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[RAPID COMMUNICATION]

Neurotrophin-3 Augments Steroid Secretion by Hamster Ovarian Follicles *In Vitro*

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ABSTRACT—Innervation of the ovary is known to be affected by neurotrophic factors; mRNA for some of these molecules and their low-affinity receptors have been identified in ovarian follicular tissue. We attempted to determine whether neurotrophin (NT-3) exerts a non-neuronal impact on the ovary, affecting follicular estradiol secretion via a possible humoral action. Ovaries were excised from mature Golden Syrian Hamsters (*Mesocricetus auratus*) and large preovulatory follicles were removed by microdissection. Follicles were incubated for 72 hr in control medium, or medium containing 10 ng, 100 ng, or 1000 ng/ml NT-3. After culture, medium samples were assayed for estradiol content. NT-3 exerted dichotomous effects, depending on concentration: NT-3 augmented mean estradiol output two-fold at 100 ng/ml, while 1000 ng/ml NT-3 decreased estradiol secretion back to control levels. By 48–72 hr in culture, all groups exhibited a significant decline in mean estradiol secretion due presumably to diminished viability, independent of neurotrophin action. These data suggest a humoral role for NT-3 in estradiol secretion of preovulatory follicles in culture.

INTRODUCTION

The nerve growth factor (NGF) family of neurotrophic factors consists of compounds previously implicated in the development and maintenance of a variety of neural fiber types, in both the central and peripheral nervous systems [8, 19, 20, 24, 29]. The mammalian ovary is a target for sympathetic and sensory neurons of the peripheral nervous system, and mRNA for NGF and neurotrophin (NT-3), two members of this neurotrophin family, is known to be transcribed there [9, 21]. It has recently been demonstrated, however, that mRNA encoding receptors of these neurotrophic factors has also been identified in the ovary [6, 15, 17]; the low-affinity neurotrophin receptor (which appears to bind all members of the nerve growth factor family [3, 8, 26, 28, 31]) has been localized to the thecal cells of developing follicles [6]. The presence of neurotrophin mRNA in the ovary and in most other tissues analyzed is expected due to these molecules being synthesized in target tissues; they are subsequently taken up by synaptic terminals and transported retrogradely to the neural cell body [12, 20, 23]. While it is known that neurotrophic factors support sympathetic innervation to the ovary [19] and are therefore integral in acquiring and maintaining reproductive function [2, 18, 19], the presence of both neurotrophin and receptor mRNA in the follicle leads to the hypothesis that these growth-promoting factors exert a non-neuronal, autocrine/paracrine impact on the ovary and its hormonal function [6, 9].

To date, there has been no comprehensive research on the non-neuronal effects of such compounds. The aim of our study was to evaluate the effects of neurotrophins in non-neuronal tissue, utilizing series of experiments designed to determine what effects neurotrophin has on follicular steroidogenesis. Healthy, developing follicles are characterized as secreting large quantities of estradiol [5]; by monitoring changes in estradiol output from healthy preovulatory follicles *in vitro*, we have attempted to assess one of many possible effects elicited by these growth factors.

MATERIALS AND METHODS

Neurotrophin preparation

The NT-3 was generously supplied by Regeneron Pharmaceuticals (Tarrytown, NY) and kept frozen at −80°C until used. When needed, small amounts of NT-3 were diluted to appropriate concentrations (10 ng, 100 ng and 1000 ng per 50 μl aliquot) under sterile conditions (and laminar flow), using 0.1% protease-free bovine serum albumin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in phosphate-buffered saline (vehicle). The diluted NT-3 was aliquoted into gas-sterilized microcentrifuge tubes under sterile conditions and stored at 4°C for a maximum of two weeks.

Animals

The present experimental protocols were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and

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Use Committee. Thirty mature (3–8 months of age, weighing ≥185 g) female golden Syrian hamsters (*Mesocricetus auratus*; Sasco, Omaha, NE) were used in the study. Animals were regarded as mature when they exhibited a regular, 4-day estrous cycle for at least one month; days of the reproductive cycle were determined in reference to a vaginal plug identifiable on estrus. On the morning of proestrus, the animals were euthanized by decapitation under ether anesthesia. Trunk blood was collected and centrifuged (500×g) to obtain serum. Ovaries were removed and prepared for dissection; the 5–6 large preovulatory follicles (≥500 μm) were excised from each ovary under a dissecting microscope, using a 23-gauge needle and microforceps.

Culture

Using a random-number generating table, five follicles were allocated randomly to one of four organ culture dishes (VWR, Chicago, IL) and cultured with medium comprised of the following: Dulbecco's modified Eagle's medium (DMEM) mixed one-to-one with Ham's F-12 (Gibco, Grand Island, NY), and supplemented with heparin (1 U/ml), and gentamycin (50 μg/ml). Dishes were allocated sequentially to control and incremental treatment groups. Previous experiments have shown that follicle viability is maintained through culture supplementation with heat-inactivated, filtered homologous serum [14]; we performed preliminary experiments comparing estradiol output by a control system versus culture systems supplemented with an aliquot of unmodified serum (bovine FCS, Sigma, St. Louis, MO) or serum that had been previously heat-inactivated and subsequently charcoal stripped to remove steroids. Results indicated that charcoal treatment removed additional substances (presumably lipid) necessary for maintenance of follicle viability. We therefore included in all subsequent experiments a serum supplement that had been heat-inactivated and filtered, but not charcoal stripped. In our experimental protocol, then, control groups were incubated with 950 μl culture medium supplemented with 50 μl heat-inactivated and filtered homologous serum (0.22 mm Millipore filter, Fisher Scientific, Pittsburgh, PA). Treatment dishes were comprised of 900 μl culture medium, 50 μl serum and 50 μl of a fixed quanitity of neurotrophin-3 in vehicle, resulting in final concentrations of 10 ng, 100 ng and 1000 ng NT-3 per ml of culture medium (molarity $=7.4\times10^{-10}$, $\times10^{-9}$ and $\times10^{-8}$, respectively). Vehicle and control medium were identical in their lack of effect on steroidogenesis. Dishes were incubated at 37°C, 5% CO_2 and 98% relative humidity, and maintained for 72 hr with all culture medium removed and frozen (−20°C) at the following time intervals: 3, 6, 9, 12, 24, 48 and 72 hr. The culture medium and any supplements were replaced in identical volumes and concentrations under sterile conditions. All cultures were terminated at 72 hr.

Radioimmunoassay

Aliquots of frozen culture medium were assayed for estradiol (E_2) concentration using prepared kits (Diagnostic Products Corporation, Los Angeles, CA) and a Packard Cobra Series Auto-Gamma counter (Meriden, CT). Kitzman and Hutz [14] validated these assay kits for hamster by demonstrating parallelism between dilutions of serum aliquots (1:0, 1:1, 1:4, 1:9, 1:49, 1:99) and authentic standards. Each assay performed included samples of pooled homologous hamster serum to monitor interassay variation. A total of seven assays were performed, encompassing ten experiments. The intraassay coefficient of variation was determined to be <8%; assay sensitivity was 20 pg E_2 per ml. Crossreactivity with estrone was 10%; with estriol 0.32%; and with other native serum estrogens, androgens, and progestins, less than 0.017%.

Statistical analyses

In order to account for interassay variation (which ranged up to 30%), all data were normalized prior to further analysis as follows: adjustments were made by multiplying data by the percent difference between serum pool values from the individual E_2 assays and the grand mean. Additionally, all experimental groups were included in each assay; with the percent change from control induced by experimental treatment being identical within each assay. Of the 280 possible samples, 12 were missing due to contamination of the cultures during the experiment; prior to computer analysis, these values were interpolated by determining the mean of all other samples at that particular dosage and time period. The calculated mean was substituted as the missing value. Following normalization and interpolation of missing values, all data were analyzed using the SPSS/PC+ statistics package (Chicago, IL). Initial analysis regarding mean estradiol output consisted of a multivariate analysis of variance (MANOVA, factors being time and concentration of NT-3) with repeated measures for time; the individual factors were subsequently evaluated for significance with the Student-Newman-Keuls multiple-comparison test. In addition, a one-way analysis of variance (ANOVA) and subsequent Student-Newman-Keuls multiple-comparison test were performed in regard to total estradiol output over 72 hr in relation to NT-3 concentration. In all cases, $p<0.05$ was considered to be significant.

RESULTS

Effect of NT-3 on follicular E_2 secretion

Follicular incubation with neurotrophin-3 significantly increased E_2 output with regard to both time course and concentration ($p<0.05$); we found no evidence of interaction between the two factors ($p>0.05$). As illustrated in Figure

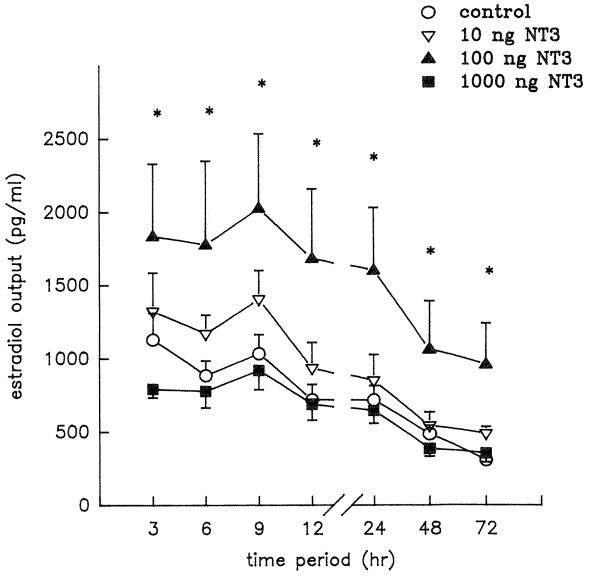

FIG. 1. Mean estradiol output (pg/ml ± SEM) of follicles in culture, NT-3 concentration versus time. Time and concentration of NT-3 were both found to be significant ($p<0.05$); there was no interaction between the two factors. *Significantly different from control and 1000 ng NT-3 groups ($p<0.05$).

1, all four plots followed similar patterns of estradiol secretion over time, regardless of NT-3 concentration. Estradiol, output was highest during the first 24 hr, displaying a peak at 9 hr; secretion decreased during the 48- and 72-hr time blocks ($p<0.05$). In addition, Figure 1 indicates a dose-dependent dichotomous relationship. Relative to control, cultures supplemented with 10 ng and 100 ng NT-3 displayed increased steroid output ($p<0.05$); culture dishes supplemented with 1000 ng NT-3 mirrored the control values.

DISCUSSION

We have demonstrated a non-neuronal effect of neurotrophin-3 (NT-3) on the mammalian ovary. We present evidence for a dichotomous effect: an NT-3 concentration of 100 ng/ml was correlated with estradiol output from preovulatory hamster follicles *in vitro* at levels significantly higher than control; while at 1000 ng/ml, E_2 concentrations experienced a decline back to control levels. Such a parabolic response curve is also shown for explant cultures of sensory ganglia; with NGF stimulating neurite outgrowth at pg concentrations and inhibiting at μg amounts: [22, 23]. In the present study, the relationship of the dosages to circulating concentrations is unknown; there are currently no antibodies to NT-3 that have allowed development of a sensitive ELISA, and therefore physiologic values for the neurotrophin have yet to be determined. Although the concentrations present *in vivo* are as yet unknown, previous research indicates NT-3 exerts *in-vitro* effects at concentrations similar to those used in our cultures [16, 29].

A dose-dependent dichotomous effect has been also described previously in regard to NT-3: when treating *trk*C-expressing NIH 3T3 cells with doses ranging from 0.01 ng-1000 ng/ml, Ip *et al.* [13] found increasing cell growth over controls to 100 ng. A dose of 1000 ng resulted in growth higher than controls, but below that correlated with 100 ng. While no hypothesis was put forward for this observation, a tentative correlation was made with transforming growth factor-β (TGF-β), one of several other peptide growth factors produced by and having effects in the reproductive system. TGF-β also demonstrates a dose-related dichotomous effect on steroid secretion, specifically on testosterone secretion by rat Leydig cells in culture [16]; it has been theorized that this effect could be due to an action on enzymes involved in steroid synthesis and/or catabolism. While it is possible that the differential effect on estradiol output associated with NT-3 is due to differential enzyme modification at this dose, an additional theory proposes that large doses of NT-3 function to desensitize/down-regulate ovarian cell cognate receptors and thereby modify the response to neurotrophic stimulation. There is currently no evidence to support this theory in regard to the high-affinity tyrosine kinase receptors; however, homologous receptor desensitization is most commonly associated with G-protein activated cascades that influence intracellular levels of cAMP, a mechanism of action proposed for the low affinity neurotrophin receptor [3]. While modificaton of cellular sensitivity is one possible effect of NT-3 in the follicle, there are other hypotheses which may be put forward.

If cellular sensitivity is not being affected, it is possible that the effects observed are due to induction of follicular cell differentiation by high doses of NT-3. NT-3 has been associated with differentiation of neural tissue [24,29]; and, in the follicle, it may function to induce luteinization of granulosa and/or thecal cells. Luteinization of follicular cells is associated with a shift in steroid synthesis, favoring progesterone rather than estrogen production [26, 27]; if the cells have differentiated, there might be a noticeable decrease in the amount of estradiol secreted into the culture medium. Experiments similar to those conducted here with subsequent assay for progesterone secretion would provide more evidence as to possible effects of NT-3 on follicular cell differentiation, with regard to both dose and time.

Over the 72-hr time course of our experiments, we noted significant differences in estradiol output, although there was no interaction between dose of NT-3 and time. Estradiol output by follicles in control dishes that did not receive additional supplementation beside serum followed the same general pattern as those in dishes receiving NT-3; we believe that this reduction was due to a decline in health of follicular cells resulting in an inability to effectively manufacture steroids. In designing our experiments, we opted to supplement our dishes with a small amount of homologous, heat-inactivated and filtered serum rather than attempt to use a serum-free system. Serum contains a number of different constituents which function in cell survival; it is a common supplement to prepared culture medium and is considered to be essential for proper growth of most cell types [7, 34]. It is possible that the amount of serum supplement was not high enough to maintain the health of large follicles for the entire 72 hours, manifesting itself via the decline in steroid secretion. However, as the pattern of estradiol output by follicles in control dishes mirrored that of follicles receiving NT-3, we are confident that if the decrease was due to a decline in cell viability, it occurred independently of neurotrophin action.

While researchers have previously identified the low-affinity NT-3 receptor to the thecal cells of developing follicles [6], we are fortunate to have received information regarding the high-affinity receptors specific to our animal model. Using proestrous ovarian tissue from hamsters in our study, northern blot analyses were performed and we identified mRNA for full-length *trk*A, and truncated forms of *trk*B and *trk*C (unpublished). While our analyses identified only the truncated version of *trk*C (the receptor to which NT-3 preferentially binds, but the significance of which remains unclear), the possibility remains that full-length *trk*C could be present in levels that are simply too low for detection by northern blot analysis, but adequate for signal transduction [35]. The information provided through these analyses has served to identify several possible receptors for NT-3 in the hamster ovary; identification of the primary receptor associated with signal induction and its specific location in the

ovary will require further investigation.

To date, research in our laboratory has focused on examining the many processes affecting ovarian function and follicular development. Investigations by Ernfors [9] and Dissen [6], specifically associating a neurotrophin and its receptor with ovarian follicles, compelled us to further examine its role in the reproductive system. Data obtained from these analyses prompted speculation in regard to a non-neuronal function for neurotrophins in the ovary. We then undertook investigations designed to identify one of many possible non-neuronal functions of NT-3. Through our research, we have demonstrated an influence of NT-3 on estradiol secretion from hamster follicles *in vitro*. This information further supports the theory that NT-3, known to be produced in the granulosa cells of developing follicles, may exert a paracrine effect in the reproductive system by stimulating thecal androstendione synthesis, for subsequent aromatization to estradiol in the follicular granulosa cells. The list of other growth factors known to be produced by and function in the ovary to alter steroidogenesis is already quite extensive, including EGF, bFGF, the IGFs and TGF-β [1, 4, 11, 30, 32]; the addition of NT-3 increases the complexity of possible control mechanisms and regulatory functions.

Collectively, these data strongly support a humoral role for the neurotrophin NT-3 in steroid secretion by ovarian follicles *in vitro*; it is therefore conceivable that NT-3, and possibly other factors previously presumed only to function in the nervous system, play important regulatory roles in normal follicular development and ovarian function in general. With this study we have identified NT-3 as a legitimate player in the dynamic environment of the mammalian ovary; further research will serve to elucidate its specific action and, hopefully, provide new information regarding the interaction between the nervous and reproductive systems.

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