

# A Trophoblast-Specific Factor(s) Suppresses Circulating Prolactin Levels and Increases Tyrosine Hydroxylase Activity in Tuberoinfundibular Dopaminergic Neurons\*

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## ABSTRACT

Rat choriocarcinoma (Rcho) cells, which are morphologically similar to trophoblast giant cells of the normal placenta and produce placental lactogen-I *in vivo*, were used to investigate placental feedback on PRL secretion and tuberoinfundibular dopaminergic neuronal activity. Rcho cells were injected into female rats either intracerebroventricularly 60–65 h before use or under the kidney capsule 10–14 days before use. The following endocrine conditions were used: 1) ovariectomized rats with or without bromocriptine treatment, 2) immature (40–44 days old) rats, 3) adult cycling (diestrous) rats, and 4) pregnant rats. Serum PRL levels in ovariectomized, diestrous, and immature female rats were suppressed to less than 20% of control levels by secretions from the Rcho cells. Tyrosine hydroxylase (TH) activity in the stalk-median eminence (SME) was increased 2-fold above control activity in Rcho-treated ovariectomized and immature female rats. When TH activity was reduced to 40% of control levels by 50 h of bromocriptine treatment, secretions from Rcho cells increased TH activity 3.5-fold to levels similar to those for Rcho alone. Even though Rcho treatment suppressed PRL levels, TH activity in the SME of cycling (diestrous) rats was not altered after either central (65 h) or peripheral (12 days) administration of cells. TH mRNA levels in the arcuate nuclei were unaltered by Rcho cells in immature female and adult cycling rats. TH

mRNA levels in ovariectomized rats were markedly reduced 75% by 50 h of bromocriptine treatment and modestly reduced 33% 65 h after injection of Rcho cells. However, Rcho cells partially reversed the bromocriptine-induced decline in TH mRNA to levels seen for Rcho cells alone. On day 7 of pregnancy, secretions from Rcho cells abolished the nocturnal and diurnal PRL surges characteristic of early pregnancy and suppressed circulating PRL levels throughout the day to less than 20% of intersurge PRL levels. Rcho cells eliminated the semicircadian rhythm in TH activity in the SME, which was out of phase with the twice daily PRL surges of early pregnancy. TH activity was increased by Rcho factor(s) at 0330 h (nocturnal surge) and 1800 h (diurnal surge), but not at 1000 h (intersurge). MMQ cells, pituitary-derived clonal PRL-secreting cells, similarly terminated the biphasic rhythm of PRL release and tuberoinfundibular dopaminergic neuronal activity during early pregnancy. These data indicate that a factor(s) secreted by Rcho cells markedly suppresses circulating PRL levels, in part by increasing tuberoinfundibular dopaminergic neuronal activity. However, the dopaminergic neurons are probably not the sole site for Rcho action. Furthermore, these results suggest that a factor(s) secreted by trophoblast cells, possibly placental lactogen-I, contributes to the elimination of the semicircadian rhythm in TH activity and, thus, to the termination of the PRL surges at midpregnancy. (*Endocrinology* 131: 105–113, 1992)

**E**ARLY pregnancy in the rat is characterized by daily nocturnal and diurnal PRL surges (1, 2). A semicircadian rhythm in tuberoinfundibular dopaminergic neuronal activity, which is out of phase with the PRL surges, also occurs during this time (3–7). Although the reduction of the inhibitory dopaminergic tone may contribute to the twice daily PRL surges, a PRL-releasing factor(s) of posterior pituitary and/or hypothalamic origin probably provides a major positive input to the PRL surges (8–10). The twice daily PRL surges are terminated at midpregnancy, coinciding with the extinction of the biphasic pattern of tuberoinfundibular dopaminergic activity (5, 7). Dopaminergic neuronal activity remains consistently high throughout the day during middle and late pregnancy, whereas PRL levels are suppressed to very low levels throughout the day (5–7). These events are dependent on uterine-placental feedback, and are temporally correlated with the midpregnancy rise in placental lactogen

(2, 6, 11–13). Indeed, incubation medium containing placental lactogen, hypothalamic implants of ovine (o) PRL, and peripheral rat choriocarcinoma (Rcho) tumors inhibit the nocturnal PRL surge (14–16). However, the hypothalamic/pituitary sites at which these agents act to terminate the PRL surges are not completely understood.

The rat placenta produces a family of protein hormones, which are similar in sequence to PRL from the anterior pituitary (17–22). The temporal and tissue-specific expression of the known members of this placental PRL family has been identified (21–25). Placental lactogen-I is expressed exclusively by trophoblast giant cells for a brief postimplantation period, whereas placental lactogen-II is expressed by this same cell type from middle until late pregnancy (23, 24). The generation of a transplantable Rcho trophoblast tumor, which elicits actions characteristic of PRL-like hormones, *i.e.* extensive mammary development in female host or testicular atrophy in male hosts (26–28), provides a tool to examine the effects of placental PRL hormones. Rcho tumors, which are maintained under the kidney capsule, produce placental lactogen I *in vivo*, but not other known members of the placental PRL family (28). The expression of placental lactogen-I mRNA and protein is restricted to a cell type morphologically similar to the normal trophoblast giant cell (28,

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29).

The role of dopamine as a PRL-inhibiting hormone is well established (30). PRL, in turn, exerts a negative feedback on its own release, in part by altering dopamine synthesis and secretion. Hyperprolactinemia increases and hypoprolactinemia decreases dopaminergic neuronal activity (31–35). The bromocriptine-induced decrease in dopamine synthesis is reversed by coadministration of PRL (34, 35). The dopamine that subserves this neuroendocrine role is released from the tuberoinfundibular dopaminergic neurons, which have their cell bodies in the arcuate nuclei and nerve terminals in the median eminence (36). Tyrosine hydroxylase (TH) catalyzes the formation of L-dihydroxyphenylalanine (DOPA) from L-tyrosine and is the rate-limiting enzyme in the catecholaminergic biosynthetic pathway (37). The catalytic activity of this enzyme in the stalk-median eminence (SME) provides a useful index of tuberoinfundibular dopaminergic neuronal function.

Given the importance of a uterine-placental feedback to the hypothalamo-pituitary axis during pregnancy, we hypothesized that secretions from Rcho cells, which have characteristics of trophoblast giant cells of the placenta, would inhibit PRL release and stimulate tuberoinfundibular dopaminergic neurons. The objectives of this study were 1) to examine the effect(s) of Rcho cells on circulating PRL levels in female rats during several endocrine states, 2) to evaluate whether substances from Rcho cells can increase TH activity and reverse the bromocriptine-induced decrease in TH mRNA levels and enzyme activity, and 3) to determine whether Rcho cells eliminate the semicircadian rhythm of TH activity and abolish PRL surges during early pregnancy and compare the results to those elicited by MMQ cells, a pituitary derived clonal cell line that secretes PRL (38).

## Materials and Methods

### *Maintenance of Rcho, HRP-1, and MMQ cells in culture*

A Rcho cell line (Rcho-1) established by Faria and Soares (29) and a HRP-1 line described by Soares *et al.* (39) were maintained in culture as previously described by these investigators. The HRP-1 cells, which do not differentiate into giant cells or express known members of the placental PRL family, were used as control cells. Briefly, cells were cultured in RPMI-1640 culture medium (JRH Bioscience, Lenexa, KS) supplemented with 20% heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), 50  $\mu$ M  $\beta$ -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 C under a humidified atmosphere of 95% air-5% CO<sub>2</sub>. Rcho or HRP-1 cells were passaged by brief exposure (30 sec) to 0.25% trypsin-0.02% EDTA, followed by mechanically scraping cells from the culture dish. Cells were plated at a density of  $1 \times 10^6$  cells/75-cm<sup>2</sup> tissue culture flask (Fisher Scientific, St. Louis, MO). Cells were briefly trypsinized, scraped, centrifuged, and resuspended in culture medium before injection into rats.

A PRL-secreting clonal cell line (MMQ) was generously provided by Drs. R. M. MacLeod and A. M. Judd (University of Virginia, Charlottesville, VA) and was maintained in culture as described by Judd *et al.* (38). Briefly, MMQ cells were cultured in RPMI-1640 medium supplemented with 7.5% horse serum (Gibco, Grand Island, NY), 2.5% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The MMQ cells were maintained at a density of  $5 \times 10^5$  cells/ml to  $1 \times 10^6$  cells/ml. Cells were centrifuged and resuspended into fresh medium before injection into rats.

### *Experimental groups of animals*

Adult female (200–250 g) and male Sprague-Dawley (300–350 g) rats were obtained from Sasco (Omaha, NE). Immature female Lewis rats (28 days old) were obtained from Harlan (Indianapolis, IN). Rats were housed under controlled temperature (22 C) and lighting (lights on, 0600–1800 h) and supplied with food and water *ad libitum*. Rats were assigned to various experimental groups, as described below.

1) Rats were ovariectomized 2 weeks before use. Either Rcho cells (100,000 cells/15  $\mu$ l) or medium was injected intracerebroventricularly (icv) between 1700–1800 h approximately 65 h before death. Intracerebroventricular injections were made with a 22-gauge needle along the bregma, 1 mm from the midline to a depth of 4 mm. Rcho- or medium-treated rats were injected with either bromocriptine (3 mg/kg, sc) or its vehicle (30% ethanol-0.3% tartaric acid) 50 h before death and at 12-h intervals (0900–2100 h) thereafter. Rats were killed at 1100 h, 65 h after the injection of Rcho cells. Brains, anterior pituitaries, and trunk blood were collected, and TH activity in the SME, TH mRNA signal levels in the arcuate nuclei, TH protein in the arcuate-median eminence region, pituitary PRL content, and serum PRL levels were determined as described below. The presence of cells in the lateral ventricle of the brain with morphological characteristics of trophoblast giant cells was verified histologically in some animals.

2) To establish the specificity of the response, ovariectomized rats were injected icv with Rcho cells (100,000 cells/15  $\mu$ l), HRP-1 cells (100,000 cells/15  $\mu$ l), or medium. Brains, anterior pituitaries, and trunk blood were collected 65 h after cell injection for determination of TH activity in the SME, pituitary PRL content, and serum PRL levels.

3) Rcho cells ( $1 \times 10^6$  cells/50  $\mu$ l) or medium were injected under the kidney capsule of immature female rats on day 30 of age. After vaginal opening, estrous cycles were followed by daily vaginal lavage. The rats were killed 10–14 days after injections of cells between days 40–44 of age. Brains, anterior pituitaries, and trunk blood were collected, and TH activity, TH mRNA signal levels, TH protein, pituitary PRL content, serum PRL, and serum progesterone were determined. The extent of tumor formation under the kidney capsule was examined at the time of death.

4) Cycling rats that had displayed at least two cycles were injected icv with either Rcho cells (100,000 cells/15  $\mu$ l) or medium at 1800 h on proestrus. The stage of the estrous cycle was monitored by daily vaginal lavage. Rcho- and medium-treated rats were killed 65h after cell injections at 1100 h on diestrus day 2. Brains and trunk blood were collected, and TH activity, serum PRL levels, and serum progesterone levels were determined.

5) The estrous cycles of rats were monitored by daily vaginal lavage, and rats that had shown at least two cycles were used. Rcho cells ( $1.5 \times 10^6$  cells/50  $\mu$ l) or medium were injected under the kidney capsule of cycling rats on diestrus day 2. The estrous cycle was followed subsequent to cell injection, and the rats were killed at 1100 h on diestrus, 12 days after injections of cells. Brains and trunk blood were collected, and TH activity, TH mRNA signal levels, serum PRL, and serum progesterone were determined. The extent of tumor formation under the kidney capsule was verified at the time of death.

6) The estrous cycles of female rats were followed by daily vaginal lavage. Each female was placed with a single male on the day of proestrus for mating purposes. If sperm were present in the vaginal lavage, the following day (estrus) was designated day 0 of pregnancy. Rcho cells (100,000/15  $\mu$ l), MMQ cells (100,000/15  $\mu$ l), or medium were injected icv at 1530 h on day 4 of pregnancy, at 2200 h on day 4 of pregnancy, or at 0600 h on day 5 of pregnancy. Rats were killed 60 h after cell injections at 0330, 1000, or 1800 h on day 7 of pregnancy. Pregnancy was confirmed by examination of the uterus for implantation sites at the time of death. Brains and trunk blood were collected, and TH activity and serum PRL and serum progesterone levels were determined.

### *TH activity*

TH activity was determined as described previously (7, 35). The medial basal hypothalamus was preincubated under 95% O<sub>2</sub>-5% CO<sub>2</sub> for 15 min at 37 C in 300  $\mu$ l Earle's Balanced Salt Solution containing 20  $\mu$ M tyrosine. The preincubation medium was removed, and 200  $\mu$ l

medium containing 100  $\mu\text{M}$  brocresine (4-bromo-3-hydroxybenzyl-oxyamine; gift from American Cyanamid Co., Pearl River, NY), an aromatic L-amino acid decarboxylase inhibitor (40), were added. After a 30-min incubation period, the SME was dissected with fine scissors under a dissecting microscope, then homogenized in 60  $\mu\text{l}$  0.1 N perchloric acid and centrifuged at  $10,000 \times g$  for 2 min. The pellet was solubilized in 0.5 N sodium hydroxide and analyzed for protein content by the method of Bradford (41). The protein content of the SME was 15–25  $\mu\text{g}$  protein/SME.

The tissue content of DOPA was determined by HPLC (CR4A, Shimadzu, Kyoto, Japan). The mobile phase consisted of 30 mM citric acid, 50 mM dibasic sodium phosphate, 0.1 mM EDTA, 1.62 mM sodium octyl sulfate, and 15% methanol. The effluent from the column (1 ml/min) was passed through an electrochemical detector (LC-4B, Bioanalytical Systems, West Lafayette, IN) set at 0.65 V and 2 namp. The retention time for DOPA was 5.2 min. The limit of detectability was between 10–20 pg. The amount of DOPA was quantitated with a built-in integrator by comparing the peak area of the unknown sample with the peak area of a known amount of standard.

### *In situ hybridization for TH mRNA*

The method for *in situ* hybridization has been described previously (7, 35, 42). Brains were quickly removed after decapitation, frozen in liquid freon ( $-70^\circ\text{C}$ ), and stored at  $-70^\circ\text{C}$  until sectioning. Coronal sections (15  $\mu\text{m}$ ) were cut through the arcuate nucleus at  $-20^\circ\text{C}$  and thaw-mounted on poly-L-lysine-coated slides. Alternate sections were used for *in situ* hybridization to assess TH mRNA and immunocytochemistry to evaluate TH protein. Slides were stored at  $-70^\circ\text{C}$ . On the assay day, one rat from each group was processed for the *in situ* hybridization procedure.

After prehybridization steps, described previously (35), the fixed brain sections were hybridized for 4 h at  $45^\circ\text{C}$  with 0.05  $\mu\text{g}/\text{ml}$   $^{35}\text{S}$ -labeled cRNA probe for TH. The probe was a 1.1-kilobase *Bam*HI/*Eco*RI insert subcloned into a pSP65 vector (Promega Biotech, Madison, WI). This probe has previously been shown to hybridize to a single mRNA band of approximately 1.9 kilobases on Northern blots (42). A single stranded  $^{35}\text{S}$ -labeled antisense RNA probe complementary to TH mRNA was synthesized using a SP6 RNA polymerase. The probe had a specific activity of about  $2 \times 10^9$  dpm/ $\mu\text{g}$ . After RNAase treatment and a series of posthybridization washes that increased in stringency, the slides were dipped in Ilford emulsion (K-5, Polysciences Inc, Warrington, PA) and diluted with 0.25 g/ml water. The autoradiographs were exposed at  $4^\circ\text{C}$  for 21 days. After development using standard photographic techniques, the sections were poststained lightly with hematoxylin.

The anatomical locations of the tissue sections were determined using the rat brain atlas of Paxinos and Watson (43). Twenty alternate sections per animal through the arcuate nuclei were used for quantitation of mRNA signal levels. Approximately 12 cells/tissue section (*i.e.* 240 cells/rat) were analyzed. TH mRNA-containing cells were identified under darkfield optics as a cluster of reduced silver grains with an identifiable cell nucleus. The number of grains in individual TH mRNA-containing cells were measured under  $\times 400$  darkfield illumination by a computerized image-processing system (Analytical Imaging Concepts, Irvine, CA).

### *Immunocytochemistry for TH*

The method for TH immunocytochemistry has been described previously (7, 35, 42). The sections from the various experimental groups were processed simultaneously. Briefly, after fixing the tissue, the endogenous peroxidase activity was quenched using 0.6% hydrogen peroxide in methanol. Then, the sections were incubated for 20 h at room temperature with a rabbit antibody to rat TH (East Acres Biologicals, Southbridge, MA), diluted 1:2600. Brain sections were incubated successively with biotinylated goat antirabbit immunoglobulin for 30 min and with avidin DH-biotinylated horseradish peroxidase-H complex (Vector Laboratories, Burlingame, CA) for 60 min. The slides were reacted for 5 min with 3,3'-diaminobenzidine (0.6 mg/ml) and 0.03% hydrogen peroxide for the peroxidase reaction.

### *Hormone determinations*

Anterior pituitaries were homogenized in 200  $\mu\text{l}$  50 mM Tris buffer (pH 8.8) and diluted with 1% BSA in 10 mM PBS. Serum and pituitary PRL levels were determined by the rat PRL RIA provided by the NIDDK. PRL RP-1 was used as a reference preparation, and the limit of sensitivity for the assay was 50 pg. The intra- and interassay coefficients of variation were 12.1% and 9.1%, respectively. Serum progesterone levels were measured by RIA, using no. 337 antiprogestosterone-11-BSA serum, described by Gibori *et al.* (44). The limit of sensitivity for the assay was 10 pg, and the intra- and interassay coefficients of variation were 14.1% and 7.4%, respectively.

### *Statistical analysis*

Results are expressed as the mean  $\pm$  SE. The n for all groups refers to the number of experimental animals. For cellular TH mRNA signal level determinations, the mean number of grains per cell in a given anatomical area was first calculated for individual animals. The individual means were used to calculate the mean  $\pm$  SE of each group. Data were evaluated by *t* test when two groups were being compared (45). Data were evaluated by analysis of variance when three or more groups were being compared, and multiple comparisons were made with Fisher's least significant procedures (45, 46). Immunocytochemistry for TH was evaluated qualitatively.

## Results

### *Rcho cells reversed the bromocriptine-induced decrease in TH*

TH catalytic activity in the SME (Fig. 1, *left panel*) was decreased by 40% in ovariectomized rats after 50 h of bromocriptine treatment. Sixty-five hours after Rcho cells were injected into the lateral ventricle of adult ovariectomized rats, secretions from Rcho cells resulted in a 2-fold increase ( $P < 0.05$ ) in TH activity in the SME. When bromocriptine treatment was initiated 15 h after icv injection of Rcho cells, secretions from the Rcho cells prevented the effect of bromocriptine and resulted in elevated TH activity similar to that observed with Rcho cells alone.

TH mRNA signal levels (Fig. 1, *middle panel*, and Fig. 2, *top panel*) in the arcuate nuclei were decreased ( $P < 0.05$ ) by 75% in ovariectomized rats after 50 h of bromocriptine treatment. Secretions from Rcho cells resulted in a significant ( $P < 0.05$ ) 33% reduction in TH mRNA signal levels within 65 h after injection of cells. However, when the injection of Rcho cells was combined with the administration of bromocriptine, secretions from the Rcho cells maintained TH mRNA signal levels similar to those observed with Rcho cells alone and at significantly ( $P < 0.05$ ) higher levels than bromocriptine treatment alone. The numbers of detectable TH mRNA-containing cells per tissue section ( $\sim 19/\text{section}$ ) were similar among groups. TH immunostaining in the median eminence (Fig. 2, *lower panel*) appeared to be modestly reduced after 50 h of bromocriptine treatment. Although the Rcho cells alone had no obvious effect on immunoreactive TH in the arcuate nuclei or median eminence, secretions from the Rcho cells appeared to reverse the bromocriptine-induced decrease in TH immunostaining.

Serum PRL levels (Fig. 1, *right panel*) were reduced to less than 10% of control levels after 50 h of bromocriptine treatment. Central administration of Rcho cells also resulted in a marked decrease in serum PRL levels to less than 10%

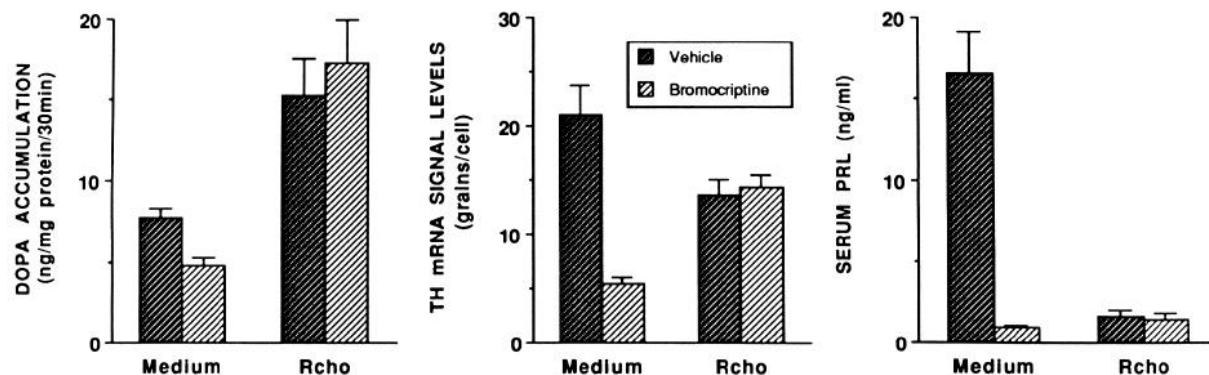
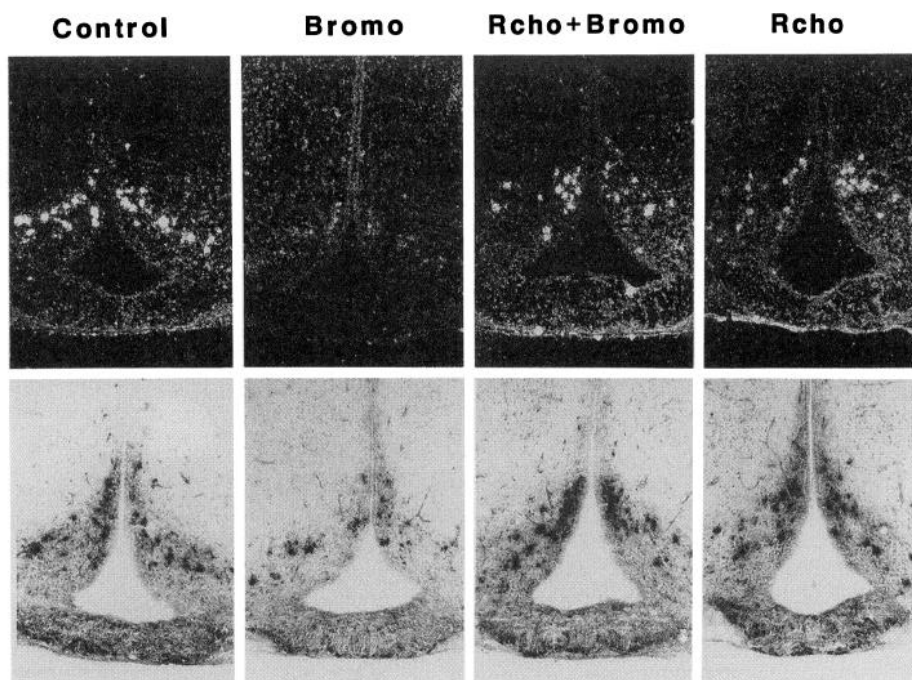


FIG. 1. *In vitro* DOPA accumulation in the SME (left panel), TH mRNA signal levels in the arcuate nuclei (middle panel), and serum PRL levels (right panel) in Rcho-treated and/or bromocriptine-treated ovariectomized rats. Intracerebroventricular injection of 100,000 Rcho cells/rat for 65 h increased ( $P < 0.05$ ) the rate of DOPA accumulation, modestly decreased ( $P < 0.05$ ) TH mRNA levels, augmented ( $P < 0.05$ ) the rate of DOPA accumulation reduced by bromocriptine treatment, and partially reversed the bromocriptine-induced decline in TH mRNA levels. Circulating PRL levels were suppressed by Rcho and/or bromocriptine treatment. Each value is the mean  $\pm$  SE of determinations from 8–10 rats for DOPA accumulation and PRL levels and 6 rats for TH mRNA signal levels.

FIG. 2. TH mRNA-containing cells (top panel) and TH-immunoreactive cells (bottom panel) in the arcuate nuclei of the hypothalamus in Rcho-treated (Rcho) and/or bromocriptine-treated (Bromo) ovariectomized rats. Darkfield (top panel) and lightfield (bottom panel) photomicrographs show coronal sections through the arcuate nuclei at the levels of the mid-median eminence. The brightness of TH mRNA-containing cells and the intensity of immunostaining in the median eminence were reduced by 50 h of bromocriptine treatment. Intracerebroventricular injection of Rcho cells reversed the bromocriptine-induced decline in TH mRNA signal levels and immunostaining.



of control levels. When bromocriptine treatment was initiated subsequent to the injection of Rcho cells, serum PRL levels were similar to levels observed after Rcho cells alone. The PRL content (micrograms of PRL per mg protein) of the anterior pituitary was significantly ( $P < 0.05$ ) suppressed from control levels ( $71.0 \pm 6.8$ ) after either bromocriptine treatment ( $47.6 \pm 3.5$ ) or injection of Rcho cells ( $54.6 \pm 7.7$ ) alone and after the combined treatment with bromocriptine and Rcho cells ( $46.6 \pm 2.9$ ).

#### Effects on TH and PRL were specific to Rcho cells

To determine if the effects observed after the injection of Rcho cells were specific to secretions from these cells, we used the HRP-1 cell line, a rat placenta-derived cell line that does not express any known members of the placental PRL

family (39). Within 65 h after icv injection of Rcho cells into ovariectomized rats, TH activity in the SME (Fig. 3, left panel) was increased ( $P < 0.05$ ) 2-fold above the control value, and serum PRL levels (Fig. 3, right panel) were reduced ( $P < 0.05$ ) to less than 20% of control levels. In contrast, icv injection of HRP-1 cells for 65 h did not alter either TH activity in the SME or serum PRL levels. The PRL content (micrograms of PRL per mg protein) of the anterior pituitary was also significantly ( $P < 0.05$ ) reduced from control levels ( $83.9 \pm 7.8$ ) after the injection of Rcho cells ( $59.4 \pm 9.3$ ), but not after the injection of HRP-1 cells ( $88.6 \pm 7.5$ ).

#### Rcho cells increased TH in immature female rats

Rcho cells were injected under the kidney capsule of 30-day-old rats, and the stage of the estrous cycle was followed

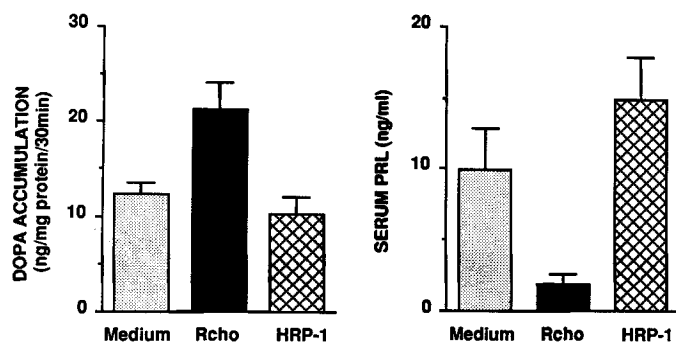


FIG. 3. *In vitro* DOPA accumulation in the SME (left panel) and serum PRL levels (right panel) 65 h after icv injection of 100,000 Rcho or HRP-1 cells into ovariectomized rats. The rate of DOPA accumulation was increased ( $P < 0.05$ ), and circulating PRL levels were decreased ( $P < 0.05$ ) in Rcho-treated rats, but were unaltered in HRP-1-treated rats. Each value is the mean  $\pm$  SE of determinations from eight rats.

by daily vaginal lavage after the day of vaginal opening. The day of vaginal opening for those rats treated with Rcho cells tended to be earlier than that for controls from the same group. Seventy percent of the Rcho-treated rats displayed vaginal opening on day 33 of age, whereas only 25% of the controls experienced vaginal opening. After an initial estrous day, the Rcho-treated rats remained in constant diestrus, whereas the age-matched controls showed somewhat erratic estrous cycles. Medium-treated controls were killed on the first diestrus day 2 after day 40. A number of Rcho-treated rats equivalent to the number of control rats were killed between days 40 and 44 after at least 8 days of constant diestrus. Visual inspection of the mammary tissue revealed extensive growth and differentiation of this gland in the Rcho-treated rats compared to those in controls. TH activity in the SME (Fig. 4, upper left panel) of Rcho-treated rats was increased ( $P < 0.05$ ) 2-fold above control levels, whereas TH mRNA signal levels in the arcuate nuclei (Fig. 4, upper right panel) were not significantly altered by Rcho treatment. Serum PRL levels (Fig. 4, lower left panel) after Rcho treatment were reduced to less than 10% of control levels. The PRL content (micrograms of PRL per mg protein) of the anterior pituitary was significantly ( $P < 0.05$ ) reduced from  $135.7 \pm 13.8$  in medium-treated controls to  $87.9 \pm 6.6$  after injection of Rcho cells. Serum progesterone levels (Fig. 4, lower right panel) in the Rcho-treated rats were increased ( $P < 0.05$ ) 3-fold above control levels.

#### Rcho cells did not alter TH in adult cycling rats

Rcho cells were injected either icv 65 h before death or under the kidney capsule 12 days before death. When Rcho cells were injected under the kidney capsule, rats with marked tumor formation completed one cycle and subsequently displayed 10 days of constant diestrus. Medium-treated control rats continued to cycle normally. Extensive growth of the mammary tissue in the Rcho-treated group was noted by visual inspection. When Rcho cells or medium were injected icv on proestrus, all rats continued to cycle and were killed on the next diestrus day 2. TH activity in the SME (Table 1) was not altered by secretions from the Rcho cells after either central or peripheral administration. Like-

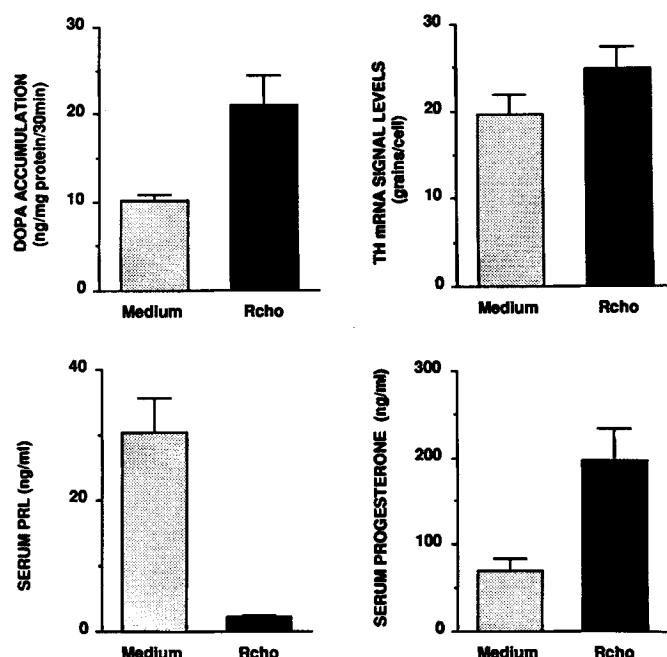


FIG. 4. *In vitro* DOPA accumulation in the SME (upper left panel), TH mRNA signal levels in the arcuate nuclei (upper right panel), serum PRL levels (lower left panel), and serum progesterone levels (lower right panel) in immature female rats. Medium or  $1 \times 10^6$  Rcho cells were injected under the kidney capsule on day 30 of age. The rate of DOPA accumulation and serum PRL and progesterone levels were increased ( $P < 0.05$ ) 10–14 days after Rcho injection, whereas TH mRNA signal levels were unaltered. Each value is the mean  $\pm$  SE of determinations from 6–10 rats.

wise, TH mRNA signal levels in the arcuate nuclei were not altered after peripheral injection of Rcho cells. However, both central and peripheral administration of Rcho cells reduced circulating PRL levels to less than 20% of control levels. Serum progesterone levels were decreased to half of control levels after central administration of Rcho cells, but were increased 5-fold above control levels after peripheral administration of Rcho cells.

#### Rcho and MMQ cells altered TH and PRL during pregnancy

Rcho cells, a placenta-derived cell line that expresses placental lactogen-I *in vivo* (28), MMQ cells, a pituitary-derived cell line that secretes PRL (38), or medium were injected icv to pregnant rats 60 h before death. A semicircadian rhythm in TH activity in the SME (Fig. 5, left panel) was observed in the medium-treated controls, with lower ( $P < 0.05$ ) activity during the nocturnal PRL surge (0330 h) and the diurnal PRL surge (1800 h) than during an intersurge period (1000 h). The semicircadian rhythm in TH activity was abolished 60 h after icv injection of either Rcho or MMQ cells. TH activity in the SME of Rcho-treated and MMQ-treated rats was significantly ( $P < 0.05$ ) elevated above levels in medium-treated controls at 0330 and 1800 h, but was not significantly altered at 1000 h. Serum PRL levels in control rats (Fig. 5, middle panel) were higher ( $P < 0.05$ ) at 0330 h (nocturnal surge) and 1800 h (diurnal surge) than at 1000 h. Secretions from Rcho cells within 60 h after cell injection abolished both the nocturnal and diurnal PRL surges and reduced

TABLE 1. TH catalytic activity in the SME, TH mRNA signal levels in the arcuate nuclei, and circulating hormone levels

	DOPA accumulation (ng/mg protein · 30 min)	TH mRNA levels (grains/cell)	Serum PRL (ng/ml)	Serum progesterone (ng/ml)
icv injection				
Medium	17.34 ± 2.06		11.94 ± 2.65	25.10 ± 4.50
Rcho	22.09 ± 4.18		1.35 ± 0.10 <sup>a</sup>	11.21 ± 4.09 <sup>a</sup>
Kidney capsule				
Medium	17.23 ± 1.15	38.81 ± 5.10	12.02 ± 2.18	15.04 ± 3.26
Rcho	16.77 ± 1.73	33.93 ± 3.37	2.31 ± 0.26 <sup>a</sup>	80.53 ± 10.58 <sup>a</sup>

Cycling (diestrous) rats were injected either icv with Rcho cells or medium 65 h before death or with Rcho cells or medium under the kidney capsule approximately 12 days before death. Each value is the mean ± SE of determinations from 6–12 rats.

<sup>a</sup> Significantly different ( $P < 0.05$ ) from medium-treated controls.

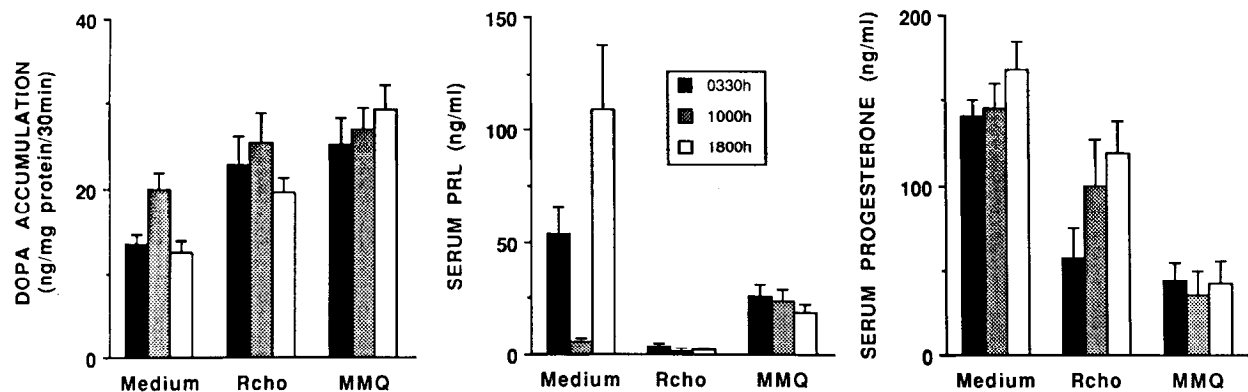


FIG. 5. *In vitro* DOPA accumulation (left panel) in the SME, serum PRL levels (middle panel), and serum progesterone levels (right panel) at 0330, 1000, or 1800 h on day 7 of pregnancy 60 h after icv injection of medium, 100,000 Rcho cells, or 100,000 MMQ cells. The rate of DOPA accumulation in the SME of Rcho- and MMQ-treated rats was increased ( $P < 0.05$ ) above that in medium-treated control at 0330 and 1800 h, but was unaltered at 1000 h. The nocturnal (0330 h) and diurnal (1800 h) PRL surges were abolished after both Rcho and MMQ treatments. Circulating PRL levels after Rcho treatment were suppressed ( $P < 0.05$ ) at all times below control intersurge values (1000 h), and PRL levels after MMQ treatment were moderately elevated ( $P < 0.05$ ) above control intersurge levels. Serum progesterone levels were suppressed ( $P < 0.05$ ) after both Rcho and MMQ treatments. Each value is the mean ± SE of determinations from 9–11 rats.

intersurge PRL levels to less than 20% of the control level. Secretions from MMQ cells also prevented the rhythm in PRL release. However, circulating PRL levels throughout day 7 were elevated 4- to 5-fold above the intersurge levels of medium-treated controls, probably as a result of PRL released by the MMQ cells reaching the peripheral circulation. Serum progesterone levels (Fig. 5, right panel) were similar throughout day 7 of pregnancy in control rats. Intracerebroventricular injection of Rcho cells modestly reduced serum progesterone levels within 60 h, and icv injection of MMQ cells reduced progesterone levels to 25% of control levels. As assessed by visual examination, pregnancy was maintained in 29 of 30 rats after icv injection of Rcho cells, whereas approximately 50% of MMQ-treated rats displayed signs of fetal reabsorption.

### Discussion

We are reporting that a factor(s) secreted from Rcho cells markedly suppressed circulating PRL levels under a number of endocrine conditions. The degree of suppression was similar to that induced by bromocriptine, a dopamine agonist. The reduction in anterior pituitary PRL content concomitant with decreased PRL release suggests that other aspects of lactotroph function may also be altered, e.g. decreased syn-

thesis and/or increased degradation of PRL. The reduction in circulating PRL levels occurred when Rcho cells were injected peripherally under the kidney capsule and allowed to grow for 10–14 days until a tumor approximately 50% the size of the kidney itself was formed. Likewise, circulating PRL was suppressed within a relatively short time (60–65 h) when Rcho cells were injected centrally into the lateral ventricles of the brain. This mode of administration allows for a factor(s) secreted from Rcho cells to reach high levels within close proximity of the hypothalamic neurons controlling PRL release. This method has been used successfully with anterior pituitary fragments to increase PRL levels in the cerebrospinal fluid (47). However, we cannot conclude that the effect on circulating PRL was exclusively a result of alterations in the hypothalamic input to the lactotrophs. When MMQ cells were injected into the lateral ventricular system of pregnant rats, circulating PRL levels were elevated above basal levels. Thus, it is possible that a factor(s) released from Rcho cells can reach the anterior pituitary to act directly on lactotrophs. Indeed, placental extracts have previously been shown to suppress PRL release from hemipituitaries or dispersed cells (48, 49). The Rcho-induced reduction in circulating PRL levels appeared to be relatively specific, since another placental cell line (HRP-1) had no effect. The Rcho cells have a trophoblast cell lineage capable of differentiating

into giant cells and subsequently producing members of the placental PRL family (28, 29), whereas HRP-1 cells apparently lack this differentiation capacity (39). Therefore, it is reasonable to hypothesize that the factor(s) responsible for this marked suppression of PRL release is produced by trophoblast cells committed to a giant cell pathway.

Factor(s) released from the Rcho cells increased the activity of the tuberoinfundibular dopaminergic neurons under most, but not all, endocrine conditions. Thus, it is likely that an increase in dopaminergic input may at times play a role in suppressing PRL release. TH catalytic activity in the SME was increased after Rcho treatment in pregnant rats during the nocturnal and diurnal PRL surges and in ovariectomized adult rats and immature female rats. Alterations in the expression of the TH gene apparently do not contribute to the augmented TH activity, since there was no increase in TH mRNA levels in the arcuate nuclei. Indeed, TH mRNA signal levels were modestly reduced by Rcho cells in ovariectomized rats, but were unaltered in immature female rats. Although it is not clear if the increase in enzyme activity and the decrease in mRNA levels are mediated by the same or different factors, it is notable that TH mRNA levels in the arcuate nuclei decrease during late pregnancy (7) when circulating levels of placental lactogen-II are very high (12, 17, 50). TH activity was not altered in pregnant rats during an intersurge time or in adult diestrous rats. An effect on TH activity was lacking in diestrous rats whether the Rcho cells were administered centrally for a short (65-h) period or peripherally for a long (12-day) period. The marked suppression of PRL release without a concomitant increase in tuberoinfundibular dopaminergic neuronal activity would suggest that Rcho cells have additional hypothalamic and/or pituitary sites of action. Moreover, the differential responsiveness of ovariectomized and diestrous rats would suggest that an ovarian input prevents the action of the Rcho factor(s). Progesterone may not be the ovarian substance responsible, as the dopaminergic neurons were unresponsive whether progesterone levels were decreased after icv injection of Rcho cells or increased after kidney capsule injection of Rcho cells. Furthermore, elevated progesterone levels did not prevent the increase in TH activity in immature female rats.

The ability of Rcho cells to reverse the bromocriptine-induced decrease in TH activity and TH mRNA levels suggests that the factor(s) secreted by the Rcho cells has lactogenic qualities. This action of the factor(s) on the tuberoinfundibular dopaminergic neurons resembles the action of PRL on TH activity and TH mRNA levels in these neurons (34, 35). Demarest *et al.* (6) previously reported that a uterine-placental factor, not suppressed by bromocriptine, acts to stimulate TH activity and, thus, abolish the PRL surges. The factor(s) released from the Rcho cells resembles the uterine-placental feedback of pregnancy, in that it is also able to increase TH activity in spite of bromocriptine administration. When a Rcho tumor is allowed to develop under the kidney capsule, giant cells, which are morphologically similar to trophoblast giant cells, express placental lactogen-I, but not other known members of the placental PRL family (28).

Thus, it is likely that placental lactogen-I may be the lactogenic species responsible for the effects of TH neurons. When the Rcho cells were injected under the kidney capsule, we observed peripheral effects that are associated with high circulating levels of PRL-like hormones (51). As has been reported previously by other investigators (26–28), extensive development of the mammary tissue was noted. Circulating progesterone levels were elevated in both immature and adult female rats, indicative of the luteotropic action of lactogenic hormones.

The placenta is a transient endocrine organ that appears only in the female rat for the defined period of pregnancy. Relatively little is known about placental feedback on the hypothalamo-pituitary axis during this time. Rcho cells provide a unique tool to examine the feedback of secretions from a specialized placental cell type, since Rcho cells appear to develop along a trophoblast cell lineage (28, 29). *In vivo* Rcho tumors contain both giant cells, which are morphologically and hormonally similar to the trophoblast giant cells, and small cells, which do not express known placental PRLs (28). During normal placental development, the trophoblast giant cells appear shortly after the initiation of implantation and remain until the end of pregnancy (52). The trophoblast giant cells begin expressing mRNA for placental lactogen-I by day 6, and placental lactogen-I reaches high circulating levels by days 9–10 of pregnancy (12, 13, 17, 23). In the present study Rcho cells were injected into lateral ventricles of the brain on day 4 or 5 of pregnancy, and the rats were killed on day 7 before the midpregnancy rise in circulating levels of placental lactogen-I (13, 17). Using this paradigm, pregnancy was maintained throughout the experiment, although circulating progesterone levels were somewhat attenuated. A semicircadian rhythm in TH activity in the SME was observed in the medium-treated controls, as has been previously reported for early pregnancy (5–7). TH activity was lower during the nocturnal (0330 h) and diurnal (1800 h) PRL surges than during the intersurge period (1000 h). A factor(s) secreted from Rcho cells increased TH activity during the times when the nocturnal and diurnal PRL surges would normally occur, but did not alter TH activity during the intersurge time. Both PRL surges were abolished by secretions from the Rcho cells, and circulating PRL levels were suppressed throughout the day to values much lower than those observed during the intersurge times of early pregnancy. This response is similar to the events that occur during midpregnancy (5–7), coincident with the rise in placental lactogen-I (12, 13, 17, 50). PRL surges as well as the biphasic rhythm in TH activity are abolished by day 11, and TH activity remains throughout the day at levels similar to the intersurge levels on day 7 (5, 7). The data in the present study indicate that a factor(s) released by trophoblast cells may contribute to the extinction of the PRL surges by increasing tuberoinfundibular dopaminergic neuronal activity. Given its PRL mimetic action, placental lactogen-I, which is produced by Rcho tumors *in vivo* (28), is a likely candidate.

Given that oPRL can also abolish the nocturnal PRL surge, the ability of MMQ cells, a pituitary-derived clonal line (38), to alter TH activity and PRL secretion was examined. Secre-

tions from MMQ cells abolished the rhythm in PRL secretion. In contrast to the extremely low PRL levels after Rcho treatment, circulating PRL levels were moderately elevated above basal levels after MMQ treatment. Since hypothalamic administration of oPRL markedly reduces circulating PRL levels (15), it is likely that some PRL released from the MMQ cells reaches the peripheral circulation. These levels of PRL were not capable of maintaining pregnancy in 50% of the rats, as corroborated by the low levels of circulating progesterone. Similar to the Rcho cells, MMQ cells abolished the semicircadian rhythm in tuberoinfundibular dopaminergic activity. TH activity in the SME was elevated by secretions from the MMQ cells during the times of the nocturnal and diurnal PRL surges, but not during an intersurge period. The present data are in contrast to studies by Demarest *et al.* (53), in which after a single injection of PRL, the rhythm in TH activity was still evident within 12 h, although all levels throughout the day were increased. The mode and duration of PRL administration are the most probable explanation for the difference between those observations and the present data. Indeed, 12 h after PRL infusion, the nocturnal and diurnal PRL surges, although attenuated, were not abolished (53), whereas the PRL surges were abolished within 60 h after injection of MMQ cells.

In summary, a placental factor(s), which is probably of trophoblast cell origin, has a profound inhibitory effect on circulating PRL levels in female rats. An increase in tuberoinfundibular dopaminergic neuronal activity may have at times contributed to the Rcho-induced decrease in PRL levels. However, since PRL was reduced to very low levels in diestrous rats, in spite of no change in dopaminergic activity, nondopaminergic inputs to the regulation of PRL secretion are probably altered as well. The factor(s), possibly placental lactogen-I, responsible for the increase in TH activity mimicked PRL's effect on the tuberoinfundibular dopaminergic neurons. When placental feedback has a physiologically important function, a factor(s) from the Rcho cells eliminated the semicircadian rhythm in TH activity and abolished the diurnal and nocturnal PRL surges of early pregnancy. These events are similar to the transition from early to midpregnancy when trophoblast giant cells normally appear and begin producing placental lactogen-I.

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