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Neuregulins Signaling via a Glial erbB-2–erbB-4 Receptor Complex Contribute to the Neuroendocrine Control of Mammalian Sexual Development

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Activation of erbB-1 receptors by glial TGFα has been shown to be a component of the developmental program by which the neuroendocrine brain controls mammalian sexual development. The participation of other members of the erbB family may be required, however, for full signaling capacity. Here, we show that activation of astrocytic erbB-2/erbB-4 receptors plays a significant role in the process by which the hypothalamus controls the advent of mammalian sexual maturation. Hypothalamic astrocytes express both the erbB-2 and erbB-4 genes, but no erbB-3, and respond to neuregulins (NRGs) by releasing prostaglandin E_2 (PGE_2), which acts on neurosecretory neurons to stimulate secretion of luteinizing hormone-releasing hormone (LHRH), the neuropeptide controlling sexual development. The actions of TGFα and NRGs in glia are synergistic and involve recruitment of erbB-2 as a coreceptor, via erbB-1 and erbB-4, respectively. Hypothalamic expression of both erbB-2 and erbB-4 increases first in a gonad-independent manner before the onset of puberty, and then, at the time of puberty, in a sex steroid-dependent manner. Disruption of erbB-2 synthesis in hypothalamic astrocytes by treatment with an antisense oligodeoxynucleotide inhibited the astrocytic response to NRGs and, to a lesser extent, that to TGFα and blocked the erbB-dependent, glia-mediated, stimulation of LHRH release. Intracerebral administration of the oligodeoxynucleotide to developing animals delayed the initiation of puberty. Thus, activation of the erbB-2–erbB-4 receptor complex appears to be a critical component of the signaling process by which astrocytes facilitate the acquisition of female reproductive capacity in mammals.

Key words: astroglial cells; tyrosine kinase receptors; glial growth factors; female sexual development; hypothalamus; puberty

Mammalian sexual development and adult reproductive function depend on the functional integrity of a group of specialized neurosecretory neurons that produce the neuropeptide luteinizing hormone-releasing hormone (LHRH). LHRH-secreting neurons are located in the basal forebrain and send their axons to the median eminence (ME) of the hypothalamus (Silverman et al., 1994) in which they release their secretory products into the portal vasculature for delivery to the anterior pituitary gland.

The regulation of LHRH secretion is exceedingly complex, because it involves multiple transsynaptic inputs, the modulatory influence of gonadal steroids, and the regulatory participation of astroglial cells (Brann and Mahesh, 1994; Ojeda, 1994; Crowley et al., 1995; Ojeda and Ma, 1995; Terasawa, 1995). Hypothalamic astrocytes are in intimate contact with LHRH neurons, because astrocytic processes appose most of the LHRH cell membrane, particularly along the secretory axons (Witkin et al., 1991; King and Letourneau, 1994; Silverman et al., 1994). Astroglial cells affect LHRH neuronal function by both remodeling the delivery sites of LHRH neurosecretory axons in the median eminence (Witkin et al., 1991; King and Letourneau, 1994) and the production of trophic (Melcangi et al., 1995; Tsai et al., 1995; Voigt et al., 1996) and neuroactive substances (Gallo et al., 1995; Ma et al., 1997a) able to affect the release of LHRH.

Evidence now exists that trophic factors signaling through receptor tyrosine kinases play a pivotal role in the cell–cell interactive mechanism by which astrocytes regulate LHRH neuronal function (Olson et al., 1995; Tsai et al., 1995; Voigt et al., 1996). For instance, transforming growth factor α (TGFα), a member of the epidermal growth factor (EGF) family (Massague, 1990), is synthesized in hypothalamic astrocytes and specialized glioependymal cells lining the third ventricle of the brain (Ma et al., 1992, 1994a), and promotes LHRH release indirectly, via juxtacrine–paracrine stimulation of glial cells containing EGF (erbB-1) receptors (Ma et al., 1994a, 1997a; Voigt et al., 1996). The ME appears to be an important site at which TGFα exerts its neuroendocrine actions. When cells genetically modified to secrete the growth factor are grafted into this region, puberty is advanced (Rage et al., 1997). Conversely, puberty is delayed by inhibition of erbB-1 tyrosine kinase activity targeted to the median eminence (Ma et al., 1992).

Signaling through erbB receptors is not, however, an isolated process involving the activation of a single receptor by a single ligand. Recent studies have shown that ligand binding to a particular erbB receptor involves the recruitment of related erbB receptors (Carraway and Cantley, 1994; Burden and Yarden, 1997). In addition to erbB-1 that binds EGF, TGFα, and four other EGF-related ligands (Carpenter and Cohen, 1990), the family of erbB tyrosine kinase receptors includes erbB-2 (Barg-

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mann et al., 1986b), erbB-3 (Kraus et al., 1989; Plowman et al., 1990), and erbB-4 (Plowman et al., 1993a). Whereas erbB-3 and erbB-4 bind a large group of structurally related, EGF-like peptides collectively known as neuregulins (NRGs) (Wen et al., 1992; Marchionni et al., 1993; Burden and Yarden, 1997; Carraway et al., 1997; Chang et al., 1997), no ligand for erbB-2 has yet been identified (Carraway and Cantley, 1994). Rather than acting as a typical receptor, erbB-2 appears to function as an auxiliary molecule (Karunagaran et al., 1996) recruited by ligand-induced activation of both erbB-1 and NRG (erbB-3 and erbB-4) receptors (Akiyama et al., 1988; Beerli et al., 1995; Karunagaran et al., 1996; Riese et al., 1996; Zhang et al., 1996). We show here that expression of the genes encoding erbB-2 and erbB-4 within the hypothalamus is mostly astroglial and that it selectively increases in this brain region during the phases of development that precede and accompany the advent of female sexual maturation. This increase in gene expression is sequentially determined by both gonad-independent and sex steroid-regulated mechanisms. Both NRGs and TGFα are equally effective in stimulating astrocytic release of PGE_2, an eicosanoid involved in mediating neurotransmitter-induced LHRH release. Disruption of erbB-2 synthesis by an antisense oligodeoxynucleotide not only prevents PGE_2 release in response to NRG stimulation and the ability of astrocytes to stimulate LHRH release via diffusible factors, but significantly, it also delays the onset of puberty.

Parts of this work have been published previously (Ma et al., 1997b).

MATERIALS AND METHODS

Animals

Immature and pregnant female rats of the Sprague Dawley strain were purchased from B & K Universal, Inc. (Fremont, CA). They were housed in a room with controlled photoperiod (14/10 hr light/dark cycle; lights on from 5:00 A.M. to 7:00 P.M.) and temperature (23–25°C). Animals were allowed access to tap water and pelleted rat chow *ad libitum*.

Cell culture

Hypothalamic and cerebrocortical astrocytes were purified from 1- to 2-d-old rats, as described previously (Ma et al., 1994a). In brief, brain tissues were mechanically dissociated using a Stomacher 80 blender (Tekmar, Cincinnati, OH) at 80% power for 2–3 min. The cell suspension was filtered first through a 230 μm metal sieve (Bellco, Vineland, NJ) and then through a 130 μm nylon mesh filter (Nitex, Elmsford, NJ). The cells were plated in 75 cm culture flasks (Corning Costar Co., Acton, MA) and cultured in DMEM–F-12 medium (1:1, vol/vol) supplemented with 10% calf serum under an atmosphere of 5% CO_2–95% air at 37°C. Upon reaching confluency (8–10 d), the astrocytes were isolated from other contaminating cells by first shaking the flasks at 250 rpm for 6 hr, replacing the medium, and then shaking again for another 18 hr. Thereafter, the astrocytes were replated in six-well plates at 800,000 cells per well. Upon reaching 80–90% confluency, the medium was replaced with a serum-free, astrocyte defined medium (ADM), and the astrocytes were used 1–2 d later for the experiments. ADM consisted of DMEM (lacking glutamate and phenol red) supplemented with L-glutamine (2 mM), HEPES (15 mM), insulin (5 μg/ml), and putrescine (100 μM). The cultures were more than 95% pure, as assessed by the number of cells immunopositive for the astrocytic marker, glial fibrillary acidic protein (Ma et al., 1994a).

The immortalized LHRH-producing cell line GT1–1 (kindly provided by Dr. Richard Weiner, University of California at San Francisco, San Francisco, CA) was used to assess the effect of astrocyte-derived substances on LHRH release. The cells were seeded in 24-well plates (100,000 cells per well) and cultured in DMEM containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Upon reaching 50–60% confluency, they were transferred to a serum-free, neuronal defined medium (NDM) for 24 hr, before being used for the experiments. NDM consisted of glutamate-free DMEM supplemented with transferrin (100 μg/ml), putrescine (100 μM), L-glutamine (2 mM), sodium selenite (30 nM), and insulin (5 μg/ml) (Berg-von der Emde et al., 1995).

MCF-7 cells (American Type Culture Collection, Manassas, VA; HTB-22, human breast adenocarcinoma), used as a positive control to verify the ability of NRG1 to induce erbB-2 tyrosine phosphorylation, were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Cell treatments

Astrocytes and MCF-7 cells. (1) To document the ability of TGFα and NRG1 to induce erbB-1, erbB-2, or erbB-4 tyrosine phosphorylation, hypothalamic and cortical astrocytes and MCF-7 cells were seeded into 100 mm tissue culture dishes and grown to ~80% confluency. The cells were then transferred to serum-free medium and cultured for 24 hr before treating them with TGFα or the NRG1, neu differentiation factor-β2 (NDF-β2; 100 ng/ml, 5 min). Thereafter, the cells were washed with PBS and solubilized in radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 1 μM PMSF, 20 μg/ml aprotinin (0.09 IU/ml), and 1 mM sodium orthovanadate. The solubilized proteins were stored at −85°C until immunoprecipitation and electrophoretic separation of the tyrosine phosphorylated species (see below). (2) The ability of NRG1 to stimulate the release of PGE_2 from hypothalamic astrocytes was examined by treating the cells with the NDF isoforms β1, β2, and α2 and comparing the response with that to TGFα administered at a similar dose. The possibility that NRG1 may facilitate the effect of TGFα on PGE_2 release was also examined by treating astrocytes with an ineffective concentration of NDF-β2 (10 ng/ml) in combination with increasing doses of TGFα (5, 10, and, 20 ng/ml). (3) To determine the importance of erbB-2 in erbB-4- mediated NRG signaling in hypothalamic astrocytes, the astrocytes were treated with NDF-β2 (50 ng/ml) for 16 hr in the presence of a 21-mer antisense oligodeoxynucleotide [erbB-2 oligodeoxynucleotide (ODN); 5′-CATGATGATCATTGCGGCTCC-3′; 1 μM] encompassing the translation initiation codon [nucleotides (nt) −9 to +12] of rat erbB-2 mRNA (Suen and Hung, 1990). At the end of the treatment, the medium was collected for PGE_2 measurement (Campbell and Ojeda, 1987). An oligodeoxynucleotide containing the same nucleotides but in a random order was used as a control. The sequence of this oligodeoxynucleotide has no similarity to any other mammalian sequence thus far deposited in GenBank. The effectiveness of the erbB-2 ODN to selectively disrupt erbB-2 synthesis was assessed by treating hypothalamic astrocytes for 16 hr with either the ODN or the scrambled sequence and then subjecting the cells to a 5 min exposure to NDF-β2. Thereafter, the cells were processed as outlined above for subsequent immunoprecipitation of erbB-2 and erbB-4 and determination of phosphorylated receptor content.

GT1–1 cells. These cells were used to determine whether NRG is able to act directly on LHRH-secreting neurons to stimulate LHRH release or whether it does so via a glial intermediacy. The cells were treated with either NDF-β2 (50 ng/ml) or a culture medium conditioned by hypothalamic astrocytes treated for 16 hr with NDF-β2 alone, or NDF-β2 in the presence of the above described erbB-2 antisense oligodeoxynucleotide. After treating the GT1–1 cells for 30 min, the medium was collected for LHRH measurement (Ojeda et al., 1986a). A conditioned medium derived from hypothalamic astrocytes treated with TGFα, known to induce LHRH release via its content of PGE_2 (Ma et al., 1997a) was also applied to GT1–1 cells as a positive control for LHRH release.

Cell transfection

Because NRG1 induces erbB-2 tyrosine phosphorylation in hypothalamic astrocytes, which contain erbB-4 receptors, but not in cerebrocortical astrocytes, which lack these receptors, cortical astrocytes were transfected with cNHER4, a plasmid that encodes the human erbB-4 receptor, to document the requirement of erbB-4 for NRG1 signaling in astrocytes. The cells were seeded into 60 mm plates, grown to 80% confluency, and transfected for 5 hr with Lipofectamine (Life Technologies, Grand Island, NY), as described previously (Mayerhofer et al., 1996). Forty-eight hours after transfection, the astrocytes were treated with NDF-β2 (100 ng/ml, 5 min) and analyzed for erbB-2 tyrosine phosphorylation.

Reverse transcription-PCR of NRGs and erbB receptors

NRGs. A 251 bp NRG1 cDNA fragment was amplified from total RNA from either hypothalamic tissue or hypothalamic astrocytes. The primers used were 20-mer oligodeoxynucleotides corresponding to nt 532–551 (5′

primer) and 764–783 (3′ primer) in the extracellular-encoding region of the NRG1 gene (Wen et al., 1992) that is common to all described NRG mRNA isoforms (Wen et al., 1994). To obtain cDNAs complementary to the α and β forms of NRG2 mRNA (314 and 270 bp, respectively), we used 5′ primers, specific for each form: 5′-AAACGGATTCTTC-GGACAGA-3′, corresponding to nt 247–266 in the α form; 5′-CGAAGGCATCAACCAACTCT-3′, corresponding to nt 989–1008 in the β form; and a common 3′ primer, 5′-TGGTGGGCCGGACA-CATGTT-3′, complementary to nt 541–560 in the α form (Chang et al., 1997). Total RNA isolated from the hypothalamus or hippocampus was used as the template. To isolate an NRG3 cDNA, we used RNA from the hypothalamus, hypothalamic astrocytes, human keratinocytes, and rat liver, and 21-mer primers that amplify a region (nt 1260–1627) including the entire transmembrane-encoding portion of murine NRG3 mRNA (Zhang et al., 1997). The 5′ primer used was 5′-CTACCAAGGAGTC-CGTTGTGA-3′; the 3′ primer was 5′-TTGACTCCATTATTTT-CTCCA-3′.

ErbBs. A 322 bp erbB-2 and a 168 bp erbB-4 cDNA fragment were generated from total RNA from either hypothalamic tissue or hypothalamic astrocytes. A 331 bp erbB-3 DNA was obtained from liver RNA. The 5′ primer (5′-CAGTGTGTCAACTGCAGTCA-3′) used to amplify erbB-2 corresponds to nucleotides 1610–1629 in the rat erbB-2 mRNA sequence (Bargmann et al., 1986b). The 3′ primer (5′-CAGGAG-TGGGTGCAGTTGAT-3′) is complementary to nucleotides 1913–1932. The primers used to amplify erbB-3 and erbB-4 DNA fragments were synthesized based on human erbB-3 and erbB-4 sequences. The 5′ primer is a common 20-mer oligonucleotide (5′-AACTGCACCC-AGGGGTGTAA-3′) corresponding to a highly conserved region (nt 1891–1910 in the human erbB-4 gene) in the extracellular domain-encoding portion of both genes (Kraus et al., 1989; Plowman et al., 1993a). In the rat sequence, nt 12 of this primer (G) is substituted for an A. The 3′ primers used are specific to each mRNA. The erbB-3 3′ primer (20-mer; 5-AAATCCCCTTGTGGACAGTT-3′) is complementary to nt 2361–2380 in the intracellular domain of the erbB-3 mRNA sequence (Kraus et al., 1989). The 20-mer 3′ primer of erbB-4 (5′-AACATAAACAGCAAATGTCA-3′) is complementary to nt 2039–2058 in the transmembrane domain of human erbB-4 (Plowman et al., 1993a). Nucleotides 1 and 10 in this primer differ from the recently published rat erbB-4 sequence at positions 2049 and 2058 (GenBank accession number AF041838).

RNase protection assay–solution hybridization

Immediately after decapitation of the rats, brains were removed, and the medial basal hypothalamic area including the ME, arcuate nucleus (ARC), and the ventromedial nuclei of the hypothalamus (VMH) (referred to as ME-ARC) was collected, as described previously (Ma et al., 1992). Cerebral cortex was used as a control. All dissected tissues were quickly frozen on dry ice and stored at −85°C until RNA isolation.

Total RNA from brain tissue and cultured cells was isolated as reported previously (Lara et al., 1990; Ojeda et al., 1991; Ma et al., 1994a). The RNase protection assay used has been described previously in detail (Ma et al., 1996). In brief, ^{32}P-UTP-labeled rat erbB-2 and erbB-4 antisense RNA probes were purified using a Fullengther apparatus (Biokey Co., Portland, OR), as recommended (Ma et al., 1996). Each probe (500,000 cpm) was hybridized to RNA samples (5 μg/tube) or to different amounts of *in vitro* synthesized sense RNA for 18–20 hr at 45°C. The tissue RNA samples were simultaneously hybridized to 5000 cpm of a cyclophilin antisense cRNA probe to correct for procedural variability (Ma et al., 1996), because cyclophilin mRNA is constitutively expressed in brain and other tissues (Danielson et al., 1988). Upon completion of the hybridization, the samples were treated with ribonucleases A and T1 to digest unhybridized RNA species. The protected cRNA fragments were separated by polyacrylamide gel electrophoresis (5 or 7% acrylamide, 7 M urea), and the hybridization signals were visualized by exposing the dried gels to Reflection x-ray film (NEN, Boston, MA). Quantitation of the signals was performed as described previously (Ma et al., 1994a), using an edited version of the NIH Image program (Correa-Rotter et al., 1992).

Probes

To prepare erbB riboprobes labeled with ^{32}P-UTP (for RNase protection assay) or ^{35}S-UTP (for hybridization histochemistry), we used DNA templates corresponding to sequences contained in the coding region of each erbB mRNA. An erbB-2 DNA template was prepared by cloning a *Bam*HI 422 bp fragment of a rat erbB-2 cDNA (Bargmann et al., 1986a) (pSV2NeuT; a generous gift of Dr. R. A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA) into the *Bam*HI site of pGEM-3Z. ErbB-3 and erbB-4 cDNA templates generated by Reverse transcription (RT)-PCR (see above) were cloned into the riboprobe vector pGEM-T. The cyclophilin cDNA template used for the transcription of cyclophilin cRNA probes was a 132 bp *Nco*I DNA fragment excised from a rat cyclophilin cDNA (Danielson et al., 1988) and cloned into the riboprobe vector pGEM-5zf(−).

Hybridization histochemistry

The procedure used (Simmons et al., 1989) has been reported in detail previously (Ma et al., 1992, 1994b). Briefly, the brains were transcardially perfused with 4% paraformaldehyde in borate buffer, pH 9.5. After an overnight post-fixation in the same fixative containing 10% sucrose, the brains were blocked and stored at −85°C until being coronally sectioned at 20–25 μm using a sliding microtome. The sections were then mounted on Superfrost Plus slides (Fisher Scientific, Kent, WA) and dried overnight under vacuum before hybridization. After prehybridization (Simmons et al., 1989), each slide was overlaid with 70 μl of hybridization solution containing 50% formamide, 0.25 M NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, 2× Denhardt's solution, and the riboprobe of interest (1×10^7 cpm/ml). Thereafter, the slides were hybridized for 18–20 hr at 55°C. Posthybridization washes were performed as reported previously (Simmons et al., 1989; Ma et al., 1992, 1994b). After dehydration in graded alcohols, the slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) and developed after 3 weeks of exposure. Controls sections were hybridized with erbB-2 or erbB-4 sense RNA probes.

Immunohistochemistry–confocal microscopy

The brains of peripubertal female rats were fixed by transcardiac perfusion with Zamboni's fixative (Ma et al., 1994b) and subjected to double immunohistofluorescence using 50 μm floating vibratome sections and a procedure described previously (Jung et al., 1997). ErbB-2 was detected with a monoclonal antibody (Ab) (c-neu, Ab-3; Oncogene Research Products, Cambridge, MA) diluted 1:100. ErbB-4 was detected with monoclonal antibody c-erbB-4, Ab-1 (also at a 1:100 dilution; NeoMarkers, Union City, CA). Astrocytes were identified with a monoclonal antibody to GFAP (1:1000; Sigma, St Louis, MO). After an overnight incubation at 4°C with either erbB-2 or erbB-4 antibodies, the reactions were developed with a Texas Red-conjugated goat anti-mouse gamma globulin (1:200, 1 hr at room temperature; Jackson ImmunoResearch, West Grove, PA). After extensive washes, the sections were incubated (overnight at 4°C) with the GFAP antibody. Because this antibody was also monoclonal, we did not use a secondary antibody to develop the reaction, but instead labeled the GFAP antibody directly with Oregon Green 488 (Molecular Probes, Eugene, OR), according to the manufacturer's instructions, to a specific activity of 2.7 molecules of dye per molecule of antibody protein. Immunofluorescence controls consisted of sections incubated in the absence of the primary antibodies.

Confocal images were acquired using a Leica (Nussloch, Germany) TCS NT confocal system, with a 25× NA 0.75 PL FLUOTAR objective. FITC and Texas Red were imaged simultaneously in most cases, using the 488 and 568 nm lines of an argon and krypton gas lasers, respectively, for excitation, a double dichroic at 488 and 568 nm, and a reflective mirror for wavelengths <580 nm in front of the first detection channel. A bandpass emission filter of 530 ± 30 nm was used for Oregon Green, and a long-pass filter at 590 nm was used for Texas Red. The intensity of the excitation light in each channel was adjusted so that the contribution of fluorescein to light detected in the Texas Red channel was negligible. Typically, 16 optical sections 1–3 μm apart were acquired for each image. Colors were merged and sections were projected into a single plane using MetaMorph (Universal Imaging, West Chester, PA). Images were further processed using Photoshop 5.0 (Adobe Systems, San Jose, CA).

ErbB-1, erbB-2, and erbB-4 tyrosine phosphorylation

For erbB-1, erbB-2, and erbB-4 kinase assays, lysates from treated cells were microfuged at 4°C for 5 min, and the supernatants were reacted for 3 hr at room temperature with a slurry of protein A–Sepharose that had been preabsorbed to antibodies against either erbB-1 (polyclonal Ab 1383; a gift from Shelton Earp, Department of Pharmacology, University of North Carolina, Chapel Hill, NC), erbB-2 (polyclonal Ab 1275, provided by G. Clinton, Department of Biochemistry, Oregon Health Sciences University, Portland, OR; or SC-284, purchased from Santa Cruz Biotechnology, Santa Cruz, CA), or erbB-4 (SC-283; Santa Cruz Bio-

technology). Immunoprecipitated proteins were electrophoresed on a 8% SDS-polyacrylamide minigel and then transferred onto a nitrocellulose membrane. After blocking for 1 hr with 2% BSA–0.2% Tween 20 in Tris-buffered saline (TBS), the membranes were probed with a monoclonal phosphotyrosine antibody (4G10, kindly provided by Dr. David Kaplan, Montreal Neurological Institute, Montreal, Canada; or PY-20, purchased from Santa Cruz Biotechnology) and then with an anti-mouse HRP-linked antibody (Boehringer Mannheim, Indianapolis, IN), as described previously (Ma et al., 1994b). After several extensive washes, tyrosine phosphorylated proteins were detected using the Enhanced Chemiluminescence system from Amersham Pharmacia Biotech (Arlington Heights, IL).

Cross-linking of erbB-2 and erbB-4 receptors

Hypothalamic astrocytes were maintained in serum-containing medium in 100 mm culture dishes until they reached ~ 90% confluency. They were then cultured in ADM (see above) for 48 hr before treatment with NDFβ2 (500 ng/ml) for 3 min at 37°C. At the end of this treatment, the astrocytes were exposed to bis (sulfosuccinimidyl) suberate (BS^3) (2 mM; Pierce, Rockford, IL), a noncleavable, amine-reactive cross-linker (Staros and Kakkad, 1983), for 2 min at room temperature, followed by incubation on ice for 30 min. Thereafter, the cells were washed with ice-cold PBS, collected into PBS, pelleted by brief centrifugation, and lysed in RIPA buffer. The lysates were immunoprecipitated overnight at 4°C using rabbit polyclonal antibodies to either erbB-2 (C-Neu, Ab-1; Oncogene Research Products) or erbB-4 (SC-283; Santa Cruz Biotechnology) at 1:200 dilution. For each sample tube, 80 μl of protein A–Sepharose (1:1 slurry, in water) was added, and the tubes were tipped for an additional 3 hr. Immunoprecipitates were pelleted by microcentrifugation and washed once with ice-cold RIPA and once with ice-cold PBS. Each immunoprecipitate was suspended in 30 μl of H_2O and 15 μl of a 3× concentrated sample buffer (final concentration of 0.0625 M Tris, pH 6.8, 3% SDS, 5% glycerol, and 5% β-mercaptoethanol). The protein samples were denatured at 100°C for 5 min, and 15 μl of each sample was used for SDS-PAGE on a 6% polyacrylamide gel. Separated proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ), and blocked with 2% BSA and 0.2% Tween 20, in TBS for 1 hr at room temperature. The membranes were then incubated with antibodies, either erbB-2 (mouse monoclonal C-Neu, Ab-3; Oncogene Research Products) or the same erbB-4 used previously for immunoprecipitation (each at 1:200 dilution). After 4 hr incubation at room temperature, the membranes were washed three times (10 min each) with TBS–Tween 20 at room temperature and then incubated with a horseradish peroxidase-linked secondary antibody, diluted 1:5000 with TBS–Tween 20, for 1 hr at room temperature. After washing, the reactions were developed using the SuperSignal Ultra Chemiluminescent Substrate (Pierce).

Ovariectomy and steroid treatment

The ovaries from early juvenile (22-d-old) rats were removed via a dorsal approach, as described previously (Andrews et al., 1981). Five days after surgery, the rats received a subcutaneously SILASTIC capsule containing corn oil or 17β-E_2 (Sigma) dissolved in corn oil at a concentration of 400 μg/ml (Andrews et al., 1981). These capsules have been shown (Andrews et al., 1981) to produce circulating levels of E_2 similar to those that precede the first preovulatory surge of gonadotropins at puberty in female rats (Andrews et al., 1980). Some animals received a single subcutaneous injection of progesterone (P) (1 mg/rat) 50 hr (12:00 P.M.) after implantation of the E_2-containing capsules, to simulate the abrupt increase in plasma P that occurs in the afternoon of first proestrus, at the time of the first preovulatory surge of gonadotropins (Andrews et al., 1980). Brain tissues were collected 4 hr after the P injection, i.e., after 54 hr of estradiol exposure.

Intracerebroventricular infusion of an erbB-2 antisense oligodeoxynucleotide

To determine the importance of a functional hypothalamic erbB-2 for the timing of puberty, *in vivo* experiments were performed. The same antisense oligodeoxynucleotide found to be effective in inhibiting the astrocyte response to NRG1 *in vitro* was chronically infused into the third ventricle of the brain via a stereotaxically implanted infusion cannula (Plastic One, Roanoke, VA) connected to a subcutaneously implanted Alzet mini-osmotic pump (Alzet Corporation, Palo Alto, CA). The pumps (model 2002) have a flow rate of 0.5 μl/hr and a capacity of 200 μl, resulting in a delivery period of 14 d. Each pump was loaded with artificial CSF (Dalva and Katz, 1994) containing either the erbB-2 antisense oligodeoxynucleotide or the scrambled sequence at 5 μg/ml. Upon connection to the infusion devise and a 4 hr preincubation at 37°C, the assembly was implanted into 25-d-old juvenile intact animals. At this time, the prepubertal increase in hypothalamic erbB-2 mRNA levels had not yet begun. Starting on day 30, the animals were monitored daily for vaginal opening (see below). Once vaginal opening occurred, vaginal lavages were obtained to estimate the time of first ovulation (Ojeda and Urbanski, 1994). All animals were killed on the day of first diestrus (defined by the presence of a predominance of leukocytes in the vaginal lavage) after an estrous type of vaginal cytology. This change in vaginal cytology has been shown to be an accurate indication that the first ovulation has taken place (Rage et al., 1997). In all cases, ovulation was confirmed by visual inspection of the ovaries to verify the presence of corpora lutea.

Phases of puberty

The developmental changes in hypothalamic erbB-2 and erbB-4 gene expression were examined at ages shown previously to correspond to key stages of sexual maturation in the rat (for review, see Ojeda and Urbanski, 1994). The different stages of puberty were defined according to established criteria (Ojeda and Urbanski, 1994). According to these criteria, the juvenile period in the female rat extends from postnatal days 21–30. Thus, the juvenile animals used in this study can be considered as mid-to-late juveniles. Their vaginae were closed, and their uteri weighed 60 mg or less, with no accumulation of intrauterine fluid. Animals with enlarged uteri and detectable intrauterine fluid (an indication of E_2 secretion) are considered to be in the early phases of puberty and, thus, are classified as being in an early proestrous (EP) stage, which precedes the day of the first preovulatory surge of gonadotropins. Animals showing a uterus "ballooned" with fluid and a uterine weight of at least 200 mg were considered to be in late proestrus (LP), the phase of puberty during which LHRH and gonadotropins are for the first time discharged as a preovulatory surge. Ovulation occurs in the early morning of the next day (first estrus; E). At this time, the vagina becomes patent and exhibits a cytology of cornified cells. Formation of the first corpora lutea leads to the first diestrus phase of puberty, characterized by a vaginal cytology showing a predominance of leukocytes and the presence of fresh corpora lutea in the ovaries.

Statistics

Changes in erbB-2 or erbB-4 mRNA levels during different stages of sexual development or in response to gonadal steroid treatments were analyzed by a one-way ANOVA, followed by the Student–Neuman–Keuls multiple comparison test for unequal replications.

RESULTS

NRGs and erbB mRNAs are expressed in the hypothalamus of immature female rats

To determine whether members of the NRG–erbB signaling complex are expressed in the hypothalamus of prepubertal female rats, total RNA from this region was subjected to RT-PCR using primers complementary to sequences contained within the NRG (NRG1, NRG2, and NRG3), erbB-2, erbB-3, and erbB-4 mRNA coding regions. With the exception of the NRG2 and erbB-3 genes, all other components of the signaling module were found to be expressed in the hypothalamus, as evidenced by the amplification of cDNA fragments of the expected size (Fig. 1) and their identification by sequencing (data not shown). RT-PCR amplification of RNA from isolated hypothalamic astrocytes yielded an identical expression profile (data not shown). Although the primers used to detect NRG2 mRNA in hypothalamic tissue yielded PCR products of a size similar to the NRG2α and β cDNAs amplified from hippocampus (Fig. 1), sequencing of several of these hypothalamic DNA fragments revealed that they did not contain the NRG2 sequence. On the other hand, failure to detect erbB-3 mRNA in the hypothalamus was not because of ineffective primers or inadequate PCR conditions, because an erbB-3 cDNA was readily amplified from tissues known to express the erbB-3

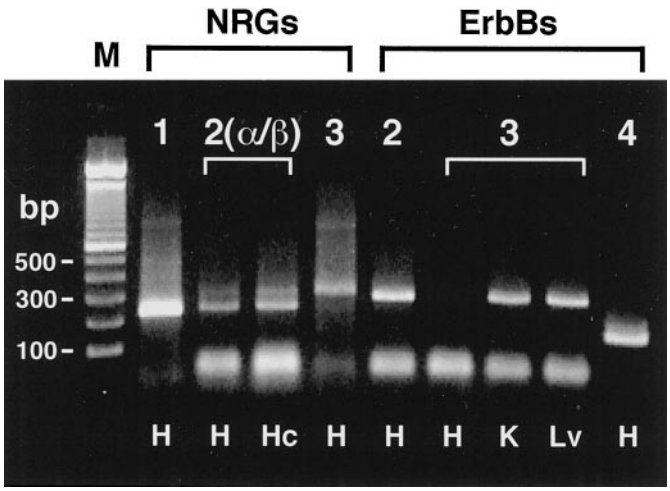

Figure 1. Detection by RT-PCR of NRGs and erbB mRNAs in the medial basal hypothalamus of late juvenile 28- to 30-d-old female rats. *M*, DNA molecular size markers. NRG1, 268 bp; NRG2α, 314 bp; NRG2β, 270 bp; NRG3, 371 bp; erbB-2, 323 bp; erbB-3, 337 bp; and erbB-4, 168 bp. *H*, Hypothalamus; *Hc*, hippocampus; *K*, human keratinocytes; *Lv*, rat liver; *bp*, base pairs.

gene, such as the liver and skin keratinocytes (Fig. 1). Thus, neither the hypothalamus of immature rats nor isolated hypothalamic astrocytes contain the NRG2 and erbB-3 mRNAs, indicating that, in this region of the brain, NRG-dependent signaling is effected exclusively via NRG1 and NRG3 acting on erbB-2–erbB-4 receptors.

Astroglial expression of erbB mRNAs is region-specific

It was shown previously that hypothalamic astroglial cells differ both molecularly and functionally from astrocytes of regions not involved in neuroendocrine regulation (Ma et al., 1992, 1994a). To determine whether these differences are also manifested in the case of NRG receptors, the relative abundance of the mRNAs encoding erbB-2, erbB-3, and erbB-4 in hypothalamic and cerebrocortical astrocytes in culture was assessed by RNase protection assay. Figure 2 shows that (1) erbB-2 mRNA is much more abundant in hypothalamic than cortical astrocytes, (2) erbB-4 mRNA is only detected in hypothalamic astrocytes, and (3) neither subpopulation of astrocytes expresses the erbB-3 gene. Thus, as suggested by the PCR data, NRG signaling in hypothalamic astrocytes appears to exclude an involvement of erbB-3 receptors.

Localization of erbB-2 and erbB-4 mRNA in the peripubertal female hypothalamus

Hybridization histochemistry of the brain from prepubertal (28-to 37-d-old) rats demonstrated that erbB-4 mRNA transcripts were more clearly detected in the paraventricular (Fig. 3*A*, *arrows*), the arcuate nucleus (ARC), and the ventromedial (VMH) and dorsomedial (DMH) nuclei (*B*) of the hypothalamus. Although in all of these regions some of the hybridization signal, analyzed under bright-field illumination, was seen in neurons, a substantial fraction of the signal was associated with small, dark nuclei, suggesting an astroglial localization. Such a localization was clearly evident in the subependymal region of the third ventricle (*A*, *B*, *small arrowheads*) and the median eminence (*B*, *large arrowheads*). This is a region devoid of neuronal cell bodies that serve as a final common pathway for neurosecretory nerve terminals converging to release their products into the portal vasculature. ErbB-4 mRNA was detected in glial cells located in the intermediate and external layer of the median eminence (*B*, *C*, *small* and *large arrowheads*, respectively). In addition to the hypothalamus, erbB-4 mRNA was abundant in the piriform cortex and hippocampus (data not shown). In both of these regions, the mRNA-containing cells appeared to be mostly neurons.

In contrast to erbB-4 mRNA, the hypothalamic distribution of erbB-2 mRNA transcripts was more circumscribed. ErbB-2 mRNA was predominantly detected in ependymal cells lining the third ventricle (*E*, *F*, *arrowheads*) and glia of the median eminence itself (*F*, *arrows*). Adjacent tissue sections incubated with the respective sense RNA probes showed no hybridization signals (*D*, *G*).

Localization of erbB-2 and erbB-4 proteins in the peripubertal female hypothalamus

Because the medial basal hypothalamic–median eminence region showed an abundance of erbB-2 and erbB-4 mRNA transcripts, we examined this region for the presence of erbB-2- and erbB-4-immunoreactive cells using immunohistofluorescence followed by confocal microscopy. Both receptor proteins were found in glial cells, in addition to some neurons. ErbB-2 immunoreactivity was abundant in tanycytes lining the wall of the third ventricle (Fig. 4*A*,*B*,*F*), with immunoreactive material distributed throughout the length of the processes that these cells send to the base of the brain (*B*, *arrows*). Astrocytes of the ventral aspect of the median eminence, identified by their content of GFAP (*green*), were also rich in erbB-2 immunoreactivity (*C–E*), as were astrocytes located along the walls of the third ventricle adjacent to tanycytes, which were negative for GFAP (*F–I*). In addition to this location, erbB-2 immunoreactivity was also observed in astrocytes of the medial basal hypothalamus away from the median eminence, especially those surrounding blood vessels (*J–L*). Scattered neurons containing erbB-2 were also observed (*A*, *B*, *short arrows*).

In agreement with the mRNA localization, erbB-4 immunoreactivity was observed in glial cells of the median eminence (Fig. 5*A*,*C*, *arrowheads*) and neurons of the arcuate nucleus (*A*, *B*, *arrows*). As in the case of erbB-2, astrocytes of the ventral aspect of the median eminence were found to contain erbB-4 (*D–F*), as were astrocytes adjacent to the wall of the third ventricle (*G–I*), and astrocytes of the medial basal hypothalamus (*J–L*), including those associated with blood vessels (*M*). No erbB-4 immunoreactivity was detected in tanycytes (*G–I*), a predominant site of erbB-2 expression.

Hypothalamic levels of erbB-2 and erbB-4 mRNA increase during juvenile and peripubertal development

To determine whether changes in the hypothalamic gene expression of erbB-2 and erbB-4 may occur in association with female sexual maturation, the tissue content of the encoding mRNAs in this brain region was quantitated by RNase protection assay. The hypothalamic levels of erbB-2 and erbB-4 mRNA increased for the first time during late juvenile development (postnatal day 28) and then again during the peripubertal period, reaching maximal values in the afternoon of the first proestrous day (LP), i.e., coinciding with the time of the first preovulatory surge of gonadotropins (Fig. 6, *top* and *bottom*), previously shown to occur in the afternoon of this day (Ojeda and Urbanski, 1994). The cerebral cortex, a brain region irrelevant to neuroendocrine control, displayed low and unchanging levels of erbB-2 mRNA throughout the juvenile and peripubertal period (Fig. 6, *top*). Although the

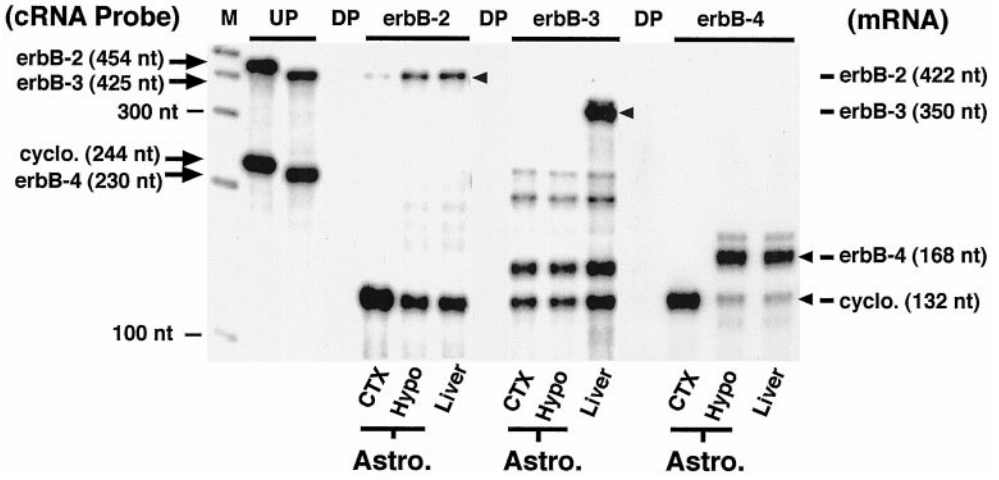

Figure 2. Detection of erbB-2 and erbB-4 mRNAs and absence of erbB-3 mRNA in isolated hypothalamic (*Hypo*) and cerebrocortical (*CTX*) astrocytes (Astro.), as assessed by RNase protection assay. *M*, ^{32}P-UTP-labeled RNA molecular size marker; *UP*, undigested cRNA probes; *DP*, digested probes; *cyclo*, cyclophilin.

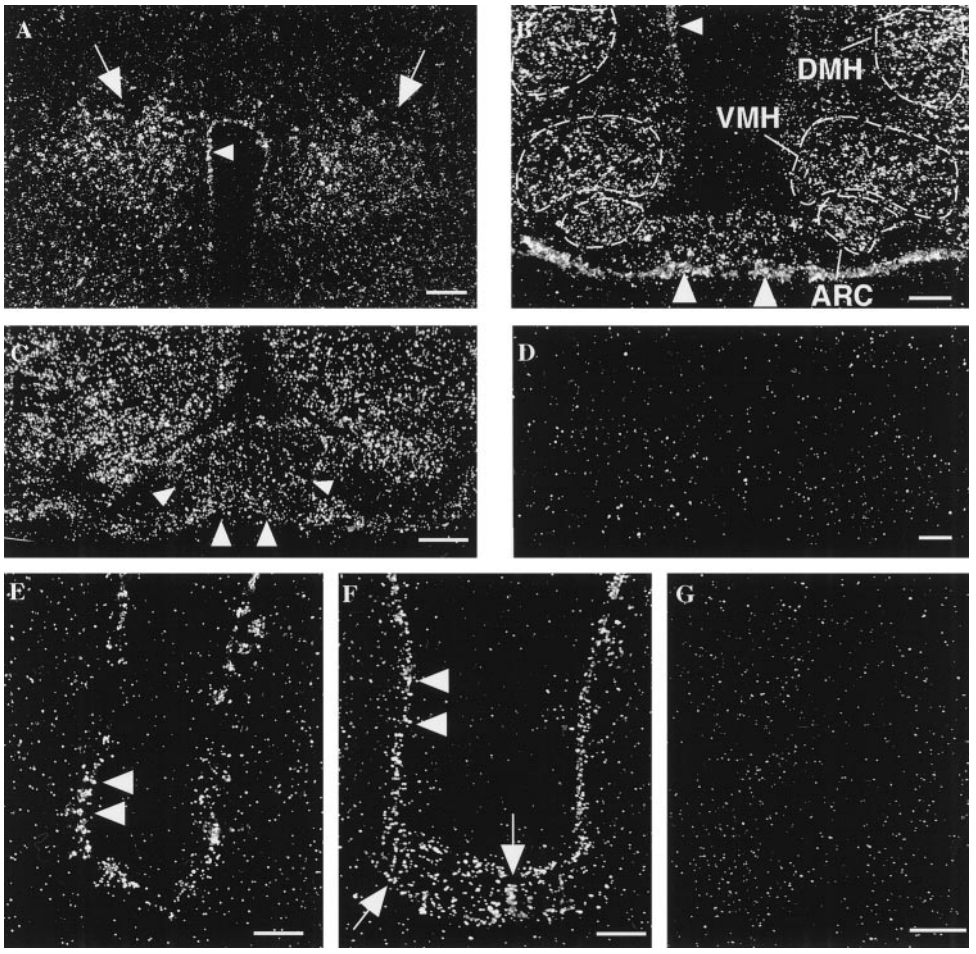

Figure 3. Cellular localization of erbB-4 and erbB-2 mRNAs in the brain of peripubertal female rats, as detected by *in situ* hybridization using ^{35}S-UTP-labeled cRNA probes. Within hypothalamic nuclei, the mRNA was found to be more abundant in cells of the paraventricular nuclei (*A*, *arrows*) and the ARC, VMH, and DMH nuclei (*B*). ErbB-4 mRNA is also diffusely detected throughout the medial basal hypothalamus (*B*, *C*) and is more abundantly present in cells of the subependymal region (*A*, *B*, *small arrowheads*) and glial cells in the intermediate and external layers of the median eminence (*B* and *C*, *small* and *large arrowheads*, respectively). ErbB-2 transcripts were more abundantly expressed in ependymal cells lining the third ventricle (*E*, *F*, *arrowheads*) and glial cells of the median eminence (*F*, *arrows*). *D* and *G* depict sections adjacent to *C* and *F* hybridized with sense erbB-4 (*D*) and erbB-2 (*G*) RNA probes. Scale bars: *B*, 200 μm; *A*, *C–G*, 100 μm.

cortical levels of erbB-4 mRNA were higher than those seen in the hypothalamus during midjuvenile development (postnatal day 24–26), they did not increase with the advent of sexual maturity (Fig. 6, *bottom*).

The juvenile increase in hypothalamic erbB-2 and erbB-4 mRNA levels is gonad-independent, but the peripubertal increase is an ovarian steroid-regulated event.

The juvenile increase in hypothalamic levels of erbB-2 and erbB-4 mRNA occurs at the time when circulating levels of ovarian steroids are low (Table 1). Thus, this initial increase in expression appears to be a centrally driven, gonad-independent event. In contrast, the changes in mRNA content observed during normal puberty occur at the time when the plasma levels of E_2 first, and progesterone (P) later, are elevated (Ojeda and Urbanski, 1994), suggesting that at least part of the peripubertal changes in mRNA levels is caused by the rising circulating levels of these ovarian steroids. Mimicking in ovariectomized juvenile rats the preovulatory increase in plasma E_2 that occurs at puberty (Andrews et al., 1980), via implantation of 17β-E_2-containing capsules (Andrews et al., 1981), resulted 2 d later in a significant

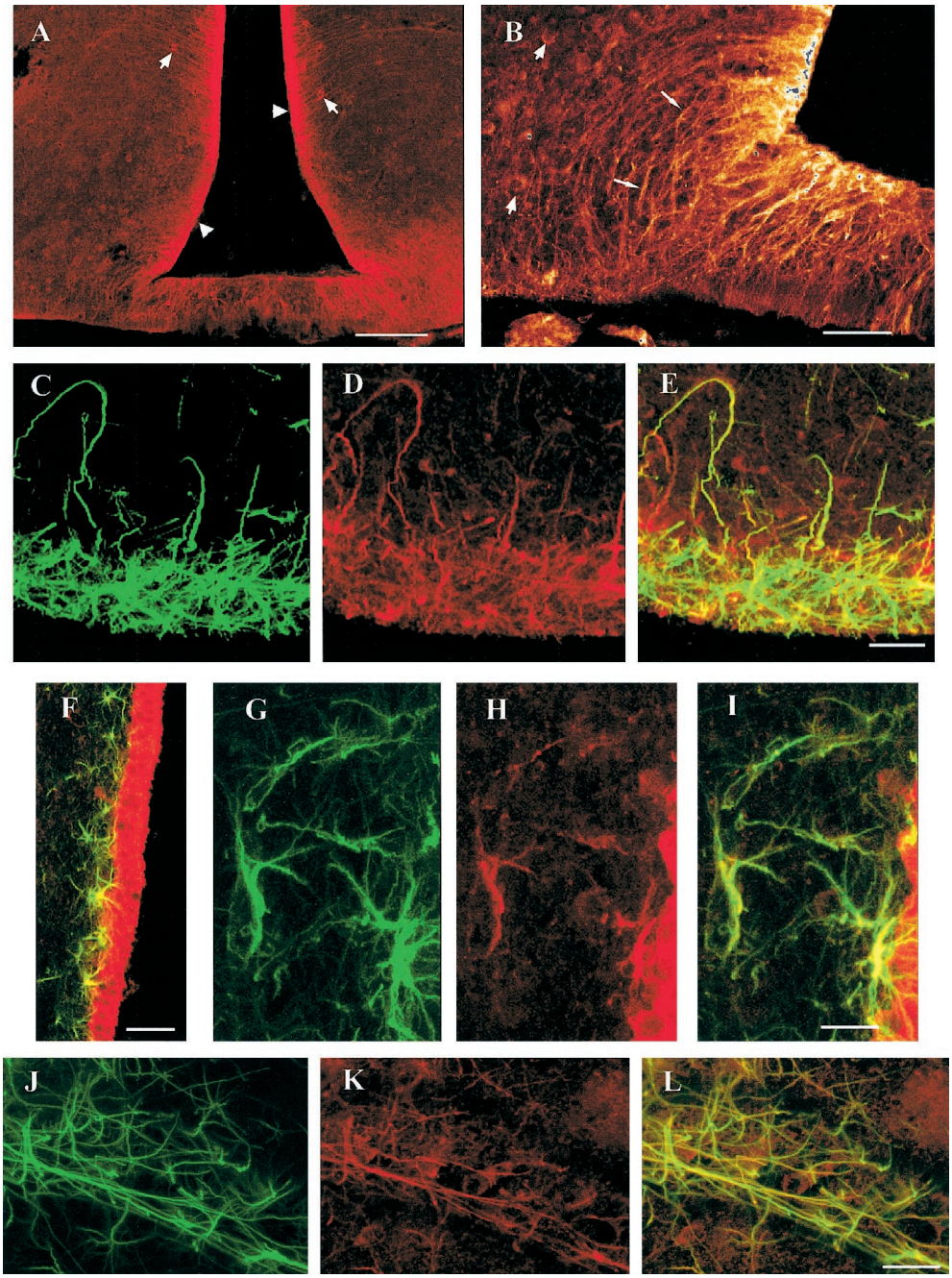

Figure 4. Immunofluorescent confocal microcopy localization of erbB-2 in the medial basal hypothalamus–median eminence region of peripubertal female rats. ErbB-2 was visualized with a monoclonal antibody to an amino acid sequence contained in the human protein, and the reaction was developed with a second antibody conjugated to the fluorochrome Texas Red (*red*). Astrocytes were identified with a monoclonal antibody to GFAP directly labeled with the fluorochrome Oregon Green 488 (*green*). *A, B*, Low- and high-magnification views of the medial basal hypothalamus–median eminence region showing abundant erbB-2 immunoreactivity in tanycytes lining the wall of the third ventricle (*A*, *arrowheads*) and the processes that these cells send to the median eminence (*B*, *arrows*). Some neurons are also labeled (*A*, *B*, *short arrows*). *C–E*, Presence of erbB-2-immunoreactive material (*D*, *red*) in astrocytes (*C*, *green*) of the ventrolateral aspect of the median eminence (images merged in *E*). *F*, Presence of erbB-2 in astrocytes adjacent to ependymal cells of the third ventricle. *G–I*, Higher magnification view of erbB-2-positive (*H*) astrocytes (*G*) adjacent to the third ventricle (images merged in *I*). Notice the abundant erbB-2 immunoreactivity in GFAP-negative tanycytes. *J–L*, Detection of erbB-2 in astrocytes of the medial basal hypothalamus associated with blood vessels. Scale bars: *A*, 100 μm; *B*, 25 μm; *C–E*, *G–L*, 10 μm; *F*, 40 μm.

increase in hypothalamic erbB-4 mRNA levels but not in erbB-2 mRNA content (Fig. 7), thus reproducing the changes in mRNA content observed during normal puberty before the afternoon preovulatory discharge of gonadotropins (Fig. 6). Administration of a single dose of P- to E_2-treated animals, to produce plasma P levels similar to those found at the time of the gonadotropin surge (Andrews et al., 1980), resulted in a marked increase in erbB-2 mRNA content but no further change in erbB-4 mRNA levels compared with animals treated with E_2 alone (Fig. 7). The changes caused by this sequential E_2 plus P treatment were again similar to those observed in the afternoon of the first proestrus during normal puberty (Fig. 6). P alone was ineffective. Thus, most of the increase in hypothalamic erbB-2 and erbB-4 gene expression at the time of puberty appears to be a gonad-dependent event.

NRG-dependent phosphorylation of erbB-2 in astroglia requires the participation of erbB-4

The mammary tumor cell line MCF-7 that lacks erbB-1 but contains erbB-2 receptors (Karunagaran et al., 1996) failed to respond to EGF with either erbB-1 or erbB-2 tyrosine phosphorylation (Fig. 8*A*, *left*) but showed a strong increase in erbB-2 phosphorylation upon stimulation with the NRG NDF-β2. In contrast to MCF-7 cells, hypothalamic astrocytes, which contain functional erbB-1 receptors (Ma et al., 1994a, 1997a), responded to the EGF relative TGFα, with tyrosine phosphorylation of both erbB-1 and erbB-2 (Fig. 8*A*, *right*). In addition, they showed erbB-2 and erbB-4 tyrosine phosphorylation when exposed to NDF-β2 (Fig. 8*A*, *right*). Cerebrocortical astrocytes, on the other hand, responded to TGFα with erbB-1 and erbB-2 phosphoryla-

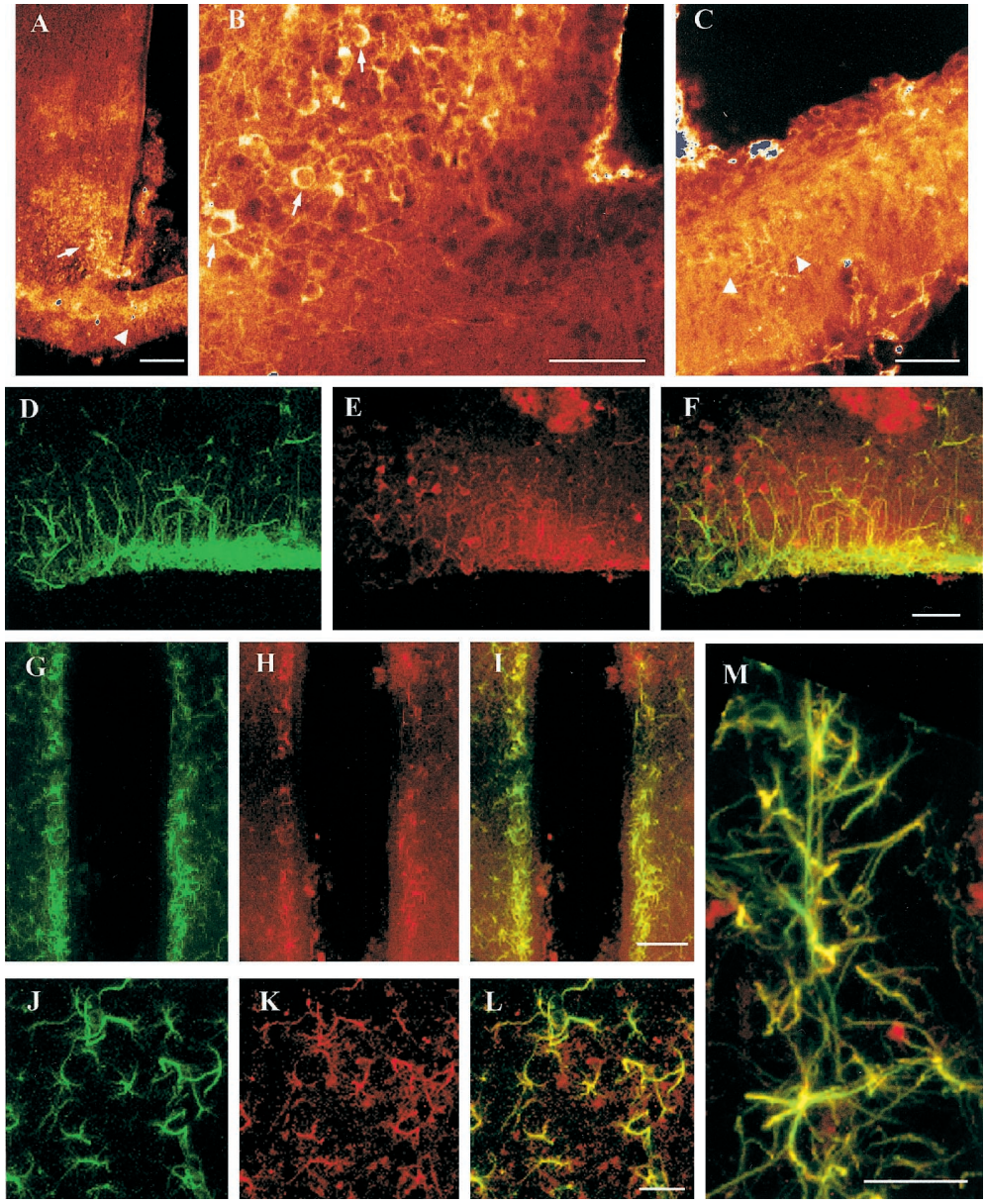

Figure 5. Immunofluorescence confocal microscopy localization of erbB-4 in the medial basal hypothalamus–median eminence region of peripubertal female rats. ErbB-4 was detected with a monoclonal antibody directed against an epitope in the human erbB-4 sequence, and the reaction was developed with a Texas Red-conjugated secondary antibody. GFAP was detected with a monoclonal antibody directly labeled with Oregon Green 488. *A*, Low-magnification view showing erbB-4-immunoreactive cells in the arcuate nucleus (*arrow*) and glial cells of the median eminence (*arrowhead*). *B*, Higher magnification view of the arcuate nucleus showing erbB-4-positive neuron-like cells (*arrows*). *C*, High-magnification view of erbB-4-positive astrocyte-like cells of the median eminence (*arrowheads*). *D–F*, Presence of erbB-4 in astrocytes of the ventrolateral aspect of the median eminence. Notice that not all astrocytes contain erbB-4 immunoreactivity. *G–I*, Presence of erbB-4 in astrocytes adjacent to tanycytes of the third ventricle. *J–L*, Presence of erbB-4 in astrocytes of the medial basal hypothalamus. *M*, Higher magnification view of erbB-4-positive astrocytes associated with blood vessels in the median basal hypothalamus, away from the median eminence. Scale bars: *A*, 100 μm; *B*, *C*, 25 μm; *D–I*, 40 μm; *J–L*, 20 μm; *M*, 30 μm.

tion (Fig. 8*B*, *left*) but showed no erbB-2 phosphorylation upon exposure to NDF-β2. Because cortical astrocytes express the erbB-2 but not the erbB-3 or erbB-4 genes (Fig. 2), we reasoned that the inability of NDF-β2 to induce erbB-2 phosphorylation was because of the lack of appropriate neuregulin receptors in these cells. Hypothalamic astrocytes, which express the erbB-4 gene, respond to NDF-β2 with erbB-2 phosphorylation (Fig. 8*B*, *middle*). We, therefore, tested the possibility that the lack of NRG-dependent erbB-2 phosphorylation in cortical astrocytes was caused by the absence of erbB-4. Expression of erbB-4 in these cells, via transient transfection with cNHER4 (Plowman et al., 1993b), a plasmid that contains a cDNA encoding the human erbB-4 receptor, led to an marked increase in both basal and NDF-β2-induced erbB-2 phosphorylation (Fig. 8*B*, *right*).

NRG-dependent activation of erbB-4 in hypothalamic astrocytes involves formation of erbB-4–erbB-2 heterodimeric complexes

To determine whether exposure of hypothalamic astrocytes to neuregulins results in receptor heterodimerization as shown in cell lines (Spivak-Kroizman et al., 1992; Cohen et al., 1996), astrocytic cultures were treated with NDF-β2 (3 min at 37°C) followed by cross-linking with bis (sulfosuccinimidyl) suberate (Staros and Kakkad, 1983). Immunoprecipitation of the reactive species with monoclonal antibodies to either erbB-2 or erbB-4, followed by Western blot analysis using antibodies to erbB-2, demonstrated in both cases the presence of a high molecular weight complex of ~365 kDa (Fig. 9, *left* and *middle*). To verify the presence of erbB-4 in this complex, the cross-linked species were immunoprecipitated with antibodies to erbB-2, and the Western blot was developed with monoclonal antibodies to erbB-4. The results showed the presence of erbB-4 in a high molecular weight species identical in size to that detected by the erbB-2 antibodies (Fig. 9, *right*).

Effect of NRG1 isoforms on the secretion of PGE2 from hypothalamic astrocytes

In a previous study, we showed that TGFα-dependent activation of erbB-1 receptors in hypothalamic astrocytes results in the release of PGE_2 (Ma et al., 1997a), a prostaglandin involved in

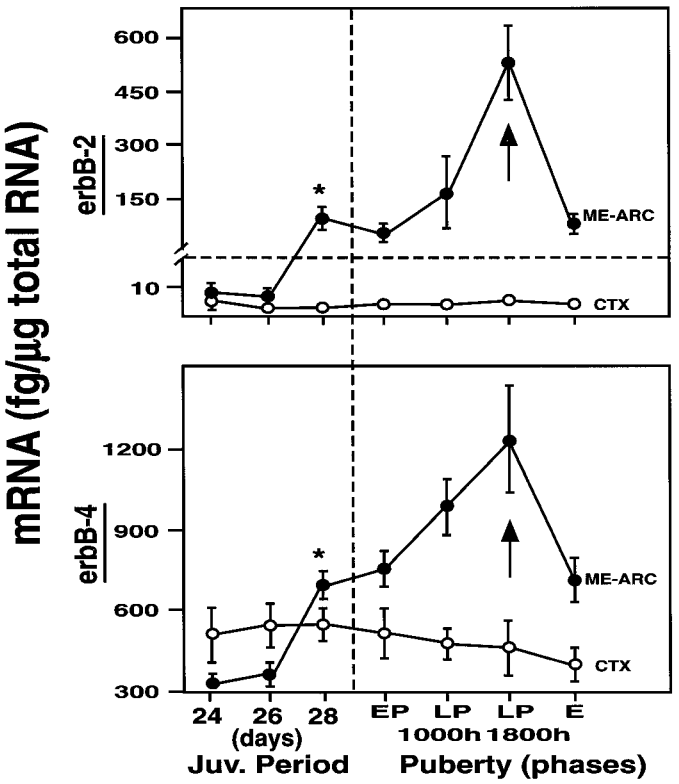

Figure 6. Developmental changes in erbB-2 (*top*) and erbB-4 (*bottom*) mRNA content in the medial basal hypothalamus (median eminence–arcuate nucleus, ME-ARC region) during juvenile and peripubertal development of the female rat. The increase in gene expression associated with the advent of sexual maturation is contrasted with the lack of changes in the cerebral cortex (*CTX*), a region of the brain irrelevant to reproductive neuroendocrine control. The *dotted vertical line* separates the juvenile from the peripubertal periods. *EP*, Early proestrous phase of puberty; a time during which the first morphological manifestations of puberty become evident as an increase in uterine weight and accumulation of uterine fluid. *LP*, The first proestrus; the first preovulatory discharge of gonadotropins (*arrows*) takes place in the afternoon of this day. *E*, The first estrus; ovulation occurs in the early morning hours of this day. Each *point* represents the mean ± SEM mRNA values (*vertical lines*) of three independent observations. Each observation derives from hypothalamic tissue pooled from three rats. $^{*}p < 0.05$, first significant increase over early (24-d-old) juvenile values.

Table 1. Serum levels of sex steroid in juvenile female rats at the time when hypothalamic erbB-2 and erbB-4 mRNA content first increases before puberty

	Age (days)		
Steroid	24 (n = 10)	26 (n = 10)	28 (n = 10)
Estradiol (pg/ml)	5.5 ± 0.34	3.4 ± 0.27	4.0 ± 0.21
Progesterone (ng/ml)	1.39 ± 0.42	0.94 ± 0.35	1.98 ± 0.38
Androstenedione (pg/ml)	37.7 ± 5.7	20.0 ± 0.2	23.0 ± 3.4

mediating neurotransmitter-induced LHRH secretion from the hypothalamus (Ojeda et al., 1986b). To determine whether NRG-1 exerts a similar stimulatory effect, cultured hypothalamic astrocytes were treated with three different isoforms of NDF, including NDF-β1, β2, and α2 (Wen et al., 1994). These isoforms

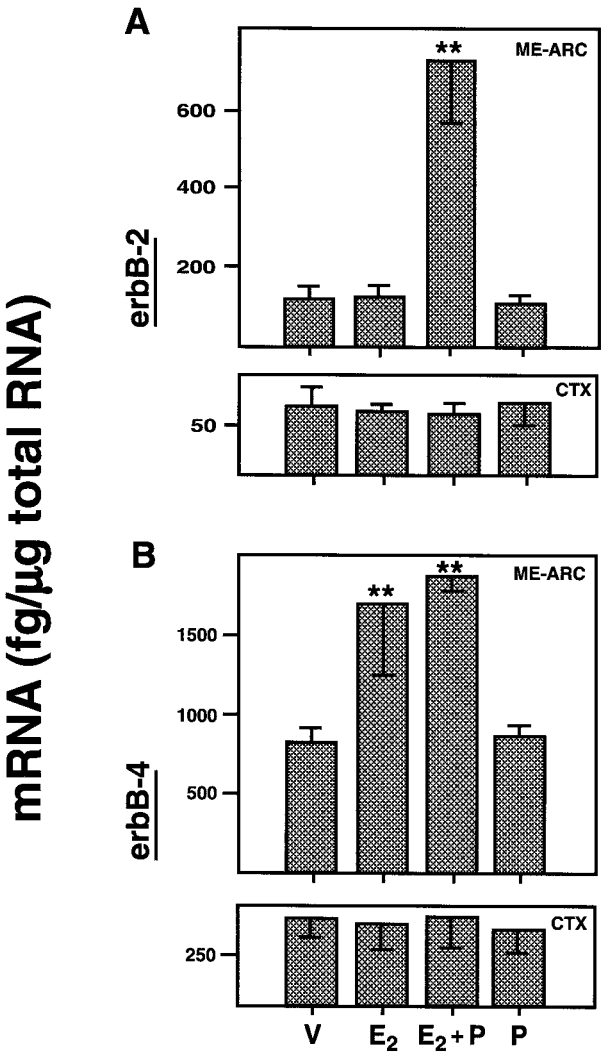

Figure 7. Increase in steady-state levels of erbB-2 and erbB-4 mRNA in the medial basal hypothalamus (ME-ARC region) of juvenile ovariectomized rats induced by E_2 or the sequential combination of E_2 plus P. The animals were ovariectomized on postnatal day 22 and received 5 d later a subcutaneous SILASTIC capsule containing 17β-E_2 at a concentration that produces preovulatory levels of serum E_2. Control animals received a capsule filled with the vehicle (*V*, corn oil). P was administered 50 hr later (at 12:00 P.M.) as a single subcutaneous injection. All animals were euthanized 4 hr after the P injection (i.e., at 4:00 P..M., 54 hr after receiving the E_2-containing capsule). Each *bar* represents the mean ± SEM of three independent observations (*vertical lines*). Each observation derives from hypothalamic tissue pooled from three rats. $^{**}p < 0.01$ versus V-treated group. *CTX*, Cerebral cortex.

were selected because of their proven ability to promote glial cell function. Thus, the β1 and α2 forms induce astrocyte maturation (Pinkas-Kramarski et al., 1994), and the β2 form facilitates the survival and maturation of glial cell precursors (Dong et al., 1995). Figure 10 (*left*) shows that NDF-β2 and NDF-α2 were as effective as TGFα in stimulating PGE_2 release. Surprisingly, NDF-β1, the reported major neuronal NDF isoform (Wen et al., 1994), was ineffective (Fig. 10, *left*).

Neuregulins facilitate the stimulatory effect of TGFα on PGE2 release from hypothalamic astrocytes

Because neuregulins bind only to erbB-3 and erbB-4 receptors but recruit erbB-2 and erbB-1 receptors for expanded signal

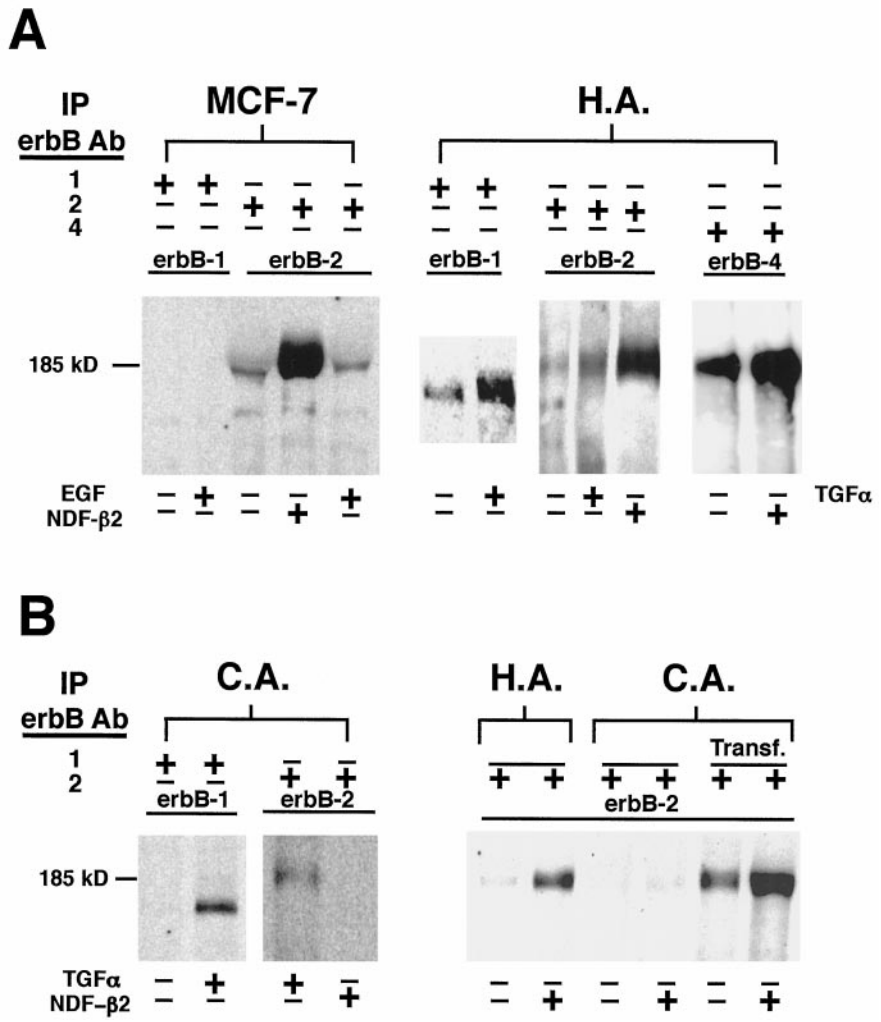

Figure 8. *A*, *Left* to *right*, Tyrosine phosphorylation of erbB-1 and erbB-2 receptors in MCF-7 cells induced by EGF and NDF-β2, and phosphorylation of erbB1, erbB-2, and erbB-4 receptors in hypothalamic astrocytes (*H.A.*) induced by TGFα or NDF-β2. The cells were exposed for 5 min to each ligand (100 ng/ml) before lysis. The erbB receptors were immunoprecipitated (*IP*) with antibodies specific to each protein, and the tyrosine phosphorylated receptors were identified by Western blots using phosphotyrosine antibodies. *B*, *Left* to *right*, Phosphorylation of erbB-1 and transphosphorylation of erbB-2 in cerebrocortical astrocytes (*C.A.*) by TGFα; inability of NDF-β_2 to phosphorylate erbB-2 in cortical astrocytes; phosphorylation of erbB-2 in hypothalamic astrocytes by NDF-β2; and effectiveness of NDF-β2 to induce erbB-2 phosphorylation in cortical astrocytes after transient overexpression (*Transf.*) of erbB-4.

transduction (Beerli et al., 1995; Karunagaran et al., 1996; Lemke, 1996; Burden and Yarden, 1997), we sought to determine whether neuregulins would be able to potentiate the effect of low concentrations of TGFα on glial PGE_2 release. Exposure of hypothalamic astrocytes to a low, ineffective dose of NDF-β2 (10 ng/ml) markedly facilitated the effect of marginally effective doses of TGFα (Fig. 10, *right*), suggesting that the two peptides are required for full erbB receptor-dependent activation of eicosanoid synthesis in hypothalamic astrocytes.

Neuregulin-induced activation of glial PGE_2 release requires erbB-2 receptors

In a variety of cell types, erbB-2 is required for neuregulin-dependent signaling via erbB-4 receptor activation (Carraway and Cantley, 1994; Beerli et al., 1995). ErbB-2 also functions, via receptor–receptor interactions, as an auxiliary subunit in erbB-1-mediated signal transduction (Karunagaran et al., 1996). It was, therefore, important to determine whether targeted inactivation of erbB-2 receptors would affect NRG and/or TGFα signaling capacity in hypothalamic astrocytes, as assessed by the ability of these growth factors to stimulate glial PGE_2 release in erbB-2-deficient cells. Because inactivation of erbB-2 receptors can be efficiently achieved by either administration of antisense ODNs (Colomer et al., 1994) or the intracellular expression of a recombinant single-chain antibody (Berrli et al., 1994; Beerli et al., 1995), we chose one of these approaches for our studies. Figure 11 (*top left*) demonstrates the effectiveness of an erbB-2 ODN treatment (1 μM for 16 hr) to reduce the levels of phosphorylated erbB-2 in hypothalamic astrocytes exposed to a 5 min NDF-β2 pulse. The inhibitory effect of the erbB-2 ODN was not seen when the cells were treated with an oligodeoxynucleotide containing the same base composition but in a scrambled order. No effect of the erbB-2 ODN on phosphorylated erbB-4 content was observed (Fig. 11, *top right*), indicating that the ODN selectively targets erbB-2. NDF-β2-induced PGE_2 release from hypothalamic astrocytes was abolished by exposing the cells to the same dose of erbB-2 ODN for the duration (16 hr) of the NRG treatment (Fig. 11, *middle*). The scrambled sequence was ineffective. The ODN partially blocked the effect of TGFα, further indicating that, as in immortalized cell lines (Beerli et al., 1995), erbB-2 is required for full expression of ligand-initiated, erbB-1-dependent signaling in primary astrocytes. The specificity of the erbB-2 ODN effect was further demonstrated by its failure to alter the stimulatory effect of basic FGF, or the lack of effect of IGF-I, on PGE_2 release (Fig. 11, *middle*). Thus, its inhibitory effect on NRG and TGFα signaling is not a result of a general inactivation of receptor tyrosine kinases in glial cells.

Neuregulins induce LHRH release via a glial intermediacy

Direct exposure of the LHRH-producing GT1–1 cells to NDF-β2 failed to stimulate LHRH release (Fig. 11, *bottom*). In contrast,

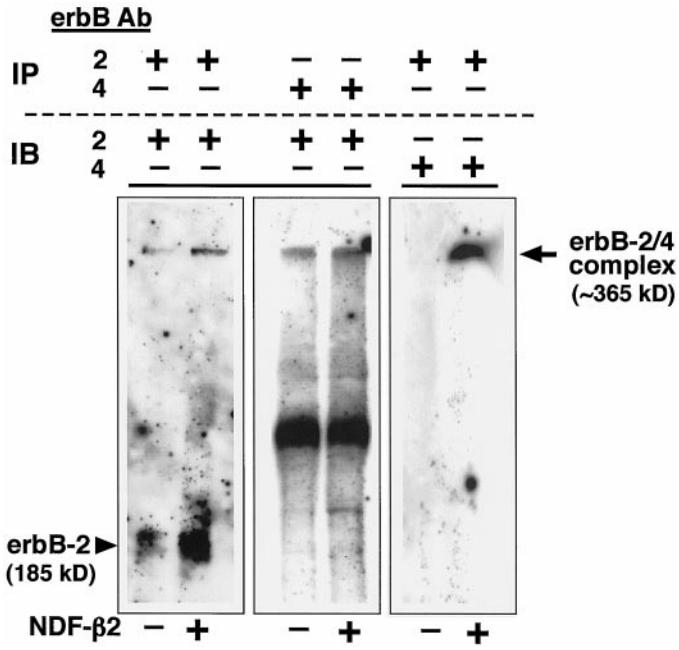

Figure 9. Heterodimerization of erbB-4 with erbB-2 receptors in hypothalamic astrocytes. After exposure to NDF-β2 (500 ng/ml, 3 min), the cells were exposed to the cross-linker BS^3, lysed, immunoprecipitated (*IP*) with either erbB-2 or erbB-4 antibodies, and blotted (*IB*) with erbB-2 or erbB-4 antibodies. Notice the formation of a high molecular weight complex containing both erbB-2 (*left* and *middle*) and erbB-4 (*right*).

the culture medium of hypothalamic astrocytes treated with NDF-β2 (N-CM) increased the release of LHRH more than threefold over control values. The changes observed were similar to those induced by the culture medium of astrocytes treated with TGFα (T-CM) (Fig. 11, *bottom*), a conditioned medium previously shown to stimulate LHRH secretion via its PGE_2 content (Ma et al., 1997a). Concomitant treatment of the astrocytes with either NDF-β2 or TGFα and the erbB-2 ODN abolished the effect of the NDF-β2-conditioned medium on LHRH release and partially suppressed that of the TGFα-conditioned medium (Fig. 11, *bottom*). Culture medium from astrocytes treated with either NDF-β2 or TGFα in the presence of the scrambled erbB-2 ODN sequence was as effective in stimulating LHRH release as culture medium from astrocytes treated with the growth factors alone. Direct application of culture medium from astrocytes treated with the scrambled sequence to GT1–1 cells also failed to affect basal LHRH release (Fig. 11, *bottom*). Thus, neuregulins appear to stimulate the secretory activity of LHRH-producing neurons via an astroglial-dependent, erbB-mediated activation of PGE_2 synthesis.

In vivo disruption of erbB-2 receptor synthesis by central administration of an erbB-2 ODN delays the onset of female puberty

To determine the physiological importance of hypothalamic erbB-2 receptor signaling in the central control of sexual maturation, juvenile female rats were treated with the same erbB-2 ODN used in the *in vitro* studies. The ODN was administered into the third ventricle of the brain via a cannula connected to a subcutaneously implanted osmotic minipump, delivering its content at a rate of 2.5 μg/hr. The ODN infusion was initiated on postnatal day 25, i.e., before the first increase in hypothalamic erbB-2 mRNA expression that occurs between postnatal day 26 and 28. Control animals received an infusion of the scrambled ODN or were left intact.

Both control groups reached puberty at a very similar age (Fig. 12), so that by postnatal day 37 all of them had ovulated (mean age at first ovulation, 35.5 ± 0.27 and 36.0 ± 0.30 d for intact and scrambled ODN-treated groups, respectively). In striking contrast, the animals infused with the erbB-2 ODN did not ovulate until after the content of the pump was exhausted (i.e., after 14 d of infusion) (Fig. 12). Ovulation occurred within 1–4 d after termination of the infusion, so that the mean age at first ovulation was 41.6 ± 0.43 d ($p < 0.01$ vs both control groups). Although these results indicate that erbB-2 receptors, and thus NRG-initiated signaling, is important for the timely initiation of female reproductive capacity, they also demonstrate that the central regulatory component that controls the onset of puberty can fully, and expeditiously, recover from the inhibitory effect of the erbB-2 ODN upon termination of the treatment.

DISCUSSION

In keeping with previous observations demonstrating that astroglial cells are targets of NRG action (Pinkas-Kramarski et al., 1994; Rio et al., 1997), our findings show that hypothalamic astrocytes contain functional NRG receptors of the erbB-2–erbB-4 subtypes. Ligand-dependent activation of this erbB-2–erbB-4 complex leads to the formation of substances involved in facilitating release of LHRH. Disruption of the complex abolishes the astrocytic response to NRGs and delays the onset of puberty when effected *in vivo*. Thus, regardless of any direct effect of NRGs on hypothalamic neurons, our findings indicate that astrocytic erbB-2–erbB-4 receptors are intrinsic components of the cell–cell signaling process that underlies the initiation of female puberty.

Although in several other cellular systems, NRGs use erbB-3 receptors to affect cellular function (Carraway and Cantley, 1994; Lemke, 1996; Burden and Yarden, 1997), neither the hypothalamus as a whole nor isolated hypothalamic astrocytes express the erbB-3 gene. Surprisingly, erbB-4 receptors found in hypothalamic astrocytes were not detected in astrocytes of the cerebral cortex, a region of the brain irrelevant to neuroendocrine control. Thus, as previously shown for the autoregulatory control of TGFα gene expression and the expression of estrogen receptors (Ma et al., 1994a), hypothalamic astrocytes appear to be molecularly different from astrocytes of brain regions not involved in neuroendocrine regulation.

The essential role of erbB-4 in mediating the effects of NRGs on hypothalamic astrocytes is indicated by three findings: (1) NRG1promotes heterodimerization of erbB-4 and erbB-2 in hypothalamic astrocytes, (2) NRG1 induces phosphorylation of erbB-2 in hypothalamic astrocytes (which contain erbB-4) but not cortical astrocytes (which lack these receptors), and (3) cortical astrocytes respond to NRG1 with erbB-2 phosphorylation only after gene transfer-mediated expression of erbB-4 receptors. That a normal contingent of functional erbB-4 receptors in astroglial cells is important for normal neuroendocrine reproductive development is suggested by the decreased plasma follicle-stimulating hormone levels observed in transgenic mice expressing an erbB-4 dominant negative mutant gene in astroglial cells (V. Prevot, C. Rio, Y. J. Ma, W. L. Dees, S. R. Ojeda, and G. Corfas, unpublished observations).

Earlier observations have led to the concept that TGFα is a physiological component of the glia-to-neuron signaling process that regulates LHRH neuronal activity during sexual develop-

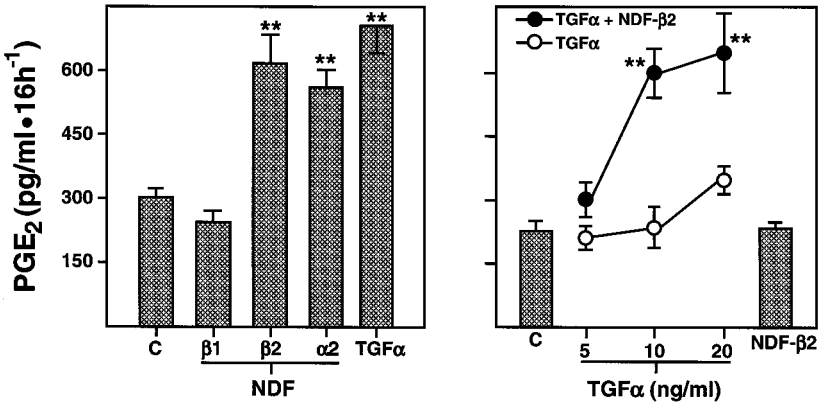

Figure 10. *Left*, Stimulatory effect of neuregulin isoforms (each at 50 ng/ml) on PGE_2 release from cultured hypothalamic astrocytes and comparison with the effect of TGFα (50 ng/ml). *Right*, Potentiation of the effect of TGFα on PGE_2 release from hypothalamic astrocytes by a dose of NDF-β2 (10 ng/ml) that by itself is ineffective. *Bars* or *circles* represent the mean of five to nine culture wells per group. *Vertical lines* are SEM. $^{**}p < 0.01$ versus control (*C*) group.

ment (Ma et al., 1992; Ojeda, 1994). The present findings indicate that actions of NRGs in hypothalamic astrocytes are closely coordinated with those of TGFα. On the one hand, and as shown in cell lines (King et al., 1988; Goldman et al., 1990; Wada et al., 1990; Riese et al., 1996), TGFα shares with NRG-1 the ability to induce phosphorylation of erbB-2. On the other, both growth factors can act independently on cultured astrocytes to stimulate release of PGE_2; when given together at ineffective doses, their individual effects are potentiated. Disruption of erbB-2 synthesis via an antisense oligodeoxynucleotide approach abolished the effect of NRG1 on PGE_2 release and reduced that of TGFα, implying that the recruitment of erbB-2 by activation of erbB-1 and erbB-4 receptors activates at least one common intracellular signal transduction pathway leading to eicosanoid formation. Although transduction of erbB-1-mediated signaling has been shown to involve metabolism of arachidonic acid to oxygenated products (Takasu et al., 1987), the pathways that may lead to prostaglandin formation upon erbB-2–erbB-4 receptor activation are not known.

The initiation of puberty is thought to be determined by events that occur within the brain independently of changes in gonadal steroid output (Ojeda and Urbanski, 1994; Terasawa, 1995). Once the change in "central drive" is initiated, the attendant changes in the secretion of pituitary gonadotropin hormones lead to activation of gonadal hormone secretion. Ovarian estrogen, in particular, becomes a prominent player in the process, because it not only promotes maturation of the reproductive organs but also facilitates further neuroendocrine development and, eventually, triggers the first preovulatory surge of gonadotropins. The present findings show that the hypothalamic content of the mRNAs encoding both erbB-2 and erbB-4 increases in two stages: first, during juvenile days, in the face of unchanging plasma steroid levels, and then at the time of puberty when ovarian steroid secretion is elevated. This latter increase in erbB receptor expression was reproduced by mimicking in immature rats the changes in circulating estrogen and progesterone levels seen at the time of puberty. It is thus likely that the earlier activation of hypothalamic erbB-2–erbB-4 gene expression is part of the gonad-independent increase in central drive that initiates puberty, whereas the subsequent, peripubertal increase is a steroid-dependent phenomenon. We do not know, however, whether these changes in erbB mRNA abundance are because of more cells expressing the receptors or a greater density of receptors per cell.

The existence of a functional relationship between the erbB signaling network and estrogen was initially recognized by the demonstration that estrogen can acutely upregulate the level of erbB-1 in uterus (Mukku and Stancel, 1985). More recently, the two systems were shown to be linked by cross talk mechanisms involving the transcriptional activation of estrogen responsive elements by both EGF and TGFα (Ignar-Trowbridge et al., 1993), the direct activation of erbB-2 phosphorylation by estrogen binding to the erbB-2 extracellular domain (Matsuda et al., 1993), and the mediatory contribution of the estrogen receptor to erbB-1 receptor signaling (Curtis et al., 1996). Earlier work demonstrated that estrogen increases TGFα gene expression in hypothalamic astrocytes (Ma et al., 1994a). The present findings extend these observations to include two more members of the erbB signaling network, erbB-2 and erbB-4, as targets of estrogen action. It is possible that activation of the hypothalamic erbB-2–erbB-4 complex before any increase in estrogen secretion occurs sets in motion signaling pathways specific to the NRG-erbB network and estrogen-dependent events able to accelerate the tempo of the pubertal process. During the onset of puberty itself the actions of estrogen may be potentiated by an increased activation of the erbB signaling module. Such an interaction has been demonstrated by the inability of EGF to exert estrogen-like effects in mice carrying a null mutation of the estrogen receptor (Curtis et al., 1996).

The present study does not identify the NRGs physiologically responsible for the prepubertal activation of the hypothalamic erbB-2–erbB-4 complex. To date, three subfamilies of NRGs have been identified. Members of the original family, now termed NRG1, consist of alternatively spliced products of a single gene (Marchionni et al., 1993; Wen et al., 1994). The second group, known as NRG2, is comprised of two members, NRG2α and NRG2β, which are encoded by a gene different from that encoding NRG1 (Carraway et al., 1997; Chang et al., 1997). An additional NRG, termed NRG3, is preferentially expressed in nervous tissue (Zhang et al., 1997). Our study shows that only NRG1 and NRG3 are produced in the hypothalamus as a whole and in astrocytes in particular. Thus, both of them may serve as physiological ligands for the astrocytic erbB-2–erbB-4 complex.

Several studies have demonstrated the importance of erbB-2 in erbB receptor-mediated signaling. ErbB-2 enhances the binding affinities of EGF to erbB-1 and NRGs to erbB-3 and erbB-4 via deceleration of ligand dissociation rates (Karunagaran et al., 1996). Although in some cellular contexts erbB- 2 is not required for NRG action (Beerli et al., 1995), heterodimeric receptor complexes that include erbB-2 have a greater affinity for NRGs than those complexes not containing erbB-2 (Tzahar et al., 1997). Moreover, NRGs appear to prefer the recruitment of erbB-2 for

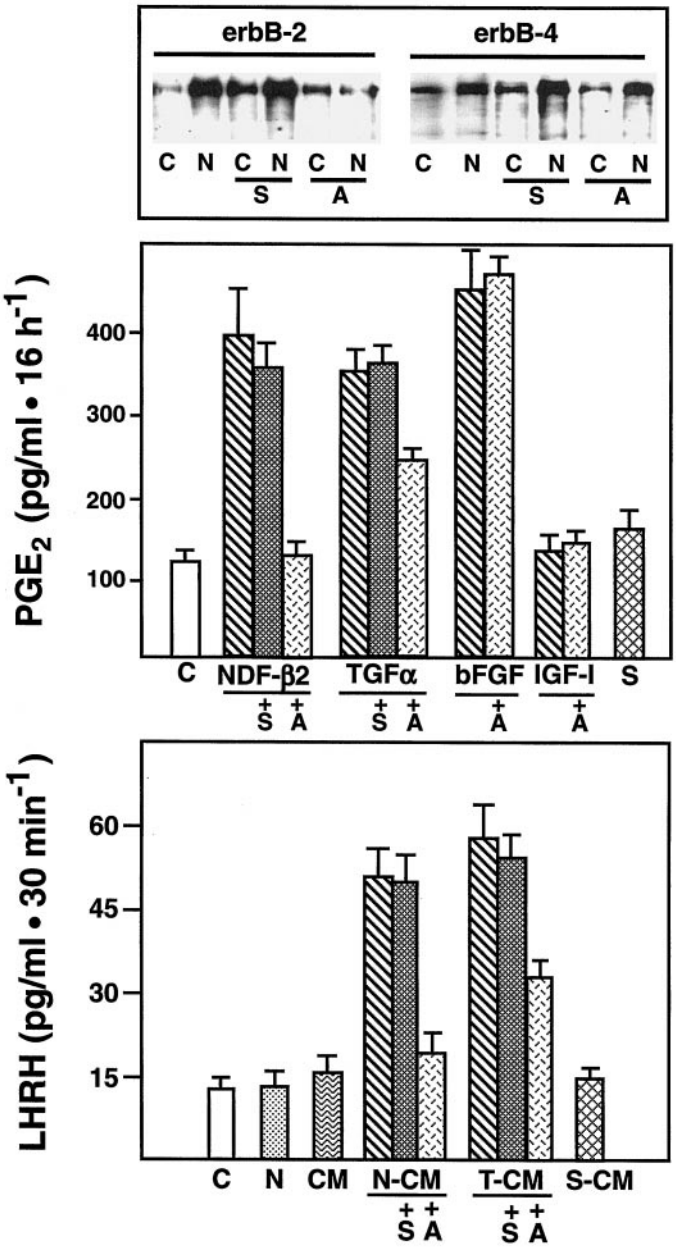

Figure 11. *Top left*, Selective decrease of phosphorylated erbB-2 in hypothalamic astrocytes treated with an antisense oligodeoxynucleotide to erbB-2 (erbB-2 ODN). The cells were treated with the ODN (*A*, 1 μM) or a scrambled sequence (*S*) for 16 hr. Then, some dishes were left untreated (*C*) and others were exposed to NDF-β2 (*N*, 50 ng/ml) for 5 min before immunoprecipitation and electrophoretic separation of phosphorylated erbB receptors. The phosphorylated receptors were detected with an antiphosphotyrosine monoclonal antibody. *Top right*, Failure of erbB-2 ODN to alter NDF-β2-induced erbB-4 receptor phosphorylation. *Middle*, Blockade of the stimulatory effect of NDF-β2 and partial inhibition of the effect of TGFα on PGE_2 release from cultured hypothalamic astrocytes by ODN-mediated inhibition of erbB-2 synthesis. Each growth factor was tested at 50 ng/ml; the erbB-2 ODN was used at a 1 μM concentration. *bFGF*, Basic fibroblast growth factor, 50 ng/ml; *IGF-I*, insulin-like growth factor I, 50 ng/ml; *C*, untreated controls; *A*, erbB-2 ODN; *S*, scrambled erbB-2 ODN sequence. *Bottom*, Stimulatory effect of a culture medium conditioned by exposure of hypothalamic astrocytes to NDF-β2 or TGFα on LHRH release from the GT1–1 LHRH-producing cells and blockade of this effect by treating the astrocytes with an erbB-2 ODN. *N*, GT1–1 cells treated directly with NDF-β2 (50 ng/ml); *CM*, cells treated with the conditioned medium from astrocytes cultured in the absence of added growth factors; *N-CM*, astrocyte culture medium conditioned by treating the astrocytes with NDF-β2 (50 ng/ml) for 16 hr; *T-CM*, astrocyte culture medium conditioned by TGFα treatment (50 ng/ml, 16 hr); *S-CM*, culture medium conditioned by treating the astrocytes with the scrambled ODN sequence. *Bars* are mean of six wells per group; *vertical lines* are SEM.

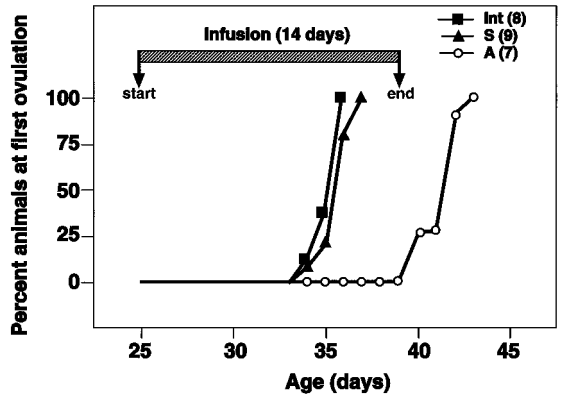

Figure 12. Delay of female puberty by targeted disruption of erbB-2 receptor synthesis via central administration of an erbB-2 ODN to developing rats. The erbB-ODN (*A*) or its scrambled sequence (*S*) were infused into the third ventricle of the brain via a stainless steel cannula connected to a subcutaneously implanted Alzet minipump delivering its content at the rate of 0.5 μl/hr for 14 d. The amount of ODN delivered was of 2.5 μg/hr. *Numbers* in parentheses are number of animals per group.

the formation of ligand-driven heterodimeric receptors (Tzahar et al., 1997), indicating that, in cells expressing erbB-2 and erbB-4 receptors, such as hypothalamic astrocytes, recruitment of erbB-2 to form a heterodimeric complex may be the preferred mechanism used by NGRs to achieve high-affinity binding and activation of effector signaling pathways.

The importance of erbB-2 for the initiation of puberty is suggested by the delay in puberty caused by the central inactivation of erbB-2 receptors via administration of an antisense ODN. That this delay was not caused by a toxic effect of the ODN is indicated by the normal growth of the animals during treatment and by the rapid initiation of reproductive development after termination of the ODN infusion. The ODN tested *in vitro* selectively reduced ligand-dependent erbB-2 phosphorylation without affecting that of erbB-4 and blocked the stimulatory action of NRG1 on astrocytic release of PGE_2, without affecting that of bFGF, which also acts via a receptor tyrosine kinase. An intact bFGF signaling system did not appear to compensate *in vivo* for the deficiency in erbB-2-dependent signaling during puberty (this study) or during early nervous system development (Morris et al., 1999).

Together, the present results indicate that NRG-mediated activation of an erbB signaling module composed of erbB-2 and erbB-4 receptors is an important component of the glia-to-neuron communication system controlling the secretory activity of LHRH neurons. Activation of this receptor complex appears to be involved in both the initial gonad-independent events that set in motion the pubertal process and the progression of steroid-regulated changes leading to the completion of puberty. The results are consistent with a model in which LHRH secretion is stimulated via a two-step mechanism. The initial step would involve a juxtacrine–paracrine stimulation of erbB receptors in astroglial cells via cell contact-dependent signaling (Fagotto and

Gumbiner, 1996). The second would involve release of neuroactive substances, such as PGE_2, capable of inducing the secretory activity of neighboring LHRH neurons. This mechanism is not exclusive to NRGs, because it also appears to operate in the case of the TGFα–erbB-1 signaling complex (Ojeda, 1994). The requirement of erbB-2 for both signaling processes strongly suggests that the activation of these two systems is a highly coordinated and interactive process involved in controlling the onset of mammalian puberty.

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