Molecular characterisation and functional interrogation of a local natriuretic peptide system in rodent pituitaries, α T3-1 and L β T2 gonadotroph cells

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Abstract

In the pituitary, C-type natriuretic peptide (CNP) has been implicated as a gonadotroph-specific factor, yet expression of the CNP gene (*Nppc*) and CNP activity in gonadotrophs is poorly defined. Here, we examine the molecular expression and putative function of a local gonadotroph natriuretic peptide system. *Nppc*, along with all three natriuretic peptide receptors (*Npr1*, *Npr2* and *Npr3*), was expressed in both α T3-1 and L β T2 cells and primary mouse pituitary tissue, yet the genes for atrial-(ANP) and B-type natriuretic peptides (*Nppa* and *Nppb*) were much less abundant. Putative processing enzymes of CNP were also expressed in α T3-1 cells and primary mouse pituitaries. Transcriptional analyses revealed that the proximal 50 bp of the murine *Nppc* promoter were sufficient for GNRH responsiveness, in an apparent protein kinase C and calcium-dependent manner. Electrophoretic mobility shift

assays showed Sp1/Sp3 proteins form major complexes within this region of the *Nppc* promoter. CNP protein was detectable in rat anterior pituitaries, and electron microscopy detected CNP immunoreactivity in secretory granules of gonadotroph cells. Pharmacological analyses of natriuretic peptide receptor activity clearly showed ANP and CNP are potent activators of cGMP production. However, functional studies failed to reveal a role for CNP in regulating cell proliferation or LH secretion. Surprisingly, CNP potently stimulated the human glycoprotein hormone α -subunit promoter in L β T2 cells but not in α T3-1 cells. Collectively, these findings support a role for CNP as the major natriuretic peptide of the anterior pituitary, and for gonadotroph cells as the major source of CNP expression and site of action.

Journal of Endocrinology (2009) **203**, 215–229

Introduction

The major members of the mammalian natriuretic peptide family consist of atrial-, B-type and C-type natriuretic peptides (ANP, BNP and CNP respectively). Of these, ANP and BNP have well-characterised effects on the cardiovascular system and on renal function (Baxter 2004, Potter *et al.* 2006); yet, despite being identified nearly three decades ago, the biological roles for CNP have remained relatively enigmatic. However, recent transgenic models involving the deletion of the CNP gene (*Nppc*) in mice have revealed a critical role of CNP in endochondral ossification, as these mice are dwarf and suffer from severe skeletal abnormalities (Chusho *et al.* 2001). Additionally, these mice also exhibit female infertility and early lethality (Chusho *et al.* 2001), but investigation of their neuroendocrine tissues has not been

reported. Early studies characterising the expression profile of CNP suggested that the CNS and anterior pituitary are rich sources of CNP (Sudoh *et al.* 1990, Komatsu *et al.* 1991). Subsequent investigations revealed a gonadotroph-specific expression profile of CNP in the anterior pituitary (McArdle *et al.* 1993, 1994), and functional studies in α T3-1 gonadotrophs implicated a role for CNP in regulating GNRH signalling (Fowkes *et al.* 1999). However, the molecular and functional characterisation of the natriuretic peptide system in gonadotrophs has remained poorly defined.

All three natriuretic peptides exert the vast majority of their effects via their specific particulate guanylyl cyclase receptors, GC-A and GC-B (encoded by the *Npr1* and *Npr2* genes respectively), leading to the generation of cGMP as a second messenger (for review see Potter *et al.* 2006). In addition, the Npr3/Npr-C receptor (encoded by the *Npr3* gene) binds all

three peptides to act predominantly as a clearance receptor (Potter *et al.* 2006). Deletion or mutation of the GC-B gene (*Npr2*) in both mice and humans essentially phenocopies the *Nppc*^{-/-} models (Tamura *et al.* 2004), supporting the requirement for GC-B in the mediation of most CNP effects. As well as the profound effects on endochondral ossification, these models of disrupted CNP/GC-B pathways suggest potentially important roles of this system in pituitary and gonadal tissues as *Npr2*^{-/-} mice are GH deficient and exhibit female infertility (Tamura *et al.* 2004). Further support for CNP as a regulator of reproductive function comes from several studies that have shown CNP to potently stimulate cGMP production in GNRH neurons (Olcese *et al.* 1994), pituitary gonadotrophs (McArdle *et al.* 1993), and endocrine cells of the testis, ovaries, placenta and uterus (Khurana & Pandey 1993, Huang *et al.* 1996, Middendorff *et al.* 1996, Acuff *et al.* 1997, Jankowski *et al.* 1997, Gutkowska *et al.* 1999, Stepan *et al.* 2001, Walther & Stepan 2004), implicating CNP function at all levels of the hypothalamo-pituitary-gonadal (HPG) axis. Furthermore, cGMP is a useful pharmacological target in the treatment of male reproductive disorders (Rosen & McKenna 2002, Christ & Hodges 2006). However, despite an ever-increasing literature detailing CNP effects in HPG tissues, understanding of the regulation and function of CNP and GC-B in neuroendocrine tissues remains poor.

In this study, we examine the molecular and functional characterisation of the natriuretic peptide system in gonadotroph cells lines (α T3-1 and L β T2) as well as primary rat and mouse pituitaries. Our studies reveal that CNP and GC-B are the predominant members of the natriuretic peptide and natriuretic peptide receptor families to be expressed in gonadotrophs, and demonstrate regulation by GNRH. Finally, we present evidence for a possible transcriptional effect of CNP on the expression of the glycoprotein hormone α -subunit gene (α GSU) in L β T2 gonadotrophs.

Materials and Methods

Materials

All chemicals were purchased from Sigma or BDH-Merck unless otherwise stated. The long-acting GNRH synthetic analogue (des-Gly¹⁰, [D-Ala⁶] GNRH ethylamide), ANP-26 (ANP) and CNP-22 (CNP) were obtained from Sigma. Nifedipine and GF109203X were purchased from CN Biosciences, Nottingham, UK, and were prepared as stock solutions in Me₂SO and applied to the cells in culture media. Cells were exposed to <0.01% Me₂SO, and these concentrations of vehicle had no effect on responses of α T3-1 or L β T2 cells. All concentrations of inhibitors were previously optimised (Fowkes *et al.* 2001). All treatments were diluted directly into culture medium (see below) before each experiment. Antibodies directed against GC-A, GC-B (Fabgennix, Frisco, TX, USA), Npr-C, cJun, ATF-2,

CREB (Santa Cruz Biotech, Santa Cruz, CA, USA), Sp1 and Sp3 (Upstate (Millipore), Watford, UK) were stored appropriately (either -20 or 4 °C).

Cell culture

α T3-1, L β T2 and ArT-20 cells were grown in monolayer culture in DMEM supplemented with high glucose (4500 mg/l) containing 10% (v/v) FCS, penicillin (100 IU/ml) and streptomycin (100 mg/l) (Sigma) (hereafter referred to as culture medium). Cells were passaged twice weekly and incubated at 37 °C in a humidified 5% (v/v) CO₂/95% (v/v) air incubator.

RNA extraction and RT-PCR

Total RNA was extracted from 1×10^6 cells (α T3-1 or L β T2), or C57B6 male mouse pituitaries using Tri-reagent (Sigma), and subjected to DNase treatment (Qiagen), before generation of first-strand cDNA (Applied Biosystems, Warrington, UK). PCR was performed for a range of targets for 30 cycles, using the primers and conditions listed in Table 1.

Plasmids and transient transfection studies

The reporter construct -5.5CNP-LUC contains 5500 bp of the 5'-flanking sequence of the mouse *Cnp* gene, linked to the luciferase (LUC) reporter gene in the plasmid pXp2 (Nordeen 1988). Deletions of the 5500 bp 5'-flanking sequence linked to a LUC reporter gene and termed -0.45CNP-LUC and -0.05CNP-LUC have been characterised and described previously (Huang *et al.* 1996). The deletion constructs encoding the proximal human α GSU promoter in pA3LUC have been described previously (Maxwell *et al.* 1989, Holdstock *et al.* 1996). The internal control plasmid Bos β Gal contains the promoter of the human elongation factor 1 gene driving expression of β -galactosidase (Mizushima & Nagata 1990), which was to normalise transfection efficiencies in some instances. All constructs were verified for orientation and correct sequence by restriction endonuclease digests. Large-scale preparation and purification of plasmids were performed by alkaline lysis and resin purification (Qiagen Ltd). For transfections, 3×10^5 cells/well were plated in 24-well plates and transfected by the calcium phosphate method as described previously (Graham & van der Eb 1973) without glycerol shock, typically with 1 μ g/well of each reporter construct. The cells were stimulated for 24 h in culture medium without (control) or with 100 nM GNRH prior to being harvested. Cellular extracts were assayed for luciferase and β -galactosidase activity as described previously (Holdstock *et al.* 1996). Luciferase data from separate experiments were pooled by normalising the data to the level of β -galactosidase activity. Each treatment group contained triplicate cultures and experiments were repeated at least twice.

Table 1 Oligonucleotide primers used for the RT-PCR analyses

Gene	Forward primer (5'–3'), reverse primer (5'–3')	Product size (bp)	Annealing temperature (°C)
<i>Nppa</i>	ATCTGCCCTCTTGAAAAGCA ACACACCACAAGGGCTTAGG	213	55.3
<i>Nppb</i>	CAGCTCTTGAAAGGACCAAGG AGACCCAGGCAGAGTCAGAA	242	57.3
<i>Nppc</i>	CGGCACCATGCACCTCTCCAGCTGAT GCTGCACTAACATCCAGACCGC	393	66.5
<i>Npr1</i>	AAGCTTATCTGGAGGAGAAGCGCA TCAGCCTCGAGTGCTACATCCCCG	677	63.3
<i>Npr2(GC-B1/2)</i>	GAAGCTGATGCTGGAGAAGGAGC GACAATACTCGGTGACATGCAG	362 (GC-B1) 287 (GC-B2)	60
<i>Npr2(GC-B1/3)</i>	AACGGGCGCATTGTGTATATCTGCGGC TTATCACAGGATGGGTCGTCCAAGTCA	692 (GC-B1) 872 (GC-B3)	57
<i>Npr3</i>	CCCTCCAAACAGTCACCCTA CCATCCTTCTTGCTGTAGCC	202	57.3
<i>Gucy1a</i>	AGCAGTGTGGAGAGCTGGAT ATCTCGGGGTGAACACAAAG	368	56.3
<i>Gucy1b</i>	GCCAATGAGCTGAGACAAA TTGACCAGCAATTTCCATCA	320	53.3
<i>Corin</i>	TCCTTCTCCAGAGGACCAGA TGATAGCAACTGAGGCGATG	180	56.3
<i>Furin</i>	AGTGGCCAACAATGGTGTCT AACGTGCCTCTACTGCATCA	104	55.3
<i>PAM</i>	CAAGCATGCAGTTTCCTTCA CTTCCTCTCTCCCTGCTTT	191	55.3
<i>Cga</i>	CTTCATTCTTCTCTGATGG TAACAAGTGCTACAGTGGCA	448	53.3
<i>Fshb</i>	GCTGCAATAGCTGTGAGC CTGAATCATGATGGAGTC	258	52.2
<i>Lhb</i>	GCTCCAGGGGCTGCTGCT CGACAGCTGAGAGCCACAGGG	302	62.2
<i>Nr5a1</i>	GCCAGGAGTTTCGTCTGTCTC TCAATGAGAAGGTTGTTGCG	248	56.3
<i>Rpl19</i>	CTGAAGGTCAAAGGGAATGTG GGACAGAGTCTTGATGATCTC	194	55.9

Nuclear protein extraction and electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using 1–3 µg nuclear extract per reaction. Nuclear protein extracts were prepared from 1×10^6 α T3-1 cells using a modification of a method described previously (Schreiber *et al.* 1989). Briefly, the media were removed and the cells washed with PBS and scraped in to 2 ml ice-cold PBS. Following centrifugation (5 min, 1500 *g*, 4 °C), the cells were resuspended in 400 µl ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)) and transferred to a cold microfuge tube. Having left the cells to swell on ice for 15 min, 25 µl of a 10% solution of Nonidet P-40 (made in buffer A) were added to each sample followed by vortexing for 10 s. Following microcentrifugation (10 000 *g*, 30 s), the pellets were resuspended in 300 µl buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF) before vigorous rocking on a shaking platform at 4 °C for 15 min. The nuclear extract was centrifuged (10 000 *g*, 5 min), and stored at –70 °C prior to protein determination

by the Bradford assay. Probes were created by filling in the 5' AGCT overhangs of the annealed *Nppc* oligonucleotides with Klenow polymerase using a mixture of dATP, dGTP, dTTP and α^{32} P-dCTP (ICN, Hampshire, UK; see Table 2 for sequences). Three microlitres of nuclear extracts were incubated at RT for 5 min in a 20 µl volume of 20 mM Tris (pH 8.0), 60 mM KCl, 2 mM MgCl₂, 1.2 mM DTT, 12% glycerol and 2.5 µg (poly(dI.dC).poly(dI.dC); Amersham-Pharmacia). For gel shifts, 2 µl specific antibodies were added to the reaction to identify components of the specific protein complexes, and these samples were incubated on ice for 60 min. The reactions were then incubated for 15 min at 30 °C in the presence of 1 ng probe. Complexes were electrophoresed on a 5% native acrylamide gel, dried and visualised by autoradiography.

Electron microscopy of rat pituitary tissue sections

Pituitaries were removed from 45-day-old female Sprague–Dawley rats killed by cervical dislocation, and fixed in 4% paraformaldehyde (PFA) with 0.1% glutaraldehyde in PBS

Table 2 The sequences of the electrophoretic mobility shift assay oligonucleotides from the promoter region of mouse *Nppc*, human *STAR* and human α *GSU* genes

	Sequence (5'–3')
Probe	
Nppc	AGCTGGTGGGCGGGAAGATGACATCAGC AGCTGCTGATGTCATCTTCCCGCCACC
WT-STAR	AGCTCCCCTGCACCTCCCCGCCCCAAGCT AGCTAGCTTGGGGCGGGGGAGGGTGCAGGGG
MUT-STAR	AGCTCCCCTGCACCTCCC AAG CCCCAAGCT AGCTAGCTTGGGGCT T GGGAGGGTGCAGGGG
WT CRE	AGCTAAATTGACGTCATGGTAAAAATTGACGTCAT- GGTA AGCTTACCATGACGTCAATTTTACCATGACGTCA- ATTT

AGCT is a HindIII site placed at the 5'-end of each sense and antisense oligonucleotides. Underlined bold text represents the mutations in the complementary oligonucleotides.

and washed in 0.1 M acetate buffer, and the tissue prepared as described previously (Christian & Morris 2002). Anterior pituitary sections were incubated in primary antibody at high titre (1:100 for CNP-22 (Peninsula Laboratories, Wirral, UK) and 1:500 for LH (National Hormone and Pituitary Program, Gaithersburg, MD, USA)) for 2 h at room temperature, and stained with 2% uranyl acetate. The stained tissue was examined using an electron microscope (JEOL, Welwyn Garden City, UK) at 5000 $\times$ magnification. Endocrine cells were identified on the basis of their secretory granule populations (i.e. their shape, electron density, size and distribution), nucleus size and organelle structures (Nogami & Yoshimura 1982). Observations were made from at least eight different cells, from sections prepared from at least four different animals.

Extraction and measurement of pituitary ANP, CNP and LH, and primary cultures

Following cervical dislocation, pituitaries were removed from male and female Sprague–Dawley and Wistar rats, between the ages of 21 and 90 days. The samples were sonicated, and 100 μ l of the resulting suspension were removed for protein analysis. The suspension was centrifuged at 450 *g* and 4 $^{\circ}$ C for 5 min. The supernatants were removed in to fresh tubes and then evaporated to dryness in a rotary evaporator under vacuum. The samples were resuspended with 500 μ l of the commercially purchased RIA buffer for CNP and stored at -20° C to await assay. CNP-22 and ANP-27 were measured using a kit from Peninsula Laboratories. Pituitary LH content was determined using reagents kindly provided by the NIDDK (Bethesda, MD, USA), using a RIA described previously (McArdle *et al.* 1994). For primary culture experiments, pituitaries were removed from 45-day-old random cycling female Sprague–Dawley rats (20 per experiment), and subjected to enzymatic dispersion as described previously (McArdle & Poch 1992). Dispersed cells were then cultured in M199 (containing 2 mM glutamine, 20 mM HEPES–NaOH

(pH 7.4), 0.3% (w/v) BSA, 2.5% (v/v) FCS, 10% (v/v) horse serum and 20 μ g/ml gentamicin sulphate) for 3 days in either 24-well plates (for static incubation) or 6-well plates in the presence of Cytodex beads (Sigma–Aldrich; for superfusion). For the static cultures, 1 ml/well of spent media was collected after 3 h incubation. For superfusion experiments, the cells were transferred to superfusion columns and allowed to equilibrate. A fraction collector was used to collect $\sim$ 1 ml samples, with media passing over the cells via peristaltic pump set to deliver 0.5 ml/min. Spent media were stored at -20° C prior to assay for LH.

Immunoblotting

Cell lysates (from 1×10^6 cells), or two pooled mouse pituitaries, were harvested in lysis buffer (0.5 M Tris (pH 6.8), 10% glycerol and 1% SDS). Protein lysates were sonicated, boiled at 95 $^{\circ}$ C for 5 min, resolved using 10% SDS–PAGE, transferred to PVDF membrane and probed for GC-A, GC-B or Npr-C expression. Appropriate HRP-conjugated secondary antibodies were purchased from DAKO (Cambridge, UK).

cGMP enzymeimmunoassay

α T3-1 and L β T2 cells were plated at a density of 2.5×10^5 cells/well in 24-well plates and left to adhere overnight. Cell treatments were conducted in physiological saline solution (NaCl (127 mM); CaCl₂ (1.8 mM); KCl (5 mM); MgCl₂ (2 mM); NaH₂PO₄ (0.5 mM); NaHCO₃ (5 mM); glucose (10 mM); HEPES (10 mM) pH 7.4; BSA (0.1% (w/v)) in the presence of 1 mM of the non-specific phosphodiesterase inhibitor, 3-isobutyl 1-methylxanthine (IBMX). Stimulations were terminated by the addition of ice-cold absolute ethanol and total (intra- and extracellular) cGMP concentrations determined as per the manufacturer's instructions (R&D Biosystems, Abingdon, UK).

Cell proliferation and FACS analyses

For FACS analysis, α T3-1 and L β T2 cells were seeded at a density of 2×10^5 cells into six-well plates in DMEM media supplemented with 2% (v/v) FCS (control), 2% (v/v) FCS containing 100 nM CNP or 10% (v/v) FCS (positive control). Cells were cultured for 48 h, harvested and resuspended in 500 μ l PBS containing 0.1% (w/v) glucose. Cells were fixed in 70% (v/v) ice-cold ethanol, washed and resuspended in a propidium iodide solution (69 μ M propidium iodide, 38 mM sodium citrate and 20 mg/ml RNase (pH 7.4)) and incubated for 40 min at 37 $^{\circ}$ C. Cells were subjected to FACS analysis using a Beckman Coulter (High Wycombe, UK) EPICS XL-MCL flow cytometer and Beckman Coulter Expo 32 software. For cell counting, α T3-1 and L β T2 cells were seeded at a density of 3×10^5 cells into six-well plates in DMEM media supplemented with 2% (v/v) FCS (control), 2% (v/v) FCS containing 100 nM CNP

or 10% (v/v) FCS (positive control) and cultured for 48 h. Following this, cells were harvested and cell number was determined by 0.4% (w/v) trypan blue exclusion methodology.

Statistical analysis and data presentation

All experiments were performed a minimum of three times, and autoradiographs are representative of three experiments. For luciferase activities, the data shown are either representative of at least three independent experiments, or have been normalised and pooled to represent the mean $\pm$ S.E.M. of at least three independent experiments, each performed in triplicate. Numerical data were subjected to ANOVA and were followed, where appropriate, by Bonferonni's multiple comparisons test, accepting $P < 0.05$ as significant. Estimates of EC₅₀ values were determined using in-built equations in GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

Molecular characterisation of gonadotroph natriuretic peptide system

Our previous expression and functional studies have suggested that CNP and GC-B are the predominant members of the natriuretic peptide system expressed in the anterior pituitary (McArdle *et al.* 1993, Fowkes & McArdle 2000, Fowkes *et al.* 2000), and that gonadotrophs are the major source of pituitary CNP (McArdle *et al.* 1994). We extended these initial studies with a comprehensive RT-PCR screen for components of the natriuretic peptide system in α T3-1 and L β T2 gonadotrophs, as well as primary mouse pituitaries. As expected, expression of the *Nppc* gene was found in both cell lines and in mouse pituitaries, but *Nppa* expression was restricted to L β T2 cells and primary tissue, and *Nppb* was not detected at all (Fig. 1). As the peptide-processing enzymes Corin and Furin have been implicated in natriuretic peptide processing (Potter *et al.* 2006), we next examined the expression of these genes. *Corin* was absent from all cDNAs, but *Furin* was detected in both cell lines and primary pituitaries (Fig. 1). As previous studies had found that peptidyl α -amidating monooxygenase enzymes (PHM/PAL or PAM; involved in N-terminal amidation of regulatory peptides) were expressed in gonadotroph cells of the anterior pituitary (El Meskini *et al.* 1997), we also examined whether *Pam* could be detected in α T3-1 and L β T2 cells. Interestingly, α T3-1 cells and primary pituitaries expressed *Pam*, but no transcripts were detected from L β T2 cells (Fig. 1).

Having established the presence of transcripts for some of the natriuretic peptides and their putative processing enzymes, we next examined expression of natriuretic peptide receptors and soluble guanylyl cyclases. Transcripts for all three natriuretic peptide receptors (*Npr1*, *Npr2* and *Npr3*) were detected across all samples, although *Npr2* expression appeared less intense in L β T2 cells (Fig. 1). Using specific

Figure 1 Expression of natriuretic peptide components in immortalised gonadotroph cell lines and primary mouse pituitaries. Total RNA was extracted from α T3-1 and L β T2 gonadotrophs and primary mouse pituitaries before subsequent cDNA synthesis and PCR (30 cycles) for natriuretic peptides, natriuretic peptide receptors, processing enzymes and gonadotroph-specific markers. Specific amplification details are described in Table 1. Each gel represents at least three similar experiments.

primers that differentiated between previously characterised *Npr2* splice variants (Tamura & Garbers 2003) we were able to detect expression of GC-B1, 2 and 3 in α T3-1 and primary pituitary, but abundance of the GC-B1 transcript in L β T2 cells was low, with no apparent GC-B2/3 expression. In addition, all cDNAs expressed soluble guanylyl cyclase

α - and β -subunits (*Gucy1a* and *Gucy1b* respectively). Finally, the expression of a range of gonadotroph markers was also examined in all samples. As expected, the primary mouse pituitary expressed all gonadotrophin subunits (*Cga*, *Lhb* and *Fshb*) as well as steroidogenic factor-1 (*Nr5a1*), and the α T3-1 and L β T2 cells expressed *Nr5a1* as well as specific

gonadotrophin subunits appropriate to their developmental stage at immortalisation (Alarid *et al.* 1996). The reduced expression of *Nr5a1* transcript in primary mouse pituitary is in keeping with SF-1 being a gonadotroph-specific gene, and hence only expressed in ~10% of the anterior pituitary population. In all cases, the gene encoding the ribosomal protein L19 (*Rpl19*) was used as a housekeeping gene and showed comparable expression.

Transcriptional regulation of the murine *Nppc* gene in gonadotroph cell lines

As our RT-PCR screen had confirmed that *Nppc* was the most abundant natriuretic peptide transcript expressed in the pituitary and gonadotroph cell lines, we next examined the transcriptional regulation of the murine *Nppc* gene. α T3-1 and L β T2 gonadotrophs, and AtT-20 corticotroph cells were transiently transfected with an *Nppc* reporter gene construct encoding -450 bp of the proximal promoter upstream of luciferase (-0.45Nppc-Luc). As shown (Fig. 2A), basal promoter activity was enhanced in α T3-1 cells compared with L β T2 and AtT-20 cells; hence, subsequent experiments were performed in α T3-1 cells alone. We next examined whether the *Nppc* promoter was influenced by GNRH treatment in α T3-1 cells (Fig. 2B). All *Nppc* promoter constructs examined (-5.0Nppc-Luc, -0.45Nppc-Luc and -0.05Nppc-Luc) were significantly stimulated following exposure to 100 nM GNRH for 24 h (by 2.8 ± 0.3 -, 3.4 ± 0.5 - and 3.3 ± 0.3 -fold respectively; *** $P < 0.001$). As the smallest promoter construct examined appeared sufficient to maintain GNRH responsiveness, we examined the potential signalling mechanisms involved in regulating GNRH-stimulated *Nppc* transcription. As shown (Fig. 2C), pretreatment of transiently transfected α T3-1 cells with inhibitors of protein kinase C (PKC) or calcium entry

Figure 2 (A–C) Transcriptional regulation of the *Nppc* promoter in α T3-1 gonadotrophs. (A) α T3-1, L β T2 (gonadotrophs) and AtT-20 (corticotrophs) were transiently transfected with either pXp2 (empty vector) or the -0.45Nppc-LUC construct and total proteins harvested 24 h post-transfection. The data shown are means $\pm$ S.E.M. of triplicate transfections expressed as arbitrary light units (representative of at least three similar experiments). (B) Effect of GNRH on *Nppc* promoter activity in α T3-1 cells. *Nppc* promoter deletion constructs were transiently transfected into α T3-1 cells prior to stimulation with 100 nM GNRH for 24 h. The data shown are means $\pm$ S.E.M. of three independent experiments, each expressed as fold increase over the respective basal promoter activity. *** $P < 0.001$, significantly different from basal promoter activity. (C) Effect of inhibitors of PKC and calcium entry on GNRH-stimulated -0.05Nppc-LUC activity. α T3-1 cells were transiently transfected with the -0.05Nppc-LUC construct, then pretreated for 30 min with either 0 or 1 μ M GF109203X or nifedipine prior to stimulation with 0 or 100 nM GNRH in the continued absence or presence of inhibitor. The data shown are means $\pm$ S.E.M. of three independent experiments, each expressed as fold increase over the respective basal promoter activity. *** $P < 0.001$, * $P < 0.05$, significantly different from control response to GNRH.

(GF109203X and nifedipine respectively) significantly attenuated the GNRH effect on *Nppc* promoter activity (from 3.8 ± 0.6 -fold (control) to 1.2 ± 0.2 -fold ($***P < 0.001$, GF109203X) and 2.0 ± 0.4 -fold ($*P < 0.05$, nifedipine) over basal). In an attempt to identify putative GNRH-responsive sites within this short 50 bp region of the *Nppc* promoter, we performed EMSA analyses using nuclear extracts from α T3-1 cells. This region of the murine *Nppc* promoter has previously been shown to be GC rich and to bind TSC-22 (Ohta *et al.* 1996). However, consistent with our preliminary *in silico* screen of potential transcription factor-binding sites (Fig. 3A), at least three distinct complexes were detected, which upon antibody interrogation appeared to contain Sp1/Sp3 proteins in homo- and heterodimer configurations (Fig. 3B and C). Despite also containing putative AP-1 and cAMP response element (CRE) sites, cJun, ATF-2 or CREB antisera failed to disrupt complex formation, suggesting that these proteins are not involved in

gonadotroph-specific *Nppc* expression. A role for Sp1/Sp3 proteins was further confirmed using a cold-competition approach with consensus wild-type (WT) or mutant Sp1 oligonucleotides from the human StAR promoter. As shown (Fig. 3C), the WT-StAR competitor markedly inhibited complex formation, but the Sp1 mutant probe failed to do so. Furthermore, an oligonucleotide encoding the consensus CRE from the human α GSU promoter also failed to disrupt complex formation. Collectively, these data suggest that the murine *Nppc* gene is responsive to GNRH and that regions conferring Sp1/Sp3 binding may act to regulate *Nppc* transcription.

Determination and localisation of CNP immunoreactivity in primary rat pituitaries

We next examined the expression of CNP at the protein level in pituitaries from 40-day-old random cycling female

Figure 3 (A–C) EMSA analyses of the proximal *Nppc* promoter in α T3-1 gonadotrophs. (A) Diagram of the proximal human/rodent *Nppc* promoter (–50 bp), showing predicted transcription factor-binding sites. (B and C) Nuclear extracts were prepared from α T3-1 cells prior to incubation with 32 P-labelled oligonucleotides representing the proximal murine *Nppc* promoter. (B) Supershift reactions were performed using antibodies specific for Sp1, cJun, ATF-2 or CREB, prior to the addition of probe. (C) Supershift and competitor reactions to determine the identity of specific complexes. Supershift reactions were performed in the presence of antibodies specific for either Sp1, Sp3 or both. In addition, some reactions contained 100X excess unlabelled oligonucleotides encoding the proximal murine *Nppc* promoter, Sp1-containing WT-StAR promoter, MUT-StAR promoter of the WT CRE from the human α GSU promoter. Autoradiographs are representative of at least three similar experiments.

Sprague–Dawley rats, by immunoelectron microscopy and RIA. Modest CNP immunoreactivity (CNP-ir) was observed over secretory granules in gonadotroph cells (Fig. 4A), yet as expected, considerably more LH-ir was detected (Fig. 4B). This difference in the relative expression of CNP and LH in the pituitary was confirmed by RIA (Fig. 4C; CNP: 1.8 ± 0.4 pmol/g wet weight cf LH: 66.7 ± 12.1 pmol/g per wet weight). To determine whether pituitary CNP content varied according to the age, sex or strain of rat, we measured pituitary CNP and ANP levels in male and female Sprague–Dawley and Wistar rats, between the ages of 21 days and 90 days. As shown (Supplementary Figure 1A–D, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/cgi/content/full/JOE-09-0189/DC1>), there was no appreciable evidence of sexual dimorphism in terms of CNP expression. In all cases, pituitary ANP content was below the limit of detection for the RIA (which was 0.05 pmol/g wet weight). These findings further suggest CNP, rather than ANP, is the major pituitary natriuretic peptide and that gonadotroph cells are a likely source of pituitary CNP.

Functional effects of natriuretic peptides in gonadotroph cell lines

Having established that both α T3-1 and L β T2 cells express many components of the natriuretic peptide system, we next determined the protein expression of natriuretic peptide receptors in α T3-1, L β T2 cells and primary mouse pituitaries using western blotting. As shown (Fig. 5A), protein expression of GC-A, GC-B and Npr3 (the clearance receptor) was detected in all samples, although there was less Npr3 expression in primary mouse pituitary samples. We next examined the activity of particulate and soluble

guanylyl cyclases in both cell lines, using cGMP accumulation as an indication of activity. α T3-1 and L β T2 cells were stimulated with the indicated concentrations of ANP, CNP or the nitric oxide donor, sodium nitroprusside (SNP), for 15 min in physiological saline solution (PSS) containing 1 mM IBMX, prior to termination of the reaction. As shown (Fig. 5B and C), both ANP and CNP potently stimulated cGMP accumulation in both cell lines. As described previously (McArdle *et al.* 1993, Fowkes *et al.* 2000), CNP was more potent than ANP in α T3-1 cells (Fig. 5B, estimated EC₅₀ values of ~ 579 and ~ 48 nM for ANP and CNP respectively) but ANP was a more effective stimulator of cGMP in L β T2 cells (Fig. 5B, estimated EC₅₀ values of ~ 1.12 and ~ 157 nM for ANP and CNP respectively), suggesting the predominant expression of GC-A receptors in these cells. In contrast, cGMP accumulation in response to SNP was less pronounced, particularly in L β T2 cells, suggesting that particulate guanylyl cyclases may be the major regulators of cGMP levels in gonadotrophs. Interestingly, cGMP accumulation in response to maximal concentrations of all three ligands was significantly greater in α T3-1 cells compared with L β T2 cells (Fig. 5D; ANP, 1710.2 ± 545.3 cf 434.4 ± 52.6 , $\ast P < 0.05$; CNP, 1749.9 ± 633.7 cf 176.7 ± 63.0 , $\ast P < 0.05$; SNP, 376.9 ± 23.9 cf 38.1 ± 3.1 , $\ast P < 0.05$; for α T3-1 and L β T2 cells respectively).

As several studies have reported a potent anti-proliferative effect of CNP (Hagiwara *et al.* 1994, Tao *et al.* 1999, Simpson *et al.* 2002), we next examined whether CNP could alter proliferation or cell cycle distribution in α T3-1 or L β T2 cells. Cells were plated in low-serum medium (2% v/v) for 48 h in the absence or presence of the indicated concentrations of either CNP or GNRH prior to cell counting or FACS analyses. In all cases, a 10% (v/v) serum-containing

Figure 4 (A–C) Expression and localisation of CNP in rat anterior pituitary gonadotrophs. Immunoelectron microscopy was performed on anterior pituitary sections taken from randomly cycling female Sprague–Dawley rats, using specific antibodies against CNP-22 (A) or LH (B). The arrows indicate immunogold labelling of CNP-ir. The data shown are representative of gonadotroph cells observed from at least four animals. (C) Pituitary CNP and LH protein content in female Sprague–Dawley rats, as determined by RIA. The data shown are means $\pm$ s.e.m. of four independent experiments.

Figure 5 (A–D) Functional analyses of receptor guanylyl cyclase activity in α T3-1 and L β T2 gonadotrophs. (A) Detection of GC-A, GC-B and Npr3 proteins by western blotting. Total protein extracts were prepared from α T3-1 and L β T2 cells, and primary mouse pituitaries. The images shown are representative of at least three independent experiments. (B–D) α T3-1 cells (B) or L β T2 cells (C) were plated at a density of 3×10^5 prior to a 15 min stimulation with the indicated concentrations of ANP, CNP or the nitric oxide donor, SNP, in PSS containing 1 mM IBMX. Total cGMP accumulation was determined by enzyme immunoassay. The data shown are means $\pm$ S.E.M. expressed as % of the control response to 10^{-6} M CNP, from at least four independent experiments, each performed in triplicate. (D) Comparison of cGMP accumulation between α T3-1 and L β T2 cells. The data shown are means $\pm$ S.E.M. expressed as pmol/mg protein, pooled from at least four independent experiments, each performed in triplicate. * $P < 0.05$, significantly different from α T3-1 cells.

media was used as a positive control. As shown (Fig. 6A and B), CNP failed to alter cell number or cell cycle distribution in either α T3-1 or L β T2 cells. As expected, 10% (v/v) serum-treated cells showed significant proliferation (Fig. 6A and B; to 226.5 ± 7.5 and $211.6 \pm 11.5\%$ of control, *** $P < 0.001$; for α T3-1 and L β T2 cells respectively) and

redistribution into G2/M (Fig. 6C and D; from 14.9 ± 1.4 to $27.5 \pm 0.7\%$ in α T3-1 cells, *** $P < 0.001$; from 20.8 ± 4.4 to $38.9 \pm 4.8\%$ in L β T2 cells, *** $P < 0.001$) in both cell lines. Collectively, these data reveal no effect of CNP on cell proliferation in spite of its pronounced effect on cGMP signalling in the cells.

Figure 6 (A–D) Lack of effect of CNP on cell proliferation or cell cycle distribution in α T3-1 and L β T2 cells. (A and B) Cells were plated at a density of 2×10^5 cells/well in 2% FCS-containing media with 0 or 100 nM CNP, GNRH or both peptides. A 10% FCS treatment was included as a positive control. After 48 h, cells were harvested and counted by trypan blue exclusion. The data shown are means $\pm$ S.E.M. of three independent experiments each performed in triplicate. *** $P < 0.001$, significantly different from basal. (C and D) Cells were plated at a density of 3×10^5 cells/well in 2% FCS-containing media with 0 or 100 nM CNP, GNRH or both peptides. A 10% FCS treatment was included as a positive control. After 48 h, cells were harvested and fixed prior to staining with propidium iodide and analyses of cell cycle distribution using flow cytometry. The data shown are means $\pm$ S.E.M. of three independent experiments each performed in triplicate. *** $P < 0.001$, significantly different from basal distribution.

Effect of natriuretic peptides on LH secretion from rat anterior pituitary cells

A few studies report effects of natriuretic peptides on the secretion of GH and ACTH *in vitro* (Gilkes *et al.* 1994, Shimekake *et al.* 1994, Guild & Cramb 1999), and soluble guanylyl cyclase activity has been implicated in regulating gonadotrophin secretion (Ceccatelli *et al.* 1993). To examine the potential effects of ANP and CNP on LH secretion, we cultured freshly dispersed primary male rat anterior pituitary cells under two experimental paradigms; static culture in 24-well plates and under superfusion conditions following culture on cytodex beads. Rat anterior pituitary cells were treated with the indicated concentrations of GNRH in the absence or presence of 100 nM ANP or CNP for 3 h before removing the spent media and assaying for LH content. As shown (Fig. 7A), GNRH caused a concentration-dependent

increase in LH secretion, and this was unaffected by either ANP or CNP. To establish whether this failure to affect GNRH-stimulated LH secretion was associated with a lack of guanylyl cyclase signalling in primary rat anterior pituitary cells, we examined cGMP accumulation in primary rat anterior pituitary cells in static culture. As shown (Supplementary Figure 2, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/cgi/content/full/JOE-09-0189/DC1>), CNP caused a concentration-dependent increase in cGMP accumulation, and exposure to 1 mM SNP also enhanced cGMP accumulation. Even repeating the static incubation experiments in the presence and absence of IBMX to potentiate any possible effect of elevated cGMP failed to reveal a role for CNP or SNP-stimulated cGMP accumulation in regulating GNRH-stimulated LH secretion (Supplementary Figure 2). Finally, to determine whether

Figure 7 (A and B) Lack of effect of CNP on LH secretion from primary rat pituitary cells in static or superfusion cultures. Primary cultures of rat pituitary cells were prepared by enzymatic dispersion before determining the effect of CNP on basal or GNRH-stimulated LH secretion over 24 h. (A) Cells were plated in 24-well plates and left to attach overnight before stimulation with the indicated concentrations of GNRH in the absence (control) or presence of 100 nM ANP or CNP. LH concentrations in spent media were determined by RIA. The data shown are means $\pm$ S.E.M. of three independent experiments, each performed in triplicate and expressed as the % control response to 1 μ M GNRH. (B) Cells were prepared as before, except they were cultured on sterile cytodex glass beads before transferring to superfusion columns. Fractions of spent media were collected every minute following exposure to 0 or 100 nM CNP and stimulation with 0 or 100 nM GNRH, before determination of LH concentrations by RIA. The data shown are means $\pm$ S.E.M. of duplicate cultures, representative of at least three independent experiments, and expressed pg/100 μ l of media.

CNP had any subtle effects on the kinetics or magnitude of GNRH-stimulated LH secretion, we used a superfusion system. Following a period of equilibration, rat anterior pituitary cells were superfused with media containing 0 or 100 nM CNP for 20 min prior to a 10 min exposure to 100 nM GNRH in the continued absence or presence of CNP. As shown (Fig. 7B), neither the kinetics nor magnitude of GNRH-stimulated LH secretion was affected by CNP. Collectively, these data suggest that despite causing enhanced cGMP accumulation, CNP and SNP fail to alter pituitary LH secretion *in vitro*.

Effect of CNP on α GSU gene transcription

We next examined whether CNP could affect gene transcription in gonadotroph cell lines. Our previous RT-PCR screen (Fig. 1) had shown that the *Cga* was the only gonadotrophin subunit common to both α T3-1 and L β T2 cells. Therefore, we chose to examine the potential role of CNP on the human α GSU promoter as we have previously published comparative activity of this construct in α T3-1 and L β T2 cells (Fowkes *et al.* 2002). Cells were transiently transfected with a full range of deletion constructs encoding the proximal human α GSU promoter (from -846 to -195 bp). As reported previously (Fowkes *et al.* 2002), promoter constructs ranging between -244 and -846 bp were active in both cell lines (Fig. 8A and B). However, the presence of 100 nM CNP for 8 h failed to alter promoter activity of any construct in α T3-1 cells. Interestingly, CNP caused a significant increase in constructs ranging

from -244 to -846 bp in L β T2 cells (by 3.9 ± 6.0 -, 5.1 ± 0.2 -, 5.9 ± 0.7 -, 4.5 ± 0.3 - and 5.2 ± 0.2 -fold for -244, -346, -442, -517 and -846 bp respectively; $***P < 0.001$). We further examined the properties of this CNP effect by investigating the concentration-dependent effects of CNP on -244 α LUC, the shortest construct to significantly respond to CNP. As shown (Fig. 8C), CNP caused a concentration-dependent increase of -244 α LUC activity in L β T2 cells (by 5.9 ± 1.5 - and 8.4 ± 2.5 -fold for 100 nM and 1 μ M CNP respectively; $*P < 0.05$). Collectively, these data suggest that there are different downstream targets of CNP in α T3-1 and L β T2 cells, some of which lead to an increase in α GSU gene transcription.

Discussion

Despite there being growing evidence that CNP and GC-B are major regulators of the HPG axis (Fowkes & McArdle 2000, Walther & Stepan 2004), molecular and functional characterisation of the local natriuretic peptide system in these HPG tissues has been poor. As early reports of CNP expression in mice suggested some of the highest tissue concentrations were found in the anterior pituitary (Komatsu *et al.* 1991), our recent investigations have focused on natriuretic peptides in the pituitary. Our current studies clearly show evidence of a local natriuretic peptide system in rat and mouse pituitaries, and in particular in gonadotroph cell lines. Specifically, our data strengthen the case for CNP and GC-B as being the major natriuretic peptide components

Figure 8 (A–C) CNP stimulates promoter activity of the human α GSU gene in L β T2 cells but not α T3-1 cells. (A and B) Cells were transiently transfected with the indicated deletion constructs of the human α GSU promoter or with promoterless control (pA3LUC) and stimulated with 0 or 100 nM CNP for 24 h post-transfection. The data shown are means $\pm$ S.E.M. of triplicate transfections, representative of at least three independent experiments, and expressed as % of relevant basal promoter activity. *** $P < 0.001$, significantly different from basal. (C) Concentration dependence of CNP-stimulated α GSU promoter activity in L β T2 cells. Cells were transfected with -244 α LUC and stimulated with the indicated concentrations of CNP for 24 h. The data shown are means $\pm$ S.E.M. of three independent experiments, each performed in triplicate and expressed as fold increase over basal.

of the anterior pituitary, and our functional data reveal a potential role in regulating gonadotroph gene expression.

The mRNA expression of all three natriuretic peptide receptors (*Npr1*, *Npr2* and *Npr3*) as well as splice variants of *Npr2* (GC-B1, 2 and 3) in all samples examined suggests that the anterior pituitary (gonadotroph cells in particular) is major target for natriuretic peptide activity. This supports our previous protein and pharmacology studies that suggested gonadotrophs are the predominant cell type to express CNP (McArdle *et al.* 1993, 1994) and are the major producers of cGMP in response to CNP (Fowkes *et al.* 2000). Although our PCR studies were purely qualitative, it appears that there is abundant expression of the *Nppc* transcript, yet little *Nppa* and no *Nppb*. While it is clear from our functional studies that gonadotrophs remain potential target cells of ANP, it is less likely that ANP and GC-A are major regulators of pituitary function compared with CNP and GC-B. Certainly, the pituitary phenotype of the *Npr2*^{-/-} mouse models that suggest a GH deficiency (Tamura *et al.* 2004) is not apparently phenocopied by *Nppa*^{-/-} or *Npr1*^{-/-} models (John *et al.* 1995, Lopez *et al.* 1995). The detection of transcripts for the processing enzymes *Furin* and *Pam* in α T3-1 cells and primary mouse pituitaries supports previous suggestions that CNP is an autocrine regulator of gonadotrophs (McArdle *et al.* 1994), although it remains to be demonstrated as to whether proCNP is cleaved by *Furin*, and subject to α -amidation by PAM in gonadotrophs. Nevertheless, gonadotroph cells are the major anterior pituitary cell type that expresses the PAM enzymes (El Meskini *et al.* 1997, 2000), intriguingly supporting a potential role for the PAM modification of CNP, as has been implicated by recent studies suggesting that ANP is modified by PAM in cardiac atria (Labrador *et al.* 2004).

Given the relative abundance of the *Nppc* transcript in the gonadotroph cell lines and mouse pituitaries, we investigated the transcriptional regulation of the *Nppc* gene using

previously generated reporter gene constructs (Huang *et al.* 1996). Basal promoter activity was greatest in the two gonadotroph cell lines, compared with the AtT-20 corticotroph cells that were previously used to examine *Nppc* transcription (Ohta *et al.* 1993). Our novel observations that the proximal *Nppc* promoter appears responsive to GNRH build upon our earlier suggestion that GNRH and CNP are part of a reciprocal pathway, whereby one affects the expression and signalling of the other (McArdle *et al.* 1994, Fowkes *et al.* 1999, 2000, Fowkes & McArdle 2000). As expected, the stimulatory effect of GNRH on *Nppc* promoter activity involves PKC activity as well as calcium entry, in common with other gonadotroph-specific genes regulated by GNRH (Halvorson 2000). Furthermore, our EMSA analyses implicated Sp1 and Sp3 proteins as the major transcriptional regulators of this proximal *Nppc* promoter. While we have no direct evidence as yet to link GNRH signalling to Sp1/Sp3 transcriptional activity, the LH β gene is partially regulated by GNRH in an Sp1/PKC-dependent manner in L β T2 cells (Kaiser *et al.* 2000, Weck *et al.* 2000), and Sp1 sites are involved in the GNRH regulation of neuronal nitric oxide synthase expression in gonadotrophs (Bachir *et al.* 2003). The novel implication that Sp1/Sp3 is involved in regulating *Nppc* transcription contrasts a previous study investigating the role of TSC-22 as a major regulator of *Nppc* expression in pituitary GH3 cells (Ohta *et al.* 1993, 1996). Although at least one additional complex remains to be identified in our EMSA analyses, the predominant complexes all contain Sp1, Sp3 or a combination of these proteins. We have detected mRNA transcripts of *Tsc22d1* in a range of anterior pituitary cell lines (IR Thompson & RC Fowkes unpublished observations), but currently have no data to suggest its involvement in gonadotroph expression of CNP. The only other study to examine the transcriptional regulation of the *Nppc* gene demonstrated a role for Wnt4/ β -catenin/TCF/LEF pathway, with multiple TCF/LEF sites

implicated (Surendran & Simon 2003). We have not examined the role of Wnt signalling in regulating CNP expression in the pituitary, yet recent observations of GNRH-dependent β -catenin signalling in gonadotrophs (Salisbury *et al.* 2008) suggest this pathway could be an alternative regulator of *Nppc* transcription in the pituitary.

In support of our studies of *Cnp* gene expression, we also demonstrated CNP protein expression and localisation in normal rat pituitaries. There appears to be no evidence of sexually dimorphic CNP changes in the anterior pituitary of Sprague–Dawley or Wistar rats, or dramatic alterations in pituitary CNP content over time. However, the levels of CNP were consistently 10 to 20-fold greater than ANP, whose expression levels were, at best, at the limit of the assay detection. This further supports a predominant role for local CNP, rather than ANP, in the anterior pituitary. Electron microscopy revealed modest CNP-ir over dense-core secretory granules in gonadotrophs, suggesting that CNP is potentially secreted by regulated exocytosis from gonadotrophs. Similar subcellular localisation of CNP has previously been reported in GT1–7 GNRH neurons (Middendorff *et al.* 1997) and in cells of the rat inner ear (Suzuki *et al.* 2000). Interestingly, in cardiac tissues, ANP and BNP are colocalised with chromogranin A (CgA) in secretory granules (Tota *et al.* 2008). As CgA and secretogranin II are implicated in gonadotrophin secretion (Nicol *et al.* 2002, 2004), it is tempting to speculate that CNP may well be cosecreted with gonadotrophins in response to GNRH.

We characterised the cGMP responsiveness of both α T3-1 and L β T2 cells to natriuretic peptides and nitric oxide donors in the current study, and found both CNP and ANP potently stimulated cGMP accumulation in both cell lines. This represents the first observation of functional receptor guanylyl cyclases in L β T2 gonadotrophs, although the effects of CNP and ANP on cGMP accumulation in α T3-1 cells confirm our previous findings (McArdle *et al.* 1993, Fowkes *et al.* 2000). Interestingly, cGMP production in response to CNP was much greater in α T3-1 cells than L β T2 cells, and estimated EC₅₀ values altered accordingly, suggesting that different populations of particulate guanylyl cyclases exist in these two cell lines. The apparent discrepancy between the relative contribution of GC-A and GC-B signalling between α T3-1 and L β T2 cells might simply reflect a phenotypic difference between the two cell lines, as we have no evidence to suggest that it could be a genuine reflection of gonadotroph responsiveness to CNP during embryonic development. Despite there being a functional CNP/GC-B system in rat primary pituitary cells and both immortalised gonadotroph cell lines, we failed to see any effect of CNP on cell proliferation and cell cycle distribution, although the interpretation of such studies is always hampered by the limitations of examining cell proliferation in SV40 large T-expressing cell lines. In addition, we also failed to see any effect of CNP (or ANP) on LH secretion. This apparent lack of effect of cGMP activators on gonadotrophin secretion is in partial agreement with other studies in rat pituitary cells (Pinilla *et al.*

1999), although this group had previously reported that nitric oxide donors stimulate LH secretion via a non-cGMP-dependent mechanism (Pinilla *et al.* 1998).

In L β T2 cells, CNP clearly stimulated the activity of the human α GSU promoter, suggesting a potential role for CNP in regulating gonadotroph gene transcription. It is unclear as to why CNP failed to alter α GSU promoter activity in α T3-1 cells, but presumably this reflects key differences in the expression of transcriptional regulators in these cell lines. The role of the CRE in mediating α GSU transcription is well characterised (Heckert *et al.* 1996, Burrin *et al.* 1998), and we have previously shown an altered response to cAMP-mediated transcription of the human α GSU promoter between α T3-1 and L β T2 cells (Fowkes *et al.* 2003). In our current study, CNP was able to significantly stimulate the proximal –244 bp construct of the human α GSU promoter in L β T2 cells, which is known to contain a tandem CRE, the gonadotroph-specific element that binds SF-1, and a consensus GATA site (Aylwin & Burrin 1996, Jorgensen *et al.* 2004). Of these sites, the CREs are potential targets for CNP action, given that cGMP is able to enhance CREB phosphorylation (Lu & Hawkins 2002). If CRE-mediated regulation of the human α GSU promoter is involved in CNP-stimulated α GSU promoter activity, then our previous description of altered expression of PKA subunits in L β T2 cells compared with α T3-1 cells (Fowkes *et al.* 2003) might explain the differential effects of CNP reported in the current study. Furthermore, there is growing evidence for direct effects of cGMP and PKG on gene transcription in other systems (Zhao *et al.* 2005, Broderick *et al.* 2007), and PKG isoforms may be expressed in anterior pituitary cells (Li *et al.* 1996). However, we have no conclusive evidence for PKG expression or activity in either α T3-1 or L β T2 cells, and its potential role in the regulation of gonadotroph gene transcription remains to be investigated.

In summary, we report the first comprehensive molecular and functional characterisation of a gonadotroph natriuretic peptide system, and show novel evidence for transcriptional regulation of *Nppc* by GNRH. It appears that CNP fails to alter conventional gonadotroph function, such as gonadotrophin secretion, although CNP can stimulate α GSU reporter gene activity in L β T2 cells. Although the physiological relevance of the gonadotroph natriuretic peptide system, and hence cGMP production, remains enigmatic, our data suggest that gonadotroph cells are a major source of CNP production and are major targets for CNP-stimulated cGMP production. In addition to the role of CNP as a putative autocrine regulator of gonadotrophs (McArdle *et al.* 1994, Fowkes *et al.* 2000), the paracrine regulation of other anterior pituitary cell types (Denef 2008) is likely to involve a contribution from gonadotroph-derived CNP.

Declaration of interest

The authors have nothing to disclose.

Funding

This study was supported in part by a post-graduate Cadogan Fellowship from the Royal Veterinary College (to support I R T), a BBSRC Project grant BB/D001560/1 (to R C F) and a Needham-Cooper Fellowship (to C A M).

Acknowledgements

We thank Prof. Pamela Mellon for provision of the α T3-1 and L β T2 cells, Prof. John Morris and Dr Helen Christian (Department of Human Anatomy, University of Oxford) for assistance with the EM studies, and Dr Danielle Aw (RVC) for technical assistance with flow cytometry.

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Received in final form 29 July 2009

Accepted 7 August 2009

Made available online as an Accepted Preprint
7 August 2009