Body and Brain Growth Following Continuous Perinatal Administration of Arginine- and Lysine-Vasopressin to the Homozygous Brattleboro Rat

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Using a recently developed controlled-drug-delivery implantation technique, arginine-vasopressin (AVP) or lysine-vasopressin (LVP) was administered to homozygous (HOM) Brattleboro rats throughout pregnancy in order to study the influence of compensation for the deficiency of AVP on body and brain development in their HOM offspring. This mutant is retarded in both body and brain growth from the neonatal period onwards. In one subgroup the LVP-treatment was continued postnatally by means of subcutaneous implantation in the pups. AVP treatment had no growth-stimulating effect either on pup body weight at day one or on postnatal body growth, nor did it affect noticeably the day of eye opening, or a number of brain parameters measured at one month of age. LVP treatment, in contrast, resulted in higher body weights at birth, which could be maintained postnatally if the pups were reared with a Wistar foster-mother. At one month of age body as well as brain weights were still larger in the treated pups. Although cerebellar weight was larger than in untreated Brattleboro pups in this group, cerebellar DNA content or gross morphology, known to be impaired in HOM rats, were not changed. LVP treatment of the pups, as well as maternal AVP-treatment beginning on day 15 of pregnancy, had inhibiting rather than growth-stimulating effects, high-lighting the different effects created by these two peptides at different stages of development.

INTRODUCTION

Homozygous (HOM) Brattleboro rats have a recessive brain deficiency for the synthesis of arginine-vasopressin (AVP). The hereditary defect is due to a single deletion in the sequence encoding the neurophysin part of the AVP precursor giving rise to a frame shift and an open reading frame³³. Normally, the vasopressin gene is transcribed within magnocellular neurones in the paraventricular and supraoptic nuclei of the hypothalamo-neurohypophyseal system. Neural lobe AVP is released into the circulation mainly to control antidiuresis³⁰. The Brattleboro rats suffer, therefore, from severe diabetes insipidus (DI) resulting in a daily water turnover up to 110% of their body weight³⁸. DI can be corrected by subcutaneous administration of vasopressin^{14,24,35,43}.

AVP is also believed to act centrally as a neurotransmitter, via a network of nerve fibers from the suprachiasmatic and paraventricular nuclei¹² and

from the bed nucleus of the stria terminalis, locus coeruleus and amygdala^{13,39}. By immunological methods, the presence of AVP has recently been demonstrated in mammalian ovary⁴¹, testis²⁵ and adrenal gland¹, including the adrenal²⁶ and ovary²¹ of HOM Brattleboro rats. These findings suggest that non-neural tissues may have a different posttranslational modification of the AVP precursor than does the brain.

When compared with heterozygous (HET) Brattleboro rats, which are capable of producing AVP and which have nearly normal diuresis³⁷, the HOM animals exhibit retarded body and brain development^{4,8,43}. In the brain the most severely and persistently affected area appears to be the cerebellum⁸. A trophic role of AVP during brain development has been postulated on the basis of: (a) the particular pattern of the stunted brain growth among other known developmental disturbances^{3,7}; (b) the mitogenic potency of vasopressin both in vitro^{23,29,31,40,42} and in

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vivo^{19,28,32}; and (c) the growth-promoting effect on neurites in cell culture¹⁸. In theory, the proper administration of this peptide to HOM animals should correct the growth anomalies in the body and brain. AVP, applied systemically fails to increase postnatal body^{7,35,43} or brain growth^{5,7}, even when applied continuously^{6,20}. Since reduced brain growth is already evident at birth⁴, it has been argued⁹ that AVP supplementation in the above experiments was given too late during development, and that earlier administration of AVP might well correct the growth anomalies in HOM animals. Since in the normal rat the AVP-generating neurons of the hypothalamus appear immunocytoologically already on prenatal day 16 (ref. 11), while AVP can be detected radioimmunochemically as early as prenatal day 14 (ref. 34), this hypothesis is quite plausible. The present study, therefore, describes the effects on the postnatal development of continuously applied AVP or LVP to the Brattleboro mothers throughout pregnancy.

MATERIALS AND METHODS

Animals and animal treatment

HOM Brattleboro rats (*di/di*), weighing 200–250 g, were obtained from TNO-CPB (Zeist, The Netherlands). All animals were kept in large cages, received tap water and standard chow ad libitum, and experienced light between 07.00 and 19.00 h. For each experiment 70 female and 25 male HOM Brattleboro rats were used to produce sufficient litters. If sperm were present in the morning vaginal smear, day postcoitus (dpc) 0, the females received Accurel/collodion tubing (see below) subcutaneously in the neck loaded with AVP, LVP or water (untreated rats). The pregnant HOM Brattleboro rats were placed in metabolism cages on dpc 7 and 16 to measure urine production and urine osmolality. Urine AVP and LVP were assayed radioimmunochemically¹⁶ (LVP could be measured by its cross-reaction in the AVP assay since no endogene AVP is available in urine of HOM rats), to control for proper functioning of the drug-delivery device (detection levels were 1 and 2 pg/ml urine respectively). On dpc 17 the rats were placed in small individual plastic cages (35 × 25 × 18 cm) and kept on ample and daily changed sawdust bedding. On day 1 postpartum (dpp 1, i.e. dpc 23), the Accurel/collodion tubing was removed and

the litters were aselectively adjusted to a litter size of 8 pups. Mothers with less than 6 pups were not used. On dpp 12, the mother and litter were placed in larger plastic cages (60 × 25 × 18 cm) in which water nipples were made available for the pups. Body weights of the pups were regularly recorded during the first month of life, after which the animals were sacrificed by decapitation.

Vasopressin/Accurel/collodion device implantations

AVP and LVP treatment of the pregnant rats and the pups was performed with a subcutaneously implantable device^{6,20}. For application in the mothers, pieces of microporous Accurel polypropylene hollow fiber (P78/15/2, o.d. 1.6 mm), 15 mm in length, were loaded with 20 or 100 µg AVP (Sigma Grade VIII, 403 IU/mg) or 100 µg LVP (Sigma Grade IX, 231 IU/mg) and coated with a layer of nitrocellulose (Necol Collodion solution for preparing permeable membranes, British Drug Houses). For neonatal Brattleboro pups small tubing (Accurel P9/80/1, o.d. 0.95 mm), 5 mm in length and loaded with 20 µg LVP, were prepared similarly and sterilized by γ -radiation (25 kGy; Gammaster B.V. Ede, The Netherlands). The in vivo release, tested with [¹²⁵I]AVP and with [³H]AVP, is approximately 1% of the total load per 24 h for at least 3 weeks²⁰ (Kruisbrink and Boer, in preparation).

Experimental series

The sperm-positive HOM rats received on dpc 0 devices loaded with 20 µg AVP in experiment 1, 100 µg AVP in experiment 2 and 100 µg LVP in experiment 4 and 5. In experiment 3 the implantations (100 µg AVP loads) were performed on dpc 15. The untreated females received devices filled with water. In experiment 5, HOM litters were fostered by Wistar mothers of the same postpartum stage. In a subgroup (experiment 5A), LVP treatment was continued in the pups: small Accurel/collodion tubing loaded with 20 µg LVP or water (untreated pups) were implanted subcutaneously on dpp 1. In all series between day 12 and 15 at 10.00 h the number of opened eyes within a litter was counted, while the urine production of the pups was measured over a two-day period in a metabolism cage between day 30 and 34. The young animals were sacrificed on day 33 or 34 by means of decapitation and the brains were

immediately isolated and weighed.

Cerebellum and medulla oblongata were quickly dissected and also weighed. Brain parts were frozen and kept at -25°C , and later lyophilized for dry weight, DNA² and protein content²² determinations.

Morphology of the cerebellum

A non-selectively obtained number of cerebelli from 7 untreated and 5 prenatally LVP-treated animals of experiment 5 were fixed in 4% paraformaldehyde and after dehydration embedded in paraffin. Sagittal sections ($5\text{ }\mu\text{m}$) near the midline of the vermis were stained with cresyl violet. The midsagittal section (Fig. 1) was selected and the morphology quantified. Total surface and the areas of the molecular, granular and white matter layers were measured with a digitizer (Calcomp 2000) using 30-fold magnified photographs. The sulcus depth of lobe VI_d was also measured (Uylings and Van Norde, in preparation).

Statistics

All data were statistically evaluated using Student's *t*-test, taking $P < 0.05$ (2-tailed) as the level of significance. The non-parametric Mann-Whitney U-test (two-tailed, corrected for ties) was used for the sulcus depth of lobe VI_d.

RESULTS

Diuresis pregnant mothers

A week after implantation of the Accurel/collodion devices in the sperm-positive HOM rats, the

continuous release of AVP and LVP had significantly decreased the diuresis to about 10% and 15% of levels of untreated rats, respectively (Table I). The DI slowly returned during pregnancy: on dpc 17 the urine production of the rats with the 20 and 100 μg AVP and 100 μg LVP loads was, respectively, approximately 40, 20 and 35% of untreated HOM rats. Urine osmolality reflected the urine excretion data.

Prenatal supplementation of AVP (experiments 1, 2 and 3)

The body weights of the pups on dpp 1 did not differ from untreated HOM rats in any of the prenatal supplementation experiments using AVP. There were also no differences in the moment of eye opening (Table II). Body weights remained unaffected up to one month postnatally (experiments 1 and 2), except when AVP treatment started at dpc 15 (experiment 3). These animals showed lower postnatal growth rate and a 10% lower body weight at one month (Table III). At that time, total brain weight, cerebellar and medulla oblongata weight also did not differ from untreated rats, except again in experiment 3 where the brain weight was significantly lower ($P < 0.005$).

Prenatal treatment with LVP (experiment 4 and 5)

Treatment with LVP resulted in increased body weights of the pups on dpp 1 (Table II), although in experiment 4 this effect did not become statistically significant ($0.2 < P < 0.3$) as it did in experiment 5

TABLE I

Urine production and osmolality of pregnant HOM Brattleboro rats after implantation of the Accurel/collodion devices on dpc 0

Urine production and osmolality are given as average $\pm$ S.E.M.

Experiment	n	Urine production (ml/100 g b. wt./day)		Urine osmolality (mOsm/kg)	
		dpc 8	dpc 17	dpc 8	dpc 17
1 Untreated	2	83 $\pm$ 7	101 $\pm$ 11	140 $\pm$ 30	125 $\pm$ 25
AVP (20 μg)	3	9 $\pm$ 1	43 $\pm$ 6	1110 $\pm$ 80	500 $\pm$ 125
2 Untreated	3	96 $\pm$ 3	100 $\pm$ 8	133 $\pm$ 3	115 $\pm$ 10
AVP (100 μg)	4	9 $\pm$ 1	18 $\pm$ 6	1150 $\pm$ 60	765 $\pm$ 70
3* Untreated	4	119 $\pm$ 9	114 $\pm$ 4	124 $\pm$ 4	140 $\pm$ 20
AVP (100 μg)	5	102 $\pm$ 7	10 $\pm$ 1	131 $\pm$ 4	1090 $\pm$ 80
4 Untreated	6	79 $\pm$ 3	96 $\pm$ 4	—	—
LVP (100 μg)	7	13 $\pm$ 1	37 $\pm$ 5	—	—
5 Untreated	24	98 $\pm$ 3	105 $\pm$ 2	146 $\pm$ 4	143 $\pm$ 2
LVP (100 μg)	24	14 $\pm$ 2	35 $\pm$ 5	1130 $\pm$ 55	630 $\pm$ 70

* Implantation dpc 15.

TABLE II

Influence of continuous treatment of AVP or LVP to pregnant HOM Brattleboro rats on day 1 weight and eye opening of their HOM offspring

Data show average $\pm$ S.E.M. (number of animals).

Experiment	Wistar foster	Weight day 1 (g)	Day of eye opening
1 Untreated	—	6.9 $\pm$ 0.1 (16)	13.4 $\pm$ 0.1 (16)
AVP (20 μ g)	—	6.8 $\pm$ 0.1 (23)	13.5 $\pm$ 0.1 (22)
2 Untreated	—	6.9 $\pm$ 0.1 (41)	13.0 $\pm$ 0.1 (40)
AVP (100 μ g)	—	6.7 $\pm$ 0.1 (92)	13.0 $\pm$ 0.1 (85)
3* Untreated	—	6.6 $\pm$ 0.1 (31)	13.3 $\pm$ 0.1 (28)
AVP (100 μ g)	—	6.7 $\pm$ 0.1 (43)	13.3 $\pm$ 0.1 (40)
4 Untreated	—	6.4 $\pm$ 0.3 (54)	13.1 $\pm$ 0.1 (18)
LVP	—	6.7 $\pm$ 0.1 (59)	13.1 $\pm$ 0.1 (24)
5 Untreated	+	6.3 $\pm$ 0.1 (98)	13.8 $\pm$ 0.1 (37)
LVP	+	6.6 $\pm$ 0.1 (97)***	13.7 $\pm$ 0.1 (24)**
			14.3 $\pm$ 0.1 (22)***

* Implantation dpc 15.

** Experiment 5A, pups from untreated mothers received control Accurel implants on dpp 1, whereas pups from LVP-treated mothers received LVP/Accurel implant on dpp 1.

*** Statistically significant at $P < 0.001$.

($P < 0.001$). The day of eye opening was not affected by the prenatal treatment (Table II).

The increase in body weight was not maintained (experiment 4, Table III) except when Wistar fosters

were introduced after birth (experiment 5, Table III). In this instance, total brain weight and cerebellar weight were also higher than in untreated rats. Medulla oblongata weight did not differ. For cerebel-

TABLE III

Effect of the continuous treatment of AVP and LVP during pregnancy of HOM Brattleboro rats on body and brain weights of their HOM offspring as measured on day 34

Data show average $\pm$ S.E.M.

Experiment	Number of pups	Body (g)	Brain (g)	Cerebellum (mg)	Medulla oblongata (mg)
1* Untreated	16	76 $\pm$ 2	1.52 $\pm$ 0.02	186 $\pm$ 3	124 $\pm$ 3
AVP (20 μ g)	22	79 $\pm$ 2	1.54 $\pm$ 0.02	193 $\pm$ 3	127 $\pm$ 4
2 Untreated	31	83 $\pm$ 1	1.57 $\pm$ 0.01	192 $\pm$ 2	123 $\pm$ 3
AVP (100 μ g)	85	80 $\pm$ 1	1.55 $\pm$ 0.01	192 $\pm$ 1	120 $\pm$ 1
3** Untreated	15	79 $\pm$ 2	1.56 $\pm$ 0.01	188 $\pm$ 2	118 $\pm$ 3
AVP (100 μ g)	29	71 $\pm$ 1***	1.49 $\pm$ 0.01***	184 $\pm$ 2	118 $\pm$ 2
4 Untreated	18	72 $\pm$ 3	1.50 $\pm$ 0.02	186 $\pm$ 3	114 $\pm$ 3
LVP	24	70 $\pm$ 2	1.50 $\pm$ 0.02	184 $\pm$ 3	111 $\pm$ 3
5§ Untreated	37	80 $\pm$ 2	1.53 $\pm$ 0.01	188 $\pm$ 3	122 $\pm$ 3
LVP	31	86 $\pm$ 2***	1.57 $\pm$ 0.01***	197 $\pm$ 2***	124 $\pm$ 2
5A Untreated	24	81 $\pm$ 2	1.54 $\pm$ 0.01	188 $\pm$ 3	119 $\pm$ 3
LVP	22	86 $\pm$ 2	1.49 $\pm$ 0.02***,§§	187 $\pm$ 3§§	112 $\pm$ 3

* Experiment 1 data are obtained on day 33.

** Implantation dpc 15.

*** Statistically significant at $P < 0.05$.

§ In experiment 5 and 5A pups were fostered by Wistars and in experiment 5A the pups received on day 1 postnatal an Accurel device subcutaneously (cf. legends Table II).

§§ Statistically significant difference as compared to LVP group of experiment 5.

TABLE IV

DNA and protein contents of the cerebellum after continuous treatment of LVP given prenatally to the HOM mother and postnatally to the HOM Brattleboro pup

Data are average $\pm$ S.E.M.

Experiment	n	Dry weight (mg)	DNA		Protein	
			(mg)	(%)	(mg)	(%)
5 Untreated	24	38.9 $\pm$ 0.6	2.26 $\pm$ 0.07	5.8 $\pm$ 0.2	22.7 $\pm$ 1.0	58 $\pm$ 2
LVP	20	42.4 $\pm$ 0.4*	2.23 $\pm$ 0.05	5.3 $\pm$ 0.1*	25.8 $\pm$ 0.8*	61 $\pm$ 2
5A Untreated	14	40.3 $\pm$ 0.5	1.75 $\pm$ 0.11	4.3 $\pm$ 0.2	23.8 $\pm$ 0.5	59 $\pm$ 1
LVP	14	41.2 $\pm$ 0.5	1.80 $\pm$ 0.16	4.4 $\pm$ 0.1	24.1 $\pm$ 0.6	60 $\pm$ 1

* Statistically significant at $P < 0.05$.

lum, dry weight and protein content were higher, whereas DNA content was not influenced by the treatment (Table IV). Consequently, the ratio DNA/dry weight was significantly decreased.

Pre- and postnatal treatment with LVP (experiment 5A)

Pups, from a LVP-treated mother and postnatally treated with LVP, experienced eye opening one day later ($P < 0.001$). The postnatal treatment impaired growth since the higher body weight at birth had disappeared at one month of age. This was true for total brain and cerebellum weight as well, whereas medulla oblongata weight became significantly lower as compared with pups from an LVP-treated mother only (Table III). DNA and protein content of cerebellum was, however, not changed (Table IV).

Cerebellar morphology (experiment 5)

Measurements of total surface area and of the molecular, granular and white matter zones of the prenatal LVP-treated and untreated animals of experiment 5, revealed no significant differences (Table V). In both groups, sublobe VI_d was either absent or had variable surface areas. No difference of the average values of depth of sulcus VI_d was observed (Table V, Fig. 1).

Diuresis pups

Neither prenatal AVP or LVP treatment of the mother had any influence on the diuresis of the pups as measured between postnatal day 30 and 34 (data not presented).

DISCUSSION

Diuresis

Kruisbrink and Boer²⁰ found that implantation of Accurel tubing containing 20 μ g AVP in male Brattleboro rats caused a decrease in diuresis to the normal (heterozygous) level for more than 35 days. In the present study, the pregnant females, and also those females that failed to become pregnant (data not presented in Table I), showed an initial decrease in diuresis followed by a slow increase. This was also observed, though to a lesser extent, when 100 μ g AVP or LVP devices were implanted. The secondary increase in diuresis could be due to a drop in AVP or LVP release from the implant over time. However, according to Cheng et al.¹⁴, there is a relation between urine and plasma AVP levels. Since the LVP excretion in experiment 5 was almost identical on dpc 8 and 17 (13.8 $\pm$ 2.1 vs 10.9 $\pm$ 1.8 ng/day, $n = 24$), it

TABLE V

Quantitative morphology of the midsagittal section of the cerebellum of 34-day-old HOM Brattleboro rats after continuous treatment of LVP, given prenatally to the mother

Data are expressed in square mm $\pm$ S.E.M. Depth of sulcus VI_d expressed in mm $\pm$ S.E.M.

	Untreated	LVP-treated
Number	7	5
Total area	16.7 $\pm$ 0.3	16.0 $\pm$ 0.3
Molecular layer	8.4 $\pm$ 0.2	8.0 $\pm$ 0.3
Granular layer	7.3 $\pm$ 0.2	7.2 $\pm$ 0.2
White matter	1.01 $\pm$ 0.07	0.93 $\pm$ 0.06
Sulcus VI _d	0.19 $\pm$ 0.08	0.30 $\pm$ 0.05

Fig. 1. Midsagittal section of the cerebellum of a 34-day-old HOM Brattleboro rat. Inset shows lobe VIId of another untreated HOM Brattleboro rat (also control of experiment 5) which has a much more pronounced appearance, illustrating the great variability within groups.

may also be possible that, as a consequence of kidney adaptation to continuous vasopressin exposure, increasing amounts of AVP or LVP are necessary for normalizing diuresis.

Body growth

Maternal treatment with LVP, a natural analog of AVP, improved prenatal body growth to some extent when measured on dpp 1 (Table II), whereas similar treatment with AVP failed in this respect. This discrepancy might be due to differences in biological ac-

tivity. The vasopressor activity of LVP (285 U/mg) and its antidiuretic activity (212 U/mg) are lower than that of AVP (respectively 369 U/mg and 323 U/mg). Considering the difference in the hydrophobic nature of the two peptides, it is probable that if AVP and LVP are able to cross the placental barrier^{27,36}, then LVP would pass more readily than AVP. This possibility is currently under investigation.

AVP supplementation which started on dpc 15 instead of dpc 0 (experiment 3), had no effect on dpp 1

weight, but showed an inhibitory effect on body development postnatally. Oosterbaan et al.²⁷ have reported disturbed fetal body and brain growth when Wistar mothers received 200 μ g AVP-loaded Accu-rel devices on day 14 of pregnancy. Apparently the present dosage rate of AVP from the device did not impair fetal growth in HOM rats. It is likely, however, that AVP treatment, during the last week of pregnancy, caused metabolic disturbances in the mother, which may influence lactation and rearing care and consequently postnatal body development.

The pups of LVP-treated Brattleboro mothers retain their increased body weight only if the pups were reared by a Wistar foster mother (experiment 4 vs 5). Since LVP treatment was terminated after birth by removal of the drug-delivery device, the failure to maintain body weight gain with their own mother may be due simply to postnatal conditions unfavorable for rearing and nursing by the again polyurinating HOM rat⁷.

Brain development

Previous studies showed a 15–20% diminished DNA content in the HOM cerebellum as compared with HET controls⁷. The present improvement in cerebellar growth induced by prenatal LVP treatment was not reflected in the DNA content, since the values at one month of age did not differ from those of untreated animals. Since water content did not differ, the average cell size in the cerebellum, as reflected in protein/DNA ratio, was 13% higher (experiment 5, Table IV).

Because an increased cell loss appears to take place in the postnatal VP-deficient brain¹⁰, the effect of continuing the LVP treatment directly on the pups (experiment 5A) was explored with the aim of further improving the observed growth-stimulating effects. Neonatal implantation of the Accurel/colloid mini-preparation itself did not disturb general growth, since comparison of body, brain, cerebellum and medulla oblongata weights of untreated animals with or without such implants failed to show any significant change (Table III). However, postnatal LVP treatment decreased brain weight and delayed eye opening, the latter effect being in agreement with postnatal vasotocin and AVP treatment in the cat¹⁷.

Postnatal supplementation with AVP also failed to promote growth of the HOM Brattleboro pups⁵. Hence, if any effect of postnatal LVP treatment is to be concluded from this series, it is one of an inhibition of brain growth.

Quantitative morphology of midsagittal sections of the cerebellum showed that the molecular, granular and white matter zones were reduced in size and that lobe VI_d was absent in most of the HOM Brattleboro rats⁷ (Uylings and Van Norde, in preparation). We found no morphological improvement in the midsagittal sections of cerebelli of prenatally LVP-treated HOM Brattleboro rats (experiment 5). The morphological variability, in particular in the size of lobe VI_d (Fig. 1), which was larger than Uylings and Van Norde observed in their HOM rats, might be explained by the fact that in our experiment the rats were raised by Wistar foster instead of HOM Brattleboro mothers.

Concluding remarks

The beneficial effects of AVP or LVP treatment on the pregnant HOM Brattleboro mother may indirectly be of benefit to the fetus as well. Such treatment may effect general metabolism by reducing drinking requirements while also normalizing sleep patterns¹⁵. These differences in prenatal circumstances might theoretically explain the higher body weight of the pups dpp 1 after prenatal treatment with LVP. This possibility is unlikely to be the explanation, however, since the even more efficient suppression of the DI by AVP (Table I) failed to be reflected in increased fetal growth. Therefore, LVP most probably exerts a direct effect upon the fetus. This possibility might best be studied by the administration of AVP and LVP directly to the fetus.

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