IMMUNOCYTOCHEMICAL INVESTIGATION OF SEROTONERGIC INPUTS TO VASOACTIVE INTESTINAL PEPTIDE (VIP)-CONTAINING NEURONS IN THE RAT SUPRACHIASMATIC NUCLEUS

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ABSTRACT

The influence of serotonergic inputs on vasoactive intestinal peptide (VIP)-containing neurons in the rat suprachiasmatic nucleus (SCN) was examined using peroxidase-antiperoxidase immunocytochemistry. Serotonergic inputs were blocked by 1) chemical destruction of the ascending serotonergic pathway by 5,6-dihydroxytryptamine injected into the medial forebrain bundle, 2) intraperitoneal injections of reserpine for successive 7 days, and 3) intraperitoneal injections of methysergide for successive 7 days. These treatments caused a marked depletion of VIP-like immunoreactivity in neuron perikarya in the ventral portion of SCN, with a concomitant decrease in the number of serotonin-immunoreactive fibers and terminals. The findings suggest the possibility that VIP-containing neurons may play a role in regulating the circadian rhythm in SCN under a strong influence of serotonergic innervation.

The suprachiasmatic nucleus (SCN) plays an important role as the center of circadian rhythm in mammals (20, 21, 30). The ascending innervation of the serotonergic neuron system is one of the major afferent projections to SCN (8, 28). Recent progress in radioimmunoassay and immunocytochemistry has demonstrated several kinds of peptide-containing neurons with characteristic distributions in SCN (17, 35). Vasoactive intestinal peptide (VIP) is one of the representative peptides in SCN (17, 27, 31), and suggestions have been made that VIP may act as a neurotransmitter (3, 7, 9, 18, 32). The distribution of VIP-containing neurons is similar to that of the serotonergic terminals in the nucleus. In addition, serotonergic terminals were found to form synaptic contacts with VIP-like immunoreactive perikarya using a combination of autoradiography and immunocytochemistry

at the electron microscopic level (15).

Catecholaminergic inputs have previously been reported to affect the peptide-containing neurons in the paraventricular nucleus of the hypothalamus (14, 19) and the central nucleus of the amygdala (13). The purpose of this study is to ascertain the influence of ascending serotonergic inputs on VIP-containing neurons in SCN by analyzing alterations in VIP-like immunoreactivity caused by blocking serotonergic inputs to this nucleus.

MATERIALS AND METHODS

Seventy-five male albino Wistar rats were divided into the following four groups: 1) untreated controls, 2) rats that received chemical transection of the ascending serotonergic pathway, 3) rats that received intraperitoneal injections of reserpine, and 4) rats that

received intraperitoneal injections of methysergide.

Chemical Transection

To destroy the ascending serotonergic pathway, 5,6-dihydroxytryptamine (5,6-DHT), a false transmitter of serotonin (4, 6, 25), was slowly infused bilaterally into the medial forebrain bundle (MFB) at the caudal hypothalamic level using a stereotaxic instrument. Infusion of 5,6-DHT into the unilateral MFB decreased insufficiently the number of serotonin-immunoreactive terminals in the bilateral SCN. The dose of 5,6-DHT was 25 μ g, dissolved in 10 μ l Ringer solution containing 0.2 mg/ml ascorbic acid (6). The control rats received 10 μ l Ringer solution containing 0.2 mg/ml ascorbic acid. The coordinates were determined according to König and Klippel (16): A, 3.4; L, ± 1.5 ; and V, -7.6 from the surface of the brain. The operation was performed under pentobarbital anesthesia (60 mg/kg body wt.; intraperitoneal injection). Animals were killed 7 days after the operation.

Drug Administration

Reserpine (5 mg/kg) or methysergide (2 mg/kg) was injected intraperitoneally once daily for 7 days. The control rats daily received one intraperitoneal injection of saline for 7 days. All injections were given at 10 a.m. Animals were killed 24 h after the last injection.

Immunocytochemistry

All animals were perfused via the left cardiac ventricle with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by a fixative consisting of 4% paraformaldehyde, 0.35% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The brains were immediately removed and immersed in the same fixative without glutaraldehyde for 48 h at 4°C.

Frontal cryostat sections, 20 μ m thick, were made serially and divided into two groups (*vide infra*). All sections were immersed in PBS containing 0.3% Triton X-100 for 4 days at 4°C, and then in bovine serum albumin (dilution 1:100) for 60 min. Then, the first group of sections was incubated with anti-VIP antiserum (R502; reference 36; dilution 1:5,000) for 7 days at 4°C, with goat anti-rab-

bit IgG (Miles-Yeda, dilution 1:200) for 120 min, and with the rabbit peroxidase-anti-peroxidase (PAP) complex solution (Dakopatts a/s, dilution 1:200) for 90 min at room temperature. The second group of sections was incubated with anti-serotonin antiserum (Sera-Laboratories, YC/HLK; reference 5; dilution 1:100) for 7 days at 4°C, with goat anti-rat IgG (Immuno Nuclear Corp.; dilution 1:100) for 120 min, and finally with the rat PAP complex solution (Immuno Nuclear Corp.; dilution 1:100) for 90 min at room temperature. For a detailed comparison of the distribution between serotonergic terminals and VIP-containing neurons, thin serial sections, 4 μ m thick, were made from some tissue blocks of the untreated rat brains including the hypothalamus. These sections were incubated alternately with anti-serotonin antiserum or anti-VIP antiserum for 12 h at room temperature, and processed with anti-rabbit IgG or anti-rat IgG, and finally with the PAP complex solution. All sera were diluted with 0.1 M PBS containing 0.3% Triton X-100, and between each step the sections were washed with the same solution. Finally, all sections were allowed to react with 0.02% 3,3'-diaminobenzidine-4HCl in 50 mM Tris buffer (pH 7.5) containing H_2O_2 . The sections were mounted on glass slides and examined under a light microscope.

The specificity of anti-VIP antiserum was confirmed by preabsorption with synthetic VIP (1–100 μ g/ml), and the specificity of anti-serotonin antiserum by preabsorption with serotonin (10 μ g/ml). No positive immunoreactivity was detected in any section after the absorption test. The specificity and cross-reactivity of these antisera have been described in detail elsewhere (5, 36).

RESULTS

Serotonin-Immunoreactive Terminals and VIP-like Immunoreactive Perikarya in SCN of the Control Rat

The rostral portion of SCN contained many serotonin-immunoreactive terminals mainly in its ventral portion. The proportion of serotonin-immunoreactive terminals was increased in the central SCN (Fig. 1a). The highest population of serotonin-immunoreactive terminals was observed in the central SCN, particularly in its ventral half. The number of serotonin-immunoreactive terminals was decreased in the dorsal portion of SCN

Fig. 1 Serotonin-immunoreactive terminals in the rostral (a), central (b) and caudal (c) SCN of untreated rats. Many serotonin-immunoreactive terminals were observed in the ventral portion. $\times 85$

Fig. 2 VIP-like immunoreactive neuron perikarya, fibers and terminals in the rostral (a), central (b) and caudal (c) SCN of the untreated rat. Many VIP-like immunoreactive neuron perikarya were observed in the ventral portion. VIP-like immunoreactive fibers and terminals were seen in the dorsal portion; some fibers passed SCN and extended toward the other hypothalamic areas (c). a and b, $\times 85$. c, $\times 60$

(Fig. 1b). Serotonin-immunoreactive terminals were fewer in the caudal SCN than in the central SCN (Fig. 1c). Serotonin-immunoreactive terminals in the ventral portion of the caudal SCN were not as densely stained as those in the central SCN.

A small number of VIP-like immunoreactive perikarya were distributed in the ventral

portion of the rostral SCN. However, VIP-like immunoreactive fibers and terminals were relatively abundant in the ventral and dorsal portions of SCN (Fig. 2a). The central SCN contained most of the VIP-like immunoreactive perikarya, particularly in its ventral portion. In the dorsal SCN, VIP-like immunoreactive fibers and terminals were abundant

(Fig. 2b). Some fibers passed through the dorsal portion (Fig. 2c) and extended toward the paraventricular nucleus and other parts of the hypothalamus.

In thin sections ($4\ \mu\text{m}$), VIP-like immunoreactivity was located in perikarya of a smaller size and of oval or spherical shape. Comparison with adjacent sections stained with the anti-serotonin antiserum revealed that serotonin-immunoreactive varicosities were located around the VIP-immunoreactive neuronal somata (Fig. 3, a and b).

Alterations in Serotonin and VIP-like Immunoreactivity after the Treatments

No differences in serotonin or VIP-like immunoreactivity were observed among untreated, sham-operated, and saline-injected animals.

After the 5,6-DHT treatment, the number of serotonin-immunoreactive terminals in SCN was markedly decreased (Fig. 4b) compared with the control (Fig. 4a). Some swollen serotonin-immunoreactive fibers and termi-

nals were also observed. The 5,6-DHT treatment caused a marked decrease in the VIP-like immunoreactivity of neuron perikarya in the ventral portion of SCN and in the number of VIP-like immunoreactive varicosities in the dorsal portion of SCN (Fig. 5b), compared with the control (Fig. 5a). At a higher magnification, a marked depletion of the VIP-like immunoreactivity in each VIP-like immunoreactive perikaryon was seen.

In the reserpine-treated rat, serotonin-immunoreactive terminals were almost completely absent throughout the SCN (Fig. 4c). The depletion of serotonin-immunoreactivity in terminals was more pronounced than in 5,6-DHT-treated rats. Reserpine caused a marked decrease in the VIP-like immunoreactivity in each perikaryon in the ventral portion, and a decrease in the number of VIP-like immunoreactive fibers and terminals in the dorsal portion of SCN (Fig. 5c). However, in the entire SCN of reserpine-treated rats, a larger number of neuronal perikarya still retained weak VIP-like immunoreactivity than

Fig. 3 Higher magnification picture of serotonin-immunoreactive terminals (a) and VIP-like immunoreactive perikarya (b) in serial thin sections ($4\ \mu\text{m}$ thick) from the untreated rat. VIP-like immunoreactive perikarya were surrounded by serotonin-immunoreactive terminals. The site surrounded by serotonin-immunoreactive terminals (a) and the corresponding perikarya showing VIP-like immunoreactivity (b) are indicated by asterisks. Arrows, blood vessels. $\times 1,300$

Fig. 4 Alterations in serotonin-immunoreactive terminals in SCN. a: Abundant varicosities in the untreated rat. b: The 5,6-DHT treatment markedly decreased the number of serotonin-immunoreactive terminals. c: Practically no serotonin-immunoreactive terminals were detected after the reserpine treatment. d: The methysergide treatment caused a slight decrease in the number of serotonin-immunoreactive terminals. $\times 125$

in 5,6-DHT-treated rats.

In the methysergide-treated rats, fewer serotonin-immunoreactive terminals were observed throughout the SCN (Fig. 4d). However, the decrease in the number of serotonin-immunoreactive terminals caused by this agent was less than under two other experimental conditions. VIP-like immunoreac-

tivity in each immunoreactive perikaryon was reduced compared with the control (Fig. 5d), but the depletion was also less pronounced than under two other conditions. In the dorsal portion of SCN, no marked differences in VIP-like immunoreactive fibers and terminals were detected between the methysergide-treated rat and the control.

Fig. 5 Changes in VIP-like immunoreactivity in SCN. a: The untreated rat; pronounced VIP-like immunoreactivity is seen in the neuron perikarya in the ventral portion, and in fibers and terminals in the dorsal portion. b: The 5,6-DHT treatment markedly decreased VIP-like immunoreactivity in the neuron perikarya in the ventral portion, and VIP-like immunoreactive varicosities in the dorsal portion. c: Reserpine decreased VIP-like immunoreactivity in SCN, but to a lesser extent than 5,6-DHT. d: Methysergide caused a moderate depletion of VIP-like immunoreactivity in the neuron perikarya in the ventral portion. No appreciable difference was seen between the control in VIP-like immunoreactive fibers and terminals in the dorsal portion. $\times 125$

DISCUSSION

In the control rat, serotonin-immunoreactive terminals were mainly located in the ventral portion of SCN throughout the rostral to caudal portions, and serotonin-immunoreactive varicosities were decreased in number toward the dorsal portion. VIP-like immunoreactive perikarya were located in the ventral portion of SCN from rostral to caudal parts, and this pattern was similar to that of the serotonin-immunoreactive terminals. Autoradiography and immunocytochemistry at the electron microscopic level demonstrated synaptic contacts between serotonergic terminals and VIP-like immunoreactive perikarya (15). In agreement with this study, the present observations have shown a similar distribution pattern of serotonin- and VIP-like immunoreactivity in SCN. In addition, careful comparison of alternately immunostained sections strongly supports that serotonin-immunoreactive varicosities are located around VIP-containing somata. It thus appears reasonable to assume that VIP-like immunoreactive perikarya receive inputs from serotonin neurons.

The treatment with 5,6-DHT markedly decreased the number of serotonin-immunoreactive terminals in SCN by selective degeneration of the serotonin pathway (4, 6, 25). After the reserpine treatment, almost all the serotonin-immunoreactive terminals also disappeared in SCN by depletion of serotonin in presynaptic terminals. Decrease in the number of serotonin-immunoreactive terminals was moderate in the methysergide-treated rat, presumably because this agent, a specific antagonist against serotonin (1, 2, 11), accelerates the turnover of serotonin and decreases serotonin in terminals. Both reserpine and methysergide exert an influence not only on the serotonergic system but also on the catecholaminergic system. However, these drugs are considered to affect mainly the serotonergic system in case of SCN since most of the monoamine-containing terminals in this nucleus of rats belong to serotonin neurons (8, 26, 33, 34).

All the treatment used in this study markedly decreased VIP-like immunoreactivity in the neuron perikarya in SCN compared with the control. However, effects of the 5,6-DHT treatment were stronger than under the two other experimental conditions. In the dorsal portion of SCN, a marked decrease in the number of VIP-like immunoreactive varicos-

ities was observed after the 5,6-DHT treatment and a moderate decrease after the reserpine treatment. The methysergide treatment caused only a slight decrease in the number of VIP-like immunoreactive varicosities in the dorsal portion of SCN, and the decrease in VIP-like immunoreactivity was restricted to the neuron perikarya in the ventral portion of SCN. Blockade of serotonergic inputs by methysergide was considered to be less complete compared with the two other experimental conditions.

SCN in mammals plays an important role as a pacemaker of circadian rhythm, as evidenced by impairment of hormonal activity (21), pineal *N*-acetyltransferase activity (23), drinking behavior and locomotor activity (29) caused by lesions on SCN. SCN is divided into two portions (20): the ventral portion where neuronal inputs from a number of brain regions arrive (10, 22) and are integrated with intrinsic neuronal circuit in SCN; and the dorsal portion whose outputs affect the circadian rhythm. Some VIP-like immunoreactive neurons have been suggested to be intrinsic neurons by immunoelectron microscopy (24).

The present results strongly suggest that serotonergic inputs facilitate synthesis and/or transport of VIP in SCN. It is also suggested that the VIP-like immunoreactive neurons in the ventral portion of SCN are mainly intrinsic neurons that act on other neurons in the dorsal portion such as arginine-vasopressin-containing neurons which may project outside SCN (12). It is also possible that VIP-containing neurons directly project outside, *e.g.* to the paraventricular nucleus of the hypothalamus, since immunocytochemical investigation suggest such features. In conclusion, the present study indicates that VIP-containing neurons participate in the regulation of the circadian rhythm under the strong influence of serotonergic innervation.

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