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The neuropeptide Y antagonist PYX2 decreases lordosis behavior

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Neuropeptide Y (NPY) has been localized to noradrenergic neurons and both the noradrenergic system and NPY play a facilitatory role in the control of luteinizing hormone-releasing hormone (LHRH) and luteinizing hormone (LH) release. The present experiments examined whether NPY also plays a role in the control of lordosis. Adult female guinea pigs were ovariectomized (ovx) and implanted with a cannula into the lateral ventricle. In Experiment 1, intracerebroventricular (ICV) administration of the NPY antagonist PYX2 (0, 0.5, 2.0 or 10.0 μg) caused a dose-dependent decrease in lordosis behavior in ovx, estrogen and progesterone-primed guinea pigs. In addition to an effect on the mean lordosis response, PYX2 also decreased the percent of animals showing lordosis and the maximum lordosis response. In Experiment 2, NPY administration (25 μg, ICV) 30 min after PYX2 (2 μg, ICV) to ovx estrogen and progesterone-primed females significantly reversed the effect of the PYX2. Because the NPY antagonist PYX2 reversibly decreased lordosis behavior this suggests that NPY plays a facilitatory role in the control of lordosis behavior.

Keywords: Neuropeptide Y; NPY; lordosis; guinea pigs; noradrenergic, sexual behavior; PYX2; NPY antagonist

Introduction

Neuropeptide Y (NPY), is a tyrosine-rich, 36 amino acid peptide that is highly conserved across species (Tatemoto *et al.*, 1982; Allen & Balbi, 1993). NPY is one of the most abundant peptides in the mammalian brain (Tatemoto, 1989) and modulates a variety of processes including effects on neuroendocrine systems (Kalra & Crowley, 1984; McDonald *et al.*, 1985; Wahlestedt *et al.*, 1987; McDonald & Koenig, 1993), food intake (Clark *et al.*, 1984; Levine & Morley, 1985; Stanley, 1993) and memory (Flood *et al.*, 1987).

High levels of neuropeptide Y are found in several hypothalamic nuclei implicated in the regulation of neuroendocrine function and sexual behavior. NPY perikarya are densely distributed in the arcuate nucleus and the dorsomedial hypothalamus (Chronwell *et al.*, 1985; deQuidt *et al.*, 1986). Neuropeptide Y immunoreactive fibers are found throughout the medial preoptic area and in a number of hypothalamic areas including the paraventricular, arcuate and periventricular nuclei and in the lateral hypothalamus, ventromedial nuclei and median eminence (Allen *et al.*, 1983; Chronwell *et al.*, 1985; Nakagawa *et al.*, 1985; deQuidt *et al.*, 1986).

Neuropeptide Y immunoreactivity in the hypothalamus has been colocalized with a number of other substances including catecholamines. Many of the NPY-containing fibers in the hypothalamus arise from cell bodies located in the adrenergic and noradrenergic nuclei of the brainstem and NPY is coexpressed in adrenergic neurons (Everitt *et al.*, 1984; Sawchenko *et al.*, 1985; Holets *et al.*, 1988).

The noradrenergic/adrenergic system plays an important facilitatory role in the control of both LHRH/LH release (e.g. Barraclough & Sawyer, 1957; Kalra *et al.*, 1972; Gallo, 1980; Alder *et al.*, 1983; Kalra & Kalra, 1983) and lordosis behavior (e.g. Crowley *et al.*, 1976; Nock & Feder, 1984; Thornton *et al.*, 1989; Vincent *et al.*, 1989). Since NPY and NE are colocalized, it is possible that NPY and NE would be co-released and might both act to modulate the preovulatory LHRH and LH surge and/or lordosis behavior. Recent data indicate that NPY does indeed play a stimulatory role in the preovulatory control of LHRH and LH release (Kalra & Crowley, 1984; McDonald *et al.*, 1985; Sabatino *et al.*, 1990; Bauer-Dantoin *et al.*, 1992). Whether NPY also facilitates lordosis is still unclear.

The present studies examined whether NPY plays a role in the control of sexual behavior in the female guinea pig. This is the first report on the effects of NPY on lordosis in the guinea pig. The small amount of data available in the rat suggests that NPY might decrease female typical sexual behavior in that species (Clark *et al.*, 1985; Clark, 1992). Although these findings are intriguing, the authors were hampered by the fact that no specific NPY antagonists were available. Consequently this research was done with either NPY itself or with the antagonist benextramine. The interpretation of these results is problematic. In the case of NPY administration, the animals already had endogenous levels of NPY, so any administered NPY was in excess of physiological levels. In the case of benextramine administration, this NPY antagonist is also an irreversible alpha 1 adrenergic antagonist (Clark, 1992). Consequently, any effects could be due to an interference with adrenergic neurotransmission rather than a specific effect on NPY. Recently a more specific NPY receptor antagonist, PYX2, which appears to act at both Y1 and Y2 NPY receptors (Tatemoto *et al.*, 1992) has become available. The present experiments used PYX2 to evaluate whether NPY plays a role in the control of lordosis behavior in the female guinea pig.

Results

Experiment 1: Dose-dependent effects of the NPY antagonist PYX2 on lordosis

When the lordosis response was examined across time, it was apparent that although there was no difference between groups prior to drug treatment (Figure 1, hours 0 and −1), administration of the neuropeptide Y antagonist PYX2 caused a significant dose-dependent decrease in lordosis responding (Figure 1). The 0.5 μg dose of PYX2 caused a small decrease in the lordosis response which reached statistical significance on hours 2 and 3. Both the 2 μg and the 10 μg PVX2 dose also caused a decrease in the lordosis response. This effect was apparent by 30 min and lasted throughout the 5 h of testing. Additionally, the 2 and 10 μg dose of PYX2 suppressed lordosis significantly below the levels seen with 0.5 μg PYX2 (hours 0.5, 1.0, 1.5, 2, 3, 4 and 5, for 2 μg, and hours 0.5, 1.0, 4 and 5, for 10 μg). There were no statistically significant differences between the 2 μg and 10 μg PYX2 dose for any of the hours tested.

This effect of the NPY antagonist PYX2 was partly mediated via a decrease in the number of animals that showed lordosis. As shown in Figure 2, both the 2 μg and the

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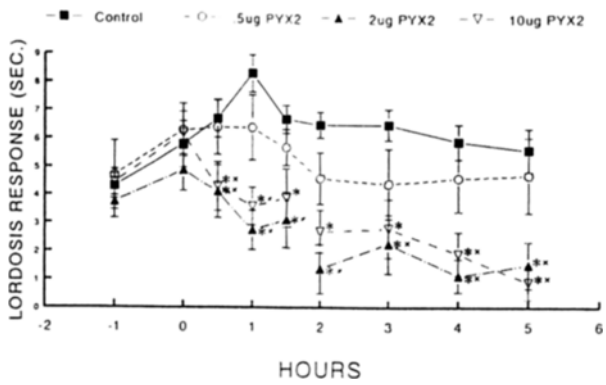

Figure 1 The NPY antagonist PYX2 caused a dose-dependent decrease in lordosis responding. Prior to drug infusion there were no significant differences between the groups (hours −1 and 0). When 0.5 μg PYX2 was infused, it significantly decreased the lordosis response (* = $p < 0.05$ compared to control at same hour). The 2 μg and 10 μg doses of PYX2 decreased the lordosis response even further (* = $p < 0.05$ compared to control at same hour, x = $p < 0.05$ compared to 0.5 μg PYX2 dose at same hour)

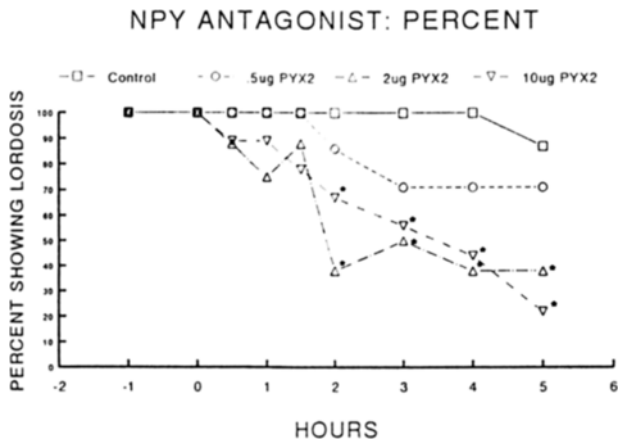

Figure 2 The NPY antagonist PYX2 decreased the percent of animals that showed lordosis. Although there was no statistically significant effect of the 0.5 μg dose, both the 2 μg and the 10 μg dose of PYX2 significantly decreased the percent of animals that showed lordosis on hours 2, 3, 4 and 5 of testing (* = 0.002–0.042 compared to Control group at same time point)

10 μg dose of PYX2 significantly decreased the percent of females that showed lordosis on hours 2, 3, 4 and 5 of testing (Fishers Exact $p = 0.002–0.042$ compared to Control group at same hour). However, it appeared that the effect of PYX2 was not just on the percent of animals responding. As seen in Figure 1, there was a significant decrease in the lordosis response as early as 0.5, 1.0 and 1.5 h after PYX2, at which time there was no significant effect on the percent of animals that responded (Figure 2). Additionally, even when only those animals which showed a response at hour 1 were examined, it was found that PYX2 still decreased the lordosis response (for hour 1: Control group = 8.27 ± 0.66 s, $n = 15$ *vs* 2 μg PYX2 group = 3.67 ± 0.49 s, $n = 6$, $p < 0.001$ compared to Control group, 10 μg PYX2 group = 4.0 ± 0.57 s, $n = 8$, $p < 0.001$ compared to Control group).

The maximum lordosis response was also decreased by PYX2 administration. As shown in Figure 3, the maximum lordosis response shown by the Control group during the course of testing was 9.53 ± 0.64 s. Although these was no significant effect of the 0.5 μg dose of PYX2 on this measure

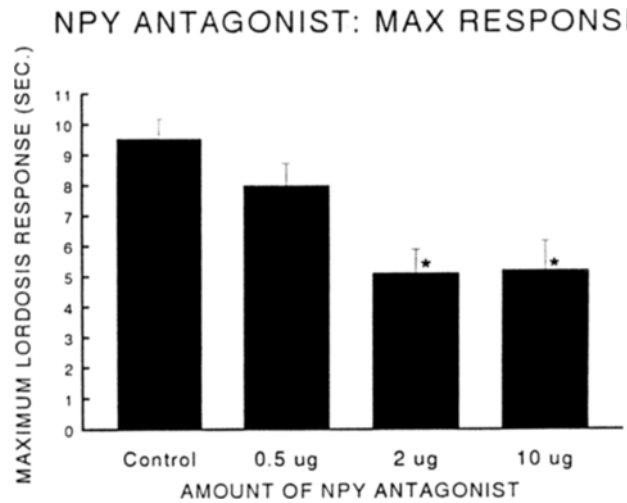

Figure 3 The NPY antagonist PYX2 decreased the maximum lordosis response. Although there was no statistically significant effect of the 0.5 μg dose, both the 2 μg and the 10 μg dose of PYX2 significantly decreased the maximum lordosis response shown (* = $p < 0.001$ compared to Control)

(0.5 μg PYX2 group = 8.0 ± 0.72 s NS compared to Control group), both the 2 μg dose (2 μg PYX2 group = 5.13 ± 0.77 s, $p < 0.001$ compared to Control group) and the 10 μg dose (10 μg PYX2 group = 5.22 ± 0.95 s, $p < 0.001$ compared to Control group) significantly decreased the maximum lordosis response shown.

Experiment 2: Reversal of the effects of PYX2 with NPY

Consistent with Expt. 1, the infusion of PYX2 significantly decreased the lordosis response across time. Prior to drug infusion there were no significant differences between the groups (Figure 4, hours −1 and 0). Subsequent infusion of the NPY antagonist PYX2 significantly decreased the lordosis response across the 5 h of testing relative to control females (Figure 4, PYX2 + Saline females, $p < 0.05$ for hours 1–5 compared to Controls). Once again, PYX2 affected the percent of animals which showed lordosis across time (Figure 5): only 4/10 females that were infused with PYX2 showed lordosis for hours 2–5 whereas 7/7 Control females showed lordosis at the same hours (Fishers Exact $p = 0.035$). Even when only those animals which showed a response were examined at hour 1, there was still a significant effect of the PYX2 infusion (PYX2 + Saline group = 3.83 ± 0.75 s, $n = 6$ *vs* control group = 8.57 ± 0.76 s, $n = 7$, $p < 0.001$). The maximum response was also significantly decreased in those animals infused with PYX2 (Figure 6; PYX2 + Saline group = 3.1 ± 0.80 s *vs* Control group = 9.43 ± 0.78 s, $p < 0.005$).

Administration of neuropeptide Y 30 min after PYX2 administration counteracted much of the effect of the PYX2. As shown in Figure 4, NPY after PYX2 infusion significantly increased the lordosis response over that seen with PYX2 + Saline (PYX2 + NPY females, $p < 0.05$ compared to PYX2 + Saline females for hours 1–4). However, the lordosis response of the females given both PYX2 and NPY was still decreased relative to Control females (Figure 4, $p < 0.05$ for hours 1 and 3–5). This same trend of an intermediate response in animals given NPY after PYX2 as compared to Control animals and PYX2 + Saline treated animals can be seen in both the percent of animals which showed lordosis (Figure 5; for PYX2 + NPY, $p \geq 0.05$ compared to Control and PYX2 + Saline groups), and the maximum lordosis response (Figure 6: for PYX2 + NPY *vs* Control, $p = 0.354$, NS; for PYX2 + NPY *vs* PYX2 + Saline, $p < 0.05$).

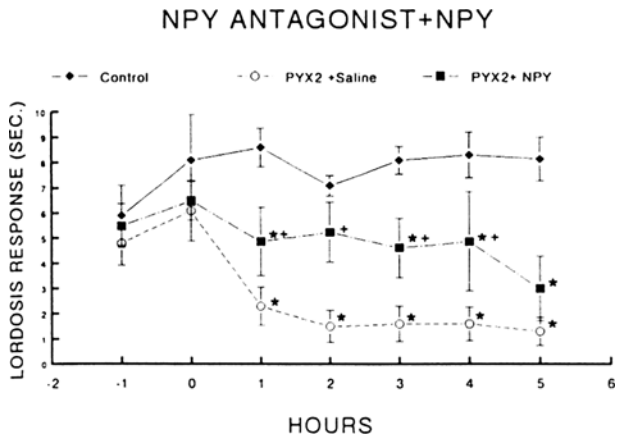

Figure 4 NPY reversed the effects of the NPY antagonist PYX2. The NPY antagonist PYX2 (2 μg) significantly decreased lordosis behavior across the hours of testing (PYX2 + Saline animals, * = $p < 0.05$ compared to Controls at same hour). Administration of Neuropeptide Y significantly reversed the effect of this putative NPY antagonist (PYX2 + NPY animals; + = $p < 0.05$ compared to PYX2 + Saline group at same hour). However, the lordosis response of the PYX2 + NPY females remained significantly shorter than the Control females for hour 1 and 3–5 (* = $p < 0.05$ compared to Control)

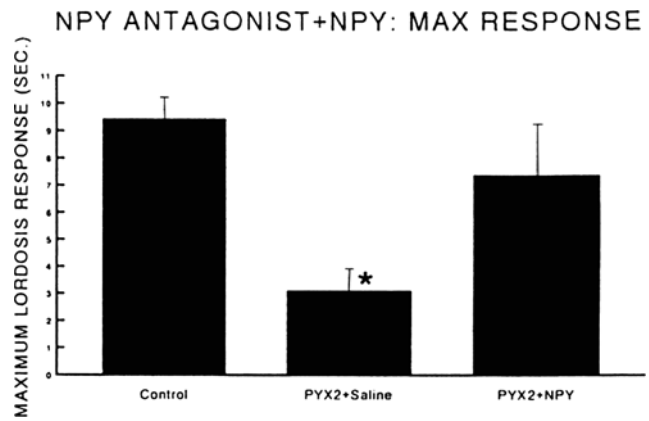

Figure 6 NPY reversed the effect of PYX2 on the maximum lordosis response shown. The maximum lordosis response of the PYX2 + Saline treated animals was significantly shorter than that of the Control females. NPY after PYX2 treatment blocked the effects of PYX2 so that the PYX2 + NPY females showed a significantly longer maximum lordosis response than the PYX + Saline animals and were not different from the Controls (* = $p < 0.05$ compared to Control and PYX2 + NPY females)

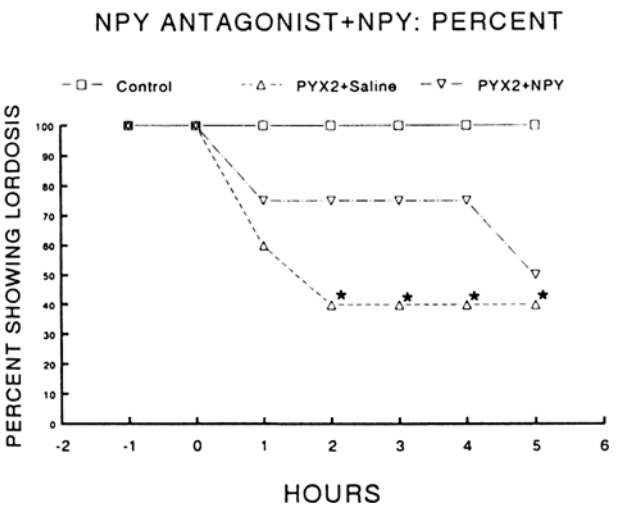

Figure 5 Effect of PYX2 and PYX2 + NPY on the percent of animals showing lordosis across the hours of testing. PYX2 + Saline administration significantly decreased the percent of animals that showed lordosis for hours 2–5 (* = $p < 0.035$ compared to Control). Animals treated with PYX2 + NPY showed an intermediate response, which was not statistically different from either the controls or the PYX2 + Saline treated animals ($p > 0.05$, NS)

Discussion

The neuropeptide Y antagonist PYX2 significantly decreased lordosis behavior in ovx estrogen and progesterone treated guinea pigs in a dose dependent manner. A small decrease was seen with 0.5 μg PYX2 whereas maximal suppression was seen with either 2.0 or 10.0 μg PYX2. PYX2 decreased not only the mean lordosis response across time, but also significantly decreased the percent of animals that showed lordosis and the maximum lordosis response. These data suggest that normally NPY plays a facilitatory role in the control of lordosis behavior. Administration of NPY counteracted much of the effect of the NPY antagonist PYX2 on lordosis behavior. This suggests that the effect of PYX2 was indeed via an effect on neuropeptide Y receptors rather than through some other, nonspecific action.

The present studies are consistent with what is known about the effects of ovarian hormones on NPY and lordosis, and suggest that one of the ways that ovarian hormones act on the brain to produce lordosis may be by increasing NPY. That is, it has been well established for many years that ovarian hormones induce lordosis behavior in female rodents (e.g. Young, 1961), and there is also good evidence that ovarian hormones increase NPY. For example, ovariectomy decreases and estradiol replacement restores NPY levels in the arcuate and ventromedial hypothalamus (Crowley *et al.*, 1985; Kalra & Crowley, 1992). Furthermore, progesterone injection to estrogen primed female rats induces further rapid increases in NPY levels in the median eminence (Crowley *et al.*, 1985). Ovarian hormones have also been shown to increase the levels of pre-pro-NPY mRNA in the mediobasal hypothalamus (Sahu *et al.*, 1994). That ovarian hormones can act directly on NPY cells is indicated by the demonstration that NPY immunoreactive cells in the arcuate contain estradiol receptors (i.e. accumulate ^{3}H-estradiol; Sar *et al.*, 1990).

Because the PYX2 and NPY were given intracerebroventricularly in the present studies, rather than to a particular nucleus of the brain, we do not know the precise locus of action of the NPY that was blocked with PYX2. However, it is now clear that the endogenous actions of NPY can be blocked with PYX2 and that this has an effect on lordosis. Because NPY is found in the hypothalamus and ovarian hormones increase hypothalamic NPY levels, and because the ventromedial hypothalamus (VMH) is involved in the control of lordosis behavior (e.g. Rubin & Barfield, 1983; Pfaff & Schwartz-Giblin, 1988) we speculate that NPY is acting at the VMH to affect lordosis. Further work is needed to test this hypothesis.

The present experiments which indicate that NPY increases lordosis behavior are congruent with data that indicate that NPY increases LHRH/LH. Rodent females show lordosis only during a circumscribed period of time during the estrous cycle, around the time of the LH surge and ovulation and obviously, both ovulation and sexual receptivity (i.e. lordosis) need to occur for reproduction to occur. Consequently because NPY increases preovulatory LH release, it is not surprising that NPY also increases lordosis behavior. To facilitate the LH surge, it appears that NPY may act at both the hypothalamic and pituitary levels. That is, NPY can stimulate LHRH release in estrogen and progesterone primed

ovx rats (Crowley & Kalra, 1987; Sabatino *et al.*, 1989, 1990) and NPY may also act at the pituitary to enhance the LH release induced by LHRH (Crowley *et al.*, 1987). Consistent with this, ovarian hormones increase the concentration of NPY mRNA and NPY in discrete hypothalamic regions of the ovx rat concomitant with increases in LHRH and prior to the LH surge (Crowley *et al.*, 1985; Bauer-Dantoin *et al.*, 1992). Thus, ovarian hormones appear to act to increase NPY which then increases LHRH/LH release during the preovulatory period. The present studies suggest that at this same time NPY might also facilitate sexual receptivity (i.e. lordosis behavior).

These data are in contrast to the small amount of data available on the effects of NPY on sexual behavior in the female rat (Clark *et al.*, 1985; Clark 1992). Clark *et al.* (1985) suggested that NPY plays an inhibitory role in the control of lordosis in female rats. That is, NPY administration to the third ventricle significantly decreased sexual receptivity of ovx females treated with estrogen followed by progesterone, and administration of the NPY antagonist Benextramine facilitated lordosis behavior. However, their conclusions were hindered by the fact that the experiments was done using either NPY itself, on top of physiological NPY levels (Clark *et al.*, 1985), or an NPY antagonist that is also an alpha adrenergic antagonist (Clark, 1992). At this point we do not know if the difference is a species or a procedural one.

It is possible that the effects of NPY on lordosis are actually mediated by the effects of NPY on LHRH. In the rat, there is data to suggest that LHRH can act at the midbrain central grey to facilitate lordosis behavior (Pfaff, 1973; Sakuma & Pfaff, 1980). There is no published data on whether LHRH might also stimulate lordosis in the female guinea pig.

Although NPY is colocalized in adrenergic neurons, and the adrenergic system plays an important role in the control of lordosis, is it unknown whether the facilitatory effects of NPY on lordosis seen in the present study are reflective of NPY release from NPY/adrenergic neurons. Although it has been estimated that up to 90% of certain noradrenergic cell groups contain NPY (Kalra & Crowley, 1992) not all NPY cells contain norepinephrine/epinephrine. That is, transection of ascending noradrenergic/adrenergic fibers does not completely eliminate NPY immunoreactivity in the hypothalamus (Holets *et al.*, 1988; Sahu *et al.*, 1988). However, whether or not NE and NPY are released from the same cell, numerous studies have indicated that the noradrenergic system and NPY can interact in a number of different areas. For example, NPY may exert presynaptic inhibition at sympathetic NE nerve terminals (Toth *et al.*, 1993), depress the response of cells in the supraoptic nuclei to A1 synaptic input (Khanna *et al.*, 1993), and act prejunctionally on vascular smooth musculature to decrease norepinephrine release, and postjunctionally to potentiate NE evoked response (Martire & Pistritto, 1992). The noradrenergic system and NPY may also interact in the control of LHRH/LH release. For example, both NPY and alpha 1 receptor stimulation may be needed to induce LHRH release in rabbits (Berria *et al.*, 1991), and the alpha 2 antagonist yohimbine blocks the effects of NPY on LH in rats (Allen *et al.*, 1987). In regards to the control of lordosis, although both the noradrenergic system and NPY appear to play a role in the control of lordosis, it remains to be determined whether they are interacting with each other to affect lordosis behavior, and if so, how.

Materials and methods

General

Adult female Hartley guinea pigs (Hilltop Lab Animals, Inc) were group housed with food and water freely available. Animals were allowed to adapt for at least one week before surgery. For surgery, animals were anesthetized with sodium pentobarbital, and ovariectomized (ovx) and implanted under stereotaxic guidance with a 20 g guide cannula directed at the lateral ventricle (incisor bar = −10 mm, anterior/posterior coordinate = +9.6 mm from the intraaural line, medial/lateral coordinate = 2.5 mm from intraaural line and dorsal/ventral coordinate = 3 mm from dura). Stereotaxic coordinates were determined using the guinea pig stereotaxic atlas of Luparello (1967) and preliminary studies. At the time of surgery, the correct placement of cannulae into the lateral ventricle was verified by infusing a small amount of physiological saline using gravity. Cannulae were attached with dental acrylic, and wires cut 1 mm longer than the guide tubes were inserted into the cannulae to keep them patent. Animals were allowed to recover for at least 1 week.

All hormones were dissolved in sesame oil and injected subcutaneously (0.1 ml volume). Both the NPY antagonist PYX2 (Sigma Chemical Co.) and Neutropeptide Y (Sigma Chemical Co.) were dissolved in sterile physiological saline.

Lordosis in the female guinea pig is generally measured using a manual stimulation technique (Young *et al.*, 1937; Goy & Young, 1957; Thornton *et al.*, 1987, 1989). This technique consists of stimulating the animals by moving one's hand along the animal's hindquarters and moving rostrally partway along the animal's back. Females that are behaviorally receptive will readily adopt the lordosis posture whereas unresponsive females will squat or run and vigorously resist stimulation attempts. The time period over which lordosis can be elicited in this manner corresponds to the period of time that the female will be receptive to a male (Young *et al.*, 1935; Goldfoot & Goy, 1970). Two measures are taken at each time point tested and the longest value is used in the analysis. From data collected in this manner the following behavioural measures were derived: The lordosis response across time (i.e. the mean time in seconds that animals exhibited a lordosis response across the hours of testing. All animals, including both responders and nonresponders, were used for each time point). The percent responding across time (i.e. the perecent of animals at each time point that showed a lordosis response). The lordosis response using responders only (in this case, the mean time in seconds that lordosis was shown was calculated using only those animals which showed a response at that time point). The maximum lordosis response (i.e. the longest response shown by an animal at any time during the course of testing subsequent to drug or comparable control treatment).

Experiment 1

Ovx cannulated females were injected with 20 μg estradiol benzoate (EB). Sixteen hours later they were again injected with 20 μg EB. Forty hours after the first EB, they were injected with 1 mg Progesterone (P). Just before P and hourly afterwards, females were checked for lordosis using the manual stimulation technique (Young *et al.*, 1937; Thornton *et al.*, 1987). If a female showed lordosis for two consecutive hourly tests, she was injected intracerebroventricularly (ICV) with either 0.5 μg ($n = 7$), 2.0 μg ($n = 8$) or 10.0 μg ($n = 9$) PYX2 in 10 μl sterile saline. These doses were chosen based on work by Leibowitz *et al.* (1992) showing that doses of 50–900 pmoles (0.5 μg equals approximately 300 pmoles) PYX2, when administered to the paraventricular nucleus of rats, can affect carbohydrate feeding (Leibowitz *et al.*, 1992), and on preliminary studies. Control females were injected ICV with either the sterile saline vehicle alone (10 μl) or were not injected. As data from these two control groups did not differ, they were combined ($n = 15$). Animals were tested every half hour for 2 h and then hourly for a total of 5 h after drug treatment.

Experiment 2

Ovx cannulated females were injected with 20 μg EB and 40 h later were injected with 0.5 mg P. Just before P and

hourly afterwards females were checked for lordosis. Females that showed lordosis on two consecutive hourly tests were either fused ICV with the NPY antagonist PYX2 (2 μg in 5 μl sterile saline) or they were not infused (Controls; $n = 7$). Thirty minutes later females that had received the PYX2 were fused ICV with either NPY (25 μg in 5 μl; PYX2 + NPY group, $n = 8$) or with the saline vehicle (5 μl; PYX2 + Saline group, $n = 10$). This dose and timing was chosen because work on rats has shown that a fourfold level of NPY relative to PYX2 can counteract the effects of PYX2 on carbohydrate feeding when the NPY is given 30 min after the PYX2 (Leibowitz *et al.*, 1992). All females were then tested for lordosis for 5 h after the PYX2.

Statistics

The mean lordosis response across time data when all the animals were included in the analysis were analysed with a two way (treatment by time) analysis of variance (ANOVA) with repeated measures on time. Planned comparisons were then run using a *t*-Test for Differences Among Several Means (Bruning & Kintz, 1968). The planned comparisons consisted of comparisons between the different treatments within each hour. The percent of animals that showed lordosis across time data were analysed using a two-tailed Fishers Exact Test. The lordosis response data using responders only, and the maximum lordosis response data were analysed with two-tailed independent groups *t*-tests. Any comparisons that did not reach a p value of at least 0.05 were considered statistically nonsignificant (NS).

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