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Neuropeptide S Stimulates the Hypothalamo-Pituitary-Adrenal Axis and Inhibits Food Intake

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Neuropeptide S (NPS) is a recently discovered peptide shown to be involved in the modulation of arousal and fear responses. It has also been shown that lateral ventricle administration of NPS causes a significant decrease in food intake. Neuropeptides involved in the modulation of arousal have been shown to be involved in the regulation of the hypothalamo-pituitary adrenal (HPA) axis and food intake. In this study, we have examined the effect of intracerebroventricular (ICV) administration of NPS on behavior, regulation of the HPA axis, and food intake. ICV NPS significantly increased plasma ACTH and corticosterone 10 and 40 min after injection, respectively. A single ICV injection of NPS caused a significant increase in rearing activity as well as ambulatory movement for up to 45 min after injection. We then studied the effect of paraventricular nucleus (PVN) administration of NPS on the regulation of the HPA axis, behavior, and food intake. There was a significant increase in plasma ACTH and corticosterone after a single NPS PVN injection. Incubation of hypothalamic explants with increasing concentrations of NPS caused a significant increase in CRH and arginine vasopressin release. In addition, PVN administration of NPS dose-dependently inhibited food intake in the first hour after injection, although no effect on food intake was seen after this time. PVN administration of NPS caused a significant increase in rearing activity. These data demonstrate a novel role for NPS in the stimulation of the HPA axis. (*Endocrinology* 147: 3510–3518, 2006)

NEUROPEPTIDE S (NPS) is a recently identified 20-amino-acid peptide that has been shown to modulate arousal and fear responses. In the rat, the NPS precursor mRNA has been found to be expressed in a large number of tissues with the highest level of expression found in the brain and the thyroid, salivary, and mammary glands (1). Within the brain, the highest level of expression is found in the brain stem principally in the locus ceruleus (LC), principle sensory 5 nucleus, and the lateral parabrachial nucleus. Low-level expression is also found in the dorsomedial hypothalamic nucleus and the amygdala. The effects of NPS are mediated via the previously orphan G protein-coupled receptor, the NPS receptor (NPS-R). NPS-R mRNA is expressed throughout the central nervous system with the highest levels of expression found in the cortex, thalamus, hypothalamus, and amygdala (1). Intracerebroventricular (ICV) administration of NPS in mice significantly increases locomotion while decreasing the amount time spent in slow-wave sleep. In addition, ICV NPS increases exploratory behavior in the open-field, light-dark box, and elevated-plus-maze paradigms, models for the study of anxiety-related behavior (1).

A number of hypothalamic neuropeptides involved in the modulation of arousal or anxiety including neuropeptide Y (2), nociceptin (3), and orexin A (4) play a role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis (5–7) and food intake (8, 9). It has recently been shown that lateral ventricle (LV) administration of NPS caused a significant reduction in food intake in previously fasted Long Evans rats. The effect on cumulative food intake was seen up to 6 h after injection. In addition, LV administration of NPS significantly inhibited voluntary food intake in rats freely feeding on a palatable diet (10). In these current studies, we examine the effect of ICV NPS on the regulation of the HPA axis, behavior, and food intake in male Wistar rats. The paraventricular nucleus (PVN) is an important hypothalamic nucleus involved in the regulation of the HPA axis and food intake. We therefore also examined the effect of intra-PVN (iPVN) administration NPS on the HPA axis, behavior and food intake.

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Abbreviations: aCSF, Artificial cerebrospinal fluid; AVP, arginine vasopressin; CLAMS, comprehensive lab animal monitoring system; HPA, hypothalamo-pituitary-adrenal; ICV, intracerebroventricular; iPVN, intraparaventricular; IR, immunoreactivity; LC, locus ceruleus; LV, lateral ventricle; NDP-MSH, [Nle^4, D-Phe^7]-α-MSH; NPS, neuropeptide S; NPS-R, NPS receptor; NPY, neuropeptide Y; PVN, paraventricular nucleus.

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Materials and Methods

Materials

Human NPS was custom synthesized by Bachem (St. Helen's, UK). The product was purified to homogeneity by reverse-phase HPLC to give greater than 95% purity. Cannulation materials were purchased from Plastic One, Inc. (Roanoke, VA). Reagents for hypothalamic explant experiments were purchased from BDH (Poole, UK).

Animals

Male Wistar rats (specific pathogen free; Charles River, Margate, UK), weighing 250–300 g, were maintained in individual cages under controlled temperature (21–23 C) and light (12-h light, 12-h dark cycle; lights on at 0700 h) with *ad libitum* access to food (RM1 diet; SDS Ltd., Witham, UK) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project License 70/5516).

ICV cannulation and injection

Animals were anesthetized with a mixture of ketamine HCl (60 mg/kg Ketalar; Parke-Davis, Pontypool, UK) and xylazine (12 mg/kg Rompun; Bayer Corp., Bury St. Edmunds, UK). Prophylactic antibiotics flucloxacillin (37.5 mg/kg) and amoxicillin (37.5 mg/kg) were administered before surgery. Animals were implanted with a 22-gauge stainless steel guide cannula projecting into the third cerebral ventricle using the coordinates calculated from the rat brain atlas of Paxinos and Watson (11) (0.8 mm posterior to bregma in the midline and implanted 6.5 mm below the outer surface of the skull). Briefly, a Kopf stereotactic frame (David Kopf Instruments, Tujunga, CA) was used and three stainless steel screws inserted into the cranium. The cannula was fixed to these with dental cement. After surgery, the animals were given 5 ml of 0.9% saline for circulatory support and buprenorphine (45 μg/kg; Schering-Plough Corp., Welwyn Garden City, UK) for analgesia. The animals were allowed 7 d recovery after surgery. They were then accustomed to handling on a daily basis. All compounds were injected using a 28-gauge stainless steel injector placed in and projecting 1 mm below the tip of the cannula. Cannula placement was confirmed by a positive dipsogenic response to angiotensin II (150 ng). Only those animals with positive dipsogenic response were included in the data analysis. All animals were habituated to the injection process by a subsequent saline injection.

iPVN cannulation and injection

Animals were implanted with a 26-gauge stainless steel guide cannula projecting immediately above the PVN using coordinates calculated from the rat brain atlas of Paxinos and Watson (11) (1.8 mm posterior to the bregma, 0.5 mm laterally, and implanted 7 mm below the outer surface of the skull) as previously described (12). After a 7-d recovery period, animals received two saline injections to habituate them to the injection procedure. All compounds were dissolved in 0.9% saline and administered in a 1-μl volume via a 33-gauge stainless steel injector projecting 1 mm into the PVN over 1 min. The spread of a 1-μl injection into the PVN is reported to be limited to 1 mm^3 (13).

Study 1a: effect of ICV NPS on plasma corticosterone and ACTH

Ad libitum-fed rats received a single ICV injection of saline, 0.1, 1, or 10 nmol NPS (n = 10 per group) in the early light phase (0900–1000 h). Rats were killed by decapitation 10 and 40 min after injection. Trunk blood was collected in plastic lithium heparin tubes containing 4200 kIU aprotinin (Bayer Corp., Haywards Heath, UK) for corticosterone analysis and in plastic EDTA tubes for ACTH analysis. Plasma was separated by centrifugation, frozen on dry ice, and stored at −20 C until assayed. Plasma TSH and LH were also measured.

Study 1b: effect of ICV NPS on behavior

Two behavioral analysis studies were carried out in ICV cannulated animals. In the first study, animals received a single ICV injection of either saline or 1, 3, or 10 nmol NPS (n = 8 per group) in the early light phase (0900–1000 h). After injection, behavioral patterns were monitored continuously for 60 min after injection by observers blinded to the experimental treatment. Behavior was classified into eight different categories: feeding, drinking, grooming, burrowing, rearing, locomotion, head down, and sleeping adapted from Fray *et al.* (14). These methods have previously been used to demonstrate abnormal behavior after central nervous system administration of peptides (15). During the analysis, each rat was observed for 15 sec every 5 min. This 15-sec period was subdivided into three, and the behavior of the rat during each time period was scored. In the second behavioral study, animals received a single ICV injection of either saline or 0.03, 0.1, or 0.3 nmol NPS (n = 8 per group) in the early light phase (0900–1000 h). After injection, behavioral analysis was carried out as described above.

Study 1c: effect of ICV NPS on activity

ICV cannulated animals were monitored using a 24-chamber open-circuit Oxymax comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH). Rats were maintained at 24 C under a 12-h light, 12-h dark cycle (light period, 0700–1900 h). Powdered RM1 diet and water were available *ad libitum* unless otherwise stated. Animals were individually housed in special Plexiglas cages, through which air was passed at a flow rate of 2.5 liters/min.

All rats were acclimatized to their cages for 2 d and were fasted 24 h before the study. Animals received a single ICV injection of either saline (n = 12) or 10 nmol NPS (n = 12) in the early light phase (0900–1000 h). This dose of NPS was chosen because it is similar to the dose given previously, which caused a reduction in food intake (10). Animals were returned to their home cage with *ad libitum* access to food. During CLAMS monitoring, the ambulatory activity of each individually housed animal was measured simultaneously using the optical beam technique (Opto M3; Columbus Instruments). Consecutive photo-beam breaks were scored as an ambulatory movement. Cumulative activity counts in x- and z-axes were recorded every minute for 120 min and were used to determine horizontal (XAMB) and rearing (ZTOT) movement, respectively.

Study 1d: effect of ICV administration of NPS on food intake

Groups of rats were fasted for 24 h (n = 10–12) and injected with saline, NPS (0.1, 1, or 10 nmol), or 3 nmol NDP-MSH (a stable analog of α-MSH) in the early light phase (0900–1000 h). This dose of NDP-MSH has previously been shown to inhibit food intake (16). In a separate experiment, groups of rats were fasted for 24 h (n = 11 per group) and injected with saline, NPS (30 nmol), or 5 nmol NDP-MSH in the early light phase (0900–1000 h). Animals were returned to their home cages with a preweighed amount of rat chow. Food intake was measured at 1, 2, 4, and 24 h after injection.

Study 2a: effect of iPVN NPS on plasma corticosterone and ACTH

Animals received a single iPVN injection of saline or 0.1 or 1 nmol NPS in the early light phase (0900–1000 h). These doses of NPS were chosen based on previous studies that show that 1/10 of the ICV dose is appropriate for intranuclear injection (12). Rats were killed by decapitation 10 and 40 min after injection (n = 9 per group per time point). Trunk blood was collected in plastic lithium heparin tubes containing 4200 kIU aprotinin for corticosterone analysis and in plastic EDTA tubes for ACTH analysis. Plasma was separated by centrifugation, frozen on dry ice, and stored at −20 C until assayed. Plasma TSH and LH were also measured. In a second study, animals received a single iPVN injection of saline or 0.01 or 0.3 nmol NPS in the early light phase (0900–1000 h). Rats were killed by decapitation, and plasma ACTH and corticosterone were measured as described above.

Study 2b: effect of iPVN NPS on behavior

Two studies to examine behavior were carried out on iPVN cannulated animals. In the first study, animals received a single iPVN injection of saline, 0.1 or 1 nmol NPS (n = 10 per group) in the early light phase (0900–1000 h). These doses of NPS were chosen based on previous studies that show that 1/10 of the ICV dose is appropriate for intranuclear injection (12). In the second study, animals received a single iPVN injection of saline or 0.003, 0.01, or 0.03 nmol NPS. In both studies, after injection, behavioral analysis was carried out as described above.

Study 2c: effect of iPVN NPS on food intake

Groups of rats were fasted for 24 h (n = 10–12) and injected with saline, NPS (0.1, 0.3, or 1 nmol) or 0.5 nmol NDP-MSH in the early light phase (0900–1000 h). This dose of NDP-MSH has previously been shown to inhibit food intake (17). In a separate experiment, groups of rats were fasted for 24 h (n = 11 per group) and injected with saline, NPS (0.003, 0.01, or 0.03 nmol) or 0.5 nmol NDP-MSH in the early light phase (0900–1000 h). In both studies, the animals were returned to their home cages with a preweighed amount of rat chow. Food intake was measured at 1, 2, 4, and 24 h after injection.

PVN cannula placement was verified at the end of the study by the injection of black ink (18). Data from an animal were excluded if its injection site extended more than 0.2 mm outside the PVN.

Study 3: effect of NPS on the release of CRH, arginine vasopressin (AVP), and neuropeptide Y (NPY) from hypothalamic explants in vitro

The static incubation system was used as described previously (19). Briefly, *ad libitum*-fed male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with the ventral surface uppermost and placed in a vibrating microtome (Microfield Scientific Ltd., Dartmouth, UK). A 1.8-mm slice was taken from the basal hypothalamus and blocked lateral to the circle of Willis to include the PVN. The hypothalamic slice was incubated in individual chambers containing 1 ml artificial cerebrospinal fluid (aCSF) (20 mM $NaHCO_3$, 126 mM NaCl, 0.09 mM Na_2HPO_4, 6 mM KCl, 1.4 mM $CaCl_2$, 0.09 mM $MgSO_4$, 5 mM glucose, 0.18 mg/ml ascorbic acid, and 100 μg/ml aprotinin) equilibrated with 95% O_2 and 5% CO_2.

The tubes were placed on a platform in a water bath maintained at 37 C. After an initial 2-h equilibration period, the hypothalami were incubated for 45 min in 600 μl aCSF (basal period) before being challenged with NPS (at doses of 10, 100, and 1000 nM) in 600 μl aCSF for 45 min. The viability of the tissue was tested by 45 min exposure to aCSF containing 56 mM KCl. Hypothalamic explants that failed to show peptide release above the basal level in response to aCSF containing 56 mM KCl were excluded from the data analysis. Isotonicity was maintained by substituting K^+ for Na^+. Nine to 12 hypothalamic slices were used for each dose of peptide administered. At the end of each period, aCSF was collected and stored at −20 C until measurement of CRH, AVP, and NPY by RIA.

Study 4: effect of NPS on ACTH release from pituitary quarters

The effect of NPS on pituitary ACTH release was determined using anterior pituitary segments. The method was a modification of that previously described (20). Rats were decapitated, and anterior pituitary glands were harvested immediately and then divided into four pieces of approximately equal size. The segments were randomly placed (one segment per well) into the wells of a 48-well tissue culture plate (Nunc International, Roskilde, Denmark) and incubated in 500 μl aCSF. The anterior pituitary segments were maintained at 37 C in a humidified environment saturated with 95% O_2 and 5% CO_2 for 2 h with the medium changed every hour. The segments were then incubated in aCSF alone (control), 100 or 1000 nM NPS, or 100 nM CRH, a positive control, for 4 h (n = 10 per group). At the end of this period, the aCSF was collected and stored at −20 C until RIA for ACTH.

RIA

CRH immunoreactivity (IR), AVP-IR, and NPY-IR were measured using established RIA methods (12, 21). The intra- and interassay coefficients of variation were less than 10% for the CRH RIA, 11 and 20% for the AVP RIA, and less than 10% for the NPY RIA, respectively. Plasma corticosterone was measured using an RIA kit from MP Biomedicals, Inc. (Orangeburg, NY), for which the intra- and interassay coefficients of variation were less than 10 and 7%, respectively. Plasma ACTH was measured by immunoradiometric assay purchased from Euro-Diagnostica B.V. (Arnhem, The Netherlands). The intra- and interassay coefficients of variation were both less than 4%. Plasma TSH and LH were measured using in-house RIAs with reagents obtained from the National Hormone and Pituitary program (Dr. A. Parlow University of California, Los Angeles, Harbor Medical Center, Los Angeles, CA) methods previously described (22, 23). The intra- and interassay coefficients of variation were 8 and 9% for TSH and 9 and 12% for LH, respectively. ACTH release from pituitary fragments was measured by RIA using methods and reagents provided by the National Hormone and Pituitary program. The intra- and interassay coefficients of variation were 9 and 12%, respectively.

Statistics

Statistical advice was provided by J. Elialoo at the Statistical Advisory Service, Imperial College London. Data for the feeding, hypothalamic explant, and chop studies are presented as the mean ± SEM. Data for behavioral analysis are presented as median and interquartile range. For both the plasma ACTH and corticosterone studies and feeding studies, groups were compared by one-way ANOVA, followed by *post hoc* Dunnett's test (Systat, Evanston, IL). Data from hypothalamic explant release experiments were analyzed using paired Student's *t* test between the basal period and the test period. For behavioral studies, data were compared using Kruskal-Wallis one-way ANOVA on ranks. For the CLAMS activity studies, differences between the treatment groups were determined using the Mann-Whitney *U* test (Stata 9; StataCorp, College Station, TX). In all cases, $P < 0.05$ was considered to be statistically significant.

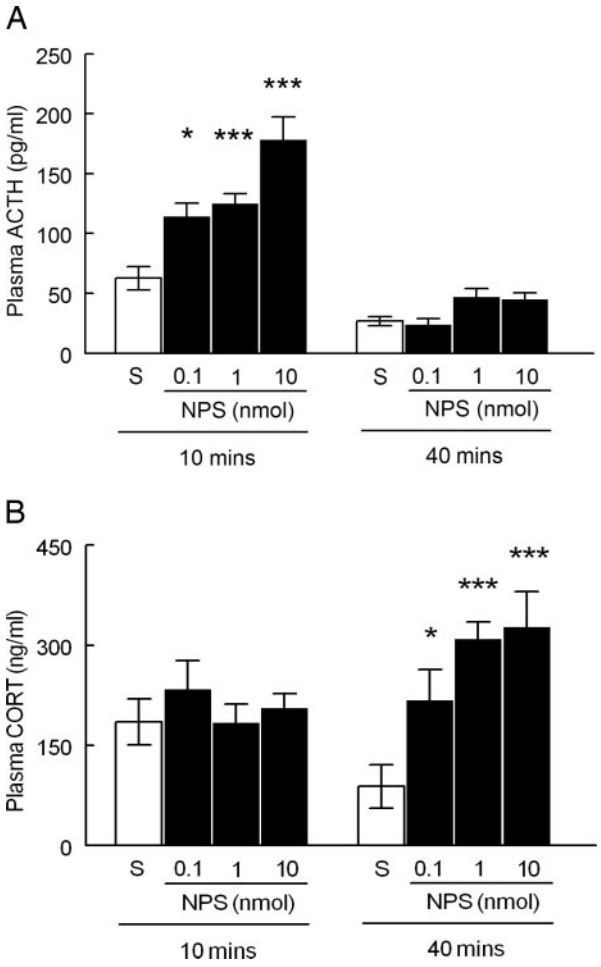

FIG. 1. Effect of a single ICV injection of NPS (0.1, 1, or 10 nmol) or saline in *ad libitum*-fed male rats on plasma ACTH (A) and corticosterone (B) at 10 and 40 min after injection. *, $P < 0.05$; ***, $P < 0.005$ *vs.* saline (n = 10 per group). Results are mean ± SEM.

Results

Study 1a: effect of ICV NPS on plasma ACTH and corticosterone

ICV administration of NPS caused a significant increase in plasma ACTH 10 min after injection compared with saline (plasma ACTH, in pg/ml, was 62.6 ± 9.9 after saline, 114.2 ± 11.0 after 0.1 nmol NPS, 125.0 ± 8.2 after 1 nmol NPS, and 178.4 ± 19.0 after 10 nmol NPS; $P < 0.05$, 0.1 nmol *vs.* saline; $P < 0.005$, 1 nmol and 10 nmol *vs.* saline; n = 10). There were no significant differences in plasma ACTH by 40 min after injection. ICV administration of NPS had no effect on plasma corticosterone at 10 min after injection. However, by 40 min after injection of NPS there was a significant increase in

plasma corticosterone (plasma corticosterone, in ng/ml, was 88.8 ± 32.6 after saline, 217.5 ± 46.3 after 0.1 nmol NPS, 309.3 ± 25.6 after 1 nmol NPS, and 327.6 ± 52.6 after 10 nmol NPS; $P < 0.05$, 0.1 nmol NPS *vs.* saline; $P < 0.005$, 1 nmol and 10 nmol NPS *vs.* saline; n = 10) (Fig. 1). No significant changes in plasma TSH or LH were observed at either time point.

Study 1b: effect of ICV NPS on behavior

ICV administration of 1 nmol NPS caused a significant increase in rearing activity compared with saline-treated animals up to 1 h after injection, with median (interquartile range) of 4 (2–7) after saline and 26 (22–27) after 1 nmol NPS ($P < 0.05$ *vs.* saline; n = 8). ICV administration of 3 and 10 nmol NPS caused a significant increase in locomotor activity compared with saline-treated animals up to 1 h after injection, with median (interquartile range) as follows: 4 (2–5) after saline, 6 (3–7) after 1 nmol NPS, 7 (6–13) after 3 nmol NPS, and 9 (7–10) after 10 nmol NPS ($P < 0.05$, 3 and 10 nmol NPS *vs.* saline; n = 8). NPS also caused a significant reduction in sleeping compared with saline-treated controls, with median (interquartile range) as follows: 20 (13–24) after saline, 0 (0–0) after 1 nmol NPS, 1.5 (0–6) after 3 nmol NPS, and 0 (0–0) after 10 nmol NPS ($P < 0.05$, 1 and 10 nmol NPS *vs.* saline; n = 8). There were no significant changes in any other behaviors (Table 1). ICV administration of lower doses of NPS had no effect on any behaviors compared with saline controls, although there was a trend toward a reduction in sleeping (published as supplemental Table 1 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Study 1c: effect of ICV NPS on activity

A single ICV injection of 10 nmol NPS caused a significant increase in horizontal movement (XAMB, horizontal beam breaks) and rearing activity (ZTOT, vertical beam breaks). There was a significant increase in horizontal movement from 8 min after injection, and the effect remained significant up to 44 min after injection (Fig. 2A). NPS also significantly increase rearing activity between 8 and 34 min after injection (Fig. 2B). There were no significant differences in activity at any other time points.

Study 1d: effect of ICV NPS on food intake

ICV administration of NPS, 0.1 and 1 nmol, had no effect on food intake. However, 10 nmol NPS showed a trend toward an inhibition in food intake 1 h after injection (0–1 h food intake was 6.3 ± 0.6 g after saline, 6.3 ± 0.6 g after 0.1 nmol NPS, 6.5 ± 0.4 g after 1 nmol NPS, and 5.5 ± 0.5 g after 10 nmol NPS). In the same experiment, 3 nmol NDP-MSH caused a significant reduction in food intake at 1 h after injection (0–1 h food intake was 6.3 ± 0.6 g after saline and 2.9 ± 0.8 g after 3 nmol NDP-MSH; $P < 0.01$; n = 6–11). In another experiment, 30 nmol NPS showed a trend toward an inhibition in food intake (0–1 h food intake was 6.8 ± 0.5 g after saline *vs.* 6.1 ± 0.2 g after 30 nmol NPS).

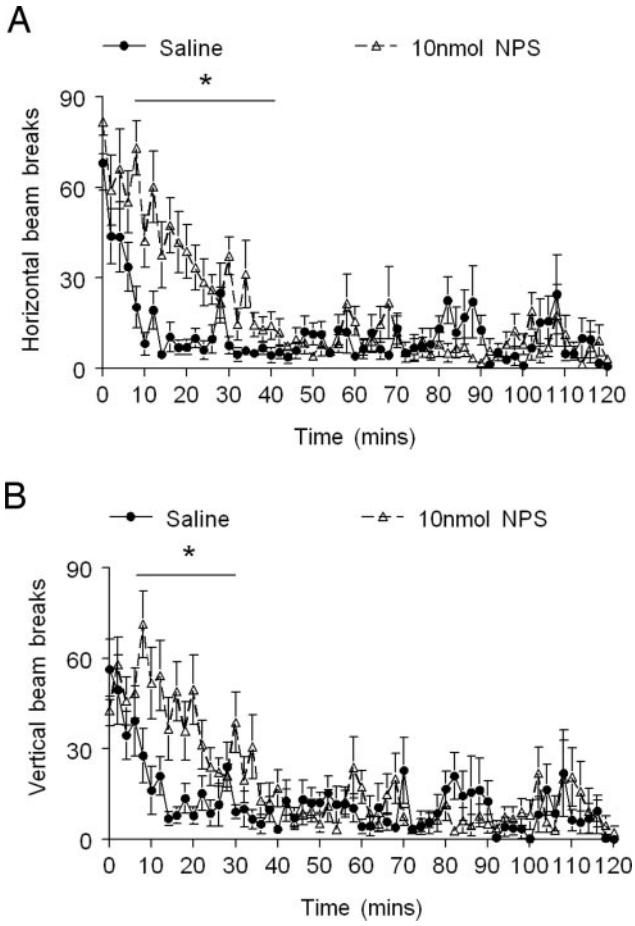

FIG. 2. Effect of ICV NPS on activity. Rats fasted for 24 h received a single ICV injection of saline or 10 nmol NPS (n = 12 per group). The ambulatory activity of each individually housed animal was measured simultaneously using the optical beam technique. The effect of NPS on movement along the x-axis (horizontal beam breaks, A) and rearing activity (vertical beam breaks, B) were determined. *, $P < 0.05$ *vs.* saline. Results are mean ± SEM.

Study 2a: effect of iPVN NPS on plasma ACTH and corticosterone

NPS significantly increased plasma ACTH 10 min after injection. In one experiment, plasma ACTH, in pg/ml, was

TABLE 1. Effect of a single ICV injection of NPS (1, 3, or 10 nmol) or saline in *ad libitum*-fed male rats on behavior

	Feeding	Drinking	Grooming	Burrowing	Rearing	Locomotion	Sleep	Head down
Saline	0 (0–0)	0 (0–0)	2 (0–3)	1 (0–2)	4 (2–7)	4 (2–5)	20 (13–24)	3 (3–7)
1 nmol NPS	0 (0–1)	0 (0–0)	1 (0–3)	0 (0–0)	26[a] (22–27)	6 (3–7)	0[a] (0–0)	2 (0–6)
3 nmol NPS	0 (0–0)	0 (0–0)	3 (2–5)	0 (0–1)	12 (8–13)	7[a] (6–13)	1.5 (0–6)	12 (8–13)
10 nmol NPS	0 (0–0)	0 (0–0)	1 (1–3)	1 (0–2)	17 (10–21)	9[a] (7–10)	0[a] (0–0)	7 (2–18)

Animals were observed for 15 sec every 5 min. This 15-sec period was subdivided into three, and the behavior of the rat during each time period was scored. Data are presented as median (interquartile range).

[a] $P < 0.05$ *vs.* saline.

42.6 ± 5.1 after saline, 106.7 ± 32.7 after 0.1 nmol NPS, and 161.8 ± 29.5 after 1.0 nmol NPS ($P = 0.09$, 0.1 nmol NPS *vs.* saline; $P < 0.005$; 1.0 nmol NPS *vs.* saline; n = 9), and in a second experiment, plasma ACTH, in pg/ml, was 30.4 ± 5.5 after saline, 69.4 ± 19.0 after 0.01 nmol NPS, and 117.3 ± 11.3 after 0.3 nmol NPS ($P < 0.05$, 0.01 nmol NPS *vs.* saline; $P < 0.005$, 0.3 nmol NPS *vs.* saline) (Fig. 3, A and B). iPVN administration of NPS resulted in a significant increase in plasma corticosterone 40 min after injection. In one experiment, plasma corticosterone, in ng/ml, was 125.1 ± 35.8 after saline, 260.9 ± 57.7 after 0.1 nmol NPS, and 358.4 ± 72.7 after 1.0 nmol NPS ($P = 0.1$, 0.1 nmol NPS *vs.* saline; $P < 0.01$, 1.0 nmol NPS *vs.* saline; n = 9), and in a second experiment, the values were 36.3 ± 12.5 after saline, 174.3 ± 47.4 after 0.01 nmol NPS, and 272.4 ± 34.7 after 0.3 nmol NPS ($P < 0.05$, 0.01 nmol NPS *vs.* saline; $P < 0.005$, 0.3 nmol NPS *vs.* saline; n = 9) (Fig. 3, C and D). There were no significant differences in plasma TSH or LH at either time point.

Study 2b: effect of iPVN NPS on behavior

iPVN administration of both 0.1 and 1 nmol NPS caused a significant increase in rearing activity up to 1 h after injection, with a median (interquartile range) of 5 (4–6) after saline, 16 (13–23) after 0.1 nmol NPS, and 14 (9–19) after 1.0 nmol NPS ($P < 0.05$, 0.1 and 1.0 nmol NPS *vs.* saline; n = 10 per group) (Table 2). NPS (1 nmol) caused a significant decrease in grooming activity, with a median (interquartile range) of 22 (16–24) after saline *vs.* 1 (0–1) after 1.0 nmol NPS ($P < 0.05$ *vs.* saline). No other significant differences in behavior were observed between the three groups (Table 2). iPVN administration of lower doses of NPS (0.003, 0.01, and 0.03 nmol) showed no differences in behavior compared with saline-treated controls (supplemental Table 2).

Study 2c: effect of iPVN NPS on food intake

NPS significantly reduced food intake in the first hour after iPVN injection in male Wistar rats fasted for 24 h (0–1 h food intake was 8.9 ± 0.5 g after saline, 7.0 ± 0.7 g after 0.1 nmol NPS, 6.6 ± 0.3 g after 0.3 nmol NPS, and 6.0 ± 0.5 g after 1.0 nmol NPS, $P < 0.05$ for all doses *vs.* saline; n = 10 per group) (Fig. 4A). There were no significant differences in food intake between any of the groups at 2, 4, or 24 h after injection (data not shown). In a separate experiment, lower

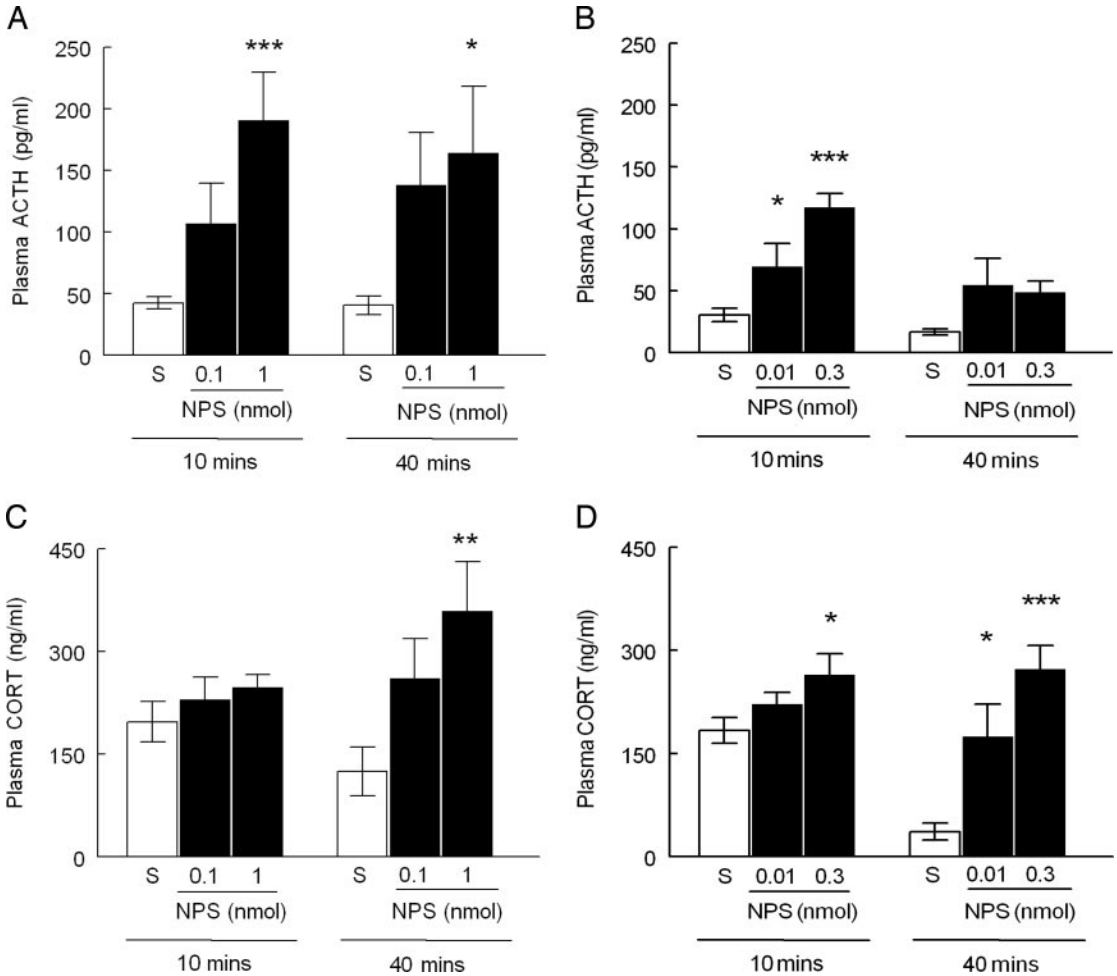

FIG. 3. Effect of a single iPVN injection of NPS (0.1 or 1 nmol) or saline (A and C) or NPS (0.01 or 0.3 nmol) or saline (B and D) in *ad libitum*-fed male rats on plasma ACTH (A and B) and corticosterone (C and D) at 10 and 40 min after injection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ *vs.* saline (n = 9 per group). Results are mean ± SEM.

TABLE 2. Effect of a single iPVN injection of NPS (0.1 or 1 nmol) or saline in *ad libitum*-fed male rats on behavior

	Feeding	Drinking	Grooming	Burrowing	Rearing	Locomotion	Sleep	Head down
Saline	0 (0–2)	0 (0–2)	22 (16–24)	0 (0–1)	5 (4–6)	4 (4–6)	3 (1–8)	15 (8–17)
0.1 nmol NPS	1 (0–3)	0 (0–0)	2 (0–3)	0 (0–1)	16[a] (13–23)	8 (3–10)	0 (0–0)	7 (2–10)
1 nmol NPS	0 (0–0)	0 (0–0)	1[a] (0–1)	0 (0–3)	14[a] (9–19)	7 (5–8)	0 (0–0)	11 (2–18)

Animals were observed for 15 sec every 5 min. This 15-sec period was subdivided into three, and the behavior of the rat during each time period was scored. Data are presented as median (interquartile range).

[a] $P < 0.05$ *vs.* saline.

doses of NPS (0.03, 0.01, and 0.003 nmol) showed no effect on food intake at any time point (Fig. 4B).

Study 3: effect of NPS on the release of CRH, AVP, and NPY from hypothalamic explants in vitro

NPS caused a significant increase in CRH and AVP release from hypothalamic explants. There was no change in NPY release from hypothalamic explants. Actual values are presented in Table 3 and graphically as a percentage of basal release in Fig. 5, A–C.

Study 4: effects of NPS on ACTH release from anterior pituitary fragments

NPS had no significant effect on ACTH release from pituitary segments. However, there was a significant increase in ACTH release from pituitary incubated in 100 nM CRH (positive control). ACTH release, in pg/ml, was 41.0 ± 5.5 for control, 59.5 ± 8.2 for 100 nM NPS, 62.5 ± 11.3 for 1000 nM NPS, and 115.3 ± 9.4 for 100 nM CRH ($P = 0.1$, 1000 nM NPS *vs.* control; $P = 0.09$, 100 nM NPS *vs.* control; $P < 0.01$, CRH *vs.* control; n = 10 per group).

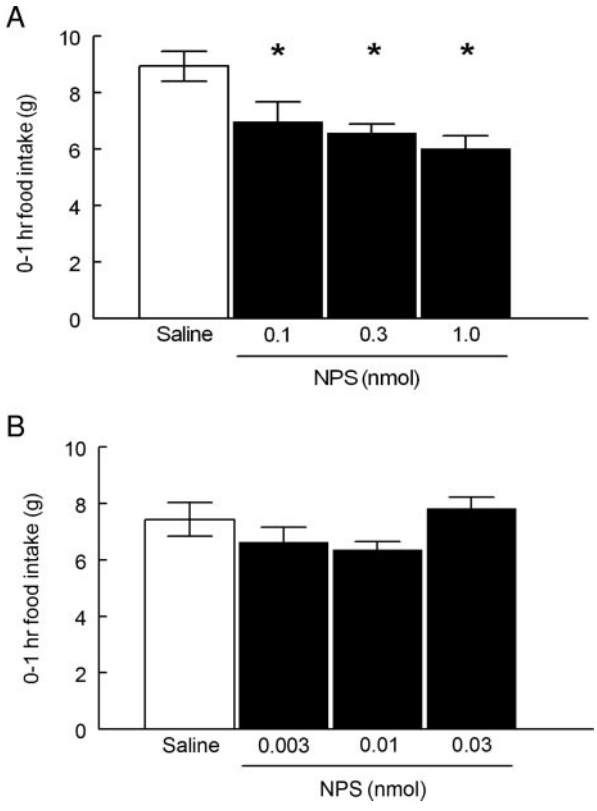

FIG. 4. Effect of iPVN NPS on food intake. Rats fasted for 24 h received a single iPVN injection of saline or NPS (0.1, 0.3, or 1 nmol) (A) or saline or NPS (0.003, 0.01, or 0.03 nmol) (B) (n = 9 per group). Food intake was measured 1 h after injection. *, $P < 0.05$ *vs.* saline. Results are mean ± SEM.

Discussion

NPS is a recently discovered peptide that has been shown to modulate arousal and anxiety-related behavior. ICV administration of 0.1 or 1 nmol NPS to mice caused a significant increase in locomotor activity. ICV administration of NPS also significantly increased wakefulness and reduced the amount of slow-wave sleep (1). A number of peptides involved in the modulation of arousal via the LC also stimulate the HPA axis (24–26). We examined the effect of ICV administration of NPS on the HPA axis. NPS caused a significant stimulation of the HPA axis with an increase in plasma ACTH 10 min after injection and plasma corticosterone 40 min after injection.

The PVN is rich in CRH and AVP neurons (27) and is important in the control of the HPA axis. Direct injection of NPS into the PVN caused a significant increase in plasma ACTH and corticosterone. Our *in vitro* studies demonstrate that NPS stimulates the release of CRH and AVP from hypothalamic explants. These data therefore suggest that NPS stimulates the HPA axis via the release of CRH and AVP. Neither ICV nor iPVN administration of NPS stimulated the release of either LH or TSH, suggesting a direct and specific effect of NPS on the HPA axis. In addition, treatment of pituitary segments with NPS did not alter ACTH release, suggesting that NPS does not have a direct effect on the pituitary gland. It is therefore likely that the effects of NPS on the HPA axis are mediated via the hypothalamus through the release of CRH and AVP. NPY plays an important role in the regulation of appetite (28) as well as in the control of arousal and anxiety (2, 29). Furthermore, it has been shown that exogenous NPY stimulates CRH neurons in the PVN and may contribute to the activation of the HPA axis. It may therefore be hypothesized that the effects of NPS on both food intake and arousal may be mediated via an NPY pathway. However, Beck *et al.* (10) have previously shown that NPS is unable to block NPY-stimulated food intake, suggesting that these peptides may work through different pathways. In agreement with this, we have shown that NPS does not affect the release of NPY from hypothalamic explants.

To further elucidate the role of NPS in arousal, we examined the effects of ICV NPS on activity. NPS caused a significant increase in both horizontal movement, *i.e.* movement around the cage and in rearing activity. These effects were rapid (occurring within 10 min of injection) but short lived with no significant differences in activity seen by 45 min after

TABLE 3. Effect of NPS (10, 100, or 1000 nM) on the release of CRH, AVP, and NPY from hypothalamic explants

Peptide release	Dose of NPS administered to hypothalamic explants					
	10 nM		100 nM		1000 nM	
	Basal	NPS	Basal	NPS	Basal	NPS
CRH	65.3 ± 23.7	79.7 ± 25.7[a]	48.2 ± 16.6	67.5 ± 26.4	64.3 ± 20.5	138.0 ± 38.6[a]
AVP	9.5 ± 2.4	15.3 ± 6.0	10.4 ± 3.1	14.0 ± 4.7[a]	9.7 ± 2.2	16.8 ± 4.0[a]
NPY	21.8 ± 4.0	30.2 ± 5.8	33.2 ± 8.1	36.0 ± 6.6	27.8 ± 3.8	32.3 ± 4.9

CRH, AVP, and NPY release are expressed as femtomoles per explant; n = 9–12 per treatment.
[a] $P < 0.05$.

injection. Formal behavioral analysis adapted from Fray *et al.* (14) showed that ICV administration of NPS caused a significant increase in rearing and locomotor activity with a significant reduction in sleeping. Having established a role for ICV NPS in activity and behavior, we examined the effect of iPVN NPS on behavior. Injection of NPS directly into the PVN caused a significant increase in rearing activity. These data are in agreement with a previous study that has shown an increase in exploratory activity in mice after NPS injection (1). The LC in the brainstem plays an important role in the regulation of arousal (30) and in particular in the regulation of the sleep-wake cycle (31). Afferent projections to the LC from a number of hypothalamic nuclei have been described (32). Recently, a monosynaptic pathway between the parvocellular region of the PVN and the LC has been demonstrated (24). Therefore, it is possible that the effects of NPS on arousal after both ICV and iPVN administration may be mediated through the LC.

Recently, it has been shown that lateral ventricle administration of 1 or 10 μg (~0.5 and 5 nmol) NPS in previously fasted Long Evans rats caused a significant decrease in food intake (10). A number of neuropeptides including NPY (33), orexin A (7), neuromedin U (34), and galanin (35) which modulate arousal and regulate the HPA axis are also important peptides in the hypothalamic control of food intake. In addition to its roles in the regulation of the sympathetic and parasympathetic nervous systems and pituitary hormone secretion, the PVN is an important nucleus in the regulation of energy homeostasis. Lesioning the PVN results in hyperphagia and weight gain (36). Furthermore, injection of a number of orexigenic peptides directly stimulates food intake, whereas injection of anorectic peptides inhibits food

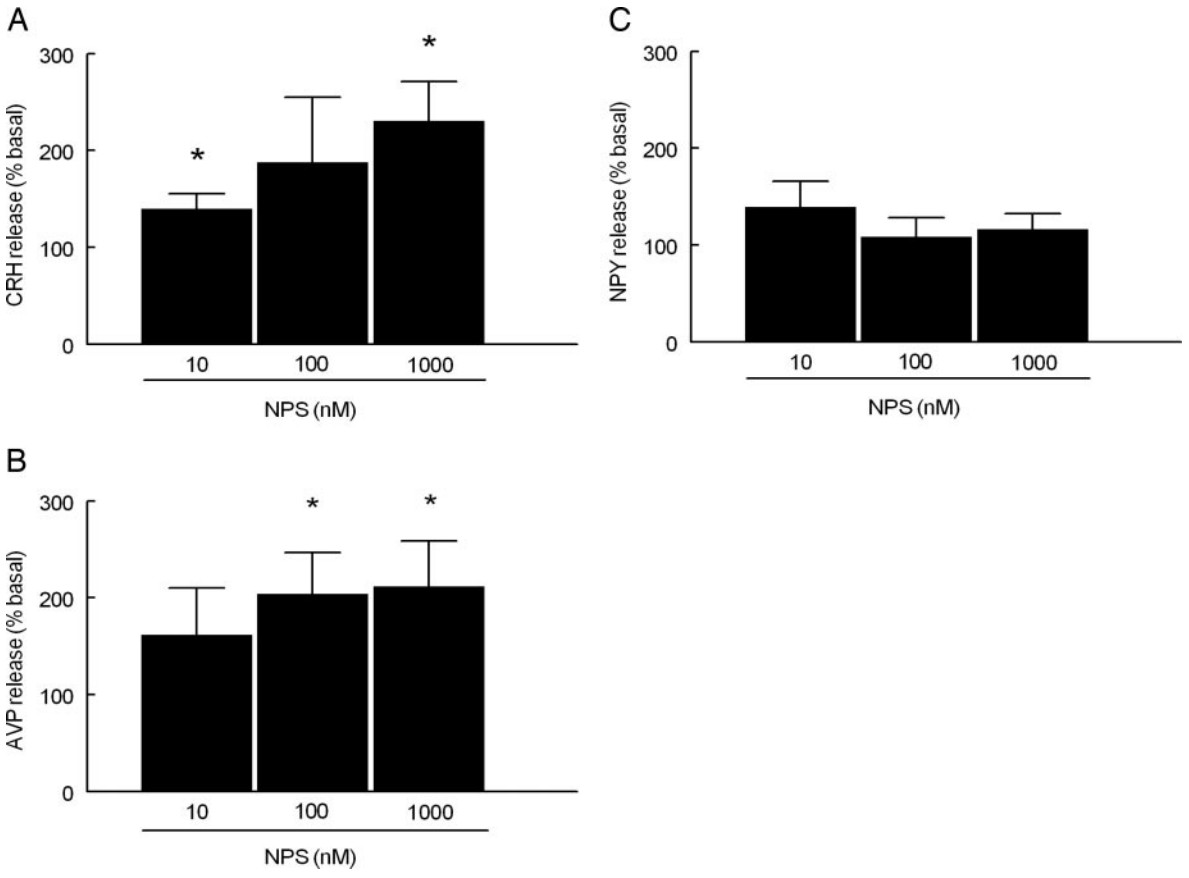

FIG. 5. Effect of NPS (10, 100, or 1000 nM) on CRH (A), AVP (B), and NPY release (C) from hypothalamic explants. Data are presented as percentage of basal release. *, $P < 0.05$ *vs.* basal release. Results are mean ± SEM.

intake (37). Therefore, the effects of ICV and iPVN administration of NPS on food intake were investigated. In our studies, ICV administration of 10 and 30 nmol NPS to 24-h-fasted male Wistar rats showed a trend toward a reduction in food intake. iPVN injection of NPS significantly inhibited food intake 1 h after injection. This effect was short lived, with no significant differences in food intake between the groups after the first hour. The effects on food intake after both ICV and iPVN administration are less potent and of shorter duration than the previous report of the anorectic effects of NPS (10). The reason for this difference in the effect of NPS on food intake in our studies compared with previous studies is not clear. However, it should be noted that the experiments of Beck *et al.* (10) were carried out in Long Evans rats that were fasted overnight, whereas the current study was carried out in 24-h-fasted male Wistar rats. It is therefore possible that there is a strain difference in the food intake response to NPS (10). In addition, the difference in the fasting period between the studies may have altered the sensitivity of the effects of NPS on food intake. It is also possible that the effects of NPS may differ when administered in the LV as opposed to directly in the third ventricle.

In conclusion, we have identified NPS as a novel stimulator of the HPA axis. Our study shows that NPS causes a significant increase in rearing and locomotor activity and stimulates the HPA axis in male Wistar rats at lower doses than are required to inhibit food intake. This may suggest that the effects of NPS on the stimulation of activity, the HPA axis, and food intake may be occurring via different circuits. Additional work is required to determine the precise mechanism by which NPS mediates these effects.

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