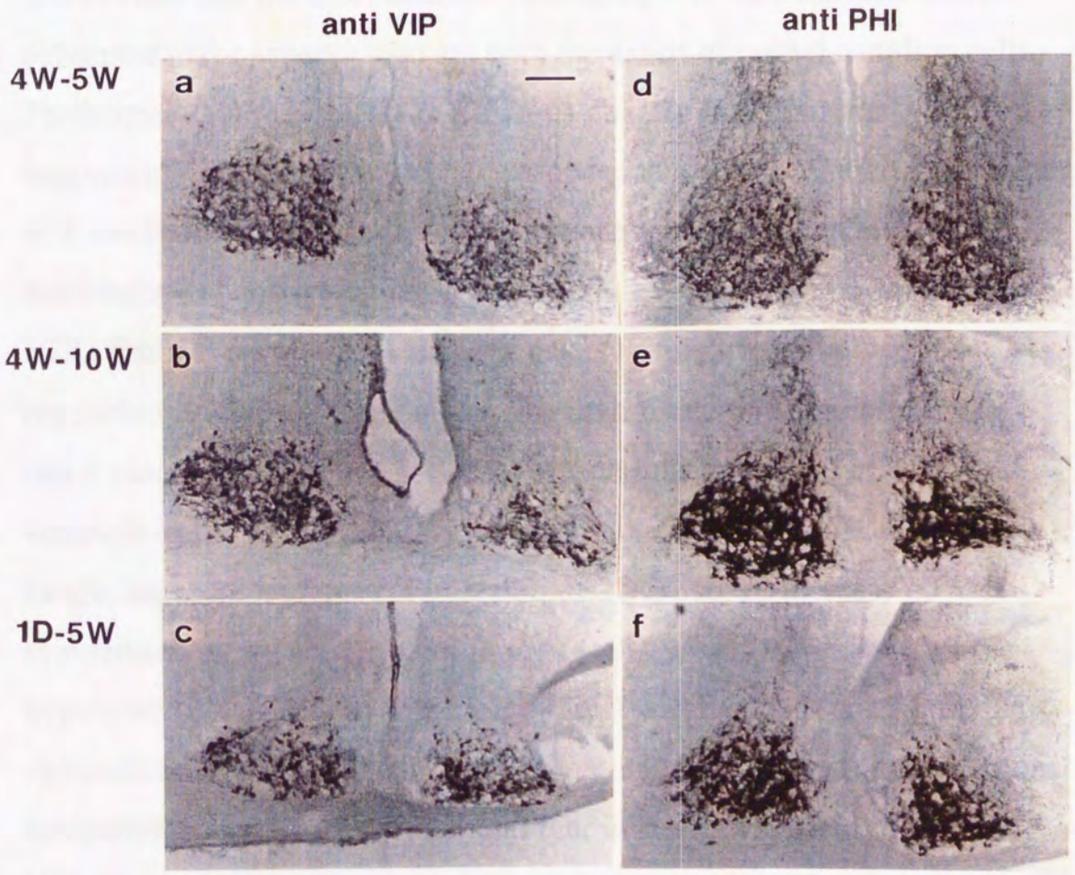


Fig. 11. Photomicrographs of coronal brain sections. Immunohistologically stained with anti-VIP and PHI antisera in the caudal part of the SCN in the three groups of rat after unilateral orbital enucleation. (Bar = 100 μm)



Chapter 2

Regulation of plasma glucose concentration by VIP included in the SCN

SUMMARY

We have presented several lines of evidence suggesting that in rats the neurons in the SCN are involved in the regulation of glucose metabolism. It was clear that the SCN neurons containing VIP-like immunoreactive substance make synaptic contact with the axons of retinal ganglion cells. Furthermore, it was shown in previous chapter that temporary suppression of the hyperglycemic response induced by 2DG from 4 weeks to 6 weeks after orbital enucleation were observed in parallel with morphological change of the SCN, especially those neurons containing VIP. From these results, I thought that VIP might be involved in regulation of glucose metabolism. To investigate this possibility, using rats I examined the effect of VIP injection into the lateral cerebral ventricle or the peritoneal cavity on the blood glucose level. On the other hands, intracerebroventricle injection of 2DG caused increase of sympathetic nerve activity. From the results, it was suggested that the hyperglycemia caused by injection of 2DG was induced through the sympathetic nervous system. I also examined the effect of VIP on adrenal sympathetic nerve activity. I found that in rats intracranial injection of VIP elicited hyperglycemia and hyperglucagonemia without hyperinsulinemia, although these responses showed no photoperiodicity, but intraperitoneal injection of the same dose of VIP had no effect on the plasma concentrations of glucose and insulin. It was also found that intracranial injection of VIP increased adrenal sympathetic nerve activity. Furthermore, I examined the effect of intracranial injection of VIP and VIP antagonist on the hyperglycemia and increase of sympathetic nerve activity caused by 2DG. It was found that the hyperglycemia and the

hyperglucagonemia caused by 2DG were enhanced synergistically by co-injection of VIP without the hyperinsulinemia, and that pre-injection of a VIP antagonist suppressed the hyperglycemia and the hyperglucagonemia caused by 2DG and also that increase of sympathetic nerve activity induced by 2DG was enhanced by injection of VIP. These facts suggest that endogenous VIP in the brain synergistically facilitate the hyperglycemia induced by 2DG through the regulation of sympathetic nerve activity.

INTRODUCTION

It has been shown in our laboratory that the SCN is involved in generation of circadian rhythms [19], and also that it is involved in regulation of glucose metabolism [11, 12, 27-29]. It has been shown that in rats the retinal neural input terminates in the ventrolateral part of the SCN [8], that the SCN neurons containing VIP-like immunoreactive substance (VIP-neurons) are localized in the area receiving the retinal neural input [8], and that the retinal ganglion cells make axo-somatic and axo-dendritic synaptic contacts with VIP-neurons in the SCN [4]. Furthermore, It was also found that the hyperglycemia induced by intracerebral injection of 2DG was temporarily suppressed in surgically blinded rats [29] in which the SCN, especially location of the VIP-neurons was morphologically abnormal [16], and that it was not observed in hereditary microphthalmic rats [13] in which the SCN is also abnormal [23]. These findings tempted me to speculate that the SCN neurons, especially VIP-neurons, are involved in the regulation of glucose metabolism. On the other hand, it was known that peripheral [17] and central [12] injections of 2DG induced increase of sympathetic nerve activities in rat liver, pancreas and adrenal. These findings suggest that

hyperglycemia induced by 2DG is caused through increase of sympathetic nerve activity. To clarify the role of the SCN neurons containing VIP-like immunoreactive substance, I examined the effect of intracranial injection of VIP on plasma glucose concentration and on sympathetic nerve activity. Furthermore, I examined the effects of VIP and VIP antagonist on hyperglycemia and the change in the sympathetic nerve activity induced by intracranial injection of 2DG.

MATERIALS AND METHODS

Animals

Forty-five male Wistar strain rats weighing 250-300 g were used. They were maintained at 24 ± 1 °C with lighting for 12 hours a day (0700-1900h) from fluorescent light bulbs (mean light intensity, 80 lux). Food (MF, Oriental Yeast, Co., Tokyo) and water were given freely available. The rats were adapted to the environmental conditions for more than one week.

Measurements of plasma glucose, glucagon and insulin concentrations

Three days before the experiments, a polyethylene cannula (PE-10) was inserted into the right lateral cerebral ventricle (LCV) by the method of Altaffer et al. [2], and another catheter made of silastic and polyethylene (PE-50) tubing was inserted into the right atrium of the heart by the method of Steffens [20] under pentobarbital anesthesia. VIP, a VIP antagonist (Sigma Chemical Co., St. Louis) and 2DG were injected in rats after they were dissolved in artificial cerebrospinal fluid (aCSF) [18]. The numbers of rats used in each experiment are indicated in the legends of figures. In the first experiment, 6 nmol of VIP (Peptide Institute Inc., Osaka) dissolved in a volume of 10 μl of aCSF or 10 μl of aCSF alone was injected into the LCV through the brain cannula or into the intraperitoneal cavity of animals that had been deprived of food for 2

hours from 1000h (light period) or 2200h (dark period). For measurements of concentrations of the plasma glucose and insulin , 200 μl of blood samples were withdrawn through the heart catheter 0, 30, 60 and 90 min after the injection. The effects of intraperitoneal injection of VIP on the plasma concentrations of glucose and insulin in the light period were also examined. For measurements of plasma concentrations of glucose, glucagon and insulin, blood samples (300 μl each) were withdrawn through the heart catheter 0, 5, 10, 20 and 30 min after VIP injection in the light period. On the second experiment, both 40 μmol of 2DG and 4 nmol of VIP dissolved in a final volume of 20 μl aCSF or each of them dissolved in a final volume of 20 μl aCSF or 20 μl of aCSF alone were injected through brain cannula of animals that had been deprived of food for 2 hours from 1000h. For measurements of plasma concentrations of glucose and glucagon, blood samples (200 μl each) were withdrawn through the heart catheter 0, 5, 10, 20 and 30 min after injection in the light period. On the third experiment, 1.5 μmol of VIP antagonist dissolved in a final volume of 20 μl of aCSF or 20 μl of aCSF were injected 30 min before the injection of 80 μmol of 2DG dissolved in a volume of 20 μl of aCSF through brain cannula to animals that had been deprived of food for 2 hours from 1000 h. No food available during the experiment. For measurements of plasma concentrations of glucose, glucagon and insulin, blood samples (200 μl each) were withdrawn through the heart catheter 30 min before and 0, 5, 10, 20 and 30 min after 2DG injection in the light period. Plasma glucose concentration was measured by the glucose oxidase method using a Fuji Dri-chem system (Fuji Film, Co., Tokyo). Plasma insulin and glucagon concentration were determined by radioimmunoassay using kits (Pharmacia Insulin RIA 100, Pharmacia, Uppsala, and Glucagon Kit "Daiichi", Daiichi Radioisotope, Tokyo) with human insulin and glucagon as standards. Data are

expressed as means ± standard errors of means. The significance of differences was assessed by analysis of variance by repeated measure of ANOVA.

Measurement of sympathetic nerve activity

The experiments were done during the period between 1200h and 1800 h. After the insertion of polyethylene cannula (PE-10) into the right LCV on the way that is described above under urethane anesthesia (1g/kg) on rat, nerve filaments were isolated from the central cut end of the adrenal branch of the splanchnic (sympathetic) nerve and located on a pair of silver wire electrodes for recording the efferent nerve activity. VIP (6 nmol), 2DG (80 μmol) and VIP (3 nmol) after 2DG (80 μmol) dissolved in aCSF were injected into the right lateral cerebral ventricle through brain cannula. Electrical activity of the nerve was amplified and displayed on an oscilloscope and stored on digital audio tape. The activity was analyzed after conversion of raw data to standard pulse with a window discriminator that separated discharges from background noise. A rate-meter with a 5-s reset time was used to observe the time course of the nerve activity, which was recorded with a pen recorder.

RESULTS

Effect of intracranial injection of VIP on plasma glucose, glucagon and insulin concentrations

I examined the effect of intracranial VIP injection on the plasma concentrations of glucose and insulin in the light and dark periods. As shown in Fig. 1, VIP injection caused significant, photoperiod-independent hyperglycemia (light=dark). The plasma insulin level did not change and was also independent of the light phase. The glucose level reached a peak at 30 min after VIP injection and then decreased to the preinjection level. I also examined the effect of VIP injection on the

plasma glucagon concentration. Injection of VIP into LCV elicited a marked hyperglucagonemia with a maximum at 10 min after injection (Fig. 2B), while the plasma glucose level reached a peak 20 to 30 min after injection in this experiment (Fig. 2A). The plasma insulin concentration showed a slight, but insignificant increase 5 min after VIP injection (Fig. 2C).

Effect of intraperitoneal injection of VIP on plasma glucose, glucagon and insulin concentrations

Next, it was examined the problem whether the effect of intracranial injection of VIP was elicited through its action in the brain or peripheral organs. As shown in Fig. 3, the same dose of VIP as injected into the brain (6 nmol) did not have any significant effect on either the plasma glucose or insulin level when injected intraperitoneally.

Effect of VIP on sympathetic nerve activity

Figure 4 shows the effect of the intracerebroventricle injection of VIP on adrenal sympathetic nerve activity. After the injection, sympathetic nerve activity was increased. The increase was gradual and continued for more than 150 min.

Effects of VIP on plasma glucose, glucagon and insulin responses induced by 2DG

In Fig. 5, the effects of intracranial injection of VIP on hyperglycemia (Fig. 5A) and hyperglucagonemia (Fig. 5B) due to intracranial injection of 2DG are shown. Changes in the plasma glucagon are shown by percentages of the levels before injection. Injection of 40 μmol of 2DG caused only a slight increase in the plasma glucose level up to 150 mg/dl (30 min after injection), and injection of 4 nmol of VIP also caused a slight increase in it up to 145 mg/dl (20 min after injection). However, coinjection of 2DG and VIP induced a marked hyperglycemia that has a maximum of 250 mg/dl 30 min after the injection. The hyperglycemia

induced by coinjection of 2DG and VIP was significantly greater than the hyperglycemia induced by injection of 2DG or VIP alone. Injection of aCSF did not affect the plasma glucose concentration.

Furthermore, 2DG (40 μmol) injection caused a slight increase in the plasma glucagon level that has a peak of 130 % 10 min after injection and VIP injection caused increase that has a peak of 190 % 5 min after injection, however, coinjection of these two caused a dramatic and synergistic increase in the glucagon level that has a peak of 420 % 10 min after injection. The hyperglucagonemia induced by coinjection of 2DG and VIP was significantly greater than hyperglucagonemia induced by injection of 2DG or VIP alone. Injection of aCSF did not affect the plasma glucagon concentration.

Effects of a VIP antagonist on plasma glucose, glucagon and insulin responses induced by 2DG

In Fig. 6, the effects of pre-injection of a VIP-antagonist on the plasma level of glucose (Fig. 6A), glucagon (Fig. 6B) and insulin (Fig. 6C) after intracranial injection of 2DG are shown. Responses of the plasma glucagon and insulin were shown by percentages of the levels before injection. Intracranial injection of 80 μmol of 2DG caused hyperglycemia that has a maximum of 270 mg/dl 60 min after injection. The injection of VIP-antagonist alone did not affect the plasma glucose level 30 min after its injection, however, it suppressed hyperglycemia and the peak value was 185 mg/dl 60 min after 2DG-injection (Fig. 6A). The 2DG injection also caused hyperglucagonemia that has a peak of 480 % 20 min after injection. The injection of VIP-antagonist did not affect plasma glucagon level 30 min after its injection, however, it remarkably suppressed hyperglucagonemia caused by 2DG and the peak value was 190 % 90 min after the 2DG injection (Fig. 6B). Plasma insulin concentration showed a slight increase and the maximum level was 200%

90 min after 2DG-injection, which was paralleled with the increase of the plasma glucose concentration. Injection of the VIP-antagonist did not affect the increase in the plasma insulin level after the 2DG injection (Fig. 6C).

Effect of VIP on the changes in the sympathetic nerve activity induced by 2DG

Figure 7 shows the effect of the intracerebroventricle injection of 4 nmol of VIP on the increase in the electrical activity of the sympathetic efferents to the adrenal after injection of 80 μmol of 2DG. Injection of 2DG alone increased the activity. This increase was gradual and observed for more than 120 min (Fig. 7A). Furthermore, the increase in the electrical activity of the sympathetic efferents to the adrenal induced by 2DG was immediately enhanced by the injection of VIP (Fig. 7B).

DISCUSSION

As described in the previous chapter, we have obtained evidence that the SCN neurons which receive retinal neural inputs might be involved in the regulation of the plasma glucose concentration [11, 12, 27-29]. There are reports that retinal ganglion cells innervate the SCN directly [3, 9], and that some of their projections make axo-somatic and axo-dendritic synaptic contacts with VIP-neurons in the SCN [4]. From these findings, I speculated that the VIP-neurons in the SCN might be involved in the regulation of glucose metabolism.

VIP is a brain-gut peptide and receptors for VIP exist in peripheral organs as well as in the brain [5]. VIP is thought to function as a hormone in peripheral organs [21], and as a neurotransmitter or a neuromodulator at nerve terminals [6, 7]. In this study, I found that intracranial injection of VIP caused a hyperglycemia when injected into the LCV (Fig. 1 A,B), but not when injected into the peritoneal cavity

(Fig. 3). These findings suggest that VIP is involved in the regulation of glucose metabolism in the central nervous system. Furthermore, from the present results that the hyperglycemic and the hyperglucagonemic responses to 2DG were synergistically enhanced by co-injection of VIP (Fig. 5 A,B) and suppressed by pre-injection of a VIP-antagonist (Fig. 6 A,B), it was suggest that VIP has a role to facilitate 2DG-hyperglycemia.

Since in preliminary experiments I observed synergistic effects of VIP and suppressive effects of a VIP-antagonist on the hyperglycemic and hyperglucagonemic responses to intracranial injection of 2DG, I used submaximal doses of 2DG (40 μmol) and VIP (4 nmol) in the second experiment (Fig. 5) and a higher dose of 2DG (80 μmol) in the third experiment (Fig. 6), in order to obtain clear results. In the second experiment 2DG or VIP alone did not induce a marked hyperglycemia and hyperglucagonemia.

We reported previously that the hyperglycemia induced by intracranial injection of 2DG showed a photoperiodicity (light period > dark period) [15]. However, hyperglycemia induced by VIP did not show a photoperiodicity (light = dark) (Fig. 1). These results might indicate that in the neural network responsible for the induction of hyperglycemia VIP acts at the point downstream to circadian oscillator in the SCN. Furthermore, it was found in our laboratory that the hyperglycemia and hyperglucagonemia induced by 2DG was not observed in rats with bilateral lesions of the SCN [27, 28]. It was reported that the amount of VIP in the SCN decreased during the light period under a light-dark cycle with a period of about 24 hours [1, 10, 24] and that VIP content in the medium of the rat SCN-slice preparation increased after the electrical stimulation of the optic nerve (Shibata et al., personal communication). From these findings it is likely that the VIP-neurons in the SCN modulate the hyperglycemic and hyperglucagonemic responses to 2DG; that is, the

disappearance and the suppression of these responses in rats with bilateral lesions of the SCN [27,28] and in surgical [14, 29] and congenital [13, 14] blind rats were ascribed to defect and functional deterioration of these VIP-neurons in the SCN, and that the photoperiodicity in the hyperglycemic response to 2DG [15] was due to the amount of VIP released from the terminal of the VIP-neurons.

It was shown that peripheral and central injections of 2DG enhanced the activity of sympathetic nerves [12, 17], and that this increase of sympathetic activity might increase glucagon release from the pancreas through direct neural input to the pancreas or through the enhancing release of adrenaline from the adrenal medulla. In this experiment I found that in intact rats intracerebroventricle injection of VIP increased the sympathetic efferents to the adrenal (Fig. 4). Furthermore, the increase in the neural activity of the sympathetic efferents to the adrenal was also observed by the intracranial injection of 2DG and this increase was exaggerated by the intracranial injection of VIP (Fig. 7). From the findings that VIP containing neurons in the SCN projected to the ventromedial nucleus, dorsomedial nucleus and paraventricular nucleus of the hypothalamus which are involved in the regulation of the autonomic nervous system [25, 26], it is possible that VIP acts on these nuclei and enhances the hyperglucagonemic response to 2DG through the regulation of the sympathetic nervous system. In this connection, we observed that exposure of the left eye of rats to light (2,000 lux, 10 min) increased the sympathetic nerve activities for more than 2 hours and these changes were eliminated by bilateral lesion of the SCN [18], and it was known that VIP immunoreactive neurons in the SCN are innervated directly form retinal ganglion cells [4]. These lines of evidence support the above possibility.

In regard to the regulatory mechanism of VIP on glucose metabolism, it is likely that VIP essentially stimulates 2DG-hyperglycemia from the following our preliminary results. 1) The increase in the plasma glucose concentration of intact rats due to intracranial injection of VIP was not dose-dependent. 2) Intracranial injection of either VIP or 2DG alone into the rats with bilateral lesions of the SCN did not elevate the activity of the sympathetic efferents to the adrenal, while, coinjection of these two did. If this is the case, the hyperglycemia elicited by VIP injection into the lateral cerebral ventricle may be explained by the cooperation of VIP and the stress induced by the intracranial injection itself.

In conclusion, these results suggest that VIP-neurons in the SCN has a role to facilitate the hyperglycemic and hyperglucagonemic responses induced by intracranial injection of 2DG through the stimulatory modulation of sympathetic activity.

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Fig. 1. Effects of intracranial injection of VIP on the plasma concentrations of glucose (A,B) and insulin (C,D) in the light (A,C) and dark (B,D) periods.

VIP (6 nmol) in artificial cerebrospinal fluid (aCSF) only (as a control) was injected into the right lateral cerebral ventricle. Each group consisted of 5 rats. The significance of differences between groups (0-120 min) were analyzed by ANOVA. N.S., not significant

comparison	factor	materials	significance	
aCSF vs. VIP	light	glucose	$P < 0.05$	($F = 5.407$)
		insulin	N.S.	($F = 0.082$)
	dark	glucose	$P < 0.005$	($F = 23.696$)
		insulin	N.S.	($F = 0.038$)
light vs. dark	VIP	glucose	N.S.	($F = 0.284$)
		insulin	N.S.	($F = 0.968$)
	aCSF	glucose	N.S.	($F = 1.104$)
		insulin	N.S.	($F = 2.884$)

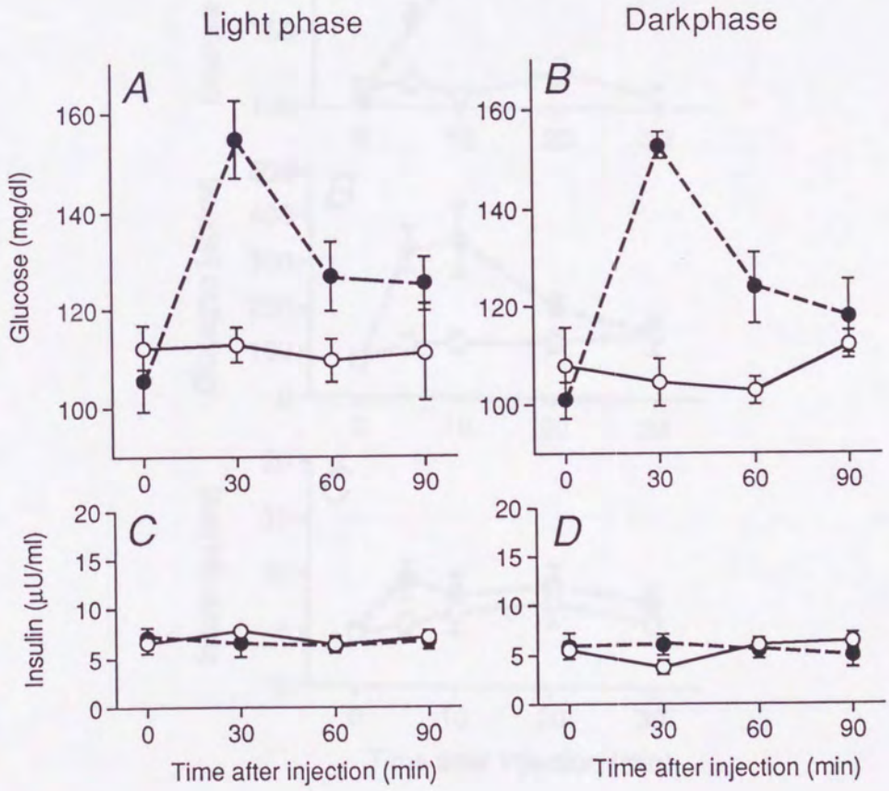


Fig. 2. Effects of intracranial injection of VIP on the plasma concentrations of glucose (A), glucagon (B) and insulin (C) in the light period.

VIP (6 nmol) or artificial cerebrospinal fluid (aCSF) only (as a control) was injected into the right lateral cerebral ventricle. The VIP- and aCSF-treated groups consisted of 6 and 5 rats, respectively. The significance of differences between groups (0-30 min) were analyzed by ANOVA. N.S., not significant

comparison	material	significance	
aCSF vs. VIP	glucose	$P < 0.001$	($F = 26.753$)
	glucagon	$P < 0.05$	($F = 7.923$)
	insulin	N.S.	($F = 1.074$)

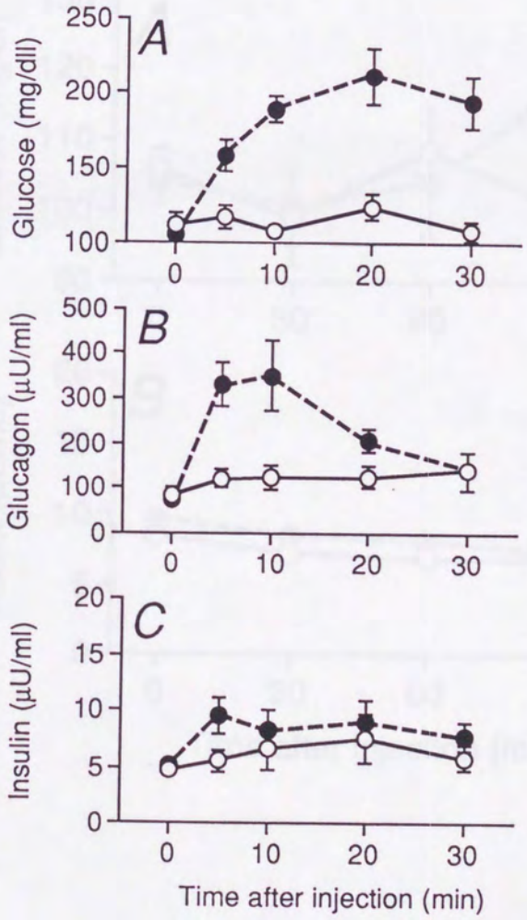


Fig. 3. Effects of intraperitoneal injection of VIP on the plasma concentrations of glucose (A) and insulin (B) in the light period.

VIP (6 nmol) or artificial cerebrospinal fluid (aCSF) only (as a control) was injected into the peritoneal cavity. Each group consisted of 5 rats. The significance of differences between groups (0-120 min) were analyzed by ANOVA. N.S., not significant

comparison	material	significance
aCSF vs. VIP	glucose	N.S. (F =0.37)
	insulin	N.S. (F=4.119)

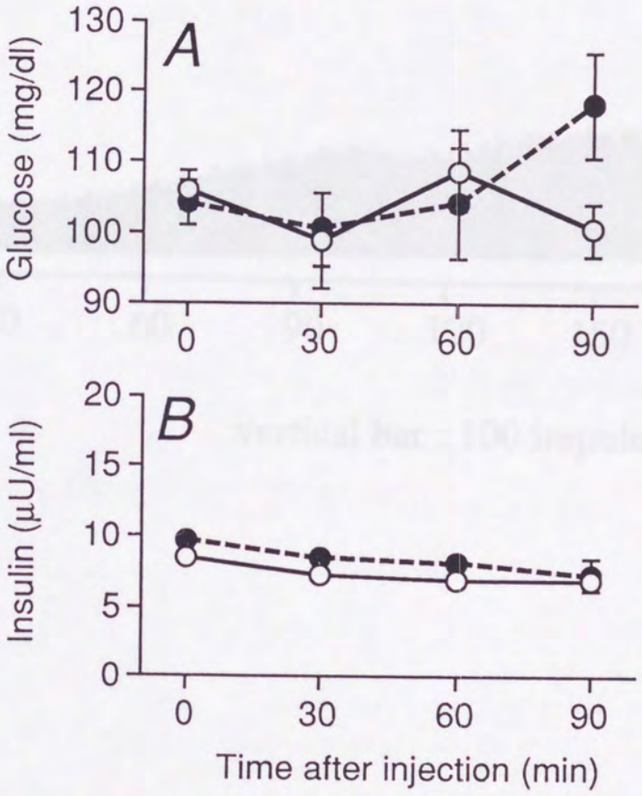


Fig. 4. Effect of intracerebroventricle injection of VIP on the efferent activity of the adrenal branch of splanchnic nerve.

VIP (6 nmol) was injected into the lateral cerebral ventricle. Horizontal bar : 10 min, vertical bars : 100 impulses / 5 s.

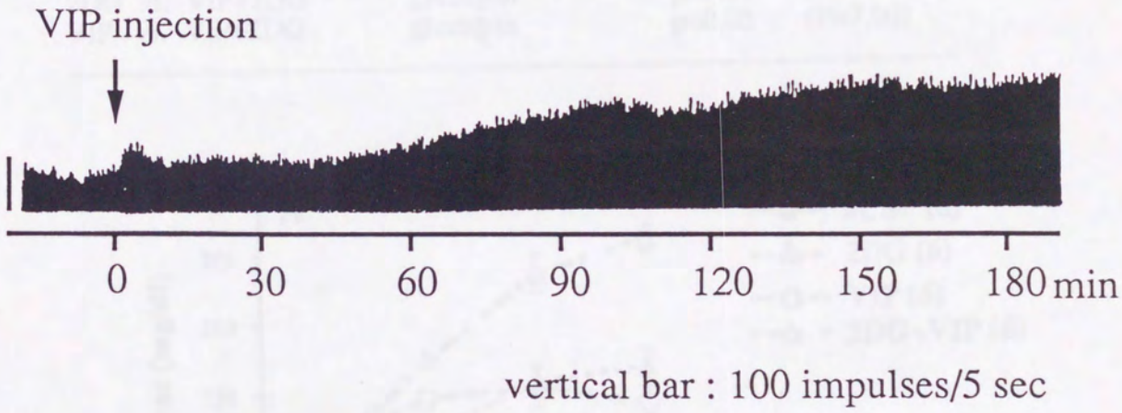


Fig. 5. Effects of intracerebroventricle injection of 2DG (40 μmol) plus VIP (4 nmol) on the plasma concentrations of glucose (A) and glucagon (B)

Data are expressed as means ± S. E. M. for groups of 6 animals. Data on plasma glucagon concentrations are shown as percentages of the values at 0 min, which were 85 ± 12 pg/ml (aCSF), 90 ± 13 pg/ml (2DG), 98 ± 22 pg/ml (VIP), and 93 ± 17 pg/ml (2DG + VP), respectively. Differences between these initial values were not statistically significant by the Mann-Whitney *U*-test. Other explanations are given in the text. The significances of differences between groups (5-30 min) were analyzed by ANOVA.

Comparison	Material	Significance	
aCSF *vs.* 2DG	glucose	0.05<p<0.1	(F=4.704)
aCSF *vs.* VIP	glucose	p<0.05	(F=6.815)
aCSF *vs.* VIP+2DG	glucose	p<0.0001	(F=107.6)
2DG *vs.* VIP+2DG	glucose	p<0.0005	(F=31.01)
VIP *vs.* VIP+2DG	glucose	p<0.001	(F=25.9)
aCSF *vs.* VIP+2DG	glucagon	p<0.05	(F=8.212)
2DG *vs.* VIP+2DG	glucagon	p<0.01	(F=11.16)
VIP *vs.* VIP+2DG	glucagon	p<0.05	(F=7.00)

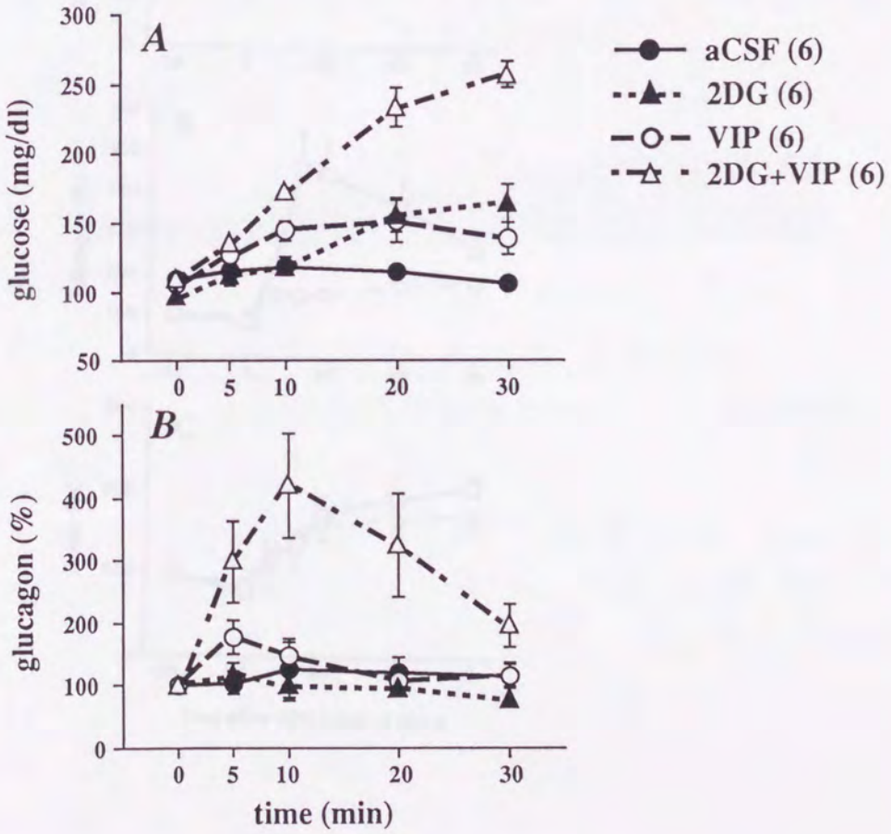


Fig. 6. Effects of intraperitoneal injection of 2DG and VIP-antagonist (VIPa) on the plasma concentrations of glucose (A), glucagon (B) and insulin (C).

2DG (40 μmol) plus VIPa (4 nmol) or 2DG alone was injected into the lateral cerebral ventricle. Each group consisted of 5 rats. The significance of differences between groups (0-120 min) were analyzed by ANOVA. N.S., not significant

Material	Significance	
glucose	$p<0.05$	($F=8.61$)
glucagon	$p<0.001$	($F=25.53$)

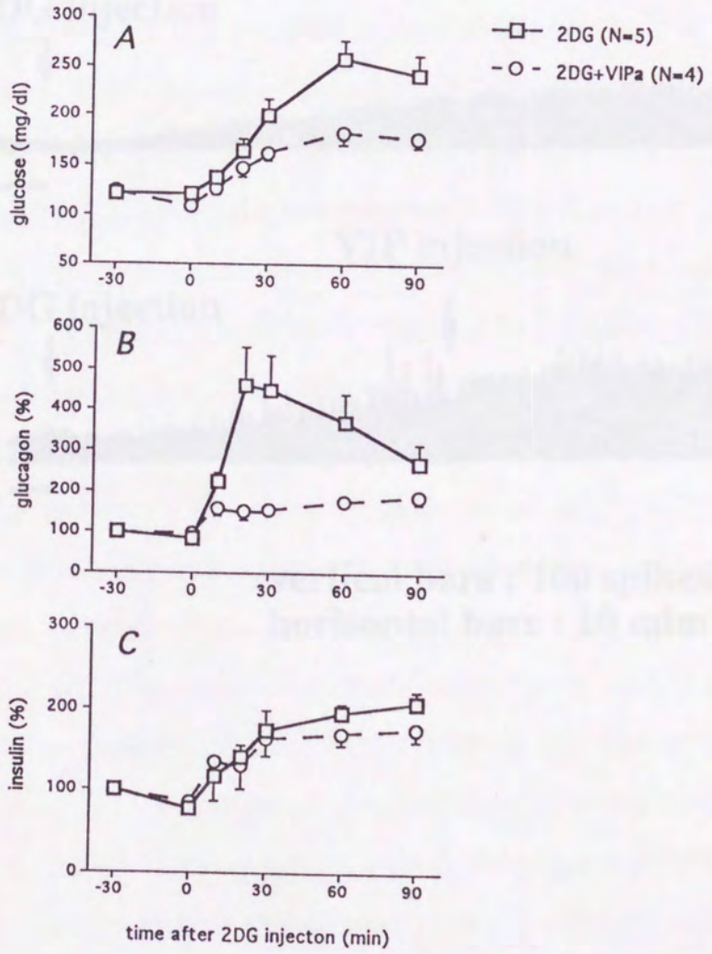
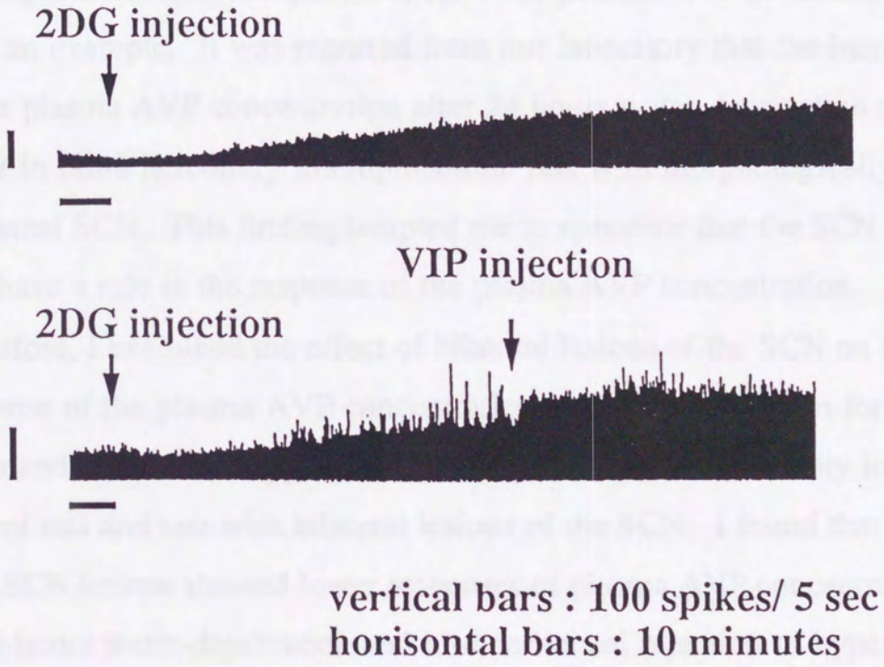


Fig. 7. Effects of intracerebroventricle injection of 2DG and VIP on the activity of the sympathetic efferents to the adrenal in intact rats.

2DG (80 μmol) and VIP (4 nmol) were injected into the lateral cerebral ventricle. Horizontal bar : 10 min, vertical bars : 100 impulses / 5 s.



Chapter 3

Modulation by VIP of the response of plasma AVP concentration to water-deprivation and osmotic challenge

SUMMARY

In the previous chapters, it was shown that VIP in the SCN has a stimulatory role on the hyperglycemia after intracranial injection of 2DG. On the other hands, it is known that various internal parameters change with circadian rhythms, e.g. the plasma glucose concentration, blood pressure, body temperature, etc. The plasma AVP concentration is such an example. It was reported from our laboratory that the increase in the plasma AVP concentration after 24 hours water-deprivation did not occur in blind hereditary microphthalmic rats with morphologically abnormal SCN. This finding tempted me to speculate that the SCN might also have a role in the response of the plasma AVP concentration. Therefore, I examined the effect of bilateral lesions of the SCN on the response of the plasma AVP concentration to water-deprivation for 24 hours and injection of hypertonic saline into the peritoneal cavity in control rats and rats with bilateral lesions of the SCN. I found that rats with SCN lesions showed lower responses of plasma AVP concentration to 24 hours water-deprivation and intraperitoneal injection of hypertonic saline. Furthermore, in order to clarify the role of VIP, I examined the effects of intracerebroventricular injections of VIP and a VIP-antagonist on the plasma AVP response after injection of hypertonic saline. In this experiment, it was found that increases in the plasma AVP concentration after 24 hours water-deprivation and osmotic challenge were significantly suppressed in rats with SCN lesions. Moreover, I found that the plasma AVP response after injection of hypertonic saline was synergistically enhanced by intracerebroventricle injection of VIP and suppressed by the

intracranial injection of a VIP-antagonist . These results suggest that the SCN has an important role in the regulation of plasma AVP concentration, and show a possibility that VIP-neurons in the SCN modulate the plasma AVP response to these stressful challenges.

INTRODUCTION

In mammals, the SCN act as an oscillator of the circadian rhythms which are synchronized by the light-dark cycle with a period of about 24 hours [9, 15]. In the previous chapters, I presented evidences suggesting that the SCN might be involved in the control of glucose metabolism [11, 20, Chapters 1 & 2] and that the neurons containing VIP in the SCN have an essential role in the hyperglycemic response to intracerebroventricular injection of 2DG [Chapter 2]. On the other hand, it was reported that water-deprivation and administration of hypertonic saline caused an increase in the plasma AVP concentration [4]. AVP acting on peripheral organs as an antidiuretic hormone is synthesized in magnocellular part of the PVN and SON and is transported to the posterior pituitary and released into the blood. We reported previously that the increase in the plasma AVP concentration after 24 hours water-deprivation was suppressed in hereditary microphthalmic rats with morphologically abnormal SCN [13]. Furthermore, it was reported that intracerebroventricle injection of VIP induced an increase in the plasma AVP concentration [3, 10]. To confirm the role of the VIP-neurons in the SCN on regulation of plasma AVP concentration, I examined whether intraperitoneal injection of hypertonic saline increased the plasma AVP concentration in rats with SCN lesions and also whether intracerebroventricular injection of VIP had any effect on the plasma AVP response to the hypertonic saline injection.

MATERIALS AND METHODS

Animals

Forty-five male Wistar strain rats weighing 250-300 g were used. They were kept in a room maintained at 24 ± 1 °C illuminated for 12 hours a day (0700-1900) by fluorescent light bulbs (mean light intensity, 80 lux). Food (MF, Oriental Yeast, Co., Tokyo) and water were given freely. The rats were adapted to the environmental conditions for more than one week. Bilateral electrolytic lesions of the SCN were done in 10 rats as described previously [12]. In brief, under pentobarbital anesthesia rat head was fixed in a stereotaxic apparatus (Narisige, Tokyo). After making a hole in the skull near the bregma, an electrode was inserted in the SCN, and the SCN was lesioned by applying a direct current of 1 mA for 5 sec. After lesioning, locomotor activity of these rats were monitored under constant dark condition for more than 4 days. Only rats showing the complete loss of the circadian rhythm more than 2 weeks after the lesioning were used in this study. After the experiments, their brains were histochemically examined and the position of lesions were identified.

Measurement of plasma AVP concentration

Three day before the experiments, a polyethylene cannula (PE-10) was inserted into the right lateral cerebral ventricle (LCV) by the method of Altaffer et al. [2] and another catheter made of silastic and polyethylene (PE-50) tubes was inserted into the right atrium of the heart by the method of Steffens [16] under pentobarbital anesthesia. The numbers of rats used in each experiment are indicated in the legends of figures. In the first experiment, 4 ml of 3.6 % saline were injected into the intraperitoneal cavity of normal rats and rats with SCN lesions which had been deprived of water and food for 2 hours from 1000 h. For the measurement of the plasma AVP concentration, 400 μl of blood samples

were withdrawn through the heart catheter 0, 15, 30, 60 and 90 min after injection. In the second experiment, VIP or aCSF was injected into rats through the brain cannula soon after the intraperitoneal injection of 3.6 or 0.9% saline. Seven rats were injected with VIP after the injection of 3.6 % saline, 6 rats with aCSF after the injection of 3.6 % saline , 8 rats with VIP after injection of 0.9 % saline and 4 rats with aCSF after the injection of 0.9 % saline. Blood samples were withdrawn at the same time points as in the first experiment. In the third experiment, rats were injected with a VIP-antagonist or aCSF through brain cannula soon after the intraperitoneal injection of 7.2 % saline. No food was available during the experiment. Blood samples were also withdrawn at the same time points as in the first experiment.

After centrifuged at 3.000 x g for 5 min, plasma samples were obtained and stored at -20 ^{o}C until the measurement of AVP concentration. The plasma AVP concentration was determined by radioimmunoassay using a kit (Mitsubisi Kagaku, Tokyo). Data are expressed as means ± standard errors of means. The statistical significances of differences were assessed by analysis of variance (ANOVA) by repeated measure.

RESULTS

Figure 1 shows changes in the plasma AVP concentration after water-deprivation for 24 hours in control and SCN-lesioned rats. In control rats, the plasma AVP level was 1.2 ± 0.3 pg/ml and 10.8 ± 1.2 pg/ml, before and after the water-deprivation, respectively, and these values showed statistically significant difference. In SCN-lesioned rats, the plasma AVP level was similar as normal, 0.8 ± 0.2 pg/ml and 4.8 ± 1.2 pg/ml before and after the water-deprivation, respectively, and difference of these values were also statistically significant. However, the difference

between the plasma AVP level before and after the water-deprivation was significantly lower in the SCN-lesioned rats than in the control rats.

Figure 2 shows changes in the plasma AVP level after intraperitoneal injections of hypertonic (3.6 %) saline solutions in control and SCN-lesioned rats. In control rats, the plasma AVP concentration before injection was 0.7 ± 0.2 pg/ml, and after the injection it markedly increased and a peak value (12.2 ± 1.5 pg/ml) was observed 15 min after injection. In SCN-lesioned rats, the AVP concentration was 0.9 ± 0.7 pg/ml before injection and it elevated and a peak value (6.0 ± 1.0 pg/ml pg/ml) was also observed 15 min after injection. However, the net increase in plasma AVP concentration from 0 min to 15 min was significantly lower in SCN-lesioned rats than in control rats.

Effect of intracerebroventricular injection of VIP on the increase in the plasma AVP concentration after intraperitoneal injection of hypertonic (3.6 %) saline is shown in Fig. 3. A combination of intracranial aCSF and intraperitoneal isotonic (0.9 %) saline injections had almost no effect on the plasma AVP concentration. As shown in Fig. 2, the addition of peripheral injection of hypertonic saline (3.6 %) to intracranial injection of aCSF induced a statistically significant increase in the plasma AVP concentration, a peak value (9.5 ± 1.3 pg/ml) was observed 15 min after the saline injection, and the AVP level stayed at a higher level until 90 min after the injections. A combination of intracranial injections of VIP and intraperitoneal injection of isotonic saline also induced a significant increase in the plasma AVP concentration, a peak value (4.4 ± 1.4 pg/ml) was observed 15 min after injections, and the AVP level returned to the level before injection within 30 min after injections. Furthermore, a combined injections of VIP and hypertonic saline induced a statistically significant increase in the plasma AVP concentration, a peak value (13.1 ± 1.7 pg/ml) was observed 15 min after injections, and AVP stayed a

higher level until 90 min after injections. However, the increase after addition of VIP injection to hypertonic saline injection was much greater than those after combined injections of VIP+isotonic saline and aCSF+hypertonic saline, and the effect of VIP was not additive but synergistic.

Figure 4 shows the effect of intracerebroventricle injection of a VIP-antagonist on the increase in the plasma AVP concentration after intraperitoneal injection of hypertonic (7.2 %) saline. Intraperitoneal injection of hypertonic saline with aCSF induced a striking increase in the plasma AVP concentration, a peak value (20.5 ± 1.1 pg/ml) was observed 15 min after injections, and the AVP level stayed at a higher level until 90 min. Intracranial injection of the VIP-antagonist significantly suppressed this increase in the AVP concentration, even though the peak value (14.1 ± 2.3 pg/ml) was observed 15 min after injections and the AVP level stayed at a higher level until 90 min.

DISCUSSION

AVP acts as an antidiuretic hormone in peripheral organs. It was reported that osmotic stimulations such as water-deprivation and hypertonic saline injection induced an increase in the plasma AVP concentration [4]. In this paper, the results were confirmed (Fig. 1, 3). However, in SCN-lesioned rats the increase in the plasma AVP concentration induced by these stimulations was lower than in normal rats (Fig. 1,3). In SCN-lesioned rats the plasma AVP value before the stimulations was from 0.9 to 1.8 pg/ml and this value was similar to that in control rats (Fig. 1,3). In this connection, the increase in the plasma AVP concentration after 24 hours water-deprivation was lost in congenital blind rats, hereditary microphthalmic rats, which had morphologically abnormal SCN. In these blind rats, however, the AVP

concentration before water-deprivation was also similar to the level observed in control rats [13]. These results suggest that the SCN is not essential for maintaining the basal level of the plasma AVP. From the results that the increase in the plasma AVP to these challenges were significant but lower in SCN-lesioned rats, it is likely that the SCN has a role to enhance the response of plasma AVP by these osmotic challenges through the neural pathways terminating to outside of the SCN.

In this study it was also observed that intracerebroventricular injection of VIP itself induced the increase in the plasma AVP concentration, and that it synergistically elevated the plasma AVP concentration after intraperitoneal injection of hypertonic saline (Fig. 3). On the other hand, intracerebroventricular injection of a VIP-antagonist significantly reduced the increase in the plasma AVP concentration after intraperitoneal injection of hypertonic saline (Fig. 4). These results suggest that endogenous VIP has a role to stimulate the response of the plasma AVP. Considering this effect of VIP and the potential role of the SCN in the response of the blood AVP to osmotic challenges, it is likely that the neurons containing VIP (VIP-neurons) in the SCN are involved in the regulation of the plasma AVP concentration under these stressful condition.

It is considered that the information about osmotic condition in the blood is transmitted to the hypothalamic PVN and SON through the osmoreceptors that exist in organum vasculosum of the lamina terminalis, medial preoptic area and subfornical organ [6-8, 17]. It is reported in rats that VIP-neurons in the SCN project to the ventral border of the hypothalamic PVN (termed as subparaventricular zone, sPVHz) , that from sPVHz there are neural connections to the preoptic area, retrochiasmatic area and dorsomedial nucleus of the hypothalamus, and that the latter three nuclei send efferent fibers to all parts of the PVN

[20]. Therefore, it is possible that in rat the innervations of VIP-neurons in the SCN to PVN with nebular pathways like these modulate the neural network responsible for the regulation of the plasma AVP level.

It has been reported that central administration of VIP affect the secretion of pituitary hormones such as prolactin [5, 18], growth hormone [18], luteineizing hormone [18] and adrenocorticotropic hormone [1]. However, it is not clear that VIP endogenously regulates these hormone secretion from the pituitary. From the evidence the VIP-neurons in the SCN project to the hypothalamic PVN indirectly, it might also be possible that secretion of these hormones were regulated by VIP contained in the neurons in the SCN like AVP.

In conclusion, the SCN has a role to enhance the plasma AVP concentration. Furthermore, it is likely that VIP-neurons in the SCN is the mediator of this role.

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Fig. 1. Effect of 24 hours water-deprivation on the plasma AVP concentration in control and rats with bilateral lesions of the SCN.

Plasma AVP concentration before and after water deprivation were measured in control (open column) and SCN-lesioned rats (closed column). Data are expressed as means ± S.E.M. for groups of rats. In control group and SCN-lesioned group consisted of 5 and 6 rats. The significance of differences were analyzed by ANOVA. $*: p < 0.05$

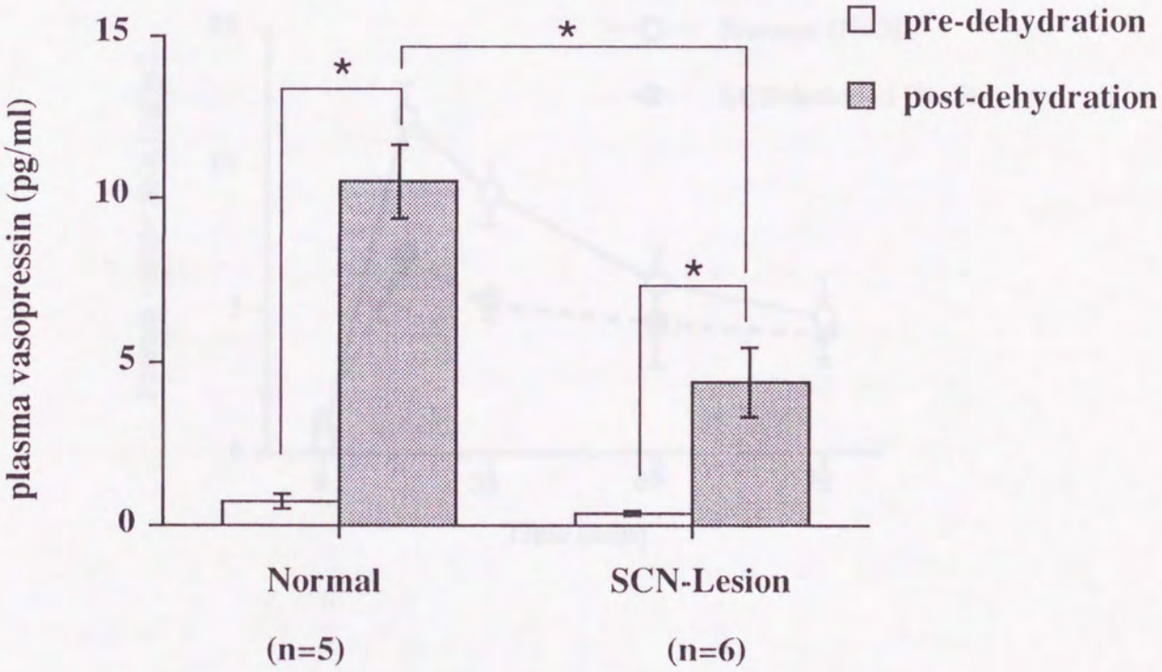


Fig. 2. Effect of intraperitoneal injection of hypertonic saline on the plasma AVP concentration in control rats and rats with bilateral lesions of the SCN.

Hypertonic (3.6 %) saline was injected intraperitoneally in control rats and rats with SCN lesions. Data are expressed as means ± S.E.M. for group of rats. In control group and SCN-lesioned group consisted of 3 rats. Differences between these initial values were not statistically significant by the Mann-Whitney *U*-test. The significant difference between control and SCN-lesioned groups (0-30 min) were $p<0.05$ ($F=9.366$ by ANOVA).

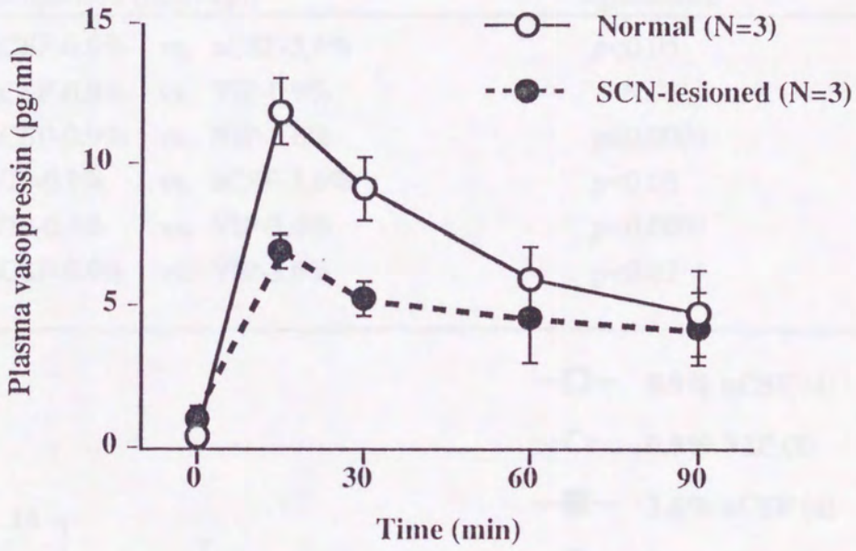


Fig. 3. Effect of VIP on increase of plasma AVP concentration induced by hypertonic saline injection.

Hypertonic (3.6 %) or isotonic (0.9 %) saline was injected intraperitoneally with intracerebroventricular injection of VIP (3 nmol) or aCSF. Data are expressed as means ± S.E.M. for group of rats. Group of hypertonic saline plus VIP consisted of 7 rats, group of isotonic saline plus VIP consisted of 8 rats, group of hypertonic saline plus aCSF consisted of 6 rats and group of isotonic saline plus aCSF consisted 4 rats. Differences between these initial values were not statistically significant by the Mann-Whitney *U*-test. The significance of differences between groups (0-90 min) were analyzed by ANOVA.

comparison (i.c.v.-i.p.)			significance
aCSF-0.9%	vs.	aCSF-3.6%	$p<0.05$
aCSF-0.9%	vs.	VIP-0.9%	N.S.
aCSF-0.9%	vs.	VIP-3.6%	$p<0.0001$
VIP-0.9%	vs.	aCSF-3.6%	$p<0.05$
VIP-0.9%	vs.	VIP-3.6%	$p<0.0001$
aCSF-0.9%	vs.	VIP-3.6%	$p<0.02$

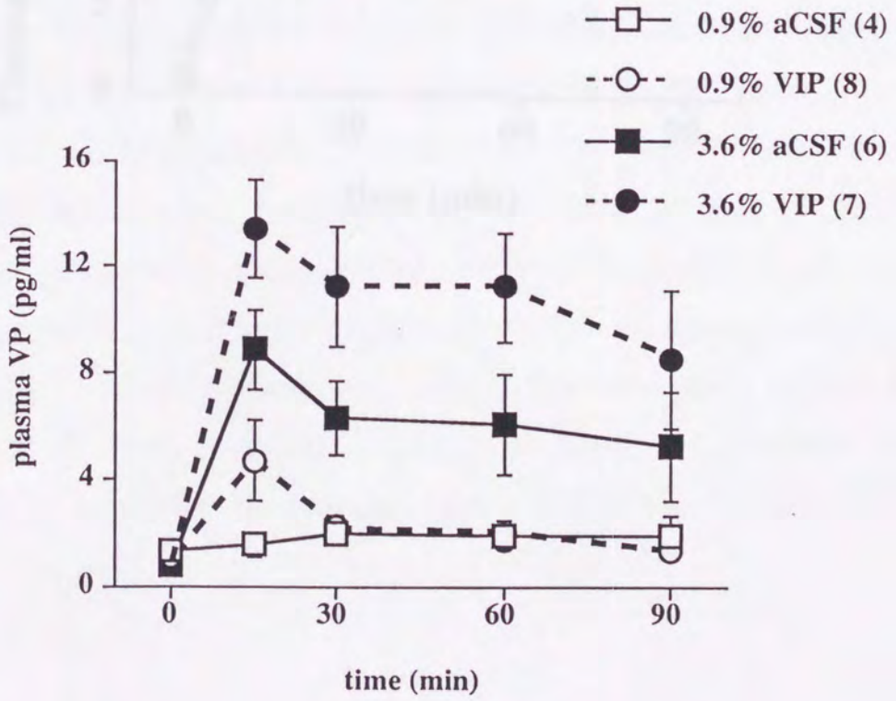
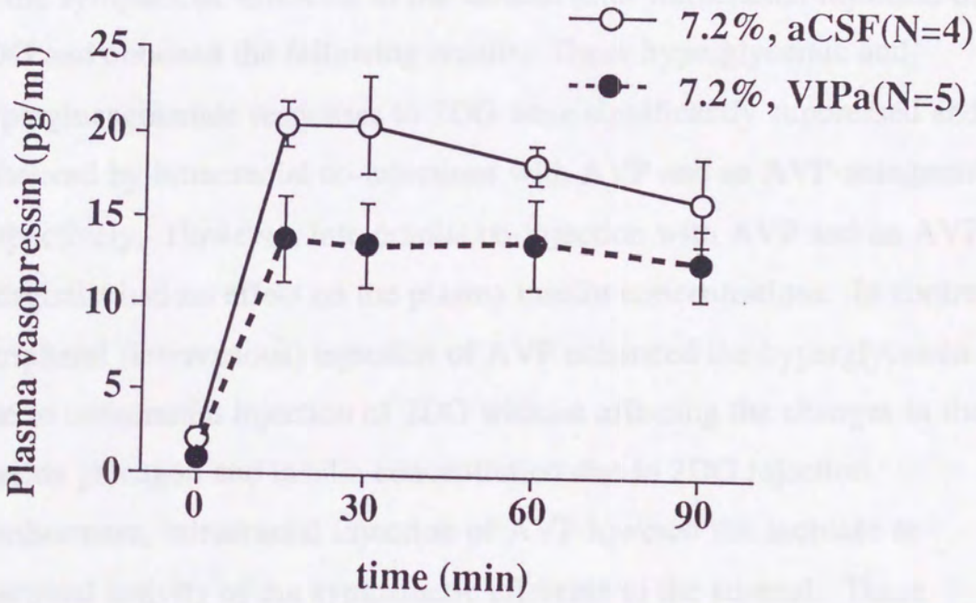


Fig. 4. Effects of a VIP-antagonist on increase in the plasma AVP concentration induced by hypertonic saline injection.

Hypertonic (3.6 %) saline was injected intraperitoneally with intracerebroventricular injection of a VIP-antagonist (1.5 nmol) or aCSF. Group of VIP-antagonist consisted of 5 rats and group of aCSF consisted of 4 rats. Differences between these initial values were not statistically significant by the Mann-Whitney *U*-test. The significant difference between these two groups (0-90 min) were $p<0.05$ ($F=7.47$ by ANOVA)



Chapter 4

Suppressive effect of central AVP on the hyperglycemic response to intracranial injection of 2DG

SUMMARY

Previously, we presented evidence that in rat the SCN is involved in glucose homeostasis. Neurons in the dorsomedial parts of the SCN are known to contain AVP-like immunoreactive substance (AVP-neurons). To clarify the role of these neurons in the SCN, I examined the effect of intracranial injection of AVP on the hyperglycemia and electrical activity of the sympathetic efferents to the adrenal after intracranial injection of 2DG and obtained the following results. These hyperglycemic and hyperglucagonemic responses to 2DG were significantly suppressed and enhanced by intracranial co-injections with AVP and an AVP-antagonist, respectively. However, intracranial co-injection with AVP and an AVP-antagonist had no effect on the plasma insulin concentrations. In contrast, peripheral (intravenous) injection of AVP enhanced the hyperglycemia due to intracranial injection of 2DG without affecting the changes in the plasma glucagon and insulin concentration due to 2DG injection. Furthermore, intracranial injection of AVP lowered the increase in electrical activity of the sympathetic efferents to the adrenal. These findings suggest that endogenous AVP plays a suppressive role in the hyperglycemic and hyperglucagonemic responses to intracranial injection of 2DG probably through modulating sympathetic nerve activity, and it is possible that AVP-neurons in the SCN are involved in the mechanism of glucose homeostasis in the opposite way of VIP-neurons in the SCN.

INTRODUCTION

In the previous chapter, I showed that hyperglycemic and the hyperglucagonemic responses to intracranial injection of 2DG were enhanced by simultaneous injection of VIP, but that they were suppressed by preinjection of a VIP-antagonist into the brain.

AVP has been shown to act as a neurotransmitter or neuromodulator on memory and learning processes in the central nervous system [1] and exist in the neurons in the dorsomedial part of the SCN [15]. These findings prompted me to examine whether AVP is also involved in the central regulation of glucose metabolism. In this chapter, I describe a suppressive effect of centrally administered AVP on the hyperglycemic response and the increase in neural activity of the sympathetic efferents to the adrenal after intracranial injection of 2DG.

MATERIALS AND METHODS

Animals

Twenty three male Wistar strain rats weighting 280-350 g were used. They were housed in a room maintained at 24 $\pm$ 1 ^{o}C and illuminated for 12 hours (0700-1900h) by fluorescent light bulbs (mean light intensity, 80 lux). Food (type MF; Oriental Yeast Co., Tokyo) and water were freely available. Rats were adapted to the environmental conditions described above for at least one week before the experiment.

Administration of drugs and blood sampling

Three days before the experiment, a catheter made of silastic and polyethylene tube (PE-50; Clay Adams, NJ) was inserted into the right atrium of the heart through the subclavian vein and another cannula (PE-10; Clay Adams, NJ) was inserted into the right lateral cerebral ventricle (LCV) of rats under pentobarbital anesthesia (40 mg/kg , i.p.) as previously described [9]. For the experiment, rats were divided into 4

groups and deprived of food for 2 hours from 1000 h. At 1200 h, the first group was given an injection of 2DG (80 μmol) plus AVP (100 pmol) (Peptide Institute Inc., Osaka) and the second group was given only 2DG (80 μmol) in a volume of 20 μl via the cannula in the LCV. The third group received 2DG (20 μmol) plus a AVP-antagonist, [Pmp^1, $Tyr(Me)^2$]-Arg^8-vasopressin, (100 pmol) (Peptide Institute Inc., Osaka) and the fourth group received 2DG (20 μmol) in a volume of 10 μl via the cannula. The agents used were dissolved in artificial cerebrospinal fluid (aCSF) [12]. Solutions were injected at a rate of 2 μl/sec. Blood samples (200 μl each) were withdrawn through the heart catheter 0, 10, 20, 30, 60 and 90 min after the injections, and mixed with aprotinin (100 kIU) (Boehringer-Mannheim, Mannheim) and EDTA (300 nmol) in a volume of 10 μl. The samples were centrifuged, and the plasma separated were stored at -60 ^{o}C for assays of glucose, glucagon and insulin. Food was withheld until the end of blood sampling.

Measurements of plasma glucose, glucagon and insulin concentrations

The plasma glucose concentration was measured by the glucose oxidase method with a Fuji Dri-chem system (Fuji Film Co., Tokyo). The plasma glucagon and insulin concentrations were determined by radioimmunoassays with a Daiichi Glucagon Kit (Daiichi Radioisotope, Tokyo) and a Shionoria Insulin Kit (Shionogi Pharmaceuticals, Osaka), respectively. Data are expressed as means $\pm$ S.E.M. Statistical significance was assessed by analysis of variance (repeated measurements by ANOBA) and the Mann-Whitney *U*-test.

Measurement of sympathetic nerve activity

The experiments were done during 1200h and 1800h. After the insertion of polyethylene cannula (PE-10) into the right LCV of rats as described above under urethane anesthesia (1g/kg). Electrical activity was recorded as described in previous chapter. 2DG (80 μmol) and AVP

(100 pmol) dissolved in aCSF were injected into the right LCV through the brain cannula.

RESULTS

Figure 1 shows changes in the plasma concentrations of glucose, glucagon and insulin after intracranial injection of 2DG or 2DG+AVP. Injection of 2DG (40 μmol) into the LCV resulted in a marked increase in the plasma glucose concentration with a peak value (193 mg/ml) 60 min after injection (Fig. 1A). It also caused dramatic increases in the plasma glucagon and insulin concentrations with peak values (about 15-fold and 2.5-fold) 10 and 30 min after injection (Fig. 1B & C), respectively. Additional injection of AVP (100 pmol) significantly suppressed the hyperglycemia ($p<0.05$ by ANOVA) and hyperglucagonemia ($p<0.05$ by ANOVA) induced by intracranial injection of 2DG, but it had no effect on the plasma insulin level after 2DG injection.

Figure 2 shows changes in the plasma concentrations of glucose, glucagon and insulin after injections of 2DG and 2DG+AVP-antagonist. 2DG (20 μmol) induced only a slight increase in the glucose concentration and the highest value was 141 mg/ml 90 min after injection (Fig. 2A), a slight elevation in the glucagon concentration with a peak value (122 %) 60 min after injection (Fig. 2B), and had no effect on the plasma insulin concentration (Fig. 2C). The intracranial injection of AVP-antagonist significantly enhanced both the hyperglycemic and hyperglucagonemic responses ($p<0.05$ by ANOVA) due to the 2DG injection, but did not affect the plasma insulin level after injection.

Changes in the plasma concentrations of glucose, glucagon and insulin after intracerebroventricular injection of 2DG with or without intravenous injection of AVP are shown in Fig. 3. Injection of 2DG (80

μmol) into the LCV elicited a marked hyperglycemia and a peak value (211 mg/dl) was observed 60 min after injection. It also caused increases in the glucagon and insulin concentrations up to 90 min after injection. Intravenous injection of AVP (100 pmol) significantly ($p<0.05$ by ANOVA) enhanced the increase in the plasma glucose level after intracranial 2DG injection without affecting the changes in the glucagon and insulin concentrations.

Figure 4 shows effects of intracranial injection of AVP (100 pmol) on the elevation of neural activity of the sympathetic efferents to the adrenal after intracranial 2DG (80 μmol) injection. 2DG injection induced a gradual and prolonged increase in the electrical activity of the sympathetic efferents. This increase was observed for more than 150 min after 2DG injection. AVP was given twice at about 54 and 154 min after 2DG injection. The first AVP injection slightly inhibited the increase in neural activity, and the second one elicited a clear inhibition of the increase.

DISCUSSION

In the present study, the following findings were obtained: 1) Intracranial injection of AVP suppressed the hyperglycemic and hyperglucagonemic responses to intracranial injection of 2DG. 2) Intracranial injection of an AVP-antagonist enhanced the hyperglycemic and hyperglucagonemic responses to intracranial injection of 2DG. 3) Peripheral (intravenous) injection of AVP enhanced the hyperglycemic response but not the hyperglucagonemic one to intracranial injection of 2DG. 4) Elevation in electrical activity of the sympathetic efferents to the adrenal was clearly suppressed by intracranial injection of AVP. These results suggest that endogenous AVP in the brain, not in the peripheral site, plays a suppressive role in the hyperglycemic and

hyperglucagonemic responses and the increase in neural activity of the sympathetic efferents to the adrenal which are induced by intracranial injection of 2DG. Furthermore, these findings also suggest that suppression of the hyperglycemic response due to the intracranial injection of AVP is closely related with suppression of the hyperglucagonemia after intracranial injection of 2DG.

In rats, the hyperglycemic response to 2DG has been shown to be associated with the activation of the sympathetic nervous system; that is, intracranial injection of 2DG increased activities of the sympathetic efferents to the pancreas, liver and adrenal and the hyperglycemia was blocked by adrenergic blockers [17]. Considering this, it is likely that the suppression of the 2DG-hyperglycemia by AVP was elicited through the suppression of the sympathetic nerve activity to these organs. Since it is suggested that in rats the neurons containing AVP in the SCN suppress the release of corticosterone under ether exposure [4], and that glucocorticoids have a "permissive" role in the sympathetic action of catecholamines [2], the suppressive effect of central AVP might be due to not only the reduction of sympathetic nerve activity, but the reduction in the blood corticosterone level due to it, the latter resulting which results in a decrease in the "permissive" action of the corticosterone on sympathetic activity.

Intracranial injection of an AVP-antagonist enhanced the hyperglycemic and hyperglucagonemic responses to 2DG (Fig. 2). In our preliminary experiments, I observed that the AVP-antagonist had an enhanced effect on the hyperglycemia, thus submaximal dose of 2DG (20 μmol) was injected for obtaining clear results on the effects of the AVP-antagonist.

Peripheral administration of AVP enhanced the hyperglycemic response to 2DG (Fig. 3). In accordance with this, it was reported that

peripheral administration of AVP caused a hyperglycemia by enhancing glycogenolysis and gluconeogenesis in the liver [3]. In contrast, central administration of AVP enhanced the hyperglycemic response to 2DG rather than suppressed it (Fig. 1). Thus the suppressive effect of AVP on the hyperglycemic response seems to be induced in the central nervous system.

In the previous chapter, I showed that VIP had a synergistically stimulatory effect on the hyperglycemia induced by 2DG [13]. Therefore, the effect of central AVP seems to be opposite to that of central VIP in regard to the regulation of glucose metabolism. Similarly, it has been reported that central administration of these two peptides have opposite effects on the blood concentration of corticosterone; that is, VIP elevates [5], and AVP lowers [4] its concentration. These facts might suggest opposite functions of these two in the brain of other physiological functions except for those of the blood glucose and glucocorticoid.

It is not clear yet where AVP acts in the brain to enhance the hyperglycemic response to 2DG. In this connection, it was reported that in rats sympathetic nerve activity was enhanced by injection of AVP into the medulla oblongata and the spinal cord [6, 10] and that it was suppressed by intravenous injection of AVP [14]. In this study, it was found that intracerebroventricular injection of AVP suppressed the increase in neural activity of the sympathetic efferents to the adrenal after intracranial injection of 2DG (Fig. 4). From these facts, it can be speculated that AVP has a reciprocal actions on the activity of the sympathetic nerve, depending on which it acts centrally or peripherally, and that the suppressive role of AVP might be elicited through the brain sites near the lateral cerebral ventricle such as the thalamus and hypothalamus.

It is not clear yet where is the origin of AVP that suppressed 2DG-hyperglycemia endogenously in central nervous system. It is known that AVP exists in the thalamus and hypothalamus including dorsolateral part of the SCN [15]. However, it can be speculated that a suppressive role of AVP on 2DG-hyperglycemia is played by AVP contained in the neurons in the SCN. In the previous chapter, I presented the evidence suggesting that the VIP-neurons in the SCN is involved in the central regulation of the blood glucose level and showed that intracranial injection of VIP into rats elicited a long-lasting increase in activity of the sympathetic efferents to the adrenal. Therefore, AVP suppressed the 2DG-hyperglycemia (Fig. 1) and increase in activity of the sympathetic efferents to adrenal by 2DG (Fig. 4). These findings suggest that both VIP- and AVP-neurons in the SCN are candidates that are involved in reciprocal control of the functions regulated by the sympathetic nervous system such as maintaining glucose homeostasis and other homeostatic mechanisms. The investigation of the problem of whether this is the case is now under progress.

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Fig. 1. Effects of intracranial injection of AVP on changes in the plasma concentrations of glucose (A), glucagon (B) and insulin (C) after intracranial injection of 2DG.

Data are expressed as mean ± S.E.M.. Groups of 4 and 5 rats received injections of 2DG and 2DG+AVP, respectively. Data on the plasma glucagon concentrations are shown as percentages of the values at 0 min (41.2 ± 6.5 pg/ml for 2DG and 56.4± 3.2 pg/ml for 2DG+AVP). Data on the plasma insulin concentrations are also shown as percentages of the values at 0 min (49.7± 2.6 μU/ml for 2DG and 38.7 ± 4.9 μU/ml for 2DG+AVP). Difference between these initial values were not statistically significant by the Mann-Whitney U-test. Other explanations are given in the text. The significances of differences between groups (10-90 min) were analyzed by ANOVA.

Material	Significance
glucose	$p < 0.05$ ($F = 6.143$)
glucagon	$p < 0.05$ ($F = 6.269$)
insulin	not significant ($F = 1.528$)

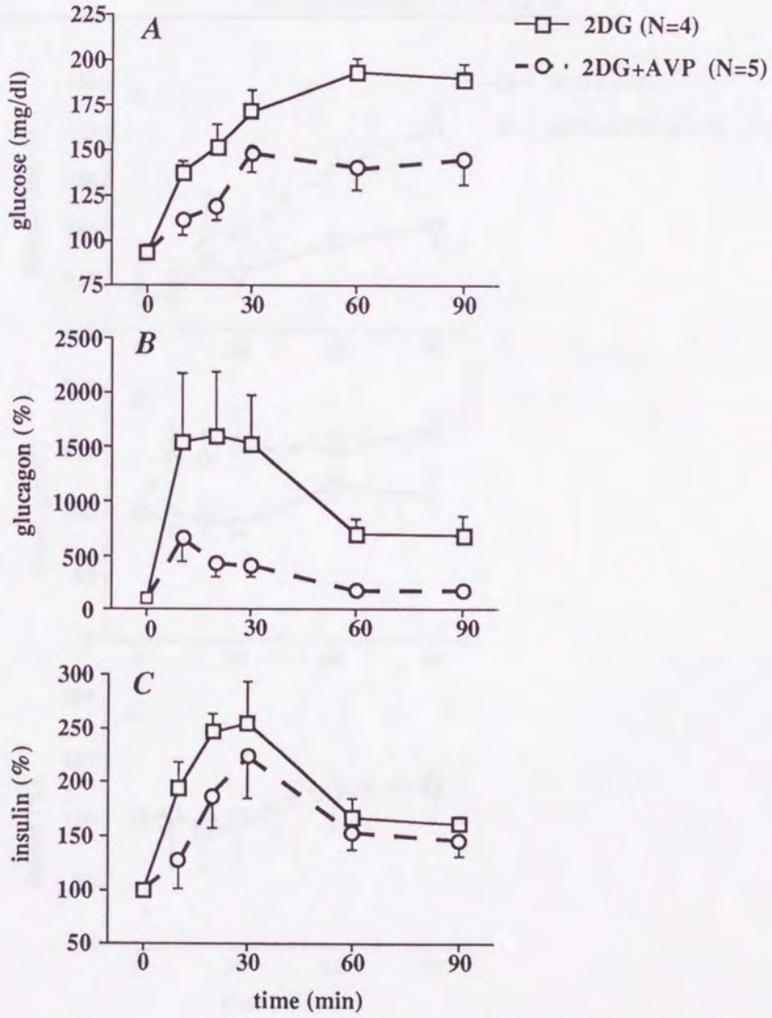


Fig. 2. Effects of intracranial injection of a AVP-antagonist on changes in the plasma concentrations of glucose (A), glucagon (B) and insulin (C) after intracranial injection of 2DG.

Data are expressed as means ± S.E.M.. Groups of 7 rats received injections of 2DG and 2DG+AVP-antagonist. Data on the plasma glucagon concentrations are shown as percentages of the values at 0 min (159.4 ± 14.2 pg/ml for 2DG and 182.2 ± 68.2 pg/ml for 2DG+AVP-antagonist). Data on the plasma insulin concentrations are shown as percentages of the values at 0 min (89.7± 36.3 μU/ml for 2DG and 83.3 ± 6.1 μU/ml for 2DG+AVP-antagonist). Differences between these initial values were not statistically significant by the Mann-Whitney U-test. Other explanations are given in the text and legend to Fig. 1. The significances of differences between groups (10-90 min) were analyzed by ANOVA.

Material	Significance
glucose	$p < 0.05$ ($F = 10.088$)
glucagon	$p < 0.05$ ($F = 5.207$)
insulin	not significant ($F = 0.527$)

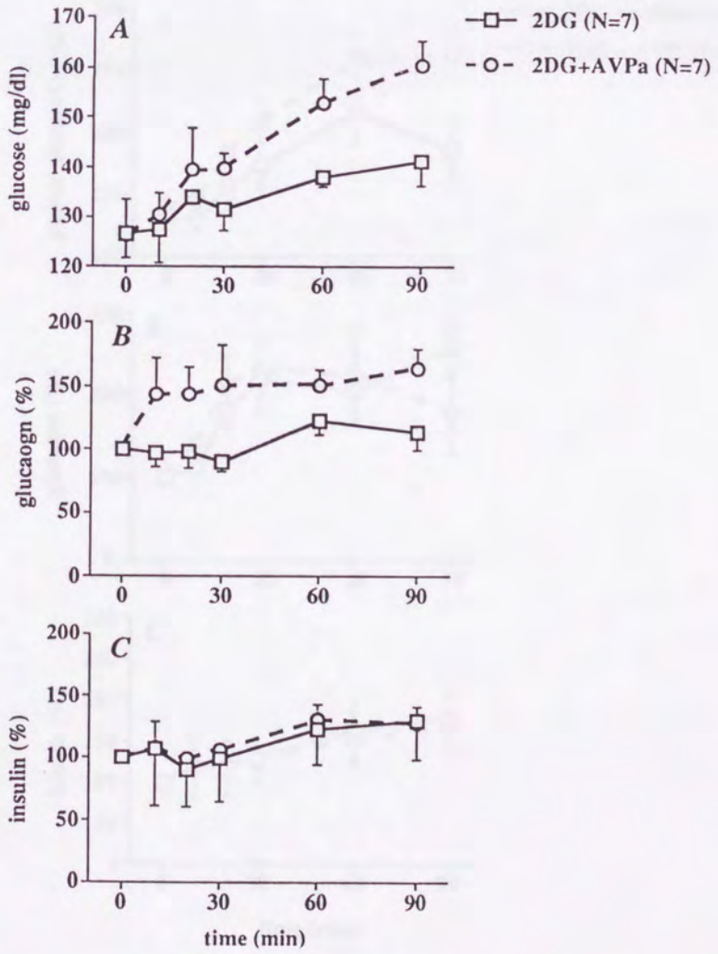


Fig. 3. Effects of intravenous injection of AVP on the plasma concentrations of glucose (A), glucagon (B) and insulin (C) after intracranial injection of 2DG.

Data are expressed as means ± S.E.M.. 4 rats received injections of 2DG and 5 rats received 2DG+AVP. Data on the plasma glucagon concentrations are shown as percentages of the values at 0 min (134.3 ± 15.3 pg/ml for 2DG and 114.3 ± 38.7 pg/ml for 2DG+AVP-antagonist). Data on the plasma insulin concentrations are shown as percentages of the values at 0 min (41.0 ± 8.1 μU/ml for 2DG and 53.0 ± 10.2 μU/ml for 2DG+AVP-antagonist). Differences between these initial values were not statistically significant by the Mann-Whitney U-test. Other explanations are given in the text and legend to Fig. 1. The significances of differences between groups (10-90 min) were analyzed by ANOVA.

Material	Significance
glucose	$p < 0.05$ ($F = 5.608$)
glucagon	not significant ($F = 0.142$)
insulin	not significant ($F = 0.012$)

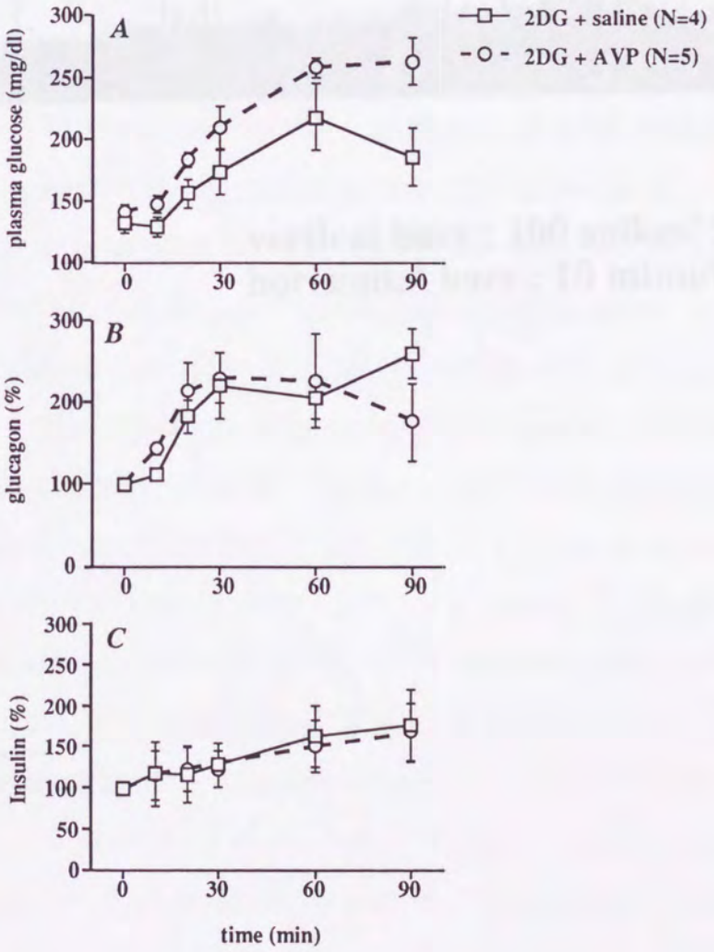
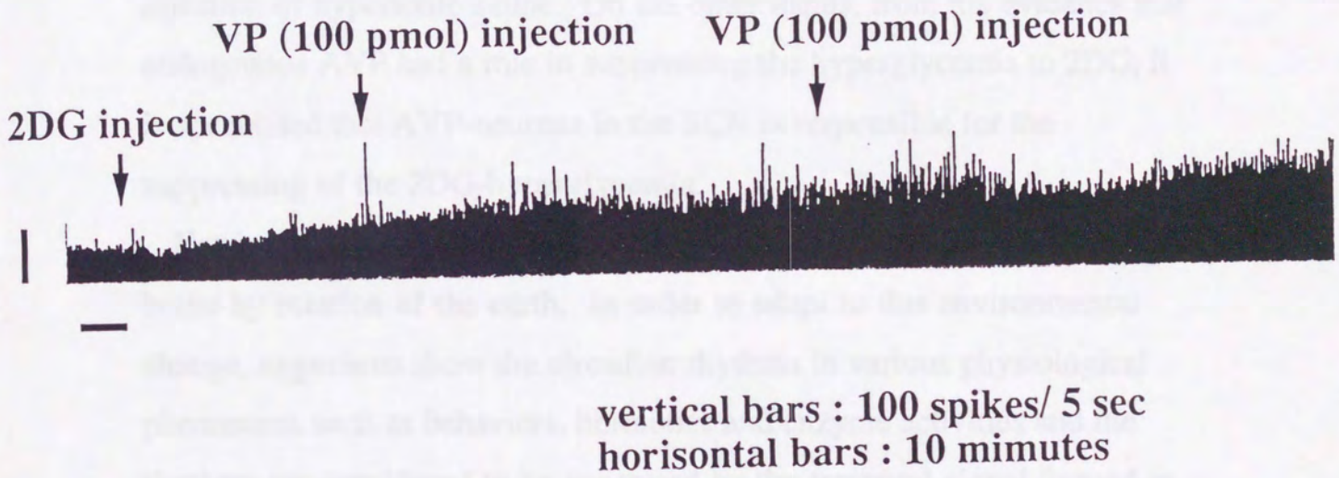


Fig. 4. Effects of intracerebroventricular injection of 2DG and AVP on electrical activity of the sympathetic efferents to the adrenal.

2DG (80 μmol) and AVP (100 pmol) were injected into the lateral cerebral ventricle. Horizontal bar : 10 min, vertical bars : 100 impulses / 5 s.



General Discussion and Conclusion

It was shown in our laboratory that the SCN is involved in the hyperglycemic response and increase in neural activity of the sympathetic efferents to the adrenal after intracranial injection of 2DG that causes energy deficiency in the brain. In this study, it was shown that the SCN is involved in increase in the plasma AVP concentration after stressful osmotic stimulations. Furthermore, it was suggested that the VIP-neurons in the SCN had roles in enhancing the hyperglycemia and increase of sympathetic nerve activity after intracranial injection of 2DG, and increase in the plasma AVP concentration after intraperitoneal injection of hypertonic saline. On the other hands, from the evidence that endogenous AVP had a role in suppressing the hyperglycemia to 2DG, it is speculated that AVP-neurons in the SCN is responsible for the suppressing of the 2DG-hyperglycemia.

Natural environmental lighting condition is changed with a period of 24 hours by rotation of the earth. In order to adapt to this environmental change, organisms show the circadian rhythms in various physiological phenomena such as behaviors, hormones and enzyme activities and the rhythms are considered to be generated by the temporal signal formed in the SCN. In addition to adapt to this environmental change brought about rotation of the earth, organisms should respond to various external and internal stressful challenges for maintaining homeostasis of their internal conditions. Taken these facts into consideration, it seems quite reasonable that the SCN involved in the generation of the circadian rhythms and also related with the mechanisms responding to the stressful challenges such as 2DG injection and water-deprivation. In this study, I found that VIP had a role in facilitating the hyperglycemic response to 2DG through enhancing sympathetic activity and in enhancing the increase of the plasma AVP concentration induced by osmotic stimulations. From

several lines of evidence, it can be speculated that VIP contained in the neurons in the SCN works as an enhancing mediator in the response to the stressful challenges as described above. On the other hand, I found that in the brain AVP suppressed the hyperglycemic and hyperglucagonemic responses and the increase of sympathetic nerve activity due to 2DG. These findings suggest that AVP-neurons in the play a critical role in counteracting the function of VIP-neurons in the SCN. However, it remains unsolved yet , where VIP and AVP work in eliciting these functions and how their signals are transduced in their target neurons. Further works must be required for detailed elucidation of these problems.

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