Stress-dependent and gender-specific neuroregulatory roles of the apelin receptor in the hypothalamic–pituitary–adrenal axis response to acute stress

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Abstract

The neuropeptide apelin is expressed in hypothalamic paraventricular and supraoptic nuclei and mediates its effects via activation of the apelin receptor (APJ). Evidence suggests a role for apelin and APJ in mediating the neuroendocrine response to stress. To understand the physiological role of APJ in regulation of the hypothalamic–pituitary–adrenal (HPA) axis, we measured ACTH and corticosterone (CORT) plasma levels in male and female mice lacking APJ (APJ knockout, APJ KO) and in wild-type controls, in response to a variety of acute stressors. Exposure to mild restraint, systemic injection of lipopolysaccharide (LPS), insulin-induced hypoglycaemia and forced swim (FS) stressors elevated plasma ACTH and CORT levels in wild-type mice. Acute mild restraint significantly increased plasma ACTH and CORT to a similar level in APJ KO mice as in wild-type mice. However, an intact APJ was required for a conventional ACTH, but not CORT, response to LPS administration in male mice and to insulin-induced hypoglycaemia in male and female mice. By contrast, APJ KO mice displayed an impaired CORT response to acute FS stress, regardless of gender. These data indicate that APJ has a role in regulation of the HPA axis response to some acute stressors and has a gender-specific function in peripheral immune activation of the HPA axis.

Introduction

Apelin, a 36 amino acid peptide, is synthesised as part of a 77 amino acid prepropeptide precursor (Tatemoto *et al.* 1998) in specific hypothalamic neurones, and appears to mediate its effects via a single G protein-coupled receptor subtype, the apelin receptor (APJ). In addition to apelin-36, other possible isoforms of the apelin peptide, including apelin-17, apelin-13 and the pyroglutamyl form of apelin-13 ([Pyr[1]]-apelin-13), also bind to and activate APJ (Zou *et al.* 2000,

Medhurst *et al.* 2003). Apelin and APJ mRNA and protein are expressed in a variety of organs including brain, pituitary gland, heart, lung, adipose tissue and gastrointestinal tract, and are known key regulators of central and peripheral responses to multiple homeostatic perturbations. These include regulation of fluid (O'Carroll & Lolait 2003) and cardiovascular homeostasis (Ishida *et al.* 2004), the stress response (O'Carroll *et al.* 2003), food intake

(Taheri *et al.* 2002), gastric cell proliferation (Wang *et al.* 2004) and angiogenesis (Cox *et al.* 2006).

The apelinergic system has a widespread but selective expression in the CNS. APJ mRNA expression and apelin-immunoreactivity (apelin-ir) are present in the medial parvocellular regions of the hypothalamic paraventricular nucleus (pPVN) and scattered magnocellular neurones of the PVN (mPVN) and supraoptic nucleus (SON; Lee *et al.* 2000, O'Carroll *et al.* 2000), as well as in extrahypothalamic structures, in particular the cerebroventricular system (Lee *et al.* 2000, Reaux *et al.* 2002), lower brainstem structures (Reaux *et al.* 2002), and also in the anterior lobe of the pituitary where both receptor and ligand are expressed in corticotrophs (Reaux-Le Goazigo *et al.* 2007). The *APJ* (*APLNR*) gene is also expressed within a proportion of mPVN and SON vasopressinergic neurones projecting to the posterior pituitary (Reaux *et al.* 2001, O'Carroll & Lolait 2003).

Many of the central effects of apelin are attributed to its expression in these PVN hypothalamic neuronal circuits that are key structures in the regulation of endocrine and autonomic responses for the maintenance of homeostasis. Homeostatic challenges trigger a number of physiological responses that are critical to life and normally allow adaptation to cope with the disturbance. The major endocrine response to stressful events is activation of the hypothalamic–pituitary–adrenal (HPA) axis, and the key CNS site integrating neuroendocrine adjustments to stress is the hypothalamic PVN. The presence of APJ transcripts and apelin-ir in the pPVN and in the pituitary suggested that the peptide may be involved in adenohypophysial hormone release.

A role for apelin as a neurohormone pivotal to the neuroendocrine response to stress has been suggested by observations that central administration of apelin increases c-*Fos* expression in the PVN (Kagiyama *et al.* 2005) and increases the secretion of both plasma ACTH and corticosterone (CORT; Taheri *et al.* 2002, Jaszberenyi *et al.* 2004). Apelin may potentially stimulate ACTH secretion either directly at the pituitary corticotroph level (Reaux-Le Goazigo *et al.* 2007), or via an indirect action on the hypothalamus involving the cooperative action of arginine vasopressin (AVP) and corticotropin-releasing-hormone (CRH). Apelin stimulates both AVP and CRH release from hypothalamic explants *in vitro* (Taheri *et al.* 2002), while we have shown that the effects of apelin on HPA axis neuroendocrine function are mediated through both CRH- and AVP-dependent mechanisms (Newson *et al.* 2009). While a role for APJ in facilitating parvocellular function under conditions of stress is supported by studies showing increased levels of APJ

mRNA expression in the pPVN resulting from acute and chronic stress, and following adrenalectomy (O'Carroll *et al.* 2003), the direct effect of a targeted disruption of APJ on HPA axis activity has not been investigated.

To test the hypothesis that activation of the apelin/APJ system is important in the HPA axis response to acute stress, we investigated the pituitary–adrenal response (measured by plasma ACTH and CORT levels) in APJ knockout (APJ KO) and wild-type mice to four acute stressors from a range of stressor categories: mild restraint (physical), lipopolysaccharide (LPS) challenge (immune), insulin-induced hypoglycaemia (metabolic) and forced swim (FS) (physical/psychological).

Materials and methods

Animals

Adult male and female littermates (a mix of the C57BL/6J and 129X1/SvJ strains) of crosses using mice heterozygous for the APJ mutation (Roberts *et al.* 2009; Deltagen, San Mateo, CA, USA) were bred and maintained on site. All female mice were used at random stages of the oestrous cycle. Before the experimental period, mice were housed under a 12 h light:12 h darkness cycle (lights on 0700 h) and controlled temperature ($21 \pm 2\,^{\circ}C$) with free access to standard laboratory chow and water. Approximately, 24 h before experimentation, mice were individually housed. All measurements and procedures were conducted between 0900 and 1200 h. All procedures were conducted in accordance with the Animal Scientific Procedures Act (1986) United Kingdom and the appropriate University of Bristol Ethical Review Process. Offspring were genotyped at weaning using PCR analysis of DNA isolated from tail clips, as described previously (Roberts *et al.* 2009).

Experiment 1: basal HPA axis parameters

For basal histology, morning plasma CORT levels, and *in situ* hybridisation histochemistry, male and female wild-type and APJ KO mice were killed 1.5 h after lights on. Trunk blood was collected by decapitation (within 5 s after removal from the home cage). Brains were dissected, frozen on dry-ice, and stored at $-80\,^{\circ}C$ until sectioning. Pituitaries and adrenals from male mice were post-fixed in Bouin's solution (Sigma–Aldrich) for 4 h at RT and destained in 70% ethanol. The tissue was embedded in paraffin and sections (6 μm) cut for staining by haemotoxylin-eosin (Veterinary Diagnostic Histopathology Service, Department of Pathology and Microbiology,

University of Bristol, UK). For evening plasma CORT levels, mice were killed 1.5 h before lights off.

Experiment 2: mild restraint stress

Mild restraint stress was performed using 50-ml (Falcon, BD Biosciences, Oxford, UK) plastic tubes to achieve a comparable degree of restraint for each animal. Age-matched male and female wild-type or APJ KO mice were restrained for 30 min before being killed. Control groups were handled before being killed. Based on previous studies on acute stress in mice (Lolait et al. 2007, Stewart et al. 2008) and the number of mice at our disposal, a single time point (30 min restraint) was chosen to optimise detection of both restraint-induced plasma ACTH and CORT.

Experiment 3: LPS challenge

LPS from Escherichia coli (serotype 055:B55; Sigma) was diluted in sterile, apyrogenic 0.9% saline and aliquots (10 mg/ml) stored at $-80\,°C$. The same serotype and lot number was used for each experiment. Frozen aliquots were thawed on ice and further diluted in saline to 500 μg/ml. Age-matched male and female wild-type or APJ KO mice ($\sim$30 g) were injected i.p. with diluted LPS (200 μl; 100 μg) or vehicle (0.9% saline), and killed by decapitation 30 min later. The 30 min time-point was chosen, so the extent of HPA axis activation could be compared with that elicited by other stressors (e.g. restraint) used on mice in our laboratory (Lolait et al. 2007). The dose of LPS used in this study is higher than the $\sim$83 μg/30 g mouse often employed (Schotanus et al. 1994, Beishuizen & Thijs 2003); however, no sickness behaviour was observed in animals 30 min post injection.

Experiment 4: insulin-induced hypoglycaemia

Age-matched male and female wild-type or APJ KO mice were fasted overnight (12 h) with water freely available. On the day of experimentation mice were injected i.p. with insulin (3.0 IU/kg diluted in vehicle; Actrapid human insulin, 100 IU/ml, from Novo Nordisk, Bagsvaerd, Denmark) or vehicle (0.9% saline; controls) and killed 1 h later. Blood glucose levels were measured using a commercially available glucose meter (Contour, Bayer).

Experiment 5: FS

Age-matched male and female wild-type or APJ KO mice were placed into a cylindrical Pyrex beaker (height 35 cm, diameter 24 cm) filled with water to a depth of 20 cm at $22\pm2\,°C$ for 5 min. Mice were killed by decapitation immediately after FS. Control groups were handled and killed immediately.

Hormone analysis

All experiments were performed at least twice and hormone concentrations in samples were measured in triplicate. Mice were killed by decapitation and trunk blood was collected into chilled heparinised tubes. Plasma was obtained by centrifugation and stored at $-20\,°C$ until assayed for ACTH and CORT. Total plasma ACTH concentration was quantified using a two-site ELISA kit (IDS, Tyne & Wear, UK) with a sensitivity of 0.46 pg/ml and intra-assay variation of <10% (supplier's product data (http://www.idsltd.com/Downloads/DZ-7023.pdf)). Plasma concentrations of CORT were measured using an enzyme immunoassay (EIA) kit (IDS), with a sensitivity of 0.55 ng/ml with an intra-assay variation of <10% (product data at: http://www.idsltd.com/Downloads/AC-14PL.pdf). For both ACTH ELISA and CORT EIA, absorbance readings were taken at 450 nm using a Versamax plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

In-situ hybridisation histochemistry

Sections (12 μm) of tissue were cut, thaw-mounted onto poly-L-lysine-coated slides (VWR, Lutterworth, UK) and stored at $-80\,°C$ until hybridisation. Mouse CRH and AVP primers, end-labelled with ^{35}S-dATP (Perkin Elmer, Cambridge, UK), were as described previously (Lolait et al. 2007). Hybridisation was performed as described in detail (http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html). Slides were exposed to Amersham Hyperfilm MP film together with ^{14}C-labelled standards (GE Healthcare, Buckinghamshire, UK) for 2 days for AVP and 2 weeks for CRH at RT. No specific hybridisation was detected with sense probes.

Statistical analysis

All results are expressed as the mean$\pm$S.E.M. Plasma hormone concentrations were analysed by two-way ANOVA followed by Bonferroni's post-hoc analysis using GraphPad Prism (version 4.0b) software (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

Experiment 1: basal HPA axis parameters

No differences were seen in the expression levels or distribution of AVP or CRH mRNA expression in the PVN of wild-type and APJ KO mice (Fig. 1A, B, C and D). Histological analysis of haematoxylin and eosin-stained

Figure 1

Basal HPA axis parameters in APJ KO mice. Expression of AVP (A and B) and CRH (C and D) mRNAs in the PVN (arrowed) of wild-type (WT, left panels) or APJ KO (KO, right panels) mice. A, B, C and D images are scanned directly from X-ray film. There was no gross histological difference in the appearances of the pituitary (E and F) or adrenal glands (G and H) between the two genotypes (E and G, WT; F and H, APJ KO). Diurnal variation in plasma CORT secretion in male (I) and female (J) WT and APJ KO mice. Values are mean ± s.e.m.; $n = 3$–6 samples for each time point. Statistical analysis: two-way ANOVA: male plasma CORT: genotype effect $F_{(1,9)} = 3.74$, $P = 0.0851$; time effect $F_{(1,9)} = 56.80$, $P < 0.0001$; interaction of genotype × time $F_{(1,9)} = 0.01$, $P = 0.9252$. Female plasma CORT: genotype effect $F_{(1,12)} = 0.71$, $P = 0.4146$; time effect $F_{(1,12)} = 7.94$, $P = 0.0155$; interaction of genotype × time $F_{(1,12)} = 0.15$, $P = 0.7044$. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as $**P < 0.001$, $*P < 0.01$, NS, not significant. i, intermediate lobe of the pituitary; a, anterior lobe of the pituitary; p, posterior lobe of the pituitary; cx, adrenal cortex; m, adrenal medulla.

sections from pituitary and adrenal glands did not reveal any apparent morphological differences in APJ KO mice compared with wild-type littermates (Fig. 1E, F, G and H). The characteristic diurnal rise in circulating CORT that occurs in the afternoon was intact in male (Fig. 1I) and female (Fig. 1J) APJ KO mice. Plasma CORT levels in the morning and afternoon were indistinguishable between the genotypes. Basal morning and afternoon plasma CORT levels were not significantly different between male and female mice, irrespective of genotype.
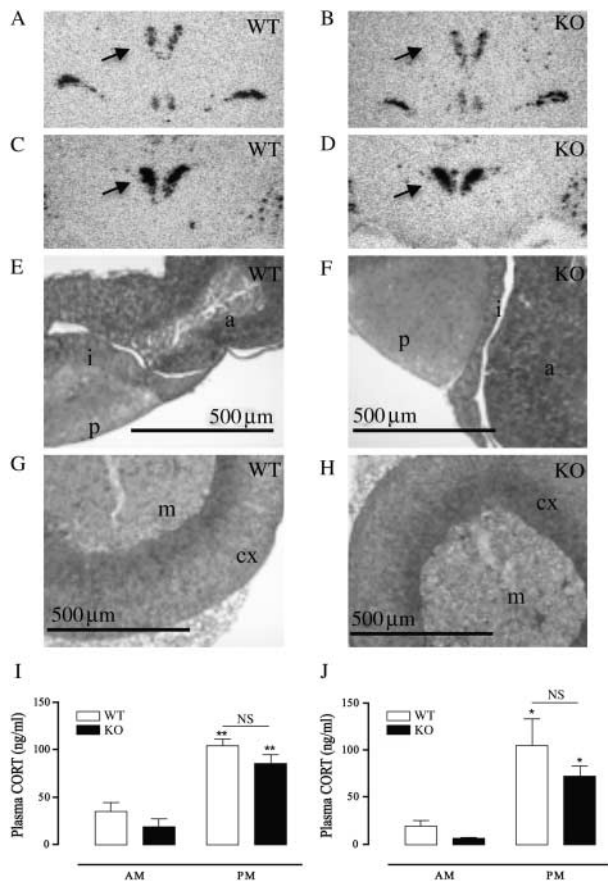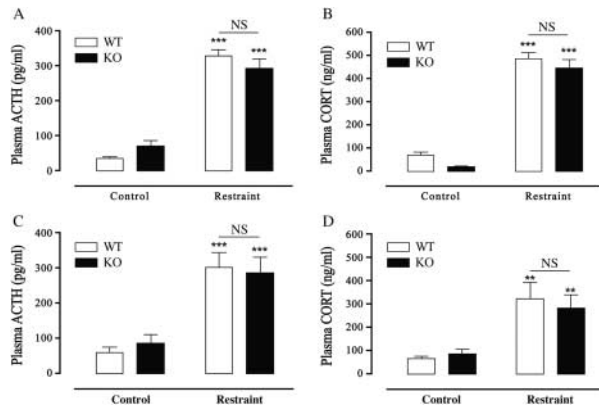
Experiment 2: effect of acute mild restraint on HPA axis activity in male and female wild-type and APJ KO mice

Single exposure to mild restraint in male wild-type mice showed a significant increase in plasma ACTH concentrations immediately after 30 min mild restraint (Fig. 2A), in keeping with a strong HPA axis response to acute stress. Plasma ACTH concentrations in male APJ KO mice also showed an increase after 30 min mild restraint, and were not significantly different from those observed in the wild-type mouse. Plasma CORT concentrations in response to acute mild restraint stress within the male wild-type group were also increased compared with wild-type handled controls (Fig. 2B). There was a similar response pattern in plasma CORT concentrations within the male APJ KO group, with plasma CORT concentrations not significantly different to their wild-type counterparts.

The plasma ACTH response to 30 min restraint stress in female wild-type and APJ KO mice paralleled that observed in male mice. There was a significant increase in wild-type and APJ KO plasma ACTH in response to restraint as compared with the control groups (Fig. 2C). Similarly, a robust CORT response was generated by 30 min restraint in both female wild-type and APJ KO mice that was not significantly different (Fig. 2D).

Experiment 3: effect of acute LPS challenge on HPA axis activity in male and female wild-type and APJ KO mice

LPS evoked a significant increase in plasma ACTH levels in male wild-type mice, 30 min after peripheral (i.p.) challenge (Fig. 3A). By contrast, plasma ACTH levels in LPS-challenged male APJ KO mice were significantly attenuated compared with LPS wild-type mice (∼38%). However plasma ACTH levels were still significantly increased in male APJ KO mice after LPS challenge compared with saline-treated APJ KO controls. Plasma CORT levels were also increased in male wild-type LPS-challenged mice (Fig. 3B). However, in contrast to

Figure 2

The effect of acute mild restraint stress on plasma ACTH (A and C) and CORT (B and D) levels in male (A and B) and female (C and D) wild-type (WT) and APJ KO (KO) mice. Mice were restrained for a period of 30 min and killed immediately. CONT indicates naïve mice briefly handled 30 min before being killed. Values are expressed as the mean ± s.e.m.; $n = 5$–7 mice/group. Statistical analysis: two-way ANOVA: male plasma ACTH: genotype effect $F_{(1,17)} = 0.19$, $P = 0.6707$; treatment effects $F_{(1,17)} = 165.35$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,17)} = 2.37$, $P = 0.1421$. Male plasma CORT: genotype effect $F_{(1,23)} = 3.77$, $P = 0.0644$; treatment effects $F_{(1,23)} = 326.45$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,23)} = 0.05$, $P = 0.8335$. Female plasma ACTH: genotype effect $F_{(1,15)} = 0.05$, $P = 0.8346$; treatment effects $F_{(1,15)} = 55.04$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,15)} = 0.48$, $P = 0.4998$. Female plasma CORT: genotype effect $F_{(1,9)} = 0.03$, $P = 0.8576$; treatment effects $F_{(1,9)} = 22.08$, $P < 0.001$; interaction of genotype × treatment $F_{(1,9)} = 0.36$, $P = 0.5642$. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as ***$P < 0.0001$, **$P < 0.001$, NS, not significant.

the response profile observed in plasma ACTH concentrations, plasma CORT concentrations were increased in APJ KO mice in response to LPS challenge and were not significantly reduced in comparison with their wild-type counterparts (Fig. 3B).

The plasma ACTH response to LPS challenge in female APJ KO mice differed to that observed in male APJ KO mice. Plasma ACTH concentrations in response to LPS challenge within the wild-type group significantly increased compared with wild-type handled controls (Fig. 3C), and, unlike male APJ KO mice, there was a similar response pattern in the female APJ KO group, with plasma ACTH concentrations not significantly different to their wild-type counterparts. Similarly, there was a significant increase in female wild-type and APJ KO plasma CORT in response to LPS challenge as compared with the control groups (Fig. 3D), with plasma CORT concentrations in APJ KO mice not significantly different to their wild-type counterparts.

Experiment 4: effect of Insulin-induced hypoglycaemia on HPA axis activity in male and female wild-type and APJ KO mice

Insulin (3.0 IU/kg) reduced plasma glucose by ∼80% in male and female wild-type and APJ KO mice (Fig. 4A and B). The hypoglycaemia induced by insulin treatment was associated with significant increases in plasma ACTH levels in male (Fig. 5A) and female (Fig. 5C) wild-type mice. However, no significant increase in plasma ACTH levels was seen in male or female insulin-injected APJ KO mice when compared with vehicle-injected APJ KO mice (Fig. 5A and C). There were no significant differences in basal saline-injected or insulin-induced ACTH levels between genotypes.

Plasma CORT concentrations in response to insulin treatment in male and female wild-type and APJ KO mice were significantly increased compared with wild-type saline-injected controls (Fig. 5B and D). There were
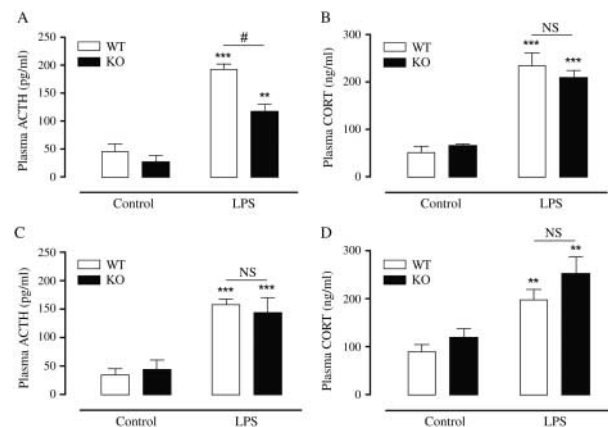
Figure 3

The effect of LPS administration on plasma ACTH (A and C) and CORT (B and D) levels in male (A and B) and female (C and D) wild-type (WT) and APJ KO (KO) mice. Animals were killed 30 min following LPS (100 μg i.p.) or saline challenge. Values are mean ± s.e.m., $n = 4$–5 mice/treatment group. Statistical analysis: two-way ANOVA: male plasma ACTH: genotype effect $F_{(1,12)} = 13.38$, $P = 0.0033$; treatment effects $F_{(1,12)} = 88.99$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,12)} = 5.02$, $P = 0.0447$. Male plasma CORT: genotype effect $F_{(1,16)} = 1.32$, $P = 0.7940$; treatment effects $F_{(1,16)} = 89.55$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,16)} = 1.32$, $P = 0.2675$. Female plasma ACTH: genotype effect $F_{(1,20)} = 0.01$, $P = 0.9144$; treatment effects $F_{(1,20)} = 41.74$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,20)} = 0.49$, $P = 0.4940$. Female plasma CORT: genotype effect $F_{(1,18)} = 2.95$, $P = 0.1031$; treatment effects $F_{(1,18)} = 23.14$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,18)} = 0.26$, $P = 0.6168$. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as ***$P < 0.0001$, **$P < 0.001$, #$P < 0.05$, NS, not significant.
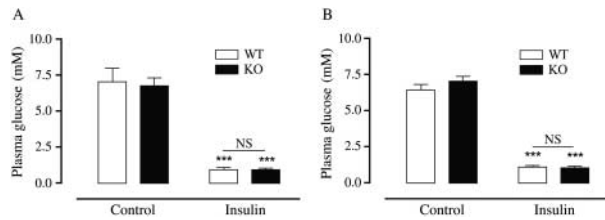
Figure 4

Blood glucose levels in male (A) and female (B) wild-type (WT) and APJ KO (KO) mice. Trunk blood was collected 1 h following i.p. injection of 0.9% saline or insulin (3.0 IU/kg). Values are mean±s.e.m., n=5–6 mice/treatment. Data shown are mean±s.e.m.; n=5–6 mice/treatment group. Statistical analysis: two-way ANOVA: male blood glucose: genotype effect $F_{(1,21)}$=0.12, P=0.7370; treatment effects $F_{(1,21)}$=153.67, P<0.0001; interaction of genotype×treatment $F_{(1,21)}$=0.07, P=0.7955. Female blood glucose: genotype effect $F_{(1,23)}$=1.02, P=0.3222; treatment effects $F_{(1,23)}$=419.82, P<0.0001; interaction of genotype×treatment $F_{(1,23)}$=1.13, P=0.2986. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as ***P<0.0001, NS, not significant.

no significant differences in basal saline-injected or insulin-induced plasma CORT levels between genotypes.

Experiment 5: effect of acute FS on plasma ACTH and CORT concentrations in male and female wild-type and APJ KO mice

In male wild-type and APJ KO mice, single exposure to FS for a period of 5 min resulted in significant increases in plasma ACTH levels compared with handled controls (Fig. 6A). In contrast to plasma ACTH levels, however, CORT levels following acute FS were significantly attenuated in male APJ KO mice compared with the wild-type group (∼29%) (Fig. 6B).

The plasma ACTH response to a single exposure to FS in female APJ KO mice paralleled that observed in male APJ KO mice. A significant increase in both wild-type and APJ KO plasma ACTH levels was seen in response to FS compared with handled controls (Fig. 6C). Plasma CORT concentrations following acute FS within the wild-type group were also significantly increased compared with wild-type handled controls (Fig. 6D). However, plasma CORT concentrations were significantly attenuated in the female APJ KO mice compared with the wild-type group (∼37%).

Discussion

We have used APJ KO mice to investigate the role of apelin/APJ in the HPA axis response to acute stress. Male and female APJ KO mice have normal basal levels of *Avp*

and *Crh* mRNA expression in the hypothalamic PVN; normal gross anatomy of the pituitary and adrenal glands; and normal levels of circulating CORT and an intact diurnal CORT rhythm, suggesting resting levels of CRH, AVP and/or other ACTH secretagogues that are sufficient to maintain normal basal HPA axis activity. The present study reveals a stress-dependent and a gender-specific neuroregulatory role for APJ in the HPA axis response to acute stress (see Table 1 for a summary of results). Whereas the ACTH and CORT response to acute mild restraint stress was not compromised in either male or female APJ KO mice, an intact APJ was required for a conventional ACTH, but not CORT, response to LPS administration in male mice and to insulin-induced hypoglycaemia in both male and female mice. Finally, APJ KO mice displayed an impaired CORT response to acute FS stress, regardless of gender. There were no significant differences in basal ACTH or CORT levels between genotypes in any stressors investigated. Thus, while APJ may have a role in the integration of HPA axis responses to some acute stressors,
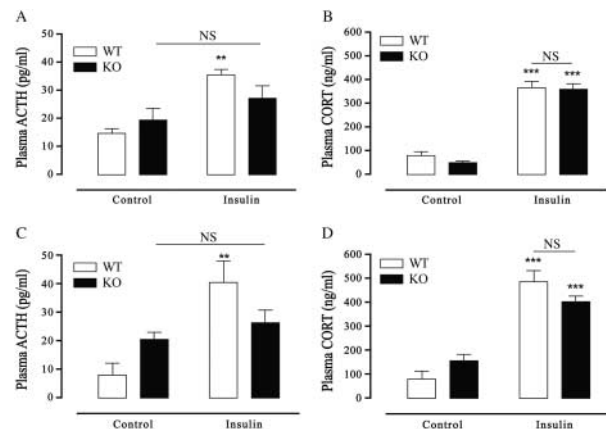
Figure 5

The effect of insulin-induced hypoglycaemia on plasma ACTH (A and C) and CORT (B and D) levels in male (A and B) and female (C and D) wild-type (WT) and APJ KO (KO) mice. Trunk blood was collected 1 h following i.p. injection of 0.9% saline or insulin (3.0 IU/kg). Values are mean±s.e.m., n=5–6 mice/treatment. Statistical analysis: two-way ANOVA: male plasma ACTH: genotype effect $F_{(1,15)}$=0.22, P=0.6473; treatment effects $F_{(1,15)}$=12.34, P=0.0031; interaction of genotype×treatment $F_{(1,15)}$=2.55, P=0.1309. Male plasma CORT: genotype effect $F_{(1,18)}$=0.58, P=0.4552; treatment effects $F_{(1,18)}$=169.41, P<0.0001; interaction of genotype×treatment $F_{(1,18)}$=0.26, P=0.6165. Female plasma ACTH: genotype effects $F_{(1,17)}$=0.02, P=0.8793; treatment effects $F_{(1,17)}$=9.64, P=0.0064; interaction of genotype×treatment $F_{(1,17)}$=4.68, P=0.0451. Female plasma CORT: genotype effects $F_{(1,22)}$=0.01, P=0.9255; treatment effects $F_{(1,22)}$=81.60, P<0.0001; interaction of genotype×treatment $F_{(1,22)}$=4.94, P=0.0368. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as ***P<0.0001, **P<0.001, NS, not significant.
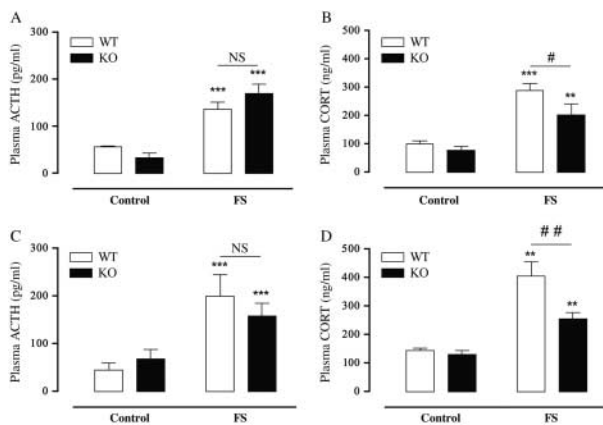
Figure 6

The effect of acute stress on plasma ACTH (A and C) and CORT (B and D) concentrations in male (A and B) and female (C and D) wild-type (WT) and APJ KO (KO) mice. Mice were exposed to a single FS for 5 min at 22 °C and killed immediately; CONT indicates mice briefly handled 5 min before killed. Results are expressed as the mean ± s.e.m.; $n = 4$–7 mice/group. Statistical analysis: two-way ANOVA: male plasma ACTH: genotype effects $F_{(1,17)} = 0.07$, $P = 0.7958$; treatment effects $F_{(1,17)} = 33.60$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,17)} = 0.07$, $P = 0.7958$; interaction of genotype × treatment $F_{(1,17)} = 2.20$, $P = 0.1562$. Male plasma CORT: genotype effects $F_{(2,18)} = 3.69$, $P = 0.0454$; treatment effects $F_{(1,18)} = 71.56$, $P < 0.0001$; interaction of genotype × treatment $F_{(2,18)} = 1.76$, $P = 0.1999$. Female plasma ACTH: genotype effects $F_{(1,18)} = 0.10$, $P = 0.7509$; treatment effects $F_{(1,18)} = 18.52$, $P = 0.0004$; interaction of genotype × treatment $F_{(1,18)} = 1.34$, $P = 0.2623$. Female plasma CORT: genotype effects $F_{(1,17)} = 6.74$, $P = 0.0188$; treatment effects $F_{(1,17)} = 36.85$, $P < 0.0001$; interaction of genotype × treatment $F_{(1,17)} = 4.72$, $P = 0.0443$. Significant differences between treatments to vehicle control or between conditions (line) as derived from Bonferroni *post-hoc* analysis are denoted as ***$P < 0.0001$, **$P < 0.001$, ##$P < 0.01$, #$P < 0.05$, NS, not significant.

this role appears not to be specific to a particular category of stress but may be associated with stressors with established vasopressinergic involvement.

It is well documented that acute restraint stress strongly stimulates the HPA axis, transiently increasing plasma ACTH and CORT levels (Harbuz & Lightman 1992), and inherent to this response is the synergistic action of CRH and AVP. CRH appears to be the dominant ACTH secretagogue and, in response to an acute restraint stress, CRH mRNA and peptide expression increase in the hypothalamus (Moldow *et al.* 1987, Harbuz & Lightman 1992). However, AVP is also involved in ACTH release (Linton *et al.* 1985, Guillaume *et al.* 1992), and our studies with V1bR-deficient mice have shown that a functional V1bR is required for an intact pituitary ACTH response to acute mild restraint (Stewart *et al.* 2008), as used in this study. Our data show a normal pituitary–adrenal response to acute restraint in APJ KO, suggesting that the major ACTH secretagogues override any contribution of APJ to

HPA axis responses to this stress. We cannot discount, however, that alterations in ACTH release and CORT secretion may have occurred at time points other than the time point investigated.

In contrast to our studies on restraint stress, hormone- or gender-specific deficits were observed in the HPA axis response to the acute challenges of LPS administration, insulin-induced hypoglycaemia, and FS in APJ KO mice. These three stressors have common and stress-specific peripheral and/or central hierarchal pathways converging on CRH- and AVP-expressing neurones in the pPVN (see Pacak & Palkovits (2001) for review). Peripheral administration of endotoxins such as LPS, a broad-acting immune stressor, is frequently used to examine the HPA axis response to an immune stimulus. LPS stimulates both cytokines and tumour necrosis factor-α (Konsman *et al.* 2002), and has been shown to potentially activate the HPA axis by a variety of mechanisms. While these mechanisms have been outlined elsewhere (e.g. see Lolait *et al.* (2007)), the consensus view is that, in the acute phase of the response, LPS acts to stimulate the activity of CRH or AVP neurones, either directly or indirectly via cytokines or other afferent mechanisms (Aubry *et al.* 1997, Turnbull & Rivier 1999, Rivest *et al.* 2000, Beishuizen & Thijs 2003, John & Buckingham 2003). Evidence from studies on the rat have suggested that higher doses of LPS, as used in this study, may activate hypothalamic AVP synthesis and release (Xia & Krukoff 2003), and that AVP is a major contributor to LPS-induced HPA axis activity (Zelena *et al.* 2009).

In this study we measured plasma ACTH and CORT levels in APJ KO and wild-type control mice in response to LPS administration. Our data support previous studies showing increased HPA axis activity following LPS administration (Rivier 1993). APJ KO male mice had markedly compromised plasma ACTH responses to acute

Table 1   Summary of results. Plasma ACTH and CORT response to the stressors employed in this study between APJ KO mice and their wild-type counterparts

APJ KO mice vs wild-type mice acute stressor	ACTH	CORT
Mild restraint	NS (♂+♀)	NS (♂+♀)
LPS	↓ (♂); NS (♀)	NS (♂+♀)
Insulin-induced hypoglycaemia	↓ (♂+♀)	NS (♂+♀)
FS	NS (♂+♀)	↓ (♂+♀)

NS denotes no significant differences in the ACTH and CORT response between stress-induced APJ KO and wild-type mice. ↓ illustrates a significant attenuation in the ACTH or CORT response in stress-induced APJ KO male (♂) or female (♀) mice when compared with their wild-type counterparts.

LPS when compared with wild-type littermates – the first demonstration, to our knowledge, of a role for APJ in peripheral immune activation of the HPA axis. A role for apelin in regulating immune responses in neonates has previously been suggested (Habata *et al.* 1999). Activation of APJ has been shown to stimulate both AVP and CRH release *in vitro* from hypothalamic explants (Taheri *et al.* 2002). The loss of functional APJs in APJ KO mice may thus act to remove a stimulatory drive to AVP and/or CRH neurones within the hypothalamus, resulting in a reduced ACTH response to LPS. We hypothesise that, in response to LPS injection, there is a rapid release of AVP that may act synergistically with CRH to drive the early phase of ACTH release – a response that is attenuated in the male APJ KO mouse.

In contrast to that seen in male APJ KO mice, the ACTH response to LPS administration was not impaired in female APJ KO mice. While randomly cycling females were used in our study, the low variability of values within the female groups in all experiments performed suggests little influence of oestrous cycle differences on the HPA axis measurements. Many autoimmune diseases exhibit a significant sexual dimorphism, including autoimmune thyroiditis, systemic lupus erythematosus and rheumatoid arthritis, which have been associated with dysregulation of the HPA axis (Sternberg 1997, Chrousos 2009). There is unequivocal evidence that the HPA and immune axes functionally overlap (Wilder 1995), and our results suggest that APJ has a gender-specific endocrine function in the regulation of neuroendocrine-immune responsiveness.

Conversely, the lack of apelin activity at APJ had no effect on the CORT response to acute LPS administration in either gender. ACTH and CORT responses can dissociate depending on the context of the stressor (Zelena *et al.* 2009), and a similar dissociation between these hormone levels has been previously described in a study on the role of AVP in LPS-induced stress responses (Spiga *et al.* 2009). The low ACTH levels found in LPS-challenged APJ KO mice may be sufficient to induce a normal CORT response. It is also possible that the residual adrenal activity may be mediated by ACTH-independent CORT modulators. Stimulation of ACTH secretion from the anterior pituitary by CRH and AVP following stressful stimuli is the major driving force behind subsequent CORT release from the adrenal cortex; however, CORT secretion can also be influenced by pituitary-independent factors, including adrenal splanchnic innervation (Ulrich-Lai & Engeland 2002), sympathetic activity (Bornstein & Chrousos 1999, Droste *et al.* 2007) and cytokines such as interleukin-6 (Bornstein & Chrousos 1999). In addition to these

peripheral pathways, apelin may activate APJ in the adrenal cortex to stimulate CORT release as, in a recent study, we have shown expression of APJ mRNA and $I^{125}[Pyr^1]$apelin-13-binding sites throughout the mouse adrenal cortex, with little to no presence in the medulla (Pope *et al.* 2012).

We also investigated the effect of a metabolic stress (insulin-induced hypoglycaemia) and a physical/psychological stress (FS) on the HPA axis response in APJ KO mice. Insulin-induced hypoglycaemia has been reported to differentially activate AVP release over that of CRH into the hypophysial portal system (Plotsky *et al.* 1985). In this study, the hypoglycaemia associated with peripheral insulin administration resulted in significantly increased plasma ACTH levels in wild-type but not in APJ KO male and female mice, demonstrating that an intact APJ is required for a conventional acute ACTH response to this stressor. Insulin increases plasma levels of AVP (Baylis & Robertson 1980) as well as stimulating apelin production in adipocytes (Boucher *et al.* 2005), suggesting that both direct and indirect pathways may be involved in the ACTH response observed in APJ KO mice. Dissociation between ACTH and CORT responses was also apparent in the hypoglycaemia studies, with normal CORT responses maintained in APJ KO mice. Our results are similar to those found for AVP in a study on Brattleboro rats, where a major role for AVP in the regulation of hypoglycaemia-induced ACTH, but not CORT, levels was described (Zelena *et al.* 2009). It has been suggested that pathways affecting AVP/CRH release may be differentially activated depending on the dose of insulin administered, with mild hypoglycaemia activating CRH neural pathways and more severe hypoglycaemia (as in this study) mediating HPA responses from more than one pathway. The normal CORT response seen in APJ KO mice may be due to humoral activation of the HPA axis, either by systemic mechanisms that act directly on the medial basal hypothalamus (Weidenfeld *et al.* 1982) or by β-adrenergic, hypothalamic-independent but pituitary-dependent neuronal activity (Mezey *et al.* 1984).

Single exposure to acute FS strongly activates the HPA axis-dependent release of ACTH and CORT (Linthorst *et al.* 2008, Stewart *et al.* 2008) and increases the expression of AVP and perhaps CRH (Wotjak *et al.* 2001, Jiang *et al.* 2004) in the PVN. Our study shows that the ACTH response to an acute FS stress was not compromised in APJ KO mice; however, the CORT response was significantly reduced in both male and female APJ KO mice. A similar, and unexplained, decrease in CORT levels following an increased ACTH response was seen in rats

after acute foot shock stress following V1bR antagonist pretreatment (Zhou *et al.* 2008). The inverse relationship between CORT and ACTH suggests that adrenal activity may not solely depend on the current plasma ACTH levels but may be mediated by ACTH-independent CORT modulators e.g. evidence supports a role for sympathetic innervation in modulation of adrenal glucocorticoid secretion (Engeland & Arnhold 2005). Moreover, the FS paradigm contains both an anxiogenic and a physical component (including effects on body temperature) that involve changes in central neurotransmitter release, which may impinge upon, or otherwise activate, peripheral or sympathetic endocrine responses that a more specific psychological stressor may not affect.

This study represents the first investigation using APJ KO mice to study the role of apelin/APJ in response to acute stressors and suggests that APJ may have a neuromodulatory role in modifying HPA axis activity in response to acute stimuli. We describe a role for APJ in the hypothalamic–pituitary response to LPS and insulin-induced hypoglycaemia, stressors with established vasopressinergic regulation of ACTH secretion, and in the adrenal response to FS, a stressor with strong vasopressinergic involvement. Our data indicate that apelin, acting via APJ, modulates ACTH and CORT release depending on the stressor employed, which may reflect a role in modulation, primarily, of AVP release, and suggest that other peptides cannot compensate for the loss of APJ activity to regulate HPA axis responses to these stressors. Apelin/APJ may therefore act in concert with AVP/V1bR and CRH/CRH type 1 receptor to ensure a normal hypothalamic–pituitary response to some acute stressors, while the role of APJ in the adrenal component of the axis may reflect paracrine/autocrine effects of apelin in the adrenal cortex.

Thus, our study, using targeted deletion of APJ, has established a role for APJ in the integration of neuroendocrine responses to acute stress and has demonstrated that APJ has a gender-specific function in peripheral immune activation of the HPA axis. The APJ KO mice may provide a useful model for further studies to investigate the role of apelin/APJ in HPA axis responses to chronic stress.

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