Ontogeny of Vasopressin and Oxytocin in the Fetal Rat: Early Vasopressinergic Innervation of the Fetal Brain

R. M. BUIJS¹, D. N. VELIS AND D. F. SWAAB

Netherlands Institute for Brain Research, IJdijk 28, 1095 KJ Amsterdam, The Netherlands

Received 1 August 1980

BUIJS, R. M., D. N. VELIS AND D. F. SWAAB. *Ontogeny of vasopressin and oxytocin in the fetal rat: Early vasopressinergic innervation of the fetal brain.* PEPTIDES 1(4) 315-324, 1980.—The content and distribution of vasopressin and oxytocin were determined during fetal development in the rat brain and pituitary by means of radioimmunoassay and immunocytochemistry. The vasopressin content in the fetal brain showed a gradual rise from day 16 of pregnancy onwards, while pituitary vasopressin rapidly increased from fetal day 19 until birth. The oxytocin content in the fetal brain was considerably lower than the vasopressin content. A decrease in oxytocin content was seen between day 16 and day 18 while from day 18 of pregnancy onwards a slight increase was found. The pituitary oxytocin content starts to rise between day 17 and 18 of pregnancy, but at term the pituitary oxytocin content was only 1/20 of the vasopressin value. Immunocytochemistry revealed that vasopressin levels in the fetal rat brain were not only due to the presence of the classical hypothalamo-neurohypophyseal system, but also to the early development of exohypothalamic fibers. Vasopressin containing cells were seen from fetal day 16 in the supraoptic nucleus, and from fetal day 18 in the paraventricular nucleus. The fiber outgrowth of these cells towards the pituitary and extrahypothalamic brain sites seems to be well synchronized, as on day 17 vasopressin containing fibers could be demonstrated in the olfactory bulb as well as in the median eminence. No positive staining for oxytocin could be obtained in the fetal rat, while during the entire fetal period no positive staining was found in cell bodies in the region of the suprachiasmatic nucleus. The early peptidergic innervation of the brain, which enabled the tracing of the source of some exohypothalamic fibers, might be related to several central processes among which brain development itself is included.

Vasopressin	Oxytocin	Fetus	Development	Immunocytochemistry	Supraoptic nucleus
Paraventricular nucleus		Extrahypothalamic innervation			

THE two neurohypophyseal hormones, arginine-vasopressin (AVP) and oxytocin (OXT), are synthesised in the magnocellular paraventricular (PVN) and supraoptic nucleus (SON) of the hypothalamus. In addition to their well-known peripheral endocrine functions, these hormones are thought to be implicated in various central processes. As has been demonstrated in the adult rat, these peptide hormones can reach, via a number of exohypothalamic pathways, brain sites far beyond the hypothalamus from the olfactory bulb to the spinal cord [5, 7, 9, 16, 32, 33, 36]. Immunoelectron microscopy demonstrated that these neurohypophyseal hormone containing fibers terminate on neuronal structures by means of true synapses, which were morphologically indistinguishable from the classical aminergic synapses [8]. No correlation was found between AVP-blood and cerebrospinal fluid levels and avoidance behavior [15]. Moreover, there is a remarkable similarity between the active sites for the behavioral effects of neurohypophyseal hormones [26,41] and the sites of extra-hypothalamic fiber termination [5, 7, 9]. These observations make it plausible that the extra-hypothalamic AVP and OXT innervation is the anatomical basis for the de-

scribed effect of these peptides on avoidance behavior [40], monoamine metabolism [26,37] and temperature regulation [11]. AVP and OXT might also be involved in various processes during development. Fetal AVP is thought to be involved in the regulation of the amniotic fluid balance and in the restriction of blood loss during labour (for references [35]), while fetal OXT is released during birth and might thus accelerate the course of labour [4].

In addition to these peripheral endocrine effects during fetal development it has recently been suggested that AVP is involved in normal brain development. Brattleboro rats, homozygous for diabetes insipidus, which are genetically incapable of synthesising AVP, were found to show a protracted brain development, which is already apparent at birth and persists into adulthood ([3], Boer personal communication).

These putative functions of neurohypophyseal hormones during development raised the question as to when the hypothalamo-neurohypophyseal system (HNS) and its exohypothalamic fibers might develop in the fetal rat brain. Although the HNS in early development has already been the

¹Send reprint requests to: R. M. Buijs, Netherlands Institute for Brain Research, IJdijk 28, 1095 KJ Amsterdam, The Netherlands.

subject of several studies [10, 19, 42] a detailed description of time of appearance of the AVP and OXT cells in the SON and PVN was not available while information on the development of the vasopressin and oxytocin pathways in the brain was totally absent. Consequently, the amount and distribution of AVP and OXT was determined using specific and sensitive radioimmunoassay (RIA) and immunocytochemical techniques.

METHOD

Animals

Adult Wistar virgin female rats and adult Wistar male rats (TNO-CPB Zeist) were placed into the same cage between 5 p.m. and 9 a.m. of the following day. The day of sperm-positive identification was designated as gestational day 0. Fetuses were collected starting with gestational day 14. The pregnant female was sacrificed by decapitation, always between 9 and 11 a.m. The peritoneal cavity was immediately opened, the uterus exposed, and the number of fetuses counted and recorded. Three fetuses from the left horn were used, only if there were at least 8 fetuses present in the uterus. One-week-old Wistars and pups homozygous for diabetes insipidus were used as experimental controls in the immunocytochemical procedure.

Radioimmunoassay

Maximally 3 fetuses per pregnant animal were removed from the left uterine horn, weighed and decapitated. The number of fetuses thus harvested on the various experimental days was: day 16: $n=15$; day 17: $n=26$; day 18: $n=15$; day 19: $n=15$; day 20: $n=18$; day 21: $n=15$; and the first postnatal day: $n=15$.

The fetal brain and pituitary were dissected under a stereomicroscope within 5 minutes after decapitation of the mother. Immediately after dissection the fetal brains were put into 1.1 ml of ice-cold HCl (0.1 N), and homogenized by means of a pestel homogenizer. The homogenates were subsequently sonicated for 30 sec, after which the extracts were neutralized by means of NaOH and centrifuged at 3000 G for 30 min at 4°C. The supernatant was stored at -20°C until assayed. The fetal pituitaries were dealt with in the same way except for deletion of the pestel homogenization step. Five adult male rats (2000 g body weight) were decapitated after which the brains were placed in 10.5 ml HCl (0.1 N) and the pituitaries in 5.5 ml HCl (0.1 N), pestel homogenized, sonicated and treated as described before.

OXT and AVP were measured in the same samples, which were prepared by diluting the neutralized pituitary homogenates in assay buffer, or by vycor extraction of the brain homogenates. Some fetal pituitaries in early developmental stages contained so little hormone that they were re-assayed after extraction by means of vycor. The radioimmunoassays were performed as described earlier [15,17].

Tissue Fixation

Three fixatives were compared: (1) freshly made glutaraldehyde 2.5%-paraformaldehyde 1% in 0.1 M cacodylate buffer, pH 7.35; (2) Formalin 4% (all reagents Merck) and (3) Bouin's fixative without acetic acid. Every first and fourth fetus was fixed in glutaraldehyde- and paraformaldehyde, the second in Formalin, and the third in Bouin's fluid. At

least 3 fetuses per age group and per fixative were embedded in paraffin. The same applied for the Wistar and Brattleboro neonates which were used as controls. Fetuses of gestational age 14 and 16 days were immersed in fixative "in toto." Fetuses of gestational age 17 days were decapitated, after which the entire head was fixed by immersion. Fetuses of gestational age 18 days or older were decapitated, the calvarium was carefully removed and the whole brain, including olfactory bulbs and chiasma opticum, was then removed and fixed by immersion. The fixative was changed after 2 hr. The minimum duration of fixation was 18 hr and the maximum 2 months. Then the specimens were embedded in paraffin or sectioned on the vibratome. Fixed brains were cut by free hand into slices of approximately 2 mm, and subsequently sectioned at 100 μ m on an Oxford Instruments vibratome.

Tissue Staining

Each section was processed as follows: 0.05 M Tris 0.9% NaCl (Merck), pH 7.6 wash, followed by methanol 100% for at least 30 min, in order to eliminate the pseudoperoxidase activity of red blood cells, and Tris-saline wash. Immunoperoxidase staining was accomplished by sequential application of: (a) rabbit anti-AVP-serum (125) 1:800, or OXT antiserum (0-2-c) 1:400 in 0.5% Triton X-100 (Sigma) for 18-72 hr at 4°C; (b) washing in Tris-saline 0.5% Triton X-100 for 1 hr; (c) swine anti-rabbit IgG (Fc fragment) serum 1:40 (Nordic) in Tris-saline 0.5% Triton X-100 for 2 hr; (d) washing in Tris-saline 0.5% Triton X-100 for 1 hr; (e) peroxidase-antiperoxidase (PAP) 1:200 in Tris-saline 0.5% Triton X-100 for 2 hr; (f) washing in Tris-saline for 1 hr; (g) 0.5 mg/ml 3,3-diamino-benzidine (Sigma) 0.01% H_2O_2 (Merck) in Tris-saline, pH 7.6 for 10-20 min; (h) wash Tris-saline; (j) dehydration and mounting of the section on glass slides and embedding in malinol.

Controls included: (1) replacement of the first antiserum with normal rabbit serum; (2) application of purified AVP antiserum on sections obtained from Brattleboro tissue. The antisera were purified and tested according to the method described in Swaab and Pool [34]. At least 3 fetuses per gestational day were studied with purified anti-AVP and 3 with purified anti-OXT serum. In addition, at least 3 animals per gestational day were studied using a mixture of non-purified anti-AVP 1:1000 and non-purified anti-OXT 1:800 serum.

The immunostaining in the paraffin sections was performed according to Buijs *et al.* [9].

RESULTS

Radioimmunoassay

Fetal weight increased 12.5 times and brain weight 4.4 times from 16 days of pregnancy to one day postnatally (Fig. 1). The assay data are expressed as mean $\pm$ SEM. If not stated otherwise, the median values gave similar results (Fig. 2).

The AVP content in the fetal brain increased during development gradually from 93 pg up to 3.6 ng per brain. The concentration of this hormone followed a similar course and reached the adult range in the day 20 fetal brain. The hypophyseal AVP content remained low until days 18-19 when a sharp rise was noted. In the same period a slight drop in brain AVP was observed. The pituitary data followed a similar course, when expressed per fetal body weight. The slightly higher concentrations on days 16 and 17 of preg-

FIG. 1. Body and brain weight development of the rats in Fig. 2 (mean $\pm$ SEM). Note the dramatic increase in body weight from fetal day 16 until birth.

nancy were not encountered when the median values were used.

The OXT values in the brain showed a different course. During the entire fetal period OXT values remained much lower than the AVP levels and did not rise above approximately 120 pg/brain which is about 1/50 of adult values. From fetal days 16–18 the amount of OXT in the brain gradually drops from 130 pg to 60 pg, which is even more pronounced in the concentration curve because of the increasing brain weight. A steady rise was observed in the fetal OXT content of the brain from day 18 of pregnancy onwards, which was proportional to the weight increase, as constant OXT concentrations were revealed in this period. A rise in pituitary OXT was found in the same period that brain OXT dropped (16–18 days), reaching a peak value at day 18, after which the values dropped again up to day 20. A gradual rise was found in the pituitary OXT content which was proportional to the rise in body weight in the last two days studied. However, OXT pituitary content was at term 1/1500 of that found in the adult pituitary.

Immunocytochemistry

Fetal day 16. Using glutaraldehyde-paraformaldehyde fixed fetal brains in vibratome sections, the first AVP-containing cell bodies could be visualized in the caudal parts of the SON. These faintly stained cells of approximately 6 μ m were irregularly shaped with very small processes (Fig. 3). Discernible AVP containing fibers were absent.

Fetal day 17. The number of AVP-containing cells in the caudal SON increases rapidly, while in addition, faintly stained AVP cells appear between the SON and the caudal part of the PVN (Fig. 4). Cells are still irregular in shape and the first AVP-containing fibers can be visualized both in the median eminence and reach via a ventral route the olfactory bulb, where they branch in the central area.

Fetal day 18. The SON now expands over a rostro-caudal region of approximately 1 mm, while AVP-containing cells that form the first beginning of the PVN can be seen over a rostro-caudal distance of ± 0.2 mm (Fig. 5). An increasing number of AVP-containing cells are seen between the SON and the PVN. Cells and fibers are also found in the region of the stria terminalis. AVP-containing fibers reach—via the stria terminalis—the amygdala for the first time.

The ventral pathway towards the olfactory bulb is more distinct from one day earlier; fibers run rostrally just above the optic chiasm and reach the olfactory bulb via the medial olfactory tract. Other fibers reach the corpus callosum via the medial septum or the claustrum. In addition, in the region of the third ventricle fibers are seen to penetrate the ependyma (Figs. 7, 10, 11).

Fetal day 19. The AVP cells now cover the whole region of the SON from caudal to rostral, while the PVN assumes its prismatic form (Fig. 6). The ventro-rostral bundle to the olfactory bulb has become more pronounced while in the rostral part of the third ventricle many fibers penetrate the ependyma (Fig. 10). Increasing amounts of AVP containing fibers reach the amygdala where they form ramifications (Figs. 8, 9). AVP containing fibers are noted in the posterior commissure, possibly projecting into the pineal gland.

Fetal day 20. There is little change in the shape of the PVN and SON although gradually more AVP-containing cells become scattered over the region between the PVN and SON. The HNS is firmly established with massive fiber projections (Fig. 7). The first AVP containing fibers are noted in the region of the angular bundle in the ventral hippocampus. In addition, the first AVP-containing fibers are demonstrated in the vicinity of the solitary tract and in the ventral part of the brain stem.

Fetal day 21. Except for increasing numbers of AVP-containing fibers in all areas described in the previous days no important changes have been observed in HNS, nor in the extrahypothalamic system. In none of the fetuses were any AVP-containing cell bodies found in the region of the supra-chiasmatic nucleus.

Using purified anti-AVP serum no positive reaction was obtained in hypothalamus sections of the Ho-Di Brattleboro rat. Using purified OXT antiserum a positive reaction was obtained in cell bodies and fibers of the PVN and SON in the week-old-Wistar and Brattleboro neonates.

Fetal days 16–17. No AVP or OXT containing cell body or fiber could be demonstrated in fetal rats of this age, using paraffin-embedded fetal brains, fixed in paraformaldehyde-glutaraldehyde in Bouin or in Formalin.

Fetal days 16–21. No positive reaction was obtained using oxytocin antisera in either of the fetuses, irrespective of the fixatives or sectioning procedures used.

The staining pattern obtained in the fetal rats in the initial experiment, using a combination of anti-AVP and anti-OXT sera in order to obtain maximal results, was similar to that obtained using purified AVP antiserum during the whole fetal period.

DISCUSSION

The use of vibratome sections improved the sensitivity of the unlabelled antibody enzyme method considerably. AVP containing cell bodies were already found by means of this procedure on fetal day 16, whereas no staining was found until day 18 in paraffin-embedded material, irrespective of

FIG. 2. Vasopressin and oxytocin levels and concentrations in fetal brain and pituitary (mean $\pm$ SEM). Note the low oxytocin content of the brain; these values are expressed in pg/brain whereas the other values are given in ng per brain or pituitary.

FIGS. 3-7. Transversal sections through the hypothalamus of fetal rats illustrating the various stages in development of the vasopressin containing cells and fibers in the supraoptic (SON) and paraventricular nucleus (PVN).

FIG. 3. Fetal day 16, the first faintly stained vasopressin containing cells appear along blood capillaries in the caudal SON (Bar=10 μ m) (860 $\times$).

FIG. 4. Fetal day 17, the vasopressin cells in the SON are preferentially located along blood vessels (arrows) (Bar=20 μ m) (690 $\times$).

FIG. 5. Fetal day 18, the PVN is now present as a small round nucleus of approximately 200 μ m in diameter (Bar=50 μ m) (144 $\times$).

FIG. 6. Fetal day 19, note the prismatic shape of the PVN (Bar=50 μ m) (154 $\times$).

FIG. 7. Fetal day 20, numerous but thin fibers are visible between the PVN and SON, some fibers of the PVN cell bodies penetrate the ependyma of the third ventricle (arrow) (Bar=50 μ m) (154 $\times$).

FIGS. 8-9. Transversal sections through the brain of a 19-day-old fetus demonstrating the most rostral part of the pathway via the stria terminalis (ST) towards the amygdala (Fig. 8) and the caudal part of this projection with fibers entering the amygdala via the stria terminalis (Fig. 9). Fiber branching and perineuronal structures (arrows) indicate termination of these vasopressinergic fibers already in the fetal amygdala (Bar=50 μ m) (180 $\times$).

FIGS. 10-11. Vasopressin fibers of the rostral bundle in a 19-day-old (Fig. 10) (344 $\times$) and 20-day-old (Fig. 11) (450 $\times$) fetus. Fibers are seen to penetrate the ependyma in the rostral part of the third ventricle. Note the vasopressin fibers in the optic nerve (arrows) (Bar=50 μ m).

the fixatives used. Similar improvements were obtained using vibratome sections in a study on the development of the SCN and its projections [39] and in a study of extrahypothalamic AVP and OXT pathways in adult tissue [7].

Until recently the high AVP/OXT ratio in the fetus was explained by the supposed presence of vasotocin in the mammalian fetus [29,30]. Recent studies in our and other laboratories demonstrated, however, that neither in the fetal nor in the adult mammalian brain, pineal or pituitary, true vasotocin immunoreactivity could be demonstrated [16,28].

Also, since in our RIA system for AVP vasotocin is not cross-reacting [16], it can be concluded that the demonstrated AVP and OXT in this study is not due to the presence of arginine vasotocin. A good correlation was obtained between the immunocytochemical and RIA results. Using RIA, small amounts of AVP in brain and pituitary could already be demonstrated on fetal day 14 [31]. E. L. Baruch, personal communication), a distinct rise in fetal brain AVP content occurred from fetal day 16 which is the day the first AVP-containing perikarya were demonstrated in the region of the SON.

The lack of any demonstrable OXT containing cell bodies in the fetal rat and the absence of AVP cell bodies before fetal day 16 is in agreement with the low amounts of radioimmunoassayable OXT and AVP on the respective days. This, and the presence of AVP and OXT in the pituitary suggest that these peptides are present but diffusely distributed over a relatively large number of cell bodies. Thymidine labelling studies [1, 2, 24] have demonstrated that the germinal layer, the wall of the third ventricle, is the origin of the neurosecretory cells and that the first cells of the SON and PVN are formed on fetal days 12 and 13, respectively, while all neurosecretory cells of these nuclei have been formed before fetal day 17. It seems probable, therefore, that the radioimmunoassayable quantities of AVP before day 16 and

the small amounts of OXT before birth are already present in the cell bodies of the PVN and SON, yet in so small a quantity that demonstration of the peptide by immunocytochemistry was impossible. In addition, at fetal day 21 the PVN and SON already have the adult features [1,21], while cells containing OXT can only be demonstrated at day 2 or 3 postnatally ([10] and our own preliminary results).

An even larger time lapse occurs between the birth of the suprachiasmatic nucleus which is already present at fetal day 15 [1,2] but where AVP can only be detected 2 days postnatally [39]. The time-lapse between the presence of the cell and its hormonal content raises the question whether a particular stimulus for peptide production is necessary. For the suprachiasmatic nucleus the appearance of AVP coincides with the onset of innervation of this nucleus [19,20], which suggests that this might be a trigger for AVP synthesis. In the SON and PVN, almost simultaneously with the immunocytochemical detection of AVP, acetylcholinesterase has been demonstrated histochemically from fetal day 17, while an adult-like distribution pattern of this enzyme was already observed at birth [12,23]. Combining these data it is feasible that cholinergic innervation may trigger the AVP synthesis in the magnocellular nuclei. The catecholaminergic innervation of the SON and PVN largely develops shortly after birth [22, 25, 27]. As in adult rats the SON and PVN are densely innervated by these catecholaminergic fibers [22,25]. The monoaminergic innervation of the magnocellular nuclei might be the trigger for the postnatal OXT synthesis. In this respect it is likely that in the fetal rat the effective innervation of the future OXT-containing cells in the hypothalamus only takes place postnatally, resulting in no synthesis or a very low synthesis of this peptide in the fetus.

The present results and those obtained studying the development of the suprachiasmatic nucleus and its processes

the first place possible in the cell body after which the staining appears more and more distally in the fiber processes. The innervation of the brain stem and spinal cord with OXT-containing fibers in the fetal rat was absent, while only at fetal days 19-20 the first AVP-containing fibers in the medulla oblongata could be detected, well after the AVP-containing part of the PVN and SON was demonstrated. As OXT predominates over AVP in the medulla and spinal cord [5,33] the bulk of the neurohypophyseal hormone innervation in these parts will also only develop well after birth. In addition, by means of immunocytochemistry at fetal day 16 only cell bodies of the SON were positive with no staining observed in fiber processes. As the first extrahypothalamic fibers were already demonstrated in the olfactory bulb on fetal day 17, when staining in the PVN is still absent it can be concluded that these fibers are derived from the SON, which also establishes this nucleus for the first time as being a source of the extrahypothalamic fibers. However, the fact that staining in the olfactory bulb appears only one day after staining of the first cells in the SON suggests that the fibers may grow out over considerable distances without yet containing any stainable amount of peptide.

The onset of extrahypothalamic innervation seems to be well synchronized with the first demonstration of neurohypophyseal hormone-containing fibers in the neurohypophysis, also on fetal day 17 [13]. Whether this also means that the establishment of the first functional contacts for neuro-hypophyseal AVP release will develop simultaneously with those for central release will be a point of future research.

In the adult rat it has been proven by immunoelectron microscopy that perineuronal structures and fiber branching indeed represent innervation since in the nuclei of the amygdala, where such structures could frequently be found, vaso-

pressin and OXT containing synapses have been demonstrated [6,8]. Although fiber branching makes it likely that already very early in development the amygdala and olfactory bulb are already directly innervated by vasopressinergic fibers, the presence of peptidergic synapses in the fetal brain still has to be established.

In the fetal rat much more fibers seem to penetrate the ependyma than in adult rat. This might be a repetition of the phylogenesis in ontogeny, as in the lower vertebrates such processes are commonly found [14,38]. The same might be true for the fibers in the optic nerve which were also demonstrated in lower vertebrates [14] and are found in higher amounts in the fetus than in the adult animal. The fibers penetrating the ependyma are supposed to fulfill a receptive function, but might alternatively serve to release AVP directly into the CSF, thus providing a mechanism via which neurohypophyseal hormones may reach their central sites of action before the extrahypothalamic system is fully mature. If so, the CSF transport route in the fetus might be essential for the effect of vasopressin on brain growth, particularly since vasopressin deficiency seems to especially affect the cerebellum [3], which is not densely innervated by these peptidergic fibers directly [7].

The dominance of AVP in the fetal rat is, in addition, an argument for the idea that this peptide, rather than OXT, might be an important factor for the functioning of the central nervous system early in development.

ACKNOWLEDGEMENTS

The authors wish to thank C. v. Rheenen-Verberg and J.J. v. Heerikhuizen for their technical assistance, A.T. Potjer for the photography and P.J. v. Nieuwkoop for typing the manuscript.

REFERENCES

- Altman, J. and S. A. Bayer. Development of the diencephalon in the rat. I. Autoradiographic study of the time of origin and settling patterns of neurons of the hypothalamus. *J. comp. Neurol.* **182**: 945-972, 1978.
- Anderson, C. H. Tissue of neuron origin in the anterior hypothalamus of the rat. *Brain Res.* **154**: 119-122, 1978.
- Boer, G. J., D. F. Swaab, H. B. M. Uylings, K. Boer, R. M. Buijs and D. N. Velis. Neuropeptides in rat brain development. In: *Progress in Brain Research, Vol. XX, Adaptive Capabilities of the Nervous System*, edited by P. McConnell, G. J. Boer, N. E. van de Poll, H. R. Romijn and M. A. Corner. Amsterdam: Elsevier, in press, 1980.
- Boer, K., J. Dogterom and H. F. Pronker. Pituitary content of oxytocin, vasopressin and α -melanocyte stimulating hormone in the fetus of the rat during labour. *J. Endocr.*, in press.
- Buijs, R. M. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tiss. Res.* **192**: 423-435, 1978.
- Buijs, R. M. Immunocytochemical demonstration of vasopressin and oxytocin in the rat brain by light and electron microscopy. *J. Histochem. Cytochem.* **28**: 357-360, 1980.
- Buijs, R. M. Vasopressin and oxytocin innervation of the rat brain. A light- and electronmicroscopical study, MD Thesis GU Amsterdam, The Netherlands, 1980.
- Buijs, R. M. and D. F. Swaab. Immuno-electron microscopical demonstration of vasopressin and oxytocin synapses in the limbic system of the rat. *Cell Tiss. Res.* **204**: 355-365, 1979.
- Buijs, R. M., D. F. Swaab, J. Dogterom and F. W. van Leeuwen. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. *Cell Tiss. Res.* **186**: 423-433, 1978.
- Choy, V. J. and W. B. Watkins. Maturation of the hypothalamo-neurohypophysial system. I. Localization of neurophysin, oxytocin and vasopressin in the hypothalamus and neural lobe of the developing rat brain. *Cell Tiss. Res.* **197**: 325-336, 1979.
- Cooper, K. E., N. W. Kasting, K. Lederis and W. L. Veale. Evidence supporting a role for endogenous vasopressin in natural suppression of fever in the sheep. *J. Physiol.* **295**: 33-45, 1979.
- Daniłova, O. A. Cholinesterase activity of the hypothalamo-hypophysial neurosecretory system in rats during the ontogenic development. *Histochemie* **28**: 255-264, 1971.
- Dellmann, H. D., M. Castel and J. G. Linner. Ultrastructure of peptidergic neurosecretory axons in the developing neural lobe of the rat. *Gen. comp. Endocr.* **36**: 477-486, 1978.
- Dierickx, K. The dendrites of the preoptic neurosecretory nucleus of rana temporaria and the osmoreceptors. *Archs Int. Pharmacodyn.* **140**: 707-725, 1962.
- Dogterom, J. The release and presence of vasopressin in plasma and cerebrospinal fluid as measured by radioimmunoassay; studies on vasopressin as a mediator of memory processes in the rat. PhD Thesis, State Univ. Utrecht, The Netherlands, 1977.
- Dogterom, J., F. G. M. Snijdwint and R. M. Buijs. The distribution of vasopressin and oxytocin in the rat brain. *Neurosci. Lett.* **9**: 341-346, 1978.
- Dogterom, J., F. G. M. Snijdwint, P. Pévet and D. F. Swaab. Studies on the presence of vasopressin, oxytocin and vasotocin in the pineal gland, subcommissural organ and fetal pituitary gland: failure to demonstrate vasotocin in mammals. *J. Endocr.* **84**: 115-123, 1980.

18. Gash, D., C. Sladek and D. Scott. Cytodifferentiation of the supraoptic nucleus correlated with vasopressin synthesis in the rat. *Brain Res.* **181**: 345-355, 1980.
19. Güldner, F. H. Synaptology of the rat suprachiasmatic nucleus. *Cell Tiss. Res.* **165**: 509-544, 1976.
20. Güldner, F. H. Synapses of optic nerve afferents in the rat suprachiasmatic nucleus. *Cell Tiss. Res.* **194**: 17-35, 1978.
21. Hyypä, M. Differentiation of the hypothalamic nuclei during ontogenetic development in the rat. *Z. Anat. Entwickl. Gesch.* **129**: 41-52, 1969.
22. Hyypä, M. A histochemical study of the primary catecholamines in the hypothalamic neurons of the rat in relation to the ontogenetic and sexual differentiation. *Z. Zellforsch.* **98**: 550-560, 1969.
23. Hyypä, M. Histochemically demonstrable esterase activity in the hypothalamus of the developing rat. *Histochemie* **20**: 29-39, 1969.
24. Ifft, J. D. An autoradiographic study of the time of final division of neurons in rat hypothalamic nuclei. *J. comp. Neurol.* **144**: 193-204, 1972.
25. Khachaturian, H. and J. R. Sladek, Jr. Simultaneous monoamine histochemistry and neuro-peptide immunocytochemistry: III. Ontogeny of catecholamine varicosities and neurophysin neurons in the rat supraoptic and paraventricular nuclei. *Peptides* **1**: 77-95, 1980.
26. Kovacs, G. L., B. Bohus, D. H. G. Versteeg, E. R. de Kloet and D. de Wied. Effect of oxytocin and vasopressin on memory consolidation: sites of action and catecholaminergic correlates after local microinjection into limbic-midbrain structures. *Brain Res.* **175**: 303-314, 1979.
27. Loizou, L. A. The postnatal ontogeny of monoamine-containing neurones in the central nervous system of the albino rat. *Brain Res.* **40**: 395-418, 1972.
28. Negro-Vilar, A., F. Sanchez-Franco, M. Kwiatkowski and W. K. Samson. Failure to detect radioimmunoassayable arginine vasotocin in mammalian pineals. *Brain Res. Bull.* **4**: 789-792, 1979.
29. Pavel, S. Evidence for the presence of lysine-vasotocin in the pig pineal gland. *Endocrinology* **77**: 812-817, 1965.
30. Perks, A. M. and E. Vizolyi. Studies on the neurohypophysis of foetal mammals. In: *Foetal and Neonatal Physiology*, edited by R. S. Comline, K. W. Cross, G. S. Dawes and P. W. Nathanielsz. London: Cambridge Univ. Press, 1973, pp. 430-438.
31. Sinding, C., A. G. Robinson, S. M. Seif and P. G. Schmid. Neurohypophyseal peptides in the developing rat fetus. *Brain Res.*, in press.
32. Sofroniew, M. V. and A. Weindl. Extrahypothalamic neurophysin-containing perikarya, fiber pathways and fiber clusters in the rat brain. *Endocrinology* **102**: 334, 1978.
33. Sofroniew, M. V. Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human. *J. Histochem. Cytochem.* **28**: 475-478, 1980.
34. Swaab, D. F. and C. W. Pool. Specificity of oxytocin and vasopressin immunofluorescence. *J. Endocr.* **66**: 263-272, 1975.
35. Swaab, D. F., G. J. Boer, J. Dogterom, F. W. van Leeuwen and M. Visser. Fetal neuroendocrine mechanisms in development and parturition. In: *Maturation of the Nervous System, Progress in Brain Research*, edited by M. A. Corner, R. E. Baker, N. E. van de Poll, D. F. Swaab and H. B. M. Uylings. Amsterdam/New York/Oxford: Elsevier, 1978, p. 48.
36. Swanson, L. D. Immunohistochemical evidence for a neurophysin-containing autonomic pathway arising in the paraventricular nucleus of the hypothalamus. *Brain Res.* **128**: 346-353, 1977.
37. Versteeg, D. H. G., E. R. de Kloet, T. van Wimersma Greidanus and D. de Wied. Vasopressin modulates the activity of catecholamine containing neurons in the specific brain regions. *Neurosci. Lett.* **11**: 69-73, 1979.
38. Vigh-Teichmann, I., B. Vigh and S. Koritsanszky. Liquorkontaktneurone im Nucleus paraventricularis. *Z. Zellforsch.* **103**: 483-501, 1970.
39. Vries, G. J. de, R. M. Buijs and D. F. Swaab. Ontogeny of the vasopressinergic neurons of the nucleus supraopticus and their exohypothalamic projections in the rat brain: presence of a sex difference in the lateral septum. *Brain Res.*, in press.
40. Wied, D. de and B. Bohus. Modulation of memory processes by neuropeptides of hypothalamic neurohypophyseal origin. In: *Brain Mechanisms in Memory and Learning: From the Single Neuron to Man*, edited by M. A. B. Brazier. New York: Raven Press, 1979, pp. 139-149.
41. Wimersma Greidanus, T. B. van, G. Croiset, E. Bakker and H. Bouman. Amygdaloid lesions block the effect of neuropeptides (vasopressin, ACTH₄₋₁₀) on avoidance behavior. *Physiol. Behav.* **22**: 291-295, 1979.
42. Wolf, G. and B. Trautmann. Ontogeny of the hypothalamo-neurohypophyseal system in rats—an immunohistochemical study. *Endokrinologie* **69**: 222-226, 1977.