The role of neuropeptide Y and interaction with leptin in regulating feed intake and luteinizing hormone and growth hormone secretion in the pig

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Abstract

Two experiments (EXP) were conducted in ovariectomized prepubertal gilts to test the hypothesis that neuropeptide Y (NPY) stimulates appetite and modulates LH and GH secretion, and that leptin modifies such acute effects of NPY on feeding behavior and LH and GH secretion. In EXP I, gilts received intracerebroventricular (ICV) injections of 0.9% saline (saline; $n=6$), or 10 μg ($n=7$), 50 μg ($n=5$) or 100 μg ($n=7$) NPY in saline and blood samples were collected. In EXP II, gilts received ICV injections of 0.9% saline ($n=4$), or 50 μg leptin ($n=4$), or 100 μg NPY ($n=4$) or 100 μg NPY + 50 μg leptin ($n=4$) in saline, and feed intake was measured at 4, 20 and 44 h after feed presentation and blood samples collected. In EXP I, NPY suppressed LH secretion and the 100 μg dose stimulated GH secretion. In EXP II, NPY reversed the inhibitory effect of leptin on feed intake and suppressed LH secretion, but serum GH concentrations were unaffected. These results support the hypothesis that NPY modulates feed intake, and LH and GH secretion and may serve as a neural link between metabolic state and the reproductive and growth axis in the pig.

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Introduction

Several reports demonstrated that neuropeptide Y (NPY) is a potent regulator of feeding behavior as well as energy expenditure and fat storage (Clark *et al.* 1984, Billington & Levine 1992). In addition, evidence indicates that hypothalamic NPY is responsive to changes in energy balance and blood leptin concentrations (Blum 1997). Leptin administration increased serum concentrations of luteinizing hormone (LH) and growth hormone (GH) in the rodent (Carro *et al.* 1997, Yu *et al.* 1997) and in the feed-restricted ruminant (Nagatani *et al.* 2000, Amstalden *et al.* 2002). We previously reported that intracerebroventricular (ICV) administration of leptin suppressed feed intake, increased serum GH concentrations (Barb *et al.* 1998), and stimulated hypothalamic gonadotropin-releasing hormone (GnRH) release *in vitro* (Barb *et al.* 2004). Central administration of NPY suppressed LH secretion in the monkey (Kaynard *et al.* 1990), ewe (Malven *et al.* 1992) and heifer (Thomas *et al.* 1999). In contrast, ICV infusion of NPY acutely stimulated secretion of GH in sheep (Morrison *et al.* 2003) and cattle (Thomas *et al.* 1999). Garcia *et al.* (2004) reported that leptin pretreatment attenuated the acute effect of

NPY on GH, but not LH secretion in the heifer. In the rat, leptin suppressed but did not block the hyperphagic response to NPY (Yokosuka *et al.* 1998), and NPY suppressed the leptin-induced increase in GH secretion in the fasted rat (Carro *et al.* 1998).

Neuropeptide Y plays a role in relaying the metabolic state of the animal to hypothalamic neurons (White 1993). The majority of NPY innervations reaching the hypothalamus arise from the arcuate nucleus (Grove & Smith 2003) with some of these NPY neurons located outside the blood–brain barrier (Peruzzo *et al.* 2000). Some of the NPY fibers are adrenergic and noradrenergic nerve fibers originating in the brain stem (Fetissov *et al.* 2004) and the medulla oblongata (Everitt *et al.* 1984). The NPY receptors are distributed throughout the hypothalamus, with individual distribution patterns (Fetissov *et al.* 2004). Campbell *et al.* (2001) reported that half of GnRH-immunopositive neurons in the medial preoptic area (MPOA) were immunoreactive for the NPY5 receptor.

Taken together the above reports support the idea that NPY could modulate hypothalamic neuronal activity and serve as a putative link between nutritional status and the growth and reproductive axis in the pig. Thus,

changes in hypothalamic NPY concentrations as a result of feeding, fast and (or) altered metabolism may play a role in regulating appetite, LH and GH secretion.

The goal of the present study was twofold: to determine if NPY stimulates feed intake and GH secretion and suppresses LH secretion and to determine if leptin modifies such effects of NPY on feeding behavior and LH and GH secretion in the pig.

Materials and Methods

Experiment (EXP) I

Eight ovariectomized (OVX) crossbred prepubertal gilts, 90 ± 4 kg body weight (BW) and 150 days of age were surgically implanted with lateral ICV cannulas using the stereotaxic procedure of Estienne *et al.* (1990) and Barb *et al.* (1993). Animals were individually penned in an environmentally controlled building at a constant temperature of 22 °C and artificial 12 h:12 h light:darkness photoperiod. Pigs were fed to appetite daily, at 08 00 and 17 00 h, a corn-soybean meal ration (14% crude protein) supplemented with vitamins and minerals, according to the National Research Council guidelines (NRC 1998). One week after the last ICV surgery, all pigs were fitted with an indwelling jugular vein cannula (Barb *et al.* 1982) 24 h before treatment. On the day of the EXP, pigs were fed at 07 30 h and blood sampling started at 08 00 h. Blood samples were collected every 15 min for 3 h before and 4 h after ICV injections of 150 µl 0.9% saline (saline, $n=2$), or 10 µg ($n=2$), 50 µg ($n=1$) or 100 µg ($n=2$) porcine NPY (Sigma Chemical Co., St. Louis, MO) in 150 µl saline. Second and third replicates were conducted as described above with an additional 8 and 12 OVX prepubertal gilts averaging 94 ± 6 kg and 68 ± 2 kg BW and 150 and 130 days of age respectively. One animal which received saline and two which received 50 µg NPY were eliminated from the study due to loss of patency of the jugular cannula. This resulted in the following groups for the three replicates; saline ($n=6$), 10 µg ($n=7$), 50 µg ($n=5$) and 100 µg ($n=7$) NPY. Serum was harvested and stored at -20 °C until assayed for LH and GH by radioimmunoassay (RIA). All procedures were approved by the Richard B Russell Agriculture Research Center Committee on Animal Care and Use.

Experiment II

Eight OVX crossbred prepubertal gilts, 88 ± 5 kg BW and 150 days of age were surgically implanted with ICV cannulas, fitted with indwelling jugular vein cannulas and housed and fed as described above. On the day of the EXP, pigs were fed at 07 30 h and blood sampling started at 08 00 h. Blood samples were collected every 15 min for 3 h before and 4 h after ICV injections of 150 µl 0.9% saline ($n=4$), or 50 µg recombinant human leptin ($n=4$; R&D Systems, Minneapolis, MN, USA), or 100 µg NPY

($n=4$) or 100 µg NPY + 50 µg leptin ($n=4$) in 150 µl of saline. Four days later, the EXP was replicated with pigs reassigned to treatment such that no pig received the same treatment a second time, resulting in 4 pigs/treatment. After the last sample was collected, feeders were placed in all pens and feed intake monitored at 4, 20 and 44 h after feed presentation. Serum was harvested and stored at -20 °C until assayed for LH and GH by RIA.

Radioimmunoassays

Serum samples were assayed for GH (Barb *et al.* 1991) and LH (Kesner *et al.* 1987) as previously described. Sensitivity of the assays was 0.4 ng/ml and 0.15 ng/ml for GH and LH respectively. Intra- and interassay coefficients of variation were 4.5 and 10.2% for LH and 3.5 and 13% for GH respectively.

Statistical analysis

To determine the effect of NPY on feed intake and LH and GH secretion, data were subjected to the general linear model split plot-in-time ANOVA procedure of the Statistical Analysis System (SAS 1999). The statistical model included dose, pig, time and replicate. Effects of treatment and replicate were tested using replicate $\times$ treatment as the error term. Replicate $\times$ treatment was tested using pig within treatment $\times$ replicate as the error term. Time and time $\times$ treatment were tested using treatment $\times$ time $\times$ replicate as the error term. Differences between treatment means within a time were determined by least-squares contrasts (SAS 1999). For each gilt in EXP I and II, mean serum hormone concentrations, basal hormone concentrations, the number of hormone pulses and hormone pulse amplitude were determined for LH and GH by Pulsar analysis, using a 1% criterion of variation (Merriam & Wachter 1982) during the post-treatment period. Data were then subjected to one-way ANOVA.

Results

Experiment I

A treatment $\times$ time interaction was detected for serum LH ($P<0.0005$) and GH ($P<0.06$) concentrations. Serum LH levels decreased ($P<0.05$) by 45 min after injection of 10 µg (0.9 ± 0.2 ng/ml), 50 µg (0.8 ± 0.2 ng/ml) and 100 µg (0.9 ± 0.2 ng/ml) NPY compared with saline (1.4 ± 0.2 ng/ml) treatment (Fig. 1). Injection of 100 µg NPY suppressed mean ($P<0.04$) and basal serum LH ($P<0.02$) concentrations and LH pulse frequency ($P<0.04$; Table 1). Only the 100 µg dose of NPY increased ($P<0.05$) GH secretion by 15 min (4.6 ± 0.5 ng/ml) after treatment compared to saline (2.2 ± 0.5 ng/ml; Fig. 2), while the indices of GH secretion were unaffected by NPY treatment (Table 2).

Figure 1 Serum LH concentrations (means $\pm$ S.E.M.) for OVX prepubertal gilts receiving ICV injections of saline (saline; $n=6$), or 10 μ g NPY ($n=7$), 50 μ g NPY ($n=5$), or 100 μ g NPY ($n=7$) at time 0 h (arrow). A treatment $\times$ time interaction was detected ($P < 0.0005$). *Times at which effects of treatment were different from saline-treated animals ($P < 0.05$).

Experiment II

A treatment $\times$ time interaction was detected for feed intake ($P < 0.001$). At 4 h, feed intake was lower ($P < 0.04$) in NPY- and NPY+leptin-treated animals compared with saline-treated pigs (Fig. 3). By 20 h, feed intake was lower ($P < 0.04$) in leptin-treated pigs, but higher ($P < 0.04$) in NPY- and NPY+leptin- treated

pigs ($P < 0.04$). A treatment $\times$ time interaction was detected for LH ($P < 0.004$). Serum LH concentrations decreased ($P < 0.01$) by hour 2 after NPY and NPY+leptin (Fig. 4). Administration of NPY and NPY+leptin suppressed LH pulse frequency ($P < 0.02$; Table 3). Serum GH concentrations (Fig. 5) and indices of GH secretion (Table 4) were unaffected by treatment.

Table 1 Mean and basal serum LH concentrations, LH pulse frequency and LH pulse amplitude after intraventricular injection of saline ($n=6$), or 10 ($n=7$), 50 ($n=5$) or 100 μ g neuropeptide Y (NPY; $n=7$). Values expressed as mean $\pm$ S.E.M.

Parameter	Treatment				P value
	Saline	10 μ g NPY	50 μ g NPY	100 μ g NPY	
Mean LH (ng/ml)	1.5 ± 0.2^b	1.1 ± 0.2^b	0.9 ± 0.2^b	0.6 ± 0.2^c	0.04
Basal LH (ng/ml)	1.2 ± 0.1^b	0.9 ± 0.1^b	0.6 ± 0.1^c	0.4 ± 0.1^c	0.02
LH pulse/4 h	3.9 ± 0.6^b	2.7 ± 0.6^b	2.4 ± 0.6^b	1.1 ± 0.6^c	0.04
LH pulse amplitude (ng/ml) ^a	1.0 ± 0.5	1.1 ± 0.5	1.2 ± 0.5	0.6 ± 0.5	0.6

^aAmplitude = pulse height – basal concentration. ^{b,c}Means in a row differ P -values present in table.

Figure 2 Serum GH concentrations (means $\pm$ S.E.M.) for OVX prepubertal gilts receiving ICV injections of saline (saline; $n=6$), or 10 μ g NPY ($n=7$), 50 μ g NPY ($n=5$), or 100 μ g NPY ($n=7$) at time 0 h (arrow). A treatment $\times$ time interaction was detected ($P<0.06$). *Times at which effects of treatment were different from saline-treated animals ($P<0.05$).

Discussion

In the present study, central administration of NPY reduced serum LH concentrations and LH pulse frequency in EXP I and II. This is in agreement with previous reports in the OVX cow (Thomas *et al.* 1999) and the OVX oestradiol-implanted ewe (Morrison *et al.* 2003).

Furthermore, central administration of NPY in the OVX and OVX oestradiol-implanted cow resulted in a marked reduction in GnRH and LH secretion (Gazal *et al.* 1998) suggesting a hypothalamic site of action. However, a pituitary site of action of NPY on LH secretion has been reported for cattle (Denniston *et al.* 2003). Thus, at least in

Table 2 Mean and basal serum GH concentrations, GH pulse frequency and GH pulse amplitude after intraventricular injection of saline ($n=6$), or 10 ($n=7$), 50 ($n=5$) or 100 μ g neuropeptide Y (NPY; $n=7$). Values expressed as mean $\pm$ S.E.M.

Parameter	Treatment				P value
	Saline	10 μ g NPY	50 μ g NPY	100 μ g NPY	
Mean GH (ng/ml)	2.4 $\pm$ 0.8	3.2 $\pm$ 0.8	3.4 $\pm$ 0.9	3.7 $\pm$ 0.8	0.4
Basal GH (ng/ml)	2.3 $\pm$ 0.7	3.2 $\pm$ 0.8	3.2 $\pm$ 0.8	3.7 $\pm$ 0.8	0.5
GH pulse/4 h	0.7 $\pm$ 0.3	0.6 $\pm$ 0.3	1.2 $\pm$ 0.4	0.4 $\pm$ 0.3	0.6
GH pulse amplitude (ng/ml) ^a	0.6 $\pm$ 0.4	0.4 $\pm$ 0.4	0.9 $\pm$ 0.4	1.7 $\pm$ 0.4	0.5

^aAmplitude=pulse height – basal concentration.

Figure 3 Cumulative feed intake (mean $\pm$ S.E.M.) for OVX prepubertal gilts receiving ICV injections of saline (saline; $n=4$), or 100 μ g NPY ($n=4$), or 50 μ g leptin ($n=4$), or NPY (100 μ g) + leptin (50 μ g; LEP+NPY; $n=4$). Feed intake was monitored at 4, 24 and 44 h after feed presentation. ^{a,b,c}Times at which effects of treatment differ ($P<0.04$).

part, the action of NPY on LH secretion may occur at the anterior pituitary in cattle. In contrast to the bovine, we reported that NPY failed to affect LH secretion in porcine anterior pituitary cells in culture (Barb & Barrett 2005), supporting the idea of a hypothalamic site of action in the

pig. The observed action of NPY on LH secretion and feed intake in the present study along with reports that nutritional status modulated hypothalamic NPY expression (Brady *et al.* 1990, Adam *et al.* 1997) supports the notion that NPY serves as a link between metabolic state and the reproductive axis in the pig.

The presence of biologically active leptin receptors (Lin *et al.* 2001), NPY and its receptor (Busch-Sorensen *et al.* 1989) and GnRH (Kineman *et al.* 1988, Lin *et al.* 2001) in the porcine hypothalamus suggest that NPY and leptin could interact to modulate GnRH/LH pulse generation. The failure of leptin to block the NPY-induced decrease in LH secretion is similar to that reported for the OVX cow in which leptin pretreatment failed to prevent the NPY-induced inhibition of LH secretion (Garcia *et al.* 2004). These observations suggest that NPY is acting post-synaptically to overcome the leptin-induced reduction in NPY and other leptin-sensitive pathways that influence GnRH neuronal activity. This would suggest that NPY plays a primary role in the leptinergic pathways modulating LH secretion. Furthermore, administration of NPY counteracted the inhibitory action of leptin on feed intake by 20 h post-treatment in the present study. It is possible that NPY could be acting through parallel pathways to alter

Figure 4 Serum LH concentrations (means $\pm$ S.E.M.) for OVX prepubertal gilts receiving ICV injections of saline (saline; $n=4$), or 100 μ g NPY ($n=4$), or 50 μ g leptin ($n=4$), or NPY (100 μ g) + leptin (50 μ g; $n=4$) at time 0 h (arrow). A treatment $\times$ time interaction was detected ($P < 0.004$). *Times at which effects of treatment were different from saline-treated animals ($P < 0.05$).

Table 3 Mean and basal serum LH concentrations, LH pulse frequency and LH pulse amplitude after intraventricular injection of saline ($n=4$), or leptin (50 μg ; $n=4$), neuropeptide Y (100 μg NPY; $n=4$) or NPY + leptin ($n=4$). Values expressed as mean $\pm$ S.E.M.

Parameter	Treatment				P value
	Saline	Leptin	NPY	NPY + Leptin	
Mean LH (ng/ml)	1.3 ± 0.3	0.9 ± 0.3	0.6 ± 0.3	1.3 ± 0.3	0.5
Basal LH (ng/ml)	1.0 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.3
LH pulse/4 h	2.8 ± 0.4^b	2.3 ± 0.4^b	0 ^c	1.3 ± 0.4^c	0.02
LH pulse amplitude (ng/ml) ^a	1.3 ± 1.0	1.7 ± 1.0	0	3.9 ± 1.0	0.2

^aAmplitude = pulse height – basal concentration. ^{b,c}Means in a row differ P -values present in table.

feeding behavior and LH secretion. Thus, NPY could act directly or indirectly to inhibit GnRH/LH release and stimulate feeding behavior. A similar hypothesis has been proposed for the interaction of leptin and NPY in the regulation of GH secretion in the rat (Carro *et al.* 1998). In support of this hypothesis, co-localization of leptin receptor mRNA with NPY gene expression was found in the arcuate nuclei (ARC; Cunningham *et al.* 1999). In addition, the paraventricular nucleus (PVN), the feeding center, receives neural projections from the arcuate nuclei (Kalra *et al.* 1999). This is an area rich in NPY-containing pre-synaptic nerve terminals and

post-synaptic NPY receptors (Bai *et al.* 1985, Chronwall *et al.* 1985). NPY could act post-synaptically to suppress ARC GnRH neuronal activity and stimulate NPY sensitive neurons in the PVN, thereby counteracting the leptin-induced reduction in NPY within the PVN. This may also account for the delayed hyperphagic response to NPY observed in the NPY + leptin-treated animals.

Several reports demonstrated that central administration of NPY stimulates feeding behavior in the rodent (Clark *et al.* 1984, McDonald *et al.* 1989), monkey (Kaynard *et al.* 1990) and human (Kaye *et al.* 1990). This

Figure 5 Serum GH concentrations (means $\pm$ S.E.M.) for OVX prepubertal gilts receiving ICV injections of saline (saline; $n=4$), or 100 μg NPY ($n=4$), or 50 μg leptin ($n=4$), or NPY (100 μg) + leptin (50 μg ; $n=4$) at time 0 h (arrow).

Table 4 Mean and basal serum GH concentrations, GH pulse frequency and GH pulse amplitude after intraventricular injection of saline ($n=4$), or leptin (50 μg ; $n=4$), neuropeptide Y (100 μg NPY; $n=4$) or NPY + leptin ($n=4$). Values expressed as mean $\pm$ S.E.M.

Parameter	Treatment				P value
	Saline	Leptin	NPY	NPY + Leptin	
Mean GH (ng/ml)	6.7 $\pm$ 1.2	4.9 $\pm$ 1.2	6.9 $\pm$ 1.2	4.7 $\pm$ 1.2	0.4
Basal GH (ng/ml)	6.3 $\pm$ 1.1	4.5 $\pm$ 1.1	6.9 $\pm$ 1.1	4.1 $\pm$ 1.1	0.3
GH pulse/4 h	2.0 $\pm$ 0.5	1.0 $\pm$ 0.5	0.6 $\pm$ 0.5	1.3 $\pm$ 1.2	0.3
GH pulse amplitude (ng/ml) ^a	3.8 $\pm$ 2.0	1.0 $\pm$ 2.0	0.5 $\pm$ 2.0	5.5 $\pm$ 2.0	0.3

^aAmplitude = pulse height – basal concentration.

is the first report in the pig to demonstrate that acute ICV administration of NPY increased cumulative feed intake. In satiated operant trained pigs, central injection of NPY increased the total number of operant feeding responses during the 30 min post-treatment period (Parrott *et al.* 1986). However, the temporal pattern of feeding responses measured at 5 min intervals revealed an apparent reduction in feeding responses by 20 min after treatment. In the present study the stimulatory effect of NPY on feeding behavior was not observed until 20 h after treatment. The apparent dichotomy between the studies may be accounted for, at least in part, by the methods used to measure feed intake and duration of the measurement period. Work in the rodent demonstrated different hypothalamic sites of action for NPY and leptin (Kalra *et al.* 1999). The leptin-induced suppression of NPY-stimulated feeding observed in the rodent was associated with attenuation of a subset of magnocellular PVN neurons and activation of dorsomedial neurons (DMN; Yokosuka *et al.* 1998) and a reduction in the availability of NPY at the nerve terminal in the PVN (Schwartz *et al.* 1996). Since the orexigenic and anorexigenic signal pathways are apparently linked, this may account for the delay in the ability of NPY to reactivate the appetite-stimulating signal.

The inability of leptin to increase LH secretion in the present study was not unexpected and may be attributed to nutritional status. Similar reports demonstrated that chronic ICV administration of leptin failed to stimulate LH secretion in well-nourished OVX ewes with no steroid replacement (Henry *et al.* 1999), and in intact ewe lambs (Morrison *et al.* 2001), whereas, in the feed-restricted OVX cow (Amstalden *et al.* 2002) and ewe (Henry *et al.* 2001), central administration of leptin stimulated LH secretion. It is possible that the dose of leptin was inadequate to elicit a response, but this is unlikely since the same dose of leptin suppressed feed intake in the present study. A more plausible explanation for the failure of leptin to increase LH secretion may be attributable to the use of satiated animals.

Stimulation of GH secretion by NPY in EXP I is similar to that reported in the OVX ewe (Morrison *et al.* 2003) and cow (Thomas *et al.* 1999, Garcia *et al.* 2004). Thomas *et al.* (1999) reported that NPY stimulated GH

secretion in OVX cows but did not affect tonic secretion patterns of GH, which is similar to results reported in the present study. These reports differ from rats, in which NPY inhibited the secretion of GH via stimulation of somatostatin secretion (Quintela *et al.* 1997). In contrast, NPY appears to stimulate growth hormone-releasing hormone (GHRH) and somatostatin release in ruminants (McMahon *et al.* 2001). McMahon *et al.* (2001) proposed that different subpopulations of NPY neurons may mediate secretion of GHRH and somatostatin independently, either via the same or different receptor subtypes. This may account for the diverse effects of NPY on GH secretion between species and physiological states. Alternatively, a pituitary site of action cannot be discounted, since NPY stimulated GH secretion from porcine pituitary cells in culture (Barb & Barrett 2005).

The almost total lack of GH response to NPY observed in EXP II may, in part, be due to the inability of NPY to exceed a putative stimulatory threshold of hypothalamic GHRH and somatostatin neurons to effect a release of GH. This is consistent with the observation that pretreatment serum GH concentrations were approximately threefold greater in EXP II pigs compared with serum GH levels in EXP I animals. Moreover, pretreatment serum GH concentrations in EXP II were similar to the maximal GH concentration in response to 100 μg NPY in EXP I, suggesting a possible change in the activity and (or) sensitivity of GHRH and somatostatin neurons. McMahon *et al.* (2000) observed a reduction of somatostatin and GHRH neuronal activity associated with decreasing GH concentrations during feeding. Thus, serum GH concentrations may reflect changes in the activity and (or) sensitivity of GHRH and somatostatin neurons. Furthermore, the effects of NPY on LH secretion lasted longer than those for GH secretion in EXP I, indicating a difference in the sensitivity of the LH and GH secretory axes to NPY.

In conclusion, the presence of NPY and its receptor (Busch-Sorensen *et al.* 1989), GnRH (Kineman *et al.* 1988, Lin *et al.* 2001) and GHRH (Leshin *et al.* 1994) in the porcine hypothalamus coupled with current results support the hypothesis that NPY may serve as a neural link between metabolic state and the reproductive and growth axis in the pig.

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