Vasopressin Fiber Pathways in the Rat Brain Following Suprachiasmatic Nucleus Lesioning

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The contribution of the suprachiasmatic nucleus to the vasopressinergic innervation of the rat brain was determined by following the changes in the immunocytochemical localization of vasopressin-containing fibers at various intervals between 2 days and 12 weeks after bilateral lesioning of this nucleus. The disappearance of the vasopressinergic fibers makes it plausible that vasopressin-containing pathways run from the suprachiasmatic nucleus towards the periventricular nucleus, the dorsomedial nucleus of the hypothalamus and the organum vasculosum laminae terminalis. Since after lesioning the vasopressinergic fibers remained unaltered in the lateral septum, the lateral habenular nucleus, the nucleus of the amygdala, the diagonal band of Broca, the nucleus of the solitary tract, the interpeduncular nucleus and the dorsal raphe nucleus, the suprachiasmatic nucleus sends no or only minor projections to these areas. In contrast to the literature, these findings indicate that the paraventricular nucleus and possibly the supraoptic nucleus form the major source for vasopressinergic pathways in the brain.

INTRODUCTION

The neurohypophyseal hormones arginine-vasopressin (AVP) and oxytocin are synthesized in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON)²⁴. More recently, AVP-containing neurons have been demonstrated in the suprachiasmatic nucleus (SCN)^{32,36}. Specific and sensitive immunocytochemical techniques enabled, in the rat, the description of an extensive exohypothalamic AVP fiber system originating from the SCN, the PVN and possibly from the SON^{7,11,28,29}. By means of immunoelectron microscopy in various structures of the limbic system it was demonstrated that these fibers terminate synaptically on neurons and dendrites¹⁰. These AVP-containing synapses are morphologically indistinguishable from the classical transmitter-containing ones. These vasopressinergic fibers are thought to be the morphological basis⁹ for the described effects of vasopressin on central processes. Until now, the description of the exohypo-

thalamic vasopressinergic and oxytocinergic fiber systems has been performed almost exclusively by the use of the intricate technique of tracing fibers in serial sections^{7,11,28,29}. This procedure, however, is not reliable if fibers from the various nuclei intermingle. Another attempt to specify the nucleus of origin was to distinguish SCN from PVN fibers by their caliber^{28,29}. Any support for this distinction, however, is not available. Based on these two methods, the SCN is thought to innervate various regions of the limbic system^{7,11,28,29} and even the dorsal raphe nucleus and NTS. The establishment of the origin of these pathways awaits, therefore, other, more appropriate, techniques. By means of retrograde transport techniques, the presence of a connection between the PVN and the NTS has not been firmly established^{2,25,33}, while the evidence for this connection regarding the vasopressinergic and oxytocinergic cells of the PVN was demonstrated²⁷.

In the present study an approach was chosen of lesioning the SCN neurons, after which the dis-

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appearance of the AVP fibers in the different brain areas, determined by means of a sensitive and specific immunocytochemical procedure, was an indication for the projections of this nucleus.

MATERIALS AND METHODS

Thirty-three young adult male rats (160–200 g) of the Wistar strain (TNO, Zeist, the Netherlands) were used in this study.

Stereotaxic lesioning

Under intramuscularly applied Hypnorm anesthesia (1 ml/kg body weight) the rats were mounted horizontally in a David Kopf stereotact. The midline of the earbars served as a reference for the anterior–posterior and the dorsal–ventral positionings. The anterior–posterior position of the electrode ranged from 6.35 to 6.90 mm rostral to the reference point, directly proportional to the body weight. Using the midsagittal bloodsinus as a midline reference, the electrodes were inserted 1.15 mm lateral to this line at an angle of 6° and at a depth of 0.4 mm above the midline of the earbars. Coagulative lesions were made by applying radiofrequency energy (Radionics) via the approximately 1.5 mm long uninsulated ends of the thermocouple electrodes (0.7 mm in diameter) for 45 s at a constant temperature of 43 °C. After bilateral SCN lesioning, the rats were housed in individual cages with free access to food and water under a light–dark cycle of 12 h.

Fixation and staining

The rats were divided into 5 different groups, each of which consisted of 1 control rat and 4–5 rats of 2 days, 1, 2, 4 and 12 weeks after lesioning. All animals of one group were sacrificed, fixed and stained simultaneously. Each experimental rat could thus be compared with a control rat that had been treated simultaneously and similarly. After intraperitoneal administration of Nembutal (1 ml/kg body weight) the rats were perfused intracardially with 0.9% saline followed by 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.35 (all reagents Merck). The brains were dissected and immersed in the same fixative for at least 2 h at 4 °C. Vibratome sections (Oxford instruments) of 100 µm were cut transversally and collected into 0.05 M Tris-HCl, pH 7.6, in 0.9%

NaCl. The immunocytochemical staining, using the unlabeled antibody enzyme method³⁰, was accomplished according to Buijs et al.¹¹. Vasopressin antiserum (125) was used in a 1:1000 dilution with diaminobenzidine (Sigma) as chromogen. After staining the sections were mounted on glass slides and dried overnight at room temperature, after which dehydration and embedding in malinol followed. For details about the specificity and production of the antisera the reader is referred to other papers^{11,31}. The number of SCN neurons still staining after lesion is expressed as the percentage of the AVP neurons in the SCN of the control rats. AVP fiber density was estimated in a semi-quantitative manner distinguishing three categories: ++, as many fibers as in the control rats; +, obviously less fibers; —, no fibers or a single fiber.

RESULTS

Extent of the lesion

In all rats the site and size of the lesion were rather constant (approximately 1.5 mm in diameter; Fig. 1A, B). Table I shows that after 1, 2, 4 and 12 weeks the lesions generally resulted in a complete or almost complete destruction of the vasopressinergic SCN neurons. Only in a few animals some AVP cells (ranging from 2 to 100 per animal), remained visible after lesioning in the ventrolateral part of the SCN. At 2 days after lesioning, however, the extent of the lesion is hard to define because many other cells are still degenerating and are causing non-specific staining.

TABLE I

Extent of the lesion

Each column (I through V) represents the animals of one group which have been treated simultaneously. In these columns the number of the remaining AVP neurons in the SCN in the left (l) and right (r) side of the brain is expressed as a percentage of the number of AVP neurons in the SCN of the control rats, at the various time intervals after lesioning.

Survival time	Group									
	I		II		III		IV		V	
	l	r	l	r	l	r	l	r	l	r
2 days	0	0	0	0	0	0	0	0	0	0
1 week	17	0	0	3	0	0.3	5	0.17	0	0
2 weeks	0	0.7	3	0	1	10	0	0	0	0.7
4 weeks	0	0	0	0	0	0.17	0	0	0	0
12 weeks	0	0	0	0	0	0				

Hypothalamic areas

The vasopressin fiber density was screened in the entire rat brain but most attention was paid to those areas which were expected to be innervated by SCN fibers. In all rats, an AVP fiber density similar to that of the staining procedure control, persisted after 2 days, 1 week, 4 weeks and 12 weeks in the following brain areas: LS (Fig. 2A, B), LH, amygdala, diagonal band of Broca, NTS, interpeduncular nucleus and dorsal raphe nucleus. However, in all rats, already 1 week following the lesion a decrease in fiber density was visible in the organum vasculosum laminae terminalis (OVLT) (Table IIA; Fig. 3A, B), the dorsomedial nucleus of the hypothalamus (DMH) (Table IIB; Fig. 4A, B) and in the periventricular nucleus (PVS) (Table IIC; Fig. 5A, B). Besides a decrease in density of the fibers, degeneration could not be demonstrated by morphological changes. After a survival period of two weeks and longer all or nearly all AVP fibers were absent in the PVS, in the DMH and in the OVLT, except, sometimes, for a few fibers in the latter area. In some animals of different survival times vasopressin-positive cell bodies could be demonstrated in the region of the electrode tracts. Fiber outgrowth from these cell bodies could, however, not be visualized.

Fig. 1A and B: transverse sections of the hypothalamus of a control rat (A) and 1 week after suprachiasmatic nucleus (SCN) lesioning (B). Note the intact other hypothalamic nuclei stained for vasopressin after SCN lesioning. PVN, most rostral part of the paraventricular nucleus; SON, supraoptic nucleus, V, ventricle. Bar = 250 μ m.
 Fig. 2A and B: transverse sections for the lateral septum (LS) of a control rat (A) and 12 weeks after SCN lesioning (B). Note the similar density of vasopressin fibers in these two animals. Arrows point to the electrode tract. Bar = 100 μ m.
 Fig. 3A and B: transverse sections of the organum vasculosum laminae terminalis of a control rat (A) and 12 weeks after SCN lesioning (B). Arrow indicates a single remaining vasopressin fiber. Bar = 50 μ m.
 Fig. 4A and B: transverse sections in the region of the dorsomedial nucleus of the hypothalamus (DMH) of a control rat (A) and 12 weeks after SCN lesioning (B). Note the total absence of vasopressin fibers in the DMH of the lesioned animal. V, ventricle. Bar = 200 μ m.
 Fig. 5A and B: transverse sections in the region of the lateral habenular nucleus (LH) and periventricular nucleus of a control rat (A) and 4 weeks after SCN lesioning (B). Note the vasopressinergic fibers in the periventricular nucleus of the control rat (arrow) (A) and the absence of these fibers in the same region of the lesioned animal. V, ventricle. Bars = 100 μ m for A and 50 μ m for B.

TABLE II

Density of AVP fibers in the organum vasculosum laminae terminalis (OVLT) (A), in the periventricular nucleus (PVS) (B) and in the dorsomedial nucleus of the hypothalamus (DMH) (C), at various times after lesioning

The two entries in each column correspond with the two sides of the brain as shown in Table I; ++, as many fibers as the staining control; +, obviously less fibers; —, a single or no fibers.

(A) AVP fiber density in the OVLT

Survival time	Group									
	I		II		III		IV		V	
	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>
2 days	++	++	++	++	++	++	++	++	++	++
1 week	+	—	—	—	—	+	+	—	—	—
2 weeks	—	—	—	—	+	+	—	—	—	—
4 weeks	—	—	—	—	—	—	—	—	—	—
12 weeks	—	—	—	—	—	—	—	—	—	—

(B) AVP fiber density in the PVS

Survival time	Group									
	I		II		III		IV		V	
	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>
2 days	++	++	++	++	++	++	++	++	++	++
1 week	+	+	+	+	+	+	+	+	+	+
2 weeks	—	—	—	—	—	—	—	—	+	+
4 weeks	—	—	—	—	—	—	—	—	—	—
12 weeks	—	—	—	—	—	—	—	—	—	—

(C) AVP fiber density in the DMH

Survival time	Group									
	I		II		III		IV		V	
	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>	<i>l</i>	<i>r</i>
2 days	++	++	++	++	++	++	++	++	++	++
1 week	+	—	—	—	+	+	—	—	—	—
2 weeks	+	+	—	—	—	—	—	—	—	—
4 weeks	—	—	—	—	—	—	—	—	—	—
12 weeks	—	—	—	—	—	—	—	—	—	—

DISCUSSION

The immunocytochemical localization of AVP provides a good possibility to check the extent of the lesion and to establish simultaneously whether the AVP containing part of the SCN has indeed been lesioned. The lesion procedure as performed in this study appears to be very reliable since, in most animals, the lesion resulted in a complete or almost complete destruction of the vasopressinergic SCN neurons.

The time course of the decreasing AVP fiber density in OVLT, DMH and PVS indicates that fiber degeneration occurs between 2 days and 2 weeks after lesioning. After 2 weeks or longer all AVP fibers in these regions have disappeared in most animals. This makes it very plausible that the SCN projects with AVP fibers towards the OVLT, the DMH and the PVS (Fig. 6). On the other hand, the destruction of possible AVP fibers, originating from the PVN and/or the SON, and possibly passing through the lesioned area, cannot be excluded by the

Fig. 6. Illustration of major vasopressinergic pathways of the suprachiasmatic nucleus (SCN) in the rat brain projected in the sagittal plane. OVLT, organum vasculosum laminae terminalis; PVS, periventricular nucleus; DMH, dorsomedial nucleus of the hypothalamus.

present experiments. However, irrespective of the survival time, no indication was found of fibers accumulating AVP at the border of the lesion. This makes it very likely that the PVN and SON have no or only a very small amount of fibers passing through this area.

An interesting finding is that the AVP fibers in the PVS appear to degenerate some days later than the fibers in the DMH and OVLT. This might be due to the greater distance from the SCN to the PVS, resulting in a slightly delayed degeneration of these fibers in this more distant region. For the time span of the experiment (up to 12 weeks) it was demonstrated, in addition, that no reinnervation occurs either from surviving SCN cells or from the other AVP producing nuclei.

No difference was found in the AVP fiber density pattern in the LH, the LS, the amygdala, the NTS, the dorsal raphe nucleus, the diagonal band of Broca and in the interpeduncular nucleus between SCN-lesioned and non-lesioned animals. Since very small changes in the AVP fiber patterns will not be detectable by the semi-quantitative methods used, our results suggest that the SCN has no or only minor projections to these areas. The previous interpretation^{7,11,28,29} that these areas were innervated by SCN fibers thus has to be explained by intermingling of SCN fibers with PVN fibers. This means

also that fiber caliber, in contrast to the suggestion of Sofroniew and Weindl^{28,29}, is no safe indication for the origin of AVP fibers.

The present results are in agreement with the retrograde labeling experiments that established SCN projections to the DMH¹⁹. In addition, electrophysiological studies, which demonstrated that PVN neurons have efferent connections to the LS and amygdala²¹, indirectly support our findings and suggest that at least part of these AVP fibers are derived from the PVN.

The present results consequently also change the interpretation of de Vries et al.¹³, who correlated a sex difference in AVP fiber density of the LS and the LH to a sex difference in the density of SCN neurons¹⁶. At present these sex differences in the vasopressinergic innervation of the LS and the LH may thus be due to an influence of steroid hormones on the PVN or the SON. This hypothesis is supported by the finding of Rhodes et al.²³, who reported the distribution of estrogen-concentrating neurophysin-containing neurons within the PVN. Although most studies now point to the PVN as a major source for the extrahypothalamic AVP-containing fibers, this opinion certainly awaits further investigation, such as by means of lesioning the PVN or combining retrograde transport techniques with immunocytochemistry¹⁷.

Centrally released vasopressin is thought to be involved in various processes such as the regulation of blood pressure^{4,18}, temperature regulation¹², brain water balance²² and avoidance behavior¹⁵. Assuming that the PVN indeed contributes to the exohypothalamic AVP fibers this nucleus might be crucial for the integration of information on these processes. From this point of view it is interesting that the possibility of feedback from all vasopressin-innervated areas to the PVN exists; by injecting HRP into the PVN^{26,34} retrogradely labeled neurons in the LS, LH, amygdala and NTS were demonstrated. That this feedback is in fact on vasopressinergic and oxytocinergic neurons can be concluded from studies in which stimulation of the septal area or amygdala resulted in an inhibition or excitation of these neurosecretory nuclei²¹.

The numerous ramifications of fibers and the appearance of perineural structures in the DMH, PVS and OVLT suggest that these AVP fibers, which originate from the SCN, terminate in these regions, especially since AVP-containing synapses could be demonstrated in regions with a similar AVP fiber pattern¹⁰. One can speculate, therefore, what the physiological role of AVP from the SCN might be. The OVLT and the PVS are considered to be of importance for the regulation of the water

balance^{1,5,6} while the involvement of the DMH in the regulation of food intake and body weight was demonstrated additionally³. AVP fibers from the SCN towards the OVLT, the PVS and the DMH may influence these processes, since in experiments in which the SCN was lesioned, a disturbance of the circadian rhythmicity of water intake and feeding behavior ensued^{20,35}. The homozygous Brattleboro rat, which genetically lacks AVP, could be expected to offer a simple and ideal model for central effects. However, since it was demonstrated that, in addition, the serotonergic innervation is different (Steinbusch, unpublished results), the Brattleboro is not just an AVP-less animal, but one with a very different brain. Consequently, as long as one is not able to lesion the AVP-containing neurons alone, it will be extremely difficult to establish the exact function of these peptidergic neurons.

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