Energetic dependence of NPY-induced LH secretion in a teleost fish (Dicentrarchus labrax)

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NEUROPEPTIDE Y (NPY) belongs to a 36-amino acid peptide family with COOH-terminal amidation. This peptide has been shown to be the most conserved neuroendocrine peptide for its size, as well as one of the most widely distributed throughout the central nervous system (CNS) of vertebrates (24). In mammals, NPY has been suggested to be a substrate of the central-effector anabolic pathways of feeding (20). Intracerebroventricular NPY injections increased food intake in satiated rats, whereas fasting-induced NPY expression in the arcuate nucleus (50). Hypothalamic NPY expression is regulated by circulating levels of insulin and glucocorticoids. Central administration of insulin inhibits NPY expression in the hypothalamus and decreases food intake in rats, whereas glucocorticoids promote the opposite effects (20, 50). NPY also plays a physiological role in the regulation of pituitary hormones (28). It has been shown to modulate the preovulatory luteinizing hormone (LH) surge by affecting gonadotropin releasing hormone (GnRH) discharge (21). However, chronic intracerebroventricular administra-

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and D-glucose were dissolved in 0.6% saline immediately before use for all experiments. NPY500 (500 ng/g pNPY), NPY500G (500 ng/g pNPY and 0.3 mg/g glucose), NPY500G (500 ng/g pNPY and 0.3 mg/g glucose), and NPY100G (100 ng/g pNPY and 0.3 mg/g glucose) were dissociated in the same media. Both L-15 and SBR (both 320 mosmol) contained 0.1% bovine serum albumin, 1% penicillin-streptomycin, and 0.1 mg/ml gentamicin. The following experimental groups (24 animals/group) were included: control saline-fed, NPY500-fed, and NPY100-fed groups, and control fasted animals. Sample size was also reduced to five groups of five animals were included for each experimental day and considered time 0. Two days before the experiment, 50 fed and 18 fasted juvenile sea bass were selected by weight (141.9 ± 0.67 g body wt). The experiment was performed as described above, but sampling times were reduced to 1, 3, and 6 h postinjection for fed animals and only 3 h postinjection for control fasted animals. Sample size was also reduced to five animals per sampling time, with the exception of the NPY500-fast group, which was reduced to three animals.

In vitro experiments. Fed and fasted juvenile fish were anesthetized using ice, and pituitaries were removed and pooled. Cells were enzymatically dispersed using a trypsin/DNAse II digestion method modified from Chang et al. (12). The cell dispersion method yielded cells of 96–98% viability using trypan blue exclusion techniques. Because the cell dispersion method described results in a mixed population of pituitary cells, immunocytochemistry using specific antibodies for LH (trout, tilapia, and stripped bass) was performed to confirm that secretory gonadotrophs were present in the populations (data not shown).

Dispersed cells were cultured (2.5 × 10^5 cells·well^-1·ml^-1) overnight at 20°C in L-15 + serum or restricted media [sea bass Ringer (SBR); Ref. 52]. SBR consisted of the following (in mM): 137 NaCl, 5.0 KCl, 1.0 Na2PO4, 1.0 MgSO4, 2.0 CaCl2, NaHCO3, and HEPES titrated to pH 7.4 with NaOH. Cells from fed and fasted animals that were to be incubated in SBR were dissociated in the same media. Both L-15 and SBR (both ~320 mosmol) contained 0.1% bovine serum albumin, 1% penicillin-streptomycin, and 0.1 mg/ml gentamicin. The following day, media were replaced with serum-free L-15 or SBR and cells were allowed to stabilize for 30 min before treatment; pNPY was prepared immediately before use. Media was removed 1, 3, 6, and 12 h after administration of pNPY and stored at −80°C until further analysis for LH content. All treatments were performed in triplicate. The basic differences between the two culture media were the presence (L-15) or absence (SBR) of amino acid, vitamins, and glucose.

Hormone and metabolite measurements. Plasma and media LH levels were assayed by an ELISA that used stripped bass LH as the standard and rabbit anti-stripped bass as antiserum (Morone saxatilis; Refs. 27, 33). Plasma insulin levels were measured by RIA using bonito insulin (Kodama, Tokyo, Japan) as the standard and a rabbit anti-bonito insulin as the

**MATERIAL AND METHODS**

Animals. Nineteen-month-old juvenile sea bass (D. labrax) were obtained from the Instituto Español de Oceanografía de Mazarrón (Murcia, Spain). Fish were maintained in 3,000-liter tanks supplied with continuously aerated, running seawater. Animals were acclimated for 3 mo under natural photoperiod and temperature conditions within the facilities of the Instituto de Acuicultura de Torre de la Sal (East coast of Spain, 40°N and 0°E) and were fed once a day to excess with a commercially prepared fish food (Ewos). Hormones and chemicals. Unless otherwise indicated, all chemicals and compounds were purchased from Sigma (St. Louis, MO). pNPY (Genosys Biotechnologies, London, UK) and D-glucose were dissolved in 0.6% saline immediately before use for all experiments.

In vivo experiments. Experiment 1 was undertaken to study the effects of pNPY on plasma LH levels of 1-mo-fasted sea bass and to examine whether the effects of NPY-induced LH could be reversed by glucose injections. The following experimental groups (24 animals/group) were included, and treatments were administered via intraperitoneal injection: control-saline (saline), control-glucose (0.3 mg/g glucose), NPY500 (500 ng/g pNPY), NPY500G (500 ng/g pNPY and 0.3 mg/g glucose), NPY100G (100 ng/g pNPY and 0.3 mg/g glucose), and NPY10G (10 ng/g pNPY and 0.3 mg/g glucose). The experiment was performed on 2 consecutive days. For day 1 (September 24, 1997), experiments included control-saline, control-glucose, and NPY500 groups and on day 2 (September 24, 1997), NPY500G, NPY100G, and NPY10G groups were used. Two days before the experiment, 156 juvenile sea bass fasted for 1 mo were selected by weight (107.6 ± 0.81 g total body wt). The day before the experiment, animals from each experimental group (24/group) were randomly divided into four 100-liter tanks (6 animals/tank) supplied with continuously aerated, running seawater. An additional group of six animals was included for each experimental day and considered time 0. On the day of experiment, animals were anesthetized with MS-222 (100 mg/l) and subsequently injected at 9:00 AM (maximum duration of time of injection was 20 min). One, three, six, and twelve hours postinjection, animals were anesthetized and weighted and blood samples were extracted by caudal puncture; blood extraction did not exceed 10 min. The last time of injections for the different groups was taken into account when calculating time of blood extraction for each group. Plasma was aliquoted and stored at −20°C until assayed.

Experiment 2 was designed to study the effects of pNPY on plasma LH levels of fed animals. The following treatment groups were included (15 animals/group): control saline-fed (saline), NPY500-fed (500 ng/g pNPY), and NPY100-fed (100 ng/g pNPY). An additional full set of control fasted animals injected with saline (control-saline-fast), pNPY 500 ng/g (NPY500-fast), and pNPY 100 ng/g (NPY100-fast) was also included. All treatments were administered via intraperitoneal injection, and the experiment was also performed on 2 consecutive days with experiments on day 1 (November 20, 1997) including control-saline-fed, NPY500-fed, and NPY100-fed groups and control-saline-fast, NPY500-fast, and NPY100-fast groups used on day 2 (November 21, 1997). Additional groups of five animals were included for each experimental day and considered time 0. Two days before the experiment, 50 fed and 18 fasted juvenile sea bass were selected by weight (141.9 ± 0.67 g body wt). The experiment was performed as described above, but sampling times were reduced to 1, 3, and 6 h postinjection for fed animals and only 3 h postinjection for control fasted animals. Sample size was also reduced to five animals per sampling time, with the exception of the NPY500-fast group, which was reduced to three animals.
antiserum (18). To evaluate the effect of fasting on gonadal steroids, plasma T and 17β-estradiol (17β-E2) levels were assayed by RIA (44) in the time 0 groups from experiment 2. Furthermore, an aliquot of plasma was deproteinized with zinc sulfate-barium hydroxide, and the supernatant was used for glucose determinations by the glucose oxidase method.

Statistical analysis. Data are expressed as means ± SE and compared by one-way ANOVA followed by Tukey’s honestly significant difference (HSD) multiple range test. Significant differences were taken at P < 0.05. For in vitro experiments, media LH values were normalized and expressed as the percent increase over basal LH secretion. Student’s t-test was used for paired data, and ANOVA was used to compare treated groups with their corresponding control groups over time of incubation. Post-ANOVA multiple comparison of means was carried out using Tukey’s HSD multiple range test.

RESULTS

Experimental protocol. No significant differences in weight were detected between animals from different sampling times. Similarly, no significant differences in plasma LH, glucose, insulin, T, or 17β-E2 steroid levels were observed between time 0 groups from the different experimental days (data not shown).

pNPY-induced LH secretion in fed and fasted animals in vivo. The effects of pNPY on LH secretion in fasted animals as well as the effect of pNPY-glucose coinjection are shown in Fig. 1A. A significant increase in plasma LH levels 3 h postinjection was observed in the pNPY (500 ng/g)-treated group. Saline and glucose injections had no effect on LH secretion. Although glucose could not reverse the pNPY-induced LH effect, a dose-dependent stimulation of LH secretion was observed. Twelve hours after injection, the group injected with the highest pNPY dose exhibited a significant decrease in basal plasma LH levels. When the active pNPY doses for fasted animals were administered to fed animals, no significant effects on basal LH secretion were observed (Fig. 2A). Furthermore, no significant differences in plasma LH levels were detected between fed and fasted animals (Fig. 2A). Results obtained from the additional groups of fasted animals treated with the same NPY doses and included in experiment 2 were not significantly different from the fasted animals of experiment 1 (data not shown).

pNPY effects on glucose plasma levels of fed and fasted animals. The saline injection in fasted animals produced a significant increase in glucose plasma levels at 1 and 3 h postinjection compared with time 0 (Fig. 1B). The glucose injection provoked the expected increase in plasma glucose levels, which was significant throughout the complete sampling cycle. However, when pNPY was injected alone, no significant variations of plasma glucose levels were found compared with the control-saline group. The combined NPY-glucose injection caused a similar increase as observed in the control-glucose group 1 h postinjection, although levels were significantly lower than those at 3 and 12 h postinjection. Only animals treated with the highest doses of pNPY + glucose displayed a significant decrease at 6 h postinjection compared with the control-glucose group. When the two maximum doses of NPY were injected alone to fed animals, no alterations in plasma glucose levels were detected. Furthermore, the plasma glucose levels of fed animals were significantly higher than those of fasted animals (Fig. 2B).

NPY effects on insulin plasma levels of fed and fasted animals. The effects of pNPY and glucose on insulin plasma levels in fasted animals are shown in Fig. 1C. Neither glucose nor pNPY alone induced any plasma insulin response. However, when glucose and pNPY
were coadministered, insulin levels decreased significantly at 6 and 12 h postinjection compared with the control-glucose group. This decrement was also apparent at 1 h postinjection for the lowest NPY dose. In fed animals, plasma insulin levels remained unaltered, even when the highest doses of pNPY were administered to fed animals (Fig 2C). Insulin levels of fasted animals were significantly lower than fed animals (Fig 2C).

Effects of fasting on plasma gonadal steroids. The plasma levels of T (0.45 ± 0.24 ng/ml) and 17β-E2 (1.02 ± 0.21 ng/ml) from fasted animals were not statistically different from those of fed animals (0.18 ± 0.15 ng/ml and 0.82 ± 0.22 ng/ml, respectively) in the time 0 group of experiment 2.

NPY-induced LH surge in pituitary cells of fed and fasted animals in vitro. Immunohistochemistry confirmed the presence of gonadotrophs in pituitary cultures (data not shown). Incubation of pituitary cells from fasted and fed animals with different concentrations of pNPY resulted in a dose-dependent stimulation of LH secretion (illustrated in Fig. 3, A and B). In both instances, LH maximal responses of ~190% at 3 h were achieved with 10 nM pNPY with half-maximal effective dose (ED50) of 0.82 and 0.93 nM for fed and fasted animals, respectively. However, if pituitary cells from fasted animals were incubated in SBR, significantly enhanced LH responses were observed at 3 h compared with L-15 incubations (Fig. 4). These increases were evident with all pNPY doses, even though basal LH levels of secretion (ng/ml) were statistically similar. Once again, the maximal LH response at 3 h was reached with 10 nM pNPY with an ED50 of 0.11 nM.

DISCUSSION

The present study describes for the first time a relationship between energetic status and gonadotropin secretion in fish. The effect of NPY on LH secretion has been widely demonstrated in both mammals and fish (4–6, 14, 19, 21, 28, 34–38). Sex steroids have been...
proposed to be responsible for this effect, and they were shown to be responsible for the observed seasonal differences (34, 38). Until now, the effects of energetic status on NPY-induced LH secretion were unexplored. The results from this study suggest that pNPY-induced LH stimulation is in fact modulated by the energetic status. Under negative energetic status imposed by chronic fasting and characterized by a significant decrease in insulin and glucose basal plasma levels, LH secretion in vivo was dose dependently enhanced in response to pNPY. In contrast, positive energetic status suppressed the ability of NPY to stimulate LH secretion. Hypothalamic NPY gene expression has been shown to increase according to periods of starvation in mammals (20, 50) and teleosts (48). Consequently, a possible explanation for the results obtained could be the presence of increased NPY levels in the hypothalamus. The effective doses of pNPY capable of modulating LH secretion in fasted animals would therefore be ineffective in fed animals. The minimum effective dose used in this experiment was lower than the NPY concentrations used in studies by Breton et al. (5) and Peng et al. (37) in which the seasonality of NPY-induced LH secretion in both Oncorhynchus mykiss and Carassius auratus, respectively, was demonstrated. Such an assumption would imply that the increased NPY levels induced by food restriction may mediate the effects of energetic status on LH secretion, and, consequently, fasted animals would display increased plasma LH levels. In the present study, however, this was not the case, because basal LH levels of fed and fasted animals were not significantly different.

In mammals, the NPY Y1-receptor is required for the physiological amplification of the preovulatory LH surge (25). Thus a second possible explanation for the differential action of pNPY observed in fed and fasted animals could be that gonadotrophs from fasted animals exhibit a higher responsiveness to pNPY due to an upregulation of NPY receptors. However, dispersed pituitary cells from fasted and fed animals incubated in L-15 were equally responsive to pNPY, further supporting a direct action of NPY on gonadotrophs (6, 34).

Glucose, injected in combination with pNPY, did not reverse the effects of pNPY on LH secretion. However, from the results obtained, a role for glucose cannot be entirely excluded, because a trend toward reduced LH secretion was observed in animals treated with pNPY + glucose, although differences were not statistically significant. Furthermore, only animals injected with the highest pNPY doses displayed significant decreases in plasma LH at 12 h postinjection; these decreases were not detected when glucose was co-injected with the same pNPY doses. Thus it appears that the absence of any influence of pNPY on LH secretion observed in fed animals could not be based on a glucose-induced LH effect.

The effect of insulin on pNPY-induced LH secretion cannot be deduced from the results of this study because no coinfusion experiments were performed with the two hormones. Although increased plasma insulin levels were observed in fed animals, it cannot be concluded that they were responsible for the differential effects of NPY-induced LH secretion. Furthermore, insulin plasma levels were not modified under pNPY or glucose administration.

Essentially, modulation of LH secretion by pNPY was only observed in animals under negative energetic conditions. Similarly, pituitary cells from fasted animals incubated in restricted SBR media were more responsive to pNPY compared with fasted cells incubated in L-15. Moreover, glucose injection was unable to reverse the effects of starvation and it is improbable that insulin mediated the observed pNPY differential effects. Therefore, the existence of a factor, expressed and/or secreted under positive energetic conditions capable of blocking or interfering with the effects of NPY on LH secretion must be considered. Because the negative energetic periods in the sea bass are associated with the reproductive period, energetic modulation of NPY effects should be taken into account when considering the seasonality of NPY-induced LH secretion. A likely candidate responsible for the regulation of the differential NPY effects could be the sex steroids. However, both T and 17β-estradiol plasma levels from fasted animals were not significantly different from those of the fed animals when the time 0 groups from experiment 2 were considered. Studies have demonstrated that an increase in ration size significantly decreases the 17β-estradiol plasma levels in mature sea bass females (9). Moreover, plasma 11-ketotestosterone levels of Opsochromis niloticus were not modified or significantly lowered in fasted male and females compared with fish fed different food rations, and 17β-estradiol plasma levels did not show any correlation with the food intake ratio (51). Similarly, plasma sex steroid binding protein (SBP) levels are known to decrease under long-term fasting conditions in rainbow trout (16). Therefore, taking into account these studies, it would be difficult to explain...
the present results based on sex steroid facilitation of NPY-induced LH secretion. Moreover, Peng et al. (38) reported that the effect of NPY in sexually regressed fish pretreated with T was enhanced compared with T-treated or untreated mature fish. The authors speculated that the existence of factors produced by mature gonads could attenuate the positive modulatory effect of T. However, the sea bass used in the present experiment were not mature animals.

Dopamine has been characterized as an LH releasing inhibitor in several teleost species (2, 46). The inhibitory effect of dopamine has been reported to display interspecific differences. This monoamine has been demonstrated to produce powerful inhibitory tone in cyprinids (2), but its effect is much less pronounced in salmonids (3, 46) and absent in the Atlantic croaker (Micropogonias undulatus; Ref. 13). Chronic food restriction decreases extracellular dopamine levels within specific areas of the rat CNS, and it has been suggested that food deprivation decreases dopamine release without modifying the rate of synthesis (41). Starvation was also shown to increase telencephalic and diencephalic dopamine levels in the lungfish (Protopterus annectens), suggesting that dopamine stores are maintained without altering biosynthetic rates (23). Furthermore, dopamine has also been shown to block in vitro NPY-induced LH secretion in the goldfish (37). The results of the present experiment suggest that starvation could reduce the inhibitory dopaminergic tone, thus allowing NPY-induced LH secretion. Whether starvation reduces dopaminergic tone and whether such a decrement selectively influences NPY-induced LH secretion remain to be determined. In support of this selectivity, it has been shown that GnRH stimulates LH secretion through vitellogenesis in the stripped bass (26). However, our experiments with fed animals (absence of NPY stimulation) were carried out during November, when sea bass are just starting vitellogenesis (28). Furthermore, dopamine has been shown to selectively attenuate NPY-induced feeding in rats (50).

Pituitary dispersed cells from fasted animals incubated in restricted media (SBR) lacking essential nutrients displayed higher responsiveness to all pNPY doses than those of fasted animals incubated in L-15. However, basal secretion levels in both L-15 and SBR were statistically similar. Preliminary results indicate that pituitary cells from fed animals respond to NPY in a manner similar to cells from fasted animals when incubated in SBR (data not shown). This suggests that a second messenger pathway mediating the effects of NPY in gonadotrophs may directly or indirectly be modified by one or more components from L-15. Such a modification would lead to a decrease in gonadotroph responsiveness to all pNPY doses. Therefore, it is possible that nutritional status may alter a second messenger pathway, thus enhancing the responsiveness to pNPY during fasting periods. The second messenger systems activated by NPY leading to stimulation of LH secretion from pituitary cells are currently unknown in the sea bass and remains a challenging area for future research.

The implications of NPY on metabolic processes in fish remain unexplored. In mammals, intracerebroventricular and paraventricular nucleus (PVN) administration of NPY resulted in increased circulating insulin levels, with minimal or no stimulatory effects on serum glucose levels. In turn, an increase in insulin could reduce plasma glucose availability. Hence, NPY may exhibit a counteracting effect (see Refs. 28 and 50 for review). When administered intravenously, NPY lowers blood glucose in rats (1). Furthermore, NPY acting directly on the pancreatic islet can decrease arginine and glucose-stimulated insulin secretion in rats (39) and, when given intravenously, glucose-induced insulin release is reduced in the mouse (40). In fish, insulin functions as a major anabolic hormone stimulating glucose uptake by the liver and skeletal muscles. However, basic amino acids, especially arginine and lysine, seem to be more potent stimulators of insulin secretion than glucose (reviewed in Ref. 31). Results from the present study demonstrate that pNPY significantly decreased both plasma insulin and glucose in fasted animals co-injected with pNPY and glucose, whereas no effect on insulin levels was observed in fed and fasted animals injected with pNPY alone. The highest dose of pNPY was shown to affect glucose plasma levels at 3 h postadministration during the complete sampling cycle and all doses modulated levels at 12 h postinjection. Furthermore, when fed animals were injected with the highest pNPY doses, glucose plasma levels decreased 3 h postadministration; however, decreased levels did not reach statistical significance (P = 0.052). These results could be explained as NPY-induced facilitation of glucose metabolism, in that NPY could be modulating glucose tissular uptake. NPY has been shown to increase insulin-stimulated glucose uptake by adipose tissue in vivo, an effect accompanied by increases in GLUT-4 mRNA (54). Insulin plasma levels of fasted animals significantly decreased 1 h after administration of the lowest doses of pNPY and after 6 and 12 h for all doses. Such decreases in insulin could be an indication of facilitation of glucose metabolism, because no glucose-stimulated increase in plasma insulin was observed after glucose administration. Arginine has been consistently reported as a major insulin secretagogue in fish (31). However, whether NPY mediates insulin secretion by modifying basic amino acid metabolism remains open to discussion. Thus, once again, the NPY effects appear to be attenuated under positive energetic status although the physiological relevance of the observed NPY effects on glucose and insulin metabolism remains to be determined.

In summary, the results obtained from this study demonstrate that the NPY-induced LH secretion is dependent on energetic status and the differential effect observed is not modified by plasma glucose availability.

**Perspectives**

In mammals, peripheral energetic information is signaled in CNS by humoral factors, such as insulin, glucocorticoids, thyroid hormones, and leptin (20). It
has been suggested that leptin could affect the neuroendocrine reproductive axis by acting on the NPY central system (28). In the sea bass, the active feeding periods are associated with the initial stages of the reproductive cycle, which appear to be regulated by FSH in salmonids (3, 43). However, reduced food intake periods are related to maturational and ovulatory phases. Therefore, it has been suggested that feeding periods could block LH secretion, thus avoiding premature maturation of the gametes. Conversely, negative energetic status could enhance gonadotroph responsiveness to NPY. The results obtained from the present study could represent an adaptive mechanism of the reproductive system in that any stimulation of NPY expression would induce an LH response exclusively during the reproductive period, suggesting an inverse effect of energetic status on FSH secretion. In salmonids, it has been shown that if spring growth is inadequate, gonadal development is physiologically switched off (45). Throughout the literature, the role of metabolic hormones in the neuroendocrine control of the reproductive process of fish is limited. However, the metabolic hormones have been shown to exhibit seasonal secretory patterns related to reproductive cycles (15). The present data suggest that metabolic hormones could modulate the main neuroendocrine systems controlling gonadotropin secretion. Such a modulation could help to explain the observed seasonal actions of the neuroendocrine factors. The recently obtained NPY probe will help to elucidate the relationship between metabolic hormones and neuroendocrine reproductive axis.

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