Molecular characterisation and functional interrogation of a local natriuretic peptide system in rodent pituitaries, αT3-1 and LβT2 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 αT3-1 and LβT2 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 αT3-1 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 LβT2 cells but not in αT3-1 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.

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 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 αT3-1 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

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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 (Olesc 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, Gutzowska 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 (αT3-1 and LβT2) 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 (αGSU) in LβT2 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 αT3-1 or LβT2 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

αT3-1, LβT2 and AtT-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 1×10⁶ cells (αT3-1 or LβT2), 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 αGSU promoter in pA3LUC have been described previously (Maxwell et al. 1989, Holdstock et al. 1996). The internal control plasmid BosβGal 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, 3×10⁵ 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 μg/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.
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 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 into 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^32P-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 MgCl_2, 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 αGSU genes

| Probe     | Sequence (5’–3’)
|-----------|------------------|
| Nppc      | AGCTGGTGGGCGCGGAAGATGACATCA
| WT-StAR   | AGCTGGTGGGCGCGGAGGACAGGG
| MUT-StAR  | AGCTGGTGGGCGCGGAGGACAGGG
| WT CRE    | AGCTGGTGGGCGCGGAGGACAGGG

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× 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 µl of the resulting suspension were removed for protein analysis. The suspension was centrifuged at 450 g and 4 °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 µl 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 (Christian & Morris 2002). 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 (Mc Ardle & 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 µg/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 ~ 1 ml samples, with media passing over the cells via peristaltic pump to deliver 0·5 ml/min. Spent media were stored at −20 °C prior to assay for LH.

Immunoblotting

Cell lysates (from 1×10⁶ 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 °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

αT3-1 and LβT2 cells were plated at a density of 2·5×10⁵ 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, αT3-1 and LβT2 cells were seeded at a density of 2×10⁵ 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 µl 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 μM propidium iodide, 38 mM sodium citrate and 20 mg/ml RNase (pH 7-4)) and incubated for 40 min at 37 °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, αT3-1 and LβT2 cells were seeded at a density of 3×10⁵ 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 ± 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 EC\(_{50}\) 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 \( \alpha T3-1 \) and \( \beta LT2 \) 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 \( \beta LT2 \) 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 \( \alpha \)-amidating monoxygenase 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 \( \alpha T3-1 \) and \( \beta LT2 \) cells. Interestingly, \( \alpha T3-1 \) cells and primary pituitaries expressed \( Pam \), but no transcripts were detected from \( \beta LT2 \) 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 \( \beta LT2 \) cells (Fig. 1). Using specific 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 \( \alpha T3-1 \) and primary pituitary, but abundance of the GC-B1 transcript in \( \beta LT2 \) cells was low, with no apparent GC-B2/3 expression. In addition, all cDNAs expressed soluble guanylyl cyclase

**Figure 1** Expression of natriuretic peptide components in immortalised gonadotroph cell lines and primary mouse pituitaries. Total RNA was extracted from \( \alpha T3-1 \) and \( \beta LT2 \) 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.
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 αT3-1 and LβT2 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. αT3-1 and LβT2 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 αT3-1 cells compared with LβT2 and AtT-20 cells; hence, subsequent experiments were performed in αT3-1 cells alone. We next examined whether the Nppc promoter was influenced by GNRH treatment in αT3-1 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 αT3-1 cells with inhibitors of protein kinase C (PKC) or calcium entry
(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 αT3-1 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 αGSU 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 rats. A proximal human/rodent Nppc promoter containing predicted transcription factor-binding sites (Fig. 3A) was used to transfect αT3-1 gonadotrophs, and nuclear extracts were prepared from these cells. EMSA analyses were performed using 32P-labelled oligonucleotides representing the proximal murine Nppc promoter. Supershift reactions were performed using antibodies specific for Sp1, cJun, ATF-2 or CREB, prior to the addition of probe (Fig. 3B). Supershift and competitor reactions were performed to determine the identity of specific complexes (Fig. 3C). 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 αGSU 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 αT3-1 and LβT2 cells express many components of the natriuretic peptide system, we next determined the protein expression of natriuretic peptide receptors in αT3-1, LβT2 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. αT3-1 and LβT2 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 (McArule et al. 1993, Fowkes et al. 2000), CNP was more potent than ANP in αT3-1 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 LβT2 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 LβT2 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 αT3-1 cells compared with LβT2 cells (Fig. 5D; ANP, 1710.2 ± 545.3 cf 434.4 ± 52.6, *P < 0.05; CNP, 1749.9 ± 633.7 cf 176.7 ± 63.0, *P < 0.05; SNP, 376.9 ± 23.9 cf 38.1 ± 3.1, *P < 0.05; for αT3-1 and LβT2 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 αT3-1 or LβT2 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

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**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 ± S.E.M. of four independent experiments.

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 αT3-1 or LβT2 cells. As expected, 10% (v/v) serum-treated cells showed significant proliferation (Fig. 6A and B; to 226.5±7.5 and 211.6±11.5% of control, ***P<0.001; for αT3-1 and LβT2 cells respectively) and redistribution into G2/M (Fig. 6C and D; from 14.9±1.4 to 27.5±0.7% in αT3-1 cells, ***P<0.001; from 20.8±4.4 to 38.9±4.8% in LβT2 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 5 (A–D) Functional analyses of receptor guanylyl cyclase activity in αT3-1 and LβT2 gonadotrophs. (A) Detection of GC-A, GC-B and Npr3 proteins by western blotting. Total protein extracts were prepared from αT3-1 and LβT2 cells, and primary mouse pituitaries. The images shown are representative of at least three independent experiments. (B–D) αT3-1 cells (B) or LβT2 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 ± 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 αT3-1 and LβT2 cells. The data shown are means ± 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 αT3-1 cells.
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 cytodes 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 6 (A–D) Lack of effect of CNP on cell proliferation or cell cycle distribution in αT3-1 and LβT2 cells. (A and B) Cells were plated at a density of 2 \times 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 ± 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 \times 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 ± S.E.M. of three independent experiments each performed in triplicate. ***P<0.001, significantly different from basal distribution.
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 αGSU 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 Cgα was the only gonadotrophin subunit common to both αT3-1 and LβT2 cells. Therefore, we chose to examine the potential role of CNP on the human αGSU promoter as we have previously published comparative activity of this construct in αT3-1 and LβT2 cells (Fowkes et al. 2002). Cells were transiently transfected with a full range of deletion constructs encoding the proximal human αGSU 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 αT3-1 cells. Interestingly, CNP caused a significant increase in constructs ranging from −244 to −846 bp in LβT2 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 −244αLUC, the shortest construct to significantly respond to CNP. As shown (Fig. 8C), CNP caused a concentration-dependent increase of −244αLUC activity in LβT2 cells (by 5.9 ± 1.5- and 8.4 ± 2.5-fold for 100 nM and 1 μM CNP respectively; *P < 0.05). Collectively, these data suggest that there are different downstream targets of CNP in αT3-1 and LβT2 cells, some of which lead to an increase in αGSU 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.
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 targets 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 αT3-1 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 γ-aminidation 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 LHβ gene is partially regulated by GNRH in an Sp1/PKC-dependent manner in LβT2 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
gonadotrophs in response to GNRH. It is tempting to speculate that CNP may well be cosecreted with ANP, which is known to modulate cGMP accumulation in both cell lines. This is consistent with our findings in the current study, and found both CNP and ANP potently stimulate cGMP accumulation in both cell lines. The apparent discrepancy between the 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 αT3-1 and LβT2 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 cGMP accumulation in αT3-1 and LβT2 cells. Although we have no conclusive evidence for PKG expression or activity in either αT3-1 or LβT2 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 αGSU reporter gene activity in LβT2 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.

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