The stimuli-specific role of vasopressin in the hypothalamus–pituitary–adrenal axis response to stress

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Abstract

Adaptation to a constantly changing environment is fundamental to every living organism. The hypothalamic–pituitary–adrenocortical (HPA) axis is a key component of the adaptation process. The present study tests the hypothesis that vasopressin (AVP) is required for the HPA response to acute stimuli. To accomplish this, naturally AVP-deficient Brattleboro rats were exposed to a wide range of stimuli and their HPA response was compared with heterozygous littermates. The circadian rhythmicity of plasma ACTH and corticosterone was not different between the two genotypes. The ACTH and corticosterone response to volume load, restraint or aggressive attack were decreased in AVP-deficient rats. The stress-induced increase in ACTH, but not corticosterone, was significantly impaired in AVP-deficient animals after novelty, elevated plus-maze, forced swim, hypoglycaemia, ulcerogenic cold immobilisation,

lipopolysaccharide, hypertonic saline and egg white injection. The HPA response to social avoidance, ether inhalation and footshock was not different between the genotypes. *In vitro*, the hypophysis of AVP-deficient animals showed a reduction in stimulated ACTH production and their adrenal glands were hyporeactive to ACTH. A dissociation between the ACTH and corticosterone response was observed in several experiments and could not be explained by an earlier ACTH peak or enhanced adrenal sensitivity, suggesting the existence of paraadenohypophyseal neuroendocrine regulators. Loss of AVP affected the HPA response to a wide variety of stressors. Interestingly, the contribution of AVP to the HPA response was not specific for, nor limited to, a known stressor category. Thus, there is a context-specific requirement for AVP in stress-induced activation of the HPA axis.

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Introduction

Maintaining homeostasis in a constantly changing environment is a fundamental process of life. Hans Selye, the father of the stress concept, defined the hypothalamic–pituitary–adrenocortical (HPA) axis as the major component required for adaptation (Selye 1937). At the time of his discovery, the central regulating molecules of the HPA axis were not established. Following its identification in 1954, arginine vasopressin (AVP) was considered as the principal regulator of ACTH release. The subsequent elucidation of the structure of corticotrophin-releasing hormone (CRH) and the domination of the ‘one neuron – one transmitter’ principle led to the replacement of AVP with CRH as the principle regulator of the HPA axis (Scott & Dinan 2002). Currently, it is thought that the effects of AVP in HPA regulation are restricted to potentiating the stimulatory effects of CRH at both the pituitary and hypothalamic levels (Buckingham 1981, Ono *et al.* 1985, Lightman & Young 1988).

The role of AVP in HPA axis regulation is thought to become more important during chronic stress (Dallman 1993, Aguilera 1994, Ma & Lightman 1998, Lightman *et al.* 2002). However, our data has shown that the lack of AVP does not affect the development of chronic hyperactivity of the HPA axis (Zelena *et al.* 2004, 2006a, Domokos *et al.* 2008). Furthermore, we found that AVP plays a prominent role in acute hormone changes, for e.g. morphine treatment and withdrawal-induced changes, suggesting that the critical role of AVP in HPA axis regulation may be during exposure to acute stimuli.

There is a wide range of studies on the role of AVP in HPA axis regulation during acute stimuli and during resting conditions. Immunoneutralisation studies revealed a role of AVP in restraint-, formalin- (Tilders *et al.* 1985) and ether stress- (Ono *et al.* 1985) induced ACTH elevations; although no role for AVP was found in basal ACTH regulation. Knocking out the V1b receptor, the main target of the vasopressinergic HPA axis regulation at the pituitary level,

resulted either in reduced (Tanoue *et al.* 2004) or normal (Lolait *et al.* 2007a) resting ACTH and corticosterone levels. Restraint- (30 min) or aggressive contact-induced release of ACTH and corticosterone was also similar in wild type and V1bR knockout (KO) mice, but the hypoglycaemia-, lipopolysaccharide (LPS)-, forced swim- and ethanol-intoxication-induced elevations were significantly diminished (Tanoue *et al.* 2004, Lolait *et al.* 2007a,b, Stewart *et al.* 2008). A selective, non-peptide V1b receptor antagonist (SSR149415) was able to diminish the restraint-induced ACTH increased by 50% (Serradeil-Le Gal *et al.* 2002) and inhibited the response to ether exposure but failed to inhibit the HPA axis response to forced swimming (Ramos *et al.* 2006). Another V1b receptor antagonist (Org) diminished the restraint- and LPS-induced ACTH release without affecting the resting levels and stress-induced corticosterone responses (Spiga *et al.* 2009).

Brattleboro rats, an AVP-deficient animal model, have been used to test the contribution of AVP to the HPA axis regulation (Valtin & Schroeder 1964, Lolait *et al.* 1986). In general, resting pituitary ACTH concentration of homozygous Brattleboro rats appears to be normal, although there are reports of impaired basal ACTH and corticosterone plasma levels (Brudieux *et al.* 1986, Eckland *et al.* 1988, Burgess & Balment 1992). In regards to the acute HPA response to stress, previous studies have shown normal stress responses (Zelena *et al.* 2003b, 2004, Mlynarik *et al.* 2007, Domokos *et al.* 2008) while other studies have shown decreased responses in the Brattleboro rat (Kjaer *et al.* 1993, Honda *et al.* 1994).

It is difficult to compare and draw overall conclusions from all these studies as the experimental and breeding conditions varied and there was not even a uniform choice for the proper control to use in studies with Brattleboro rats (Bohus & de Wied 1998, Zelena *et al.* 2003b). Thus, it is not clear whether the intensity and/or the nature of the stress or the experimental approaches/conditions are responsible for the differences observed. Therefore, to test the hypothesis that AVP augments the ACTH response to stress in a context-specific manner; this study was designed to test the Brattleboro rats in a variety of stress conditions with results being compared with the appropriate control (i.e. heterozygous littermates). We used a wide range of stimuli to find out if the intensity and/or nature of the stressors influence the participation of AVP in HPA axis activation. In addition, the responsiveness of the pituitary and adrenals to CRH and ACTH respectively, were tested *in vitro*.

Materials and Methods

Animals

Adult, male Brattleboro rats (~330 g, 10–12 weeks old) were maintained in our institute in a colony started from breeder rats from Harlan, Indianapolis, IN, USA. Rats were kept in controlled environment (23 ± 1 °C, 50–70% humidity, 12 h

light starting at 0700 h) and given commercial rat chow (Charles River, Budapest, Hungary) and tap water *ad libitum*. We standardised the colony using pair-housed di/+ female and di/di male breeders rats (Zelena *et al.* 2003b). We compared the AVP deficient homozygous (di/di) rats with diabetes insipidus to heterozygous (di/+) control rats from the same litters. Although the di/+ rats have only one functional allele their neurohypophysis contains large amounts of AVP and they do not show any signs of diabetes insipidus. Rats were tested for water consumption at the age of 6 weeks to define the diabetes insipidus phenotype and then kept two per cage until the experiment. Age-matched animals were used. To avoid the disturbance of presently untreated animals all experiments were performed on single-housed animals (max. 3–4 day isolation). Separate sets of animals were used for each stress study and all experiments were done between 0800 and 1200. The experiments were performed in accordance with regulations set by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by our Institutional Animal Care and Use Committee.

Special controls In some cases, an additional control group was also involved. To ensure that the heterozygous animals behave similarly to a wild type, we used Wistar controls (Charles River) in one experiment (ulcerogenic stimulus). Moreover, by cross-mating we have derived a +/+ line from our original colony being in close relationship with each other (heterozygous breeding mothers are the daughters of the +/+ mothers) and in one experiment (hypoglycaemia) we also used a +/+ group as a control. To exclude the influence of peripheral AVP deficiency in one experiment (forced swim), an Alzet osmotic minipump (1 ml/h, 14 days) was implanted under the skin filled with 200 µl of 1 mg/ml desmopressine (DDAVP, V2 receptor agonist; Ferring Lieciva) under ether anaesthesia (Zelena *et al.* 2006b).

In situ hybridisation

The animals were decapitated under basal conditions and the brain and hypophysis were rapidly removed, frozen on dry ice and stored at –70 °C until measurement. Brain sections of 16 µm were cut on a cryostat and hybridised as previously described (Zelena *et al.* 2006a). Briefly, *Crh* mRNA and *Pomc* (ACTH precursor) mRNA levels were quantified by means of ³⁵S-UTP containing riboprobes complementary to the exonic sequences of the gene (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 hybridisation, slides were exposed to imaging plates (Fujifilm, BAS-IP, MS 2340) for 72 (CRH) or 16 h (POMC) and the plates were scanned by a fluorescent image analyser (FLA 3000, Fujifilm, scanning resolution of 50 µm). Radiograms were evaluated by the ImageJ program (<http://rsbweb.nih.gov/ij/>). The integrated densities for the nucleus paraventricularis hypothalami (PVN) (*Crh* mRNA;

Barna *et al.* 2003, Zelena *et al.* 2003a) or the average densities for the hypophysis (*Pomc* mRNA; Zelena *et al.* 2007) were calculated. The data were summarised from three hybridisation series expressing the levels of AVP-deficient animals as a percentage of their heterozygous littermate controls.

Blood sampling

Depending on the experimental procedure, three different blood sampling techniques, as described below, were used in this study. Blood samples were collected on ice and K₂-EDTA was used as anticoagulant. After centrifugation, plasma was stored at -20 °C until hormone measurement.

- I. In most cases, the time course of the hormones was established by repeated blood sampling through i.v. catheter implanted into the right jugular vein under anaesthesia i.p. injection of ketamine (50 mg/kg, SelBruHa Allatgyogyaszati Kft, Budapest, Hungary) – xylazine (20 mg/kg, Spofa, Prague, Czech Republic) – promethazinium chloratum (0.2 ml/kg, EGIS, Budapest, Hungary) in physiological saline (Zelena *et al.* 2005). After surgery, the animals were housed singly and allowed to recover for 2 days. On the day of the experiment, the animals were connected to a long polyethylene cannula and after taking the first sample the rats were stressed and blood samples (0.4 ml/sample) were taken at respective time points without additional animal handling. Collected blood was replaced by physiological saline to avoid the additional stimulus of volume loss. The animals were killed by high-dose pentobarbital administered i.v. after the conclusion of the experiment.
- II. When mild stressors were used, blood was collected at a single time point at the expected peak of ACTH. In behavioural tests (elevated plus-maze (EPM), social avoidance), tail sampling was used at the end of the test. Animals were placed in a restraint tube and their tail was cut with a surgical knife and blood collected into Eppendorf tubes within 0.5 min of touching their cage (Mikics *et al.* 2007). The animals were killed by a high dose i.p. pentobarbital injection.
- III. In other tests (for e.g. novelty, lipopolysaccharide injection), rapid decapitation was performed within 0.5 min of removing animal from cage and trunk blood was collected. For the ether stimuli, we were able to choose a single time point based upon previous observations from our laboratory. The time point for blood collection during hypoglycaemia was chosen based upon the work of Lolait *et al.* (2007a) who suggested that there is a role of AVP in hypoglycaemia-induced HPA axis activation.

Daily rhythm A separate group of animals was placed in a room with reversed light cycle (lights off at 1000 h) and habituated for at least 2 weeks. Two days after i.v. cannula

implantation serial blood samples were taken every 2 h six times a day from both normal (for blood sampling 0–12 h) and reversed light cycle (for blood sampling 12–24 h) animals. The procedure was repeated on the next day with 12 h rest between. The data from the two series were fitted in time (the beginning of the light phase, referred to as Zeitgeber time 0) and were presented as a single line.

Type of stressors (same order as in results)

Novelty Rats were placed into an open plastic cage (40 × 36 × 20 cm) without bedding material for 10 min. This cage is bigger than the home-cage (30 × 36 × 20 cm) and was previously defecated by another rat to induce more severe anxiety. Non-handled controls and stressed animals were decapitated at the end of 10 min novelty exposure.

Social avoidance Anxiety was provoked by a two-chamber method developed in our institute (Haller *et al.* 2003). The apparatus consisted of two connected chambers, one of which contained an unfamiliar Wistar male confined in a subchamber with a perforated Plexiglas wall. The subjects were placed in the empty chamber and, after 3 min of habituation, were allowed to explore the apparatus for 5 min. Upon completion, blood was collected by tail cuts.

Elevated plus-maze Animals were placed to an EPM (arm length, 50 cm; width, 20 cm; platform height, 70 cm; 440 lux), a common procedure used for anxiety measurements (Zelena *et al.* 1999a). Test duration was 5 min and blood was taken at the end via tail cuts (Mikics *et al.* 2007).

LPS challenge Based on previous experiments (Foldes *et al.* 2000) and our preliminary data *Escherichia coli* LPS, serotype 0111:B4 (Sigma Chemical Co.), was used at a dose (300 µg/kg) that submaximally activates the HPA axis. LPS was dissolved in sterile, pyrogen-free saline and injected i.p. and rats were decapitated 1 h after injection.

Ether inhalation The animals were put in a glass jar filled with ether vapour for 1 min, and then they were kept anaesthetised for another 2 min with an ether-soaked nose cone and returned to their home cage. Animals were rapidly decapitated 10 min after the beginning of the stress (see e.g. (Zelena *et al.* 1999b)).

Hypertonic saline-volume load 1.5 mol/l NaCl (1.5 ml/100 g) was injected i.p. and blood samples collected before (0 min) and at 5, 15, 30, 60 and 120 min after injection (Ma & Aguilera 1999). This stress is a combination of osmotic and volume stimulus and pain. Control rats received an equivolume injection of 0.15 mol/l NaCl (physiological saline; ~4.5 ml/rat).

Anaphylactoid reaction Fresh, filtered egg white (500 ml/l solution in sterile saline) was slowly injected through the jugular catheter in a dose of 1 ml per kg

(Foldes *et al.* 2000). Blood samples were taken at 0, 5, 15, 30, 60, 90 and 120 min after injection.

Footshock–footshock box novelty Rats were exposed to repeated electrical footshocks (10-ms pulses of 0.8 mA at 50 Hz for 1 s, repeated every 30 s for 5 min through a grid floor) as previously described (Zelena *et al.* 1999c). Control rats were transferred to the same box without electrical impulses. Blood samples were collected from the jugular vein at 0 (before stress), 5, 15, 30, 60, 90 and 120 min.

Restraint A pre-stress blood sample was collected (0 min), then the animals were subjected to restraint stress in a polyethylene tube as previously described (Zelena *et al.* 2003a). Stressor duration was 1 h and blood samples were collected before (at 0 min), during (at 5, 15, 30, 45, 60) and after (at 120 min) restraint stress.

Social defeat The test rats were placed into the home cage of a bigger Brattleboro couple (without pups) and left there for 10 min. The test animals lost the confrontation but were not injured. Blood samples were taken before (0 min) and after stress exposure (15, 30, 60, 90 and 120 min after stressor initiation).

Ulcerogenic cold-immobilisation Cannulated rats were placed into a cold room (4 °C) and immobilised (Kvetnansky & Mikulaj 1970). Serial blood samples were taken before (0 min) and during (30, 60, 120, 180 and 240 min) the stress. The appearance of gastric erosion was visualised by opening the stomach at the end of the 4 h examination period (Filaretova *et al.* 1998). For this experiment, a Wistar control group was also used to confirm stressor efficacy to induce ulcers.

Hypoglycaemia After 18 h fasting, hypoglycaemia was induced by i.p. actrapid injection (3NE/2 ml per kg; Novo Nordisc, Bagsvaerd, Denmark) and the animals were decapitated 1 h later (Lolait *et al.* 2007a). Blood glucose levels were measured by a commercially available analyser (D-Cont Personal, 77 Elektronika Kft, Budapest, Hungary). For this experiment, a +/+ control group was also used for comparison with the heterozygous animals.

Forced swim Serial blood samples were taken before (0 min), during (5 min) and after (15, 30, 60 and 90 min) a 10 min forced swim period conducted in a glass cylinder (diameter: 30 cm; height: 50 cm) filled with tap water (24 °C ± 1 °C) to a height of 35 cm (Porsolt *et al.* 1978). After swimming, the animals were removed from the tank, carefully dried with paper towels and returned to their home cages. An additional control group (DDAVP treatment) was used in this experiment to exclude the influence of peripheral AVP deficiency. Ten days before blood sampling, half of the di/di rats were implanted with osmotic minipump

(see earlier), while other animals (both di/di and di/+) went through sham operation. Eight days later a jugular catheter was implanted and after 48 h the swim test was completed.

Anterior pituitary sensitivity to CRH

In vivo testing of anterior pituitary sensitivity to CRH To test the sensitivity of the anterior pituitary to CRH, 40 ng/kg CRH (Sigma) was injected i.v. into cannulated animals and blood samples were collected at 0, 10, 20, 30, 45 and 60 min.

In vitro testing: static incubation Pituitary ACTH secretion and cAMP content as well as the adrenal gland corticosterone secretion were assessed by static incubation (Stachura *et al.* 1985). Anterior pituitary and adrenal glands were obtained after death by decapitation between 0830 and 0930. Each gland was chopped into eight pieces and pre-incubated in 1 ml DMEM (Sigma–Aldrich) containing 2.5 g BSA/l (Fraction V, Calbiochem, La Jolla, CA, USA) at 37 °C under 95% O₂–5% CO₂ atmosphere for 2 × 1 h. After pre-incubation, the pituitary fragments were incubated for 20 min with 5 × 10^{−8} M CRH (for cAMP content) or 5 × 10^{−11} M CRH (for ACTH secretion; (Antoni & Dayanithi 1990)) containing DMEM. For adrenal segments, the medium was collected and replaced with fresh media every 15 min six times with different doses of ACTH (10^{−12}; 10^{−11}; 10^{−10} M) added to the media. In the case of serial sampling, all data were expressed as percentage of the basal secretion measured in the first 15 min sample. At the conclusion of the experiment (after 20 min for hypophysis and at the end of each 15 min fraction for the adrenal gland), media were removed, centrifuged at 3000 g for 5 min and the supernatant stored at −20 °C until analysis. Pituitary tissue from each tube was carefully removed and homogenised in 250 µl of 0.1 M HCl with 250 µl of 0.1 M HCl wash and the homogenate was centrifuged at 3000 g for 5 min. A volume of 250 µl of supernatant was stored at −20 °C, freeze dried then cAMP content was measured.

Hormone measurement

The concentrations of ACTH and corticosterone were measured by RIA, as described earlier (Zelena *et al.* 2008). The intraassay coefficients of variation for ACTH and corticosterone were 4.7 and 12.3 respectively. Immuno-reactive cAMP was determined by the modification of the specific RIA described by Brooker *et al.* (1979). Our highly specific antibody (no. 309) developed against 2-O-succinyl-cAMP-HAS in goat has a cross-reactivity of <0.001% for cGMP, ATP and AMP thereby allowing the direct determination of cAMP even in the presence of high concentration of ATP. The antibody was used in final dilution of 1:8000. 2'-O-succinyl-cAMP tyrosine methylester iodinated by the chloramine *t*-method served as radioligand. Separation of bound and free fraction was performed with 1 ml of ice-cold ethanol. The sensitivity of the assay was

Table 1 Resting hormone and mRNA levels of the HPA axis. The mentioned parameters were summarised from different experimental series

	<i>Crh</i> mRNA in PVN (% of di/+)	<i>Pomc</i> mRNA in AL (% of di/+)	ACTH (fmol/ml) decap.	ACTH (fmol/ml) cannulated	Corticosterone (pmol/ml) decap.	Corticosterone (pmol/ml) cannulated
di/+	100.0 ± 4.7	100.0 ± 3.5	23.9 ± 3.0	70.6 ± 10.5*	137.4 ± 17.6	277.4 ± 41.4*
<i>n</i>	21	28	136	83	142	66
di/di	135.4 ± 9.2†	111.6 ± 3.9*	21.0 ± 1.5	82.8 ± 10.7*	175.2 ± 13.7	332.7 ± 43.5*
<i>n</i>	24	30	135	75	140	63

* $P < 0.05$; † $P < 0.01$ versus di/+; * $P < 0.05$ versus decapitated.
decap., decapitated.

0.05 pmol/tube. The intrassay variation was 7.1%. All samples from one experiment were measured in one assay.

Statistical analysis

Data were analysed by ANOVA using the ANOVA/MA-NOVA module of the STATISTICA 6.0 software package (Tulsa, OK, USA). In experiments where serial blood samples were collected, a repeated measure ANOVA was used (effect of time). Both one-way (genotype) and two-way (1: genotype (di/+, di/di, +/+ or Wistar or desmopressin), 2: stress) ANOVAs were used to analyse data based upon the number of dependent variables in an experiment. Multiple pairwise comparisons were made by the Newman-Keuls method. Data are expressed as mean ± S.E.M. and the level of significance was set at $P < 0.05$.

Results

Basal levels

The di/di rats had significantly higher resting *Crh* mRNA levels in their PVN (genotype: $F_{(1,43)} = 14056$; $P < 0.01$; Table 1) and elevated *Pomc* mRNA level in the anterior lobe of the pituitary (genotype: $F_{(1,56)} = 1942$; $P < 0.05$; Table 1) compared with heterozygous littermates.

The resting hormone levels from different experimental series are summarised in Table 1. Cannulation of animals resulted in higher resting ACTH and corticosterone plasma levels than those following decapitation (mode of blood sampling, ACTH: $F_{(1,425)} = 78.5$; corticosterone:

$F_{(1,407)} = 32.4$; $P < 0.01$). Genotype did not have a significant effect on basal hormone levels, although there was a trend for elevated corticosterone in di/di rats following decapitation (genotype: $F_{(1,280)} = 2.87$; $P = 0.09$).

Since these data (elevated *Crh* mRNA in PVN, enhanced *Pomc* mRNA levels in anterior lobe and a tendency for elevated basal corticosterone levels) are typically observed during chronic stress. We decided to collect somatic measures to determine whether peripheral endpoints were similarly affected (Table 2). The main difference was the smaller body and tissue weight of di/di animals (genotype, body weight: $F_{(1,203)} = 55.2$; thymus: $F_{(1,203)} = 19.8$; adrenal gland: $F_{(1,183)} = 27.8$; $P < 0.01$). However, there was no significant difference in the relative organ weights between the two genotypes.

Daily rhythms

The well-established rhythmic changes were present both in ACTH and corticosterone levels with lowest levels between 2 and 6 h of the light phase and the highest levels detected between 14 and 22 h (beginning 2 h after the onset of the dark phase; Fig. 1, time, ACTH: $F_{(23,138)} = 5.44$; corticosterone: $F_{(23,184)} = 4.80$; $P < 0.01$). There was no significant difference between the two genotypes in net secretion (area under the curve (AUC) ACTH (fmol/ml): di/+ 546.4 ± 95 di/di 460.6 ± 73 corticosterone (pmol/ml): di/+ 6891.5 ± 615 di/di 6981.1 ± 492) or in maximal hormone changes during the day (ACTH (fmol/ml): di/+ 85.6 ± 24, di/di 86.4 ± 7 corticosterone (pmol/ml): di/+ 698.3 ± 100, di/di 615.0 ± 37) for both ACTH and corticosterone.

Table 2 Somatic differences between di/+ and di/di animals. The mentioned parameters were summarised from different experimental series

	Body weight (g)	Thymus weight (mg)	Relative thymus weight (mg/kg)	Adrenal gland (mg)	Relative adrenal gland weight (mg/kg)
di/+	364.5 ± 5.6	363.1 ± 9.0	1015.6 ± 28.9	42.1 ± 0.8	114.1 ± 2.2
<i>n</i>	102	102	98	92	88
di/di	309.0 ± 5.0†	306.8 ± 8.9*	996.8 ± 29.7	36.5 ± 0.8†	115.9 ± 2.1
<i>n</i>	103	103	99	93	89

* $P < 0.05$; † $P < 0.01$ versus di/+; * $P < 0.05$ versus decapitated.

Figure 1 Rhythmic changes in plasma ACTH ((A) fmol/ml) and corticosterone ((B) pmol/ml) levels in Brattleboro rats. Serial blood samples were collected on two consecutive days every 2 h via a catheter in the vena jugularis. Separate group of animals were used for light phase (lights on at 7 h; samples 0–12) and dark phase (kept in reversed cycle, lights on at 22 h; samples 12–24). The time when the lights were switched on is referred to as Zeitgeber time 0. $n=7-9$. The effect of time was statistically significant for both hormones ($P<0.01$). $^+P<0.05$, $^{++}P<0.01$ the time point versus 0 h; $^!P<0.05$; $^{!!}P<0.01$ the time point versus 2–4–6 h (lower levels).

Stress studies with single timepoint (decapitation)

Novelty stress In di/+ animals, both the ACTH and corticosterone plasma levels were elevated at the end of 10 min of novelty stress (Fig. 2; stress, ACTH: $F_{(1,34)}=9.89$; corticosterone: $F_{(1,34)}=48.88$; $P<0.01$). The resting plasma levels were similar in di/+ and di/di animals. Novelty stress did not induce ACTH elevations in di/di rats (stress-genotype interaction: $F_{(1,34)}=5.72$; $P<0.05$); however, the rise in corticosterone was similar to that seen in di/+ animals (no significant interaction).

Social avoidance There was no significant effect of genotype on the ACTH or corticosterone response to 8 min in the social avoidance box (Table 3).

Elevated plus-maze In response to the EPM, plasma ACTH levels were significantly lower in di/di rats compared with heterozygous littermates (Table 3, genotype: $F_{(1,18)}=4.91$; $P<0.05$). By contrast, the plasma corticosterone levels from the same animals were similar between the two genotypes.

LPS challenge One hour after i.p. injection of LPS (300 µg/kg) plasma ACTH and corticosterone levels were significantly elevated in di/+ rats (Table 3, treatment, ACTH: $F_{(1,36)}=9.38$; corticosterone: $F_{(1,36)}=20.3$; $P<0.01$; control levels can be found in Table 1). Although

the effect of genotype was not significant for either hormone, there was a significant increase in ACTH in di/+ animals that did not occur in di/di rats, in contrast to the big elevation in corticosterone levels observed for both genotypes.

Ether inhalation Ten minutes after the onset of ether inhalation plasma ACTH and corticosterone levels were significantly elevated (Table 3, stress, ACTH: $F_{(1,67)}=287.3$; corticosterone: $F_{(1,67)}=110.8$; $P<0.01$). The genotype had no effect on the observed elevations.

Stress studies with serial blood sampling

Hypertonic saline The combined effect of i.p. injection and volume load (controls on Fig. 3A) induced a small, although statistically significant, elevation in plasma ACTH (time: $F_{(5,85)}=8.11$; $P<0.01$) with lower levels in AVP-deficient rats (genotype: $F_{(1,17)}=8.34$; $P=0.01$). At the same time, the corticosterone elevation did not differ between genotypes (Fig. 3B controls; only the effect of time was significant: $F_{(5,85)}=21.2$; $P<0.01$).

Injecting hypertonic saline into the peritoneal cavity stimulates ACTH secretion within 5 min, and the effect persists for 2 h (treatment: $F_{(1,36)}=90.5$, time: $F_{(5,180)}=47.3$, treatment $\times$ time: $F_{(5,180)}=31.0$; $P<0.01$). In AVP-deficient animals, the hypertonic saline-induced ACTH elevation was lower only at 1 h ($P=0.05$), and there was not a significant

Figure 2 Novelty stress in Brattleboro rats. Plasma ACTH ((A) fmol/ml) levels were significantly elevated in di/+ but not in di/di rats, while the corticosterone ((B) pmol/ml) levels were elevated in both genotypes. $n=8-12$ C-control, unstressed; Nov- at the end of 10 min novelty ** $P<0.01$ versus control; ## $P<0.01$ versus di/+.

overall effect of genotype (treatment $\times$ time $\times$ genotype interaction: $F_{(5,180)}=2.77$; $P<0.05$). The accompanying corticosterone levels increased gradually until 30 min and remained elevated until the end of the examination period (treatment: $F_{(1,36)}=5.53$, time: $F_{(5,180)}=77.5$, treatment $\times$ time: $F_{(5,180)}=5.28$; $P<0.01$). Genotype had no effect on the corticosterone response to hypertonic saline.

Anaphylactoid reaction Intravenous saline injection induced a very mild ACTH secretion 1 h after its administration in both genotypes (Fig. 3C; time: $F_{(6,102)}=3.38$; $P<0.01$). The corticosterone levels were significantly elevated and did not differ between the two genotypes (Fig. 3D; time: $F_{(6,102)}=9.97$; $P<0.01$, between 30 and 120 min).

Injecting the egg white preparation into the vena jugularis induced a remarkable elevation in the ACTH and corticosterone, with a peak in ACTH at 15 and 30 min and a persisting corticosterone elevation from 15 min through 2 h (treatment, ACTH: $F_{(1,39)}=165.0$; corticosterone: $F_{(1,39)}=15.17$; time, ACTH: $F_{(6,234)}=52.7$; corticosterone: $F_{(6,234)}=41.3$; treatment $\times$ time, ACTH: $F_{(6,234)}=51.5$; corticosterone: $F_{(6,234)}=6.84$; $P<0.01$). The ACTH elevation was significantly smaller in AVP-deficient rats (genotype: $F_{(1,39)}=5.6$, treatment $\times$ genotype: $F_{(1,39)}=7.11$, time $\times$ genotype: $F_{(6,234)}=2.84$, treatment $\times$ time $\times$ genotype: $F_{(6,234)}=2.31$; $P<0.05$). In di/+ animals, the ACTH elevation disappeared at 90 min, while in di/di animals the concentration of ACTH returned to basal levels by 1 h. By contrast, the corticosterone elevation was similar between the two genotypes.

Footshock Placing the animals into the footshock box without administering shocks resulted in a significant elevation of their plasma ACTH and corticosterone levels and was not affected by genotype (Fig. 3E and F; time, ACTH: $F_{(5,110)}=22.3$; corticosterone: $F_{(5,110)}=58.9$; $P<0.01$).

Electrical shock was able to induce a further ACTH rise being similar in di/+ and di/di rats (footshock: $F_{(1,46)}=5.99$; $P<0.05$; footshock $\times$ time: $F_{(5,230)}=7.04$; $P<0.01$). At the same time, there was only a trend for a shock-induced increase in corticosterone (footshock: $F_{(1,45)}=3.13$, $P=0.08$; footshock $\times$ time: $F_{(5,225)}=2.87$, $P<0.05$) and genotype did not affect the response.

Restraint Putting the animals into a restraint chamber stimulated both ACTH and corticosterone secretion within 5 min (Fig. 4A and B; time, ACTH: $F_{(6,222)}=24.4$; corticosterone: $F_{(6,216)}=50.9$; $P<0.01$). The highest ACTH levels were visible at 15 min and remained stable until the end of the stress (1 h) but went back to basal levels by

Table 3 Plasma ACTH and corticosterone levels at one time-point after onset of a stimulus

	Time of stimulus (min)	ACTH (fmol/ml)		Corticosterone (pmol/ml)	
		di/+	di/di	di/+	di/di
Social avoidance	3+5	110.5 $\pm$ 17.6	83.5 $\pm$ 22.7	339.5 $\pm$ 76.5	326.0 $\pm$ 50.4
<i>n</i>		18	9	7	5
EPM	5	209.2 $\pm$ 31.4	121.6 $\pm$ 20.4*	499.2 $\pm$ 61.4	534.7 $\pm$ 35.6
<i>n</i>		6	14	6	14
LPS	60	295.2 $\pm$ 106.1*	192.9 $\pm$ 98.7	962.9 $\pm$ 201.4*	864.8 $\pm$ 270.5*
<i>n</i>		10	10	10	10
Ether	3+7	1027.6 $\pm$ 75.8*	999.8 $\pm$ 89.3*	721.1 $\pm$ 94.1*	791.8 $\pm$ 73.8*
<i>n</i>		19	16	19	16

* $P<0.05$; † $P<0.01$ versus control, untreated rats of the same genotype (control levels can be found in Table 1); * $P<0.05$ versus di/+ rats.

Figure 3 Serial blood sampling from cannulated Brattleboro animals after i.p. saline (1.5 ml/100 g), hypertonic saline (1.5 M) treatment (A and B; $n=7-12$), i.v. administration of saline/egg white preparation (C and D; $n=9-13$) or new box/footshock (E and F; $n=10-17$). ACTH (fmol/ml; A, C and E) levels were elevated in all five cases (volume load (control on A), hypertonic saline, egg white, new box or footshock). The di/di animals had lower elevation after volume load and at 1 h after i.p. hypertonic saline (A) and after egg white injection (C), but not after placing them into the footshock box, or after footshock (E). The corticosterone (pmol/ml, B, D and F) plasma levels were elevated throughout without any effect of genotype. $^+P<0.05$, $^{++}P<0.01$ vs 0 min; $*P<0.05$, $**P<0.01$ versus control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus di/+.

120 min (Fig. 4A). By contrast, the corticosterone levels elevated gradually throughout the stimulus and remained stable even 1 h after stressor cessation (Fig. 4B). The AVP-deficient rats revealed smaller elevations during the stress for both hormones (genotype, ACTH: $F_{(1,37)}=12.08$; corticosterone: $F_{(1,36)}=7.38$; time $\times$ genotype, ACTH: $F_{(6,222)}=5.29$; corticosterone: $F_{(6,216)}=2.93$; $P<0.01$).

Social defeat Aggressive interaction is a significant stimulus to rats inducing long-lasting ACTH (Fig. 4C) and corticosterone (Fig. 4D) elevations in the plasma visible even 110 min after stressor cessation (time, ACTH: $F_{(5,175)}=14.83$; corticosterone: $F_{(5,170)}=32.06$; $P<0.01$). The lack of AVP resulted in a reduced activation of the HPA axis, which reduced both hormone levels (time $\times$ genotype, ACTH:

$F_{(5,175)}=2.25$, $P=0.05$; corticosterone: $F_{(5,170)}=32.06$, $P<0.01$).

Special controls

Ulcerogenic cold-immobilisation Ulcerogenic stimulus (4 h cold-immobilisation) resulted in the formation of haemorrhagic erosion with similar severity in all three animal groups (erosion area in Wistar: 4.98 ± 1.0 mm²; di/+ : 5.06 ± 1.6 mm²; di/di: 4.74 ± 1.4 mm²). The ACTH levels of these animals were elevated with a peak at 30 min (Fig. 5A; time: $F_{(5,45)}=53.1$; $P<0.01$). Both di/+ and Wistar rats showed similar changes in ACTH throughout, while di/di animals had reduced ACTH at the 1 h time point (time $\times$ group: $F_{(10,45)}=4.46$; $P<0.01$). The corticosterone

Figure 4 Serial blood sampling from cannulated Brattleboro rats during 1 h restraint (A and B; $n=26-28$) and after 10 min aggressive interaction (Agr; C and D; $n=24-26$). Both ACTH (fmol/ml; A and C) and corticosterone (pmol/ml; B and D) plasma levels were elevated with smaller elevations in di/di rats. $^+P<0.05$; $^{++}P<0.01$ vs 0 min; $^{##}P<0.05$, $^{###}P<0.01$ versus di/+.

levels increased through 1 h then remained stable (Fig. 5B; time: $F_{(5,40)}=25.5$; $P<0.01$) and was not different between groups.

Hypoglycaemia The blood glucose levels of all three genotypes were similar after 18 h fasting (+/+ : 4.67 ± 0.1 mmol/l; di/+ : 4.62 ± 0.2 mmol/l; di/di : 4.45 ± 0.2 mmol/l). Actrapid injection (i.p.) significantly decreased blood glucose in all groups, with the lowest concentration in di/di rats (+/+ : 2.48 ± 0.05 mmol/l; di/+ : 2.29 ± 0.05 mmol/l; di/di : 2.0 ± 0.1 mmol/l; treatment: $F_{(1,72)}=439.1$, $P<0.01$; genotype: $F_{(2,72)}=3.52$, $P<0.05$). The fall in the blood glucose level resulted in a huge ACTH and corticosterone elevation in all three genotypes (Fig. 5C and D; treatment, ACTH: $F_{(1,71)}=359.5$, corticosterone: $F_{(1,70)}=132.5$; $P<0.01$). The Actrapid induced ACTH elevation was smaller in di/di rats compared with both +/+ and di/+ controls, while there was no difference between +/+ and di/+ rats (Fig. 5C; genotype: $F_{(2,71)}=10.66$, treatment $\times$ genotype: $F_{(2,71)}=12.48$; $P<0.01$). There was no difference in corticosterone elevation between groups.

Forced swim Replacing the peripheral AVP by an osmotic minipump leads to a significant reduction in water intake in

di/di animals (di/+ : 34.8 ± 1.9 ml/day; di/di : 103.9 ± 8.3 ml/day; di/di with DDAVP : 32.6 ± 1.9 ml/day; group: $F_{(2,22)}=5.24$; $P<0.05$). Forcing the animals to swim for 10 min induced a significant ACTH elevation with a peak at 5 min in di/+ rats (Fig. 5E; time: $F_{(5,115)}=13.6$; $P<0.01$). This elevation was significantly reduced in the absence of central AVP, as compensation of the peripheral AVP deficiency by DDAVP was unable to restore the ACTH elevation (group: $F_{(2,23)}=4.43$, time $\times$ group: $F_{(10,115)}=2.21$; $P=0.02$). Corticosterone levels were also elevated with the highest level seen 20 min after the end of the forced swimming test (Fig. 5F; time: $F_{(5,115)}=19.6$; $P<0.01$). The corticosterone reducing effect of AVP deficiency was not statistically significant (groups: $F_{(2,23)}=3.39$, $P=0.051$; time $\times$ group: $F_{(10,115)}=1.75$, $P=0.07$).

Sensitivity of the anterior pituitary

To study the *in vivo* sensitivity of the anterior pituitary we injected animals with CRH (40 ng/kg i.v.) and observed an activation of ACTH and corticosterone secretion (Fig. 6A and B; time, ACTH: $F_{(5,65)}=11.9$, corticosterone: $F_{(5,65)}=8.05$; $P<0.01$). The ACTH changes were significantly smaller in di/di rats (Fig. 6A; genotype: $F_{(1,13)}=9.51$; $P<0.01$), while the corticosterone levels were similar in both genotypes.

Figure 5 Response to 4 h cold immobilisation (A and B; $n=10-12$; Wistar), i.p. actrapid induced hypoglycaemia (C and D; $n=12-14$; +/+) and 10 min forced swimming (E and F; $n=5-12$; peripheral DDAVP in minipump). In all experiments there was at least one time point when the stress-induced ACTH (fmol/ml; A, C and D) elevations were smaller in di/di rats. Wistar (A) and +/+ rats (C) showed similar profile to di/+ animals while replacing the peripheral AVP did not restore the effect of AVP deficiency (E). The corticosterone (pmol/ml; B, D and F) response was not different between groups. $^+P<0.05$, $^{++}P<0.01$ vs 0 min; $^{**}P<0.01$ versus control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus di/+.

In an *in vitro* static incubation system the presence of CRH in the medium for 20 min resulted in a significant elevation in both cAMP content (Fig. 6C; due to 5×10^{-8} M CRH; treatment: $F_{(1,35)}=45.6$; $P<0.01$) and ACTH secretion (Fig. 6D; due to $5-10^{-11}$ M CRH; treatment: $F_{(1,38)}=107.4$; $P<0.01$) from anterior pituitary fragments. The absence of AVP in di/di rats led to reduced ACTH secretion (2.2-fold increase in di/di animals versus 3.7-fold increase in

di/+ rats; genotype: $F_{(1,38)}=18.96$, treatment $\times$ genotype: $F_{(1,38)}=16.76$; $P<0.01$) without a significant difference in cAMP levels.

Sensitivity of the adrenal gland

There was a dose-dependent increase in net corticosterone secretion (Fig. 7A; AUC; treatment: $F_{(2,63)}=24.4$; $P<0.01$)

Figure 6 Sensitivity of the anterior pituitary to CRH. *In vivo* administration of 40 ng/kg CRH (i.v.; A and B; $n=6-9$) resulted in a significant elevation of both ACTH (fmol/ml, A) and corticosterone (pmol/ml, B) with smaller ACTH rises in di/di animals. *In vitro* studies show both that cAMP content (C; due to 5×10^{-8} M CRH; $n=9-10$) and ACTH secretion (D; due to $5-10^{-11}$ M CRH; $n=10-11$) were enhanced by CRH with a smaller ACTH response in di/di rats. ⁺ $P < 0.05$, ⁺⁺ $P < 0.01$ vs 0 min; ^{**} $P < 0.01$ versus control; ^{##} $P < 0.01$ versus di/+.

in response to ACTH (10^{-12} , 10^{-11} , 10^{-10} M). The increase was significantly lower in di/di rats (genotype: $F_{(1,63)} = 17.13$; $P < 0.01$). The lowest dose significantly elevated corticosterone secretion from the adrenal gland being maximal in the post-test fraction (Fig. 7B; time: $F_{(5,105)} = 41.6$; $P < 0.01$). The increase was significantly smaller in di/di rats (genotype: $F_{(1,21)} = 8.3$, treatment $\times$ genotype: $F_{(5,105)} = 3.95$; $P < 0.01$).

Discussion

Our work is the first to use such a wide range of stressors to better define the role of AVP in the acute HPA response to stress in Brattleboro rats. The present findings show that the role of AVP is indeed important for the regulation of ACTH secretion during exposure to acute stimuli and that the magnitude of AVP contribution in stress-induced HPA activity is context dependent. Interestingly, the role of AVP in the regulation of stress-induced HPA activity was not limited to a stressor category (i.e. AVP is involved in both systemic and psychological stressors), and the requirement for AVP was not consistent within a given stressor category. In regards to HPA activity, our results show that changes

in plasma corticosterone did not consistently mirror changes in ACTH, except for a few exceptions (Table 4) and that this fact could not be explained by an earlier ACTH peak (as demonstrated by serial blood sampling) or by enhanced adrenal gland sensitivity to ACTH (as confirmed by *in vitro* studies).

The majority of the stress paradigms tested demonstrated a decreased ACTH response in di/di rats (Table 4). In fact, in over 70% of the stressors (13 out of 18) the neuroendocrine response to stress, i.e. the hypothalamo-pituitary component, was reduced in di/di rats. Interestingly, only in 30% of the cases where ACTH was reduced (4 out of 13), a decreased corticosterone response was detected as well. In some cases, only one time point was examined, suggesting that perhaps our time point was able to detect the ACTH peak but was premature to detect a difference in corticosterone. However, this explanation cannot explain the lack of difference in corticosterone in the face of different ACTH concentrations in the studies that incorporated serial blood sampling. Enhanced adrenal gland sensitivity to ACTH could have provided a simple explanation; however, a decreased adrenal sensitivity to ACTH in di/di animals was observed and is also supported by previous *in vitro* (Wiley *et al.* 1974) and *in vivo* (Brudieux *et al.* 1986) studies. Similar dissociations were

Figure 7 Sensitivity of the adrenal gland to its main secretagogue, ACTH. Corticosterone response to 10^{-12} , 10^{-11} and 10^{-10} M ACTH was measured in an *in vitro* static incubation system. Samples were collected every 15 min. The area under the curve (AUC), i.e. the total amount of secreted corticosterone during 90 min is shown on part A representing a dose–response curve with smaller elevations in di/di rats ($n=10$ – 12). The temporal evolution of corticosterone secretion after 10^{-12} M ACTH is illustrated in part B. The horizontal bar indicates the duration of ACTH treatment (15 min). ++ $P < 0.01$ versus first fraction; * $P < 0.05$, ** $P < 0.01$ versus the effect of 10^{-12} M ACTH; ## $P < 0.01$ versus di/+.

found in studies using the V1b receptor antagonist (Org) in Sprague–Dawley rats (Spiga *et al.* 2009) and in the V1b receptor KO mice (Stewart *et al.* 2008), demonstrating that phenomenon is not species specific. The fact that the discrepancy between ACTH and corticosterone could not be explained by ACTH sensitivity or missed time points raises the possibility that other peripheral mediators are involved and may be acting directly on the adrenal gland. It supports the theory of paraadenohypophyseal neuroendocrine regulation (Elifanov *et al.* 1988) and requires further studies. The discrepancy between vasopressinergic ACTH and corticosterone regulation requires further attention in the face that V1b antagonists are under development for treatment of stress-related disorders with the assumption that they will decrease the HPA axis hyperactivity.

We observed three types of HPA responses in AVP deficient rats: 1) stressors with decreased ACTH and corticosterone response (for e.g. morphine injection,

aggression, restraint), 2) stressors with decreased ACTH response but no change in corticosterone (for e.g. novelty, EPM, forced swim, hypoglycaemia, egg white), 3) stimuli without a change in ACTH or corticosterone (for e.g. social avoidance, footshock, ether inhalation; Table 4). It has been hypothesised that the brain categorises stressors and utilises neural response pathways that vary in accordance with their assigned category (Herman *et al.* 1996). There are many ways by which stressors have been categorised: 1) intensity (weak or strong), 2) psychological (novelty, social avoidance, EPM), physical (aggression, restraint, footshock, forced swim, immobilisation, ether inhalation; Dayas *et al.* 2001) or metabolic (hypertonic saline, immune exposure, hypoglycaemia; Carrasco & Van de Kar 2003), 3) cognitive (restraint) or non-cognitive (infections; Lolait *et al.* 2007a), 4) systemic (cardiovascular, osmotic and immune challenge) or neurogenic (restraint, immobilisation and electrical footshock; Sawchenko *et al.* 2000), 5) interoceptive (reflex responses) or exteroceptive (affective and visceromotor responses; for e.g. acute footshock; Sawchenko *et al.* 2000). Of the above-mentioned stressor categories, none were able to fit with it and classify the three types of responses we observed with differential roles for AVP involvement: 1) AVP regulates both ACTH secretion and corticosterone release, 2) AVP also regulates ACTH secretion but does not affect corticosterone release, 3) no obvious role of AVP in HPA axis regulation. Thus, the role of AVP in HPA regulation during stress is neither limited to nor specified for a known stressor category.

Magnocellular AVP plays an important role in fluid homeostasis and based upon the fact that *Avp* mRNA in the parvocellular PVN responds rapidly to osmotic stimulus (Lightman & Young 1988), we expected to observe a massive role of AVP in HPA axis regulation during osmotic stimulus. We were surprised that our results did not confirm this assumption, i.e. that AVP is required for the HPA response to osmotic stimuli. Water deprivation in Brattleboro rats led to a similar conclusion (Popova *et al.* 2002). It is possible that oxytocin compensates for the loss of AVP in this process as it is also stimulated by osmotic challenges (Schlosser *et al.* 1994). However, the control procedure (volume load) induced the HPA axis activation was markedly reduced in the absence of AVP. This is consistent with the fact that volume changes (bleeding) increase AVP release (Lewandowska *et al.* 1992, Lipinska *et al.* 2004).

Of the stressors tested, there were two stressors i.e. immune challenge and insulin-induced hypoglycaemia, where the contribution of AVP was of a large magnitude. An immune challenge (LPS or egg white) activates the PVN, especially the AVP-containing neurons (Foldes *et al.* 2000), and the activation is not limited to the parvocellular neurons of the PVN. During the stronger anaphylactic stimulus (egg white), the role of AVP was confirmed in ACTH regulation although there was no effect on corticosterone levels. The weaker stimulus, LPS injection, induced ACTH elevation only in di/+ rats, resembling previous studies using V1b receptor KO mice (Lolait *et al.* 2007a) and V1b receptor antagonist

Table 4 Summary of the examined hormone changes in Brattleboro rats. The last three cases are our previously published data. The peak hormone values measured in di/+ animals are presented in columns 4 and 6. The list of stresses is given in the order appearing in the text. ACTH levels are expressed as fmol/ml and corticosterone as pmol/ml. The method of blood sampling is indicated in column 3. In case of i.v. blood sampling more time points were examined. The presence of significant changes in di/di animals compared with their heterozygous littermates is indicated in columns 5 and 7. There are 18 stressors and in 13 cases the ACTH levels were significantly lower in AVP-deficient animals, while we could find impaired corticosterone elevation just in 4 cases

Stress	Source	Blood	Max. ACTH	ACTH in di/di	Max. cort.	Cort. in di/di
Novelty	Fig. 2	Decap.	79.3±16	↓	596.6±102	NO
Social avoidance	Table 3	Tail	110.4±18	NO	339.5±76	NO
Elevated plus maze	Table 3	Tail	153.4±18	↓	534.7±36	NO
LPS	Table 3	Decap.	295.2±106	(↓)	962.8±201	NO
Ether inhalation	Table 3	Decap.	1027.5±75	NO	721.1±94	NO
Volume load	Fig. 3 control	i.v.	146.3±25	↓	954.5±165	↓ at 15 min
Hypertonic saline	Fig. 3	i.v.	443.3±34	↓ at 60 min	977.9±80	NO
Anaphylactoid reaction	Fig. 3	i.v.	824.3±84	↓	1073.8±91	NO
Footshock box	Fig. 3	i.v.	360.2±51	NO	979.4±67	NO
Footshock	Fig. 3	i.v.	575.4±64	NO	961.1±76	NO
Restraint	Fig. 4	i.v.	544.4±55	↓	1162.1±64	↓
Social defeat	Fig. 4	i.v.	464.9±85	↓	918.0±114	↓
Ulcerogenic cold immobilisation	Fig. 5	i.v.	887.1±53	↓ at 60 min	1701.8±221	NO
Hypoglycaemia	Fig. 5	Decap.	802.9±45	↓	2622.5±232	NO
Forced swim	Fig. 5	i.v.	602.3±45	↓ at 5 min	1943.9±152	NO
Morphine s.c.	Domokos <i>et al.</i> (2008)	i.v.	276.0±38	↓	1144.9±113	↓
NMDA i.v.	Zelena <i>et al.</i> (2005)	i.v.	339.7±48	NO	870.7±77	NO
Kainate i.v.	Zelena <i>et al.</i> (2005)	i.v.	384.6±49	↓ at 5 min	1008.2±82	NO

Cort., corticosterone

(Org; Spiga *et al.* 2009), further confirming the role of AVP during immune stress. The dissociation between ACTH and corticosterone has been observed before (Spiga *et al.* 2009), although not by all (Lolait *et al.* 2007a).

During insulin-induced hypoglycaemia a preferential release of AVP over CRH into the hypophysial portal blood was described (Plotsky *et al.* 1985). The role of AVP was further confirmed in V1b receptor KO mice not only on blood glucose regulation (Fujiwara *et al.* 2007), but also directly on the HPA axis (Fujiwara *et al.* 2007, Lolait *et al.* 2007a). Our results suggest a prominent, although not exclusive, role for AVP in the regulation of ACTH secretion during insulin-induced hypoglycaemia. In contrast to results in V1b receptor KO mice, Brattleboro animals did not demonstrate a corticosterone change following ACTH diminution.

The *in vivo* and *in vitro* experiments demonstrate that the ACTH response to CRH is lower in AVP-deficient rats (Buckingham 1981). This observation highlights the prominent role of AVP in ACTH secretion during acute challenges via supporting the effect of CRH (Buckingham & Leach 1980, Ono *et al.* 1985, Lightman & Young 1988). The lack of effect in di/di animals on the CRH-induced cAMP signalling demonstrates that the CRH signalling cascades remained effective while the ACTH response was dampened, suggesting that our effect was not due to loss of CRH signalling capacity.

The role of AVP in maintaining the basal hormonal activity of the HPA axis (ACTH and corticosterone secretion) is not supported by our results, consistently with previous reports

(Zelena *et al.* 2004, 2006a, Domokos *et al.* 2008) and also human data from diabetes insipidus patients (Itagaki *et al.* 2001). Moreover, the secretion pattern of the two hormones was also similar between the two genotypes. It was surprising as AVP shows *circadian rhythms* of synthesis and release within the suprachiasmatic nucleus (Engelmann *et al.* 1998), so its absence could have disturbed the daily rhythms of HPA axis hormone production as well. However, on the basis of the present results it seems that the lack of AVP in Brattleboro rats does not influence the pattern of the secretion, similar to results in V1b receptor KO mice (Lolait *et al.* 2007a) and in rats given the V1b receptor antagonist (Spiga *et al.* 2009).

In resting conditions, *Crh* mRNA in the PVN was higher in di/di animals (Mlynarik *et al.* 2007). In the Brattleboro rats, several molecules might serve to compensate for the lack of AVP, thereby reducing the magnitude of our observed effects. The observed *Crh* mRNA elevation in the PVN might be one of them, although it is worth noting that hypothalamic CRH content, as well as the concentration of CRH in portal blood, has been found to be unchanged (Kjaer *et al.* 1993). The structurally related other nonapeptide oxytocin, is another possible candidate which can act also on V1b receptors (Schlosser *et al.* 1994). In our recent study, we have established that although oxytocin mRNA level is elevated in the PVN, the functional replacement remained incomplete (Zelena *et al.* 2009). The adjacent stress regulatory system, the sympatho-adrenomedullary axis is also functioning at an elevated level in homozygote Brattleboro rats (Kvetnansky *et al.* 1990). Besides these well-known

components involved in stress regulation some minor elements may play a prominent role during AVP deficiency. Circulating atrial natriuretic peptide concentration was found to be elevated in di/di animals (Burgess & Handa 1992) and there is a physiological correlation between hypothalamic histamine and AVP systems (Correa & Saavedra 1983). The fact that during the perinatal period the lack of AVP completely abolished the maternal separation induced ACTH release (Zelena *et al.* 2008) suggests that the appearance of a compensatory element in adult Brattleboro rats may hide the importance of AVP in acute HPA axis regulation. However, the results with other tools (immune- or pharmacological blockade), consistent with the results presented in this study support that, in contrast to pups, the role of AVP in HPA axis regulation is not exclusive during adulthood.

For proper interpretation of our results we incorporated additional control groups, as previously discussed (Bohus & de Wied 1998, Zelena *et al.* 2003b). To exclude the consequences of the heterozygous state in our di/+ control animals in one experimental series we used Wistar rats as a control group and in another experiment a+/+ line breed out from our Brattleboro colony was used. These studies supported the assumption that one functional allele in di/+ animals are sufficient to allow for full HPA axis activation, as no difference was revealed between di/+ and Wistar or +/+ rats even during strong stress exposure, such as ulcerogenic stimulus. Peripheral AVP deficiency leads to diabetes insipidus and might result in a chronic stress state with reduced stress reactivity to further stimuli (Aguilera 1994). The unchanged somatic parameters (Table 2) as well as the retained corticosterone elevations did not support this assumption. The influence of peripheral AVP deficiency was excluded by using DDAVP containing osmotic minipumps (Zelena *et al.* 2006b) thereby demonstrating that the changes observed in the present study were due to the loss of central vasopressinergic regulation.

Immune (egg white, LPS) and metabolic (hypoglycaemia) challenges are two prominent areas with important vasopressinergic ACTH secretion regulation. Although glucocorticoids are the main end-product of the HPA axis, changes in ACTH were not always accompanied by changes in corticosterone, thereby raising the question of the importance of ACTH secretion. In addition to its well-established role in glucocorticoid release, ACTH has a trophic effect in the mammalian adrenal cortex, allowing the expression of genes encoding steroidogenic enzymes (Saez *et al.* 1989). In addition to the adrenal cortex (Voisey *et al.* 2003), ACTH receptors are also found on adipocytes (Boston & Cone 1996), skin cells (Kapas *et al.* 1998) and sympathetic ganglia (Nankova *et al.* 1996). Furthermore, it has been proposed that ACTH can also participate in immunomodulation (Weigert & Blalock 1987).

As a conclusion, our results support the important role of AVP in the regulation of ACTH secretion during acute stress. The requirement for AVP in acute stress regulation of HPA

activity was observed in a variety of stressors and was not specified for nor limited to a known stressor category.

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

Planning the experiments: D Z, L F; conducting the experiments: D Z, Á D, S K J, L F; analysing the data: D Z, Á D, S K J, L F; preparing the manuscript D Z, S K J, R J, L F.

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