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Use of antiserum to neurotensin reveals a physiological role for the peptide in rat prolactin release

(third-ventricle injection/intravenous injection/dispersed-anterior pituitary cell incubation/prolactin-inhibiting factor/prolactin-releasing factor)

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ABSTRACT **Previous studies have indicated that the brain peptide neurotensin can stimulate prolactin release by direct action on the pituitary gland, whereas its action within the hypothalamus is inhibitory. The inhibitory action is mediated by the release of dopamine into the hypophyseal portal veins, which deliver the neurotransmitter to the anterior pituitary gland to inhibit prolactin release. Our experiments were done to evaluate the physiologic significance of these neurotensin actions by injecting the globulin fraction of highly specific neurotensin antiserum either intravenously or intraventricularly. Injection into the third ventricle of either 1 or 3 μl of neurotensin antiserum significantly increased plasma prolactin concentrations in (*i*) ovariectomized and (*ii*) ovariectomized estrogen- and progesterone-primed rats within 1 hr of injection. The response was more pronounced in the ovariectomized than in the ovariectomized estrogen- and progesterone-treated animals and was dose related. Intraventricular injection of these doses of neurotensin antiserum also evoked elevations in plasma prolactin in intact males, which were significant but smaller in magnitude than those seen in female rats. To evaluate the effect of the antiserum on the pituitary directly, the antiserum was injected intravenously at a dose of 40 μl, which was sufficient to block the blood pressure-lowering effect of neurotensin. After the intravenous injection of antiserum, a highly significant suppression of plasma prolactin occurred, detectable when first measured at 1 hr after injection in both ovariectomized and ovariectomized estrogen- and progesterone-treated animals; however, the intravenous injection of antiserum had no significant effect on the prolactin release in males. These data indicate the physiological significance of the hypothalamic inhibitory actions of neurotensin on prolactin release, which are probably mediated by its stimulation of dopamine release that in turn, inhibits prolactin secretion by the lactotropes. The direct stimulatory effect of the peptide on prolactin release after its presumed release into portal vessels also appears to be physiologically significant in female but not in male rats.**

Neurotensin, a tridecapeptide first isolated from bovine hypothalamus, fulfills many of the criteria required for a neurotransmitter or neuromodulator. Neurotensin is localized in discrete populations of neuronal cell bodies and terminals in the hypothalamus, median eminence, thalamus, and brain stem (1–3) and can be released from brain slices by depolarizing stimuli in a calcium-dependent manner (4). High-affinity binding sites for neurotensin have been demonstrated in synaptic membranes (5), and these sites are unevenly distributed in the central nervous system (6). Because high concentrations of neurotensin and its receptor(s) are present in hypothalamic tissue, this tissue is a likely site for some of the neuroendocrine effects of this peptide (7).

In an earlier study we demonstrated the ability of neurotensin to block the release of prolactin in conscious ovariectomized (8) and male (9) rats after its injection into the third ventricle. On the other hand, intravenous injection of the peptide significantly elevated prolactin levels and significantly increased prolactin release by pituitaries incubated *in vitro* (8). These results indicated that neurotensin had opposite actions on prolactin release—an inhibitory effect at a hypothalamic site and an excitatory effect at the pituitary.

Experiments detailed in this report were designed to examine the possible role of endogenous neurotensin on prolactin release by injecting a highly specific antiserum to neurotensin (NT-AS) into the third ventricle or i.v. into unanesthetized (*i*) intact male, (*ii*) ovariectomized female, and (*iii*) ovariectomized estrogen- and progesterone-primed (OEP) female rats. Additionally, *in vitro* incubation of dispersed pituitary cells from intact male rats with NT-AS was done to evaluate the significance of potential neurotensin concentrations within these cells. Portions of these data have appeared previously in abstract form.¶

MATERIALS AND METHODS

Virgin female and male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 220–240 g were housed under controlled conditions of light (on 0500–1900 hr) and temperature (24 ± 1°C) with free access to rat chow and water. One week after arrival the female animals were gonadectomized while lightly anesthetized with ether. Groups of intact males and females 3 weeks after gonadectomy were used for experiments.

Experimental Procedure. One week before experimentation a 23-gauge stainless steel cannula was implanted in the third ventricle, and 1 day before the experiment an indwelling catheter was placed in the external jugular vein as described (8). On the day of the experiment an extension of polyethylene tubing (PE50, 30.5 cm long) filled with heparin (20.0 units) in 0.9% NaCl (saline) was attached to the distal end of the i.v. cannula, and the animals were left undisturbed in individual cages for 60–120 min. During this time, a preinjection blood sample (0.6–0.8 ml) was withdrawn over a period of 60 sec just before injection of NT-AS or the control normal rabbit serum.

Several groups of ovariectomized rats were injected s.c. with 50 μg of estradiol benzoate and 25 mg of progesterone

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Abbreviations: NT-AS, specific antiserum to neurotensin; OEP, ovariectomized estrogen- and progesterone-treated.

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¶Vijayan, E., Carraway, R., Leeman, S. E. & McCann, S. M., 69th Endocrinology Society Meeting, Indianapolis, IN, June 1987, abstr. 213.

dissolved in oil 72 hr before experiment as described (8). These OEP rats were evaluated to determine the effects of priming the rats with gonadal steroids on the responses.

NT-AS. Antiserum TH-6 was a serum pool obtained from four rabbits injected with synthetic neurotensin conjugated to succinylated bovine thyroglobulin or keyhole limpet hemocyanin using carbodiimide (10). Antiserum TH-6 was shown to be directed towards the C-terminal six residues of neurotensin (11), which is the biologically active portion (12). Before use, the antibodies were precipitated using ammonium sulfate (40% saturation) and dialyzed exhaustively against saline. Control normal rabbit serum was treated similarly. When given i.v. to 250-g anesthetized female rats 5 min before challenge, TH-6 blocked the hypotensive effect of synthetic neurotensin (0.5 nmol/kg, $n = 3$). NT-AS was microinjected into the third ventricle of conscious rats in volumes of 1 or 3 μl with a 10-μl Hamilton microsyringe as described (13). Intravenous injections delivered a 40-μl volume of NT-AS. Control rats received an equal volume of normal rabbit serum by the appropriate route of injection. In all cases injection time was ≈60 sec, and all experiments were begun between 800 and 900 hr. Blood samples (0.6–0.8 ml) were collected from the external jugular vein catheter in heparinized syringes at various intervals (see *Results*) while the animal was freely moving in the cage. The volume of each blood sample was replaced immediately after bleeding by an equivalent volume of saline. Plasma was separated by centrifugation at low speed at 4°C and stored frozen until the day of assay.

Dispersed Pituitary Cell Incubations. Anterior pituitaries were removed from adult male rats after decapitation and dispersed in the presence of trypsin (Difco) as described (14). Cells were incubated overnight in medium 199 containing 20 mM Hepes buffer (GIBCO), 10% horse serum, and penicillin/streptomycin (1 ml per 100 ml of medium) at 37°C. Cells were then pelleted on the day of experimentation, preincubation medium was withdrawn, and the cells were resuspended prior to 1-hr incubation (37°C) in either 1 ml of medium 199 or 1 ml of the same medium containing appropriate test substances. Incubations were terminated by centrifugation (600 × g for 10 min at 25°C), and the media were stored frozen.

RIA. Prolactin concentrations were measured by RIA using the kits provided by the National Institute of Diabetes, Digestive and Kidney Diseases and expressed in terms of the RP-3-NIH prolactin standard. Control experiments were performed to demonstrate that the doses of NT-AS injected intravenously did not interfere with the prolactin RIA. To eliminate interassay variation, all samples from each experiment were run in the same assay at two different dilutions in duplicate. The quality of assays was controlled according to the criteria proposed by Rodbard *et al.* (15).

Statistics. Data were analyzed for statistical significance by analysis of variance for repeated measures followed by the Student Newman–Keuls test and are expressed as mean ± SEM.

RESULTS

Effects of NT-AS on Plasma Prolactin Levels in Ovariectomized or OEP Rats. Plasma prolactin levels were elevated in OEP rats above those of the ovariectomized animals in all experiments (Figs. 1–3). Injection into the third ventricle of either 1 or 3 μl of NT-AS significantly increased plasma prolactin levels in ovariectomized as well as OEP rats (Figs. 1 and 2); the increase was evident within 1 hr after injection of either dose of NT-AS in ovariectomized animals, whereas in OEP rats prolactin levels had increased significantly at 1 hr after the 3-μl dose only. There was a dose-related increase of prolactin levels in ovariectomized animals at 1 hr after the antiserum injection. Thereafter both doses were nearly equally effective in ovariectomized rats, whereas in OEP rats the 3-μl dose produced significantly higher increases in prolactin levels than those produced by the 1-μl dose. The stimulatory effect of the 3-μl dose persisted for the 5-hr duration of the experiment in OEP rats, whereas the stimulatory effect of the 1-μl dose was evident only at 2–3 hr after injection (Fig. 2).

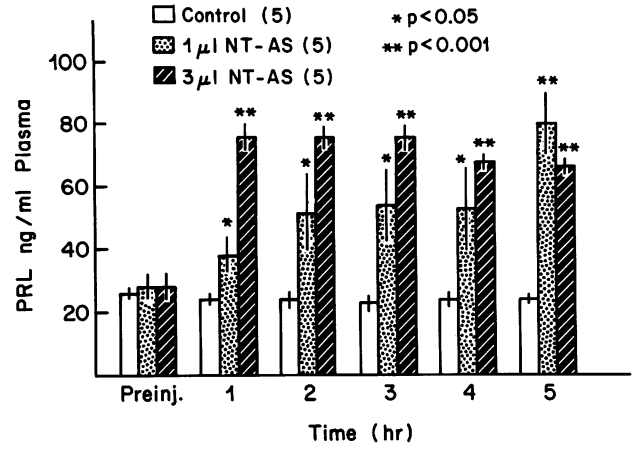

FIG. 1. Plasma prolactin (PRL) levels in ovariectomized rats after injection of NT-AS into the third ventricle. Each column represents the mean ± SEM. Numbers in parentheses represent the number of animals per group. P values refer to significance of differences from preinjection (Preinj.) levels; *, $P < 0.05$; **, $P < 0.01$.

To determine whether the effects of intraventricular NT-AS on plasma prolactin were mediated centrally, the antiserum was administered systemically by intravenous pulse injection at a 40-μl dose. This dose was chosen because of its ability to block the hypotensive effect of neurotensin (see *Materials and Methods*). Intravenous injection of NT-AS significantly suppressed plasma prolactin levels in ovariectomized as well as OEP animals, effects that were detectable when first measured at 1 hr after injection and that persisted for the 5-hr duration of the experiment (Fig. 3). The decrease in plasma prolactin induced by intravenous NT-AS con-

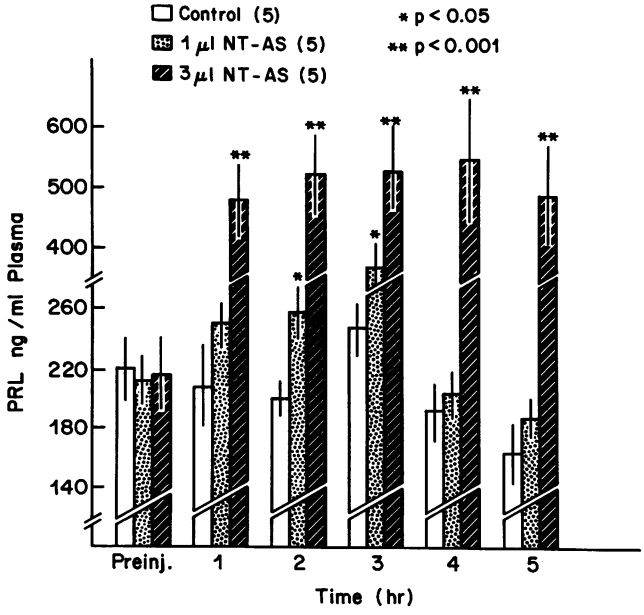

FIG. 2. Plasma prolactin levels in OEP rats after injection of NT-AS into the third ventricle. Each column represents the mean ± SEM. P values refer to significance of differences from preinjection (Preinj.) levels.

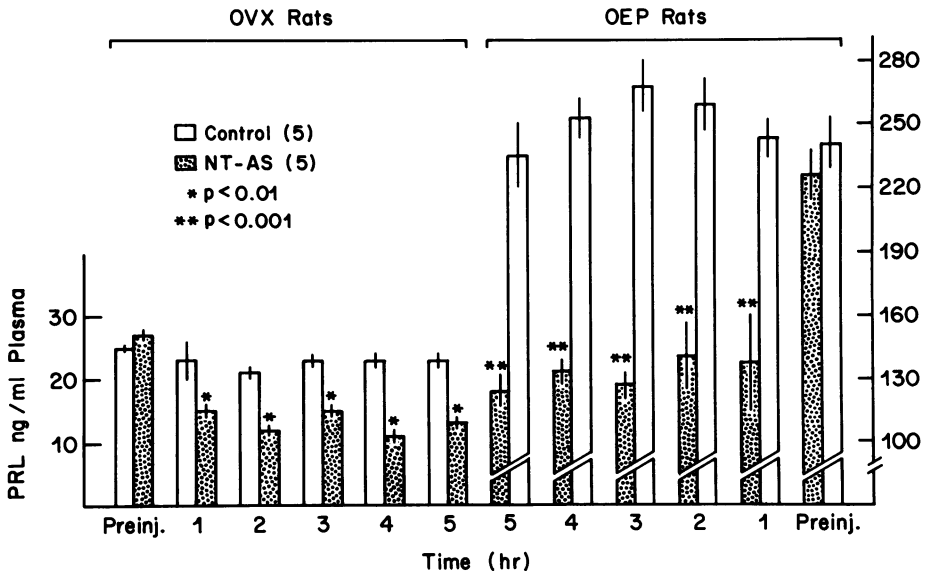

FIG. 3. Plasma prolactin levels in ovariectomized (OVX) and OEP rats after intravenous injection of NT-AS. Each column represents the mean ± SEM. *P* values refer to significance of differences from preinjection (Preinj.) levels.

trasted with the significant increase induced by the antiserum after intraventricular injection.

Effects of NT-AS in Intact Male Rats. Injection into the third ventricle of 1- or 3-μl doses of NT-AS in intact male rats produced significant increases of similar magnitude in plasma prolactin levels (Fig. 4), but the elevations evoked by NT-AS in intact males were smaller than those seen in ovariectomized or OEP animals. These increases were detectable on first measurement at 1 hr after injection and persisted for the 5-hr duration of the experiment. However, intravenous injection of NT-AS to intact male rats had no significant effect on prolactin levels (Fig. 5).

***In Vitro* Effects of NT-AS on Dispersed Pituitary Cells.** The effects of NT-AS on prolactin release from dispersed pituitary cells are shown in Table 1. Neither control normal rat serum nor NT-AS, when added to the incubation medium, had any effect on prolactin release.

DISCUSSION

Using highly specific antiserum directed against the tridecapeptide, the present study indicates that endogenous neurotensin plays a physiological role in the control of prolactin release in rats in several hormonal states. In an earlier study we demonstrated that injection of neurotensin into the third ventricle suppressed prolactin release, an action opposite to the stimulation of release seen after its intravenous injection or its incubation with pituitaries *in vitro* (8). Thus, the intraventricularly administered peptide suppressed prolactin release by stimulation of prolactin-inhibiting factor release, inhibition of prolactin-releasing factor release, or by a combination of both actions. Because blockade of dopaminergic transmission abolished the prolactin-lowering action of intraventricular neurotensin (16), it appears that neurotensin stimulates the tuberoinfundibular dopaminergic neurons to release dopamine into the hypophysial portal vessels. The released dopamine then acts directly on the lactotropes to inhibit prolactin release. On the other hand, intraventricular injections of NT-AS produced a response opposite to that of the peptide itself and significantly elevated plasma prolactin in intact male, ovariectomized, and OEP rats. The ability of the intraventricularly injected antiserum to elevate plasma prolactin suggests that endogenous neurotensin, presumably released from terminals of neurotensin-containing neurons within the hypothalamus, plays a physiologically significant role to hold prolactin release in check. This action is present in ovariectomized rats with relatively low plasma prolactin

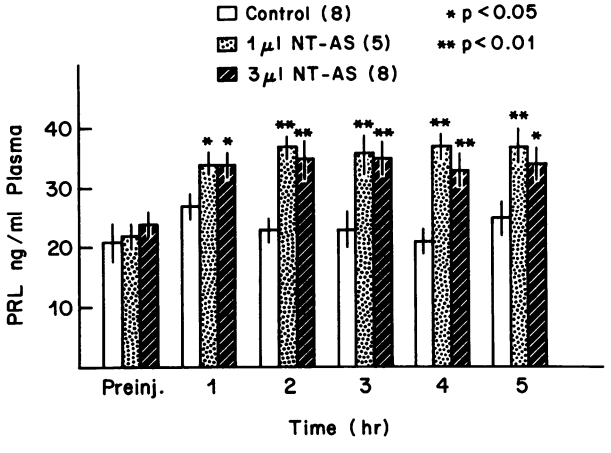

FIG. 4. Plasma prolactin levels in intact male rats after injection of NT-AS into the third ventricle. Each column represents the mean ± SEM. *P* values refer to significance of differences from preinjection (Preinj.) levels.

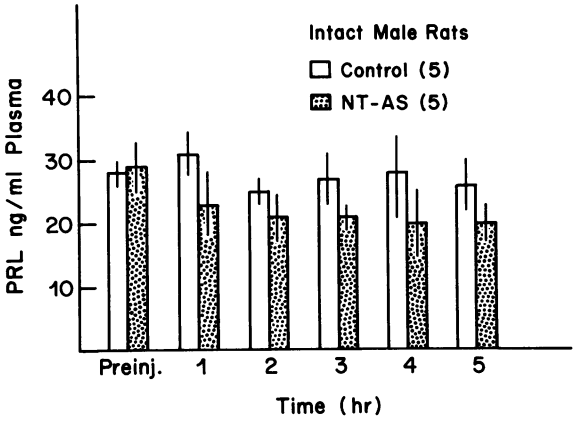

FIG. 5. Plasma prolactin levels in intact male rats after intravenous injection of NT-AS. Each column represents the mean ± SEM. *P* values refer to significance.

Table 1. Prolactin release from dispersed anterior pituitary cells of male rats

Treatment	Prolactin released, ng/ml
Control (medium only)	58.6 ± 1.3
Control serum (10 μl)	61.7 ± 1.8
NT-AS (10 μl)	61.6 ± 1.8

Concentration of pituitary cells was 5×10^5 cells per tube. Results are reported as mean ± SEM.

levels and in the estrogen- and progesterone-primed rats with very high initial prolactin levels as a result of ovarian steroid stimulation. This action was also present in males under the influence of androgens and with low plasma prolactin levels.

Intravenous injection of NT-AS lowered plasma prolactin in ovariectomized and OEP rats, an effect opposite to that of intraventricular NT-AS in these rats. In contrast, intravenous injection of neurotensin elevated plasma prolactin levels, probably by direct stimulation of the lactotrophs, because as little as 300 nM neurotensin was sufficient to increase prolactin release by anterior pituitaries incubated *in vitro* (8). The ability of intravenously injected antisera to lower plasma prolactin suggests that neurotensin is acting tonically on the pituitary to stimulate the release of prolactin from the lactotrophs. The concentration of neurotensin in systemic blood is much lower than that required to stimulate prolactin release (17). Therefore, we postulate that the peptide is released into hypophysial portal vessels so that the concentrations in pituitary sinusoidal blood are sufficiently high to stimulate the lactotropes.

Thus, it appears that neurotensin has dual roles under resting conditions—to inhibit prolactin release at a hypothalamic site and to stimulate prolactin release at a pituitary site. This duality is analogous to the actions of most other peptides that directly affect pituitary hormone release: Their action within the hypothalamus is opposite to their action on the pituitary. These dual actions are all examples of ultrashort-loop negative feedback (18). Another example of this type of activity with regard to prolactin release is oxytocin, which also stimulates at the lactotroph but inhibits within the brain (19).

When neurotensin antisera were incubated with dispersed pituitary cells *in vitro*, there was no effect on prolactin release. These results indicate that neurotensin is not released from pituitary cells *in vitro*, at least in amounts sufficient to stimulate dispersed lactotrophs. Presumably *in vivo* the peptide is released into the portal vessels and is delivered to the gland. The results with intravenous injection of NT-AS suggest that the direct action of neurotensin plays a physiological role in stimulating prolactin release in ovariectomized and OEP rats, but not in the male animal because no significant lowering of plasma prolactin occurred after injection of the antiserum in males.

The relative importance of the intrahypothalamic and pituitary effects of the peptide remains to be determined.

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