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The Hypothalamic-Pituitary-Adrenal Axis Response to Stress in Mice Lacking Functional Vasopressin V1b Receptors

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Abstract

The role of arginine vasopressin (Avp) as an adrenocorticotropin (ACTH) secretagogue is mediated by the Avp 1b receptor (Avpr1b) found on anterior pituitary corticotropes. Avp also potentiates the actions of corticotropin-releasing hormone (Crh) and appears to be an important mediator of the hypothalamic-pituitary-adrenal (HPA) axis response to chronic stress. To investigate the role of Avp in the HPA axis response to stress, we measured plasma ACTH and corticosterone (CORT) levels in Avpr1b knockout (KO) mice and wild-type controls in response to two acute (restraint and insulin administration) and one form of chronic (daily restraint for 14 days) stress. No significant difference was found in the basal plasma levels of ACTH and CORT between the two genotypes. Acute restraint (30 min) increased plasma ACTH and CORT to a similar level in both the Avpr1b mutant and wild-type mice. In contrast, plasma ACTH and CORT levels induced by hypoglycemia were significantly decreased in the Avpr1b KO mice when compared to wild-type littermates. There was no difference in the ACTH response to acute and chronic restraint in wild-type mice. In the Avpr1b KO group subjected to 14 sessions of daily restraint, plasma ACTH was decreased when compared to wild-type mice. On the other hand, the CORT elevations induced by restraint did not adapt in the Avpr1b KO or wild-type mice. The data suggests that the Avpr1b is required for the normal pituitary and adrenal response to some acute stressful stimuli, and is necessary only for a normal ACTH response during chronic stress.

Keywords

Avp 1b receptor; adrenocorticotropin hormone; corticosterone; hypoglycemia; restraint stress

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Introduction

The neurohypophysial hormone vasopressin (Avp) is the hormonal regulator of water homeostasis and has major effects on behaviour and vascular tone (e.g. see 1-5). In addition to this, it is also a key regulator of the hypothalamic-pituitary-adrenal (HPA) axis (6,7). These actions are mediated through a family of G protein-coupled receptors; the Avp 1a receptor (Avpr1a) which regulates vascular tone and has many putative roles in the central nervous system, the Avp 2 receptor (Avpr2) which controls renal collecting duct water permeability, and the Avp 1b receptor (Avpr1b or V3R) which is predominantly found in the corticotropes of the anterior pituitary (8), where it is involved in the regulation of adrenocorticotropin (ACTH) release.

Diverse homeostatic challenges including cognitive (e.g. restraint) and noncognitive (e.g. infection) stressors activate the HPA axis and sympathoadrenal systems. The key CNS site integrating the neuroendocrine adjustments to stress is the hypothalamic paraventricular nucleus (PVN). This nucleus is comprised of two major neurosecretory components: the magnocellular (mPVN) and parvocellular (pPVN) subdivisions (6,9). The mPVN together with the supraoptic (SON) nuclei of the hypothalamus constitute the neurohypophysial system that is the primary source of Avp and the related peptide oxytocin (Oxt) released into the systemic circulation from neurons terminating in the posterior pituitary. Neurons in the more medially situated pPVN are the principal CNS source of corticotropin-releasing hormone (Crh), which is a major physiological regulator of pituitary ACTH secretion. Under basal conditions about 50% of these neurons also express Avp (10). Numerous neural inputs and blood- and CSF-borne factors dynamically influence the activity of pPVN Crh and Avp neurons (11,12). Moreover, there is evidence that the neurohypophysial and HPA axes may functionally overlap but the extent of this interaction is not fully understood (6,7,13,14). Crh neurons originating from the pPVN project to the external zone of the median eminence from where Crh is released into the portal vessels bathing the anterior pituitary. Crh and Avp stimulate ACTH secretion by interacting with the Crh-type 1 receptor (Crhr1) and Avpr1b, respectively, on the pituitary corticotrope. ACTH in turn potently induces the secretion of glucocorticoids (corticosterone (CORT) in rodents) from the adrenal cortex, which exert a negative feedback action on the pituitary, PVN and other brain regions such as the hippocampus to restrict the dramatic initial release of ACTH and CORT (6).

Regulation of CORT (via ACTH) secretion is critical for life and is necessary for the mammalian response to stress. Although Crh seems to be the dominant ACTH secretagogue in rodents in response to most acute stressors (e.g. restraint), Avp synergizes with Crh in activating the release of ACTH (6), and in both Crh- and Crhr1-deficient mice, Avp seems to be sufficient to maintain adequate HPA activity for survival (15-17; although for the Crh mutants, exogenous CORT is needed for lung maturation at birth (15)). In fact, there is a compensatory increase in the basal hypothalamic vasopressinergic system in Crhr1 KO animals (18). However, the precise role of Avp release in the response to stress remains controversial because the vast majority of studies looking at Avp have used indirect or correlative measurements of Avp- pPVN neuron activation after a stress challenge (19). Some of the best evidence for Avp involvement in the HPA axis response to stress derives from studies demonstrating that Avp immunoneutralization inhibits the rise in plasma ACTH produced by diverse stressors such as restraint, insulin-induced hypoglycemia and lipopolysaccharide (20-22). Additional studies (in particular those in which Avp levels in pituitary portal blood have been sampled) indicate that Avp may be released, sometimes preferentially over Crh, in response to some acute stressors such as insulin-induced hypoglycemia (23-32).

There is evidence that Avp may become the dominant ACTH secretagogue in some chronic stress situations (33,34). If an acute stress is repeated over a number of days, adaptation or

desensitization of the HPA axis can occur resulting in diminished responsiveness to this (homotypic) stressor (33,34). Avp and the pituitary Avpr1b appear to play major roles in this adaptive response. Repeated restraint stress in rats results in increased expression of Avp in pPVN Crh-containing neurons (35). Both pituitary Crhr1 and Avpr1b are activated and undergo regulatory variations during stress, but only the changes in Avpr1b levels parallel the changes in pituitary ACTH responsiveness (34,36). In addition, exogenous Avp but not Crh alters ACTH levels in chronically restrained rats even though these animals are able to respond to an acute heterotypic stress (37). Furthermore, recent studies have shown that an acute restraint episode following repeated restraint results in a rapid increase in Avp- but not Crh-hnRNA in the pPVN (38).

Avpr1b-deficient mice generated by us exhibit markedly reduced aggression and modestly impaired social recognition (5). The Avpr1b knockout (KO) mice exhibited a normal CORT response to an acute physical-psychological stress (resident-intruder)(5). In a subsequent study, the ACTH and CORT responses to forced swim were substantially reduced in another line of Avpr1b KO mice (39). The present experiments were designed to test the hypothesis that a functional Avpr1b is required for a normal HPA axis response (as measured by plasma ACTH and/or CORT levels) to acute and repeated restraint and insulin-induced hypoglycemia in mice.

Materials and Methods

Animals

Adult (8-12 weeks) littermates (a mix of the C57BL/6J and 129X1/SvJ strains) of crosses using mice heterozygous for the Avpr1b mutation (5) were group housed (three to four per cage) under controlled light and temperature (21 ± 2 C) with food and water available *ad libitum* and maintained on a 14 h light, 10 h dark cycle (lights on at 0500h). Male mice were used for all studies with the exception of the diurnal CORT measurements, where plasma from both males and females was collected. Studies were performed between 0900-1200h. All procedures were conducted in accordance with the Animal Scientific Procedures Act (1986) United Kingdom and the appropriate University of Bristol Ethical Review Process.

Basal measurements

For basal histology, *in situ* hybridization histochemistry and plasma CORT (am) levels, animals were sacrificed 1.5 h after lights on. Trunk blood was collected by decapitation (within 5 sec after removal from the home cage). Brains and pituitaries were dissected, frozen on dry-ice and stored at -80 C until sectioning. Adrenals from male mice were post-fixed in Bouin's solution (Sigma-Aldrich, UK) for 4 h at RT and destained in 70% ethanol. The tissue was embedded in paraffin and 6 μ m sections cut for staining by haemotoxylineosin (Veterinary Diagnostic Histopathology Service, Dept. Pathology & Microbiology, University of Bristol, UK). For pm plasma CORT levels, animals were sacrificed 1.5 h before lights off.

Pituitary bioassay

KO and wild-type mice were sacrificed by cervical dislocation. Pituitaries were removed and placed into ice-cold Krebs-Ringer buffer (Sigma #K4002) containing 15mM sodium bicarbonate, 2.6mM calcium chloride, 0.1% bovine serum albumin and 100 μ g/ml ascorbic acid (incubation buffer). The neural/intermediate lobes were carefully dissected away from the anterior lobe under a dissecting microscope (magnification, x10) to yield hemisected pituitaries - these were further divided into two to yield four pieces of approximately equal size. Two pituitary quarters (randomly selected from either pooled KO or wild-type pituitary tissue) were equilibrated in 1.5 ml incubation buffer in the wells of a 12-well plate (Netwells 15mm, 74 μ m pore size mesh grid, Corning) for 1 h. The equilibration buffer was removed and the tissue incubated with 1.5 ml fresh incubation buffer with or without 10nM Avp (Bachem,

UK), 1nM Crh (Bachem, UK) or 10nM Avp + 1nM Crh for 2 h. At the end of the experiment, buffer was removed, microfuged for 2 min at 13,000×g (4 C) and supernatant promptly frozen at -20 C. Total ACTH/well was measured (see below) in unextracted incubation buffer. All incubations were at 37 C in a 95% O₂/5% CO₂ atmosphere.

Restraint stress

Restraint stress was performed using 50 ml (Falcon) plastic tubes in which packing with paper tissue at the rear was used to achieve a comparable degree of restraint for each animal. Age-matched Avpr1b KO and wild-type mice were single-housed and divided into four groups (4-6 mice/group). One control group of mice was handled daily and sacrificed on the morning of day 14. A second group of mice were handled daily for 13 days and sacrificed immediately after 30 min restraint on day 14 (acute restraint). The two other groups were restrained daily for 30 min for 13 days and sacrificed either without further restraint on day 14 or immediately after a final restraint episode on day 14 (repeated restraint). Based on previous studies in restrained rats (e.g., see ref.35) and acute stress in mice (40), and the number of mice at our disposal, we chose to investigate a single time point (30 min restraint) to optimize detection of both restraint-induced plasma ACTH and CORT. This precluded any time-course study on the dynamics of ACTH and CORT secretion in the Avpr1b KO and wild-type mice.

Insulin-induced hypoglycemia

Single-housed mice were fasted overnight (12 h) with water freely available. KO and wild-type animals (4-5/group) were injected i.p. with 175-200 µl vehicle (0.9% saline; controls) or insulin (0.75 or 3.0 IU/kg diluted in vehicle; Actrapid (human insulin; 100IU/ml from Novo Nordisk, Denmark)) and sacrificed 1 h later.

Hormone analysis

All experiments were performed at least twice and samples measured in duplicate or triplicate (CORT RIA). Plasma obtained from trunk blood collected into heparinized tubes was used for all hormonal measurements. Total plasma CORT was measured (10 µl plasma diluted in 500 µl assay buffer) using antiserum kindly supplied by Dr G. Makara (Institute of Experimental Medicine, Budapest, Hungary) as described previously (41). Each experiment was processed in the same assay with intra-assay variation of less than 10%. The tracer was [¹²⁵I]-corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2-3 mCi/µg. The sensitivity of the assay was 10 ng/ml. Plasma ACTH was measured as described previously (42) using a rabbit anti-rat ACTH primary antibody (donated by G. Makara) and [¹²⁵I]ACTH (Amersham Biosciences, Little Chalfont, UK). Glucose was measured by an automated enzymatic assay (hexokinase method; courtesy Dept. Clinical Biochemistry, Bristol Royal Infirmary, Bristol, U.K.).

In situ hybridization histochemistry (ISHH)

Coronal PVN and axial pituitary sections (12µM) were thaw-mounted onto polylysine-coated slides and stored at -80 C until hybridization. The Avp, Otx and Crh oligonucleotide probes used in this study were B50 (5'-GTAGACCCGGGGCTTGGCAGAATCCACG GACTCCCGTGTCCAGCCAG-3'; bp 3928-3345 corresponding to the carboxy peptide after neurophysinII in the Avp precursor; GenBank Acc#M88354), B51 (5'-CAAGCAGG CAGCAAGCGAGACTGGGGCAGGCCATGGCATTGGTGCTCA-3'; bp1072-1119 just prior to the start of the signal peptide and terminating before mature Oxt in the Oxt precursor; GenBank Acc#M88355) and Crh (5'-CAGTTTCCTGTTGCTGTGAGCTTG CTGAGCTAACTGCTCTGCCCGGGC-3'; bp 1685-1732 of GeneBank Acc#AY128673), respectively. The specificity of the probes has been described previously (43,44). Proopiomelanocortin (Pomc) mRNA was detected with a riboprobe, generated by PCR

essentially as described previously (45) using reverse-transcribed 129Sv mouse pituitary mRNA as template, 2.5 units of Amplitaq polymerase (Applied Biosystems, CA, USA), and two primers encompassing bp 147-622 (B56, upstream: 5'-GCGAATTCGG CCCAGGAACAGCAGC-3'; B57, downstream: 5'-GCAAGCTTGAATGAGACCC CTG-3') of exon 3 of the mouse *Pomc* gene (46; GenBank Acc#V01529). The primers contain the recognition sequences for the restriction endonucleases *EcoRI* (B56) and *HindIII* (B57) to facilitate subcloning of the PCR product into the RNA-generating vector pGEM4Z (Promega, WI, USA). The integrity of the probe was verified by DNA sequencing. The oligonucleotides were end-labelled with ³⁵S-dATP as described (47) and sense and antisense *Pomc* riboprobes were generated using T7 and SP6 polymerases with ³⁵S-UTP and the MAXIScript in vitro transcription kit (Ambion, TX, USA). Biotin-labelling of the *Pomc* riboprobe was carried out using biotin-16-UTP (Roche Applied Science, Germany) in place of the radiolabelled nucleotide. All *in situ* hybridization experiments were performed as described in detail (<http://intramural.nimh.nih.gov/lcmr/snge/protocols/ISHH/ISHH.html>). ³⁵S-labelled sections were exposed to Kodak XAR film together with ¹⁴C-labelled standards (Amersham Biosciences, UK) for 2 (Avp and Oxt) or 14 (Crh) days. Biotin-labelled pituitary sections were processed using the TSA amplification kit (Perkin Elmer Life Sciences, MA, USA) according to the manufacturer's instructions. After the final wash, slides were coverslipped using Vectashield (Vector Laboratories Inc., CA, USA) and viewed under a fluorescence microscope. No specific hybridization was detected with the sense probe.

Data analysis

For ISHH data, analysis of the autoradiographic images of probe bound and visualized on film was measured using NIH image analysis software (W.Rasband, version 1.62; rsb.info.nih.gov/nih-image/) and values were calibrated with respect to the autoradiographic images of the C-14 standards. For each animal (total of four KO and four wild-type mice), four adjacent sections of PVN or pituitary/slide for each probe were measured. *Pomc* mRNA was measured only in the anterior lobe of the pituitary - the intermediate (and posterior) lobe was excluded from analysis. The relative number of *Pomc* mRNA-expressing cells in anterior pituitaries from KO (n = 3) or wild-type (n = 3) mice was determined by capturing images from three axial sections under x100 magnification. The total number of *Pomc*-positive cells was counted for the entire field-of-view of each area.

ISHH data was statistically compared using one-way ANOVA followed by Fisher's PLSD *post-hoc* test. The statistical differences between hormone measurement groups were determined by two-way ANOVA followed by Bonferroni's *post-hoc* test using GraphPad Prism (version 4.0b) software. $P < 0.05$ was considered statistically significant.

Results

Basal HPA axis parameters

Loss of *Avpr1b*-dependent pathways in the *Avpr1b* KO mice may result in compensatory changes in the distribution or expression levels of components of the HPA axis. ISHH analyses showed no differences in the level or spatial distribution of *Crh*, *Avp* or *Oxt* mRNA expression in the PVN of naïve, wild-type and KO mice (Fig.1A-F; Table 1). There were no changes in the level of *Pomc* mRNA expression, or in the relative number of *Pomc*-mRNA-expressing cells in the anterior pituitary of mutant compared to wild-type animals (Fig.1G,H; Table 1). Histological analysis of adrenal glands from male mice revealed no gross morphological changes in the medulla, or zona glomerulosa, zona reticularis or zona fasciculata (the major site of CORT production) regions of the cortex (Fig.1I,J).

To prove that the Avpr1b in the Avpr1b mutants was specifically impaired, cultured pituitary segments were stimulated with Avp, Crh or a combination of both peptides and ACTH release was measured. Fig.2 shows that there is no difference in basal or Crh (1nM)-stimulated ACTH release from anterior pituitary tissue from Avpr1b KO or wild-type mice. Avp (10nM) stimulated ACTH release (although to a significantly lower level than the smaller Crh (1nM) dose) from wild-type but not Avpr1b KO pituitaries. Basal and Avp-stimulated ACTH release from pituitaries of Avpr1b KO mice was of a similar magnitude. Fig. 2 also shows that Avp had a small but statistically significant potentiating effect on Crh-stimulated ACTH release in pituitaries from wild-type animals: in contrast, there was no difference between Crh/Avp- or Crh-stimulated ACTH release from Avpr1b KO pituitaries. Thus Avp does not stimulate ACTH release or potentiate Crh-induced ACTH release from Avpr1b-deficient pituitaries.

Fig. 3 shows that the characteristic diurnal rise in circulating CORT that occurs in the afternoon is intact in naïve, male and female Avpr1b KOs. Plasma CORT levels in the morning or afternoon were indistinguishable between the genotypes. Basal morning and afternoon plasma CORT levels were significantly increased ($P < 0.05$) in female, compared to male mice, irrespective of the genotype. Sexual dimorphism is a well-known feature of the HPA axis, with higher plasma CORT levels consistently reported for female compared with male rodents (e.g. see ref.48).

Hormone levels following stress

The effect of acute and repeated restraint on the plasma levels of ACTH and CORT in Avpr1b KO and wild-type mice is shown in Fig. 4 (A,B). In naïve wild-type mice, 30 min restraint increased plasma ACTH and CORT - there was no significant difference ($P > 0.05$) between these values and those obtained for Avpr1b KO animals (see Fig.4A,B - acute). Irrespective of genotype, there was no statistical difference between basal ACTH level after 13 d of repeated restraint and the level found in the control, handled groups of mice. After restraint on day 14 plasma ACTH levels were dramatically reduced (but not absent) in Avpr1b KO but not wild-type mice (Fig.4A - 14d). In contrast, the plasma CORT levels in repeatedly restrained mice were similar in each genotype (Fig.4B - 14d).

Since there was no difference in plasma ACTH or CORT between acutely restrained Avpr1b KO and wild-type mice, we tested the effects of another acute stressor (hypoglycemia) on HPA axis activation in the animals. Fig. 5A shows that 0.75iu/kg insulin reduces ($P < 0.01$) plasma glucose by approx. 33% and 38% in Avpr1b KO and wild-type mice, respectively. A higher dose of insulin (3.0iu/kg) causes a further fall in plasma glucose such that levels are now approx. 60% lower than saline-injected control animals for both genotypes. As described in many studies (see refs.19,22-31), the hypoglycemia associated with peripheral insulin administration activated the HPA axis, as shown by the increase in plasma ACTH (approx. 3-5-fold; see Fig. 5B) and CORT (approx. 4-5-fold; see Fig.5C) levels in wild-type mice (Fig.5B). In contrast, the 0.75iu/kg insulin dose did not increase plasma ACTH or CORT levels in the Avpr1b KO animals - the CORT levels in Avpr1b KO plasma following the higher insulin dose (3.0iu/kg) were approx. 40-50% lower ($P < 0.001$ for ACTH levels; $P < 0.01$ for CORT levels) than those found in the plasma of wild-type mice. The results show that an intact Avpr1b is required for a normal HPA axis response to acute insulin-induced hypoglycemia but not acute restraint.

Discussion

In previous studies mice with a targeted disruption of the Avpr1b have been shown to exhibit markedly reduced male–male territorial aggression (in the resident-intruder paradigm)(5) and impaired social motivation (49). In the present study we have verified that the pituitary Avpr1b is not functional in the Avpr1b mutants - Avp does not stimulate ACTH release or potentiate Crh-induced ACTH release from Avpr1b-deficient pituitaries - and have used the KO animals

to further investigate the role of Avp in the HPA axis response to stress. Basal levels of Avp, Oxt and Crh gene expression in the hypothalamic PVN, and Pomc (the precursor for ACTH and β -endorphin) gene expression in the anterior pituitary are normal in the Avpr1b KO mice. In the absence of any gross anatomical deficit in the adrenal, male and female Avpr1b KO mice have normal levels of circulating CORT and an intact diurnal CORT rhythm. The main finding(s) of this study are that an intact Avpr1b is required for an HPA axis response to acute insulin-induced hypoglycemia but not acute restraint in male mice. In addition, our results show a lack of adaptation in plasma CORT levels to repeated restraint in wild-type and Avpr1b mutant mice, and suggest that Avp (and its cognate Avpr1b) only seems necessary for a full ACTH response to the final episode of restraint in a 14 d 'chronic' (repeated) restraint paradigm.

There is a wealth of data suggesting that Avp participates in the HPA axis response to acute stressors, including restraint or immobilization stress (e.g., see refs. 35,38,66,71 and Introduction). Many of these studies indicate that the co-operation between pPVN Crh and Avp (often indirectly assessed by measuring peptide hnRNA/mRNA levels by ISHH following a stress) is required for a normal ACTH/CORT response to some, but not all acute stressors. We have previously shown that Avpr1b KO mice exhibit a normal CORT response to a brief physical-psychological stress (exposure to an intruder mouse)(5). However, there is evidence that in acute stress, ACTH responses do not always parallel CORT responses (e.g., ref.50), and that ACTH-independent activation of adrenal CORT secretion through neural or humoral factors can occur (51,52). Such factors may also act in concert to modulate adrenocortical sensitivity to ACTH and be stressor-specific, and include immune- or adrenal-produced cytokines such as interleukin-6 (53,54), sympathetic (e.g., splanchnic nerve) activity (55) and locally produced peptides and other factors that may have a paracrine function (51). Results in another Avpr1b KO line demonstrated that while the HPA response to forced swim is reduced in Avpr1b KO mice, stress-stimulated ACTH release appears more closely related to Avp stimulation than CORT release (39). In the present study, the HPA axis response to acute restraint stress was not compromised in male Avpr1b KO mice and suggests that Avp is not required for the acute restraint-induced elevation in ACTH and CORT secretion. It is possible that the restraint procedure employed in this study was sufficiently stressful to override any contribution from Avp - e.g., ACTH secretion may be entirely (over)driven by the major ACTH secretagogue Crh. This concept is supported by a study (38) that found that there was mainly a pPVN Crh (and not Avp) hnRNA and mRNA response to acute restraint in naïve rats. In addition, there are many other 'minor' agents with potential ACTH releasing activity (such as vasoactive intestinal peptide, angiotensin, ghrelin, orexins, isatin (56-59)) that may compensate in part for the lack of Avp action at the Avpr1b. The normal ACTH response to acute restraint in our Avpr1b KO mice cannot be accounted for by substitutive or compensatory effects of Oxt stimulation of corticotrope function because Oxt's action on corticotrope ACTH release are mediated by the Avpr1b (60,61). It should be noted that an Avpr1b antagonist SSR149415 blocks acute restraint-induced ACTH secretion in rats (62), although the specificity of this compound has been questioned by others (63).

Our results on restraint stress are compatible with those of a previous study in the Avp-deficient Brattleboro rat by Zelena and coworkers (64). In their study, normal ACTH and CORT responses to acute restraint were maintained in Avp-deficient animals. These authors also found that the fast ACTH response to the 11th and the 15th restraint (but not 5th or 8th) was significantly lower in male, but not female Brattleboro rats compared to that obtained for acute restraint or to the 11th restraint in control animals. In our repeated restraint study only one time point (14 d) was investigated - it is conceivable that, as in the Brattleboro rat, a deficit in the ACTH response may be observed in the last session of a shorter period of repeated restraint. In both the Brattleboro rat and Avpr1b KO mice it appears that there is sufficient ACTH remaining after (and secreted during) the final restraint session to elicit a full CORT response. It is important to note that the glucocorticoid response to ACTH saturates at low circulating

ACTH levels (65). In addition, our results may reflect increased adrenocortical sensitivity to ACTH with or without the concomitant participation of ACTH-independent neural or humoral factors as already outlined above. The extrapituitary regulation of adrenocortical function is known to be very pertinent to chronic forms of stress (52). Future studies may determine whether there are changes in adrenocortical function (e.g., is there evidence of increased expression of cytokines and/or cytokine receptors or an increased sympatho-adrenomedullary drive?) in the Avpr1b KO subjected to chronic stress. Notwithstanding the above considerations, it should be emphasized that there are alternative explanations for the discordant ACTH and CORT responses to repeated restraint stress in the Avpr1b KO mice, e.g., ACTH levels (driving a normal CORT response) may have peaked earlier than the one time point (30 min) assayed in this study.

Assuming that the dynamics of restraint-induced release of ACTH secretagogues is similar in Avpr1b KO and wild-type mice, our findings also suggest that Crh (and/or other stimulators of ACTH secretion) does not compensate for the reduced pituitary ACTH responsiveness to the final acute restraint session in the repeated restraint experiments in the Avpr1b KOs. This conclusion is supported by the studies of Ma and coworkers (38) which showed that a final episode of acute restraint following 13 d of repeated daily restraint in rats increased the expression of pPVN Avp hn- and m-RNAs but not pPVN Crh hn/mRNAs. In addition, a 'related' stress such as repeated immobilization increases Avp expression in Crh-containing fibres in the external zone of the median eminence without altering Crh levels (66). A critical difference between our results and those of others using repeated restraint procedures (as outlined in Ma and coworkers (38)) is that the ACTH and CORT responses in wild-type mice, and CORT responses in the Avpr1b KOs did not adapt to the final restraint stress. In the Avpr1b KOs the ACTH response adapted after 14 d of daily, repeated restraint stress. When rodents are repeatedly subjected to the same stressor on a daily basis, a reduction of the ACTH or CORT response with days is frequently, but not always observed (67). Adaptation appears to be clearer and more consistent with low to middle intensity stressors that do not possess a physical component (67), and its extent can be strain-dependent (68).

In contrast to our results on acute restraint, the HPA response (ACTH and CORT - see Fig.5) to hypoglycemia evoked by acute peripheral insulin administration is dramatically reduced in the Avpr1b KOs. Insulin-induced hypoglycemia is a potent physiological stimulus of the HPA axis and both Crh and/or Avp appear to act as ACTH secretagogues in studies on man (25-27), sheep (28,29,32), horse (31) and rat (21,23,24). The relative roles of Crh and Avp in mediating the ACTH and CORT response to insulin-induced hypoglycemia are uncertain, and although one study suggested that there was a preferential release of Avp over Crh and Oxt into hypophysial portal blood in rats (23), most studies interpret their findings as suggesting a synergistic effect of Crh and Avp in simulating ACTH release. The origin of Avp was not addressed in this study – insulin increases plasma levels of Avp (69), presumably derived from the magnocellular vasopressinergic neurons in the PVN or SON, which may act on the corticotrope Avpr1b (6,13,14). On the other hand, others have shown that insulin increases Avp (and Crh) turnover in the external zone of the median eminence (30), suggesting that the peptide originates from the pPVN. It is clear that low and high doses of insulin may have different effects on Crh/Avp release (32) depending on the pathway(s) that are activated. Insulin-induced hypoglycemia may induce HPA axis responses via two major pathways - neuronal components that impinge on the Crh neuron and humoral activation of the HPA axis via a pituitary-dependent pathway (11). It is possible that there is less inhibition of CORT release with the high (3.0iu/kg) compared with low (0.75iu/kg) insulin doses in the Avpr1b KOs because a more Avp-independent pathway(s) is being activated. Additional studies using different stressors with varying levels of intensity (e.g., more moderate forms of restraint) and that activate different hierarchal pathways will be required to shed more understanding on the role of the Avpr1b in acute stress.

In conclusion, the data suggests that the Avpr1b is required for the normal pituitary and adrenal response to some acute stressful stimuli, and is necessary only for a normal ACTH response during chronic restraint stress, and that other hypothalamic peptides such as Crh do not compensate for the loss of the Avpr1b in maintaining the normal response to these stressors. The Avpr1b KO should provide a useful model to investigate the role of Avp in other aspects of HPA axis function, for example facilitation and sensitization, where Avp has been implicated (70,71).

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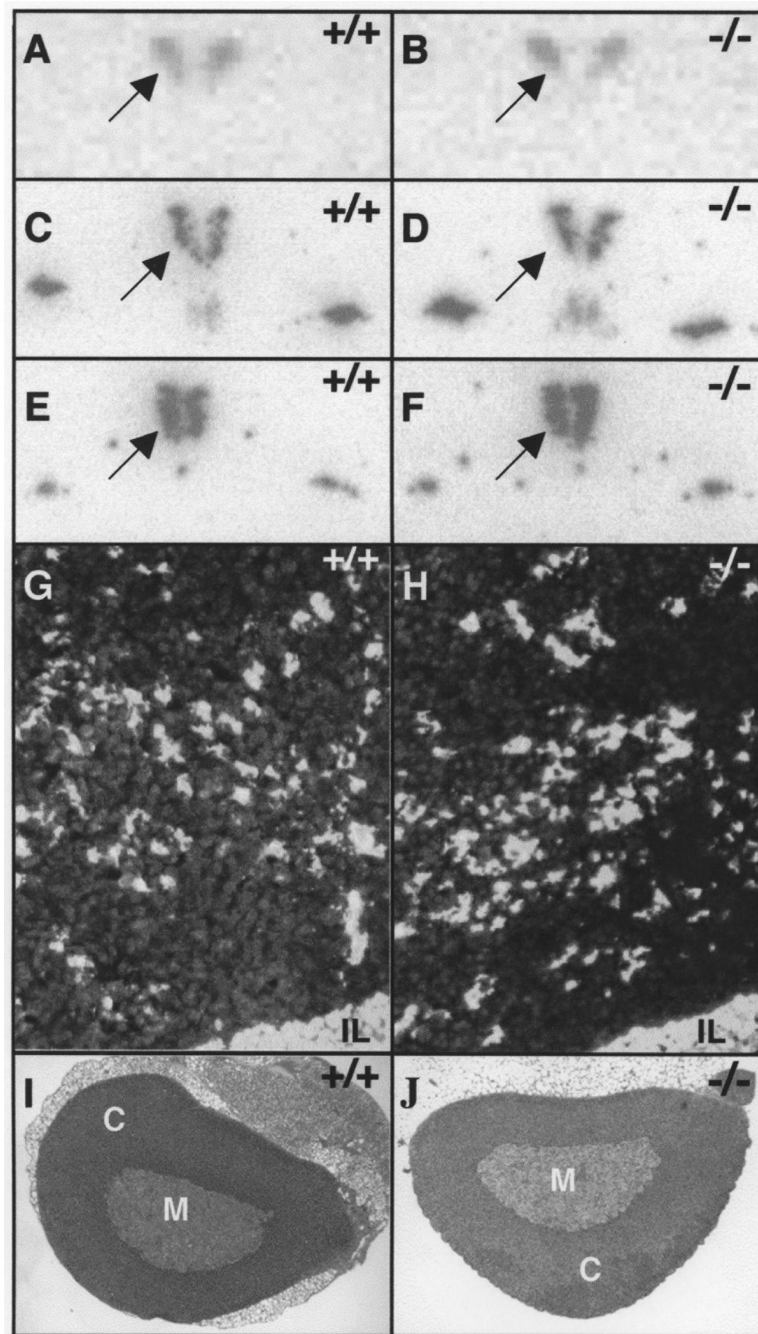


Figure 1.

Basal expression of neuropeptide mRNAs in PVN and pituitary and histology of the adrenal in Avpr1b KO mice.

There was no difference in the expression (dark labelling on light background) of Crh (A,B), Avp (C,D) or Oxt (E,F) mRNAs in the PVN (arrowed) of wild-type (left panels) or Avpr1b KO (right panels) mice. A-F are images scanned directly from X-ray film and have been graphically adjusted in Adobe Photoshop CS to optimise brightness and contrast. The level of Pomc mRNA expression and relative numbers of Pomc mRNA-expressing cells (white labelling on dark background) were similar in the anterior pituitaries from wild-type (G) or Avpr1b KO (H) mice. There was no gross histological differences in the appearances of the

adrenal glands between the two genotypes (I, wild-type; J, KO). IL, intermediate lobe of the pituitary; M, adrenal medulla; C, adrenal cortex.

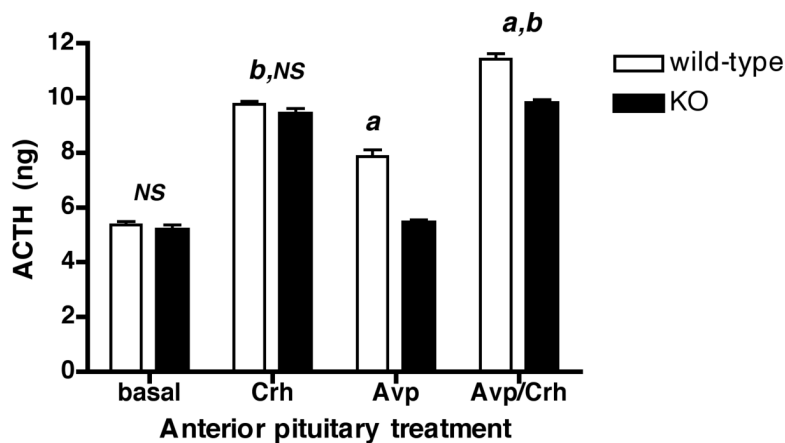


Figure 2.

Vasopressin does not stimulate ACTH secretion from anterior pituitary segments obtained from Avpr1b KO mice.

Isolated anterior pituitary segments from wild-type or Avpr1b KO mice were incubated in vitro with buffer alone (basal), Crh (1nM), Avp (10nM), or a combination of both ACTH secretagogues (Avp/Crh). Values are mean total ACTH (ng)/well \pm SEM, n = 4 separate pituitary preparations. **a**, $P < 0.001$ Avp KO vs. Avp wild-type; Avp/Crh KO vs. Avp/Crh wild-type; Avp wild-type vs. basal wild-type; Avp/Crh wild-type vs. Crh wild-type. **b**, $P < 0.0001$ Avp/Crh KO vs. Avp KO; Avp/Crh KO vs. basal KO; Crh KO vs. basal KO; Crh wild-type vs. Crh basal; Avp/Crh wild-type vs. Avp wild-type; Avp/Crh wild-type vs. basal wild-type. **NS**, not significant ($P > 0.05$) KO vs. wild-type basal; Crh wild-type vs. Crh KO; Crh KO vs. Avp/Crh KO; basal KO vs. Avp KO.

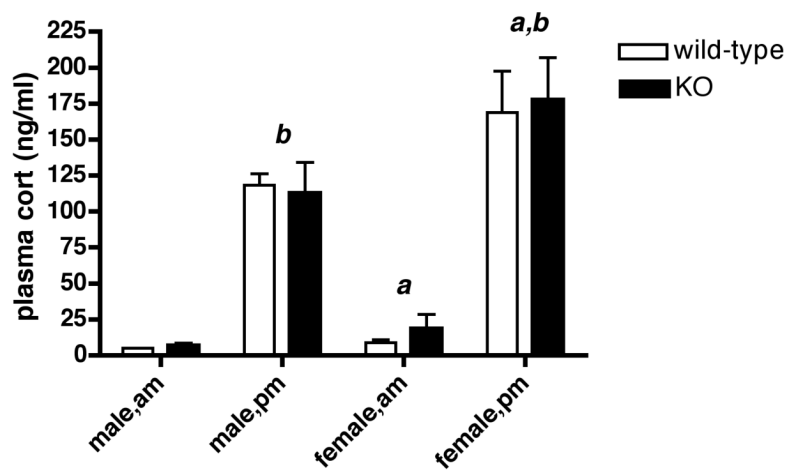


Figure 3.

Diurnal variation in plasma CORT secretion in wild-type and *Avpr1b* KO mice.

Values are mean \pm SEM, $n = 3-5$ samples for each time-point. *a*, $P < 0.05$ female am vs. male am; female pm vs. male pm. *b*, $P < 0.0001$ male am vs. male pm; female am vs. female pm.

There were no significant differences ($P > 0.05$) between am or pm values for male or female mice of both genotypes.

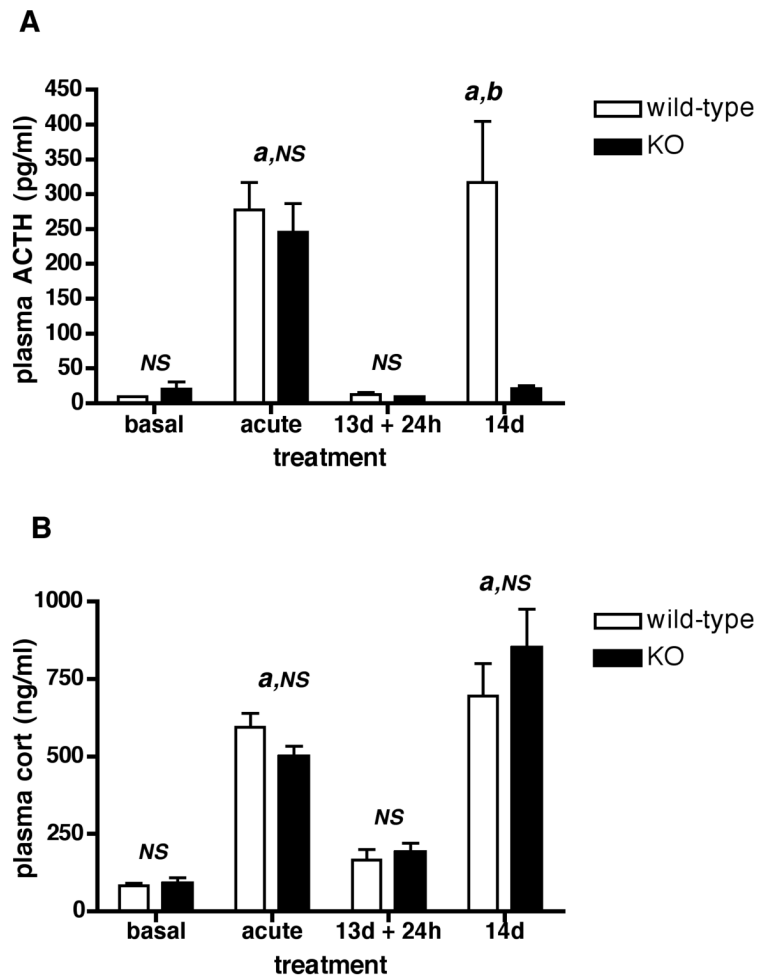


Figure 4.

Effect of acute and repeated restraint stress on plasma ACTH (A) and CORT (B) levels in Avpr1b mutant and control mice.

basal = control, naïve mice briefly handled 30 min prior to sacrifice; acute = mice subjected to 30 min acute restraint; 13d + 24h = 24 h after 13 daily 30 min restraint sessions; 14d = daily restraint for 14 d, sacrifice immediately following the last 30 min restraint session. Values are mean \pm SEM, $n = 4-5$ mice/group. Four out of five 14d restraint Avpr1b KO samples had detectable ACTH; in contrast, the vast majority (14/16) of samples in the Avpr1b KO and wild-type basal and 13d + 24h groups had no measurable ACTH (the assay limit of 10 pg/ml was recorded for these samples). **a**, $P < 0.001$ 13d restraint + 24h wild-type vs. 14d restraint KO; **b**, $P < 0.0001$ acute restraint vs. basal; 14d restraint wild-type vs. 14d restraint KO (ACTH only). *NS*, not significant ($P > 0.05$) basal wild-type vs. basal KO; acute wild-type vs. acute KO; 13d + 24h wild-type vs. 13d + 24h KO; 14d wild-type vs. 14d KO (CORT only).

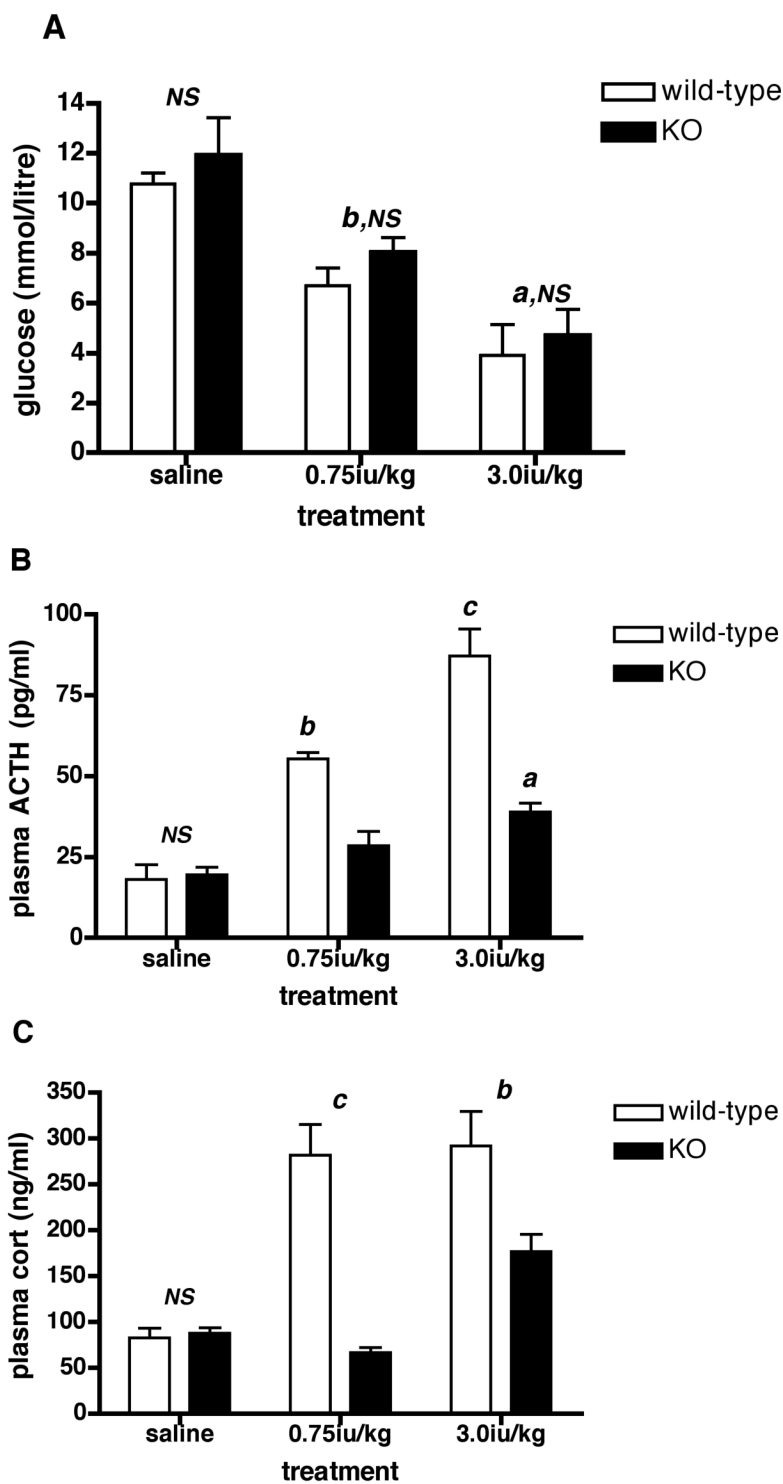


Figure 5. Levels of plasma glucose (A), ACTH (B) and CORT (C) following peripheral (ip) administration of insulin (Actrapid) to Avpr1b-deficient mice. Mice were sacrificed 1 h after treatment with saline vehicle, 0.75iu/kg or 3.0iu/kg insulin. Values are mean \pm SEM, n = 3-4 mice/treatment. Plasma glucose = A: a, $P < 0.05$ 0.75iu/kg

insulin vs. 3.0iu/kg insulin. **b**, $P < 0.01$ saline vs. insulin-injected. **b**, $P < 0.01$ saline vs. insulin-injected. *NS*, no significant difference ($P > 0.05$) saline wild-type vs. saline KO; 0.75iu/kg insulin wild-type vs. 0.75iu/kg insulin KO; 3.0iu/kg insulin wild-type vs. 3.0iu/kg insulin KO. Plasma ACTH = **B**: **a**, $P < 0.05$ 3.0iu/kg insulin KO vs. saline KO. **b**, $P < 0.01$ 0.75iu/kg insulin wild-type vs. 0.75iu/kg insulin KO; 0.75iu/kg insulin wild-type vs. saline wild-type; 3.0iu/kg insulin wild-type vs. 0.75iu/kg insulin wild-type. **c**, $P < 0.001$ 3.0iu/kg insulin wild-type vs. 3.0iu/kg insulin KO; 3.0iu/kg insulin wild-type vs. saline wild-type. *NS*, no significant difference ($P > 0.05$) saline wild-type vs. saline KO. Plasma CORT = **C**: **b**, $P < 0.01$ 3.0iu/kg insulin wild-type vs. 3.0iu/kg insulin KO; 0.75iu/kg insulin KO vs. 3.0iu/kg insulin KO. **c**, $P < 0.001$ 0.75iu/kg insulin wild-type vs. 0.75iu/kg insulin KO. *NS*, no significant difference ($P > 0.05$) saline wild-type vs. saline KO.

Table 1

Mean PVN Avp, Oxt, and Crh, and anterior pituitary pomc mRNA levels as a percentage of the wild-type, control values (n = 4 mice/group)

Group	Avp mRNA	Oxt mRNA	Crh mRNA	pomc mRNA
wild-type	100 (2)	100 (4)	100 (13)	100 (4)
KO	105 (3)	94 (4)	109 (12)	95 (7)

There are no significant differences ($P > 0.05$) for relative Avp, Oxt, Crh or pomc mRNA levels between un-stressed, adult (8-12 weeks), male Avpr1b KOs and wild-type mice. Results are expressed as % mean (\pm SEM).