

erbB-1 and erbB-4 Receptors Act in Concert to Facilitate Female Sexual Development and Mature Reproductive Function

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Glial erbB-1 and erbB-4 receptors are key components of the process by which neuroendocrine glial cells control LHRH secretion and the onset of female puberty. We now provide evidence that these two signaling systems work in a coordinated fashion to control reproductive function. To generate animals carrying functionally impaired erbB-1 and erbB-4 receptors, we crossed Waved 2 (*Wa-2*^{+/+}) mice harboring a point mutation of the erbB-1 receptor with mice expressing a dominant-negative erbB-4 receptor in astrocytes. In comparison to single-deficient mice, double-mutant animals exhibited a further delay in the onset of puberty and a strikingly diminished adult reproductive capacity. Ligand-dependent erbB receptor phosphorylation and erbB-mediated MAPK (ERK 1/2) phosphorylation were impaired in mutant astrocytes. *Wa-2*^{+/+} or double-mutant astrocytes failed to respond

to TGF α with production of prostaglandin E₂, one of the factors mediating the stimulatory effect of astroglial erbB receptor activation on LHRH release. Medium conditioned by *Wa-2*^{+/+} or double-mutant astrocytes treated with TGF α failed to stimulate LHRH release from GT1-7 cells. The LH response to ovariectomy was significantly attenuated in mutant mice in comparison with wild-type controls. Although the *Wa-2* mutation affects all cells bearing erbB-1 receptors, these results suggest that a major defect underlying the reproductive defects of animals with impaired erbB signaling is a decreased ability of glial cells to stimulate LHRH release. Thus, a coordinated involvement of erbB-1 and erbB-4 signaling systems is required for the normalcy of sexual development and the maintenance of mature female reproductive function. (*Endocrinology* 146: 1465–1472, 2005)

FEMALE SEXUAL MATURATION and adult reproductive function require the coordinated and timely activation of LHRH neurons. These neurons, which in rodents are located in the preoptic region, extend their neurosecretory axons to the median eminence, where LHRH is released into the pituitary portal blood vessels for delivery to the adenohypophysis. There, LHRH binds to specific receptors to stimulate the secretion of the gonadotropins LH and FSH. In turn, these hormones promote gonadal development and support reproductive physiology.

Activation of LHRH neurons is thought to occur both in response to changing inputs from synaptically connected neuronal networks and via the activation of growth factor-dependent glia-to-neuron signaling pathways (for review see Ref. 1). One of these pathways uses the epidermal growth factor (EGF)-related peptides, TGF α and neuregulins (NRGs), and their cognate receptors, erbB-1 and erbB-4, to set in motion astroglial-LHRH neuron communication (reviewed in Ref. 2). *In vitro* studies showed that TGF α and NRGs produced by glial cells do not stimulate LHRH secretion directly; instead, they do so by acting in an autocrine and/or paracrine fashion on astrocytes themselves where

activation of erbB signaling results in prostaglandin E₂ (PGE₂) release. Upon release from astrocytes, PGE₂ acts on LHRH neurons to elicit LHRH secretion (3–5). In line with these studies, pharmacological blockade of erbB-1 receptor at the median eminence (6) or antisense oligonucleotide-mediated inhibition of erbB-2 receptor synthesis (5) delayed the onset of female puberty in rats. Although erbB-2 receptors do not bind any known member of the EGF family of ligands, they are recruited by activated erbB-1 or erbB-4 receptors to form heterodimeric complexes that enhance downstream signaling (7). To assess the role of glial erbB-3, erbB-4 and erbB-2 receptors in female sexual development, we engineered transgenic mice in which the function of these receptors is disrupted specifically in astroglial cells by the targeted expression of a dominant-negative (DN) truncated form of the receptor (DNerbB-4) (8). Because hypothalamic astrocytes do not express erbB-3 receptors (5), the mutant erbB-4 protein is expected to only affect astroglial erbB-4/erbB-2 signaling. Expression of this truncated form in astrocytes reduces the ability of these cells to respond to NRG stimulation with production of PGE₂ and also impairs NRG-induced LHRH release from neuroendocrine terminals of the median eminence, without affecting the ability of these nerve endings to respond to PGE₂ with LHRH release (8). In accordance with this deficit in LHRH secretion, female mice carrying the DNerbB-4 transgene exhibited delayed sexual maturation and a diminished reproductive capacity in early adulthood (8).

Despite the unavailability of an animal model in which the

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Abbreviations: ACM, Astrocyte-conditioned medium; DM, dominant-negative; EGF, epidermal growth factor; HRP, horseradish peroxidase; NRG, neuregulin; PGE₂, prostaglandin E₂.

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function of erbB-1 signaling is compromised in a cell-specific manner, alternative genetic approaches have been used to gain insights into the involvement of TGF α /erbB-1 signaling in the control of female sexual maturation. Thus, the transgenic overexpression of TGF α , and the intrahypothalamic grafting of cells engineered to produce TGF α have been shown to advance female puberty in rats (9, 10). In keeping with these observations, mice harboring the naturally occurring recessive *Wa-2* point mutation in the tyrosine kinase domain of the erbB-1 receptor (11, 12) have also been shown to have delayed puberty (13).

Here we report that mice carrying both the *Wa-2* mutation and a DNerbB-4 mutant receptor selectively expressed in astrocytes exhibit a more pronounced delay in the onset of puberty and a dramatically impaired adult reproductive function in comparison to single-mutant mice and wild-type animals. These defects appear to be caused, to a significant extent, by loss of erbB receptor-mediated astrocyte-to-neuron communication. These results indicate that the glial erbB-1/erbB-2 and erbB-4/erbB-2 receptor systems act coordinately in the hypothalamic control of reproductive function.

Materials and Methods

Transgenic mice

The generation of animals expressing a DN form of the erbB4 receptor (DNerbB-4) in astrocytes has been previously reported (8). *Wa-2*^{+/-} mice carrying a point mutation in the tyrosine kinase domain of the erbB-1 receptor (11) were obtained from The Jackson Laboratory (Bar Harbor, ME).

To generate double mutant mice, homozygous *Wa-2* male mice were crossed to homozygotes DNerbB-4 to obtain double heterozygous mice. DNerbB-4 homozygous mice were identified by crossing DNerbB-4-positive animals with wild-type mice. After genotyping the resulting progenies, those parents that produced three consecutive litters in which all pups were positive for the transgene were considered to be homozygous. The double heterozygous DNerbB-4⁺/*Wa-2*^{+/-} animals were bred among themselves to obtain the four desired genotypes: wild-type, DNerbB-4⁺/*Wa-2*^{-/-}, DNerbB-4^{-/-}/*Wa-2*^{+/+}, and DNerbB-4⁺/*Wa-2*^{+/+} (double mutant). Although animals carrying the DNerbB-4 transgene driven by the GFAP promoter were identified by genotyping (8), *Wa-2*^{+/+} mice were identified by their curly whiskers, vibrissae, and guard hairs, a pleiotropic recessive effect of the erbB-1 receptor point mutation (<http://jaxmice.jax.org/library/notes/index.html>) (11).

Evaluation of sexual maturation and adult reproductive function

To determine whether the astrocytic blockade of erbB4 signaling combined with a defective erbB-1 receptor have a cumulative effect on female sexual maturation, wild-type, single mutant and double mutant littermates were inspected daily for imperforation of the vaginal membrane (vaginal opening), starting on postnatal d 26. Thereafter, vaginal lavages were performed daily to identify the occurrence of the first estrus, which in rodents is manifested by a predominance of cornified cells (14). Although ovulation normally occurs on the day of estrous (14), detection of cornified cells in mice cannot be assumed to indicate that ovulation has occurred, unless vaginal cornification is followed by the appearance of a predominance of leukocytes (15). This feature defines the diestrous phase of the estrous cycle, and indicates that a functional corpus luteum was formed after ovulation. Therefore, a true first estrus (and thus the age at first ovulation) was considered to have occurred only when the cornified cells were replaced by at least 2 d of lavages containing mostly leukocytes (8).

To examine the effect of defective erbB-4 and erbB-1 signaling on adult reproductive capacity, young adult (50 d old) female mice of each of the four aforementioned genotypes were exposed to a fertile male of identical genotype (one male per female). Thereafter, litter birth and size

were recorded for at least 6 consecutive months for each breeding pair. The fertility index was calculated by dividing the number of litters recorded by the number of months during which each dam was monitored. The mean number of pups per litter was also calculated for each dam. The figures obtained from each independent breeding pair of each genotype were then collapsed into single means, which were used as independent observations to generate the overall mean for each group. The breeding capacities of single and double mutant male mice were assessed by the ability of these animals to produce two litters of normal size within 50 d of exposure to a wild-type female.

Antibodies, growth factors, and prostaglandins

Human recombinant TGF α was supplied by Upstate (Lake Placid, NY), NRG β 1 was purchased from Neomarkers (Union City, CA), β -cellulin from R&D Systems (Minneapolis, MN), and PGE₂ was obtained from Sigma (St. Louis, MO). erbB-1 receptors were immunoprecipitated using sheep polyclonal antibodies (Fitzgerald Industries, Concord, MA) and were detected in Western blots with goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as described (8). erbB-2 receptors were both immunoprecipitated and detected in immunoblots with rabbit polyclonal antibodies (SC-284, Santa Cruz). Phosphorylated erbB-1 and erbB-2 were detected with an antiphosphotyrosine monoclonal antibody (4G10) generously provided by Dr. Brian Drucker (Oregon Health & Sciences University, Portland, OR). The antibodies used to detect ERK1/2 (p44/p42 MAPK), and phospho-ERK1/2 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The secondary antibody, antimouse IgG peroxidase [horseradish peroxidase (HRP)], and the antirabbit IgG peroxidase (HRP) used in Western blots were from Pierce (Rockford, IL) and from Zymed Laboratories (South San Francisco, CA), respectively. The Anti-Goat/Sheep peroxidase (HRP) (Clone GT-34) was from Accurate Chemicals & Scientific Co. (Westbury, NY).

Cell culture

Astrocytes were isolated from the whole brain of 1- to 2-d-old wild-type and mutant mice and cultured as previously described (5, 8). Initially, each culture derived from a single brain. Once the genotype of the donor animal was established, the cultures to be employed for Western blots (see *Immunoprecipitation and Western blots*) were kept as individual cultures until conducting the experiment proper. The cultures to be used for PGE₂ and astrocyte-conditioned medium (ACM)-evoked LHRH release were pooled (two to three brains from the same genotype) before replating the astrocytes before the experiment (see below). In all cases, the cells were dispersed upon brain collection, and were first grown for 8–10 d in 75-cm culture flasks containing DMEM-F12 medium supplemented with 10% donor calf serum (Hyclone, Logan, UT). At this time, the astrocytes were isolated from other cell types by overnight shaking at 250 rpm and replated in either 15-cm dishes for immunoblots or 12-well plates for prostaglandin release experiments. After reaching 80–90% confluence, the medium was replaced with a serum-free, astrocyte-defined medium (DMEM supplemented with putrescine-100 μ M and insulin (5 μ g/ml) (5), and the cultures were used 4 d later for the experiments. To examine the effect of TGF α on PGE₂ release, the cells were incubated with the growth factor (at 100 ng/ml) for 16 h at 37 C. For erbB1/erbB2 and ERK 1/2 phosphorylation studies, TGF α , NRG β 1, and β -cellulin were added to the cultures for 5 min, and the cells were snap-frozen for subsequent protein extraction and SDS-PAGE analysis.

To assess the effect of astrocyte-derived substances on LHRH release, the GT1-7 immortalized LHRH-secreting cell line was used. The cells were plated (100,000 cells per well) in a 24-well plate and cultured with DMEM containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) for 24 h. Thereafter, the medium was replaced with serum-free neuronal defining medium for 24 h. Neuronal defined medium consisted of glutamate-free DMEM with transferrin (100 μ g/ml), putrescine (100 μ M), L-glutamine (2 mM), sodium selenite (30 nM), and insulin (5 μ g/ml). The cells were exposed to 0.5 ml of culture medium conditioned by brain astrocytes treated for 16 h with or without TGF α ACM. After a 30-min exposure to ACM, the medium was collected, boiled, and stored at -20 C until LHRH measurement.

Immunoprecipitation and Western blots

Cell cultures were lysed in immunoprecipitation assay buffer, and the extracted proteins were size-fractionated and transferred to polyvinylidene difluoride (Millipore, Billerica, MA) membranes, as described (5, 16). To identify phosphorylated erbB-1 and erbB-2 receptors, the receptors were immunoprecipitated using the polyclonal antibodies sc-03-G and sc-284 (Santa Cruz), respectively, electrophoresed, and immunoblotted with phosphotyrosine antibody 4G10, exactly as reported (8). To develop the immunoreaction, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA), developed using enhanced chemiluminescence (PerkinElmer, Boston, MA), and exposed to film. After stripping (8), the membranes were reprobbed with the same erbB-1 or erbB-2 antibodies used for immunoprecipitation to control for procedural variability.

To detect ERK1/2 phosphorylation, protein extracts (20 μ g protein per well) were loaded into an SDS-PAGE 4–20% polyacrylamide gel (Invitrogen Life Technologies, Carlsbad, CA), electrotransferred to a polyvinylidene difluoride membrane, blocked with 2.5% gelatin, and blotted with phospho ERK1/2 antibodies (Cell Signaling) diluted 1:1000. After incubation with horseradish peroxidase-conjugated secondary antibodies (Zymed, 1:5000), the membranes were treated with enhanced chemiluminescence reagents (PerkinElmer) and exposed to film. Thereafter, the membranes were reprobbed with antibodies against nonphosphorylated ERK1/2 (Cell Signaling).

Ovariectomy

Ovariectomy was performed on postnatal d 24. The ovaries were aseptically removed from animals anesthetized with isoflurane, via a single dorsal skin incision followed by blunt separation of the underlying muscle-aponeurosis interface. Different groups of mice were killed 24 and 96 h later.

Measurements of LHRH, PGE₂, and serum LH

PGE₂ released from astrocytes in response to TGF α , and LHRH released from GT1-7 cells in response to ACM, were detected by RIA, as described previously (8, 17). Serum levels of LH were measured by RIA as reported (8).

Statistics

The differences between several groups were analyzed by ANOVA followed by the Student-Newman-Keuls' multiple comparison test for unequal replications. The Student's *t* test was used to compare two groups. When comparing percentages, groups were subjected to arc-sine transformation before statistical analysis to convert them from a binomial to a normal distribution (18).

Results

Effect of the *Wa-2* mutation and/or astrocytic *DNerbB-4* expression on the onset of female puberty

To determine whether erbB-1 and erbB-4 receptor signaling cooperate to facilitate female sexual maturation, we assessed the time of puberty in the progeny of *Wa-2* and *DNerbB-4* double hemizygous breeding pairs. In agreement with earlier findings (13), vaginal opening was significantly delayed in *Wa-2*^{+/+} mice ($P < 0.05$ vs. wild-type controls). This delay was accentuated by the simultaneous expression of the *DNerbB-4* transgene ($P < 0.01$, Fig. 1A), which by itself did not affect the age at vaginal opening (8) (Fig. 1A). The time of puberty (defined by the age at first ovulation) was significantly delayed in *Wa-2*^{+/+} and double mutant *Wa-2*^{+/+}-*DNerbB-4*⁺ mice (Fig. 1B). In accordance with our previous study (8), the first ovulation was also delayed in *DNerbB-4*⁺ mice (Fig. 1B, $P < 0.05$). Because in the earlier study we used FVB mice, and in the present study the animals were of a

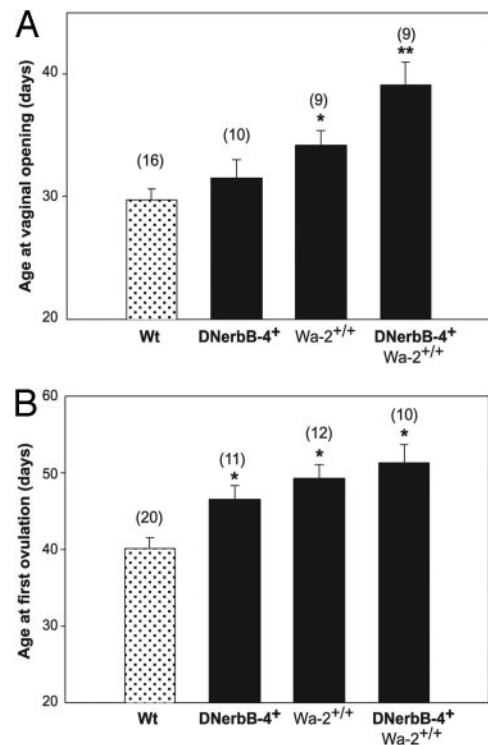


FIG. 1. Combining a generalized impairment of erbB-1 function with a selective blockade of astrocytic erbB-4 signaling results in a more pronounced delay of female sexual development. A, The age at vaginal opening is delayed in *Wa-2*^{+/+} mice, and it is further delayed in double-mutant *Wa-2*^{+/+}-*DNerbB-4* mice. B, The age at first ovulation is delayed in both single- and double-mutant mice. In all panels: *, $P < 0.05$ vs. wild-type group; **, $P < 0.01$ vs. all other groups. In this and subsequent figures, columns are means and error bars indicate SEM. Numbers on top of bars are number of animals per group.

mixed FVB (*DNerbB-4*) and B6EiC³H (*Wa-2*^{+/+}) background, these results indicate that the effects of disrupting astrocytic erbB-4 function on female sexual development are not dependent on the genetic background of the animals. Monitoring animal weight at the end of the second week (PN12) of postnatal life revealed a small, but significant, reduction in body weight for *Wa-2*^{+/+} and double mutant mice when compared with wild-type and *DNerbB-4*⁺ animals (Fig. 2A). Such a deficit was no longer observed in double mutant mice during the peripubertal period (PN36) (Fig. 2B), suggesting that delayed puberty in these animals was unlikely due to growth deficits.

Effect of the *Wa-2* mutation and/or astrocytic *DNerbB-4* expression on female fertility

Breeding young adult (50–60 d of age) *Wa-2*^{+/+} animals demonstrated that they deliver litters at longer intervals than wild-type controls because their fertility index (number of litters per month) is significantly reduced (Fig. 3A). This decrease was accentuated by the simultaneous expression of the *DNerbB-4* transgene ($P < 0.05$, Fig. 2A), which by itself did not affect litter frequency (Fig. 3A). In addition to this defect, *Wa-2*^{+/+} mice displayed a significant reduction in the size of their litters in comparison to wild-type and *DNerbB-4* animals (Fig. 3B). Although *DNerbB-4* mice had normal size

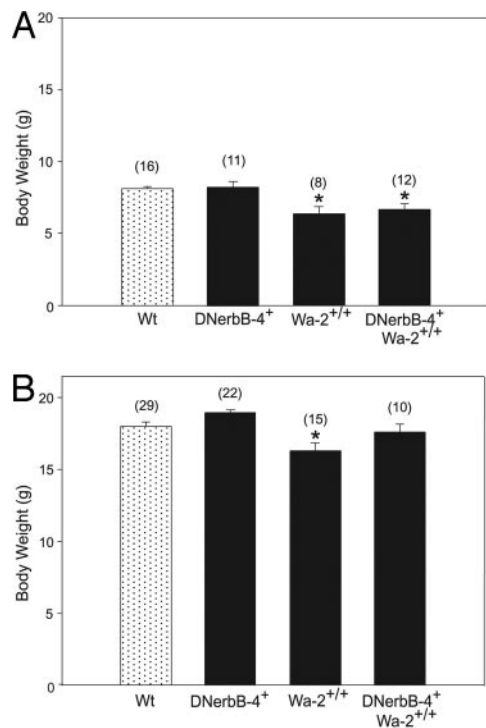


FIG. 2. Monitoring changes in body weight in wild-type, DNerbB-4⁺, Wa-2^{+/+}, and double mutant female mice during the infantile (PN12; A) and peripubertal (P36; B) periods of postnatal sexual development. In all panels: *, $P < 0.05$ vs. wild-type group. Numbers above bars are number of animals per group.

litters (Fig. 3B), expression of the DNerbB-4 transgene in the Wa-2^{+/+} background resulted in a striking reduction of litter size in comparison to all other groups (Fig. 3B). To determine whether this defect is gender specific, we bred Wa-2^{+/+} and double mutant males to wild-type females and found that both groups were capable of producing normal size litters (Fig. 3B). Thus, the impaired reproductive capacity observed in Wa-2^{+/+} and double mutant mice is due to a female defect.

The Wa-2 mutation impairs TGF α -dependent erbB-1/erbB-2 signaling in astrocytes

Although the Wa-2 mutation impairs EGF-dependent erbB-1 receptor phosphorylation and receptor tyrosine kinase activity in several peripheral tissues (11, 12), it is not known whether the ability of TGF α to signal via erbB-1 receptors in astrocytes is also affected. To address this issue, we used primary cultures of brain astrocytes. Immunoprecipitation combined with Western blot analysis showed that TGF α -induced phosphorylation of astrocytic erbB-1 receptor was diminished by 50% in astrocytes from Wa-2^{+/+} mice in comparison to wild-type astrocytes (Fig. 4A). Because erbB-1 heterodimerizes with erbB-2 in astroglial cells (8), we determined whether the Wa-2 mutation also affect erbB2 phosphorylation. As shown in Fig. 4B, the ability of erbB-1 to dimerize with erbB-2 receptors was not affected in Wa-2^{+/+} astrocytes, as indicated by the coimmunoprecipitation of equal amounts of erbB-1 receptors with anti-erbB-2 antibodies in both wild-type and Wa-2^{+/+} cells. In contrast, tyrosine phosphorylation of

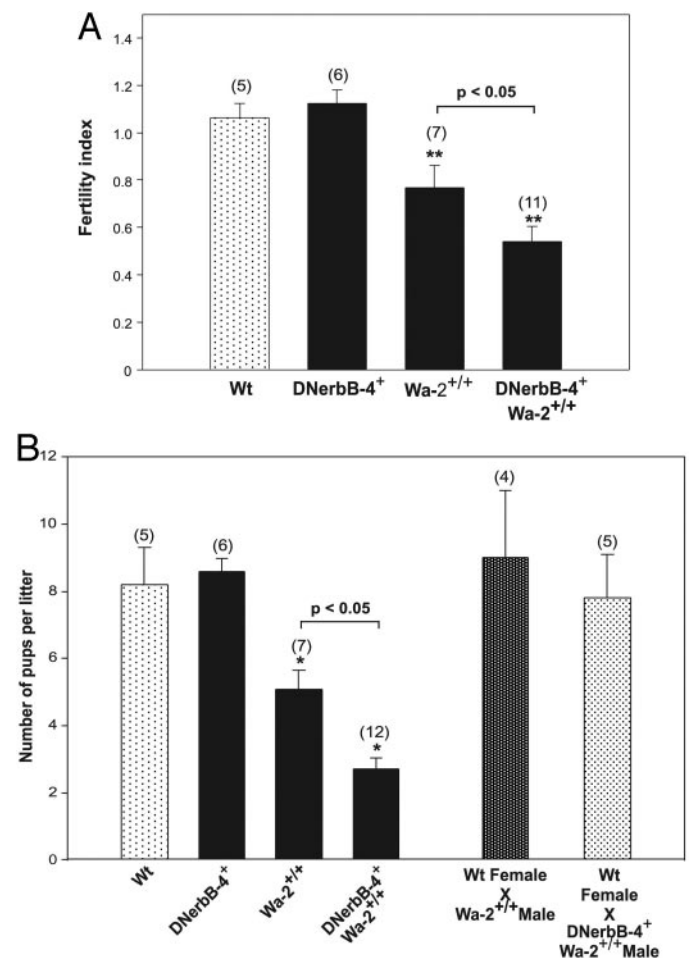


FIG. 3. Combining a Wa-2 mutation of the erbB-1 receptor with the selective expression of a DN form of the erbB-4 receptor in brain astrocytes disrupts adult female reproductive function. A, The fertility index is significantly reduced in Wa-2^{+/+} mutants and additional expression of DNerbB-4⁺ in these mice further accentuates the defect. To calculate this index, animals of each of the four genotypes were paired, and the date of birth and size of their litters were recorded for at least 6 consecutive months for each breeding pair. Thereafter, the number of litters delivered by each dam were collapsed into a single number, and these numbers were used to calculate the mean fertility index for each group (number of litters recorded per dam divided by the number of months during which each dam was monitored). B, The number of pups per litter is reduced in Wa-2^{+/+} animals and is dramatically reduced in double-mutant mice. Note that both Wa-2^{+/+} and double mutant males produce normal-sized litters when mated with wild-type females. In all panels: *, $P < 0.05$ vs. wild-type group; **, $P < 0.01$ vs. all other groups. Numbers on top of bars are number of dams per group.

erbB-2 receptors was decreased in mutant astrocytes (Fig. 4B), indicating that the Wa-2 mutation impairs the ability of erbB-1 receptors to cross-phosphorylate erbB-2 coreceptors in astrocytes.

The Wa-2 mutation combined to DNerbB-4 expression blunts ligand-dependent erbB activation of ERK1/2 phosphorylation in astrocytes

Having shown that ligand-dependent phosphorylation of both erbB-1 and erbB-2 is impaired in Wa-2^{+/+} astrocytes, and having recently demonstrated that transgenic

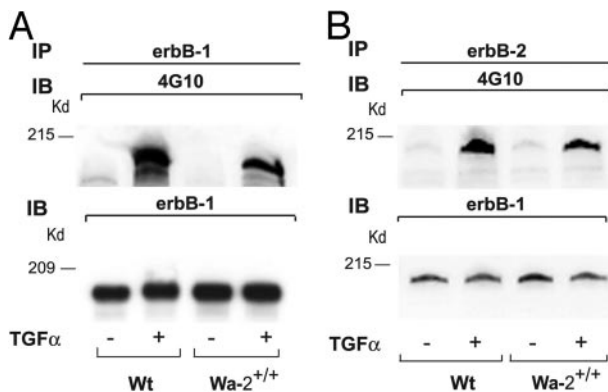


FIG. 4. The *Wa-2* point mutation of erbB-1 receptors in brain astrocytes diminishes ligand-induced erbB-1 and erbB-2 phosphorylation without affecting erbB-1/erbB-2 heterodimerization. A, The *Wa-2* mutation decreases ligand-dependent erbB-1 phosphorylation in brain astrocyte cultures. After treating the cells with TGF α (50 ng/ml for 5 min), erbB-1 receptors were immunoprecipitated (IP) and subjected to SDS-PAGE separation on two different gels. After transfer, one of the membranes was immunoblotted (IB) with 4G10 antiphosphotyrosine antibodies (*top panel*) to assess erbB-1 phosphorylation, and the other membrane was probed for erbB-1 (*bottom panel*) to define the relative amount of receptor immunoprecipitated in each sample. B, The *Wa-2* mutation diminishes TGF α -induced erbB-2 activation, without affecting erbB-1/erbB-2 heterodimerization. Cultured astrocytes were treated with TGF α (50 ng/ml for 5 min), lysed, and immunoprecipitated with erbB-2 antibodies before SDS-PAGE, and immunoblotting with 4G10 (*top panel*). The membrane was stripped and incubated with erbB-1 antibodies to determine the relative amount of coimmunoprecipitated receptors. Each lane was loaded with astrocytic proteins derived from a single brain of each genotype (for additional details see *Materials and Methods*). The experiment was repeated twice and one of the blots was selected for presentation.

expression of DNerbB-4 disrupts ligand-induced erbB-4/erbB-2 signaling in brain astrocytes (8), we asked the question as to whether the combined presence of *Wa-2* mutant erbB-1 receptors and the DNerbB-4 transgene in astrocytes would further disrupt erbB signaling in these cells. We used the phosphorylation of ERK1 and ERK2 as an end point, because the extent and kinetics of ERK1/2 activation has been shown to be similar in both erbB-1 and erbB-4 ligand-activated signaling (19). Primary cultures of astrocytes obtained from individual brains from wild-type, DNerbB-4⁺, *Wa-2*^{+/+}, and double-mutant littermates were treated for 5 min with TGF α , NRG β_1 , or β -cellulin (at 50 ng/ml each) to differentially activate erbB-1, erbB-4, or both signaling pathways, respectively. Treatment of wild-type astrocytes with each of these ligands resulted in the expected increase in ERK1/2 phosphorylation (Fig. 5A). DNerbB-4 astrocytes responded to TGF α and β -cellulin, but not to NRG β_1 (Fig. 5B). Conversely, *Wa-2*^{+/+} astrocytes fail to respond to TGF α but responded well to both NRG β_1 and β -cellulin (Fig. 5C). In contrast to this selectivity, the ERK1/2 phosphorylation response of astrocytes from double-mutant mice to each of the three ligands was markedly impaired (Fig. 5D). Thus, a combined defect in erbB-1 and erbB-4 receptor function significantly reduces the signaling capabilities of erbB ligands in astroglial cells.

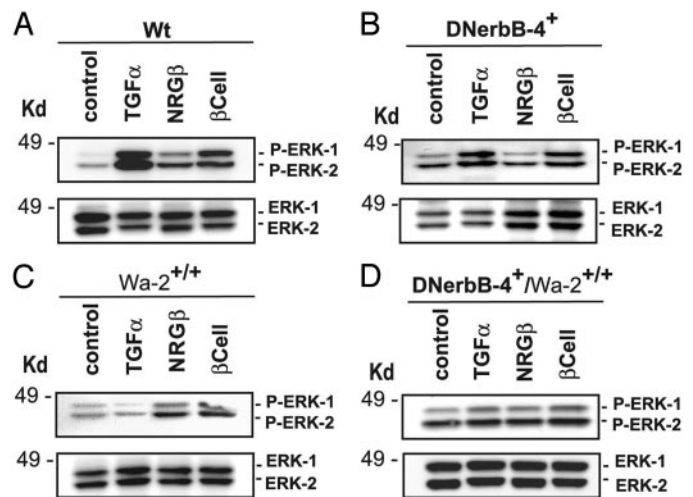


FIG. 5. Differential effect of the *Wa-2*^{+/+} mutation and/or DNerbB-4 expression on ligand-dependent erbB stimulation of ERK1/2 phosphorylation in cultured astrocytes. Astrocyte cultures were obtained from brains of individual 2-d-old littermates derived from double hemizygous crosses. Although the occurrence of the *Wa-2*^{+/+} mutation in donor mice was determined by visually inspecting the whiskers before preparation of the cultures, the presence of DNerbB-4 was ascertained a few days after initiating the culture by genotyping the donors. Astrocytes were treated with 50 ng/ml TGF α , NRG β_1 , or β -cellulin (β -cell) for 5 min to differentially activate erbB-1 or erbB-4, or both signaling pathways, respectively. Equal amounts of lysates were blotted with antibodies specific for phosphorylated ERK 1/2 (*upper panels*). Membranes were stripped and reprobed with antibodies to nonphosphorylated ERK1/2 to determine the relative amount of protein loaded (*lower panels*). The extent of ERK 1/2 phosphorylation subsequent to stimulation with EGF-related peptides is shown for wild-type (Wt) (A), DNerbB-4⁺ (B), *Wa-2*^{+/+} (C), and DNerbB-4⁺/*Wa-2*^{+/+} (D) astrocytes. Each lane was loaded with astrocytic proteins derived from a single brain of each genotype (for additional details see *Materials and Methods*). Each experiment was repeated two to three times and one of the blots was selected for presentation.

The *Wa-2* mutation blocks TGF α -induced release of PGE₂ from astrocytes and ACM-induced LHRH release from GT1-7 cells

We previously showed that TGF α stimulates LHRH release via a glial intermediacy that involves activation of astrocytic erbB-1 receptors and the subsequent release of PGE₂ (3), which then binds to specific receptors on LHRH neurons (4) to elicit LHRH release. To determine whether this pathway was affected in astrocytes from *Wa-2*^{+/+} mice, we assessed the PGE₂ response of these cells to TGF α , and the LHRH response of GT1-7 cells to the culture medium of astrocytes treated with TGF α . Although, as shown previously (8), both wild-type and DNerbB-4 astrocytes responded to TGF α (100 ng/ml, 16 h exposure) with a significant increase in PGE₂ release, *Wa-2*^{+/+} astrocytes failed to do so (Fig. 6A). Moreover, GT1-7 cells incubated for 30 min in ACM derived from astrocytes exposed or not to TGF α released LHRH in response to the ACM derived from wild-type and DNerbB-4 astrocytes treated with TGF α but failed to release the decapeptide when exposed to ACM derived from TGF α -treated *Wa-2*^{+/+} or double mutant astrocytes (Fig. 6B). Thus, disruption of erbB-1 receptor function by the *Wa-2* mutation significantly impairs the ability of TGF α to

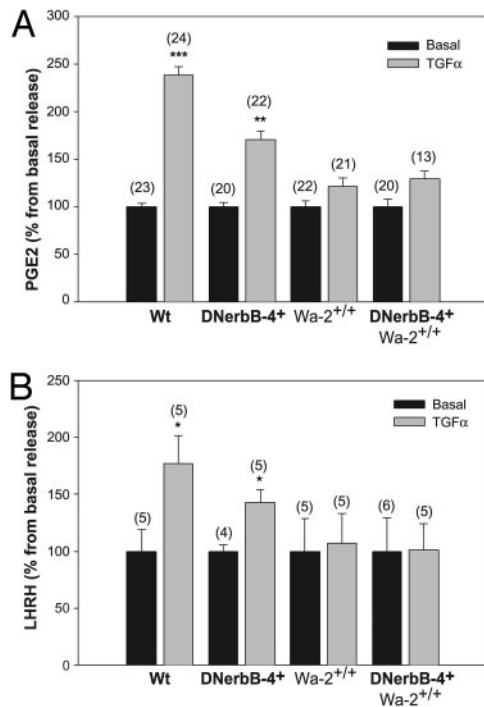


FIG. 6. *Wa-2* astrocytes fail to respond to TGF α with PGE₂ release, and the astrocyte conditioned medium produced by these astrocytes fails to stimulate LHRH release from GT1–7 cells. A, TGF α -induced PGE₂ release is suppressed in *Wa-2*^{+/+} astrocytes. ***, $P < 0.001$; **, $P < 0.01$ vs. basal release. B, The conditioned medium (ACM) of TGF α -treated *Wa-2*^{+/+} astrocytes does not elicit LHRH release from GT1–7 cells. *, $P < 0.05$ vs. basal release.

activate the PGE₂-dependent glia-to neuron communication required for glial control of LHRH secretion.

Wa-2^{+/+} and DNerbB-4 mutant mice exhibit deficits in the LH response of the hypothalamic-pituitary unit to removal of ovarian negative feedback control

To determine in an *in vivo* context the ability of the hypothalamic-pituitary unit to respond with gonadotropin release to the loss of a hormonal restraint, we compared the LH response to ovariectomy in wild-type and mutant mice. Because puberty is delayed in both *Wa-2*^{+/+} and DNerbB-4 mice, we performed this experiment in juvenile animals in an attempt to detect a central defect in gonadotropin secretion near the time of puberty. The results showed that the LH response to ovariectomy was significantly blunted in the mutant animals, a change that was initiated 24 h after ovariectomy and became unmistakable at 96 h (Fig. 7). Thus, in both *Wa-2*^{+/+} and DNerbB-4 females the ability of the hypothalamic-pituitary unit to increase its LH output in response to removal of gonadal inhibitory control is compromised.

Discussion

The present results demonstrate that the glial erbB-1 and erbB-4 signaling systems are not only independently involved in the glial-to-neuron communication process that control LHRH release, but also act in a complementary fashion to promote normal female sexual maturation and adult

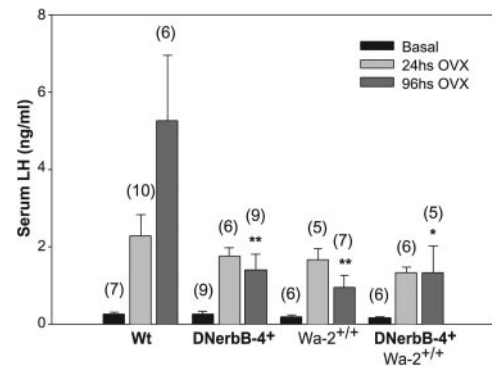


FIG. 7. The LH response to ovariectomy (OVX) is blunted in *Wa-2*^{+/+}, DNerbB-4 and double-mutant mice 96 h after removal of the gonads. **, $P < 0.01$; *, $P < 0.05$ vs. LH levels in wild-type mice 96 h after OVX. All mice were ovariectomized on postnatal d 24.

reproductive function. In agreement with previous findings (8, 13), mice carrying either the *Wa-2* mutation or a DNerbB-4 receptor selectively expressed in astrocytes exhibited delayed puberty and decreased fertility. Importantly, the combination of these two deficits resulted in a more pronounced effect on female reproductive function. This accentuated defect was related to a diminished ligand-dependent activation of erbB signaling in astrocytes. Thus, although the *Wa-2* mutation is not cell specific and, therefore, can affect reproductive function at every level of the hypothalamic-pituitary-ovarian axis, our findings are consistent with the notion that an adequate level of glial erbB-1 and erbB-4 activity is required for normal female sexual development and adult reproductive function. It is noteworthy that the use of astrocytes from only the hypothalamus instead of the entire brain may have resulted in an even greater effect of disrupting erbB-4 receptor function on ligand-induced ERK phosphorylation in the DNerbB-4 and double-mutant groups. This is because hypothalamic astrocytes contain erbB-4 receptors, but other brain regions such as the cerebral cortex do not (5).

The complementary involvement of glial erbB-1 and erbB-4 signaling pathways in the paracrine control of LHRH release was first suggested by *in vitro* experiments demonstrating that cotreatment of hypothalamic astrocytes with low doses of NRG and TGF α , that by themselves were ineffective, resulted in PGE₂ production (5). Additional evidence suggesting that coregulation of glial erbB-1 and erbB-4 signaling system by physiological stimuli is an important component of neuroendocrine neuron-glia communication was provided by the demonstration that concomitant activation of metabotropic and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors promotes ligand-dependent activation of both erbB1 and erbB-4 receptors in hypothalamic astrocytes (20). Because an increase in glutamatergic neurotransmission appears to be one of the primary events underlying the advent of puberty (1), these findings suggested that the facilitatory control exerted by glutamatergic neurons on LHRH release also involves the coordinated activation of a dual, erbB-1/erbB-4-dependent glia-to-neuron communication pathway.

The close coordination of ligand-induced erbB signaling in astrocytes may be directly linked to the ability that erbB-1

shares with erbB-4 to induce phosphorylation of erbB-2 coreceptors. Ligand-promoted phosphorylation of erbB-2 via the formation of erbB-4/erbB-2 heterodimers is abrogated in DNerbB-4 astrocytes (8). Here we show that the ability of erbB-1 receptors to transphosphorylate erbB-2 coreceptors is also impaired in *Wa-2^{+/+}* astrocytes. The physiological importance of these effects is demonstrated by the delay sexual maturation observed in rats in which erbB-2 synthesis was selectively inhibited using antisense oligodeoxynucleotides (5). Interestingly, and as shown for other glial cell types (21, 22), a certain degree of erbB-2 heterodimerization with erbB-1 (this study) and erbB-4 receptors (5) occurs in astrocytes in the absence of exogenous ligand stimulation. This physical association might be driven by endogenous erbB ligands produced by astrocytes themselves (5, 23). Such an interaction might enable hypothalamic astrocytes to sense microchanges in their extracellular environment like the processing of erbB ligand precursors by matrix metalloproteinases (24, 25). An increase in metalloproteinase activity has been shown to be required for glutamate receptors to transactivate both erbB-1 and erbB-4 receptors in hypothalamic astrocytes (20).

Because the *Wa-2* mutation affects all cells containing erbB-1 receptors, it is unlikely that the defects in reproductive function detected in these and double-mutant mice are only the consequence of a central defect. However, the alterations in glial erbB-1 signaling, astrocytic PGE₂ release, neuronal LHRH release in response to ACM, and LH response to ovariectomy clearly demonstrate the existence of a central defect. A previous study arrived to a similar conclusion by demonstrating that the *Wa-2* mutation disrupts the synchrony that exists between behavioral estrus and ovulation, and by showing that central, pharmacological blockade of erbB-1 receptors resulted in similar abnormalities (13). Again, it is possible that the diminished fertility observed in *Wa-2^{+/+}* and double-mutant mice are secondary to defects in sexual behavior. Without denying the potential contribution of such defects, the clear-cut deficiency in astrocyte-induced LHRH release associated with the *Wa-2^{+/+}* phenotype argues strongly for the existence of a central, neuroendocrine defect contributing to the delay in puberty and the diminished fertility observed in these mutant mice. This conclusion is further supported by the impaired ability of *Wa-2^{+/+}*, DNerbB-4⁺ and double-mutant juvenile mice to respond with LH release to removal of the ovaries. The lack of alterations in body weight in double-mutant mice reinforces this view. Because this is the group exhibiting the most pronounced alterations in reproductive function, and yet it grows normally, it would not appear that alterations in the output of pituitary hormones either controlling or influencing growth are responsible for the reproductive defects observed. A very interesting aspect of the present study is the finding that male reproductive function was not affected by the disruption of erbB-1/erbB-4 receptors. A strong precedent for this notable sex difference does indeed exist because several null mutations that cause female infertility have no effect on male reproductive capacity. Prominent examples can be found in the targeted disruption of the cyclooxygenase (26), progesterone receptor (27), and FSH (28) genes.

In summary, the present results demonstrate that the func-

tional integrity of both erbB-1 and erbB-4 signaling system in hypothalamic astrocytes is critical for glial cells to engage in neuron-glia interactions able to facilitate LHRH secretion at the time of puberty. This functional integrity also appears to be an important component of the central mechanism controlling female reproductive function in adulthood.

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