Blunted HPA axis response in lactating, vasopressin-deficient Brattleboro rats

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Abstract

Adaptation to stress is a basic phenomenon in mammalian life that is mandatorily associated with the activity of the hypothalamic–pituitary–adrenal (HPA) axis. An increased resting activity of the HPA axis can be measured during pregnancy and lactation, suggesting that these reproductive states lead to chronic load in females. In this study, we examined the consequences of the congenital lack of vasopressin on the activity of the HPA axis during lactation using vasopressin-deficient Brattleboro rats. Virgin and lactating, homozygous vasopressin-deficient rats were compared with control, heterozygous rats. In control dams compared with virgins, physiological changes similar to those observed in a chronic stress state (thymus involution, adrenal gland hyperplasia, elevation of proopiomelanocortin mRNA levels in the adenohypophysis, and resting plasma corticosterone levels) were observed. In vasopressin-deficient dams, adrenal gland hyperplasia and resting corticosterone level elevations were not observed. Corticotropin-releasing hormone (Crh) mRNA levels in the hypothalamic paraventricular nucleus were elevated in only the control dams, while oxytocin (OT) mRNA levels were higher in vasopressin-deficient virgins and lactation induced a further increase in both the genotypes. Suckling-induced ACTH and corticosterone level elevations were not observed. Corticotropin-releasing hormone (Crh) mRNA levels in the hypothalamic paraventricular nucleus were elevated in only the control dams, while oxytocin (OT) mRNA levels were higher in vasopressin-deficient virgins and lactation induced a further increase in both the genotypes. Suckling-induced ACTH and corticosterone level elevations were blunted in vasopressin-deficient dams. Anaphylactoid reaction (i.v. egg white) and insulin-induced hypoglycemia stimulated the HPA axis, which were blunted in lactating rats compared with the virgins and in vasopressin-deficient rats compared with the controls without interaction of the two factors. Vasopressin seems to contribute to the physiological changes observed during lactation mimicking a chronic stress state, but its role in acute HPA axis regulation during lactation seems to be similar to that observed in virgins. If vasopressin is congenitally absent, OT, but not the CRH, compensates for the missing vasopressin; however, the functional restitution remains incomplete.

Key Words

- lactation
- vasopressin
- Brattleboro
- ACTH
- corticosterone
- Crh mRNA
- Pomc mRNA
- oxytocin mRNA
- suckling, egg white

Introduction

Adaptation to stress is a basic phenomenon in mammalian life that is mandatorily associated with the activity of the hypothalamic–pituitary–adrenal (HPA) axis. Extracellular signaling molecules that stimulate the HPA axis at the brain level are the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Both neuropeptides are synthesized in and released from the parvocellular neurons of the hypothalamic paraventricular nucleus...
(PVN; Aguilera 1994). After being released from the axon terminals of these neurons into the portal blood, AVP and CRH stimulate – in a dual potentiating manner – the proopiomelanocortin (POMC)-producing cells of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). Under chronic stress conditions, the activity of the HPA axis is maintained despite a constant inhibitory feedback by sustained elevations of glucocorticoid levels. Because the AVP-induced ACTH release is less sensitive to glucocorticoid feedback inhibition, it has been suggested that AVP is the principal secretagogue to maintain high ACTH levels during chronic stress (Dallman 1993, Aguilera 1994, Chowdrey et al. 1995). Indeed, previous studies using hypothalamic Avp-deficient male Brattleboro rats have suggested an increasing impact of AVP on the activation of the HPA axis after a 4-week-lasting stressor exposure (repeated restraint (Zelena et al. 2007) and ‘mild chronic stress’ (Varga et al. 2011)). This was detectable by the presence of significantly reduced ACTH and/or corticosterone levels in the Avp-deficient mutants when compared with the control animals. By contrast, the application of similar stressors for only 2 weeks (i.e. repeated restraint (Zelena et al. 2003), streptozotocin-induced diabetes mellitus (Zelena et al. 2006), and repeated morphine withdrawal (Domokos et al. 2008)) failed to reveal significant differences between the Brattleboro and control rats, indicating that AVP did not essentially contribute to the state of the HPA axis at this time point.

During lactation, female rats undergo numerous neuromorphological, neuroendocrine, metabolic, and behavioral changes, which favor beneficial interactions with the offspring. The modifications of the HPA axis include an increase in Cth and Avp mRNA levels in the PVN neurons (da Costa et al. 2001) and elevated plasma ACTH and corticosterone levels (Lightman 1992). As suckling has been shown to be a constant stimulus for the activity of the HPA axis in lactating rats (Walker et al. 1992), one would expect that the changes in the HPA axis signaling that occur during lactation are similar to those induced by chronic stress in male or virgin female rats (Toufexis et al. 1999). Interestingly, when exposed to defined stressors, lactating rats exhibit – compared with virgin females – reduced ACTH, corticosterone, prolactin, catecholamine, and oxytocin (OT) responses. In the PVN of lactating rats, Cth mRNA levels fail to increase in response to a defined stressor exposure, in contrast to both Avp mRNA levels (da Costa et al. 2001) and the number of neurons exhibiting the co-localization of AVP and CRH (Walker et al. 2001). During lactation, the ACTH response to CRH is blunted, whereas AVP triggers an increased ACTH release (Toufexis et al. 1999). This suggests a shift in the sensitivity of the pituitary corticotrophs from CRH to AVP in response to additional stressors. This hypothesis would fit the observation that maternal adaptations, such as decreased anxiety and attenuated stress responsiveness, are necessary to enable the successful postnatal development of the offspring (Hillerer et al. 2011), and a shift from a CRH- to an AVP-stimulating ACTH release could be one of the most important components of this adaptation.

An appropriate reactivity of the HPA axis is also indispensable for normal lactation (Cowie & Folley 1947). OT, the twin neuropeptide of AVP, governs milk ejection after its release from the axon terminals of hypothalamic magnocellular neurons into the blood stream (Bisset et al. 1970). During lactation, OT gene expression and OT immunoreactivity are increased in the magnocellular neurons of both the PVN and supraoptic nucleus (Wigger & Neumann 2002). Within the brain, OT acts as an extracellular signal suggested to play an important role in social attachment (Weisman et al. 2012).

The present study was designed to test the hypothesis that AVP is necessary for the physiological changes mimicking a chronic stress state and the maintenance of normal adrenocortical reactivity during lactation. To do so, we used females of the Avp-deficient Brattleboro rat strain. This strain has congenital diabetes insipidus due to a deletion of a nucleotide within the Avp gene (Schmale & Richter 1984). Consequently, the effect of AVP is absent in the whole body. Besides the basal comparison of virgin and lactating, control and Avp-deficient females, we used different acute stimuli as well. In case AVP signaling is instrumental for the maintenance of adrenocortical responsiveness during lactation, the Avp-deficient dams are expected to develop fewer symptoms of chronic stress and to exhibit markedly impaired adrenocortical responses to acute stimuli. Because of structural similarity, OT might compensate for the absence of AVP; therefore, we measured its levels as well.

Materials and methods

Animals

Brattleboro rats were maintained in our institute in a colony originating from commercially available breeder rats (Harlan, Indianapolis, IN, USA). The rats were kept in a controlled environment (23 ± 1 °C, 50–70% humidity, and 12 h light starting at 0700 h) with ad libitum access to standard rat chow (Charles River, Budapest, Hungary) and tap water. We compared Avp-deficient homozygous (di/di)
rats with diabetes insipidus with heterozygous (di/di) control rats from the same litters (Bohus & de Wied 1998). Although the di/+ rats have only one functional allele, their neurohypophysis contains large amounts of AVP and the rats do not show signs of diabetes insipidus. Young rats were tested for water consumption at the age of 6 weeks to define the diabetes insipidus phenotype and then maintained two per cage until the start of the experiments. Female rats from the same litter were randomly distributed into various treatment groups when they were 75–115 days old, mated and a few days before delivery individually housed. Different stimuli were applied during the postpartum week (i.e. when the pups were 7–11 days old; Olah et al. 2009). Rats of a separate group were decapitated under resting condition at the end of the lactation period (i.e. when the pups were 20–22 days old). Control virgin rats were also individually housed. All the experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary.

Blood sampling

Blood sampling was carried out by either using a chronically implanted venous (i.v.) catheter (in case of suckling or egg white injection) or collecting trunk blood after decapitation (in case of insulin injection). The samples were placed on ice and K$_2$-EDTA was used as the anticoagulant. After centrifugation, plasma was stored at −20 °C until the measurement of hormone levels.

The time course of the hormone levels was established by repeated blood sampling using an i.v. catheter implanted into the right jugular vein under anesthesia (i.p. injection of ketamine (50 mg/kg, SelBruHa Allatgyogyaszati Kft, Budapest, Hungary)–xylazine (20 mg/kg, Spofa, Praha, Czech Republic)–promethazine (0.2 ml/kg, EGIS, Budapest, Hungary) in physiological saline) (Zelena et al. 2005). After surgery, the rats were allowed to recover for 2 days. On the day of the experiment, the rats were connected to a long piece of polyethylene tubing. After collection of the first sample, the rats were exposed to defined stimuli (see below), and blood samples (0.4 ml/sample) were collected at defined time points without additional animal handling. Collected blood was instantly replaced by physiological saline to avoid volume loss. After the conclusion of the experiment, the rats were killed using a high dose of i.v. pentobarbital.

Stimuli

Suckling  On the day of the experiment, the dams were separated from their pups 4 h prior to their exposure to the suckling stimulus (Olah et al. 2009). The first blood sample was collected before separation, and 4 h later another blood sample was collected and the pups were returned to the dams. Full pup attachment was achieved within 5 min of reunion. Subsequent blood samples were collected at 15, 30, 60, and 90 min after reunion. The body mass of the litter was measured before and after suckling. The data reported here are only those collected from mothers whose litter’s body mass gain reached at least 1 g during the 90-min suckling period (15/19 and 13/17 in di/+ and di/di mothers respectively).

Egg white injection  Fresh, filtered egg white (500 ml/l solution in sterile saline) was slowly injected through the jugular catheter at a dose of 1 ml/kg (Foldes et al. 2000). Blood samples were collected immediately before (at 0 min) and 15, 30, 60, 90, and 120 min after injection.

Insulin injection  After 18 h of fasting, hypoglycemia was induced by i.p. injection of Actrapid (rapid insulin, 3NE/2 ml/kg; Novo Nordisk, Bagsvaerd, Denmark). One hour later, the rats were decapitated (Lolait et al. 2007), and glucose levels in the trunk blood were measured using a commercially available analytical device (D-Cont Personal, 77 Elektronika Kft, Budapest, Hungary).

In situ hybridization

The rats were decapitated under basal conditions at the end of the lactation period; the brain and hypophysis were rapidly removed from the skull, frozen on dry ice, and stored at −70 °C until measurement. Brain sections of 16 μm were cut in a cryostat and hybridized as described previously (Zelena et al. 2006). Briefly, the respective slices containing the PVN were selected with the help of a microscope and a rat brain atlas (Paxinos & Watson 1998). After successful hybridization, Crh (brain) and Pomc (ACTH precursor; hypophysis) mRNA levels were quantified using 35S-UTP-containing riboprobes complementary to the exonic sequences of the genes (the Crh probe was obtained from Dr D Richter, University of Hamburg, Germany, while the plasmid containing the Pomc template was a generous gift from Dr J Eberwine, University of Pennsylvania). After hybridization, the slides were exposed to imaging plates (Fujiﬁlm, BAS-IP, MS 2340)
for 72 h (Crh) or 16 h (Pomc), and the plates were scanned using a fluorescent image analyzer (FLA 3000, Fujifilm, scanning resolution 50 μm). Radiograms were evaluated using the ImageJ program (http://rsbweb.nih.gov/ij/). The average grayness density of three sections on both hemispheres (Crh) or six sections (Pomc) taken at 80 μm intervals was used for analysis (Zelenà et al. 2007, Fodor et al. 2012).

Quantitative real-time PCR

Brains were cut in the frontal plane into 200 μm slices. Corresponding brain slices containing the PVN were selected with the help of a rat brain atlas (Paxinos & Watson 1998). Working in the cryostat chamber at –10 °C, PVN tissue was punched out from the respective slices with a punching needle (0.8 mm diameter) from three consecutive slices, resulting in a total of six punches. Total RNA was isolated using the RNeasy Micro Kit (Qiagen) including the on-column DNaseI treatment. RNA was converted to cDNA using the RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas, St Leon Roth, Germany). One-fourth of the RNA sample containing column eluate (5 μl) was used per 20 μl reaction mixture and the reaction mixture was oligo(dT)18 primed. Real-time PCR was carried out using the MX3005P device (Stratagene, La Jolla, CA, USA). The reaction mixtures contained 1 × Brilliant SYBR Green QPCR Master Mix (Stratagene), 30 nM ROX reference dye, each primer at 200 nM, and 0.5 μl of cDNA at a volume of 25 μl. After an initial denaturation step at 95 °C for 10 min, amplification was carried out with 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s, and extension at 72 °C for 35 s. Amplification was followed by a melting curve analysis to confirm the specificity of the PCR products. No signals were detected in no-template controls. The experimental threshold (Ct) was calculated using the MxPro Mx3005P v3.00 Software (Stratagene). All samples were run in duplicate, and the mean value of each duplicate was used for all calculations. Primers for the reference gene Gapdh were selected using the RTPrimer Database (http://medgen.ugent.be/rtprimerdb/). ID3243 was selected and adapted to fit the rat sequence to 100%. OT primers were constructed using Primer 3 (http://primer3.wi.mit.edu/). Because of the high similarity between OT and Avp mRNA sequences, primers were positioned in the C-terminal, OT-specific sequence of rat OT sequence (accession no. M25649) in order to be OT specific (f, bp 380–397; r, bp 469–451).

Primer sequences for the following genes were as follows: GAPDH, product size 118 bp – f, 5′-CAAC-TCCCTCAAGATTGTCAAGAG-3′ and r, 5′-GGCATG-GACTTGCTGAT-3′; and OT, product size 90 bp – f, 5′-CCTGCGACCCAGAAGTG-3′ and r, 5′-GTAAGGCCCATGGGAATG-3′.

Measurement of hormone concentrations

The concentrations of ACTH and corticosterone were measured using RIA, as described previously (Zelenà et al. 2009b). The intra-assay coefficients of variation for ACTH and corticosterone were 4.7 and 12.3% respectively.

Statistical analysis

Data are reported as means ± S.E.M. Data were analyzed by ANOVA using the ANOVA/MANOVA module of the STATISTICA 11.0 Software Package (Tulsa, OK, USA). Completely randomized two-way ANOVAs (factors: ‘genotype’ and ‘reproductive state’) were used to analyze the data on body mass at weaning and the adrenal and thymus mass as well as those on Crh, Pomc, and OT mRNA measurements and corticosterone levels in the trunk blood. In the pup separation experiment, data were analyzed using a two-way ANOVA (factors: ‘genotype’ and ‘time’) with repeated measures on the second factor. In the ‘egg white’ experiment, data were analyzed using a three-way ANOVA (factors: ‘genotype’, ‘reproductive state’, and ‘time’) with repeated measures on the factor ‘time’. The same analysis was used for the analysis of body mass and water intake within 5 weeks of mating. In the Actrapid treatment experiment, data were analyzed using a completely randomized three-way ANOVA (factors: ‘genotype’, ‘reproductive state’, and ‘treatment’). If allowed, ANOVAs were followed by multiple pairwise comparisons using the Newman–Keuls method. The level of significance was set at P ≤ 0.05.

Results

Development of chronic stress state

Somatic parameters  Body mass changed in parallel in di/+ and di/di rats; however, the heterozygous rats exhibited higher levels of change (‘genotype’: F1,48S = 43.43, P<0.01 and ‘genotype’×‘time’: F1,4,3440 = 6.04, P<0.01; Fig. 1A). Mated females continuously gained body mass during pregnancy, which also remained elevated during the lactation period (‘reproductive state’):
AVPergic HPA axis regulation during lactation

**Research**

A. Food and others

**AVPersic HPA axis regulation during lactation**

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Figure 1

Physiological parameters in female Brattleboro virgins and dams compared with those in heterozygous control rats. (A) Body mass changes during pregnancy and lactation (n=18–32, left panel) and at weaning (n=9–10, right panel) (g). (B) Water intake during pregnancy and lactation (n=7–13). (C) Mass of both adrenal glands (n=9–10). (D) Mass of the thymus (n=9–10). *P<0.05, **P<0.01 vs virgin; *P<0.05, **P<0.01 vs heterozygous control rat.

HPO axis  *Crh* mRNA levels in the PVN were significantly lower in di/di rats than in the di/+ ones (‘genotype’: F(1,24)=16.5, P<0.01; Fig. 2A). Lactation was able to significantly increase *Crh* mRNA levels (‘reproductive state’: F(1,24)=9.44, P<0.01), but a closer look revealed that this increase was detectable in only the di/+ mothers (‘genotype’×‘reproductive state’: F(1,24)=7.52, P=0.01).

The expression of *OT* mRNA in the PVN was significantly increased in mothers than in the virgins (Fig. 2B). Furthermore, the expression was significantly enhanced in di/di rats without showing a significant interaction with the factor ‘reproductive state’ (‘genotype’: F(1,21)=9.53, P=0.01 and ‘reproductive state’: F(1,21)=15.56, P<0.01).

Both the factors ‘genotype’ and ‘reproductive state’ significantly affected the expression of *Pomc* mRNA in the anterior lobe of the pituitary without showing a significant factor interaction (‘genotype’: F(1,24)=4.39, P<0.05 and ‘reproductive state’: F(1,24)=8.83, P<0.05; Fig. 2C).

We failed to detect a significant difference in plasma ACTH levels at the time of weaning either between di/+ and di/di dams or between virgin (V) and lactating (L)

$F$ (1,85) = 28.19, P<0.01; ‘reproductive state’×‘time’: $F$ (4,340) = 90.01, P<0.01. Body mass gain in di/di mothers was lower than that in di/+ ones (‘genotype’×‘reproductive state’: $F$ (1,85) = 8.21, P<0.01). At weaning (20–22 days of lactation), lactating mothers had higher body mass than the control, virgin females; furthermore, di/di mothers had significantly lower body mass than the di/+ ones (‘genotype’: $F$ (1,36) = 12.78, P<0.01 and ‘reproductive state’: $F$ (1,36) = 4.55, P<0.05).

As expected, di/di rats drank more water than di/+ virgins; lactation was able to induce a further rise in water intake not only in heterozygous mothers, but also in Avp-deficient mothers (‘genotype’: $F$ (1,22) = 304.19, P<0.01; ‘reproductive state’: $F$ (1,22) = 22.05, P<0.01; and ‘genotype’×‘reproductive state’: $F$ (1,22) = 5.65, P<0.05; Fig. 1B).

The Avp-deficient rats had smaller adrenals (‘genotype’: $F$ (1,36) = 23.74, P<0.01; Fig. 1C). Maternity resulted in a significant increase in adrenal mass in di/+ rats, but not in di/di rats (‘genotype’×‘reproductive state’: $F$ (1,36) = 5.31, P<0.05). There was no significant difference in thymus mass between the control and lactating females or di/+ or di/di rats (Fig. 1D).
High corticosterone levels were detected in lactating di/C rats compared with the virgins, but this rise was absent in di/di dams (‘genotype’ × ‘reproductive state’: \( F_{(1,58)} = 5.86, P = 0.01 \); Fig. 2D).

**Response to defined stimuli** **Suckling**

There was no difference in the body mass gain of the litters during the 90-min suckling period between di/+ and di/di mothers (di/+, 2.70 ± 0.26 g/eight pups and di/di, 2.98 ± 0.39 g/eight pups).

The 4-h-lasting separation of the pups from the mothers was unable to influence the plasma ACTH and corticosterone levels of the mothers (Fig. 3). The reunion of the pups with their mothers significantly increased HPA axis hormone levels (ACTH ‘time’: \( F_{(5,125)} = 12.88, P < 0.01 \) and corticosterone ‘time’: \( F_{(5,120)} = 11.65, P < 0.01 \)). This increase was smaller in the Avp-deficient mothers for ACTH (‘genotype’ × ‘time’: \( F_{(5,125)} = 3.02, P = 0.01 \)), whereas the statistical analysis revealed no effect of the factor ‘genotype’ on corticosterone levels (\( F_{(1,24)} = 2.24, P = 0.14 \)). A detailed post hoc analysis revealed that ACTH level elevation was lower in the samples from di/di rats collected at 15 min. Moreover, compared with the samples collected at 0 min, only samples collected at 90 min from di/di rats had significantly increased levels of both hormones.
both di/di mothers (no significant interactions). In all the groups, i.v. injection of egg white resulted in a rapid induction of ACTH secretion (‘time’: $F_{(4,200)} = 48.23$, $P < 0.01$; Fig. 4A). The peak was reached during the 15-min sample collection period, and then ACTH concentrations decreased within 30–60 min of injection; values obtained for the samples collected at 120 min reached those of samples collected at 0 min for all the four groups. The Avp-deficient rats had lower responses compared with di/+ rats (‘genotype’: $F_{(1,50)} = 3.95$, $P = 0.05$). The anaphylactoid stimulus induced a smaller increase in ACTH levels in lactating dams compared with the virgins (‘reproductive state’: $F_{(1,50)} = 13.36$, $P < 0.01$) in both di/+ and di/di mothers (no significant interactions).

The effect of egg white injection on plasma corticosterone levels was similar to that observed for ACTH levels, with a less precipitous time curve being obtained in the first 15–30 min (‘time’: $F_{(4,192)} = 25.95$, $P < 0.01$; Fig. 4B). There was a – nonsignificant – tendency for a smaller increase in corticosterone levels in di/di rats (‘genotype’: $F_{(1,48)} = 3.28$, $P = 0.07$). We could detect a smaller increase in corticosterone levels in dams compared with the virgins without any influence of the genotype (‘reproductive state’: $F_{(1,48)} = 5.88$, $P < 0.05$; no significant interactions).

**Insulin injection** In the fasting rats, i.p. injection of Actrapid significantly reduced the blood glucose levels (‘treatment’: $F_{(1,55)} = 105.13$, $P < 0.01$; Table 1). There was no significant difference in Actrapid-induced blood glucose reduction between the two genotypes and between the virgin and lactating mothers.

As shown in Fig. 4C, hypoglycemia induced an elevation of ACTH levels compared with the levels in the saline-treated rats (‘treatment’: $F_{(1,55)} = 131.96$, $P < 0.01$). This increase was significantly smaller in di/di females (‘genotype’: $F_{(1,55)} = 7.15$, $P < 0.01$ and ‘treatment’×‘genotype’: $F_{(1,55)} = 12.34$, $P < 0.01$). Although lactation per se had no effect on ACTH levels, it reduced the hypoglycemia-induced elevation (‘treatment’×‘reproductive state’: $F_{(1,55)} = 12.43$, $P < 0.01$). There was no interaction between the factors ‘genotype’ and ‘reproductive state’.

The administration of Actrapid increased corticosterone levels significantly compared with the levels in the control, saline-treated groups (‘treatment’: $F_{(1,55)} = 172.74$, $P < 0.01$; Fig. 4D). The factor ‘genotype’ had no effect on corticosterone levels in this experiment, in contrast to the factor ‘reproductive state’, which reduced the stress-induced elevations (‘reproductive state’: $F_{(1,55)} = 3.58$, $P = 0.06$ and ‘treatment’×‘reproductive state’: $F_{(1,55)} = 16.62$, $P < 0.01$). There was no interaction between the factors ‘genotype’ and ‘reproductive state’.

**Discussion**

Data obtained in this study reveal that in the control dams lactation induces chronic stressor exposure-like HPA axis changes when compared with the virgins (Walker et al. 1992). Significant changes in body mass may also be a sign of a chronic stress state (Tamashiro et al. 2011). Moreover, elevated adrenal gland mass, increased mRNA levels of *Crh* in the PVN and *Pomc* in the adrenohypophysis, and resting plasma corticosterone levels were observed in heterozygous Brattleboro mothers. Interestingly, di/di mothers failed to exhibit an increase in body mass, adrenal gland hyperplasia, or increased *Crh* mRNA and resting corticosterone levels. The levels of *OT* mRNA were increased in the PVN of di/di rats, possibly to compensate for the congenital absence of Avp. In addition, we found that suckling significantly elevated stress hormone levels and di/di mothers exhibited a blunted response to this stimulus. The induction of anaphylactoid or hypoglycemic responses by the administration of egg white and insulin respectively was paralleled by elevated ACTH and corticosterone levels, but these effects were smaller in mothers vs virgins and blunted in di/di vs di/+ rats. These
results suggest that AVP may play a significant role in the maintenance of the resting HPA axis hyperactivity in dams, but its role in HPA axis reactivity might not be more pronounced in dams when compared with that in virgins.

Lactation is associated with hypothalamic reorganization and alterations in basal HPA axis functions (Walker et al. 1992). In this context, during pregnancy CRH is secreted by the placenta (Makrigiannakis et al. 1995), and after delivery, it is rapidly eliminated from the peripheral circulation paralleled by a decline in glucocorticoid secretion. As a consequence, the hypothalamic feedback on CRH secretion is removed, which leads to an enhanced activation of brain CRH peptide synthesis (O’Keane et al. 2011). Our finding of an enhanced expression of Crh mRNA in the PVN is similar to the findings of a study carried out in 3-day-old lactating rats of the Sprague Dawley strain (da Costa et al. 2001) and are in accordance with higher Crh mRNA levels found in the PVN of chronically stressed animals (Pournajafi-Nazarloo et al. 2009). However, other authors have reported a reduction in the hypothalamic Crh mRNA levels under chronic stress.

Table 1 Blood glucose levels (mmol/l) 1 h after Actrapid (3NE/2 ml/kg i.p.) injection

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<td>Virgin</td>
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<td>Lactating</td>
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<tr>
<td>Control</td>
<td>5.10 ± 0.28</td>
<td>4.90 ± 0.18</td>
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<td>Stressed</td>
<td>2.98 ± 0.33</td>
<td>2.19 ± 0.12</td>
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Figure 4
Effect of i.v. egg white (A and B; n = 12–15) and i.p. Actrapid (C and D; n = 5–8; C, control and S, stressed) injections on plasma ACTH (A and C) and corticosterone (B and D) levels in virgin (V) and lactating (L) Brattleboro rats. *P < 0.05, **P < 0.01 vs virgin; *P < 0.05, **P < 0.01 vs di/+; *P < 0.05, **P < 0.01 vs 0 min, or control, unstressed rat.
conditions (Fischer et al. 1995, Brunton et al. 2008). As the CRH levels were found to be low during pregnancy (Neumann et al. 1998) and HPA axis reactivity was restored within 2 days of pup removal (Walker et al. 1992, Toufexis et al. 1999), it can be concluded that the stage of the lactation period obviously influences the outcome of this type of analysis. Nevertheless, we detected elevated Crh mRNA levels in the PVN during weaning and at 20–22 days of lactation. These increased Crh mRNA levels could also be part of a compensatory mechanism involving reduced pituitary CRH sensitivity, which in turn is responsible for the stress hyporesponsivity of lactating rats (Neumann et al. 1998, Toufexis et al. 1999). We observed no further diminution of ACTH and corticosterone reactivity in Avp-deficient lactating rats compared with the control dams. This suggests that neither AVP nor an enhanced AVP sensitivity is able to compensate for the reduced stimulatory effect of CRH in lactating dams in response to the defined stimuli.

OT circulating in the blood stream has been suggested to be able to trigger ACTH secretion from the adenohypophysis after interaction with V1b receptors (Schlosser et al. 1994). Thus, OT may have compensated for – at least partially – the absence of AVP in our Brattleboro rats (Zelena et al. 2009a). Indeed, we found an enhanced expression of OT mRNA in the PVN in both the lactating and Avp-deficient rats (Fig. 2B). The changes observed between the genotypes and the reproductive state for OT mRNA levels in the PVN could be paralleled by the Pomc mRNA levels in the anterior lobe of the pituitary (Fig. 2; correlation of means: $F_{1,2} = 22.9, P < 0.05; \beta = 0.96$), which implies that OT participates in the stimulation of POMC synthesis in the corticotrophs. Together, these findings suggest a regulatory role of OT originating in the PVN for the activity of the HPA axis. Indeed, OT, in concert with endogenous opioids and prolactin, was thought to contribute to diminished HPA axis hormonal responses during the peripartum period (Slattery & Neumann 2008).

Our data show a significant elevation of Pomc mRNA levels at the end of the lactation period in the pituitaries of rats of both genotypes (Fig. 2). Fischer et al. (1995) reported that Pomc mRNA levels in the adenohypophysis of Wistar rats are elevated only during the first day of lactation and then return to levels observed in virgin rats. At present, it is difficult to explain the difference between the two observations. A possible explanation may allow the use of different rat strains; this, however, needs to be confirmed in additional experiments. Independently from this, our observation corresponds with data obtained from chronically stressed male rats (Zelena et al. 2006) exhibiting higher Pomc mRNA levels in the anterior lobe of the hypophysis. It is worth noting that although ACTH is derived from POMC, we failed to detect significant differences in resting plasma ACTH levels. Obviously, the increased synthesis of the precursor mRNA is not necessarily paralleled by a similarly increased release of ACTH, as has been suggested earlier (Fischer et al. 1995). Further studies have to determine whether an enhanced synthesis of Pomc mRNA may provide an alternative marker for a chronic stress state (Makara et al. 2012). The discrepancy between hypophyseal Pomc mRNA and plasma glucocorticoid concentrations indicates that additional regulatory mechanisms control the adrenal steroid hormone secretion (Bornstein et al. 2008).

We found that lactation significantly increased resting blood corticosterone levels in only di/+ mothers (Fig. 2D; Fischer et al. 1995). The absence of this increase in Avp-deficient mothers suggests a relevant role for hypothalamic AVP in the maintenance of hypercorticism during lactation. Indeed, at least during the early stages of the lactation period (to day 10), increased Avp mRNA concentrations in the PVN of Wistar rats were measured (Fischer et al. 1995).

Suckling stimulates the adrenocortical system and probably accounts for the tonically elevated corticosterone concentrations (Walker 1995). When – after separation – we returned the pups to their mothers, ACTH and corticosterone levels increased significantly in mothers of both the genotypes. Interestingly, in Avp-deficient mothers, the initial peak in ACTH levels (see Fig. 3A, sample collected at 15 min) was missing, and instead a constant increase over the time was monitored. This suggests that not only the suckling-induced prolactin release (Nagy et al. 1989), but also ACTH release is controlled by, among others, AVP.

During lactation, the exposure of mothers to different acute stressful stimuli results in a blunted response of the HPA axis when compared with that in virgin rats (Walker et al. 1992, Windle et al. 1997, Brunton et al. 2008). In lactating women, ACTH and cortisol responses to treadmill exercise have been found to be lower within the first 2 h of suckling but not subsequently (Altemus et al. 1995). This suggests that suckling results in a short-term suppression of HPA axis responses to stressful stimuli (Heinrichs et al. 2001). We chose two different stimuli known to trigger the activity of the HPA axis: egg white and insulin injections (Zelena et al. 2009a). The insulin-induced hypoglycemia test is widely regarded as a ‘gold standard’ for testing the ability of the HPA axis to respond
appropriately to stimuli in patients who either suffer from diseases known to involve a dysregulation of the HPA axis or who have received a long-term glucocorticoid therapy (Erturk et al. 1998). AVP – as an ACTH secretagogue – controls HPA axis responses to inflammatory stimuli (Chowdrey et al. 1995) and is involved in the regulation of glucose homeostasis (Alexander et al. 1997, Nakamura et al. 2011). Injections of both egg white and insulin significantly triggered the activity of the HPA axis with lower elevations in dams than in virgins, which is in line with a blunted FOS expression/Fos synthesis in the PVN in response to lipopolysaccharide (Shanks et al. 1999) and egg white (Monasterio et al. 2008) injections. The administration of egg white and insulin in the present study resulted in a blunted ACTH response, but not in a blunted corticosterone response, in Avp-deficient Brattleboro rats compared with the controls. Previously, we found a similar dissociation of ACTH and corticosterone secretion in male rats (Zelena et al. 2009a), suggesting alternative pathways for the regulation of adrenal glucocorticoids (Bornstein et al. 2008). Although the end hormone of the HPA axis is corticosterone, we think that ACTH also has a strong influence on stressor-induced homeostatic changes (Makara et al. 2012). Nevertheless, in the present study, the lack of an interaction between the factor ‘reproductive stage’ and the factor ‘genotype’ on ACTH and corticosterone levels suggests that the regulatory role of AVP in the activity of the HPA axis did not become more pronounced in dams when compared with that in the virgins. These observations confirm previous findings in males that the increased vasopressinergic activity characteristic to chronic stress played roles other than mediating the hypersensitivity of the HPA axis to a novel stressor (Chen et al. 2008, Spiga et al. 2009, Makara et al. 2012).

AVP plays an important role in the regulation of body water retention; therefore, di/di rats suffering from diabetes insipidus consume more water than the control di/+ rats (Zelena et al. 2006). In our experiments, di/di rats consumed six- to sevenfold more water than the di/+ ones; furthermore, the increase in consumption was more prominent in pregnant di/di rats, where it reached a maximum in the third week of pregnancy with a value of 348 ml/day per rat (Hyde et al. 1989). The elevation (~65–70%) was similar in di/+ and di/di mothers compared with respective virgins. The fact that pregnancy and lactation are able to induce an increase in water intake even in di/di animals suggests that circulating AVP is not essential for the physiological adaptation of water balance during pregnancy and lactation.

Taken together, the results of the present study suggest that endogenous AVP supports the changes in resting HPA axis activity during lactation that mimic – partially – those observed under chronic stress conditions (Makara et al. 2012). During lactation, the primary function of AVP in acute HPA axis regulation may be similar to that in males and virgins, namely restricted to ACTH secretion.

However, in dams, AVP of PVN origin might play a different role in the control of the activity of the HPA axis when compared with that played in male animals (i.e. maintaining basal HPA axis hyperactivity vs promoting corticosterone level normalization after stress exposure in male rats (Zelena et al. 2009b)) or virgins. Not the elevation of CRH levels, but that of OT levels is aimed to compensate for the missing Avp in this mutant strain; however, the functional restitution remains incomplete.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
A F analyzed the data and wrote the paper; O P and A D planned and conducted the experiments and analyzed the data; K L carried out the RT-PCR; I B conducted the in situ hybridization experiments; M E planned the experiments and wrote the paper; and D Z planned and conducted the experiments, analyzed the data, and wrote the paper.

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