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ORIGINAL ARTICLE

Central Administration of Aminoprocalcitonin Inhibits Food Intake and Stimulates the Hypothalamic–Pituitary–Adrenal Axis in Rats via the Corticotrophin-Releasing Factor System

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Aminoprocalcitonin (N-PCT), a neuroendocrine peptide derived from procalcitonin, reduces food intake and body weight when administered centrally in rats. We have recently shown that N-PCT is expressed in brain areas known to be involved in energy homeostasis, including the paraventricular nucleus (PVN) of the hypothalamus, which contains a prominent population of corticotrophin-releasing factor (CRF)-synthesising neurones. CRF plays a pivotal role in the regulation of the hypothalamic-pituitary adrenal (HPA) axis and food intake. However, little is known about functional interactions of N-PCT and CRF. In the present study, we found endogenous N-PCT protein in the rat PVN. We also showed N-PCT immunoreactivity in PVN co-localised with NeuN, a neuronal marker, or glial fibrillary acidic protein, an astrocyte marker. Double staining immunohistochemistry revealed that N-PCT co-localised with CRF in parvocellular neurones of the PVN. Intracerebroventricular N-PCT administration increased CRF mRNA and content in the hypothalamus, suggesting that N-PCT stimulates the HPA axis and suppresses food intake and body weight via CRF-dependent pathways. In keeping with this, i.c.v. co-injection of D-Phe-CRF_{12-41}, a CRF receptor antagonist, significantly attenuated N-PCT-induced reduction in food intake and body weight in a dose-dependent manner. Furthermore, i.c.v. administration of N-PCT increased plasma adrenocorticotrophic hormone and corticosterone concentrations and induced the expression of Fos protein, a marker of neuronal activity, in parvocellular CRF neurones. These data collectively support the hypothesis that N-PCT inhibits food intake and body weight and stimulates the HPA axis via CRF-mediated pathways.

Key words: N-procalcitonin, CRF, hypothalamus, paraventricular nucleus, feeding.

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Aminoprocalcitonin (N-PCT), a 57-amino acid protein, was initially described as a neuroendocrine peptide derived from the amino-terminal half of rat prohormone procalcitonin with bone-cell mitogenic properties (1). It belongs to a group of related proteins including calcitonin, calcitonin gene-related peptides, amylin and adrenomedullin that are encoded on either the calcitonin (CALC)-I or CALC-II gene on chromosome 11 (2, 3). This chromosome is associated with body metabolism and obesity in humans (4, 5). The expression of these peptides is widespread, and several functions have been ascribed to these molecules, mediated via binding to calcitonin receptor family complexes, including hormone secretion regulation, growth modulation and energy homeostasis, amongst others (6). Furthermore, systemic bacterial infections induce an ubiquitous expression of CALC genes with a sustained release of calcitonin peptides, namely N-PCT, procalcitonin, calcitonin-gene related peptide and adrenomedullin (7, 8). Recent *in vitro* (9) and *in vivo* investigations suggest that N-PCT, similar to other peptides of the calcitonin gene family, has bioactivity at calcitonin receptors (CT-Rs) and plays a role in the central nervous system (CNS) regulating energy homeostasis in normal metabolic conditions and systemic inflammatory responses (10, 11).

We have proposed a role for N-PCT in energy homeostasis and neuroendocrine regulation because N-PCT-immunoreactive fibres and/or cell bodies are detected in several brain regions of primary importance in the regulation of feeding and energy balance. N-PCT-like immunoreactivity (IR) is observed in tanycytes, a type of specia-

lised glial cell that interacts in neuroendocrine functional dynamics, located in zones at the interface between brain and cerebrospinal fluid, such as the ependymal layer and ventral glia limitans (12). N-PCT-immunoreactive cells are also present in several hypothalamic nuclei, including the arcuate (ARC), paraventricular (PVN), preoptic, supraoptic, ventromedial (VMN) and dorsomedial (DMN) nuclei and lateral hypothalamic area (10, 12, 13). Furthermore, the hypothalamic expression of N-PCT is reduced, typically for an anorectic peptide, in states of negative energy balance (fasting and sepsis) in rats (10, 14). Intracerebroventricular administration of N-PCT significantly reduces both spontaneous nocturnal feeding and body weight gain in rats fed *ad lib.* (10, 13). In addition, N-PCT does not appear to affect feeding via nonspecific or nonhomeostatic actions because it does not support conditioned taste aversion or disrupt the behavioural satiety sequence (10). However, the mechanisms of action of N-PCT on appetite are unknown.

The regulation of energy balance is a complex process involving the interplay of neuronal circuitries controlling food intake and energy expenditure with endocrine secretions modulating the activity of the neurones making up those circuitries. Besides the classical role of CRF in hypothalamic-pituitary-adrenal (HPA) axis control, CRF is an important anorectic neuropeptide (15) and plays a critical role in these neuronal circuitries (16–18). Neurones from the dorsal part of the medial parvocellular PVN in the hypothalamus project to the median eminence and are involved in the control of pituitary function, whereas neurones in the ventral and the posterior parvocellular PVN are concerned with autonomic function, projecting to the brainstem and spinal cord (19).

There is some evidence that the actions of N-PCT in the brain may involve CRF-synthesising neurones located in the PVN of the hypothalamus. Similar to CRF, the central administration of N-PCT causes inhibition of feeding and body weight gain, and hyperthermia (10). Previous studies also demonstrated that i.c.v. administration of N-PCT increased the expression of Fos (a measure of neuronal activation) in the PVN of the hypothalamus (10, 13). Furthermore, N-PCT (10, 13) and CT-Rs are expressed in the PVN (20, 21), and activation of these receptors induces anorexia in rats (22, 23). These results suggest that i.c.v. administration of N-PCT may excite CRF-containing neurones in the CNS, in particular the parvocellular neurones in the PVN. Taken together, these data indicate that N-PCT may signal in the hypothalamus through the modulation of neuronal CRF activity, leading to energy homeostasis control. The present study therefore aimed to determine whether the anorectic effect of N-PCT in rats could be mediated via the CRF/CRF receptor neuronal system.

Although N-PCT is expressed in the PVN, its distribution and cellular localisation in this hypothalamic nucleus have not been fully explored. Therefore, in the present study, we attempted to characterise cell types expressing N-PCT in the PVN and the role of PVN neurones of the rat hypothalamus in the anorectic effect of N-PCT by combining biochemical, molecular, immunohistochemical and pharmacological approaches. Because PVN CRF neurones play a pivotal role in the regulation of the HPA axis and food intake, we hypothesised that N-PCT participates in regulating HPA axis activity and affects food intake via CRF/CRF-receptor-mediated pathways. In support of our hypothesis, we found that: (i) N-PCT is present in the PVN of the hypothalamus; (ii) N-PCT is expressed in astrocytes and CRF neurones located in the parvocellular subdivisions of PVN; (iii) N-PCT affects food intake and body weight via CRF-receptor mediated pathways; (iv) i.c.v. administration of N-PCT significantly increased hypothalamic expression of CRF mRNA, hypothalamic CRF content, blood adrenocorticotrophic hormone (ACTH) concentrations and the adrenocorticosterone response to the circulating ACTH, an effect that is dependent on CRF receptor activation; and (v) i.c.v. injection of N-PCT significantly increased Fos expression, a functional marker of neuronal activity (24), in PVN neuronal cell bodies containing CRF.

Materials and methods

Materials

Synthetic human N-PCT ([1]APFRSAL ESSPADP ATLSEDE ARLLLAA LVQDYVQ MKASELE QEQEREG SLDSPRS[57]) and D-Phe-CRF_{12-41} [D-Phe12, Nle21.38-CRF (12–41)] were purchased from Bachem (Bubendorf, Switzerland) and were dissolved in rat artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA, USA). This purified human N-PCT was used because the sequence of human N-PCT is highly conserved in rodents (25) and, in addition, previous studies have used this form of N-PCT (10, 11, 13), therefore enabling us to compare our results with previous work. The same batches and solutions were used for all experiments and contained an endotoxin level < 0.1 ng/ml of protein (i.e. below the detection limit of endotoxin assay), as determined by the limulus lysate assay (ICN Biomedicals, Costa Mesa, CA, USA). Fresh aliquots were thawed before each experiment, briefly sonicated and warmed to 37 °C just before use. All solutions were passed through 0.22 μm pore-size Millipore filters and stored at −80 °C.

For *in vitro* studies, we used antiserum against N-PCT raised from mouse (clone 44d9, NB120-14817; Novus Biologicals, Littleton, CO, USA) and tested previously in rats (10, 11, 13). This monoclonal antibody has been shown to stain a western blot band corresponding to N-PCT (manufacturer's technical information). Other rabbit polyclonal antibodies used were: anti-neuronal nuclei (NeuN; Chemicon International Inc., Temecula, CA, USA), anti-glial fibrillary acidic protein (GFAP; Dakocytomation, Carpinteria, CA, USA), anti-CRF and c-Fos antibodies (Peninsula Laboratories, Belmont, CA, USA) and anti-cFos (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animals

Male Wistar rats (Harlan Laboratories, Barcelona, Spain) weighing 250–300 g were used in all the experiments. Before surgery, animals were housed in separate cages (n = 3 per cage) under a 12 : 12 h light/dark cycle (lights on 08.00 h) at a constant ambient temperature of 24 ± 1 °C. After surgery, rats were individually housed under a reversed 12 : 12 h light/dark cycle (lights off 08.00 h) and habituated to the experimental condition by training them for single housing and handling for at least 1 week before the experiments. Water and standard laboratory rat chow pellets (Harlan Teklad 2014; Harlan Laboratories, Barcelona, Spain) were provided *ad lib.* except where noted. All experiments were carried out during the dark phase, the normal (physiological) feeding time for rodents (26). All procedures were performed in accordance with institutional guidelines for animal care at the University of Seville.

Surgical and stereotaxic procedures

Rats anaesthetised with a ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride solution (5 mg/kg, i.p.; Sigma-Aldrich, Madrid, Spain) were

implanted stereotaxically with 22-gauge stainless steel guide cannulas aimed at the right lateral cerebral ventricle. Coordinates were 0.9 mm posterior to bregma, 1.5 mm lateral to medline and 3.5 mm ventral to dura, as described previously (10, 13). Cannulas were then cemented into place using dental cement anchored by skull screws. A 29-gauge obturator was inserted into the guide cannula to maintain patency. Prophylactic antibiotics (ceftriaxone, 25 mg/kg; Roche Laboratories, Madrid, Spain) and meloxicam (1 mg/kg, sc; Boehringer Ingelheim, San Cugat del Vallés, Spain) for analgesia postoperatively were administered at completion of surgery. The animals were allowed 1 week of recovery after surgery. They were then accustomed to handling on a daily basis. Only animals that showed progressive weight gain after the surgery were used in subsequent experiments. Proper placement of the cannulas was verified at the end of experiments by dye administration. Animals that did not have proper cannula placement were eliminated from the study.

Intracerebroventricular injections

After a 7-day recovery period, animals were sham-injected before the study, then weighed, and handled daily. Rats were adapted to the injection procedure by removing the dummy cannula and connecting the injection device to the i.c.v. guide cannula every 24 h for 3 day. On the last day of the adaptation (day of the experiment), the dummy cannula was removed and replaced with a 28-gauge stainless injector cannula connected to polyethylene tubing attached to a 25-μl Hamilton microsyringe. All drugs were given i.c.v. in lightly restrained, conscious rats. Each microinjection was completed over 60 s; the injector was held stationary for another 60 s and slowly removed over the following 60 s. The total volume injected was always made up to 5 μl with aCSF. After injections, rats were immediately returned to their own cages, and were left undisturbed in their home cages.

N-PCT administration

To determine the effects of N-PCT on food intake, Fos and CRF expression in the PVN, hypothalamic CRF content and plasma ACTH and corticosterone levels, separate groups of rats matched for body weights were injected through chronic i.c.v. cannulas as described above. Intracerebroventricular injections (5 μl) of either N-PCT (1 μg/μl) or vehicle (aCSF) were given at the onset of the active dark phase in *ad lib.* fed rats. The dose of N-PCT (5 μg/rat = 1 nmol) used in the present study is know to inhibit food intake and is based on previous dose–response studies performed after i.c.v. injection in *ad lib.*-fed rats (10, 13). Controls include heat-denatured N-PCT (1 μg/μl protein; 90 °C for 1 h) or the vehicle solution (artificial CSF with a protein content matched to that in the N-PCT preparation).

Experimental design

Study 1: Specificity of the N-PCT antibody in rat brain and characterisation of cell types expressing N-PCT in the PVN

Four male Wistar rats (290–300 g) of freely fed singly-housed rats adapted to the injection procedure were injected i.c.v. with aCSF vehicle (5 μl) at the onset of the dark phase (n = 4). Thirty minutes after injection, rats were anaesthetised by an i.p. injection of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4% formaldehyde. Brains were removed and conserved in the same fixative solution at 4 °C. The hypothalamus was cut into 30 μm serial sections and free-floating sections were collected in PBS.

The monoclonal anti-N-PCT antibody used in these studies was raised in mouse against human N-PCT (Novus Biologicals). Because the sequence of N-PCT is highly conserved in all mammalian species, including humans and rodents (25), we used this antiserum to establish specificity to stain rat neuronal tissue. The specificity of this antibody against N-PCT was histochemically demonstrated on rat brain sections by pre-absorption with its blocking peptide, which totally prevented the staining (Fig. 1). N-PCT antibody was diluted in 5% bovine serum albumin–PBS either alone or with 5 μg of corresponding blocking peptide and incubated with brain sections at 4 °C for 72 h. This solution was then used as the primary antibody in accordance with the immunohistochemistry protocol outlined below. The specificity of N-PCT antibody was also confirmed in hypothalamus homogenates by western blotting. Western blot analysis revealed a single approximately 8.5 kDa, consistent with the deduced molecular mass of the N-PCT protein. Taken together, our data supported the specificity of the anti-N-PCT antibody in the rat hypothalamus.

Immunohistochemistry

After quenching peroxidase activity with 0.3% H_2O_2 for 30 min, hypothalamic sections were blocked by 3% normal horse serum (Vector Laboratories, Burlingame, CA, USA)-PBS-0.25% Triton X-100 for 1 h at room temperature. Sections were then incubated in the presence of monoclonal anti-N-PCT antibody (1 : 200 diluted in the same blocking solution) for 72 h at 4 °C. After washing, sections were incubated with biotinylated horse anti-mouse immunoglobulin (Ig)G (dilution 1 : 200; Vector Laboratories) for 1 h at room temperature and finally incubated in peroxidase-conjugated streptavidin (dilution 1 : 500; Jackson Laboratories, West Grove, PA, USA) for 1 h at room temperature. Specificity of labelling was ascertained with omission of primary antibody or by using blocking peptide. Peroxidase activity was revealed using diaminobenzidine and nickel chloride method (DAB substrate kit for peroxidise; Vector Laboratories). Sections were mounted on slides, air-dried, coverslipped with permanent mounting media and examined under a transmitted-light microscope.

Multiple fluorescence immunohistochemistry

To determine the cell types expressing N-PCT in the PVN, hypothalamic sections were subjected to multiple immunohistochemistry for a neuronal (NeuN, Neuronal Nuclei) or glial marker (GFAP, glial fibrillary acidic protein), or CRF. After quenching autofluorescence with 0.3 M glycine for 20 min, sections were blocked in the presence of 3% normal donkey serum (Jackson Laboratories)-PBS-0.3% triton X100 for 1 h at room temperature, and then anti-N-PCT antibody was applied (dilution 1 : 100; Novus Biologicals) during 72 h at 4 °C. After washing, free-floating sections were incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse antibody (dilution 1 : 200; Jackson Laboratories) for 1 h at room temperature and then polyclonal rabbit antibodies were added overnight at 4 °C for NeuN (dilution 1 : 500; Chemicon International, Inc.), GFAP (dilution 1 : 500; Dakocytomation), or CRF (dilution 1 : 1000; Peninsula Laboratories). Finally, sections were incubated with TRITC-conjugated donkey anti-rabbit (dilution 1 : 200; Jackson Laboratories). Specificity of labelling was ascertained with omission of primary antibodies on one section for each brain or by incubating some sections with the specific blocking peptides. Sections were mounted on slides, coverslipped with anti-fading mounting media (Vectashield; Vector Laboratories), and examined under a fluorescent Olympus microscope (Olympus, Tokyo, Japan). Co-localisation images were the combination of each corresponding fluorescent signals. Image editing software (ADOBE PHOTOSHOP; Adobe Sysyems, Inc., San Jose, CA, USA) was used to combine photomicrographs into plates. Only the sharpness, contrast and brightness were adjusted.

Study 2: Effects of i.c.v. administration of N-PCT on food intake and body weight

Three groups of rats of equal body weight were studied (n = 10–12/group): (i) vehicle, (ii) N-PCT and (iii) heat-denatured N-PCT. Animal received an i.c.v.

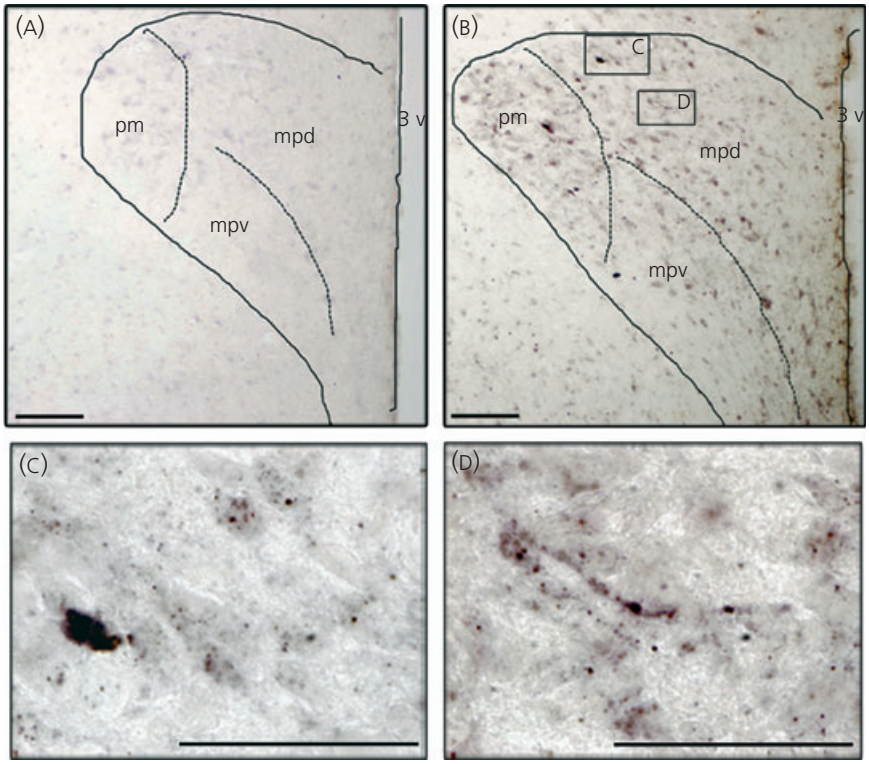

Fig. 1. Specificity of aminoprocalcitonin (N-PCT) antibody shown by immunostaining and pre-absorption in rat brain. Immunohistochemical staining of the rat paraventricular nucleus (PVN) with the N-PCT antibody and after pre-absorption with N-PCT polypeptide. (A) No immunostaining can be detected in the medial parvicellular zone of the PVN after the N-PCT antibody was pre-absorbed with N-PCT polypeptide. (B) Immunohistochemical staining of the PVN with the N-PCT antibody. (C, D) Enlargements from the boxed areas in (B). Scale bars = 50 μm (n = 4 per group). 3v, third ventricle; pm, posterior magnocellular PVN; mpv, ventral medial parvocellular PVN; mpd, dorsolateral medial parvocellular PVN.

injection of N-PCT (5 μg) or an equivalent volume of heat-denatured N-PCT (1 μg/μl protein; 90 °C for 1 h) or vehicle (aCSF) at the onset of dark phase when rats are most active and tend to eat their largest meals (27). This dose of N-PCT has been reported to inhibit nocturnal increase in food intake in rats with *ad lib.* access to standard rat chow (10, 13). After injection, rats were returned to their individual cages and given *ad lib.* access to both food and water as described above. Cumulative food intake was quantified (measurement accuracy = 0.01 g) at 1, 2, 4 and 8 h after i.c.v. injection. Food intake and body weight were also measured at 24 h to verify that the animals responded normally to N-PCT. Visual examination of the cage bottom revealed negligible spillage, and there were no apparent differences in spillage within or between treatment groups. The results obtained from N-PCT or vehicle injections from these studies are similar to those reported previously (10, 13), suggesting that the spillage had minimal impact on the results.

Study 3: Effect of central blockade of CRF receptors on N-PCT-induced change in food intake and body weight

Intracerebroventricular injections of D-Phe-CRF_{12-41}, at dosed between 1 and 5 μg, have been shown to inhibit CRF-induced actions, including effects on food intake and body weight (28, 29). Six groups of freely fed rats (n = 10–12/group) matched for body weights were randomly assigned to the groups: (i) vehicle (aCSF) + vehicle (aCSF); (ii) D-Phe-CRF_{12-41} + vehicle; (iii) vehicle + N-PCT; (iv) D-Phe-CRF_{12-41} (1 μg) + N-PCT; (v) D-Phe-CRF_{12-41} (2.5 μg) + N-PCT; and (vi) D-Phe-CRF_{12-41} (5 μg) + N-PCT. Animals were i.c.v. injected with D-Phe-CRF_{12-41} or an equivalent volume of vehicle (5 μl aCSF) 30 min before the onset of dark. At the onset of dark cycle, rats received either vehicle or N-PCT (5 μg/rat). After injection, rats were returned to their individual cages and given *ad lib.* access to both food and water. Food was removed from cages 1 h before N-PCT injection and returned to the cages with a pre-weighed amount of rat chow 15 min after i.c.v. infusion. Cumulative food intake was measured at 1, 2, 4 and 8 h into the dark phase and subsequently at 24 h when body weight was also measured.

Study 4: Effects of i.c.v. administration of N-PCT on hypothalamic CRF mRNA expression

Separate groups of rats (n = 5/group) were randomly assigned to receive either vehicle or 5 μg N-PCT via i.c.v. injection at the onset of the dark cycle and given *ad lib.* access to both food and water post-injection. Four hours after i.c.v. injection, rats were killed by cervical dislocation. The brain was quickly removed after retraction of the calvarium, placed in a brain matrix (Harvard Apparatus, Natick, MA, USA) on dry ice with the dorsal aspects upward. To remove the hypothalamus, we used the optic chiasm and the rostral edge of the mammillary bodies as rostral and caudal limits, respectively. The hypothalamic sulci were used as lateral limits. The fresh hypothalamic block was immediately frozen in liquid nitrogen and stored at −80 °C until mRNA extraction and real-time quantitative polymerase chain reaction (qPCR).

CRF mRNA expression

Frozen hypothalamic blocks containing the PVN were homogenised in Tripure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) for total

RNA isolation and measurement of CRF mRNA in accordance with the manufacturer's instructions. For each 10 mg of hypothalamic tissue, 0.2 ml of Tripure was used and other volumes were adjusted accordingly. RNA strand was reverse transcribed into its DNA complement (cDNA) using the Quantitect Enzyme Reverse Transcriptase kit (Qiagen, Hilden, Germany). A reverse transcriptase (RT) primer mix and 5 μl of a 1/10 RNA dilution as template were used in accordance with the manufacturer's instructions. The thermal cycling for cDNA synthesis consisted of 15 min of RT at 42 °C and 3 min of RT-inactivation at 95 °C. To rule out the possibility that PCR products would result from the amplification of genomic DNA contaminating the RNA samples, samples were treated with the RNase-free DNase kit (Roche Diagnostics).

PCR analysis of CRF was performed using GAPDH as a housekeeping gene. The forward and reverse primers used for CRF (assay identification no. Rn_CRF_1_SG) and GAPDH (assay identification no. Rn_Gapd_1_SG) were purchased from Qiagen (Quantitect Primer Assay). Each primer assay was derived from gene sequences contained in NCBI Reference Sequence Database. Each specific gene product was amplified by qPCR using the Mx3005P system (Agilent Technologies Inc., Santa Clara, CA, USA) and the SensiMix Sybr Low-Rox kit (Bioline, Berlin, Germany). Briefly, the hot-start DNA polymerase was activated by incubation for 10 min at 95 °C. PCR amplifications (reaction volume 25 μl) were performed for 40 cycles consisting of 15 s of denaturation at 94 °C, 15 s of primer annealing and 15 s of primer extension at 72 °C. Before each assay, a standard curve was constructed using different increasing amounts of cDNA to determine the optimal cDNA quantity. The optimal qPCR conditions were obtained using as template 2.5 μl of a 1/10-cDNA dilution. The amplification of the housekeeper was performed in parallel with the gene to be analysed. Threshold cycle values were calculated using AMIPRO QPCR, version 3.20 (Stratagene. La Jolla, CA, USA). The comparative $\Delta\Delta$Ct method was employed to measure fold changes in expression of RNA transcript levels between untreated and drug-treated rats.

Study 5: Effect of i.c.v. administration of N-PCT on hypothalamic CRF content and plasma ACTH and corticosterone levels

Separate groups of freely fed singly-housed rats and adapted to the injection procedure were injected i.c.v. with N-PCT (5 μg/rat) or heat-denatured N-PCT (1 μg/μl protein; 90 °C for 1 h) or an equivalent volume of vehicle at the onset of the dark phase and decapitated 15 and 30 min after injection (n = 6/group). Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes at the time of sacrifice and removal of brain tissue and placed on ice until plasma separation. The hypothalamus was immediately dissected out, weighed and frozen in liquid nitrogen for the assay of hypothalamic CRF content. Plasma was separated by centrifugation, frozen on dry ice and stored at −20 °C until assayed.

Measurement of CRF, ACTH and corticosterone

Plasma levels of ACTH and the adrenal glucocorticoid hormone, corticosterone, were measured using commercially available rat enzyme immunoassay (EIA) kits (ACTH: Phoenix Pharmaceuticals Inc., Belmont, CA, USA; corticosterone: Immunodiagnostic Systems Ltd, Tyne and Wear, UK) in accordance with the manufacturer's instructions. Plasma ACTH was extracted using a 1% trifluoroacetic acid buffer and C-18 column technique. Extracted samples were dehydrated using an Eppendorf vacufuge, and pelleted samples were stored at −80 °C. Samples were reconstituted 24 h before the EIA in assay buffer. The range of detection for the ACTH kit was 0–25 ng. The intra- and interassay coefficients of variation were < 5% and 14%, respectively. The corticosterone EIA had a sensitivity of ≤ 0.55 ng/ml. The intra- and interassay coefficients of variation were < 4% and 8%, respectively. All plasma samples were analysed in duplicate and repeated freeze–thaw cycles for reagents and samples were avoided.

For CRF analysis, each frozen hypothalamus was homogenised in 30-fold volume of extraction solution (10 mM PBS containing 0.2% Nonidet P-40; pH 7.2) in an ice bath. The homogenate was centrifuged (15 000 *g* for 20 min) at 4 °C. An aliquot of the supernatant was assayed for CRF; another aliquot was used for determination of protein. CRF concentrations were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit for rats (YK131; Yanaihara Institute Inc., Shizuoka, Japan) in accordance with the manufacturer's instructions. This assay can measure rat CRF within the range of 0.078–2.5 ng/ml. The intra- and interassay coefficients of variation were < 7% and 4%, respectively. Protein concentration of each hypothalamic sample was measured by the method of Bradford (30).

Study 6: Effect of i.c.v. administration of N-PCT on CRF-containing neurones in the PVN

Separate groups of rats of equal body weight were randomly assigned to receive either vehicle or N-PCT (5 μg) via i.c.v. injection (n = 6/group) and given *ad lib.* access to both food and water post-injection as described above. Rats were left undisturbed in their home cages. Ninety minutes after i.c.v. injection, rats were deeply anaesthetised using a standard ketamine cocktail (1 ml/kg body weight ip) and perfused transcardially with heparin solution (1000 U/l, 0.9% saline), followed by ice-cooled 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and post-fixed in the same fixative for 24 h at 4 °C, then cryoprotected in phosphate-buffered 30% sucrose solution with 0.1% sodium azide for 24–48 h. Next, brains were frozen and cut in the coronal plane (six series of 40-μm thick sections) and collected in 0.1 M PBS with 0.1% sodium azide.

Immunohistochemistry

Immunohistochemical visualisation of Fos and CRF was performed on free-floating sections using a sequential two-colour immunoperoxidase dual-labelling technique (13, 31–34). Briefly, brain sections are incubated in two sequences of avidin-biotin immunoperoxidase reagents using a nickel-intensified diaminobenzidine (Ni-DAB) solution to visualise the first antigen (Fos), and DAB alone to visualise the second antigen (CRF). With these chromogens, black and amber reaction products are generated at the locations of the first and second antigens, respectively.

After blocking endogenous peroxidase and preincubation in 10% normal goat serum, brain sections were incubated in primary rabbit polyclonal anti-Fos antiserum (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 3000 in 0.1 M PBS with 0.1% Triton X-100 (PBS-TX) for 48 h at 4 °C. After rinsing three times for 5 min in PBS-TX, sections were further incubated in secondary biotinylated donkey anti-rabbit IgG (AP182B, dilution 1 : 500; Chemicon International, Inc.) for 90 min at room temperature and rinsed three times for 5 min in PBS-TX. Sections were then incubated for 90 min in avidin–biotin–horseradish peroxidase complex (Vectastain ABC kit, 1 : 500; Vector Laboratories) before being exposed for 20 min to a Ni-DAB solution (nickel ammonium sulphate, 0.02% DAB in 0.1 M Tris–HCl buffer (pH 7.6) and 0.01% H_2O_2) to allow visualisation of Fos protein as a black nuclear deposit.

For dual immunostaining for CRF, sections were first immunoreacted for Fos using the ABC method and the Ni-DAB development as described above. To avoid fading of the Ni-DAB reaction, all subsequent steps were carried out using 0.1 M Tris–HCl buffer (pH 8.5) as diluent. As a first step, the sec-

tions were treated for 30 min with 10% methanol-3% hydrogen peroxide to inhibit the peroxidase activity due to the Fos immunostaining. After blocking in 10% normal goat serum, sections were incubated overnight in rabbit anti-CRF antibody (T-4037, dilution 1 : 5000; Peninsula Laboratories). CRF was detected using the ABC method as described for the Fos immunohistochemistry. However, to obtain an amber cytoplasmic deposit and thus visualise immunolabelled CRF and Fos, section were exposed to DAB alone (DAB in 0.1 M Tris–HCl buffer without nickel sulphate) as chromogen instead of Ni-DAB. After the immunohistochemical procedures, sections were washed, mounted on gelatinised slides, air-dried, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA, USA). In some animals, adjacent sections were counterstained with cresyl violet to identify nuclear borders. Sections were analysed using an Olympus BX41 microscope connected to an Olympus C3030 zoom digital camera. Brightness and contrast were adjusted using ADOBE PHOTOSHOP, version 6.0.

Cell counts and quantification

Immunopositive cells were identified in the PVN on sections between −0.8 and −2.12 from the bregma, in accordance with the coordinates from the rat brain atlas of Paxinos and Watson (35). To analyse the division of the PVN, representative sections were stained with cresyl violet. Medial and posterior parvocellular subdivisions of the PVN nucleus were considered to be at −1.80 and −2.12 mm from the bregma, respectively. Quantitative analysis of Fos and Fos–CRF double labelling was performed using an Olympus BX-41 microscope equipped with a C3030 digital camera attached to a contrast enhancement device. Fos-positive cells, as indicated by the black staining, were identified when the nuclear structure demonstrated a clear immunoreactivity compared with the background level. The cytoplasmic CRF (brown) labelling was counted if it had a clearly labelled cell body surrounding a nucleus. Counting of immunolabelled cells was obtained as a mean of positive labelling from two to three sections from each animal. Two participants who were blinded to the experimental protocols performed the visual counting of neurones in sections of six animals in each set of experimental conditions. Image editing software (ADOBE PHOTOSHOP) was used to combine photomicrographs into plates. Only the sharpness, contrast and brightness were adjusted.

Study 7: Hypothalamic N-PCT protein levels in 24-fasted and ad lib.-fed rats

Rats were subdivided into two groups of equal body weight: fed and fasted. The first group was fed *ad lib.* (n = 7). The second group was fasted for 24 h (n = 11). Rats were killed by decapitation at the beginning of the dark phase, and hypothalami quickly removed and frozen. Using a rat brain slicer (Harvard Apparatus) a 2-mm-thick slice, including the entire PVN, was taken from the basal hypothalamus using a microtome. Based on anatomical landmarks (the apex of the third ventricle), both paraventricular nuclei, adjoining the third ventricle were obtained by punching the bilateral PVN region with a hollow needle (diameter 1000 μm), stored immediately in liquid nitrogen, and kept at −80 °C until processing for protein extraction.

Protein extraction and quantification

Frozen hypothalamic tissues were homogenised at 4 °C in a lysis buffer containing 50 mM Tris–HCl, 5 mM EDTA, 2% sodium dodecyl sulphate (SDS) and a protease inhibitor cocktail (Roche Diagnostics). Tissue homogenates were centrifuged at 5000 *g* for 15 min at 4 °C to clear the cellular debris. Total protein content in the homogenate was extracted using the Tripure Isolation Reagent (Roche Diagnostics) in accordance with the manufacturer's instructions. This procedure allows the isolation of protein fractions from a single sample. The protein pellets, obtained using the Tripure Isolation Reagent, were re-suspended in 4% SDS and 8 M urea in 40 mM Tris–HCl (pH 7.4) and rotated overnight at room temperature for total dilution. The protein content in the soluble fractions was determined by spectrophotometry at 280 nm. N-PCT content was analysed by western blotting as described below, using β-actin as an internal control.

Analysis of N-PCT protein

Western immunoblots using a mouse monoclonal anti-human N-PCT antibody (NB120-14817; Novus Biologicals) were used to analyse endogenous N-PCT in the rat hypothalamus. Samples of the hypothalamus (100 μg total protein) were analysed by 18% SDS-PAGE and transferred onto a nitrocellulose membrane (Protran; Whatman GmbH, Dassel, Germany). Membranes were blocked for 1 h at 37 °C in PBS–Tween buffer (0.01 M PBS and 0.1% Tween 20) containing 5% nonfat milk and incubated for 2 h at room temperature with the primary antibody diluted in PBS–Tween buffer with 0.5% nonfat milk. We used the anti-N-PCT antibody (1 : 500; Novus Diagnostic), and anti-β-actin antibody ACTBD11B7 (dilution 1 : 1000; Santa Cruz Biotechnology). After washing, membrane was incubated for 1 h at room temperature with goat anti-mouse IgG conjugated with horseradish peroxidase (Chemicon International Inc.). After rinsing, subsequent detection was performed using an enhanced chemiluminiscence detection kit (Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions. Intensities of the bands were quantified and normalised using PCBAS, version 2.08e (Raytest Inc., Dusseldorf, Germany).

Statistical analysis

Data are shown as the mean ± SEM and were analysed using parametric statistics (SIGMA STAT, version 3.1; Systat Software Inc., Chicago, IL, USA). Two-way repeated-measures ANOVA followed by a Student–Newman–Keuls test was used for the analysis of food intake. For other comparisons (i.e. N-PCT, Fos or CRF expression), either a one-way ANOVA (for more than a two-group comparison) followed by a Student-Newman–Keuls post-hoc test or an unpaired Student's t-test (for two-group comparison) was used. $P < 0.05$ was considered statistically significant.

Results

Specificity of the N-PCT antibody to label rat brain tissue

Specificity of the monoclonal mouse anti-human N-PCT was tested in the rat hypothalamus by immunohistochemistry and western blotting. Immunohistochemical staining of N-PCT in rat hypothalamus results in an intense staining of neurones in the PVN (Fig. 1). We demonstrated that, after pre-absorption of this antibody (Fig. 1A), no immunostaining could be detected in the PVN, whereas intense immunostaining was observed with the nonpre-absorbed N-PCT antibody (Fig. 1B). The immunohistochemical analysis of hypothalamic sections suggests that N-PCT-IR cells were neurones and astrocytes localised in the medial parvocellular divisions of the PVN (Fig. 1C,D, respectively). We also found N-PCT-IR in other hypothalamic regions, including the supraoptic, ARC, DMN and VMN and lateral hypothalamic areas (data not shown). Specificity of the monoclonal mouse anti-human N-PCT was also tested in the rat hypothalamus homogenates by western blotting (Fig. 9). Taken together, our data supported the specificity of the anti-N-PCT antibody in the rat hypothalamus.

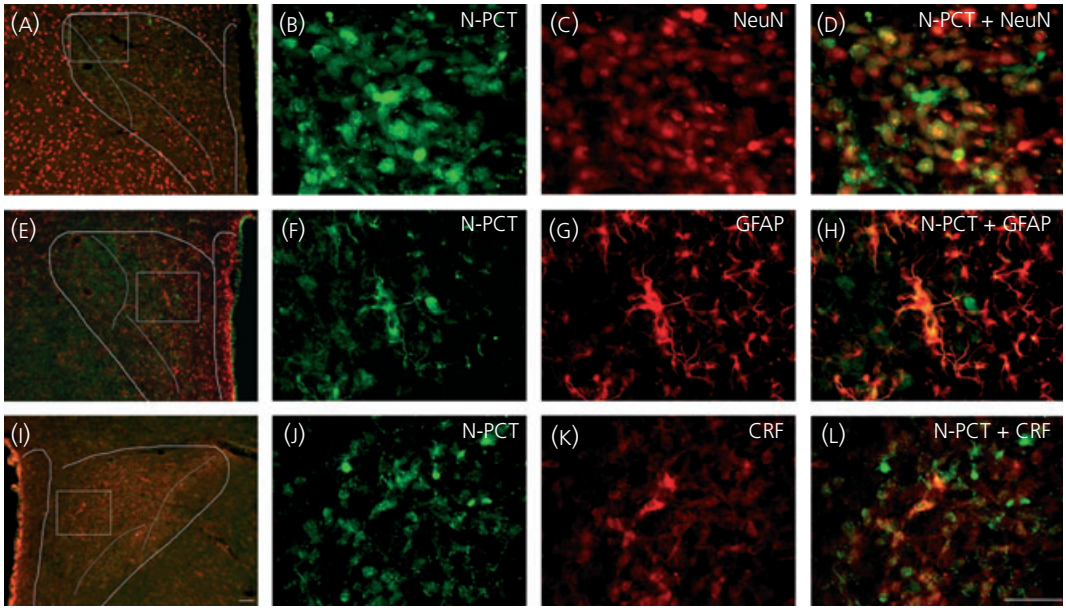

Fig. 2. Characterisation of cell types expressing aminoprocalcitonin (N-PCT) in the rat paraventricular nucleus. Fluorescence micrographs show endogenous N-PCT, neuronal nucleus (NeuN; a neuronal marker), glial fibrillary acid protein (GFAP) (an astrocyte marker), or corticotrophin-releasing factor (CRF) immunoreactivity in the PVN of adult male rats. Green fluorescence reveals N-PCT presence (B, F, J), whereas red fluorescence represents NeuN-, GFAP- or CRF-immunoreactivities (C, G, K, respectively). Co-localisation appears as yellow (D, H, L). (B-D) Enlargements from the boxed areas in (A). (F-H) Enlargements from the boxed areas in (E). (J-L) Enlargements from the boxed areas in (I). Data are representative of four rats. Bregma: −1.80 according to the rat brain atlas of Paxinos and Watson (35). Scale bars = 50 μm. NeuN, neuronal nucleus.

Characterisation of cell types expressing N-PCT in the PVN

To characterise cell types expressing N-PCT-IR in the PVN, we performed single and double immunohistochemistry. Figure 2 depicts a representative section with co-localisation of CRF and N-PCT in the PVN at bregma −1.80. The immunohistochemical analysis of hypothalamic sections indicates that N-PCT-IR cells were neurones (Fig. 2A-D) and astrocytes (Fig. 2E-H) localised in the medial parvocellular divisions of the PVN, where CRF-synthesising neurones reside. Double staining immunofluorescence confirmed that N-PCT co-localised with CRF-positive neurones in the PVN (Fig. 2I-L). We also found N-PCT-IR in fibres and/or terminals in contact or close proximity with CRF neurones in the PVN (Fig. 2L), suggesting that N-PCT efferents exert excitatory actions on CRF PVN neurones. This is consistent with the presence of N-PCT-IR in other hypothalamic regions, including the supraoptic, ARC, DMN, VMN and lateral hypothalamic areas (data not shown).

Structural integrity of N-PCT is required for N-PCT-induced reduction of dark-phase food intake and body weight

The effect of i.c.v. N-PCT on food intake and body weight is shown in Fig. 3. Compared to an artificial CSF vehicle, N-PCT suppressed dark-phase cumulative food intake from the first (57%; $P < 0.001$) to the 8 h (52%; $P < 0.01$) (Fig. 3A). Cumulative food intake over the 24-h period after i.c.v. N-PCT was reduced by 29% compared to controls ($P < 0.001$; Fig. 3B). Intracerebroventricular N-PCT injection also caused marked loss of body weight gain (−7.5 ± 1.3 g) over 24 h compared to vehicle-treated controls (6.5 ± 1.2 g; $P < 0.001$; Fig. 3C). By comparison, i.p. administration of the same dose of N-PCT did not significantly alter food intake at 1, 2, 4, 8 or 24 h time points (data not shown). To determine whether the structural integrity of N-PCT is required for the observed effects, and that the effects are not a result of nonspecific antigenicity associated with the foreign peptide administered, N-PCT was inactivated by heat-denaturation. Intracerebroventricular injection of this solution at the onset of dark-phase did not influence the 8-h dark-phase feeding response or the 24-h food intake and body weight gain (Fig. 3A-C).

N-PCT-induced reduction of food intake and body weight was attenuated by central blockade of CRF receptors

To determine whether N-PCT affects food intake via CRF-receptor-mediated pathways, we co-administered the nonspecific $CRF_{1/2}$ receptor antagonist D-Phe-CRF_{12-41} before N-PCT (Fig. 4). Intracerebroventricular administration of D-Phe-CRF_{12-41} (5 μg/rat) alone did not significantly affect food intake in vehicle-treated animals as previously reported (28). Co-administration of D-Phe-CRF_{12-41} with N-PCT (5 μg) attenuated the effects of N-PCT on dark-phase food intake in a dose-dependent manner (Fig. 4A), such as the food intake of these animals treated with the high doses of D-Phe-CRF_{12-41} was returned to 83% of the intake of vehicle-treated controls at 24 h (Fig. 4B). N-PCT injection also caused marked loss of body weight (−7.1 ± 1.2 g) over 24 h, which was dose-dependently attenuated by co-treatment with the CRF receptor antagonist used,

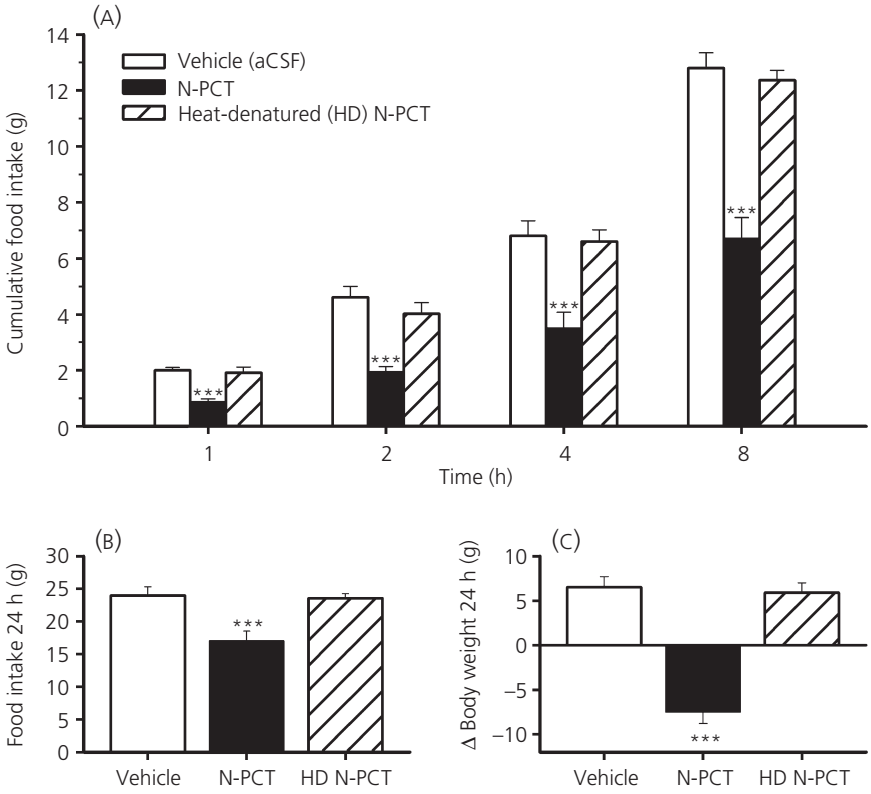

Fig. 3. Intracerebroventricular administration of aminoprocalcitonin (N-PCT) inhibits dark phase food intake and body weight gain in rats. Vehicle (artificial cerebrospinal fluid; aCSF), N-PCT (5 μg/rat) or heat-denatured N-PCT (HD N-PCT) were injected at the onset of the dark phase in *ad lib.* fed rats chronically implanted with cannula into the lateral brain ventricle. N-PCT decreased the dark-phase (A) and the 24-h (B) cumulative food intake after i.c.v. injection. The i.c.v. injection of N-PCT decreased body weight gains 24 h after injection (C). Each bar represents the mean ± SEM (n = 10–12 rats per group). ***P < 0.001 versus vehicle.

such as the body weight gain of these animals treated with the high dose of D-Phe-CRF_{12-41} was returned to 65% of the body weight gain of vehicle-treated controls (Fig. 4C).

N-PCT induced CRF mRNA expression and CRF production in the hypothalamus

To determine whether N-PCT-induced anorexia is associated with the CRF system, we used qPCR to measure levels of mRNA CRF and ELISA to measure CRF content in the hypothalamus after i.c.v. injection of N-PCT. Compared with control aCSF- and heat-inactivated N-PCT-treated rats, N-PCT-treated rats had significantly increased hypothalamic CRF gene expression (Fig. 5A) and CRF levels (Fig. 5B).

N-PCT increased plasma ACTH and corticosterone levels

To determine whether the effect of i.c.v. N-PCT administration to increase hypothalamic CRF mRNA and CRF levels could have resulted from a response to nonspecific stress or toxicity associated with i.c.v. N-PCT injection, we also measured plasma ACTH and corticosterone levels after i.c.v. injection of heat-denatured N-PCT under the same experimental conditions. Intracerebroventricular administration of N-PCT resulted in a significant increase in plasma ACTH (133%) and corticosterone (142%) compared to control aCSF-treated rats (P < 0.001; Fig. 6A,B). By contrast, i.c.v. administration of heat-denatured N-PCT did not raise hypothalamic CRF content, nor did it alter plasma levels of ACTH and corticosterone.

Intracerebroventricular administration of N-PCT stimulated CRF neurones in the PVN

To determine whether a neuroanatomical framework exists for direct N-PCT activation of CRF neurones in the PVN, we used Fos as a marker of neuronal activation (24). Intracerebroventricular administration of N-PCT (5 μg) caused activation of CRF-containing neurones in the parvocellular divisions of the PVN (Fig. 7). We found that i.c.v. administration of N-PCT increases the number of Fos-positive and CRF-immunoreactive neurones in the parvocellular divisions of the PVN compared to i.c.v. vehicle, mainly in the medial subdivisions of the PVN (P < 0.001; Fig. 7A–E). A discrete, but significant increase in the number of Fos-positive cells was also observed in the magnocellular part of the PVN (P < 0.05 versus vehicle control; Fig. 7E). Double labelling revealed that, after i.c.v. N-PCT, 25% of all CRF-immunoreactive cells in the medial parvocellular PVN were activated by N-PCT (P < 0.001 versus control; Fig. 7F). After a pre-absorption test with anti-Fos and anti-CRF, there was no specific immunostaining in the sections (data not shown). Figure 8 shows a topographical mapping

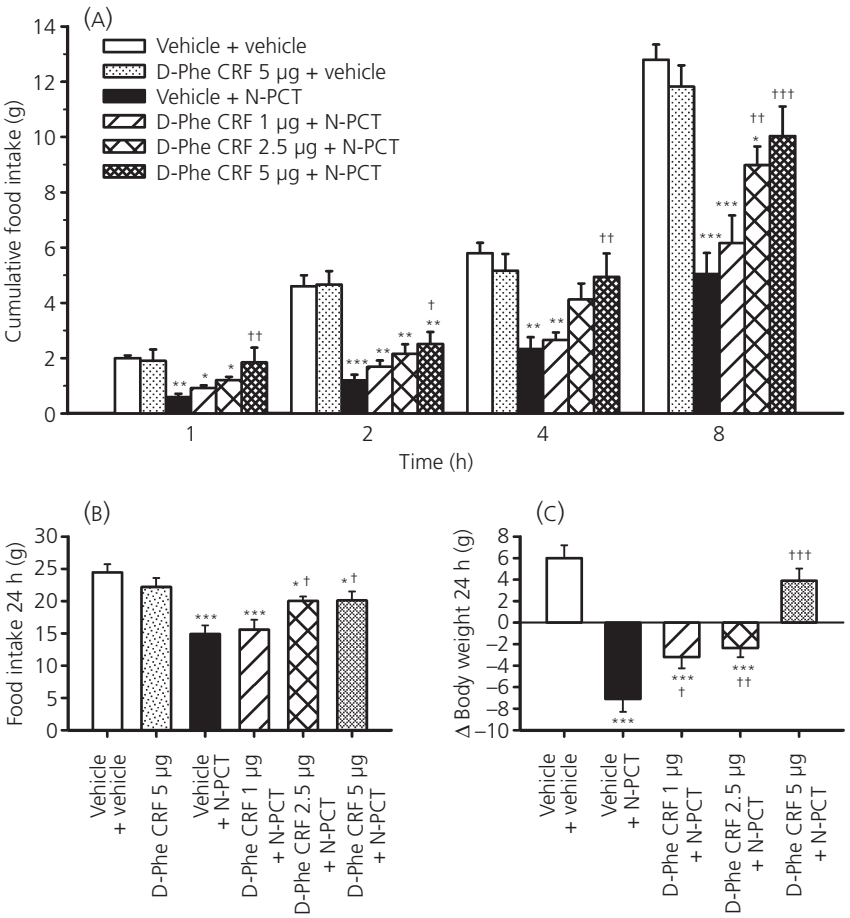

Fig. 4. Intracerebroventricular injection of a corticotrophin-releasing factor (CRF) receptor antagonist attenuates aminoprocalcitonin (N-PCT)-induced reduction in food intake and body weight. The nonspecific $CRF_{1/2}$ receptor antagonist D-Phe-CRF_{12-41} (1, 2.5 or 5 μg/rat, i.c.v.) was administered 1 h before the onset of the dark-phase and 30 min before N-PCT (5 μg/rat, i.c.v.) in *ad-lib.* fed rats. Intracerebroventricular D-Phe-CRF_{12-41} (5 μg/rat) alone did not significantly affect food intake in vehicle-treated animals. Co-administration of D-Phe-CRF12-41 with N-PCT attenuated the effects of N-PCT on dark-phase food intake (A and B) and body weight gain (C) in a dose-dependent manner. Data are the mean ± SEM (n = 10–12 rats per group). *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle + vehicle. †P < 0.05; ††P < 0.01; †††P < 0.001 versus vehicle + N-PCT.

of Fos- and CRF-IR in the parvocellular divisions of the PVN after i.c.v. administration of vehicle or N-PCT.

N-PCT is present in the PVN and is regulated by fasting

Using N-PCT antiserum, western immunoblotting of hypothalamus proteins revealed an immunoreactive band of weight 8.5 kDa, corresponding to the molecular weight of rat N-PCT (Fig. 9, upper panel). Signal abolition by pre-absorbing the antibody with purified N-PCT confirmed antibody specificity and the presence of N-PCT protein in the rat PVN of the hypothalamus. We also found that N-PCT protein levels were significantly reduced in the PVN of 24-h fasted rats compared to those in control animals (P < 0.05, Fig. 9, lower panel).

Discussion

We have reported previously that i.c.v. administration of N-PCT at the onset of the active dark cycle in rodents (26) reduces food intake acutely (within 1 h) and body weight gain over a 24-h period in a dose–response manner (10). Taken with the fact that i.c.v. N-PCT is not aversive to rats and that comparable doses of N-PCT administered systemically have no effect on food intake, this anorectic action probably occurs within the brain (10, 12). N-PCT immunoreactivity is found primarily in the CNS, including the PVN and the ARC nucleus of the hypothalamus, which are areas of the brain that have been implicated in the control of appetite (10, 12, 13). However, these studies did not identify the specific hypothalamic cells expressing N-PCT or molecular mechanisms mediating the anorectic effect of N-PCT.

In the present study, we extend these observations. Based on these previous dose–response studies (10, 12), we confirmed that a single dose of N-PCT (5 μg = 1 nmol) injected i.c.v. before the onset of the dark-phase inhibits food intake in freely fed rats, with a cumulative food intake reduction still maintained for 24 h after injection and without inducing unusual changes in behaviour. Although this dose of N-PCT did not modify body temperature at

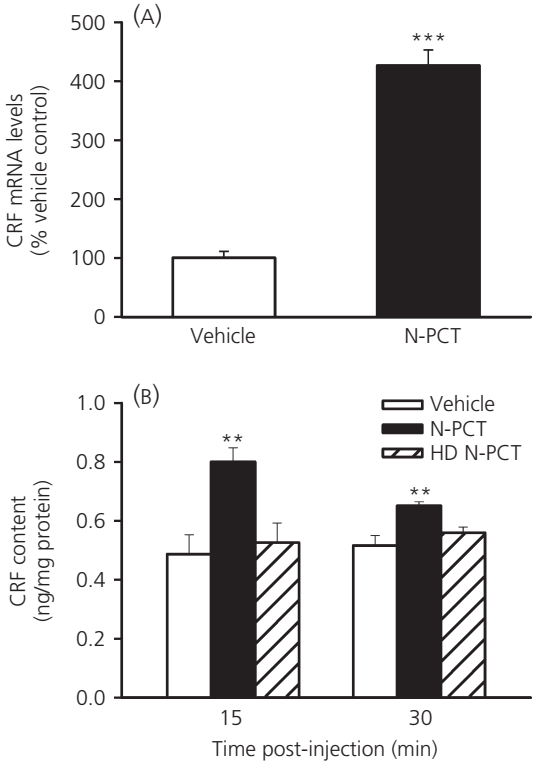

Fig. 5. Intracerebroventricular injection of aminoprocalcitonin (N-PCT) increases the expression of corticotrophin-releasing factor (CRF) mRNA and the CRF content in the paraventricular nucleus (PVN). Expression of CRF mRNA (A) and CRF content (B) in the PVN of *ad lib.* fed rats injected i.c.v. with vehicle (artificial cerebrospinal fluid; aCSF), N-PCT (5 μg/rat) or heat-denatured N-PCT at the onset of the dark phase. Data are the mean ± SEM (n = 5 per group). **P < 0.01; ***P < 0.001 versus vehicle.

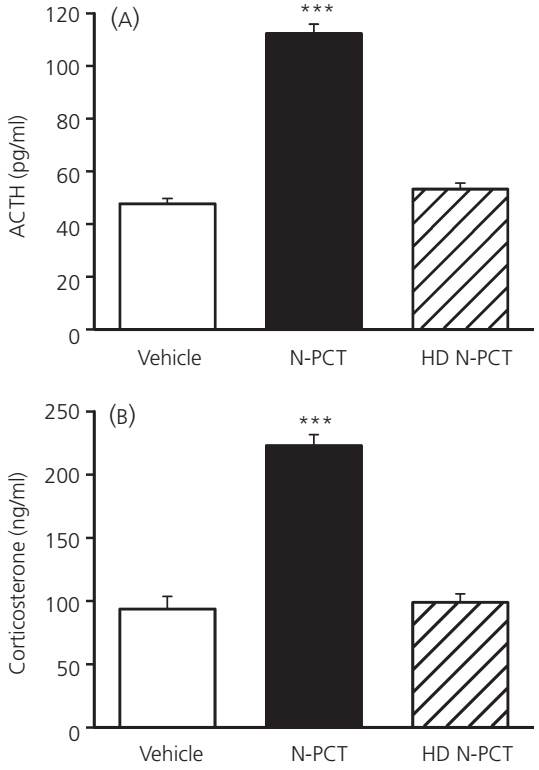

Fig. 6. Effect of aminoprocalcitonin (N-PCT) on plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels. (A) Plasma levels of ACTH 15 min after the administration of vehicle (artificial cerebrospinal fluid; aCSF), N-PCT (5 μg) or heat-denatured N-PCT (HD N-PCT). (B) Plasma levels of corticosterone 30 min after the administration aCSF, N-PCT (5 μg) or HD N-PCT. Drugs were injected i.c.v. at the onset of the dark phase to freely fed singly housed rats adapted to the injection procedure. Data are the mean ± SEM (n = 6 per group). ***P < 0.001 versus aCSF control.

24 h (10), body weight gain at 24 h after injection was significantly decreased, indicative of increased energy expenditure. These data suggest that N-PCT acts directly in the brain to reduce food intake, consistent with its proposed role in the negative-feedback regulation of energy homeostasis. We also found that i.c.v. administration of heat-denatured N-PCT failed to affect nocturnal food intake, suggesting that structural integrity of N-PCT is required for the observed biological effects and is not a result of nonspecific antigenicity associated with the foreign peptide administered. In addition to this examination, conditioned taste aversion test is also useful for excluding loathing induced by N-PCT. Utilising a conditioned taste aversion paradigm, we have previously shown that i.c.v. N-PCT is not aversive to rats and does not affect feeding via nonspecific actions (10). Combined with the observations that central immunoneutralisation of endogenous N-PCT increased nocturnal feeding and that i.c.v. administration of a specific N-PCT antibody inhibits the anorectic effect N-PCT (11), these data give additional support to the role of N-PCT as an important mediator of satiety-related responses.

Although N-PCT receptors has been not demonstrated *in vivo*, a receptor for procalcitonin has been identified in COS-7 cells (9). Recent data (11, 13) suggest that N-PCT may induce inhibition of feeding in rats via activation of calcitonin-receptors, which are highly expressed in key hypothalamic nuclei involved in the control of feeding such as PVN and ARC (20, 21). Activation of these receptors decreases food intake in rats (23, 36, 37) and stimulates hypothalamic CRF release, as well as plasma ACTH and cortisol secretion (38). Thus, it is plausible that the anorectic effect of N-PCT could be mediated through the activation of CT-Rs on CRF-containing neurones in the PVN and the subsequent activation of CRF receptors.

According to this hypothesis, we showed that N-PCT affects food intake via a CRF-receptor-mediated pathways. Besides the classical role of CRF in HPA axis control, an anorectic effect of CRF is well established. Central administration or direct administration into the PVN inhibits night-time feeding in freely fed rats, whereas its antagonists increase it (16, 18, 39). Thus, it is possible that i.c.v. N-PCT inhibits food intake through the stimulation of hypothalamic CRF pathways. We found that i.c.v. pre-treatment with D-Phe-CRF $_{12-41}$, a nonspecific CRF receptor antagonist, attenuated the actions of N-PCT on food intake and body weight. D-Phe-CRF $_{12-41}$ has been used widely at doses similar to those employed in the present study to inhibit the actions of CRF (28) and leptin (29) on food

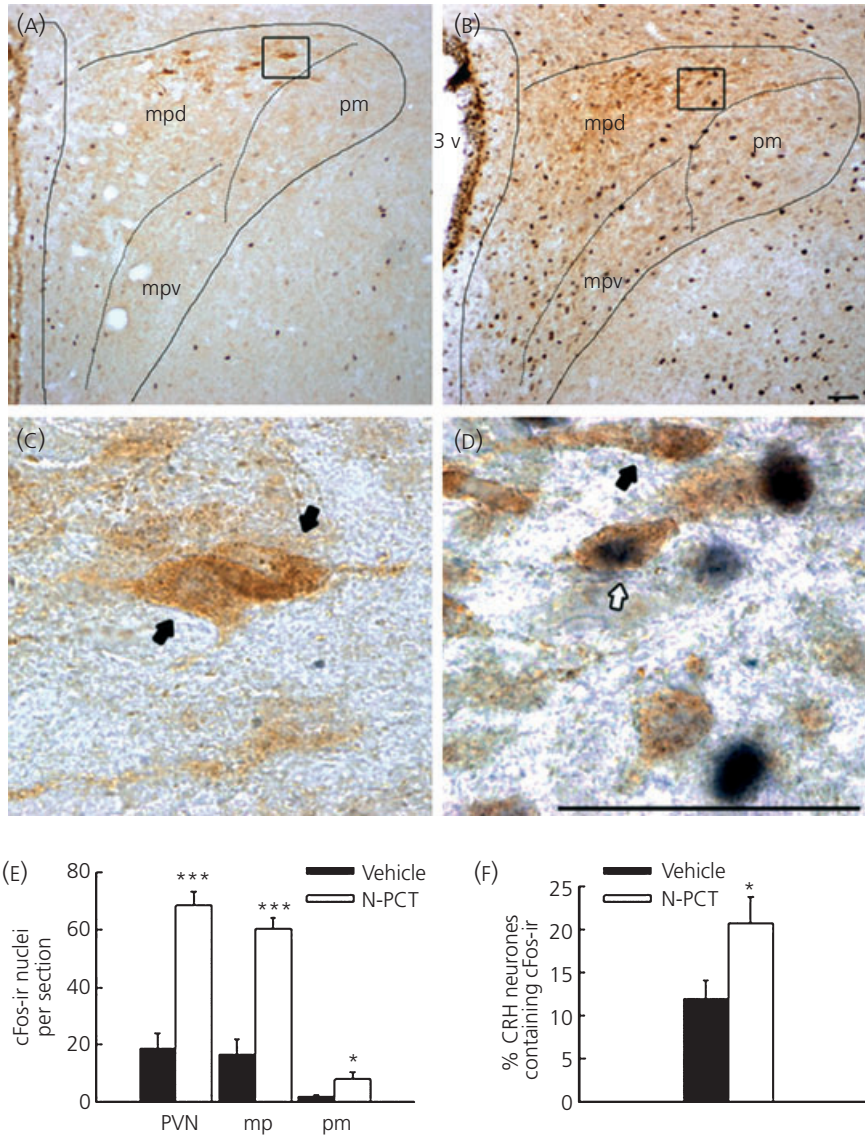

Fig. 7. Intracerebroventricular aminoprocalcitonin (N-PCT) administration induces Fos expression in corticotrophin-releasing factor (CRF)-immunoreactive (IR) neurones of the paraventricular nucleus (PVN). *Ad lib.* fed rats were injected i.c.v. with control artificial cerebrospinal fluid (aCSF) vehicle (A) or N-PCT (5 μg/rat) (B) at the onset of the dark phase. N-PCT increased the number of Fos-positive neurones (black) in the medial parvicellular zone of the PVN (B, E) and the percentage of these activated neurones colocalised with CRF (brown) (D, F). (C, D) Enlargements from the boxed areas in (A) and (B), respectively. White arrowheads indicate co-existence of nuclear Fos-IR and CRF-IR. Black arrowhead indicates CRF-IR without Fos-IR. Scale bars = 50 μm. Data are the mean ± SEM. n = 6 rats per group. *P < 0.05; ***P < 0.001 versus i.c.v. vehicle (E, F). 3v, third ventricle; pm, posterior magnocellular PVN; mpv, ventral medial parvocellular PVN; mpd, dorsolateral medial parvocellular PVN.

intake and body weight. Furthermore, central administration of CRF reduced nocturnal food intake in freely fed rats through activation of hypothalamic CRF-receptors (16, 40, 41). These data suggest that N-PCT-induced anorexia could be mediated, at least in part, via CRF-receptor-mediated pathways.

These data are consistent with the hypothalamic CRF signalling system being part of underlying mechanisms of N-PCT-induced reduction of food intake; however, the CRF receptor subtype involved in the anorectic effects of N-PCT cannot be ascertained from the present study because the antagonist D-Phe-CRF_{12-41} has a similar affinity for type CRF-1 and CRF-2 receptors (42). Similarly, the actions of N-PCT cannot be conclusively ascribed to CRF because there are several other endogenous ligands that act at CRF receptors, such as urocortin, which also is a more potent suppressor of appetite than CRF (43). This stated involvement of CRF is supported by the observation that 25% of CRF-containing neurones in the PVN were activated by i.c.v. injection of N-PCT. Furthermore, because the effects of N-PCT on food intake and body weight were attenuated but not abolished by the CRF antagonist, CRF may act in conjunction with other mediators implicated, such as melanocortins in the central regulation of appetite (44, 45). Using a similar approach, we recently demonstrated that the anorectic effect of N-

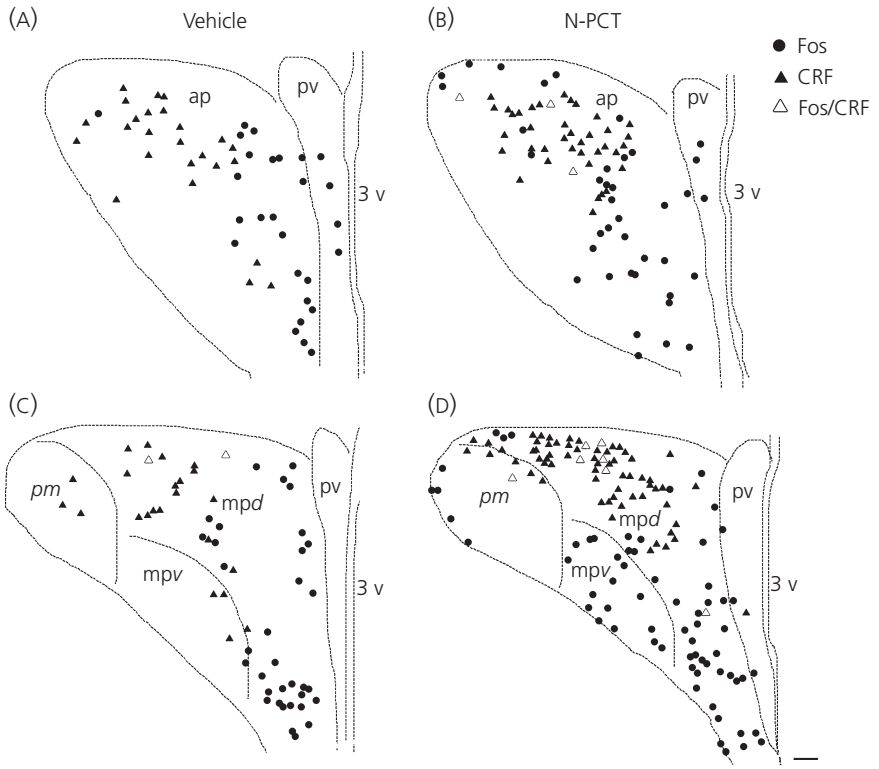

Fig. 8. Topographical mapping of Fos- and corticotrophin-releasing factor (CRF)-immunoreactivity (IR) in the paraventricular nucleus (PVN) after i.c.v. administration of vehicle (artificial cerebrospinal fluid; aCSF) (A, C) or aminoprocalcitonin (N-PCT) (5 μg/rat) (B, D). (A–D) Co-existence of Fos-IR and CRF-IR (open triangle). The closed circle indicates a Fos-positive cell and the closed triangle indicates a CRF-positive cell. Scale bars = 50 μm. 3v, third ventricle; ap, anterior parvocellular PVN; pm, posterior magnocellular PVN; mpv, ventral medial parvocellular PVN; mpd, dorsolateral medial parvocellular PVN; pv, periventricular nucleus.

PCT is mediated at least partly through the stimulation of arcuate POMC neurones and subsequent activation of brain melanocortin 3/4-receptors (E. Tavares , R. Maldonado and F.J. Miñano, unpublished data). Because axons from the ARC innervate anorectic neurones containing CRF in the PVN and hypothalamic melanocortin 3/4 receptors are responsible for the central control of appetite and energy expenditure (46), these findings support the potential involvement of CRF neurones in the anorectic actions of N-PCT.

We have demonstrated previously in rats that N-PCT is expressed in hypothalamic nuclei involved in the regulation of energy homeostasis and appetite, including the ARC, VMN, DMN, supraoptic and PVN (10, 12, 13). Among them, the PVN governs the neuroendocrine cascade (HPA axis) and plays a crucial role in the control of eating and regulation of body weight (16–18). However, these studies did not identify the specific hypothalamic cells expressing N-PCT. Because the PVN contains a prominent population of CRF-synthesising neurones, in the present study, we aimed to characterise cell types expressing N-PCT in the rat PVN and to demonstrate that N-PCT activates the downstream signalling of CRF as mirrored by ACTH secretion.

To characterise the cell types expressing N-PCT in the PVN, we first ascertained the specificity of the commercially available monoclonal N-PCT antiserum to stain N-PCT in the rat brain. Using immunohistochemistry, we found that this antibody detects N-PCT protein. Furthermore, when this antibody was pre-absorbed with N-PCT, immunostaining was completely abolished in brain sections. In addition, western immunoblots using confirmed the specificity of this mouse monoclonal anti-human N-PCT antibody and showed that N-PCT protein is present in the PVN. Therefore, the present expression pattern in rats is likely to reflect N-PCT-IR. Those immunoreactive cells are located in the feeding regulatory hypothalamic nuclei previously reported to be N-PCT positive (10, 12). In particular, we showed that at the cellular level N-PCT-IR was confined to astrocytes and neurones of the parvocellular divisions of the PVN, where CRF-synthesising neurones reside. Double staining immunohistochemistry revealed that N-PCT was co-expressed with CRF in parvocellular PVN neurones. We also found N-PCT fibres and/or terminals in contact or close proximity of CRF neurones, suggesting that N-PCT axon terminals have synaptic connections with parvocellular CRF neurones in the PVN. Taken together, these observations indicate that N-PCT exerts excitatory actions on CRF PVN neurones, and that endogenous N-PCT in the PVN may be released together with CRF. Because CRF neurones play a pivotal role in the regulation of ACTH synthesis and secretion in the anterior pituitary (47), these observations suggest that N-PCT may also play a regulatory role on the basal activity of the HPA axis.

Consistent with this hypothesis, i.c.v. administration of N-PCT induced hypothalamic expression of CRF mRNA with a subsequent

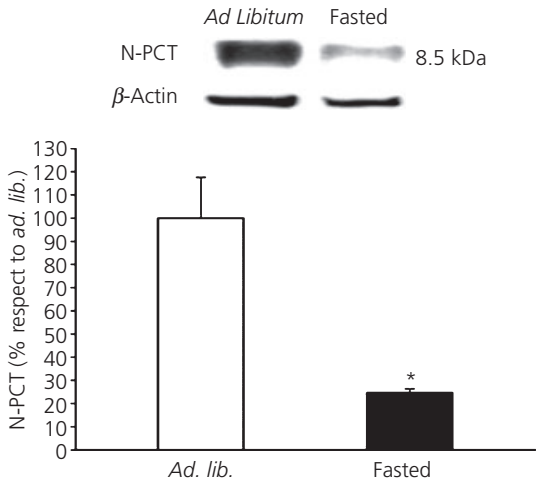

Fig. 9. Expression of aminoprocalcitonin (N-PCT) protein in the hypothalamus of *ad lib.*-fed and 24-fasted rats. Western blotting of rat paraventricular nucleus (PVN) tissue with N-PCT showing a band near 8.5 kDa, confirming the specificity of the monoclonal mouse anti-human N-PCT antibody used (upper panel). Supernatant protein from PVN was separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and blotted on membranes for immunodetection. To verify the protein samples that were loaded, the parallel membranes were probed with anti-β-actin antibody as the internal control. The values were first normalised to β-actin levels and then expressed as relative to the *ad lib.*-fed group (lower panel). Values are the mean $\pm$ SEM (n = 9–11 animals per group). *P < 0.05 versus vehicle *ad lib.*-fed rats.

increase in hypothalamic CRF production. These changes were associated with an increase in plasma concentrations of corticosterone and ACTH, an effect that is dependent of CRF receptor activation (48), and were not attributable to a nonspecific response associated with stress or peptide infusion because hypothalamic CRF content and plasma levels of ACTH were not significantly affected by i.c.v. injection of control aCSF or heat-denatured N-PCT under the same conditions. Because CRF neurones in the parvocellular region of the PVN are the major source of tuberoinfundibular CRF neurones (49, 50), we speculate that N-PCT may stimulate the HPA axis via activation of CRF neurones and subsequent stimulation of CRF receptors that results in the release of ACTH. It is worth noting that many Fos-IR cells without CRF-IR were also observed in the medial parvocellular divisions of the PVN that contain heterogeneous populations of neurosecretory neurones, such as CRF and arginine vasopressin (AVP). Therefore, N-PCT may activate other neurones in addition to CRF-containing neurones. AVP, in addition to CRF, is known to be a potent stimulant of secretion of ACTH from the anterior pituitary (51).

The findings of the present study also show that N-PCT is expressed on astrocytes surrounding CRF neurones. The physiological relevance of this finding remains unclear. In the hypothalamus, astrocytes regulate the secretory activity of neuroendocrine neurones (52). N-PCT is expressed in glial cells that interact in neuroendocrine functional dynamics, located in zones at the interface between brain and cerebrospinal fluid, and within hypothalamic areas such as the ARC and the median eminence (10, 12, 13). Functional CT-Rs have been identified by cDNA cloning in the rat hypothalamus (53) and in cultured rat astrocytes (54). Astrocytes contain the enzyme cyclooxygenase, which, under stimulation, mediates the synthesis of prostaglandins that are involved in HPA axis activation and food intake regulation (55, 56). Activation of central CT-Rs causes a potent reduction in food intake in rats mediated by the release of prostaglandins (57). This is consistent with our studies in rats in which pre-treatment with ibuprofen, an inhibitor of prostaglandin synthesis, was found to block the effects of N-PCT on food intake and prevent an increase in Fos expression induced by N-PCT in key hypothalamic areas regulating appetite (13). These observations indirectly suggest a role of astrocyte N-PCT in the regulation of neuroendocrine responses, although further research is needed to confirm this hypothesis.

In the present study, N-PCT was administered into a lateral cerebral ventricle. Thus, it is difficult to determine whether N-PCT activated CRF-containing neurones in the PVN directly or indirectly. The present study did not address these issues, although the response occurs rapidly, supporting the notion of a direct action of N-PCT on specific receptors localised in hypothalamic cells. The detailed molecular mechanisms of how N-PCT decreases food intake and increases CRF expression require further study.

Fasting in rats is known to decrease the hypothalamic expression of anorectic peptides (58). Therefore, it might be expected that hypothalamic N-PCT expression is reduced during fasting. Earlier reports showed that 24 h of fasting reduces the number of N-PCT immunoreactive cells in the ARC (10). Similar to CRF mRNA expression in the PVN (59), we demonstrated that N-PCT protein levels also decreased in the PVN of the hypothalamus when the animals were fasted for 24 h compared to those in *ad lib.*-fed rats. These observations are potentially critical and support N-PCT as a central satiety factor involved in the control of food intake and the adaptive response to negative energy balance. Further studies investigating the effects of longer-term administration of N-PCT on food intake and body weight will help our understanding of physiological feeding mechanisms and should facilitate the study of eating disorders.

In conclusion, our data indicate that N-PCT affects food intake and body weight partly via CRF-receptor-mediated pathways and suggest that endogenous N-PCT, produced by neurones and astrocytes, stimulates the HPA axis via a direct and/or indirect interaction with CRH-producing neurones of the PVN, resulting in ACTH release and corticosterone secretion. Whether N-PCT exerts similar effects in other species is not known and further investigations will be necessary to establish and characterise the relevance of these findings.

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