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Transforming growth factor α contributes to the mechanism by which hypothalamic injury induces precocious puberty

(brain injury/sexual precocity/glial cells)

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ABSTRACT **It has long been known that lesions of the hypothalamus lead to female sexual precocity. While an increased production of luteinizing hormone-releasing hormone (LHRH), the neurohormone that controls sexual development, appears to mediate the advancement of puberty induced by these lesions, little is known about the mechanism(s) by which hypothalamic injury activates LHRH secretion. Since brain lesions result in accumulation of neurotrophic/mitogenic activities in the injured area, we tested the hypothesis that transforming growth factor α (TGF-α), a mitogenic polypeptide recently shown to stimulate LHRH release, is produced in response to hypothalamic injury and mediates the effect of the lesion on puberty. Radiofrequency lesions of the preoptic area–anterior hypothalamic area (POA–AHA) of 22-day-old female rats resulted in precocious puberty within 7 days after the operation. RNA blot hybridization revealed that lesion-induced puberty was preceded by an increase in TGF-α mRNA levels in the POA–AHA. Epidermal growth factor (EGF) mRNA was undetectable in both intact and lesioned hypothalami. TGF-α mRNA levels, quantitated by RNase protection assays, were 3.5-fold greater in lesioned animals approaching puberty than in age-matched controls. Immunohistochemical studies, utilizing single- and double-staining procedures, demonstrated the presence of TGF-α precursor-like immunoreactivity in reactive astrocytes surrounding the lesion site. Hybridization histochemistry showed increased TGF-α mRNA expression in cells of the same area, further implicating reactive astrocytes as a site of TGF-α synthesis. The actions of TGF-α are mediated by its interaction with EGF receptors. Continuous infusion of RG-50864, an inhibitor of EGF receptor kinase activity, at the site of injury prevented the advancement of puberty induced by the lesion. These results suggest that TGF-α acting via EGF-like receptors contributes to the acceleration of puberty induced by anterior hypothalamic lesions. They also indicate that activation of TGF-α gene expression in glial cells is a component of the hypothalamic response to injury.**

The onset of mammalian puberty depends on the activation of neurons that are located in the diencephalon and secrete the neurohormone luteinizing hormone-releasing hormone (LHRH) (for review, see ref. 1). Although most cases of precocious puberty of cerebral origin occur in girls and are diagnosed as idiopathic (2), computed axial tomography has revealed that an increasing number of these cases are associated with discrete hypothalamic pathology (2, 3). Experimentally, female sexual precocity can be induced in both primates and rodents by lesions of the hypothalamus (4, 5). Lesions of the anterior hypothalamus in rodents (5) result in true sexual precocity, as animals bearing these lesions mate and rear litters normally (6). The mechanisms underlying this phenomenon are unknown, but the notion that lesions accelerate sexual development by eliminating a site(s) for steroid inhibitory control (5) has gained acceptance. To date, an involvement of neurotrophic/mitogenic activities produced locally in response to brain injury (7) has not been considered in lesion-induced precocious puberty. We have taken advantage of this unique model (5, 6) to test the hypothesis that neurotrophic/mitogenic factors, thought to be involved in the reorganization of the brain that follows injury (7), are responsible for the advancement of puberty caused by hypothalamic lesions. We focused our attention on transforming growth factor α (TGF-α) because of its potential neurotrophic/mitogenic activity in brain (8, 9), the prevalence of its mRNA in the developing female hypothalamus (10), and its ability to stimulate LHRH secretion (10). TGF-α, a mitogenic polypeptide involved in cell proliferation and wound healing (11), is structurally and functionally related to epidermal growth factor (EGF) and interacts with the same receptor (11).

MATERIAL AND METHODS

Animals. Twenty-two-day-old Sprague–Dawley female rats were subjected to bilateral radiofrequency lesions (5 mA; 25 sec; Grass LM4 lesion maker) with a unipolar stainless steel electrode (anode) isolated with epoxylite resin except for 0.5 mm at the tip (6). The tip of the electrode was positioned between the posterior aspect of the preoptic area and the anterior hypothalamic area (6). The lesion coordinates were 0.8 mm anterior to bregma, 8 mm ventral to the skull surface, and 0.4 mm lateral to the midline. In sham-operated rats, the electrode was lowered at the appropriate coordinates but no current was delivered.

Assessment of Puberty Onset. The time of puberty was determined by using previously described criteria (1). These are: (*i*) canalization of the vaginal orifice, an estrogen-dependent phenomenon that usually occurs on the day of first ovulation; (*ii*) sequential manifestation after vaginal opening of the estrous and diestrous phases of the first estrous cycle; and (*iii*) confirmation of the presence of corpora lutea at the time of necropsy.

Tissue Dissection. Animals were euthanized between 1000 and 1200 h. A hypothalamic region comprising the preoptic area and the anterior hypothalamic area (POA–AHA) was dissected as follows: rostrally, the anterior border of the optic chiasm; laterally, the hypothalamic sulci; caudally, 2 mm in front of the mammillary bodies; depth, 2 mm.

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Abbreviations: LHRH, luteinizing hormone-releasing hormone; TGF-α, transforming growth factor α; EGF, epidermal growth factor; POA–AHA, preoptic area–anterior hypothalamic area; cRNA, complementary RNA; GFAP, glial fibrillary acidic protein.

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RNA Preparation. Total RNA and poly(A)$^+$ RNA were prepared as reported (12, 13).

Northern Blot Analysis. The poly(A)$^+$ RNA was size-fractionated in agarose/formaldehyde gels and blotted onto nitrocellulose membranes (12). TGF-α mRNA was identified by hybridization to a [^{32}P]CTP-labeled rat TGF-α complementary RNA (cRNA), as described (12, 13). The membranes were then hybridized to a p1B15 cDNA probe complementary to cyclophilin mRNA, which is constitutively expressed in brain (14). The blots were exposed to Kodak XAR-5 film and the autoradiograms were analyzed by laser densitometry using the cyclophilin mRNA signal to standardize TGF-α mRNA values.

Solution Hybridization/RNase Protection Assay. [^{32}P]CTP-labeled TGF-α cRNA (500,000 cpm) was hybridized with various amounts of *in vitro* synthesized TGF-α sense mRNA or with 5 μg of total RNA extracted from two POA–AHAs. The tissue RNA was simultaneously hybridized to 5000 cpm of [^{32}P]CTP-labeled cyclophilin cRNA. After overnight hybridization at 45°C, the samples were treated with RNase A and T1 and further processed as described (15). The samples were then electrophoresed through a 7 M urea/5% acrylamide gel and the gel was exposed to XAR-5 film. The autoradiograms were analyzed as described above.

Probes. The antisense TGF-α cRNA probe used was obtained by *in vitro* transcription (12, 13) of a 400-base-pair (bp) *Eco*RI/*Hin*dIII cDNA fragment (16) linearized with *Sac* II to yield a 272-nucleotide [^{32}P]CTP-labeled cRNA. The same template was used for hybridization histochemistry but uridine 5′-[α-[^{35}S]thio]triphosphate was substituted for [^{32}P]CTP. The cyclophilin cDNA used in Northern blots was a 700-bp *Bam*HI/*Pst* I DNA fragment complementary to nucleotides 1–743 in cyclophilin mRNA (14). The cDNA was labeled by the random primer method as reported (13).

The TGF-α sense mRNA used to obtain standard curves in RNase protection assays was obtained by *in vitro* transcription of the above-mentioned *Eco*RI/*Hin*dIII cDNA fragment linearized with *Hin*dIII and transcribed with T7 RNA polymerase. The 182-bp cDNA fragment used to synthesize labeled cyclophilin cRNA was PCR generated from a cyclophilin cDNA cloned into the pSP65 vector. The primers were a 17-mer oligonucleotide identical to the SP6 promoter sequence and a 24-mer oligonucleotide complementary to the antisense cyclophilin cDNA sequence 543–567.

Hybridization Histochemistry. The procedure followed is that described by Simmons *et al.* (17) with minor modifications. The hybridization reaction took place overnight at 55°C in a 50% formamide solution containing 0.4 M NaCl, 10 mM Tris·HCl, 10 mM EDTA, and 2× Denhardt's solution; each section was overlaid with 50 μl of this solution containing 250,000 cpm of labeled probe. Posthybridization washes were as described (17). The sections were then dehydrated and dipped in Kodak NTB-2 emulsion for signal detection. After 2 weeks, the slides were developed, counterstained with thionin, and analyzed under dark-field illumination. Controls included treatment of the sections with RNase A before hybridization and hybridization with a sense RNA probe.

Immunohistochemistry. Brains were fixed by intracardiac perfusion of Zamboni's fixative. Immunocytochemistry was performed on vibratome sections (75 μm) using an ABC immunoperoxidase technique (18). TGF-α-immunoreactive cells were identified with polyclonal antibody 1296 (1:1500). This antiserum recognizes a peptide sequence (amino acids 137–151) contained within the cytoplasmic domain of the TGF-α precursor (19). Adjacent sections were stained for glial fibrillary acidic protein (GFAP) (using antiserum R77, 1:2000). Control experiments included substitution of the primary antiserum to TGF-α with (*i*) serum from nonimmunized animals and (*ii*) the primary antiserum preabsorbed with its antigen (10 μg/ml). Colocalization of TGF-α and GFAP was studied by a double-labeling technique (20).

For immunohistochemistry of LHRH neurons the rats were perfused with a solution of 4% paraformaldehyde in phosphate buffer containing 2.5% acrolein. Immunohistochemistry was performed on cryostat sections (25 μm) using the antiserum LR-1 (1:100,000), as reported (21).

***In Vivo* Pharmacological Manipulations.** Immediately after lesion, the animals were implanted with a stainless steel cannula (30 gauge, 15 mm long) at the lesion site. The cannula was connected through a PE-10–PE-50 polyethylene tubing assembly to an Alzet osmotic minipump implanted subcutaneously. The pump, catheter, and cannula were filled with either a 1% ethanol solution in 0.9% saline (pH 5.5) or a 240 μM solution of RG-50864 (22) in saline. RG-50864 is an inhibitor of EGF/TGF-α receptor tyrosine kinase that blocks

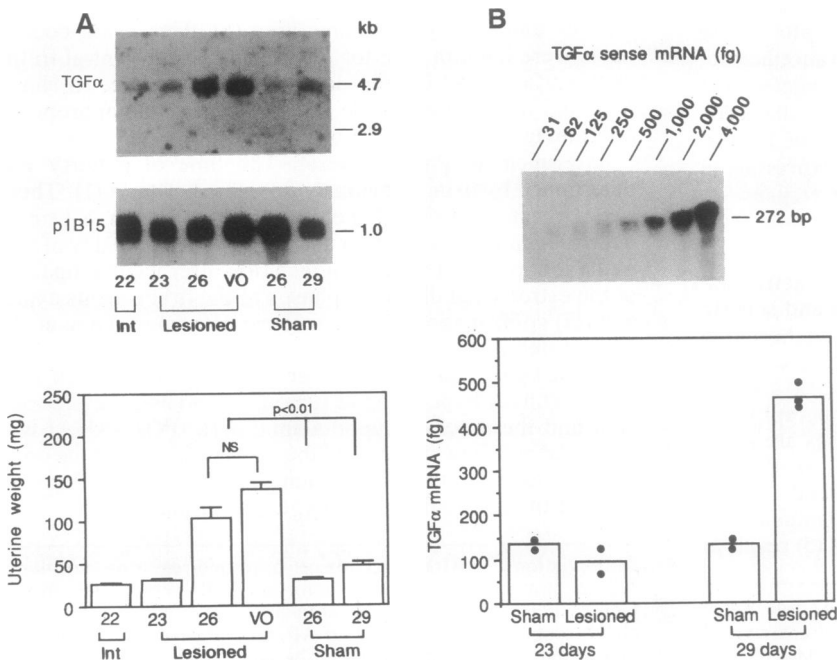

FIG. 1. (*A*) (*Upper*) TGF-α mRNA levels in the POA–AHA at different times after lesion [2.5 μg of poly(A)$^+$ RNA per lane]. The results depicted represent one of three separate experiments. (*Lower*) Mean uterine weight of lesioned rats used to obtain the Northern blot results depicted in *Upper*. Uterine weight in POA–AHA lesioned rats was similar on the day of precocious vaginal opening to that of control rats undergoing normal puberty (138.5 ± 10.2 mg vs. 136.3 ± 8.2 mg, respectively; $n = 6$; mean ± SEM). Numbers under the abscissa indicate age of rats. VO, vaginal opening; Int, intact rats; lesioned, rats bearing POA–AHA lesions; sham, sham-operated rats. (*B*) (*Upper*) Autoradiogram of a gel showing increasing concentrations of TGF-α sense mRNA protected from RNase digestion by hybridization to ^{32}P-labeled TGF-α cRNA. The standard curve generated was used to quantitate TGF-α mRNA levels in the tissue samples. (*Lower*) TGF-α mRNA levels in the tissues surrounding the lesion, 1 and 7 days after the lesion, as assessed by RNase protection assay. Each circle represents an independent sample (two POA–AHA per point). Mean uterine weight 7 days after the lesion was 151 ± 7 mg in lesioned rats ($n = 6$) compared to 33 ± 3 mg in control rats (mean ± SEM; $n = 4$).

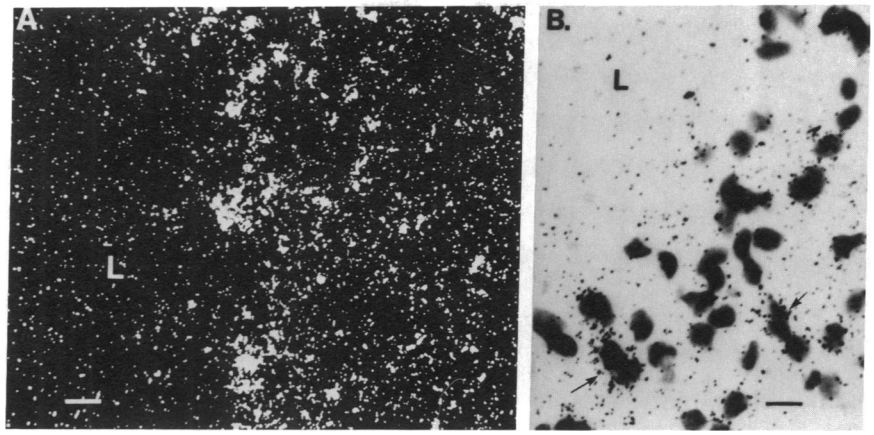

FIG. 2. Hybridization histochemistry of a ^{35}S-labeled cRNA probe complementary to TGF-α mRNA. (*A*) Photomicrograph using dark-field optics of a section through the area of a POA–AHA lesion (L). (Bar = 50 μm). (*B*) High magnification bright-field micrograph of TGF-α-labeled cells (arrows) next to the site of the lesion. Similar results were obtained with three different brains. (Bar = 10 μm.)

ligand-dependent activation of the receptor without interfering with ligand binding (22). RG-50864 was dissolved in absolute ethanol before final dilution.

Statistics. Differences between groups were analyzed by a one-way analysis of variance followed by the Newman–Keul's test for multiple comparisons.

RESULTS

Effect of POA–AHA Lesions on the Onset of Puberty. As shown by others (5, 6), lesions of the POA–AHA advanced the onset of puberty [age at vaginal opening; 34.7 ± 0.7 (n = 6) vs. 29.1 ± 0.1 (n = 10) days in sham-operated and lesioned rats, respectively; $P < 0.01$]. Immunohistochemistry of LHRH neurons at the time of vaginal opening showed that at least 90% of the neurons were spared by the lesion (data not shown). The pattern of estrous cyclicity (monitored for three consecutive cycles after vaginal opening) was similar in lesioned and control animals (data not shown).

Expression of TGF-α mRNA in the Lesioned POA–AHA. A maximal increase in TGF-α mRNA levels was seen 4–6 days postinjury (Fig. 1*A Upper*). This change was correlated with an increase in uterine weight (an index of estrogen secretion) (Fig. 1*A Lower*). EGF mRNA measured 6 days after lesion was undetectable in control and injured rats (data not shown). Quantitation of the changes in TGF-α mRNA by RNase

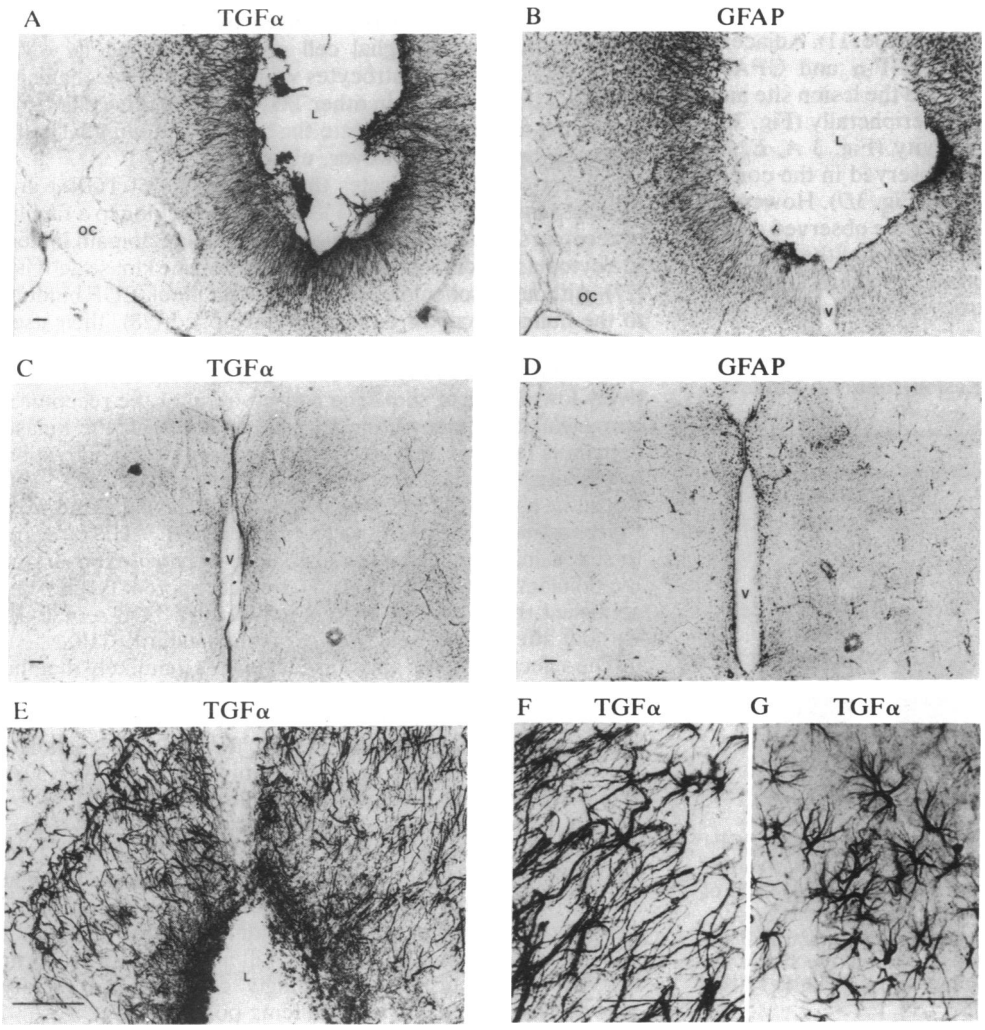

FIG. 3. Immunocytochemical detection of TGF-α in lesioned and control hypothalami. (*A*) Photomicrograph of cells with TGF-α precursor-like immunoreactivity around the site of the lesion (29-day-old rat, day of vaginal opening). (*B*) Adjacent section stained for GFAP. Pattern of staining suggests that TGF-α-positive cells in *A* are astrocytes. (*C*) TGF-α immunoreactivity in the same region of the normal hypothalamus (35-day-old rat, day of vaginal opening). (*D*) Control brain stained for GFAP. (*E–G*) Higher magnification of reactive astrocytes near the site of the lesion. Note two distinct morphological types of astrocytes, one with long processes directed toward the site of the lesion (*E* and *F*), and the other with a typical stellate shape (*G*) located distal to the lesion's site (*E*). L, lesion; OC, optic chiasm; V, third ventricle. (Bars = 100 μm.)

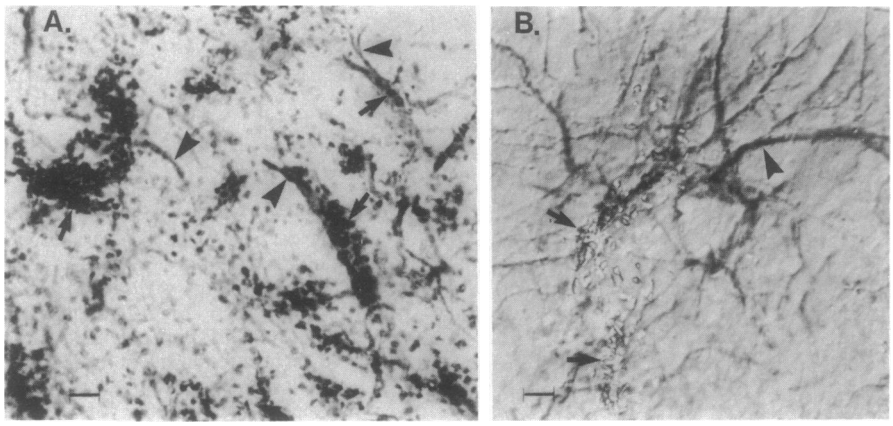

FIG. 4. Localization of TGF-α and GFAP in reactive astrocytes around the site of POA–AHA lesions. (*A*) In the original sections GFAP staining had a brown color/smooth appearance (arrowheads). TGF-α staining had a blue color/punctate appearance (arrows). (*B*) Cells labeled with GFAP (arrowheads) and TGF-α (arrows) antibodies photographed by using Nomarski optics. (Bars = 10 μm.)

protection assay demonstrated that TGF-α mRNA levels increased 3.5-fold within a week after the lesion (Fig. 1*B Lower*).

Cellular Localization of TGF-α mRNA and Protein. *In situ* hybridization experiments performed 6 days after the lesion revealed that TGF-α mRNA was highly expressed in cells surrounding the site of injury (Fig. 2). The same region of control brains exhibited little specific hybridization signal (data not shown), in agreement with the low levels of TGF-α precursor protein detected by immunohistochemistry in this region (see below; Fig. 3*D*). The cell type in which TGF-α gene expression was activated by the lesion was localized by immunohistochemistry using antibodies to GFAP, which identify astrocytes, and antibodies to the TGF-α precursor (19), which identify cells that synthesize TGF-α, as opposed to those that may bind mature TGF-α, which derives from the extracellular domain of the precursor molecule (11). Adjacent sections were alternately stained for TGF-α and GFAP. Astrocytes sending long processes toward the lesion site and those with stellate shape located more peripherally (Fig. 3*B*) exhibited strong TGF-α immunoreactivity (Fig. 3 *A, E, F,* and *G*). No reactive astrocytes were observed in the corresponding intact region of control brains (Fig. 3*D*). However, scattered TGF-α immunoreactive fibers were observed along the third ventricle (Fig. 3*C*) and in astrocytes of the external capsule (data not shown), as reported by others (23). The expression of TGF-α in reactive astrocytes was confirmed by double-labeling studies (Fig. 4).

Effect of the Inhibition of TGF-α/EGF Receptor Tyrosine Kinase Activity on POA–AHA Lesion-Induced Puberty. Chronic delivery of RG-50864, a selective blocker of TGF-α/EGF receptor tyrosine kinase activity (22), to the lesion site via an osmotic minipump prevented the advancement of puberty induced by the lesion (Fig. 5).

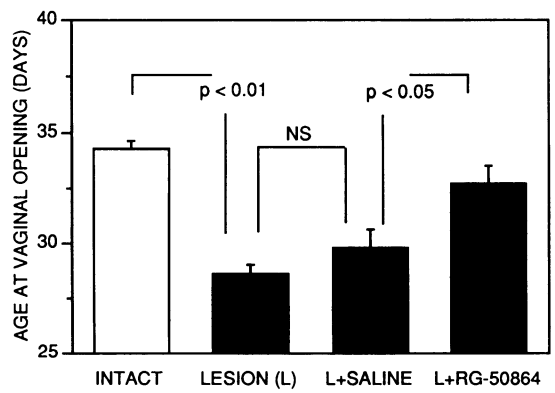

FIG. 5. Central infusion of RG-50864, a blocker of the EGF/TGF-α receptor protein kinase activity, prevented the advancement of puberty induced by hypothalamic lesions. The combined results of two independent experiments are shown. Intact, intact rats (n = 7); L, POA–AHA lesioned rats (n = 7); L + saline, lesioned rats infused with vehicle (0.9% saline containing 1% ethanol) at the site of the lesion (n = 6); L + RG-50864, lesioned rats infused with RG-50864 (n = 6).

DISCUSSION

The present results provide evidence that increased TGF-α gene expression in reactive astrocytes surrounding the site of injury is a component of the mechanism by which hypothalamic lesions induce sexual precocity. They also indicate that this action of TGF-α is exerted through EGF/TGF-α-like receptors.

Astrocytic proliferation is an integral component of the brain's response to injury (7, 24). Since activation of EGF/TGF-α receptors stimulates glial cell proliferation (25) and TGF-α gene expression is increased by TGF-α itself (26), TGF-α may stimulate glial cell proliferation and its own expression in reactive astrocytes via an autocrine mechanism similar to that observed in other TGF-α-producing cells (26). The initial factors that activate the TGF-α gene in glial cells following injury are, however, unknown.

Current evidence indicates that the actions of TGF-α are mediated by EGF receptors (11, 27). These belong to a family of receptors that have an extracellular binding domain linked to a cytoplasmic domain endowed with tyrosine kinase activity (27). Although monoclonal antibodies that block EGF binding to the human receptor have been developed (28), their usefulness in manipulations *in vivo* is diminished by factors such as receptor glycosylation, tissue penetration, and the relatively low degree of sequence conservation of the receptor's extracellular domain among species. In contrast, the kinase domain is more conserved (29). Its ligand-induced activation is essential for the receptor's biological activity (27, 29). Recently, a family of low molecular weight substances termed "tyrphostins" have been shown to block EGF/TGF-α receptors in a highly selective manner (22). *In vitro* exposure of hypothalamic tissue to RG-50864, the most potent tyrphostin, abolished the release of LHRH induced by TGF-α or EGF without affecting that elicited by prostaglandin E_2 (10).

The selectivity of tyrphostins (22) makes it unlikely that the inhibitory effect of RG-50864 on lesion-induced puberty is caused by preferential blockade of receptor tyrosine kinases such as the insulin, insulin growth factor I, and platelet-derived growth factor receptors, which share only ≈30% homology with the EGF receptor kinase domain (29). However, we cannot exclude the possibility that RG-50864 interacts with EGF receptor relatives such as HER2/neu (30) or HER3/ERBB3 (31, 32), both of which are expressed in brain (31, 32) and share 60% sequence homology with the EGF receptor kinase domain. It is also difficult to rule out that RG-50864 may have blocked a still unidentified receptor tyrosine kinase of this exceedingly complex and rapidly growing family of signal-transducing polypeptides.

We have previously shown that TGF-α can stimulate LHRH release from the median eminence and that this effect is presumably mediated by prostaglandin E_2 (10). Since most EGF/TGF-α receptors are in glial cells (9), the mechanism by which TGF-α accelerates puberty may involve a glial–neuronal interaction. According to this concept, TGF-α produced by reactive astrocytes would stimulate, through paracrine/autocrine actions, the release of prostaglandins from glial cells. Prostaglandins, in turn, would stimulate LHRH release by acting on LHRH cell bodies neighboring the lesion and/or LHRH nerve terminals traveling toward the median eminence near the ventral aspect of the injured area.

The present results provide an explanation for the earlier finding that lesions affecting different hypothalamic regions were similarly effective in eliciting precocious puberty (33). If an increased TGF-α expression always occurred after brain injury regardless of the site of the lesion, the only limiting factor for TGF-α to stimulate LHRH release would be the relative vicinity of the lesion to LHRH neurons and their secretory axons. Thus, hypothalamic lesions located in a broad region extending from the posterior preoptic area to the premammillary area (33) would be expected to advance puberty, provided that they damage neither the perikarya nor the axons of LHRH neurons.

The ability of lesioned rats to maintain estrous cyclicity is difficult to explain if the assumption is made that after the lesion the LHRH neuronal system becomes subjected to an exclusive, and sustained, TGF-α-driven stimulation. While an explanation is not at hand, the activation of other regulatory mechanisms, particularly those relevant to neuronal plasticity and gonadal steroid control, can be invoked (for further discussion, see ref. 34). Relevant to this issue is our recent finding that activation of c-fos expression in LHRH neurons does not occur as an acute response to the lesion, but rather it is seen during the actual initiation of puberty, when estradiol levels are elevated (M.-P.J., A. Wolf, G.H., Y.J.M., and S.R.O., unpublished data). Importantly, the loss of c-fos expression observed in LHRH neurons after ovulation in the normal estrous cycle (35) also occurred in lesioned rats.

Taken altogether our results raise the possibility that an inappropriate TGF-α expression may contribute to the etiology of human sexual precocity of cerebral origin, both idiopathic and caused by hypothalamic lesions. They also suggest that TGF-α is involved in repair of the central nervous system. Whether TGF-α is involved in the normal initiation of puberty remains to be elucidated. Recent experiments (36) demonstrating increased TGF-α mRNA levels in the female hypothalamus at developmental phases when gonadotropin output is increased (postnatal day 12 and first proestrus) suggest that this may be the case.

Note Added in Proof. After 6 days at room temperature, RG-50864 diluted in saline was found to retain 50% of its initial inhibitory activity, as determined by its ability to block ligand-induced EGF receptor-mediated phosphorylation of the substrate poly(Glu_6-Ala_3-Tyr_1).

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