Relaxin-3 stimulates the neuro-endocrine stress axis via corticotrophin-releasing hormone

B M McGowan¹, J S Minnion², K G Murphy², D Roy², S A Stanley³, W S Dhillo², J V Gardiner², M A Gbatei² and S R Bloom²

¹Department of Diabetes and Endocrinology, Guy's and St Thomas' NHS Foundation Trust, Westminster Bridge Road, London SE1 7EH, London

²Section of Investigative Medicine, Department of Medicine, Imperial College London, 6th Floor Commonwealth Building, Hammersmith Campus, Du Cane Road, London W12 0NN, UK

³Molecular Genetics, Rockefeller University, New York, NY 10065, USA

Correspondence should be addressed to S R Bloom
Email
s.bloom@imperial.ac.uk

Abstract

Relaxin-3 is a member of the insulin superfamily. It is expressed in the nucleus incertus of the brainstem, which has projections to the hypothalamus. Relaxin-3 binds with high affinity to RXFP1 and RXFP3. RXFP3 is expressed within the hypothalamic paraventricular nucleus (PVN), an area central to the stress response. The physiological function of relaxin-3 is unknown but previous work suggests a role in appetite control, stimulation of the hypothalamic–pituitary–gonadal axis and stress. Central administration of relaxin-3 induces c-fos expression in the PVN and increases plasma ACTH levels in rats. The aim of this study was to investigate the effect of central administration of human relaxin-3 (H3) on the hypothalamic–pituitary–adrenal (HPA) axis in male rodents *in vivo* and *in vitro*.

Intracerebroventricular (i.c.v.) administration of H3 (5 nmol) significantly increased plasma corticosterone at 30 min following injection compared with vehicle. Intra-PVN administration of H3 (1.8–1620 pmol) significantly increased plasma ACTH at 1620 pmol H3 and corticosterone at 180–1620 pmol H3 at 30 min following injection compared with vehicle. The stress hormone prolactin was also significantly raised at 15 min post-injection compared with vehicle. Treatment of hypothalamic explants with H3 (10–1000 nM) stimulated the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), but H3 had no effect on the release of ACTH from *in vitro* pituitary fragments. These results suggest that relaxin-3 may regulate the HPA axis, via hypothalamic CRH and AVP neurons. Relaxin-3 may act as a central signal linking nutritional status, reproductive function and stress.

Key Words

- ▶ relaxin-3
- ▶ RXFP3
- ▶ RXFP1
- ▶ paraventricular nucleus
- ▶ ACTH
- ▶ corticosterone

Journal of Endocrinology
(2014) 221, 337–346

Introduction

Relaxin-3, also known as insulin-like peptide 7, is a member of the insulin superfamily (Bathgate *et al.* 2002). Before the discovery of the relaxin-3 gene (*RLN3*) and its peptide, only one other relaxin gene, *RLN1*, had been characterised in most species, although two relaxin genes *RLN1* and *RLN2* are found in humans and higher primates

(Hudson *et al.* 1983, 1984, Soloff *et al.* 2003). The human gene product of *RLN2*, human relaxin-2 (H2), is the functional ortholog of the *RLN1* gene in other mammals. Unlike relaxin, relaxin-3 is highly homologous across species. It is expressed at greatest levels in the CNS and expression is localised to the nucleus incertus (NI) in the

caudoventral region of the pontine periventricular gray (Burazin *et al.* 2002, Tanaka *et al.* 2005). Anatomical studies suggest that this nucleus is involved in a midbrain behaviour control network that regulates locomotion, attention and learning processes, and that responds to stress-related neuro-endocrine signals (Goto *et al.* 2001). Tracing studies have shown that the neurons of the NI project extensively to hypothalamic areas, including the lateral hypothalamic area, the posterior hypothalamic nucleus and the medial and periventricular zones (Goto *et al.* 2001). Relaxin-3 immunoreactivity has been described in hypothalamic areas important in the regulation of the stress axis, including the paraventricular nucleus (PVN), as well as areas important in feeding such as the arcuate nucleus (Tanaka *et al.* 2005).

The cognate receptor for relaxin-3 is RXFP3 (formally known as GPCR135; Liu *et al.* 2003a, Chen *et al.* 2005, Sutton *et al.* 2005), although it can also bind and activate RXFP1 and RXFP4 with lesser affinity (Liu *et al.* 2003b,b, Sudo *et al.* 2003). RXFP3 is expressed predominantly in the CNS, and in particular within the hypothalamic PVN and supraoptic nucleus (SON; Liu *et al.* 2003a, Sutton *et al.* 2004). RXFP3 has been found to be expressed in both the lateral magnocellular neurons (which secrete the hormones oxytocin (OXT) and arginine vasopressin (AVP)) and medial parvocellular neurons (which secrete hormones including corticotrophin-releasing hormone (CRH)) of the PVN (Watanabe *et al.* 2011). RXFP1, the receptor for which relaxin is the physiological ligand, is expressed peripherally and centrally, including in the hypothalamic PVN, ARC and SON (Burazin *et al.* 2005, Ma *et al.* 2006). RXFP4 is not expressed in the rat (Chen *et al.* 2005), but does contribute to relaxin-3 binding in the mouse brain (Liu *et al.* 2003b, Chen *et al.* 2005, Sutton *et al.* 2005).

Relaxin has been classically associated with female reproductive physiology, but the physiological functions of relaxin-3 are less well characterised. Previous work suggested that the relaxin-3/RXFP3 system may play a role in appetite control, stimulation of the reproductive axis, spatial working memory and arousal (McGowan *et al.* 2005, 2006a,b, 2008, Ma *et al.* 2009, Ganella *et al.* 2012, 2013a). More recent work has suggested a role for relaxin-3 in the modulation of anxiety and depression and alcohol-seeking behaviour (Ryan *et al.* 2013a,b), and there is emerging evidence to suggest that relaxin-3 may regulate the neuro-endocrine stress axis (Ganella *et al.* 2013b). CRH is the major central signalling factor regulating the neuro-endocrine stress axis. CRH stimulates adrenocorticotrophic hormone (ACTH) release from the anterior pituitary, an effect that is potentiated by

AVP (Volpi *et al.* 2004). ACTH in turn stimulates corticosterone release from the adrenal cortex. A further pituitary hormone, prolactin, so-called because of its well-characterised role in lactation in mammals, has also been shown to play an important role in the stress axis (Noel *et al.* 1972). It has been reported that most relaxin-3 immunoreactive neurons in the NI co-express the CRH type 1 receptor (Tanaka *et al.* 2005). Central administration of CRH results in the expression of the early gene *c-fos* (*fos*) in relaxin-3 neurons in the NI, and similar *c-fos* expression is achieved under stress conditions (Tanaka *et al.* 2005). Exposure of rats to a repeated forced swim for 10 min markedly increases relaxin-3 mRNA levels in the NI at 30–60 min after the second swim (Banerjee *et al.* 2010). Central administration of relaxin-3 has been reported to induce *c-fos* expression in the PVN and to increase *Crh* and *c-fos* mRNA in the PVN (Watanabe *et al.* 2011). Recently, a study has shown sex differences in hypothalamic expression of CRH and relaxin-3 in the NI between male and female rats in response to chronic stress and food restriction (Lengos *et al.* 2013).

The aim of the current study was, therefore, to further characterise the effect of relaxin-3 on the neuro-endocrine stress axis in rats. We investigated the effect of intracerebroventricular (i.c.v) and intra-PVN (iPVN) administration of H3 on stress hormones corticosterone and prolactin (Noel *et al.* 1972) and the effect of H3 on ACTH release from pituitary explants and on CRH and AVP release from hypothalamic explants. These studies suggest that H3 stimulates the hypothalamic–pituitary–adrenal (HPA) axis by modulating hypothalamic CRH and AVP release.

Materials and methods

Materials

H3 (studies 1, 3–5) was purchased from The Howard Florey Institute (Melbourne, VIC, Australia); H3 for study 2 was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). H2 was kindly donated by Prof. John Wade at The Howard Florey Institute. Reagents for basal hypothalamic explant studies and pituitary fragment experiments were supplied by VWR (Leicestershire, UK).

Animal studies

Male Wistar rats (Specific pathogen free, Charles River, Margate, Kent, UK) weighing 250–300 g were maintained in individual cages for all *in vivo* studies. Male Wistar rats weighing 200–220 g were caged in groups of five for use

in hypothalamic and pituitary explant experiments. All animals were kept under controlled temperature (21–23 °C) and light (12 h light:12 h darkness lights on at 0700 h) with access to food (pelleted RM1 chow diet, Special Diet Services, Witham, Essex, UK) and water *ad libitum* unless otherwise stated. All procedures undertaken were approved under the British Home Office Animals (Scientific Procedures) Act 1986 (project license 70/5516).

I.c.v. and iPVN cannulation and injection

Male Wistar rats underwent i.c.v. or unilateral iPVN cannulation 7–10 days before the studies and were habituated to regular handling and injection, as previously described (Wren *et al.* 2001). For i.c.v. cannulation, permanent 22-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA, USA) were placed into the third cerebral ventricle (0.8 mm posterior to the bregma on the mid-sagittal line 6.5 mm below the outer surface of the skull, coordinates calculated using atlas of Paxinos & Watson (1998)). For iPVN cannulation, permanent 26-gauge stainless steel guide cannulae were implanted in the animals projecting into the PVN (1.8 mm posterior from bregma, 0.5 mm lateral from midline and 8.0 mm in depth), according to the coordinates of Paxinos & Watson (1998). Central injections (5 µl (i.c.v.) or 1 µl (iPVN)) were administered over 1 min via stainless steel injectors (27-gauge (i.c.v.) or 31-gauge (iPVN)), placed in and projecting 1 mm below the end of the cannula. Spread of a 1 µl injection into the PVN is reported to be limited to 1 mm³ (Ward *et al.* 2003). H3 and H2 studies 1 and 3–5 were dissolved in 0.9% saline. H3 for study 2 was dissolved in 10% acetonitrile in 0.9% saline (McGowan *et al.* 2005). Thus, vehicle for experiments 1 and 3–5 was 0.9% saline, and vehicle for experiment 2 was 10% acetonitrile in 0.9% saline. Studies were carried out in rats fed *ad libitum* ($n=10$ – 12) in the early light phase (0900–1000 h) unless otherwise stated.

I.c.v. cannula position was verified by a positive dipsogenic response to angiotensin II (150 ng/rat). Only those animals with correct cannula placement were included in the data analysis. For the PVN-cannulated animals, cannula position was verified histologically at the end of the study (Wren *et al.* 2001). Immediately following decapitation, 1 µl India ink was injected into the cannula. The brains were removed and fixed in 4% paraformaldehyde, dehydrated in 40% sucrose, frozen and stored at –70 °C. Brains were sliced on a cryostat (Bright, Huntingdon, UK) into 15-µm coronal sections and correct PVN placement was determined by microscopy according to the position of the India ink.

In vivo effects of relaxin-3 on the HPA axis

Study 1: effect of i.c.v. H3 and H2 on plasma corticosterone Male Wistar rats received a single i.c.v. injection (5 µl) of vehicle, H3 (5 nmol) or H2 (5 nmol) ($n=10$ per group). This dose was used because it is the effective i.c.v. dose of relaxin-3 known to stimulate the hypothalamic–pituitary–gonadal (HPG) axis in male Wistar rats (McGowan *et al.* 2008). The effect of H3 was compared with that of H2 to investigate the receptor responsible for the H3-induced stimulation of the HPA axis. Thirty minutes following i.c.v. administration, animals were killed by decapitation and plasma was collected into plastic lithium heparin tubes containing 4200 KIU aprotinin (Bayer). Plasma was separated by centrifugation, frozen and stored at –20 °C until the measurement of corticosterone.

Study 2: time course effect of iPVN H3 on ACTH, corticosterone and prolactin Male Wistar rats ($n=10$ – 12 per group) received a single iPVN injection (1 µl) of vehicle or H3 (540 pmol). At 15 or 30 min following administration, animals were killed by decapitation and plasma was collected, separated and stored as described above until measurement of plasma ACTH by solid-phase IRMA and plasma corticosterone and prolactin by RIA.

Study 3: dose-response of iPVN H3 on plasma ACTH and corticosterone Male Wistar rats ($n=8$ – 10 per group) received a single iPVN injection (1 µl) of vehicle, H3 at 1.8, 18, 180, 540 and 1620 pmol or H2 (540 pmol). This iPVN dose of H2 (tenfold lower vs H2 i.c.v. dose) was used based on i.c.v. results from experiment 1, which revealed effective stimulation of the HPA axis at 5 nM. Thirty minutes following iPVN administration, animals were killed by decapitation and plasma was collected, separated and stored as described above until measurement of plasma ACTH by solid-phase IRMA and plasma corticosterone by RIA.

In vitro effects of relaxin-3 on the HPA axis

Study 4: effects of relaxin-3 on ACTH release from anterior pituitary quarters The effects of relaxin-3 on pituitary ACTH release were determined using anterior pituitary quarters. This method was a modification of that previously described (Buckingham & Hodges 1977). Rats were decapitated and anterior pituitary glands were harvested immediately and then divided into four pieces

of approximately equal size. The segments were randomly placed (one segment per well) in the wells of a 48-well tissue culture plate (Nunc International, Loughborough, Leicestershire, UK) and incubated in 500 μ l of artificial cerebrospinal fluid (aCSF) (20 mM NaHCO_3 , 126 mM NaCl, 0.09 mM Na_2HPO_4 , 6 mM KCl, 1.4 mM CaCl_2 , 0.09 mM MgSO_4 , 5 mM glucose, 0.18 mg/ml ascorbic acid and 100 μ g/ml aprotinin). The quarters were maintained at 37 °C in a humidified environment saturated with 95% O_2 and 5% CO_2 for 2 h with the medium changed every hour. The segments were then incubated in aCSF alone (control), H3 at 10, 100, 1000 or 100 nM CRH as a positive control for 4 h ($n=20$ per group). At the end of this period, the aCSF was collected and stored at -20°C until measurement of ACTH by solid-phase IRMA.

Study 5: effect of relaxin-3 on hypothalamic CRH and AVP release The static incubation system was used as previously described (Stanley *et al.* 1999). Briefly, male Wistar rats were killed by decapitation and the brain was immediately removed. The brain was mounted with ventral surface uppermost and placed in a vibrating microtome (Microfield Scientific Ltd, Dartmouth, UK). A 1.7 mm slice to include the medial preoptic area (MPOA) was taken from the basal hypothalamus and incubated in an individual chamber containing 1 ml aCSF (as described above) equilibrated with 95% O_2 and 5% CO_2 . Then the tubes were placed on a shaking platform in a water bath maintained at 37 °C. After an initial 2 h equilibration period, the hypothalami were incubated for 45 min in 600- μ l aCSF (basal period) before being challenged with H3 (10, 100 or 1000 nM) for 45 min ($n=18$ –27 per group). Finally, the viability of the tissue was verified by a 45 min exposure to 56 mM KCl; isotonicity was maintained by substituting K^+ for Na^+ . At the end of each period, the aCSF was removed and frozen at -20°C until RIA for measurement of CRH and AVP.

Hormone assays

ACTH was measured using a solid-phase IRMA (Euro Diagnostica, Huntingdon, Cambridgeshire, UK). Corticosterone was measured using a commercial RIA Kit (MP Biomedicals, Loughborough, Leicestershire, UK). Prolactin was analysed by RIA using reagents and methods provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program as previously described (Beak *et al.* 1998). Hypothalamic hormones (CRH and AVP) were assayed using in-house assays as previously described (Wren *et al.* 2002).

Statistical analysis

Results are shown as mean $\pm$ s.e.m. *In vivo* studies (studies 1–3) and *in vitro* study 4 were analysed by ANOVA with *post-hoc* Tukey's test (Systat, Evanston, IL, USA and SPSS 20). Hypothalamic explant data (study 5) between control and treated groups were compared by paired Student's *t*-test. In all cases, $P<0.05$ was considered to be statistically significant.

Results

In vivo effects of H3 and H2 on the HPA axis

Study 1: effect of i.c.v. relaxin-3 on plasma corticosterone A single i.c.v. injection of H3 (5 nmol) to male Wistar rats significantly increased plasma corticosterone at 30 min post-injection (225.0 ± 39.0 ng/ml (vehicle) vs 371.0 ± 35.7 ng/ml (H3), $P<0.05$ vs vehicle) (Fig. 1). A single i.c.v. injection of H2 to male Wistar rats also significantly increased plasma corticosterone at 30 min post-injection (225.0 ± 39.0 ng/ml (vehicle) vs 376.0 ± 38.7 ng/ml (H2), $P<0.05$ vs vehicle; Fig. 1).

Study 2: time course effect of iPVN H3 on plasma ACTH, corticosterone and prolactin The time course effect of iPVN H3 administration on the HPA axis was examined. A single iPVN injection of H3 (540 pmol) to male Wistar rats significantly increased plasma ACTH at 15 min post-administration of H3. ACTH was also elevated at 30 min post-injection of H3, but this effect was not statistically significant (51.4 ± 4.8 pg/ml (vehicle)

Figure 1

Effect of i.c.v. administration of H3 (5 nmol) and H2 (5 nmol) on plasma corticosterone (ng/ml) at 30 min post-injection in male adult rats. $*P<0.05$ vs vehicle, by ANOVA with *post-hoc* Tukey's test ($n=10$ per group).

vs 114.8 ± 18.0 pg/ml (H3) at 15 min, $P < 0.05$ vs vehicle and 61.4 ± 10.4 pg/ml (vehicle) vs 104.5 ± 14.4 pg/ml (H3) at 30 min, $P = 0.212$ vs vehicle; Fig. 2A). Plasma corticosterone was elevated at 15 min post-injection but was only significantly increased at 30 min post-injection (252.8 ± 46.8 ng/ml (vehicle) vs 424.3 ± 47.3 ng/ml (H3) at

15 min, $P = 0.09$ vs vehicle and 122.0 ± 32.2 ng/ml (vehicle) vs 497.6 ± 60.3 ng/ml (H3) at 30 min, $P < 0.001$ vs vehicle; Fig. 2B). Plasma prolactin was significantly elevated at 15 min post-injection and remained elevated at 30 min, although this effect was not statistically significant (4.1 ± 1.0 ng/ml (vehicle) vs 27.0 ± 5.0 ng/ml (H3) at 15 min, $P < 0.001$ vs vehicle and 4.9 ± 1.0 ng/ml (vehicle) vs 12.3 ± 3.2 ng/ml (H3) at 30 min, $P = 0.34$; Fig. 2C).

Figure 2

Effect of iPVN administration of H3 (540 pmol) on (A) plasma ACTH (pg/ml), (B) plasma corticosterone (ng/ml) and (C) plasma prolactin (ng/ml) at 15 and 30 min post-injection in male adult rats. * $P < 0.05$ and *** $P < 0.001$ vs vehicle by ANOVA with *post-hoc* Tukey's test ($n = 10$ –12 per group).

Study 3: dose-response of iPVN H3 on plasma ACTH and corticosterone

To investigate the effects of increasing doses of iPVN H3 on ACTH and corticosterone release, a single iPVN injection of H3 was given to male Wistar rats. IPVN H3 was found to dose-dependently increase plasma ACTH at 30 min post-injection. Plasma ACTH was significantly increased following iPVN injection of 1620 pmol H3 (67.8 ± 8.0 pg/ml (vehicle) vs 178.8 ± 29.8 pg/ml (1620 pmol H3), $P < 0.01$; Fig. 3A). There was no statistically significant effect on plasma ACTH at lower doses of H3 (1.8–540 pmol), although there was a trend for increased plasma ACTH concentrations with increasing doses of H3 (67.8 ± 8.0 pg/ml (vehicle) vs 79.7 ± 9.9 ng/ml (1.8 pmol H3, $P = 0.99$), 86.4 ± 10.8 pg/ml (18 pmol H3, $P = 0.99$), 142.6 ± 27.9 pg/ml (180 pmol H3, $P = 0.08$), 156.1 ± 31.2 pg/ml (540 pmol H3, $P = 0.23$)) (Fig. 3A). A single iPVN injection of H2 (540 pmol) to male Wistar rats increased plasma ACTH at 30 min post-injection, but this did not achieve statistical significance (67.8 ± 8.0 pg/ml (vehicle) vs 119.3 ± 13.3 pg/ml (540 pmol H2), $P = 0.44$; Fig. 3A).

Plasma corticosterone was significantly increased at 30 min following iPVN injection of 180, 540 and 1620 pmol H3 (174.2 ± 34.5 ng/ml (vehicle) vs 474.7 ± 84.9 ng/ml (180 pmol H3), $P < 0.05$, 505.9 ± 64.2 ng/ml (540 pmol H3), $P < 0.01$ and 602.5 ± 88.8 ng/ml (1620 pmol H3), $P < 0.001$; Fig. 3B). There was no statistically significant effect on plasma corticosterone at lower doses of H3 (1.8–18 pmol) (174.2 ± 34.5 ng/ml (vehicle) vs 170.8 ± 48.5 ng/ml (1.8 pmol H3, $P = 1.0$) and 279.6 ± 65.1 ng/ml (18 pmol H3, $P = 0.89$)) (Fig. 3B). A single iPVN injection of H2 (540 pmol) to male Wistar rats increased plasma corticosterone at 30 min post-injection, but this did not achieve statistical significance (174.2 ± 34.5 ng/ml vehicle) vs 409.9 ± 48.0 ng/ml (540 pmol H2), $P = 0.138$) (Fig. 3B).

In vitro effects of relaxin-3 on the HPA axis

Study 4: effect of relaxin-3 on ACTH release from anterior pituitary fragments Administration of H3 (10–1000 nM) had no effect on the release of ACTH

Figure 3

Effect of iPVN administration of H3 (1.8–1620 pmol; $n=10-12$ per group) and H2 (540 pmol), (A) plasma ACTH (pg/ml) and (B) plasma corticosterone (ng/ml) at 30 min post-injection in male adult rats. * $P<0.05$ vs vehicle, ** $P<0.01$ and *** $P<0.001$ vs vehicle, by ANOVA with *post-hoc* Tukey's test ($n=10-12$ per group).

from *in vitro* pituitary fragments (Fig. 4), suggesting that relaxin-3 alone is not acting at the level of the pituitary to influence the HPA axis (100 ± 12.5 basal vs 124.0 ± 10.0 (H3 10 nM), 122.7 ± 11.1 (H3 100 nM) and 122.0 ± 11.9 (H3 1000 nM)), data shown as percentage of basal release, with basal aCSF indicated as 100% (Fig. 4). The positive control CRH significantly increased ACTH release (100 ± 12.5 basal vs 348.2 ± 12.1 (CRH 100 nM), $P<0.001$), data shown as percentage of basal release, with basal aCSF indicated as 100% (Fig. 4).

Study 5: effect of relaxin-3 on hypothalamic CRH and AVP release Administration of H3 (10–1000 nM) to hypothalamic explants stimulated the release of CRH (100 ± 9.0 basal vs 128.2 ± 21.8 (10 nM H3), $P<0.01$ vs basal aCSF; 132.4 ± 26.5 (100 nM H3), $P<0.05$ vs basal aCSF and 137.3 ± 33.3 (1000 nM H3), $P<0.05$ vs basal aCSF, data shown as percentage of basal release with basal aCSF indicated as 100%) (Fig. 5A). The positive control

(aCSF with 56 nM K^+) increased CRH release (100 ± 9.0 basal vs 159.0 ± 22.7 (aCSF+ with 56 nM K^+)) (Fig. 5A).

Administration of H3 (10–1000 nM) to hypothalamic explants stimulated the release of AVP (100 ± 11.2 basal vs 188.5 ± 44.9 (10 nM H3), $P<0.05$ vs basal aCSF; 220.7 ± 39.2 (100 nM H3), $P<0.01$ vs basal aCSF and 186.1 ± 43.1 (1000 nM H3), $P<0.05$ vs basal aCSF, data shown as percentage of basal release with basal aCSF indicated as 100%) (Fig. 5B). The positive control (aCSF with 56 nM K^+) increased AVP release (100 ± 11.2 basal vs 242.0 ± 26.9 (aCSF+ with 56 nM K^+)) (Fig. 5B).

Discussion

We have previously shown that the central administration of H3 increases food intake in rats and stimulates the HPG axis (McGowan *et al.* 2005, 2006a,b, 2008). Subsequent work has revealed a possible role for relaxin-3 in the stress response, with central administration of relaxin-3 inducing *c-fos* and CRH expression in the PVN, a hypothalamic area which plays a crucial role in the regulation of the stress response (Watanabe *et al.* 2011). Recently, a study investigating the effect of chronic stress and repeated food restriction in rats has shown increased body weight, increased plasma corticosterone levels, lower PVN *Crh* mRNA expression and increased NI relaxin-3 mRNA expression in stressed female rats compared with both female controls and male rats subjected to the same repeated stress (Lenglos *et al.* 2013). These studies extend previous work to demonstrate that H3 is involved

Figure 4

Effect of H3 (10, 100 and 1000 nM) on the release of ACTH from *in vitro* anterior pituitary fragments (data shown as percentage of basal release with basal aCSF indicated as 100%). CRH was used as a positive control ($n=20$ per group).

Figure 5

Effect of H3 (10, 100 and 1000 nM) on stimulation of (A) CRH and (B) AVP release from *in vitro* hypothalamic explants from adult male rats (data shown as percentage of basal release with basal aCSF indicated as 100%). The positive control was artificial cerebrospinal fluid (aCSF) containing 56 nM K⁺. * $P < 0.05$ vs aCSF control and ** $P < 0.01$ vs aCSF control by paired *t*-test ($n = 18$ –27 per group).

in the regulation of the HPA axis via CRH and suggest sex differences in the effects of chronic stress and food restriction.

Relaxin-3 immunoreactivity is found in PVN, and its cognate receptors RXFP3 and RXFP1, to which relaxin-3 can also bind and activate (Liu *et al.* 2003a, Sudo *et al.* 2003), are expressed at high concentrations in this hypothalamic nucleus. We have shown that the central administration of H3 in male rats significantly increases plasma corticosterone at 30 min following i.c.v. injection. This evidence prompted us to investigate the role of intraparenchymal H3 on the HPA axis. First we investigated the time course effects of a high dose (540 pmol) of H3 on ACTH, corticosterone and the stress hormone prolactin. Our results showed a maximum corticosterone response at 30 min, with the pituitary hormones ACTH and prolactin reaching an earlier peak

at 15 min. We were interested in the downstream corticosterone response, and hence a time point of 30 min was chosen to investigate the iPVN dose–response to H3. The effects of iPVN H3 on ACTH and corticosterone were greater with increasing doses of H3, with an intranuclear dose of at least 540 pmol required to elicit a significant response in plasma ACTH 15 min post-injection, and 180 pmol H3 for plasma corticosterone at 30 min post-injection.

We hypothesised that relaxin-3 may stimulate the HPA axis via modulation of the hypothalamic CRH pathways. In accord with this hypothesis, it has been shown that i.c.v. administration of relaxin-3 enhanced *c-fos* and *Crh* mRNA transcription in PVN 2 h after i.c.v. injection (Watanabe *et al.* 2011). Our *in vitro* studies support this hypothesis. H3 stimulated the release of CRH from *in vitro* hypothalamic explants at doses of 10–1000 nM of H3. It is possible that the stimulatory effects of relaxin-3 on CRH release are via a direct effect on CRH neurons. Further studies, such as dual immunohistochemistry/*in situ* hybridisation for CRH and RXFP3/RXFP1 receptors, would be required to further investigate the possibility of a direct effect of relaxin-3 on CRH neurons.

AVP has a well-characterised potentiating effect on CRH-stimulated ACTH release (Volpi *et al.* 2004), and evidence suggests that AVP plays a physiological role in the stress response (Plotsky *et al.* 1985, Engler *et al.* 1989, Bartanusz *et al.* 1993, Herman 1995, Muller *et al.* 2000). Our *in vitro* studies show that in addition to stimulating CRH release from *in vitro* hypothalamic explants, H3 also stimulated the release of AVP, suggesting that relaxin-3 may stimulate the HPA axis via vasopressinergic pathways in addition to stimulating CRH release. Interestingly, the expression of hypothalamic *Avp* mRNA, in addition to hypothalamic *Oxt*, has been shown to be down-regulated following chronic rAVV expression of a relaxin-3 agonist in the rat hypothalamus (Ganella *et al.* 2013a). The results of this chronic study are in contrast to our results showing acute stimulation of AVP. These differences may reflect distinct effects of relaxin-3/RXFP3 activation in the acute and chronic setting and also under different physiological states of stress and feeding. OXT has also been implicated in the stress response (Robinson *et al.* 2002), and it would be interesting to measure OXT release from *in vitro* hypothalamic explants and assess whether OXT may be implicated in relaxin-3/RXFP3 signalling.

Expression of RXFP3 in the human pituitary has been previously demonstrated by RT-PCR (Matsumoto *et al.* 2000). RXFP1 has been shown to be expressed in the pituitary glands of pregnant mice, but not in those of male

or non-pregnant female mice (Krajnc-Franken *et al.* 2004). In our studies, H3 had no effect on the release of ACTH from anterior pituitary fragments harvested from male rats, suggesting that relaxin-3 is unlikely to mediate its effect on the HPA axis via the anterior pituitary. This is in accord with our hypothesis that the effects of relaxin-3 are mediated at the level of the hypothalamus.

IPVN injections of H3 (540 pmol) stimulated production of prolactin, an anterior pituitary hormone so named for its ability to stimulate lactation and mammary gland development, with a maximal response at 15 min post-IPVN injection. Prolactin is one of the first hormones shown to increase in plasma serum in response to acute physical or psychological stress (Noel *et al.* 1972), although the mechanism by which this response is mediated remains uncharacterised. Many peptides have been implicated in prolactin release, including thyrotrophin-releasing hormone, vasoactive intestinal peptide, prolactin-releasing peptide and AVP, OXT and dopaminergic pathways (Shin 1982, Freeman *et al.* 2000, Maixnerova *et al.* 2011, Kennett & McKee 2012). AVP has been shown to have a direct effect on prolactin release in male rats (Shin 1982), and OXT is emerging as an important regulator of prolactin secretion (Kennett & McKee 2012). In our studies, there was no direct H3 effect on ACTH production from anterior pituitary fragments. It is possible that the increase in prolactin following administration of H3 may have been mediated indirectly via an increase in hypothalamic AVP. It would also be interesting to assess in future studies whether OXT may also be responsible for the H3-induced increase in plasma prolactin concentrations.

We have shown that an i.c.v. injection of H2 (5 nmol) significantly increased corticosterone levels at 30 min after injections, suggesting that H2 may also stimulate the HPA axis. IPVN injection of H2 increased corticosterone at 30 min following injection compared with vehicle, although this effect did not reach statistical significance at 540 pmol compared with an equimolar dose of H3. It is possible that higher doses of IPVN H2 may have significantly stimulated the HPA axis. Both RXFP1 and RXFP3 are abundant in the PVN (Sutton *et al.* 2004, Ma *et al.* 2006). H2 and H3 bind to RXFP1 with high affinity, and initial binding studies suggested that only H3 binds to RXFP3 (Liu *et al.* 2003a). However, recent work demonstrates that H2 can also bind and activate human RXFP3 (van der Westhuizen *et al.* 2010), although currently there is no evidence of H2-binding activity to rodent RXFP3 *in vitro* or *in vivo*. H3 also binds RXFP4, but this is a pseudogene in the rat (Bathgate *et al.* 2005, Chen *et al.* 2005). Relaxin-3 and relaxin-mediated stimulation of the

HPA axis may involve both RXFP1 and RXFP3. Further studies requiring specific relaxin receptor agonists or antagonists are necessary to determine the receptor system or systems that mediate the effects of the relaxins on the HPA axis. Such studies have been carried out to investigate other relaxin-3-mediated actions, including the central effects of an RXFP3 antagonist on alcohol-seeking behaviour (Ryan *et al.* 2013b) and those of a newly developed RXFP3-selective agonist on anxiety- and depressive-like behaviours in the rat (Ryan *et al.* 2013a). The latter study demonstrated that i.c.v. administration of the selective RXFP3 agonist decreased anxiety-like behaviour, suggesting a potential role for RXFP3 agonists as anxiolytic and anti-depressant agents. These studies highlight the potential use of selective agents to tease out the potential physiological effects of the relaxin-3/RXFP3 system.

In summary we have investigated the effects of relaxin-3 on the HPA axis. IPVN administration of H3 increased plasma ACTH and corticosterone in a dose-response at 30 min following injection compared with vehicle. Treatment of hypothalamic explants with H3 stimulated the release of CRH and AVP, but H3 had no effect on the release of ACTH from *in vitro* pituitary fragments. These results suggest that relaxin-3 may regulate the HPA axis via hypothalamic CRH and AVP neurons. Relaxin-3 may act as a central signal linking nutritional status, reproductive function and stress.

Declaration of interest

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

Funding

The section is funded by grants from the MRC, BBSRC, NIHR, an Integrative Mammalian Biology (IMB) Capacity Building Award, an FP7-HEALTH-2009-241592 EuroCHIP grant and is supported by the NIHR Imperial Biomedical Research Centre Funding Scheme. B M McGowan was funded by an MRC Clinical Fellowship.

References

- Banerjee A, Shen PJ, Ma S, Bathgate RA & Gundlach AL 2010 Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF1 activation. *Neuropharmacology* **58** 145–155. (doi:10.1016/j.neuropharm.2009.06.019)
- Bartanusz V, Aubry JM, Jezova D, Baffi J & Kiss JZ 1993 Up-regulation of vasopressin mRNA in paraventricular hypophysiotrophic neurons after acute immobilization stress. *Neuroendocrinology* **58** 625–629. (doi:10.1159/000126602)

- Bathgate RA, Samuel CS, Burazin TC, Layfield S, Claasz AA, Reyntomas IG, Dawson NF, Zhao C, Bond C, Summers RJ *et al.* 2002 Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. *Journal of Biological Chemistry* **277** 1148–1157. (doi:10.1074/jbc.M107882200)
- Bathgate RA, Ivell R, Sanborn BM, Sherwood OD & Summers RJ 2005 Receptors for relaxin family peptides. *Annals of the New York Academy of Sciences* **1041** 61–76. (doi:10.1196/annals.1282.010)
- Beak SA, Heath MM, Small CJ, Morgan DG, Ghatei MA, Taylor AD, Buckingham JC, Bloom SR & Smith DM 1998 Glucagon-like peptide-1 stimulates luteinizing hormone-releasing hormone secretion in a rodent hypothalamic neuronal cell line. *Journal of Clinical Investigation* **101** 1334–1341. (doi:10.1172/JCI610)
- Buckingham JC & Hodges JR 1977 The use of corticotrophin production by adenohypophysial tissue *in vitro* for the detection and estimation of potential corticotrophin releasing factors. *Journal of Endocrinology* **72** 187–193. (doi:10.1677/joe.0.0720187)
- Burazin TC, Bathgate RA, Macris M, Layfield S, Gundlach AL & Tregear GW 2002 Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *Journal of Neurochemistry* **82** 1553–1557. (doi:10.1046/j.1471-4159.2002.01114.x)
- Burazin TC, Johnson KJ, Ma S, Bathgate RA, Tregear GW & Gundlach AL 2005 Localization of LGR7 (relaxin receptor) mRNA and protein in rat forebrain: correlation with relaxin binding site distribution. *Annals of the New York Academy of Sciences* **1041** 205–210. (doi:10.1196/annals.1282.031)
- Chen J, Kuei C, Sutton SW, Bonaventure P, Nepomuceno D, Eriste E, Sillard R, Lovenberg TW & Liu C 2005 Pharmacological characterization of relaxin-3/INSL7 receptors GPCR135 and GPCR142 from different mammalian species. *Journal of Pharmacology and Experimental Therapeutics* **312** 83–95. (doi:10.1124/jpet.104.073486)
- Engler D, Pham T, Fullerton MJ, Ooi G, Funder JW & Clarke IJ 1989 Studies of the secretion of corticotropin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep. I. Effect of an audiovisual stimulus and insulin-induced hypoglycemia. *Neuroendocrinology* **49** 367–381. (doi:10.1159/000125141)
- Freeman ME, Kanyicska B, Lerant A & Nagy G 2000 Prolactin: structure, function, and regulation of secretion. *Physiological Reviews* **80** 1523–1631.
- Ganella DE, Ryan PJ, Bathgate RA & Gundlach AL 2012 Increased feeding and body weight gain in rats after acute and chronic activation of RXFP3 by relaxin-3 and receptor-selective peptides: functional and therapeutic implications. *Behavioural Pharmacology* **23** 516–525. (doi:10.1097/FBP.0b013e3283576999)
- Ganella DE, Callander GE, Ma S, Bye CR, Gundlach AL & Bathgate RA 2013a Modulation of feeding by chronic rAAV expression of a relaxin-3 peptide agonist in rat hypothalamus. *Gene Therapy* **20** 703–716. (doi:10.1038/gt.2012.83)
- Ganella DE, Ma S & Gundlach AL 2013b Relaxin-3/RXFP3 signaling and neuroendocrine function – a perspective on extrinsic hypothalamic control. *Frontiers in Endocrinology* **4** 128. (doi:10.3389/fendo.2013.00128)
- Goto M, Swanson LW & Canteras NS 2001 Connections of the nucleus incertus. *Journal of Comparative Neurology* **438** 86–122. (doi:10.1002/cne.1303)
- Herman JP 1995 *In situ* hybridization analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids. *Journal of Comparative Neurology* **363** 15–27. (doi:10.1002/cne.903630103)
- Hudson P, Haley J, John M, Cronk M, Crawford R, Haralambidis J, Tregear G, Shine J & Niall H 1983 Structure of a genomic clone encoding biologically active human relaxin. *Nature* **301** 628–631. (doi:10.1038/301628a0)
- Hudson P, John M, Crawford R, Haralambidis J, Scanlon D, Gorman J, Tregear G, Shine J & Niall H 1984 Relaxin gene expression in human ovaries and the predicted structure of a human preprorelaxin by analysis of cDNA clones. *EMBO Journal* **3** 2333–2339.
- Kennett JE & McKee DT 2012 Oxytocin: an emerging regulator of prolactin secretion in the female rat. *Journal of Neuroendocrinology* **24** 403–412. (doi:10.1111/j.1365-2826.2011.02263.x)
- Krajnc-Franken MA, van Disseldorp AJ, Koenders JE, Mosselman S, van Duin M & Gossen JA 2004 Impaired nipple development and parturition in LGR7 knockout mice. *Molecular and Cellular Biology* **24** 687–696. (doi:10.1128/MCB.24.2.687-696.2004)
- Lenglos C, Mitra A, Guevremont G & Timofeeva E 2013 Sex differences in the effects of chronic stress and food restriction on body weight gain and brain expression of CRF and relaxin-3 in rats. *Genes, Brain, and Behavior* **12** 370–387. (doi:10.1111/gbb.12028)
- Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Jornvall H, Sillard R & Lovenberg TW 2003a Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *Journal of Biological Chemistry* **278** 50754–50764. (doi:10.1074/jbc.M308995200)
- Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R & Lovenberg TW 2003b Identification of relaxin-3/INSL7 as a ligand for GPCR142. *Journal of Biological Chemistry* **278** 50765–50770. (doi:10.1074/jbc.M308996200)
- Ma S, Shen PJ, Burazin TC, Tregear GW & Gundlach AL 2006 Comparative localization of leucine-rich repeat-containing G-protein-coupled receptor-7 (RXFP1) mRNA and [33P]-relaxin binding sites in rat brain: restricted somatic co-expression a clue to relaxin action? *Neuroscience* **141** 329–344. (doi:10.1016/j.neuroscience.2006.03.076)
- Ma S, Olucha-Bordonau FE, Hossain MA, Lin F, Kuei C, Liu C, Wade JD, Sutton SW, Nunez A & Gundlach AL 2009 Modulation of hippocampal theta oscillations and spatial memory by relaxin-3 neurons of the nucleus incertus. *Learning & Memory* **16** 730–742. (doi:10.1101/lm.1438109)
- Maixnerova J, Spolcova A, Pychova M, Blechova M, Elbert T, Rezacova M, Zelezna B & Maletinska L 2011 Characterization of prolactin-releasing peptide: binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. *Peptides* **32** 811–817. (doi:10.1016/j.peptides.2010.12.011)
- Matsumoto M, Kamohara M, Sugimoto T, Hidaka K, Takasaki J, Saito T, Okada M, Yamaguchi T & Furuichi K 2000 The novel G-protein coupled receptor SALPR shares sequence similarity with somatostatin and angiotensin receptors. *Gene* **248** 183–189. (doi:10.1016/S0378-1119(00)00123-2)
- McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, Gardiner JV, Murphy KG, Ghatei MA & Bloom SR 2005 Central relaxin-3 administration causes hyperphagia in male Wistar rats. *Endocrinology* **146** 3295–3300. (doi:10.1210/en.2004-1532)
- McGowan BM, Stanley SA, Smith KL, Minnion JS, Donovan J, Thompson EL, Patterson M, Connolly MM, Abbott CR, Small CJ *et al.* 2006a Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. *Regulatory Peptides* **136** 72–77. (doi:10.1016/j.regpep.2006.04.009)
- McGowan BM, Stanley SA, White NE, Spangeus A, Patterson M, Thompson EL, Smith KL, Donovan J, Gardiner JV, Ghatei MA *et al.* 2006b Hypothalamic mapping of orexigenic action and fos-like immunoreactivity following relaxin-3 administration in male Wistar rats. *American Journal of Physiology. Endocrinology and Metabolism* **292** E913–E919. (doi:10.1152/ajpendo.00346.2006)
- McGowan BM, Stanley SA, Donovan J, Thompson EL, Patterson M, Semjonous NM, Gardiner JV, Murphy K, Ghatei MA & Bloom SR 2008 Relaxin-3 stimulates the hypothalamic–pituitary–gonadal axis. *American Journal of Physiology. Endocrinology and Metabolism* **295** E278–E286. (doi:10.1152/ajpendo.00028.2008)

- Muller MB, Landgraf R, Preil J, Sillaber I, Kresse AE, Keck ME, Zimmermann S, Holsboer F & Wurst W 2000 Selective activation of the hypothalamic vasopressinergic system in mice deficient for the corticotropin-releasing hormone receptor 1 is dependent on glucocorticoids. *Endocrinology* **141** 4262–4269. (doi:10.1210/endo.141.11.7767)
- Noel GL, Suh HK, Stone JG & Frantz AG 1972 Human prolactin and growth hormone release during surgery and other conditions of stress. *Journal of Clinical Endocrinology and Metabolism* **35** 840–851. (doi:10.1210/jcem-35-6-840)
- Paxinos G & Watson C 1998 In *The Rat Brain in Stereotactic Co-ordinates*, 4th edn. San Diego, CA, USA: Academic.
- Plotsky PM, Bruhn TO & Vale W 1985 Hypophysiotropic regulation of adrenocorticotropin secretion in response to insulin-induced hypoglycemia. *Endocrinology* **117** 323–329. (doi:10.1210/endo-117-1-323)
- Robinson DA, Wei F, Wang GD, Li P, Kim SJ, Vogt SK, Muglia LJ & Zhuo M 2002 Oxytocin mediates stress-induced analgesia in adult mice. *Journal of Physiology* **540** 593–606. (doi:10.1113/jphysiol.2001.013492)
- Ryan PJ, Buchler E, Shabanpoor F, Hossain MA, Wade JD, Lawrence AJ & Gundlach AL 2013a Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive-like behaviours in the rat. *Behavioural Brain research* **244** 142–151. (doi:10.1016/j.bbr.2013.01.034)
- Ryan PJ, Kastman HE, Krstew EV, Rosengren KJ, Hossain MA, Churilov L, Wade JD, Gundlach AL & Lawrence AJ 2013b Relaxin-3/RXFP3 system regulates alcohol-seeking. *PNAS* **110** 20789–20794. (doi:10.1073/pnas.1317807110)
- Shin SH 1982 Vasopressin has a direct effect on prolactin release in male rats. *Neuroendocrinology* **34** 55–58. (doi:10.1159/000123277)
- Soloff MS, Gal S, Hoare S, Peters CA, Hunzicker-Dunn M, Anderson GD & Wood TG 2003 Cloning, characterization, and expression of the rat relaxin gene. *Gene* **323** 149–155. (doi:10.1016/j.gene.2003.09.015)
- Stanley SA, Small CJ, Kim MS, Heath MM, Seal LJ, Russell SH, Ghatei MA & Bloom SR 1999 Agouti related peptide (Agrp) stimulates the hypothalamo-pituitary gonadal axis *in vivo* & *in vitro* in male rats. *Endocrinology* **140** 5459–5462. (doi:10.1210/endo.140.11.7248)
- Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RA & Hsueh AJ 2003 H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exolooop 2. *Journal of Biological Chemistry* **278** 7855–7862. (doi:10.1074/jbc.M212457200)
- Sutton SW, Bonaventure P, Kuei C, Roland B, Chen J, Nepomuceno D, Lovenberg TW & Liu C 2004 Distribution of G-protein-coupled receptor (GPCR)135 binding sites and receptor mRNA in the rat brain suggests a role for relaxin-3 in neuroendocrine and sensory processing. *Neuroendocrinology* **80** 298–307. (doi:10.1159/000083656)
- Sutton SW, Bonaventure P, Kuei C, Nepomuceno D, Wu J, Zhu J, Lovenberg TW & Liu C 2005 G-protein-coupled receptor (GPCR)-142 does not contribute to relaxin-3 binding in the mouse brain: further support that relaxin-3 is the physiological ligand for GPCR135. *Neuroendocrinology* **82** 139–150. (doi:10.1159/000091267)
- Tanaka M, Iijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H & Ibata Y 2005 Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. *European Journal of Neuroscience* **21** 1659–1670. (doi:10.1111/j.1460-9568.2005.03980.x)
- Volpi S, Rabadan-Diehl C & Aguilera G 2004 Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis and stress adaptation. *Stress* **7** 75–83. (doi:10.1080/10253890410001733535)
- Ward H, Gardiner J, Kong WM, Murphy K & Bloom S 2003 Distribution of fluorescence following injection of recombinant adeno-associated virus encoding green fluorescent protein into the paraventricular nucleus. *Neuroendocrinology* **77** 100–104. (doi:10.1159/000068651)
- Watanabe Y, Miyamoto Y, Matsuda T & Tanaka M 2011 Relaxin-3/INSL7 regulates the stress-response system in the rat hypothalamus. *Journal of Molecular Neuroscience* **43** 169–174. (doi:10.1007/s12031-010-9468-0)
- van der Westhuizen ET, Christopoulos A, Sexton PM, Wade JD & Summers RJ 2010 H2 relaxin is a biased ligand relative to H3 relaxin at the relaxin family peptide receptor 3 (RXFP3). *Molecular Pharmacology* **77** 759–772. (doi:10.1124/mol.109.061432)
- Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA *et al.* 2001 Ghrelin causes hyperphagia and obesity in rats. *Diabetes* **50** 2540–2547. (doi:10.2337/diabetes.50.11.2540)
- Wren AM, Small CJ, Abbott CR, Jethwa PH, Kennedy AR, Murphy KG, Stanley SA, Zollner AN, Ghatei MA & Bloom SR 2002 Hypothalamic actions of neuromedin U. *Endocrinology* **143** 4227–4234. (doi:10.1210/en.2002-220308)

Received in final form 7 February 2014

Accepted 25 February 2014

Accepted Preprint published online 27 February 2014