The Origin of the Vasopressinergic and Oxytocinergic Innervation of the Rat Brain with Special Reference to the Lateral Septum

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The origin of the vasopressin-containing fibers in the rat lateral septum was studied by means of lesioning specific areas, in which vasopressin-containing cells are found, or by surgically separating the septum from the underlying structures. Following these procedures sections of the brain were stained immunocytochemically for the presence of vasopressin. In addition, retrograde labeling tracers were injected in the lateral septum.

Lesioning of the paraventricular nucleus did not result in the disappearance of vasopressin fibers from the lateral septum, nor from the various other areas studied. It did, however, cause the disappearance of fibers from the nucleus of the solitary tract and the nucleus ambiguus. By contrast, after the same lesion practically the whole oxytocinergic innervation of the brain disappeared. Injection of tracers into the lateral septum revealed retrograde labeled cells, e.g. in the bed nucleus of the stria terminalis, but not in the paraventricular and supraoptic nucleus.

Horizontal cuts under the lateral septum, intersecting the diagonal band of Broca, resulted in a dramatic decrease of the vasopressin fibers in the lateral septum, suggesting that the fibers enter the septum via this structure. Moreover, since the vasopressin fiber density was found to decrease drastically in the lateral septum after lesioning the bed nucleus of the stria terminalis, the vasopressin cells found in this area are probably the source of these fibers. Other areas where fibers were seen to decrease after lesions of the bed nucleus are the diagonal band of Broca, the area of the anterior amygdala, the lateral habenular nucleus, the periventricular gray, and the locus coeruleus.

INTRODUCTION

Immunocytochemical studies have demonstrated that various areas of the brain are innervated by vasopressin (VP) containing fibers^{1,22}. An intriguing aspect of the VP system is the sexually dimorphic innervation of the lateral septum, i.e. the VP fiber density in this area is higher in male than in female rats⁸. This sex difference appeared to be dependent on the neonatal presence of androgens⁹. Several arguments plead for a neurotransmitter role of vasopressin in the lateral septum. For instance, immuno-electron-microscopy has revealed that VP fibers terminate synaptically on neuronal elements³, while in vitro experiments performed on slices of the lateral septum have demonstrated that VP can be released upon a depolarizing stimulus⁴. In addition, micro-iontophoretic application of lysine-vasopressin to cells in the lateral septum has demonstrated the ability of this peptide to alter neuronal firing patterns¹¹. All these findings point to an important central role for VP in

the lateral septum, where it might be involved in functions such as regulation of body temperature⁵ and water balance^{1,2}.

Although many features of the VP innervation of the lateral septum are gradually becoming clearer, its site of origin is still obscure. Thus far the VP fibers in the brain have been thought to be derived from the paraventricular (PVN), supraoptic (SON) and supra-chiasmatic nuclei (SCN)^{1,22}. Since lesion studies involving the SCN have revealed that this nucleus did not contribute to the VP innervation of the lateral septum¹⁰, the PVN was lesioned in the present study. In addition, retrograde labeling tracers were injected into the lateral septum. The results of these experiments make it clear that neither the PVN nor the SON send VP fibers to the lateral septum. In a parallel study Van Leeuwen and Caffé²⁷ have detected in colchicine-treated rats, the presence of VP-containing cells in several areas outside the hypothalamus. Since even in the rostral part of the lateral septum some VP-containing cells were found, horizontal

cuts were made, separating the septum from underlying structures to see whether the vasopressin fibers in the lateral septum were derived from outside the septum. In addition, the bed nucleus of the stria terminalis was lesioned since cells could be labeled in this area retrogradely from the septum and because this nucleus, in particular, appeared to contain a great number of VP cells²⁷.

MATERIALS AND METHODS

Retrograde labeling studies

In 37 male Wistar rats, WU:Cpb (250–300 g), anesthetized with Hypnorm (1 ml/kg body weight) tracer injections were made successfully in that area of the lateral septum which is most densely innervated by VP fibers. This area is caudally bordered by the septum fimbrialis and extends about 1 mm rostrally, occupying a crescent-shaped area directly lining the ependyma (Fig. 1). Different types of tracing techniques were used in order to increase the chance of finding labeled cells. The injections were placed according to the coordinates of Pellegrino et al.¹⁶, using the bregma as a zero reference point for the anterior–posterior coordinates.

Horseradish peroxidase (HRP), Sigma Grade VI, was either injected stereotactically (4 rats) via a 5 μ l Hamilton syringe (0.5–1 μ l 30% HRP in an aqueous solution), or applied iontophoretically, (10 rats) using glass micropipettes (tip diameter 10–30 μ m) filled with 10% HRP in 0.01 M NaCl. HRP was ejected by means of a positive current of 1 μ A for 20 min, alternating 5 s on, 5 s off. Nine rats were injected with 0.5 μ l of a 3% aqueous solution of wheat germ agglutinin, conjugated to HRP (WGA–HRP), Sigma Grade VI. Twenty-four or 48 h after injection the rats were anesthetized with Nembutal and the brains were fixed by perfusion fixation with 1.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer according to Rosene and Mesulam¹⁷. After dissection the brains were kept overnight in 10% sucrose in 0.1 M phosphate buffer at 4 °C. Subsequently, 50 μ m thick frozen sections were cut transversally and collected in phosphate buffer. In order to visualize the presence of HRP activity the sections were either processed with the diaminobenzidine method of Malmgren and Olsson¹⁴ or with the tetramethylbenzidine method of Mesulam¹⁵.

Fluorescent tracers were injected stereotactically (0.3 μ l through a 5 μ l Hamilton). The tracers used were True Blue ($\pm$ 3% aqueous solution, 5 rats) or Granular Blue ($\pm$ 3% aqueous solution, 9 rats). Following survival times of 2–5 days, the animals were anesthetized with Nembutal and perfused intracardially with 0.9% saline, followed by 10% formaline. After dissection, the brains were kept overnight at 4 °C in a phosphate-buffered, 10% sucrose solution. Subsequently the brains were frozen and 50 μ m-thick transverse sections were cut, which were mounted immediately on glass slides. Air-dried sections were studied with a Leitz fluorescence microscope.

Lesion studies

Male Wistar rats, WU:Cpb (160–190 g), were anesthetized with Hypnorm (1 ml/body weight) and then mounted in a David Kopf stereotactic apparatus with the incisor bar placed level with the intra-aural line. The midpoint of the two centered earbars was used as a zero reference point. The angle of the lesion electrodes was 6° from the vertical axis and rectangular to the anterior–posterior axis. The following coordinates were used: for the PVN lesions, bilaterally: AP +5.4 to +5.9 mm (directly proportional to the body weight), L +1.2 mm, V +1.3 mm; for lesions of the bed nucleus of the stria terminalis, unilaterally, AP +6.8 to +7.3 (directly proportional to the body weight), L +1.6 mm, V +3.3 mm. Lesions were made by applying radiofrequency energy using the 1.5 mm long uninsulated ends of the thermocouple electrodes (diameter 0.7 mm) for 1 min at a constant temperature of 50 °C (small PVN lesions, in 5 rats), 60 °C (large PVN lesions, in 5 rats), or 65 °C (lesions of the bed nucleus of the stria terminalis, in 6 rats).

Six rats weighing 160–190 g were anesthetized with Hypnorm and mounted in the stereotaxic apparatus in the same way as described for the radiofrequency lesions. A Halasz-type knife (2 mm radius) was inserted vertically in the rat: 1.6 mm rostral to the bregma and 1.2 mm lateral to the midline. By using these coordinates, and inserting the knife parallel to the longitudinal axis via the lateral ventricle, the tissue was damaged as little as possible. At a depth of 6 mm, the knife was rotated 180°, by which an incision was made on the medial side, thus separating the septum unilaterally from the underlying structures.

After a survival time of 2.5–3 weeks the animals

Fig. 1. Frontal planes reproduced from the atlas of König and Klippel¹², demonstrating the distribution of labeled cells (dots) after injection of WGA-HRP into the lateral septum (LS). Black spot on the right side indicates injection site, the hatched area on the opposite side represents that part of the lateral septum, which is most densely innervated by vasopressin-containing fibers. BNST, bed nucleus of the stria terminalis; PVN, paraventricular nucleus; SON, supraoptic nucleus.

Fig. 2. Transverse sections of the brain after injection of WGA-HRP into the lateral septum, showing retrogradely labeled cells (arrows) in (A) the region of the optic chiasm (OC), and (B) the bed nucleus of the stria terminalis (BNST). Note the absence of labeled cells in the supraoptic nucleus (SON). F, fornix. Bar: 100 μ m.

were sacrificed. Following perfusion fixation 100 μm thick, transverse sections of the entire brain were cut with a vibratome (Oxford Instruments), which were stained immunocytochemically for the presence of VP or oxytocin. For details about the fixation and the staining procedure see De Vries et al.⁸. The VP and oxytocin fiber density in various brain areas in lesioned animals was compared with that of control rats, which had not been treated at all.

RESULTS

Retrograde tracing studies

The size of the VP network in the lateral septum being relatively large (Fig. 1), injection of the tracer proved to be more useful than iontophoresis in this study. The latter procedure rendered only small spots, thereby reducing the chance of finding retrogradely labeled cells. This became apparent in studying the labeling in the hippocampus and ventral tegmental area, which regions were used as references to control the efficacy of the retrograde tracing techniques used, because they are known to project to the lateral septum. In the hippocampal formation, labeled cells were found bilaterally in CA3 and ipsilaterally only in CA1 and in the ventral part of the subiculum, conforming to the anterograde labeling studies of Swanson and Cowan²⁴. In the ventral tegmental area a predominantly ipsilateral labeling was found, probably of dopamine neurons that project to the lateral septum¹³.

In our hands the use of the tetramethylbenzidine method¹⁵ proved to be a far more sensitive method for visualizing retrogradely transported HRP or WGA-HRP than was the diaminobenzidine method¹⁴. The latter method was more useful for delineating the site of injection. Both retrograde and

anterograde labeling turned out to be very intense in WGA-HRP-injected rats. When fluorescent tracers were used, Granular Blue and True Blue proved to be even more efficient than WGA-HRP, as judged from the number of labeled cells per section. Granular Blue, in particular, showed bright retrograde and, to a lesser extent, anterograde labeling. The pattern of labeling was not essentially different from that found in the HRP studies.

The results of the retrograde tracing studies are summarized in Fig. 1. In none of the experiments were labeled cells found in the SCN, PVN or SON (Fig. 2A). At the level of the PVN cells were found just lateral to the PVN, lining the fornix. Many labeled cells were present in the lateral hypothalamic area, just dorsal to the SON. Some labeled cells were also found in the ventral part of the bed nucleus of the stria terminalis (Fig. 2B).

Lesion studies

The VP fiber density following lesioning was screened in the whole brain, with special attention being paid to the innervation in the structures listed in Table I.

In the first series of PVN lesions the diameter of the lesioned area was approximately 1.5 mm. These lesions included the delta-shaped part of the PVN in which the highest concentration of VP cells are found (Fig. 3A). After this lesion, VP fibers were seen to decrease only in the nucleus of the solitary tract and the nucleus ambiguus. In a second series, the size of the lesion was approximately 2.5 mm. These lesions not only destroyed the entire area of the PVN, as defined by Swanson and Kuypers²⁵, but also a large area around the nucleus, eliminating many of the small islands with VP and oxytocin cells (Fig. 3B). This treatment resulted both in a dramatic decrease

Fig. 3. Transverse sections of the hypothalamus stained for the presence of vasopressin, showing examples of lesions of the paraventricular nucleus (*) from the first (A) and second series (B). BNST, bed nucleus of the stria terminalis; F, fornix; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus. Bar: 500 μm .

Fig. 4. Transverse sections in the region of the nucleus of the solitary tract (NTS) stained specifically for the presence of oxytocin in a control rat (A) and in a rat from the second PVN lesion series (B). Arrows indicate the few remaining oxytocin fibers in the lesioned rat. Bar: 150 μm .

Fig. 5. Transverse sections in the region of the periventricular and the lateral habenular nucleus (LH) in a control rat (A) and in a rat from the second PVN lesion series (B). Note that the vasopressin fiber network in the periventricular nucleus has disappeared in the lesioned rat (arrows), whereas the fiber staining in the LH remains unimpaired. V, ventricle. Bar: 150 μm .

TABLE I

Effect of different lesions on vasopressin fiber density in termination area

Each vertical column indicates whether vasopressin fiber density was diminished (+) or unimpaired (—) after a series of small PVN lesions (PVN1), a series of large PVN lesions (PVN2), or after unilateral lesions of the bed nucleus of the stria terminalis (BNST) in the diagonal band of Broca (DBB), lateral septum (LS), organum vasculosum laminae terminalis (OVLT), periventricular nucleus (PVS), dorsomedial nucleus of the hypothalamus (DMH), area of the anterior amygdala (AA), lateral habenular nucleus (LH), medial nucleus of the amygdala (MA), hippocampus (HIP), ventral tegmental area (VTA), periventricular grey (PVG), nucleus of the dorsal raphe (NDR), locus coeruleus (LC), nucleus of the solitary tract (NTS) and the nucleus ambiguus (NA).

	DBB	LS	OVLT	PVS	DMH	AA	LH	MA	HIP	VTA	PVG	NDR	LC	NTS	NA
PVN1	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+
PVN2	—	—	—	+	+	—	—	—	—	—	—	—	—	+	+
BNST	+	+	—	—	—	+	+	—	—	—	+	—	+	—	—

in VP fibers within the nucleus of the solitary tract and the nucleus ambiguus, and in the disappearance of the fiber staining in the periventricular (Figs. 5A and B) and the dorsomedial hypothalamic nuclei. The same lesion resulted in an almost complete disappearance of the oxytocinergic innervation of the brain (Figs. 4A and B).

In rats that had been subjected to unilateral horizontal knife cuts immediately ventral to the septum, the VP fiber density was examined only in the lateral septum and the diagonal band of Broca. In all rats the septum on the operated side was completely severed caudally from underlying structures. Variation was found only in the extent of the lesion in the rostral direction.

In those rats where the lesion extended far enough rostral to intersect the diagonal band of Broca a pronounced unilateral loss of VP fibers was seen within the lateral septum (Figs. 6A and C). In rats with more caudally situated knife cuts, only a slight reduction was seen in the VP fiber density (Figs. 6B and D). Ventral to the cut, the intersected VP fibers were seen to be swollen as if VP was accumulating proximal to the transection. This phenomenon was especially clear in the diagonal band of Broca.

Lesioning of the bed nucleus of the stria terminalis was performed unilaterally, since bilaterally lesioned rats failed to survive operation. The lesioned area had a diameter of about 2.5 mm (Fig. 7) and resulted in a clear decline in VP fiber density within the ipsilateral diagonal band of Broca, the lateral septum, the lateral habenular nucleus, the area of the anterior amygdala, the periventricular gray and the locus coeruleus (Figs. 8, 9, 10A and B). In no other areas

studied was any diminution of VP fiber density noted (Table I).

DISCUSSION

Since lesioning the SCN failed to affect the VP fiber density within the lateral septum, Hoorneman and Buijs¹⁰ concluded that the PVN, and perhaps the SON, probably constitute the source of the VP fibers in the lateral septum. In contrast to the PVN, however, the SON does not seem to be a likely candidate for being the site of origin of VP fibers within the lateral septum or within any other part of the brain. Injection of tritiated amino acids into the SON followed by autoradiography has demonstrated only fibers running to the median eminence and the neurohypophysis²⁶, while in a similar experiment the PVN was found to project to many other brain areas, including the lateral septum⁷. Other data pointing to the PVN rather than the SON as a source for extrahypothalamic VP fibers were obtained by injection of retrograde tracers into the nucleus of the solitary tract, which contains predominantly oxytocin, but also some VP fibers^{1,22}. This resulted in the labeling of cells in the PVN but not in the SON^{18,20}. When this technique was combined with immunocytochemistry, part of this projection from the PVN was indeed found to contain VP and oxytocin^{19,21}.

In the present study, the injection of tracers into that part of the lateral septum which is most densely innervated by VP fibers, resulted in the labeling of cells in many places known to project to the lateral septum, but not in the PVN nor in the SON. The finding of labeled cells immediately lateral to the PVN

Fig. 6. Transverse sections of the septum, demonstrating that unilateral horizontal knife cuts, which extended far enough rostral to intersect the diagonal band of Broca (arrows in A) were able to reduce the vasopressin fiber density in the lateral septum drastically (arrows in C), in contrast to cuts that did not intersect the diagonal band of Broca (B and D). CA, anterior commissure; DBB, diagonal band of Broca; LV, lateral ventricle; PVS, periventricular nucleus. Bar: 500 μ m.

might explain the finding of the aforementioned projection from the PVN to the lateral septum⁷.

The results of the lesion experiments are in line with the retrograde tracing studies. Although lesioning the PVN did result in the disappearance of VP fibers in the nucleus of the solitary tract and the nucleus ambiguus, it did not impair the VP fiber density in the lateral septum. Because the VP cells found in clusters scattered throughout the hypothalamus might also be the source of VP fibers in the lateral septum, a much larger part of the hypothalamus around the PVN was lesioned. This lesion probably included the fibers of passage from the SCN towards the periventricular and the dorsomedial nuclei of the hypothalamus¹⁰, since VP fibers disappeared not only in the nucleus of the solitary tract and the nucleus ambiguus, but also in the former two regions. The disappearance of VP and oxytocin fibers from the nucleus of the solitary tract following lesioning of the area of the PVN confirms the results of the retrograde tracing studies mentioned before^{19,21}. Since the VP fiber density remained unaffected in most other areas of the brain, the PVN and its accessory cell groups do not contribute for a major part to the VP innervation of the brain. This conclusion is corroborated even more convincingly by the results of experiments in which the injection of fluorescent tracers in the lateral septum was combined with immunofluorescence staining for VP. These experiments did not reveal any retrograde labeling of VP containing neurons in the entire hypothalamus, whereas application of the same procedure reveals the presence of such cells in the PVN after injection of tracer in the nucleus of the solitary tract (unpublished results of P. Vallet in our laboratory).

Consequently, as far as the VP fibers in the lateral septum are concerned, the aforementioned results of the tracing and lesion studies make it unlikely that the PVN, SON, SCN or the hypothalamic island of VP cells from the source of these fibers. No VP immunopositive cells had previously been reported in other areas. However, in an immunocytochemical study which was performed parallel to the present study, treating rats with colchicine resulted in the appearance of VP-immunopositive perikarya, not only in the hypothalamus, but also in a number of other brain regions. The largest number of extrahypothalamic VP cells was found in the bed nucleus of the stria

terminalis. In addition, VP-immunopositive cells were revealed in other areas, e.g. in the rostral part of the lateral septum, the amygdala and the locus coeruleus²⁷.

Since unilateral horizontal cuts that separated the septum from the underlying structures were able to reduce the VP fiber density in the lateral septum, the major source of VP fibers in this area almost certainly lies outside the septum itself. As this reduction of VP fibers was substantial only when the diagonal band of Broca was intersected, the majority of the fibers seems to enter the septum via this route, the site of origin probably being the bed nucleus of the stria terminalis. Indeed, after unilateral lesioning of this structure VP fiber density was drastically reduced in the lateral septum as well as in various other areas. It is noteworthy in this respect that not all fibers in these areas disappeared on the lesioned side, suggesting that some of these fibers cross over to the contralateral side. Another possibility is that additional sources are present that were not included in the lesion.

Of course, one cannot exclude the possibility that the VP fibers in the lateral septum originate from areas situated outside the bed nucleus of the stria terminalis and only pass through it. This problem can only be solved by a combination of immunocytochemistry and retrograde tracing studies. However, in favor of the bed nucleus of the stria terminalis as the actual source of the VP innervation of the lateral septum is the correspondence of the position of VP immunopositive cells seen in colchicine-treated rats²⁷ and the retrograde labeled cells seen in the present study after injection of tracers into the lateral septum. Still another argument in favor of the bed nucleus of the stria terminalis is the demonstration of projections from this nucleus to all areas where VP fibers were seen to decrease, including the lateral septum, the lateral habenular nucleus and the locus coeruleus⁶.

It is interesting to note that the bed nucleus of the stria terminalis is an area containing a high density of steroid receptors²³. It is therefore tempting to speculate that steroid receptors are present also in the VP-immunopositive cells, and that these receptors play a role in the sexual differentiation of the VP innervation of the lateral septum and the lateral habenular nucleus⁸.

Fig. 7. Transverse section of the brain, stained specifically for the presence of vasopressin, demonstrating the extent of the unilateral lesion (*) of the bed nucleus of the stria terminalis (BNST). V, ventricle. Bar: 150 μ m.

Fig. 8. Transverse section in the region of the periventricular (PVS) and lateral habenular nucleus in a rat with a lesion of the bed nucleus of the stria terminalis on the left side. Note that the vasopressin fiber density in the lateral habenular nucleus is decreased unilaterally (arrows), whereas that of the PVS is not. Bar: 150 μ m.

Fig. 9. Photomontage of a transverse section of the septum of a rat with a lesion of the bed nucleus of the stria terminalis on the left side. Note that the vasopressin fiber density in the left lateral septum (LS) is much lower than in the right LS. Arrows point to individual vasopressin fibers. LV, lateral ventricle. Bar: 150 μ m.

Fig. 10. Photomicrographs of a transverse section of the medulla at the level of the locus coeruleus (LC) of a rat with a unilateral lesion of the bed nucleus of the stria terminalis. Note that the vasopressin fiber density is much lower on the lesioned side (A) than on the intact side (B). Arrows point to individual vasopressin fibers. PCS, superior cerebellar peduncle. Bar: 50 μ m.

Although the present study has clarified the sites of origin of VP fibers in many areas, the origin of VP fibers within the dorsal raphe nucleus, the nucleus of the medial amygdala, the hippocampus and the ventral tegmental area is still quite obscure, because in none of the lesion experiments mentioned were VP fibers seen to decrease in these areas. It may well be that the source of these fibers lies in the other areas where VP-immunopositive cells are found after colchicine treatment²⁷.

In sharp contrast to the vasopressinergic innervation of the brain, which clearly has multiple sites of origin, the oxytocinergic fibers seem to be derived predominantly from the PVN. After lesioning the PVN the oxytocin fiber density decreases dramatically in all the oxytocin projection sites in the brain. This is in line with the observation of Van Leeuwen and Caffé²⁷, who could not find any additional areas in the brain containing oxytocin-immunopositive cells after colchicine treatment.

The present findings, which indicate that both cell bodies and terminal fields of the VP system in the brain are far more diverse than those of the oxytocin system, have far-reaching implications for the functioning of the vasopressinergic and oxytocinergic innervation of the central nervous system. Since oxytocin fibers find their origin in the PVN, the actions of

oxytocin in the various parts of the central nervous system might be triggered by the same stimuli and probably all occur at much the same time. Furthermore, individual cells in the PVN send collaterals to different areas known to contain oxytocin fibers²⁵. If such cells include oxytocinergic neurons, a tight synchronization of the central actions of oxytocin would be even more likely. The actions of VP, in turn, are probably linked in the various areas of which the VP fibers have a common origin, but could be independent when the fibers are derived from different nuclei. For example, the VP cells of the bed nucleus of the stria terminalis probably receive a totally different input than the VP cells in the PVN or SCN, which obviously causes the output to be different as well.

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