ONTOGENY OF THE VASOPRESSINERGIC NEURONS OF THE SUPRACHIASMATIC NUCLEUS AND THEIR EXTRAHYPOTHALAMIC PROJECTIONS IN THE RAT BRAIN — PRESENCE OF A SEX DIFFERENCE IN THE LATERAL SEPTUM

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SUMMARY

Immunocytochemical studies have revealed the presence of extensive vasopressinergic projections from the suprachiasmatic nucleus to the limbic system and other brain areas. Vibratome sections and the unlabeled antibody enzyme method were used to investigate the ontogeny of the vasopressinergic neurons of the suprachiasmatic nucleus and their exohypothalamic fibers in the rat brain. The first immunopositive neurons of this nucleus were revealed on the 2nd postnatal day. An adult appearance of the suprachiasmatic nucleus was detected on day 14. Although fibers appeared in the periventricular nucleus already on the 7th postnatal day, such fibers were visible in the lateral septum and lateral habenular nucleus only on day 10. From the 12th postnatal day onwards a marked sex difference developed with respect to the density of the vasopressin fibers in the lateral septum and, to a lesser extent, in the lateral habenular nucleus. In male rats the fiber density was higher in both areas. This sex difference persisted in adulthood.

INTRODUCTION

The neurohypophyseal hormones vasopressin (AVP) and oxytocin are synthesized in the magnocellular paraventricular (PVN) and supraoptic nuclei (SON). In addition, on the basis of immunocytochemical evidence the parvocellular suprachiasmatic nucleus (SCN) appeared to contain AVP^{38,39}, an observation that has been confirmed by radioimmunoassay¹⁶. Fibers of the SON and PVN run to the classical neurohemal areas, i.e. the median eminence and the neurohypophysis⁴⁰. In addi-

tion to this well-known pathway, Barry⁴ observed, as early as 1954, Gomori-positive fibers running from the PVN to a number of brain regions outside the hypothalamus. The presence of these exohypothalamic fibers was confirmed and their hormonal content was established by means of immunocytochemical studies using specific antibodies against AVP and oxytocin^{8,10,34}. From the PVN both AVP and oxytocin containing fibers run to various extrahypothalamic brain areas, e.g. in the limbic system, the medulla oblongata and the spinal cord^{8,10}. In addition, AVP-containing fibers could be traced from the SCN projecting rostrally to the organum vasculosum laminae terminalis, rostr dorsally towards the lateral septum and dorsocaudally via the periventricular nucleus towards the lateral habenular nucleus. Some minor projections run to the posterior hypothalamus, the interpeduncular nucleus and the mesencephalic central gray^{8,34}.

In the lateral septum and the lateral habenular nucleus the fibers form numerous ramifications and punctate perineuronal structures^{8,34}, suggesting the termination of fibers in these areas. Immunoelectron-microscopical studies revealed indeed the presence of AVP-containing synapses in these brain regions which were morphologically indistinguishable from the classical transmitter-containing synapses⁹. Push-pull experiments in the sheep septal region demonstrated that AVP is released in this area in direct relation to body temperature changes¹³. Since, in addition, the electrical activity of molluscan neurons can be altered by AVP³, this peptide seems to act as a central neurotransmitter.

Boer et al.⁵ reported that in Brattleboro rats, which are genetically unable to synthesize AVP, brain development is permanently stunted. Thus AVP might, besides affecting certain central processes in the adult brain, play a role in brain development. This has focused our interest on the development of the AVP-containing cells and their exohypothalamic fibers. Some information was available about the time of appearance of the first AVP-containing cells in the SCN^{12,42}, but literally nothing was known about the development of the massive AVP projections of this nucleus. Since Buijs et al.¹¹ were not able to demonstrate AVP in the SCN of the fetal rat, we studied the postnatal development of the AVP-containing cells of the SCN and their exohypothalamic fibers. Special attention is given to the occurrence of a sex difference in the density of AVP-containing fibers in the lateral septum and the lateral habenular nucleus.

MATERIALS AND METHODS

In a first series, infant Wistar rats of either sex were studied from days 1 to 8 and on days 10, 12, 14, 17 and 21 of age, the day of birth being day 0. Per age group 3 rats were used. In order to study possible sex differences a second series was used in which the animals were sacrificed on days 3, 10, 12, 14, 17, 21, 27, 35 and 55 days of age. Two to 6 rats of each sex were studied at each age group (see Table I). Rats were only used from litters which contained at least 8 pups, if more pups were present the size of the litter was reduced to 8 on the day of birth. In order to compare the findings in these infants to the adult condition, 3 male and 3 female rats weighing 230–250 g were used.

TABLE I

Density of AVP fibers in the lateral septum during development

Each entry represents one rat; +, very few fibers; ++, moderate number of fibers; ++++, fibers form a network; +++++, fibers form a very dense network.

<i>Postnatal day: 10 12 14 17 21 27 35 55 Adult</i>									
Male rats	+	+	+	++++	++++	++++	++++	++++	++++
	+	+	+	++++	++++	++++	++++	++++	++++
Female rats	+	+	+	++++	++++	++++	++++	++++	++++
	+	+	+	++++	++++	++++	++++	++++	++++

The stages of the estrous cycle of the female rats were proestrus, estrus and metestrus. After intraperitoneal administration of Nembutal (0.1 mg/100 g body weight) the rats were perfused intracardially with 0.9% saline followed by 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.35; (chemicals derived from Merck). The brains were dissected and immersed in the same fixative for an additional 24 h at 4 °C¹⁰. Sections of 100 µm were cut transversely with an Oxford Instruments vibratome¹¹ and brought into phosphate-buffered saline (PBS), pH 7.45. Immunocytochemical staining was accomplished according to the unlabeled antibody enzyme method (for details see ref. 10). The sections were incubated in the following solutions: (a) 30–40 min in 100% methanol in order to eliminate pseudoperoxidase activity (modification from Streifler³⁹); (b) washing in PBS, 30 min; (c) 10% swine serum, 0.5% Triton X-100, 1 h; (d) rabbit AVP antiserum (# 125) 1:800 in PBS, 0.5% Triton X-100 16 h, 4 °C; (e) washing in PBS, 1.5 h; (f) swine-anti-rabbit IgG (Fc fragment) serum (Nordic) 1:40 in PBS, 0.5% Triton X-100, 2 h; (g) washing in PBS, 1.5 h; (h) peroxidase antiperoxidase (PAP) 1:200 in PBS, 0.5% Triton X-100, 2 h; (i) washing in PBS, overnight; (j) 0.05 M Tris-HCl, pH 7.6, 30 min; (k) 0.5 mg/ml 3,3'-diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH 7.6, 0.01% H₂O₂, 10–20 min; (l) water to stop the peroxidase activity; (m) graded ethanol series followed by xylene and embedding in malinol.

The controls included: replacement of AVP antiserum by (1) normal rabbit serum, (2) AVP antiserum adsorbed to AVP on solid phase, (3) AVP antiserum adsorbed to oxytocin on solid phase, (4) oxytocin antiserum adsorbed to AVP on solid phase. The antibodies were purified and tested by means of immunofluorescence, according to Swaab and Pool³⁷. The specificity for the AVP and oxytocin antisera was tested in the sections of the brains of 1, 2 and 3 weeks old and adult Brattleboro rats and the SCN of an adult Wistar rat, respectively.

The density of AVP-containing fibers in the lateral septum and lateral habenular

Figs. 1-4. Four stages in the development of the SCN. 100 μ m thick frontal sections of the brain stained immunocytochemically for vasopressin. SCN, supraoptic nucleus; OC, optic chiasm; V, third ventricle; m, immunopositive magnocellular neuron.

Fig. 1. 2-day-old rat: only very few immunopositive cells are visible (arrows). Bar = 30 μ m.

Fig. 2. 7-day-old rat: more immunopositive cells are present in the SCN. Note that only few fibers are visible (arrows). Bar = 50 μ m.

Fig. 3. 10-day-old rat: many immunopositive cells (arrows) and fibers are present in the SCN. Many fibers leave the area of the nucleus in a dorsal direction. Bar = 50 μ m.

Fig. 4. 14-day-old rat: the SCN has an adult appearance. The fibers are spread over the entire area of the nucleus while many fibers fan out in a dorsal direction (arrows). Bar = 50 μ m.

nucleus was estimated in a semi-quantitative manner in a blind study distinguishing 4 categories: +, very few fibers; ++, a moderate number of fibers; +++, fibers form a network; +++++, fibers form a very dense network (cf. Fig. 5–10).

RESULTS

Suprachiasmatic nucleus

In the area of the SCN no cells were seen which stained positively for AVP on the first postnatal day. In one out of three 2-day-old rats a few immunopositive cells could be detected (Fig. 1). On postnatal day 3, in 6 out of 8 rats and from the 4th to 6th day in all animals, immunopositive cells were visible in the SCN, revealing only very few or no fibers. AVP-containing fibers and cells were spread all over the SCN area on the 7th and 8th days (Fig. 2), while only very few fibers left the SCN running into rostral or dorsorostral directions. On the 10th day the number of cells and the density of fibers fanning out the SCN were dramatically increased (Fig. 3). From the 14th postnatal day onwards the appearance of the SCN was similar to that of the adults (Fig. 4). The immunopositive cells were scattered over the entire SCN but the highest concentration was found at the medial and mediodorsal border of the SCN. Numerous fine caliber fibers were homogeneously distributed over the SCN while vast projections fanned out in rostral, rostradorsal and dorsocaudal directions. No sex difference was noted in the development of the AVP-containing cells and in the density of fibers in the immediate vicinity of the SCN.

Extrahypothalamic areas

In the first series, in which no distinction was made between sexes, it was found that AVP-containing fibers were visible in the lateral septum only on the 10th postnatal day. After this day an enormous individual variation in the density of the developing network was found. In the second series this variation appeared to be attributable to a sex difference (Table I). In male and female rats generally only very few AVP-containing fibers were found in the lateral septum on days 10 and 12. Some more fibers, already forming a network, were visible in 3 out of 6 male rats of day 14 (Fig. 5) and in all male rats of days 17, 21, 27, 35 and 55 (Fig. 7). The density of fibers was only clearly larger in the adult male rats in which a very dense network of fibers could be demonstrated (Fig. 9). While in male rats the fiber density increased dramatically from the 12th to 17th day, no such change was seen in female rats (Fig. 6). Only on the 55th day was a discernibly larger, but still moderate number of fibers found, comparable to the number of fibers in the lateral septum of male rats of 12–14 days of age (Fig. 8), while in the adult female rats the fibers had formed a network (Fig. 10). Although the sex difference in the density of the fiber network persisted in adulthood (Figs. 11 and 12), this difference was not as striking as between days 17 and 35.

Very few fibers were visible in the periventricular nucleus, medioventral to the lateral habenular nucleus on the 7th and 8th days, but many fibers were detected on the 10th and 12th days. From the 14th day onwards the periventricular nucleus had an adult appearance. No sex differences were found in this brain region.

Figs. 5-10. Three stages in the development of the vasopressinergic fiber network in the lateral septum of male and female rats. Frontal sections just dorsal to the anterior commissure stained immunocytochemically for vasopressin. LV, lateral ventricle. Bar = 20 μ m.

Fig. 5. 14-day-old male rat, noted as + + +. Many fibers are visible (arrows), forming a network.

Fig. 6. 14-day-old female rat, noted as +. Only very few fibers are present (arrows).

Fig. 7. 55-day-old male rat, noted as + + +.

Fig. 8. 55-day-old female rat, noted as + +. Few fibers are visible.

TABLE II

Density of AVP fibers in the lateral habenular nucleus during development

Each entry represents one rat. Meaning of the plus signs as in Table I.

	Postnatal day: 10	12	14	17	21	27	35	55	Adult
Male rats	+	++	++	+++	+	+++	+++	+++	+++
	++	++	++	+++	++	+++	+++	+++	+++
	++		+++	+++	++	+++	+++	+++	++++
			+++						
			+++						
			+++						
Female rats	+	+	++	++	+	++	++	++	+++
	++	+	++	++	+	++	++	++	+++
			++	++	++	+++	+++	+++	+++
			++						
			++						

The results of the observations made in the lateral habenular nucleus are summarized in Table II. On the 10th postnatal day the first AVP-containing fibers were visible in this area, while a practically adult appearance was found in male rats on the 14th day and in female rats on the 17th day. The variation in the fiber density within the groups was larger in this area throughout development than in the lateral septum. Although generally female rats showed a smaller density of fibers in the lateral habenular nucleus than male rats of the same age, the overlap was considerable.

As soon as fibers were visible in the lateral habenular nucleus and the lateral septum, branching of fibers and punctate perineuronal structures could also be detected.

DISCUSSION

In the present study the first AVP-containing cells in the SCN were found on the 2nd postnatal day, which is earlier than in other studies. Wolf and Trautmann⁴² observed SCN cells immunopositive for neurophysins only on day 7 postnatally, while Burlet et al.¹² demonstrated, on the 6th postnatal day, AVP-containing SCN cells. The earlier demonstration of AVP in the SCN reported in this study can probably be attributed to the use of vibratome sections instead of sections derived from paraffin embedded tissue, since Buijs et al.¹¹ demonstrated in fetal rats that the former method enhances the sensitivity of the staining procedure.

Although AVP-containing cells appear in the SCN as early as the 2nd postnatal day, this is still considerably later than the time of birth of the neurons of this nucleus as determined by means of thymidine labelling on the 15th and 16th prenatal days^{2,22}. This suggests that there is a delay of more than a week before AVP synthesis starts in detectable amounts. The appearance of AVP in the SCN coincides with the onset of the innervation of this structure. The first fibers derived from the retina reach the SCN on the 2nd–3rd postnatal day³⁵. In addition, while on the day of birth almost no synapses can be found in the SCN, the number of synapses increases progressively

Fig. 9. Adult male rat, noted as + + + +. The fibers form a very dense network. Arrow indicates a punctate perineuronal structure.

Fig. 10. Adult female rat, noted as + + + +.

from the 4th postnatal day onwards²⁶. The formation of the synaptic contacts might thus trigger AVP synthesis.

An unexpected finding in the present study was that the density of AVP-containing fibers in the lateral septum and, to a lesser extent, in the lateral habenular nucleus was higher in male rats than in female rats from the 12th day onwards. Although Gorski et al.²⁰ demonstrated the existence of a sex difference in the relative volume of the SCN, in our study no obvious difference was seen in the morphology of the SCNs of male and female rats. However, the procedure used in this study was not suited to a quantitative approach and for this reason a sex difference in the number of AVP-containing cells can certainly not be excluded.

One can only speculate how the observed sex differences are reflected in functional differences, because very little is known about the possible functions of the exohypothalamic AVP-containing fibers. The presence of AVP-containing fibers and synapses in limbic brain structures^{8,9} seems to form the morphological basis for the observed effects of AVP on avoidance behavior⁶. This idea is sustained by the observation that injection of AVP in the dorsal septal nucleus facilitated passive avoidance behavior²⁴. A simple relationship between the vasopressin fibers in the septum and the passive avoidance behavior can, however, not be expected since Van Oyen⁴¹ has observed that 30-day-old female rats perform better than males in the

Figs. 11 and 12. Survey of the lateral septum of an adult male and female rat. Frontal sections just dorsal to the anterior commissure stained immunocytochemically for vasopressin. LV, lateral ventricle; ls, lateral septum; cp, nucleus caudatus putamen. Bar = 100 μ m.

Fig. 11. Male rat. Very dense network of immunopositive fibers (arrows).

Fig. 12. Female rat. The fibers form a network (arrows), but not as dense as in the male rat.

passive avoidance behavior paradigm, while in the adult rat this sex difference has switched in the opposite direction.

Several lesion studies have demonstrated the involvement of the SCN in the control of circadian rhythms²⁸. The retinohypothalamic tract is considered to synchronize the oscillatory activity of the SCN with the day/night cycle^{29,33}. Since AVP-containing fibers are spread over the entire area of the SCN and the terminals of the retina form axodendritic synapses in this nucleus²¹, a direct innervation of the AVP-containing cells by the retinohypothalamic tract is certainly possible. The AVP-containing SCN fibers might thus be associated with the regulation of certain circadian rhythms.

AVP fibers in the lateral septum might, in addition, play a role in the physiology of temperature regulation. Administration of AVP in the lateral ventricle of the rat causes a sharp decrease in body temperature²³, a similar phenomenon as has been observed by Cushing¹⁴ as early as 1932, when he injected posterior pituitary extract into the lateral ventricle of patients. Cooper et al.¹³ demonstrated that AVP exerted an antipyretic action when perfused into the septal area of the sheep and that, in addition, the amount of AVP released from the septal area correlated negatively with changes in body temperature. Fowler and Kellog¹⁷ found a clear onset of the development of

thermoregulation in rats at 9 days of age which is about the time the first AVP fibers are visible in the lateral septum. These observations are consistent with lesion studies that demonstrated the involvement of the SCN in the circadian rhythm of body temperature^{1,32}.

Another function of AVP fibers of the SCN might be an involvement in the regulation of water balance. The SCN fibers are abundant in the organum vasculosum laminae terminalis and the periventricular nucleus⁸, both areas which are considered to be of importance for the regulation of water balance⁷. The AVP-containing fibers in the lateral septum might also participate in this regulation by influencing indirectly the release of AVP in the neurohypophysis. Stimulation of the medial septum changes the spike frequency of the neurosecretory cells of the PVN and SON^{30,31}. In addition, Garriss¹⁸ has demonstrated by means of autoradiography that most efferent fibers of the lateral septum projected to the medial septum, and that a part of the projections of both septal regions could be traced into the PVN and SON. The AVP-containing fibers of the SCN projecting to the lateral septum might influence neurohypophyseal function via these pathways. This idea is supported by the synchronization of the development of the AVP projection from the SCN to the lateral septum and the onset of the osmoregulation which starts only after the 10th day and becomes clearly apparent on the 14th day¹⁵. In man, plasma AVP levels show a day/night rhythm¹⁹, while indications for diurnal fluctuations in plasma AVP concentrations have also been found in rats²⁷, which is in agreement with the suggested connection between the SCN and the hypothalamo-neurohypophyseal system.

If AVP fibers in the lateral septum are involved in processes such as thermoregulation and osmoregulation, then it is difficult to interpret the lower density of AVP fibers, especially during development, in the female rats, unless a compensatory mechanism were to exist. Since, in Brattleboro rats, which lack AVP, the serotonin innervation of the septum is denser than in non-diabetic insipidus rats (H. W. M. Steinbush, pers. commun.), serotonin fibers might balance out the paucity of AVP fibers in female rats. This is consistent with the higher levels of serotonin in the female brain, a sex difference which is only measureable from the 12th postnatal day²⁵, on which day the sex difference in the density of AVP fibers in our study also became noticeable. So far it is not known what the influence of AVP is on possible target neurons in extrahypothalamic areas. Further investigation will have to clarify whether the sex difference in the density of AVP-containing fibers is reflected in a sex difference in the responsiveness of neuronal elements to AVP. In addition, it has to be settled to what extent the sex difference presented in this paper is dependent on the organizational effect of steroid hormones during perinatal development and what the influence of circulating sex steroids is on the density of AVP-containing fibers in the septum of the adult rat.

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