Neuropeptide Y Stimulates *in Vivo* Gonadotropin Secretion in Teleost Fish

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The effects of the neuropeptide Y (NPY), alone or in combination with a gonadotropin-releasing hormone analogue, D-Ala⁶-desGly¹⁰-Pro⁹-Net LHRH (LHRHa), have been studied on the *in vivo* secretion of the maturational gonadotropin (GtH₂) in the rainbow trout *Oncorhynchus mykiss* and the common carp *Cyprinus carpio*. Depending on the species, two routes of administration were used: in trout, intraperitoneal injection (20 µg/kg body wt); in carp, direct infusion (3 µg/kg body wt) into the third ventricle via a temporary brain cannula. In both cases NPY alone induced a twofold increase in GtH₂ secretion and peaked 2 to 4 hr after administration regardless of the route of injection. The plasma gonadotropin levels returned to basal within 8 hr. The relative increases (peaked secretion/basal secretion) did not differ with the route of injection. When the animals were first treated with NPY and then LHRHa (20 µg/kg) 1 hr later, the magnitude of the response to LHRHa was greater in the animals pretreated with NPY, indicating either a potentiation of LHRHa action by NPY or additive effects of the two peptides. The return to basal levels also took longer in fish receiving NPY first. NPY may act directly at the pituitary level or activate central neuro-mediatory systems. © 1991 Academic Press, Inc.

Neuropeptide Y (NPY) has been now identified in the brain and the pituitary gland in several fish species including an elasmobranch (Vallarino *et al.*, 1988) and teleosts including anglerfish *Lophius americanus* (Noe *et al.*, 1989), goldfish *Carassius auratus* (Pontet *et al.*, 1990), and rainbow trout *Oncorhynchus mykiss* (Danger *et al.*, 1990). In these last two species NPY stimulates *in vitro* gonadotropin (GtH₂) secretion from perifused pituitary fragments (Kah *et al.*, 1989; Breton *et al.*, 1989). In the rainbow trout its *in vitro* action is pleiotropic (Breton *et al.*, 1990). NPY also acts directly on dispersed pituitary cells, or indirectly via GnRH (Breton *et al.*, 1990). Moreover the peptide's *in vitro* action depends on the *in vivo* steroidogenic environ-

ment (Breton *et al.*, 1989). In fish, unlike in mammals, all results to date concern NPY's *in vitro* actions. The aim of this study was to assess *in vivo* effects of NPY in two teleosts, the common carp *Cyprinus carpio* and the rainbow trout injected alone or in combination with GnRH.

MATERIAL AND METHODS

Two protocols were followed.

A. Intracerebroventricular (icv) Infusion in Carp

Experiments were performed at the experimental fish farm of the Department of Ichthyobiology, Krakow, Poland. Five- to 6-year-old sexually mature female common carp weighing 2.5 to 4.5 kg were used. During the experiment they were kept in 800-liter tanks in which the water temperature was maintained at 20 ± 1°, under a 16L-8D light regime.

They were prepared for icv infusion 8 days before beginning treatment by placing a cannula into the third cerebral ventricle using a stereotaxic approach. The

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stereotaxic positions were determined following Sokolowska (1982), using histological preparations after vital dye injections. Position 0 was at the level of the anterior commissure.

Under anesthesia (2-phenoxyethanol, 2.4×10^{-6} M) the brain was exposed after drilling a median 1-cm² aperture in the skull between the telencephalon and the mesencephalon. Adipose tissue was removed and a guide, a 1-mm-external-diameter metal canula, was slowly inserted 2 mm toward the anterior commissure. It reached the third ventricle 3 mm from the base of the brain. During the surgical operation hemorrhage was stemmed using a sterile, water-insoluble hemostatic gelatine sponge (Spongostan, Denmark). The guide was then fixed 0.5 cm above the brain surface using dental cement (Duracryl, Chemapol, Czechoslovakia). After the cement had hardened fish were returned to aquaria to recover. Throughout the surgery fish were perfused through the gills with anesthetic (2-phenoxyethanol, 2.4×10^{-7} M).

Drug infusions were also done under anesthesia, with the fish supine. A needle (external diameter 0.5 mm) connected to a Zimmerman microsyringe via a 0.58-mm-diameter polyethylene tube was introduced into the guide. Each fish received 10 μ l delivered within 10 sec. According to the group ($n = 8$ fish per group), fish received either a saline solution or NPY (Bachem, Switzerland) (3 μ g/kg body wt). Thirty minutes later they were injected intraperitoneally with saline or a LHRH analogue, D-Ala⁶-desGly¹⁰-Pro⁹-Net LHRH (LHRHa; Sigma, St. Louis, MO) (20 μ g/kg body wt).

Group Ic	Saline	Saline
Group Iic	NPY	Saline
Group IIic	Saline	LH-RHa
Group IVc	NPY	LH-RHa

Blood samples were taken by caudal venous puncture using heparinized syringes at Time 0, 30 min, and 1, 2, 4, 8, 24, and 48 hr after infusion. The plasma gonadotropin contents were assayed using a specific RLA for carp GtH (Breton *et al.*, 1971). Ovulation was checked at times 24 and 48 hr by abdominal palpation.

B. Intraperitoneal Injections (ip) into Rainbow Trout

These experiments were performed in August on rainbow trout (body weight 870 ± 370 g) kept at 12° in a recirculating water system (summer spawning strain). Fish were 2 years old and were reproducing for the first time. They were chosen at the end of vitellogenesis when follicles can be hand stripped. Fish were divided into four experimental groups of eight fish per group.

Group It. Control fish received saline injections at Time 0 and 2 hr.

Group IIt. These fish received an injection of NPY at Time 0 (20 μ g/kg body wt) and a saline injection at Time 2 hr.

Group IIIt. Fish were treated with saline at Time 0 and by LH-RHa (20 μ g/kg body wt) at Time 2 hr.

Group IVIt. NPY (20 μ g/kg body wt) was given at Time 0 and LHRHa (20 μ g/kg body wt) 2 hr later.

After anesthesia using the same anesthetic as in carp, blood samples were taken from a venous caudal vessel using a heparinized syringe at Time 0, 30 min, and 1, 2, 2.5, 3.5, 5, 7.5, 10, 24, and 48 hr after the first injection. Plasma levels of GtH₂ were measured by a specific RIA for salmonid GtH₂ (Breton *et al.*, 1978). Data of ovulation was noted.

In both experiments, statistical analyses were done by Mann-Whitney *U* test. Results are presented as means $\pm$ SEM.

RESULTS

A. NPY Infusion in Carp

In the control group (Fig. 1) plasma GtH₂ levels did not change over the first 8 hr after saline infusion, but there was a significant decrease after 24 hr ($P < 0.01$) which was maintained 1 day later. After 3 μ g/kg NPY infusion, GtH₂ blood plasma levels increased and were statistically higher 2 and 4 hr after treatment reaching 35 ± 15.8 ng/ml. They returned to basal levels at 8 hr (Fig. 2). In fish receiving LHRHa alone (Fig. 3, white bars) mid basal GtH₂ levels were 23.9 ± 12.5 ng/ml. They increased to reach 88 ± 34 ng ($P < 0.05$) 7.5 hr after injection. When fish were pretreated with NPY, the re-

FIG. 1. Blood plasma GtH₂ level in the common carp *Cyprinus carpio*, after intracerebroventricular (icv) infusion of saline. Group Ic. Arrows indicate the time of injections: first, icv; second, ip.

FIG. 2. Effects of icv infusion of neuropeptide Y (NPY) on the GtH₂ secretion in the common carp *Cyprinus carpio*. Arrows indicate the time of injection: first, icv; second, saline ip. Group IIc. **Statistically higher than Time 0; $P < 0.01$.

sponse to GnRH injection was more pronounced than when the decapeptide was used alone. Whatever time after GnRH treatment, the plasma GtH levels were higher after the combined treatment with NPY-LHRHa than with LHRHa alone (Fig. 3). The return to basal levels was more rapid when fish were treated with LHRHa

FIG. 3. Effect of icv infusion of neuropeptide Y (NPY) on the response to intraperitoneally injected LHRHa in the common carp *Cyprinus carpio*. The arrows indicate the time of treatment. Hatched bars correspond to Group IVc receiving first NPY and then LHRHa. Open bars correspond to Group IIIc receiving first icv injected saline and then LHRHa. *Statistically higher in Group IVc than in Group IIIc; $P < 0.05$.

alone. In this group two fish ovulated after 24 hr; and in another group oocytes were entering maturation. In the other groups ovulation and maturation did not occur.

B. NPY Intraperitoneal Treatment in Trout

As in the carp in the control group GtH₂ plasma levels remained stable at around 2 ng/ml (Fig. 4). NPY injection induced a gradual increase in plasma GtH₂ levels which peaked 2.5 hr after treatment rising from 3.72 ± 0.69 to 8.76 ± 3.05 ng/ml. GtH₂ levels then returned to basal at 7.5 hr (Fig. 5). LHRHa induced a rapid increase in plasma GtH₂ levels 30 min after treatment (Fig. 6) to peak 8 hr after injection. The stimulation of the GtH₂ secretion was maintained 24 hr later, but GtH₂ levels returned to basal 48 hr after the beginning of the experiment. In fish first treated with NPY (Fig. 6), there was, as before, a gradual increase in GtH₂ secretion up to 2 hr, the time at which they received LHRHa; there was a more pronounced stimulation of the GtH₂ release twice that induced by LHRHa alone. This stimulation also peaked 1.5 hr after treatment and was maintained for longer; plasma GtH₂ levels had not returned to basal levels 48 hr later.

In control fish, two out of eight ovulated

FIG. 4. Changes in blood plasma GtH₂ levels in the rainbow trout receiving two ip injections of saline. Group It. Arrows indicate the time of injections.

FIG. 5. Effect of intraperitoneal injection of neuropeptide Y (NPY) on the GtH₂ secretion in the rainbow trout. Arrows indicate the time of injection: first, NPY injection 20 µg/kg body wt, second, saline injection. *Statistically higher than Time 0; $P \leq 0.05$

at Day 8 (one at Day 4, plus one at Day 8). In fish receiving NPY only six out of eight ovulated at Day 8 (two at Day 2, plus two at Day 4, plus two at Day 8). GnRH induced ovulation in six fish out of eight (one at Day 2, plus two at Day 4, plus three at Day 8). The combined treatment (NPY ± GnRH) resulted in eight ovulations out of eight fish (four at Day 4, plus four at Day 8).

DISCUSSION

In both carp and rainbow trout, NPY stimulates *in vivo* GtH secretion in mature fish. This agrees with *in vitro* results in the goldfish (Kah *et al.*, 1989) and the rainbow trout (Breton *et al.*, 1989). There were no apparent differences in the dynamics of the responses between these two species. Maximal GtH₂ levels were seen 2 hr after treatments. Since these effects are observed after both icv and ip injections, it might be postulated that in fish NPY affects GtH release in areas which lack the blood-brain barrier as suggested in mammals (Kerkerian *et al.*, 1985). In gonadectomized rats, generally NPY had opposite *in vivo* and *in vitro* effects. It stimulated *in vitro* LH secretion (McDonald *et al.*, 1985; Chabot *et al.*, 1988), whereas it inhibited *in vivo* LH secretion (McDonald *et al.*, 1985; Kerke-

rian *et al.*, 1985; Crowley *et al.*, 1985; Guy *et al.*, 1988). This *in vivo* inhibitory effect can be suppressed by estrogen-progesterone treatment (Crowley *et al.*, 1985) and was not apparent in intact animals, in which NPY stimulated *in vivo* LH secretion, but did not affect its *in vitro* release (Rodriguez-Sierra, 1987). Thus in mammals NPY modulation of LH secretion depends on the steroidogenic environment, especially estradiol (Crowley *et al.*, 1985; Crowley and Kahra, 1987; Wehrenberg *et al.*, 1989), under which NPY stimulates LH secretion. In previous *in vitro* studies (Breton *et al.*, 1989) the findings were opposite to those in mammals; estradiol inhibited GtH₂ secretion under the action of NPY. The present *in vivo* experimental conditions in the rainbow trout gave estradiol levels that were lower around ovulation (Fostier *et al.*, 1978), and also a stimulatory effect of NPY on GtH secretion was observed, indicating possibly different modulatory actions of NPY and estradiol in fish and mammals.

From the present experiments it is not possible to assess modes and levels of action of NPY. *In vitro* NPY stimulation of GtH secretion from pituitary dispersed cells in the trout (Breton *et al.*, 1990) suggests similarities with *in vivo* actions, as has also been postulated in mammals (Chabot *et al.*, 1988). The most plausible hypothesis in mammals is that NPY is a component of an excitatory neural circuit responsible for the induction of preovulatory LH release (Sahu *et al.*, 1989) mainly interacting with the LHRH system, on which it could have a dual action. An initial stimulation of LHRH release from the median eminence (Khorram *et al.*, 1987; Allen *et al.*, 1987; Pau *et al.*, 1989) is one about which no information is available in fish. A second action postulates a direct effect on pituitary gonadotrophs to activate and/or potentiate LH release induced by LHRH (Crowley *et al.*, 1987; Sutton *et al.*, 1987; Sahu *et al.*, 1989). Previously Breton *et al.* (1990) dem-

FIG. 6. Effects of intraperitoneal injection of neuropeptide Y (NPY) on the response to intraperitoneally injected LHRHa in the rainbow trout. The arrows indicate the time of treatments. Hatched bars correspond to Group IVt receiving first NPY then LHRHa alone at Time 2 hr. Statistically higher in Group IVt than in Group IIIIt; ** $P < 0.01$, * $P < 0.05$.

onstrated that the combined effects of GnRH and NPY on the stimulation of gonadotropin secretion by trout pituitary fragments *in vitro* were greater than the cumulative effects of each secretagogue individually. These results have now been confirmed on dispersed trout pituitary cells using NPY in doses (10^{-8} M) noneffective in themselves (Breton, unpublished data), indicating a possible sensitization of pituitary gonadotrophs to the action of GnRH by NPY. The *in vivo* responses to the combined treatment NPY–GnRH were also greater than the additive effects of the separate peptides. It seems that the two actions of NPY demonstrated in mammals may also be present in fish. In mammals the action of NPY on pituitary gonadotrophs involves extracellular Ca^{2+} influx through voltage-sensitive channels (Crowley *et al.*, 1990) to increase a Ca^{2+} influx which is also stimulated by LHRH (Conn *et al.*, 1987). In fish it is now established that GnRH action also requires calcium ions (Levasi-Sivan and Yaron, 1989; Jamaluddin *et al.*, 1989) of extracellular origin (Mikolajczyk *et al.*, 1990a) and involves voltage-dependent calcium channels (Mikolajczyk *et al.*, 1990b). In fish the potentiation of the GnRH action by NPY at the pituitary level might also reflect a dual action of both NPY

and GnRH to increase extracellular calcium influx.

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