Prenatal Development of the Brattleboro Rat Is Influenced by Genotype and Lysine Vasopressin Treatment of the Mother

F.G.M. Snijdwint, G.J. Boer, D.F. Swaab

Netherlands Institute for Brain Research, Amsterdam, The Netherlands

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Abstract. Homozygous (Hom) Brattleboro rats suffer from severe diabetes insipidus (DI) as a consequence of the lack of arginine-vasopressin (AVP) in the brain. Compared with heterozygous (Het) AVP-synthesizing Brattleboro rats, Hom rats show disturbed body and brain development. In this study breeding experiments with Het and Hom rats were performed to determine whether prenatal conditions might contribute to the developmental disturbances in Hom pups. For this purpose Het and Hom females were mated with Hom and Het males, respectively. In addition lysine-vasopressin (LVP) was administered to half of the pregnant females, since this has previously been shown to stimulate birth weight of Hom pups. On day 1 postnatally the body and brain weight of Hom pups of nontreated Hom mothers was significantly smaller than that of the Het litter mates, whereas no difference was found between the weight of Het or Hom pups of nontreated or LVP-treated Het mothers. These results indicate an important role of the genotype of the mother in prenatal development of Hom pups. LVP administration failed to diminish the growth deficits, but increased protein and DNA content of the cerebellum of both Het and Hom pups. Notwithstanding the improved prenatal growth of Hom pups from Het mothers, postnatally retarded development was still observed: at 1 month of age there was a significant difference between the body, brain and cerebellar weight of Het and Hom pups from Het mothers. It was therefore concluded that the prenatal situation of the Hom mother, i.e. AVP-deficiency, significantly contributes to the developmental disturbance of the Hom pup, but also that growth impairment is linked to the presence of the mutation in the Hom pups themselves.

Introduction

The Brattleboro rat stems from a diabetes insipidus (DI) mutant discovered in a strain of Long-Evans rats [27]. The DI is the result of a deficiency in the synthesis of arginine-

vasopressin (AVP) in the brain due to a single deletion in the DNA sequence encoding the neurophysin part of the AVP precursor [21]. Homozygous (Hom) Brattleboro rats have a disturbed body growth and brain development as compared with heterozygous

(Het) Brattleboro rats, which synthesize AVP in the brain [8]. The cerebellum of the Hom rat seems to be especially affected [8, 26]. There are various environmental factors which have been shown to affect postnatal growth differences between Hom and Het Brattleboro rats [e.g. genotype of the mother, prenatal conditions, fostering, litter size, cage conditions, etc.; for a review, see Boer, 3]. In order to determine how important the lack of AVP might be in relation to the other factors, all additional breeding variables should be excluded. For this purpose homogeneously genotyped litters were previously bred by mating Hom females with either Hom or outbred normal (+/+) Brattleboro males. Using this breeding scheme, body weights were lower for Hom pups at birth as compared with Het pups [8]. Furthermore, when litters with 9 pups were examined, both brain weight and cerebellar DNA content of the Hom pups were affected [5]. These observations indicated that growth-reducing factors are probably already expressed during intrauterine development.

Administration of AVP to Hom animals should, theoretically, correct the growth anomalies of body and brain, provided AVP is the missing trophic factor [3, 8]. However, AVP or lysine-vasopressin (LVP) applied systemically to the neonate failed to increase postnatal body weight [6, 23, 31] or brain growth [6], even when applied continuously by means of a drug delivery device or by injection into the brain [6]. Moreover, AVP supplementation of the Brattleboro mothers throughout pregnancy with the same drug delivery technique did not increase postnatal body or brain weight of the pup [22]. However, the same maternal treatment with LVP resulted in increased body weights of the Hom pups at birth, which could be main-

tained postnatally if the pups were reared with a Wistar foster mother. At 1 month of age, body as well as brain weight was then still greater in the pups from treated mothers [22].

The purpose of the present study is to establish to what degree AVP deficiency of the mother affects prenatal growth in the pup and how LVP treatment interferes with it. This was accomplished by examining genotypically mixed litters taken from both Het (control) and Hom Brattleboro females, half of which treated throughout pregnancy with LVP. The weight of body, brain and cerebellum (the most affected brain area [8]) of the pups were then measured after birth. The results indicate a clear-cut effect of the genotype of the mother on the prenatal development of Hom pups. Prenatal LVP treatment exerted small effects on both Het and Hom pups, whereas a growth disturbance remains related to the AVP defect in the Hom pups.

Materials and Methods

Prenatal Development

Hom (di/di) and Het (+/di) Brattleboro rats, weighing 200–250 g, were obtained from CPB-TNO (Zeist, The Netherlands). All animals were kept in large cages, received tap water and standard chow ad libitum and the light was on between 07.00 and 19.00 h. Mating of the Brattleboro rats with their genetic counterparts (for breeding scheme see table I) for 4 nights within 2 weeks resulted in 21 sperm-positive Het and 21 sperm-positive Hom females. When sperm were present in the morning vaginal smear (prenatal day 0), about half of each group received a subcutaneous Accurel/collodion implant loaded with 100 µg LVP (see below) in the neck, whereas the other half received a placebo implant. The rats were placed in metabolism cages for 24 h at prenatal day 7 and 15 to measure urine production. From prenatal day 20 onwards the pregnant rats were housed individually

Table I. Survey of the breeding of the mixed Het/Hom nests, the diuresis of the sperm-positive females and litter size of the pregnant females

Parents (female × male) ^a	Implant in sperm-positive females ^b	Diuresis, ml/100 g body weight/day		Litter size
		day 8 p.n.	day 16 p.n.	
30 Het × 10 Hom	10 Het (control)	10.6 ± 1.3	12.4 ± 1.4	10.0 ± 1.8 (4)
	11 Het (LVP)	6.8 ± 0.8 ^c	11.2 ± 0.9	10.1 ± 0.8 (10)
30 Hom × 10 Het	9 Hom (control)	109 ± 3	106 ± 3	9.3 ± 0.6 (8)
	12 Hom (LVP)	10.2 ± 1.5 ^c	23.9 ± 6.5 ^c	8.9 ± 0.7 (10)

Data are means ± SEM. The figures in parentheses indicate number of nests. p.n. = Postnatal.

^a Females and males were placed together for 4 nights within 2 weeks.

^b Sperm-positive females received on prenatal day 0 either a control or a 100-μg LVP-loaded Accurel drug delivery device.

^c Statistically significantly different from control values.

in small plastic cages and kept on ample sawdust bedding, which was changed daily.

The day after delivery (prenatal day 23; postnatal day 1) the pups were weighed and sacrificed by means of decapitation, whereafter the brains were immediately isolated and weighed. The pituitaries were subsequently removed and immersed in 100 μl 0.1 N HCl to determine the genotype of the pups by measuring the presence or absence of AVP radioimmunologically [11]. The cerebella were then quickly dissected from the brain and weighed as well. Brain parts were frozen on dry ice and kept at -25 °C. After lyophilization, cerebellar dry weight, DNA [2] and protein content [17] were determined. To exclude as much as possible the influence of litter size on the interpretation of neonatal body and brain weight data [4, 5], measurements were only taken from nests with 9–12 pups.

Postnatal Development

In a second series, 20 Hom and 10 Het female rats were mated as described above, resulting in 4 nests from Het and 6 nests from Hom mothers. On day 1 postnatally all nests were randomly adjusted to 8 pups and the mothers were replaced by Wistar foster mothers of the same postnatal stage in order to eliminate differences due to postnatal maternal effects. During development, body weight of individual pups was regularly recorded using toe marking for identification, and between postnatal day 30 and 32 diuresis of the

pups was measured in metabolism cages. Discrimination between Het and Hom was established on the basis of polyuria [25]. On day 32 the pups were decapitated. Brain and cerebellar weight and DNA content of the cerebellum were measured as described above.

Vasopressin/Accurel/Collodion Device

LVP treatment of the pregnant rats was accomplished by subcutaneously implanting a peptide delivery device [7, 15]. Pieces of microporous Accurel polypropylene hollow fiber (P78/15/2, outside diameter 1.6 mm), 15 mm in length, were lumen-loaded with a 7.5-μl solution containing 100 μg LVP (Sigma Grade IX, 231 IU/mg), heat-sealed at both ends and coated with a layer of nitrocellulose (Necol Collodion solution for preparing permeable membranes; The British Drug Houses Ltd). The *in vivo* release, tested with [¹²⁵I]AVP and with [³H]AVP, is approximately 1% of the total load/24 h for more than 3 weeks [14, 15].

Statistics

Data from the pups, expressed as means ± SEM, of postnatal day 1 (table II) were statistically evaluated using a three-way analysis of variance (SPSSx program) with mother genotype, LVP treatment and pup genotype as variables. In the case of significant F values, individual comparisons were made according to Winer [30]. Data from the 1-month-old Het and

Table II. Body and brain weight, and cerebellar dry weight, DNA and protein content of 1-day-old Het and Hom pups derived as mixed litters from control or prenatally LVP-treated Het and Hom Brattleboro mothers

	Pup genotype	Mother			
		Het	Het (LVP)	Hom	Hom (LVP)
Number of pups	Het	10	28	20	29
	Hom	11	30	29	31
Body weight, g	Het	7.1 ± 0.2	6.9 ± 0.1	7.0 ± 0.1	6.8 ± 0.1
	Hom	7.0 ± 0.2	6.9 ± 0.1	6.4 ± 0.1 ^{a, b}	6.5 ± 0.1 ^b
Brain weight, mg	Het	277 ± 4	274 ± 3	270 ± 4	267 ±
	Hom	274 ± 4	272 ± 1	257 ± 4 ^{a, b}	260 ± 3 ^b
Cerebellar dry weight, mg	Het	1.33 ± 0.05	1.36 ± 0.04	1.29 ± 0.03	1.35 ± 0.03
	Hom	1.40 ± 0.05	1.43 ± 0.03	1.28 ± 0.03 ^b	1.31 ± 0.03 ^b
Cerebellar protein, µg	Het	425 ± 20	536 ± 20 ^c	437 ± 16	500 ± 20 ^c
	Hom	462 ± 18	567 ± 20 ^c	426 ± 11	500 ± 17 ^c
Cerebellar DNA, µg	Het	24 ± 3	29 ± 3	26 ± 2	27 ± 2
	Hom	24 ± 3	31 ± 3 ^c	23 ± 1	30 ± 2 ^c

The litter size was 9–12 pups. Data (means ± SEM) are analyzed by a three-way analysis of variance. $MS_{w, cell}$ values, used for further individual comparisons according to Winer [30], were, respectively, for body and brain weight and cerebellar protein and DNA contents 0.343, 312, 61.8 and 101.

^a Significant difference for genotype pup.

^b Statistically significant difference for genotype mother.

^c Significant difference for treatment of the mother.

Hom rats (table III) were analyzed for differences between the genotype of the pups using Student's *t* test. χ^2 test was used to determine the significance in the difference in pregnancy rate of the Het and Hom mothers. $p < 0.05$ (two-tailed) was taken as the level of significance in all cases.

Results

Prenatal Influences on Pups at Postnatal Day 1 (tables I and II)

The LVP treatment strongly reduced the DI of the pregnant Hom rats, and slightly reduced the diuresis of the pregnant Het rats

(table I). Eighteen out of twenty-one sperm-positive Hom females became pregnant compared to 14 out of 21 for the Het females (χ^2 test; $0.20 < p < 0.30$). Litter size ranged from 4 to 13 pups (Het mothers 10.1 ± 0.7 and Hom mothers 9.0 ± 0.4 ; $0.10 < p < 0.20$; table I). After selection of nests of 9–12 pups (see Materials and Methods), average litter size of Het and Hom mothers was, respectively, 10.6 ± 0.4 ($n = 8$) and 10.0 ± 0.3 ($n = 11$; $0.20 < p < 0.30$). The total number of male (96) and female pups (95) did not differ within these litters. Within the litters of nontreated Hom mothers, the body

Table III. Body and brain weight, and cerebellar dry weight and DNA content of 32-day-old Het and Hom pups derived as mixed litters from Het and Hom Brattleboro mothers, and postnatally reared by Wistar foster mothers

	Pup genotype	Mother	
		Het	Hom
Number of mothers		4	6
Number of pups ^a	Het	12	22
	Hom	17	24
Body weight day 1, g	Het	7.2 ± 0.1	6.9 ± 0.1
	Hom	7.1 ± 0.1	6.6 ± 0.1 ^b
Diuresis day 32, ml/100 g/day	Het	10 ± 1	12 ± 1
	Hom	68 ± 4 ^b	72 ± 5 ^b
Body weight day 32, g	Het	84 ± 4	89 ± 3
	Hom	65 ± 2 ²	69 ± 3 ^b
Brain weight day 32, g	Het	1.59 ± 0.02	1.62 ± 0.02
	Hom	1.53 ± 0.02 ^b	1.53 ± 0.02 ^b
Cerebellar dry weight, mg	Het	47 ± 1	48 ± 1
	Hom	42 ± 1 ^b	43 ± 1 ^b
Cerebellar DNA, mg	Het	1.80 ± 0.08	1.71 ± 0.06
	Hom	1.83 ± 0.06	1.68 ± 0.06

Data are means ± SEM.

^a On day 1 postnatally litter size was randomly adjusted to 8 pups.

^b Statistically significant difference between Het and Hom pups within litter.

weights of the male pups were greater than those of the females. However, the remainder of the body weight data and all brain weight data were not significantly different for male and female neonates. The data in table II are, therefore, averages of male and female figures.

Analysis of variance of the day 1 body and brain weight of the pups showed a statistically significant difference for the genotype of the mother (body and brain weight $F[1,188] = 11.2$ and 14.5 , respectively; both $p < 0.001$) and for the genotype of the pup (body and brain weight $F[1,188] = 8.5$ and

8.2 , respectively, with $p = 0.004$ and 0.005) but no difference for the treatment of the mother (body and brain weight $F[1,188] = 0.58$ and 0.17 , respectively, with $p = 0.448$ and 0.680). The analysis gave no significant two- or three-way interactions. Individual comparisons using $MS_{w,cell}$ (see table II) showed significantly lower body and brain weight of Hom pups of nontreated Hom mothers compared with Het litter mates (both $p < 0.001$), but no difference between Hom pups taken from LVP-treated Hom mothers and Het litter mates (both $p > 0.05$). Analysis of variance of the ratio be-

tween brain and body weight revealed no difference for the influence of the genotype of the mother, genotype of the pup or treatment of the mother ($F[1,188] = 0.399, 1.837$ and 0.246 , respectively, with $p = 0.528, 0.177$ and 0.620). Individual comparison of the brain/body ratio of the pups showed that in all groups brain weight was 3.9–4.0% of the total weight.

Cerebellar dry weight of the pups only showed an influence of the genotype of the mother (table II). Analysis of the protein and DNA contents of the cerebellum showed a difference for the genotype of the mother (protein and DNA $F[1,188] = 10.5$ and 1.48 , respectively, with $p = 0.002$ and 0.225). The protein and DNA content of the cerebellum were different for the LVP treatment of the mother ($F[1,188] = 47.9$ and 5.13 , respectively, with $p < 0.001$ and $= 0.025$), but had no difference for the genotype of the pup (protein and DNA $F[1,188] = 2.59$ and 0.40 , respectively, with $p = 0.110$ and 0.530). There was a two-way interaction between the genotype of the mother and the genotype of the pup ($F[1,188]$ with $p = 0.024$; table II).

Prenatal Influences on Pups at 1 Month of Age (table III)

In the breeding series without LVP treatment (table III), the 4 Het mothers had average litter size of 11.8 ± 1.0 pups (range 9–13), whereas the 6 Hom mothers had a smaller litter size of 8.3 ± 0.8 pups (range 6–11; $p < 0.05$). For the randomly adjusted 8-pup litters of the Hom mothers, the body weights of day 1 postnatally were significantly lower for the Hom pups than for the Het pups. As shown in table II, this was not true for the neonates within the litters of Het mothers (table III). Following the course of body development of these pups (reared by

Wistar foster mothers), it was found that body weights of Hom pups from Het mothers were already significantly lower by day 3 postnatally (8.2 ± 0.5 vs. 11.1 ± 0.4 ; $p < 0.001$). At 1 month of age body, brain and cerebellar weight of the Hom pups were significantly lower as compared with Het pups, though there was no difference in DNA content of the cerebellum (table III).

Discussion

Reproductive Aspects

In earlier reports on breeding with Hom Brattleboro rats a high incidence of semisterility, fetal death, stillbirth and runts was reported [20, 27]. In later studies the breeding results were reported to be impaired mainly by lower fertility rate of Hom females as compared with Het females [4, 10]. This was supposed to be due to the irregular cyclicity of the female [10]. In the present study, Hom females mated with Het males had a pregnancy quotient (the number of sperm-positive females that became pregnant) similar to that of Het females mated with Hom males. This is not only in contrast to the above studies but also to our own previous experiences with HOM $\times$ HOM breeding in which a 40–50% pregnancy quotient was routine [22]. Since upon mating with outbred normal (+/+) Brattleboro males, Hom females were found to have a higher pregnancy quotient [4], a male factor related to AVP was thought to have some influence on reproduction. It could be that the reproductive tract, reported to be under influence of these peptides in both sexes [28], is disturbed. It is also possible that selection by breeding of the Brattleboro rat for more than

25 years reduced the influence of the mutation on reproduction.

As has been reported previously [4, 10] and reconfirmed in the present study, the litter size of Het mothers was consistently larger than that of Hom mothers. Nestling of impregnated egg cells in Hom females might be less efficient. In agreement with earlier reports on maternal AVP [13, 22] and LVP treatments [22], the present LVP treatment throughout pregnancy failed to increase the litter size. The reduced litter size does not seem to be due to a poor prenatal survival of Hom fetuses in a mixed litter, since in the present study the number of Hom pups (42) was higher than that of the Het pups (30) for nontreated Hom mothers [see also 4].

Developmental Aspects

In the present study we have established that the prenatal body and brain growth of Hom fetuses was significantly improved when a Het instead of a Hom female was the mother. The ratio between brain and body weight of the day 1 pups of these two types did not differ (brain/body ratio 0.0395 vs. 0.0401; $p = 0.394$). This indicates that prenatal growth stimulation due to the Het genotype of the mother is not specific for brain but on overall growth stimulation. Whether the intrauterine development of the Hom fetus in Het mothers is improved by (natural) circulating AVP of the Het mother can as yet only partially be answered. It seems unlikely since, firstly, supplementation of AVP to pregnant AVP-deficient Hom mothers abolished their polyurea but failed to improve prenatal body growth of their Hom offspring [22]. Therefore, neither the absence [or low presence, 1, 16, 28, 29] of maternal AVP nor the polyurea of the Hom mothers seems to be a direct explanation for

the growth retardation observed in the offspring. Secondly, a direct effect of maternal AVP on the fetus is difficult to accept since efficient placental transfer of AVP has been repeatedly reported to be absent [18, 24]. Thus unknown indirect effects, possibly related to the mutation but not restorable by AVP supplementation, would be involved.

Although a role of maternal AVP in fetal growth seems unlikely, treatment of Hom mothers with LVP throughout pregnancy had growth-promoting effects on birth weight in homogeneous Hom litters [3, 22]. Such an effect was not found in the present study for the Hom pups in the heterogeneously genotyped litters. However, stimulatory effects of the exogenously applied LVP to the mother were observed in the protein and DNA content in the immature cerebellum. This, moreover, occurred upon treatment of pregnant (AVP-synthesizing) Het females (table II). Therefore, it remains to be elucidated whether LVP treatment brings about maternal changes that improve intrauterine conditions (see above) or affect fetal growth directly by passage into the intrauterine compartment. Pilot studies have shown that the amniotic fluid of Hom pups of nontreated Hom mothers contains an AVP-like compound, which might be related to the possible presence of AVP of a peripheral origin [1, 16, 19, 29]. This compound made it difficult to conclusively demonstrate the passage of LVP into the amniotic compartment of Hom fetuses of Hom mothers implanted with LVP/Accurel devices.

LVP, AVP and related polypeptides can also inhibit growth. A 20 μg LVP/Accurel implant inhibits postnatal brain development [22], whereas a similar AVP implant had no effect [6]. The present study suggests that maternal LVP treatment may have in-

hibiting effects on body and brain weights for Het pups (table II). Moreover, high dosages of AVP (200 µg AVP/Accurel implant) given to pregnant Wistar rats also induce stunted intrauterine fetal growth [18]. Finally, lasting brain growth disturbance has been found in kittens exposed neonatally to vasotocin [12]. It could, therefore, well be that LVP and AVP have different profiles of action with regard to their effect on development depending on developmental stage, dosage, etc.

Cerebellar DNA content of 1-day-old Hom pups was previously reported to be smaller than that of Het pups when both were born in genetically homogeneous litters from Hom mothers [5]. In the present study using heterogeneous litters from nontreated Hom mothers cerebellar DNA content was not significantly different between pup genotypes. This may indicate that the mere presence of Het fetuses can influence the prenatal development of Hom fetuses in Hom Brattleboro mothers.

The data obtained in the present study lead us to conclude that prenatal development of the Hom fetus is influenced by the genotype of the mother. However, when Het and Hom pups of Het mothers were reared under identical conditions, differences were revealed. Thus, the Het/Hom growth differences must be coupled to the mutation underlying the AVP deficiency of the Hom pup. Because postnatal and maternal treatments with AVP or LVP largely failed to bring about a growth stimulus, it might be concluded that AVP is the missing factor in the intrauterine compartment of the Hom fetus and that this peptide should have been given to the fetus itself. However, it is still possible that administration of AVP (or LVP) to the pregnant mother or the growing

pup does not result in adequate amounts of peptide being present at the right moment in the appropriate area of the brain. For Hom neonates, it may be more advantageous to supplement AVP by means of transplanting AVP neurons into the brain [3]. In addition, blocking endogenous AVP action by specific antagonists in normal rats might be more appropriate. Both possibilities are currently under investigation. We have also initiated studies to determine whether AVP (in oil) can be injected into the fetus on prenatal day 16. Such a treatment resulted, however, in high prenatal mortality or strong growth reduction of both treated and untreated fetuses. At present, therefore, implants of a miniaturized Accurel/collodion device [9] appear to be more successful in this respect [cf. 15]. We have been able to place such devices within the amniotic compartment from prenatal day 16 onwards. In this way continuous intrauterine treatment with AVP can be attained and growth parameters tested postnatally.

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F.G.M. Snijdwint
Netherlands Institute for Brain Research
Meibergdreef 33
NL-1105 AZ Amsterdam (The Netherlands)