Evidence That the Posterior Pituitary Plays a Role in Neuropeptide Y and Luteinizing Hormone–Releasing Hormone–Stimulated Gonadotropin Secretion *In Vitro* (44036)

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Abstract. **Neuropeptide Y (NPY) has been shown to increase gonadotropin secretion directly at the level of the anterior pituitary (AP) in both the absence and the presence of luteinizing hormone–releasing hormone (LHRH). This is interesting because high-affinity ^{125}I-NPY–binding sites have not been found in the AP. However, high-affinity ^{125}I-NPY–binding sites have been localized in the posterior pituitary (PP), and it has been shown that removal of the PP alters luteinizing hormone (LH) secretion *in vivo*. The following studies were conducted to determine if gonadotropin responsiveness to either NPY alone or to NPY in combination with LHRH was significantly different in AP cells cultured in the presence compared with the absence of PP cells. The studies indicated that NPY-induced LH secretion was significantly greater in the presence of PP cells, while follicle-stimulating hormone (FSH) secretion was not significantly affected. LHRH-induced LH secretion was also significantly greater in the presence of PP cells; however, LHRH-induced FSH secretion was significantly decreased. NPY potentiated LHRH-induced LH secretion in AP cells cultured in both the presence and the absence of PP cells; however, the degree of potentiation was not significantly different whether PP cells were present or absent. These results indicate that the PP may play a role in the responsiveness of the AP to NPY and to LHRH but plays no apparent role in NPY potentiation of LHRH responsiveness.** [P.S.E.B.M. 1996, Vol 213]

Neuropeptide (NPY) plays a significant role in reproduction by acting as a peptidergic neurotransmitter which regulates the secretion of hypothalamic luteinizing hormone–releasing hormone (LHRH) (1, 2); it also acts as a neuromodulator which increases gonadotropin secretion directly at the level of the anterior pituitary (AP) in the presence (3–7) as well as in the absence (7–9) of LHRH. The manner in which NPY acts alone at the level of the pituitary to modulate gonadotropin secretion is poorly understood because high-affinity NPY-binding sites have not been detected in the AP in studies utilizing ^{125}I-NPY in quantitative autoradiography as well as competition binding assays (10–12). However, high-affinity ^{125}I-NPY–binding sites (10–12) and NPY immunoreactivity (NPY-IR) (13–15) have been detected in the posterior pituitary (PP). NPY can reach the PP through a variety of hypothalamic neuronal projections (11, 13–15), and substances can be translocated between the AP and the PP by way of intercellular diffusion (16) or by short interconnecting portal vessels (17, 18). In addition, the *in vivo* removal of the PP increases luteinizing hormone (LH) secretion in intact female rats (19, 20) and inhibits the post-ovariectomy increase in LH secretion as well as the estrogen-induced LH surge in ovariectomized rats (21), thereby indicating a possible role of the PP in regulating AP gonadotropin secretion. These previous observations suggest that the ability of NPY alone to modulate gonadotropin secretion may possibly be mediated through an interaction with NPY receptors on the PP. The current studies utilized AP

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cells cultured in either the presence or the absence of PP cells to pursue the following two objectives. Objective 1 was to determine whether NPY-induced gonadotropin secretion was significantly different in AP cells cultured in the presence compared with the absence of PP cells. Objective 2 was to determine whether NPY potentiation of LHRH-induced gonadotropin secretion was significantly different in AP cells cultured in the presence compared with the absence of PP cells.

Materials and Methods

Animals. Adult female Sprague-Dawley rats were obtained from Sasco Co. (Omaha, NE) at 57 days of age and housed under controlled temperature and lighting conditions (lights on at 0700 hr and off at 1700 hr). Food and water were supplied *ad libitum*. At 60 days of age, pituitaries were collected for subsequent dispersal and culture. All experimental animal protocols were approved by the institutional Committee on Animal Use for Research and Education (CAURE) prior to the initiation of any studies.

Pituitary Dispersal and Culture. Pituitary tissue for a given experiment was collected from a single group of randomized rats without regard to stage of the estrous cycle. The pituitary tissue was randomized at collection and was dispersed in two separate pools: the first pool contained AP cells dispersed with the PP *in situ*, including both the intermediate and neural lobes (AP cells cultured in the presence of PP cells); the second pool contained AP tissue dispersed subsequent to the removal of the PP lobe (AP cells cultured in the absence of PP cells). The dispersal procedure has been previously described (22). The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% charcoal-treated serum (5% fetal bovine serum and 5% horse serum) as previously described (22–28). The cells were plated at the equivalent of one-half anterior pituitary per well utilizing six-well (3.5 × 1–cm) culture dishes. This resulted in a cell density of 1.2×10^6 cells/well for cultures containing both AP and PP cells and 1.0×10^6 cells/well for cultures containing AP cells only. The total culture medium volume per well was 1 ml. At 72 hr postdispersal, DMEM with serum was replaced with DMEM without serum and the cultures were stimulated for 5 hr with either vehicle (PBS + gelatin), 10^{-10} *M* LHRH alone, 10^{-7} *M* NPY (low level NPY) alone, 10^{-6} *M* NPY (high level NPY) alone, low level NPY (10^{-7} *M*) + LHRH (10^{-10} *M*), or high level NPY (10^{-6} *M*) + LHRH (10^{-10} *M*); all molarities indicate final in-culture concentrations. Trypan blue exclusion indicated that cell viability exceeded 90%–95% both immediately following dispersal and following stimulation on Day 3 in culture.

Following the stimulation period, media were collected for subsequent radioimmunoassay (RIA) of LH and follicle-stimulating hormone (FSH) utilizing reagents provided by the National Hormone and Pituitary Program as previously described (22); gonadotropin secretion data were expressed in reference to RP-1.

Data Analysis. To compensate for differences in gonadotropin secretion which might be exhibited by randomly cycling rats, data were expressed and statistically analyzed in Objective 1 as percentage change compared with basal LH and FSH secretion (Fig. 1, A and B, respectively) and, in Objective 2, as percentage change compared with LH and FSH secretion induced by LHRH alone (Fig. 2, A and B, respectively). The experiments were performed in triplicate ($n = 3$), and the results presented in Figure 1 and 2 were obtained from one randomized pool of rats and are representative of results obtained from three experiments. Experimental group differences were assessed by one-way analysis of variance (ANOVA). Comparisons within a group were performed utilizing Duncan's multirange test. Comparisons between groups were performed utilizing Student's *t* test. Data were plotted in Figure 1 and 2 as mean ± SEM and $P < 0.05$ was considered significant.

Results

Objective 1. *Response to 10^{-10} M LHRH alone.* LH secretion was significantly increased above basal by LHRH alone in AP cells cultured in both the presence (Fig. 1A, Bar 1; 744%; $P < 0.01$) and the absence (Fig. 1A, Bar 2; 515%; $P < 0.01$) of PP cells. When LHRH-induced LH secretion exhibited by AP cells cultured in the presence and absence of PP cells were directly compared, a significantly greater increase in LHRH-induced LH secretion was observed in AP cells cultured in the presence of PP cells (Fig. 1A, Bar 1 vs 2; 145%; $P < 0.05$). FSH secretion was also significantly increased above basal by LHRH alone in AP cells cultured in both the presence (Fig. 1B, Bar 1; 167%; $P < 0.01$) and the absence (Fig. 1B, Bar 2; 203%; $P < 0.01$) of PP cells. When LHRH-induced FSH secretion exhibited by AP cells cultured in the presence and absence of PP cells were directly compared, significantly less LHRH-induced FSH secretion was observed in AP cells cultured in the presence of PP cells (Fig. 1B; Bar 1 vs 2; 82%; $P < 0.01$).

Response to low NPY (10^{-7} M NPY). LH secretion was significantly increased above basal by 10^{-7} *M* NPY alone in AP cells cultured in the presence (Fig. 1A, Bar 3; 220%; $P < 0.05$), but not the absence (Fig. 1A, Bar 4) of PP cells. FSH secretion was not significantly increased above basal by 10^{-7} *M* NPY alone in AP cells cultured in either the presence (Fig. 1B, Bar 3) or absence (Fig. 1B, Bar 4) of PP cells.

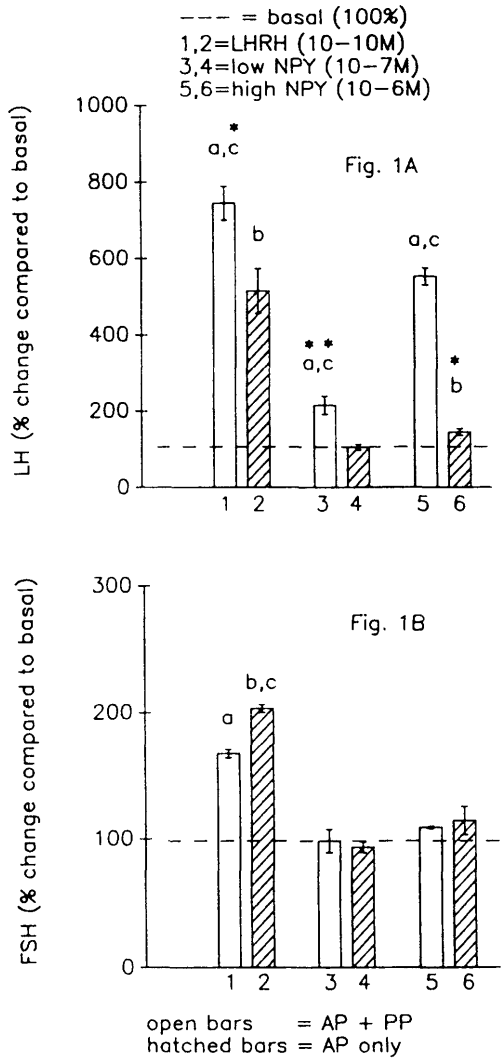

Figure 1. LH (A) and FSH (B) secretion exhibited by monolayer cell cultures composed of AP cells cultured in the presence of PP cells (AP + PP, open bars) or AP cells cultured in the absence of PP cells (AP only, hatched bars). Pituitary tissue was derived from randomly cycling 60-day-old rats. At 72 hr postdispersal, cultures were stimulated for 5 hr with either 10^{-10} *M* LHRH alone, 10^{-7} *M* NPY alone or 10^{-6} *M* NPY alone; control cultures were treated with vehicle alone (PBS + gelatin). Media were subsequently collected for RIA estimation of LH and FSH secretion. Cultures treated with vehicle alone were considered as basal; stimulated cultures were expressed as percentage change compared with basal. Each bar represents the mean (±SEM) of triplicate determinations from a representative experiment. [a]Significantly greater than basal in the AP + PP group; [b]significantly greater than basal in the AP only group; [c]significantly greater than the same treatment in the AP only group; *$P < 0.05$; all others, $P < 0.01$.

Response to high NPY (10^{-6} M NPY). LH secretion was significantly increased above basal by 10^{-6} *M* NPY in AP cells cultured in both the presence (Fig. 1A, Bar 5; 550%; $P < 0.01$) and the absence (Fig. 1A, Bar 6; 144%; $P < 0.05$) of PP cells. When 10^{-6} *M* NPY-induced LH secretion exhibited by AP cells cultured in the presence and absence of PP cells were directly compared, LH secretion was significantly greater in AP cells cultured in the presence of PP cells (Fig. 1A, Bar 5 vs 6; 393%; $P < 0.01$). FSH secretion was not significantly increased above basal by 10^{-6} *M* NPY alone in AP cells cultured in either the presence (Fig. 1B, Bar 5) or the absence (Fig. 1B, Bar 6) of PP cells.

Objective 2. *Response to 10^{-7} M NPY + 10^{-10} M LHRH.* LH secretion induced by 10^{-7} *M* NPY in combination with LHRH was not significantly different when compared with LH secretion induced by LHRH alone in AP cells cultured in either the presence (Fig. 2A, Bar 1) or the absence (Fig. 2A, Bar 2) of

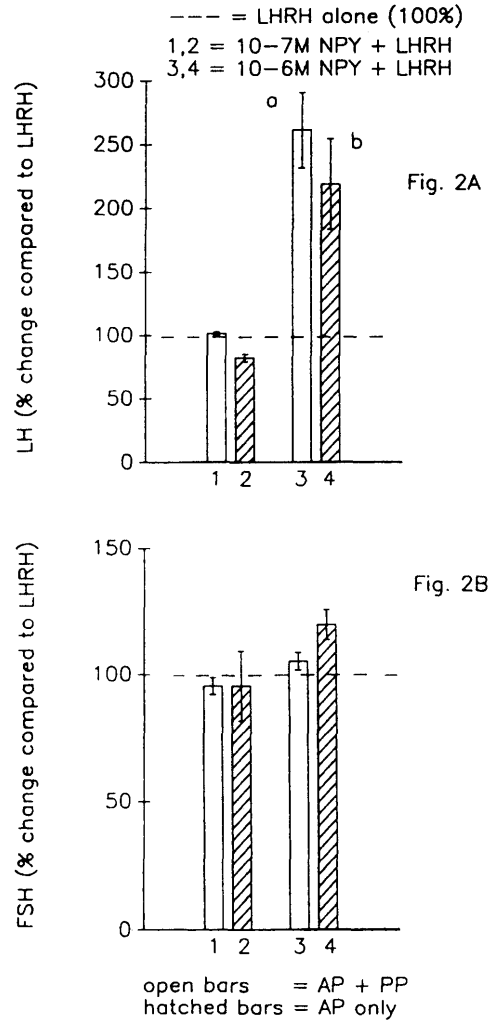

Figure 2. LH (A) and FSH (B) secretion exhibited by monolayer cell cultures composed of AP cells cultured in the presence of PP cells (AP + PP, open bars) or AP cells cultured in the absence of PP cells (AP only, hatched bars). Pituitary tissue was derived from random cycling 60-day-old female rats. At 72 hr postdispersal, cultures were stimulated for 5 hr with either 10^{-7} *M* NPY in combination with 10^{-10} *M* LHRH or 10^{-6} *M* NPY in combination with 10^{-10} *M* LHRH. Control cultures were stimulated with 10^{-10} *M* LHRH alone. Media were subsequently collected for RIA estimation of LH and FSH secretion. LH and FSH secretion exhibited by cultures stimulated with both 10^{-7} *M* NPY and 10^{-6} *M* NPY in combination with LHRH were expressed as percentage change compared with LHRH alone. (A) [a]Significantly greater than LHRH alone in the AP + PP group ($P < 0.01$); [b]significantly greater than LHRH alone in the AP only group ($P < 0.01$).

PP cells. FSH secretion induced by 10^{-7} *M* NPY in combination with LHRH also was not significantly different when compared with FSH secretion induced by LHRH alone in AP cells cultured in either the presence (Fig. 2B, Bar 1) or the absence (Fig. 2B, Bar 2) of PP cells.

Response to 10^{-6} M NPY + 10^{-10} *M* LHRH. LH secretion induced by 10^{-6} *M* NPY in combination with LHRH was significantly greater than LH secretion induced by LHRH alone in AP cells cultured in both the presence (Fig. 2A, Bar 3; 260%; $P < 0.01$) and absence (Fig. 2A, Bar 4; 220%; $P < 0.01$) of PP cells. However, there was no significant difference when LH secretion induced by 10^{-6} *M* NPY in combination with LHRH exhibited by AP cells cultured in the presence and absence of PP cells were directly compared (Fig. 2A, Bar 3 vs 4). FSH secretion induced by 10^{-6} *M* NPY in combination with LHRH was not significantly different when compared with FSH secretion induced by LHRH alone in AP cells cultured in either the presence (Fig. 2B, Bar 3) or the absence (Fig. 2B, Bar 4) of PP cells.

Discussion

It is well established that NPY plays a role in regulating gonadotropin secretion by acting at the hypothalamus (1, 2, 29) and at the pituitary (3–9). The observation that NPY alone modulates gonadotropin secretion directly at the level of the AP is particularly interesting since high affinity ^{125}I-NPY–binding sites have not been detected in the AP (10–12). However, high-affinity ^{125}I-NPY–binding sites have been detected in the neural lobe of the PP (10–12). Further, NPY-IR has been localized in the PP in nerve terminals projecting from the arcuate nucleus (13, 14) and in magnocellular neurosecretory neurons projecting from the paraventricular nucleus and the supraoptic nucleus during periods of osmotic stimulation (15); NPY may also be secreted from peripheral sympathetic ganglia (11). Thus NPY reaches the PP from a variety of sources where it potentially binds to high-affinity NPY-binding sites. As a result of this binding, NPY may subsequently induce the secretion of one or more PP substances which have been reported capable of modulating gonadotropin secretion; these substances include oxytocin (30, 31), galanin (32), dopamine (33), endothelin (34), and β-endorphin (35). These substances can be carried from the PP to the AP by way of diffusion into intercellular spaces (16) or through short portal vessels connecting the PP and AP lobes (17) which are known to carry 20%–30% of the total blood flow to the AP (18). Because of this significant potential for communication between the two lobes, it was suggested some time ago that the PP might play a physiologically relevant role in modulating the secretion of AP hormones (36–38). For example, a role for the PP in regulating the secretion of prolactin from the AP has been established (30, 31, 39–41). Previous studies suggest that the PP may also play a role in the regulation of gonadotropin secretion. Specifically, removal of the PP *in vivo* results in rapid elevation of LH release (19, 20), attenuates the postovariectomy rise in LH secretion (21), and decreases both the magnitude and duration of the LH surge observed in the ovariectomized estrogen-primed rat (21). However, it is not known whether the PP plays a role in the regulation of LHRH and NPY-stimulated gonadotropin secretion from the AP.

Results from the current study indicate that LH responsiveness, not only to LHRH, but to 10^{-7} *M* NPY and to 10^{-6} *M* NPY as well were all significantly greater in AP cells cultured in the presence compared with the absence of PP cells. In attempting to understand the role of PP cells in these results, one might speculate that these differences may have been due to the action of one or more of the previously described PP substances which are capable of modulating gonadotropin secretion. It must also be considered that the *in vitro* co-culture of dispersed AP and PP cells is not the equivalent of the relationship that exists between the AP and the PP lobes *in vivo*. Although the melanotrophs of the intermediate lobe and the pituicytes of the neural lobe are maintained in culture, nerve terminals ordinarily do not survive and the vascular connections between the two lobes no longer exist. However, it is still possible in a cell culture system for substances, such as α-MSH and β-endorphin from melanotrophs (35), to be secreted from PP cells into the culture medium where these substances would then be able to interact directly with AP cells and thus potentially modulate gonadotropin secretion. In addition, oxytocin from nerve terminals in the neural lobe has been determined to be present in cultured PP cells even 7 days after dispersal (42).

Under the conditions employed in the current studies, PP cells played a significant role in the LH response of AP cells to NPY alone and to LHRH alone, while they appeared to play no role in the LH response of AP cells to NPY in combination with LHRH. This was indicated by the finding that although NPY potentiation of LHRH-induced LH secretion was observed in both the presence and absence of PP cells, the degree of potentiation was not significantly different. This would suggest that the PP plays no role in the NPY potentiation of LHRH responsiveness which has been observed both *in vivo* and *in vitro* (3–7).

It is also of potential physiological relevance that whereas the presence of PP cells in AP cell cultures affected LHRH-induced and NPY-induced LH secretion positively, FSH secretion induced by LHRH was affected negatively; NPY did not affect FSH secretion at all. The observation that the presence of PP cells

affects LH and FSH secretion differently may suggest that the PP plays a role in modulating the divergence of LH and FSH secretion which has been observed *in vivo* and *in vitro* under a number of widely recognized circumstances (28, 43–46).

The current studies observed that 10^{-6} *M* NPY (but not 10^{-7} *M*) induced the secretion of LH from AP cells in the absence as well as the presence of PP cells. This result leads to some interesting speculation concerning the receptor dynamics which are potentially involved in NPY action at the level of the AP. While numerous studies utilizing ^{125}I-NPY have been unable to detect high-affinity NPY-binding sites in the AP, one study utilizing ^{125}I-PYY has reported the presence of PYY binding sites which were of primarily low affinity (47). This report suggests that NPY receptors may be present in the AP because PYY and NPY have been shown to bind to the same receptor (48, 49). However, PYY binds to the PYY/NPY receptor with much greater sensitivity than does NPY (50), suggesting that the primarily low-affinity PYY receptors in the AP may exhibit even less affinity for NPY. In fact, competition binding assays have indicated that there is an inherent difference in the nature of NPY binding to PYY/NPY receptors on the AP when compared with NPY binding to the high-affinity PYY/NPY receptors in the hypothalamus (47). Thus, the low affinity of AP PYY/NPY receptors for NPY may explain why in the current and previously reported studies (7–9), LH secretion was significantly increased by NPY only when a super-physiological level (10^{-6} *M*) of NPY (4, 47) was used in AP cells cultured in the absence of PP cells. The importance of the presence of high-affinity NPY receptors in the PP is supported by the observation that LH secretion was significantly increased by a physiological level (10^{-7} *M*) of NPY (51, 52) only in AP cells cultured in the presence of PP cells. These observations suggest that the presence of the PP may facilitate NPY responsiveness of the AP to NPY levels reported to normally occur *in vivo* (51, 52).

These are the first studies reported that have utilized pituitary cell cultures to examine directly the role of PP cells in modulating the gonadotropin response of AP cells to NPY alone and to NPY in combination with LHRH. While previous studies have shown that NPY modulates LH secretion in AP tissue preparations that were presumably free of PP tissue (3, 4, 7–9), the current studies show that the response to NPY is significantly greater in AP cells cultured in the presence of PP cells. Thus, the current studies provide evidence that the PP may play a significant role in modulating gonadotropin responsiveness of the AP to NPY and to LHRH but not in NPY potentiation of LHRH responsiveness. These studies also suggest that the PP may contribute to the divergence of LH and FSH secretion that is frequently observed *in vivo*.

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