The effects of apelin on hypothalamic–pituitary–adrenal axis neuroendocrine function are mediated through corticotrophin-releasing factor- and vasopressin-dependent mechanisms

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Abstract

The apelinergic system has a widespread expression in the central nervous system (CNS) including the paraventricular nucleus, supraoptic nucleus and median eminence, and isolated cells of the anterior lobe of the pituitary. This pattern of expression in hypothalamic nuclei known to contain corticotrophin-releasing factor (CRF) and vasopressin (AVP) and to co-ordinate endocrine responses to stress has generated interest in a role for apelin in the modulation of stress, perhaps via the regulation of hormone release from the pituitary. In this study, to determine whether apelin has a central role in the regulation of CRF and AVP neurones, we investigated the effect of i.c.v. administration of pGlu-apelin-13 on neuroendocrine function in male mice pre-treated with the CRF receptor antagonist, α-helical $CRF_{9–41}$, and in mice-lacking functional AVP V1b receptors (V1bR KO). Administration of pGlu-apelin-13 (1 mg/kg i.c.v.) resulted in significant increases in plasma ACTH and corticosterone (CORT), which were significantly reduced by pre-treatment with α-helical $CRF_{9–41}$, indicating the involvement of a CRF-dependent mechanism. Additionally, pGlu-apelin-13-mediated increases in both plasma ACTH and CORT were significantly attenuated in V1bR KO animals when compared with wild-type controls, indicating a role for the vasopressinergic system in the regulation of the effects of apelin on neuroendocrine function. Together, these data confirm that the *in vivo* effects of apelin on hypothalamic–pituitary–adrenal neuroendocrine function appear to be mediated through both CRF- and AVP-dependent mechanisms.

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Introduction

The apelin peptide, isolated from extracts of bovine stomach (Tatemoto *et al.* 1998), selectively binds to the G-protein-coupled apelin receptor (APJ; O'Dowd *et al.* 1993). Apelin, a 36-amino acid peptide, is derived from a 77-amino acid precursor, preproapelin (Tatemoto *et al.* 1998, Lee *et al.* 2000). Although initial studies suggested that apelin-36 was the predominant cleavage product *in vivo*, subsequent studies have suggested that other cleavage products, notably the pyroglutamyl form of apelin-13 (pGlu-apelin-13) and apelin-17, may be more abundant in the rat (De Mota *et al.* 2004, Reaux-Le Goazigo *et al.* 2004). These cleavage products have greater affinity for APJ (Medhurst *et al.* 2003) and exhibit greater biological activity than the parent peptide on extracellular acidification rates, promote APJ internalisation and strongly inhibit forskolin-stimulated cAMP production (Medhurst *et al.* 2003, El Messari *et al.* 2004).

The apelinergic system has a widespread expression in the CNS, including the paraventricular nucleus (PVN), preoptic area, periventricular hypothalamus and ventromedial and dorsomedial nuclei (Lee *et al.* 2000), while high densities of apelin immunoreactive fibres and cell bodies have been detected in the PVN, supraoptic nucleus (SON), the median eminence (Reaux *et al.* 2002) and in the posterior pituitary (Brailoiu *et al.* 2002). Outside the CNS, apelin immunoreactivity and mRNA are present in vascular endothelial cells (Kleinz & Davenport 2004) and other peripheral tissues such as heart, mammary gland, lung and adrenal (Habata *et al.* 1999, O'Carroll *et al.* 2000). The pattern of expression of the rat receptor implies that APJ has tissue-specific functions in adult animals. High levels of APJ mRNA expression are found in the brain, the pineal gland, the parenchyma of the lung, the heart and in isolated cells of the anterior lobe of the pituitary (O'Carroll *et al.* 2000). APJ expression is restricted in the CNS and is particularly striking in the medial parvocellular regions of the hypothalamic PVN (pPVN) and scattered magnocellular neurones of the PVN (mPVN) and SON (De Mota *et al.* 2000, O'Carroll *et al.* 2000), sites that control hypothalamic–pituitary–adrenal (HPA) axis and hypothalamo-neurohypophysial system activity respectively (Ynag *et al.* 1977). APJ mRNA and apelin

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immunoreactivity are expressed within a proportion of vasopressinergic neurones originating from the SON and mPVN and projecting to the posterior pituitary (Reaux *et al.* 2001, O'Carroll & Lolait 2003), where the two peptides are segregated within distinct subcellular compartments (Reaux-Le Goazigo *et al.* 2004). Extrahypothalamic structures such as the piriform cortex, the dentate gyrus, the nucleus of the lateral olfactory tract and the dorsal raphe nucleus also express APJ mRNA (De Mota *et al.* 2000).

The key CNS site integrating neuroendocrine adjustments to stress is the hypothalamic PVN. This nucleus is comprised of two major neurosecretory components – the mPVN and pPVN subdivisions. Neurones originating from the more medially situated pPVN (where vasopressin (AVP) is co-localised in a proportion of corticotrophin-releasing factor (CRF) neurones; Tramu *et al.* 1983) project to the median eminence where, in response to stress, CRF and AVP are released from nerve terminals into the portal vessels bathing the anterior pituitary. CRF and AVP stimulate ACTH secretion by interacting with the CRF-type 1 (CRFR1) and AVP V1b (V1bR) receptors respectively, on the pituitary corticotrope (Gillies *et al.* 1982). ACTH in turn potently induces the secretion of glucocorticoids (corticosterone (CORT) in rodents) by the adrenal cortex. Regulation of CORT via ACTH secretion is critical for life and is necessary for the mammalian response to stress.

In the HPA axis, the presence of apelin and APJ transcripts and immunoreactivity in hypothalamic nuclei known to contain AVP and CRF and to co-ordinate endocrine responses to stress suggests that apelin may play a role in the control of anterior hormone release. Central, i.e. administration (i.c.v.) of apelin increases c-*fos* expression in the PVN (Kagiyama *et al.* 2005), and appears to have a role in the regulation of AVP and CRF neurones as it has been found to stimulate both AVP and CRF release from hypothalamic explants *in vitro* (Taheri *et al.* 2002), effects consistent with activation of PVN neurones and stimulation of the stress axis. This role was confirmed by increased levels of APJ mRNA resulting from acute and chronic stress and following adrenalectomy (O'Carroll *et al.* 2003). The involvement of apelin in the regulation of CRF neurones was substantiated by physiological experiments showing apelin-stimulated basal CORT secretion in rats to be partially blocked by pre-treatment with a CRF antagonist, α-helical $CRF_{9–41}$ (Jaszberenyi *et al.* 2004); however, the role of additional mediators such as AVP cannot be excluded.

In this study, to determine whether apelin has a central role in the regulation of CRF and AVP neurones, we investigated the effect of i.c.v. administration of pGlu-apelin-13 on neuroendocrine function in male mice pre-treated with the CRF receptor antagonist, α-helical $CRF_{9–41}$, and in mice lacking functional AVP V1bRs (V1bR KO).

Materials and Methods

Animals

Male adult (8–12 weeks) mice (a mix of the C57BL/6J and 129×1/SvJ strains) were used for *in vivo* experiments. For V1bR KO experiments, male adult littermates of crosses using mice heterozygous for the AVP V1bR mutation were used. Animals were group-housed (two to four per cage) under controlled light conditions (12 h light:12 h darkness cycle, lights on at 0700 h) and temperature (21±2 °C) with free access to standard laboratory chow and water. Studies were performed 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.

Surgical procedure

Mice were anaesthetised with isoflurane. Permanent 23-gauge stainless steel guide cannulae (Coopers Needleworks, Birmingham, UK) were stereotactically placed into the lateral ventricle (AP, −0·8; LM, +1·0; DV, −2·5 mm from bregma). Guide cannulae were secured with dental cement and two tether screws (Precision Technology Supplies, East Grinstead, UK). A 29-gauge stainless steel stylet was inserted down the guide cannula to ensure patency. On completion of surgery, animals were single housed in a clean cage, placed on a heatpad (36±1 °C) and administered antibiotics (Baytril, Bayer UK). Animals were allowed to recover for a minimum of 7 days before being used in experiments. To verify correct positioning, methylene blue was subsequently injected into decapitated heads and the brains dissected.

On the day of injection, stylets were removed from the guide cannulae and a 30-gauge stainless steel injection needle (40 mm long, Coopers Needleworks) inserted down the guide cannula, projecting 0·5 mm below the tip. The injection needle was attached to a gas-tight 10 μl Hamilton microsyringe (Fisher Scientific, Loughborough, UK) via 30 cm of flexible portex tubing (0·28 mm internal diameter, Southern Scientific Supplies, Lancing, UK). The animal was returned to the home cage and allowed to move freely during the injection and diffusion process. All substances were injected in a total volume of 2 or 5 μl (depending on solubility) over a period of 30 s, with a further 30 s allowed for complete diffusion. After injection, the injection needle was removed from the guide cannula and the home cage returned to the holding room.

Experiment 1 Mice (n=12–16/group) were administered either vehicle (0·9% saline; 5 μl i.c.v.) or α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.), followed 30 min later by administration of pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) or vehicle (0·9% saline; 2 μl i.c.v.) and killed 30 min later.

Experiment 2 V1bR KO and wild-type (WT) mice (n=9–12/group) were administered pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) or vehicle (0·9% saline; 2 μl i.c.v.) and killed 30 min later.

Experiment 3 V1bR KO mice ($n=12$–16/group) were administered either vehicle (0·9% saline; 5 μl i.c.v.) or α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.), followed 30 min later by administration of pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) or vehicle (0·9% saline; 2 μl i.c.v.) and killed 30 min later.

Peptides

pGlu-apelin-13 was purchased from Bachem (St Helens, UK). α-helical $CRF_{9–41}$ was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). All peptides were made up fresh in 0·9% sterile physiological saline (pH 5) on the day of the experiment and kept on ice until use.

Hormone analysis

All experiments were performed at least twice and hormone concentrations in samples were measured in duplicate. 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/)). Plasma concentrations of CORT were measured using an enzyme immunoassay (EIA) kit (IDS). The sensitivity is 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).

Statistical analysis

Hormone concentrations are expressed as a percentage of baseline of saline-treated control animals. All results are expressed as mean±S.E.M. Where appropriate, plasma hormone concentrations were analysed by one-way ANOVA followed by Dunnett's *post hoc* using GraphPad Prism (version 4.0b) software (GraphPad Software, San Diego, CA, USA). $P<0·05$ was considered statistically significant. Effects of genotype and treatment were determined by two-way ANOVA with repeated measures and subsequent *post hoc* tests as appropriate.

Results

Experiment 1: effect of α-helical $CRF_{9–41}$ on pGlu-apelin-13 stimulated plasma ACTH and CORT

To investigate whether apelin has a central role in regulating CRF neurons, the effect of i.c.v. administration of pGlu-apelin-13 on neuroendocrine function in mice pre-treated with the CRF receptor antagonist, α-helical $CRF_{9–41}$, was determined. Absolute levels of ACTH secreted after administration of saline were 50±24 pg/ml. Administration of pGlu-apelin-13 (1 mg/kg i.c.v.) resulted in a statistically significant increase in ACTH (202·2±29·3% of saline control; see Fig. 1A). This effect was blocked by pre-treatment with α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.), which reduced the ACTH response to 97·4±8·3% of saline-treated animals. Administration of α-helical $CRF_{9–41}$ alone had no significant effect on plasma ACTH (77±12·0%), when compared with saline controls.

The effect of pGlu-apelin-13 on plasma CORT is shown in Fig. 1B. Absolute levels of CORT secreted after administration of saline were 73±17 ng/ml. pGlu-apelin-13 administration (1 mg/kg i.c.v.) resulted in a significant increase in CORT (189·5±15·3%) compared with saline-injected controls. This increase in plasma CORT was significantly reduced by pre-treatment with α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.) to 129±16% of saline-treated control.

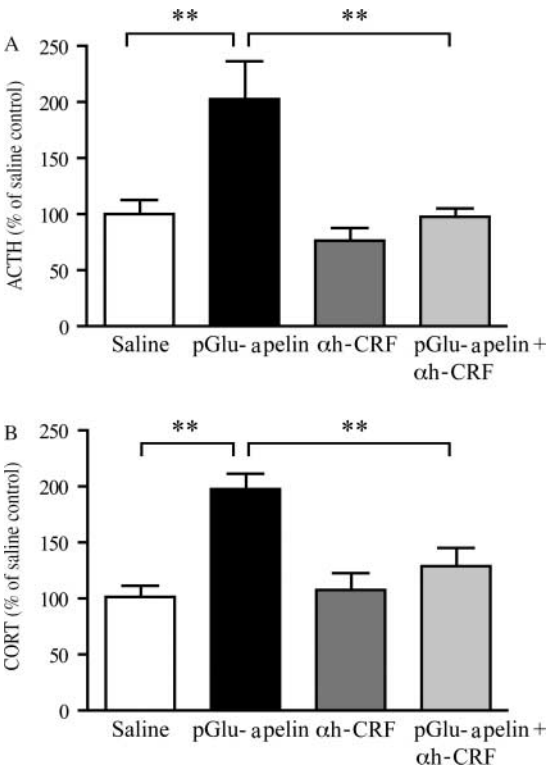

Figure 1 The effect of α-helical $CRF_{9–41}$ on pGlu-apelin-13 stimulated plasma ACTH and CORT. Pre-treatment with α-helical $CRF_{9–41}$ (αh-CRF) blocked the pGlu-apelin-13-induced increase in (A) ACTH and (B) CORT concentrations in male mice. Animals received either α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.) or vehicle (0·9% saline; 5 μl i.c.v.), followed 30 min later by administration of pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) or vehicle (0·9% saline; 2 μl i.c.v). Data are expressed as mean±S.E.M. $^{**}P<0·01$, one-way ANOVA followed by Dunnett's *post hoc* test.

Pre-treatment with α-helical $CRF_{9–41}$ (25 μg; 5 μl i.c.v.) alone had no significant effect on plasma CORT (107·4 ±15·4%) when compared with saline controls.

Experiment 2: effect of pGlu-apelin-13 on plasma ACTH and CORT in WT and V1bR KO mice

The potential role of apelin in regulating AVP neurones was investigated by determining the effect of i.c.v. administration of pGlu-apelin-13 on neuroendocrine function in V1bR KO mice (Fig. 2). There was no significant effect of genotype on the plasma ACTH response to administration of saline. Absolute levels of ACTH secreted after administration of saline were WT 50±24 versus KO 59±16 pg/ml plasma; $P>0·05$, two-way ANOVA. After administration of pGlu-apelin-13 (1 mg/kg i.c.v.), a significant increase in plasma ACTH was seen in WT (231·9±24·4%) but not in V1bR KO (119·5±16·6%) mice compared with WT control (Fig. 2A).

The effect of pGlu-apelin-13 administration (1 mg/kg i.c.v.) on plasma CORT in WT and V1bR KO mice is shown in Fig. 2B. As for ACTH, there was no significant effect of genotype on plasma CORT response to the administration of saline, WT 73±17 ng/ml plasma versus KO 109±24 ng/ml plasma; $P>0·05$, two-way ANOVA. Administration of pGlu-apelin-13 (1 mg/kg i.c.v.) resulted in a significant increase in plasma CORT in both WT and V1bR KO mice; however, this CORT response to pGlu-apelin-13 was significantly reduced in V1bR KO compared with WT mice (WT 554·3±41·1% versus V1bR KO 402·3±28·9%; $P<0·01$).

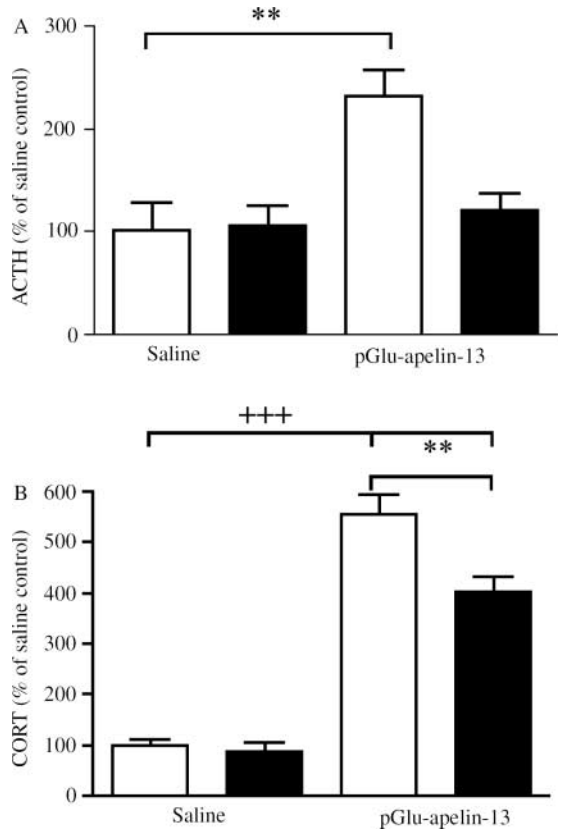

Figure 2 Effect of pGlu-apelin-13 on plasma ACTH and CORT in wild-type (white bars) and V1bR KO mice (black bars). (A) Administration of pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) resulted in a significant increase in plasma ACTH in wild-type but not in V1bR KO mice. (B) Administration of pGlu-apelin-13 (1 mg/kg; 2μl i.c.v.) resulted in a significant increase in plasma CORT in both wild-type and V1bR KO mice; however, the CORT response to pGlu-apelin-13 was significantly reduced in V1bR KO compared to wild-type mice. Data are expressed as mean±S.E.M. $^{+++}P<0·001$, $^{**}P<0·01$ two-way ANOVA using Bonferroni *post hoc* test.

Experiment 3: effect of α-helical $CRF_{9–41}$ on pGlu-apelin-13 stimulated plasma CORT in V1bR KO mice

To investigate whether the CORT response seen after the administration of pGlu-apelin-13 to V1bR KO mice is due to an involvement of CRF-mediated actions, the effect of i.c.v. administration of pGlu-apelin-13 on the CORT response of V1bR KO mice pre-treated with α-helical $CRF_{9–41}$ was determined. The CORT response to pGlu-apelin-13 alone was similar to that seen in experiment 2 with an increase to 408·8±63·3% of baseline control. This increase in CORT secretion was unaffected by pre-treatment with α-helical $CRF_{9–41}$ (359·0±30·7%; $P<0·001$; Fig. 3).

Discussion

The present study demonstrates that the *in vivo* effects of apelin on HPA axis neuroendocrine function are mediated through both CRF- and AVP-dependent mechanisms. pGlu-apelin-13 activation of the HPA axis, as evidenced by an increase in plasma ACTH and CORT in control animals, was significantly attenuated both in mice pre-treated with the CRF receptor antagonist, α-helical $CRF_{9–41}$, and in mice lacking functional AVP V1b receptors.

In the HPA axis, localisation of apelin and APJ mRNA and protein to hypothalamic nuclei known to contain AVP and CRF, and to modulate HPA endocrine responses to stress, has generated interest in a role for apelin in the regulation of HPA axis activity. Apelin may possibly act by controlling hypothalamic neuropeptide synthesis or release in parvocellular AVP and/or CRF neurones, to directly or indirectly alter ACTH secretion. However, apelin may also have a direct effect on HPA axis activity at the pituitary corticotroph level, as the peptide and receptor are both co-localised with ACTH in corticotrophs suggesting autocrine and/or paracrine interactions between apelin and ACTH. Our previous work suggested aspects of the apelinergic system present in the hypothalamus to be involved in the modulation of stress responses, as restraint stress was shown to be associated with

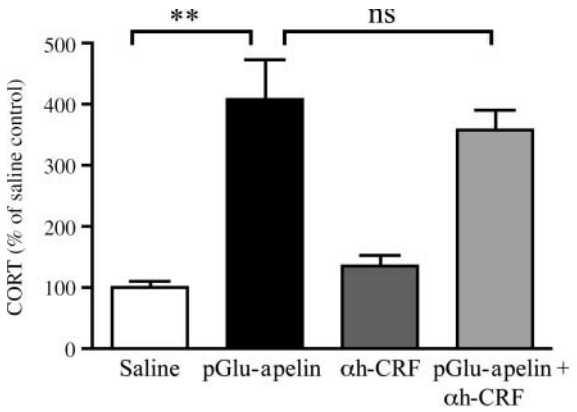

Figure 3 Effect of α-helical $CRF_{9–41}$ on pGlu-apelin-13 stimulated plasma CORT in V1bR KO mice. Administration of pGlu-apelin-13 (1 mg/kg; 2 μl i.c.v.) resulted in a significant increase in plasma CORT in V1bR KO mice that was not blocked by pre-treatment with α-helical $CRF_{9–41}$ (αh-CRF) (25 μg; 5 μl i.c.v.). Data are expressed as mean ± S.E.M. $^{**}P<0{\cdot}01$ one-way ANOVA followed by Dunnett's *post hoc* test; ns, not significant.

a significant increase in APJ mRNA expression in the pPVN (O'Carroll *et al.* 2003). Removal of endogenous glucocorticoids by adrenalectomy resulted in an increased expression of APJ mRNA in the pPVN, implying negative regulation of APJ mRNA expression by glucocorticoids (O'Carroll *et al.* 2003). After repeated restraint stress, there was a sustained increase in expression of APJ mRNA in the pPVN, similar to that observed for AVP mRNA (De Goeij *et al.* 1992), suggesting that apelin acting through APJ facilitates parvocellular function under conditions of repeated stress. While CRF is the major activator of pituitary corticotroph ACTH secretion in acute stress, AVP appears to be important in maintaining the HPA axis response to a number of repeated and chronic stressors (Harbuz & Lightman 1992, Aguilera 1994), and, although both pituitary CRFR1 receptors and AVP V1b receptors are activated and undergo regulatory variations during stress, only the changes in V1b receptor levels parallel the changes in pituitary ACTH responsiveness (Rabadan-Diehl *et al.* 1995, Aguilera & Rabadan-Diehl 2000). Recent studies, however, in V1bR KO receptor-deficient mice, have substantiated the role of AVP in the HPA axis response to some acute stressors (Lolait *et al.* 2007*a*,*b*, Stewart *et al.* 2008).

Previous studies involving central administration of pGlu-apelin-13 have focussed on the activity of this peptide in the regulation of a broad range of homeostatic behaviours in the rat, notably food (Sunter *et al.* 2003) and water intake (Reaux *et al.* 2001,Taheri *et al.* 2002), or cardiovascular effects (Kagiyama *et al.* 2005). Few studies have investigated the role of central apelin and APJ in neuroendocrine activation as reflected by ACTH and CORT secretion (Taheri *et al.* 2002, Jaszberenyi *et al.* 2004). Central administration of pGlu-apelin-13 has been shown to activate PVN neurones and to stimulate the HPA axis as indicated by increased c-*fos* expression in the PVN (Kagiyama *et al.* 2005), and increased secretion of both ACTH and CORT into the plasma (Taheri *et al.* 2002). Additionally, apelin appears to have a central role in the regulation of AVP and CRF neurones, as apelin stimulated release of both of these peptides from hypothalamic explants *in vitro* (Taheri *et al.* 2002). Recently, in an *in vitro* perfusion system of anterior pituitaries, apelin-17 significantly increased basal ACTH secretion (Reaux-Le Goazigo *et al.* 2007).

This study represents the first *in vivo* investigation of the mechanism of action of pGlu-apelin-13 on the secretion of ACTH and CORT in the mouse. We investigated whether pGlu-apelin-13 may exert its actions via stimulation of the release of CRF from neurones of the pPVN, which acts on the anterior pituitary to stimulate the secretion of ACTH into the periphery, resulting in the secretion of CORT from the adrenal glands. We show that pre-treatment of mice with the CRF receptor antagonist, α-helical $CRF_{9–41}$, attenuated the pGlu-apelin-13-induced elevations in plasma ACTH and CORT observed in control mice to levels seen in saline controls. This result is in agreement with a study performed on rats where apelin-evoked CORT secretion was diminished by pre-treatment with α-helical $CRF_{9–41}$ (Jaszberenyi *et al.* 2004). Our data clearly indicate that apelin may exert its effects through CRF-dependent mechanisms; however, the role of additional mediators such as AVP cannot be excluded. The relative contributions of AVP and CRF in activation of the HPA axis depend on the nature of the stimulus, but it is well established that both peptides are required for a full ACTH response to many acute stressors (Kjaer *et al.* 1992, Suda *et al.* 1992).

In the neurohypophysial system, the physiological effects of apelin appear to be mediated by AVP. We and others have found apelin and APJ to be expressed within a proportion of magnocellular PVN (mPVN) and SON vasopressinergic neurones (Reaux *et al.* 2001, O'Carroll & Lolait 2003), where the two peptides are segregated within separate subcellular compartments (Reaux-Le Goazigo *et al.* 2004), suggesting an interaction between the apelinergic and vasopressinergic systems in the regulation of body fluid homeostasis. Recent studies report a cross-regulation of endogenous levels of apelin and AVP (Reaux-Le Goazigo *et al.* 2004), and that apelin acts to regulate AVP actions through modulation of AVP neurone activity and AVP release (Reaux-Le Goazigo *et al.* 2004, Tobin *et al.* 2008). Interestingly, there is unequivocal evidence that the neurohypophysial and HPA axes functionally overlap and that AVP produced in the mPVN contributes to the regulation of HPA axis activity (secretion of ACTH; reviewed in Engelmann *et al.* 2004). Moreover, some stressors that activate the HPA axis appear to preferentially activate mPVN more than pPVN AVP neurones e.g. forced swim test (Engelmann *et al.* 2004).

In order to determine the role of AVP in the mechanism of action of pGlu-apelin-13 in activation of the HPA axis, pGlu-apelin-13 was administered to both WT and V1bR KO mice. These mice have impaired HPA responses to a number of stress models (Lolait *et al.* 2007*a*,*b*, Stewart *et al.* 2008),

indicating a key role for V1bR in HPA axis activation. However, we have shown no difference in basal or CRF-stimulated ACTH release from anterior pituitary tissue from V1bR KO or WT mice, suggesting that there is no difference in pituitary sensitivity to CRF between genotypes (Lolait *et al.* 2007*a*). Additionally, in the same study, we have shown basal levels of AVP, oxytocin and CRF gene expression in the hypothalamic PVN, and pro-opiomelanocortin (the precursor for ACTH) gene expression in the anterior pituitary to be similar in V1bR KO and WT mice implying no evidence of compensatory gene expression to the genetic change. In this present study, administration of pGlu-apelin-13 resulted in a significant increase in plasma ACTH in WT mice, an effect that was abolished in V1bR KO mice, indicating a significant role for the V1bR in the ACTH response to apelin administration. The absence of an ACTH response in these mutant mice suggests that the stimulatory effect of apelin on CRF release cannot by itself manifest a full ACTH response to apelin administration. Interestingly, V1bR KO mice had a markedly diminished, although not absent, CORT response to pGlu-apelin-13 administration. Dissociation between ACTH and CORT responses to stimulation in V1bR KO animals has been observed previously, albeit in different experimental models (acute lipopolysaccharide challenge (Lolait *et al.* 2007*b*), acute and chronic forced swimming and acute and chronic restraint stress (Stewart *et al.* 2008)). It is possible that ACTH levels in V1bR KO mice, however low, are still high enough to induce a normal CORT response to stress, or that the residual adrenal activity may be mediated by ACTH-independent CORT modulators. Pituitary-independent factors that may modulate adrenal CORT secretion include sympathetic activity (Bornstein & Chrousos 1999, Droste *et al.* 2007) and cytokines such as interleukin-6 (Bornstein & Chrousos 1999). Levels of CORT secretion in V1bR KO mice did not change when animals were pre-treated with α-helical $CRF_{9–41}$, indicating that the remaining levels of CORT cannot be attributed to the action of CRF on CRF pituitary receptors.

In conclusion, our data suggest that apelin, mediating its effects through activation of paraventricular CRF and AVP neurones, requires both CRF and AVP for a full ACTH and CORT response and that neither can compensate for the loss of the other in maintaining a normal HPA axis response to apelin. We cannot exclude the possibility, however, that apelin injected i.c.v. in these studies reaches the anterior pituitary and/or other sites that may influence HPA axis activity to exert a direct effect. Our results emphasise an interaction between apelin and paraventricular AVP and CRF and suggest that the integration of neurobehavioural responses to stress is more complicated than previously envisioned.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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