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Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food

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ABSTRACT **Feeding in mammals is a periodic behavior; however, knowledge of how the brain signals an intermittent eating pattern is scanty. Recent indirect evidence indicates that one of the signals encoded in the structure of neuropeptide Y (NPY) is to stimulate robust feeding. Therefore, two series of experiments were undertaken to characterize NPY secretion within the paraventricular nucleus (PVN) in association with eating behavior in the rat. Dynamic changes in NPY concentration in several hypothalamic sites and release in the PVN were assessed before and during the course of food consumption in rats trained to eat daily only for 4 h. Only in the PVN were NPY concentrations elevated before the introduction of food and, thereafter, levels decreased significantly during the course of eating. A similar temporal pattern in NPY release into the PVN interstitium was evident in samples collected by push–pull cannula perfusion in unrestrained rats. In addition, in food-deprived rats displaying a robust drive for feeding, NPY release in the PVN was also markedly enhanced in the shape of high-amplitude secretory episodes as compared to a lower release rate in rats receiving food ad libitum. The higher rate of NPY release in fasted rats returned to the control range after 24 h of ad libitum food supply. These findings of intense and dynamic NPY neurosecretory activity within a discrete hypothalamic site in association with an increased drive for food consumption demonstrate that NPY release in the PVN is an important orexigenic signal for periodic eating behavior. These results have important global implications for elucidating the underlying causes of the pathophysiology of eating disorders—anorexia nervosa, bulimia, and obesity—as well as constituting a specific contextual model for the formulation and testing of suitable NPY receptor agonists and antagonists for therapeutic intervention.**

Neural circuits that integrate metabolic, neural, and hormonal signals leading to intermittent motivation to eat reside in the hypothalamus. There is now a consensus that stimulation of hunger or appetite for food is encoded in a few specific signals in the hypothalamus. Among peptides and amines that stimulate feeding, neuropeptide Y (NPY) is found to be the most potent enhancer of consummatory behavior in a large number of species (1–4). NPY is produced in the arcuate nucleus (ARC) of the hypothalamus and other regions of the brain, including discrete cell groups in the brainstem. The fiber systems from the ARC and brainstem project into various hypothalamic sites previously implicated in regulation of feeding behavior (5–8). In fact, not only administration into the cerebroventricular system (3, 9, 10) but microinjection of NPY into various hypothalamic sites (11–13) rapidly elicited robust feeding responses in rats. Continuous NPY infusion into the third cerebroventricle evoked continuous episodic feeding during the infusion and postinfusion intervals (14). Multiple daily injections of NPY into the paraventricular nucleus (PVN) of the hypothalamus also increased daily food intake and body weight (15). Although suggestive of the powerful orexigenic effects of NPY, this evidence is indirect and pharmacological, involving NPY administration at concentrations far exceeding the endogenous levels at the putative target sites of action, and thereby fails to distinguish direct activation by NPY of hunger or appetite for food from that produced by a general activation of the hypothalamic circuit that regulates ingestive behavior.

Whether or not NPY is the final common signal that normally evokes feeding is unknown. To address this issue, it is imperative to document experimentally that dynamic changes in the release of NPY at a discrete site(s) in the hypothalamus closely follow sensations of hunger and satiety. Therefore, we adopted the push–pull cannula (PPC) technique to measure NPY release *in vivo* from hypothalamic sites in response to physiological demands in unrestrained rats (16). In this study, PPCs were aimed at the PVN because it receives heavy NPY projections from the ARC and brainstem (5–8), and NPY concentrations fluctuated selectively in this nucleus in response to sensations of hunger and satiety evoked by food deprivation and refeeding in rats (17).

Rats generally eat intermittently during the dark phase of the light/dark cycle (18, 19). Because of the extensive technicalities involved in the operation of the PPC technique, it was not feasible to assess NPY release associated with normal nighttime feeding. Consequently, we examined the pattern of NPY release in the PVN during the day before and during feeding sessions in two experimental paradigms—scheduled daily feeding and food deprivation.

MATERIALS AND METHODS

Male Sprague–Dawley rats (250–400 g; Crl:CD$^{(R)}$BR, Charles River Breeding Laboratories) were housed individually under controlled conditions (22°C–23°C; lights on 0500–1900 h) with ad libitum access to Purina rat chow pellets and water.

Experiment 1. *Effects of a scheduled feeding (SF) regimen on NPY levels in various microdissected brain regions and NPY release from the PVN* in vivo. Rats were weighed and assigned to either a SF or ad libitum feeding group. SF rats (ad libitum access for 4 h/day) were provided with food from 1100 to 1500 h. The ad libitum controls had their food removed and replaced with fresh food at both 1100 and 1500 h. Water was continuously available to both groups.

Experiment 1A. *Effects of SF on NPY levels.* After 3 weeks (20), rats from each group were killed by decapitation (*i*) just

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Abbreviations: NPY, neuropeptide Y; ARC, arcuate nucleus; PVN, paraventricular nucleus; PPC, push–pull cannula; SF, scheduled feeding; CSF, cerebrospinal fluid; FD, food deprived.

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before presentation of rat chow (1100 h), (*ii*) 2 h after food presentation (1300 h), and (*iii*) at the end of the 4-h feeding period (1500 h). Brains were quickly dissected out and frozen on dry ice; serial coronal sections (300 μm thick) were cut in a microtome cryostat at −10°C (17). Six brain nuclei—medial preoptic area, PVN, median eminence, ARC, ventromedial nucleus, and dorsomedial nucleus—were microdissected and processed for NPY RIA.

Experiment 1B. *Effects of SF on NPY release from the PVN* in vivo. Samples for *in vivo* release of NPY from the extracellular compartment of the PVN were obtained by using PPC assemblies (Plastics One, Roanoke, VA) implanted in the PVN after the start of SF. In addition to ad libitum controls and SF rats provided with food, a SF group that received no food during the perfusion period was included.

On the day of the experiment, rats were weighed and connected to the PPC system. Artificial cerebrospinal fluid (CSF) was perfused between 1020 and 1500 h at a rate of 20 μl/min and 200-μl samples were collected on ice, acidified, lyophilized, and stored at −80°C for NPY determination.

Experiment 2. *Effect of food deprivation on NPY release from the PVN* in vivo. Rats bearing a stereotaxically implanted PPC were assigned to one of the two experiments described below.

Experiment 2A. The rats underwent PPC perfusion after being food deprived (FD) for 3 days (deprived group) or were maintained with ad libitum food access before PPC perfusion (control group).

Experiment 2B. The rats were FD for 3 days, followed by ad libitum access to food for 24 h, and then perfused with PPC (refed group), or they were continued on ad libitum food access for 4 days and then perfused (control group).

The FD rats lost 63.0 ± 1.97 g in 3 days and with 24-h ad libitum access to food, they regained 34.3 ± 3.07 g. Control groups gained 19.6 ± 1.81 g and 21.6 ± 2.63 g at the end of 3 or 4 days of the experiment.

During perfusion, the control and refed groups had ad libitum access to food. The FD group was given a preweighed amount of food 6 h after lights-on (≈1 h after the start of perfusion), while the control and refed groups had their food replaced by preweighed food. All animals had ad libitum access to water.

NPY Radioimmunoassay. NPY was determined by RIA as described (17, 21). NPY antibody R-31, raised against human NPY, was generously donated by Harold Spies (Oregon Regional Primate Research Center, Beaverton). This antibody cross-reacts 100% with porcine and rat NPY and 14% with PYY (21). Assay sensitivity was 1.95–3.90 pg per tube and the intraassay coefficient of variation in assays for PPC samples was 13.4%; for hypothalamic micropunched tissues it was 4.3%. Various volumes of CSF from pooled PPC PVN samples produced inhibition curves parallel to the NPY standard.

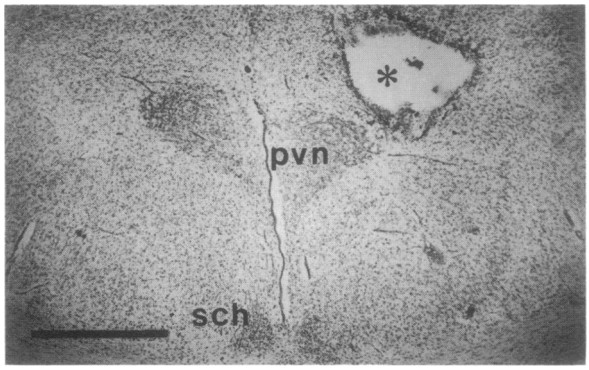

FIG. 1. Low-power view of a representative PPC perfusion site (*), the center of which is near the lateral magnocellular part of the PVN of the hypothalamus. sch, Suprachiasmatic nucleus. (Bar = 1 mm.)

Histology. After PPC perfusion, the animals received an overdose of pentobarbital and were intracardially perfused with formol saline. The fixed brains were frozen, sectioned, and stained with carbol fuchsin. The perfusion sites were identified by direct projection of the section onto the Paxinos and Watson atlas (22). Only those animals whose perfusion sites were located within or contiguous to the PVN and showed no evidence of mixing CSF from the third ventricle based on the absence of perfused dye were selected for analysis. A representative PPC perfusion site near the lateral magnocellular part of the PVN is shown in Fig. 1.

Statistical Analysis. For analysis of *in vivo* NPY release in the SF and FD experiments, the data were grouped into 40-min time blocks and the values were expressed as pg of NPY release per 10 min. One-way analysis of variance, followed by Duncan's multiple range test, was used to analyze the responses in the SF experiment within each treatment the time and between the treatments at each time point. Two-way analysis of variance (time by deprivation condition) was applied for analysis of the 3-day FD experiment followed by Fisher's protected least significant difference for post hoc comparisons. Student's *t* test (two-tailed) was used when comparisons were made between two groups.

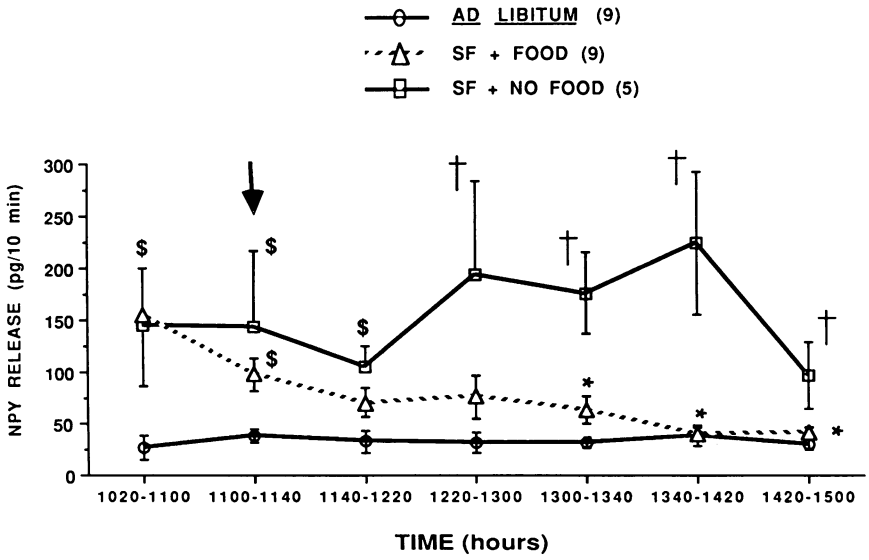

FIG. 2. NPY release in the PVN of male rats maintained on SF. ○, Control rats with ad libitum food supply. △, rats on SF with ad libitum food for 4 h (1100–1500 h) daily and during perfusion; □, SF rats did not receive food during the experiment. Arrow indicates the time food was supplied. Values are means ± SEM and parentheses denote the number of rats per group in this and subsequent figures. *, $P < 0.05$ vs. values during 1020–1100 and 1100–1140 h ($F(5,25) = 5.028$; $P < 0.01$); $, $P < 0.05$ vs. ad libitum at that point; †, $P < 0.05$ vs. other groups at the same time point.

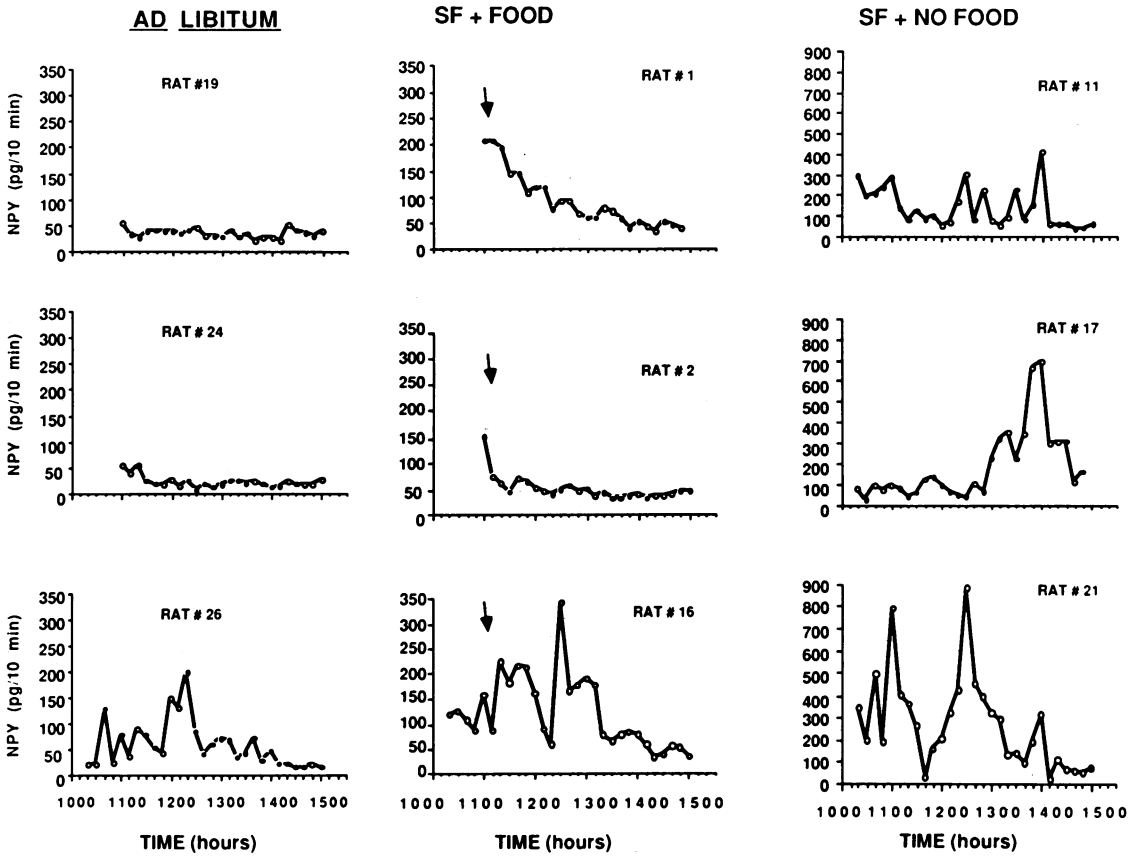

FIG. 3. Representative profiles of PVN NPY release in three groups of rats. Note change in the *y* axis for rats in SF + NO FOOD group.

RESULTS

Changes in NPY Release and Content in the PVN of Rats Maintained on a SF Regimen. NPY release in the PVN of control rats receiving food ad libitum was at a stable low rate throughout the period of observation [Figs. 2 and 3; $F(5,20) = 0.874$; $P > 0.05$]. The NPY concentration in the PVN also remained unchanged during this period [Fig. 4; $F(2,21) = 0.541$; $P > 0.05$]. In contrast, significant differences in the PVN content [$F(2,21) = 6.501$; $P < 0.01$] and release [$F(5,25) = 5.028$; $P < 0.01$] were evident in rats maintained on daily SF regimens. First, the rate of NPY release in the PVN before presentation of food was significantly higher than that found in the ad libitum control group. Second, in response to the availability of food, the NPY release rate in the PVN fell significantly after 2 h and remained low thereafter. Third, NPY content in the PVN was significantly elevated before and decreased progressively during the course of food consumption with a time course quite similar to that of NPY release in the PVN (Fig. 4). Unlike the picture in the PVN, NPY levels in other sites displayed no significant pattern of change in these groups. Inexplicably, NPY levels in the dorsomedial nucleus of SF and not ad libitum controls were significantly elevated at 1500 as compared to 1100 h (Table 1).

That these dynamic decreases in the release of NPY in the PVN were produced by food consumption is indicated by the group of rats maintained on the scheduled feeding regimen but not given food during the period of observation. In these rats, the high initial rate of NPY release was maintained [$F(6,24) = 0.747$; $P > 0.05$] through the entire period of

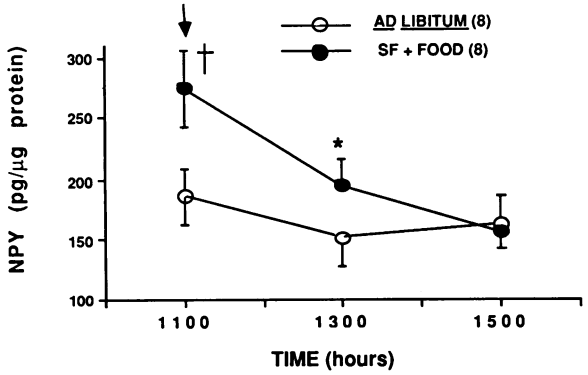

FIG. 4. NPY concentration in the PVN of rats with ad libitum food (○) and on SF given food (●) at 1100 h as indicated by arrow. Note that 1100 h values in the SF + FOOD group represent values before food was presented to these rats. *, $P < 0.05$ vs. 1100 h; †, $P < 0.05$ vs. AD LIBITUM group.

Table 1. NPY levels (pg per μg of protein) in hypothalamic sites of rats maintained on SF regimen

Site	Group	Time, h 1100	1300	1500
MPOA	Ad Lib	57.8 ± 7.2	77.4 ± 10.1	77.9 ± 12.1
	SF	77.3 ± 3.5	67.3 ± 10.3	75.4 ± 7.8
ME	Ad Lib	69.1 ± 7.2	64.3 ± 6.7	69.2 ± 5.9
	SF	78.6 ± 7.3	83.5 ± 9.9	68.6 ± 5.4
ARC	Ad Lib	159.6 ± 12.0	165.0 ± 10.0	197.4 ± 15.4
	SF	216.0 ± 15.2	205.5 ± 13.8	204.0 ± 12.2
VMN	Ad Lib	60.0 ± 9.1	90.9 ± 10.6	95.3 ± 14.5
	SF	86.4 ± 8.5	87.4 ± 12.8	80.1 ± 5.3
DMN	Ad Lib	129.7 ± 16.4	162.7 ± 17.8	194.3 ± 22.8
	SF	171.6 ± 19.8	168.7 ± 11.0	258.6 ± 30.3*

Results are expressed as mean ± SEM for eight rats in each group at each time. MPOA, medial preoptic area; ME, median eminence; VMN, ventromedial nucleus; DMN, dorsomedial nucleus.
*$P < 0.01$ vs. 1100 h.

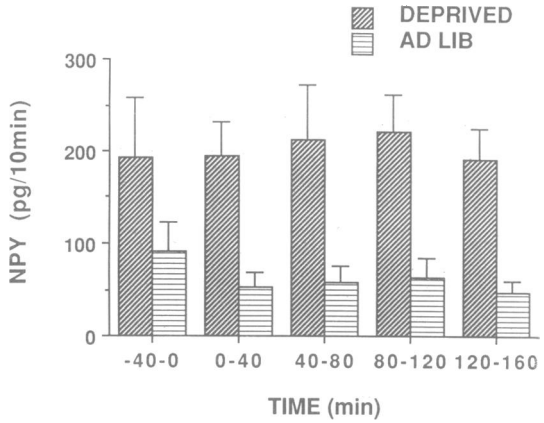

FIG. 5. NPY release in the PVN of rats FD for 3 days and ad libitum fed control rats. Data are presented as means of 10-min samples in blocks of 40 min. Food was given to FD rats at time 0. NPY release was significantly higher throughout the period of observation in the PVN of FD ($n = 6$) as compared to ad libitum fed ($n = 5$) rats [$F(1,6) = 18.96$; $P < 0.01$].

observation (Fig. 2). Interestingly, NPY release in the PVN appeared to occur in an episodic manner, and, particularly in the group of rats not given food, high amplitude secretory episodes were consistently evident (Fig. 3).

NPY Release in the PVN of FD Rats. As shown in Fig. 5, the high NPY release rate in FD rats was maintained throughout the 160 min of observation. During this time, the rats ate 5.3 ± 0.4 g as compared to the 1.0 ± 0.8 g eaten by the control rats. In addition, the pattern of NPY release in the control group and FD rats was markedly different (Fig. 6). Whereas in control rats NPY release occurred at a low basal rate with occasional episodes of high secretion, two types of NPY secretion patterns were observed in FD rats; either a steady high rate was maintained (Fig. 6, rat 59) or high amplitude, fast frequency NPY episodes were evident (rats 61 and 65). However, when given access to food for 24 h, the rate of NPY release returned to the range seen in control rats receiving food ad libitum throughout (mean, 46.9 ± 11.4 and 36.2 ± 15.9 pg per 10 min, respectively; $n = 6$ rats per group).

DISCUSSION

These experiments have allowed us to characterize the temporal pattern of NPY changes in the PVN in two experimental models that have been previously used to examine the effects of drugs, neurotransmitters, and neuromodulators on ingestive behavior in rats (1, 4). The results presented here clearly indicate that changes in NPY measured in the PVN interstitium with the aid of PPC can be detected with accuracy under experimental conditions that reliably affect appetite in the rat. A remarkable outcome of these investigations is that the change detected in the rate of NPY release in the PVN was rapid and correlated with the nutritional demands created in rats that were maintained on a daily SF regimen. Our basic underlying assumptions for this model were 3-fold. We assumed that appetite or sensation of hunger increases sometime prior to the SF time, it gradually subsides with food consumption, but it remains at high levels if food supply is withheld during the SF time. If NPY is a physiological stimulator of appetite, then it is imperative that the assumed sequential changes in appetite in this model should reflect alterations in NPY efflux in the PVN for local stimulatory action (17). Indeed, we observed a tight correlation between appetite and NPY release, the release rate was high before the SF time, and as the rats consumed food over the 3-h period, NPY efflux gradually decreased to the range seen in the control rats receiving food ad libitum. On the other hand, if food was withheld, the high rate of NPY secretion was sustained during the entire course of observation. In addition, the regional content of NPY was elevated selectively in the PVN before and decreased in association with food consumption in these rats.

To explain the mechanism whereby these rapid concomitant changes in NPY content and release responses occur in the PVN, we speculate that the onset of food consumption itself signals either an abrupt cessation or retardation in the availability or transport of NPY to nerve terminals for release at this site. Current immunocytochemical and other experimental evidence (5–8) shows that the PVN receives projections from NPY-producing perikarya located in the brainstem and ARC. However, transection of brainstem projections to the PVN has only a slight impact on feeding behavior in the rat (8, 23). Thus, it is likely that the projections of ARC perikarya to the PVN are the primary pathways mediating the close correlation between food consumption and the PVN NPY response. On the other hand, the afferent pathways to the ARC–PVN NPYergic axis that presumably transmit signals for cessation of NPY release in response to food consumption are unknown.

The second experiment, involving food deprivation to elicit robust behavioral signs of hunger, showed that NPY release

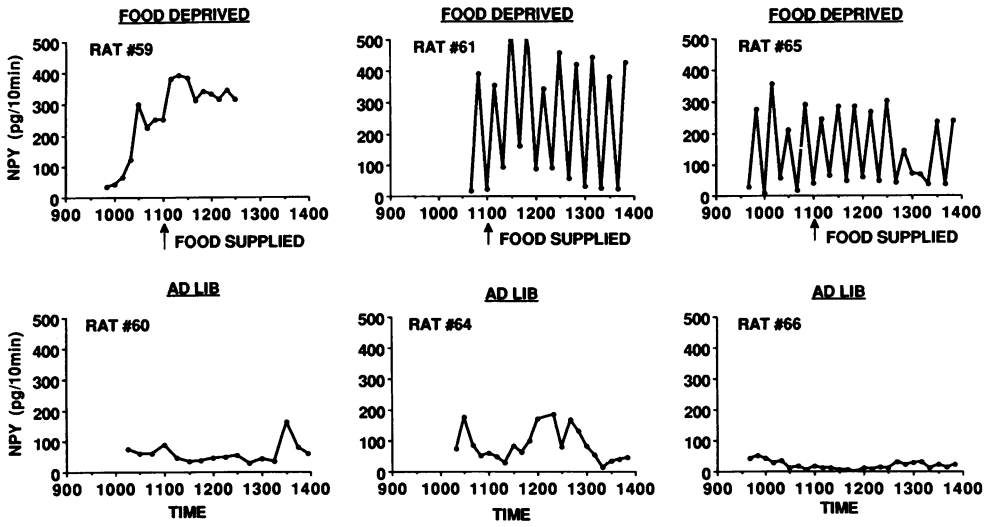

FIG. 6. Representative profiles of PVN NPY release in FD and ad libitum fed rats. Food was presented to FD rats at 1100 h.

in the PVN was increased, but the time course of return to the normal basal condition was different. Decrease in NPY secretion upon initiation of eating, as observed in the SF experiment, did not occur in these rats. Instead, high rates of NPY secretion in the PVN were sustained in conjunction with continuous eating for 4 h, but NPY efflux eventually returned to the control range after 24 h of ad libitum food supply. A similar delayed return in the PVN NPY content was also seen in these rats (17). Thus, one suspects that under these more extreme conditions of fasting, rats require many hours of food consumption in order to affect a return of PVN responses reflecting satiety and replenishment of the attendant nutritional deficits. Overall, fluctuations in NPY release and contents in the PVN (17) and an increase in the prepro-NPY mRNA in the ARC (24, 25) are in harmony with the proposal that robust appetite induced by fasting is also intimately linked with the activation of the ARC–PVN NPY-ergic axis in the brain.

The observation of NPY release in discrete episodes, which were prominent both in food-deprived rats when they were eating and in rats not given food at the scheduled time, was unexpected. Although additional studies for rigorous quantitative reevaluation of amplitude and frequency pattern of NPY discharge under these conditions are necessary, these findings, nevertheless, imply that synchronized increases in the amplitude and frequency of NPY discharge in the PVN may be associated with enhanced appetite.

Thus, a close temporal relationship exists between eating and the output of an excitatory neuropeptidergic signal in a discrete site in the rat brain. The coincidence between high rates of NPY secretion with increased appetite and a progressive return to lower control release rates upon initiation of feeding lend credence to the notion that NPY is the final common neurochemical signal released into the PVN when feeding is desired. Since the structure of NPY is highly conserved in vertebrates (2, 26) and it induces similar robust feeding in other species (1, 4), a universal physiological role of NPY within the neural network that regulates ingestive behavior in vertebrates is possible. Furthermore, experimental hyperphagia in diabetic rats is also associated with a general activation of hypothalamic NPYergic neurons (27–29), and concentrations of NPY are significantly altered in the CSF of patients with eating disorders (30). It is, therefore, possible that abnormal shifts in either secretion or expression of NPY action selectively at discrete anatomical substrates in the brain, which include the PVN, underlie the etiology of clinical eating disorders—namely, anorexia nervosa, bulimia, and obesity.

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