Development/Plasticity/Repair

# Activation of erbB-1 Signaling in Tanycytes of the Median Eminence Stimulates Transforming Growth Factor $\beta_1$ Release via Prostaglandin $E_2$ Production and Induces Cell Plasticity

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**The activation of transforming growth factor $\alpha$ (TGF$\alpha$)– erbB-1 and neuregulin– erbB-4 signaling pathways in hypothalamic astrocytes has been shown to play a key role in the process by which the neuroendocrine brain controls luteinizing hormone-releasing hormone (LHRH) secretion. Earlier studies suggested that tanycytes, an ependymoglial cell type of the median eminence, regulate LHRH release during the estrous cycle by undergoing plastic changes that alternatively allow or prevent direct access of the LHRH nerve terminals to the portal vasculature. Neither the molecules responsible for these plastic changes nor the underlying controlling mechanisms have been identified. Here we show that cultured tanycytes express erbB-1 and erbB-2, two of the four members of the erbB receptor family, and respond to TGF$\alpha$ with receptor phosphorylation, release of prostaglandin $E_2$ ($PGE_2$), and a $PGE_2$-dependent increase in the release of TGF$\beta_1$, a growth factor previously implicated in the glial control of LHRH secretion. Blockade of either erbB-1 receptor signal transduction or prostaglandin synthesis prevented the stimulatory effect of TGF$\alpha$ on both $PGE_2$ and TGF$\beta_1$ release. Time-lapse studies revealed that TGF$\alpha$ and TGF$\beta_1$ have dramatically opposite effects on tanycyte plasticity. Whereas TGF$\alpha$ promotes tanycytic outgrowth, TGF$\beta_1$ elicits retraction of tanycytic processes. Blockade of metalloproteinase activity abolished the effect of TGF$\beta_1$, suggesting that TGF$\beta_1$ induces tanycytic retraction by facilitating dissolution of the extracellular matrix. Prolonged (>12 hr) exposure of tanycytes to TGF$\alpha$ resulted in focal tanycytic retraction, an effect that was abolished by immunoneutralization of TGF$\beta_1$ action, indicating that the retraction was attributable to TGF$\alpha$-induced TGF$\beta_1$ formation. These *in vitro* results identify tanycytes as targets of TGF$\alpha$ action and demonstrate that activation of erbB-1-mediated signaling in these cells results in plastic changes that, involving $PGE_2$ and TGF$\beta_1$ as downstream effectors, mimic the morphological plasticity displayed by tanycytes during the hours encompassing the preovulatory surge of LHRH.**

*Key words:* **neuroendocrine; TGF$\alpha$; TGF$\beta$; ependymoglial cells; LHRH; cell plasticity; hypothalamus**

## Introduction

Essential for normal sexual development and reproductive cyclicity is the timely release of the neuropeptide luteinizing hormone-releasing hormone (LHRH) into the portal blood vessels of the median eminence. Once delivered by the portal vasculature to the pituitary gland, LHRH binds to specific receptors to stimulate both the secretion and synthesis of the gonadotropins luteinizing hormone and follicle-stimulating hormone. In turn, these hormones promote gonadal steroid secretion and gametogenesis.

LHRH neurosecretion is controlled by transsynaptic inputs of both inhibitory and excitatory natures and by growth factor-dependent glia-to-neuron signaling pathways (for review, see Ojeda and Terasawa, 2002). One of these pathways uses transforming growth factor $\alpha$ (TGF$\alpha$) and neuregulins (NRGs), two members of the epidermal growth factor (EGF) family of growth factors, and their erbB-1 and erbB-4 receptors for astroglial–LHRH neuron communication (for review, see Ojeda et al., 2001). Another pathway uses TGF$\beta_1$ and TGF$\beta_2$ and their cognate receptors (for review, see Prevot, 2002). An important difference between the two systems is the absence of erbB receptors in LHRH neurons (Voigt et al., 1996), which are, however, endowed with TGF$\beta$ receptors (Prevot et al., 2000). Consistent with this distribution, TGF$\alpha$ and NRGs do not stimulate LHRH release directly; instead, they do so via a juxtacrine mechanism that involves the activation of astrocytic erbB receptors and the subsequent release of prostaglandin $E_2$ ($PGE_2$), which then acts on LHRH neurons to induce LHRH secretion (Ma et al., 1997; Rage et al., 1997). TGF$\beta$s, on the other hand, can act directly on LHRH neurons to elicit changes in both LHRH gene expression and LHRH output (Melcangi et al., 1995; Galbiati et al., 1996; Buchanan et al., 2000). In contrast to erbB ligands, which act on

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both immortalized GT1–7 LHRH neurons and median eminence LHRH nerve terminals to elicit LHRH release (Ojeda et al., 1990; Ma et al., 1997), TGFβs are only effective in GT1 cells (Ojeda et al., 1990; Melcangi et al., 1995; Buchanan et al., 2000) (S. Bouret and V. Prevot, unpublished data), suggesting that, instead of acting on the secretory terminals, these growth factors facilitate LHRH secretion mostly via actions exerted on the LHRH neuronal cell bodies.

The specialized ependymal cells of the median eminence known as tanycytes (Leonhardt, 1966; Knowles, 1972), however, express abundant levels of TGFβ type I receptor mRNA (Prevot et al., 2000), suggesting the participation of TGFβ-dependent mechanisms in the regulation of tanycyte physiology. Tanycytes and astrocytic tanycytes, an intermediate cell type of the median eminence (Záborszky and Schiebler, 1978; Rützel and Schiebler, 1980), are also rich in erbB-1 and erbB-2 but lack erbB-4 receptors (Ma et al., 1994a, 1999), indicating that erbB-1 and TGFβ receptor-mediated signaling mechanisms converge in these specialized cells to regulate specific aspects of tanycytic function. A well characterized morphological association of tanycytes in the median eminence is that with LHRH nerve terminals (Kozlowski and Coates, 1985; Ugrumov et al., 1985, 1989). Tanycytic processes closely appose LHRH nerve terminals traveling down to the external layer of the median eminence. They also intervene between the nerve endings and the endothelial wall via tanycytic "end feet," which essentially prevent direct access of the terminal to the portal vasculature (Ugrumov et al., 1985, 1989; King and Letourneau, 1994; Prevot et al., 1999). This intervention is, however, highly plastic because it changes according to the steroid milieu present (King and Letourneau, 1994), and it becomes noticeably less pronounced during the preovulatory surge of gonadotropins. At this time, tanycytic end feet retract, allowing a significant fraction of LHRH nerve endings to directly contact the pericapillary space (King and Rubin, 1996; Prevot et al., 1998, 1999). A similar dynamic relationship between glial cells and vasopressin nerve terminals has been described in the posterior pituitary (for review, see Hatton, 2002) (for additional references, see Miyata et al., 2001).

The cell–cell communication molecules that may contribute to this glial plasticity are unknown. In the present report, we identify an erbB-1-dependent signaling pathway associated with activation of TGFβ$_1$ release as one of the underlying mechanisms. We show that stimulation of erbB-1 receptors in ependymoglial cells results in biphasic plastic changes characterized by an initial phase of outgrowth and a secondary phase of retraction. Although the initial outgrowth is independent of the TGFβ system and partially requires formation of $PGE_2$, the subsequent retraction requires $PGE_2$ synthesis and a $PGE_2$-dependent increase in the production of TGFβ$_1$.

## Materials and Methods

*Growth factors, inhibitors, and antibodies*. Human recombinant NRGβ$_1$ was purchased from Neomarkers (Union City, CA), TGFα was from Becton Dickinson Biosciences (Belford, MA), and betacellulin and TGFβ$_1$ were purchased from R & D Systems (Minneapolis, MN). The erbB-1 receptor inhibitor AG1478 was from Calbiochem (La Jolla, CA); the cyclooxygenase inhibitor indomethacin was from Sigma (St. Louis, MO); and the metalloproteinase inhibitor GM6001 (Ilomastat) was from Chemicon (Temecula, CA). TGFβ-neutralizing antibodies were purchased from Oncogene Research Products (Cambridge, MA). The sheep polyclonal antibody used for the immunoprecipitation of erbB-1 was obtained from Fitzgerald Industries (Concord, MA). Polyclonal antibodies used for Western blot detection of erbB-1 (sc-03-G), erbB-2 (sc-284), erbB-3 (sc-285), and erbB-4 (sc-283) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody used for the detection of phospho-Tyr (4G10) levels was a generous gift from Dr. Brian Drucker (Oregon Health Science University, Portland, OR). The secondary antibodies anti-mouse and anti-rabbit peroxidase conjugate (HRP) were from Pierce (Rockford, IL), and the anti-goat and anti-sheep HRP (GT-34) was obtained from Sigma.

*Cell culture*. Hypothalamic astrocyte cultures were prepared from 2-d-old Sprague Dawley rats as recommended by McCarthy and De Vellis (1980), with the modifications reported previously by us (Ma et al., 1994b; Prevot et al., 2003). Primary cultures of tanycytes were prepared using median eminences dissected from 10-d-old rats. At this age, cell cultures from the median eminence contain numerous tanycytes and only a few astrocytes (Chauvet et al., 1996). After a growing period of 8–10 d in 75 cm culture flasks containing DMEM-F-12 high-glucose medium supplemented with 10% calf serum and 2 mM L-glutamine, the astrocytes and tanycytes were isolated from contaminant cells by overnight shaking at 250 rpm and were plated either in six-well plates (at 400,000 cells per plate) for TGFβ$_1$ and prostaglandin release experiments or in 15 cm dishes (1–1.5 million cells per dish) for immunoblot analysis. For immunohistochemistry and cell motility studies, the cells were seeded in six-well plates on poly-L-ornithine (100 μg/ml)- and laminin (2 μg/ml)-coated coverslips. After reaching 90% confluence, the medium was replaced with a serum-free, astrocyte- and tanycyte-defined medium (ADM) consisting of DMEM devoid of phenol red, supplemented with 2 mM L-glutamine, 15 mM HEPES, 5 μg/ml insulin, and 100 μM putrescine. The cells were used 48 hr later for experiments. To examine the effect of NRGβ$_1$ and TGFα on TGFβ$_1$ release, astrocytes and tanycytes were incubated in the presence of either peptide (at 100 ng/ml each) for 16 hr at 37°C. Time course experiments were also performed to determine the effect of TGFα (100 ng/ml) on $PGE_2$ and TGFβ$_1$ secretion from tanycytes after 4, 8, 12, 16, and 24 hr of treatment.

*Immunohistochemistry*. Immunohistochemistry was used to characterize tanycyte cell cultures from the median eminence and astrocyte cultures from the hypothalamus. When the cells reached 70% confluence, they were transferred to ADM. Two days later, they were washed twice with PBS, pH 7.4, and fixed in 4% paraformaldehyde for 15 min at room temperature. After extensive washes in 0.02 M potassium phosphate buffer containing 0.9% NaCl (KPBS), the cells were incubated in KPBS containing 0.3% heat-inactivated goat serum and 0.1% Triton X-100 for 30 min. Thereafter, they were incubated overnight at 4°C with the primary antibody diluted in KPBS.

Both tanycytes and hypothalamic astrocytes cultures were incubated with a rabbit polyclonal antibody to the tanycyte marker (Meister et al., 1988) dopamine- and cAMP-regulated phosphoprotein (DARPP-32, 1:200; Chemicon). A monoclonal antibody was used to detect the astrocytic marker glial fibrillary acidic protein (GFAP, 1:1000; Sigma), whereas Texas Red-X phalloidin (Molecular Probes, Eugene, OR) was used to visualize F-actin in tanycytes. The DARPP-32 immunoreactivity was developed with FITC-conjugated goat anti-rabbit IgG (1:250; Jackson ImmunoResearch, West Grove, PA), and GFAP was detected with Texas Red-conjugated goat anti-mouse IgG (1:250; Jackson ImmunoResearch).

Confocal images were acquired using a Leica TCS NT confocal system (Leica Microsystems, Exton, PA), with a 25×, numerical aperture 0.75, Plan Fluotar objective as described previously (Ma et al., 1999; Prevot et al., 2003).

*Immunoprecipitation and Western blots*. After 2 d in ADM, the cells seeded in 10 cm dishes were placed in fresh medium and were then treated with 100 ng/ml TGFα, 50 ng/ml betacellulin, or 1 μM tetradecanoyl-phorbol-13-acetate (TPA; Sigma) for 5 min. Some dishes were preincubated with GM6001 (50 μM, 30 min), a broad-spectrum inhibitor of metalloproteinase activity (Galardy et al., 1994), and stimulated with TPA as indicated. After treatment, the cells were briefly washed with ice-cold PBS and snap-frozen on dry ice. They were lysed in 500 μl of freshly prepared lysis buffer (25 mM Tris, pH 7.4, 50 mM β-glycerophosphate, 1% Triton X-100, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and Pepsamin A, 10 μg/ml aprotinin, and 100 μg/ml PMSF). Protein concentrations of the cell lysates were determined using a Bio-Rad (Hercules, CA) protein assay kit. For immunoprecipitation of erbB receptors, equal amounts of protein (750–1000 μg) in a total volume of

750 μl of lysis buffer were incubated with 1.5 μg of antibody with gentle rocking for 90 min at 4°C. Thereafter, the antigen–antibody complexes were incubated with 60 μl of protein A-Sepharose (Sigma) 1:1 slurry in lysis buffer for 45 min. The Sepharose beads were collected by centrifugation, washed two times in lysis buffer, resuspended in 50 μl of 2× sample buffer, boiled 5 min, and stored at −85°C. For Western blotting, samples were boiled again on thawing and fractionated in 8% polyacrylamide-SDS precast gels (Gradipore; Frenshs Forest, Australia). After electrophoresis at 130 V for 2 hr, the proteins were transferred for 4 hr at 4°C onto polyvinylidene difluoride membranes. The membranes were blocked in 2.5% enzyme immunoassay grade gelatin (Bio-Rad) in Tris buffered saline (TBS) for 1 hr at 37°C and subjected to immunoblotting using the monoclonal phosphotyrosine antibody 4G10 (1.5 μg/ml). After overnight exposure to the primary antibody at room temperature, the blots were washed in 0.05% Tween 20 and TBS, treated with anti-mouse horseradish peroxidase-conjugated secondary antibodies, and developed using enhanced chemiluminescence (Renaissance; PerkinElmer Life Sciences, Boston, MA). After stripping (62.5 mM Tris HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol, 30 min at 60°C), the membranes were reprobed with erbB-1 or erbB-2 antibodies.

*Determination of cell shape and motility.* Monolayer migration assays with cultured tanycytes were performed as described by Berens et al. (1994) and Zhou and Skalli (2000) for immortalized cell lines with some modifications. Two hundred thousand tanycytes contained in 70–120 μl of DMEM plus 10% calf serum were plated on poly-L-ornithine- and laminin-coated coverslips within an area delimited by a glass cloning cylinder (Bellco, Vineland, NJ) with an inner lumen diameter of 7 mm and an outer diameter of 8 mm; each cylinder was placed on the center of the coverslip. After a 4 hr incubation at 37°C in an atmosphere containing 95% $O_2$ and 5% $CO_2$, the cells formed a confluent circular monolayer on the surface of the coverslip encircled by the cloning cylinder. The cylinders were then removed, and 2 ml of astrocyte- and tanycyte-defined medium with or without test substances was added to the culture wells. The cells were then cultured for 8, 12, and 24 hr.

For morphological analysis, the cultures were fixed for 15 min at room temperature by adding 2 ml of 8% paraformaldehyde to the culture medium. Thereafter, the cells were gently rinsed in PBS and incubated in Texas Red-X palloidin (1:150) prepared in PBS containing 0.3% Triton X-100 for 20 min at room temperature. After one rinse in PBS, the coverslips were mounted on glass slides using PermaFluor (Immunon, Pittsburgh, PA). The circular area occupied by attached cells in each well was imaged using a CoolPix camera (Roper Scientific, Tucson, AZ) attached to a Zeiss (Thornwood, NY) Axioscope fluorescence microscope and analyzed using MetaMorph5.0 (Universal Imaging, West Chester, PA). Four images from random uniformly distributed fields of cells were acquired per circular area, with a 5× 0.16 Plan Apochromat objective. The length of cellular processes, cell motility, or both were measured as the distance between the preset location of the cells just after the removal of the glass cylinder and the edge of the migration front after 8, 12, or 24 hr of incubation with or without a treatment. For illustration purposes, a photo montage of each circular area occupied by cells was prepared with the help of Photoshop 5.0 (Adobe Systems, San Jose, CA) using 14–20 digitalized images acquired with a 20× 0.60 Plan Apochromat objective.

*Time-lapse recording experiments.* Tanycytes were seeded in cloning cylinders positioned on 37 mm culture chambers (MatTek Corp., Ashland, MA) containing poly-L-ornithine- and laminin-coated glass coverslips, as described above. After removal of the cylinder, the cells were maintained in Leibovitz's L15 medium without phenol red containing 5 μg/ml insulin and 100 μM putrescine. The dishes were then perifused (5 ml/hr at 37°C) in a temperature-controlled microscope stage microincubator (Harvard Apparatus, Holliston, MA). Living cells were imaged every 2 min for 12 hr using a Leica TCS SP confocal system with a 10× 0.30 Plan Fluotar objective using differential interference contrast optics and the 647 nm line of a HeNe laser. Stack images were built in Metamorph.

At the end of the experiments, cell viability was assessed using the Live/Dead viability and cytotoxicity kit (Molecular Probes, OR). This kit enables simultaneous determination of live and dead cells with two probes that measure two parameters of cell viability: intracellular esterase activity using the polyanionic dye calcein and plasma membrane integrity using ethidium homodimer. Whereas polyanionic dye calcein produces an intense green fluorescence in live cells, ethidium homodimer produces a bright red fluorescence in dead cells.

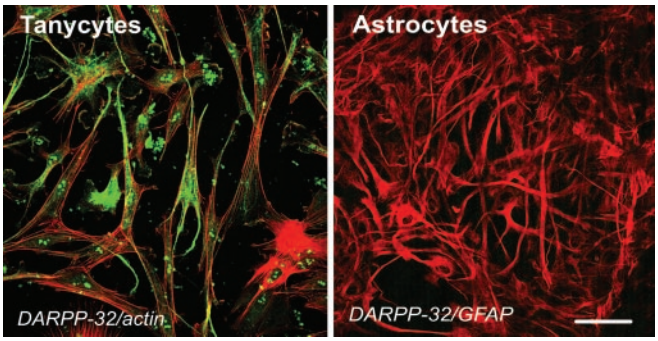


**Figure 1.** DARPP-32 immunostaining in primary cultures of tanycytes and astrocytes, the two major glial cell types present in the median eminence of the hypothalamus. Although high levels of immunoreactive DARPP-32 are detected in tanycytes cultured in ADM (left panel, green), DARPP-32-immunoreactivity is absent in GFAP-positive cultures of hypothalamic astrocytes (right panel, red). F-actin was visualized in tanycytes (left panel, red) by exposing the fixed and permeabilized cells to phalloidin (diluted 1:150) for 20 min at room temperature. Scale bar, 10 μm.

*Bromodeoxyuridine-labeling index.* To determine whether TGFα or β treatment affected cell proliferation, replicating cells were identified by their ability to incorporate bromodeoxyuridine (BrdU) into their DNA. Two hundred thousand tanycytes were seeded onto coverslips coated with poly-L-ornithine and laminin. After the cells adhered to the substrate (4 hr), the medium was replaced with ADM containing 5 μg/ml BrdU (Roche Molecular Biochemicals , Indianapolis, IN). The coverslips ($n = 6$ per condition) were then incubated at 37°C for 24 hr. After 15 min fixation in 4% paraformaldehyde, the cells were rinsed and subjected to an antigen retrieval protocol consisting of microwaving the coverslips for 3 min in sodium citrate buffer (Antigen Retrieval Citra; BioGenex Laboratories Inc., San Ramon, CA), as recommended (Shi et al., 1991), before incubation with the primary antibody. Cells were then incubated with a monoclonal antibody to BrdU (diluted at 1:1000; Sigma) overnight at 4°C, and the immunoreaction was developed the next day using a Texas Red-conjugated goat anti-mouse IgG (1:400; Jackson ImmunoResearch). Cell nuclei were stained with Hoescht (Molecular Probes). Four images from random fields were acquired per coverslip using a 20× objective, and colors were merged using Photoshop 5.0. For each image, the number of double-labeled nuclei and the total number of cells were counted.

*Measurements of $TGF\beta_1$ and $PGE_2$.* $TGF\beta_1$ released into the culture medium by astrocytes and tanycytes was measured using an enzyme-linked immunosorbent assay kit (TGFβ Emax; Promega, Madison, WI) according to the manufacturer's instructions. The sensitivity of this assay is 32 pg/ml, and the linear range of determinations is between 32 and 1000 pg/ml. $PGE_2$ released from tanycytes was measured by radioimmunoassay, as described previously (Ojeda et al., 1986). This assay detects 3.5 pg/ml.

*Statistics.* The differences between several groups were analyzed by ANOVA followed by the Student–Newman–Keuls multiple-comparison test for unequal replications.

## Results

### Purity of glial cell cultures

To study the physiology of tanycytes *in vitro*, we prepared primary cultures from 10-d-old rat median eminences. As previously described by others (Chauvet et al., 1996), >80% of the cells in the resulting cultures displayed intense immunoreactivity for DARPP-32 (Fig. 1*A*), a phosphoprotein that in the hypothalamus is specifically expressed in tanycytes (Meister et al., 1988) and particularly in tanycytic processes in contact with LHRH nerve endings (Meister et al., 1988). In contrast, primary cultures

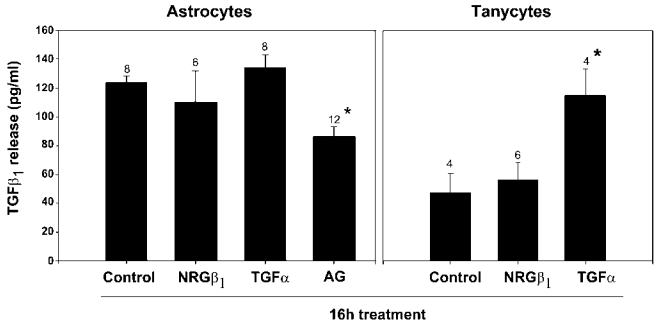


**Figure 2.** TGFα (100 ng/ml) stimulates TGFβ1 release from tanycytes (left panel) but not astrocytes (right panel) cultured in ADM for 16 hr as assessed by ELISA. AG1478 (left panel, AG, 50 μM), an inhibitor of erbB-1 receptor-tyrosine kinase activity, significantly reduces TGFβ1 production by hypothalamic astrocytes, indicating that activation of erbB-1 signaling is required for basal TGFβ1 release in these glial cells. NRGβ1, another member of the EGF family, is ineffective in stimulating TGFβ1 secretion from either hypothalamic astrocytes or tanycytes. *$p < 0.05$ versus basal release (control). In this and subsequent figures, columns are means; error bars indicate SEM; and the numbers on top of the vertical lines are the numbers of independent observations per group.

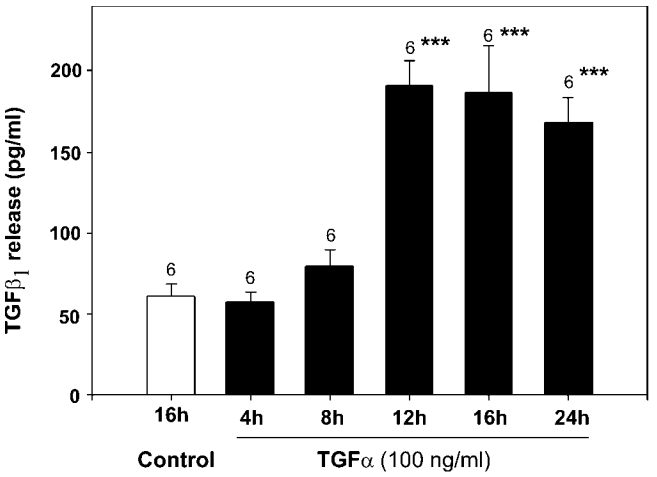


**Figure 3.** Time course of the stimulatory effect of TGFα (100 ng/ml) on TGFβ1 release from cultured tanycytes. ***$p < 0.001$ versus control.

of hypothalamic astrocytes that were strongly immunoreactive for GFAP (>95% of the culture) were devoid of DARPP-32 immunoreactivity (Fig. 1*B*). Neither culture contained cells immunoreactive for the neuronal marker NeuN, and very few cells (<1%) were immunoreactive for O4 and von Willerbrand factor, suggesting that the cultures were almost devoid of oligodendrocytes and endothelial cells, respectively (data not shown).

### Tanycytes respond to TGFα with increased formation of TGFβ1

It is now clear that a glia-to-neuron signaling pathway regulates LHRH release within the hypothalamus (Ojeda et al., 2000; Dhanpapani et al., 2003). This communication pathway is initiated by glial cells using a paracrine–juxtacrine mode of communication that requires the participation of TGFα and NRGβ1, two members of the EGF family (Ojeda et al., 2000). It has also been suggested that, in addition, astrocytes might use TGFβ1, a member of the TGFβ superfamily, to facilitate LHRH secretion (Melcangi et al., 1995; Galbiati et al., 1996). Whether these systems also operate in tanycytes of the median eminence is not known. Because TGFα has been shown to upregulate TGFβ gene expression in astrocytes (Melcangi et al., 2000), we asked whether EGF-related peptides might affect the release of TGFβ1 from astrocytes and tanycytes. Primary cultures of astrocytes and tanycytes were treated with TGFα or NRGβ1 (100 ng/ml) for 16 hr, and the resulting conditioned medium was assayed for TGFβ1. Figure 2 shows that both hypothalamic astrocytes and tanycytes release TGFβ1 under basal unstimulated conditions (control), that the basal release of TGFβ1 is greater in astrocytes than tanycytes, and that TGFα stimulates TGFβ1 release in cultured tanycytes but not in hypothalamic astrocytes. In contrast to TGFα, NRGβ1 did not increase TGFβ1 secretion in either hypothalamic astrocytes or tanycytes. Exposing astrocytic cultures to AG1478, an inhibitor of erbB-tyrosine kinase activity, partially reduced TGFβ1 levels in the culture medium (Fig. 2), suggesting that the greater TGFβ1 release observed in astrocytes than tanycytes is in part attributable to higher basal erbB-1 activity. TGFβ1 release from tanycyte cultures begins to increase in response to TGFα after 8 hr, reaching maximal values by 12 hr, and remains elevated for at least the next 12 hr (Fig. 3).

### TGFα-dependent stimulation of TGFβ1 release in tanycytes involves activation of the erbB-1–erbB-2 signaling module

To determine whether the erbB receptor proteins are expressed in tanycyte cultures and are functionally active, we performed immunoblots and coimmunoprecipitation assays. Whereas erbB-1–erbB-4 receptors were present in protein extracts from the median eminence, cultured tanycytes contained mostly erbB-1 and erbB-2 receptors. These cells also showed low levels of erbB-3, but they lacked detectable erbB-4 (Fig. 4*A*). Because previous immunohistochemical and *in situ* hybridization studies showed the presence of erbB-1 and erbB-2 but not erbB-4 in tanycytes of prepubertal female rats (Ma et al., 1994a, 1999), the present results indicate that the expression pattern of these receptors in tanycytes of the median eminence is identical *in vitro* and *in vivo*. The presence of erbB-3 in these cells is also consistent with the detection of erbB-3 mRNA in tanycytes of the adult rat brain (Steiner et al., 1999). Because of the greater abundance of erbB-1 and erbB-2 than erbB-3 receptors in cultured tanycytes and the lack of effect of NRGβ1 on TGFβ1 release, we focused our attention on the erbB-1–erbB-2 system in subsequent experiments. Figure 4*B–D* shows that activation of tanycytic erbB-1 and erbB-2 receptors with either TGFα or betacellulin, another natural ligand of erbB-1 receptors, results in tyrosine phosphorylation of the receptors. Coimmunoprecipitation assays (Fig. 4*B–D*) demonstrated that erbB-1 and erbB-2 receptors are physically associated, and this heterodimeric complex is constitutive in tanycytes because immunoprecipitation of erbB-1 receptors coprecipitates erbB-2 receptors and vice versa, even in the absence of ligand stimulation.

Having demonstrated that tanycytes in culture contained functional erbB-1 receptors, we next investigated whether the stimulation of TGFβ1 release by TGFα is mediated by the activation of these receptors. Incubation of the cells with the erbB-1 inhibitor AG1478 abolished the ability of TGFα to stimulate TGFβ1 secretion in tanycytes after 12 hr of treatment and significantly reduced basal TGFβ1 release (Fig. 4*E*). Thus, cultured tanycytes appear to have active erbB-1 receptors, likely because of the ability of these cells to produce TGFα (Ma et al., 1992).

To determine whether tanycytes, as recently shown for astrocytes (Dziedzic et al., 2003), have all the molecular components required for the juxtacrine and paracrine ligand-dependent activation of erbB-1 signaling, we treated tanycyte cultures with TPA,

an activator of PKC that promotes the shedding of erbB ligands through the activation of metalloproteinases (Pandiella and Massague, 1991; Prenzel et al., 1999). Figure 4*F* shows that within 5 min of treatment, TPA induced the phosphorylation of tanycytic erbB-1 receptors, and this effect was obliterated when the cells were pretreated for 30 min with GM6001, a broad-spectrum inhibitor of metalloproteinase activity. Thus, cleavage of the erbB-1-ligand through metalloproteinase-dependent mechanisms is likely to be an integral component of the process by which erbB-1 receptors are activated in cultured tanycytes.

### TGF$\beta_1$ secretion resulting from the activation of the TGF$\alpha$–erbB-1 signaling pathway in tanycytes requires $PGE_2$ release

In previous studies, we showed that TGF$\alpha$-induced erbB-1 activation in the median eminence of the hypothalamus results in the production of $PGE_2$, and $PGE_2$ induces LHRH release from median eminence fragments (Ojeda et al., 1990; Prevot et al., 2003). A strong body of evidence indicates that $PGE_2$ is, at least in part, secreted by astrocytes in response to erbB signaling (Ma et al., 1997; Prevot et al., 2003). To determine whether TGF$\alpha$ is also able to stimulate $PGE_2$ production by tanycytes, cultured tanycytes were exposed to 100 ng/ml TGF$\alpha$ for various periods. Figure 5*A* shows that although $PGE_2$ levels remained low during the first 8 hr of treatment, they increase dramatically after 12 hr, declining gradually thereafter. Inhibition of erbB-1 receptor-tyrosine kinase activity with AG1478 abolished the stimulatory effect of TGF$\alpha$ on $PGE_2$ release (Fig. 5*B*), whereas blockade of cyclooxygenase, the rate-limiting enzyme in prostaglandin synthesis, with indomethacin abolished both basal and TGF$\alpha$-stimulated $PGE_2$ release (Fig. 5*B*).

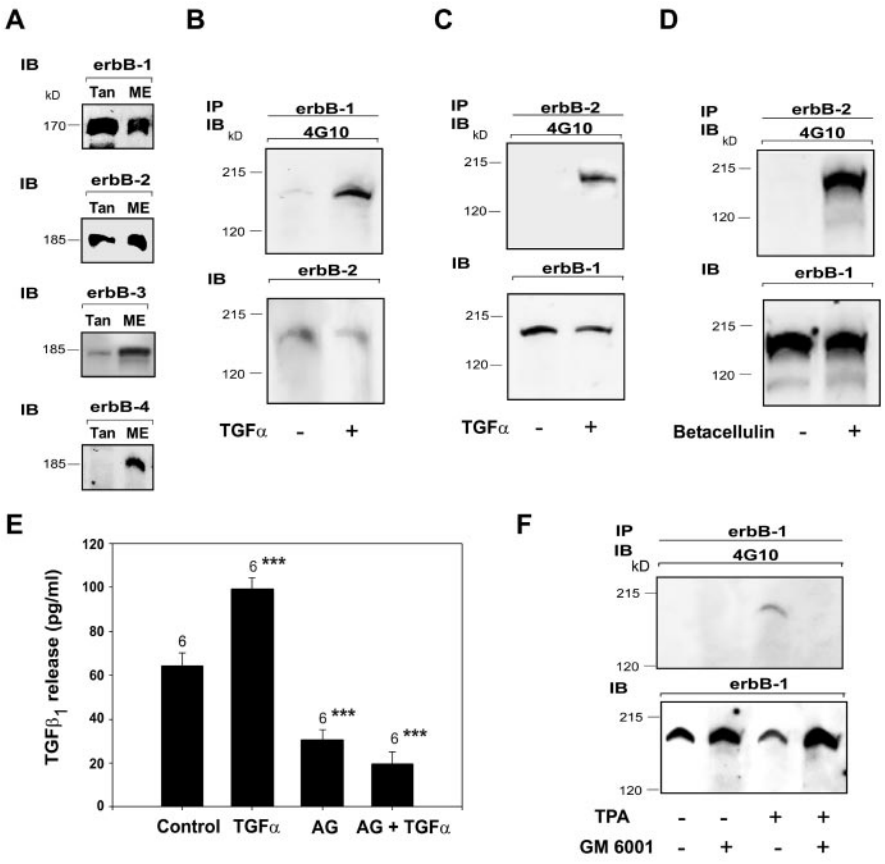


**Figure 4.** Tanycytes (Tan) of the median eminence (ME) contain erbB-1, erbB-2, and low levels of erbB-3 but not erbB-4 receptors (*A*). For erbB-1 and erbB-2 detection, each lane was loaded with 25 $\mu$g of protein; for erbB-3 detection, the tanycyte lane was loaded with 50 $\mu$g of protein, and the ME lane was loaded with 100 $\mu$g. Activation of erbB-1 signaling with either TGF$\alpha$ (*B, C*) or betacellulin (*D*) results in tyrosine phosphorylation of the erbB-1–erbB-2 heterodimeric complex. Tanycytes were cultured in defined medium and were exposed to erbB-1 ligands (100 ng/ml) for 5 min. Proteins were collected after treatment and immunoprecipitated (IP) with specific erbB-1 (*B*) or erbB-2 (*C, D*) antibodies, electrophoresed to size fractionate the immunoprecipitated species, and immunoblotted (IB) with antibodies to phosphotyrosine (4G10). Then immunoblots were stripped and reprobed with antibodies to erbB-2 (*B*) or erbB-1 (*C, D*). Note that immunoprecipitation of erbB-1 receptors coimmunoprecipitates erbB-2 receptors (*B*), and, conversely, immunoprecipitation of erbB-2 receptors coimmunoprecipitates erbB-1 (*C, D*), even in the absence of ligand stimulation. *E*, Exposure of tanycytic cultures to AG1478 (50 $\mu$M), an inhibitor of erbB-1 signaling, significantly blocks both basal TGF$\beta_1$ production (control) and TGF$\alpha$-induced TGF$\beta_1$ release (***$p < 0.001$ vs control). *F*, Activation of PKC with TPA results in the metalloproteinase-dependent transphophorylation of erbB-1 receptors in tanycytes of the median eminence. Tanycytes were treated for 5 min with TPA (1 $\mu$M). To block metalloproteinase activity, some cultures were pretreated for 30 min with GM6001 (50 $\mu$M). After treatment, erbB-1 receptors were immunoprecipitated, and the phosphorylated species were detected by immunoblotting with antibody 4G10. Then the immunoblot was stripped and reprobed with antibodies to erbB-1 to ensure that equal amounts of erbB-1 protein had been immunoprecipitated and loaded on the gel.

Because the profile of $PGE_2$ release in response to TGF$\alpha$ treatment (Fig. 5*A*) was strikingly similar to that of TGF$\beta_1$ (Fig. 3) in tanycytes, we performed experiments to determine whether these two secretory events are causally related. Cultured tanycytes were exposed to TGF$\alpha$ and indomethacin for 16 hr, and TGF$\beta_1$ levels were measured at this time. Figure 5*C* shows that indomethacin not only suppressed the stimulatory effect of TGF$\alpha$ on TGF$\beta_1$ secretion but also significantly reduced basal TGF$\beta_1$ output.

### TGF$\alpha$ and TGF$\beta_1$ exert dramatically opposite effects on tanycyte plasticity

Previous electron microscopy studies suggested that tanycytes regulate the direct access of LHRH neuroendocrine terminals to the pericapillary space in the external zone of the median eminence through plastic remodeling of their end feet processes that envelop LHRH nerve endings (King and Letourneau, 1994; Prevot et al., 1999). To investigate whether TGF$\alpha$ and TGF$\beta_1$ signaling would be involved in the remodeling of the tanycyte cytoarchitecture, we tested the ability of both growth factors to induce plastic rearrangements in isolated tanycytes in culture. Confluent circular monolayers of tanycytes were subjected to different treatments for various periods, and morphometric analyses were performed to measure tanycytic process extension, tanycyte motility, or both from preset locations in the culture toward more peripheral locations (outgrowth and migration) or inward (ingrowth and retraction).

As shown in Figure 6, TGF$\alpha$ (50 ng/ml) promoted both cell migration and outgrowth of tanycytic processes when compared with control cultures. TGF$\alpha$ induced extension of tanycyte processes in most parts of the circular cell monolayer (Fig. 6*B*). The growing processes exhibited bundles of actin filaments arranged in parallel along the entire longitudinal axis of the process (Fig.

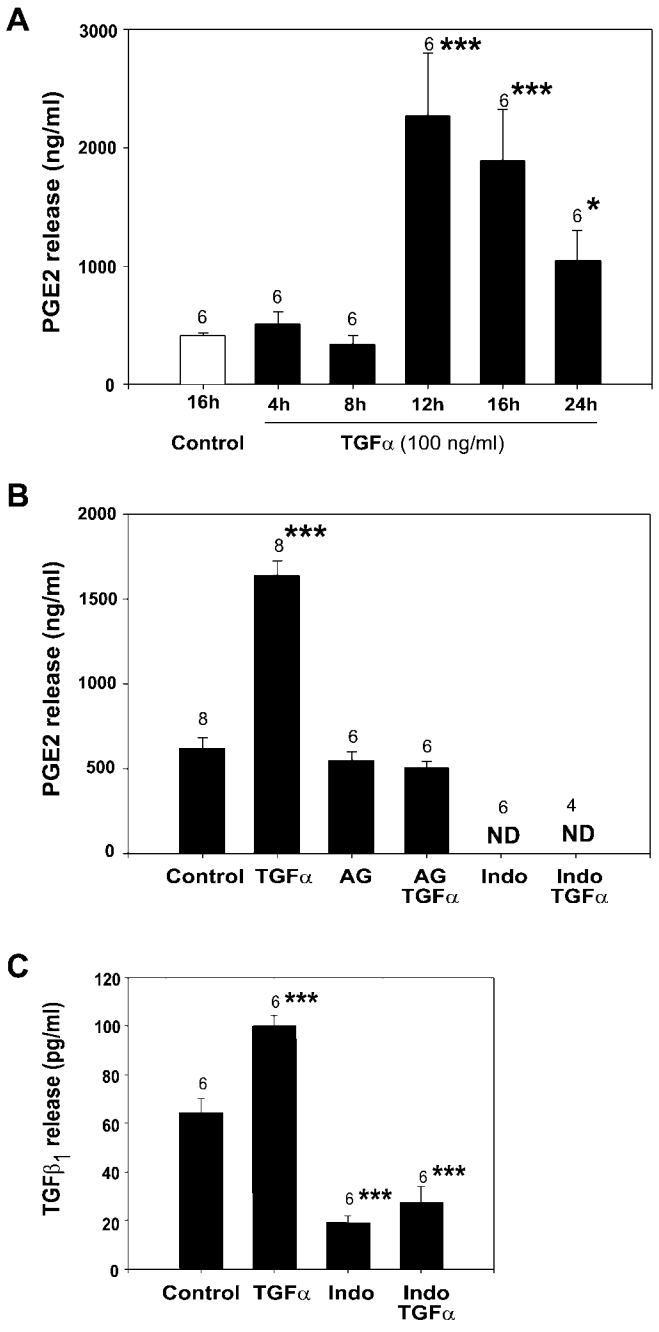


**Figure 5.** TGFα-induced TGFβ$_1$ secretion in tanycytes of the median eminence requires $PGE_2$ release. *A*, TGFα (100 ng/ml) increases $PGE_2$ production in tanycytes cultured in ADM after 12 hr of treatment as assessed by RIA. *B*, TGFα-induced $PGE_2$ release is blocked by the addition of AG1478 (50 μM), an erbB-1-tyrosine kinase blocker, to the culture medium and abolished by the addition of indomethacin (50 μM), an inhibitor of cyclooxygenase activity. *C*, Indomethacin obliterates both basal and TGFα-induced TGFβ$_1$ secretion from tanycytes (50 μM). $^*p < 0.05$; $^{***}p < 0.001$ versus control.

6*B*, inset). Morphometric analysis of these changes performed on circular monolayer cultures fixed after 8, 12, and 24 hr incubation periods revealed that TGFα treatment induced an increase in tanycytic outgrowth, motility, or both by 100% at 8 and 12 hr of treatment and by >300% after 24 hr of treatment when compared with controls ($p < 0.01$; Fig. 7). In contrast to TGFα, treatment of cultured tanycytes with TGFβ$_1$ (0.4 nM) caused a dramatic cell retraction within 8–12 hr (Fig. 6*C*). Quantitation of this effect is shown in Figure 7. To further understand the TGFα and TGFβ$_1$ effects on tanycytic plasticity, we performed time-lapse experiments during 12 hr periods. Figure 8*A–F* shows that cells in control cultures exhibited definite but limited linear motility. TGFα had a pronounced effect on cell motility and cell shape remodeling in tanycytes located at the edge of the culture (Fig, 8*G–L*, arrowheads) and promoted the formation of filopodial, finger-like extensions from tanycytic end feet (Fig. 8*H–J*, arrows) that eventually resulted in process outgrowth (Fig. 8*K,L*, arrows). TGFβ$_1$ had no overt effect on tanycyte motility during the first 2–4 hr of treatment. Thereafter, and within a few minutes, the cell monolayer began to move centripetally (Fig. 8*M–R*). The few cells that maintained their focal contacts on the laminin-coated coverslip at the preset location of the culture were then stretched between their adhesion points and the new inward location of the monolayer (Fig. 8*P*, arrows). These tanycytic processes eventually retracted when stretching forces appeared to exceed the adhesive strength of the focal contacts (Fig. 8*Q*, arrows). Sometimes the whole circular monolayer of tanycytes was released from the substrate and became detached from the coverslip (Fig. 8*R*). Assessment of cell viability at the end of each time-lapse experiment showed that the ratio of live/dead cells was identical in control conditions and in both TGFα- and TGFβ$_1$-treated conditions (data not shown).

Unexpectedly, we also noticed the presence of areas of cell retraction in 60% of the cultures treated with TGFα for 24 hr ($n = 26$; Fig. 9), whereas no retraction was noticed at shorter treatment times. Because TGFα stimulates TGFβ$_1$ release from tanycytes (Figs. 2–5), and because TGFβ$_1$ induces a dramatic retraction in circular tanycyte cultures (Figs. 6–9) we performed experiments to determine whether TGFα-induced tanycyte retraction involved TGFβ$_1$ release. As before, exposing the cells to TGFβ$_1$ alone resulted in a striking tanycyte retraction (Fig. 9). Exposure of TGFα-treated cultures to either TGFβ$_1$-neutralizing antibodies (Fig. 9) or indomethacin (data not shown), which inhibits the $PGE_2$ production required for TGFα-stimulated TGFβ$_1$ release (Fig. 5), abolished the focal retraction of tanycytes seen in these cultures ($n = 6$ for each condition). Interestingly, whereas TGFβ$_1$-neutralizing antibodies did not affect TGFα-promoted tanycytic outgrowth, motility, or both, indomethacin did inhibit these effects partially (by 20%; $p < 0.01$; data not shown).

Calculation of the BrdU-labeling index for tanycytes cultured in control conditions (2.5 ± 0.5%) or after treatment with either TGFα (2.7 ± 0.6%) or TGFβ$_1$ (2.2 ± 0.9%) showed that the proliferative capacity of tanycytes was not affected by these growth factors within a 24 hr period. Thus, the differential effect of TGFα and TGFβ$_1$ on cell migration is neither caused nor influenced by changes in cell proliferation.

### TGFβ$_1$-mediated retraction of tanycytic processes requires matrix metalloproteinase activity

To determine whether TGFβ$_1$-induced retraction of tanycytes requires extracellular matrix remodeling, degradation, or both, we subjected circular monolayers of cultured tanycytes to concomitant treatment with TGFβ$_1$ (0.4 nM) and GM6001 (50 μM), a broad-spectrum inhibitor of matrix metalloproteinases, the major extracellular matrix-degrading enzymes (Yong et al., 2001). Figure 10 shows that inhibition of matrix metalloproteinase activity abrogated the ability of TGFβ$_1$ to promote tanycytic retraction.

## Discussion

For >30 years, it has been recognized that the median eminence of the hypothalamus is a region of the brain endowed with an unusual degree of cellular plasticity (Kobayashi et al., 1972). Numerous studies (Leonhardt, 1966; Knowles, 1972; Rützel and Schiebler, 1980; Kozlowski and Coates, 1985; Prevot et al., 1999) have shown that tanycytes, the specialized ependymoglial cells that line the ventral surface of the third ventricle (Leonhardt, 1966; Knowles, 1972; Kozlowski and Coates, 1985; King and Letourneau, 1994), play a central role in this process. Morphological documentation of the changes in the relationship that exists between neuronal neurosecretory processes and tanycytes during both postnatal development and adulthood have demonstrated that a prominent site of neuronal–glial plasticity in the median eminence resides in the association between tanycytes and neurosecretory LHRH nerve terminals (Meister et al., 1988; Ugrumov et al., 1989). Although tanycytes also establish contact associations with other neuronal systems projecting to the median eminence, such as dopaminergic neurons (Meister et al., 1988; Ugrumov et al., 1989; Chauvet et al., 1995), their relationship with LHRH nerve terminals is perhaps one of the best characterized features of median eminence morphology (Kozlowski and Coates, 1985; Meister et al., 1988; Ugrumov et al., 1989). Indeed, the relationship that exist between tanycytes and LHRH nerve terminals does not appear to be shared by tanycytic processes and nerve fibers belonging to other peptidergic systems in the median eminence (Liposits et al., 1983; Beauvillain et al., 1984; Jew et al., 1984; Hisano et al., 1986; Ibata et al., 1986). In rodents, LHRH fibers travel caudally until they reach the level of the median eminence region. There, instead of continuing toward the neurohypophysis, they travel in close association with tanycytic processes toward the external layer of the median eminence (Kozlowski and Coates, 1985; Silverman et al., 1991), where they gain access to the portal vasculature that drains the median eminence of their neurosecretory products. Electron and confocal microscopy studies aimed at defining the dynamics of this neuro–hemal–tanycytic relationship either during the estrous cycle or after manipulation of the steroid milieu have led to the concept that tanycytic plasticity is a required component of reproductive cyclicity (Kozlowski and Coates, 1985; King and Letourneau, 1994; King and Rubin, 1996; Prevot et al., 1998, 1999), and cycling changes in the association of tanycytes to LHRH neurosecretory processes defines the capability of the terminals to access the endothelial wall of the portal vessels and, thus, to release LHRH into the portal circulation (King and Letourneau, 1994; Prevot et al., 1999). Under conditions of low gonadotropin output, most LHRH nerve endings are separated from the pericapillary space by tanycytic end feet, which prevent their direct access to the endothelial wall. At the time of the preovulatory surge (King and Rubin, 1996; Prevot et al., 1999) or after gonadectomy (King and Letourneau, 1994), two conditions of enhanced gonadotropin release, this relationship becomes less conspicuous. The nerve endings sprout toward the basal lamina of the endothelial wall (Prevot et al., 1999); the tanycytic end feet retract (King and Letourneau, 1994); and evaginations of the parenchymatous basal lamina overlaying the pericapillary space become prominent (for review, see Prevot, 2002).

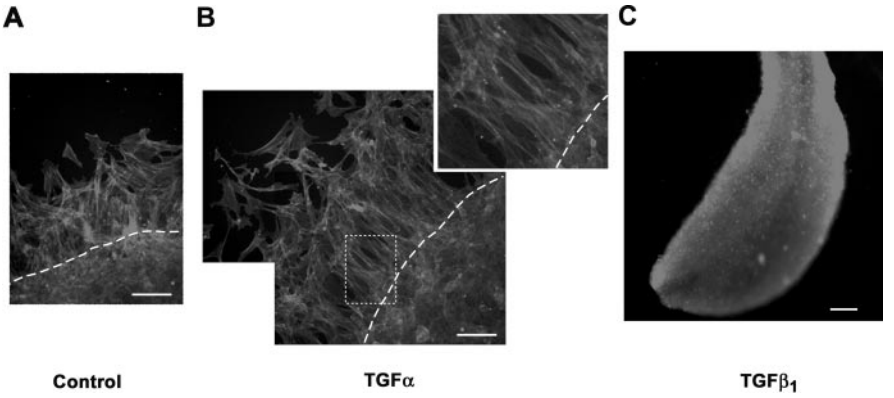


**Figure 6.** Ability of TGF$\alpha$ and TGF$\beta_1$ to induce plastic rearrangements of isolated tanycytes in culture as assessed by monolayer migration assays. Under basal unstimulated conditions, circular monolayers of tanycytes cultured in ADM for 24 hr exhibit a definite but limited ability to move from preset locations in the culture well (dotted lines) toward more peripheral locations (*A*). TGF$\alpha$ (50 ng/ml, 24 hr) dramatically increases the ability of tanycytes to extend processes and to migrate (*B*). Note that tanycytic processes exhibited bundles of actin filaments (red) arranged in parallel along the entire longitudinal axis of the process (*B*, inset). Activation of TGF$\beta$ signaling with TGF$\beta_1$ (0.4 nM) elicits a complete retraction of tanycytes within 12 hr (*C*). In all panels the actin cytoskeleton of tanycytes was labeled using Texas Red-labeled phalloidin and was visualized by fluorescent microscopy. Scale bars, 200 $\mu$m.

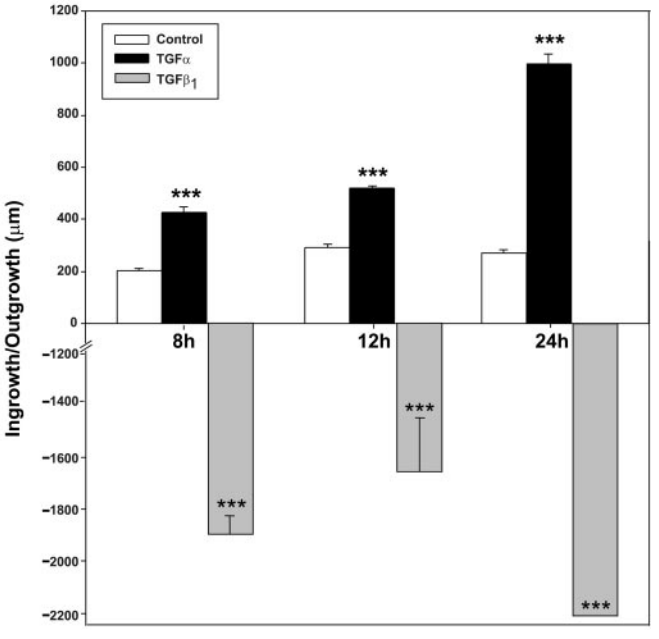


**Figure 7.** Quantitative morphometric analysis of the plastic changes elicited by TGF$\alpha$ and TGF$\beta_1$ on circular monolayers of tanycytes fixed after 8, 12, or 24 hr of treatment. ***$p < 0.001$ versus control.

Despite such detailed morphological characterization and the identification of several mechanisms controlling astrocyte–neuron communication in the neuroendocrine hypothalamus (Garcia-Segura et al., 1996; Miyata et al., 2001; Hatton, 2002; Oliet, 2002; Ojeda et al., 2003), there is a paucity of information concerning the cell–cell communication molecules that may underlie tanycytic plasticity. The present results provide evidence for the integrated role of two growth factor-dependent signaling systems in the regulation of this process. Because tanycytes contain TGF-$\beta$ receptor mRNA type I (Prevot et al., 2000) and the

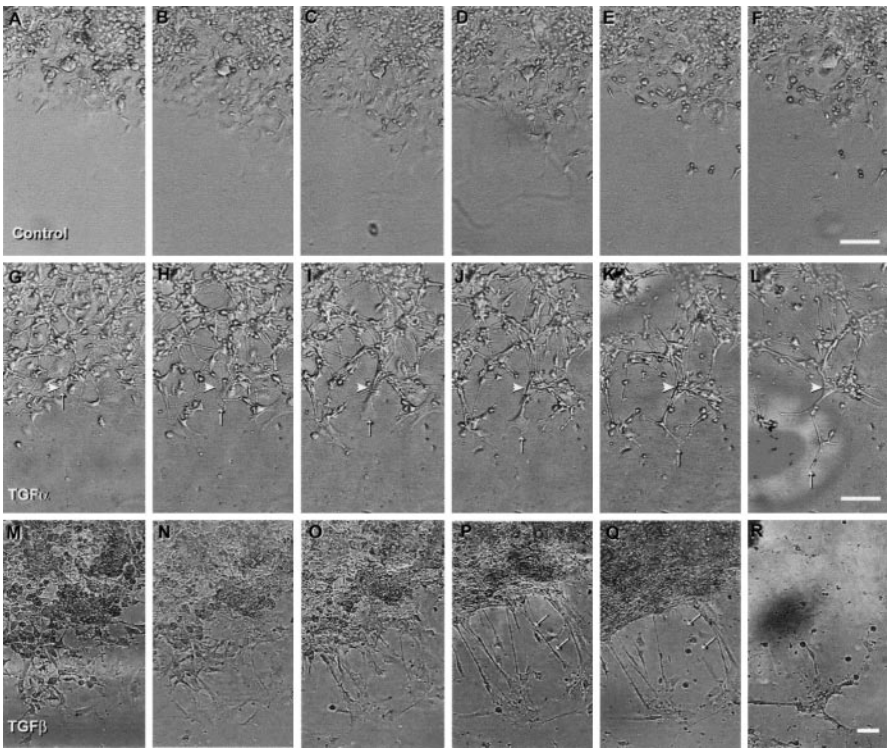


**Figure 8.** Time-lapse recording of TGF$\alpha$ and TGF$\beta_1$ effects on tanycytic morphology in circular cell monolayers perifused with Leibovitz's L15 medium for 12 hr. Under basal unstimulated conditions, tanycytes exhibit motility, but the overall movement of the cells at the edge of the circular monolayer is very limited (*A–F*). Treatment of tanycytes with TGF$\alpha$ (50 ng/ml) strongly stimulates cell motility and induces pronounced cell shape remodeling (*G–L*). Arrowheads in *G–L* point to a tanycytic end foot specialization that undergoes filopodial formation (*H–J*, arrows) and process extension (*K, L*, arrows) during the treatment period. When tanycytic cultures are exposed to TGF$\beta_1$ (0.4 nM), circular cell monolayers undergo dramatic retraction (*M–R*). In *P*, arrows point to tanycytes whose processes stretch between their initial adhesion point on the laminin-coated glass coverslip and the new inward location of the monolayer. These processes eventually retract in *Q* (arrows). Scale bars, 200 $\mu$m.

mRNAs encoding TGF$\alpha$ and its erbB-1–erbB-2 receptor complex, in addition to their immunoreactive proteins (Ma et al., 1992, 1994a, 1999), we considered the possibility that either one or both of these signaling systems might play a role in tanycytic plasticity. Consistent with the aforementioned histochemical findings, the results of our Western blot analysis show that tanycytes in culture contain erbB-1 and erbB-2 receptors but lack erbB-4 receptors, which are prominent in hypothalamic astrocytes (Ma et al., 1999). Activation of erbB-1 receptors with TGF$\alpha$, a specific erbB-1 ligand, or betacellulin, a peptide of the EGF family that activates all erbB receptors and their various heterodimeric forms (Dunbar and Goddard, 2000), resulted in both erbB-1 and erbB-2 receptor phosphorylation, indicating that the receptors are functional. Moreover, coprecipitation assays revealed that these receptors are physically associated both in basal and TGF$\alpha$-stimulated conditions, suggesting that production of TGF$\alpha$ and, perhaps, other erbB-1 ligands by cultured tanycytes may maintain a certain degree of receptor activity in the absence of added growth factors to the culture medium. Supporting this idea is the previous demonstration of both TGF$\alpha$ protein and its encoding mRNA in tanycytes *in situ* (Ma et al., 1992).

Like hypothalamic astrocytes (Ma et al., 1997), tanycytes respond to TGF$\alpha$ with release of $PGE_2$, but, in addition, they also release TGF$\beta_1$. Because basal TGF$\beta_1$ release is much higher in astrocytes than tanycytes, and these elevated levels are reduced to some extent by blocking erbB receptors, it might be concluded that cultured astrocytes exhibit a high level of ligand-dependent erbB-1 receptor activity, which is at least in part responsible for the increased basal output of TGF$\beta_1$ and the lack of a TGF$\beta_1$ response to exogenous TGF$\alpha$. Because other authors have shown that erbB-1 receptor activation increases TGF$\beta_1$ mRNA levels in cultured astrocytes (Lindholm et al., 1992), it is also possible that the dominant effect of TGF$\alpha$ on the astroglial TGF$\beta_1$ system might be to upregulate TGF$\beta_1$ gene expression rather than to increase release of the mature peptide. In contrast to astrocytes, tanycytes respond to TGF$\alpha$ with TGF$\beta_1$ release. Although tanycytes express low levels of the neuregulin receptor erbB-3, they lack erbB-4. Consistent with this absence, they fail to respond to NRG$\beta_1$ with changes in TGF$\beta_1$ release. These features and the ability of the tyrosine kinase blocker AG1478 to prevent the stimulatory effect of TGF$\alpha$ on TGF$\beta_1$ release indicate that the functional connection that exists between the EGF and TGF$\beta$ signaling systems in tanycytes is exclusively mediated by activation of erbB-1 receptors.

The intracellular mechanisms underlying the stimulatory effect of TGF$\alpha$ on TGF$\beta_1$ release involve the formation of $PGE_2$, an eicosanoid previously shown to mediate the stimulatory effects of TGF$\alpha$ on LHRH release (Ma et al., 1997; Rage et al., 1997). In addition to a temporal correlation between the release of $PGE_2$ and TGF$\beta_1$ after the initial exposure of the cells to TGF$\alpha$, blockade of cyclooxygenase activity abolishes the increase in both $PGE_2$ and TGF$\beta_1$ release, indicating that an increase in prostaglandin synthesis is a required step in the signaling pathway used by TGF$\alpha$ to stimulate TGF$\beta_1$ release. This effect of TGF$\alpha$ is likely mediated by an erbB-1-dependent stimulation of arachidonic acid release from the cell membrane, previously shown to occur in other cell types (Nolan et al., 1988; Handler et al., 1990). This release may result not only from an increased activity of phospholipase A2 (Nolan et al., 1988; Handler et al., 1990) but also from the phospholipase C-dependent metabolism of phosphatidyl inositols (Prescott and Majerus, 1983; Lapetina, 1984), which are key components of erbB signaling (Schlessinger, 2000). The involvement of additional downstream molecules known to participate in erbB-1-mediated cell signaling (Schlessinger, 2000) remains a distinct possibility.

On the basis of the above-described findings, any effect of TGF$\alpha$ or TGF$\beta_1$ on tanycytic plasticity could have been predicted as being unidirectional and sequentially controlled, first by TGF$\alpha$ and then by TGF$\beta_1$. Contrary to this expectation, the exposure of tanycytes to either growth factor resulted in diametrically opposite results: whereas TGF$\alpha$ enhanced tanycytic migration from their site of seeding, TGF$\beta_1$ elicited a dramatic retraction of the cells. The increased tanycyte motility and migration induced by TGF$\alpha$ is consistent with earlier observations in glioma cell lines showing that TGF$\alpha$ not only affects the expression of intermediate filament proteins (Zhou and Skalli, 2000) but also increases cell motility and induces changes in cell morphology (El-Obeid et al., 1997; Zhou and Skalli, 2000). As shown in time-lapse recordings, (Fig. 8*G–L*), tanycytes exposed to TGF$\alpha$ exhibit remarkable

changes in motility, extending and retracting filopodia as they migrate outwardly from their initial site of seeding. Our results are also consistent with earlier *in vivo* findings showing that infusion of EGF, another erbB-1 ligand, into the ventricular system of the mouse brain results in migration of subependymal cells away from the lateral ventricle into the adjacent parenchymal tissue (Craig et al., 1996). The main difference between these findings and ours is that tanycytes do not proliferate in response to TGFα, whereas proliferation is a prominent feature of the response of subependymal cells to erbB-1 receptor stimulation (Weickert and Blum, 1995; Craig et al., 1996; Tropepe et al., 1997). Because tanycytes have neither the constitutive proliferative capacity of subependymal progenitor cells nor the pluripotential capability of subependymal stem cells (Chiasson et al., 1999), their lack of proliferation in response to TGFα, a prominent feature of subependymal cell physiology (Craig et al., 1996; Tropepe et al., 1997; Junier, 2000), is not surprising. In this regard, the behavior of tanycytes *in vitro* is similar to that of subependymal progenitor cells that differentiate in response to TGFα into a limbic molecular phenotype but do not proliferate in the presence of the growth factor, regardless of the adhesive substrate used (Ferri et al., 1996). It thus appears that the migratory response of tanycytes to erbB-1 receptor activation represents more a growth factor-induced differentiated function than a developmental feature of cell migration. As such, it emphasizes the notion that the periodic changes in tanycyte morphology observed during the rodent adult estrous cycle (King and Rubin, 1996; Prevot et al., 1999) are differentiated events controlled at least in part by the erbB-1 signaling system.

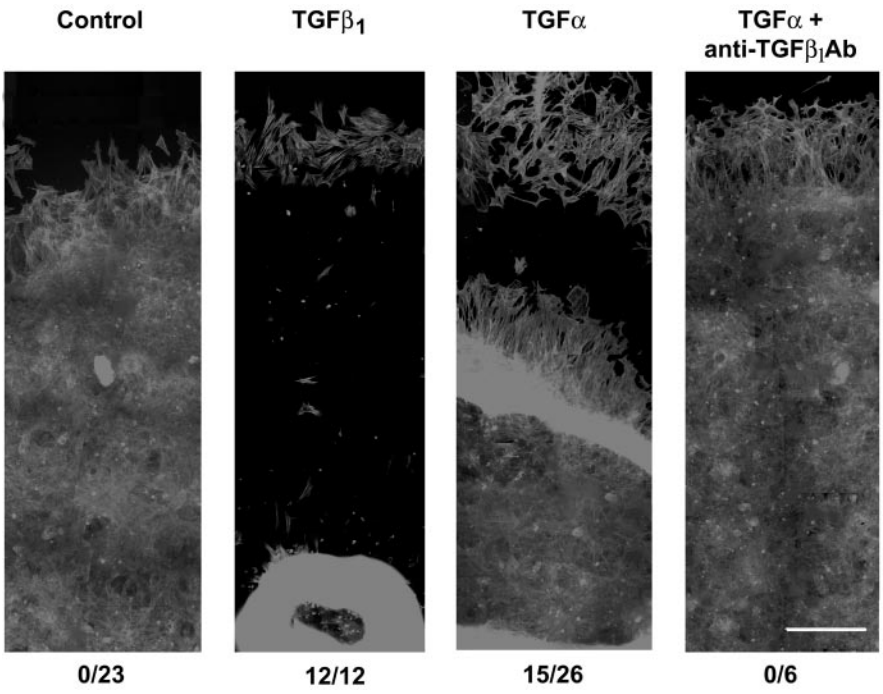


**Figure 9.** Exposure of circular monolayers of tanycytes to TGFα for 24 hr results in a TGFβ1-dependent retraction of the cells in 60% of the cultures. Photo montages of representative cultures illustrate these findings. Under basal unstimulated conditions (control), no retraction of the circular monolayer was observed in 23 cultures examined (0/23). TGFα (50 ng/ml, 24 hr) and TGFβ1 (0.4 nM, 24 hr) induced retraction in 60% (15/26) and 100% (12/12) of the cultures, respectively. When neutralizing antibodies to TGFβ1 (5 μg/ml) were added for 24 hr to tanycytic cultures treated with TGFα (50 ng/ml), none of the cultures (0/6) showed monolayer retraction. In all panels, the actin cytoskeleton of tanycytes was labeled using Texas Red-labeled phalloidin and was visualized by fluorescent microscopy. Scale bars, 800 μm.

Our results also show that, in contrast to their response to TGFα, tanycytes retract in the presence of TGFβ1, indicating that these two growth factor-signaling systems exert antagonistic effects on tanycytic plasticity. Cultured tanycytes differ from tanycytes *in situ* in that they do not appear to form an epithelium with polarized cells and junctional complexes. Their ability to respond to TGFα with TGFβ1 release, however, suggests that a similar relationship may exist *in vivo* because hypothalamic TGFα expression increases before that of TGFβ1 during the period encompassing the preovulatory surge of gonadotropins (Ma et al., 1992; Galbiati et al., 2002). The mechanism by which TGFβ1 induces tanycytic retraction is likely to involve the activation of extracellular matrix metalloproteinases because overall inhibition of metalloproteinase activity prevented the TGFβ1 effect. Although the metalloproteinases involved remain to be identified, there is a clear precedent that TGFβ1 is a potent inducer of extracellular matrix gene expression, and its ability to do so depends on the degree of cell differentiation at the time of exposure (Vollberg et al., 1991). A TGFβ1 effect on the extracellular matrix is not, however, the only mechanism by which the growth factor may affect tanycytic plasticity. Direct TGFβ1 effects on erbB-1 receptor physiology (Baskin et al., 1981) and erbB-1-dependent signaling events (Pientenpol, 1990) have been de-

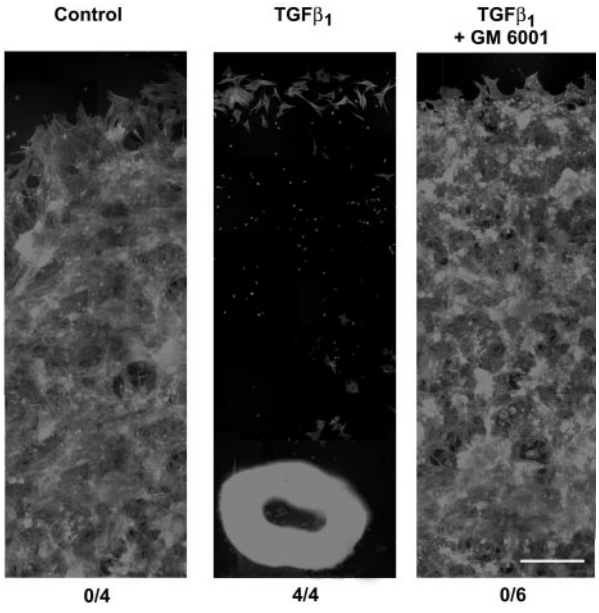


**Figure 10.** Blockade of matrix metalloproteinase activity abolishes the effect of TGFβ1 on tanycyte retraction, as assessed by migration assays. To block matrix metalloproteinase activity, we treated cultures with GM6001 (50 μM). Cotreatment of tanycytic cultures with TGFβ1 (0.4 nM) and GM6001 (50 μM) for 24 hr abrogated the ability of TGFβ1 to promote tanycyte retraction. In all panels, the actin cytoskeleton of tanycytes was labeled using Texas Red-coupled phalloidin and was visualized by fluorescent microscopy. At the bottom of each panel, the left number indicates the number of cultures that retracted under the given experimental condition, and the right number indicates the total number of cultures that were analyzed. Scale bars, 800 μm.

scribed, and, perhaps more importantly, the intracellular signaling pathways activated by stimulation of both TGF$\alpha$ and TGF$\beta$ receptors have been found to converge antagonistically on the Smad family of proteins originally found to mediate signals derived from the activation of TGF$\beta$ family receptors (Kretzschmar et al., 1997; de Caestecker et al., 1998). Regardless of their respective mechanisms of action, our results demonstrate that both TGF$\alpha$ and TGF$\beta_1$ exert profound, although antagonistic, effects on tanycytic plasticity. By showing that these effects occur sequentially and follow a TGF$\alpha$–TGF$\beta_1$ hierarchy, our results provide a framework toward an understanding of the cell–cell signaling mechanisms underlying ependymoglial plasticity in the adult neuroendocrine brain. Whether the main function of tanycyte plasticity is to directly control the access of LHRH to the portal vasculature or to regulate neurosecretion by controlling glia–glia or glia–neuron communication or both remains to be determined.

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