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NEUROPEPTIDE Y-1 RECEPTORS MEDIATE THE SUPPRESSION OF LH SECRETION BY NPY IN CASTRATED MALE SHEEP

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ABSTRACT

GnRH and, thus, LH secretion is decreased during undernutrition. Neuropeptide Y (NPY) is increased during undernutrition and NPY administration inhibits LH secretion, leading to the idea that NPY is an important component in nutrition-induced suppression of reproduction. The purpose of this study was to determine if the Y1 receptor isoform mediates the inhibitory effects of NPY on LH secretion. Four castrated male sheep were each surgically fitted with a cannula in the third cerebral ventricle. Blood samples were collected every 12 minutes for 4h after icv injection (50 ul) of either: sterile water, NPY (50 ug), or NPY+BIBP3226 (50 ug; a specific Y1 receptor antagonist). Each animal randomly received all treatments with treatments delivered at least one week apart. Water had no effect on mean LH whereas NPY decreased mean LH concentrations by 2h postinjection. Mean LH levels in males receiving NPY+BIBP3226 were similar to those observed during NPY treatment alone. Because of the lack of effect of BIBP3226, a second study was performed using these same males, except that they received a higher dose (100 ug) of BIBP3226 injected 30 min prior to injection of NPY (50 ug). Blood samples were collected at 10 minute intervals for 30 min prior to BIBP3226 injection and for 4h after NPY injection. In three of four animals, the suppression of mean LH by NPY was blocked. Cortisol was also measured and BIBP3226 had no effect on the NPY-induced increase in cortisol release, suggesting that the effects of the antagonist were at least somewhat specific for LH. Based on these data, we suggest that NPY inhibits GnRH release in male sheep via the Y1 receptor. Whether these effects are generated directly at the GnRH neuron or indirectly via other intermediate neurons, or whether this receptor subtype is involved in nutrition-induced suppression of GnRH release, remains to be determined.

Key words: sheep NPY, GnRH, LH, Y1 receptor

INTRODUCTION

Nutrition is one of the most important variables influencing reproductive proficiency in mammals (Bronson, 1989). Undernutrition can delay the onset of puberty, interrupt the expression of estrous or menstrual cycles, and delay the postpartum return to fertility (Schillo, 1992). While inadequate nutrition may influence all aspects of the hypothalamic-pituitary-gonadal axis, of primary importance is the suppression of GnRH. However, GnRH neurons themselves are functional during restricted feeding (I'Anson *et al.*, 1993), suggesting that the influence of undernutrition on GnRH secretion is mediated by increased inhibitory and/or decreased stimulatory neural input to GnRH neurons. In addition, although chronic food restriction reduces GnRH or LH secretion in the absence of gonadal steroids (Hall *et al.*, 1992; Hileman *et al.*, 1993), long-term undernutrition also enhances sensitivity to steroid-negative feedback (Beckett *et al.*, 1997; Dong *et al.*, 1994; Glass *et al.*, 1982; Howland, 1979; Piacsek *et al.*, 1986; Sprangers & Piacsek, 1988). The neural mechanisms underlying the nutrition-induced inhibition of GnRH release remain to be fully elucidated.

One candidate for mediating the effects of undernutrition on GnRH secretion is neuropeptide PY

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(NPY). NPY is one of the most potent endogenous orexigenic agents known (Levine & Morley, 1984) and is highly expressed within the arcuate nucleus of the hypothalamus (ARC) (Gray & Morley, 1986). NPY-containing neurons synapse directly onto GnRH neurons (Tillet *et al.*, 1989) with the majority of input arising from the ARC (Turi *et al.*, 2003). In species other than ruminant livestock, NPY can stimulate or inhibit LH secretion (Crowley & Kalra, 1987; Kalra *et al.*, 1992; Pau *et al.*, 1995; Sabatino *et al.*, 1989; Sahu *et al.*, 1987; Woller *et al.*, 1993) depending on the steroidal environment or avenue of delivery (i.e. intracerebroventricular vs. intrahypothalamic). In stark contrast, the majority of reports in sheep and cattle show that NPY inhibits LH secretion in ewes or cows that are ovariectomized or ovariectomized and implanted with estradiol (Barker-Gibb *et al.*, 1995; Gazal *et al.*, 1998; Malven *et al.*, 1992; McShane *et al.*, 1992; Morrison *et al.*, 2003; Thomas *et al.*, 1999). An inhibitory effect of NPY is consistent with the elevated NPY gene expression normally observed during feed restriction in both male (Adam *et al.*, 2002) and female sheep (McShane *et al.*, 1993), and during an inhibitory photoperiod in testosterone-treated male sheep (Dobbins *et al.*, 2004).

As a first step in examining the mechanism whereby NPY may inhibit GnRH release, we examined the ability of a NPY Y1 receptor antagonist to block the inhibitory effects of NPY on LH release. There have been 6 NPY receptor isoforms identified (Y1-Y6). Some of these particular subtypes have been found to be expressed on GnRH neurons (Campbell *et al.*, 2001; Dufourny & Skinner, 2004; Li *et al.*, 1999) within the POA and have been shown to influence pulsatile LH secretion or the LH surge (Barker-Gibb *et al.*, 1995; Leupen *et al.*, 1997; Raposinho *et al.*, 1999). In addition, Y1 receptor mRNA expression and immunopositive cells are evident in the ARC and stalk-median eminence (SME) (Li *et al.*, 1999), two areas important in the regulation of GnRH release. NPY has also been shown to stimulate cortisol release (Brooks *et al.*, 1994; Liu *et al.*, 1994; Wahlestedt *et al.*, 1987), thus we also investigated the role of the Y1 receptor in this response.

MATERIALS AND METHODS

General

Four castrated male sheep (wethers) were used at approximately 9 months to 1 year of age. Animals were housed in an environment where lighting was adjusted to simulate natural photoperiod and temperatures were maintained at approximately 25°C. Sheep were fed ad libitum a diet of alfalfa pellets supplemented with corn. Free access to water was provided at all times. Animals were purchased from the Davis College of Agriculture, Forestry and Consumer Sciences at West Virginia University (Morgantown, WV). All procedures were approved by the West Virginia University Animal Care and Use Committee and conducted according to NIH Guidelines for the use of animals in research.

Neurosurgery

Implantation of guide tubes into the third cerebroventricle (3V) was done as previously described (Anderson *et al.*, 2001). Briefly, animals were anesthetized, their heads placed in a stereotaxic apparatus, a 2-cm diameter hole drilled in the top of the skull, and the sagittal sinus ligated and cut. Radio-opaque dye was injected into the lateral ventricle and a guide tube lowered into place with the aid of lateral and frontal X-rays of the third ventricle. After placement of the guide cannula into the 3V, the surface of the brain was covered with gel foam and the skin sutured. The guide cannula was then attached to the skull with dental acrylic, a protective cap cemented around it, and animals allowed to recover for at least 2 weeks.

NPY Y1 Receptor Antagonist

BIBP3226 is a selective Y1 antagonist that displaces NPY in a dose-dependent fashion in CHO-K1 cells expressing human Y1 receptors, but not from a neuroblastoma cell line expressing Y2 receptors (Wieland et al., 1995). In HEK cells, BIBP3226 inhibits binding of a specific Y1 agonist at 1.2 nM, but did not affect binding of Y2, Y4, or Y5 agonists. BIBP3226 inhibits 1) the Y1 receptor-mediated decrease in the twitch response in rabbit vas deferens (Doods *et al.*, 1995), 2) increases in blood pressure induced by NPY in rats (Doods *et al.*, 1995), and 3) the contractile effect of NPY in the rabbit saphenous vein (Jacques *et al.*, 2003). BIBP3226 treatment accelerates puberty (Pralong *et al.*, 2000) and attenuates the proestrus LH surge (Leupen *et al.*, 1997). BIBP3226 was purchased from Sigma (St. Louis, MO).

Design

In the first study, males were assigned to receive each of the following treatments in random order with each treatment period separated by at least one week; (1) a single injection of 50 ul of sterile water, (2) 50 ug NPY in 50 ul of sterile water, or (3) 50 ug NPY+50 ug BIBP3226 in 50 ul of sterile water. On each treatment day, injections were made and blood samples were collected beginning immediately after injection at 12-minute intervals for 4 hours. Sera were collected and every sample assessed for LH while cortisol was evaluated every 36 minutes (or every third sample). Both were measured by radioimmunoassay.

Due to the results of the first study, a second study was performed in which a higher dose of BIBP3226 was used in the same four wethers. BIBP3226 (100 ug in 50 ul of water) was injected and then NPY (50 ug in 50

ul water) was injected a half-hour later. Jugular blood samples were collected every 10 min beginning a half-hour prior to BIBP3226 injection and continuing for 4 hours following the NPY injection. LH was assessed in all samples and cortisol was assessed at half-hour intervals.

Assays

LH was measured in all samples by RIA according to previously described procedures (Anderson *et al.*, 2001; Havern *et al.*, 1994). Intra- and interassay coefficients of variation were 10.4% and 15.3%, respectively. Sensitivity, defined by the 95% confidence interval at 0 ng.ml^{-1}, was 0.1 ng. ml^{-1} Cortisol was measured using a commercially available radioimmunoassay (MP Biomedicals, Costa Mesa, CA). Samples were run in two assays with intra- and interassay coefficient of variation being 7.6% and 6.8%, respectively, with a sensitivity of 0.13 ng.ml^{-1}.

Statistics

LH pulses were identified according to the methods of Goodman and Karsch (1980). Mean LH for each treatment period was compared by ANOVA, followed by paired t-test. Changes over time in mean LH and cortisol were compared by repeated measures ANOVA, followed by repeated measures for one variable within group or one-way ANOVA within time period to determine differences between groups. LH pulse frequencies were compared by the Friedman test. $P<0.05$ was considered significant.

RESULTS

LH

As mentioned above, there were 4 wethers used for this study. However, after examination of the LH data, it was clear that one wether did not respond as the others. That wether showed a short-lived decrease in LH in response to NPY that returned to baseline by 1.5- to 2 hours postinjection. Thus, the LH data described below reflects the averages of the remaining 3 wethers.

Figure 1A shows results for mean LH during each experimental period. NPY reduced mean LH in comparison to water control injection and coadministration of BIBP3226 (50 ug) had no effect on this inhibition. Mean LH following NPY+BIBP (100 ug) treatment was not significantly different than levels observed during water treatment and were higher than those observed during NPY or NPY+BIBP (50 ug) treatment. Changes over time in mean hourly LH concentrations for the four groups are shown in Figure 1B. Repeated measures ANOVA revealed that mean LH did not change significantly over time following water injection, but decreased in wethers treated with either NPY or NPY+BIBP3226 (50 ug). Repeated measures ANOVA revealed that mean LH did not change significantly with time following NPY+BIBP3226 (100ug). Indeed, the shape of the response curve was almost identical to that of wethers receiving water alone. LH pulse frequency during water (1.7±0.5 pulses/4h), NPY (2.0±0.9 pulses/4h) or NPY+BIBP3226 at 50ug (2.0±0.7 pulses/4h) did not differ significantly. While LH pulse frequency in wethers treated with NPY+BIBP3226 (100ug) was numerically higher (3.3±1.2 pulses/4h) than the other groups, post-hoc testing showed no significant difference when compared to either water treatment or NPY treatment.

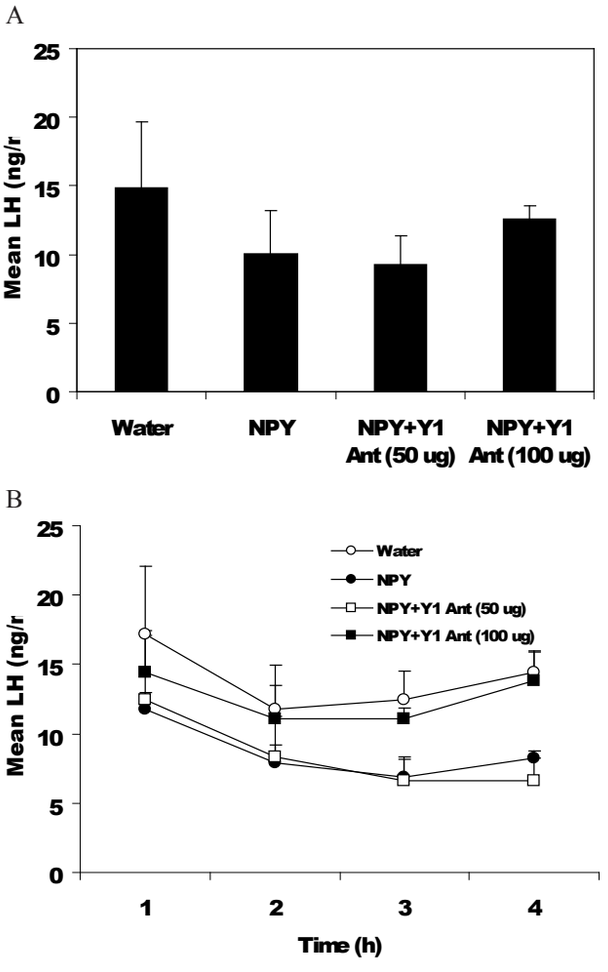
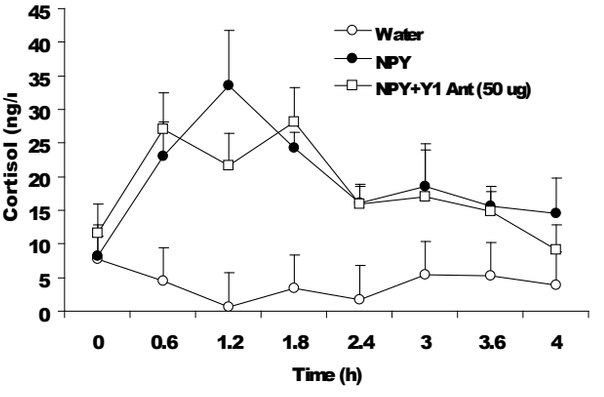


Fig. 1. A) Mean±SEM LH in wethers injected with either water, NPY (50 ug), NPY+BIBP3226 (50 ug), a specific Y1 receptor antagonist or NPY+BIBP3226 (100 ug)
B) Hourly changes in mean±SEM LH following either water, NPY, NPY+BIBP3226 (50 ug), or NPY+BIBP3226 (100 ug)

Cortisol

Data from all 4 wethers were used for the evaluation of cortisol. Changes in cortisol over time for wethers receiving water, NPY (50ug) or NPY+BIBP3226 (50ug) are shown in Figure 2a. NPY increased circulating cortisol concentrations and this effect was not influenced by coadministration of BIBP3226 (50 ug). Mean concentrations of cortisol for wethers receiving NPY+BIBP3226 (100 ug) are shown in Figure 2b. NPY increased circulating cortisol and the magnitude of this increase was very similar to that observed with NPY treatment alone (see Figure 2a), strongly indicating that BIBP3226 was without effect.

A



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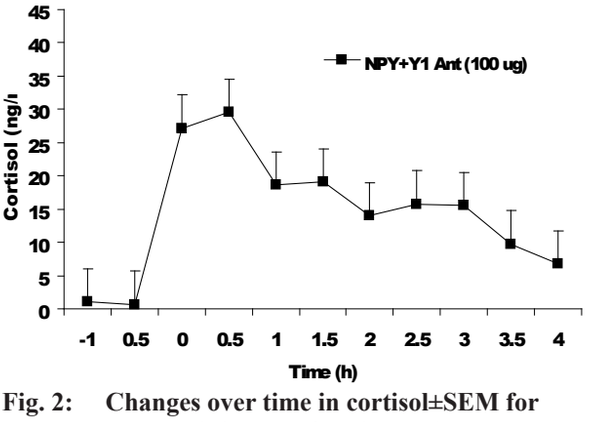

Fig. 2: Changes over time in cortisol±SEM for wethers injected with either A) water, NPY (50 ug), or NPY+BIBP3226 (50 ug), a specific Y1 receptor antagonist, or B) NPY+BIBP3226 (100 ug)

DISCUSSION

Feed restriction reduces LH secretion, a response normally associated with increased NPY mRNA expression. Combined with the fact that NPY administration suppresses LH secretion, it is reasonable to speculate that increased NPY input plays an important role in undernutrition-induced inhibition of GnRH release. GnRH neurons have been reported to express the Y1, Y2, and Y5 NPY receptor subtypes. Our study suggests that the Y1 receptor may, at least in part, mediate the effects of NPY on GnRH/LH secretion. In contrast, we would suggest that this receptor subtype may not play an important role in the regulation of cortisol secretion by NPY.

NPY is a 36 amino acid peptide localized largely to the arcuate nucleus of the hypothalamus in sheep. Agouti-related peptide is colocalized exclusively with NPY neurons of the ARC and Turi et al. (2003) used this characteristic to determine that in mice, approximately 50% of the NPY input to GnRH neurons of the preoptic area arises from the ARC. NPY neurons of the ARC show a marked increase in mRNA expression during feed restriction, supporting the idea that these neurons are important in mediating the effects of nutrition on GnRH release. Our study provides evidence to suggest a role for the NPY Y1 receptor subtype in mediating the effects of NPY on GnRH release. This is consistent with previous work showing a colocalization of Y1 receptors with GnRH neurons (Li *et al.*, 1994), an acceleration of puberty onset with BIBP3226 in rodents (Pralong *et al.*, 2000), and an increase in GnRH mRNA expression following administration of a Y1 receptor antagonist (Li *et al.*, 1994).

While our data provides evidence of a role for the Y1 receptor isoform, this does not rule out possible roles for the other NPY receptor subtypes. Both the Y2 and Y5 receptor subtypes have been colocalized with GnRH (Campbell *et al.*, 2001; Dufourny & Skinner, 2004). There is a paucity of data regarding the role of these receptors in reproduction, but Pinilla et al. (2007) reported that a Y2 agonist decreased GnRH secretion in male rats while a Y2 antagonist increased GnRH release. Based on pharmacological screening of various NPY receptor antagonists, Raposinho et al. (1999) suggested that reductions in LH secretion by NPY in rats were mediated by the Y5 isoform. The Y4 isoform has also been implicated in playing a role in the regulation of GnRH by NPY. Lin et al. (2007) showed that fasting-induced reductions in GnRH mRNA expression were blocked in Y4-deficient mice. However, they suggested that this effect was indirect since they found Y4-receptor expression in close proximity to GnRH neurons, but no colocalization with GnRH itself. Given that the role for NPY may depend upon several variables, including sex, species, route of delivery and endocrine milleiu (Crowley & Kalra, 1987; Kalra *et al.*, 1992; Pau *et al.*, 1995; Plant, 2001; Sabatino *et al.*, 1989; Sahu *et al.*, 1987; Terasawa & Fernandez, 2001; Woller *et al.*, 1993), much more work is needed to unravel the specific role of each isoform in regulating reproduction.

We noted a decrease in mean LH in response to NPY, but surprisingly did not find a decrease in

pulsatile LH secretion. One possible explanation is that NPY delivered to the third ventricle was reaching and influencing pituitary function. An influence of NPY at the pituitary has been reported previously (Crowley *et al.*, 1987; Parker *et al.*, 1991). However, a more likely explanation is that LH pulse patterns showed a large number of low amplitude excursions from baseline, making it very difficult to accurately identify pulses. This may be due to the fact that the wethers used were long-term castrates and in our previous experience, these types of patterns of LH secretion are not unusual. Indeed, we shortened our sampling interval in the second study to try and better identify pulses, but this did not improve the ability to do so.

Food restriction is associated with increased cortisol or corticosterone secretion and elevated NPY input. NPY administration results in elevated cortisol or corticosterone release with actions predominantly occurring within the hypothalamus via increased CRH (Brooks *et al.*, 1994; Liu *et al.*, 1994; Wahlestedt *et al.*, 1987). In rodents, CRH is reported to play a role in the food restriction-induced inhibition of reproduction (Maeda *et al.*, 1994). Even though we saw a moderation of the effects of NPY on LH secretion with coadministration of the Y1 antagonist, we did not observe a similar effect on the NPY-induced increase in cortisol secretion. Changes in LH secretion in response to cortisol have been reported in sheep (Breen & Karsch, 2004; Breen *et al.*, 2005; Breen & Karsch, 2006; Stackpole *et al.*, 2006). However, at least in sheep and primates, the involvement of the stress axis in mediating the response to feed restriction may not be as important as suggested for rodents (Cameron *et al.*, 1993; Helmreich *et al.*, 1993; Hilton & Loucks, 2000; I'Anson *et al.*, 1994; Schreihofer *et al.*, 1993). Nonetheless, we would speculate that an NPY-induced rise in cortisol may be an important adaptation to feed restriction to insure adequate metabolic fuel mobilization and supply.

CONCLUSION

In conclusion, our data support a role for the NPY Y1 receptor isoform in mediating the effects of NPY on GnRH and LH secretion. A proposed model for the involvement of NPY in undernutrition-induced impairment of GnRH release is shown in Figure 3. Feed restriction causes a marked decrease in leptin secretion. NPY neurons express the signaling form of the leptin receptor and are generally considered to be a direct target of leptin. Because of the fall in leptin, and perhaps other metabolic signals, NPY input increases. In addition, our previous work (McManus et al., 2005) showed that feed restriction enhances the ability of estradiol to inhibit pulsatile LH secretion and that this effect is mediated through actions of estradiol within the mediobasal hypothalamus. A subset of NPY neurons express estrogen receptor-α. Thus, based on our data and the preceding discussion, we suggest that the actions of leptin and estradiol are mediated, at least in part, through ARC NPY neurons. Because GnRH neurons express NPY receptors and receive NPY-containing synapses, NPY may act directly to inhibit GnRH release. Our data would raise the possibility that the Y1 isoform may play a role in this direct inhibition. However, other neurons in the POA and ARC/stalk median eminence (SME) also express NPY receptors, including the Y1 isoform. Thus, inhibitory effects of NPY on GnRH release may also occur through interneurons (depicted as a question mark in Fig. 3).

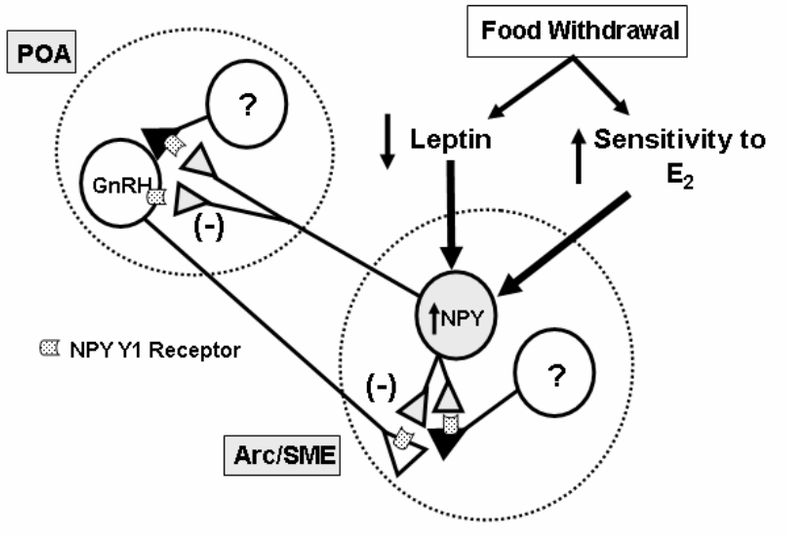

Fig. 3: Proposed model for NPY involvement in undernutrition-induced impairments in GnRH release. Abbreviations: POA, preoptic area; Arc/SME, arcuate nucleus of the hypothalamus/stalk median eminence; E_2, estradiol

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