EXPRESSION AND FUNCTION OF BRAIN-DERIVED NEUROTROPHIN FACTOR AND ITS RECEPTOR, TrkB, IN OVARIAN FOLLICLES FROM THE DOMESTIC HEN (*GALLUS GALLUS DOMESTICUS*)

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Summary

This report summarizes patterns of mRNA expression for the brain-derived neurotrophic factor (BDNF) together with its high-affinity neurotrophin receptor trkB within the hen ovary during follicle development, describes hormonal mechanisms for the regulation of trkB gene expression and provides preliminary evidence for a novel function for BDNF-mediated TrkB signaling within the granulosa layer. Levels of BDNF mRNA in the thecal layer and of trkB mRNA within the granulosa cell layer increase coincident with entrance of the follicle into the preovulatory hierarchy. Localization of the BDNF mRNA transcript correlates with expression of BDNF protein within the theca interna of preovulatory follicles, while localization of trkB mRNA and protein occurs extensively within the granulosa cell layer of preovulatory follicles. This pattern of expression suggests a paracrine relationship between theca and granulosa cells for BDNF signaling *via* TrkB. Vasoactive intestinal peptide and gonadotropin treatments stimulate increases in levels of trkB mRNA within cultured granulosa cells derived from both prehierarchal and preovulatory follicles, and this response is increased by co-treatment with 3-isobutyl-1-methylxanthine. Finally, BDNF treatment of cultured granulosa cells from preovulatory follicles results in a modest, but significant, reduction in basal progesterone production, whereas this effect was reversed by k252a, an inhibitor of Trk kinase activity. These results support the proposals that BDNF functions as a paracrine signal in hen granulosa cells and that its physiological functions may include the modulation of steroidogenesis.

Key words: neurotrophin, neurotrophin receptor, ovarian follicle, granulosa, theca, chicken, *Gallus gallus domesticus*.

Introduction

Expression of the neurotrophin brain-derived neurotrophic factor (BDNF) and of its high-affinity receptor TrkB have previously been described in the avian embryo. During early embryonic development, BDNF mRNA expression was identified by reverse transcriptase/polymerase chain reaction (RT-PCR) beginning at stage 1 (3–4 h of embryo incubation), while trkB expression was not detected until the eight-somite stage (29 h) (Baig and Khan, 1996). These observations compare favorably with those of Yao et al. (Yao et al., 1994), who described early (embryonic day 1 and 2) expression of neurotrophin in quail embryos. Further analysis of this neurotrophin in the chick embryo by northern blot analysis and *in situ* hybridization during embryonic development showed a peak of expression of BDNF at day 4.5 (Hallbook et al., 1993). BDNF has been reported to serve as a cell survival factor for chick embryonic motor neurons (Becker et al., 1998), and its expression has been associated with stato-acoustic ganglion development within the inner ear (Hallbook and Fritzsch,

1997). The results from these and other studies largely emphasize the role of BDNF as a target-oriented factor that promotes the development of neuronal tissues in the developing avian embryo, yet also support the emerging view that BDNF can modulate the development and differentiation of non-neuronal tissues.

The expression and function of several neurotrophins and neurotrophin receptors, including BDNF and TrkB, within the rat ovary have previously been associated with the initial (pubertal) ovulation (Dissen et al., 1996). Moreover, expression of trkB mRNA has been demonstrated at the time of primordial follicle organization in the rat ovary, and it has been proposed that such expression is required for the organization of somatic and germ cells and/or for the initiation of follicle growth (Dissen et al., 1995; Ojeda et al., 1996). In contrast, the presence of BDNF and trkB has not previously been described in the adult avian ovary. Furthermore, neither the regulation nor the site of function of this neurotrophin

ligand/receptor pair within the ovary has been fully elucidated for any vertebrate species.

In the hen ovary, developing follicles (within which the granulosa cell layer is steroidogenically incompetent) are known as prehierarchal follicles, while the 5–9 largest steroidogenically active follicles are called hierarchal or preovulatory follicles. Several dramatic changes occur coincident with the time at which a follicle enters into the preovulatory hierarchy (rapidly growing follicles >9 mm in diameter). Some of the more important changes occur within granulosa (follicular epithelial) cells and include the initiation of steroidogenesis and the development of resistance to apoptosis (Johnson, 1999; Johnson et al., 1996b). In addition, there occurs a transition from dependence upon follicle-stimulating hormone (FSH) to dependence upon luteinizing hormone (LH), as demonstrated by the decrease in FSH receptor (FSH-R) mRNA levels and the increase in LH receptor (LH-R) mRNA levels within the granulosa layer following entrance into the preovulatory hierarchy (Johnson, 1999; You et al., 1996; Johnson et al., 1996a). One important consequence of this transition in gonadotropin dependence is the dramatically increased potential for progesterone production, which is prerequisite for inducing ovulation of the largest (F1) preovulatory follicle (Tilly et al., 1991a; Johnson and van Tienhoven, 1984).

Previous studies have shown that hen granulosa cell differentiation and progesterone production are attenuated by agents, such as phorbol 12-myristate-13-acetate (PMA), that induce protein kinase C (PKC) activation. The inhibitory effects of PKC activation have been shown to occur *via* suppression of adenylyl cyclase activity and inhibition of cytochrome P_{450} side-chain cleavage (P450scc) mRNA expression and enzyme activity (Tilly et al., 1991b; Tilly and Johnson, 1989). Moreover, it has previously been found that phorbol ester treatment mimics the effects of BDNF/TrkB signaling in rat cerebellar granule cells, indicating that PKC activation is either a component of, or feeds into, the intracellular pathways that promote cell survival in this cell type (Coffey et al., 1997).

One additional neuropeptide unrelated to neurotrophins, vasoactive intestinal peptide (VIP), is known to be present within the thecal layer of hen follicles at all stages of follicle development (Johnson et al., 1994). It has been demonstrated *in vitro* that VIP is capable of promoting granulosa cell steroidogenesis primarily by increasing cyclic AMP (cAMP) formation (Johnson and Tilly, 1988; Johnson, 1996). Moreover, VIP treatment protects against the initiation of programmed cell death of granulosal cells of prehierarchal follicles and, together with gonadotropins, may serve as an important cell survival signal (Flaws et al., 1995; Johnson et al., 1996b).

Given the absence of information regarding the expression or function of neurotrophins within hen ovarian follicles, the present studies were conducted to characterize the tissue distribution and expression levels of the neurotrophin BDNF and its high-affinity receptor TrkB at distinct stages of follicle maturation. Additional experiments were initiated to evaluate the regulation of trkB mRNA expression and to evaluate potential physiological actions resulting from TrkB signaling in hen granulosal cells.

Materials and methods

Animals and reagents

Hens of the single comb white Leghorn breed (H&H Poultry, Portland, IN, USA) were used at 25–35 weeks of age and were producing at least 5–6 eggs in regular sequences. Birds were housed individually in laying batteries, provided with free access to food (Purina Lay Mash; Purina Mills, St Louis, MO, USA) and water, and exposed to a photoperiod of 15 h:9 h L:D (lights on at midnight). Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed by cervical dislocation approximately 16–18 h prior to a mid-sequence ovulation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human BDNF used in culture experiments was purchased from PeproTech (Rocky Hill, NJ, USA), and the putative Trk kinase inhibitor k252a, which acts by inhibiting receptor tyrosine kinase autophosphorylation, was from Alexis Corp. (San Diego, CA, USA). The TrkB antiserum was a gift from Dr F. Lefcort (University of California, San Francisco, CA, USA). Chicken VIP, 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor) and PMA were from Sigma Chemical Co. (St Louis, MO, USA). Ovine LH (lot 26) and recombinant human FSH (lot R1) were from the National Hormone and Pituitary Program, progesterone used to generate the radioimmunoassay standard curve was obtained from Steraloids Inc. (Wilton, NH, USA) and [1,2,6,7-^{3}H]progesterone was from Amersham (3.1 TBq mmol^{-1}; Arlington Heights, IL, USA).

Primer design and polymerase chain reaction (PCR) amplification

Oligonucleotide primers for PCR amplification used to produce BDNF and trkB cDNA probes were designed using the MacVector sequence analysis program (v. 4.5.3; Eastman Kodak, Rochester, NY, USA). Primers for BDNF were based upon the homologous chicken sequence (GenBank accession no. M83377; Maisonpierre et al., 1992) and produced a 475 base pair (bp) product (corresponding to base pairs 214–688); trkB primers yielded a 439 bp product from within the extracellular coding region (corresponding to base pairs 553–981) of the chicken cDNA (GenBank accession no. X74109; Dechant et al., 1993). The products produced were predicted not to cross-hybridize with any other known chicken transcript.

Trizol Reagent (Gibco BRL, Richmond, CA, USA) was utilized to isolate total RNA from brain cortex tissue and thecal or granulosal layers of prehierarchal and preovulatory follicles

(Gilbert et al., 1977; Tilly et al., 1991a). This RNA was subsequently used as template to produce reverse-transcribed cDNA using random hexamer primers (Perkin-Elmer, Norwalk, CT, USA). Following PCR amplification, the DNA fragments were gel-purified, ligated into the PCRII or PCR2.1 vector and subcloned using the TA-cloning kit (Invitrogen, Carlsbad, CA, USA). Nucleic acid sequence analysis was conducted for verification of the PCR product. Products used for probe templates in either northern blot hybridization or *in situ* hybridization were cut from the vector with the *Eco*RI restriction enzyme and gel-purified prior to use.

Northern blot analysis

Total RNA was isolated from ovarian stroma, granulosa and theca tissues from the largest (F1), second largest (F2) and third largest (F3) follicles, from 9–12 mm diameter preovulatory (hierarchal) follicles and from prehierarchal follicles 3–5 mm and 6–8 mm in diameter. Northern blot analysis was accomplished by electrophoretic separation of 10–15 μg of total RNA on a 1 % agarose gel containing 6 % formaldehyde, followed by transfer to nitrocellulose Nitro ME membranes (MSI, Westboro, MA, USA) by capillary action overnight (Johnson et al., 1997b).

Complementary DNA probes were synthesized by random-primed labeling (Megaprime DNA labeling system, Amersham, Arlington Heights, IL, USA) of gel-purified PCR products with $[\alpha\text{-}^{32}P]dCTP$ (92.5 TBq mmol^{-1}; Amersham). Prehybridization, hybridization and washing of membranes was conducted as described previously (You et al., 1996). Briefly, membranes were prehybridized for 1 h at 60 °C in prehybridization buffer, followed by overnight hybridization in hybridization buffer containing approximately 2×10^6 cts min^{-1} ml^{-1} probe. The blots were washed under stringent conditions at 60 °C (two 15 min washes in 2× SSC (1× SSC is 150 mmol l^{-1} sodium chloride and 15 mmol l^{-1} sodium citrate, pH 7.0) plus 1 % SDS and one wash for 15 min in 0.1× SSC plus 0.1 % SDS), followed by exposure for 7–12 days to phosphorimager plates and analysis using the ImageQuant data analysis program (Storm 840 Phosphoimager system; Molecular Dynamics, Sunnyvale, CA, USA).

Production of anti-chicken BDNF antibody

A BDNF polyclonal antiserum was generated in a rabbit against a 56 amino acid residue fusion protein based upon the deduced chicken amino acid sequence (Maisonpierre et al., 1992). The fusion protein was produced by expressing a BDNF cDNA, corresponding to residues 171–226, ligated into the pCAL-n vector, in BL21(DE3)pLysS competent cells (Stratagene, La Jolla, CA, USA). The over-expressed protein was gel-purified on a 12 % SDS–polyacrylamide gel, eluted in water and mixed 1:1 (v:v) with complete Freund's adjuvant (total of 120 μg protein in 1.0 ml). The rabbit was given at least five injections of 0.2 ml or less, subcutaneously, along the back. Booster injections were made in a similar fashion using incomplete Freund's adjuvant. The injection regime consisted of the first injection, followed by four booster injections each

separated by 14 days. The antiserum was affinity-purified by transferring antigen protein from a 12 % SDS–PAGE gel to a nitrocellulose membrane. The strip of membrane containing the fusion protein was cut and incubated with the polyclonal antiserum at a dilution of 1:5000. The strip was washed three times in Tris-buffered saline (TBS; 0.1 % Tween 20, 5 % milk), followed by elution (two washes of 10 min each in 100 mmol l^{-1} glycine, pH 2.5) of the purified antibody and then buffered with 1 mol l^{-1} Tris (pH 7.5). Antibody specificity was verified by western blot prior to use in immunocytochemical studies, and was incubated with tissue sections at a dilution of 1:200.

In situ *hybridization*

Tissues used for *in situ* analysis were dissected from the hen and immediately fixed in Dietrick's fixative (4 % formaldehyde, 28 % ethanol, 58.8 % glacial acetic acid) overnight, dehydrated by ethanol washes of increasing concentration (50 %, 70 %, 90 %, 100 %), cleared in xylene and embedded in paraffin (Paraplast, Fisher Scientific, Pittsburgh, PA, USA). Subsequently, tissues were sectioned at 4 or 6 μm, fixed to slides (Fisherbrand superfrost plus, Fisher) for 1 h at 55 °C on a slide warmer, cooled to room temperature (20 °C) and used immediately. The cDNA probes were biotin-labeled using the BioPrime labeling system (GibcoBRL, Grand Island, NY, USA). *In situ* hybridization was conducted using a modified version of the protocol provided with the DAKO *in situ* hybridization detection system (Dako, Carpinteria, CA, USA). Briefly, sections were deparaffinized in xylene and rehydrated in several changes of graded ethanols, followed by digestion for 2 or 5 min with proteinase K (40 mg ml^{-1}; Fisher) at room temperature. They were then post-fixed in 4 % paraformaldehyde for 7 min at room temperature, followed by several washes in phosphate-buffered saline (PBS). The DAKO biotin blocking system was used to block endogenous biotin. Prehybridization [(23 mg of herring sperm DNA per 100 ml of 10 % dextran sulfate, 53 % deionized formamide, 47 % hybridization buffer (4× SSC, 0.2 mol l^{-1} sodium phosphate, pH 6.5, 2× Denhardt's solution)] was conducted at 43 °C for 2 h. Hybridization (in prehybridization buffer containing 30 μg of biotin-labeled probe per 100 ml and 20 mg of yeast transfer RNA per 100 ml) was performed at 43 °C for 12–20 h. Following two stringent washes in 0.2× SSC at 43 °C for 15 min, detection of the probe was accomplished using the DAKO *in situ* hybridization detection system. Levamisole (200 μg ml^{-1}; Sigma), an inhibitor of endogenous alkaline phosphatase activity, was used in the final TBS wash as well as in the substrate solution. The substrate solution consisted of a combination of Nitroblue Tetrazolium (NBT) and 4-bromo-5-chloro-3-indolylphosphate (BCIP). Slides were dehydrated through a graded series of ethanol concentrations and mounted in Permount (Fisher).

Immunocytochemistry

Tissues for immunocytochemical analysis were dissected from the hen, immediately fixed and subsequently paraffin-

embedded, as described above. The tissues were sectioned at 4 or 6 µm, fixed to slides for 1 h at 55 °C on a slide warmer, cooled to room temperature and used immediately. Slides were deparaffinized in three changes of xylene (each 10 min in duration), followed by rehydration through a graded series of ethanol concentrations and TBS buffer. The tissue sections were incubated in blocking solution (TBS buffer containing 1.5 % serum and 0.2 % Triton-X 100) for 1 h and incubated overnight at 4 °C in blocking buffer containing a 1:100 or 1:200 dilution of the primary antisera. Slides were rinsed three times for 5 min in PBS buffer including 0.3 % Triton-X 100 (PBT), then incubated for 30 min at room temperature in secondary HRP- (horseradish peroxidase) or FITC- (fluoroscein isothiocyanate) conjugated goat anti-rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in PBT plus 1.5 % goat serum. The secondary antibody was visualized using either the Rabbit ABC immunostaining system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a light microscope for the HRP-conjugated secondary antibody or by laser confocal microscopy (Bio-Rad MRC 1024, Bio-Rad Hercules, CA, USA) using the Lasersharp software package (BIO-Rad) for the FITC-conjugated secondary antibody.

Cell culture

Immediately following isolation of granulosal cell layers from either prehierarchal (3–8 mm diameter) or preovulatory (F1 or F2) follicles, cells were dispersed by treatment in medium containing 0.3 % Type 2 collagenase (Worthington Biochemical, NJ, USA) for 10–15 min. To evaluate the regulation of trkB mRNA levels by various factors, cells were plated overnight in six-well tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) with M199/Hepes medium containing 2.5 % fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) (Johnson et al., 1996b). Following pre-culture, the medium was changed, and the cells were incubated with VIP ($1 \mu\mathrm{mol\,l^{-1}}$), IBMX ($0.01 \mu\mathrm{mol\,l^{-1}}$) or VIP plus IBMX for 18 h. Since granulosal cells from preovulatory follicles preferentially express LH-R, F1 follicle granulosa treatments also included LH ($100 \mathrm{ng\,ml^{-1}}$) and LH plus IBMX. As granulosa cells from prehierarchal follicles also preferentially express FSH-R, treatments for 3–8 mm follicle granulosa included FSH ($100 \mathrm{mi.u.\,ml^{-1}}$) and FSH plus IBMX. Doses and treatment times were chosen on the basis of previously published studies (Johnson et al., 1994; Johnson et al., 1996b; Johnson et al., 1997a; Johnson et al., 1997b). Non-cultured cells used for controls (T0) were frozen at −70 °C following dispersion but before culture, while cultured control cells were treated with the vehicle (dimethylsulfoxide, DMSO, $5 \mu\mathrm{l\,ml^{-1}}$) for 18 h. Following culture, cells were collected and stored at −70 °C until processed for total cellular RNA.

To test the effects of various factors on preovulatory granulosal cell basal progesterone production, cells were pre-cultured overnight. Following this pre-culture, the medium was changed, and the cells were pretreated for 15 min with k252a ($100 \mathrm{nmol\,l^{-1}}$) or its vehicle ($5 \mu\mathrm{l\,ml^{-1}}$ DMSO), then treated without or with BDNF (25, 50 or $100 \mathrm{ng\,ml^{-1}}$) for 18 h. A final set of experiments was designed to compare the inhibitory effects on progesterone production of PMA ($167 \mathrm{nmol\,l^{-1}}$) and BDNF ($50 \mathrm{ng\,ml^{-1}}$) or a combination of these two agents. In both experiments, media were collected and stored at −70 °C until assayed for progesterone.

Radioimmunoassay (RIA) for progesterone

The progesterone polyclonal antibody and RIA protocol have been described previously (Tilly et al., 1991a). Media from three replicate experiments were quantified within the same assay to eliminate inter-assay variation. Briefly, $10 \mu\mathrm{l}$ of medium from treated cultures was added to $90 \mu\mathrm{l}$ of $0.01 \mathrm{mol\,l^{-1}}$ PBS, 0.1 % gelatine and $100 \mu\mathrm{l}$ of anti-progesterone serum diluted 1:10 000 in $100 \mu\mathrm{l}$ of $0.1 \mathrm{mol\,l^{-1}}$ PBS for 15 min at room temperature, followed by addition of labeled progesterone for 15 min at room temperature. Assay tubes were then incubated at 4 °C for 18 h. Free progesterone was separated from antibody-bound progesterone by the addition of 0.75 ml of dextran-covered charcoal (Fisher; $250 \mathrm{mg\,100\,ml^{-1}}$) in $0.1 \mathrm{mol\,l^{-1}}$ PBS containing 0.1 % gelatine and 0.025 % dextran for 15 min at 4 °C. Tubes were centrifuged at $600\,g$ for 15 min at 4 °C, and the supernatant was decanted into vials containing 7 ml of scintillation fluid (Scintiverse, Fisher SX 18-4) and quantified using a scintillation counter (Beckman LS 5000TD) to an accuracy of ±2 %. The percentage binding from samples was compared with a progesterone standard curve.

Data analysis

Analysis of mRNA data was accomplished following standardization of the target signal to 18S ribosomal RNA (rRNA) (Johnson et al., 1996a; Johnson et al., 1997b; You et al., 1996). All northern blot hybridization experiments were performed using a minimum of three independent replicates. Levels of mRNA are presented as the mean fold difference compared with a reference tissue (not included in statistical analyses), and analyzed by one-factor analysis of variance (ANOVA) followed by Fisher's PLSD test using Statview II (Abacus Concepts inc. Berkeley, CA, USA). Radioimmunoassay data were analyzed following log/logit transformation, and treatment effects were expressed as the fold difference compared with control (untreated) cells. Treatment differences in progesterone levels were analyzed by ANOVA. *Post-hoc* analysis was conducted on selected data by paired *t*-test using original (untransformed) data (e.g. T0 *versus* cultured control cells; see Fig. 4). Results are expressed as means ± S.E.M. (*N*).

Results
Northern blot analysis

Two BDNF mRNA transcripts corresponding to approximately 4.5 kb and 1.5 kb were detected in thecal tissue (Fig. 1A). Although neither transcript was detected in the granulosal layer by northern analysis, low-level expression of BDNF was detected following RT-PCR amplification (data not
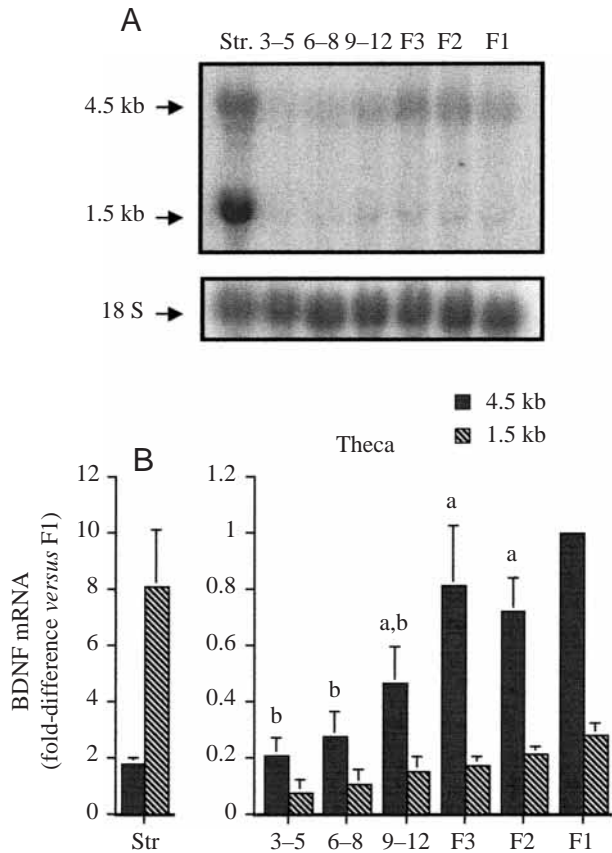
Fig. 1. Representative northern blot of brain-derived neurotrophic factor (BDNF) mRNA in theca cells (A) reveals two transcripts of approximately 4.5 kb and 1.5 kb. Levels of mRNA were measured by densitometric scans of northern blots and were standardized to 18S rRNA (B). Data are expressed as fold difference compared with F1 follicle theca. Values are means + S.E.M. (N=3 replicate experiments). a and b denote significant differences ($P<0.05$) in levels of the 4.5 kb transcript. Str, stromal tissue; 3–5 and 6–8 refer to the diameter (in mm) of prehierarchal follicles, while 9–12 (follicle diameter 9–12 mm) and F3, F2 and F1 represent preovulatory follicles, with F1 representing the next follicle to ovulate.

shown). Levels of the 4.5 kb BDNF transcript in thecal tissue were relatively low in prehierarchal follicles and increased by 4.7±1.3-fold in the F1 preovulatory follicles (compared with 3–5 mm follicles). Expression of the 1.5 kb transcript was low except in stromal tissue, where higher levels of the 1.5 kb transcript than of the 4.5 kb transcript were transcribed (Fig. 1B).

By comparison, analysis of the corresponding trkB receptor mRNA showed three transcripts: a major 9.0 kb transcript together with less abundant 6.3 kb and 5 kb transcripts (Fig. 2A). Levels of the 9.0 kb trkB transcript increased by six- to ninefold ($P<0.05$) in preovulatory (9–12 mm through F1) follicle granulosa compared with prehierarchal (3–5 mm and 6–8 mm) follicles (Fig. 2B). The low, but detectable, levels of trkB expression in theca did not change during follicle development (Fig. 2C).
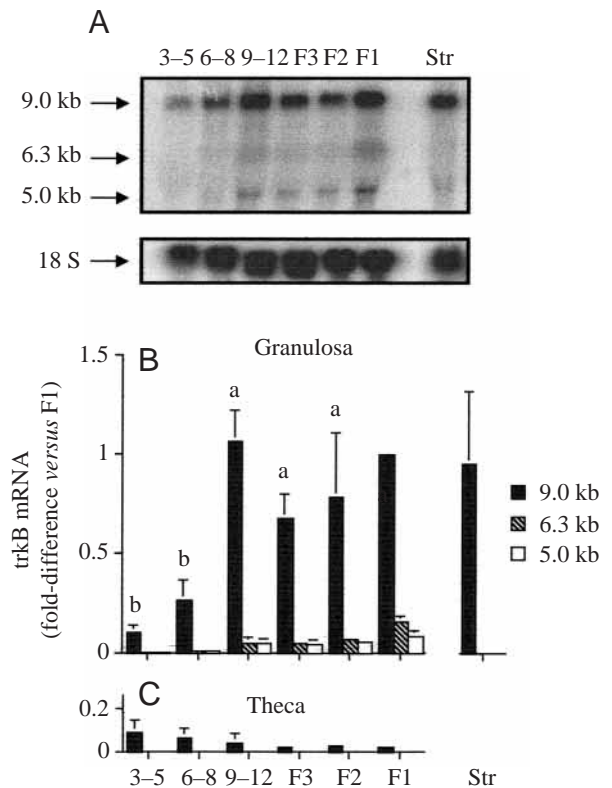

Fig. 2. Representative northern blot of trkB (the high-affinity neurotrophin receptor of brain-derived neurotrophic factor) mRNA in granulosa cells (A) reveals three transcripts of approximately 9, 6.3 and 5 kb. Levels of mRNA from granulosa and stroma tissues (B) and theca (C) were standardized to 18S rRNA and are expressed as fold difference compared with F1 follicle granulosa. Values are means + S.E.M. (N=3 replicate experiments). a and b denote significant differences ($P<0.05$) in levels of the 9 kb transcript. Abbreviations are as described in the legend to Fig. 1.

In situ *hybridization and immunocytochemical detection*

In situ hybridization (Fig. 3A) and immunocytochemical analysis (Fig. 3B) of BDNF shows localization of BDNF mRNA and protein, respectively, within cells from the theca interna of the F1 preovulatory follicle, immediately adjacent to the granulosal layer. In contrast, trkB mRNA (Fig. 3C) and protein (Fig. 3D) are localized to the granulosal cell layer plus isolated groups of cells within the theca interna adjacent to the basement membrane.

Regulation of trkB mRNA levels in cultured granulosa

Levels of trkB mRNA decreased significantly in granulosal cells from both prehierarchal and preovulatory follicles following pre-culture and a subsequent culture in the absence of treatment for 18 h ($P<0.05$ *versus* T0; Fig. 4A,B). Since the three different transcripts detected by northern blot analysis (9.0, 6.3 and 5.0 kb) appeared to be coordinately regulated both during follicle development (Fig. 2) and following culture (data not shown), only the 9.0 kb transcript was quantified during the analysis of these culture experiments. Transcript levels in prehierarchal follicle granulosa were increased by

8.1±2.5-fold (N=3) and 8.7±2.1-fold (N=3) when cultured for 18 h in the presence of VIP and FSH, respectively, compared with control cultured cells (P<0.05). This increase was potentiated by co-treatment with IBMX to 26.7±9.2-fold (N=3) and 22.1±1.9-fold (N=3) control levels, respectively (Fig. 4A). In contrast, levels of trkB mRNA in preovulatory follicle granulosa were increased by 4.6±1.5-fold (N=3; P<0.05) and 2.2±0.5-fold (N=3; P=0.07) in cultured granulosal cells treated with VIP and LH, respectively, and these effects were potentiated in the presence of IBMX (to 5.5±1.2-fold, N=3, for VIP plus IBMX and to 4.4±1.1-fold, N=3, for LH plus IBMX; Fig. 4B).

Basal progesterone production is suppressed by BDNF treatment

Treatment of F1 follicle granulosal cells with 50 ng ml^{-1} BDNF (Fig. 5A) resulted in a 34 % decrease in progesterone levels compared with levels in control cultures (P<0.05, by paired t-test). This BDNF-mediated inhibition of progesterone levels was effectively prevented by pre-culture with the putative inhibitor of tyrosine kinase k252a. Addition of 25 ng ml^{-1} BDNF yielded a less robust suppression (23 %) of progesterone levels, whereas the 100 ng ml^{-1} treatment showed a level of suppression similar to the 50 ng ml^{-1} treatment. Treatment of cultured cells with both BDNF (50 ng ml^{-1}) and PMA (167 nmol l^{-1}) showed no additional suppression of progesterone levels compared with PMA or BDNF treatments alone (P>0.2; Fig. 5B).

Discussion

In the hen ovary, theca interna cells express BDNF mRNA and protein, while the corresponding high-affinity TrkB receptor is highly expressed within the adjacent epithelial granulosal cell layer. Moreover, increases in constitutively expressed BDNF and trkB mRNA in theca and granulosa, respectively, occur coincident with entry of a prehierarchal follicle into the preovulatory hierarchy. Since BDNF treatment was found to attenuate basal progesterone production by cultured preovulatory follicle granulosal cells, these results provide evidence for a paracrine action of BDNF-producing thecal cells upon TrkB-expressing granulosal cells within the hen ovary.

Each of the BDNF and trkB transcripts detected by northern blot analysis (Fig. 1, Fig. 2) has previously been described in the literature (Maisonpierre et al., 1992; Dechant et al., 1993), and all are sufficiently large to encompass the entire 738 bp and 2456 bp coding sequences for the ligand and receptor, respectively. To date, however, the specific transcript(s) that encode the functional proteins for chicken BDNF and TrkB have yet to be identified. Our results suggest that the two (BDNF) or three (trkB) transcripts are coordinately regulated both *in vivo* (Fig. 1, Fig. 2) and *in vitro* (data not shown).

Of interest was the finding that the initial increase in expression of BDNF and its high-affinity receptor TrkB correlates with entry of the follicle into the preovulatory hierarchy (e.g. 9–12 mm follicles; Tilly et al., 1991a). Several genes show elevated expression within granulosal cells coincident with this stage of development, including genes associated with resistance to apoptosis (ita/ciap1, Johnson et al., 1998; bcl-xLong, Johnson et al., 1997b) and the initiation of progesterone production (Bauer et al., 2000; Li and Johnson, 1993). The coordinately increased expression of BDNF and trkB RNA in preovulatory follicles suggests a role for BDNF/trkB signaling during the final stages of granulosal cell development.

The localization of BDNF protein within the theca interna adjacent to the granulosal layer and expression of TrkB within the adjacent granulosa cells suggest a paracrine relationship between the theca interna and granulosal cell layers. In addition, the presence of a few theca interna cells demonstrating TrkB protein suggests an autocrine feedback control of BDNF expression. Both positive and negative feedback regulation of BDNF on trkB expression have been described previously (Knusel et al., 1997; Ferrer et al., 1998). Unlike VIP expression, which is revealed by immunocytochemical staining to be localized to nerve terminals in both the theca externa and interna (Johnson et al., 1994), BDNF mRNA and protein are diffusely distributed within most cells of the theca interna and focally distributed in a few cells of the theca externa (Fig. 3). Although not dismissing the possibility of BDNF being expressed by neuronal cells within the theca, this pattern of expression suggests that BDNF is produced by cells of non-neuronal origin. Moreover, the granulosal layer represents a tissue that is completely devoid of neuronal cells. Thus, these data support a role for BDNF/TrkB signaling in modulating the function of cells outside the nervous system.

The rapid progression of granulosal cell differentiation that occurs following follicle recruitment is driven largely by intracellular signaling *via* the protein kinase A (PKA) pathway. For instance, expression of the genes *ita*, *bcl-x*, *StAR* and *P450scc* has previously been documented to be upregulated *in vitro* following treatment of prehierarchal follicle granulosal cells with VIP, the stable cAMP analog 8-bromo-cyclic AMP and/or gonadotropins (Bauer et al., 2000; Johnson et al., 1994; Johnson et al., 1997b; Johnson et al., 1998; Li and Johnson, 1993). In the present experiments, the decrease in levels of trkB mRNA found in control cultured cells from both prehierarchal and preovulatory follicle granulosa, compared with non-cultured (T0) cells (Fig. 4), suggests the removal of an expression maintenance signal in cultured cells that occurs tonically *in vivo*. Significantly, activation of PKA signaling following treatment with VIP or gonadotropin in the presence of IBMX restored (preovulatory granulosa), or nearly restored (prehierarchal granulosa), levels of expression of trkB mRNA in cultured cells to those found in uncultured (T0) cells. The efficacy of FSH in prehierachal follicle granulosa and of LH in preovulatory granulosa is consistent with the stage-dependent expression of gonadotropin receptors, as reported previously (Johnson et al., 1996a; You et al., 1996).
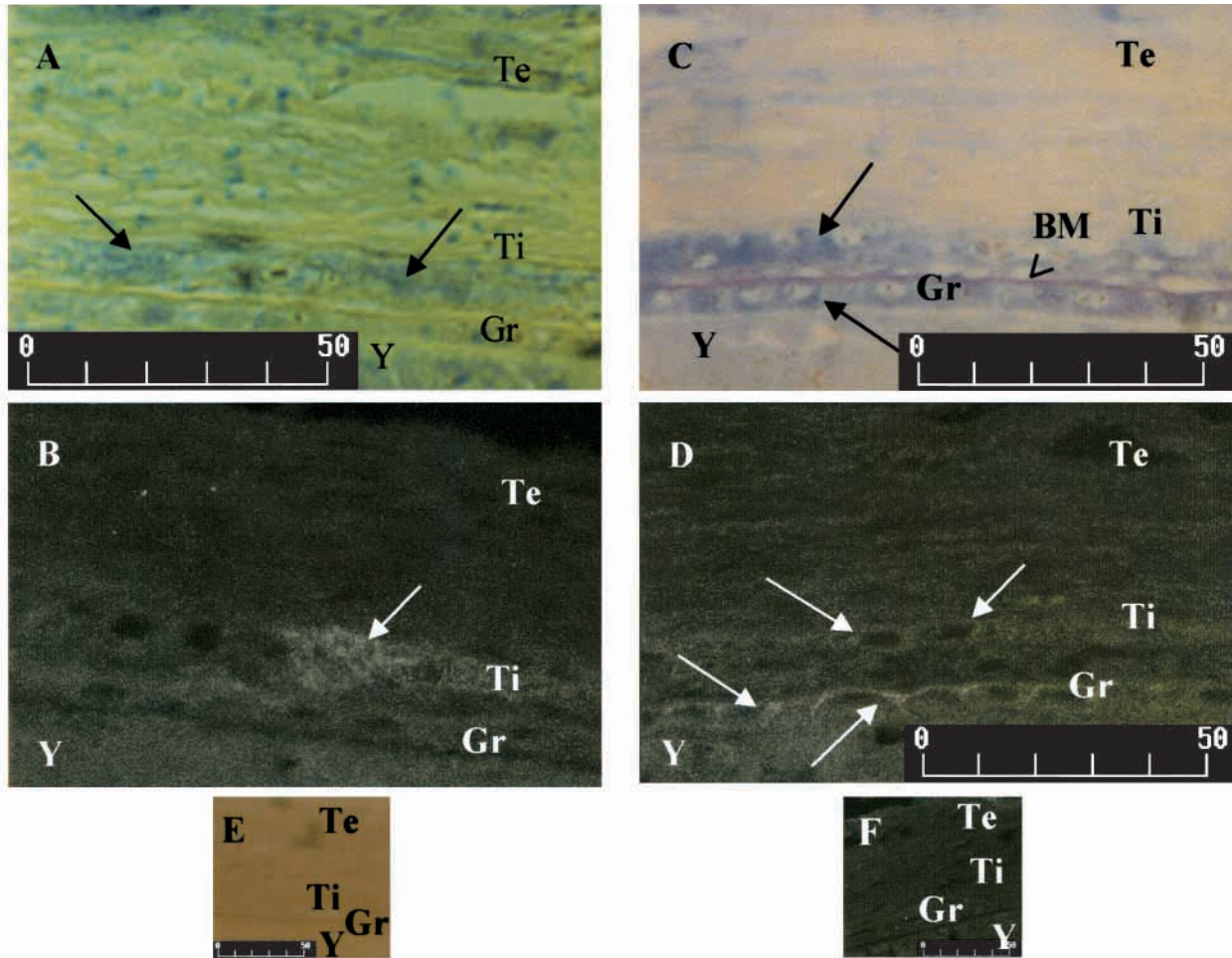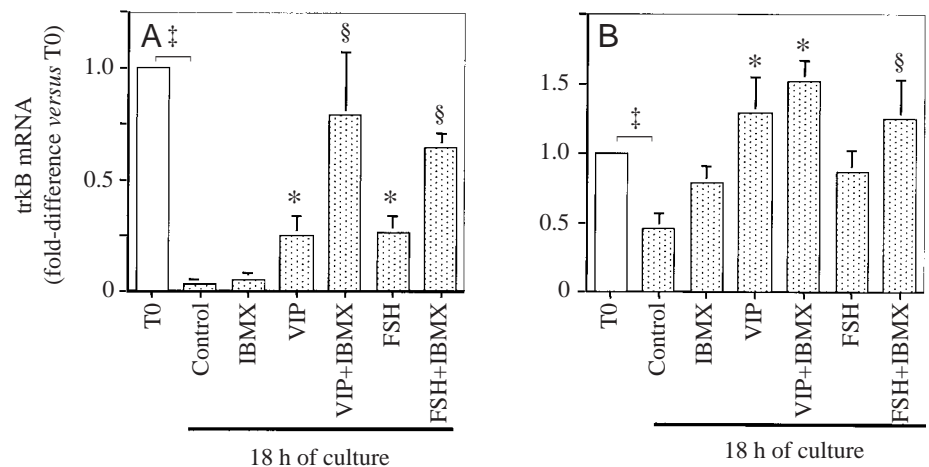
Fig. 3. Localization of brain-derived neurotrophic factor (BDNF) and trkB (the high-affinity neurotrophin receptor of brain-derived neurotrophic factor) mRNA and protein within the preovulatory follicle. *In situ* hybridization and immunocytochemical detection shows BDNF mRNA (A) and protein (B) expression, respectively, within the theca interna of the F1 follicle (arrows). By comparison, *in situ* hybridization shows trkB mRNA (C) localized within the theca interna and granulosa layers (arrows), while expression of the TrkB protein (D) is localized to the basement membrane side of the granulosa cell layer and to a lesser extent within the theca interna (arrows). (E) A negative control for *in situ* hybridization using a non-sense cDNA probe; (F) a negative control for immunolocalization using preimmune serum. The scale bars are graded in μm. Y, yolk; Gr, granulosa layer; BM, basement membrane (arrowhead); Ti, theca interna; Te, theca externa.

Fig. 4. (A) Levels of the 9.0 kb trkB (the high-affinity neurotrophin receptor of brain-derived neurotrophic factor) transcript in cultured prehierarchal follicle granulosa cells following overnight pre-culture and subsequent treatment for 18 h without (control) or with vasoactive intestinal peptide (VIP; $1\,\mu mol\,l^{-1}$) or follicle-stimulating hormone (FSH; $100\,ng\,ml^{-1}$) in the absence or presence of the phosphodiesterase inhibitor IBMX ($0.01\,\mu mol\,l^{-1}$). (B) Levels of trkB mRNA in cultured preovulatory follicle granulosa cells following treatment for 18 h without or with VIP or luteinizing hormone (LH) in the absence or presence of 3-isobutyl-1-methylxanthine (IBMX). trkB mRNA was standardized to 18S rRNA and expressed as fold difference compared with non-cultured (T0) cells. ‡$P<0.05$ compared with T0 by a *post-hoc* paired *t*-test; *$P<0.05$ compared with control; §$P<0.05$ compared with the respective treatment in the absence of IBMX. Values are means + s.e.m. ($N=3$ replicate experiments).

Fig. 5. (A) Progesterone (P4) production in preovulatory follicle granulosa cells following treatment with brain-derived neurotrophic factor (BDNF), k252a (100 nmol l^{-1}), BDNF (50 ng ml^{-1}) plus the Trk (the high-affinity neurotrophin receptor of brain-derived neurotrophic factor) kinase activity inhibitor k252a or vehicle (control) for 18 h following overnight preplating. Levels of progesterone are expressed as fold difference compared with the control. Values are means + S.E.M. (N=4 or 5 replicate experiments). [a,b]P<0.05; *P<0.05 for BDNF at 50 ng ml^{-1} compared with the control, as determined by a *post-hoc* paired *t*-test. (B) Progesterone production in preovulatory follicle granulosa cells following treatment with BDNF (50 ng ml^{-1}), phorbol 12-myristate-13-acetate (PMA; 167 nmol l^{-1}), BDNF plus PMA or vehicle (control) for 18 h after overnight preplating. Levels of progesterone, expressed as fold difference compared with control cells, are not different among the three treatment groups. Values are means + S.E.M. (N=3 replicate experiments). *P<0.025 compared with the control, as determined by a *post-hoc* two-tail paired *t*-test.
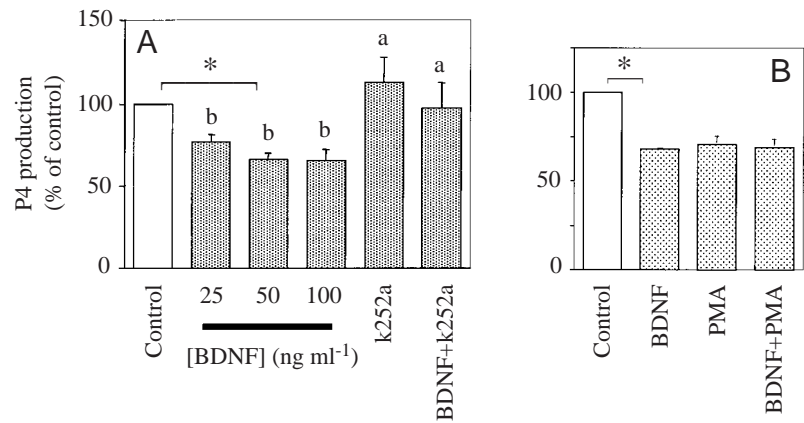
As the potential for paracrine signaling is enhanced simultaneously with the commencement of steroidogenesis, it was hypothesized that BDNF/TrkB expression might be involved in the regulation of steroid production within preovulatory granulosal cells. In fact, treatment of cultured preovulatory follicle granulosal cells with BDNF decreased basal progesterone production compared with control cells or with cultures containing BDNF in the presence of the putative Trk kinase inhibitor k252a. These data indicate that the BDNF ligand can function as a subtle negative modulator of progesterone levels in preovulatory follicle granulosal cells in culture through the TrkB receptor-mediated signaling pathway.

The decrease in progesterone induced by treatment with 50 ng ml^{-1} BDNF is comparable with the level of inhibition exerted by activation of the PKC intracellular signaling pathway using the phorbol ester PMA (Fig. 5; Tilly and Johnson, 1991). Furthermore, the present experiments show that there is no further change in the level of negative regulation when granulosal cells were co-cultured with PMA plus BDNF, indicating that TrkB- and PMA-induced signaling may occur through the same pathway. Thus, it is proposed that PKC activation represents at least one intracellular pathway activated by BDNF-induced TrkB signaling, and this is consistent with results from previous studies documenting the ability of TrkB signaling to activate PKC in mammalian cells (rat cerebellar granule neurons, Zirrgiebel et al., 1995; Coffey et al., 1997; PC12 cells, Williams et al., 1998). In contrast, from related experiments, it was determined that extracellular-signal-regulated kinase (Erk) phosphorylation was absent from BDNF-treated granulosal cells (data not shown), indicating that TrkB signaling does not occur through this mitogen-activated protein kinase (MAP kinase) pathway. This finding apparently contrasts with results from PC12 cells, in which TrkB activation results in cell signaling *via* MAP kinase signaling (Williams et al., 1998).

Although the present studies do not rule out the potential for additional actions of BDNF/TrkB signaling within the granulosal layer, it is interesting to speculate on the possible physiological relevance of BDNF-mediated attenuation of progesterone production in preovulatory follicle granulosa. It is unlikely that BDNF plays any role in modulating the gonadotropin-induced preovulatory progesterone surge because preliminary studies (T. Jensen and A. L. Johnson, unpublished results) show no inhibitory effects of BDNF treatment on LH-induced progesterone production after a 3 h incubation. Instead, it is suggested that, since gonadotropins and VIP have been shown to induce steroid synthesis and have been proposed to maintain trkB mRNA expression, a constant low level of BDNF secretion by the thecal layer may act as a negative feedback mechanism to regulate basal progesterone production and prevent increases in progesterone levels other than those associated with the preovulatory LH surge. For instance, there are reports of a small crepuscular peak in LH levels (P. A. Johnson and van Tienhoven, 1984), which does not appear to be associated with an increase in serum progesterone levels. Indeed, granulosa progesterone secretion has been demonstrated to be inhibited by perifusion in the presence of the F2 theca layer, presumably in specific response to some, as yet unidentified, secreted factor (P. A. Johnson et al., 1987). Finally, in the light of recent data that clearly show a direct relationship between steroidogenic acute regulatory protein (StAR) expression and progesterone production by preovulatory follicle granulosal cells (Johnson and Bridgham, 2001), it will be of interest to evaluate whether BDNF treatment is capable of modulating StAR expression. Alternatively, TrkB signaling may promote cytochrome P_{450} 17α-hydroxylase activity and the conversion of progesterone to androgens, which in turn would decrease basal secretion of progesterone. Neither of these proposals was tested experimentally in the present studies.

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