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Neuropeptide Y in Central Control of Feeding and Interactions with Orexin and Leptin

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Neuropeptide (NPY) increases feeding when injected into the brain. In this study, we tested the hypothesis that its action might be related to feeding regulation of the orexin and leptin systems in rats. Intracerebroventricular administration of NPY (1 nmol/5 µL) stimulated feeding in rats. Injection of an antibody to orexin-A inhibited feeding, suggesting that endogenous orexin exerts a stimulatory tone on feeding. Intracerebroventricular injection of orexin antiserum before injection of NPY significantly attenuated the feeding response to NPY. On the other hand, ip pretreatment with leptin (2 mg/kg) significantly decreased food intake and inhibited NPY-induced feeding. We then examined whether orexin-containing neurons are activated under the stimulation of feeding in response to intracerebroventricular NPY or suppression of feeding in response to ip leptin, using Fos-like immunoreactivity (FLI) as a marker of neural activation. We observed that FLI was induced in the paraventricular, supraoptic, and dorsomedial nuclei as well as the lateral hypothalamic area (LHA) following administration of NPY. Double staining with anti-Fos and anti-orexin antibodies revealed that 23.4% of the orexin-containing neurons in the LHA expressed FLI after NPY injection. Approximately 7.8% of the orexin-positive neurons in the LHA coexpressed Fos after leptin plus NPY. Our data indicate that a functional interaction among NPY, orexin, and leptin exists that may contribute to the central regulation of appetite.

Key Words: Neuropeptide Y; orexin; leptin; food intake; Fos; immunohistochemistry.

Introduction

The central mechanisms coordinating energy homeostasis reside primarily in the hypothalamus, where hormonal and visceral inputs are integrated *(1)*. Neuropeptide Y (NPY) is the most powerful stimulant of feeding known *(2,3)* and has been shown to interact functionally with other hypothalamic neuropeptides, such as galanin and dynorphin, in the central control of food intake *(1,4)*. Recently, a new class of orexigenic peptides, orexin-A and orexin-B, was isolated from the hypothalamus *(5,6)*. Orexin-producing neurons are localized in the lateral hypothalamic area (LHA). NPY-immunoreactive axons are abundant in the LHA in both rat and primate brains. NPY-expressing cells in the arcuate nucleus (ARC) of the hypothalamus make synaptic contacts with orexin neurons in the LHA *(7)*. Some LHA neurons show Fos-like immunoreactivity (FLI) after central administration of NPY *(8)*. These observations suggest the functional cooperation of NPY neurons and orexin neurons in the brain.

On the other hand, leptin is a hormone synthesized in white adipose tissue and released into the circulation that participates in the regulation of food intake and metabolism *(9)*. The anorectic effects of leptin are mediated via specific leptin receptors (Ob-R) in the hypothalamus *(10)*. The long leptin receptor isoform (Ob-Rb) mRNA and Ob-R-immunoreactive cells have been demonstrated in several hypothalamic nuclei *(11–13)*. Double-labeling studies have shown that Ob-R are present in NPY-containing neurons of ARC *(13)*. The administration of leptin has been shown to suppress NPY gene expression in the ARC *(14–16)*, suggesting that suppression of feeding by leptin may, in part, be mediated by the hypothalamic NPY system. In addition, Ob-R have been localized in orexin perikarya, suggesting a direct action of leptin on these neurons *(7,17)*.

In the present study, we show that NPY acts in the brain to stimulate food intake and may have functional interactions with the orexin and leptin systems in rats. Furthermore, we investigated the neural activity of the orexin-immunoreactive (ir) neurons under stimulation of feeding in response to intracerebroventricular NPY or suppression of feeding in response to ip leptin using FLI as a marker of neuronal activation.

Results

Effects of Orexin Antiserum on NPY-Induced Food Intake

Cumulative food intake over a 4-h period (2 h in the dark phase) after intracerebroventricular NPY injection in rats

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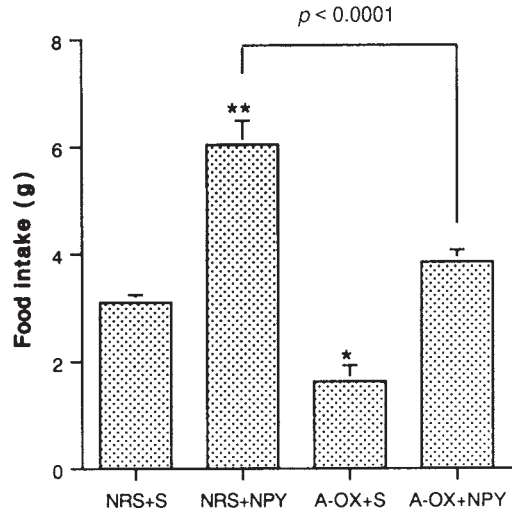

Fig. 1. Effects of NPY and antiserum to orexin-A (A-OX) on food intake measured over 4 h (2 h in the dark phase). The histogram represents the mean ± SEM ($n = 5$ rats/group). *$p < 0.01$ vs NRS + S group; **$p < 0.0001$ vs normal rabbit serum (NRS) + saline (S) group.

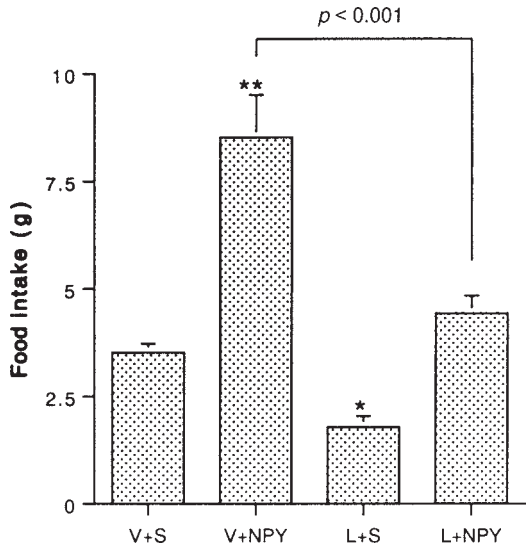

Fig. 2. Effects of leptin (L) and NPY on food intake measured over 4 h ($n = 5$/group, mean ± SEM). *$p < 0.05$ vs V + S group; **$p < 0.0001$ vs vehicle (V) + saline (S) group.

was significantly increased by 195% compared with that in controls (Fig.1). Intracerebroventricular injection of orexin antibody suppressed food intake by 52% compared with controls. Intracerebroventricular injection of orexin antibody before injection of NPY significantly attenuated the feeding response to NPY.

Effects of Leptin on NPY-Induced Food Intake

NPY alone elicited a feeding response that was significantly greater than that seen in control rats (Fig. 2). Intraperitoneal pretreatment with leptin significantly reduced food intake by 51% compared with the control group. Rats injected with leptin before receiving NPY consumed 52% less food than those receiving NPY alone.

Table 1
Rate of Fos-ir Neurons
Within Orexin-ir Neurons of Rats in Four Treated Groups

	Total orexin-ir cells	Orexin and Fos-ir/orexin-ir (%)
Vehicle + saline ($n = 4$)	539 ± 74	0
Vehicle + NPY ($n = 4$)	480 ± 32	23.4 ± 2.1
Leptin + saline ($n = 4$)	551 ± 40	0
Leptin + NPY ($n = 4$)	442 ± 36	7.8 ± 1.9[a]

[a] $p < 0.01$ vs vehicle + NPY group.

Immunohistochemical Identification

Administration of 1 nmol of NPY intracerebroventricularly induced expression of FLI in the paraventricular nucleus (PVN), supraoptic nucleus (SON), and dorsomedial nucleus (DMN) of the hypothalamus and LHA (data not shown). Orexin-ir was clearly identified in the perikarya within and around the LHA. No significant difference was observed among the four treated groups of animals with respect to the number of orexin-containing neurons (Table 1). Double staining revealed that FLI was present in many orexin-containing neurons of the LHA in the NPY-treated group (Fig. 3). The percentage of orexin neurons double labeled for FLI was 23.4 ± 2.1 in the NPY-treated group. In the saline-treated control rats, the proportion of orexin neurons coexpressing Fos was 0% in the LHA. On the other hand, in rats injected intraperitoneally with leptin, many Fos-positive cells were present in the ventromedial hypothalamic (VMN), the posterior part of the DMN, and the arcuate nuclei, but Fos-positive cells in the LHA were very scarce, as previously reported *(18)*. The proportion of orexin-positive neurons in the LHA coexpressing Fos after leptin plus NPY was significantly smaller than that seen in the vehicle + NPY-treated group. The percentage of orexin neurons double labeled for Fos was 0% in the leptin + saline group.

Discussion

NPY has been established as a powerful orexigenic agent, and we have shown that intracerebroventricular injection of NPY (1 nmol) dramatically increases 4 h food intake. It has recently been shown that orexins play a role as mediators in the central mechanisms that regulate feeding behavior and sleep control *(6,19,20)*. The administration of orexins increases food intake and locomotor activity in rats *(6,21)*. In the present study, injection of an orexin antibody inhibited 4 h food intake, suggesting that endogenous orexin exerts a stimulatory tone on feeding. The anorectic action of orexin antibody with a decrease in activity and drinking behavior was observed within 40 min after the

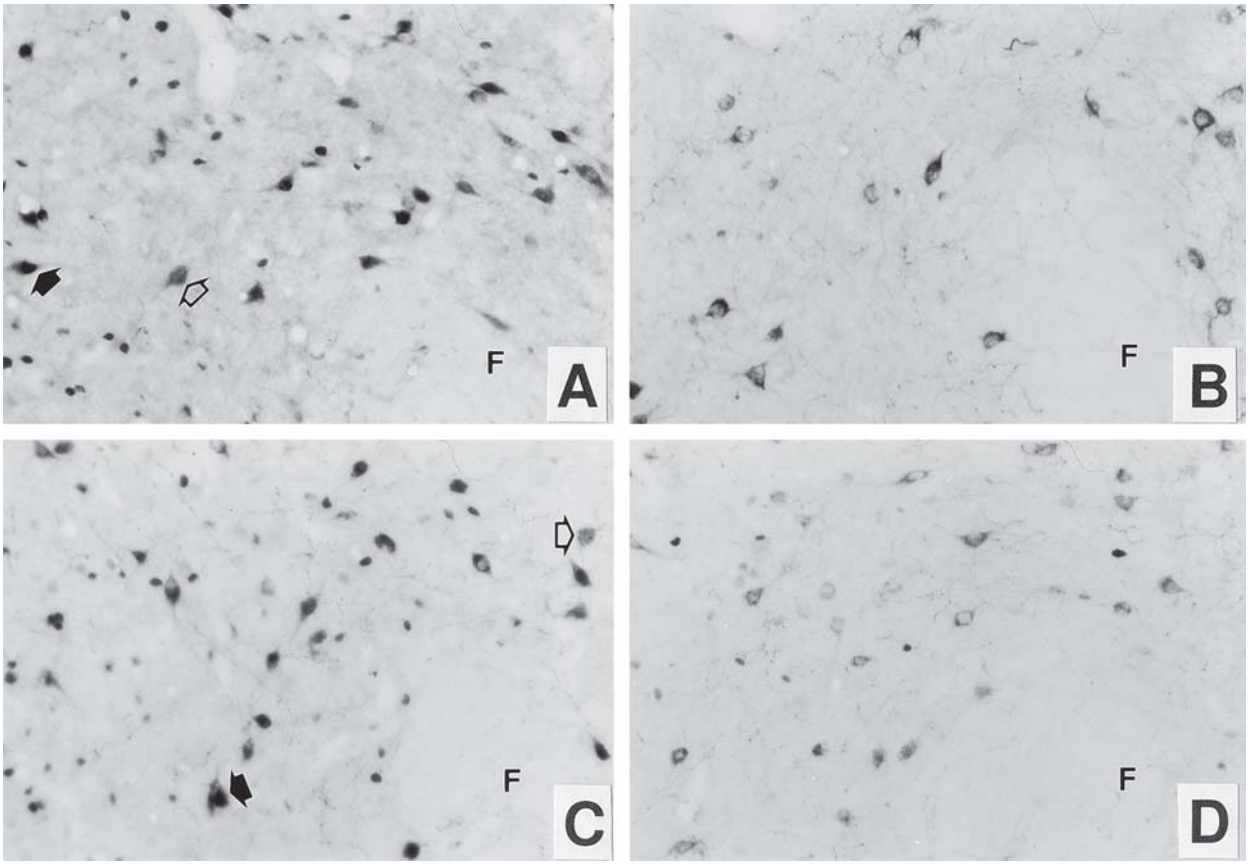

Fig. 3. Representative section of the LHA stained immunocytochemically for orexin (cytoplasm) and Fos (nucleus) in (**A**) vehicle + NPY-injected or (**B**) vehicle + saline-injected group. Solid arrows indicate double-labeled cells; open arrows indicate orexin-ir, but Fos-negative cells. Magnification: ×200. (**C**) A few orexin contain neurons in the LHA expressed Fos in the leptin + NPY-injected group. (**D**). No orexin-producing neurons showed FLI in the leptin+saline group. Magnification: ×200. F, Fornix.

intracerebroventricular injection. We also found that the orexin antibody–treated rats showed periods of reduced activity during the light phase, and we speculated that orexin's primary effect on food intake is on arousal and motivation. Similarly, Yamada et al. *(22)* reported that intracisternal but not ip injection of the orexin antibody suppressed feeding in a dose-dependent manner. Further study will be needed to examine the role of orexins in the relationship between the regulation of feeding and sleep. Intracerebroventricular injection of orexin antiserum before injection of NPY attenuated the feeding response to NPY. These results suggest that stimulation of feeding by NPY may, in part, be mediated by the hypothalamic orexin system. Since the actual increase in food intake in grams in response to NPY was similar to that for the normal rabbit serum vs the orexin antibody–treated groups, the second possibility is that most of NPY's downstream actions may be independent of orexin. Therefore, we examined whether orexin-containing neurons responded to NPY using immunohistochemical staining for Fos and orexin.

Intracerebroventricular administration of NPY stimulated FLI in neurons located in the SON, PVN, DMN, and LHA, as shown in previous investigations *(8,23)*. This was supported by the localization of NPY Y_1 and Y_5 receptor subtypes, putative receptors mediating stimulation of feeding by NPY *(24–26)*. Yokosuka et al. *(27)* demonstrated that pretreatment of rats with the NPY Y_1 receptor antagonist 1229U91 attenuated both NPY-induced feeding and Fos activation in the magnocellular regions of the PVN (mPVN). These results suggest that a subpopulation of Y_1 receptor-containing neurons in the mPVN may, in part, mediate the orexigenic effects of NPY. An additional site of action may be the LHA. It was evident that intracerebroventricular injection of NPY triggered Fos expression in some of the orexin-containing perikarya within the LHA. These results do not prove that NPY has a direct effect on orexin cells. However, a previous report *(7)* has shown synaptic interaction between NPY nerve boutons and orexin-containing neurons in LHA by electron microscopic immunostaining. The synaptic contact clearly suggests the direct innervation of orexin-containing neurons by NPY fibers. The ARC-LHA in addition to ARC-PVN NPY pathways may be activated in response to signals associated with a decline in body fat stores.

Leptin previously has been shown to inhibit NPY-induced food intake in rats *(23,28)*, and the present study also

demonstrates this. In addition, we observed that the orexin neurons of the LHA containing NPY-induced Fos-positive nuclei were significantly reduced by leptin pretreatment. Recent studies have shown that orexin-containing neurons express Ob-R immunoreactivity and Ob-R mRNA *(7,17)*. Therefore, orexin neurons are able to detect this signal from adipose tissue. Interestingly, Moriguchi et al. *(29)* reported that some populations of neurons that contain orexins in the LHA are activated under hypoglycemic conditions. These reports suggest that orexin neurons themselves may express both glucose and leptin receptors. The excitatory actions of NPY could increase orexin release, resulting in enhanced feeding behavior, whereas leptin would have the opposite effect on the same neurons, leading to a decrease in orexin-mediated hypothalamic functions.

Materials and Methods

Animals

All experiments were performed on adult male Sprague-Dawley rats weighing 250–300 g. They were kept under controlled lighting (light on, 6:00 AM to 6:00 PM) and temperature (22ºC). Free access to laboratory chow and tap water was provided.

Experiment 1

A lateral ventricle cannula was implanted in each rat, as described previously *(30)*. Following surgery, rats were placed in individual cages and were handled for about 10 min/d between 3:00 PM and 5:00 PM. Ten days after the operation, the rats were divided into four groups and food was withdrawn at 2:30 PM. Half of the rats were injected intracerebroventricularly with antiserum to orexin (5 µL) (Peptide Institute, Osaka, Japan), and the other half received normal rabbit serum (5 µL) alone at 3:30 PM. Thirty minutes later, the antiserum-injected rats were subdivided into two groups; one group received intracerebroventricular NPY (1 nmol/5 µL in saline) (Peptide Institute), and the other group received saline (5 µL). Similarly, normal rabbit serum–injected rats were divided into two subgroups; one group was injected with NPY intracerebroventricularly, and the other group was injected with saline at 4:00 PM. After injection, the rats were returned to their home cages, which contained a known amount of rat chow. At 4 h after injection, the remaining food was weighed. The experiment to determine its effect on food intake, which was performed only once in each rat, was then conducted.

Experiment 2

To investigate the effects of leptin on NPY-induced food intake, rats were preimplanted with permanent lateral ventricle cannulae as described in experiment 1. On the day of the experiment, rats were divided into four groups and food was withdrawn at 2:30 PM. The rats were injected intraperitoneally with 2.0 mg/kg of recombinant mouse leptin (Pepro Tech EC, London, UK) dissolved in vehicle (0.05 *M* Tris-HCl, pH 8.0) or vehicle alone at 3:00 PM. These rats received NPY (1 nmol/5 µL in saline) or saline intracerebroventricularly at 4:00 PM and 4 h food intake was measured.

Experiment 3

The rats implanted with an intracerebroventricular cannula were divided into four groups and food was withdrawn at 2:30 PM. Half of the rats were injected intraperitoneally with recombinant leptin (2 mg/kg); the other half received vehicle alone at 3:00 PM. The rats were returned to their home cages. One hour later, these rats were injected with NPY (1 nmol/5 µL in saline) or saline intracerebroventricularly at 4:00 PM. Two hours later, the rats were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde. The brains were removed, postfixed overnight in the same fixative, and then placed in 15% sucrose in phosphate-buffered saline (PBS) for 24 h. The brains were frozen, 25-µm transverse sections were cut by cryostat, and every third sections was collected into PBS.

For Fos and orexin double immunohistochemistry, the sections were first stained for Fos. Fos primary antiserum (1:2000) (Ab-2, rabbit polyclonal IgG; Oncogene Science, NY) was used for 48 h at 4ºC. This antiserum was prepared against residues 4–17 of human *c-fos* and not contained in Fra proteins. The subsequent procedure for the immunohistochemistry was followed by Vector's protocol (Vectastain ABC kit; Vector) using previously described methods *(14, 27)*. Fos was visualized after the addition of 3,3'-diaminobenzidine, nickel ammonium sulfate, and 0.03% hydrogen peroxide in 50 m*M* Tris. Before staining for orexin, the sections were incubated with 3% hydrogen peroxide to prevent any further reaction of the Fos-linked peroxidase. Sections were then incubated in normal goat serum and rabbit antihuman orexin IgG (1:2500) (Peptide Institute). After approx 24 h of incubation with the primary antibody, the sections were rinsed with PBS and Vector's protocol was carried out. Peroxidase linked to orexin was visualized with 3,3'-diaminobenzidine and hydrogen peroxide. Preadsorption of orexin antiserum with 10 µ*M* orexin-A or rat orexin-B (Peptide Institute) antigen completely blocked orexin-ir.

The number of FLI nuclei particularly in the orexin-ir neurons was counted in five coronal sections throughout the hypothalamic region containing these neuronal populations. Counting was performed on sections from identical retrocaudal levels, making direct comparison between animals possible. For each field, the number of FLI nuclei belonging to orexin-ir neurons was scored.

Statistical Analyses

Data are presented as mean ± SEM. Food intake was analyzed using one-way analysis of variance followed by Fisher's PLSD test. The cell count data in the third experimental group were analyzed using the unpaired student's *t*-test.

References

1. Kalra, S. P., Dube, M. G., Pu, S., Xu, B., Horvath, T. L., and Kalra P. S. (1999). *Endocr. Rev.* **20,** 68–100.
2. Clark, J. T., Kalra, P. S., Cowley, W. R., and Kalra, S. P. (1984). *Endocrinology* **115,** 427–429.
3. Lambert, P. D., Wilding, J. P. H., Al-Dokhayel, A. A., Bohuon, C., Comoy, E., Gilbey, S. G., and Bloom, S. R. (1993). *Endocrinology* **133,** 29–32.
4. Lambert, P. D., Wilding, J. P. H., Al-Dokhayel, A. A., Gilbey, S. G., and Bloom, S. R. (1993). *Brain Res.* **629,** 146–148.
5. de Lecea, L., Kilduff, T. S., Peyron, C., et al. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 322–327.
6. Sakurai, T., Amemiya, A., Ishii, M., et al. (1998). *Cell* **92,** 573–585.
7. Horvath, T.L., Diano, S., and van den Pol, A.N. (1999). *J. Neurosci.* **19,** 1072–1087.
8. Li, B-H., Xu, B., Rowland, N. E., and Kalra, S. P. (1994). *Brain Res.* **665,** 277–284.
9. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994). *Nature* **372,** 425–432.
10. Tartaglia, L.A., Dembski, M., Weng, X., et al. (1995). *Cell* **83,** 1263–1271.
11. Schwarz, M. W., Seeley, R. J., Campfield, L. A., Burn, P., and Baskin, D. G. (1996). *J. Clin. Invest.* **98,** 1101–1106.
12. Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R., and Friedman, J. M. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 7001–7005.
13. Hakansson, M. L., Brown, H., Ghilardi, N., Skoda, R. C., and Meister, B. (1998). *J. Neurosci.* **18,** 559–572.
14. Stephens, T. W., Basinski, M., Bristow, P. K., et al. (1995). *Nature* **377,** 530–532.
15. Schwartz, M. W., Baskin, D. G., Bukowski, T. R., et al. (1996). *Diabetes* **45,** 531–535.
16. Schwartz, M. W., Erickson, J. C., Baskin, D. G., and Palmiter, R. D. (1998). *Endocrinology* **139,** 2629–2635.
17. Hakansson, M. L., de Lecea, L., Sutcliffe, J. G., Yanagisawa, M., and Meister, B. (1999). *J. Neuroendocrinol.* **11,** 653–663.
18. Niimi, M., Sato, M., Yokote, R., Tada, S., and Takahara, J. (1999). *J. Neuroendocrinol.* **11,** 605–611.
19. Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P. J., Nishino, S., and Mignot, E. (1999). *Cell* **98,** 365–376.
20. Chemelli, R. M., Willie, J. T., Sinton, C. M., et al. (1999). *Cell* **98,** 437–451.
21. Hagan, J. J., Leslie, R. A., Patel, S., et al. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 10,911–10,916.
22. Yamada, H., Okumura, T., Motomura, W., Kobayashi, Y., and Kohgo, Y. (2000). *Biochem. Biophys. Res. Commun.* **267,** 527–531.
23. Yokosuka, M., Xu, B., Pu, S., Kalra, P. S., and Kalra, S. P. (1998). *Physiol. Behav.* **64,** 331–338.
24. Larsen, P. J., Sheikh, S. P., Jakobsen, C. R., Schwartz, T. W., and Mikkelsen, J. D. (1993). *Eur. J. Neurosci.* **5,** 1622–1637.
25. Kanatani, A., Ishihara, A., Asahi, S., Tanaka, T., Ozaki, S., and Ihara, M. (1996). *Endocrinology* **137,** 3177–3182.
26. Gerald, C., Walker, M. W., Criscione, L., et al. (1996). *Nature* **382,** 168–171.
27. Yokosuka, M., Kalra, P. S., and Kalra, S. P. (1999). *Endocrinology* **140,** 4494–4500.
28. Sahu, A. (1998). *Endocrinology* **139,** 795–798.
29. Moriguchi, T., Sakurai, T., Nambu, T., Yanagisawa, M., and Goto, K. (1999). *Neurosci. Lett.* **264,** 101–104.
30. Niimi, M., Sato, M., Tamaki, M., Wada, Y., Takahara, J., and Kawanishi, K. (1995). *Neurosci. Res.* **23,** 361–364.