Immunohistochemical Studies on the Distribution of Neuropeptides and Serotonin in the Suprachiasmatic Nucleus of the Brattleboro Rat

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Summary. The distribution of vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP)/bombesin, somatostatin, vasopressin, neuropeptide Y (NPY) and serotonin was examined immunohistochemically in the suprachiasmatic nucleus (SCN) of male rats genetically deficient for vasopressin (Brattleboro strain). VIP-containing neurons and their varicose fibers were preferentially distributed in large numbers in the ventromedial part of the SCN. GRP/bombesin-containing neurons and their fibers were also gathered in the ventral part of the SCN, particularly in the ventromedical region of the nucleus. Somatostatin-containing neurons and their fibers were prominent in the rostral and middle portions of the SCN, where the highest concentration of immunoreactivity was restricted in their ventromedial part. No vasopressin-immunoreactivity was found at all throughout the SCN. Profuse NPY-containing varicose fibers were observed in the ventrolateral part of the SCN, but no immunoreactive neurons were distributed in this nuclear region. Serotonergic fibers showed a topographic arrangement in the SCN: a serotonin-immunoreactive nerve plexus was predominantly distributed in the ventrolateral part. These findings indicate that the SCN of Brattleboro rats is composed of distinct subdivisions of immunoreactive cell bodies and fibers. The distribution of the five peptides and indoleamine within the SCN in the Brattleboro strain was compared with that in normal Long-Evans rats. Furthermore, both strains of rats were exogenously administered with arginine-vasopressin, but no conspicuous difference in the regional patterns of immunoreactivity was detected. The possible role of vasopressin in the SCN is discussed.

The suprachiasmatic nucleus (SCN) of the hypothalamus in mammals, particularly rodents, has been recognized as playing a crucial role in the maintenance of circadian rhythms (MOORE and EICHLER, 1972; STEPHAN and ZUCKER, 1972; IBUKA and KAWAMURA, 1975; NISHINO et al., 1976; RUSAK and ZUCKER, 1979). It has been shown both morphologically and electrophysiologically that the SCN consists of a heterogeneous population of neurons, and that various circadian rhythms of behavior and metabolism, such as drinking, locomotor activity, sleeping and waking, heart rate, estrous cycle, feeding, plasma corticosterone, and pineal serotonin-N-acetyltransferase are regulated by different types of neurons and afferent inputs (MOODY et al., 1979). Recent immunohistochemical studies have identified that some of the afferent fibers in the rat SCN contain avian pancreatic polypeptide (CARD and MOORE, 1982), neuropeptide Y (NPY) (ALLEN et al., 1983; UEDA et al., 1986) and serotonin (STEINBUSCH, 1981; TAKEUCHI and SANO, 1983; UEDA et al., 1983), and that neuronal cell bodies within the SCN contain vasopressin and neurophysin (VANDESANDE et al., 1975; ZIMMERMAN, 1976; SOFRONIEW...
and WEINDL, 1978, 1980; UEDA et al., 1983), somatostatin (DIERICKX and VANDESANDE, 1979), vasoactive intestinal polypeptide (VIP) (ROBERTS et al., 1980; SIMS et al., 1980; CARD et al., 1981), gastrin-releasing peptide (GRP)/bombesin (ROTH et al., 1982) and glutamate decarboxylase (VINCENT et al., 1982). These results show the topographic and preferential distributional pattern of each peptide and amine within the SCN.

The Brattleboro rat, a mutant of the Long-Evans strain, is characterized by its inability to synthesize vasopressin and neurophysin, and consequently to display diabetes insipidus (SOKOL and VALTIN, 1965; RICHTER and SCHMALE, 1984). Although no vasopressin-immunoreactivity was detected throughout the central nervous system of this mutant rat, the cytoarchitecture of the SCN as well as magnocellular paraventricular and supraoptic nuclei in the hypothalamus remains intact (PETERSON et al., 1980; MORRIS, 1982). The present investigation was undertaken to determine by use of the immunohistochemical method whether the principles of organization in the SCN of the normal Long-Evans rat, which contains vasopressin, somatostatin, VIP, GRP/bombesin, NPY and serotonin, can be extended to the Brattleboro rat with homozygous for diabetes insipidus. This was done by analyzing the distributional pattern of immunoreactivity within the nucleus.

MATERIALS AND METHODS

Animals
Seven male rats of the Brattleboro strain weighing 150-200g, which are homozygous for diabetes insipidus, and seven male Long-Evans rats as controls were used in this study. The animals were housed individually in cages maintained under controlled air circulation and lightning conditions in which the period was from 6.00 p.m. to 6.00 a.m. was kept dark. Food was available ad libitum. The water intake and urine output were monitored daily. Two Brattleboro and Long-Evans rats received arginine-vasopressin (Pitressin tannate in peanut oil, Park-Davis, Morris Plains, N. J., U. S. A.) 0.5 U/100 g body weight by placing subcutaneously of the mini-osmotic pump (Alzet, model 2001, Alza, Palo Alto, C. A., U. S. A.) for one week. Two Brattleboro and two Long-Evans rats received only the vehicle.

Tissue preparation
The animals were perfused under sodium pentobarbital anesthesia via the left cardiac ventricle with a fixative consisting of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) at 4°C between 1.00 to 2.00 p.m. The animals, which were implanted with the mini-osmotic pump, were perfused by the same fixative after 7 days of sub-cutaneous placing. The brains were removed from the skull, cut into blocks containing hypothalami and stored in an ice-cold fixative which contained 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer for 24 hr. After immersion in 0.1 M phosphate buffer containing 20% sucrose at 4°C, the blocks were frozen with CO₂ gas and cut into 15 μm section on the frontal plane using a cryostat. The serial sections were stored in 0.1 M phosphate buffered saline containing 0.3% Triton X-100 at 4°C in preparation for the immunohistochemical staining. The sections were separated into six groups by selecting every sixth section, so that each container held sections from all parts of the mass.
Immunohistochemical staining
All immunohistochemical staining was done on free-floating sections. The peroxidase-antiperoxidase method and the avidinbiotin-peroxidase complex technique used in this study were slight modifications of methods described in detail elsewhere (Kawata and Sano, 1982; Takeuchi et al., 1982; Kawata, 1983; Ueda et al., 1983; Kawata et al., 1984). The sections were incubated for 24-48 hr at 4°C in six different antisera at dilutions between 2,000 and 20,000. The antisera solution for dilution was 0.1 M phosphate buffered saline containing 0.3% Triton X-100. The chromogen used in this study was 3,3'-diaminobenzidine hydrochloride (0.5 mg/ml) and the peroxidase reaction product was intensified by immersing tissue slides in a 0.1% osmium tetroxide solution for 1 min.

Antisera
The present immunohistochemical study was carried out using six antisera generated by rabbits against arginine-vasopressin, somatostatin, vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP)/bombesin, neuropeptide Y (NPY) and serotonin. The vasopressin antiserum was a gift from Dr. Yamaguchi et al., (1985). Serotonin antiserum was raised in our laboratory (Takeuchi et al., 1982). Somatostatin antiserum was obtained from the Immuno Nuclear Corporation (Minneapolis, Minn., U. S. A.). All other antisera were purchased from Amersham International (Buchinghamshire, U. K.). The testing for antisera specificity except for serotonin was histochemically performed by preabsorption of each antiserum with a 10 μM concentration of appropriate antigen which was purchased from Peptide Institute, Inc. (Minohshi, Osaka, Japan). The specificity of serotonin antiserum was tested by preabsorbing the aliquot of antiserum with 20 μg of serotonin creatinine sulphate. Crossreactivity tests for antisera were carried out by preabsorbing each antiserum with a 10 μM concentration of all other antigens used in this analysis.

RESULTS

A. Brattleboro rat
Cytoarchitecture
The suprachiasmatic nucleus (SCN) of Brattleboro rats was characterized by densely packed groups of small neurons lying above the dorsal ridge of the optic chiasma on the bilateral side of the third ventricle (Fig. 1). In cryostat sections the rostrocaudal length of the SCN was approximately 720 μm. At the middle level of the SCN in the frontal plane, the greatest height of the nuclear mass was 290 μm and the width was 260 μm. The SCN of the Brattleboro rat showed an oval conformation. The SCN at the middle level was typically divided into two distinct subpopulations by a cell-sparse zone, the dorsomedial and ventrolateral parts. The neuronal somata in the dorsomedial part were packed tightly and they were smaller in diameter (10×10 μm), whereas those in the ventrolateral part were segregated and appeared relatively larger (15×15 μm). In the most rostral and caudal portions of the SCN there was almost no conspicuous subdivision. The rostral portion of the nucleus consisted of only larger neurons (15×13 μm). The caudal portion, on the contrary, contained smaller neurons and the exact dorsal border of the SCN was difficult to identify.
Fig. 1. Photomicrograph of a frontal section through the suprachiasmatic nucleus (SCN) of a Brattleboro rat stained with cresyl violet. ×125

Fig. 2-6. The SCN of a Brattleboro rat stained immunohistochemically with anti-VIP serum (Fig. 2), anti-GRP serum (Fig. 3), anti-somatostatin serum (Fig. 4), anti-NPY serum (Fig. 5) and anti-serotonin serum (Fig. 6). ×125
Peptides and Amine in SCN of Brattleboro Rat

Immunohistochemistry

Animals under normal conditions

Vasoactive intestinal polypeptide (VIP): VIP-immunoreactive neuronal somata and fibers were distributed in the SCN. At the most rostral level of the nucleus a few VIP-containing varicose fibers were observed, but no immunoreactive cell bodies occurred within the nucleus. More caudally, immunoreactive fibers appeared to be more copious and there was a dense concentration of VIP-containing cell bodies. At the middle level of the SCN a large number of immunoreactive cell bodies and fibers were preferentially distributed in the ventral part, particularly in the ventromedial region (Fig. 2). The ventrolateral region at this level contained a moderate number of immunoreactive cell bodies with varicose fibers, while the dorsal half of the SCN was supplied with a few fibers. Near the midline beneath the bottom of the third ventricle decussating VIP-containing fibers had a distinct beaded appearance. In the caudal portion of the nucleus, most of the VIP-immunoreactive neurons were located in the ventromedial part; the rest of the SCN was characterized by profuse immunoreactive fibers, which intermingled with the fibers distributed in the dorsal aspect of the SCN.

Gastrin-releasing peptide (GRP)/bombesin: GRP-immunoreactivities were found in the cell bodies and fibers of neurons in the SCN. The rostral portion of the nucleus contained a large number of GRP-immunoreactive varicose fibers, while immunoreactive cell bodies were not observed. In the middle portion of the SCN the highest number of cell bodies, along with an extensive and dense plexus of varicose fibers displaying GRP-immunoreactivity were present in the ventromedial part of the nucleus. These immunoreactive cell bodies were oval in shape and 10-15 µm in diameter (Fig. 3). Scattered GRP-containing varicose fibers at this level were observed in the ventrolateral and dorsal parts of the nucleus. Immunoreactive fibers also crossed the midline to form a commissural connection between each nucleus. At the caudal level, dense accumulations of GRP-containing cell bodies and varicose fibers were distributed in the ventrolateral part of the SCN, whereas the rest of the nucleus was filled with a moderate number of immunoreactive fibers, some of which were extended out of the dorsal aspect of the nucleus.

Somatostatin: In the rostral portion of the SCN, small numbers of somatostatin-immunoreactive cell bodies and varicose fibers were distributed in the ventral part. The highest concentration of cell bodies and fibers which contain somatostatin-immunoreactivity was observed in the ventromedial part of the middle portion of the SCN (Fig. 4). The somata were oval in shape and 10 µm in diameter. More caudally, the number of the immunoreactive cell bodies and fibers appeared to decrease, and at the caudal end of the SCN little immunoreactivity could be found.

Vasopressin: No immunohistochemically demonstrable cell bodies and fibers were present within the SCN from the rostral portion to the caudal end.

Neuropeptide Y (NPY): NPY-immunoreactivity was restricted to the varicose fibers within the nucleus. In the rostral portion of the nucleus a great accumulation of immunoreactive fibers was seen in its ventral part immediately above the optic chiasma. More caudally, these profuse fibers expanded dorsally and laterally to form a dense plexus. At the middle level the highest number of NPY-containing fibers was demonstrated in the ventrolateral part of the nucleus, while the dorsomedial part of the SCN was almost devoid of immunoreactive fibers which stood out conspicuously amid the profusion of the NPY-containing fibers in the periventricular area of the third
ventricle (Fig. 5). This distributional pattern continued to the caudal end of the SCN, and there was a large number of communicable immunoreactive fibers between each nucleus.

Serotonin: No immunoreactive cell bodies were present in any portion of the SCN. At the rostral level, serotonin-immunoreactive varicose fibers were restricted to the ventral part of the nucleus, just dorsal to the optic chiasma. More caudally, the density of serotonin-containing fibers increased dorsally. At the middle level, the highest concentrations of serotonergic fibers were observed in the ventral half of the SCN, in contrast to scant immunoreactivity in the dorsal half of the nucleus (Fig. 6). The caudal portion of the SCN, particularly the ventrolateral part, contained a moderate number of serotonin-immunoreactive fibers, some of which appeared to penetrate laterally and intermingle with an abundant serotonergic plexus around the nucleus. At all levels sectonin-containing fibers exhibited a pericellular arrangement around the non-immunoreactive neurons.

Animals implanted subcutaneously with vehicles and arginine-vasopressin
In these animals there was no conspicuous difference in the distributional patterns of VIP-, GRP-, somatostatin-, NPY, and serotonin-immunoreactivity. In addition, no immunoreactivity of vasopressin was recognized in these animals.
The SCN of Long-Evans rats was located bilaterally in the wall of the third ventricle immediately dorsal to the optic chiasma. In the frontal sections the total length of the SCN was 810 μm, with a 310 μm-height and 290 μm-width size. Two distinct subpopulations were discernible in the SCN of this species, i.e., dorsomedial and ventrolateral subdivisions, although there was no clear-cut separation between them at the most rostral portion of the nucleus. The former subdivision contained tightly packed small neurons, whose diameters were calculated to be 9×10 μm, while the latter was characterized by a relatively sparse arrangement of neurons with a diameter of 14×15 μm.

**Immunohistochemistry**

*Animals under normal conditions*

VIP: The distributional pattern of VIP-containing neurons in the SCN was quite comparable to those in previous reports on normal Wistar rats (Sims et al., 1980). Throughout the SCN, the ventral part, particularly the ventromedial part contained a large number of immunoreactive cell bodies which were oval in shape and 10–12 μm in diameter, in addition to the profusion of VIP-containing varicose fibers in the same area.
Fig. 12-17. The SCN of a Long-Evans control rat stained immunohistochemically with anti-VIP serum (Fig. 12), anti-GRP serum (Fig. 13), anti-somatostatin serum (Fig. 14), anti-vasopressin serum (Fig. 15), anti-NPY serum (Fig. 16) and anti-serotonin serum (Fig. 17). \( \times 125 \)
Peptides and Amine in SCN of Brattleboro Rat

GRP: GRP-immunoreactivity was found in the cell bodies and varicose fibers throughout the SCN. The highest concentration of immunoreactive cell bodies (12-14 \( \mu \text{m} \) in diameter) was seen in the ventromedial part of the middle portion of the SCN. In addition, the greatest accumulation of GRP-containing varicose fibers was present in the ventromedial subdivision of the nucleus.

Somatostatin: Throughout the SCN, the more rostral portion contained larger numbers of immunoreactive cell bodies (9 \( \mu \text{m} \) in diameter) and fine varicose fibers, particularly in the ventromedial part. The rest of the nucleus was supplied with a small number of somatostain-containing fibers.

Vasopressin: A strong concentration of vasopressin-containing cell bodies (10-12 \( \mu \text{m} \) in diameter) and varicose fibers was noticeable in the dorsomedial part of the SCN, while the ventrolateral part contained few varicose fibers.

NPY: No immunoreactive cell bodies were present in any portion of the SCN. At all levels of the nucleus the ventrolateral part was supplied with the greatest accumulation of NPY-containing fibers. In contrast, immunoreactive varicose fibers were absent in the dorsomedial part of the SCN.

Serotonin: The distributional pattern of serotonin-immunoreactive fibers within the SCN was observed to be quite similar to our previous description for Wistar rats (UEDA et al., 1983); throughout the full extent of the nucleus, profuse serotonergic fibers were preferentially distributed in the ventrolateral part, and a few immunoreactive varicose fibers were also observed in the dorsomedial part of the SCN.

**Animals implanted subcutaneously with vehicle and arginin-vasopressin**

No obvious changes in the distributional patterns of immunoreactivities for VIP, GRP, somatostatin, vasopressin, NPY, and serotonin were found lightmicroscopically in those animals with the placement mini-osmotic pump filled with or without vasopressin.

**DISCUSSION**

The present study clearly showed both differences and similarities in the distributional patterns of immunoreactivities for VIP, GRP, somatostatin, NPY, and serotonin in the SCN of Brattleboro rats, which are homozygous for diabetes insipidus. Moreover, similarities in the distribution of immunoreactive cell bodies and varicose fibers between the Brattleboro and Long-Evans rats were described, even if subjected to the administration of vasopressin. These results have several points of interests worth discussing.

Cytological studies with silver impregnation and electron microscopy have demonstrated that the SCN of the rat can be cytologically divided into two subpopulations: the dorsomedial and ventrolateral parts; the former is characterized by small neurons with a scanty cytoplasm and restricted dendritic arbor, and the latter by relatively larger neurons with more extensive dendritic arborization (VAN DEN POL, 1980; MOORE, 1982). It has been shown by autoradiography that afferent fibers to the SCN originat-ed from the retinal ganglion cells and lateral geniculate nucleus terminate in the neurons of the nucleus, particularly in the ventrolateral part (MOORE and LENN, 1972; HENDRICKSON et al., 1972; SWANSON et al., 1974; RIBAK and PETERS, 1975). Immunohistochemical investigations have clarified that the ventrolateral part of the mammalian SCN contain a neuronal structure endowed with specific peptides and amines; nerve
fibers displaying the immunoreactivity of the so-called pancreatic polypeptide family such as avian pancreatic polypeptide in rats (CARD and MOORE, 1982; CARD et al., 1983) and hamsters (CARD and MOORE, 1984), FMRF-amide in rats (WEBER et al., 1981; MOORE et al., 1984), NPY in rats (ALLEN et al., 1983; MOORE et al., 1984; UEDA et al., 1986), and serotonin in rats (STEINBUSCH, 1981; TAKEUCHI and SANO, 1983; UEDA et al., 1983), hamsters (UEDA et al., 1983; CARD and MOORE, 1984), cats (UEDA et al., 1983), and monkeys (UEDA et al., 1983; KAWATA et al., 1984). On the other hand, the dorsomedial part of the SCN contained neuronal cell bodies with processes showing the immunoreactivity of vasopressin in mice (ZIMMERMAN et al., 1975; SOFRONIEW and WEINDL, 1980), rats (VANDESANDE et al., 1975; ZIMMERMAN et al., 1975; SOFRONIEW and WEINDL, 1978; VAN LEEWEN et al., 1978; SOFRONIEW and WEINDL, 1980; KAWATA, 1983; UEDA et al., 1983), hamsters (SOFRONIEW and WEINDL, 1978; UEDA et al., 1983; CARD and MOORE, 1984), cats (SOFRONIEW and WEINDL, 1980; UEDA et al., 1983), monkeys (SOFRONIEW and WEINDL, 1980; KAWATA and SANO, 1982; UEDA et al., 1983) and humans (SOFRONIEW and

Fig. 18–23. Diagrams illustrating the distribution of VIP- (Fig. 18), GRP- (Fig. 19), somatostatin- (Fig. 20), arginine-vasopressin- (Fig. 21) NPY- (Fig. 22) and serotonin (5-HT)- (Fig. 23) immunoreactivities at the rostral, middle and caudal levels of the SCN of a Long-Evans control rat. OC optic chiasma, V third ventricle.
Peptides and Amine in SCN of Brattleboro Rat

Weindl, 1980), and somatostatin in rats (Dierickx and Vandesande, 1979) and hamsters (Card and Moore, 1984). The present study confirmed the occurrence of such peptides and amine in the SCN of both the Brattleboro and control Long-Evans rats, with minor variations in their distributional patterns. The topographic separation and morphological differentiation of these peptides and amine have suggested their involvement in maintaining different circadian rhythms (Moore, 1982).

With respect to the efferent elements of the SCN, detailed immunohistochemical studies combined with the lesionings of the nucleus have clarified that axons from the vasopressin-containing neurons within the rat SCN do not project to the neurohypophysis, but to various regions throughout the central nervous system (Buijs, 1978; Sofroniew and Weindl, 1978; Hoorneman and Buijs, 1982). In these areas a conspicuous number of axosomatic contacts formed, suggesting that the vasopressinergic neurons of the rat SCN might be involved in modulating the activities of other neurons (Sofroniew and Weindl, 1980). However, the efferent projection from the SCN of the Brattleboro rat has not been demonstrated.

Concerning the functional role of vasopressin in the SCN, Peterson et al. (1980) have succeeded in demonstrating from behavioriological viewpoints that the circadian
rhythms in locomotor activity, drinking and pineal serotonin-N-acetyltransferase in the homozygous Brattleboro rat appear the same as the normal Long-Evans rat. These results strongly suggest that the presence of vasopressin in the SCN is not essential for maintaining at least the above mentioned three rhythms. These authors have also stated that circadian rhythms are affected only when a major portion of the total neuronal population of the SCN is ablated. Van den Pol (1980) has described in detail how the neurons of the SCN have a large number of possibilities for intercellular communication between cells within the nucleus, including the frequent local circuit of the neurons. Since Brattleboro rats, as homozygous to diabetes insipidus, are totally lacking in vasopressin in the central nervous system, the possibility could be conceived that some compensatory mechanisms might be present to maintain the biorhythms in this mutant rat. At least lightmicroscopically, however, the present investigation clearly shows similar patterns in the distribution of immunoreactive peptides and amine between the Brattleboro and Long-Evans rats. Furthermore, these results were not affected by the exogeneous administration of arginine-vasopressin. The morphological findings that the absence of vasopressin from the SCN does not alter the distribution of immunoreactive neurons provides evidence supporting the behavioral observations reported by Peterson et al. (1980) in this mutant rat (vide supra).

REFERENCES


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