RESEARCH

Neuronostatin exerts actions on pituitary that are unique from its sibling peptide somatostatin

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Abstract

Neuronostatin, a somatostatin gene-encoded peptide, exerts important physiological and metabolic actions in diverse tissues. However, the direct biological effects of neuronostatin on pituitary function of humans and primates are still unknown. This study used baboon (*Papio anubis*) primary pituitary cell cultures, a species that closely models human physiology, to demonstrate that neuronostatin inhibits basal, but not ghrelin-/GnRH-stimulated, growth hormone (GH) and luteinizing hormone (LH) secretion in a dose- and time-dependent fashion, without affecting the secretion of other pituitary hormones (prolactin, ACTH, FSH, thyroid-stimulating hormone (TSH)) or changing mRNA levels. Actions of neuronostatin differs from somatostatin which in this study reduced GH/PRL/ACTH/LH/TSH secretion and GH/PRL/POMC/LH gene expression. Remarkably, we found that inhibitory actions of neuronostatin are likely mediated through: (1) the orphan receptor GPCR107 (found to be highly expressed in pituitary compared to somatostatin-receptors), (2) common (i.e. adenylyl cyclase/protein kinase A/MAPK/extra-/intracellular Ca²⁺ mobilization, but not phospholipase C/protein kinase C/mTOR) and distinct (i.e. PI3K) signaling pathways than somatostatin and; (3) dissimilar molecular mechanisms than somatostatin (i.e. upregulation of GPCR107 and downregulation of GHS-R/Kiss1-R expression by neuronostatin and, upregulation of sst1–5 expression by somatostatin). Altogether, the results of this study provide the first evidence that there is a functional neuronostatin signaling circuit, unique from somatostatin, which may work in concert with somatostatin to fine-tune hormone release from somatotropes and gonadotropes.

Key Words

- ▶ neuronostatin
- ▶ somatostatin
- ▶ somatotrope and gonadotrope cells
- ▶ primates
- ▶ growth hormone and luteinizing hormone
- ▶ GPCR107
- ▶ signaling pathways

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Introduction

Neuronostatin (NST), a recently described peptide hormone encoded in the preprosomatostatin gene but without amino-acid homology to somatostatin (SST) (Samson *et al.* 2008), has been shown to exert important physiological actions in diverse tissues (Hua *et al.* 2009,

Carlini *et al.* 2011, Yosten *et al.* 2011, Su *et al.* 2012, Salvatori *et al.* 2014). Immunoreactivity studies have demonstrated that NST and SST co-localized in a variety of cell types, including hypothalamic neurons and pancreatic cells (Samson *et al.* 2008, Dun *et al.* 2010).

However, measurement of SST and NST in the same samples indicated variations in the ratio of both peptides in different tissues, suggesting differential processing of these two peptides from the same precursor (Samson *et al.* 2008). Although it is clear that the actions of SST are mediated through activation of the SST receptor subtypes (sst1–sst5) (Ben-Shlomo & Melmed 2010, Vazquez-Borrego *et al.* 2017), it has been reported that NST failed to activate any of the sst-subtypes in an *in vitro* system (Samson *et al.* 2008), suggesting that NST and SST might exert different functions, or that the associated activities might be independent from each other. In fact, evidence indicating that NST acts through the G-protein-coupled receptor 107 (GPCR107) to exert some of its functions (Yosten *et al.* 2012, Elrick *et al.* 2016) is available.

Irrespective of the receptors involved, NST has been shown to regulate metabolic function at the central (i.e. acting as a neuropeptide in the cerebellum and hypothalamus) and the peripheral levels (i.e. heart, pancreas, gastrointestinal tract, etc.), including regulation of neuronal migration, hypothalamic neuron firing, modulation of food/water intake and blood pressure (Samson *et al.* 2008, Hua *et al.* 2009, Dun *et al.* 2010, Carlini *et al.* 2011, Yosten *et al.* 2011, Su *et al.* 2012, Salvatori *et al.* 2014). In fact, some of the actions of NST are common to SST (Samson *et al.* 2008, Hua *et al.* 2009). In spite of the genetic and putative functional link between NST and SST, the actions of NST on pituitary function, also known as the 'master gland' of the body, has not been explored in detail despite the fact that SST was originally identified based on its ability to suppress the secretion of growth hormone (GH) and subsequent studies have shown that SST also suppresses the secretion of other pituitary hormones (Brazeau *et al.* 1973, Eigler & Ben-Shlomo 2014, Vazquez-Borrego *et al.* 2017). In addition, NST induces c-Fos expression in the pituitary (Samson *et al.* 2008) and is expressed in the arcuate nucleus of the hypothalamus which represents an important site for the control of the function of different pituitary cell types (Dun *et al.* 2010). Based on these associations, the current study sought to explore for the first time the direct effects of NST on pituitary function using primary pituitary cell cultures from baboons (*Papio anubis*), a species that closely models human physiology (McClure 1984, Comuzzie *et al.* 2003).

Specifically, in this study, we compared the direct effects of NST to SST on the pituitary hormone (GH, prolactin (PRL), follicle-stimulating hormone (FSH), luteinizing hormone (LH), adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH)) release and expression. In addition, to better

understand the mechanisms behind these actions, we used pharmacological inhibitors to various intracellular signaling pathways previously shown to be important in the actions of SST (adenylyl cyclase (AC), protein kinase A (PKA), phospholipase C (PLC), protein kinase C (PKC), extracellular Ca²⁺ L-type channels, intracellular Ca²⁺ channels, mitogen-activated protein kinases (MAPK), mammalian target of rapamycin (mTOR) and phosphatidylinositol-3-kinase (PI3K)). Finally, we evaluated the impact of NST and SST on the expression of key receptors and transcriptional factors involved in the normal functioning of the pituitary cell types.

Materials and methods

Reagents

All the material (reagents, peptides, inhibitors of signaling pathways, etc.) used in this study were purchased from Sigma-Aldrich unless otherwise specified. Human amidated NST-19 (Ala-Pro-Ser-Asp-Pro-Arg-Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-NH₂) and SST-14 (SST; Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys; with a disulfide bridge: Cys3–Cys14) were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). α -Minimum essential media, HEPES, horse serum and penicillin–streptomycin were obtained from Invitrogen. U73122 was purchased from Cayman Chemical.

Animals and tissue collection

Primate (olive baboon; *Papio anubis*; *n*=5, 8–10 years of age; 15.0–31.8 kg of weight) pituitaries and hypothalami were obtained from randomly cyclic control females from a breeding colony at the University of Illinois at Chicago within 15 min after sodium pentobarbital overdose as previously reported (Cordoba-Chacon *et al.* 2012a, Sarmiento-Cabral *et al.* 2017). All animals in which tissues were taken were covered by protocols approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Hypothalami were immediately frozen in liquid nitrogen and stored at –80°C until extraction for total RNA (see the 'RNA isolation, reverse-transcription and qrtPCR of baboon transcripts' section below). Pituitaries were placed in sterile cold (4°C) α -MEM (media supplemented with 0.15% BSA, 6 nM HEPES, 10 IU/mL penicillin and 10 μ g/mL streptomycin). Then, the *pars distalis* of the pituitary was isolated, washed twice in fresh media and cut into small pieces (~20–40 mg) with

surgical blades under sterile conditions. One fragment of the pituitary was rapidly frozen in liquid nitrogen and stored at -80°C until extraction for total RNA (see the 'RNA isolation, reverse-transcription and qrtPCR of baboon transcripts' section below), while the remaining pieces of the pituitary were transported to the laboratory in sterile cold α -MEM, minced into $1\text{--}2\text{ mm}^3$ pieces under sterile conditions and then, incubated in 30-mL S-MEM medium complemented with 0.3% trypsin (Becton, Dickinson and Company, Sparks, MD, USA) in a spinner flask (Bellco Glass, Vineland, NJ, USA) for 2 h at 37°C under gentle shaking to obtain dispersed single cells for culture (see the 'Primary pituitary cell cultures' section below), as previously reported (Luque *et al.* 2006, Kineman & Luque 2007). To avoid fibroblast contamination, suspensions of dispersed pituitary cells were filtered through a nylon gauze of $130\text{-}\mu\text{m}$ -mesh, and cultured in media with D-valine (replaced for L-valine) to selectively inhibit fibroblast proliferation/overgrowth. In addition, visual inspection of primary cell cultures at the time of experimental assays showed no sign of cells displaying the typical fibroblast-like morphology.

Primary pituitary cell cultures

Single cells (50,000–200,000 cells/well) were plated onto 48- or 24-well plates in media containing 10% fetal horse serum. After 36-h incubation (37°C), media were removed and cells pre-incubated for 1 h with fresh, warm, serum-free media alone to stabilize basal hormone secretion. After this pre-incubation period, cells were incubated with serum-free media containing the following treatments: (1) NST alone (0.01–10 nM; dose–response experiment; 4 h incubation); (2) NST alone (10 nM) for 30 min, 4, 12 and 24 h (time course experiment); (3) NST or SST alone (10 nM) for 4 h and 24 h (experiment for the comparison between the two somatostatin gene-encoded peptides); (4) NST or SST alone (10 nM) or in combination with acylated-ghrelin (10 nM) or gonadotropin-releasing hormone (GnRH, 10 nM) for 4 h.

In order to study and compare the intracellular signaling pathways involved in the NST- and SST-mediated actions on GH and LH release, serum-free media containing inhibitors of different intracellular signaling pathways (AC (MDL-12330A; $10\text{ }\mu\text{M}$), PKA (H89; $15\text{ }\mu\text{M}$), PLC (U73122; $50\text{ }\mu\text{M}$), PKC (Go6983; $20\text{ }\mu\text{M}$), extracellular Ca^{2+} L-type channels (nifedipine; $1\text{ }\mu\text{M}$), intracellular Ca^{2+} channels (thapsigargin; $10\text{ }\mu\text{M}$), MAPK (PD-98059; $10\text{ }\mu\text{M}$), mTOR (rapamycin; 10 nm) and PI3K (wortmannin; $1\text{ }\mu\text{M}$)) were incubated for 90 min after the 1-h pre-incubation

period with serum-free media, and then, the media were replaced with media with the specific inhibitor alone (vehicle) or media with the inhibitor containing NST or SST (10 nM) and incubated for an additional 4 h. Additional controls consisted of serum-free media alone or media with NST or SST (in all cases without inhibitors). Doses for acylated-ghrelin, SST, GnRH or inhibitors of intracellular signaling pathways were selected based on previous studies (Kineman & Luque 2007, Cordoba-Chacon *et al.* 2011, 2012b, Luque *et al.* 2011).

At the end of the corresponding incubation periods with the different treatments, media were collected for hormone analysis using commercial ELISAs (see the 'Hormone release analysis' section below) and, in selected cases, cells were processed for total RNA recovery and assessment of mRNA levels by quantitative real-time PCR (qPCR; see the 'RNA isolation, reverse-transcription and qrtPCR of baboon transcripts' section below).

Hormone release analysis

GH, PRL, ACTH, LH, FSH and TSH hormone concentrations in the culture media were measured using human commercial ELISAs (human: GH, PRL, ACTH, LH, FSH and TSH (reference numbers: EIA-1787, EIA-1291, EIA-3647, EIA-1289, EIA-1288 and EIA-1790, respectively; DRG, Mountainside, NJ, USA)). All the assays were performed following the manufacturer's instructions where the information regarding specificity, detectability and reproducibility for each of the assays can be accessed at the websites of the indicated company.

RNA isolation, reverse-transcription and qrtPCR of baboon transcripts

Tissues (pituitary and hypothalamus) and pituitary cell cultures from baboons were processed for recovery of total RNA and the subsequent quantification of the amount of RNA recovered using kits and methods previously described (Sarmiento-Cabral *et al.* 2017). Briefly, total RNA was reverse-transcribed in a $20\text{-}\mu\text{L}$ volume using random-hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hannover, MD, USA). cDNAs were amplified by qrtPCR using a Stratagene Mx3000p real-time PCR machine and the brilliant SYBR Green QPCR Master Mix (Stratagene). To estimate mRNA copy number, samples were run against specific synthetic standards ($1\text{--}10^6$ copies of synthetic cDNA template for each transcript of interest) run on the same plate. Details regarding the development, validation and application of a

qRT-PCR to measure expression levels of baboon transcripts, including cyclophilin-A (used as a housekeeping gene), have been reported previously (Luque *et al.* 2006, Kineman & Luque 2007). Specific sets of primer sequences used in this study have been previously reported (Luque *et al.* 2006, 2011, Cordoba-Chacon *et al.* 2012a). New baboon primer sequences were used in the present study to amplify baboon *sst3* (sense: CCAGCCCTTCAGTCACCA; antisense: CCGAAGGGCCAGTAAGACA; product size: 115bp; accession number: EU156181.1), *sst4* (sense: TCTTTGTGCTCTGCTGGATG; antisense: AACCATAGAGTACGGGGTTGG; product size: 138bp; accession number: EU156182.1) and *GPCR107* (sense: GCATCAGCAACAGATGGAAA; antisense: GGAACAGCGAGTTTGAGGAG; product size: 131bp; accession number: XM_003911188.4). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy numbers of the baboon transcripts analyzed were adjusted by cyclophilin-A expression, where baboon cyclophilin-A mRNA levels did not significantly vary between experimental groups (data not shown).

Statistical analysis

Samples from all groups within an experiment were processed at the same time. To normalize values within each treatment and minimize intragroup variations in the different experiments (i.e. different age of the tissue donor, stage of the estrus cycle and/or to the metabolic environment, etc.), the values obtained were compared with vehicle-treated control cultures (set at 100%), where this style of data presentation does not alter the relative differences between NST- or SST-treated and vehicle-treated groups. Results were obtained from at least three separate independent experiments carried out on different days and with different cell preparations (3–4 replicate culture wells/treatment/experiment).

Differences between groups were assessed by one-way ANOVA (or two-way ANOVA when the intracellular signaling pathways, with treatments with and without (controls) specific inhibitors, were studied) followed by a Newman–Keuls test for multiple comparisons. $P \leq 0.05$ was considered significant. All data are expressed as means $\pm$ s.e. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Silver Spring, MD, USA).

Results

Direct effect of NST on pituitary hormone release

Incubation of cultured baboon pituitary cells with increasing doses of NST for 4h revealed clear inhibitory effects on GH and LH release in a concentration-dependent manner (at doses equal to or above 10^{-9} M for GH and 10^{-7} M for LH; Fig. 1). Conversely, NST failed to alter basal PRL, ACTH, FSH or TSH release at all the doses tested (Fig. 1). Based on these results, the dose of NST that caused a maximal decrease of both GH and LH release, 10^{-7} M, was chosen to further analyze the action of the peptide on baboon pituitary function.

Interestingly, treatment with 10^{-7} M NST, for different incubation times (30min, 4, 12 and 24h), revealed an inhibitory effect on both GH and LH, but not PRL, ACTH, FSH or TSH, release between 4h and 12h, whereas hormone release was not impacted after short-term (30min) or long-term (24h) incubation (Fig. 2).

Comparison between the effects of NST and SST on pituitary hormone expression and release

In a separate experiment, we confirmed that NST treatment (10^{-7} M) was able to significantly reduce GH and LH secretion at 4h, but not at 24h (Fig. 3A), without impacting the release of other pituitary hormones or

Figure 1

Direct effect of neuronostatin (NST) on baboon pituitary hormone secretion. Dose response (4h) of NST (dose between 10^{-7} M and 10^{-11} M; white columns) on the secretion of baboon GH, PRL, ACTH, FSH, LH and TSH. Data are expressed as percent of control (set at 100%; black columns) and represent the mean $\pm$ s.e.m. ($n=4$ individual experiments, 3–4 wells/experiment). Values that do not share a common letter (a, b, c) are statistically different.

Figure 2

Time-dependent effect of neuronostatin (NST; 10^{-7} M; 30 min, 4, 12, and 24 h; white columns) on the secretion of baboon GH, PRL, ACTH, FSH, LH and FSH. Data are expressed as percent of control (set at 100% at 30 min of incubation; black columns) and represent the mean $\pm$ s.e.m. ($n=4$ individual experiments, 3–4 wells/experiment). Values that do not share a common letter (a, b and c) are statistically different in the vehicle-treated controls at different times. Asterisks indicate values of the NST-treated groups at different times of incubation that significantly differ from their respective vehicle-treated control values (* $P<0.05$).

altering gene expression (GH, PRL, pro-opiomelanocortin (POMC, the precursor of ACTH), FSH, LH or TSH) (Fig. 3B). In this set of studies, we compared these actions to SST at the same dose (10^{-7} M) and found that SST was able to significantly reduce GH, PRL, ACTH and LH secretion

at both 4 h and 24 h, as well as TSH release at 4 h, but not at 24 h, of incubation (Fig. 3A). Interestingly, the inhibitory effect of SST on basal GH, but not LH, release was significantly higher than that exerted by NST at 4 h (Fig. 3A). Moreover, SST treatment was also able to

Figure 3

Comparison between direct effect of 4-h and 24-h treatment of neuronostatin (NST; 10^{-7} M; white columns) and somatostatin (SST; 10^{-7} M; grey columns) on the secretion of baboon GH, PRL, ACTH, FSH, LH and FSH (A) and, on the expression of GH, PRL, POMC, FSH, LH, FSH and pituitary transcription factor-1 (Pit-1) (B) in primary pituitary cell cultures from baboons. Values are expressed as percentage of controls (4 h or 24 h, set at 100% within each experiment; black columns), and represent the mean $\pm$ s.e.m. of four independent experiments (three to four wells/experiment). Values that do not share a common letter (a, b, c, d) differ significantly ($P<0.05$).

significantly reduce GH, PRL, POMC and LH, but not FSH or TSH, expression at 24 h of incubation (Fig. 3B).

It should be mentioned that the inhibitory effect of NST or SST on somatotrope function (or on lactotrope function by SST) does not seem to be mediated by a reduction in pituitary transcription factor-1 (Pit-1) mRNA production (a critical factor for normal development and function of somatotrope and lactotrope cells (Andersen & Rosenfeld 2001); Fig. 3B).

Interaction of NST or SST with key regulators of GH and LH secretion: ghrelin and GnRH in a non-human primate model

We next tested the direct effects of 4 h of incubation with NST or SST alone or in combination with a primary stimulatory factor of somatotrope (i.e. ghrelin (Kineman & Luque 2007)) or gonadotrope (i.e. GnRH (Luque *et al.* 2011)) function (Fig. 4). Specifically, we found that treatments with NST or SST alone inhibited GH and LH release, whereas ghrelin or GnRH alone stimulated

GH or LH release in primary pituitary cell cultures, respectively (Fig. 4). The stimulatory effects of ghrelin on GH and of GnRH on LH secretion were fully blocked by the co-administration with SST, but not with NST (Fig. 4). Interestingly, we found that NST augmented the inhibitory action of SST on ghrelin-stimulated GH release (Fig. 4; left panel), while it did not impact the actions of SST on GnRH-stimulated LH release (Fig. 4; right panel).

Intracellular signaling pathways involved in NST- and SST-mediated GH and LH release in a non-human primate model

The use of pharmacological inhibitors indicates that NST and SST inhibit GH and LH release through almost identical signaling pathways (Fig. 5A and B, respectively). Given the limited source of baboon cell preparations, we were able to study only some selected signaling routes. Specifically, administration of these inhibitors alone did not modify basal GH or LH release (Fig. 5A or B, respectively; control-columns). Our results indicate that the inhibitory effect of NST and SST on GH and LH release is mediated through AC/PKA, MAPK and extra-/intracellular Ca^{2+} influx because incubation with specific blockers of these routes, but not with PLC/PKC or mTOR inhibitors, completely blocked the inhibitory effect of NST on GH and LH secretion (Fig. 5A or B, respectively; NST and SST columns). Interestingly, blockade of PI3K activity completely abolished the inhibitory effect of SST, but not of NST, on GH secretion (Fig. 5A).

Presence of receptors for NST and SST in the pituitary, and homologous and heterologous regulation of these receptors

GPCR107 was highly expressed in baboon pituitaries (Fig. 6A), and this expression was higher than that found in the hypothalamus (where NST has been previously shown to be highly produced and to exert relevant functions (Samson *et al.* 2008)). Interestingly, we also found that the expression level of GPCR107 in pituitaries from female baboons was remarkably higher than that of the various sst-subtypes (sst1–5; Table 1). The relevant expression levels of the sst-subtypes shown in this study are consistent with previous reports in baboon and human (sst5 > sst2 > sst1 > sst3 > sst4; (Neto *et al.* 2009, Cordoba-Chacon *et al.* 2012b)) (Table 1).

To confirm that primary pituitary cells of baboons maintain a differentiated phenotype after dispersion and culture, absolute mRNA levels (copy numbers per 0.05 μg total RNA) of NST and SST receptors and cyclophilin-A were compared between whole-tissue extracts (*in vivo*

Figure 4

Interaction of neuronostatin (NST; 10^{-7} M) and/or somatostatin (SST; 10^{-7} M) and the main stimulatory regulator of somatotrope and gonadotrope function (ghrelin and gonadotrophin-releasing hormone (GnRH), respectively) in primary pituitary cell cultures from baboons. Effect of 4-h treatment of NST or SST and/or ghrelin (10 nM) on GH secretion and, of NST or SST and/or GnRH (10 nM) on LH secretion. Values are expressed as percentage of controls, set at 100% within each experiment, and represent the mean $\pm$ S.E.M. of three independent experiments (three to four wells/experiment). Values that do not share a common letter (a, b, c, d) differ significantly ($P < 0.05$).

Figure 5

Intracellular signaling pathways of neuronostatin (NST)- and somatostatin (SST)-inhibited baboon GH and LH secretion. Effect of the inhibition of AC (MDL-12330A; 10 μ M), protein kinase A (H89; 15 μ M), PLC (U73122; 50 μ M), protein kinase C (Go6983; 20 μ M), extracellular Ca²⁺ L-type channels (nifedipine; 1 μ M), intracellular Ca²⁺ channels (thapsigargin; 10 μ M), MAPK (PD-98059; 10 μ M), mTOR (rapamycin; 10 nM) and PI3K (wortmannin; 1 μ M) on NST- and SST-inhibited GH (A) and LH (B) secretion. On the day of the experiment, inhibitors were added to the incubation media 90 min before NST or SST treatment (4 h; 10⁻⁷ M). Values are expressed as percentage of vehicle-treated controls without inhibitor (set at 100%) within each experiment, and represent the mean $\pm$ S.E.M. of four independent experiments (three to four wells/treatment per experiment). Values that do not share a common letter (a, b, c) significantly differ ($P < 0.05$).

conditions; Table 1) and extracts prepared from pituitary cell cultures (*in vitro* conditions; 4 h after incubation in serum-free medium). Results indicated that transcript levels did not vary significantly between *in vivo* and *in vitro* samples (data not shown), indicating that the cell preparation and culture conditions did not impact the expression of pituitary receptors for NST or SST, which

support that the culture system used allows for the maintenance of correct functions of the different pituitary cell types.

As previously reported (Cordoba-Chacon *et al.* 2012a), we observed that 4 h of incubation with SST (10⁻⁷ M) markedly increased the expression of sst1, sst2 and sst5 in baboon pituitary cultures (Fig. 6B; left panel). Moreover,

Figure 6

Presence and regulation of the expression of key pituitary receptors for the regulation of somatotrope and gonadotrope function. (A) Presence of GPCR107 (absolute mRNA copy numbers) in the pituitary (PIT; white column) and hypothalamus (HPT; black column) of baboons, as determined by quantitative rtRT-PCR. (B) Effects of neuronostatin (NST; 10⁻⁷ M; white columns) and somatostatin (SST; 10⁻⁷ M; grey columns) treatment on the expression of pituitary receptors for SST (sst1–sst5), NST (GPCR107), GHRH (GHRH-R), ghrelin (GHS-R) and Kiss1 (Kiss1-R) in primary pituitary cell cultures from baboons after 4 h of treatment. Data are expressed as absolute mRNA copy numbers per 0.05 μ g total RNA in the whole pituitary of female baboons ((A); $n = 5$), or as percentage of vehicle-treated controls ((B); set at 100%; black columns) in primary pituitary cell cultures from baboons and represent the mean $\pm$ S.E.M. ($n = 4$ individual experiments; 3–4 wells/experiment). Asterisks indicate values that significantly differ from their respective control values (** $P \leq 0.01$).

Table 1 Presence of somatostatin receptor subtypes, as well as GPCR107, in the baboon pituitary.

	Pituitary
sst1	1424±256
sst2	4373±301
sst3	1318±343
sst4	56±12
sst5	6045±589
GPCR107	21,260±2753
Cyclophilin-A	125,936±9842

Absolute mRNA copy number per 0.05 µg total RNA of gene transcripts in the whole pituitary of female baboons, as determined by quantitative rtRT-PCR. Values represent means±S.E.M. (from 5 separate whole pituitary extracts).

this is the first report showing that the expression of sst3 was also elevated in response to SST treatment in primary pituitary cell cultures from baboons (Fig. 6B, left panel). In contrast, SST did not alter the expression of GPCR107, GH-releasing hormone receptor (GHRH-R), ghrelin receptor (GHS-R) or kisspeptin-1 receptor (Kiss1-R) (Fig. 6B, left/right panels). Interestingly, NST treatment (10⁻⁷M; 4h of incubation) increased the expression of its own putative receptor (GPCR107; Fig. 6B, left panel), but decreased GHS-R and Kiss1-R expression, without impacting GHRH-R expression (Fig. 6B, right panel).

Discussion

NST, a recently discovered amidated peptide encoded by the somatostatin gene, has been shown to exert diverse activities at the central and peripheral level (Samson *et al.* 2008, Hua *et al.* 2009, Carlini *et al.* 2011, Yosten *et al.* 2011, Su *et al.* 2012, Salvatori *et al.* 2014). However, information about the direct role that NST exerts at the level of the pituitary gland, a true sensor of whole body function integrating central and peripheral signals and often referred to as the 'master endocrine gland', is quite limited. Specifically, to the best of our knowledge, the effect of NST at the pituitary level has been only derived from a single series of studies showing that treatment with NST increased c-fos immunoreactivity in rat pituitaries, but NST did not impact GH, PRL, ACTH or LH release from primary rat pituitary cell cultures after a short period of incubation (30 min) (Samson *et al.* 2008). Therefore, the question remains: can NST directly regulate pituitary function (basal and stimulated hormone release and hormone gene expression) if applied for longer periods and at different doses? To address this question, in the current study, we examined whether NST could directly

impact hormone release/expression in primary pituitary cell cultures from normal baboons (*Papio anubis*), a primate model that closely models human physiology and is therefore of interest for translational medicine (McClure 1984, Comuzzie *et al.* 2003).

Our current findings are the first to show that NST inhibits somatotrope and gonadotrope function. Specifically, we showed that NST acts selectively to suppress basal GH and LH release, without altering GH and LH gene expression, whereas these selected effects differ from the broad inhibitory effects of SST on basal and stimulated pituitary hormone release and synthesis, as shown by our current studies and as previously reported (Weeke *et al.* 1975, Wildt *et al.* 1981, Cordoba-Chacon *et al.* 2011, 2012b, Luque *et al.* 2014, Sarmiento-Cabral *et al.* 2017). It should be noted that although some reports (Yu *et al.* 1997, Milosevic *et al.* 2004), including the current study, show that SST has no effect on FSH release and synthesis, other reports have shown that SST is able to inhibit FSH (Evans 1999). In this sense, although LH and FSH are known to be co-stored in a subpopulation of bihormonal gonadotropes and are often co-regulated and released in response to different factors (Evans 1999), previous studies in non-human primate models have demonstrated that FSH secretion can follow a markedly different pattern from that of LH (Wildt *et al.* 1981), as it has been observed in the current study.

To the best of our knowledge, this is the first report studying, in a cell culture system, the direct interaction between SST and NST, and also the interaction of NST and/or SST with other primary regulators of pituitary function. We found that SST, but not NST, blocked ghrelin-stimulated GH release and GnRH-stimulated LH release, which is consistent with previous studies reported with SST (Kineman & Luque 2007, Cordoba-Chacon *et al.* 2011, 2012b, Luque *et al.* 2011), and with the only report published to date indicating that neither ghrelin-stimulated GH release nor GnRH-stimulated LH release was affected by NST treatment in rats cultured anterior pituitary cells (Samson *et al.* 2008). Interestingly, NST was able to augment the inhibitory effect of SST on ghrelin-stimulated GH, but GnRH-stimulated LH release. Therefore, our data are the first to provide evidence that NST might act together with SST to fine-tune somatotrope function. These results are novel and, although the mechanisms and physiologic relevance behind these actions remain unknown, set the stage for future investigations.

Our report also demonstrates for the first time that the actions of NST are mediated by AC/PKA, MAPK and

extra-/intracellular Ca^{2+} mobilization, a complex set of second messenger pathways which almost parallels that found to be mediated by SST. We also confirm previous reports (Cordoba-Chacon *et al.* 2012b), that activation of AC and MAPK pathways is required by SST to exert its action on GH release. Interestingly, we also found that the inhibitory actions of SST, but not NST, on GH secretion also require PI3K signaling which might suggest that activation of this pathway by SST could be important in mediating some of the unique actions associated to SST (not shared with NST).

Taken together, these results strongly indicate that the mechanism of action of NST clearly differs from SST at the pituitary. This differential effect may be related to the fact that SST is a cyclic, non-amidated peptide, and NST is a non-cyclic, amidated peptide. In fact, the presence or absence of amidation enzymes or degrading enzymes could dictate the expression levels and activity of NST and SST in a tissue-specific fashion (Samson *et al.* 2008). Indeed, the amidation process of NST seems to be essential to exert some of its physiological actions, including its influence in gastric emptying and gastrointestinal transit, hypertension activity, antinociceptive effect and depressive behavior (Yang *et al.* 2011a,b, Yosten *et al.* 2011, Su *et al.* 2012). In addition, differential effects of NST vs SST on pituitary function are likely mediated by different receptors. Specifically, SST exerts its inhibitory effects on pituitary function via somatostatin receptor subtypes sst1–5 (Samson *et al.* 2008, Ben-Shlomo & Melmed 2010, Vazquez-Borrego *et al.* 2017). However, it was previously reported that NST failed to activate sst1–5 in an *in vitro* experimental system (Samson *et al.* 2008), but could interact and be functionally connected with the orphan receptor GPCR107 (Yosten *et al.* 2012, Elrick *et al.* 2016). Importantly, we found that GPCR107 is highly expressed in the baboon pituitary (mRNA levels greater than those for all sst-subtypes) and pituitary GPCR107 expression (but not sst1–5 expression) is increased by NST treatment, where ligand-mediated upregulation of canonical receptors has been observed for other pituitary regulatory peptides (i.e. SST/sst1–5 (Luque *et al.* 2004a, Cordoba-Chacon *et al.* 2012a), and as occurs in the present study; GHRH/GHRH-R and ghrelin/GHS-R (Luque *et al.* 2004b, Cordoba-Chacon *et al.* 2012a). In fact, upregulation of GPCR107 in response to NST treatment was also observed in pancreatic cell cultures (Elrick *et al.* 2016). Noteworthy is the observation that NST, but not SST, exerted a heterologous regulation on the expression of key receptors for somatotrope (GHS-R) and gonadotrope (Kiss1-R) function. These results may

be (patho)physiologically relevant in humans in that we have previously shown that the hormonal/clinical control of patients with different endocrine pathologies (i.e. pituitary adenomas, neuroendocrine and thyroid tumors, etc.) that are treated with SST analogues is directly associated with the amount of and ratio between the expression of pituitary sst-subtypes (Taboada *et al.* 2007, Puig-Domingo *et al.* 2014, Luque *et al.* 2015, Sampedro-Nunez *et al.* 2016). Most importantly, this report demonstrated for the first time that NST inhibited basal GH and LH release and that NST augmented the inhibitory action of SST on basal GH release which provide a potentially useful tool to develop new therapeutic targets to treat patients with pituitary tumors (i.e. combination of SSAs with NST or NST-analogues). In any case, our current data further extend those original observations, suggesting that the physiological actions of NST might include a pituitary site of action as well. In fact, based on the results of the present study as well as on previous results demonstrating that the actions of multiple peptides acting at the pituitary level are similar when tested *in vivo* and *in vitro* (Brazeau *et al.* 1973, Cordoba-Chacon *et al.* 2011, Kineman & Luque 2007, Luque *et al.* 2014, Vazquez-Borrego *et al.* 2017), it is tempting to speculate that NST would exert its pituitary actions on selected pituitary cell types through different, at least partially dissimilar, mechanisms and/or signaling pathways than those involved in the effects of SST.

Summary

This study supplies the first evidence that NST can directly control pituitary function. The actions of NST are distinct from its older sibling peptide, SST, in that the inhibitory actions of NST are isolated to inhibition of basal GH and LH release, whereas SST has a more broad impact on basal and stimulated pituitary hormone release and gene expression. These actions of NST may be exerted via GPCR107, based on the previous reports (Yosten *et al.* 2012, Elrick *et al.* 2016), as well as on the current finding that GPCR107 is highly expressed in the primate pituitary and is regulated by NST. Although NST and SST may signal through different receptors to suppress GH and LH release, the downstream intracellular signals required for these actions may be in large part mediated via AC/PKA, MAPK and extra-/intracellular Ca^{2+} mobilization. Given these effects on NST were observed in a primate model, that could be readily translated to human patho(physiology), these results lay a solid foundation to pursue question related to the importance of NST on

somatotrope and gonadotrope development and adult function and if alterations in NST production or signaling play a significant role in control of hormone release from pituitary adenomas.

Declaration of interest

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

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References

- Andersen B & Rosenfeld MG 2001 POU domain factors in the neuroendocrine system: lessons from developmental biology provide insights into human disease. *Endocrine Reviews* **22** 2–35. (<https://doi.org/10.1210/edrv.22.1.0421>)
- Ben-Shlomo A & Melmed S 2010 Pituitary somatostatin receptor signaling. *Trends in Endocrinology and Metabolism* **21** 123–133. (<https://doi.org/10.1016/j.tem.2009.12.003>)
- Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J & Guillemin R 1973 Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179** 77–79.
- Carlini VP, Ghersi M, Gabach L, Schioth HB, Perez MF, Ramirez OA, Fiol de Cuneo M & de Barioglio SR 2011 Hippocampal effects of neuronostatin on memory, anxiety-like behavior and food intake in rats. *Neuroscience* **197** 145–152. (<https://doi.org/10.1016/j.neuroscience.2011.09.040>)
- Comuzzie AG, Cole SA, Martin L, Carey KD, Mahaney MC, Blangero J & VandeBerg JL 2003 The baboon as a nonhuman primate model for the study of the genetics of obesity. *Obesity Research* **11** 75–80. (<https://doi.org/10.1038/oby.2003.12>)
- Cordoba-Chacon J, Gahete MD, Pozo-Salas AI, Martinez-Fuentes AJ, de Lecea L, Gracia-Navarro F, Kineman RD, Castano JP & Luque RM 2011 Cortistatin is not a somatostatin analogue but stimulates prolactin release and inhibits GH and ACTH in a gender-dependent fashion: potential role of ghrelin. *Endocrinology* **152** 4800–4812. (<https://doi.org/10.1210/en.2011-1542>)
- Cordoba-Chacon J, Gahete MD, Castano JP, Kineman RD & Luque RM 2012a Homologous and heterologous in vitro regulation of pituitary receptors for somatostatin, growth hormone (GH)-releasing hormone, and ghrelin in a nonhuman primate (Papio anubis). *Endocrinology* **153** 264–272. (<https://doi.org/10.1210/en.2011-1677>)
- Cordoba-Chacon J, Gahete MD, Culler MD, Castano JP, Kineman RD & Luque RM 2012b Somatostatin dramatically stimulates growth hormone release from primate somatotrophs acting at low doses via somatostatin receptor 5 and cyclic AMP. *Journal of Neuroendocrinology* **24** 453–463. (<https://doi.org/10.1111/j.1365-2826.2011.02261.x>)
- Dun SL, Brailoiu GC, Tica AA, Yang J, Chang JK, Brailoiu E & Dun NJ 2010 Neuronostatin is co-expressed with somatostatin and mobilizes calcium in cultured rat hypothalamic neurons. *Neuroscience* **166** 455–463. (<https://doi.org/10.1016/j.neuroscience.2009.12.059>)
- Eigler T & Ben-Shlomo A 2014 Somatostatin system: molecular mechanisms regulating anterior pituitary hormones. *Journal of Molecular Endocrinology* **53** R1–R19. (<https://doi.org/10.1530/JME-14-0034>)
- Erick MM, Samson WK, Corbett JA, Salvatori AS, Stein LM, Kolar GR, Naatz A & Yosten GLC 2016 Neuronostatin acts via GPR107 to increase cAMP-independent PKA phosphorylation and proglucagon mRNA accumulation in pancreatic alpha-cells. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **310** R143–R155. (<https://doi.org/10.1152/ajpregu.00369.2014>)
- Evans JJ 1999 Modulation of gonadotropin levels by peptides acting at the anterior pituitary gland. *Endocrine Reviews* **20** 46–67. (<https://doi.org/10.1210/edrv.20.1.0355>)
- Hua Y, Ma H, Samson WK & Ren J 2009 Neuronostatin inhibits cardiac contractile function via a protein kinase A- and JNK-dependent mechanism in murine hearts. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **297** R682–R689. (<https://doi.org/10.1152/ajpregu.00196.2009>)
- Kineman RD & Luque RM 2007 Evidence that ghrelin is as potent as growth hormone (GH)-releasing hormone (GHRH) in releasing GH from primary pituitary cell cultures of a nonhuman primate (Papio anubis), acting through intracellular signaling pathways distinct from GHRH. *Endocrinology* **148** 4440–4449. (<https://doi.org/10.1210/en.2007-0441>)
- Luque RM, Park S, Peng X-D, Delgado E, Gracia-Navarro F, Kineman RD, Malagon MM & Castano JP 2004a Homologous and heterologous in vitro regulation of pig pituitary somatostatin receptor subtypes, sst1, sst2 and sst5 mRNA. *Journal of Molecular Endocrinology* **32** 437–448. (<https://doi.org/10.1677/jme.0.0320437>)
- Luque RM, Kineman RD, Park S, Peng X-D, Gracia-Navarro F, Castano JP & Malagon MM 2004b Homologous and heterologous regulation of pituitary receptors for ghrelin and growth hormone-releasing hormone. *Endocrinology* **145** 3182–3189. (<https://doi.org/10.1210/en.2003-1626>)
- Luque RM, Gahete MD, Valentine RJ & Kineman RD 2006 Examination of the direct effects of metabolic factors on somatotrope function in a non-human primate model, Papio anubis. *Journal of Molecular Endocrinology* **37** 25–38. (<https://doi.org/10.1677/jme.1.02042>)
- Luque RM, Cordoba-Chacon J, Gahete MD, Navarro VM, Tena-Sempere M, Kineman RD & Castano JP 2011 Kisspeptin regulates gonadotroph and somatotroph function in nonhuman primate pituitary via common and distinct signaling mechanisms. *Endocrinology* **152** 957–966. (<https://doi.org/10.1210/en.2010-1142>)
- Luque RM, Cordoba-Chacon J, Ibanez-Costa A, Gesmundo I, Grande C, Gracia-Navarro F, Tena-Sempere M, Ghigo E, Gahete MD, Granata R, et al. 2014 Obestatin plays an opposite role in the regulation of pituitary somatotrope and corticotrope function in female primates and male/female mice. *Endocrinology* **155** 1407–1417. (<https://doi.org/10.1210/en.2013-1728>)
- Luque RM, Ibanez-Costa A, Neto LV, Taboada GF, Hormaechea-Agulla D, Kasuki L, Venegas-Moreno E, Moreno-Carazo A, Galvez MA, Soto-Moreno A, et al. 2015 Truncated somatostatin receptor variant sst5TMD4 confers aggressive features (proliferation, invasion and reduced octreotide response) to somatotropinomas. *Cancer Letters* **359** 299–306. (<https://doi.org/10.1016/j.canlet.2015.01.037>)

- McClure HM 1984 Nonhuman primate models for human disease. *Advances in Veterinary Science and Comparative Medicine* **28** 267–304. (<https://doi.org/10.1016/B978-0-12-039228-5.50014-0>)
- Milosevic V, Nestorovic N, Filipovic B, Velkovski S & Startcevic V 2004 Centrally applied somatostatin influences morphology of pituitary FSH cells but not FSH release. *General Physiology and Biophysics* **23** 375–380. (<https://doi.org/10.3181/00379727-214-44073>)
- Neto LV, Machado E de O, Luque RM, Taboada GF, Marcondes JB, Chimelli LMC, Quintella LP, Niemeyer PJ, de Carvalho DP, Kineman RD, et al. 2009 Expression analysis of dopamine receptor subtypes in normal human pituitaries, nonfunctioning pituitary adenomas and somatotropinomas, and the association between dopamine and somatostatin receptors with clinical response to octreotide-LAR in acromegaly. *Journal of Clinical Endocrinology and Metabolism* **94** 1931–1937. (<https://doi.org/10.1210/jc.2008-1826>)
- Puig-Domingo M, Luque RM, Reverter JL, Lopez-Sanchez LM, Gahete MD, Culler MD, Diaz-Soto G, Lomena F, Squarcia M, Mate JL, et al. 2014 The truncated isoform of somatostatin receptor5 (sst5TMD4) is associated with poorly differentiated thyroid cancer. *PLoS ONE* **9** e85527. (<https://doi.org/10.1371/journal.pone.0085527>)
- Salvatori AS, Elrick MM, Samson WK, Corbett JA & Yosten GLC 2014 Neuronostatin inhibits glucose-stimulated insulin secretion via direct action on the pancreatic alpha-cell. *American Journal of Physiology: Endocrinology and Metabolism* **306** E1257–E1263. (<https://doi.org/10.1152/ajpendo.00599.2013>)
- Sampedro-Nunez M, Luque RM, Ramos-Levi AM, Gahete MD, Serrano-Somavilla A, Villa-Osaba A, Adrados M, Ibanez-Costa A, Martin-Perez E, Culler MD, et al. 2016 Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors. *Oncotarget* **7** 6593–6608. (<https://doi.org/10.18632/oncotarget.6565>)
- Samson WK, Zhang JV, Avsian-Kretchmer O, Cui K, Yosten GLC, Klein C, Lyu R-M, Wang YX, Chen XQ, Yang J, et al. 2008 Neuronostatin encoded by the somatostatin gene regulates neuronal, cardiovascular, and metabolic functions. *Journal of Biological Chemistry* **283** 31949–31959. (<https://doi.org/10.1074/jbc.M804784200>)
- Sarmiento-Cabral A, Peinado JR, Halliday LC, Malagon MM, Castano JP, Kineman RD & Luque RM 2017 Adipokines (leptin, adiponectin, resistin) differentially regulate all hormonal cell types in primary anterior pituitary cell cultures from two primate species. *Scientific Reports* **7** 43537. (<https://doi.org/10.1038/srep43537>)
- Su S-F, Yang A-M, Yang S-B, Wang N-B, Lu S-S, Wang H-H & Chen Q 2012 Intracerebroventricular administration of neuronostatin delays gastric emptying and gastrointestinal transit in mice. *Peptides* **35** 31–35. (<https://doi.org/10.1016/j.peptides.2012.03.013>)
- Taboada GF, Luque RM, Bastos W, Guimaraes RFC, Marcondes JB, Chimelli LMC, Fontes R, Mata PJP, Filho PN, Carvalho DP, et al. 2007 Quantitative analysis of somatostatin receptor subtype (SSTR1-5) gene expression levels in somatotropinomas and non-functioning pituitary adenomas. *European Journal of Endocrinology* **156** 65–74. (<https://doi.org/10.1530/eje.1.02313>)
- Vazquez-Borrego MC, Gahete MD, Martinez-Fuentes AJ, Fuentes-Fayos AC, Castano JP, Kineman RD & Luque RM 2017 Multiple signaling pathways convey central and peripheral signals to regulate pituitary function: lessons from human and non-human primate models. *Molecular and Cellular Endocrinology* **463** 4–22. (<https://doi.org/10.1016/j.mce.2017.12.007>)
- Weeke J, Hansen AP & Lundaek K 1975 Inhibition by somatostatin of basal levels of serum thyrotropin (TSH) in normal men. *Journal of Clinical Endocrinology and Metabolism* **41** 168–171. (<https://doi.org/10.1210/jcem-41-1-168>)
- Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE & Knobil E 1981 Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. *Endocrinology* **109** 376–385. (<https://doi.org/10.1210/endo-109-2-376>)
- Yang A, Ge W, Lu S, Yang S, Su S, Mi Z & Chen Q 2011a Central administration of neuronostatin induces antinociception in mice. *Peptides* **32** 1893–1901. (<https://doi.org/10.1016/j.peptides.2011.07.010>)
- Yang A, Ji Y, Su S, Yang S, Lu S, Mi Z, Yang Q & Chen Q 2011b Intracerebroventricular administration of neuronostatin induces depression-like effect in forced swim test of mice. *Peptides* **32** 1948–1952. (<https://doi.org/10.1016/j.peptides.2011.08.012>)
- Yosten GLC, Pate AT & Samson WK 2011 Neuronostatin acts in brain to biphasically increase mean arterial pressure through sympatho-activation followed by vasopressin secretion: the role of melanocortin receptors. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **300** R1194–R1199. (<https://doi.org/10.1152/ajpregu.00849.2010>)
- Yosten GLC, Redlinger LJ & Samson WK 2012 Evidence for an interaction of neuronostatin with the orphan G protein-coupled receptor, GPR107. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **303** R941–R949. (<https://doi.org/10.1152/ajpregu.00336.2012>)
- Yu WH, Kimura M & McCann SM 1997 Effect of somatostatin on the release of gonadotropins in male rats. *Proceedings of the Society for Experimental Biology and Medicine: Society for Experimental Biology and Medicine* **214** 83–86.

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