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RESEARCH PAPER

Alarin stimulates food intake and gonadotrophin release in male rats

CK Boughton, M Patterson, GA Bewick, JA Tadross, JV Gardiner, KEL Beale, F Chaudery, G Hunter, M Busbridge, EM Leavy, MA Ghatei, SR Bloom and KG Murphy

Department of Investigative Medicine, Imperial College London, Commonwealth Building, Du Cane Road, London, UK

Correspondence
Dr KG Murphy, Department of Investigative Medicine, Imperial College London, Commonwealth Building, Du Cane Road, London, W12 0NN, UK. E-mail: k.g.murphy@imperial.ac.uk

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BACKGROUND AND PURPOSE

Alarin is a recently discovered member of the galanin peptide family encoded by a splice variant of galanin-like peptide (GALP) mRNA. Galanin and GALP regulate energy homeostasis and reproduction. We therefore investigated the effects of alarin on food intake and gonadotrophin release.

EXPERIMENTAL APPROACH

Alarin was administered into the third cerebral ventricle (i.c.v.) of rats, and food intake or circulating hormone levels were measured. The effect of alarin on the hypothalamo–pituitary–gonadal axis was investigated *in vitro* using hypothalamic and anterior pituitary explants, and immortalized cell lines. Receptor binding assays were used to determine whether alarin binds to galanin receptors.

KEY RESULTS

The i.c.v. administration of alarin (30 nmol) to *ad libitum* fed male rats significantly increased acute food intake to 500%, and plasma luteinizing hormone (LH) levels to 170% of responses to saline. *In vitro*, 100 nM alarin stimulated neuropeptide Y (NPY) and gonadotrophin-releasing hormone (GnRH) release from hypothalamic explants from male rats, and 1000 nM alarin increased GnRH release from GT1-7 cells. *In vivo*, pretreatment with the GnRH receptor antagonist cetrorelix prevented the increase in plasma LH levels observed following i.c.v. alarin administration. Receptor binding studies confirmed alarin did not bind to any known galanin receptor, or compete with radiolabelled galanin for hypothalamic binding sites.

CONCLUSIONS AND IMPLICATIONS

These results suggest alarin is a novel orexigenic peptide, and that it increases circulating LH levels via hypothalamic GnRH. Further work is required to identify the receptor(s) mediating the biological effects of alarin.

Abbreviations

aCSF, artificial CSF; CHO, Chinese hamster ovary; FSH, follicle-stimulating hormone; GALP, galanin-like peptide; GLP-1, glucagon-like peptide 1; GnRH, gonadotrophin-releasing hormone; HPG, hypothalamo–pituitary–gonadal; LH, luteinizing hormone; NDP-MSH, [Nle4D-Phe7]-α-melanocyte-stimulating hormone; NMU, neuromedin U; NPY, neuropeptide Y

Introduction

Alarin is a recently discovered member of the galanin peptide family that is encoded by a splice variant of galanin-like peptide (GALP), first found in gangliocytes of human neuroblastic tumours. This splice variant results from exclusion of exon 3, which causes a frame shift after the signal peptide sequence of GALP, and generates the 25-amino acid peptide alarin. The N-terminal ends of alarin and GALP share their first five amino acids (Table S1). The subsequent 20 amino acids of alarin have no

significant homology to any other known peptide (Santic *et al.*, 2006).

Little is known regarding the physiological role or pharmacological properties of alarin. To date, the only reported *in vivo* effects of alarin are to promote vasoconstriction and anti-oedema activity in the cutaneous microvasculature in mice (Santic *et al.*, 2007). Alarin mRNA has been detected in the mouse brain, skin and thymus (Santic *et al.*, 2007). The distribution of alarin-like immunoreactivity (LI) in the adult rat brain has been reported in abstract format only (Eberhard *et al.*, 2007). This study used an affinity-purified polyclonal antibody directed against synthetic murine alarin peptide, and detected alarin-LI mainly in the basal ganglia, but also detected alarin-LI in regions including the amygdala and hippocampus, and found relatively dense staining for alarin-LI in the hypothalamus (Eberhard *et al.*, 2007).

Other members of the galanin peptide family are relatively well characterized. Galanin is a 29 (30 in humans)-amino acid peptide highly conserved between species (Tatemoto *et al.* 1983; Vrontakis, 1987; Schmidt, 1991; Lundkvist, 1995; Kofler *et al.*, 1996) and widely distributed throughout the CNS and PNS (Melander *et al.* 1985; 1986). GALP is a 60-amino acid peptide originally discovered as another endogenous ligand for the galanin receptors (Ohtaki *et al.*, 1999; Cunningham, 2004). GALP mRNA distribution is more restricted than that of galanin. In the CNS, GALP mRNA is detected only in the hypothalamic arcuate nucleus and the median eminence of the rat (Jureus *et al.* 2000; Kerr *et al.*, 2000; Larm & Gundlach, 2000; Takatsu *et al.*, 2001). Galanin and GALP have been associated with several physiological functions within the CNS (Lang *et al.*, 2007). In particular, both peptides appear to play roles in energy homeostasis and reproduction. The i.c.v. administration of galanin or GALP to rats has been shown to stimulate feeding (Crawley *et al.*, 1990; Matsumoto *et al.* 2002) and increase circulating levels of luteinizing hormone (LH). The stimulatory effect of both galanin and GALP on LH appears to be mediated by the release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (Sahu *et al.* 1987; Lopez *et al.*, 1991; Matsumoto *et al.*, 2001).

Galanin is thought to mediate its effects via three G-protein-coupled receptors: GAL_1, GAL_2 and GAL_3 (Branchek *et al.*, 2000; nomenclature follows Alexander *et al.*, 2009). GALP also binds to all three galanin receptors, with highest affinity for the GAL_3 receptor and lowest affinity for the GAL_1 receptor, but experimental evidence suggests it may also mediate its effects via an additional and as yet unidentified receptor (Matsumoto *et al.*, 2001; Lawrence *et al.* 2003; Krasnow *et al.*, 2004; Seth *et al.* 2004).

Both galanin and GALP contain an identical sequence of 13 residues (galanin 1–13, GALP 9–21), which is thought necessary to bind to the three known galanin receptors (Land *et al.* 1991; Bloomquist *et al.*, 1998; Smith *et al.* 1998; Lang *et al.*, 2005). Alarin lacks this proposed galanin receptor-binding domain, suggesting that it may mediate its biological effects through other receptor(s). In support of this hypothesis, it has been reported that alarin is unable to bind to membrane preparations of human GAL_1 or GAL_2 receptor-expressing neuroblastoma cells (Santic *et al.*, 2007). There is an interest in the development of GALP as a therapeutic agent for the treatment of obesity due to its restricted distribution and relatively specific biological activity (Gundlach, 2002; Nonaka *et al.*, 2008). However, GALP is known to bind to multiple receptors, increasing the chance of side effects if it is to be used therapeutically. Until the complete galanin peptide family biology is understood, pharmacologically manipulating the GALP system remains problematic.

Given the well-characterized role of galanin and GALP in metabolism and reproduction, we investigated the effect of alarin on food intake and the hypothalamo–pituitary–gonadal (HPG) axis in male rats. We have also investigated the mechanisms by which alarin mediates its effects on the HPG axis. The present study demonstrated that i.c.v. administration of alarin acutely stimulated food intake and LH release in male rats. Our results also suggested that the effects of alarin on the HPG axis were mediated via hypothalamic GnRH. The receptor by which alarin mediates these effects remains unknown, but our results suggest that it is likely to be a receptor at which galanin does not bind. Determining the role of alarin may be useful in the design of therapeutic agents to treat reproductive disorders or obesity.

Methods

Animals

All animal care and experimental procedures complied with the UK Home Office Animals (Scientific Procedures) Act 1986 (Project Licence 70/6402). Adult male Wistar rats (specific pathogen free; Charles River, Margate, UK) weighing 250–300 g were individually housed for cannulation under controlled temperature (21–23°C) and light (12 h light, 12 h dark cycle; lights on at 0700 h) with *ad libitum* access to food (RM1 diet; SDS Ltd, Witham, UK) and water. Animals weighing 200–250 g group

housed (five per cage) under the same conditions were used for hypothalamic and pituitary explant studies, and membrane preparation.

i.c.v. Cannulation and injection

The rats were implanted with a permanent 22-gauge stainless steel cannula projecting to the third ventricle, as described previously (Wren *et al.*, 2001), according to the co-ordinates of Paxinos and Watson (0.8 mm caudal to bregma in the midline, and implanted 6.5 mm below the outer surface of the skull) (Paxinos and Watson, 2007). The animals were allowed to recover for 7 days after surgery. Cannula placement was confirmed by a positive dipsogenic response to angiotensin II (50 ng per rat). For all i.c.v. injections, a total volume of 5 μL was injected over 1 min via a 28-gauge stainless steel injector placed in and projecting 1 mm below the tip of the cannula.

Effect of i.c.v. alarin on food intake

A in ad libitum *fed male rats during the early light phase.* Adult male *ad libitum* fed rats received a single i.c.v. injection of either saline, alarin (3, 6 or 30 nmol) or neuropeptide Y (NPY) (2.4 nmol) (positive control) (Levine and Morley, 1984) in the early light phase (0900–1000 h) (n = 8–10 saline or alarin, n = 5 NPY). The animals were returned to their home cages with a pre-weighed amount of rat chow. Food intake was measured at 1, 2, 4, 8 and 24 h after injection, and body weight was measured at 0 and 24 h. A second study was carried out using an identical protocol, but administering higher doses of alarin (10, 30 and 90 nmol) to establish the most effective dose on food intake.

B in fasted male rats during the early light phase. Adult male rats, fasted for 12 h, received a single i.c.v. injection of either saline, alarin (3, 10, 30 or 90 nmol) or [Nle4D-Phe7]-α-melanocyte-stimulating hormone (NDP-MSH) (3 nmol) (positive control) (Brown, 1998) between 0900 and 1000 h (n = 7–9 saline and alarin, n = 4 NDP-MSH). The animals were returned to their home cages, and food intake and body weight were measured as described above.

C in ad libitum *fed male rats at the onset of the dark phase.* Adult male *ad libitum* fed rats received a single i.c.v. injection of either saline, alarin (3, 10, 30 or 90 nmol) or NDP-MSH (3 nmol) (positive control) between 1900 and 2000 h (n = 9–10 saline and alarin, n = 5 NDP-MSH). The animals were returned to their home cages, and food intake and body weight were measured as described above.

Effect of i.c.v. alarin on behaviour in ad libitum *fed male rats in the early light phase*

Adult male *ad libitum* fed rats received a single i.c.v. injection of saline, alarin (30 nmol) or neuromedin U (NMU) (3 nmol) (positive control) (Wren *et al.*, 2002) between 0900 and 1000 h (n = 9–11 saline and alarin, n = 5 NMU). Behaviour was analysed for 2 h after injection by observers unaware of the treatments, using a method adapted from Fray *et al.* (Fray *et al.* 1980; Smith, 2006; Patel *et al.*, 2008). The observers remained in the room throughout the observation period, and behaviours were recorded without disturbance to the animals. Behaviour was classified into 10 categories: feeding, drinking, grooming, rearing, locomotion, burrowing, head down, sleeping, pica and tremor. Each animal was observed for 15 s every 5 min during the test period; three separate behaviours were recorded during the 15 s observation with a total of 36 observations per hour.

Effect of i.c.v. alarin on plasma gonadotrophins and testosterone in male rats

Adult male *ad libitum* fed rats received a single i.c.v. injection of either saline, 6 nmol alarin or 2 nmol kisspeptin-10 (positive control) (Thompson *et al.*, 2004) in the early light phase (0900–1200 h). The rats were decapitated either 30 (n = 9–10 saline and alarin, n = 5 kisspeptin) or 60 min (n = 5 for each group) after injection. Trunk blood was collected in lithium heparin tubes containing 4200 kallikrein inhibitor units aprotinin (Bayer Corp., Haywards Heath, UK), and plasma was separated by centrifugation, frozen on dry ice and stored at –20°C until measurement of plasma hormones by RIA.

Effect of alarin on neuropeptide release from medial basal hypothalamic explants

The static hypothalamic explant incubation system was used as described previously (Stanley *et al.*, 1999). Briefly, *ad libitum* fed adult male rats were decapitated and the whole brain immediately removed. A 1.9 mm slice was taken from the basal hypothalamus using a vibrating microtome (Campden Instruments, Loughborough, UK), and the hypothalamus, including the medial pre-optic area, was dissected out. Following a 2 h equilibration period in artificial CSF (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^{-1} ascorbic acid and 100 g·mL^{-1} aprotinin), hypothalami were incubated in either aCSF or alarin (10 or 100 nM) in aCSF (n = 16–24 per group) for 45 min. Viability of the tissue was confirmed by a final exposure to 56 mM KCl in aCSF for 45 min.

Hypothalamic explants that failed to show peptide release above the basal level in response to aCSF containing 56 mM KCl were excluded from the data analysis. Galanin and GALP (100 nM) have previously been shown to stimulate the release of GnRH, and GALP to stimulate the release of NPY, from hypothalamic explants (Seth *et al.*, 2003; 2004). Therefore, NPY and GnRH levels in the aCSF were measured by RIA.

Effect of alarin on GnRH release from GT1–7 cells

Immortalized murine hypothalamic GnRH-producing neurons, GT1–7 cells (Mellon *et al.*, 1990) were grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine (Invitrogen Ltd, Paisley, UK), 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 IU·mL^{-1}) and streptomycin (100 µg·mL^{-1}), and maintained at 37°C in 5% CO_2. GT1–7 cells were plated on poly-L-lysine-coated 24-well plates, and incubated for 24 h before secretion experiments were performed as described previously (Patel *et al.*, 2008). For GnRH secretion experiments, cells were pre-incubated for 2 h in serum-free medium. Thereafter, the medium was discarded and the cells were incubated in 0.5 mL serum-free medium (basal); serum-free medium containing alarin (1, 10, 100, 1000 or 10 000 nM); GALP (0.1, 1, 10, 100 or 1000 nM); or glucagon-like peptide 1 (GLP-1) (100 nM) (positive control) (Beak *et al.*, 1998) (n = 14–30).

In a separate experiment, cells were incubated in 0.5 mL serum-free medium (basal); 0.5 mL serum-free medium containing alarin (6–25) (10, 100 or 1000 nM); 0.5 mL serum-free medium containing murine alarin (10, 100 or 1000 nM); or GLP-1 (100 nM) (positive control) (n = 20–24 per group). This was to determine whether the first five amino acids of alarin are necessary for its biological activity, and whether there is a difference in potency between mouse and rat alarin, because this is a cell line of murine origin. The cells were incubated at 37°C with the test substance for 240 min after which the medium was removed and stored at –20°C until measurement of GnRH by RIA.

Effect of alarin on LH release from pituitary explants

The pituitary explant system was used as described previously (Stanley *et al.*, 2004). Briefly, group housed male rats were decapitated and the anterior pituitary glands removed immediately and divided into four pieces of approximately equal size. Single quarters were placed in the wells of a 48-well tissue plate (Nunc International, Roskilde, Denmark), and incubated in 0.5 mL aCSF. They were maintained at 37°C in a humidified environment saturated with 95% O_2 and 5% CO_2 for 120 min, with the medium changed each 60 min. The segments were then incubated in 0.5 mL of aCSF alone (basal); aCSF containing alarin (10, 100 or 1000 nM); or aCSF containing GnRH (100 nM) (positive control) for 240 min (n = 10–12). The aCSF was then collected and stored at –20°C until RIA for LH.

Effect of alarin on LH release from LβT2 cells

Immortalized pituitary LH-releasing LβT2 cells (Alarid *et al.*, 1996; Thomas *et al.* 1996) were cultured and plated as described above for the GT1–7 cells. The cells were incubated in 0.5 mL serum-free medium (basal); serum-free medium containing alarin (1, 10, 100, 1000 or 10 000 nM); or GnRH (100 nM) (positive control) (n = 8–24) (Turgeon *et al.*, 1996). The cells were incubated at 37°C with the test substance for 240 min after which the medium was removed and stored at –20°C until measurement of LH by RIA.

Effect of i.c.v. alarin on plasma gonadotrophins in male rats following pretreatment with cetrorelix

Adult male *ad libitum* fed rats were given (s.c.) either saline or 200 nmol cetrorelix (GnRH receptor antagonist). The animals had been s.c. injected with saline twice in the week prior to the study to acclimatize them to the procedure. Thirty minutes later, the animals were i.c.v. injected with either saline, 30 nmol alarin or 2 nmol kisspeptin-10 (positive control) (n = 9–11 saline and alarin, n = 5 kisspeptin) (Thompson *et al.*, 2004). The animals were decapitated 30 min after i.c.v. injection, and trunk blood was collected and stored as previously described.

Preparation of membranes from galanin receptor-expressing cells, hypothalamic tissue and GT1–7 cells

Cell membranes were prepared from male rat hypothalamic tissue, Chinese hamster ovary (CHO) cells expressing GAL_1 or GAL_2 receptors and GT1–7 cells. The CHO cells were maintained in F12 nutrient mixture containing 1 mM L-glutamine (Invitrogen Ltd) and supplemented with 10% (v/v) fetal bovine serum, penicillin (100 IU·mL^{-1}) and streptomycin (100 µg·mL^{-1}) under the same conditions as GT1–7 cells described above. Membranes for receptor binding studies were prepared by differential centrifugation as described previously (Bhogal *et al.* 1993; Coppock *et al.*, 1999). Briefly, cells were washed with ice-cold phosphate-buffered saline and scraped into 50 mM HEPES (pH 7.4) (containing 30 µg·mL^{-1} aprotinin, 0.5 µg·mL^{-1} pepstatin,

0.25 μg·mL^{-1} leupeptin, 0.25 μg·mL^{-1} antipain, 0.1 mg·mL^{-1} benzamidine and 10 μg·mL^{-1} soy bean trypsin inhibitor), using an Ultra-Turrax T25 homogenizer (BDH, Poole, UK). Hypothalamic tissue from male Wistar rats was homogenized in 50 mM HEPES in the same manner as described above. Homogenates were centrifuged at 1500× *g* for 20 min at 4°C (Beckman J2-21, rotor JS-13.1, High Wycombe, UK), and the supernatant was centrifuged at 100 000× *g* at 4°C (Sorvall Ultracentrifuge OTD55B, rotor A-841, Sorvall Centrifuge, Buckinghamshire, UK). Pellets were then homogenized using a Potter-Elvehjem tissue grinder (Cole Parmer, London, UK) and re-suspended in 50 mM HEPES. For hypothalamic tissue, homogenates were further centrifuged at 100 000× *g* for 1 h at 4°C before resuspension as described above. The protein concentration in the membrane preparation was measured by Biuret assay (Gornall *et al.*, 1949), and the preparations were stored at –70 C.

Receptor binding assays

GAL_1 receptor-expressing, GAL_2 receptor-expressing, GT1–7 and hypothalamic membranes (60 μg) were incubated for 45 min in siliconized polypropylene tubes together with [^{125}I] porcine galanin (1000 Bq) and unlabelled competing peptides (as specified), at 22°C in binding buffer [20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1% BSA] in a final assay volume of 0.5 mL (Wynick *et al.*, 1993). Cell membranes expressing GAL_3 receptors were purchased from BioXtal (Mundolsheim, France), and were incubated according to the manufacturer's instructions. Briefly, membrane (1.5 μg) was incubated for 90 min in siliconized polypropylene tubes together with [^{125}I] porcine galanin (1000 Bq) and unlabelled competing peptides (as specified), at room temperature in binding buffer [50 mM Tris–HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM EDTA and 1% BSA] in a final assay volume of 0.2 mL. Galanin was iodinated using the chloramine-T method as previously described (Wood *et al.*, 1981). The reaction was centrifuged (15 874× *g*, 3 min, 4 C) (Sigma Laboratory Centrifuges 3, rotor K18, Shrewsburry, UK) to separate bound and free label. The pellets were then washed with assay buffer (0.5 mL, ice cold) and centrifuged (15 874× *g*, 3 min, 4 C). Bound radioactivity was measured by counting in a γ-counter for 240 s. Specific binding was calculated as the difference between the number of radioactive counts in 240 s in the absence (total binding) and presence (non-specific binding) of 400 nM (1 mM for GAL_3 receptor membrane) unlabelled galanin. Equilibrium competition curves were constructed with increasing amounts of unlabelled peptide (1×10^{-13} to 10^{-3} M). Binding of GLP-1 to the GT1–7 cell membranes was carried out under the conditions described above using [^{125}I] GLP-1 (1000 Bq) and unlabelled GLP-1. GLP-1 was iodinated using the iodogen method as previously described (Wood *et al.*, 1981).

All curves were performed with points in triplicate. IC_{50} values were calculated from non-linear regression analysis using the GraphPad Prism 5 program (GraphPad Software Inc., San Diego, CA, USA).

Radioimmunoassays

GnRH immunoreactivity and NPY immunoreactivity were measured using established RIAs (Allen *et al.* 1984; Patel *et al.*, 2008). LH and follicle-stimulating hormone (FSH) were measured using methods and reagents provided by the National Hormone and Pituitary program (Dr A. Parlow, University of California, Harbor Medical Center, Los Angeles, CA, USA) as described previously (Patel *et al.*, 2008). The radiolabelled peptides for the assays were prepared by the chloramine-T method (Wood *et al.*, 1981). Total plasma testosterone was measured using a commercial Coat-a-Count assay kit (Euro/DPC Ltd, Caernarfon, UK).

Data analysis

All data are presented as mean ± SEM. Data from *in vivo* HPG axis studies were analysed using a one-way ANOVA with *post hoc* Tukey's multiple comparison test. Data from the feeding studies, pituitary explant study and cell studies were analysed using a one-way ANOVA with *post hoc* Dunnett's multiple comparison test (GraphPad Prism 5). The data from cell studies were pooled from three separate experiments in order to generate an *n* of approximately 24 per group. As behavioural observation data were not normally distributed, Kruskal–Wallis one-way ANOVA on ranks was used for comparisons between treatment groups (Systat 11, San Jose, CA, USA). Data from the hypothalamic explant studies were compared by paired Student's *t*-test between the basal and the test period. These data were pooled from three separate experiments in order to generate an *n* of approximately 24 per group. In each individual experiment, explants act as their own control. In all cases, $P < 0.05$ was considered to be statistically significant.

Materials

Rat alarin (used in all studies unless specified otherwise) was synthesized by BioMol International LP (Exeter, UK). The peptide was synthesized on a TentaGel resin (Sigma-Aldrich, Gillingham, UK),

using an Fmoc/t-butyl-based solid-phase synthesis strategy. The product was purified by reversed-phase preparative HPLC followed by lyophilization. Analysis of the purified product by reversed-phase HPLC and by mass spectrometry showed above 90% purity (molecular weight: 2820.8). The peptide was not C-terminally amidated. Murine alarin and alarin (6–25) were purchased from Phoenix Pharmaceuticals Inc (Burlingame, CA, USA). Galanin, GALP, GnRH, GLP-1, NMU and NPY were purchased from Bachem UK Ltd. (Merseyside, UK). Kisspeptin-10 was purchased from Peptide Institute Inc. (Osaka, Japan). Cannulation materials were purchased from Plastics One, Inc. (Roanoke, VA, USA). Reagents for explant experiments and cell culture experiments were purchased from BDH and Invitrogen Ltd.

Results

Effect of i.c.v. alarin on food intake

A in ad libitum *fed rats during the early light phase.* The i.c.v. administration of alarin (30 nmol) and NPY (2.4 nmol) to *ad libitum* fed male Wistar rats in the early light phase significantly increased food intake five- and sixfold above that after i.c.v. saline, respectively, 0–1 h post-injection ($P < 0.01$, n = 8–10 saline and alarin, n = 5 NPY; Figure 1). There was no significant effect of alarin on food intake at any other dose or time-point studied. There was no significant effect of alarin on body weight at 24 h. The highest dose of alarin (90 nmol) caused several of the animals within this group to appear unwell immediately following the injection; therefore, 30 nmol alarin was considered to be the most effective dose without any obvious adverse behavioural effects.

B in fasted male rats during the early light phase. There was no significant effect of i.c.v. alarin (3, 10, 30 or 90 nmol) on food intake 0–1 h post-injection in fasted rats. NDP-MSH (3 nmol) significantly reduced food intake ($P < 0.05$, n = 7–9 saline and alarin, n = 4 NDP-MSH; Supporting Information Figure S1A). There was no significant effect of alarin on food intake or body weight at any other dose or time-point studied. The highest dose of alarin (90 nmol) caused several of the animals within this group to appear unwell immediately following the injection.

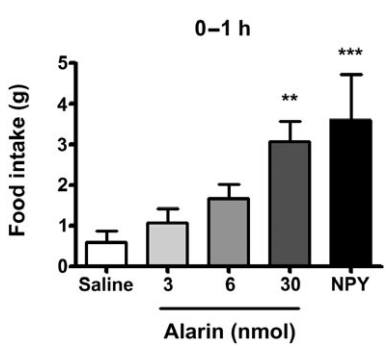

Figure 1

The effect of i.c.v. injection of saline; alarin (3, 6 or 30 nmol) (n = 8–10 per group); or NPY (2.4 nmol) (positive control) (n = 5) on 0–1 h food intake in *ad libitum* fed male rats in the early light phase. $^{**}P < 0.01$; $^{***}P < 0.001$ versus saline. Results are mean ± SEM.

C in ad libitum *fed male rats at the onset of the dark phase.* There was no significant effect of i.c.v. alarin (3, 10, 30 or 90 nmol) administered at the onset of the dark phase on food intake 0–1 h post-injection in rats. NDP-MSH (3 nmol) significantly reduced food intake ($P < 0.05$, n = 9–10 saline and alarin; n = 5 NDP-MSH; Supporting Information Figure S1B). There was no significant effect of alarin on food intake or body weight at any other dose or time-point studied. The highest dose of alarin (90 nmol) caused several of the animals within this group to appear unwell immediately following the injection.

Effect of i.c.v. alarin on behaviour in ad libitum *fed male rats in the early light phase*

The i.c.v. administration of alarin (30 nmol) had no significant effect on any behaviour recorded compared to saline-injected controls in either 0–1 or 1–2 h observation periods. NMU (3 nmol) significantly increased the frequency of grooming, locomotion, drinking and tremor behaviour, while reducing the frequency of sleeping and rearing behaviour compared to saline-injected controls (n = 9–11 saline and alarin, n = 5 NMU). Data are presented as median frequency and interquartile range (Supporting Information Table S2).

Effect of i.c.v. alarin on plasma gonadotrophins and testosterone in male rats

The i.c.v. administration of alarin (6 nmol) significantly increased plasma LH by almost 50% in *ad libitum* fed male Wistar rats 30 min after i.c.v. injection ($P < 0.05$, n = 9–10 saline and alarin; Figure 2). Alarin had no significant effect on FSH or testosterone at either 30 or 60 min after injection. Kisspeptin (2 nmol, i.c.v.) was used as a positive control and resulted in a significant increase in plasma LH, FSH and testosterone at 30 min after injection (plasma LH ng·mL^{-1}, saline 0.7 ± 0.1; kisspeptin, 7.7 ± 1.8; FSH ng·mL^{-1}; saline 10.3 ± 0.8, alarin, 11.5 ± 0.9, kisspeptin 17.3 ± 2.3 $P < 0.001$, n = 9–10 saline; n = 5, kisspeptin; plasma testosterone nmol·L^{-1}; saline 4.6 ± 0.9, alarin 4.7 ± 0.9, kisspeptin 12.3 ± 1.2 $P <$ 0.01, n = 9–10 saline and alarin, n = 5 kisspeptin).

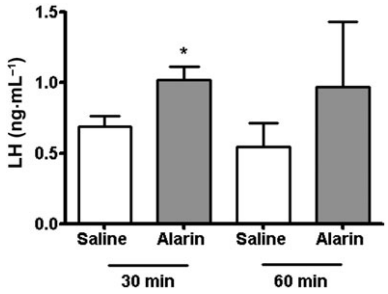

Figure 2

The effect of i.c.v. injection of saline or alarin (6 nmol) (n = 9–10 per group) on plasma LH levels 30 and 60 min after administration in *ad libitum* fed male rats. *P < 0.05; ***P < 0.001 versus saline (30 min). Results are mean ± SEM.

Effect of alarin on neuropeptide release from medial basal hypothalamic explants

Treatment with 100 nM alarin significantly increased NPY (NPY basal 100 ± 7.4%, 100 nM alarin 129.8 ± 10.2%, P < 0.05; n = 16) and GnRH release (P < 0.01; n = 24; Figure 3A) from adult male rat hypothalamic explants *in vitro* compared with basal values. At a lower dose (10 nM), alarin did not significantly influence NPY or GnRH release. Data are presented as a percentage of basal release, pooled from three separate experiments.

Effect of alarin on GnRH release from GT1–7 cells

Treatment with 1000 and 10 000 nM alarin significantly increased GnRH release from GT1–7 cells 60 and 75% above basal, respectively, after 240 min incubation (P < 0.05; n = 38, basal; n = 31, 1000 nM alarin; n = 14, 10 000 nM alarin; Figure 3B). GALP (1000 nM also significantly increased GnRH release (P < 0.05; n = 38 basal; n = 27, 1000 nM GALP; Figure 3B). GLP-1 (100 nM), used as a positive control (Beak *et al.*, 1998), significantly increased GnRH release to 270% of basal (fmol·mL^{-1}: basal 75.8 ± 6.9, 100 nM GLP-1 187.6 ± 22.1 P < 0.001, n = 38 basal, n = 39 100 nM GLP-1) These data are pooled from three separate experiments. There was no difference in the potency of rat and mouse alarin on GnRH release from GT1–7 cells. Murine alarin significantly increased GnRH release from GT1–7 cells at a concentration of 1000 nM, but lower concentrations had no effect. Truncated alarin (6–25) had no effect on GnRH release from GT1–7 cells compared to basal after 240 min incubation (n = 20–24 per group) (Supporting Information Table S3).

Effect of alarin on LH release from pituitary explants

There were no significant changes in LH release from anterior pituitary explants from adult male rats following treatment with alarin. GnRH 100 nM, the positive control, significantly stimulated LH release from pituitary explants (LH ng·mL^{-1}: aCSF 120.4 ± 15.7; 10 nM alarin, 119.7 ± 14.5; 100 nM alarin, 158.2 ± 22.0; 1000 nM alarin, 140.4 ± 11.6; 100 nM GnRH, 538.2 ± 58.0; P < 0.001, n = 10–12 per group).

Effect of alarin on LH release from LβT2 cells

There were no significant changes in LH release from LβT2 cells after 240 min incubation with alarin compared to basal. GnRH 100 nM, the positive control, significantly stimulated LH release from LβT2 cells (LH ng·mL^{-1}: basal 1.2 ± 0.1, 1 nM alarin 1.1 ± 0.1, 10 nM alarin 1.0 ± 0.1, 100 nM alarin 1.2 ± 0.1, 1000 nM alarin 1.1 ± 0.1, 10 000 nM alarin 1.4 ± 0.1, 100 nM GnRH 2.4 ± 0.4. P < 0.001, n = 23 basal, 10, 100 and 1000 nM alarin, n = 8 0.1 and 10 000 nM alarin, n = 17 100 nM GnRH). These data are pooled from three separate experiments.

Effect of i.c.v. alarin on plasma gonadotrophins in male rats after pretreatment with cetrorelix

The i.c.v. administration of 30 nmol alarin significantly increased plasma LH by 70% in *ad libitum* fed male Wistar rats 30 min after i.c.v. injection compared to saline-injected controls (P < 0.05; n = 9–11, saline or alarin; Figure 4). This effect was blocked by pretreatment with the GnRH receptor antagonist cetrorelix (200 nmol) (P < 0.01; n = 9–11, saline or alarin; Figure 4). There was a significant increase in plasma LH 30 min after i.c.v. injection of 2 nmol kisspeptin, the positive control, compared to saline-injected controls. This effect was also blocked by pretreatment with cetrorelix (LH ng·mL^{-1}; s.c. saline/i.c.v. saline 0.6 ± 0.1, s.c. saline/i.c.v. kisspeptin 3.0 ± 0.3, P < 0.001; s.c. cetrorelix/i.c.v. kisspeptin 0.6 ± 0.1, P < 0.001, n = 9–11 saline; n = 5 kisspeptin).

Galanin receptor binding studies

Alarin was unable to displace [^{125}I] galanin binding at the GAL_1, GAL_2, GAL_3 receptor or hypothalamic membranes (IC_{50} values: alarin, >1000 nM for all membranes; Figure 5). Galanin and GALP, the positive controls, were able to displace [^{125}I] galanin binding at the GAL_1 receptor (specific binding 91.6 ± 1.5%) (IC_{50} values: galanin, 1.4 ± 0.04 nM; GALP, 45 ± 0.08 nM); GAL_2 receptor (specific binding 63.0 ± 2.7%) (IC_{50} values: galanin 1.9 ± 0.07 nM: GALP, 18.7 ± 0.2 nM); GAL_3 receptor (specific binding 47.1 ± 1.8%) (IC_{50} values: galanin, 108.4 ± 0.26; GALP, 1.53 ± 0.21; alarin >1000 μM); and hypothalamic membranes (specific binding 75.8 ± 2.1%) (IC_{50} values: galanin, 0.06 ± 0.1 nM; GALP,

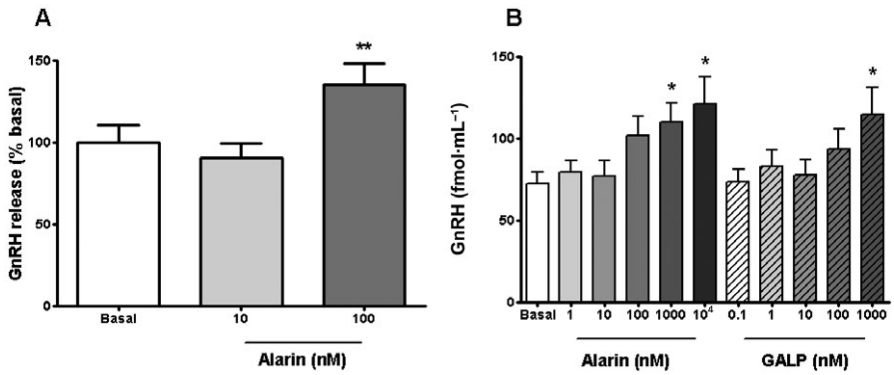

Figure 3

(A) The effect of alarin (10 or 100 nM) on GnRH release from hypothalamic explants from adult male rats ($n = 16$ per group). $^{**}P < 0.01$ versus basal release. Data are presented as percentage of basal release. (B) The effect on GnRH release from GT1–7 cells of a 240 min incubation with serum-free medium (basal); serum-free medium containing alarin (1, 10, 100, 1000, 10 000 nM) (open bars); or serum-free medium containing GALP (0.1, 1, 10, 100, 1000 nM) (hatched bars) ($n = 14$–30 per group). $^{*}P < 0.05$ versus basal release. Results are mean ± SEM.

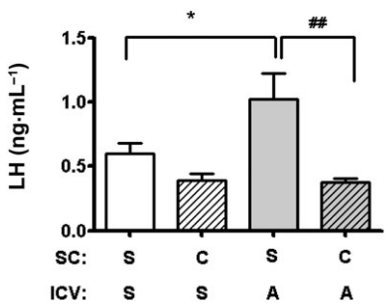

Figure 4

The effect of i.c.v. administration of saline (S) or alarin (A) (30 nmol) 30 min after s.c. pretreatment with 200 nmol cetrorelix (C) or saline (S) in *ad libitum* fed adult male rats on plasma LH levels ($n = 9$–10 per group) 30 min after i.c.v. injection. $^{*}P < 0.05$ versus saline/saline. $^{\#\#}P < 0.01$ versus saline/alarin. Results are mean ± SEM.

36.9 ± 0.09 nM). Results are from two independent experiments performed in triplicate (n = 4–6 per point; Figure 5).

[^{125}I] labelled GLP-1 was able to bind to GT1–7 membrane and was displaced with increasing concentrations of unlabelled GLP-1 (specific binding 30.0 ± 2.4%) (IC_{50} value 1.7 ± 0.1 nM), confirming the receptor bioactivity of the membrane preparation. [^{125} I] labelled galanin was unable to bind to GT1–7 membrane (specific binding less than 3%) (data not shown).

Discussion

We have demonstrated that i.c.v. administration of alarin acutely stimulates food intake and increases circulating LH levels in male rats. Our results suggest that the effects of alarin on the HPG axis are mediated via hypothalamic GnRH.

While all doses of alarin administered to *ad libitum* fed rats in the early light phase increased food intake at 1 h post-injection, only the effect of the highest dose of 30 nmol reached statistical significance, with a mean food intake fivefold greater than saline-injected animals. The orexigenic effect of alarin was not observed following i.c.v. administration to rats in fasted or early dark phase experimental paradigms. The orexigenic effect of alarin thus appears to be relatively weak compared to other, well-characterized, hypothalamic orexigenic neuropeptides such as NPY and GALP (Levine and Morley, 1984; Matsumoto *et al*. 2002). Alarin did stimulate NPY release from hypothalamic explants. The orexigenic effect of alarin may therefore be mediated, at least in part, by an increase in hypothalamic NPY release. We currently do not know which brain regions mediate the effect of alarin on food intake. Alarin-LI has been detected in the rat hypothalamus and basal ganglia (Eberhard *et al*., 2007), and both areas of the brain are implicated in the regulation of appetite (Kelley, 2004; Berthoud, 2006). It is possible that alarin may mediate effects on feeding through either or both structures. However, given the distance of the third ventricle from the basal ganglia, it appears more likely that the effects of alarin observed in our i.c.v. studies are mediated via the hypothalamus.

The galanin family of peptides are known to play a role in the regulation of the HPG axis (Gottsch *et al*. 2004; Merchenthaler, 2008). The i.c.v. administration of 6 nmol alarin significantly increased plasma LH levels by nearly 50%, 30 min after injection. To investigate the mechanism by which alarin stimulated the HPG axis, we examined the effects of alarin on hypothalamic explants and immortalized GnRH-releasing GT1–7 cells, and on pituitary

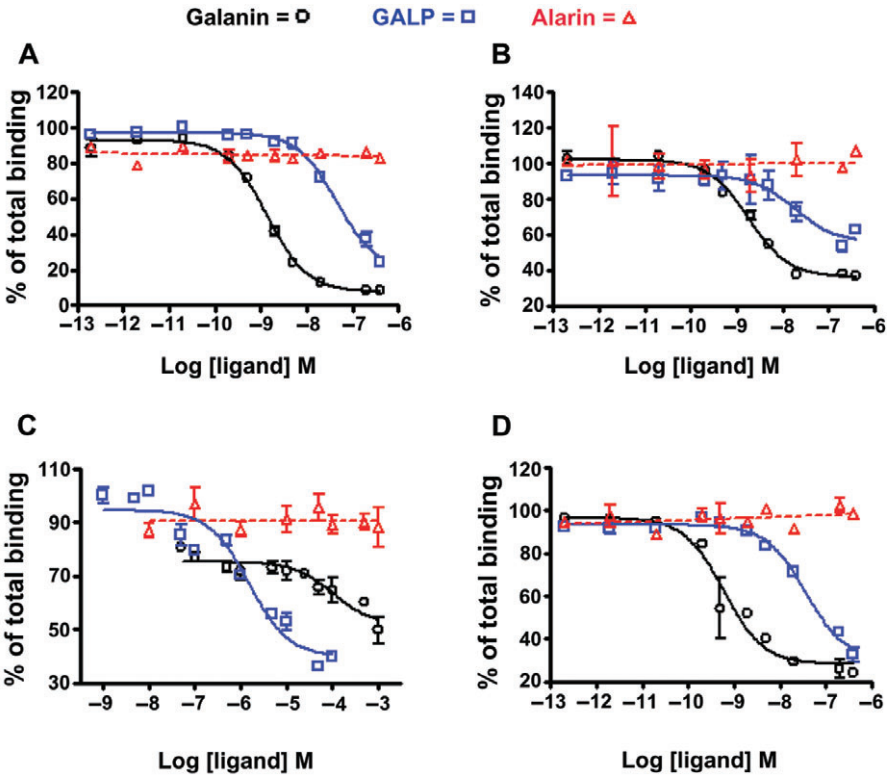

Figure 5

Competitive receptor binding of [^{125}I] radiolabelled galanin with increasing concentrations of unlabelled galanin, GALP and alarin to membranes prepared from (A) CHO cells transfected with GAL_1 receptors, (B) CHO cells transfected with GAL_2 receptors, (C) GAL_3 receptor expressing membranes and (D) male rat hypothalamic membranes. There was no significant binding of alarin to any of the membrane preparations. IC_{50} values for galanin and GALP are given in the corresponding text. Results are mean ± SEM from two independent experiments performed in triplicate (n = 4–6 per point).

explants and immortalized LH-releasing LβT2 cells. Alarin significantly stimulated GnRH release from hypothalamic explants and GT1–7 cells. Such *in vitro* systems are typically less sensitive than *in vivo* responses to the administration of neuropeptides (Wren *et al.*, 2002). Thus, relatively high concentrations and n numbers are often required to cause a significant effect, even with very potent factors (Thompson *et al.*, 2004). There is a difference in the effective dose necessary for a GnRH response between the hypothalamic explants and the GT1–7 cell line, and this is possibly due to differences in sensitivity between these two *in vitro* systems. Hypothalamic explants contain many different cell types through which alarin may act to increase GnRH release either directly or indirectly. There may only be a small direct effect of alarin on GnRH-releasing neurons themselves. However, even high concentrations of alarin had no effect on LH release from pituitary segments or LβT2 cells. These results suggest that alarin stimulates the HPG axis via an effect on GnRH. The effects of i.c.v. alarin on LH were blocked by pretreatment with the GnRH antagonist cetrorelix, providing further evidence that this effect is mediated via hypothalamic GnRH. In this study, we used a higher dose of alarin (30 nmol) than in the previous experiment. This was to ensure that alarin had a stimulatory effect on LH, as pretreatment with an s.c. injection has been reported to cause physical stress sufficient to suppress LH secretion (Li *et al.*, 2004). The effect of i.c.v. alarin on circulating LH is small, and there appears to be little difference between the magnitude of the LH response to 6 or 30 nmol alarin, suggesting perhaps a threshold effect of alarin on the HPG axis rather than a dose–response.

Alarin lacks the galanin receptor binding domain, which is thought to be responsible for the binding of galanin and GALP to the three known galanin receptors (Land *et al.*, 1991; Bloomquist *et al.*, 1998; Smith *et al.* 1998; Lang *et al.*, 2005). It has previously been reported that alarin is unable to bind to membrane preparations of human GAL_1 or GAL_2 receptor-expressing neuroblastoma cells (Santic *et al.*, 2007). Our results support this finding, demonstrating that alarin is unable to compete with

the binding of radiolabelled galanin to membranes from CHO cells expressing the GAL_1 or GAL_2 receptor, and we have shown for the first time that alarin is unable to compete with the binding of radiolabelled galanin to membranes expressing GAL_3 receptors. Galanin and GALP were able to dose-dependently displace galanin binding at all three receptors.

In addition, the hypothalamus expresses all three galanin receptors (Seth *et al.*, 2004). Our data are the first to show that alarin does not compete with radiolabelled galanin binding to male rat hypothalamic membranes. Again, GALP was able to compete with radiolabelled galanin in these experiments. Previous studies suggest that none of the known galanin receptors are expressed in GT1–7 cells, and galanin has no effect on GnRH release from GT1–7 cells (Seth *et al.*, 2004), and our binding studies suggest that radiolabelled galanin is unable to bind to GT1–7 cell membranes. The release of GnRH from GT1–7 cells in response to alarin therefore strongly suggests that an as yet unidentified receptor mediates this effect, and that this unidentified receptor is unable to bind galanin.

It has been speculated that GALP mediates some of its biological effects via a receptor or receptors distinct from the three known galanin receptors (Matsumoto *et al.*, 2001; Lawrence *et al.* 2003; Krasnow *et al.*, 2004; Seth *et al.* 2004). Alarin may act as a ligand for the same unidentified receptor or receptors. It is difficult to determine whether GALP and alarin bind to the same receptors. GALP has been reported to be very adhesive (Lang *et al.*, 2005), and I^{125} labelled GALP shows high non-specific binding to receptor membranes (C. Boughton, unpubl. obs.). However, our studies suggest that the first five amino acids of alarin, which it has in common with GALP, are essential for the stimulatory effect of alarin on GT1–7 cells *in vitro*, as alarin (6–25) had no effect on GnRH release from GT1–7 cells. Further studies are now required to determine whether GALP and alarin mediate any of their biological effects through a shared receptor.

The dose of alarin required to stimulate food intake following i.c.v. administration in rats is 100-fold greater than the dose of GALP required to elicit a similar effect, suggesting that if the effects of GALP and alarin on appetite are mediated via the same novel receptor, that alarin has relatively low affinity for this receptor (Matsumoto *et al.*, 2002). GALP stimulates NPY release from hypothalamic explants (Seth *et al.*, 2003), and it has been suggested that GALP mediates its orexigenic effects in rats in part, through activating NPY neurons in the dorsomedial nucleus (Kuramochi *et al.*, 2006). In our studies, alarin also stimulated NPY release from hypothalamic explants, suggesting that alarin may mediate its effect on feeding via similar pathways to GALP. Further work is required to identify the precise mechanism mediating the orexigenic effect of central alarin administration. It would be of particular interest to determine the neuronal populations activated by central administration of alarin, and to compare the pattern of activation with those elicited by central administration of GALP and galanin.

GALP has been shown to stimulate the HPG axis via hypothalamic GnRH release *in vivo* (Matsumoto *et al.*, 2001), and to stimulate the release of GnRH from hypothalamic explants *in vitro* (Seth *et al.*, 2004). The stimulatory effects of GALP on the HPG axis *in vivo* are observed following the administration of relatively low doses (Matsumoto *et al.* 2001; Castellano *et al.*, 2006). The principal galanin receptor expressed in GnRH neurons is the GAL_1 receptor (Mitchell *et al.*, 1999), which may mediate the effects of galanin and GALP on the HPG axis. However, we have shown that GALP also stimulates the release of GnRH from GT1–7 cells which do not express GAL_1 receptors or the other known galanin receptors (Seth *et al.*, 2004). We compared the effects of GALP and alarin on GnRH release from GT1–7 cells, and they appear to have similar potency. The difference in potency between alarin and GALP *in vivo* may be due to their different binding affinities at a novel receptor and/or the activation of different receptors. Alarin may be specific for only one receptor, whereas GALP is known to bind to at least three receptors (GAL_1, GAL_2 and GAL_3 receptors) (Lang *et al.*, 2005).

In summary, these studies demonstrate that i.c.v. alarin stimulates food intake and the HPG axis in rats, and that the stimulatory effect of alarin on circulating LH is likely to be mediated via GnRH. While these studies demonstrate the pharmacological effects of alarin *in vivo*, further studies are necessary to investigate the physiological significance of alarin. The *in vivo* potency of alarin appears relatively low, but if alarin is a specific ligand for a particular novel receptor, it may provide a tool to investigate and manipulate the specific neural pathways mediating the different actions of the galanin peptide family. Determining the role of alarin may thus be useful in the design of therapeutic agents to treat reproductive disorders or obesity. Further work is required to identify and characterize the receptor(s) through which alarin mediates its biological effects.

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Conflict of interest

The authors have no conflict of interest to disclose.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) The effect of i.c.v. injection of saline; alarin (3, 10, 30 or 90 nmol); or NDP-MSH (3 nmol) (positive control) on 0–1 h food intake in fasted male rats in the early light phase (n = 7–9 saline and alarin, n = 4 NDP-MSH). *P < 0.05 versus saline. Results are mean ± SEM. (B) The effect of i.c.v. injection of saline; alarin (3, 10, 30 or 90 nmol) (n = 8–10 per group); or NDP-MSH (3 nmol) (positive control) on 0–1 h food intake in *ad libitum* fed male rats at the onset of the dark phase (n = 9–10 saline and alarin, n = 5 NDP-MSH). *P < 0.05 versus saline. Results are mean ± SEM.

Table S1 Amino acid sequence comparison of different species homologues of alarin, GALP and galanin. Grey shading indicates conserved residues between species. Single-line rectangle indicates five amino acids common to both alarin and GALP. Double-line rectangle indicates 13 amino acids common to both GALP and galanin (galanin receptor binding domain). Alarin, GALP and galanin sequences are taken from (Tatemoto *et al.*, 1983; Vrontakis, 1987; Schmidt, 1991; Lundkvist, 1995; Cunningham, 2004; Santic *et al.*, 2007).

Table S2 The effect of i.c.v. administration of saline, alarin (30 nmol) or NMU (3 nmol) to *ad libitum* fed male rats in the early light phase on 0–1 h behaviour (n = 9–11 saline and alarin, n = 5 NMU). Data are presented as median frequency and interquartile range. *P < 0.05 versus saline; **P < 0.01 versus saline.

Table S3 The effect of serum-free medium (basal); serum-free medium containing murine alarin (10, 100 or 1000 nM); or serum-free medium containing alarin (6–25) (10, 100 or 1000 nM) on GnRH release from GT1–7 cells after a 240 min incubation (n = 20–24 per group). Results are mean ± SEM. Data are presented as percentage of basal release and are pooled from three separate experiments. Data from Figure 3B are also included in this table for comparison (rat alarin and GLP-1). *P < 0.05 versus basal release. ***P < 0.001 versus basal release.

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