Aminoprocacin-mediated suppression of feeding involves the hypothalamic melanocortin system

Eva Tavares,1 Rosario Maldonado,1 and Francisco J. Miñano1,2

1Unidad de Farmacología Experimental y Clínica, Hospital Universitario de Valme, Seville, Spain; and 2Departamento de Farmacología, Pediatría y Radiología, Facultad de Medicina, Universidad de Sevilla, Seville, Spain

Submitted 26 November 2012; accepted in final form 2 April 2013

Tavares E, Maldonado R, Miñano FJ. Aminoprocacin-mediated suppression of feeding involves the hypothalamic melanocortin system. Am J Physiol Endocrinol Metab 304: E1251–E1262, 2013. First published April 9, 2013; doi:10.1152/ajpendo.00590.2012.—Aminoprocacin (N-PCT), a neuroendocrine peptide encoded by the calcitonin-I (CALC-I) gene, suppresses food intake when administered centrally in rats. However, the neural pathways underlying this effect remain unclear. N-PCT and calcitonin receptors (CT-R) have been identified in hypothalamic regions involved in energy homeostasis, including the arcuate nucleus (ARC). Here, we hypothesized an involvement of the hypothalamic ARC in mediating the anorectic effects of central N-PCT based on its content of peptideergic neurons involved in feeding and its expression of N-PCT and CT-R. Fasting strongly reduced expression of the N-PCT precursor gene CALC-I in the ARC, and central immunoneutralization of endogenous N-PCT increased food intake. Intracerebroventricular administration of N-PCT reduced food intake in fed and fasted rats, and its effect was attenuated by a neutralizing anti-N-PCT antibody. Immunohistochemistry for N-PCT showed that it is expressed in astrocytes and neurons in the ARC and is colocalized with anorexigenic proopiomelanocortin (POMC) neurons. Fasting reduced coexpression of N-PCT and POMC, and N-PCT administration activated hypothalamic neurons, including rostral POMC neurons. We also found that N-PCT stimulates POMC mRNA expression in fed and fasted rats, whereas it reduced the expression of orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP) only in fasted rats in which those mRNAs are normally elevated. Finally, we showed that the melanocortin-3/4 receptor antagonist SHU 9119 attenuates the intake-suppressive effect of N-PCT. These data demonstrate that hypothalamic N-PCT is involved in control of energy balance and that its anorexigenic effects are mediated through the melanocortin system.

food intake; aminoprocacin; arcuate nucleus; melanocortin system; proopiomelanocortin; neuropeptide Y

AMINOPROCACIN (N-PCT) was originally described as a 57-amino acid neuroendocrine peptide derived from the amino-terminal half of rat prohormone procalcitonin with bone cell mitogenic properties (6, 7). N-PCT derives from the calcitonin-I gene (CALC-I), which is localized in chromosome 11 (21, 50), a chromosome associated with body metabolism and obesity in humans (8). N-PCT, a highly conserved amino acid peptide with >85% amino acid identity in human and rodents (41), belongs to the calcitonin (CT) peptide family, which is made up by CT, CT gene-related peptide (CGRP), amylin, adrenomedullin, intermedin, and CT receptor-stimulating protein (3). These peptides function as ligands for a complex family of G protein-coupled receptors consisting of the CT receptor (CT-R), CT-R-like receptor, and three receptor-associated modified proteins (40). CALC-I gene products and their receptors are widely distributed in the central nervous system (CNS), and several neural functions have been ascribed to these molecules, including hormone secretion regulation and energy homeostasis, among others (3, 4, 42, 60). Although CT has been considered as an enigmatic hormone (19), and there is not a true ligand for CT-R, N-PCT has the potential to be an endogenous ligand for central CT-R. Recent evidence indicates that N-PCT can function as a ligand for the CT-R and may act as an anorectic signaling molecule in the CNS (47, 54, 55).

The CT-R has been cloned and characterized in different cell types and species (40). Two CT-R isoforms, CTa and CTb, have been described in the rat brain with an overlapping distribution pattern particularly in hypothalamic nuclei involved in feeding behavior, including the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) (4, 23, 48). RT-PCR analysis in combination with laser capture microdissection, in situ hybridization, and immunocytochemistry has demonstrated that a subpopulation of proopiomelanocortin (POMC) neurons and corticotropin-releasing hormone (CRH) neurons selectively expressed the CTa isoform, whereas the CTb isoform was expressed in neuropeptide Y (NPY) neurons (23, 24). These findings suggest that the anorectic effects of N-PCT may be mediated via a possible interaction with CT-R expressed in ARC POMC/NPY neurons.

Intracerebroventricular (icv) administration of N-PCT causes a dose-dependent reduction in food intake and body weight gain in rats via a mechanism unlikely to involve taste aversion (55). N-PCT-immunoreactive cells have been found in brain areas closely associated with food intake and body weight, including the ARC and PVN (34–36). This distribution coincides with that of CT-R, which is expressed abundantly in these hypothalamic nuclei (4, 37). Recent findings show that N-PCT colocalized with CRH in parvocellular neurons of the PVN (54). Moreover, c-Fos expression, a marker of neuronal activity, was observed primarily within the ARC and PVN after icv injection of N-PCT (54, 57). In vitro and in vivo studies suggest that N-PCT, similarly to other neuropeptides of the CT family, like amylin and CT gene-related peptides, can function as a ligand for the CT-R (47, 55, 57). Together, these findings suggest that the anorectic actions of N-PCT likely occur within the hypothalamus. However, the neural pathways underlying these actions of N-PCT are not well understood. One possibility is that N-PCT, like other anorexigenic peptides, alters melanocortin (MC) signaling in the ARC.

The MC signaling in the hypothalamus plays a pivotal role in the integration of signals regulating appetite and energy balance (12). Regulation through this pathway is exerted by two functionally opposing neuronal populations in the ARC that coexpress NPY and agouti-related peptide (AgRP) to
stimulate food intake and POMC and cocaine- and amphetamine-regulated transcript (CART) to induce anorexia (1, 9, 33, 45). Activation of POMC neurons decreases food intake and increases energy expenditure through activation of target neurons expressing melanocortin-3/4 receptors (MC3/4-R) via the release of α-MSH, a posttranslational cleavage product of POMC (30). Activation of neighboring NPY/AgRP neurons counteracts the activity of POMC neurons (10) in part through the release of AgRP, a competitive inhibitor of MC3/4-R (38). Because N-PCT is found together with CT-R in the ARC, CT-Rs are expressed in hypothalamic POMC neurons, and ARC POMC neurons form an integral part of the MC system regulating feeding behavior within the CNS (9), we hypothesized that hypothalamic N-PCT is an anorectic peptide and that this function is mediated via the MC system. Therefore, we investigated the effect of nutritional status on hypothalamic CALC-I gene expression. We also characterized cell types expressing N-PCT in the ARC and explored the colocalization of N-PCT with hypothalamic POMC neurons. Changes in feeding behavior after central immunoneutralization of N-PCT were also studied. Subsequently, we investigated the potential mechanism of action by determining sites of neuronal activation and interaction between N-PCT and other hypothalamic neuropeptides regulating energy homeostasis after icv administration of N-PCT. Finally, using a MC3/4-R antagonist, we established a potential link between N-PCT and the MC system in modulating feeding behavior.

MATERIALS AND METHODS

Materials. N-PCT and the MC3/4-R antagonist SHU 9119 [Nle4,Asp5,D-2-Nal7,Lys10]-cyclo-o-MSH (4–10) amide] were purchased from Bachem (Bubendorf, Switzerland). The monoclonal antibody directed against rat N-PCT was synthesized by Abcam (Cambridge, MA), and the isotype-matched nonimmune mouse IgG2a was purchased from Biogenex (San Ramon, CA). All drugs were dissolved in a rat artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA), which served as the control vehicle. All solutions were passed through a 0.22-μm pore size Millipore filters and stored at −80°C. The same batches and solutions were used for all experiments and contained an endotoxin level of <0.1 ng/ml protein (i.e., below the detection limit of endotoxin assay), as determined by the limulus lysate assay (ICN Biomedicals, Costa Mesa, CA). Fresh aliquots were thawed before each experiment, briefly sonicated, and warmed to 37°C just before use.

Animals. Adult male Wistar rats (Harlan Iberica, Barcelona, Spain) initially weighing 250–300 g were used. The rats were initially housed in group cages (3/cage) in a room with temperature controlled at 22 ± 1°C and a 12:12-h light-dark cycle (lights on at 0900 h, lights off at 2100 h). Standard rodent chow (2014S Teklad; Harlan) and tap water were provided ad libitum throughout the experiments, except where indicated. At least 2 wk before the experiments, the rats were placed in individual cages and housed in a separate room under a reversed 12:12-h light-dark cycle (lights off at 0900 h) with temperature controlled at 25 ± 1°C, in or near the thermoneutral ambient temperature range for rats. Experiments began after 2 wk of acclimatization. Experiments were carried out during the dark phase (except where noted), the physiological feeding time for rodents (51). All experiments were performed in accordance with the Council of the European Communities’ Directive (86/609/EEC) and Spanish regulations (BOE/67:8509/1988) for the use of experimental animals. The protocol was approved by the Animal Care and Use Committee of the University of Seville.

Surgical procedures: icv cannulation and injection. The detailed procedure for implantation of guide cannulas into the rat lateral cerebral ventricle has been described previously (55). Briefly, animals were anesthetized with an intraperitoneal injection of a ketamine (100 mg/kg)-xylazine (5 mg/kg) cocktail. A 22-gauge stainless-steel guide cannula was stereotaxically implanted into the lateral ventricle. The coordinates used were 0.8 mm posterior to the bregma, 1.5 mm lateral to the medline, and 2.5 mm ventral to the skull surface (39). The cannula was attached to the skull with dental cement and bone screws, and a nonsteroidal anti-inflammatory drug, meloxicam (Metacam, 1 mg/kg; Boehringer Ingelheim, Barcelona, Spain), was injected subcutaneously. Animals were allowed ≥7 days recovery before commencement of experimentation. The animals were housed individually after surgery for ≥1 wk and were handled and habituated to the injection procedure daily. Test substances were injected icv by gravity flow (5.0-μl volume) in conscious, lightly restrained rats. Correct cannula positioning was confirmed postmortem by histological examination.

For feeding experiments, cannulated rats were isolated in individual cages without litter. Food intake of each rat was measured for ≥3 days to establish the baseline. On test days, ad libitum-fed rats were weighed, and food was removed 2 h before the beginning of the dark cycle. Other rats were fasted for 24 h. Fasted animals received water ad libitum, but food was removed from the cages 24 h before lights off. This fasting duration resulted in ∼15% weight loss. Rats in both conditions received an icv injection of the drugs tested immediately before the dark phase (except where noted). Immediately after injections, a preweighed amount of food was presented to the animals. Food consumption was measured 1, 2, and 4 h later (except where noted).

Real-time RT-PCR analysis. Four hours after the beginning of the dark phase, rats were euthanized by decapitation. The brains were immediately removed and placed in a brain matrix (Harvard Apparatus) on dry ice, with the ventral surface on top under a dissecting microscope. To remove the hypothalamus, we used the optic chiasm and rostral edge of mamillary bodies as rostral and caudal limits, respectively; the hypothalamic sulci were used as lateral limits. Brain slices containing the whole hypothalamus were prepared, and the entire ARC was excised from the left and right sides. We removed the ARC by cutting between the rostral and caudal limits of the median eminence and 0.5 mm to each lateral side of the median eminence (see Fig. 1) (39). In general, the entire ARC with minimal surrounding tissue was taken for analysis. Hypothalamic blocks containing the ARC were preserved immediately in an RNA stabilization solution (Qiagen, Hilden, Germany) and stored at −80°C until RNA extraction and RT-PCR analysis.

RT-PCR was performed essentially as described previously for CALC-I and CRH gene expression in hypothalamic rat tissue (54, 56). Total RNA was isolated using Tripure reagent (Roche Diagnostics, Basel, Switzerland) and then reverse-transcribed into cDNA using the Quantitect Enzyme Reverse Transcriptase Kit (Qiagen) according to the manufacturer’s instructions. SYBR Green real-time RT-PCR was performed with the Stratagene Mx3005P sequence detection system (Agilent Technologies) using SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany). Primers for CALC-I were designed using the software Beacon Designer (Premier Biosoft, Palo Alto, CA) according to the rat CALC-I sequence NM_017338 (National Center for Biotechnology Information, National Database). The primers were 5′-CGGCAGCAGTCAGTC-3′ (forward) and 5′-AGATGCT-GACAACCAGGAAGG-3′ (reverse) (Eurogentec, Liege, Belgium). The primers for the target rat genes (POMC: Rn_Pome2.1_SG; NPY: Rn_Npy_1_SG; AgRP: Rn_Agrp_1_SG) and the reference gene glyceraldehyde-3-phosphate dehydrogenase (rat GPDH; Rn_Gapd_1_SG) were predesigned and validated by Qiagen (QuantiTect Primer Assay). Threshold cycle values were determined automatically using the software supplied by Stratagene (MxPro Software 3.20). Primer specificity was verified by melt curve analysis. Relative gene expres-
of the 2
real-time RT-PCR experiment. ARC, arcuate nucleus; DM, dorsomedial nu-
cleus; f, fornix; OX, optic chiasm; M, mammillary bodies; ME, median
eminence; VM, ventromedial nucleus; 3V, 3rd ventricle.

Expression following PCR analysis are expressed as relative to
GAPDH mRNA content and referred to as relative expression.

Tissue preparation for immunohistochemistry. Rats were deeply
anesthetized with a ketamine (100 mg/kg)-xylazine (5 mg/kg) cocktail
and transcardially perfused with PBS (pH 7.4), followed by 4%
paraformaldehyde in 0.1 M PBS for fixation. The brains were re-
moved and postfixed in the same fixative for 2 h, cryopreserved in
30% sucrose-PBS for 48 h at 4°C, and stored at
−20°C until they were required for immunohistochemistry.

Multiple fluorescence immunohistochemistry. To characterize cell
types expressing N-PCT, hypothalamic sections were subjected to
double-labeling immunohistochemistry for a neuronal nuclei (NeuN)
or a glial marker [glial fibrillary acidic protein (GFAP)], as described
previously (54). To determine whether POMC neurons express N-
PCT, double-labeling immunohistochemistry was performed using a
rabbit anti-POMC antibody (57). Briefly, after quenching of autofluo-
rescence with 0.3 M glycine and saturation of nonspecific sites with
3% normal donkey serum (Jackson Laboratories, West Grove, CA),
sections were incubated for 72 h at 4°C with a mouse anti-N-PCT
monoclonal antibody (1:100; Abcam) or incubated overnight at 4°C
with rabbit anti-NeuN antibody (1:500; Millipore), anti-GFAP anti-
body (1:500; Dakocytomation, Carpinteria, CA), or anti-POMC anti-
body (1:1,000; Phoenix Pharmaceuticals). The specificity of the
anti-N-PCT antibody used in this study was determined previously in
the rat hypothalamus by Western blot and immunohistochemistry
experiments (54). After washing, the sections were incubated with
FITC-conjugated donkey anti-mouse IgG (1:200; Jackson Laborato-
ries) and TRITC-conjugated donkey anti-rabbit IgG (1:200; Jackson
Laboratories). Omission of primary antibodies and substitution of the
primary antibody with specific blocking peptides confirmed antibody
specificity. Immunohistochemistry was performed on sections be-
tween −1.80 and −3.60 from the bregma (39). Sections were
mounted on slides, coverslipped with anti-fading mounting medium
(Vectashield; Vector Laboratories), and examined under a fluorescent
Olympus BX41 microscope. Sections were postfixed on glass slides
under the same conditions to avoid the possibility of varied staining.

Colocalization pictures were the combination of each corresponding
fluorescent signals. Image-editing software (Adobe Photoshop) was
used to combine photomicrographs into plates. Double-stained sec-
tions were examined with a laser-scanning confocal microscope, as
described below.

Laser-scanning confocal microscopy and quantitative colocaliza-
tion analysis. Images were acquired using a confocal microscope
(Zeiss LSM 7 DUO) in sequential mode with a ×40 (NA 0.95)
objective, 12-bit resolution and two lines averaging. The conditions
for FITC channel were excitation at 488 nm, emission 493–558 nm,
pinhole at 1.03 Airy units, and section thickness of 1.1 μm. For
TRITC channel, we used the following conditions: excitation at 561
nm, emission 566–585 nm, pinhole at 0.75 Airy units, and section
thickness of 1.0 μm. We chose the most intense signal plane between
several z-axis positions of the slice as the most representative for the
analysis. Twelve images were stitched to reconstruct the whole ARC
in each slice. For quantitative colocalization analysis, we used the
corresponding ZEN 2010 imaging software module (Carl Zeiss).

Three square regions of 400 × 400 pixels were located randomly
in each nucleus of every image, and the Manders’ overlap coefficient
(29), which indicates quantitative colocalization on a scale of 0 (no
overlap) to 1 (100% overlap), was calculated for statistical analysis.

Double-labeling immunohistochemistry for c-Fos and POMC. Im-
munohistochemical staining for c-Fos and POMC was performed by
using a sequential two-color avidin-biotin immunoperoxidase com-
plex technique according to a previously described method (16, 49,
57). Briefly, endogenous peroxidase activity was blocked with 3%
hydrogen peroxide in methanol. Nonspecific binding was prevented
by using 10% normal goat serum in PBS for 1 h. Then, sections were
washed and incubated with rabbit anti-c-Fos antisemur (1:3,000,
sc-52; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-POMC
antisemur (1:2,000; Phoenix Pharmaceuticals) for 48 h at 4°C. The
secondary antibody was goat anti-rabbit IgG conjugated to biotin
(Merck Millipore, Darmstadt, Germany). The commercially available
avidin-biotin immunoperoxidase complex system (Vectastain Elite
ABC kit; Vector Laboratories, Burlingame, CA) was used to visualize
bound antibody. The color reaction was produced with nickel-intensi-
ted diaminobenzidine (Ni-DAB; Sigma) in the presence of 0.01%
hydrogen peroxide. This results in a black reaction product in singly
stained sections. For double labeling, brain sections were processed
for the localization of c-Fos, as described above. Subsequently, we
performed the second immunostaining, incubating the tissue in the
POMC antisemur to label POMC neurons. Sections were processed
using only DAB (without nickel) as chromogen, resulting in brown
staining. Cells with dark blue nuclear staining were c-Fos positive,
and cells with brown cytoplasmic staining were POMC immunoreac-
tive.

Image analyses and cell counting. For data acquisition and quanti-
fication of c-Fos and/or POMC-labeled cells in the ARC, positive
cells were counted on an Olympus BX41 microscope fitted with a grid
reticle, with the investigator blinded to study conditions. Using the
rat brain atlas of Paxinos and Watson (39) as an anatomic guide, the
ARC was divided into two areas (defined as rostral and caudal ARC)
by 10.220.33.2 on October 21, 2017 http://ajpendo.physiology.org/ Downloaded from
POMC-immunoreactive neurons, at
1.8 mm posterior to the bregma
and continued caudally to the appearance of the dorsomedial hypo-
thalamic nucleus, marking the most rostral extent of the distribution
of POMC-immunoreactive neurons, at −1.8 mm posterior to the bregma
and continued caudally to the appearance of the dorsomedial hypo-
thalamic nucleus ~−2.56 mm posterior to the bregma. Caudal ARC
 corresponded to a region from −2.8 to 3.6 mm posterior to the bregma
and included the most caudal region of the ARC and disappearance
of POMC-immunoreactive neurons. For each region, four representative
matched sections were examined bilaterally in each rat to determine

Fig. 1. Schematic figure demonstrating the brain region dissected for the
real-time RT-PCR experiment. ARC, arcuate nucleus; DM, dorsomedial nu-
cleus; f, fornix; OX, optic chiasm; M, mammillary bodies; ME, median
eminence; VM, ventromedial nucleus; 3V, 3rd ventricle.

E1253
the total number of c-Fos- or POMC-immunoreactive cells and double-labeled cells. Colocalization was expressed as a percentage of the total number of POMC-immunoreactive cells expressing c-Fos.

**Study 1: effect of nutritional status on hypothalamic CALC-I mRNA expression.** Two groups of rats (ad libitum fed and fasted for 24 h, n = 16) were euthanized 4 h after the beginning of the dark phase. Whole hypothalami were dissected, and the entire ARC was removed, preserved in an RNA stabilization solution, and stored at −80°C until it was processed for RT-PCR analysis as described above.

**Study 2: characterization of cell types expressing N-PCT.** To determine cell types expressing N-PCT, rats (n = 4) were anesthetized and transectively perfused 90 min after the beginning of the dark phase. Brains were removed and processed for double-labeling immunohistochemistry for N-PCT, and NeuN or GFAP was performed on sections, including the ARC, as described previously (54).

**Study 3: effect of nutritional status on N-PCT expression in POMC-expressing neurons.** For this study, rats were either fed ad libitum (n = 4) or fasted for 24 h (n = 6). Ninety minutes after the beginning of the dark phase, rats were anesthetized and fixed, and hypothalamic sections, including the ARC, were prepared for double-labeling immunohistochemistry for N-PCT and POMC, as described above.

**Study 4: effect of i.c.v administration of anti-N-PCT antibody on food intake.** Ad libitum-fed rats (n = 8/group) were injected i.c.v with either control IgG (2 μg protein/rat) or anti-N-PCT antibody (0.5, 1, and 2 μg protein/rat) in the early light phase, when rats normally eat very little. Food intake was measured 60 min after injections.

**Study 5: effect of i.c.v administration of anti-N-PCT antibody on N-PCT-induced suppression of food intake in ad libitum-fed and fasted rats.** Two groups of rats, ad libitum fed and fasted for 24 h (n = 10–12/group), were injected i.c.v with either 1) aCSF + aCSF, 2) anti-N-PCT antibody (2 μg protein/rat) + aCSF, 3) aCSF + N-PCT (1 nmol/rat, 5 μg/rat), or 4) anti-N-PCT antibody (2 μg protein/rat) + N-PCT (1 nmol/rat). The dose of N-PCT has been shown to suppress food intake in rats in preliminary dose-response studies (55). The first injection occurred 30 min before the beginning of the dark phase and was followed 30 min later by the second injection. After the second injections, preweighed food was returned to all subjects. Food intake was measured 1, 2, and 4 h later. Each rat received only one combination of treatments.

**Study 6: effect of i.c.v injection of N-PCT on c-Fos in ARC POMC neurons.** Two weight-matched groups, ad libitum fed and fasted for 24 h (n = 10/group), were injected into the lateral ventricle with either control aCSF or 1 nmol N-PCT at the beginning of the dark phase. To prevent the induction of c-Fos expression due to feeding, food was immediately removed from the cages of injected rats. Ninety minutes later, rats were deeply anesthetized and transectively perfused. Brains were removed and processed for double-labeling immunohistochemistry for c-Fos and POMC, as described above.

**Study 7: effect of i.c.v N-PCT on the gene expression of hypothalamic neuropeptides known to affect appetite.** Rats were separated into two weight-matched groups (n = 10/group); one was allowed ad libitum access to food and the other was fasted for 24 h before i.c.v injections. This fasting duration is effective to demonstrate coexpression of orexigenic and anorexigenic neuropeptides in fasting-activated hypothalamic neurons (2, 15, 44). On the day of the experiment, each rat received an i.c.v injection of either control aCSF or 1 nmol N-PCT immediately before the onset of the dark cycle. Rats were euthanized by decapitation 4 h after injections. The brain was immediately removed from the skull, and the ARC was dissected out from the whole hypothalamus, as described above. Hypothalamic blocks containing the ARC were preserved in an RNA stabilization solution (Qiagen) and stored at −80°C until processing for RT-PCR analysis.

**Study 8: effect of melanocortin receptor antagonism on N-PCT-elicited suppression of food intake in ad libitum-fed and fasted rats.** Two weight-matched groups of rats, ad libitum fed and fasted for 24 h (n = 10–12/group), were injected i.c.v with either 1) aCSF + aCSF, 2) SHU 9119 (0.1 nmol/rat) + aCSF, 3) aCSF + N-PCT (1 nmol/rat), or 4) SHU 9119 (0.1 nmol/rat) + N-PCT (1 nmol/rat). The dose of SHU 9119 has been reported to inhibit reductions in feeding induced by i.c.v injection of the MC receptor agonist MTH in rats but does not affect food intake or body weight alone (13). The first injection occurred 30 min before dark phase and was followed 30 min later by the second injection. Immediately after the second injection, a preweighed amount of food was presented to the animals. Food intake was measured 1, 2, and 4 h later. Every rat was subjected to each treatment, with ≥5 days between tests.

**Statistical analysis.** Data were analyzed using parametric statistics (Sigma Plot 11; Systat Software, San Jose, CA). Two-way repeated-measures ANOVA, followed by Student-Newman-Keuls test, was used for analysis of food intake. For other comparisons, i.e., mRNA levels, either a one-way ANOVA (for more than a 2-group comparison) followed by Student-Newman-Keuls test or an unpaired Student’s t-test (2-group comparison) was used. P values <0.05 were considered statistically significant.

**RESULTS**

**Expression of CALC-I gene is reduced during fasting.** The expression levels of hypothalamic neuropeptides that reduce appetite are often elevated in states of positive energy balance and reduced in states of negative energy balance (32, 33). Hypothalamic CALC-I gene expression, the gen precursor of N-PCT, was significantly reduced by 80% in rats fasted for 24 h (Fig. 2A). This is consistent with the hypothesis that N-PCT is an endogenous anorectic hypothalamic neuropeptide.

**Characterization of cell types expressing N-PCT in the ARC.** To characterize cell types expressing N-PCT in the ARC, we used multiple immunofluorescence labeling. The immunohistochemical analysis of hypothalamic sections indicates that N-PCT-immunoreactive cells were astrocytes and neurons and localized the rostral ARC (Fig. 3, A–F). Colocalization of N-PCT and GFAP immunoreactivities confirmed N-PCT expression by astrocytes (Fig. 3C). N-PCT was also colocalized with NeuN, a marker of neuronal cell bodies and nuclei in the ARC, particularly in the rostral ARC, where the majority of POMC neurons reside (10, 46) (Fig. 3F). We also found N-PCT-immunoreactive cells in other hypothalamic regions surrounding the ARC and involved in energy homeostasis, including the median eminence, the supraoptic and dorsomedial nuclei, and the ventromedial and lateral hypothalamic areas (data not shown).

Confocal immunofluorescence microscopy showed the expression of N-PCT on ARC cells in all examined samples. Quantitative colocalization analysis by Manders’ coefficient showed that 68% (overlap coefficient: 0.68 ± 0.01) of N-PCT ligands (green) overlap with GAFP ligands (red), implying that N-PCT is expressed in astrocytes. The analysis also indicated that 55% (overlap coefficient: 0.55 ± 0.01) of N-PCT ligands overlap with NeuN, suggesting that N-PCT is also expressed in ARC neurons. Fasting markedly reduced the degree of overlap between N-PCT and GAFP (28% decrease) or NeuN (16% decrease) (overlap coefficients: fed, 0.49 ± 0.01; fasted, 0.46 ± 0.01; P < 0.001 vs. ad libitum-fed rats, respectively).

These findings suggest a potential role for N-PCT on ARC cells and open the possibility that POMC neurons may mediate, at least in part, the anorexigenic effects of N-PCT.

**N-PCT is colocalized with POMC in the ARC.** To corroborate this hypothesis, we next performed colocalization studies of N-PCT and POMC, using double-immunofluorescent staining. We also determined the effect of nutritional status on...
hypothalamic N-PCT expression in ARC POMC neurons. We observed a prominent presence of N-PCT protein in the ARC (Fig. 3G). Figure 3, G–L, depicts a representative section with colocalization of POMC and N-PCT immunoreactivity in the rostral ARC at bregma −2.30 mm, where most of the population of POMC neurons resides (46). We found that in both ad libitum-fed (Fig. 3, G–I) and fasted conditions (Fig. 3, J–L), N-PCT was expressed by neurons and by cells that present glial morphology. We also found that the number of cells expressing N-PCT (Fig. 3, G and J) or POMC (Fig. 3, H and K) in the rostral ARC was markedly reduced in fasted rats (N-PCT: fed 71.8 ± 9.1 v. fasted 39.1 ± 1.2; POMC: fed 57.1 ± 4.8 vs. fasted 29.8 ± 6.0; P < 0.01). Quantitative double labeling revealed that the percentage of ARC POMC neurons that express N-PCT was reduced significantly in rats fasted for 24 h (fed 41.8 ± 2.4% vs. fasted 32.9 ± 1.5%, P < 0.01). However, the percentage of N-PCT cells expressing POMC was increased slightly (fed 40.7 ± 3.6% vs. fasted 45.7 ± 4.1%; P > 0.05). These results indicate that N-PCT is produced locally within the ARC, including within POMC neurons, astrocytes, and possibly other neurons. These data also suggest that N-PCT, acting via a paracrine or neurocrine effect, plays an important role in feeding regulation.

Intracerebroventricular administration of anti-N-PCT antibody stimulated food intake and attenuated the anorectic effect of N-PCT. To determine whether endogenous N-PCT signaling influences food intake, we administered anti-N-PCT antibody icv to ad libitum-fed rats in the early light phase. Rats injected with anti-N-PCT antibody ate significantly more than rats injected with control IgG antibody, suggesting that endogenous N-PCT may restrain appetite (Fig. 2B). No adverse reactions such as sedation or ataxia were observed.

A subsequent experiment determined whether anti-N-PCT antibody could specifically block N-PCT anorectic action. As depicted in Fig. 4, icv administration of N-PCT alone (1 nmol) decreased food intake significantly in both ad libitum-fed (Fig. 4A) and 24-h-fasted (Fig. 4B) conditions, and this effect was attenuated by the antibody at 1, 2, and 4 h. Furthermore, icv administration of anti-N-PCT antibody to ad libitum-fed and fasted rats increased food intake in the early light phase.

N-PCT activates POMC neurons in the ARC. To determine whether icv administration of N-PCT resulted in activation of arcuate POMC neurons, we performed colocalization studies of c-Fos and POMC in ad libitum-fed and 24-h-fasted rats after icv administration of N-PCT. Intracerebroventricular injection of N-PCT in fed animals decreased feeding significantly over the 90-min test period (vehicle: 3.8 ± 0.2 g; N-PCT: 0.9 ± 0.1 g; P < 0.001 vs. control). Figure 5 shows a quantitative analysis of c-Fos- and POMC-like immunoreactive cells in the rat ARC after icv administration of either vehicle (aCSF) or N-PCT in ad libitum-fed (Fig. 5A) and fasted rats (Fig. 5B). Both groups of N-PCT-treated rats showed a significant increase in the number of c-Fos, POMC, and POMC neurons expressing c-Fos in the ARC (P < 0.001, P < 0.05, and P < 0.05, respectively, vs. control aCSF). Representative images of the effects of N-PCT on the expression of c-Fos in POMC-immunoreactive neurons in the ARC are summarized in Fig. 6 (ad libitum-fed: Fig. 6, A and B; fasted: Fig. 6, D and E). The increased activation of arcuate POMC neurons induced by N-PCT was associated with a significant increase in the percentage of rostral POMC-expressing neurons exhibiting c-Fos immunoreactivity in both ad libitum-fed (Fig. 6C) and 24-h-fasted rats (Fig. 6F). However, N-PCT did not cause significant changes in c-Fos expression in POMC neurons within the caudal ARC compared with their respective controls (Fig. 6, C and F). These results suggest that POMC neurons are a target site for the anorectic effect of N-PCT.

Intracerebroventricular administration of N-PCT prevented fasting-induced changes in CALC-I, POMC, NPY, and AgRP mRNA levels in the ARC. To examine this possibility and to determine whether N-PCT alters the expression of other peptides associated with energy homeostasis, hypothalamic CALC-I, POMC, AgRP, and NPY mRNA levels were measured in N-PCT-treated rats. Intracerebroventricular administration of N-PCT to rats fed ad libitum increased POMC and CALC-I mRNA levels significantly (P < 0.001; Fig. 7, A and B, respectively) but did not alter AgRP or NPY mRNA levels in the ARC compared with aCSF-treated rats (Fig. 7, C and D, respectively). To assess whether the changes in gene expression associated with food deprivation could be influenced by N-PCT, fasted rats were infused icv with either N-PCT or
aCSF. In normal male rats, a period of 24 h of fasting reduced POMC and CALC-I mRNA levels by 40 and 80% (Fig. 7, A and B, respectively) and increased NPY and AgRP mRNA levels by 60 and 150% in the ARC (Fig. 7, C and D, respectively) compared with ad libitum-fed controls. However, icv administration of N-PCT reversed the fasting-induced increase in NPY and AgRP mRNA levels and the decrease in POMC and CALC-I mRNA levels such that there was no significant difference between the levels in these fasted rats and those that were fed ad libitum (Fig. 7, A–D). These findings suggest a hypothalamic autocrine/paracrine role for N-PCT in energy homeostasis.

**DISCUSSION**

The neuroendocrine peptides N-PCT and CT-R have been localized in rat hypothalamic structures involved in the control of feeding behavior, including the PVN and ARC (34–36). Indeed, N-PCT expression is downregulated in the hypothalamus of fasted animals (54, 55). Intracerebroventricular injection of N-PCT results in a significant decrease in food intake and body weight. In addition, N-PCT induces Fos expression in these hypothalamic nuclei (54, 55) and stimulates the hypothalamic-pituitary-adrenal (HPA) axis (22). Altogether, these observations strongly suggest that N-PCT plays a role in energy homeostasis by regulating appetite and energy expenditure. However, the mechanism by which N-PCT regulates food intake remains unknown. The presence of a high density of N-PCT in the ARC prompted us to examine whether N-PCT may interact with the NPY/POMC system to exert its anorexigenic activity.

The present data show that fasting strongly reduces expression of the N-PCT precursor gene CALC-I in the ARC and that central immunoneutralization of N-PCT increases food intake, supporting the hypothesis that hypothalamic N-PCT may act as an endogenous anorectic signal. The observation that icv administration of N-PCT reduced food intake in fed and fasted rats and that its anorectic effect was attenuated by immunoneutralization with an anti-N-PCT antibody further supports that the effect is specific to N-PCT.

To characterize cell types expressing N-PCT in the ARC, we examined the cellular localization of N-PCT in the rat ARC in...
fed and fasted rats. N-PCT-immunoreactive fibers and cells have been observed in several areas of the adult rat brain but are found in the highest concentrations in appetite control hypothalamic nuclei expressing high density of CT-R, including the ARC and PVN (4, 34, 37, 54, 57). In the present study, we found that N-PCT is produced locally within the ARC, including within POMC neurons, astrocytes, and possibly other neurons. We also found that fasting reduces the coexpression of N-PCT and POMC, thus providing anatomic and functional evidence for a possible role of N-PCT in feeding. We also found N-PCT fibers and/or terminals in contact with or in close proximity to POMC neurons, indicating that N-PCT axon terminals make synaptic contacts with POMC neurons in the ARC. These findings suggest that N-PCT may function in a paracrine manner to exert its anorectic effect in part by modulation of POMC neuron activity at sites outside of the ARC. However, the existence of morphofunctional interactions between N-PCT and POMC neurons depends on the possibility that ARC POMC neurons express CT-R. Which neurons/cells carry the N-PCT receptor that may mediate the interaction between N-PCT and the melanocortin system? Two major rat CT-R isoforms, CTa and CTb, have been described in rat ARC (23). The CTa isoform is expressed specifically in POMC neurons, whereas the CTb isoform is expressed in NPY neurons. Altogether, these observations suggest that the feeding response evoked by N-PCT may be directly or indirectly modulated by CT-R (23, 42, 55). However, there is no reliable means of pharmacologically distinguishing these receptors, and a selective antagonist for the CT-R is lacking.

The present study shows that N-PCT is expressed in neurons and astrocytes surrounding POMC neurons, suggesting that N-PCT may act as a paracrine-signaling molecule in the hypothalamus. The relevance of these findings is unclear but...
emphasizes the potential role of N-PCT in energy homeostasis (34). In the hypothalamus, astrocytes regulate the secretory activity of neuroendocrine neurons via release of prostaglandins in response to cell-cell signaling initiated by neurons and glial cells (58). The activation of astrocytes leads to the release of gliotransmitters that trigger rapid responses in neighboring cells and thus contribute to the region-specific homeostatic regulation of neuronal function (18). Consistent with these findings, we have shown previously that inhibition of prostaglandin synthesis attenuates the effects of N-PCT on food intake (57). Therefore, we speculate that astrocytes are involved, at least in part, in the anorexigenic effect of N-PCT.

The ARC is believed to be an important center for integrating circulating energy signals (9). Neuroanatomic studies have shown that a population of caudal ARC neurons is able to synthesize both an agonist for an anabolic neuroendocrine

Fig. 6. N-PCT activates rostral ARC POMC neurons. Photomicrographs of ARC coronal sections immunolabeled for c-Fos and POMC. Ad libitum-fed (A) and 24 h-fasted rats (D) were injected icv with control aCSF vehicle (A and D) or N-PCT (1 nmol/rat) (B and E) at the beginning of the dark phase and euthanized 90 min later. N-PCT increased the number of c-Fos-positive neurons (black) in the ARC of ad libitum-fed (B and C) and fasted rats (E and F). The higher magnification (boxed areas in B and E) showed that a proportion of these activated neurons colocalized with rostral POMC neurons (gray). Scale bars (A, B, D, and E), 100 μm; scale bars in B and E, insets, 50 μm. Coronal sections were cut at 30 μm. Bregma: −2.30. C and F: percentage of rostral and caudal POMC neurons containing c-Fos immunoreactivity (ir) in ad libitum-fed (C) and fasted rats (F). Each bar represents the mean ± SE of 4–6 rats/group. *P < 0.05 and ***P < 0.005 vs. control group treated with aCSF.

Fig. 7. N-PCT regulates POMC, calcitonin-I (CALC-I), neuropeptide Y (NPY), and agouti-related peptide (AgRP) mRNA expression in the ARC. Levels of POMC, CALC-I, AgRP, and NPY mRNA in the ARC were determined by RT-PCR in rats fed ad libitum (n = 10) or fasted for 24 h (n = 10). N-PCT (1 nmol) or an equivalent volume (2 μl) of aCSF was administered icv immediately before the dark phase (n = 5/group); icv administration of N-PCT prevented fasting-induced upregulation of hypothalamic AgRP and NPY mRNAs as well as downregulation of POMC and CALC-I mRNAs. ***P < 0.001 vs. aCSF-treated ad-libitum fed rats; +++P < 0.001 vs. aCSF-treated fasted rats.
system (NPY) and an antagonist for a catabolic system (AgRP). In contrast, anorexigenic POMC-expressing neurons appeared to form a separate population in the rostral ARC (14, 46). To identify specific sites in the ARC that are activated by N-PCT, we used c-Fos as a marker of neuronal activation (20). Intracerebroventricular injection of N-PCT to ad libitum-fed and fasted rats causes neuronal activation of a subpopulation of rostral ARC POMC neurons. In contrast, our neuroanatomic studies revealed that N-PCT has no significant effect in neurons located in the caudal portion of the ARC, indicating that ARC neurons exhibit regional sensitivity to N-PCT. Therefore, these findings suggest that POMC neurons in the hypothalamus may mediate N-PCT's signaling via POMC-derived peptides in the CNS.

To further investigate cellular mechanisms involved in the anorectic effect of N-PCT, the expressions of several key neuropeptides implicated in regulating feeding were measured in the ARC of ad libitum-fed and 24-h-fasted rats. Compelling evidence indicates that expression of feeding-related neuropeptides in ARC neurons is regulated by the energetic condition of the organism (17, 25, 28). In particular, the expression of orexigenic peptides NPY and AgRP is potently upregulated by fasting (5- to 10-fold increase compared with basal) (5, 32). In opposition, the levels of anorexigenic POMC are only modestly decreased with fasting (20–50% decrease compared with basal) (31, 44). N-PCT, injected icv, stimulates POMC mRNA in fed and fasted rats, whereas it reduces the expression of NPY and AgRP only in fasted rats, in which those mRNAs are normally elevated. Altogether, these data strongly suggest N-PCT exerts its anorectic action by enhancing activity of the MC system. The fact that POMC neurons express the CTα isoform, whereas NPY neurons express the CTβ isoform (23), suggests that N-PCT may exert complex effects on POMC and NPY neuron activity, depending, for instance, on the relative affinity of each receptor subtype. It is thus tempting to speculate that N-PCT may modulate, via an autocrine and/or paracrine effect, the production of these neuropeptides in the ARC according to the variations of energetic status of the animal. It is also interesting to note that the effect of N-PCT on POMC, NPY, and AgRP mRNA levels is accompanied by a significant decrease in CALC-I gene expression. These findings suggest a model in which N-PCT upregulates its own expression via an endogenous/autoregulatory loop. However, the detailed molecular mechanism of how N-PCT increases CALC-I gene expression remains unknown. Whether these findings have an impact on the feeding response to N-PCT will require further investigation.

Finally, using a MC3/4-R antagonist, we established a potential link between N-PCT and the MC system in modulating feeding behavior. We hypothesized that blockade of downstream MC receptors will block or attenuate the anorectic action of N-PCT. Intracerebroventricular pretreatment with the
MC3/4-R antagonist SHU 9119, at a dose that has no effect on its own, attenuated the intake-suppressive effect of N-PCT in both fed and fasted rats. Altogether, our results suggest that N-PCT-induced suppression of food intake is mediated through activation of the hypothalamic MC system.

POMC neurons in the ARC are known to be primary recipients of circulating nutritional information, and their output to thePVN via the MC4-R can strongly inhibit food intake (53). These neurons project to feeding-related areas of the CNS, including the PVN (11). Although melanocortin receptors are expressed in a number of distinct regions in the CNS that could mediate the effects of \( \alpha \)-MSH on energy balance, there is substantial evidence to implicate the hypothalamic PVN as a major center for the action of the MC signaling system on the regulation of appetite and satiety. Microinjections of \( \alpha \)-MSH or \( \alpha \)-MSH agonists directly into the PVN fully replicate reduced feeding responses observed after icv \( \alpha \)-MSH administration (61). Conversely, injection of the \( \alpha \)-MSH antagonist SHU 9119 into the PVN has a potent effect to increase feeding (26). Recently, a role of N-PCT as an activator of the HPA axis has been documented (54). Intracerebroventricular administration of N-PCT stimulates CRH mRNA expression in the PVN, releases CRH, and increases ACTH and glucocorticoid plasma levels in rats. However, it is not yet clear whether N-PCT’s effects on the HPA axis occur via direct regulation of CRH-producing neurons or via indirect pathways. Thus, it is also possible that N-PCT will mediate part of its anorectic effect via activation of ARC POMC neurons, which in turn project and activate MC receptors expressed in anorexigenic paravascular PVN neurons (52). Altogether, our data support a mechanistic model in which N-PCT suppresses food intake via activation of CT-R in the hypothalamus. This model suggests that ARC POMC expression is regulated in an autocrine and/or paracrine manner via presynaptic CTa receptors present in POMC neurons and acting to increase POMC expression. During fasting, N-PCT inhibits the expression of the orexigenic neuropeptides NPY and AgRP via presynaptic CTb receptors present in NPY/AgRP neurons (see Fig. 9). Although further research is required to verify this hypothesis, our observations add neuroanatomic and functional support to the concept that N-PCT is involved in the hypothalamic control of energy homeostasis.

The present data also suggest that N-PCT interacts with the hypothalamic melanocortin system in ways similar to leptin. Leptin is hypothesized to affect food intake not only by stimulating catabolic effector pathways such as the MC system but also by inhibiting anabolic signaling systems such as NPY/AgRP neurons in the ARC. Leptin receptors are expressed particularly in the ARC, particularly in neurons expressing POMC and CART, and in neurons expressing NPY and AgRP (2, 43, 59). Intracerebroventricular injection of leptin results in reduced expression of NPY and AgRP and increased expression of POMC (14). In addition, administration of a MC4-R-blocking agent prevents the ability of leptin to inhibit food intake acutely (46). The apparent redundancies in the effects of N-PCT and leptin on peptideergic neurons involved in the control of energy balance suggest that leptin and N-PCT may interact functionally to regulate food intake. However, in contrast to leptin, which is a circulating hormone produced outside the ARC, N-PCT is produced locally within the ARC, including within POMC neurons, astrocytes, and possibly other neurons. Further studies to assess whether leptin and N-PCT would act synergistically or additively when co-administered into the ventricular system of the brain are necessary.

In conclusion, the present report shows that hypothalamic N-PCT is involved in control of food intake and that its anorexigenic effects are mediated through the MC system. Our data also suggest that N-PCT-containing cells in the ARC may be part of the hypothalamic neuropeptide circuitry involved in control of energy balance. Characterization of the central effect of N-PCT on feeding should stimulate further research into the physiological functions of this peptide, and mechanistic understanding mediating the anorectic function of N-PCT may lead to the development of new treatments for metabolic disorders such as obesity.

ACKNOWLEDGMENTS

We are grateful to Ana Maria Garcia and Pablo Martinez-Aguado for technical support. Some experiments were performed in the Biology and Microscopy Central Services of the Universidad de Sevilla. We thank Dr. Juan Luis Ribas for help with the confocal microscope.

DISCLOSURES

The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

E.T. and F.J.M. contributed to the conception and design of the research; E.T. and R.M. performed the experiments; E.T., R.M., and F.J.M. analyzed the data; E.T. and F.J.M. interpreted the results of the experiments; E.T., R.M., and F.J.M. prepared the figures; E.T., R.M., and F.J.M. approved the final version of the manuscript; F.J.M. drafted the manuscript; E.T. and F.J.M. edited and revised the manuscript.

REFERENCES


