Vasopressin and Oxytocin in the Developing Rat Brain as Shown by Isoelectric Focusing of Radioimmunoassayable Peptides

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Vasopressin and oxytocin content were measured by radioimmunoassay in tissue extracts from rat pituitary, hypothalamus and the extrahypothalamic brain from gestational day 14 until postnatal day 14. Assayable compounds were subsequently characterized by isoelectric focusing in gels. Fetal day 14 extracts contained only low amounts of both hormones (around 1–2 pg per fetus). Vasopressin content rapidly increased to levels of 41 ng in pituitary, 20 ng in hypothalamus, 0.3 ng in extrahypothalamic brain on postnatal day 14. Oxytocin content showed only measurable amounts around day 20 prenatally, reaching levels of, respectively, 10, 0.5 and 0.2 ng on postnatal day 14. The presence of radioimmunoassayable vasopressin in the extrahypothalamic brain on fetal day 14 (around 2 pg) confirms the early ontogeny of vasopressinergic innervation of the brain. Whenever measurable, immunoreactivity had an isoelectric point similar to that of synthetic vasopressin or oxytocin. The possibility that the detected amounts of vasopressin were due to vasotocin, which had been claimed to be present in fetal brain, was excluded by comparison of the regression coefficients of serial dilutions of the extracts in the radioimmunoassay with those of synthetic vasotocin or vasopressin.

INTRODUCTION

The neurohypophyseal hormones vasopressin (VP) and oxytocin (OT) are involved in the regulation of kidney functions, lactation and labour. In fetal life, VP is likely to be of importance in brain development and in setting the levels of osmoregulation for later life^{4,13}, while both fetal VP and OT may be involved in the process of labour^{18,26}.

VP and OT are present in the fetal brain and pituitary of mammals^{7,20} including man^{6,19}. Using immunocytochemistry, VP-containing neurons in the rat brain first appear in the hypothalamus on fetal day 16, after which time VP fiber outgrowth towards the pituitary and extrahypothalamic brain sites has been observed⁷. OT-containing cells, however, were stained only after neonatal day 4 (refs. 7, 8). Using bioassay⁹ and radioimmunoassay (RIA)^{7,20}, a high VP/OT ratio was observed in rat fetal pituitary and brain. Early studies ascribed this to the presence of vasotocin (VT) in mammalian fetal pituitary^{23,29}. Al-

though several other RIA studies failed to confirm the presence of this peptide in either rat^{11,20,21} or man⁵, others have claimed its presence in human fetuses^{2,16,17,25}. In addition, VP and OT in the fetal rat pituitary were reported to be partly present in a high-molecular-weight form (~ 100,000 dalton), which was not found in the neonate²².

These observations raised the question of whether it was indeed genuine VP and OT which had been measured in our early developmental studies⁷ and in those of others (see above). The present study characterizes the radioimmunoassayable VP and OT in extracts of developing rat brain and pituitary by means of isoelectric focusing.

MATERIALS AND METHODS

Animals

Wistar rats (CBP/TNO, Zeist, The Netherlands) were mated overnight. Sperm in the morning vaginal smear identified day-0 pregnant females. Pregnancy

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length was 21 days and the day of birth was taken as day 0 postnatally.

Peptides

The peptide standards for the RIAs were kindly supplied by Organon (VP, batch W.S.1; OT, batch 435 AK1; VT, batch TH223 K11). Synthetic peptides used for isoelectric focusing were VP (Sigma, grade VIII) and OT (Sigma, grade X).

Tissue treatment

For the prenatal period pregnant females were decapitated and the uterus immediately isolated and transferred onto crushed ice. The fetuses were then removed from the uterine horns for further dissection in a similar way as for the neonates: decapitation, removal of the brain and dissection of the pituitary and hypothalamus. These structures were immediately transferred along with the remaining parts of the brain (extrahypothalamic brain) into ice-cold 0.1 N HCl. On fetal day 14 no pituitary could be distinguished for isolation. The dissected tissues were pooled per age group and homogenized using a Potter-Elvehjem homogenizer in 0.1 N HCl (minimum volume 5 ml/g wet tissue) and centrifuged at 2500 g for 20 min at 4 °C. The numbers of pooled animal samples were: fetal day 14, $n = 38$; fetal day 17, $n = 32$; fetal day 20, $n = 20$; day 1, $n = 10$; and day 14, $n = 4$. The neuropeptide content of the supernatants was further extracted with Vycor glass powder according to Dogterom et al.¹⁰. The dried extracts were dissolved in 50 μ l 10% (v/v) dimethylformamide (DMF, British Drug House, for gas liquid chromatography), 2% Servalyte 2-11 (Serva, Heidelberg) and stored at -80 °C until use for isoelectric focusing or RIA (see below).

Characterization of radioimmunoassayable peptides

Gel isoelectric focusing of 10 μ l of dissolved Vycor extracts was performed in a Pharmacia Flat-Bed Apparatus model FBE-3000 at 4 °C as described earlier¹⁴ with the modification that prefocusing was omitted. The 9.5-cm gel lanes were sliced into 2-mm fractions, which were eluted by rotation for 1 h at 4 °C in 500 μ l assay buffer (0.02 M 5,5-diethylbarbituric acid, 0.01 M EDTA, 0.1 M cystine, 0.14 M NaCl, 0.25% bovine serum albumin, set at pH 8.0 with NaOH) containing 0.05% Triton X-100. Next,

the eluate was lyophilized in order to remove the DMF from the focusing gel and stored at -20 °C. Before the RIA for VP and OT¹⁰, the lyophilized material was redissolved in 500 μ l distilled water under rotation for 1 h at 4 °C and 3 50- μ l aliquots were taken for the assay. The lower detection limit for both assays was between 0.5 pg and 2 pg/50 μ l sample.

In order to determine the hormone content and regression coefficients in the RIAs, 20 μ l of the dissolved 50 μ l Vycor glass extracts (see above) were lyophilized to remove DMF and then redissolved in 700 μ l assay buffer containing 0.05% Triton X-100. Dilutions with a factor 2 were made and the VP and OT RIAs were performed in triplicate with 50- μ l aliquots. Regression coefficients of these dilution curves plotted as logit bound/free values¹⁰ were calculated and compared with those of the standard curves using Student's *t*-test ($P < 0.05$ was considered to be statistically significant).

RESULTS

Table I shows the amounts of radioimmunoassayable VP and OT in the fetal and postnatal pituitary, hypothalamus and extrahypothalamic brain. Brain VP and OT were present at fetal day 14 but only in amounts in the order of magnitude of the lower detection limit of both RIAs. The OT data remained low on day 17 prenatally but the VP contents of the pituitary, hypothalamus and residual brain were readily measurable. On prenatal day 20, OT became measurable too, although the extrahypothalamic value was again just above the detection level. Contents increased rapidly with development of pituitary and hypothalamus, but less so for the extrahypothalamic brain, and earlier for VP than for OT (extrahypothalamic OT was measurable in reasonable quantities only at two weeks of postnatal age).

Gel isoelectric focusing of the extracts and subsequent RIA of the gel fractions indicates that, whenever VP or OT was detectable, the immunological activity was always found exclusively at the isoelectric points of the synthetic peptides (Fig. 1).

Since VT cannot be separated from VP in the focusing system used¹⁴, the dilution curves of the tissue extracts peptide were measured and together with the genuine VP dilution curve compared with the VT cross-reaction curve (1% cross-reaction at 50% bind-

TABLE I

Radioimmunoassayable vasopressin and oxytocin content of pituitary, hypothalamus and the extrahypothalamic brain

The data (expressed in pg/animal) are calculated using the serial dilution curves of the extracts and are corrected for the recovery of the Vycor extraction. The pooled (see Materials and Methods) and extracted tissue samples have all been taken up in 50 μ l from which 20 μ l, diluted 35-fold, served as source of the first 50- μ l aliquots of two-fold dilution series in the assays (see Table II). Data in the range of the assays, i.e., 0.5–32 and 2–128 pg/50 μ l for the VP and OT RIAs respectively, were used to calculate the tissue peptide content per animal.

Day	Vasopressin			Oxytocin		
	Pituitary	Hypothalamus	Extrahypothalamic brain	Pituitary	Hypothalamus	Extrahypothalamic brain
Fetal						
14	—*	1.0	2.1	—*	0.9	1.3
17	4.1	6.8	7.6	<3.2**	<3.2**	<3.2**
20	969	202	41.6	35.3	25.9	9.2
Postnatal						
1	4350	325	219	222	26.3	<10.4**
14	40600	20000	285	10270	482	189

* Pituitary not distinguishable.

** Below detection limit.

ing). This test showed that there existed no parallelism of VT with the standard curve for the VP RIA. The dilution curves of the extracts always showed parallelism with the VP, never with the VT curve (Table II).

DISCUSSION

The time of appearance and the developmental course of VP and OT content in the rat hypothalamus and pituitary measured with our RIA are comparable to those in reported by others^{7,20–22,24}, except that Sladek et al.²⁴ showed a fetal rise in OT of a more pronounced character. This last observation could be a result of the extraction procedure at neutral pH, since Sinding et al.²² showed a ten-fold increase in radioimmunoassayable OT as compared to acidic extraction. Although of the same order of magnitude, the presently found VP and OT contents of the pre-natal brain are somewhat lower than the values reported by Buijs et al.⁷ and Sinding et al.²⁰. These differences may again be caused by the slightly different extraction procedures used. However, we have found no marked difference in the VP and OT extraction efficiency between the present extraction procedure and that of Buijs et al.⁷. Another cause of discrepancies at early developmental stages might be the dilution of the RIA samples, which should be

TABLE II

Regression coefficients of standard curves in the vasopressin and oxytocin RIA and of serial dilution curves of extracts from pituitary, hypothalamus and extrahypothalamic brain during ontogeny

RIA data are log-logit transformed and coefficients are average $\pm$ S.E.M. (number of triplicate points), based on the best-fitted line. Data of each age group have been compared with the standard curve regression coefficient measured with the same assay.

	Vasopressin	Oxytocin
Standard	-2.67 ± 0.15 (6)	
Vasotocin	-1.61 ± 0.05 (8)*	
Fetal day 17		
standard	-2.4 ± 0.2 (6)	
pituitary	-2.0 ± 0.5 (3)	—**
hypothalamus	-2.8 ± 0.4 (4)	—
extrahypothalamic brain	-2.1 ± 0.4 (4)	—
Fetal day 20		
standard	-2.8 ± 0.2 (6)	
pituitary	-3.8 ± 0.6 (6)	—
hypothalamus	-2.7 ± 0.4 (4)	—
extrahypothalamic brain	-2.6 ± 0.4 (4)	—
Neonatal day 1		
standard	-2.4 ± 0.2 (8)	-2.3 ± 0.6 (4)
pituitary	-2.1 ± 0.2 (6)	-3.1 ± 0.7 (3)
hypothalamus	-2.2 ± 0.2 (7)	—
extrahypothalamic brain	-2.6 ± 0.3 (4)	—
Neonatal day 14		
standard	-2.8 ± 0.2 (5)	-2.7 ± 0.3 (5)
pituitary	-2.5 ± 0.2 (4)	-2.3 ± 0.2 (8)
hypothalamus	-2.9 ± 0.2 (6)	-2.3 ± 0.6 (4)
extrahypothalamic brain	-2.7 ± 0.3 (5)	-2.4 ± 0.6 (4)

* Difference statistically significant from standard curve.

** Not detectable.

Fig. 1. Polyacrylamide gel isoelectric focusing in a pH 2–11 gradient of HCl/Vycor glass extracts from pituitary, hypothalamus and extrahypothalamic brain during ontogeny and subsequent vasopressin (A) and oxytocin (B) radioimmunoassay of the 2-mm gel fractions. Assay profiles have been normalized by expressing them per single animal. Positions of synthetic peptides are indicated by arrows; for vasopressin at pH 9.5 and for oxytocin at pH 7.0, both measured at 4 °C¹⁴; dpn, days postnatal; dpc, days postcoitus.

within the range where parallelism with the standard curve holds.

The RIA data reported here are the first demonstrating the presence of extrahypothalamic VP in fetal brain, and confirm the immunocytochemically observed VP innervation of the brain as an early developmental event^{3,7}. Data also confirm the late maturation of the OT as compared to the VP system^{6–8,22,30}. They also show that both concentrations remain to increase up to postnatal day 14 at least, indicative for a still immature brain, and also seen for other brain peptides, e.g. vasoactive intestinal polypeptide (VIP), somatostatin, substance P and bombesin¹⁵.

The currently used polyacrylamide gel isoelectric focusing is able to separate in one single run very

small (pg) amounts of peptides in tissue extracts over a wide pH-interval into a complete spectrum^{12,27}. This makes the technique suitable to study the specificity of immunocytochemical localizations and RIAs when complex antigen mixtures are assayed^{27,28}. Radioimmunoassay of the gel fractions fails to elicit forms of VP and OT immunoreactivity other than those at the isoelectric points of synthetic VP and OT in any of the developmental stages presently investigated. Since isoelectric focusing is not able to distinguish between compounds with similar isoelectric point, i.e. VP and VT¹⁴, in addition regression coefficients of the dilution curves of tissue extracts were compared to those of synthetic VP, VT and OT. The regression coefficients confirm that the immunoreactive compounds focused at the positions of synthetic

VP and OT are indeed these peptides. Unfortunately, the amounts of VP immunoreaction at fetal day 14 and OT immunoreaction at fetal days 14–17 were too low to allow a similar conclusion to be drawn about these early stages of development.

Our results, therefore, do not support the idea that the initially high VP/OT ratio during ontogeny is due to the presence of VT (see Introduction) or a related compound¹, yet are in keeping with earlier results indicating a relatively late appearance of OT. The latter conclusion finds further support from immunocytochemical studies^{3,7}. Evidence has recently been provided that this phenomenon is caused by differences in the rate of maturation of VP and OT precursors^{6,30}.

The seemingly contradictory results on the presence of fetal vasotocin may be due in part to the different species used. Thus, the presence of fetal VT has never been reported for the rat. In fact, Dogterom et al.¹¹, using a specific VT RIA procedure, failed to detect any VT whatsoever.

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REFERENCES

- 1 Amico, J.A., Gore Ervin, M., Leake, R.D., Fisher, D.A., Finn, F.M. and Robinson, A.G., A novel oxytocin-like and vasopressin-like peptide in human plasma after administration of estrogen, *J. Clin. Endocrinol. Metab.*, 60 (1985) 5–12.
- 2 Artman, H.G., Leake, R.D., Weitzman, R.E., Sawyer, W.H. and Fisher, D.A., Radioimmunoassay of vasotocin, vasopressin, and oxytocin in human cerebrospinal fluid and amniotic fluid, *Dev. Pharmacol. Ther.*, 7 (1984) 39–49.
- 3 Boer, G.J., Buijs, R.M., Swaab, D.F. and De Vries, G.J., Vasopressin and the developing rat brain, *Peptides*, 1, Suppl. 1 (1980) 203–209.
- 4 Boer, G.J., Kragten, R., Kruisbrink, J. and Swaab, D.F., Vasopressin fails to restore postnatally the stunted brain development in the Brattleboro rat, but affects water metabolism permanently, *Neurobehav. Toxicol. Teratol.*, 6 (1984) 103–109.
- 5 Burford, G.D., Robinson, I.C.A.F. and Swann, R.W., Neurophysin and hormone levels in the hypothalamus, neurohypophysis and cerebrospinal fluid of the human fetus, *Acta Endocrinol.*, 97, Suppl. 243 (1981) 317.
- 6 Burford, G.D. and Robinson, I.C.A.F., Oxytocin, vasopressin and neurophysins in the hypothalamo-neurohypophyseal system of the human fetus, *J. Endocrinol.*, 95 (1982) 403–408.
- 7 Buijs, R.M., Velis, D.N. and Swaab, D.F., Ontogeny of vasopressin and oxytocin in the fetal rat: early vasopressinergic innervation of the fetal brain, *Peptides*, 1 (1980) 315–324.
- 8 Choy, V.J. and Watkins, W.B., Maturation of the hypothalamo-neurohypophyseal system. I. Localization of neurophysin, oxytocin and vasopressin in the hypothalamus and neural lobe of the developing rat brain, *Cell Tissue Res.*, 197 (1979) 325–336.
- 9 Dicker, S.E. and Tyler, C., Vasopressor and oxytocic activities of the pituitary glands of rats, guinea pigs and cats and of human foetuses, *J. Physiol. (London)*, 121 (1953) 206–214.
- 10 Dogterom, J., Van Wimersma Greidanus, Tj.B. and Swaab, D.F., Evidence for the release of vasopressin and oxytocin into cerebrospinal fluid: measurements in plasma and CSF of intact and hypophysectomized rats, *Neuroendocrinology*, 24 (1977) 108–118.
- 11 Dogterom, J., Snijdwint, G.M., Pevet, P. and Swaab, D.F., 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. Endocrinol.*, 84 (1980) 115–123.
- 12 Gianazza, E., Chillemi, F., Gelfi, C. and Righetti, P.G., Isoelectric focusing oligopeptides: detection by specific stains, *J. Biochem. Biophys. Meth.*, 1 (1979) 327–351.
- 13 Handelsmann, G.E., Russell, J.T., Gainer, H., Zerbe, R. and Bayorh, M., Vasopressin administration to neonatal rats reduces antidiuretic response in adult kidneys, *Peptides*, 4 (1984) 827–832.
- 14 Kok, T.P., Van der Sluis, P.J. and Boer, G.J., Chemical identification of the vasopressin immunoreactive material present in the rat suprachiasmatic nucleus, *Neuropeptides*, 3 (1983) 255–262.
- 15 McGregor, G.P., Woodhams, P.L., O'Shaughnessy, D.J., Ghatei, M.A., Polak, J.M. and Bloom, S.R., Developmental changes in bombesin, substance P, somatostatin and vasoactive intestinal polypeptide in the rat brain, *Neurosci. Lett.*, 28 (1982) 21–27.
- 16 Pavel, S., Vasotocin biosynthesis by neurohypophyseal cells from human fetuses. Evidence for its ependymal origin, *Neuroendocrinology*, 19 (1975) 150–159.
- 17 Pavel, S., Presence of relatively high concentrations of arginine vasotocin in the cerebrospinal fluid of newborns and infants, *J. Clin. Endocrinol. Metab.*, 50 (1980) 271–273.
- 18 Schrieffer, J.A., Lewis, P.A. and Miller, J.W., Role of fetal oxytocin in parturition in the rat, *Biol. Reprod.*, 27 (1982) 362–368.
- 19 Schubert, F., George, J.M. and Baskar Rao, M., Vasopressin and oxytocin content of human fetal brain at different stages of gestation, *Brain Res.*, 213 (1981) 111–117.
- 20 Sinding, C., Robinson, A.G., Seif, S.M. and Schmid, P.G., Neurohypophyseal peptides in the developing rat fetus, *Brain Res.*, 195 (1980) 177–186.
- 21 Sinding, C., Seif, S.M. and Robinson, A.G., Levels of neu-

- rohypophyseal peptides in the rat during the first month of life. Basal level in plasma, pituitary, and hypothalamus, *Endocrinology*, 107 (1980) 749–754.
- 22 Sinding, C., Czernichow, P., Seif, S.M. and Robinson, A.G., Quantitative changes in neurohypophyseal peptides in the developing brain, *Peptides*, 1, Suppl. 1 (1980) 45–50.
 - 23 Skowsky, W.R. and Fisher, D.A., Fetal neurohypophyseal arginine vasopressin and arginine-vasotocin in man and sheep, *Pediatr. Res.*, 11 (1977) 627–630.
 - 24 Sladek, C.D., Gash, D.M., Khachaturian, H., Scott, D. and Sladek, J.R., Jr., Maturation of the supraoptic nucleus: a multidisciplinary analysis, *Peptides*, 1, Suppl. 1 (1980) 51–67.
 - 25 Smith, A. and McIntosh, N., Neurohypophyseal peptides in the human fetus: presence in pituitary extracts of immunoreactive arginine-vasotocin, *J. Endocrinol.*, 99 (1983) 441–445.
 - 26 Swaab, D.F., Boer, G.J., Boer, K., Oosterbaan, H.P. and Oosting, P., Neurohypophysial and intermediate lobe peptides in intrauterine growth and labour. In A.J. Baertschi and J.J. Dreyfuss (Eds.), *Neuroendocrinology of Vasopressin, Corticotiberin and Opiomelanocortin*, Academic Press, London, 1982, pp. 343–352.
 - 27 Van der Sluis, P.J., Boer, G.J. and Pool, C.W., Fixation and immunoperoxidase staining of oligopeptides after isoelectric focusing in thin polyacrylamide slab gels, *Anal. Biochem.*, 133 (1983) 226–232.
 - 28 Van der Sluis, P.J. and Boer, G.J., The relevance of various tests to study specificity in immunocytochemical staining, *Cell Biochem. Funct.*, in press.
 - 29 Vizsolyi, E. and Perks, A.M., New neurohypophyseal principle in foetal mammals, *Nature (London)*, 223 (1969) 1169–1171.
 - 30 Whitnall, M.H., Key, S., Ben-Barak, J., Ozato, K. and Gainer, H., Neurophysin in the hypothalamo-neurohypophyseal system. II. Immunocytochemical studies of the ontogeny of oxytonergic and vasopressinergic neurons, *J. Neurosci.*, 5 (1985) 98–106.