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RESEARCH PAPER

Augurin stimulates the hypothalamo-pituitary-adrenal axis via the release of corticotrophin-releasing factor in rats

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Background and purpose: The functional characterization of secreted peptides can provide the basis for the development of novel therapeutic agents. Augurin is a recently identified secreted peptide of unknown function expressed in multiple endocrine tissues, and in regions of the brain including the hypothalamus. We therefore investigated the effect of hypothalamic injection of augurin on the hypothalamo-pituitary-adrenal (HPA) axis in male Wistar rats.
Experimental approach: Augurin was given as a single injection into the third cerebral ventricle (i.c.v.) or into the paraventricular nucleus (iPVN) of the hypothalamus. Circulating hormone levels were then measured by radioimmunoassay. The effect of augurin on the release of hypothalamic neuropeptides was investigated *ex vivo* using hypothalamic explants. The acute effects of iPVN augurin on behaviour were also assessed.
Key results: i.c.v. injection of augurin significantly increased plasma ACTH and corticosterone, compared with vehicle-injected controls, but had no effect on other hypothalamo-pituitary axes hormones. Microinjection of lower doses of augurin into the PVN caused a similar increase in plasma ACTH and corticosterone, without significant alteration in behavioural patterns. Incubation of hypothalamic explants with increasing doses of augurin significantly elevated corticotrophin-releasing factor (CRF) and arginine vasopressin release. *In vivo*, peripheral injection of a $CRF_{1/2}$ receptor antagonist prevented the rise in ACTH and corticosterone caused by i.c.v. augurin injection.
Conclusions and implications: These data suggest that augurin stimulates the release of ACTH via the release of hypothalamic CRF. Pharmacological manipulation of the augurin system may therefore be a novel target for regulation of the HPA axis.
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Keywords: augurin; hypothalamo-pituitary-adrenal axis; paraventricular nucleus; CRF

Abbreviations: aCSF, artificial cerebrospinal fluid; AVP, arginine vasopressin; CRF, corticotrophin-releasing factor; ECRG4, oesophageal cancer-related gene 4; HPA, hypothalamo-pituitary-adrenal; i.c.v., intracerebroventricular; PVN, paraventricular nucleus

Introduction

Novel secreted peptides represent an important field of research because delineation of their function affords new insights into the pathophysiological processes causing disease, and provides opportunities for the development of pharmacological therapies. Secreted peptide systems are particularly amenable to pharmacological modulation as their receptors are generally found on the cell surface and accessible to circulating factors. Synthetic secreted peptides or structural analogues can therefore be used directly as therapeutic agents for various diseases.

Augurin is a recently identified secreted peptide of unknown function encoded by the gene *c2orf40* (Mirabeau *et al.*, 2007), a potential tumour suppressor gene in human oesophageal epithelium (Su *et al.*, 1998). Down-regulation of *c2orf40* expression due to hypermethylation can occur in human oesophageal squamous cell carcinoma and may be an independent prognostic factor for poor survival (Yue *et al.*, 2003). The product of *c2orf40* is the highly conserved 148-amino-acid oesophageal cancer-related gene 4 protein (ECRG4 protein). ECRG4 protein has characteristic features of a preprohormone; it contains a canonical signal peptide

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sequence and at least one basic residue, prohormone cleavage, site. Augurin is produced by post-translational processing of ECRG4 protein (Mirabeau *et al.*, 2007).

The primary sequence of endogenous augurin has not been determined. ECRG4 protein contains one canonical dibasic prohormone cleavage site corresponding to residues Lys69 and Arg70. Cleavage at this site has been demonstrated *in vitro* following transfection of a pancreatic cell line with the *c2orf40* gene in which the Flag antigen sequence was inserted between Arg70 and Gln71 (Mirabeau *et al.*, 2007). Western blotting of supernatant from Flag augurin-transfected cells revealed two immunoreactive bands consistent with secretion of an Arg70 cleaved 10 kDa peptide (augurin 71–148) and a smaller 8 kDa peptide. It is suggested the 8 kDa peptide results from a second, unknown, C-terminal non-canonical cleavage site (Mirabeau *et al.*, 2007).

c2orf40 expression has been quantified in various tissues. Microarray data suggest that, in humans, the highest expression is found in the thyroid, pituitary, testis and adrenal. In mice, the highest expression is found in the adrenals, ovaries, digits, retina and trachea [GNF Symatlas: http://symatlas.gnf.org/SymAtlas/ (last accessed 12 April 2009)] (Su *et al.*, 2004). In the human brain *c2orf40* expression is highest in the olfactory bulb, cerebellum, hypothalamus and amygdala, an expression pattern mirrored in mice (Su *et al.*, 2004). *In situ* hybridization in mouse embryos has detected *c2orf40* mRNA in the intermediate lobe of the pituitary, glomerular layer of the adrenal cortex, choroid plexus and atrioventricular node of the heart (Mirabeau *et al.*, 2007).

The distribution of ECRG4-like immunoreactivity in hypothalamus has been reported in an abstract submitted for a recent scientific meeting (Roberton *et al.*, 2009). In addition to the choroid plexus, ECRG4-like immunoreactivity was detected in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus, where it colocalized with vasopressin (AVP) and oxytocin. Immunoreactivity was localized to synaptic–capillary interfaces in the median eminence, and Herring bodies in the posterior pituitary. This pattern of immunoreactivity is characteristic of a secreted peptide and suggests that ECRG4 or a product thereof may be a novel hypothalamic neuropeptide.

As *c2orf40* is expressed in the brain and endocrine organs, we hypothesized that its product may be involved in the neuroendocrine system. Substantial expression in the hypothalamus could be consistent with a physiological role in the hypothalamo-pituitary axis. We therefore investigated the effects of hypothalamic injection of augurin 71–148, a probable endogenous form, on the hypothalamo-pituitary axis.

The present study found that injection of augurin into the third cerebral ventricle (i.c.v.) or into the PVN (iPVN) elevated plasma adrenocorticotrophin (ACTH) and corticosterone compared with vehicle-injected controls, and that incubation with augurin increased the release of corticotrophin-releasing factor (CRF) and AVP from hypothalamic explants. The rise in plasma ACTH and corticosterone following i.c.v. injection of augurin was blocked by pretreatment with a CRF receptor antagonist. Dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis and of plasma glucocorticoid levels have been implicated in the pathogenesis of hypertension, obesity, type II diabetes and depression (Buckingham, 2006). These studies suggest that the augurin system may be an attractive pharmacological target for manipulation of the HPA axis.

Methods

Animals

All animal care and experimental procedures complied with the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licence 70/6402). Male Wistar rats (specific pathogen free; Charles River, Margate, UK) weighing 250–300 g were maintained in individual cages under controlled temperature (21–23°C) and light conditions (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.

Peptides

A synthetic fragment of human augurin corresponding to amino acids 71–148 of ECRG4 protein was synthesized by Bachem UK Ltd. (Merseyside, UK). The synthesis was performed using a Symphony automated peptide synthesizer (Protein Technology, Inc., Woburn, MA, USA). The product was initially purified by flash chromatography with a reversed phase resin (Daisogel), followed by reversed phase high pressure liquid chromatography (HPLC). Analyses of the purified peptide by matrix-assisted laser desorption/ionization mass spectroscopy, HPLC and amino acid analysis showed above 91% purity (average molecular weight 9689).

Rat neuromedin U (NMU) 23 and astressin were purchased from Bachem UK Ltd.

For all studies lyophilized augurin was first dissolved in a small amount of 0.05 M HCl, and then diluted in saline for *in vivo* studies or in artificial cerebrospinal fluid [(aCSF; see below for composition) for hypothalamic or pituitary explant studies] containing sufficient NaOH to neutralize the HCl. For all studies the vehicle control was prepared using identical amounts of HCl and NaOH.

Intracerebroventricular cannulation and injections

Intracerebroventricular cannulation was carried out using an established protocol (Rossi *et al.*, 1997). A 22-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) projecting into the third cerebral ventricle was stereotactically implanted into each rat using coordinates calculated from the rat brain atlas 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), as previously described (Rossi *et al.*, 1997). Prior to commencement of surgery, each rat received a single s.c. injection of buprenorphine (45 $\mu g \cdot kg^{-1}$; Schering-Plough, Welwyn Garden City, UK) for analgesia. Following surgery, rats were allowed a 7 day recovery period during which time they were checked daily. Prior to the studies, each rat received two sham injections (one of angiotensin II and one of saline) to acclimatize them to the procedure. Only animals with correct cannula placement, as confirmed by a sustained drinking response to i.c.v. angiotensin II (50 ng per rat), were included in the studies.

For i.c.v. injections, peptides were dissolved as described above and administered in 5 μL volume via a stainless steel injector projecting 1 mm beyond the tip of the cannula. Rats were returned to their own cages following the injection procedure.

Intraparaventricular cannulation and injection
Unilateral intrahypothalamic cannulation directed at the PVN (iPVN) was carried out using an established protocol (Abbott *et al*., 2003). Rats were maintained as for i.c.v. cannulation. A 26-gauge stainless steel guide cannula (Plastics One) was stereotactically implanted using established coordinates obtained from the Paxinos and Watson rat brain atlas (1.8 mm caudal to the bregma, 0.3 mm lateral, 7.5 mm below the outer surface of the skull) (Paxinos and Watson, 2007).

For iPVN injections, peptides were dissolved as described above and administered in 1 μL volume via a stainless steel injector projecting 1 mm beyond the tip of the cannula. Correct cannula placement was confirmed histologically at the end of the study period by injection of India ink as previously described (Abbott *et al*., 2003). Data from rats were excluded if the injection site extended more than 0.4 mm outside the intended injection site, or if any ink was detected in the cerebral ventricular system. In total 71% of cannulae were correctly positioned. Figure S1 shows a representative section of the PVN containing India ink.

Study 1: The effect of i.c.v. augurin on plasma hormone levels
Each rat received a single i.c.v. injection of vehicle or 5 nmol augurin (n = 9–10 per group) in the early light phase (0900–1200 h). Rats were killed by decapitation 60 min after injection. Trunk blood was collected in plastic lithium heparin tubes containing 4200 kallidinogenase inactivator units of aprotinin (Bayer Corp., Haywards Heath, UK) for luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin, testosterone, free tri-iodothyronine (T3), free thyroxine (T4) and corticosterone assays, and in plastic EDTA containing tubes for ACTH assays. Serum was immediately separated by centrifugation, frozen on dry ice and stored at −20°C (lithium heparin tubes) or −80°C (EDTA tubes).

Study 2: The effect of i.c.v. augurin on plasma corticosterone and ACTH
Each rat received a single i.c.v. injection of vehicle, 0.6, 1.7 or 5 nmol augurin in the early light phase (0900–1200 h). Rats were killed by decapitation 20 or 60 min post injection (n = 8–10 per group per time point). Trunk blood was collected and stored as above.

Study 3A: The effect of iPVN augurin on plasma corticosterone and ACTH
Each rat received a single iPVN injection of vehicle, 0.1, 0.3 or 1 nmol augurin in the early light phase (0900–1200 h). Rats were killed by decapitation 20 or 60 min post injection (n = 5–8 per group per time point). Trunk blood was collected and stored as above.

Study 3B: The effect of iPVN augurin on behaviour
Each rat received a single iPVN injection of vehicle, 1 nmol augurin or 0.3 nmol NMU 23 (n = 6–8 per group) in the early light phase (0900–1200 h). Behavioural patterns were monitored for 120 min following injection by observers unaware of the experimental treatments. Behaviour was classified into eight different categories: feeding, drinking, grooming, burrowing, rearing, locomotion, head down and sleeping, adapted from Fray *et al.* (1980). These methods have previously been used to demonstrate abnormal behaviour following iPVN injection of peptides (Wren *et al.*, 2002). During the analysis, each rat was observed for 15 s every 5 min. Each 15 s period was further subdivided into three and the predominant behaviour of the rat during each 5 s episode was noted. NMU was used as a positive control as it is known to increase grooming behaviour and decrease sleeping (Wren *et al.*, 2002).

Study 4: The effect of augurin on the release of CRF and AVP from ex vivo *hypothalamic explants.*
A static incubation system was used as described previously (Bewick *et al.*, 2005). Briefly, a 1.9 mm slice was taken from the basal hypothalamus and incubated in individual tubes containing 1 mL 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 $mg{\cdot}mL^{-1}$ ascorbic acid and 100 $\mu g{\cdot}mL^{-1}$ aprotinin) saturated with 95% O_2 and 5% CO_2 at 37°C. After an initial 2 h equilibration period, hypothalamic slices were incubated for 45 min in 600 μL aCSF (basal period), before being treated with 1, 10 or 100 nM augurin in 600 μL aCSF for 45 min (n = 13–15 hypothalami per group). Finally, tissue viability was assessed by 45 min incubation in isotonic aCSF containing 56 mM KCl. 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. At the end of each period, aCSF was collected and stored at −20°C until measurement of CRF and AVP by radioimmunoassay (RIA).

Study 5: The effect of peripheral CRF receptor antagonism on augurin-stimulated ACTH and corticosterone secretion
Intracerebroventricular cannulated rats were injected i.p. with 100 $\mu g{\cdot}kg^{-1}$ astressin or vehicle 15 min before receiving an i.c.v. injection of 5 nmol augurin or vehicle. Astressin ($cyc^{30\text{-}33}$[D-Phe^{12},$Nle^{21,38}$,Glu^{30},Lys^{33}]CRF-(12-41)) is a $CRF_{1/2}$ receptor antagonist (Gulyas *et al.*, 1995), not known to cross the blood–brain barrier. This dose of astressin has previously been used to block the increase in plasma ACTH and corticosterone caused by i.c.v. injection of GLP-1 (Kinzig *et al.*, 2003). Rats were decapitated 30 min after the i.c.v. injection (n = 7–9 per group). Trunk blood was collected and stored as above.

Study 6A: The effect of peripherally administered augurin on plasma hormone levels
Rats were handled daily and received twice weekly i.p. injections to acclimatize them to the injection procedure. On the

day of the study, rats were injected i.p. with 100 nmol·kg^{-1} augurin or vehicle during the early light phase (0900–1200 h).

Study 6B: The effect of augurin on ACTH release from pituitary segments

Static pituitary explants were performed as previously described (Smith *et al.*, 2006). Briefly, the pituitaries of *ad libitum* fed rats were harvested immediately following decapitation. The posterior pituitary was removed and discarded, and the remaining anterior pituitary was bisected along the mid-saggital line and then divided into four pieces of approximately equal size. After an acclimatization period, the segments were then incubated in one of aCSF alone, or aCSF containing 10 nM augurin, 100 nM augurin, 1000 nM augurin or 100 nM CRF for 4 h (n = 17–19 per group). At the end of this period the incubation medium was collected and stored at −20°C until assayed for ACTH.

Radioimmunoassays

RIAs for CRF immunoreactivity and AVP immunoreactivity were performed using established methods (Dhillo *et al.*, 2003). The intra- and interassay coefficients of variation were <10% for the CRF RIA, and 11% and 20%, respectively, for the AVP RIA. Plasma corticosterone was measured using an RIA kit from MP Biomedicals, Inc. (Orangeburg, NY, USA), for which the intra- and interassay coefficients of variation were less than 10% and 7% respectively. For studies 1, 2 and 3A, plasma ACTH was measured by immunoradiometric assay purchased from Euro-Diagnostica B.V. (Arnhem, the Netherlands). The intra- and interassay coefficients of variation were both less than 4%. For study 5, plasma ACTH was measured by immunoradiometric assay purchased from BioSource Europe S.A. (Nivelles, Belgium) because the previously used kit was no longer available. The intra- and interassay coefficients of variation were 6.4% and 6.2% respectively. Plasma TSH, LH, FSH, prolactin, GH and ACTH release from pituitary segments were measured, using methods and reagents provided by the National Hormone and Pituitary programme. Total plasma testosterone, free T3 and free T4 were measured using commercial Coat-a-Count assay kits (Euro/DPC Limited, Caernarfon, UK).

Statistics

Data from hypothalamic explant, pituitary explant and terminal studies are presented as mean ± SEM. Data from behavioural analyses are presented as median frequency and interquartile range for each behaviour. For plasma ACTH and corticosterone studies, values were log-transformed to homogenize variances among groups, and to improve the normality of residuals (Bland and Altman, 1996). Transformed values were then analysed by Student's *t*-test, or one- or two-way ANOVA followed by *post hoc* Holm-Sidak test (SigmaStat 3.5, San Jose, CA, USA). Data from hypothalamic explant release were analysed using paired Student's *t*-test between the basal period and the test period. Data from pituitary explant release were analysed using one-way ANOVA followed by *post hoc* Holm-Sidak test (SigmaStat 3.5, San Jose, CA, USA). Data from the behavioural study were analysed using Kruskal-Wallis one-way ANOVA on ranks (Systat 11, San Jose, CA, USA).

Nomenclature

The nomenclature of receptors and peptides described in this manuscript conform to *BJP*'s Guide to Receptors and Channels (Alexander *et al.*, 2009).

Results

Study 1: The effect of i.c.v. augurin on plasma hormone levels

Augurin significantly increased plasma ACTH 60 min after injection (Table 1). Plasma corticosterone was raised but failed to reach statistical significance. There were no significant changes in plasma LH, FSH, GH, TSH, prolactin, testosterone, free T3 or free T4 (Table 1).

Study 2: The effect of i.c.v. augurin on plasma corticosterone and ACTH

Plasma ACTH and corticosterone levels were significantly elevated following i.c.v. injection of augurin compared with vehicle-injected controls (Figure 1). In the case of ACTH, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,60)}$ = 11.789; P < 0.001), a significant main effect of time ($F_{(1,60)}$ = 7.956; P < 0.01) and a significant interaction ($F_{(3,60)}$ = 3.181; P < 0.05). *Post hoc* analysis demonstrated that i.c.v. injection of 5 nmol augurin significantly increased plasma ACTH 20 min post injection (P < 0.001). All doses of augurin significantly increased plasma ACTH 60 min post injection (P < 0.01 for all doses).

In the case of corticosterone, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,68)}$ = 8.510; P < 0.001), a significant main effect of time ($F_{(1,68)}$ = 3.799; P < 0.05) and no significant interaction ($F_{(3,68)}$ = 0.954; P = 0.420).

Table 1 Effect of a single i.c.v. injection of augurin (5 nmol) or vehicle in *ad libitum* fed rats on plasma hormone concentrations at 60 min post injection

	Vehicle		*Augurin (5 nmol)*	
	Mean	*SEM*	*Mean*	*SEM*
ACTH (pg·mL^{-1})	21.3	3.2	66.7***	16.3
Corticosterone (ng·mL^{-1})	152.8	25.9	300.0	65.1
LH (ng·mL^{-1})	0.60	0.09	0.54	0.12
FSH (ng·mL^{-1})	8.97	0.65	7.18	0.88
Testosterone (ng·mL^{-1})	4.25	0.87	4.96	1.39
TSH (ng·mL^{-1})	2.38	0.14	2.21	0.37
Free T4 (ng·L^{-1})	20.5	1.1	22.7	1.2
Free T3 (pg·L^{-1})	12.8	1.1	12.1	1.3
PRL (ng·mL^{-1})	5.28	0.79	5.15	0.81
GH (ng·mL^{-1})	20.27	4.38	22.39	11.54

Results are mean ± SEM.

***P < 0.001 versus vehicle, n = 9–10 per group.

FSH, follicle stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; T3, tri-iodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

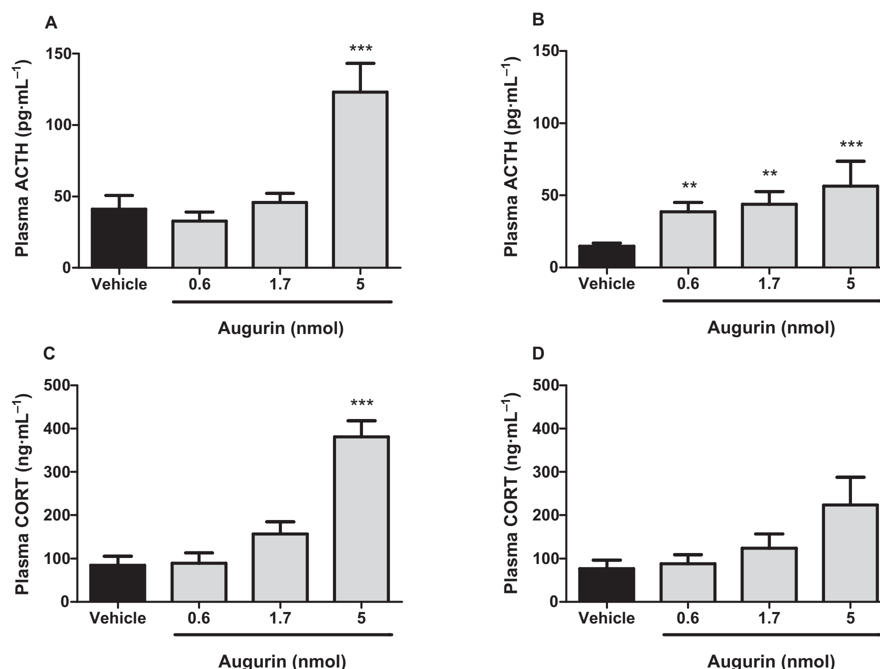

Figure 1 Effect of a single i.c.v. injection of augurin (0.6, 1.7 or 5 nmol) or vehicle in *ad libitum* fed male Wistar rats on plasma ACTH and corticosterone (CORT) at 20 (A and C) and 60 (B and D) min post injection. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle, $n = 8$–10 per group per time point. Results are mean ± SEM.

Post hoc analysis demonstrated that i.c.v. injection of 5 nmol augurin significantly increased plasma corticosterone 20 min post injection ($P < 0.001$).

Study 3A: The effect of iPVN augurin on plasma corticosterone and ACTH

Plasma ACTH and corticosterone levels were significantly elevated following iPVN microinjection of augurin compared with vehicle-injected controls (Figure 2). In the case of ACTH, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,45)} = 9.891$; $P < 0.001$), a significant main effect of time ($F_{(1,45)} = 5.363$; $P < 0.05$) and no significant interaction ($F_{(3,45)} = 0.600$; $P = 0.618$). *Post hoc* analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma ACTH 20 and 60 min post injection ($P < 0.001$ and $P < 0.01$ respectively).

In the case of corticosterone, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,45)} = 6.247$; $P < 0.001$), no significant main effect of time ($F_{(1,45)} = 2.275$; $P = 0.138$) and no significant interaction ($F_{(3,45)} = 1.129$; $P = 0.347$). *Post hoc* analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma corticosterone 20 min post injection ($P < 0.01$).

Study 3B: The effect of iPVN augurin on behaviour

There were no significant behavioural differences between iPVN augurin- or vehicle-injected rats. Injection of 0.3 nmol NMU significantly increased time spent grooming and decreased time spent sleeping or feeding (Table 2).

Study 4: The effect of augurin on the release of CRF and AVP from ex vivo *hypothalamic explants.*

Incubation of hypothalamic explants with augurin significantly increased the release of CRF and AVP (Figure 3).

Study 5: The effect of peripheral CRF receptor antagonism on augurin-induced ACTH and corticosterone secretion

Pretreatment with astressin completely blocked the increase in plasma ACTH and corticosterone 30 min post i.c.v. injection of 5 nmol augurin (Figure 4).

Study 6A: The effect of peripherally administered augurin on plasma hormone levels

There were no significant differences in plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3 or free T4, 30 minutes following i.p. injection of $100\ \text{nmol·kg}^{-1}$ augurin (Table S1).

Study 6B: The effect of augurin on ACTH release from pituitary segments

No significant change in ACTH release was seen following incubation of pituitary segments with 10, 100, or 1000 nM augurin, compared with aCSF alone. Incubation with 100 nM CRF resulted in a significant increase in ACTH release (ACTH ng/explant; vehicle 192.9 ± 23.1; CRF 100 nM 296.7 ± 36.2, $P < 0.01$) (Figure S2).

Discussion

Augurin is a recently identified secreted peptide of unknown function. It is expressed in the CNS and endocrine organs (Su

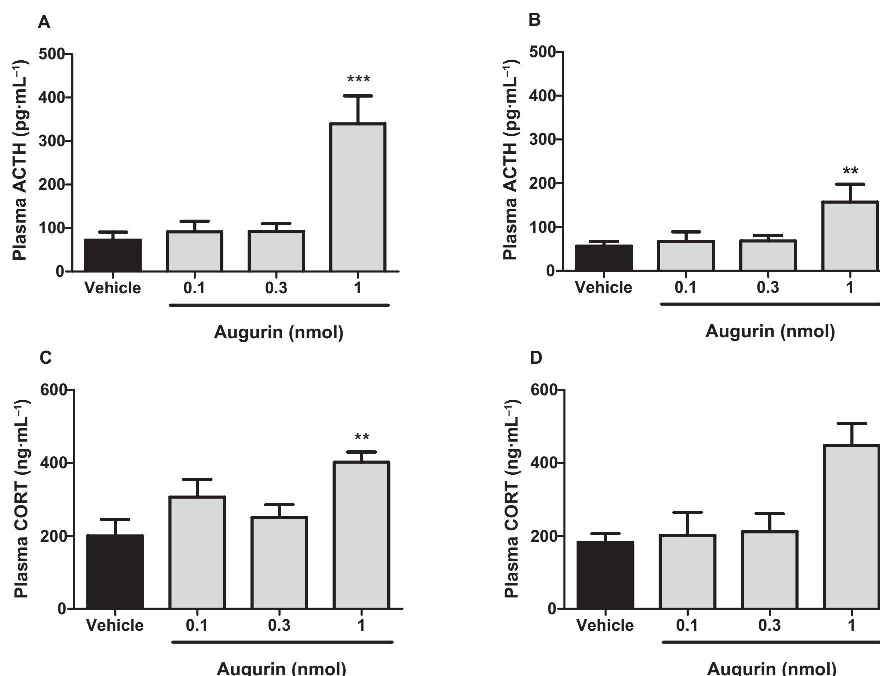

Figure 2 Effect of a single iPVN (paraventricular nucleus) injection of augurin (0.1, 0.3 or 1 nmol) or vehicle in *ad libitum* fed male Wistar rats on plasma ACTH and corticosterone (CORT) at 20 (A and C) and 60 (B and D) min post injection. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus vehicle, $n = 5–8$ per group per time point. Results are mean ± SEM.

Table 2 Effect of a single iPVN injection of augurin (1 nmol), NMU (0.3 nmol) or vehicle in *ad libitum* fed male rats on behaviour

	Feeding	*Rearing*	*Drinking*	*Locomotion*	*Grooming*	*Burrowing*	*Head down*	*Sleeping*
0–60 min								
Vehicle	2 [0:4]	8 [6:15]	0 [0:0]	5 [3:7]	6 [4:8]	0 [0:1]	1 [0:1]	11 [7:14]
Augurin 1 nmol	7 [4:9]	10 [8:13]	0 [0:0]	8 [6:9]	5 [4:8]	0 [0:0]	1 [0:3]	2 [0:9]
NMU 0.3 nmol	0 [0:0]	7 [6:9]	0 [0:0]	5 [3:6]	21 [18:24]**	1 [0:4]	1 [0:3]	0 [0:0]**
61–120 min								
Vehicle	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:2]	35 [31:36]
Augurin 1 nmol	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	2 [0:4]	32 [30:36]
NMU 0.3 nmol	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:1]	10 [2:18]*	0 [0:0]	1 [0:4]	23 [17:24]*
0–120 min								
Vehicle	3 [1:4]	8 [6:16]	0 [0:0]	5 [3:8]	6 [4:8]	0 [0:1]	3 [0:5]	47 [38:48]
Augurin 1 nmol	7 [4:9]	10 [8:14]	0 [0:0]	8 [6:9]	6 [4:9]	0 [0:0]	3 [3:6]	32 [30:45]
NMU 0.3 nmol	0 [0:0]*	7 [6:9]	0 [0:0]	5 [3:6]	30 [20:40]***	1 [0:4]	2 [1:8]	23 [17:24]*

Data presented are median frequency and interquartile range.
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus vehicle, $n = 6–8$ per group.
NMU, neuromedin U; PVN, paraventricular nucleus.

et al., 2004; Mirabeau *et al*., 2007). We therefore examined the effects of augurin on the hypothalamo-pituitary axes. Both i.c.v. and iPVN injection of augurin elevated plasma ACTH and corticosterone compared with vehicle-injected controls, and augurin increased the release of CRF and AVP from hypothalamic explants. The rise in plasma ACTH and corticosterone following i.c.v. injection of augurin was blocked by pretreatment with a CRF receptor antagonist.

Intracerebroventricular injection of 5 nmol augurin significantly increased plasma ACTH and corticosterone 20 min after injection, compared with vehicle-injected controls. Injection of 0.6 or 1.7 nmol augurin appeared to cause a delayed rise in plasma ACTH, evident only at 60 min. The reason for this delay is unclear; it is possible that it represents the time taken for the peptide to diffuse to its site(s) of action. Alternatively, small differences in plasma ACTH at 20 min may have been masked by the stress of the experimental procedure.

Corticotrophin-releasing factor and AVP are the main regulators of ACTH levels; they act synergistically on the pituitary to stimulate ACTH secretion (Whitnall, 1993). Augurin increased the release of CRF and AVP from explanted hypothalami, and pretreatment with a $CRF_{1/2}$ receptor antagonist blocked the rise in plasma ACTH and corticosterone caused by i.c.v. injection of augurin. This suggests augurin stimulates the secretion of ACTH via the release of hypothalamic CRF and AVP, although the relative contribution of AVP cannot be determined from the antagonist study as CRF is necessary for AVP stimulated ACTH release (Whitnall, 1993).

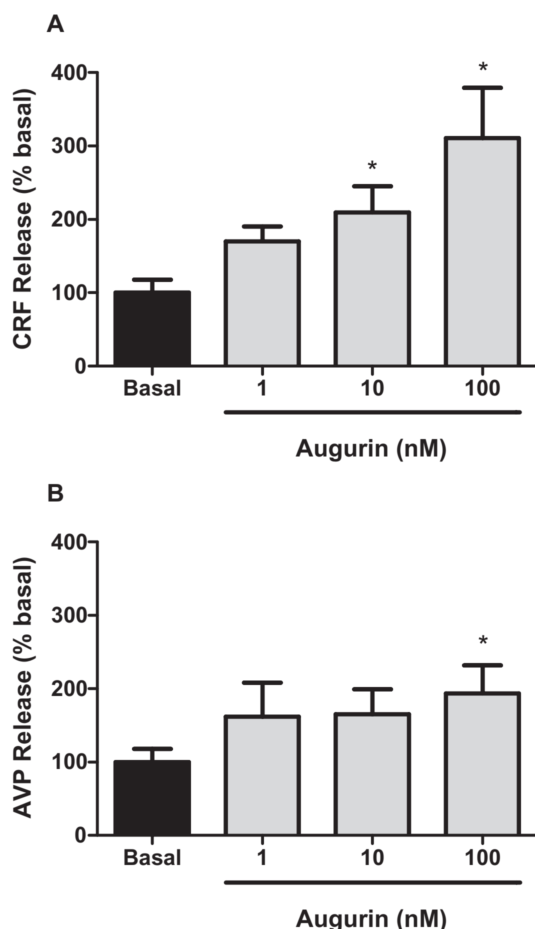

Figure 3 Effect of augurin (1, 10 or 100 nM) on corticotrophin-releasing factor (CRF) (A) and arginine vasopressin (AVP) (B) release from hypothalamic explants. Data presented as percent of basal release. $*P < 0.05$ versus basal release, n = 13–15 hypothalami per group. Results are mean ± SEM.

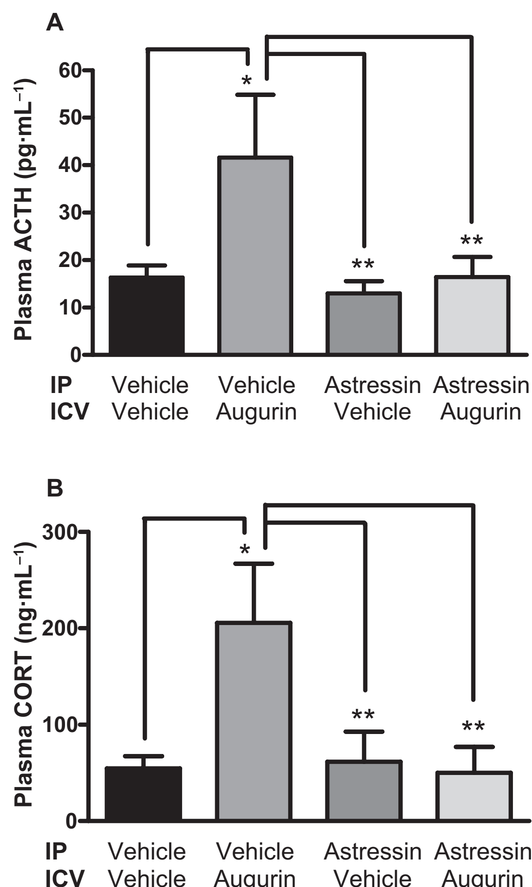

Figure 4 Effect of a single i.c.v. injection of augurin (5 nmol) or vehicle in *ad libitum* fed male Wistar rats pretreated with 100 nmol·kg^{-1} astressin on plasma ACTH (A) and corticosterone (CORT; B) 30 min post i.c.v. injection. $*P < 0.05$, $**P < 0.01$ versus vehicle, n = 7–9 per group. Results are mean ± SEM.

Corticotrophin-releasing factor neurons in the PVN are the major conduit for endocrine, autonomic and behavioural responses to stress (Herman, 1997). The PVN contains a subpopulation of hypophysiotropic neurons that constitute the primary source of CRF and AVP (Whitnall, 1993). We therefore examined the effects of injecting augurin directly into the PVN. Intranuclear injection of lower doses minimizes any non-specific side effects of peptide administration that may occur following i.c.v. injection (Dhillo *et al.*, 2003).

Microinjection of 1 nmol augurin iPVN significantly increased plasma ACTH and corticosterone, a much lower dose than required for a similar effect when given i.c.v. This could indicate that the receptors for augurin are found within or close to the PVN. It is, however, possible that lower concentrations of augurin could diffuse into adjacent nuclei, and we cannot discount administration of augurin to other hypothalamic areas having a similar effect. We did not observe any significant effects on ACTH or corticosterone following iPVN injection of 0.1 or 0.3 nmol augurin. However, we cannot rule out slightly elevated ACTH levels in the vehicle group masking minor effects at the 20 min time point. There were no significant differences in behaviour in the 2 h following iPVN microinjection of 1 nmol augurin, compared with vehicle-injected controls. In particular, no adverse behaviour was observed in the augurin group, indicating that non-specific activation of the HPA axis as a result of a noxious effect is unlikely.

Intracerebroventricular injection of 5 nmol augurin had no effect on plasma LH, FSH, testosterone, TSH, free T3, free T4, prolactin or GH at 60 min post injection, suggesting the neuroendocrine effects of augurin are specific to the HPA axis. Peripheral administration of augurin at doses of 100 nmol·kg^{-1} had no effect on plasma corticosterone at 30 min (Table S1). This dose is relatively high; i.p. injection of 4.2 nmol·kg^{-1} of CRF causes a significant rise in ACTH (Watanabe *et al.*, 1991). In addition, we have shown that incubation of pituitary segments with augurin at concentrations of up to 1000 nM has no effect on ACTH release. It therefore appears unlikely that peripheral augurin acts directly on the pituitary to release ACTH unless it is released into the circulation at very high concentrations. To date, the presence of augurin in the circulation has not been investigated.

The primary sequence or sequences of endogenous augurin are unknown. While these data show residues 71–148 have a biological effect, it is also possible shorter fragments of

augurin may be biologically active. The N-terminal segment of augurin adjacent to the dibasic cleavage site has substantially higher interspecies conservation than the rest of the peptide (Mirabeau *et al*., 2007). We hypothesize that the increase in conservation implies the N-terminal segment is important for biological activity. Identifying the endogenous forms of augurin and determining the minimum biologically active sequence would provide a basis for developing structural analogues acting as agonists or antagonists of the augurin system

Data from our studies demonstrate injection of augurin into the hypothalamus stimulates the HPA axis, culminating in the release of glucocorticoids. Various pharmacotherapies have been developed based on components of the HPA axis. Glucocorticoid receptor agonists are widely used as anti-inflammatory and immunosuppressive agents (Rhen and Cidlowski, 2005). More recently, antagonists of the CRF receptors have shown promise in the treatment of anxiety and depression, sleep disorders, addictive behaviour and preterm labour (Habib *et al*., 2000; Zoumakis *et al*., 2006). While the data presented suggest a novel role for augurin, these are preliminary studies. Currently, little is known about the physiology of the augurin system. Further characterization of the neuroanatomy, identification of receptors and the development of functional analogues would aid us in establishing whether the augurin system is a viable target for manipulating the HPA axis.

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Statement of conflict of interest

SM is an employee of Bachem UK Ltd. JAT, MP, KS, KEB, CKB, KLS, MAG and SRB have nothing to disclose.

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Supporting information

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

Figure S1 Photomicrograph of the spread of 1 μL India ink injected into the paraventricular nucleus. The section was not stained so that the ink could be clearly seen. 3V, third ventricle; ot, optic tract.

Figure S2 Effect of augurin (10, 100 or 1000 nM) or corticotrophin-releasing factor (CRF) (100 nM) on ACTH release from pituitary segments. $^{**}P < 0.01$, $n = 17–19$ per group. Results are mean ± SEM.

Table S1 Effect of a single i.p. injection of augurin (100 $nmol \cdot kg^{-1}$) or vehicle in *ad libitum* fed rats on plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3 and free T4 at 30 min post injection

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