Altered Gene Activity of Epidermal Growth Factor Receptor (ErbB-1) in the Hypothalamus of Aging Female Rat Is Linked to Abnormal Estrous Cycles

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Activation of the ErbB-1 receptor is necessary for initiating mammalian female puberty by stimulating the release of LH-releasing hormone (LHRH), the neuropeptide necessary for controlling sexual maturation and reproduction. These neurons are scattered in the basal forebrain and send their axons to the median eminence (ME), where LHRH is released. LHRH, in turn, acts on the anterior pituitary where it stimulates the release of gonadotropins (LH and FSH) that regulate ovarian function. LH surge, expression levels in the preoptic area of YA rats increased to a maximal value. No such increase in ErbB-1 mRNA was found in MA rats. This difference was confirmed by the analysis of in situ hybridization histochemistry, where a stronger mRNA signal was observed in the preoptic area of YA rats compared with MA females. ErbB-1 protein levels measured by Western blot reflected this difference. A peak level of ErbB-1 mRNA in the median eminence-arcuate nucleus was detected at 0800 h in YA rats, but it was delayed in MA animals. There were intense ErbB-1 mRNA-positive cells in the arcuate nucleus. Pharmacological blockade of ErbB-1 receptor-mediated signal transduction resulted in the disruption of estrous cyclicity in YA rats. These results indicate that ErbB-1 receptors are necessary for maintaining normal estrous cycles. Consequently, age-related alterations in hypothalamic ErbB-1 gene activity may contribute to a delayed preovulatory LH secretion in aging females. Thus, the ErbB-1 signaling system plays an important role in the control of female reproduction during adulthood. (Endocrinology 143: 577–586, 2002)

MAINTAINING FEMALE reproduction depends upon normal activities of neurons able to release LH-releasing hormone (LHRH), the neuropeptide necessary for controlling sexual maturation and reproduction. These neurons are scattered in the basal forebrain and send their axons to the median eminence (ME), where LHRH is released. LHRH, in turn, acts on the anterior pituitary where it stimulates the release of gonadotropins (LH and FSH) that regulate ovarian function. LH surge, expression levels in the preoptic area of YA rats increased to a maximal value. No such increase in ErbB-1 mRNA was found in MA rats. This difference was confirmed by the analysis of in situ hybridization histochemistry, where a stronger mRNA signal was observed in the preoptic area of YA rats compared with MA females. ErbB-1 protein levels measured by Western blot reflected this difference. A peak level of ErbB-1 mRNA in the median eminence-arcuate nucleus was detected at 0800 h in YA rats, but it was delayed in MA animals. There were intense ErbB-1 mRNA-positive cells in the arcuate nucleus. Pharmacological blockade of ErbB-1 receptor-mediated signal transduction resulted in the disruption of estrous cyclicity in YA rats. These results indicate that ErbB-1 receptors are necessary for maintaining normal estrous cycles. Consequently, age-related alterations in hypothalamic ErbB-1 gene activity may contribute to a delayed preovulatory LH secretion in aging females. Thus, the ErbB-1 signaling system plays an important role in the control of female reproduction during adulthood. (Endocrinology 143: 577–586, 2002)

LHRH neurons is important for the activation of LHRH release that leads to the onset of female puberty. The regulatory signaling events of astroglia are mediated by membrane-anchored ErbB tyrosine kinase receptors that belong to the epidermal growth factor receptor family (14). For instance, activation of ErbB-1 receptors in hypothalamic astrocytes by TGFα of astrocytic origin results in the production of PGE₂, which consequently stimulates the release of LHRH, leading to the onset of mammalian female puberty (15). It is further demonstrated that induction of ErbB-1-mediated signaling events by TGFα in the hypothalami of transgenic mice (16) or mice grafted with genetically engineered TGFα-producing cells (17) results in an enhanced LHRH release. This, in turn, leads to acceleration of sexual maturation. Conversely, blockade of ErbB-1-mediated signal transduction delays the onset of puberty due to the reduction of LHRH release (18, 19).

It is clear that activation of ErbB-1 in hypothalamic astroglial cells contributes to the initiation of mammalian female puberty by the stimulation of LHRH secretion. This view is also supported by the finding that activation of ErbB-1 receptors by TGFα occurs during the onset of puberty in rhesus monkeys (20, 21). Little is known, however, about potential roles of this and other related receptors in the maintenance of reproduction during adulthood. This is a critical issue because it is relevant to the possibility that altered capacity of the ErbB receptor signaling system may contribute to estrous acyclicity in reproductive aging females. The present study was undertaken to examine the hypothesis that age-related changes in ErbB-1 gene function may be linked to

Abbreviations: DMSO, Dimethylsulfoxide; EIA, enzyme immunoassay; LHRH, LH-releasing hormone; MA, middle-aged; ME-ARC, median eminence-arcuate nucleus; MPA, medial preoptic area; nt, nucleotides; OVLT, organum vasculosum of the lamina terminalis; P4, progesterone; POA, preoptic area; YA, young adult.
altered cyclicity in aging female rats. Semiquantitative RT-PCR and Western blot analyses were used to examine the expression of hypothalamic ErbB-1 from young adult and aging females. In situ hybridization histochemistry was also employed to examine expression of ErbB-1 mRNA in the hypothalamic areas related to control of LH-RH release. Age-related changes in ErbB-1 receptor expression occur in aging female rats and may be linked to altered gonadotropin release. Importantly, blockade of ErbB-1 receptor-mediated signal transduction via a highly specific ErbB-1 inhibitor resulted in disruption of estrous cyclicity in adult female rats.

Materials and Methods

Animals

Young adult (YA; 4–5 months old, proven breeder) and middle-aged (MA; 9–10 months old, retired breeder) female rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Additional young adult females (1–2 months old) were purchased to be used for ErbB-1 receptor blockade experiments. They were housed two per cage and maintained under a controlled temperature (23–25 C) and photoperiod environment (14 h of light, 10 h of darkness, lights on at 0500 h) with food and water available ad libitum. Estrous cyclicity was determined by daily monitoring vaginal lavage. The proestrous state was determined by 1) vaginal cytology and 2) uterine weight of at least 200 mg and a uterus “balanced” with fluid. YA rats displaying at least two or three consecutive regular 4- to 5-d estrous cycles and MA rats exhibiting regular, consecutive regular 4- to 5-d estrous cycles and MA rats exhibiting regular, altered cyclicity in aging female rats. Semiquantitative RT-PCR and Western blot analyses were used to examine the expression of hypothalamic ErbB-1 from young adult and aging females. In situ hybridization histochemistry was also employed to examine expression of ErbB-1 mRNA in the hypothalamic areas related to control of LH-RH release. Age-related changes in ErbB-1 receptor expression occur in aging female rats and may be linked to altered gonadotropin release. Importantly, blockade of ErbB-1 receptor-mediated signal transduction via a highly specific ErbB-1 inhibitor resulted in disruption of estrous cyclicity in adult female rats.

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Every experiment was performed in compliance with guidelines on the use of laboratory and experimental animals. The protocols used were approved by our institutional animal care and use committee at the University of Nebraska Medical Center (protocol 99-055-00).

Tissue dissection and RNA isolation

Immediately after decapitation, brains were removed, and two major parts were dissected from the hypothalamus as described previously (22). Briefly, one is the preoptic area (POA; −2 mm thick) comprising the region dorsal and anterior to the optic chiasm. The POA was collected by a coronal cut just posterior to the entry point of the optic chiasm and two cuts converging from the lateral edges of the optic chiasm to a point on the hypothalamic sulci on each side. A site on the anterior region of the cortex was also collected to serve as a control tissue. All collected tissues were immediately stored at −80 C until RNA extraction. Total RNA was isolated by the acid-phenol method (23).

RT-PCR assay

Oligodeoxynucleotides. All oligodeoxynucleotides used for PCR were synthesized by MWG-Biotech, Inc. (High Point, NC). An oligonucleotide containing a 15-mer polydeoxynucleotide sequence (purchased from Promega Corp., Madison, WI) was used for RT of poly(A) cellular mRNA. An extracellular portion of rat ErbB-1 cDNA (272 bp) was amplified from total RNA derived from the POA, ME-ARC, or cortex using primers corresponding to nucleotides (nt) 311–330 (5'-CGATGT- TCAAAACACTTGGA-3') and complementary to nt 563–582 (5'-AATT- TCTTGTAAATCGCCGA-3') (24). Individual sources of variability were accounted for by coamplifying cyclophilin mRNA, which is constitutively expressed in brain (25). In addition, it has been shown that no significant differences in cyclophilin mRNA levels are detectable by reproductive status or age (13, 18). Therefore, cyclophilin was used as an internal control. The cyclophilin primers were 5'-GGCAAGTCCATC- TACGG3' (corresponding to nt 265–282) and 5'-ACATGGTTGCGA- CTCACAGC3' (complementary to nt 405–422) (25).

RT-PCR procedures. The procedures have been described previously in detail (20) with minor modifications. In brief, RT reaction was carried out for 2 h at 37 C in a 20-μl volume. Each reaction mixture contained 200 ng total RNA that was ribonuclease-free and deoxyribonuclease-treated, 1× RT buffer, 0.01 M dithiothreitol, 0.5 mM of each dNTP, and 20 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). PCR was performed in a 25-μl total volume consisting of 2 μl diluted (1 μl RT/1 μl) RT mixture, 2.5 μl of 10× PCR buffer, 4 μl 25 mM MgCl2, 1 μl 10 mM dNTP, 20 pmol of each specific gene primer set including both 5'- and 3'-primers, 5 pmol of each 5'- and 3'-end cyclophilin primer, and 0.625 U Taq polymerase (Promega Corp.). After samples were treated at 94 C for 4 min to inactivate the reverse transcriptase, PCR consisted of 35 cycles of denaturing (95 C, 15 sec), annealing (55 C, 1 min), and a final extension of 7 min at 72 C.

Quantitative analysis

Amplified cDNAs were visualized by electrophoresis where 20 μl of each PCR reaction sample were run on a 3% agarose gel containing ethidium bromide (0.1 g/ml). The images were captured by photography on 555 Polaroid film (Cambridge, MA). The photos were scanned for densitometric analysis using Hewlett-Packard Co. ScanJet 6200C flat bed scanner and the computer image program written by Dr. Wayne Rasband (NIH, Bethesda, MD). We used a background-subtracted mean OD to measure each amplified cDNA signal. Mean OD values were normalized according to the coamplified cyclophilin cDNA value detected in each sample. The authenticity of each PCR-amplified product was confirmed by sequencing analysis (22).

Western blot analysis

Tissue samples (four to six ME-ARC or POA fragments per time point) were collected at 1000, 1200, and 1500 h during proestrus and stored at −80 C until preparation of the whole cell lysate using procedures previously described (22). The protein concentration of each lysate was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Immunoprecipitation was initiated by adding 10 μl ErbB-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody to each sample containing 1 mg protein in a 500-μl volume. Samples were then incubated for 12–14 h at 4 C under constant rotation. Thereafter, 35 μl of a preformed protein A-Sepharose slurry (1 μl antibody/ml of antibody in 200 μl 150 mM NaCl, 0.01% NaN3, and 0.5% BSA) was added to each sample and incubated at 4 C for an additional 4 h with rotation. The immunoprecipitates were pelleted in the microcentrifuge and washed once with 0.5 ml cold RIPA buffer (1% IGE-PA30, 630 mg/ml deoxycholic acid sodium, 0.1% SDS in 1× PBS), 10 μl 10 mg/ml phenylmethylsulfonylfluoride, 5 μl 200× protease inhibitor cocktail (0.952% leupeptin, 1.37% pepstatin, and 1.5% aprotinin), and 10 μl 100 mM sodium orthovanadate in 1 ml RIPA before use and twice with 0.5 ml cold 1× PBS (9.1 mM NaH2PO4, 1.7 mM Na2HPO4, and 150 mM NaCl). Immunoprecipitated proteins were electrophoresed on an 8% SDS-polyacrylamide mini-gel and then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) via a transfer apparatus (Bio-Rad Laboratories, Inc.). After electrotransferring, gels were stained with GelCODE Blue Stain Reagent (Pierce Chemical Co., Rock- ford, IL) to ascertain a complete transfer of protein. The membranes were blocked with 5% nonfat milk and 5% BSA in Tris-buffered saline (TBS)-Tween 20 (0.2% Tween 20, 50 mM Tris (pH 7.4), and 154 mM NaCl) for 3 h at room temperature. Each membrane was probed with a specific primary antibody (1:500 dilution) in TBS-Tween 20 solution containing 2% nonfat milk for 2 h at room temperature with gentle agitation. Membranes were then washed eight times for 8 min each time with TBS-Tween 20 at room temperature, followed by probing with an antispecies-specific horseradish peroxidase-linked antibody (1:2000 in TBS-Tween 20) for 1 h with gentle agitation. After washing again as...
In situ hybridization histochemistry

Animals were cardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were dissected out and further fixed with the same fixative for 24 h at 4 C. Thereafter, the fixative was replaced with cold (4 C) 25% sucrose in 0.1 M phosphate buffer (pH 7.4). After 24 h, the brains were transferred into new sucrose solution for an additional 1 or 2 d at 4 C. The brains were then blocked and stored at −80 C until being coronally sectioned at 20 µm using a freeze sliding microtome. The sections were then mounted on gelatin-coated Super-Frost glass slides (Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight in a vacuum hood. The dried slides were stored in slide boxes at −85 C before hybridization.

Probe preparation

To prepare the [35S]UTP-labeled ErbB-1 riboprobe, DNA templates corresponding to sequences contained in the coding region of ErbB-1 mRNA as previously reported (22) were used. Briefly, it recognizes the extracellular domain of the full-length ErbB-1 mRNA and its 3′ end of the truncated form. The cRNA probe was synthesized by in vitro transcription using 17 polymerase.

Prehybridization, hybridization, and posthybridization

The procedures were followed as previously described (26). In short, each slide was overlaid with 180 µl hybridization solution containing 50% deionized formamide, 4 × SSC, 1 × Denhardt’s solution, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml tRNA, 10% dextran sulfate, and the riboprobe of interest (5 × 106 cpm/ml). The slides were hybridized in a slide moat (model 240000, Boekel Scientific, Feasterville, PA) for 16–20 h at 56 C. Posthybridization procedures were performed as previously reported (26). After dehydration in graded alcohols, the slides were dipped in NTB3 emulsion (catalogue 1654441, Kodak, Rochester, NY) and developed after 2 wk of exposure. All slides were stained with Toluidine Blue O (Fisher Scientific, catalog no. T-161). Control sections were hybridized with a sense RNA probe.

Hormone assays

Plasma levels of LH were measured using enzyme immunoassay (EIA) detection kit (code RPN 2562) purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The sensitivity of the assay was 0.1 ng/ml, and intraassay coefficients of variation were 7.6%. Circulating E2 and progesterone (P4) concentrations were determined by EIA detection kits [catalogue DE2000 (E2), R&D Systems, Inc., Minneapolis, MN; 07B70102A (P4), ICN Pharmaceuticals, Inc., Orangeburg, NY]. The lowest detectable levels of E2 and P4 were 10.1 pg/ml and 0.05 ng/ml, respectively.

ErbB-1 receptor tyrosine kinase inhibitor treatment

To block signal transduction mediated by the ErbB-1 receptor, AG-1478 (provided by Dr. Alexander Levitzki, Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel), a quinazoline potent inhibitor of ErbB-1 tyrosine kinase (27, 28), was administered to 50- to 60-d-old YA female rats displaying at least two consecutive estrous cycles. Each rat was ip injected on the diestrous d 2 (1500 h), followed by two injections the next day (proestrus), at 1000 and 1500 h, respectively. The dosage used (50 mg/kg BW) was based on the findings that ErbB-1 tyrosine kinase activity was effectively inhibited in vivo at this concentration (28). The AG-1478 compound was dissolved in dimethylsulfoxide (DMSO) (100 mg/ml) before injection. Age-matched control animals were treated with no injection, DMSO as a vehicle, and AG-825 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), which is structurally similar to AG-1478, but not active in vivo (29).

Statistical analyses

Comparisons of changes in ErbB-1 mRNA and all hormonal levels were analyzed by two-way ANOVA, followed by Student-Newman-Keuls multiple comparison test for unequal replications. In all cases, significance was set at P < 0.05.

Results

Gene expression of hypothalamic ErbB-1 receptor is altered in aging female rats during proestrus

To determine whether changes in hypothalamic ErbB-1 gene expression may occur during proestrus (a stage before ovulation) in aging female rats, the relative tissue content of ErbB-1 mRNA in this brain region was measured using a semiquantitative RT-PCR assay as previously reported (20). The technical validation of this assay is further depicted in Fig. 1. Expression of ErbB-1 mRNA in the POA is low for both YA and MA rats in early morning (0600–0800 h) of the proestrous day. ErbB-1 gene expression significantly increases in YA rats at 1000 h and reaches a maximal level at 1500 h (Fig. 2A). In contrast, no initial elevation at 1000 h was found in MA animals (Fig. 2A). Although the level of ErbB-1 mRNA increases at 1500 h in MA rats, it is not statistically significant compared with that at other time points. Importantly, this elevation is significantly lower than that in YA animals (Fig. 2A). In YA animals, ErbB-1 mRNA expression levels in the ME-ARC are high during the early morning of the proestrous day, reach a maximal value at 0800 h, then decrease significantly to low levels during the afternoon (Fig. 2B). In comparison to YA rats, ErbB-1 mRNA levels in MA animals are lower in the morning and slowly increase to the peak level 4 h later (Fig. 2B). The levels then significantly decrease during the late afternoon. This gene expression pattern in YA animals and the differences in ErbB-1 receptor expression between YA and MA female rats appear to be limited to these specific regions of the hypothalamus. No such expression pattern and differences were observed in the region of cerebral cortex used (not shown), an brain area that has no known involvement in neuroendocrine function.

Localization of ErbB-1 mRNA in the hypothalami of YA and MA female rats by in situ hybridization histochemistry

A [35S]UTP-labeled ErbB-1 cRNA probe was used to determine the cellular expression of ErbB-1 mRNA in the hypothalami of YA and MA female rats during proestrus at 1200 h. In the POA of YA rats, the most abundant ErbB-1 mRNA-labeled cells are located around the organum vasculosum of the lamina terminalis (OVLT; Fig. 3A, a). Labeled cells are also observed in the ventral region of the medial preoptic area (MPA) and the wall of the third ventricle (Fig. 3, C, c1 and c2). In striking contrast, only a few weakly labeled cells are detected in the matched areas of MA animals (Fig. 3, B, b, D, d1 and d2; Table 1). These observations further confirm the results detected by RT-PCR. In the medial basal hypothalamus, numerous ErbB-1 mRNA positive cells are found in the ventral-lateral region of the ARC (Fig. 4C, c), which is involved in controlling LHRH release. Interestingly, intensely labeled cells are also identified around the subfornical organ (Fig. 4A, a), the area related to the regulation of water intake (30). A weaker hybridization signal is detected in aging female rats.
in the dorsal part of the paraventricular thalamic nucleus anterior part (Fig. 4B, b).

Hypothalamic ErbB-1 protein levels in the YA and MA rat during proestrus

Western blot analyses show that the mean ErbB-1 protein content in the POA from YA rats at 1000, 1200, or 1500 h of the proestrous day was higher than that in MA rats especially at the 1000 and 1500 h points (Fig. 5A). In contrast, no difference was observed in the protein levels of the ME-ARC at 1000 or 1500 h between YA and MA rats (Fig. 5B). However, at 1200 h the ErbB-1 protein level in MA animals was higher than that in YA rats (Fig. 5B). It is clear that ErbB-1 protein...
FIG. 3. Detection of ErbB-1 mRNA in the hypothalamus of YA and MA female rats at 1200 h on the proestrous day by in situ hybridization histochemistry. Representative results derived from a group of four animals are depicted (in brightfield). Magnification of A, ×100. Abundant ErbB-1 mRNA-labeled cells around the OVLT region (arrows) of a YA female rat. The labeled cells in the framed area in A are shown at a higher magnification (×400) in a (red arrowheads). Magnification of B, ×100. In contrast, fewer weakly labeled cells are found around the OVLT area (arrow) of an MA rat. The labeled cells in the framed area in B, which matches the framed area in A, are depicted at a higher magnification (×400) in b (red arrowheads). C, (magnification, ×100) shows that numerous ErbB-1 mRNA-positive cells are located on the dorsal region of the wall of the third ventricle (box 1) and in the MPA (arrows and box 2) of a YA rat. ErbB-1 mRNA-positive cells (red arrowheads) in box 1 and box 2 in C are shown at higher magnification (×400) in c1 and c2, respectively. D (magnification, ×100) represents strikingly reduced ErbB-1 mRNA-labeled signal in the dorsal area of the third ventricle box 1 and the MPA (arrows and box 2) of a MA rat. Weakly labeled cells in box 1 (red arrowheads) and no detectable labeled cells in box 2 in D are depicted in d1 and d2 (magnification, ×400), respectively. V, Third ventricle.
expression profiles parallel its gene expression pattern at these three time points. A major reason for selecting only three time points during proestrus was based on the ErbB-1 receptor mRNA expression profile described above. Specifically, mRNA in the ME-ARC of YA rats reached a maximal level at 0800 h, but in MA animals the peak level of ErbB-1 mRNA was found 4 h later. It is thus reasonable to select time points after 0800 h. The 1500-h point was selected because ErbB-1 mRNA levels at this time reached the highest value in the POA. Although it would represent a more accurate protein expression profile if more time points were included, it would be too costly due to the limited protein content that can be obtained from each POA or ME-ARC tissue fragment. This also contributes to the use of four to six pooled tissue fragments per measured time point. Nonetheless, it is clear that the protein expression profiles for both YA and MA animals are comparable to its encoding gene expression pattern.

Changes in LH secretion from YA and MA female rats during proestrus

As an altered ErbB-1 receptor expression was detected in the hypothalamus of MA female rats, it was important to determine whether this alteration is temporally associated with the changes in LH release, an index of LHRH secretion. There was no significant difference in the circulating concentration of secreted LH between YA and MA female rats from 0600–1500 h on proestrus. However, an expected pre-ovulatory LH surge was detected at 1800 h in YA, but not MA, animals (Fig. 6). Furthermore, this difference in LH secretion was probably not due to differential ovarian function, because no significant difference in the levels of E2 and P4 was found between these two age groups (Table 2).

Blockade of ErbB-1-mediated signal transduction disrupts the normal estrous cycle in YA female rats

It has been well demonstrated that pharmacological inhibition of ErbB-1 receptor tyrosine kinase activity results in 1) blockade of LHRH release induced by activation of ErbB-1 receptor (16, 31), and 2) delay of normal onset of sexual maturation due to disrupted LHRH secretion (18). One important issue is whether ErbB-1 receptor-mediated signal transduction is also necessary for maintaining normal estrous cyclicity during adulthood. To address this issue, AG-1478, a highly potent inhibitor of the ErbB-1 tyrosine kinase (28), was employed to determine whether in vivo blockade of

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**TABLE 1.** Estimated total number of ErbB-1 mRNA-labeled cells (mean ± SEM; n = 4) detected by in situ hybridization

<table>
<thead>
<tr>
<th>Age</th>
<th>OVLT</th>
<th>POA</th>
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<tbody>
<tr>
<td>YA</td>
<td>120.8 ± 9.2</td>
<td>118.8 ± 11.3</td>
</tr>
<tr>
<td>MA</td>
<td>32.5 ± 3.2</td>
<td>18.3 ± 3.1</td>
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* P < 0.05 vs. MA, by Mann-Whitney test.

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**Fig. 4.** Detection of ErbB-1 mRNA in other areas outside the hypothalamus and ARC of the hypothalamus by in situ hybridization histochemistry using the same YA animals as those described in Fig. 3. Magnification: A–C, ×100; a–c, ×400. A shows that numerous intensely labeled cells (arrows) are scattered around the dorsal and lateral edges of the subfornical organ (SFO). The magnified cells (red arrowheads) in the boxed area in A are illustrated in a. B depicts ErbB-1 mRNA-positive cells (arrows) along the paraventricular thalamic nucleus, anterior part (PVA). The positive cells (red arrowheads) in the boxed area in B are amplified and shown in b. C exhibits a wing-shape-labeling cell group covering the medial- and ventro-lateral parts of the ARC (circled and boxed areas). The labeled cells (red arrowheads) in the boxed area in C are enlarged and shown in c. DV, Dorsal third ventricle.
ErbB-1 receptors would disrupt the estrous cycle in YA female cycling rats. The administration of AG-1478 to 50- to 60-d-old female rats resulted in a striking disruption of the estrous cycle (Fig. 7). Among the five treated animals, three displayed prolonged diestrus and two exhibited prolonged estrus. In contrast, there was no impact on the cyclicity in the age-matched female rats treated with no injection, AG-825 (structurally similar to AG-1478, but inactive in vivo), or DMSO as a vehicle (Fig. 7).

**Discussion**

Little is known concerning the role of the ErbB-1 receptor in controlling reproductive function during adulthood. Involvement of ErbB-1-mediated signal transduction in the control of female sexual maturation (14) strongly suggests that cell-cell interactions mediated by ErbB-1 receptors in the hypothalamus are also necessary for maintaining reproduction during adulthood. It is thus conceivable that an alteration in ErbB-1 receptor signaling capacity may contribute to the compromised reproductive function in aging females. The present study provides initial evidence that supports this view.

Hypothalamic cellular localization of ErbB-1 mRNA in YA and MA female rats was determined employing in situ hybridization histochemistry. The results reveal that numerous ErbB-1 mRNA-labeled cells in YA female rats are observed around the OVLT area, a site scattered with LHRH cell bodies (32). This strong expression of ErbB-1 mRNA in the OVLT is consistent with high levels of ErbB-1 protein found in the same area (22). Because ErbB-1 mRNA is mostly expressed in astroglia, but not in LHRH neurons in the hypothalamus (22), these ErbB-1 mRNA-positive cells are probably astroglia. Based on the idea that ErbB-1 receptor-mediated stimulation of LHRH release in pubertal rats requires glial cells (14), it is possible that control of LHRH neuronal function by ErbB-1 receptors in adult females is also dependent at least in part on glial intermediacy. Furthermore, in YA female rats, ErbB-1 mRNA-positive cells are found in the MPA, which is known to be involved in maintenance of estrous cyclicity via control of LHRH release (33). Consistent with the previous study (22), cells scattered along the wall of the third ventricle from YA animals are also ErbB-1 mRNA positive. It is important to note that differences in cellular ErbB-1 mRNA expression in the POA of YA and MA female rats at 1200 h on proestrus were also examined. This time point was selected because ErbB-1 mRNA expression levels detected by RT-PCR (see below) were significantly different between the two age groups. Similar to the RT-PCR results, MA female rats have fewer weakly labeled ErbB-1 mRNA cells in the OVLT, MPA, and wall of third ventricle compared with the matched areas of YA animals.
TABLE 2. Serum levels (mean ± SEM) of E2 and P during proestrus in YA and MA female rats

<table>
<thead>
<tr>
<th>Time</th>
<th>YA</th>
<th>MA</th>
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<tbody>
<tr>
<td>0600 h</td>
<td>4.8 ± 1.2 (n = 5)</td>
<td>3.8 ± 1.25 (n = 4)</td>
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<tr>
<td>0800 h</td>
<td>7.9 ± 0.5 (n = 5)</td>
<td>8.3 ± 0.4 (n = 4)</td>
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<tr>
<td>1000 h</td>
<td>35.7 ± 16.5 (n = 7)</td>
<td>36.6 ± 8.6 (n = 6)</td>
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<tr>
<td>1200 h</td>
<td>25.7 ± 12.8 (n = 6)</td>
<td>21.1 ± 7.3 (n = 6)</td>
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<tr>
<td>1500 h</td>
<td>15.5 ± 6.1 (n = 7)</td>
<td>13.3 ± 4.2 (n = 4)</td>
</tr>
<tr>
<td>1800 h</td>
<td>4.5 ± 0.7 (n = 4)</td>
<td>3.8 ± 1.0 (n = 4)</td>
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ErbB-1 mRNA-positive cells are also detected in the medial basal hypothalamus, predominantly in the medial- and ventral-lateral portion of the ARC, which is known to be involved in the regulation of LHRH release (3, 34). This suggests that expression of the ErbB-1 receptor in the ARC may be functionally linked to controlling LHRH secretion. This view is supported by the evidence that activation of ErbB-1 receptors from the ME-ARC results in the release of LHRH (16, 31). Once again, studies have established the concept that the effect of ErbB-1 receptor activation on LHRH release is not exerted directly; instead, intermediate astroglial cells are required (14). ErbB-1 mRNA expressed in the ME-ARC in this study is overlapped with receptor expression found in astroglial cells of the same region in prepubertal rats (22). It is thus plausible that the effect of activation of the ErbB-1 receptor on LHRH release in adult female rats is mediated via astroglial cells. Interestingly, strong ErbB-1 mRNA signals are also detected around the subfornical organ, which is known to be involved in the control of water intake. ErbB-1 mRNA hybridization signals can be identified in the dorsal and lateral edges of the paraventricular thalamic nucleus anterior part. At present, the physiological relevance of ErbB-1 expression in these regions related to reproductive function remains unknown.

Evidence exits that an increase in ErbB-1 gene expression before and during the onset of puberty is necessary for stimulation of LHRH release, which is a key regulator of sexual maturation and reproduction. Thus, alteration in this receptor gene activity may contribute to changes in LHRH neuronal function, which, in turn, lead to the altered gonadotropin output found in aging females (35, 36). This study shows that in the POA of cycling YA rats, ErbB-1 mRNA expression starts to increase during the late morning when serum levels of E2 are high. It then reaches a maximal value at 1500 h, 3 h before the preovulatory LH surge. On the other hand, MA rats show no initial morning or late afternoon significant increase in ErbB-1 mRNA expression. Although there is an increase in mRNA levels at 1500 h, it is significantly lower than that in YA animals. This difference in mRNA levels is also reflected by higher ErbB-1 protein content in YA vs. MA animals. Importantly, these MA animals exhibit no preovulatory-like LH surge at 1800 h on proestrus compared with YA rats, suggesting a delayed LH secretion in these animals. It is well documented that the preovulatory LH surge is either delayed or reduced in MA female rats (37). This indicates that the late afternoon increase in ErbB-1 receptor expression during proestrus may be required for induction of the preovulatory LH surge. This view is supported by evidence that ligand-induced activation of ErbB-1 receptors results in stimulation of LHRH release (16, 17, 31). Conversely, disruption of the ErbB-1 receptor-mediated signal transduction leads to a decrease in LHRH (16) or LH (19) release. Whether the altered ErbB-1 receptor expression in MA rats is causally associated with the decreased or delayed release of gonadotropins found in aging female rats (35, 36) requires further studies. Furthermore, the lack of initial increase in ErbB-1 receptor expression in MA rats, which did not exhibit an LH surge at 1800 h, may be linked to the blunted elevation of receptor expression at 1500 h. This suggests that the initial increase in ErbB-1 receptor expression may be required for inducing full capacity expression in the afternoon of the proestrous day, as seen in YA animals. Importantly, induced full capacity receptor expression may be necessary for the preovulatory gonadotropin release. More studies are needed to address this issue. Interestingly, although a similar peak level of ErbB-1 mRNA in the ME-ARC is found in both proestrus YA and MA animals, it is observed at 0800 h in YA rats, but at 1200 h in MA rats. This time-postponed activation of the ErbB-1 gene in MA rats is temporally comparable to the delayed LHRH and LH release from aging animals (35, 36). These results suggest that temporally regulated activation of ErbB-1 receptors in the hypothalamus may be necessary for induction of the preovulatory release of LHRH, which leads to gonadotropin secretion. Moreover, an intriguing finding in this study is that ErbB-1 expression in the POA of YA animals is temporally different from that in the ME-ARC. In other words, expression levels in the POA start to increase from 1000–1500 h, 3 h before the LH surge. However, ErbB-1 expression levels in the ME-ARC reach the highest value at 0800 h and decrease thereafter. This difference in ErbB-1 gene activity may be required, as one of the regulatory components, for the complexity of temporal and regional control of LHRH neuronal activity. Desynchronization of this component would result in disruption of an integral control of LHRH/LH re-
clearly show that acute administration of AG-1478 to cycling YA female rats indeed disrupts the estrous cycle. Those animals treated with the blocker exhibited either prolonged diestrus or prolonged estrus, which is a typical estrous cycle displayed by MA female rats. The effect of AG-1478 appears to be specific, as no such effect was observed in animals treated with AG-825, which is an inactive tyrphostin in vivo (29). Moreover, the disruption of estrous cyclicity was short term and fully reversible after one cycle disruption. This result is consistent with a short half-life (31 min) of AG-1478 when it is used in vivo (28). The number of proestrus followed by estrus and diestrus was used to determine the number of estrous cycles. Although pharmacological study supports the specificity of AG-1478, it cannot be completely ruled out that this compound could inhibit other tyrosine kinases if administered at a relatively high concentration. However, this may not be the case in the current study because 1) the dosage of AG-1478 used is the same as in a previous study (28), in which the researchers reported a peak plasma concentration of 61 μM after a single ip injection; 2) one injection is not enough to produce the level (>100 μM) required to inhibit other tyrosine kinase receptors, such as ErbB-2 or platelet-derived growth factor receptor (27); and 3) the two injections administered with a 5-h gap on the day of proestrus are unlikely to produce a 100-μM plasma level of AG-1478, as only 1.5 μM can be detected within 4 h after a single injection (28). Thus, the impact of AG-1478 on estrous cyclicity is probably due to inhibition of ErbB-1-mediated signal transduction. Although the lipophilic nature of AG-1478 ensures its action on the central nervous system, particularly the hypothalamus, we cannot rule out its action on other peripheral tissues, such as ovaries. However, this is unlikely, because activation of ErbB-1 in ovaries results in the inhibition of LHRH secretion (31), and, consequently, blockade of female reproductive function. The present study also provides evidence that altered ErbB-1 receptor expression in transgenic female mice is disrupted (our unpublished observation). It is important to note that other related tyrosine kinases may contribute to the regulation of female reproduction. Emerging evidence shows that cellular signal transduction mediated by multi-tyrosine kinase receptors is a common feature related to controlling neuroendocrine function. For instance, the insulin receptor (39) and IGF receptor (19) are also necessary for maintaining normal reproduction.

In summary, this study demonstrates that blockade of ErbB-1 receptor-mediated signal transduction results in disruption of the estrous cycle. Therefore, the ErbB-1 signaling system is a physiological component necessary for maintaining female reproductive function. The present study also provides evidence that altered ErbB-1 receptor expression in the hypothalamus of aging female rats may be linked to changes in LHRH neuronal activities that lead to attenuated or delayed preovulatory LH surge during reproductive aging.

FIG. 7. Analysis of the disruption of estrous cyclicity in young adult cycling female rats by blockade of ErbB-1 receptor-mediated signal transduction via the receptor tyrosine kinase inhibitor (AG-1478). The top panel shows estrous cyclicity from one representative animal of each experimental group. Notice that the AG-1478-treated animals show either prolonged diestrus (PD) or prolonged estrus (PE) compared to controls. D, Diestrus; P, proestrus; E, estrus. The bottom panel depicts the percentage of animals displaying estrous cyclicity in different treatment groups. A striking alteration in estrous cyclicity is found in animals injected with AG-1478. None of these animals exhibited cyclicity 3 d after the injections. The AG-1478 and AG-825 solutions were prepared in DMSO (100 mg/ml) and administered by ip injection at the dosage of 50 mg/kg BW. The same volume of DMSO was injected into animals as a vehicle control. Each arrow represents one injection at a specific time of a defined cycle stage. For details, see Materials and Methods. Numbers in parentheses are the number of animals per group.
Acknowledgments

Received July 6, 2001. Accepted October 15, 2001.

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This work was supported by Grant ROI-AG-18078.

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