TRANSFORMING GROWTH FACTOR ALPHA (TGFα) OF GLIAL ORIGIN

IS A HYPOTHALAMIC REGULATOR OF MAMMALIAN

SEXUAL DEVELOPMENT

by

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ABSTRACT

Substantial evidence exists that mammalian puberty is initiated within the central nervous system. Unfolding of a still poorly understood series of events ultimately results in the activation of neurons that produce luteinizing hormone-releasing hormone (LHRH), the neurohormone that controls sexual development. Since these neurons can be activated prematurely by experimental manipulations, it is clear that they do not constitute a limiting factor for puberty to occur. Instead, the initiation of puberty depends on events that occur in cells functionally connected to the LHRH neuronal network. While there is little doubt that one of these events is the activation of neurotransmitter systems regulatory to LHRH neurons, the present studies suggest that the overall process may require the participation of molecules that do not act as neurotransmitters, but rather mediate cell-to-cell interactions between neurons and glia, the two major brain cell types. The results of these studies, performed in female rats and non-human primates, implicated transforming growth factor alpha (TGFα) -- a mitogenic polypeptide thought to be involved in normal embryonic development of the central nervous system and in malignant transformation -- as one of such regulatory molecules. Evidence was provided that the genes encoding TGFα and its receptor are expressed in the developing hypothalamus of both rats and monkeys, that TGFα mRNA and its protein product and the TGFα receptor are predominantly expressed in cells of hypothalamic nuclei concerned with the control of LHRH secretion, and that hypothalamic levels of TGFα mRNA increase at the time of puberty in both species. Experiments in rats showed that TGFα-induced activation of its receptor results in LHRH release via a non-genomic, prostaglandin-mediated, mechanism and that hypothalamic TGFα gene expression is up-regulated by ovarian steroids. Furthermore, blockade of TGFα

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receptors targeted to the median eminence was found to delay puberty, suggesting that a site-specific activation of TGFα receptors is an essential component of the neuroendocrine process that leads to the acquisition of female reproductive competence. *In vitro* experiments with isolated astrocytes demonstrated that TGFα acts in an autocrine/paracrine fashion to stimulate its own gene expression, and that this effect is region-specific, as it was observed with hypothalamic but not with cerebellar astrocytes. These experiments also demonstrated that hypothalamic, but not cerebellar astrocytes, express the estrogen receptor gene and respond to estradiol with an increase in TGFα mRNA levels. These findings support the concept that glial cells in the brain are functionally diverse and specialized in a region-specific fashion to subserve different functions. In the hypothalamus, one of these functions appears to be linked to the regulation of sexual development. Additional studies with transgenic mice carrying a human TGFα transgene under the control of a heavy metal inducible promoter demonstrated that activation of the promoter resulted in preferential expression of the transgene in the hypothalamus and led to precocious initiation of estrous cyclicity. Altogether, these findings provide evidence for the concept that TGFα, a growth factor molecule of glial origin, is a normal component of the regulatory process that controls LHRH neuronal function during development. They also suggest that a derangement of TGFα production, and hence of the functional relationship between glial cells and LHRH neurons, may play a pivotal role in the premature activation of LHRH secretion underlying central sexual precocity.

INTRODUCTION

Initiation of Female Puberty

There is little doubt that mammalian puberty is initiated by events that occur within the central nervous system (CNS) (for review, see 1). Unfolding of these events leads to activation of a group of highly specialized neurons which produce the decapeptide luteinizing hormone-releasing hormone (LHRH), also referred to as GnRH, the neurohormone that controls sexual development (2,3). Most LHRH neurons are located in preoptic area and the hypothalamus and project their axons to the median eminence, a highly vascularized region of the brain located in the ventral portion of the hypothalamus in close proximity to the pituitary gland. LHRH is secreted into the portal hypophyseal vessels of the median eminence, and is transported via this route to the anterior pituitary gland where it stimulates gonadotropins (LH and FSH) secretion. Gonadotropins, in turn, stimulate the development of the gonads; in the case of females, completion of ovarian development culminates with activation of the first preovulatory surge of gonadotropins and the first ovulation, which signals the initiation of reproductive competence.

Regulation of the Onset of Puberty

The role of neurotransmitters: The onset of normal puberty requires an increased secretion of LHRH. Several neurotransmitters, and the regulatory influence of gonadal steroids, have been implicated in the process that leads to the pubertal activation of LHRH secretion (1,4). In regard to neurotransmitters, experiments conducted in female rats (5) and rhesus monkeys (6) have demonstrated that pulsatile administration of the excitatory amino acid analog N-methyl-D aspartic acid (NMDA) to juvenile animals results in the synchronized activation of LHRH

secretion and leads to the initiation of puberty. Conversely, blockade of NMDA receptors inhibits pulsatile luteinizing hormone LH secretion (7) and delays onset of puberty in female rats (5). These observations suggest that excitatory amino acids acting through NMDA-like receptors are physiologically involved in the initiation of mammalian puberty. Other experiments have implicated catecholaminergic (noradrenergic) and peptidergic (neuropeptide Y)-containing neurons as physiologically involved in the transsynaptic stimulation of LHRH neurons at puberty (8,9). Evidence also exists that maturation of LHRH neurons and their associated neuronal circuitry is required for puberty to occur. It is known that mature LHRH neurons are synaptically connected to various neurotransmitter systems, and also to other LHRH neurons (10-13), but only recently has evidence been presented demonstrating their developmental plasticity (14). LHRH neurons undergo marked morphological changes during the prepubertal period (14), a time during which their catecholaminergic synaptic inputs also increase (15). This suggests that functional maturation of the LHRH secreting network is intimately related to morphological remodeling of the neurons themselves and their associated neuronal circuitry.

The role of neurotrophic factors: Until recently, no consideration had been given to the intriguing possibility that acquisition of functional competence by the LHRH secreting system may require the trophic influence of growth factors of brain origin. The first inkling that epidermal growth factor (EGF), a mitogenic polypeptide (16), may participate in the control of LHRH neurons came from the observation that EGF stimulated luteinizing hormone (LH) release *in vitro* from the anterior pituitary, in the presence of hypothalamic tissue, but not in its absence (17). Previous studies had suggested that EGF may function in the central nervous system (CNS) as a mitogenic as well as a trophic factor, because of its ability to stimulate glial proliferation (18,19) and to promote the survival and morphological

differentiation of brain neurons in culture (20). However, the exceedingly low levels of EGF messenger ribonucleic acid (mRNA) and of its translated protein products found throughout the CNS (21-23) suggest that EGF may not be the endogenous effector of the actions attributed to EGF in brain.

In regard to the ability of EGF to stimulate LHRH release, there is now evidence that transforming growth factor alpha (TGFα), a member of the EGF family, may be involved in the control of mammalian female sexual development (for review see 24). TGFα is a secreted 50-amino acid mitogenic polypeptide originally isolated from the medium of transformed fibroblasts (25). TGFα shares structural and functional homology with EGF and is recognized by the same receptor (EGFR) (26,27). Characterization of TGFα cDNA revealed that the mature 50 amino acid form of TGFα is synthesized via proteolytic cleavage of a glycosylated transmembrane precursor (28) encoded by a 4.8 kilobases (kb) mRNA species (29,30). The TGFα precursor consists of three major domains: an extracellular domain including the N-terminal signal sequence and the 50 amino acid mature form of TGFα, a hydrophobic transmembrane domain, and a cytoplasmic domain (31,32). Although in most cases EGFRs are activated by TGFα, there is evidence that the uncleaved precursor is able to activate EGFR on adjacent cells and modify cellular function via "juxtacrine" effects (28).

Although TGFα is produced by brain cells, its specific functions in the CNS remain unclear. The ability of TGFα to enhance the survival of neurons from various brain regions in culture (33) and to induce proliferation of neuroepithelial progenitor cells (34) suggest that TGFα may function in the CNS as a neurotrophic factor as well as a mitogenic factor. Moreover, the ability of glial cells to produce TGFα (35) is of particular interest, as it implies that TGFα is a peptide growth factor that may participate in interactions between neurons and glial cells.

Recent studies showed that TGFα stimulates the release of LHRH from median eminences (ME) *in vitro* in the absence of LHRH cell bodies (21). The ME is a specialized area of the hypothalamus compacted with LHRH nerve terminals from which LHRH is released. This finding suggested that TGFα may have a role in the regulation of LHRH secretion at the level of the nerve terminals. The stimulatory effect of TGFα on LHRH release appears to be mediated by prostaglandins since it was abolished by indomethacin, an inhibitor of prostaglandin cyclooxygenase. Moreover, TGFα elicits prostaglandins E_2 release from the median eminence. Blockade of the EGFR signal transduction process with a highly selective inhibitor of EGFR intrinsic tyrosine kinase activity (36) abolished the TGFα -induced increase in LHRH and PGE_2 release, but not the stimulatory effect of PGE_2 on LHRH secretion (21), indicating that the PGE_2 acts directly on LHRH neurons to stimulate LHRH release. This view is supported by the observation that PGE_2 enhances the release of LHRH in a neuronal LHRH-secreting cell line (37). These and other observations have led to the notion that TGFα may be a trophic factor physiologically involved in facilitating hypothalamic LHRH secretion via an EGFR receptor-mediated process. Subsequent studies (38) demonstrated that hypothalamic lesions able to induce sexual precocity in female rats result in activation of TGFα and EGFR gene expressions in reactive astrocytes surrounding the site of injury (38,39). The ability of hypothalamic lesions to advance puberty appears to involve activation of EGFR because inhibition of EGFR tyrosine kinase activity (36) abolishes lesion-induced sexual precocity (38). While these results suggest that TGFα is an important contributor to the neuropathological mechanisms underlying brain injury-induced sexual precocity, little is known about the involvement of hypothalamic TGFα in the normal process of female sexual development.

Essential for the resolution of this issue is the experimental examination of

several interrelated hypotheses: a) that both TGFα and EGFR are present in the normal developing female hypothalamus, b) that expression of their genes in the hypothalamus is developmentally regulated and associated with sexual maturation, c) that interfering with TGFα ligand-EGFR interactions in the hypothalamus alters the onset of puberty; d) that the ability of TGFα to induce LHRH release involves an indirect affect of TGFα on cells anatomically associated with LHRH neurons, e) that these cells are glial in nature, which implies that both the TGFα and EGFR genes are expressed in glial cells and not in LHRH neurons, f) that TGFα can regulate its own gene expression in hypothalamic glial cells acting via a paracrine/autocrine manner, g) that <u>selective</u> endogenous overexpression of the TGFα gene results in sexual precocity, and h) that the involvement of TGFα in the hypothalamic control of puberty is not a peculiarity of rodents, but instead represents a general phenomenon relevant to all mammalian species, including primates.

The present investigation was undertaken to examine these issues. Experiments have been conducted a) to characterize the presence of TGFα in the developing female hypothalamus in order to define its sites of synthesis, its developmental and hormonal regulation and its relationship to the control of LHRH secretion, b) to characterize the presence of EGFR in the developing female hypothalamus, study its pattern of expression at puberty, and examine the hypothesis that EGFR are not expressed in LHRH neurons, but in neighboring glial cells instead, c) to determine if TGFα can enhance its own gene expression on hypothalamic astrocytes via activation of EGFR, d) to determine if selective activation of TGFα gene expression, using a transgenic approach, is able to affect the pace and timing of puberty, and e) to examine the changes in hypothalamic TGFα gene expression in developing rhesus monkeys and subsequently attempt to advance the initiation of puberty in these animals utilizing a gene transfer - cell grafting

approach.

The present document consists of five manuscripts, referred to as Chapters 1 through 5. The results of these studies strongly suggest that transforming growth factor alpha of glial origin is a hypothalamic regulator of mammalian female sexual maturation.

Phases of puberty

To study the changes in hypothalamic $TGF\alpha$/EGFR gene expression that occur around the time of puberty, the animals were classified in different phases of puberty according to criteria previously proposed (1). These phases are: Anestrus (A): Originally this phase was meant to correspond to late juvenile phase, but it now appears to correspond to the phase during which the changes in mode of LH release begin to occur. Animals in this phase are 28-30 days of age, their uteri are small (wet weight less than 100 mg), and, importantly, no intrauterine fluid can be detected. The vagina is always closed. Early proestrus (EP): Animals in this phase have larger uteri with intraluminal fluid; their vagina is closed. Late proestrus (LP): This phase corresponds to the day of first proestrus. Animals have large "ballooned" uteri full of fluid, with a wet weigh greater than 200 mg. Their ovaries have large follicles. Most animals in this phase show closed vaginae. Estrus (E): This is the day of first ovulation, when uterine fluid is no longer present, fresh corpora lutea can be readily discerned, the vagina is open, and vaginal cytology shows a predominance of cornified cells. First diestrus (D_1): This phase of puberty is characterized by a vaginal cytology showing a predominance of leukocytes, as well as by the presence of mature corpora lutea within the ovaries.

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Transforming Growth Factor Alpha (TGFα) Gene Expression
in the Hypothalamus is Developmentally Regulated and
Linked to Sexual Maturation

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Summary

Hypothalamic injury causes female sexual precocity by activating luteinizing hormone releasing hormone (LHRH) neurons, which control sexual development. Transforming growth factor alpha (TGFα) has been implicated in this process, but its involvement in normal sexual maturation is unknown. The present study addresses this issue. TGFα mRNA and protein were found mostly in astroglia, in regions of the hypothalamus concerned with LHRH control. Hypothalamic TGFα mRNA levels increased at times when secretion of pituitary gonadotropins -- an LHRH dependent event -- was elevated, particularly at the time of puberty. Gonadal steroids involved in the control of LHRH secretion increased TGFα mRNA levels. Blockade of TGFα action in the median eminence, a site of glial-LHRH nerve terminal association, delayed puberty. These results suggest that TGFα of glial origin is a component of the developmental program by which the brain controls mammalian sexual maturation.

Introduction

Transforming growth factor alpha (TGFα), a 50-amino acid mitogenic polypeptide, is a member of the epidermal growth factor (EGF) family (Derynck, 1988; Massague, 1990). TGFα shares structural and functional homology with EGF and is recognized by the same receptor. While both TGFα and EGF immunoreactive material have been detected in brain (Fallon et al., 1984; Code et al., 1987; Kudlow et al., 1989; Seroogy et al., 1991), TGFα mRNA levels are one to two orders of magnitude higher than those of EGF mRNA (Lazar and Blum, 1992), suggesting that the predominant ligand for EGF receptors in brain may be TGFα. The mRNA encoding TGFα has

been detected in several brain regions, including the hypothalamus, by RNA blot hybridization (Kudlow et al., 1989; Ojeda et al., 1990), hybridization histochemistry (Wilcox and Derynck, 1988), and RNase protection assay (Junier et al., 1991; Lazer and Blum, 1992).

While these observations firmly establish the ability of brain cells to synthesize TGFα, little is known about the specific functions that TGFα may have in the CNS. The ability of EGF to promote neuronal survival (Morrison et al., 1987; Kornblum et al., 1990; Abe et al., 1990) and stimulate astrocytic proliferation (Leutz and Schachner, 1981; Simpson et al., 1982) suggests that TGFα may function in brain as a neurotrophic as well as a mitogenic factor. This view is supported by the observation that TGFα enhances the survival of neurons from various brain regions in culture (Morrison, RS. Soc. Neurosci. Abstr. 15:1361, 1989). TGFα may also play an important role during early development of the CNS as suggested by its ability to induce proliferation of neuroepithelial progenitor cells (Anchan et al., 1991).

The relative abundance of TGFα mRNA in brain, its widespread distribution, and persistent expression throughout postnatal life suggest that TGFα may have still other roles, possibly related to the maintenance and regulation of differentiated neuronal and/or glial functions. In this regard, the apparent ability of astrocytes to synthesize TGFα (Fallon et al, 1990; Junier et al., 1991) is of particular interest as it implies TGFα as a peptide growth factor involved in glial-neuronal interactions.

Recent studies have demonstrated the ability of both TGFα and EGF to stimulate LHRH release from the median eminence of the hypothalamus (Ojeda et al., 1990). That this stimulatory effect requires activation of EGF receptors and the intermediacy of prostaglandins was indicated by the ability of an inhibitor of EGF receptor tyrosine kinase activity and of a blocker of prostaglandin synthesis to prevent the increase in LHRH release elicited by either TGFα or EGF. Subsequent

studies showed that hypothalamic lesions that caused sexual precocity resulted in a dramatic activation of TGFα gene expression in reactive astrocytes surrounding the site of injury (Junier et al., 1991). The lesions also caused an increase in EGF receptor protein and its mRNA in reactive astrocytes (Junier, M-P. et al., 1992a). Since infusion of a selective inhibitor of EGF receptor kinase activity prevented the effect of the lesion on sexual maturation, the hypothesis was advanced that TGFα is an important contributor to the neuropathological mechanisms underlying injury-induced sexual precocity (Junier et al., 1991).

While, in a broader sense, these studies implicated TGFα as a component of the CNS response to injury, they also raised the possibility that, in the intact brain, TGFα may contribute to regulating LHRH neuronal function. This sub-set of highly specialized neurons located in the hypothalamus is essential for sexual maturation and maintenance of reproductive function. In their absence, sexual development fails to occur and reproductive capacity is lost (Mason et al., 1986). The present study provides evidence for the involvement of TGFα in the developmental process that leads to normal sexual maturation and indicates that in the hypothalamus, but not in brain regions insensitive to gonadal steroids, TGFα gene expression is under sex steroid control.

Results

Localization of TGFα mRNA in the Prepubertal Hypothalamus
An antisense RNA probe complementary to the coding region of TGFα mRNA was used to determine the cellular sites of TGFα mRNA expression in the hypothalamus of prepubertal female rats. Although specific labeling was detected in cells scattered throughout the hypothalamus, TGFα mRNA was more abundant in discrete

hypothalamic nuclei. These included the suprachiasmatic (SCN), paraventricular (PVN), arcuate (ARC), ventromedial (VMH) and dorsomedial (DMH) nuclei. The SCN is located directly above the optic chiasm; the PVN is located in the anterior hypothalamus, lateral to the dorsal aspect of the third ventricle; the ARC, VMH and DMH are located in the medial region of the hypothalamus, lateral to the third ventricle in a ventral, intermediate and dorsal position, respectively, along the sides of the third ventricle. Hybridization was less prominent, but still evident, in the medial preoptic area (MPOA) located in the forebrain, directly rostral to the hypothalamus. Specific labeling was also observed in tanycytes of the third ventricle and glial cells of the median eminence. The median eminence, located at the base of the hypothalamus, is a region devoid of neuronal perikarya, but packed with nerve terminals from neuroendocrine neurons. The neurosecretory products of these neurons are released into the capillary bed of the median eminence for transport to the anterior pituitary gland via the portal vasculature. Figure 1 depicts the presence of TGFα mRNA in the SCN (Figure 1A), tanycytes of the third ventricle and glial cells of the median eminence (Figure 1D and E). Fewer labeled cells were detected in the PVN, VMH and ARC (Figure 1C and D, respectively). No specific labeling was observed in adjacent sections hybridized with a sense RNA probe transcribed from the same TGFα cDNA template (Figure 1B and F).

Labeling of cells lining the third ventricle was limited to tanycytes, located in the ventral region of the ventricle (Figure 2). "Typical" ependymal cells (Knowles, 1972), located in the dorsal third of the ventricle had no detectable levels of TGFα mRNA; in fact, the hybridization signal ended abruptly in the transition between these two cell types (Figure 2A and A1). These observations were corroborated by immunohistochemistry, which demonstrated the presence of TGFα precursor protein in tanycytes, and a paucity of immunoreactivity in dorsal ependymal cells (Figure 2B

and B1).

Localization of TGFα Precursor Immunoreactivity in the Immature Hypothalamus
Immunoreactive TGFα precursor-like material was identified in the hypothalamus of juvenile female rats using polyclonal antibody 1296 which recognizes the peptide sequence 137-151 contained within the cytoplasmic domain of the TGFα precursor (Gentry et al., 1987). The highest levels of TGFα precursor immunoreactivity were found in the same areas and nuclei where TGFα mRNA was most abundant, i.e., the SCN, PVN, DMH, VMH, and ARC. Immunoreactive material was also detected in tanycytes and glial cells of the median eminence. Although some positive cells were also seen in the periventricular nucleus (PVA), most of the immunoreactive material in this region was detected in fibers running along the third ventricle (Figure 3C arrowhead). As with TGFα mRNA, some positive cells were seen in the MPOA (not shown). Figure 3 provides some examples of these localizations. The SCN appeared to be particularly rich in immunoreactive material (Figure 3A). Preabsorption of the primary antibodies with the synthetic peptide used as antigenic determinant, resulted in complete elimination of immunoreactive material, as shown in an adjacent section depicted in Figure 3B. Throughout the hypothalamus, including the nuclei where the immunoreactive material is more abundant, the TGFα precursor appeared to be localized in both neurons and astrocytes, but more predominantly in the latter. Figures 3C-F illustrate this distribution. Figure 3C depicts TGFα immunoreactive cells in the PVN at low magnification; at a higher magnification most of the positive cells appear to be astrocytes (Figure 3D and E, arrows), with only a few cells having a neuronal appearance (arrowhead). Preabsorption experiments that resulted in complete elimination of TGFα precursor immunoreactivity in astrocyte-like cells, reduced but did not eliminate the reaction in neurons. Figure 3F demonstrates,

using double immunohistochemistry, the presence of TGFα precursor immunoreactive material (arrowheads, granular appearance) in astrocytes of the arcuate nucleus, identified as such by their GFAP immunoreactivity (arrows, smooth appearance of the staining). The presence of TGFα precursor-like material in tanycytes and glial cells of the median eminence is shown in Figure 3G.

Changes in TGFα mRNA Levels During Pre- and Peripubertal Development

The assay. To determine if TGFα gene expression changes in the hypothalamus in relation to female sexual development, TGFα mRNA levels were quantitated by RNase protection assays. Figure 4A depicts standard curves constructed by simultaneously hybridizing different concentrations of *in vitro* transcribed sense TGFα or LHRH mRNA with their corresponding ^{32}P-labeled antisense RNA probes. In both cases, the assay is able to detect as little as 60 fg of sense RNA. Figure 4B depicts the regression lines and best fit for the standard curves shown in panel A. The autoradiograms shown in panel C illustrate the changes in TGFα and LHRH mRNAs detected in the POA at the time of normal puberty (lanes 1-4) and after treatment with gonadal steroids (lanes 5-8). Quantitative analysis of these changes is described below.

Prepubertal development. TGFα mRNA levels in the POA and MBH remained relatively unchanged between fetal day 18 and postnatal day 5 (Figure 5). They increased markedly on day 12, a time at which LHRH and pituitary gonadotropin secretion peak (Hompes et al., 1982; Ojeda and Ramirez, 1972), and returned to lower levels during the juvenile phase of sexual development (days 23-28, Ojeda and Urbanski, 1988). In contrast, TGFα mRNA levels in the cerebellum, used as a control tissue, did not change within this time frame (Figure 5).

The initiation of puberty. A marked increase in TGFα mRNA levels occurred

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in both the POA and the MBH during the initiation of puberty (Figure 6, upper panel). Levels increased gradually after the anestrous phase of puberty, which corresponds to the end of juvenile development (Ojeda and Urbanski, 1988), reaching peak values on the afternoon of the first proestrus, i.e., at the time of the first preovulatory surge of LHRH and gonadotropins. The elevated circulating levels of gonadotropin hormones resulting from this discharge cause the ovary to ovulate. After the first ovulation, i.e., the estrous and first diestrous phases of puberty, TGFα mRNA levels returned to lower values. No such changes were observed in the cerebellum or the lateral hypothalamus (Figure 6, upper panel). The latter, referred to as MBH(-), corresponds to tissue remaining after removal of the medial basal portion of the hypothalamus and that is limited laterally by the hypothalamic sulci. The proestrous peak in TGFα mRNA found in the POA correlated well with a similar increase in LHRH mRNA detected at this time (Figure 6, lower panel). Since most LHRH neurons are located in the POA no attempts were made to examine changes in LHRH mRNA in the MBH.

Effect of Ovarian Steroids on Hypothalamic Content of TGFα mRNA

Because the changes in TGFα mRNA observed during normal puberty occur at the time when the secretion of the ovarian steroids, estradiol (E_2) and progesterone (P), is increasing (Ojeda and Urbanski, 1988), we examined the possibility that TGFα mRNA expression in the hypothalamus is under the regulatory influence of gonadal steroids. Juvenile rats were ovariectomized at 22-days of age and 5 days later were implanted subcutaneously with a Silastic capsule containing a concentration of an E_2 dissovled in corn oil at a concentration of 400 μg/ml that results in circulating levels of the steroid similar to those seen on the day of first proestrus (Andrews et al., 1981). This dose of E_2 has been shown to be effective in eliciting a preovulatory-like

discharge of pituitary gonadotropins in prepubertal rats (Andrews et al., 1991). After two days some of the rats received a single subcutaneous injection of P at a dose (1 mg/rat) previously shown to evoke a rapid increase in gonadotropin secretion (Kalra, 1986). Neither short-term ovariectomy to remove circulating E_2 levels nor administration of E_2 alone to mimic the levels of E_2 seen during normal puberty affected TGFα mRNA levels in the ME-ARC, MBH(-) or Cb (Figure 7, left panels). However, administration of P to E_2-primed rats to mimic the changes in sex steroid levels observed at the time of the preovulatory surge of gonadotropins resulted in a five-fold increase in TGFα mRNA in the ME-ARC, 2h after the injection as compared with control animals receiving vehicle only. The effect was absolutely region-specific as it was not observed in the MBH(-) or Cb (Figure 7, left lower panels). It also required previous exposure to E_2 since P alone was ineffective. In contrast to the ME-ARC, the POA region responded to E_2 alone with a two-fold increase in TGFα mRNA levels (Figure 7, right upper panel). As in the case of the ME-ARC, P treatment following E_2 priming elicited a robust increase in POA TGFα mRNA. The increase was evident 2h after the injection, but much more prominent at 4h, a time at which levels were more than 10-fold higher than basal values (Figure 7, right upper panel). P alone was ineffective.

Similar to the changes observed during normal puberty, the increase in TGFα mRNA elicited by P in E_2-primed rats was well correlated with that in LHRH mRNA induced by the steroids (Figure 7, right lower panel).

Effect of Pharmacological Blockade of Median Eminence EGF Receptors on the Onset of Puberty

It is well established that TGFα utilizes the EGF receptor to exert its biological actions (Derynck, 1988). Blockade of EGF receptor protein kinase activity with a

newly developed family of compounds termed tyrphostins (Yaish et al., 1988) has been shown to abolish the increase in LHRH release elicited by TGFα (Ojeda et al., 1990), and to prevent the advancing effect of hypothalamic lesions on female puberty (Junier et al., 1991). Consequently, we utilized one of these tyrphostins (RG 50864) to determine if region-specific blockade of EGF receptors would delay the normal onset of puberty. The compound, delivered to the median eminence of the hypothalamus via a stereotaxically implanted stainless steel cannula, resulted in a marked delay of puberty, as determined by the age at vaginal opening (Figure 8) and first ovulation (assessed upon necropsy on the day of first diestrus after vaginal opening). Control implants containing only the vehicle were ineffective. Importantly, RG50864 implants located caudal to the median eminence area, in the region of the mammillary bodies -- which is devoid of LHRH nerve terminals -- did not reproduce the delaying effect of the median eminence implants (Figure 8).

Discussion

Little is known regarding the functions that TGFα and EGF, its structural homolog, may have in the CNS. The ability of EGF to induce glial proliferation *in vitro* (Simpson et al., 1982; Leutz and Schachner, 1981) suggests that the EGF family of mitogenic peptides, of which TGFα is a prominent member, may be involved in stimulating astroglial division during both normal development and following CNS injury. The latter role is supported by the recent observation that injury of the hypothalamus results in a striking increase in TGFα mRNA expression in reactive astrocytes surrounding the lesion (Junier et al., 1991). TGFα may also act as a neurotrophic factor to support the survival of specific neuronal populations (Morrison, RS. Soc. Neurosci. Abstr 15:1361, 1989), either directly or by stimulating

glial synthesis of neurotrophins (Spranger et al., 1990).

That TGFα and its congeners may subserve additional, region-specific functions throughout the brain is suggested by the marked regional differences in TGFα and EGF mRNA expression detected across the adult CNS (Wilcox and Derynck, 1988; Seroogy et al., 1991; Lazar and Blum, 1992). Conceivably, these functions may be relevant to the maintenance and regulation of differentiated neuronal and/or glial activities. The presence of TGFα mRNA in the adult and developing hypothalamus (Kudlow et al., 1989; Ojeda et al., 1990) and the ability of TGFα to stimulate secretion of LHRH (Ojeda et al., 1990), the hypothalamic neuropeptide controlling sexual development, suggest that one such region-specific function may be the regulation of neuroendocrine secretory activity.

In recent publications we suggested that TGFα may be an important component of the mechanism by which hypothalamic injury enhances the secretory activity of LHRH neurons and hastens sexual maturation (Junier et al., 1991). We also furnished evidence that this stimulatory effect of TGFα is mediated by EGF receptors (Junier et al., 1991), may not require activation of LHRH gene expression but, instead, is exerted via activation of LHRH release (Junier et al., 1992b) and appears to involve the intermediacy of glial cells, because astrocytes and not LHRH neurons are endowed with EGF receptors (Ojeda et al., 1990; Ma et al., Endocr. Soc. Abstr., p. 303, 1991; Junier et al., 1992a). An important issue raised by these observations concerns the role that TGFα may play in the **normal** process of hypothalamic maturation that leads to the acquisition of reproductive competence. The present findings strongly suggest that TGFα is a physiological component of this process.

Although TGFα mRNA and its protein product were detected in cells scattered throughout the hypothalamus and in several hypothalamic nuclei, they were

conspicuously present in areas concerned with the control of LHRH secretion, most noticeably, the SCN and cellular groups of the medial basal hypothalamus. Interestingly, even in discrete hypothalamic nuclei, the majority of TGFα expressing cells were astrocyte-like. This raises the intriguing possibility that astrocytes may be not only regionally diverse (Shinoda et al., 1989; Ernsberger et al., 1990), but also have specialized functional properties when interacting with anatomically discrete neuronal sub-groups.

The SCN, a nodal point of synaptic connectivity, plays a fundamental role in the maintenance of circadian rhythms, including rhythms entrained by photoperiodic cues (Meijer and Rietveld, 1989; Rusak and Zucker, 1979) and those underlying reproductive cyclicity (Raisman and Brown-Grant, 1977). Destruction of the SCN results in loss of reproductive cyclicity (Wiegand and Terasawa, 1982; Ronnekleiv and Kelly, 1986) without alterations in the number of LHRH neurons or their LHRH content (Ronnekleiv and Kelly, 1986; Ma et al., 1990). These findings have led to the conclusion that the SCN contributes to regulating LHRH release rather than LHRH synthesis. We have reached a similar conclusion regarding the mechanism by which lesion-induced TGFα synthesis in reactive astrocytes accelerates sexual maturation (Junier et al., 1992b). The high level of TGFα expression in the SCN observed in the present study suggests that some functions of this nucleus, including those affecting the LHRH neuronal network, are under TGFα regulatory influence.

Regarding the presence of TGFα in cells of the medial basal hypothalamus, detection of TGFα mRNA and protein in the ARC, VMH, tanycytes of the third ventricle and glial cells of the median eminence is of particular interest because of the postulated involvement of this region as a whole in the tonic control of LHRH secretion (for a review see Kalra, 1986). While the ARC and VMH are involved in mediating the inhibitory effect exerted by gonadal steroid on LHRH release, and

contain neuronal populations functionally coupled to LHRH neurons (for reviews see Kalra, 1986; Weiner et al, 1988), ultrastructural studies have suggested that tanycytes and glial cells of the median eminence may play a significant role in the regulation of LHRH secretion (Kozlowski and Coates, 1985). Tanycytes envelop LHRH nerve terminals throughout their entire course towards the perivascular space of the hypophyseal-portal vascular system, and prevent (via their endfeet) most LHRH neurosecretory endings from contacting the perivascular space directly (Kozlowski and Coates,1985; Ugromov et al., 1989). This arrangement seems to be particular for LHRH axons as it has not been seen with nerve endings of other neuroendocrine neurons (Ugromov et al., 1989). The importance of such a tight LHRH axon/tanycyte relationship in the control of LHRH neuronal function is supported by two recent observations. One of them showed that approximately half of glial cells in the median eminence contain estrogen receptors as opposed to glia in other hypothalamic areas (Langub et al., 1992). Since LHRH neurons do not have estrogen receptors (Shivers et al., 1983), the presence of the receptors in glial cells of the median eminence provides the anatomical substrate for a glial involvement in estrogen-dependent regulation of LHRH release. The other observation demonstrated that normal LHRH neurons transplanted to an homozygous mutant hypogonadal mouse, which lacks a functional LHRH gene, are guided to the perivascular space of the median eminence by glial/tanycytic channels (Silverman et al., 1991). As in normal animals, the axons of transplanted LHRH neurons do not contact the portal endothelial cells directly, but do so via intervening tanycytic feet.

The ability of TGFα to stimulate LHRH release from the median eminence in the absence of LHRH neuronal perikarya was attributed, not to a direct effect of the growth factor on LHRH axons, but to a mechanism involving glial intermediacy (Ojeda et al., 1990). That this may indeed be the case is suggested by the presence

of EGF receptors in tanycytes and glial cells of the median eminence, but not in LHRH neuronal perikarya or axons (Ma et al., Endocr. Soc. Abstr., p. 303, 1991). The present demonstration of TGFα gene expression in tanycytes lends credence to this view, which also proposes that TGFα produced by tanycytes/glial cells of the median eminence may stimulate release of LHRH via prostaglandins, the formation of which would be increased by TGFα through an autocrine mechanism. Initial support for this hypothesis comes from the findings that: 1) prostaglandin receptor blockade abolishes TGFα-induced LHRH release (Ojeda et al., 1990), 2) astrocytes release prostaglandins in response to neuropeptide stimulation (Katsuura et al., 1989), 3) a neuronal LHRH secreting cell line responds to prostaglandins with LHRH release, but is unable to synthesize prostaglandins on its own (Negro-Vilar et al, Endo. Soc Abstr. 1991, p. 458), and 4) TGFα does exert autocrine effects on hypothalamic astrocytes as evidenced by its ability to increase TGFα mRNA in cultured astrocytes from this brain region (Ma et al., Soc. Neurosci. Abstr., p. 1449, 1992).

Although an involvement of TGFα in other hypothalamic functions is entirely possible, the changes in hypothalamic TGFα mRNA content at times of enhanced gonadotropin secretion strongly suggest that TGFα contributes to regulating LHRH neuronal activity during sexual development. Gonadotropins are the only pituitary hormones that show a peak of secretion at the second week of postnatal life (for a review see Ojeda et al., 1980). That this enhanced secretion is due, at least in part, to an increased LHRH output is indicated by the hyperresponsiveness of LHRH neurons to stimulation *in vitro* (Hompes et al.,1982). The marked increase in POA-MBH TGFα mRNA content observed on postnatal day 12 suggests, but does not prove, that the two events may be causally related. The factors that increase TGFα gene expression at this time of development are not known, but are unlikely to be

gonadal steroids as circulating progesterone levels are exceedingly low during the first three weeks of postnatal life and the biological activity of estradiol is severely limited by its binding to alpha-fetoprotein (for a review see Ojeda and Urbanski, 1988).

In contrast to this situation, the present results strongly suggest the involvement of gonadal steroids in the increase of hypothalamic TGFα mRNA seen at the time of puberty. Since the existing literature shows that TGFα gene expression in endocrine glands is predominantly regulated by estradiol (see for instance Salomon et al., 1989; Bates et al., 1988), it was surprising to find that hypothalamic TGFα content was most effectively increased by progesterone preceded by estradiol than by estradiol alone. The ability of progestins to increase TGFα mRNA levels has been reported in breast cancer cells (Murphy and Dotzlaw, 1989), but no progesterone responsive elements have been detected in the 5' flanking region of the TGFα gene bearing the cis-acting regulatory elements responsive to estradiol (Saeki et al., 1991). This suggests that the effect of progesterone is indirect or that the DNA sequences responsive to progesterone are not located within this 5' flanking region.

The stimulatory effect of estradiol and progesterone on TGFα gene expression may be exerted on neuronal populations bearing the appropriate receptors. Nevertheless, the possibility that effects of at least one of these steroids are exerted directly on astrocytes needs to be considered. Long-term exposure of astrocytes to estradiol was recently shown to induce formation of progesterone receptors (Jung-Testas et al., 1991) and we, in our own experiments, found that hypothalamic astrocytes express estrogen receptor mRNA as assessed by RNase protection assay (Ma et al., Soc. Neurosci. Abstr., p. 1449, 1992). Moreover, treatment of purified astrocytes with estradiol resulted in increased TGFα mRNA levels within 8h of exposure.

Ligand-induced activation of the EGF receptor tyrosine kinase is essential for receptor-mediated signal transduction (Carpenter, 1987). The availability of tyrphostins, a new group of highly selective blockers of EGF receptor tyrosine kinase activity (Yaish et al., 1988), has provided a tool to block receptor activation in intact cells without having to resort to the use of antibodies (Lyall et al., 1989). We recently demonstrated that one of these tyrphostins abolishes the ability of TGFα to induce LHRH *in vitro* (Ojeda et al., 1990) and prevent the advancing effect of hypothalamic lesions on puberty *in vivo* (Junier et al., 1991). That this compound delays normal puberty when applied to the median eminence of otherwise intact animals suggests that activation of EGF receptors (or EGF receptor homologs) is an intrinsic component of the neuroendocrine process that underlies sexual maturation. The effect of RG50864 appeared to be highly region-specific because no delay in puberty was seen when the compound was implanted in the area of the mammillary bodies, which does not contain LHRH nerve terminals. The effect was also fully reversible as evidenced by the attainment of puberty after the supply of blocker to the tissue became exhausted. As previously stated (Junier et al., 1991), we cannot rule out the possibility that RG50864 may be blocking other receptor tyrosine kinases, particularly those of the EGF receptor family (Schechter et al., 1985, Kraus et al, 1989; Plowman et al., 1990). Nevertheless, the high selectivity of these compounds for EGF receptors (Yaish et al., 1988) makes it less likely that the delaying effect of RG50864 on puberty is due to inhibition of other less related receptor tyrosine kinases such as those for insulin and platelet-derived growth factor. Whether RG50864 blocks some other still unidentified receptor tyrosine kinase is not known.

Lastly, the simultaneous increases in TGFα and LHRH mRNAs observed during normal development and following estradiol/progesterone treatment may

indicate a causal relationship between the two events. While the changes in LHRH mRNA are consistent with those reported in adult animals (Kim et al., 1989; Park et al., 1990), currently we have no indications that TGFα is capable of increasing LHRH gene expression. Further experiments are necessary to resolve this issue.

Taken altogether, these and our previous findings (Junier et al., 1991) indicate that TGFα not only contributes to the neuropathological mechanisms underlying hypothalamic injury-induced sexual precocity, but -- importantly -- is a physiological component of the developmental process by which the hypothalamus controls the advent of normal female sexual maturation. The results also indicate that expression of the TGFα gene in the neuroendocrine brain is subjected to steroid regulation and that a TGFα-mediated activation of EGF receptors plays a role in the initiation of mammalian puberty.

In a more general context, the regulatory interaction between glial cells and LHRH nerve terminals via TGFα may represent an example of a common mechanism by which glial cells affect neuronal function. According to this view, trophic molecules produced by glial cells would be able to act directly on neurons bearing the appropriate receptors or, as in the case of TGFα and LHRH neurons, indirectly via paracrine mechanisms. Since the glial ensheathing of neurons includes the neuronal perikarya, processes and synaptic contacts, the regulatory effects of glial-derived trophic factors on differentiated neuronal functions may be exerted at each one of these levels. An important implication of this concept is that glial-derived trophic factors may represent highly localized regulatory components of critical importance in the continuous process of neuronal/synaptic remodeling that characterizes development of the central nervous system and that is known to persist in adulthood (Cotman and Nieto-Sampedro, 1984).

Experimental Procedures

Animals

Immature rats of the Sprague-Dawley strain were used for these studies. They were housed in a room with controlled photoperiod (14 h light-10 h darkness, lights on from 0500 to 1900h) and temperature (23-25°C), and were provided free access to tap water and pelleted rat chow. When animals younger than 21 days of age were used, they were housed with their mothers.

Surgery

Ovariectomy. Animals were ovariectomized at 22 days of age while lightly etherized. Both ovaries were removed through a single dorsal skin incision, as described (Andrews et al., 1981).

Intrahypothalamic Implants. To block the biological actions of endogenous TGFα in an anatomically circumscribed area, an inhibitor of EGFR tyrosine kinase activity was delivered to the median eminence of the hypothalamus via a stereotaxically implanted cannula containing the inhibitor at its tip. Tyrphostin RG50864 blocks ligand-induced activation of EGFR in a highly selective manner (Yaish et al. 1988; Lyall et al., 1989), and inhibits TGFα/EGF-induced LHRH release from the median eminence of immature female rats (Ojeda et al., 1990). Utilizing cultures of hypothalamic astrocytes, we verified the selectivity of RG50864, by comparing its ability to block EGFR and insulin-induced receptor-mediated phosphorylation of the artificial substrates poly(Glu_6-Ala_3-Tyr_1) and poly(Glu_4-Tyr_1), respectively (not shown). For the implantation experiments, the inhibitor was mixed with melted cocoa butter (1:2, w/w) and tapped into the open end of a stainless steel 23-gauge cannula, as described (Ojeda and Ramirez, 1969). Control cannulae

contained only the vehicle. The cannulae were stereotaxically guided to the median eminence or mammillary bodies of late juvenile 28-day-old rats according to the following coordinates: median eminence - 0.0 mm caudal to bregma, 0.0 mm lateral from the midline, and 8.5 mm vertical from the surface of the brain; mammillary bodies - 0.7 mm caudal to bregma, 0.0 mm lateral from the midline and 8.5 mm vertical, respectively. After implantation, the canulae were fixed with dental cement to the skull and the skin wound was sutured with surgical clips.

Steroid Treatment

To simulate the changes in circulating E_2 levels that accompany the initiation of female puberty, 17ß-estradiol (17ß-E_2, Sigma Chemicals Co., St. Louis, MO) was administered to ovariectomized 27-day-old rats via subcutaneously implanted Silastic capsules. The animals were ovariectomized on day 22 and five days later received a capsule (i.d., 1 mm; o.d., 2.16 mm; length, 20 mm/100g BW)) containing 17ß-E_2 dissolved in corn oil at 400 μg/ml. This concentration has been shown to reproduce the preovulatory levels of plasma E_2 found during the initiation of puberty in the rat (Andrews et al., 1981). Some animals were subjected to a combined E_2/P treatment to simulate the sequence of events observed during the afternoon of the first proestrus, a time during which elevated E_2 levels are accompanied by a marked and abrupt increase in P levels (Andrews et al., 1981). Progesterone (1 mg/rat) was injected sc at 1200 h, 48 h after E_2.

Phases of Sexual Development

Changes in hypothalamic TGFα mRNA expression were determined at ages previously shown to correspond to key phases of female sexual development in the rat (for a review see Ojeda and Urbanski, 1988). The most prominent of these

phases are the infantile period that extends between the second and third week of postnatal life, and the initiation of puberty that occurs during the fifth and sixth week after birth. Secretion of pituitary gonadotropins increases markedly during the infantile period, presumably due to an increase LHRH output that results at least partially from a relative absence of gonadal steroid negative feedback control. At puberty, the most striking neuroendocrine event is the preovulatory surge of gonadotropins brought about by the stimulatory effect of ovarian steroids on the hypothalamic-pituitary unit.

Animals studied during the initiation of puberty were classified according to previously established criteria (Ojeda and Urbanski, 1988). In brief, they were considered to be in the anestrous phase of puberty (i.e., late juvenile development) if their vagina was not patent and had a uterine weight of 60 mg or less, without accumulation of intrauterine fluid. Animals with enlarged uteri and detectable intrauterine fluid (an index of estradiol secretion) were classified in the early proestrous (EP) stage, which precedes the day of the first preovulatory surge of gonadotropins. Animals with an uterine weight of at least 200 mg and a uterus "ballooned" with fluid were considered to be in late proestrus (LP), i.e. the phase of puberty during which LHRH and gonadotropins are for the first time discharged as a preovulatory surge. The first ovulation occurs on the next day (first estrus, E). At this time, the vagina becomes patent exhibiting a cytology with a predominance of cornified cells, and the ovaries have fresh corpora lutea. The first diestrous (D1) phase, characterized by a vaginal cytology with a predominance of leukocytes, follows.

Tissue Dissection

The hypothalamus was divided in three parts: one comprising the region above the optic chiasm that includes the POA and SCN (referred to as preoptic area, POA),

another comprising the medial basal hypothalamic area that includes the median eminence, ARC, and VMH (referred to as ME-ARC), and another (referred to as MBH-) that includes the lateral basal hypothalamus dissected by one sagittal cut half-way between the median eminence and each hypothalamic sulci and a second cut along the hypothalamic sulci. When prepubertal animals were studied, the basal hypothalamus was removed *in toto* without further dissecting it into medial and lateral components. Cerebellar samples were used as a control tissue. Immediately after collection all tissues were frozen on dry ice and stored at -85°C until RNA extraction.

RNase Protection Assay

Total RNA was extracted as reported (Lara et al., 1990; Ojeda et al., 1991). The solution hybridization/RNase protection assay employed has been previously described in detail (Junier et al., 1991; Hill et al., 1992). In brief, 500,000 cpm of a gel-purified antisense ^{32}P-labeled TGFα cRNA synthesized by *in vitro* transcription (see below) were hybridized to RNA samples (3 μg/tube) or to different amounts of *in vitro* transcribed sense TGFα mRNA for 18-20 h at 45°C. The tissue RNA samples were simultaneously hybridized to 5,000 cpm of a cyclophilin antisense RNA to correct for procedural losses. Cyclophilin mRNA is constitutively expressed in brain and other tissues (Danielsson et al., 1988). After hybridization, single stranded RNA was digested with ribonucleases and the protected hybrids were isolated by polyacrylamide gel electrophoresis (5% acrylamide, 7 M urea).

Probes

The antisense TGFα cRNA probe was transcribed from a 400 bp TGFα cDNA (Lee et al., 1985) cloned into the riboprobe vector pGEM-3Z and linearized with Eco RI.

SP6 polymerase-directed transcription of this template results in a 430 bp ^{32}P-CTP labeled cRNA, of which 30 bp correspond to vector sequences. The same template was used to prepare cRNA probes for hybridization histochemistry, utilizing ^{35}S-UTP as the labeled nucleotide. Sense TGFα RNA, used to construct standard curves for the RNase protection assay or as a control for hybridization histochemistry experiments, was transcribed with T7 polymerase from the same DNA fragment linearized with Hind III. The antisense cyclophilin probe used in the RNase protection assays was prepared essentially as described (Junier et al., 1991).

Hybridization Histochemistry

The procedure employed is that described by Simmons et al. (1989) with minor modifications (Junier et al., 1991). The brains were fixed by transcardiac perfusion with 4% paraformaldehyde in borate buffer pH 9.5, followed by overnight post-fixation in the same fixative, containing 10% sucrose. After blocking the regions of interest, the tissues were frozen on dry ice and stored at -85°C until sectioning. Twenty μm sections were obtained with a sliding microtome, adhered to polylysine-coated slides and dried under vacuum overnight before hybridization. Composition of the hybridization buffer was 50% formamide, 0.25 M NaCl, 10 mM EDTA, 10 mM Tris pH 8.0, and 2x Denhardt's solution; the sections were overlaid with 70 μl of this solution containing 5 x 10^6 cpm probe/ml, and hybridized for 18-20 h at 55°C. Post-hybridization washes were those recommended (Simmons et al., 1989). Hybridization to the sense and antisense RNA probes was carried out in the same experiment, utilizing adjacent sections. All sections were dipped in NTB-2 emulsion and developed after 3 weeks of exposure.

Immunohistochemistry

The procedure used was exactly as that recently described (Junier et al. 1991), utilizing the same anti-TGFα precursor serum. For double immunohistochemistry, GFAP and TGFα precursor immunoreactivities were simultaneously detected in 75 μm vibratome sections utilizing the technique of Lakos and Basbaum (1986), as reported elsewhere (Junier et al., 1991, 1992a). Diaminobenzidine was employed as the chromogen to develop the GFAP antibody reactions to a brown color and benzidine dihydrochloride to develop the TGFα precursor antibody reaction to a blue color. Astrocytes were identified with the GFAP antiserum R77 (a generous gift from Dr. L. Eng, Stanford University, Palo Alto, CA) at 1:2000 dilution.

Statistics

The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications.

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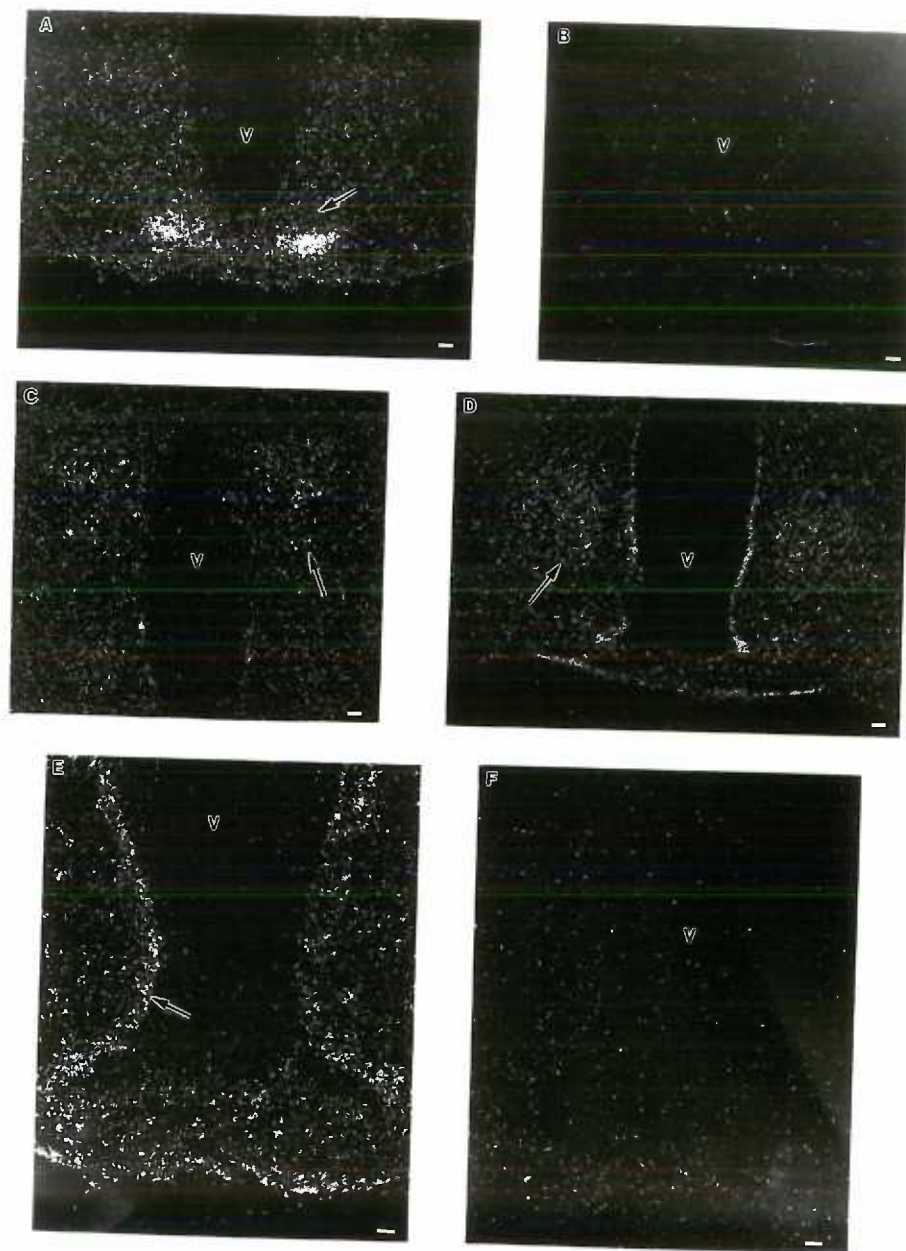
Figure 1. Detection of TGFα mRNA in the hypothalamus of juvenile 28-day-old female rats by hybridization histochemistry. Representative results from a group of 7 animals are depicted. Coronal sections were hybridized with a [35] S-labeled antisense RNA probe complementary to the coding region of TGFα mRNA. Control sections (B and F) were hybridized with a sense RNA strand transcribed from the same DNA template. **A.** Intense labeling in cells of the suprachiasmatic nucleus (arrow). **B.** Lack of specific labeling in an adjacent section hybridized with the sense RNA probe. **C.** Labeled cells in the PVN (arrow). **D.** Labeled cells in the ARC (short arrow), VMH (arrow) and tanycytes of the third ventricle. **E.** Higher magnification of the median eminence region showing TGFα mRNA expression in tanycytes of the ventral portion of the third ventricle (arrow) and glial cells of the median eminence (short arrows). **F.** Lack of specific labeling in a section of the same area hybridized to the sense RNA probe. **A-D**, bar = 200 μm; **E** and **F**, bar = 100 μm. All autoradiographic emulsions were developed after three weeks of exposure to the sections. V = third ventricle.
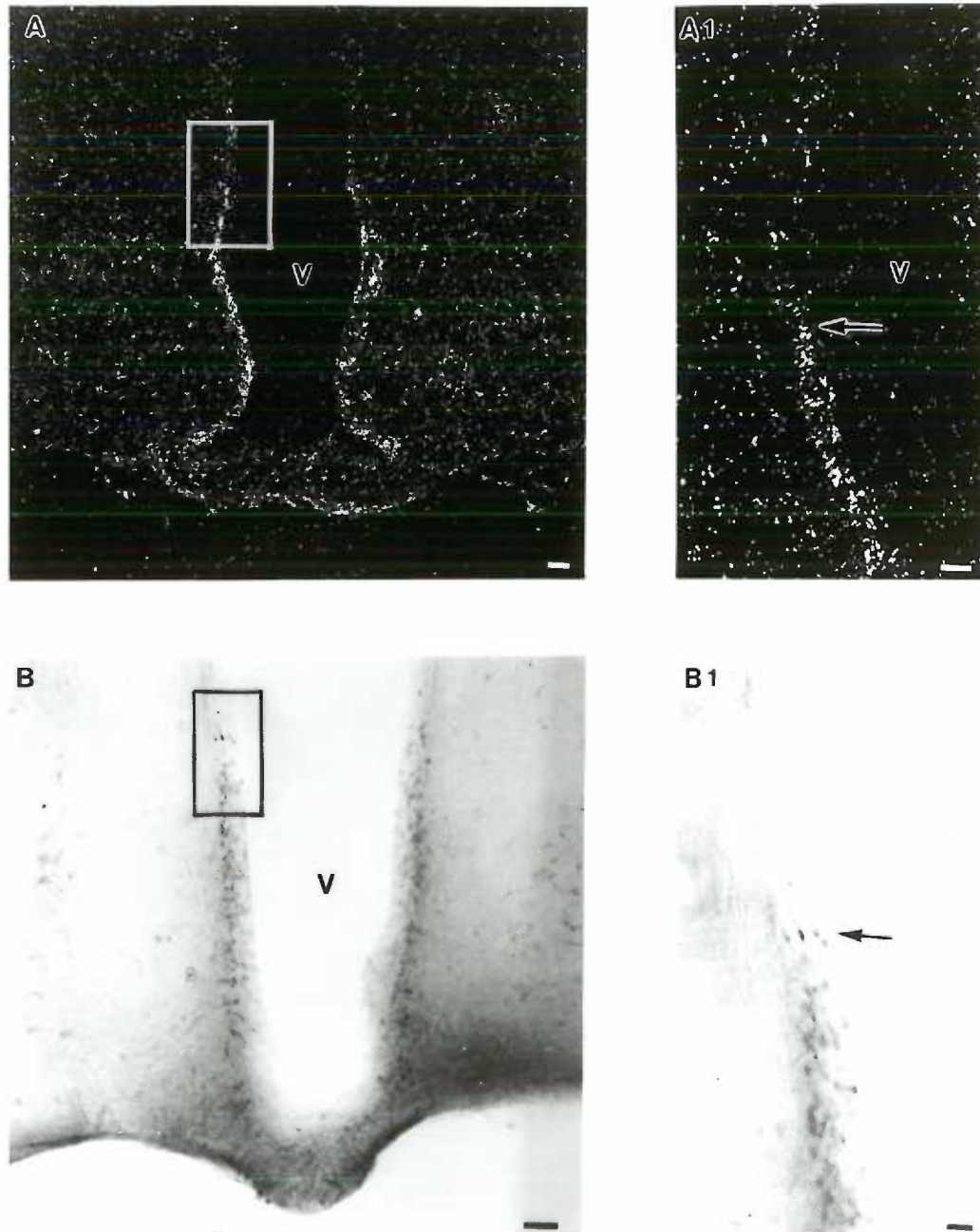
Figure 2 Detection of TGFα mRNA and TGF precursor protein in tanycytes of the third ventricle of juvenile 28-day-old female rats by hybridization histochemistry (panels **A** and **A1**) and immunohistochemistry (Panels **B** and **B1**), respectively. A total of 7 rats was used for in situ hybridization experiments (see Figure 1). An additional 8 animals were used for immunohistochemistry. Notice the abrupt loss of hybridization signal in the upper third of the ventricle (**A1**, arrow) and the corresponding disappearance of immunoreactive material in the same region (**B1**, arrow). The sections depicted are from two different brains. For in situ hybridization the sections (20μm) were obtained with a sliding microtome; the immunohistochemical reaction was performed on 75μm vibratome sections. **A**, bar = 200μm; **B**, bar = 50μm; **A1**, bar = 50μm; **B1**, bar = 20μm. V = third ventricle.
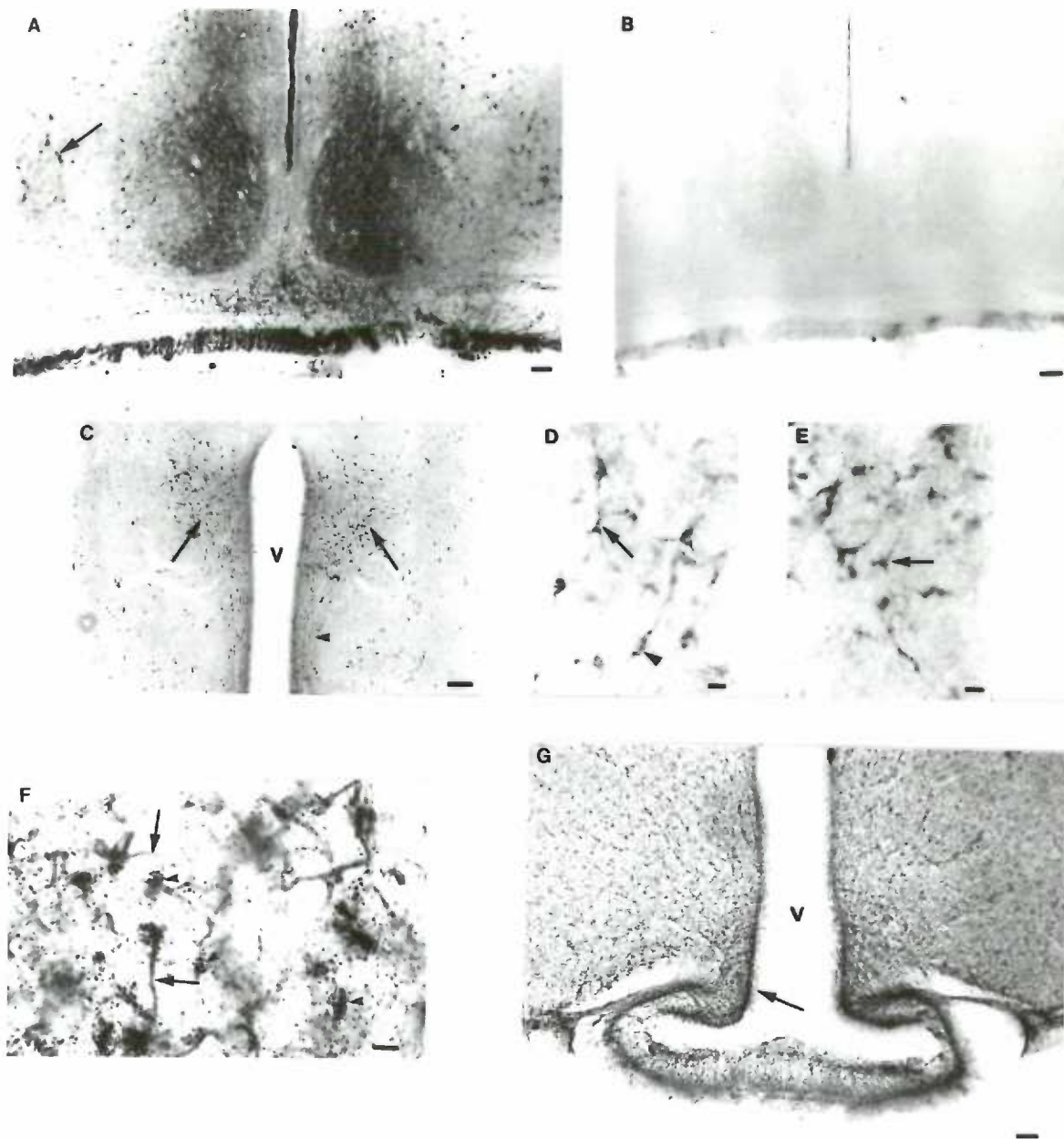
Figure 3. Detection of immunoreactive TGFα precursor-like material in selected areas of the prepubertal female hypothalamus (28-day-old rats). A total of 8 animals was employed. The immunohistochemical reaction was performed on 75 μm vibratome sections utilizing polyclonal antibodies directed against a peptide sequence contained within the intracellular domain of the TGFα precursor protein. **A.** Abundant TGFα precursor immunoreactivity in cells of the SON. Notice that other scattered cells, not contained within the SON are also immunopositive (arrow). **B.** Absence of TGFα precursor immunoreactivity in an adjacent section stained with the same antiserum preabsorbed with the peptide (10 μg/ml) used as the antigenic determinant. **C.** TGFα precursor immunoreactivity in cells of the PVN (arrows) and fibers coursing along the third ventricle in the area of the PVA (arrowhead). **D** and **E,** Higher magnification of areas from the left (D) and right (E) PVN depicted in **C.** Notice that while most of the immunopositive cells are astrocyte-like (arrows), a few appear to be neurons (arrowheads). **F.** Double immunohistochemistry demonstrating the presence of TGFα precursor immunoreactivity (arrowheads, granular appearance of the staining) in hypothalamic astrocytes (arcuate nucleus) identified by their content of GFAP (arrows, smooth aspect of the staining). In the original tissue section TGFα immunoreactivity has a dark blue color and the GFAP reaction is light brown. **G.** TGFα precursor in the median eminence. Tanycytes (arrow) as well as glial-like cells of the median eminence are immunoreactive. As in other regions of the hypothalamus, staining is also observed in scattered cells, not belonging to any particular nuclei. **A, B** and **F,** bar = 50 μm; **C,** bar = 100 μm, **D, E** and **F,** bar = 10 μm. V = third ventricle.
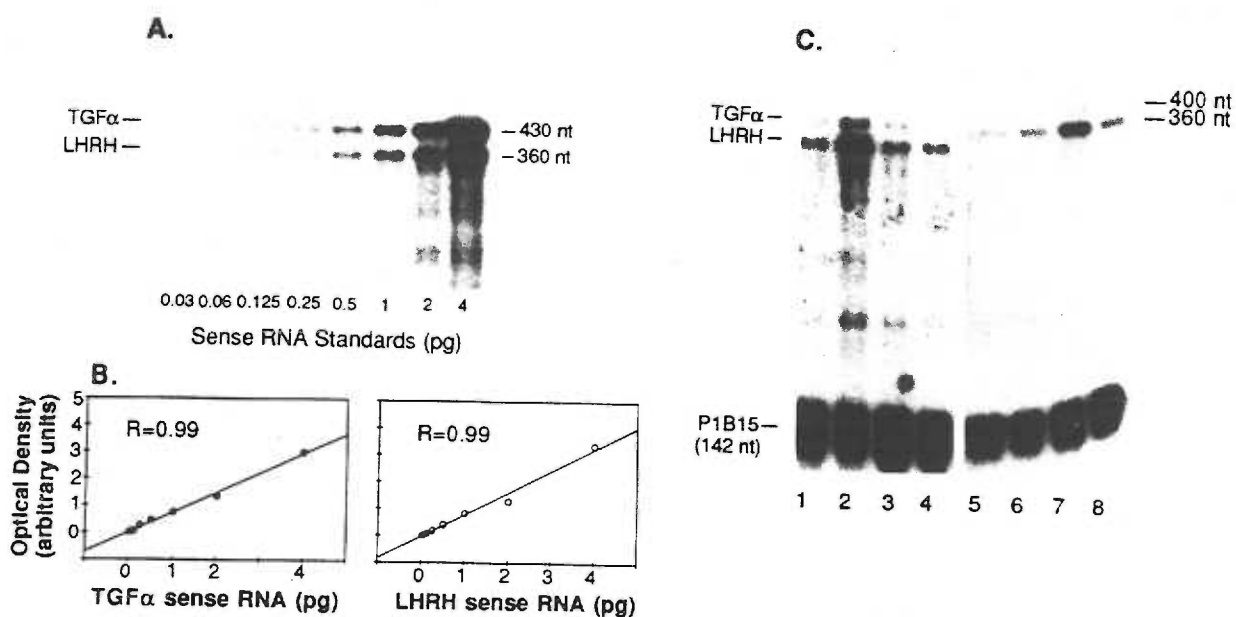
Figure 4. Quantitation of TGFα mRNA by RNase protection assay. **A.** Standard curves of TGFα and LHRH mRNA generated by hybridization of ^{32}P-cRNAs to increasing amounts of in vitro transcribed TGFα and LHRH sense mRNA followed by ribonuclease digestion (see methods). **B.** Linear regression analysis for the TGFα and LHRH mRNA standard curves depicted in **A. C.** Autoradiograms of gels illustrating the changes in TGFα and LHRH mRNA content detected in the POA of female rats by RNase protection assay during the initiation of puberty (lanes 1-4), and following treatment with gonadal steroids (lanes 5-8). Lane 1 - LP, day of the first preovulatory surge of gonadotropins at 1000 h; Lane 2 - LP at 1800 h; Lane 3 - estrus (E, day of the first ovulation); Lane 4 - diestrus 1 (D1); Lane 5 - ovariectomized (OVX) rats; Lane 6 - OVX + E_2; Lane 7 - OVX + E_2 + P; Lane 8 - OVX + P. The RNA loaded in each lane (3 μg) was obtained from a pool of 2 or 3 POAs.
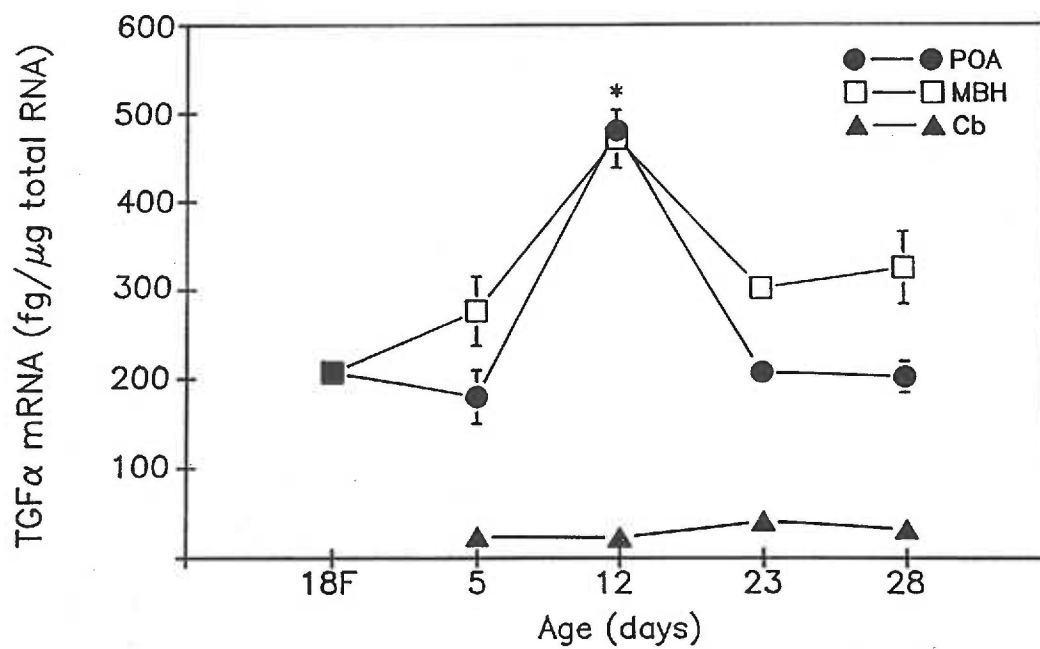
**Figure 5.** Changes in TGFα mRNA content in the POA, MBH and Cb of developing prepubertal female rats as quantitated by RNase protection assay. Each point represents the mean± SEM (fg/μ g total RNA) of three independent observations, each derived from a pool of 3 animals. Some of the standard errors are smaller than the symbols representing the group means. F = fetal. * = p < 0.01 vs all ages before and after day 12.
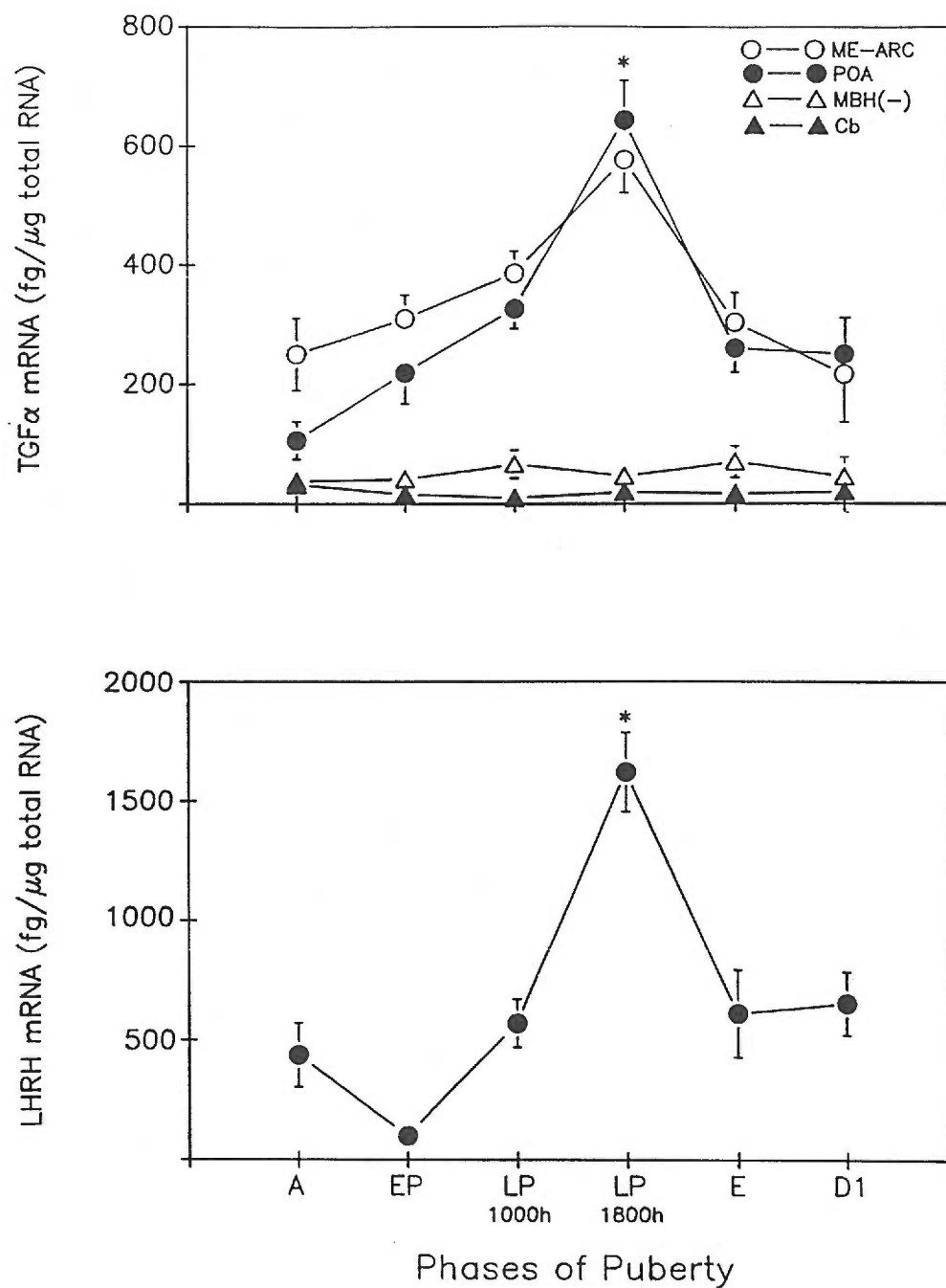
Figure 6. Changes in hypothalamic TGFα and LHRH mRNA levels at the time of female puberty quantitated by RNase protection assay. Upper panel: Changes in TGFα mRNA content detected by RNase protection assay in the ME-ARC, POA, MBH(-) and Cb of female rats during the onset of puberty. Lower panel: Changes in LHRH mRNA content in the POA of the same animals depicted in the upper panel. A = anestrus, juvenile period; EP = early proestrus, initiation of puberty. LP = late (first) proestrus, the day of the first preovulatory discharge of pituitary gonadotropins. E = first estrus. D1 = first diestrus. For further description of phases of puberty, see text. Each point represents the mean ± SEM (fg/μg total RNA) of three independent observations, each derived from a pool of 2 animals. * = p < 0.01 vs all other phases of puberty.
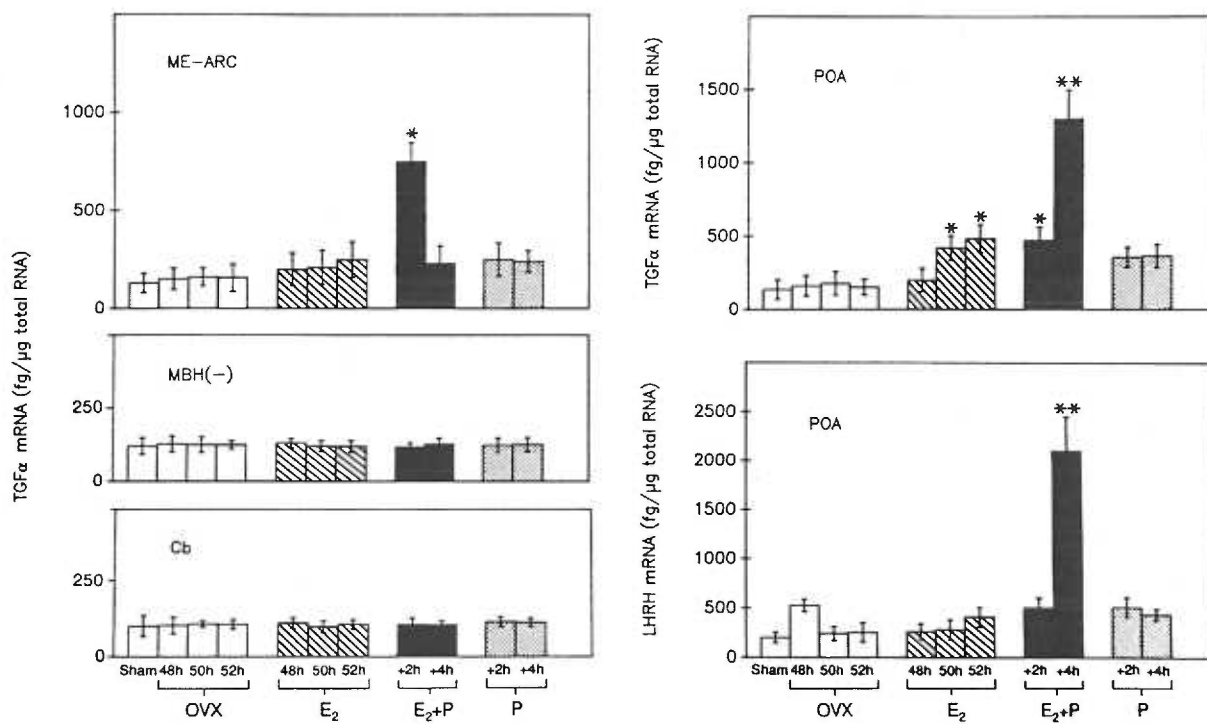
Figure 7. Effect of gonadal steroids on the TGFα mRNA content of POA, ME-ARC, MBH(-) and Cb, and LHRH mRNA content in the POA of juvenile female rats, as quantitated by RNase protection assay. The animals were ovariectomized on postnatal day 22 and provided with a s.c. silastic capsule containing E_2 (400 μg/ml corn oil) on day 27. Two days later some animals received a s.c. injection of progesterone (P, 1 mg/rat at 1200 h) or vehicle and brain tissues were collected 2 or 4 hours after P. Each bar represents the mean ± SEM (fg/μg total RNA) of four independent observations, each derived from a pool of 2 or 3 animals. * = p < 0.05 and ** = p < 0.01 vs ovariectomized controls.
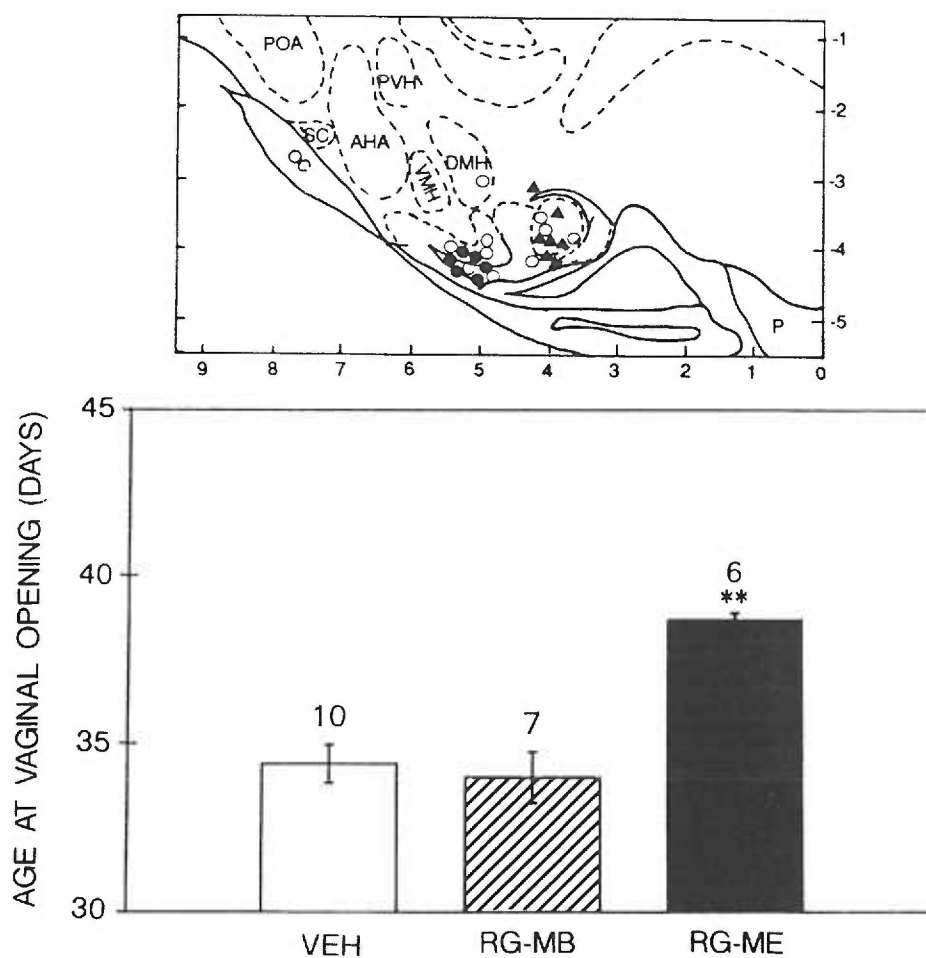
Figure 8. Effect of pharmacological blockade of EGF receptors targeted to the ME of the hypothalamus on the onset of female puberty. The upper panel depicts the anatomical localization of the implants. The blocker (RG50864, RG) or vehicle (VEH) were delivered via stereotaxically implanted stainless steel cannula (see Methods) to either the ME or the region of the mammillary bodies (MB). Closed circles and triangles represent RG50864 implants; open circles are vehicle-only implants. In the lower panel, each bar represents the age at vaginal opening in days (mean ± SEM). The numbers on top of each bar indicate the number of animals per group. ** = $p < 0.01$ vs control groups implanted with the vehicle in the ME or the inhibitor in the MB.

Expression of Epidermal Growth Factor Receptor Changes in the

Hypothalamus During the Onset of Female Puberty

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ABSTRACT

Recent findings have led to the concept that transforming growth factor alpha (TGFα) contributes to the neuroendocrine regulation of female puberty by stimulating the release of luteinizing hormone-releasing hormone (LHRH), the neurohormone controlling sexual development. It was postulated that this effect is mediated by epidermal growth factor receptors (EGFR) and that EGFR may not be located on LHRH neurons, so that TGFα-induced LHRH release would require a cell-to-cell interaction, presumably of glial-neuronal nature. The present study was undertaken to characterize the presence of EGFR in rat hypothalamus and to determine if changes in EGFR gene expression and EGFR protein occur at the time of puberty. RNA blot hybridization demonstrated that the hypothalamus expresses all mRNA species known to encode EGFR. RNase protection assays revealed that alternative splicing of the EGFR primary mRNA transcript occurs in the hypothalamus and produces a predominant transcript encoding the full-length EGFR and a much less abundant, shorter mRNA encoding a truncated, and presumably secreted form of EGFR. EGFR immunoreactive material was found in several hypothalamic regions including the organum vasculosum of the lamina terminalis (OVLT), supraoptic (SON), suprachiasmatic (SCN), and paraventricular (PVN) nuclei, ependymal cells lining the third ventricle, some astrocytes associated with blood vessels, astrocytes of the pial surface and tanycytes and glial cells of the median eminence. Low levels of EGFR mRNA were detected by hybridization histochemistry in cells of the same areas containing EGFR immunoreactivity. Double-immunohistochemistry revealed that even though LHRH neurons are in close proximity to EGFR-positive cells, they do not contain EGFR. In the median eminence, EGFR immunonegative LHRH nerve terminals tightly coexist with EGFR-positive tanycytes and glial cells. Quantitative reverse transcription-

polymerase chain reaction assay (RT-PCR) showed that EGFR mRNA levels in the hypothalamus decrease in the morning of the first proestrus, returning to basal values in the afternoon of the same day, at the time of the first preovulatory surge of gonadotropins. Functional EGFR protein levels detected by the ability of the receptor to autophosphorylate, increased markedly in the morning of proestrus in the region of the hypothalamus that contains the median eminence, despite declining EGFR mRNA expression, and remained elevated at the time of the gonadotropin surge. These results demonstrate the existence of EGF receptors in the prepubertal female rat hypothalamus and provide evidence for their involvement in the neuroendocrine process underlying the first preovulatory surge of gonadotropins. They also support the view that the stimulatory effect of TGFα/EGF on LHRH secretion is not exerted directly on LHRH neurons but rather through intermediate, EGFR-bearing cells.

INTRODUCTION

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are mitogenic polypeptides that share structural similarity and interact with the same cell membrane receptor[3,6,36]. They belong to a family of peptides that include at least seven other members, each of which is encoded by a different gene[3,14,57].

In recent years, reports have appeared showing the presence of EGF and TGFα in the central nervous system[10,54]. Both peptides appear to influence neuronal and glial function via trophic and mitogenic effects[1,29,31,40,60]. While earlier immunohistochemical studies showed a relative abundance and widespread distribution of EGF-like material in brain[10], more recent studies have demonstrated that the prevalence of EGF mRNA in nervous tissue is exceedingly low[27,28,49]. In contrast, TGFα mRNA can be readily detected by conventional RNA blot hybridization[24,49] at levels estimated to be at least one order of magnitude higher than those of EGF mRNA[27,28]. This suggests that, in brain, most of the effects attributed to EGF may be exerted by TGFα instead.

Regardless of their relative abundance, experiments performed in other cellular systems have demonstrated that EGF and TGFα initiate their biological actions by binding to a common cell membrane-spanning receptor molecule, the EGF receptor (EGFR)[3]. Although both polypeptides interact similarly with EGFR, they may lead to both similar and different cellular responses[6]. Without negating the possibility that a related receptor may be involved in mediating some of the actions of TGFα, the greater abundance of TGFα than EGF in the central nervous system suggests that in this tissue EGFR mediates a significant portion of TGFα biological actions.

Autoradiographic and biochemical methods applied to cell cultures have been used to demonstrate that most EGFR in brain are present in astroglial cells[29,60,66].

Exposure of purified astrocytes to EGF resulted in increased levels of ³H-thymidine incorporation and cell proliferation[29,60], suggesting that one of the functions of EGF in brain is to stimulate astroglial proliferation. Consistent with this view, autoradiographic localization of EGFR revealed the presence of EGFR in fetal and neonatal, but not in adult brain[53]. Immunohistochemical studies, on the other hand, showed that EGFR immunoreactive material is only transiently expressed in astrocytes during the third week of postnatal life in the rat, and that the staining becomes mostly neuronal thereafter[13]. In contrast to the autoradiographic studies, the neuronal localization of immunoreactive EGFR-like material was found to persist in adulthood. Similar immunohistochemical studies of the adult human brain also showed a predominant neuronal localization of EGFR immunoreactivity[68], and demonstrated an abundance of EGFR-like material in ependymal cells of choroidal and extra-choroidal locations. The marked increase in EGFR-like material observed in reactive astrocytes after injury[44] provided an explanation for the discrepancy between the *in situ* results showing a neuronal localization of EGFR and those obtained in culture, which demonstrated the presence of the receptors only in astrocytes (if the assumption is made that cultured astrocytes behave more as reactive than normal astrocytes).

Within the central nervous system, EGFR is likely to mediate the effects of EGF/TGFα on neuroblast differentiation[1], neuronal differentiation and survival[40], and glial proliferation[29,60]. Little is known, however, about the potential role that EGFR and its ligands may play in the regulation of differentiated neuronal functions within specific brain regions, and especially those of specific neuronal populations.

The hypothalamus contains a group of highly specialized neurons that secrete luteinizing hormone releasing hormone (LHRH), a decapeptide that controls sexual development and mature reproductive function (for reviews see[38,48]). We recently

provided evidence that the TGFα gene is preferentially expressed in the developing female hypothalamus over that of EGF, and that both EGF and TGFα are able to stimulate LHRH release from nerve terminals located in the median eminence of the hypothalamus, in the absence of the neuronal cell bodies[49]. We also showed that this stimulatory effect was blocked by tyrphostin RG-50864, a low molecular weight compound recently shown to be a highly selective blocker of EGFR tyrosine kinase activity[32,71]. In other experiments we showed that the onset of puberty was delayed by implantation of RG-50864 into the medial basal hypothalamus of peripubertal rats[33]. It was further demonstrated that electrolytic lesions of the anterior hypothalamus that result in female sexual precocity, are followed by activation of TGFα gene expression in reactive astrocytes, and that infusion of tyrphostin RG-50864 prevents the advancing effect of the lesion on puberty[19]. These findings and those showing the presence of EGFR in glial cells led us to postulate that the effect of TGFα on LHRH neuronal secretory activity is exerted via EGFR, and that these receptors are located on glial cells rather than on LHRH neurons[19,49].

In the present study, we have used RNA blot hybridization, RNase protection assays and a quantitative polymerase chain reaction (PCR) technique to demonstrate the presence of EGFR mRNA in the prepubertal female rat hypothalamus and determine if its content changes during the onset of puberty. Since the primary rat EGFR mRNA transcript has been shown to undergo alternative splicing to originate either an mRNA encoding the full-length, membrane-spanning receptor or a truncated form corresponding to the EGFR extracellular domain[50], experiments were conducted to define whether these two mRNA species were also expressed in the hypothalamus. Changes in content of biochemically active receptor molecules were assessed by the ability of immunoprecipitated EGFR to autophosphorylate via a tyrosine kinase-mediated reaction. Hybridization histochemistry utilizing a cRNA

probe and immunohistochemistry with antibodies raised against a peptide sequence located in the C-terminus of the human EGFR[23] were employed to study the cellular distribution of EGFR mRNA and the EGFR protein in the hypothalamus. A partial report of these findings has appeared[34].

MATERIALS AND METHODS

Animals

Immature rats of the Sprague-Dawley strain (Bantin and Kingman, Fremont, CA) were used. They were housed in a room with controlled temperature (23-25°C) and light-dark cycle (10 h darkness/14 h light, lights on from 0500 to 1900 h). The animals had free access to tap water and pelleted rat chow (Purina Laboratory Chow, Ralston-Purina, St. Louis, MO).

Tissue dissection

Immediately after decapitation, brains were removed and the hypothalamus (henceforth, referred to as medial basal hypothalamus [MBH]), the region dorsal to the optic chiasm (henceforth, referred to as preoptic area [POA]), cerebral cortex (Cc), hippocampus (Hc) and cerebellum (Cb) were dissected and frozen on dry ice. Liver samples were also collected. The MBH was dissected into two parts, as described[33]. One fragment, referred to as ME-ARC, included the medial eminence, arcuate nucleus and ventromedial nucleus. The other, referred to as MBH(-), included the lateral part of the medial basal hypothalamus dissected by one sagittal cut half-way between the ME and the hypothalamic sulci and another cut along the hypothalamic sulci; the thickness of the tissue fragment was about 2 mm. The POA was dissected by two cuts converging from the lateral edges of the optic chiasm to a point rostral to the decussation of the optic nerves. The thickness of such fragments was also 2 mm. All tissues were stored at -85°C.

Nucleic acid probes

EGFR mRNA was detected with an antisense RNA probe transcribed from a sequence contained within ER-ts, a 2.3 kb rat EGFR cDNA[50]. The DNA template used for transcription[46] was obtained by subcloning a blunt-ended Sau3A1 fragment (nucleotides, nt 1720-2298 of EGFR mRNA) into the Sma1 site of the riboprobe vector pBluescript SK II. This fragment spans the site of divergency (located at nt 2076) between the mRNAs encoding the full-length EGFR and the truncated EGFR form. Linearization of the template at nt 1915 with Ava II yields a 383 base pair (bp) DNA template of which 160 bp correspond to the sequence common to the external domain of the full-length EGFR mRNA and its truncated form, and 223 bp to the unique 3' end of the truncated EGFR mRNA.

In vitro transcription of this fragment was performed as reported[26], using T7 polymerase and a radiolabeled nucleotide. The nucleotides used were ^{32}P-CTP for RNA blot hybridizations and RNase protection assays, and ^{35}S-UTP for hybridization histochemistry.

RNA isolation

Total RNA was prepared by the acid-phenol method[4]; polyadenylated RNA (A $^{+}$ RNA) was isolated by a microbatch procedure[56]. Use of both procedures in our laboratory has been described previously[26,46].

RNA blot hybridization

Poly A+ RNA was size-fractionated in a 1% agarose formaldehyde gel[35], transferred to Nytran membranes (Schleicher and Schuell, Keene, NH), fixed to the membrane by cross-linking (120,000 μJoules for 35 sec) and hybridized to the EGFR cRNA probe as reported[26,46]. The blots were then exposed to Kodak XAR-5 film at -85°C for 24-48 h.

Solution hybridization/ribonuclease (RNase) protection assay

This procedure was used to identify the mRNAs encoding the full-length EGFR and its truncated form in the hypothalamus. The assay, based on the method of Gilman[12] has recently been reported[15,19]. In brief, a newly synthesized EGFR cRNA probe was isolated by electrophoresis in a 7.1 M urea, 5% polyacrylamide gel, eluted from the gel into a 2 M ammonium acetate solution containing 1% SDS and 25 μg/ml tRNA, and concentrated by ethanol precipitation. RNA samples were dried by vacuum centrifugation, reconstituted in 30 μl of hybridization buffer (80% formamide in 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA) containing 500,000 cpm of probe, and hybridized overnight at 45 °C. Thereafter, the RNA species not protected by hybridization were digested by incubation with a mixture of ribonuclease A and T1 (40 and 2 μg/ml, respectively) and the proteins were removed by digestion with proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The protected RNA fragments were then separated by electrophoresis in a 7.1 M urea, 5% polyacrylamide gel and visualized by exposure of the dried gel to Kodak XAR-5 film.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay

Preparation of polyadenylated EGFR mRNA. A synthetic polyadenylated EGFR sense RNA was used as a standard for quantitating cellular EGFR mRNA amplified by RT-PCR. To synthesize the standard mRNA, a 254 bp cDNA fragment corresponding to a sequence located in the intracellular domain of EGFR (see below) was generated and amplified by RT-PCR using oligodeoxythymidine (oligo-dT) and oligodeoxynucleotide gene-specific primers (see below). The amplified fragment was subcloned into the SmaI site of pSP64(poly A), a plasmid vector (Promega Biotech, Madison, WI) containing a multiple cloning site flanked by the SP6 polymerase promoter and a polyadenylated sequence. Linearization of

this recombinant plasmid with EcoR1, followed by SP6 polymerase-directed transcription yielded a EGFR mRNA containing a 30 nt-polyadenylated tail. The amounts of RNA produced was determined by absorbance at 260 nm and by comparison to known amounts of RNA standards in agarose gels stained with ethidium bromide.

Oligodeoxynucleotides. Oligodeoxynucleotides were synthesized in an Applied Biosystems 391 DNA synthesizer (Foster, CA). An oligonucleotide containing an 18 mer-polydeoxythymidine sequence and the Xhol and EcoRI restriction sites (5'-ACGTCTCGAGAATTCTTTTTTTTTTTTTTTTTTT-3') was used for reverse transcription of polyadenylated cellular mRNA. The EGFR sequence of interest was amplified using a sense oligonucleotide (5'-CACCAAAGCGACGTCTGGAG-3') corresponding to nt 2739-2758 in the intracellular domain of EGFR mRNA and an antisense oligonucleotide (5'-CGCTGTGGGTCTCTGGCCAT-3') complementary to a down-stream sequence spanning nt 2973-2992. Procedural variabilities due to primer efficiency and/or the structural characteristics of the cellular RNA sequence to be amplified were minimized by reverse transcription and PCR amplification of a standard EGFR mRNA identical to the target cellular sequence. "Tube effects" and other individual sources of variability[65] were accounted for by co-amplifying a fragment of cyclophilin mRNA, which is constitutively expressed in brain[5]. The primers used in this case were 5'-TGCAGACGCCGCTGTCTCTTTTCGCCG-3' (corresponding to nt 1-30 in the cyclophilin mRNA sequence) and 5'-GCATTTGCC-ATGGACAAGATGCCAGGA-3' (complementary to nt 324-350).

RT-PCR procedures. Reverse transcription was carried out for 2 h at 37°C in a 20 μl volume. The reaction mixture contained either 100 ng of total cellular RNA or different amounts (1 to 256 fg) of synthetic polyadenylated EGFR cRNA, 1x RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 0.01 M dithiothreitol

(DTT), 0.5 mM of each dNTPs, 20 units of RNasin, 25 pmol of oligo(dT) primer and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). PCR was performed in a 75 μl final reaction volume made up of two parts. Cocktail A consisted of 2 μl RT reaction mixture, 7.5 μl of 10x PCR buffer (500 mM KCl; 100 mM Tris-HCl; 1.0% Triton X-100), 3 μl of 25 mM $MgCl_2$, and 267 μM dNTPs (each) in a 60 μl volume. Cocktail B contained 150 pmoles of each 5'- and 3'-end EGFR gene specific primers, 12.5 pmoles of each 5'- and 3'-end cyclophilin primers and 3 units of Taq polymerase (Promega) in a 15 μl volume. After overlaying Cocktail A with two drops of mineral oil, the mixture was held at 94°C for 4 min to inactivate the reverse transcriptase. Thereafter, Cocktail B was added through the oil to each sample. PCR consisted of 35 cycles of denaturing (95°C, 15 sec), annealing (55°C, 1 min) and extension (72°C, 2 min) followed by a final extension of 7 min at 72°C.

Quantitative analysis. Twenty μl of each PCR reaction sample were electrophoresed in a 3% agarose gel containing ethidium bromide (0.1 μg/ml) using 50 mM Tris borate-EDTA, pH 8.0 as the running buffer. The gel was photographed using 555 Polaroid film (Cambridge, MA) and the negative was developed for densitometric analysis. The amounts of cellular EGFR mRNA were then calculated using the EGFR mRNA standard curve as the reference. Values obtained were normalized according to the cyclophilin mRNA levels detected in each sample. Authenticity of the PCR products was verified by Southern blot analysis utilizing a 5'-end ^{32}P-labeled oligonucleotide complementary to an internal sequence (nt 2819-2842) in the EGFR cDNA fragment amplified.

Hybridization histochemistry

The procedure employed, which is based on that of Simmons et al.[59], has been described elsewhere[7,19]. The brains, fixed by transcardiac perfusion with 4%

paraformaldehyde in borate buffer pH 9.5, were sectioned at 20 μm using a sliding microtome. The sections were mounted on polylysine-coated slides, dried overnight and overlaid with 70 μl of hybridization solution (50% formamide, 0.2 M NaCl, 10 mM Tris pH 8.0, 10 mM EDTA, 2 x Denhardt's solution) containing 1 x 10^7 cpm/ml of labeled EGFR cRNA probe. Hybridization was for 18-20 h at 55 °C; post-hybridization washes were as described[7] with the last wash at 65 °C for 30 min. Following dehydration in graded alcohols, the slides were dipped in Kodak NTB-2 emulsion and stored at 4 °C. After 4 weeks they were developed, counterstained with thionin and analyzed under bright and darkfield illumination. Controls consisted of sections hybridized with an EGFR sense RNA probe synthesized from the same DNA template employed to prepare the antisense RNA probe, but linearized with Hind III and transcribed with T3 polymerase.

Immunohistochemistry

Immunoreactive EGFR were visualized in 75 μm vibratome sections obtained after fixation of the brain via transcardiac perfusion of Zamboni's fixative. The immunohistochemical procedure used was an ABC peroxidase technique described by Nilaver and Kozlowski[45] with minor modifications[7,19]. The antiserum used (RK-2, 1:1000 dilution) is directed against a peptide sequence (residues 984-996) contained within the C-terminus of the human EGFR[23]. This antibody has been used to immunoprecipitate the human EGFR[23] and to detect immunoreactive EGFR in rat tissues[62]. In the present study, controls included either substitution of the primary antibody with preimmune serum or removal of the EGFR antibodies from the primary antiserum via binding to semi-purified human EGFR. The latter procedure was carried out as follows: solubilized membranes of A431 cells were incubated with wheat germ agglutinin-sepharose for 2 h at 4 °C. Following elution of bound glycoprotein with 0.5 M acetylglucosamine, the semi-purified EGFR was incubated

with an excess of biotinylated EGF (2 μg, Boehringer Mannheim, Indianapolis, IN) for 1 h at 4 °C in a 160 μl volume. Two μl of RK-2 antiserum were added, and the incubation was continued for another hour. This was followed by addition of 50 μl of avidin-agarose (Sigma Chemical Co., St. Louis, MO) and incubation of the suspension for 45 min at 4 °C, with end-over-end tipping. The tube was then microfuged to pellet the avidin-agarose-biotinylated EGF-EGFR-RK-2 complex, and the supernatant was incubated with another 50 μl of avidin agarose. After centrifugation the supernatant was diluted with the buffer for immunohistochemistry (0.05 M Tris, pH 7.6 containing 0.9% NaCl, 0.02% BSA, and 0.1% Triton X-100) to a final dilution of 1:1000. Simultaneous detection of LHRH and EGFR immunoreactivity was achieved using a technique that employs diaminobenzidine (DAB) as the chromogen to develop one of the antigen-antibody reactions and benzidine dihydrochloride (BDHC) to develop the other[25]. LHRH was identified with monoclonal antibody HU4H3 which has conformational immunospecificity and requires the entire decapeptide molecule for recognition[64]. For staining of cell bodies, the antibody was used at a 1:2,000 dilution and the reaction was developed to a brown color with DAB. As with single-staining, the EGFR antiserum RK-2 was used at 1:1,000, but the reaction was developed to a blue color with BDHC. For staining of fibers in the median eminence, the order and developing of the reactions were reversed so that the EGFR reaction was developed first to a brown color and the LHRH reaction next, to a blue color.

EGFR protein kinase assay

Each sample, in triplicate, consisted of a pool of three ME-ARC fragments which were frozen on dry ice and stored at -85° until the day of homogenization. All the following steps were performed on ice or at 4°C unless otherwise noted. Each sample was homogenized in 300 μl of TG buffer (1% Triton X-100, 10% glycerol, 10

μg/ml aprotinin, in phosphate buffered saline, pH 7.4) in a glass/glass homogenizer. After transfer of the homogenate to a microcentrifuge tube, the homogenizing tube and pestle were rinsed with an additional 300 μl of TG buffer. Homogenates were kept on ice for an additional 1 h to maximize solubilization. After microcentrifugation for 5 min, to remove nuclei or other insoluble material, the supernatants were transferred to clean tubes and a 20 μl aliquot was taken for protein analysis using the Bio-Rad method with bovine serum albumin standards. The supernatants were maintained at -85°C until the day of assay.

Sample aliquots containing 300 μg of protein were adjusted to 0.5 ml with TG buffer. Immunoprecipitation and kinase activation was initiated by adding 3 μl EGF receptor antiserum SD4/17 (the generous gift of Dr. Stuart Decker, Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI), and tubes were tipped for 1 1/2 h. Protein A-Sepharose (30 μl of a 1:1 slurry, in water) was added and tubes tipped for an additional 1 h. Immunoprecipitates were pelleted in the microcentrifuge and washed once with 750 μl TG buffer and once with 750 μl 10 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 1 mM orthovanadate. The tubes were removed from ice and the receptor autophosphorylation was initiated by the addition of 20 μl of the HEPES buffer, containing 10 μM sodium ATP. After mixing, the reaction was permitted to proceed for 7 min at room temperature and stopped by the addition of 10 μl of a 3x concentrated sample buffer (final concentration of 0.0625 M Tris, pH 6.8, 3% sodium dodecyl sulfate, 5% glycerol, 5% ß-mercaptoethanol). Samples were heated at 100°C for 10 min and cleared by centrifugation prior to SDS-PAGE on a 7% minigel (Idea Scientific, Minneapolis, MN). Separated proteins were electrophoretically transferred to nitrocellulose, blocked with 2% BSA, 0.2% Tween 20, in Tris buffered saline (TBS) for 1 h at RT, then placed in a plastic pouch containing a monoclonal antibody to phosphotyrosine residues (Mab 4G10, the

generous gift of Dr. David Kaplan, Frederick Cancer Research and Development Center, Frederick, MD) diluted 1:5 with 0.2% Tween 20/TBS. Following an overnight (with tipping) exposure to the antibody, the membrane was washed extensively in 0.2% Tween 20/TBS, then incubated with a goat anti-mouse: horseradish peroxidase-linked second antibody, diluted 1:20,000 with TBS/Tween, for 1 h at RT. Following additional extensive washes, the membrane was developed using the Enhanced Chemiluminescence system from Amersham (Arlington Heights, IL) and exposed to X-ray film.

RESULTS

Detection of EGFR mRNA by RNA blot hybridization

Using the rat EGFR cRNA probe described above, several EGFR mRNA species were detected in normal human keratinocytes and the human epidermoid carcinoma cell line A431 (Fig. 1, panel A). The predominant species found in normal keratinocytes had a size of ~10 kb, with a less abundant species of ~6.5 kb and a minor species of ~7.0 kb. In contrast, and in agreement with previous observations [63,70], the predominant RNA species found in A431 cells had a size of 2.9 kb; a 10 and a 6.5 kb species were also detected, albeit at lower levels. The abundant 2.9 kb mRNA is aberrant; it results from the amplification and rearrangement of the EGFR gene in A431 cells[30,63,70] and encodes a truncated form of EGFR that corresponds to the extracellular domain of the receptor.

Isolation of DNA sequences encoding rat EGFR mRNAs from a normal liver library showed the existence of ~9.6, 6.5, 5.0 and 2.7 Kb mRNA species[50]. The latter derives from alternative splicing of the EGFR primary mRNA transcript, and encodes a truncated and secreted form of EGFR that spans most of the extracellular domain of the receptor. In the present experiments, the 2.7 kb mRNA was found to

be predominantly expressed over the other larger species in prepubertal rat liver (Fig. 1, panel A). In addition, the abundance of the ~9.0 and ~6.5 kb mRNAs was much lower than that of both the 2.7 and 5.0 kb mRNA species. Figure 1, panels B and C shows that the most prominent mRNA species expressed in the prepubertal rat brain is the 5.0 kb mRNA. Longer exposure of film to the blots allowed detection of the ~9.0 and 6.5 kb EGFR mRNAs (not shown). Detection of the truncated 2.7 kb EGFR mRNA in the brain regions examined (POA, MBH, Cc and Cb) was equivocal (Fig. 1, panels B and C).

Detection of EGFR mRNA and its truncated form by RNase protection assay

As predicted by the region of EGFR mRNA selected for complementarity to the radiolabeled EGFR cRNA probe, hybridization of rat liver total RNA to the probe resulted in protection of two fragments; the most abundant, 383 nt in length, corresponds to the mRNA encoding the truncated EGFR, the other, 160 nt in length, represents the sequence common to the full-length EGFR and its truncated form (Fig. 2, panel A). POA-MBH collected on the day preceding the first ovulation (late proestrus, LP) or 12 h after ovulation (first estrus, E) was found to predominantly express the full-length EGFR mRNA (Fig. 2, panel B). In both developmental phases, the truncated mRNA species became detectable only upon a much longer film exposure (Fig. 2, panel C).

Localization of EGFR immunoreactivity by immunohistochemistry

The polyclonal antibody RK-2, directed against a peptide near the carboxy terminus of the human EGFR, recognized EGFR-like material in several regions of the prepubertal female hypothalamus. In the POA, the most prominent staining was observed in the regions of the organum vasculosum of the lamina terminalis (OVLT), and the suprachiasmatic (SCN) and supraoptic nuclei (SON) (Fig. 3, panels A, B and C, respectively). In all three regions, the staining appeared mostly scattered (single

and double arrows, panel B) or associated with cell processes. The latter localization was more prominent in the OVLT and SON (arrowheads, panels A and C). Small, neuron-like cells located in the periventricular area were also found to be immunoreactive (arrowheads, panel B). As reported by others using a different antiserum[13], ependymal cells of the third ventricle and glial cells of the pial surface were strongly immunopositive (double arrowheads, panels B and C respectively).

The most prominent staining in the anterior portion of the MBH was located in the paraventricular nucleus (PVN), and periventricular area (PVA), (Fig. 4, panels A and B, respectively). Again, the immunoreactivity appeared associated with a few small neuron-like cells (single arrows) and cell processes of either astrocytic or neuronal nature (arrowheads). Some astrocytes associated with blood vessels displayed intense immunoreactivity (Fig. 4, panel C). In general, however, astrocytic staining, though discernible, was weak (see for instance Fig. 4, panel B, arrow heads).

In the MBH proper, immunoreactivity was found to be mainly associated with tanycytes of the third ventricle and median eminence (Fig. 5). The staining was observed along the tanycyte processes (panel A, arrow, and panel C) and in both glial cells and tanycytes of the median eminence (panel D). Removal of EGFR antibodies from the primary antiserum prior to immunostaining by binding to purified human EGFR, resulted in complete elimination of immunoreactive material in both the POA and MBH. Figure 5 (panel B) illustrates this in a section adjacent to that depicted in panel A.

Localization of EGFR mRNA by hybridization histochemistry

With the exception of pial astrocytes, abundance of EGFR mRNA per cell was low throughout the hypothalamus. EGFR mRNA was discretely present in cells located in the same areas where the EGFR protein was detected. Examples of this localization are shown in Fig. 6. Panels A-C depict positive cells in the PVN, SCN

and SON, respectively (arrows). Panels D-F show EGFR mRNA in pial astrocytes (D), ependymal cells of the third ventricle (E), tanycytes and glial cells of the median eminence (F).

Colocalization of EGFR and LHRH

Double-immunohistochemistry demonstrated the absence of detectable EGFR immunoreactivity in LHRH neurons. Figure 7, panel A, depicts an area of the POA immediately anterior to the rostral recess of the third ventricle, in which EGFR-negative LHRH neurons (arrows) co-exist with EGFR-positive neuronal-like cells (arrowheads). The double arrow points to an EGFR-positive cell overlying a LHRH neuron. Notice that the LHRH immunoreactivity developed with DAB has a smooth, non-granular aspect (brown color in the original section), whereas the EGFR immunoreaction developed with BDHC has a crystal-like appearance (blue color in the original section). Panel B shows an LHRH neuron (arrow) next to an EGFR-positive astrocyte-like cell (arrowhead). Panel C shows a monopolar LHRH neuron (arrow) located close to the midline of the hypothalamus above the region of the OVLT, in close proximity of an EGFR-positive cell (arrowhead) and EGFR-positive cell processes (double arrowheads). Figure 8 shows that EGFR immunoreactivity is localized in tanycytes, but not in LHRH nerve terminals of the median eminence. As indicated before, in this case the immunohistochemical reactions were reversed so that EGFR immunoreactivity was developed with DAB, followed by development of the LHRH reaction with BDHC. Panel A depicts the preferential lateral localization of LHRH terminals in the ME (arrows, blue color in the original section) and the presence of a smooth, lighter EGFR immunoreaction (brown in the original section) in tanycytes (double arrowheads) and glial cells of the ME (arrowheads). Panel B shows at higher magnification the absence of EGFR immunoreactivity in LHRH terminals traveling along the wall of third ventricle (arrows) perpendicular

to the EGFR-positive tanycytes (arrowheads).

Changes in hypothalamic EGFR mRNA levels at the time of puberty

The results from a previous study[33] suggested that TGFα synthesized in the hypothalamus contributes to regulating female sexual development via activation of EGFR. Hence, it was important to determine if expression of the EGFR gene changes during the onset of puberty. To minimize the amount of tissue required for quantitation, EGFR mRNA levels were measured using the highly sensitive RT-PCR technique. Figure 9A demonstrates the linearity of the reaction following RT-PCR amplification of different amounts of *in vitro* synthesized EGFR mRNA (sense RNA). The assay detects as low as 2 fg EGFR mRNA (Fig. 9A). Examination of the changes in hypothalamic levels of EGFR mRNA at the time of puberty revealed that EGFR mRNA content in the ME-ARC region of the hypothalamus increases between the juvenile phase of development (anestrus) and the initiation of puberty (early proestrus, EP) (Fig. 9B and C), to decrease dramatically in the morning of the first (late) proestrus day, i.e., on the day preceding the first ovulation. EGFR mRNA levels returned to basal values by the afternoon of first proestrus, at the time of the first preovulatory surge of gonadotropins, remaining elevated after the first ovulation, i.e., during the first estrus and diestrus phases of puberty (Fig. 9B and C). In contrast, no such changes were found in the cerebellum (Fig 9B and C).

Changes in biologically active EGFR protein at the time of puberty

In view of the marked changes in EGFR mRNA levels detected on the day of first proestrus in the ME-ARC region, the ability of EGFR to autophosphorylate when activated by a divalent antibody[61] was analyzed on this day and compared with the EP phase of puberty. As shown in Fig. 10, there was a 2-fold increase in autophosphorylated receptor content in the ME-ARC between EP and the morning of LP, with levels remaining elevated in the afternoon of LP. While the

phosphorylated receptor protein had a molecular weight of ~170 kDa similar to, but slightly smaller than EGFR detected in the human carcinoma cell line A431, no such protein was detected in 3T3 NR 6 cells, which derive from the murine Swiss albino 3T3 cell line[52] and have been shown to lack both EGFR mRNA and a functional EGFR protein[55] (data not shown).

DISCUSSION

TGFα is synthesized in the developing rat hypothalamus where it stimulates the release of LHRH, the neurohormone that controls sexual development[49]. That TGFα mRNA content in the hypothalamus, as well as other brain areas, is much greater than that of EGF mRNA[27,28], suggests that TGFα is the prevalent physiological ligand for brain EGF-like receptors. The finding that both TGFα and EGF stimulate LHRH release and that this effect was prevented by pharmacological blockade of the EGFR protein tyrosine kinase activity[49] supports this idea, and underscores the need to provide direct evidence for the presence of EGFR in the immature hypothalamus.

The present study addresses this issue. RNA blot hybridization of hypothalamic A[+] RNA to a rat EGFR cRNA probe demonstrated that the predominant EGFR mRNA expressed in the hypothalamus (as well as in the other brain regions examined) is a 5 kb species, similar in size to that previously shown to encode EGFR in rat liver[50]. The heavier transcripts detected in human tissues[30,63,70] (Fig. 1), were also detected in the rat tissues studied, but at much less abundant levels. Since they may represent EGFR mRNA variants with longer 3' untranslated sequences[63], their low abundance in the rat suggests the existence of species-specific differences in transcriptional processing.

It has been known for several years that the A431 epidermoid carcinoma cell

line over-expresses an aberrant mRNA that encodes the extracellular domain of EGFR[30,63,70]. This truncated receptor form is secreted in substantial amounts[37,67]. Appearance of its encoding mRNA is due to a chromosomal rearrangement that results in fusion of the 5' end of the EGFR gene to an unrelated DNA sequence[39]. While normal human cells do not express this short EGFR mRNA form, a recent report showed that in the rat the normal primary transcript of EGFR mRNA undergoes alternative splicing, to originate either a full-length EGFR mRNA, or a 2.7 kb species encoding the extracellular domain of the receptor[50]. This truncated form was found to be secreted by a rat hepatic cell line in culture, suggesting that it may have a physiological function, possibly related to modulation of ligand availability to the EGFR[50].

A potential role for a secreted EGFR form in the central nervous system is suggested by the recent finding of a soluble EGFR-related peptide in rat brain, which inhibits astrocyte proliferation[43]. The fall in inhibitor levels after injury, and the ability of antibodies to EGFR to induce the appearance of reactive astrocytes in intact brain raised the possibility that this EGFR related molecule is a physiological modulator of glial proliferation and ligand-induced activation of EGFR. Our results demonstrate that the short EGFR mRNA encoding the truncated form of EGFR is indeed expressed in brain, albeit at exceedingly low levels. Quantitation of both mRNAs by RNase protection assay in lesioned hypothalami revealed no changes in short mRNA levels in the face of a significant increase in full-length EGFR mRNA content[18]. Since large changes in EGFR protein content are not necessarily accompanied by corresponding changes in steady state levels of its mRNA[63], our findings would still be consistent with the possibility that the EGFR-related astrocyte mitogen inhibitor described by Nieto-Sampedro[43] is indeed an alternative product of the EGFR gene, encoded by the short EGFR mRNA form. However, other data

argue against this possibility. The soluble extracellular domain of the human EGFR produced with recombinant technology and expressed in CHO cells or in the baculovirus system has a 200- to 300-fold lower affinity for EGF[17] as compared to membrane bound EGFR. The same is true for the extracellular domain secreted by A431 cells (DH Hurwitz, personal communication). Further, the extracellular protein does not interfere with EGF-induced aggregation and activation of membrane bound EGFR (I. Lax, personal communication). Hence, the secreted form of the receptor would have to be expressed at very high levels in order to influence physiology. Development of antibodies specific to the extracellular domain of rat EGFR should help in resolving this issue.

Immunoreactive EGFR-like material was detected at low levels in hypothalamic astrocytes, and more abundantly in nerve terminals innervating the OVLT and in a few cells and cellular processes of the SCN, SON and PVN nuclei. There was also weak, scattered staining of these nuclei, suggesting that as in the OVLT, EGFR may be contained in nerve terminals of projecting neurons. That cells in the SCN and PVN synthesize EGF-related peptides able to interact with EGFR is evidenced by the presence of immunoreactive TGFα and TGFα mRNA in these nuclei [33].

EGFR staining was also observed in small neurons of the PVA, scattered along the third ventricle, and more prominently in some astrocytes associated with blood vessels and in pial astrocytes. Similar findings were previously reported using a different antiserum[13]. Perhaps more remarkable was the detection of EGFR-like material in ependymal cells and tanycytes of the median eminence and third ventricle. The presence of EGFR immunoreactivity in ependymal cells has been described earlier by others[68]; our results show that the immunoreactivity in tanycytes was not limited to the cell body but included their entire processes which project

towards the ventral surface of the hypothalamus and capillaries of the outer surface of the median eminence. Tanycytes are specialized ependymal cells which appear to be involved in bidirectional transport of substances between the cerebro-spinal fluid and the portal vasculature of the median eminence[20]. While a close anatomical relationship exists between tanycytes and neuronal processes projecting to the median eminence[2], a particularly tight association has been found between LHRH nerve terminals and ventricular ependyma/tanycytes[21]. This has led to the suggestion that release of LHRH from the median eminence may be regulated by the extent of glial/tanycyte ensheathing of LHRH terminals[21]. The presence of EGFR immunoreactivity in tanycytes closely associated with LHRH nerve terminals coupled to our previous finding that TGFα and EGF are able to stimulate LHRH release from the median eminence in the absence of the LHRH perikarya[49], suggest that a mechanism by which tanycytes may regulate the neurosecretory activity of LHRH nerve terminals is by mediating the stimulatory effect of TGFα/EGF on LHRH release.

Hybridization histochemistry, utilizing the same EGFR cRNA employed in RNA blot hybridization and RNase protection assays, revealed that the relative abundance of EGFR mRNA per cell is low throughout the hypothalamus. Although EGFR mRNA was detected in tanycytes, and a few cells scattered throughout the hypothalamus and located within the PVN, PVA and SON, the levels were not commensurate with the intensity of the immunostaining shown by EGFR-positive cells in these areas, especially tanycytes which were all strongly immunopositive. While this discrepancy may be attributed to the recognition of an EGFR-related molecule by the RK-2 antiserum, we have found a similar pattern of staining in the median eminence-third ventricle of mice using two different polyclonal antibodies (Goldsmith and Ojeda, unpublished). It is possible, however, that all of these

antisera recognize the EGFR proper and some of the other members of the EGFR family, which share extensive sequence homology with EGFR in their intracellular domains[22,51]. Indeed, the RK-2 antiserum has been shown to immunoprecipitate the EGFR from human and avian cells, as well as the v-erbB proteins of the avian erythroblastosis virus[23].

Another explanation for the apparent discrepancy between the *in situ* hybridization and immunohistochemical results is that an increase in abundance of the EGFR protein is not always accompanied by a corresponding increase in steady state levels of EGFR mRNA. This is the situation in A431 cells which have EGFR levels 50 times higher than any other cell type, yet express comparable levels of EGFR mRNA[63]. A third explanation is that the conditions used for *in situ* hybridization were inadequate. We believe this to be unlikely, because a) EGFR mRNA was abundant in pial astrocytes, and b) in the same hybridizations the hypothalamus of newborn rats were found to contain numerous cells displaying strong EGFR mRNA hybridization signal (Hill DF, Costa ME and Ojeda SR, unpublished data). Interestingly, the pattern of distribution of these cells suggested that they are migrating (or associated with migrating cells) from the neuroepithelium lining the third ventricle.

We have previously suggested that the stimulatory effect of TGFα and EGF on LHRH release is not exerted directly, requiring instead an intermediate, presumably glial, cell[49]. The present findings, which demonstrate that EGFR are not located on LHRH neurons but rather on cells associated with them, lend credence to this view. Co-localization of LHRH and EGFR was not observed even in the OVLT, which is extensively innervated by septal-preoptic area LHRH neurons[58] and receives an abundant supply of EGFR immunopositive cell processes (this study). Although the possibility that a sub-population of LHRH neurons (or LHRH neurons

during early development) expresses EGFR cannot be ruled out, it appears clear that the majority of LHRH neurons are EGFR-negative.

While some EGFR-positive cells appear neuronal others, showing a less intense staining, were glial. The anatomical relationship between EGFR-positive astrocytes and LHRH neurons is more clearly manifested after lesions of the hypothalamus that cause sexual precocity. In this case, reactive astrocytes become strongly positive for EGFR and they appear to be in intimate contact with both LHRH neuronal perikarya and LHRH nerve terminals[18]. As in intact rats, no EGFR-positive LHRH neurons were detected in lesioned animals. Since it appears that TGFα is produced in both neurons[24,69] and astrocytes[9,19], it has been hypothesized that some of the actions of TGFα on EGFR bearing astrocytes are of a paracrine/autocrine nature[18,19,49]. This notion is supported by the presence of TGFα mRNA and EGFR mRNA in reactive astrocytes[18,19] and in tanycytes/glial cells of the median eminence, the main terminal field of LHRH neurons ([33] and this study).

We have recently shown [33] that hypothalamic TGFα mRNA levels increased on postnatal day 12, a time at which LHRH and pituitary gonadotropin secretion peak[16,47]. More importantly, hypothalamic TGFα mRNA levels were also increased during the initiation of puberty, suggesting that TGFα is a physiological component of the developmental process by which the hypothalamus controls the advent of normal female sexual maturation. Since TGFα exerts its biological actions via activation of EGFR, it was important to determine if the time of puberty is associated with changes in hypothalamic EGFR expression, or in the content of biologically active receptors. The present study demonstrates that the levels of both EGFR mRNA and EGFR protein change during the initiation of puberty. Surprisingly, EGFR mRNA levels markedly declined between the earliest phase of

puberty (EP) and the morning of the first proestrus, a time during which receptor levels were increased. Based on available evidence showing that TGFα /EGF either do not affect EGFR mRNA levels[41] or increase them[8], it would be difficult to explain the proestrus decrease in EGFR mRNA levels as due to an increased activity of the receptor. Equally unlikely is the possibility that the decrease is due to the elevated estrogen levels that characterize the proestrus phase of puberty. Estradiol has been shown to increase, rather than decrease, EGFR mRNA levels[11,42]. Thus, it would rather appear that EGFR gene expression in the morning of first proestrus is transmodulated by factors other than TGFα or EGF. While the mechanisms underlying this phenomenon remain to be identified, the restoration of EGFR mRNA levels that occur at the time of the first preovulatory surge of gonadotropins, and the elevated levels of biologically active receptors observed throughout the first proestrus day suggest that, indeed, activation of EGFR synthesis and EGFR-mediated transmembrane signalling occurs at the time of puberty.

Taken altogether, the present results demonstrate that EGFR are expressed in glial cells and discrete neuronal subsets of the developing rat hypothalamus, but not in LHRH neurons and that activation of hypothalamic EGFR receptor synthesis and EGFR-mediated transmembrane signalling occurs on the day of the first pre-ovulatory surge of gonadotropins. The close relationship between LHRH neuronal terminals and EGFR-positive tanycyte/glial cells suggest that the previously reported stimulatory effect of TGFα /EGF on LHRH release does, indeed, involve the participation of EGFR-bearing glial cells.

ACKNOWLEDGEMENTS

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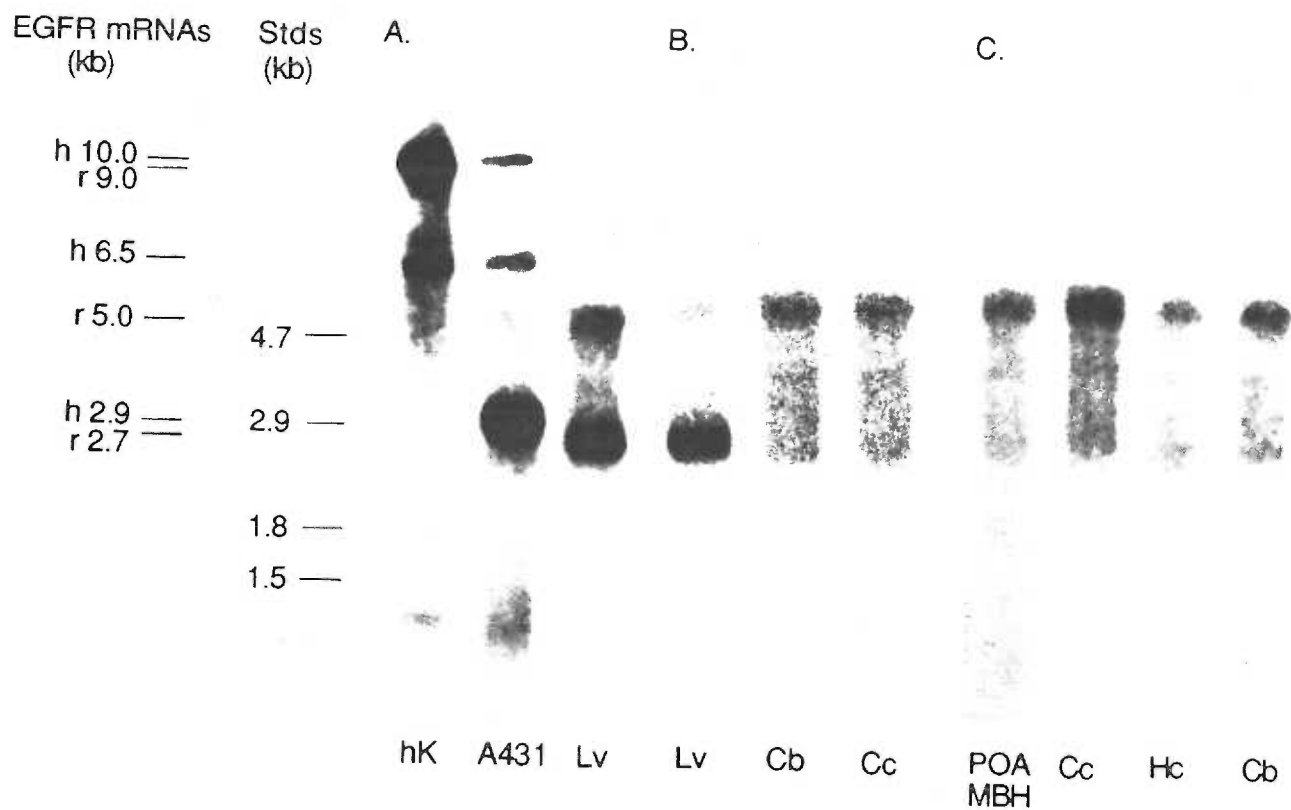
Figure 1. Detection of EGFR mRNAs in the hypothalamus and other tissues of immature female rats and in human cell lines by RNA blot hybridization of A$^+$ RNA to a ^{32}P-labeled antisense rat EGFR probe. Panel A depicts EGFR mRNAs in human keratinocytes (hK), the human epidermoid carcinoma cell line A431 and rat liver (Lv). Panel B demonstrates that the main EGFR mRNA species in brain is similar in size to that of rat liver (Cb = cerebellum; Cc = cerebral cortex). Panel C shows that a similar mRNA species is present in the POA-MBH, Cc, Cb and hippocampus (Hc). All lanes have 5 μg A$^+$ RNA.
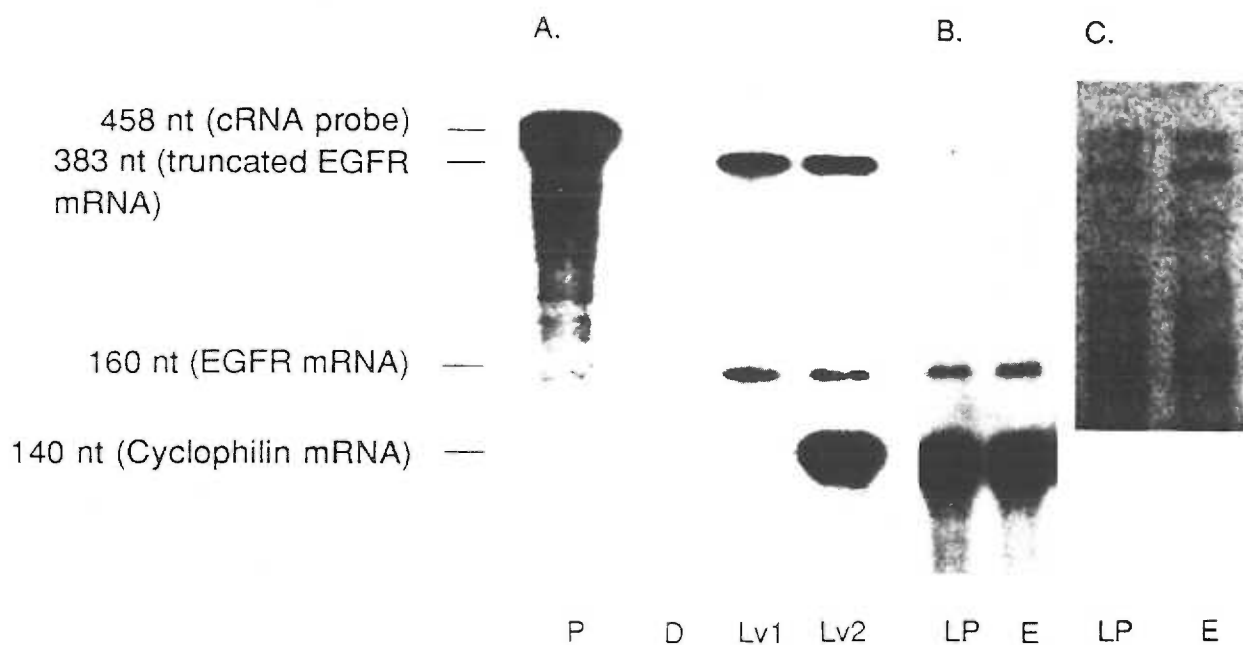
Figure 2 Detection of alternatively spliced EGFR mRNA transcripts in the immature rat hypothalamus and liver by RNase protection assay, followed by electrophoretic separation of the protected RNA species in a urea-polyacrylamide gel. Panel A shows the intact [32] P-labeled EGFR cRNA probe not exposed to ribonuclease digestion (P), the digested probe (D), and the two fragments (383 and 160 nt in length) protected by rat liver RNA (Lv). Except in one case (Lv1), the tissue RNA was simultaneously hybridized to a cyclophilin cRNA, used to assess procedural losses [19] . Panel B illustrates the detection of a 160 nt cRNA fragment protected by hypothalamic RNA that corresponds to the extracellular domain of the intact EGFR mRNA (LP = tissues collected from an animal on the day of the first preovulatory surge of gonadotropins, late proestrus; E = tissues collected on the day of first ovulation, first estrus). Panel C shows a 383 nt protected band corresponding to the truncated EGFR mRNA, detected after a seven-day exposure to X-ray film of the blot depicted on panel B. The upper band is an artifact of the assay. Lv lanes = $2\,\mu$g total RNA; hypothalamus lanes = $15\,\mu$g total RNA.
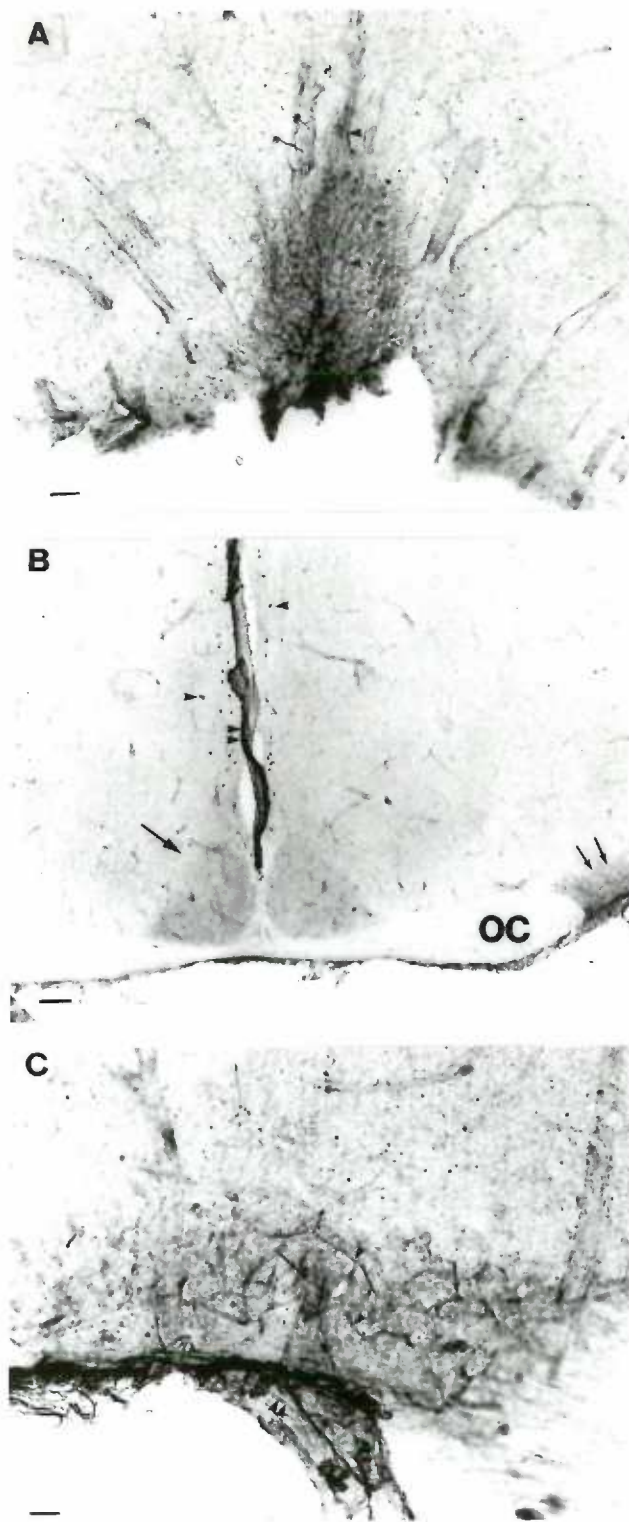
Figure 3. Detection of EGFR immunoreactivity in the hypothalamus of immature female rats. Panel A: immunostaining in fibers converging to the organum vasculosum of the lamina terminalis (OVLT). Panel B: Scattered immunoreactivity in the suprachiasmatic (SCN) and supraoptic (SON) nuclei (single and double arrows,respectively), and more intense and defined staining in small cells along the third ventricle (arrowheads) and ependymal cells of the ventricle (double arrowheads). In both panels the bars = 50 μm. Panel C: Higher magnification of the SON depicting EGFR staining in cells (arrow), processes (arrowheads), and pial astrocytes (double arrowheads). Bar = 20 μm. A total of five animals was employed.
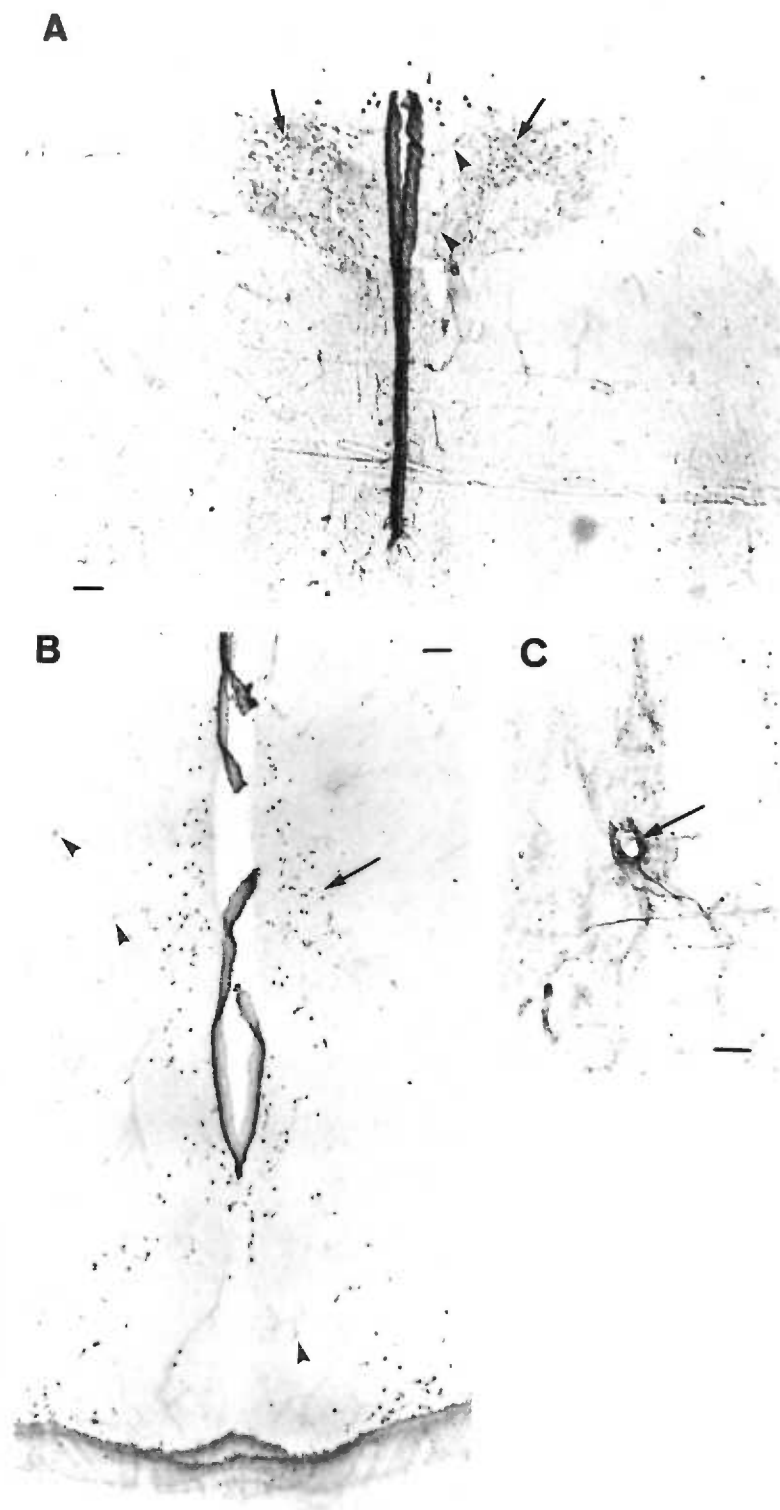
Figure 4. Panel A: EGFR immunoreactivity in the paraventricular nucleus (PVN). Notice the staining in small cells (arrows) and processes (arrowheads), as well as the more diffuse staining throughout the nucleus. Bar = 100 μm. Panel B: EGFR immunoreactivity in small, neuron-like cells (arrows) scattered along the periventricular area (PVA). Notice weak staining in astrocytes (arrowheads). Bar = 100 μm. Panel C: EGFR staining in a perivascular astrocyte. Bar = 50 μm. A total of five animals was employed.
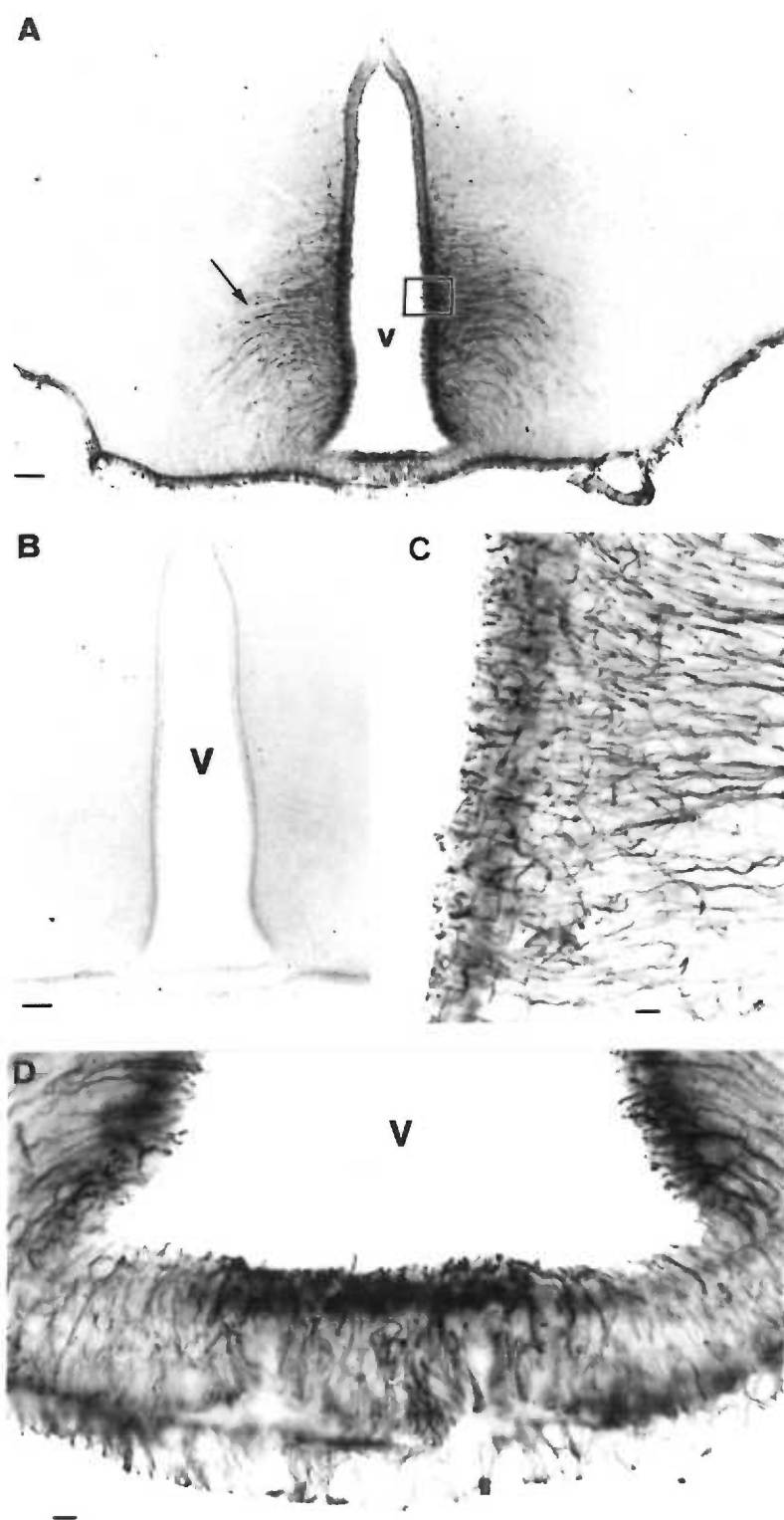
Figure 5. Panel A: EGFR immunoreactivity in tanycytes of the third ventricle (V) and its processes (arrow). Panel B: Lack of immunoreactivity in a section adjacent to that depicted in A after removal of the antibodies to EGFR by binding to partially purified EGFR. Bar = $100 \, \mu$m. Panel C = Higher magnification of immunopositive tanycytes (area boxed in A). Bar = $10 \, \mu$m. Panel D: Higher magnification of the median eminence showing EGFR immunoreactivity in tanycytes and astroglial cells. Bar = $10 \, \mu$m. A total of five animals was employed.
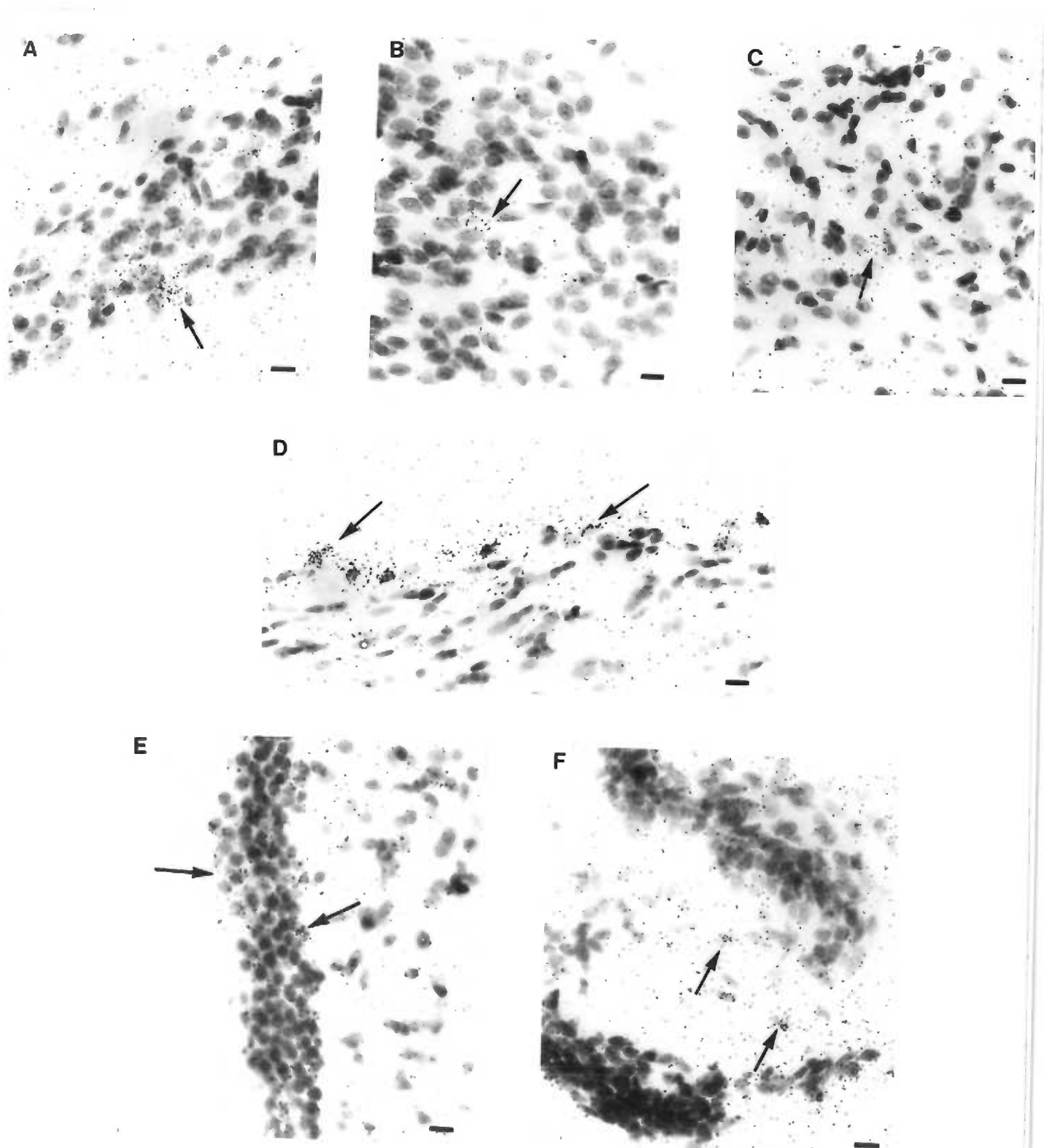
Figure 6. Detection of EGFR mRNA in the immature female rat hypothalamus by hybridization histochemistry. Panel A-C: EGFR mRNA positive cells in the PVN (A), SCN (B) and SON (C). Panel D-F: EGFR mRNA in pial astrocytes (D), ependymal cells of the third ventricle (E), tanycytes and cells (presumably glia) of the median eminence (F). Bars = 10 μm. A total of six animals was employed.

Figure 8 Panel A: Relationship between EGFR-positive tanycytes (double arrowhead)/glial cells (arrowheads) and LHRH nerve terminals (arrows) in the median eminence of the hypothalamus. Bar = 50 μm. Panel B: Lack of EGFR immunoreactivity in LHRH nerve terminals (arrows) traveling along the third ventricle perpendicular to processes of EGFR-positive tanycytes (arrowheads). In the original sections LHRH immunoreactivity has a blue color, crystal-like appearance, and EGFR staining is brown with a smooth appearance. Bar = 20 μm. A total of five animals was employed.
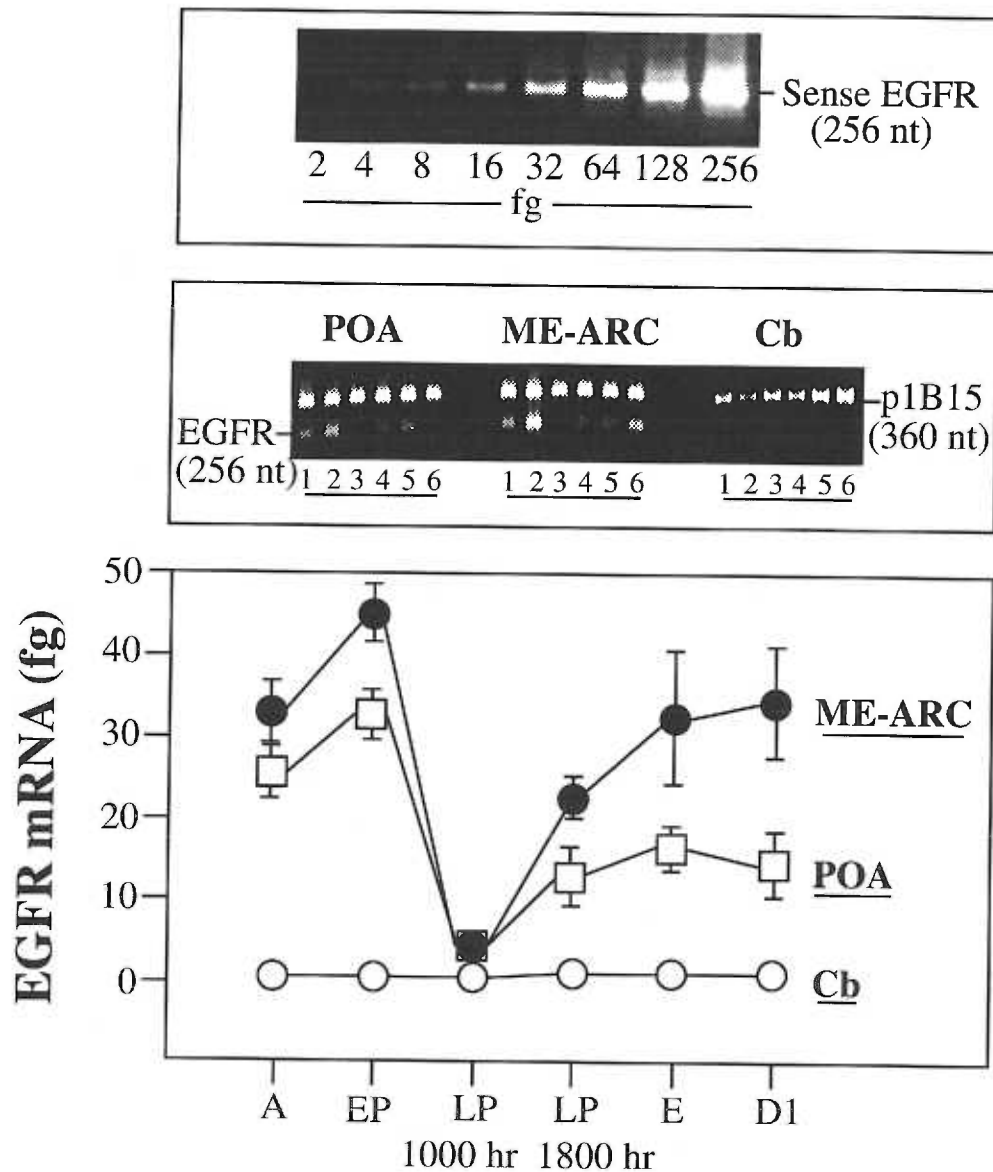
Figure 9. Changes in EGFR mRNA levels in the hypothalamus of female rats at the time of puberty quantitated by RT-PCR. The upper panel depicts a standard curve generated by reverse transcription-PCR amplification of an in vitro synthesized polyadenylated EGFR sense RNA fragment spanning the same cellular RNA sequence targeted for quantitation. The middle panel illustrates the results of one experiment; p1B15 refers to the cyclophilin mRNA sequence co-amplified with EGFR mRNA for the purpose of data normalization. 1 = A, anestrus; 2 = EP, early proestrus; 3 = LP, late proestrus 1000 hr; 4 = LP 1800 hr; 5 = E, first estrus; 6 = D1, first diestrus. The lower panel depicts the combined results of three experiments. Each point represents the mean ± SEM of three independent determinations (each derived from a pool of three animals). POA = preoptic area, ME-ARC = median eminence-arcuate nucleus region, Cb = cerebellum.
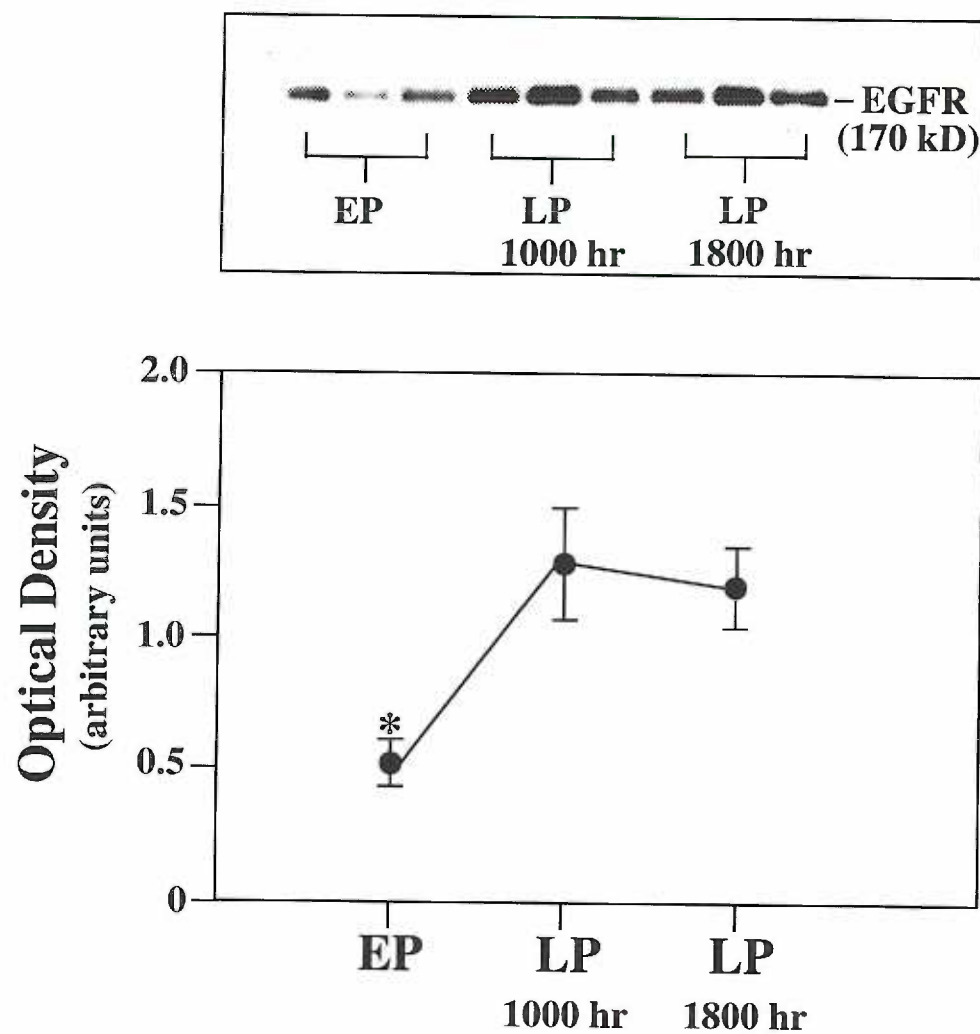
Figure 10. Changes in EGFR content at the time of puberty. The autoradiogram depicted in the upper panel demonstrates the relative abundance in the ME-ARC region of the hypothalamus of an ~170 KD autophosphorylated protein immunoprecipitated with EGFR antiserum and recognized on nitrocellulose by a Mab to phosphotyrosines. The lower panel depicts the results of the densitometric quantitation of the data; each point represents the mean ± SEM of three independent observations. Each independent observation derives from a pool of three animals.

Region-Specific Regulation of Transforming Growth

Factor Alpha (TGFα) Gene Expression in Astrocytes

of the Neuroendocrine Brain

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Abstract

It is becoming increasingly evident that glial cells of the central nervous system are functionally diverse. In the hypothalamus, a region of the brain specialized in neuroendocrine control, astrocytes have been shown to participate in the regulation of reproductive development via the release of transforming growth factor alpha (TGFα), a mitogenic polypeptide with neurotrophic activity. TGFα appears to affect female sexual development by stimulating the secretory activity of neurons producing luteinizing hormone releasing hormone (LHRH), the neurohormone that controls sexual development. Since, in contrast to glial cells, LHRH neurons do not express epidermal growth factor receptors (EGFR), which mediate TGFα actions, it has been hypothesized that TGFα facilitates LHRH release indirectly via an autocrine/paracrine mediated activation of glial function. TGFα, or its structural homolog EGF, increased TGFα mRNA levels in astrocytes isolated from the hypothalamus but not from the cerebellum, a brain region irrelevant to neuroendocrine function. The effect of TGFα or EGF was blocked by Mab425, a monoclonal antibody that binds to the EGFR extracellular domain or by RG-50864, a selective inhibitor of EGFR tyrosine kinase activity. Since ovarian steroids involved in the control of LHRH secretion increase hypothalamic TGFα mRNA levels *in vivo*, additional experiments were performed to determine if astrocytic TGFα mRNA expression is regulated by these steroids *in vitro*. RNase protection assays revealed the presence of estrogen receptor mRNA in cultured hypothalamic astrocytes, but not in cerebellar astrocytes. Estradiol-17ß (17ß-E$_2$), but not its

inactive stereoisomer 17α-E_2 or progesterone, increased TGFα mRNA levels in hypothalamic, but not cerebellar, astrocytes. These results demonstrate that astroglial cells of the neuroendocrine brain are molecularly and functionally different from those of regions not involved in neuroendocrine regulation. Such specialization includes the presence of estrogen receptor mRNA, the ability of TGFα to affect its own gene expression via an autocrine/paracrine mechanism, and the ability of estradiol to directly enhance TGFα gene expression without neuronal intermediacy.

Key Words: trophic factors, TGFα, astrocytes, hypothalamus, estradiol

Introduction

Transforming growth factor alpha (TGFα), a structural and functional homolog of epidermal growth factor (EGF), interacts with EGF receptors (EGFR) to exert its biological actions (Derynck, 1988; Massague, 1990). Although both TGFα and EGF have been shown to be present in brain (Fallon et al., 1984; Code et al., 1987; Wilcox, Derynck, 1988; Kudlow et al., 1989; Seroogy et al., 1991), TGFα mRNA levels are one to two orders of magnitude higher than those of EGF mRNA (Lazar and Blum, 1992). Moreover, EGF protein levels measured by radioimmunoassay (RIA) are exceedingly low as compared to those of TGFα (Kaser et al., 1992), suggesting that TGFα may be the predominant ligand for EGF receptors in brain.

The ability of EGF to promote neuronal survival (Morrison et al., 1987; Kornblum et al., 1990; Abe et al., 1990) and to stimulate astrocytic proliferation (Leutz and Schachner, 1981; Simpson et al., 1982) suggests that TGFα may function in brain as a neurotrophic as well as a mitogenic factor. This view is supported by the observation that TGFα enhances the survival of neurons from various brain regions in culture (Morrison, 1989). TGFα may also play an important role during early development of the CNS, as suggested by its ability to induce proliferation of neuroepithelial progenitor cells (Anchan et al., 1991).

Recent studies (Ojeda et al., 1990) have demonstrated that both TGFα and EGF stimulate the release of luteinizing hormone releasing hormone (LHRH), the neuropeptide that controls sexual development and maintains reproductive functions,

from the median eminence (ME) of the hypothalamus, and that this effect is dependent on activation of EGFR and requires the intermediacy of prostaglandin E_2. Subsequent studies suggested that activation of TGFα gene expression in hypothalamic astrocytes during both the initiation of normal puberty and after hypothalamic lesions contributes to the neuroendocrine process that underlies the initiation of normal female puberty and the neuropathological mechanism by which hypothalamic lesions induce sexual precocity (Junier et al., 1991; Ma et al., 1992). That these effects are also mediated via EGFR was indicated by the ability of an inhibitor of EGF receptor tyrosine kinase activity to delay the normal timing of puberty and prevent the advancing effect of the lesions on puberty. These studies suggested that TGFα is a polypeptide growth factor involved in glial-neuronal interactions and led to the hypothesis that TGFα facilitates LHRH release indirectly via an autocrine/paracrine mediated activation of glial function.

The results of the present study provide evidence for this concept by demonstrating that TGFα is able to act in an autocrine/paracrine manner to enhance its own gene expression in hypothalamic astrocytes via activation of EGFR. They also show that this regulatory mechanism is not operative in astrocytes of a brain region irrelevant to neuroendocrine function and that astrocytes of steroid-sensitive brain regions are targets for sex steroid action.

Materials and Methods

Materials. The basic culture medium employed consisted of DME/Ham's F-12 (1:1)

(Sigma Chemical Co., St. Louis, MO) containing 4.5 g/L glucose, 14 mM $NaHCO_3$ and 15 mM HEPES at pH 7.4. Astrocyte defined medium (ADM) was prepared by adding insulin (50 μg/ml, Sigma) and putrescine (100 μM, Sigma) to DME/F12 medium. Glial fibrillary acidic protein (GFAP) antiserum R77 was a generous gift form Dr. L. Eng (Stanford University), fetal calf serum (FCS) was from HyClone Sterile Laboratories (Logan, UT), EGF from Collaborative Biomedical Products (Bedford, MA), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) from Sigma. TGFα was a gift from Dr. D. Twardzik (Oncogene, Seattle, WA). Mab425 was a gift from Dr. U. Rodeck (Wistar Institute, Philadelphia, PA), and RG-50864 was kindly provided by Dr. A. Scheiber (Rorer Central Research, Horsham, PA).

Astrocyte culture. One to two day-old Sprague-Dawley (Bantin & Kingman, Fremont, CA) neonatal rats were used. The primary cultures were prepared from hypothalamic and cerebellar regions as reported (McCarthy and de Vellis, 1980). The dissociated cells were cultured in T-75 flasks (Nunc Inc., Naperville, IL) containing DME/F12 medium supplemented with 10% FCS, under an atmosphere of 5% CO_2/95% air at 37°C. The medium was changed every 2-3 days until the cultures became confluent (about 10 days).

Purified astrocyte cultures were obtained by the following procedure: Oligodendrocytes, neurons and microglial cells growing on the surface of the astrocyte monolayer were removed by shaking the culture flask on a rotary shaker at 250 RPM for 6 hr at 37°C. Thereafter, the medium was replaced and the cells

were shaken for an additional 18 hr. The detached cells were then removed by washing the cultures with sterile PBS. The purified cultures were trypsinized by adding 3 ml of 0.05% trypsin/0.02% EDTA in PBS. Thereafter, 10 ml of DME/F12 medium + 10% FCS were added to inactivate the trypsin. The isolated astrocytes were then subcultured into 6-well-plates (Linbro, Flow Laboratories, McLean, VA) in a 2 ml volume at the concentration of 7-8 x 10^5 cells per well. They were maintained in DME/F12 +10% FCS and the medium was changed every two days until astrocytes were about 90% confluent (3-4 days). At this time, the medium was replaced by ADM and two days later the cultures were used for the experiments.

Characterization of the astrocyte cultures. The astroglial nature of the isolated cells was defined by the immunohistochemical detection of GFAP, a cytoskeletal protein expressed exclusively by astrocytes, using an ABC immunoperoxidase technique (Nilaver and Kozlowski, 1989), as previously reported (Junier et al., 1991). The cells were fixed and permeabilized with 5% acetic acid/95% ethanol at -20°C for 10 min and GFAP-positive cells were identified with antiserum R77 at a 1:1000 dilution.

Microglial cells were identified by their content of non-specific esterases according to the method of (Koski et al., 1976) . This procedure is based on the ability of nonspecific esterases to catalyze the hydrolysis of esters to alcohol-like products which interact with hexazotized pararosaniline in the presence of α-naphthylbutyrate generating a red color. The cells were fixed with 4% paraformaldehyde in 0.1 M phosphosaline buffer (PBS) pH 7.4 for 30 min at room

astrocyte cultures were treated with 17ß-E$_2$ (274 pg/ml), its biologically inactive stereoisomer 17α-E$_2$ or progesterone (50 ng/ml) for various lengths of time (see Results). Other control cultures were left untreated. While the concentration of 17ß-E$_2$ used is about three times the preovulatory plasma levels of 17ß-E$_2$ found at puberty, the concentration of progesterone approximately corresponds to the plasma levels of the steroid detected at the time of the first preovulatory surge of gonadotropins (Ojeda and Urbanski, 1988).

RNase protection assay. Cellular RNA was extracted as reported (Peppel and Baglioni, 1990). The RNase protection assay used has been described in detail (Junier et al., 1991; Ma et al., 1992). In brief, a 400 base pair (bp) TGFα cDNA (Lee et al., 1985) cloned into the riboprobe vector pGEM-3Z was linearized with EcoRI and used to synthesize a ^{32}P-labeled TGFα cRNA via SP6 polymerase-directed transcription. A gel-purified TGFα cRNA probe (500,000 cpm) was hybridized to RNA samples (3 μg/tube) or to different amounts of *in vitro* transcribed sense TGFα mRNA for 18-20 hr at 45°C. Detection of EGFR mRNA was accomplished by using an antisense probe transcribed from a rat EGFR cDNA fragment [nucleotides(nt) 1720-2298] (Petch et al., 1990), subcloned into the SmaI site of the riboprobe vector pBluescript SK II (Junier et al., 1993). This fragment spans the site of divergence (located at nt 2076) between the mRNAs encoding the full-length EGFR and the truncated EGFR form. Linearization of the template at nt 1915 with AvaII yields a 383 bp DNA template of which 160 bp corresponds to the sequence common to

the external domain of the full-length EGFR mRNA and its truncated form, and 223 bp to the unique 3'-end of the truncated EGFR mRNA. Estrogen receptor (ER) mRNA was detected using an antisense cRNA probe transcribed from an 850 bp XboI-EcoRI ER cDNA fragment subcloned into pGEM-3Z (Koike et al., 1987). The probe recognizes the entire steroid binding coding domain of rat ER mRNA.

The cellular RNA samples were also simultaneously hybridized to 5,000 cpm of a gel-purified ^{32}P-labeled cyclophilin mRNA antisense cRNA probe. Cyclophilin mRNA was used as an internal control for normalization of procedural variabilities, because it is constitutively expressed in brain tissue (Danielsson et al., 1988). After hybridization, single-stranded RNA was digested with ribonucleases A and T1 (Sigma), and the protected hybrids were isolated by polyacrylamide gel electrophoresis (5% acrylamide, 7M urea) and visualized by autoradiography.

To quantitate the changes in mRNA levels the hybridization signals were scanned with an LKB ultrascan XL laser densitometer and compared to a standard curve (described below). The values obtained were then normalized using the levels of cyclophilin mRNA detected in each sample. Results are expressed as femtograms mRNA per microgram of total RNA. Figure 1A depicts a typical standard curve constructed by simultaneously hybridizing different concentrations of *in vitro* transcribed sense TGFα mRNA with its corresponding ^{32}P-labeled antisense RNA probe. The assay is able to detect as little as 60 fg of sense RNA. Figure 1B depicts the regression line and best fit for the standard curve shown in Figure 1A.

Statistics. The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications.

Results

Assessment of culture purity. At the time of use for the different experiments, astrocyte cultures consisted of more than 95% GFAP-positive cells (Fig. 2A). A very small fraction (about 1%) of the total cell population corresponded to microglial cells, identified by their content of non-specific esterases (Fig. 2B).

TGFα mRNA levels in hypothalamic astrocytes are increased by TGFα and EGF. Hypothalamic astrocytes responded to both TGFα and EGF with an increase in TGFα mRNA levels. The increase was apparent within 4 hr exposure and reached maximal values by 8 hr (5-fold higher than basal levels). Thereafter, the levels declined so that at 24 hr they were not significantly greater than control values (Fig. 3 A and A1). The time-course of these changes was similar to that seen after treatment with TPA (Fig. 3 A1), an activator of protein kinase C known to enhance TGFα gene expression in other cell systems (Coffey et al., 1987). In marked contrast, cerebellar astrocytes failed to respond to either TGFα, EGF or TPA with a change in TGFα gene expression (Fig. 3 B and B1). In the absence of stimulation, basal TGFα mRNA levels in cerebellar astrocytes were much lower than in hypothalamic astrocytes (Fig. 3 A and B).

The effect of TGFα on astrocytic TGFα mRNA levels is mediated by EGFR. Both hypothalamic and cerebellar astrocytes contained readily detectable levels of EGFR mRNA. In fact, the latter appear to have higher levels of both the mRNA encoding the full-length, membrane-spanning EGFR and that encoding a truncated EGFR form (Fig. 4 A). Figure 4 B and B1 depicts that the effect of TGFα on hypothalamic astrocyte TGFα mRNA levels was abolished by incubation of the cells in the presence of RG-50864, a blocker of EGFR tyrosine kinase activity (Yaish et al., 1988) or Mab425, a monoclonal antibody that antagonizes ligand-induced activation of EGFR by binding to a relevant epitope of the receptor's extracellular domain (Murthy et al., 1990).

Hypothalamic astrocytes express ER mRNA and respond to estradiol with an increase in TGFα mRNA levels. Experiments were conducted to determine if the ability of estradiol to increase hypothalamic TGFα mRNA levels *in vivo* is due, at least in part, to a direct effect on astroglial cells. RNase protection assays revealed that hypothalamic, but not cerebellar astrocytes express ER mRNA (Fig. 5, top panel). $17ß-E_2$ increased TGFα mRNA levels in hypothalamic astrocytes by about 3-fold after an 8 hr interval (Fig. 5 A and A1). In contrast, no response was observed in cerebellar astrocytes (Fig. 5 B and B1). Moreover, no effect of progesterone on TGFα mRNA levels was observed in either hypothalamic or cerebellar astrocytes (Fig. 5 A1 and B1). The stereospecificity of the $17ß-E_2$ effect on hypothalamic astrocytes was demonstrated by the inability of its stereoisomer $17α-E_2$ to affect

TGFα mRNA in these cells (control 47 ± 11 fg TGFα mRNA/μg total RNA vs. 49 ± 8 fg in 17α-E$_2$-treated cultures).

Discussion

The results of this study demonstrate that TGFα acts in an autocrine/ paracrine manner on hypothalamic astrocytes to enhance expression of its own gene, through a process that requires activation of EGF receptors. In addition, the results indicate that TGFα gene expression in hypothalamic astrocytes is up-regulated by estrogen, suggesting that astrocytes in steroid-sensitive brain regions are targets for sex steroid action.

While the physiological functions of astrocytes in the CNS are not fully understood, it has become evident that astrocytes actively interact with neurons via humoral mechanisms (Lauder and McCarthy, 1986). The ability of astrocytes to produce a variety of neuroactive substances (Martin, 1992) including neurotrophic factors such as nerve growth factor (Houlgatte et al., 1989; Spranger et al., 1990), basic fibroblast growth factor (Ferrara et al., 1988), and insulin like growth factor I (Balloti et al., 1987), and to enhance neuronal survival and differentiation (Manthorpe et al., 1986) suggests that astrocytes are involved in regulating neuronal function. Recent findings have demonstrated that TGFα produced by hypothalamic astrocytes is involved in the regulation of normal female puberty and contributes to the process by which hypothalamic lesions hasten sexual maturation (Ma et al., 1992;

Junier et al., 1991). TGFα appears to exert these effects by stimulating the secretion of luteinizing hormone releasing hormone (LHRH), the neurohormone controlling sexual development. This notion derives from the observation that TGFα stimulates LHRH release from the hypothalamus (Ojeda et al., 1990). That this stimulatory effect is not due to a direct action of TGFα on LHRH neurons, but is instead exerted via the intermediacy of glial cells is suggested by the presence of EGF receptors in astrocytes, but not in LHRH neurons (Ma et al., 1991; Ma et al., in preparation, see Chapter 2). The presence of TGFα mRNA and the TGFα precursor protein in hypothalamic astrocytes (Ma et al., 1992) has led to the suggestion that glial TGFα acting via an autocrine/paracrine mechanism enhances the release of neuroactive substances from neighboring astrocytes able to stimulate LHRH release. One such substance may be prostaglandin E_2 (PGE_2). Both TGFα and EGF elicit release of PGE_2 from the median eminence of the hypothalamus, whereas blockade of prostaglandin synthesis abolished TGFα -induced LHRH release (Ojeda et al., 1990). Brain astrocytes release prostaglandin E_2 in response to neuropeptide stimulation (Katsuura et al., 1989) and hypothalamic explants also release PGE_2 in response to neuropeptide stimulation, a response attributed predominantly to astrocytes (Navarra et al., 1992). That the ability of LHRH neurons to produce PGE_2 may be limited is suggested by the finding that a neuronal LHRH-secreting cell line responds to prostaglandins with LHRH release, but is unable to synthesize prostaglandins on its own (Negro-Vilar et al., 1991).

The presence of EGFR mRNA in hypothalamic astrocytes and the ability of

TGFα to increase its own mRNA levels, indicate that TGFα can indeed act on glial cells via an autocrine/paracrine mechanism to up-regulate its own gene expression. This mechanism appears similar to that described in keratinocytes (Coffey et al., 1987) and may have its greater impact on hypothalamic function at times when glial TGFα gene expression is enhanced, such as the onset of puberty or after hypothalamic injury (Junier et al., 1991; Ma et al., 1992).

In contrast to hypothalamic astrocytes, no effect of TGFα or the protein kinase C activator TPA on TGFα mRNA levels was observed in cerebellar astrocytes, despite the fact that they express as much EGFR mRNA as hypothalamic astrocytes. Perhaps cerebellar EGFR do not transduce-TGFα initiated signalling as efficiently as receptors located on hypothalamic astrocytes. There is evidence that in spite of binding to EGFR, TGFα biological activity may differ substantially from that of EGF on the same cell system (Chalazonitis et al., 1992; Derynck, 1988). A growing body of evidence now exists indicating that astrocytes are much more functionally diverse than originally envisioned. For instance, angiotensinogen is produced by astrocytes in some hypothalamic, midbrain and brain stem nuclei, but not in other regions (Stornetta et al., 1988). Substance P immunoreactivity is found in a subpopulation of astrocytes associated with blood vessels (Kostyk et al., 1989; Michel et al., 1986) and somatostatin is produced by cerebellar, but not striatum or cerebellar cortex astrocytes (Shinoda et al., 1989). Expression of TGFα itself is regionally diverse, as the peptide is found preferentially in astrocytes of the basal ganglia including the caudate-putamen, external capsule and globus pallidus (Fallon

et al., 1990). Within the hypothalamus, TGFα mRNA and precursor protein have been found to be predominantly expressed in the suprachiasmatic, paraventricular, arcuate, ventromedial and dorsomedial nuclei, in addition to the median eminence (Ma et al., 1992). In recent experiments, we have found that conditioned medium from hypothalamic, but not cerebellar astrocytes, is able to promote the survival of LHRH neurons in culture (data not shown), further supporting the concept that hypothalamic astrocytes are not only functionally different from astrocytes of other brain regions, but also that a sub-set of them is specialized to subserve neuroendocrine reproductive functions.

It is well established that ligand-induced activation of the EGF receptor tyrosine kinase is essential for receptor-mediated signal transduction (Carpenter, 1987). A highly selective blocker of EGFR tyrosine kinase activity (Yaish et al., 1988) has been shown to abolish the stimulatory effect of TGFα on LHRH release (Ojeda et al., 1990), delay the normal onset of puberty in female rats (Ma et al., 1992), and prevent the advancing effect of hypothalamic lesions on puberty (Junier et al., 1991). In the present study, the ability of TGFα to enhance its own gene expression in hypothalamic astrocytes was abolished by the blocker of EGFR tyrosine kinase, tyrphostin RG-50864. As previously stated (Junier et al., 1991; Ma et al., 1992), it is difficult to rule out the possibility that RG-50864 may be blocking other receptor tyrosine kinases, particularly those of the EGFR family (Schechter et al., 1985; Kraus et al., 1989; Plowman et al., 1990). Nevertheless, the high selectivity of RG-50864 for EGFR (Yaish et al., 1988) makes it less likely that the blocking effect

of RG-50864 on TGFα to enhance its own gene expression is due to inhibition of other less related receptor tyrosine kinases, such as those for insulin and platelet-derived growth factor. Whether RG-50864 blocks some yet unidentified receptor tyrosine kinases is unknown. That EGFR or EGFR-like molecules are, at least in part, involved in mediating the up-regulating effect of TGFα on its own mRNA levels is further suggested by the ability of the monoclonal antibody Mab425 to blunt the TGFα effect. This antibody, raised against the EGF receptor of human A-431 carcinoma cells, binds to peptide epitopes near and/or at the EGF binding site of the EGFR external domain in a conformational-dependent fashion (Murthy et al., 1987). The antibody has been shown to inhibit EGF binding and trigger receptor down-regulation (Murthy et al., 1987). It also blocks several EGF-dependent functions, such as receptor autophosphorylation and mitogenesis, but it has been found to be devoid of agonist properties (Murthy et al., 1987). Importantly, Mab425 was also found to inhibit TGFα biological action, as it blocked the effect of TGFα on inositol 1,4,5,-triphosphate formation and calcium mobilization in epidermoid A-431 and colorectal carcinoma SW-948 cells (Murthy et al., 1990).

Although the regulation of TGFα gene expression is complex and the underlying mechanisms are not entirely understood, it is becoming clear that estradiol is one of the hormonal components involved in the regulatory process. Estradiol has been shown to increase TGFα mRNA in breast cancer cells (Bates et al., 1988), normal mammary epithelial cells (Salomon et al., 1989), anterior pituitary gland (Borgundvaag et al., 1990) and hypothalamus (Ma et al., 1992). Molecular

characterization of the 5'-flanking region of the human TGFα gene revealed the presence of a potential estrogen-responsive element (ERE) located within 1,140 bp of the transcription start site (Saeki et al., 1991). Interestingly, the 5'-flanking region of the rat TGFα gene does not appear to contain traditional EREs (Blasband et al., 1990), suggesting that in this species estrogen actions on TGFα gene expression are indirect or that the rat TGFα gene contains imperfect, but functional, palindromic ERE-like sequences. We recently showed that 17ß-E_2 acts *in vivo* to enhance TGFα mRNA expression in the developing female hypothalamus (Ma et al., 1992). We also observed that administration of progesterone to estradiol-primed animals was much more effective than estradiol alone in increasing hypothalamic TGFα mRNA levels. While these results indicated that the increase in the hypothalamic TGFα gene expression that accompanies the initiation of puberty is caused, at least in part, by gonadal steroids, they did not inform us as to whether the stimulatory effect of 17ß-E_2 and progesterone on TGFα gene expression is exerted on neurons or astrocytes. The present study demonstrates that hypothalamic astrocytes express the ER gene, as assessed by the detection of ER mRNA by RNase protection assay. Moreover, the results show that treatment of purified hypothalamic astrocytes with 17ß-E_2 increases TGFα mRNA levels within 8 hr of exposure. In contrast to hypothalamic astrocytes, neither ER mRNA nor an effect of estradiol on TGFα mRNA levels were detected in cerebellar astrocytes. These results strongly suggest that estrogen can act directly on hypothalamic astrocytes bearing the appropriate receptors to enhance TGFα gene expression. They are also in keeping with the recent finding that a sub-

population of hypothalamic astrocytes express estrogen receptors *in vivo* (Langub, Watson, 1992). The inability of cerebellar astrocytes to respond to estradiol with changes in TGFα mRNA levels is in all likelihood due to the absence of ER gene expression. Indeed, autoradiographic studies have shown that the cerebellum is essentially devoid of estrogen receptors (Pfaff, Keiner, 1973). In addition to these differences, our results show that TGFα mRNA levels are more than 4-fold lower in cerebellar than in hypothalamic astrocytes, further suggesting that marked differences in gene expression exist between the glial cells of these two brain regions.

The presence of ER mRNA in cultures of hypothalamic astrocytes coupled to the ability of estradiol to enhance TGFα gene expression indicate that at least a subset of hypothalamic astrocytes are molecularly and functionally differentiated to subserve neuroendocrine functions relevant to the control of reproductive function. Support for this concept comes from the observation that TGFα mRNA levels in the hypothalamus vary according to the stage of sexual development in both rats and non-human primates (Ma et al., 1992; Ma et al., in preparation, see Chapter 5).

A puzzling observation of the present study was the inability of progesterone to affect TGFα gene expression in isolated hypothalamic astrocytes *in vitro* in spite of its marked effectiveness *in vivo* (Ma et al., 1992). Progesterone has been shown to increase TGFα mRNA levels in human breast cancer cells (Murphy, Dotzlaw, 1989) and long-term estradiol exposure was reported to induce the expression of progesterone receptors in cultured cortical astrocytes (Jung-Testas et al., 1991), two observations that inferentially predict a stimulatory effect of progesterone on

astrocytic TGFα gene expression. Since it is becoming increasingly evident that neurons and glial cells are normally engaged in complex cell-to-cell interactions, it may be suspected that progesterone affects glial gene expression of TGFα *in vivo* by stimulating the production of neuronal molecules that interact with neighboring glial cells to affect TGFα synthesis. Further experimentation is required to resolve this issue.

Taken altogether, the results of this study unequivocally demonstrate that the hypothalamus, a key component of the neuroendocrine brain, contains a subset of astroglial cells molecularly and functionally different from astrocytes of a brain region not involved in neuroendocrine control. This regional specialization involves the ability of performing functions which require the interactive modulation of TGFα gene expression by autocrine/paracrine mechanisms and estradiol-dependent processes. Unfolding of these interactions appears to contribute to the functional regulation of the neuronal circuitry that controls sexual development.

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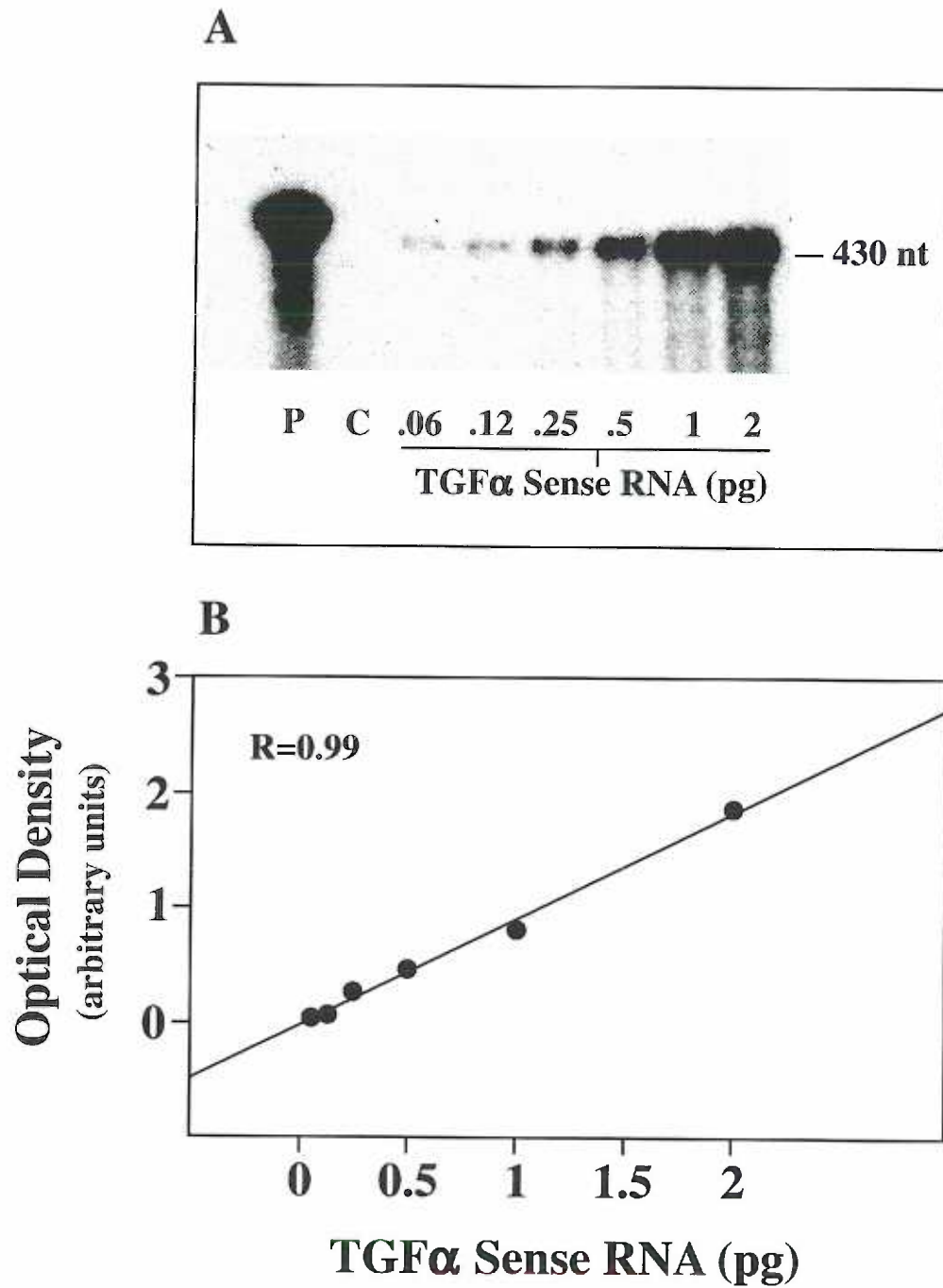
Figure 1. (A) Standard curve of TGFα mRNA generated by hybridization of a ^{32}P-labeled TGFα cRNA to increasing amounts of in vitro transcribed TGFα sense RNA followed by ribonuclease digestion (see Methods). P = intact probe; C = digested probe. (B) Linear regression analysis for the standard curve depicted in (A).
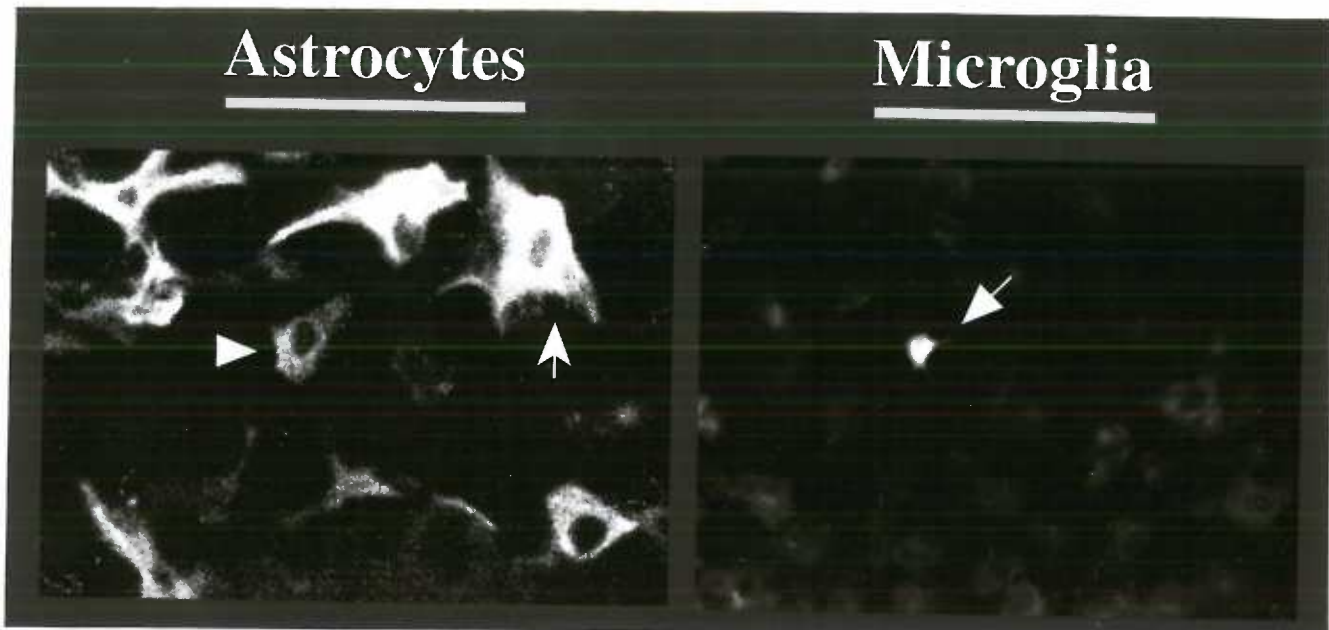
Figure 2. Left Panel: Immunohistochemical identification of hypothalamic astrocytes in culture by their content of glial fibrillary acidic protein (GFAP). More than 95% of the cultured cells were GFAP-positive, but the intensity of the staining was greater in some cells (arrow) than in others (arrowhead). In the original microphotograph GFAP immunoreactive cells have a brown color (100x). Right Panel: Identification of microglial cells in cultures of hypothalamic astrocytes by their content of non-specific esterases. Very few positive cells (arrow) were detected indicating that the cultures had a negligible degree of microglial contamination. In the original microphotograph, positive cells have a dark red color (46x).
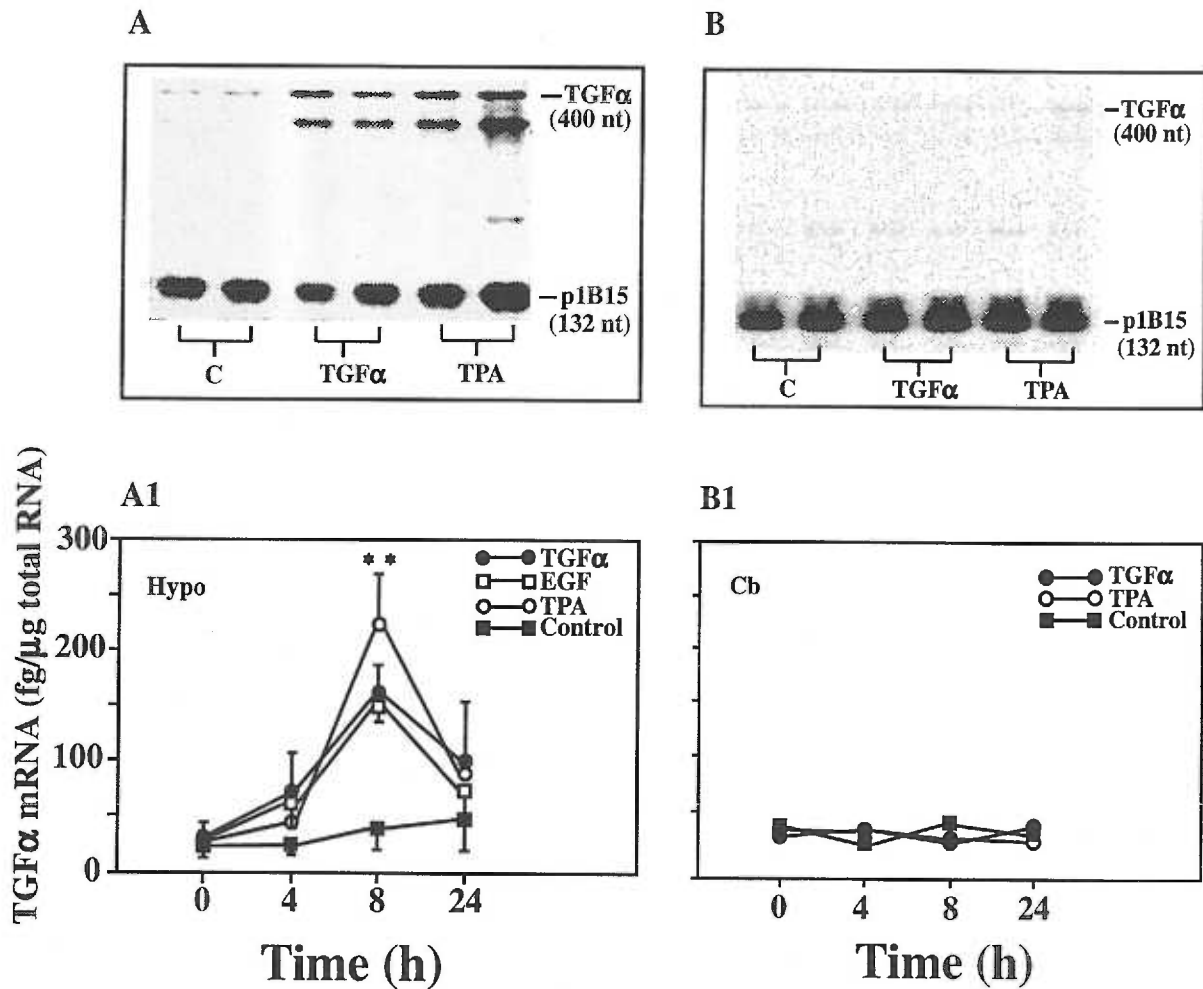
Figure 3. Ability of TGFα to increase its own gene expression in hypothalamic, but not cerebellar astrocytes in culture. (A) Representative autoradiograms showing the effect of TGFα (50 ng/ml) and TPA (10 ng/ml) on TGFα mRNA content of hypothalamic astrocytes, after 8 h of exposure. The additional small (~350 nt) band that was protected may be the product of alternative splicing or represent a highly related TGFα relative. (A1) Quantitative data showing the time-course of the effect of TGFα, EGF and TPA on hypothalamic astrocyte TGFα mRNA levels. Each point represents the mean ± SEM of 6-8 culture wells. (B) Representative autoradiogram illustrating the lack of effect of TGFα or TPA on cerebellar astrocyte TGFα mRNA levels. (B1) Quantitative data showing the lack of changes in TGFα mRNA levels in cerebellar astrocytes after different times of exposure to TGFα or TPA. Hypo = hypothalamus; Cb = cerebellum. ** = $p < 0.01$ vs controls.
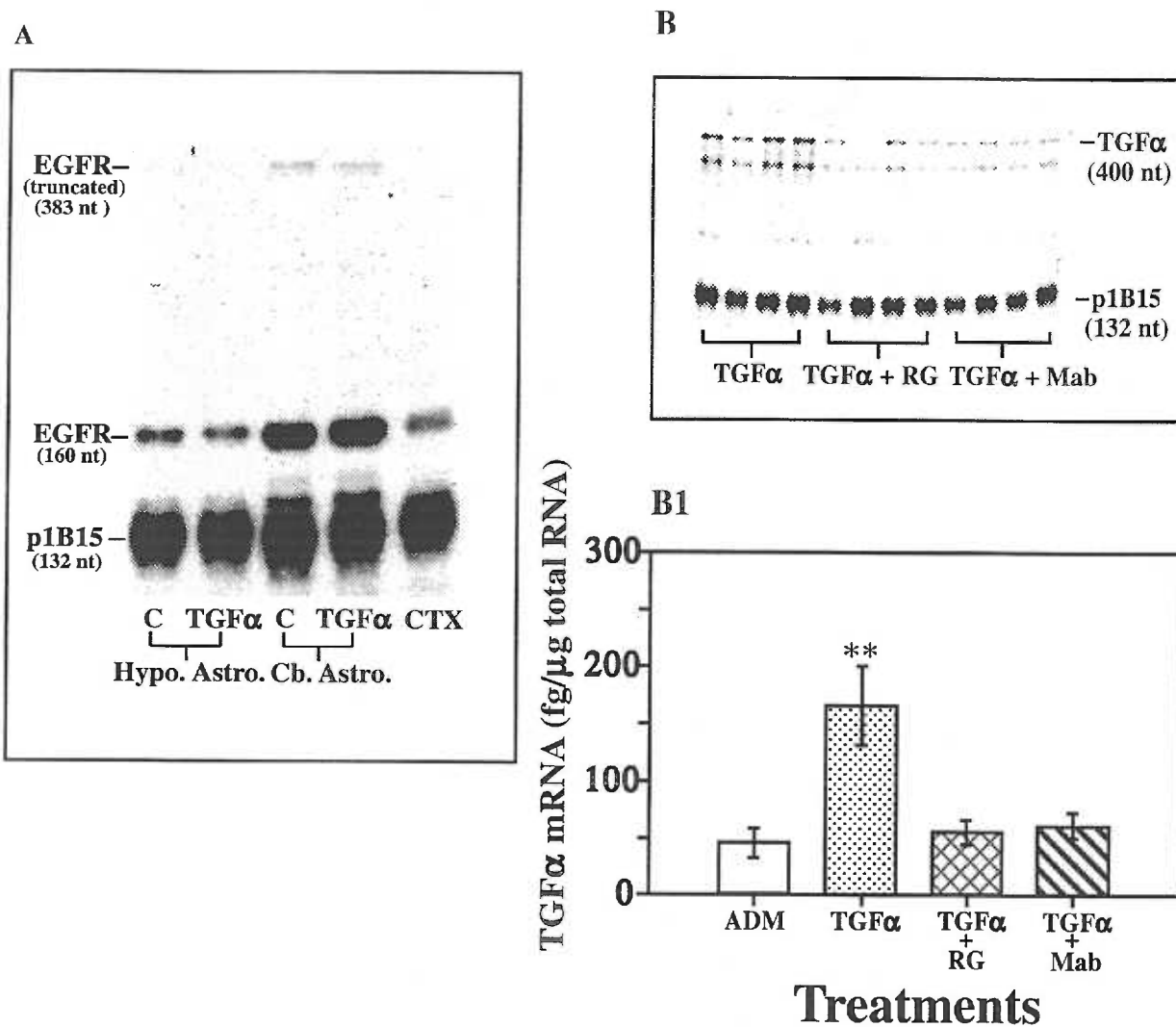
Figure 4. (A) Detection of EGFR mRNA in hypothalamic and cerebellar astrocytes. The 160 nt mRNA form corresponds to the extracellular domain encoding region of EGFR mRNA. The 383 nt protected species corresponds to the truncated EGFR mRNA form that results from alternative splicing of the EGFR mRNA primary transcript in rats. (B) Autoradiogram illustrating the ability of a blocker of EGFR tyrosine kinase (RG), or a monoclonal antibody to the extracellular domain of EGFR (Mab) to inhibit TGFα -induced increase in TGFα mRNA levels in hypothalamic astrocytes. (B1) Quantitation of the data illustrated in Panel B. Bars represent the mean ± SEM of four independent observations per group. ** = $p < 0.01$ vs all other groups.

Figure 5. Selective expression of ER mRNA in hypothalamic astrocytes in culture (upper panel) is coupled to the ability of 17β-E$_2$ to increase TGFα mRNA levels in hypothalamic, but not cerebellar astrocytes (panels A-A1 and B-B1). Panels A and B depict representative autoradiographs showing the ability of 17β-E$_2$ to increase TGFα mRNA levels in hypothalamic (panel A), but not cerebellar astrocytes (panel B). In each case, the cells were exposed to TGFα for 8 h. Panel A1 and B1 provide the quantitative data for these experiments demonstrating the effect of TGFα on TGFα mRNA levels after 4 and 8 h of exposure. Bars are mean ± SEM. Number in parentheses are number of culture wells per group. P = progesterone. ** = p < 0.01.

Classification: Neurobiology

Overexpression of a Human Transforming Growth Factor Alpha (TGFα)

Transgene Reveals a Dual Antagonistic Role of TGFα in Female

Sexual Development

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ABSTRACT

The importance of transforming growth factor alpha (TGFα) on female reproductive development was assessed using transgenic mice bearing a human TGFα cDNA under the control of a mouse metallothionein 1 (MT1) promoter. Chronic activation of the transgene expression by daily administration of zinc chloride delayed the time of first estrus (an index of peripubertal estrogen secretion) but shortened considerably the interval between the first estrus and the onset of estrous cyclicity (an index of reproductive competence). Elevated serum gonadotropin levels and a marked increase in the number of small, but not large ovarian antral follicles preceded the acquisition of ovulatory capacity suggesting a relative inability of gonadotropins to further follicular development in TGFα-overexpressing animals. Acute exposure to zinc chloride activated expression of the transgene in both the brain and ovaries; within each organ the level of expression was most prominent in the hypothalamus and ovarian follicles, respectively. *In vitro* experiments showed that acute transgene activation resulted in increased hypothalamic release of luteinizing hormone-releasing hormone (LHRH), the neuropeptide controlling sexual development, but markedly attenuated the ovarian steroidal response to gonadotropins. Normal ovaries placed under the control of a transgenic hypothalamus by heterologous grafting, rapidly ovulated and initiated estrous cyclicity. In contrast, acquisition of reproductive capacity was severely delayed in normal mice bearing transgenic ovarian grafts. The results indicated that TGFα regulates female reproductive development through two opposite mechanisms:

within the brain, it facilitates the neuroendocrine activation of the process; at the ovarian level, it modulates the stimulatory effect of gonadotropin hormones on follicular growth and steroidogenesis.

INTRODUCTION

Transforming growth factor alpha (TGFα), a 50-amino acid mitogenic polypeptide, is synthesized as part of a glycosylated transmembrane precursor encoded by a 4.8 kb mRNA species (1,2). TGFα is structurally and functionally related to epidermal growth factor (EGF), and exerts its biological effects via activation of EGF receptors (3,4). Although both TGFα and EGF have been shown to be present in brain (5-9), the levels of TGFα mRNA and its translated protein product are much higher than those of EGF mRNA (10) and EGF protein (11), suggesting that TGFα may be the predominant ligand for EGF receptors in the central nervous system. While there is evidence that TGFα may function in brain as a neurotrophic (12-14) as well as a mitogenic factor (15,16), recent studies have demonstrated that TGFα and EGF are able to affect differentiated brain functions, and in particular, the secretion of hypothalamic neuropeptides (17-20). Both trophic factors have been shown to stimulate the release of luteinizing hormone releasing hormone (LHRH), from the median eminence (ME) of the hypothalamus in a process that involves activation of EGF receptors and requires the intermediacy of prostaglandins (20). Subsequent studies implicated TGFα of glial origin in the regulatory process that controls the initiation of normal female puberty (21) and in the cell-to-cell signalling mechanism underlying the ability of hypothalamic lesions to induce sexual precocity (22). Since these studies showed that TGFα is predominantly produced in a discrete sub-set of astrocytes, the conclusion was reached that TGFα is a peptide growth factor involved in glial-neuronal interactions.

In vitro studies reported in the previous chapter supported and expanded this notion by demonstrating that TGFα is able to act in an autocrine/paracrine manner and in a region-specific fashion to enhance its own gene expression in purified hypothalamic astrocytes.

There is substantial evidence that TGFα acts on at least one other component of the hypothalamic-pituitary-ovarian axis to affect reproductive function. Several studies have made it clear that the ovary is a target for TGFα action. The predominant site of TGFα synthesis in the ovary has been localized to the thecal compartment of follicles (23,24), and its main effects have been shown to be mostly inhibitory. Thus, TGFα inhibits the ability of FSH to stimulate aromatase activity (25) and to induce LH receptors in granulosa cells (26). TGFα also suppresses gonadotropin-stimulated estradiol secretion (27,28). Nevertheless, there is contradictory evidence, as a stimulatory effect of TGFα on estrogen secretion from both granulosa and thecal cells of developing follicles has been postulated (29). Furthermore, it has been recently shown that TGFα may favor follicular development by inhibiting the process of apoptosis (30).

We have now used transgenic mice overexpressing a human TGFα gene in an attempt to define the role that this peptide may play in the developmental control of the reproductive hypothalamus and ovarian maturation. The results indicate that while TGFα facilitates hypothalamic release of LHRH, it reduces the ovarian response to gonadotropins, thus effectively modulating the progression of sexual development via two opposite but functionally interactive paracrine regulatory loops.

MATERIALS AND METHODS

Animals. The adult transgenic mice that served as breeders were obtained from the National Cancer Institute, Bethesda, MD. They were allowed to mate (1 male with 2 females per cage) in a room with controlled photoperiod (14 hr light/10 hr darkness, lights on from 0500-1900 hr) and temperature (23°C - 25°C) and were provided free access to tap water and pelleted chow. When the offspring were born, they were housed with their transgenic parents until 21 days-old. Thereafter, the females were separated for study. CD1 controls (purchased from Charles River, Wilmington, MA) were acquired at 21 days of age and were housed under identical conditions.

The Transgenic Mice. The procedure used to generate TGFα transgenic mice has been described in detail (31). As indicated by these authors, the inducible human TGFα expression vector was made by cloning a 917 bp human TGFα cDNA into the plasmid pEV142 (32), which contains the zinc inducible mouse metallothionein 1 (MT1) promoter and the human growth hormone polyadenylation signal. A 2.3 kb EcoRI MT1-hTGFα fusion gene fragment was then isolated and microinjected into outbred CD1 one-cell mouse embryos. The intact MT1-hTGFα transgene was stably integrated at a single site containing two copies per haploid genome and transmitted in typical Mendelian fashion.

RNase Protection Assay. Tissue RNA was isolated according to procedures previously reported (33,34) from 28-day-old transgenic female mice and CD1 controls, 5 hr after the injection of 25 mM zinc chloride in a 0.1 ml volume. The

RNase protection assay has been previously described in detail (22,35). In brief, a 316 bp human TGFα cDNA (31) cloned into the riboprobe vector pGEM-3 and linearized with EcoRI was used as template to synthesize a ^{32}P-labeled human TGFα cRNA probe via SP6 polymerase-directed transcription. A gel-purified TGFα cRNA probe (500,000 cpm) was hybridized to the RNA samples (3 μg/tube) for 18-20 h at 45°C. After hybridization, single-stranded RNA was digested with ribonucleases, and the protected hybrids were isolated by polyacrylamide gel electrophoresis (5% acrylamide, 7 M urea).

Hybridization Histochemistry. The procedure employed is that described by Simmons et al. (36) with minor modifications (21,22). The brains were fixed by transcardiac perfusion with 4% paraformaldehyde in borate buffer (pH 9.5), followed by overnight postfixation in the same fixative, containing 10% sucrose. After blocking the regions of interest, the tissues were frozen on dry ice and stored at -85°C until sectioning. Twenty micrometer sections were obtained with a sliding microtome, adhered to polylysine-coated slides, and dried overnight under vacuum before hybridization. The hybridization buffer contained 50% formamide, 0.25 M NaCl, 10 mM EDTA, 10 mM Tris (pH 8.0), and 2x Denhardt's solution; the sections were overlaid with 70 μl of this solution containing 1×10^7 cpm of ^{35}S-labeled TGFα cRNA probe per ml and hybridized for 18-20 hr at 55°C. Posthybridization washes were those recommended (36). All sections were dipped in NTB-2 emulsion and developed after 2 weeks of exposure.

Evaluation of Sexual Maturation. Starting on postnatal day 22, CD1 control

mice or transgenic animals were either injected once a day with zinc chloride (25 mM solution, in 0.1 ml saline) every afternoon or were provided with zinc chloride (25 mM) in the drinking water. Daily assessment of vaginal opening and vaginal cytology was initiated four days later. Vaginal opening and cornification of the vaginal epithelium are consequences of the rise in circulating estradiol levels that accompany the onset of puberty in rodents (37). The first estrus was defined as the day when vaginal cytology exhibited a preponderance of cornified cells. The onset of estrous cyclicity was defined as the first day of diestrus following a cycle less than, or equal to 6 days in length (38). Based on these criteria, the animals were sacrificed on the first diestrus day following the first 4- to 6-day estrous cycle, and their ovaries collected for histological analysis.

Ovarian Histology and Morphometric Analysis. The collected ovaries were fixed in 10% formalin, embedded in paraffin and serially sectioned at 8 μm. The sections were stained with hematoxylin-eosin and the number of follicles and degree of follicular development were analyzed using an IBAS-2000 Image Analysis System, according to a procedure previously reported (33).

***In Vitro* Incubation of Ovaries and Median Eminence-Arcuate nucleus (ME-ARC) Region.** *Tissue dissection*: ME-ARCs and ovaries from 28-day-old transgenic or CD1 normal female mice were collected 5 hr after the injection of 0.1 ml 25 mM $ZnCl_2$. The ME-ARC region was dissected out by one sagittal cut one third of the way between the ME and each hypothalamic sulci, one caudal cut in front of the mammillary bodies and one rostral cut immediately behind the optic chiasm. The

depth of the tissue fragments collected was about 1 mm. Both ME-ARC and ovaries were incubated *in vitro* under an atmosphere of 95% O_2, 5% CO_2, at 37°C in 250 μl (ME-ARC) or 500 μl (ovary) of Krebs-Ringer bicarbonate buffer (pH 7.4) containing D-dextrose at either 4.5 mg/ml (ME-ARC) or 1 mg/ml (ovary), as described (39). In the case of ovary, the medium also contained 0.1 mg/ml of BSA to improve the recovery of ovarian steroids (40).

At the time of sacrifice, trunk blood was collected for assay of LH and FSH levels. In all experiments, the ME-ARC fragments were incubated for 30 min periods up to total 90 min with media collection at the end of each period. The ovaries were preincubated for 30 min, and then for 3 hr in the presence or absence of hCG (50 ng/ml). The collected media were stored at -20°C until assay for LHRH (ME-ARC) or steroids (ovaries). The LHRH assay, which has been described in detail in earlier publications (20,41), utilizes ^{125}I-labeled LHRH and the polyclonal antibody HFU60 at a 1:25,000 dilution. The sensitivity of the assay was 0.4 pg of LHRH per tube. The RIAs for estradiol, androstenedione, testosterone and progesterone were performed by the ORPRC P-30 Center Core assay following a procedure described by Resko et al. (42).

Heterologous Ovarian Transplantation. On postnatal day 23, the animals were anesthetized with ether, and both ovaries were removed through a single dorsal midline incision. The ovaries were then transferred to sterile Dulbecco's minimal essential medium and cleaned of adherent tissue before transplantation. Three experimental groups were used: 1) CD1 mice, each of which was ovariectomized and

received ovaries from a transgenic mouse; 2) Transgenic mice, the ovaries of which were grafted into the CD1 controls and in turn received the normal ovaries from these controls; and 3) CD1 controls which were ovariectomized and had their ovaries autotransplanted. The transplantation procedure consisted of placing the ovaries next to the jugular vein through a small incision made on the fascia/adipose tissue that covers this area, as previously reported (43). Each animal received two ovaries, one on each side of the neck, using sterile procedures. The incision, made through the fascia, was closed with 3-0 suturing silk, and the skin incision was closed with wound clips. Starting 12 h after surgery, all animals were provided with zinc chloride in the drinking water and were inspected daily for vaginal opening. Following vaginal opening, vaginal cytology was daily examined by vaginal lavage. The transgenic mice receiving CD1 ovaries and CD1 controls receiving autologous ovarian grafts were sacrificed at the first diestrus following a complete 4-6 day estrous cycle. The CD1 mice grafted with transgenic ovaries were killed one week after the last CD1 control bearing ovarian autografts had shown a complete estrous cycle. The transplanted ovaries were collected and processed for histological examination using the same procedure described above.

Statistics. The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications.

RESULTS

The MT1-hTGFα Transgene is Expressed in the Hypothalamus and Ovaries.
The MT42 transgenic mice used in this study, express the MT1-hTGFα transgene in both brain and a variety of peripheral tissues (31). As expected, hTGFα mRNA was detected in the hypothalamus and ovaries of transgenic mice, but not in those of CD1 normal mice, and its steady-state levels were greatly increased 5 h after a single s.c. injection of zinc chloride. *In situ* hybridization revealed that hTGFα mRNA was preferentially expressed in the medial basal hypothalamus (Fig. 2B and C) including the ME, and arcuate, ventromedial- and dorsomedial nuclei. A strong hybridization signal was also found in the dentate gyrus of the hippocampus (Fig. 2B). Expression of hTGFα mRNA in the ovary was more prominent in granulosa and thecal cells of developing follicles (Fig. 3).

A Dual Effect of hTGFα Overexpression on Female Sexual Development.
Stimulatory effect on hypothalamic LHRH release. Recent studies (20) demonstrated that both TGFα and EGF stimulate LHRH release from the ME of the hypothalamus. In the present study, injection of zinc chloride, which markedly increased hTGFα mRNA levels in the ME-ARC of the hypothalamus 5 hr later (Fig. 2), resulted in more than a three-fold increase in LHRH released from the isolated ME-ARC region incubated *in vitro*, as compared to the release observed in CD1 controls (Fig. 4). Preliminary results show that serum gonadotropin levels are also increased at this time in transgenic mice.

Inhibitory effect of hTGFα overexpression on ovarian steroidogenesis: In contrast

to the stimulatory effect of hTGFα overexpression on LHRH release from the ME-ARC of the hypothalamus, overexpression of the hTGFα gene in transgenic ovaries was found to dramatically attenuate the hCG-stimulated release of androstenedione and estradiol, as compared to CD1 controls (Fig. 5).

Effect of hTGFα overexpression on puberty: Overexpression of hTGFα induced by adding zinc chloride to the drinking water significantly delayed the first estrus in transgenic mice as compared with CD1 (Fig. 6, upper panel). However, the interval between the first estrus and the onset of estrous cyclicity, which takes several days in normal mice, was considerably shortened in hTGFα -overexpressing mice (Fig. 6, lower panel).

Ovarian histology, analyzed on diestrous ovaries after occurrence of the first estrous cycle, showed a striking increase in the total number of antral follicles in the ovaries of hTGFα -overexpressing mice (Fig. 7). The number of small antral follicles (100-200 μm) in transgenic ovaries was about 6-fold greater than that in control ovaries (Fig. 7, lower panel). The number of medium sized follicles (201-300 μm), but not that of larger (>300 μM) follicles, was also significantly increased in transgenic ovaries (Fig. 7, lower panel).

Definition of the Antagonistic Actions of TGFα on Sexual Development by Heterologous Ovarian Transplantation. Transgenic mice receiving two non-transgenic CD1 ovaries and treated with zinc chloride to activate expression of the TGFα transgene, exhibited the first estrus almost 20 days earlier than CD1 controls grafted with transgenic ovaries and at about the same age as CD1 controls receiving

autologous ovarian grafts (Fig. 8, upper panel). On the other hand, the onset of cyclicity was markedly advanced in TGFα-overexpressing mice bearing CD1 ovaries, as compared with CD1 controls receiving autologous ovarian grafts (Fig. 8, lower panel). Although CD1 controls bearing hTGFα-overexpressing ovaries showed sporadic vaginal cornification, no regular estrous cycles were detected throughout the period studied, which extended more than one week after the last CD control bearing autologous ovarian grafts had been sacrificed (Fig. 8, lower panel). CD1 ovaries transplanted into transgenic mice exhibited numerous corpora lutea and a few number of follicles in different phases of development (Fig. 9). In contrast, transgenic ovaries transplanted into CD1 mice had fewer corpora lutea and a greater number of small antral follicles than CD1 ovaries transplanted into TGFα-overexpressing mice (Fig. 9).

DISCUSSION

Previous studies (21,22) have provided evidence for the concept that hypothalamic TGFα of glial origin is a regulator of mammalian sexual development. The results of these studies led to the conclusion that TGFα, acting via binding to EGF receptors, contributes to both the neuroendocrine process that underlies the initiation of normal female puberty and the neuropathologic mechanism by which hypothalamic lesions induce sexual precocity. TGFα appears to exert these effects by stimulating the release of luteinizing hormone-releasing hormone (LHRH), the neurohormone that controls sexual development (20,44). While these studies provide

an essential framework towards establishing the importance of TGFα in female reproductive development, they do not demonstrate that selective activation of TGFα gene expression in developing animals leads to the precocious advent of puberty. The present study addresses this issue by using transgenic mice genetically engineered to overexpress a metallothionein-human TGFα (MT1-hTGFα) fusion gene. The results provide direct evidence for the notion that activation of hypothalamic TGFα gene expression leads to enhanced LHRH release and initiation of reproductive cyclicity. They also, however, revealed that TGFα acts on the ovary to modulate the stimulatory effect that the centrally-initiated increase in gonadotropin secretion exerts on steroidogenesis and ovarian maturation.

The transgenic mice used in this study have been shown to express the MT1-hTGFα transgene in a wide variety of tissues including liver, lung, kidney, reproductive tract and brain (31). Of particular interest is the finding made in the present study, that heavy metal-induced overexpression of the transgene does not increase hTGFα mRNA levels uniformly throughout the brain but, instead, it does so more predominantly in the medial basal hypothalamus and dentate gyrus of the hippocampus. Within the hypothalamus the greatest levels of expression were seen in the internal layer of the median eminence, arcuate, ventromedial and dorsomedial nuclei, all components of the neuroendocrine anatomical complex that governs LHRH release (45). While the mechanisms responsible for this region-related activation of TGFα gene expression are not known, the mere fact that the TGFα transgene is preferentially expressed in the hypothalamus provides an important piece

of evidence in support of its involvement in reproductive development. The arcuate and ventromedial nuclei participate in mediating the inhibitory effect of gonadal steroids on LHRH release and contain neuronal populations able to affect LHRH neuronal function (45,46). On the other hand, non-neuronal glial cells in the ME may also play a role in the regulation of LHRH secretion (47). Tanycytes have been found to envelop LHRH nerve terminals and separate LHRH neurosecretory endings from the perivascular space via their endfeet (47,48). Additional evidence supporting a role for glial cells in the control of LHRH neuronal activity stems from the finding that 50% of glial cells in the ME contains estrogen receptors, in contrast to glia in other hypothalamic areas (49). Since LHRH neurons do not have estrogen receptors (50), glial cells of the ME may be involved in the estrogen-dependent regulation of LHRH release.

Hypothalamic TGFα of glial origin may stimulate the release of LHRH indirectly by enhancing, via a paracrine/autocrine mechanism, the production of neuroactive substances from glial cells adjacent to LHRH neurons and/or LHRH nerve terminals. This is suggested by the ability of exogenous TGFα to stimulate LHRH secretion from the ME (20), the absence of EGF receptors in LHRH neurons as opposed to their detection in glial cells (Ma et al., Prog. 73rd Ann. Mtg. Endocr. Soc., p. 303, 1991; Ma et al., 1993, in preparation, Chapter 2), and the response of hypothalamic astrocytes, but not cerebellar astrocytes, to TGFα with an increase in TGFα mRNA expression (Ma et al., 1993 in preparation, see Chapter 3). Prostaglandin E_2 may represent one of the neuroactive substances mediating the

effects of TGFα on LHRH neurons, since TGFα induces PGE_2 release from the ME and its effect on LHRH release is abolished by blockade of prostaglandin synthesis (20). Furthermore, in recent experiments, we have found that TGFα increases PGE_2 release from purified hypothalamic astrocytes in culture (Berg-Von der Emde et al., unpublished). We have postulated that TGFα contributes, via this mechanism, to the acquisition of female reproductive competence. That this may indeed be the case is indicated by the finding that acute activation of hTGFα transgene expression by injection of zinc chloride led to an increase in LHRH release from the ME.

Consistent with the pleiotropism of hTGFα overexpression in these transgenic animals, hTGFα mRNA was also abundantly expressed in the ovaries, a target organ of gonadotropins and a major reproductive endocrine gland. In contrast to its stimulatory effect on hypothalamic LHRH release, activation of the hTGFα gene expression in the ovary led to inhibition of gonadotropin-stimulated ovarian steroidogenesis. Thus, zinc chloride-induced activation of the transgene resulted in inhibition of *in vitro* hCG-stimulated secretion of both androstenedione, a thecal-interstitial cell product, and estradiol, a steroid produced by follicular granulosa cells. These findings are in keeping with earlier observations showing that TGFα reduces hCG-stimulated accumulation of androstenedione in bovine thecal cells (51) and that treatment with the epidermal growth factor (EGF), a structural and functional homolog of TGFα (52,53), inhibits hCG-stimulated estradiol production in porcine thecal cells (28) and rabbit ovary (54), and androgen production from rat and bovine thecal cells (51,55).

While the exact mechanism by which TGFα inhibits gonadotropin-stimulated steroidogenesis remains to be defined, evidence exists that the suppressive effect of TGFα and EGF on gonadotropin-dependent estradiol secretion is exerted via inhibition of aromatase activity (27,56,57). There is also evidence (51) that the suppressive effects of TGFα on androgen production may be related to the mitogenic activity of TGFα, which by stimulating cell proliferation, may decrease biochemical differentiation (i.e., the androgen-producing capacity of thecal-interstitial cells).

An extremely interesting outcome of TGFα chronic overexpression was the marked increase in the number of smaller ovarian antral follicles observed in mice treated with zinc chloride throughout prepubertal development. This increase may be due to the elevated serum FSH levels resulting from the activation of LHRH release, and to effects of TGFα exerted directly on ovarian cells. It has been recently shown that EGF and TGFα inhibit the spontaneous initiation of apoptosis seen in cultured granulosa cells of follicles (30), a finding suggestive of a role for EGF/TGFα in preventing follicular atresia. The increased number of small antral follicles seen in animals overexpressing TGFα is consistent with this view.

It would appear that in spite of promoting the viability of small follicles, TGFα exerts an inhibitory effect on subsequent follicular development. It is plausible that, as a consequence of this inhibition of gonadotropin-supported follicular growth, the occurrence of first estrus is delayed in TGFα-overexpressing mice. Nevertheless, TGFα-overexpressing animals initiated estrous cyclicity much more rapidly than non-transgenic controls after occurrence of the first estrus,

suggesting that as small antral follicles accumulate in the presence of elevated FSH and LH levels, the output of estradiol increases sufficiently to cornify the vaginal epithelium and trigger a preovulatory surge of gonadotropins. This surge would then trigger ovulation of those follicles that succeeded in reaching a periovulatory condition under the persistent TGFα-dependent, LHRH-driven gonadotropin stimulation.

The dual antagonist actions of TGFα on the hypothalamic-pituitary-ovarian axis of developing animals suggested by the aforementioned observations, was conclusively demonstrated by the experiments involving cross-transplantation of transgenic and normal ovaries. Non-transgenic ovaries placed under the control of a transgenic hypothalamus precociously initiated estrous cyclicity and ovulation. In contrast, the timing of first estrus was considerably delayed and normal estrous cyclicity was prevented in normal mice grafted with transgenic ovaries. These observations strongly suggest that intact TGFα-overexpressing animals initiate estrous cyclicity because the central activation of TGFα gene expression overrides, by enhancing gonadotropin secretion, the inhibition of ovarian function brought about by expression of the TGFα transgene in ovarian cells. The grafting of transgenic ovaries into normal animals is particularly enlightening, because it combines a transgenic approach that results in pleiotropic expression of the TGFα gene with a tissue grafting technique that allows the *in vivo* isolation of the organ of interest to study the consequences of gene overexpression. For all practical purposes, this procedure allows the study of organ-specific gene expression without the need of

using tissue-specific promoters in the construction of transgenes.

Taken together, these and our previous findings (21) demonstrate that hypothalamic TGFα is a physiological component of the developmental process by which the hypothalamus controls the advent of normal female sexual maturation. The results also indicate that ovarian TGFα utilizes a paracrine regulatory loop to modulate the facilitatory action of gonadotropin on ovarian growth and function.

## ACKNOWLEDGEMENTS

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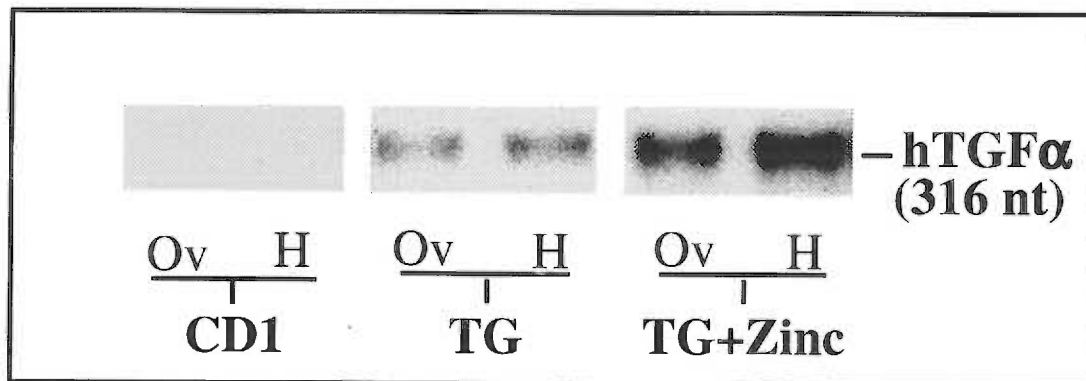
Figure 1. Detection of human (h) TGFα mRNA in the ovaries (Ov) and hypothalamus (H) of prepubertal transgenic mice (TG) carrying a hTGFα fusion gene under the control of the zinc-inducible mouse metallothionein 1 promoter. hTGFα mRNA is expressed even without zinc treatment (middle panel) and is strongly induced 5 h after a single s.c. injection of zinc chloride (right panel).
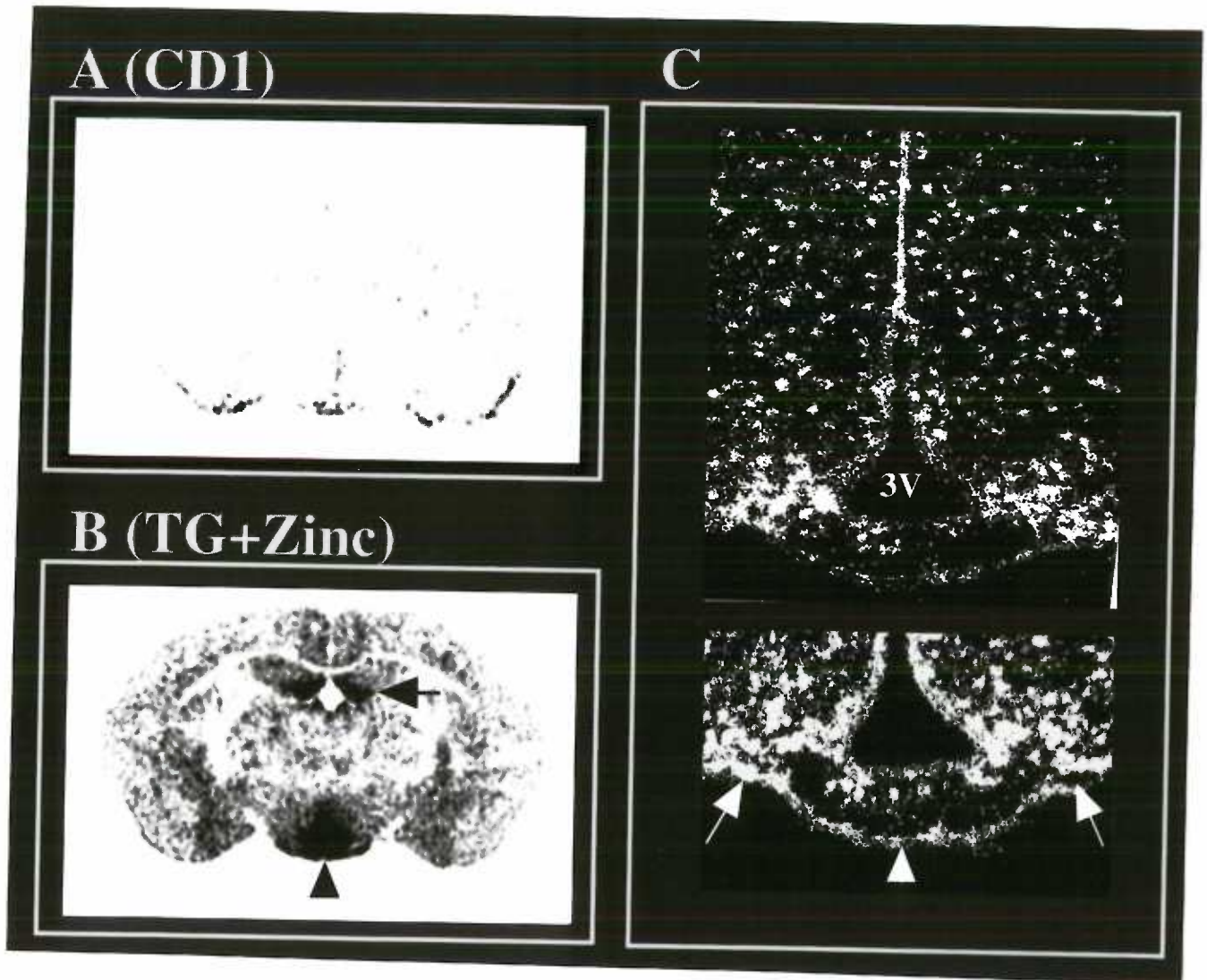
Figure 2 Preferential expression of hTGFα mRNA in the hypothalamus of 28-day-old prepubertal mice 5 hr after a single s.c. injection of zinc chloride, as assessed by hybridization histochemistry. A) Lack of specific hybridization signal in the brain of CD1 controls. B) Low magnification view of the brain showing preferential expression of hTGFα mRNA in the hypothalamus (arrowhead) and dentate gyrus of the hippocampus (arrow). C) Higher magnification view of the medial basal hypothalamus showing intense cellular hybridization of the hTGFα cRNA probe, particularly in the arcuate nucleus (arrows) - median eminence (ME) (arrowhead) region. A & B = 6.5x; C-upper panel = 27x and C-lower panel = 30x.

Figure 3. Detection of hTGFα mRNA by hybridization histochemistry in the ovary of 28-day-old transgenic mice carrying a hTGFα transgene, 5 h after induction of the transgene expression by a s.c. injection of zinc chloride. Notice the intense hybridization signal in thecal and granulosa cells of antral follicles (arrows) (17x).
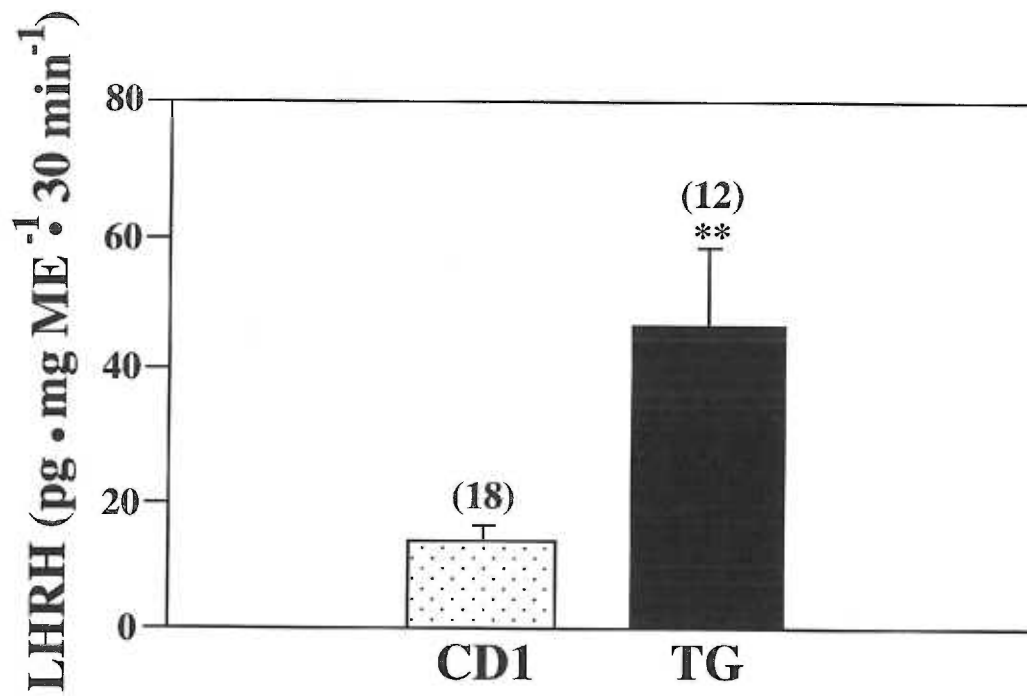
Figure 4. Increased _in vitro_ release of LHRH from the ME-ARC region of mice carrying a hTGFα fusion gene, 5 h after induction of the transgene expression by a s.c. injection of zinc chloride. CD1 = control mice; TG = hTGFα transgenic mice. In this and subsequent bar graphs, bars are mean ± SEM, and numbers in parentheses are number of animals per group. ** = $p < 0.01$ vs CD1 control group.
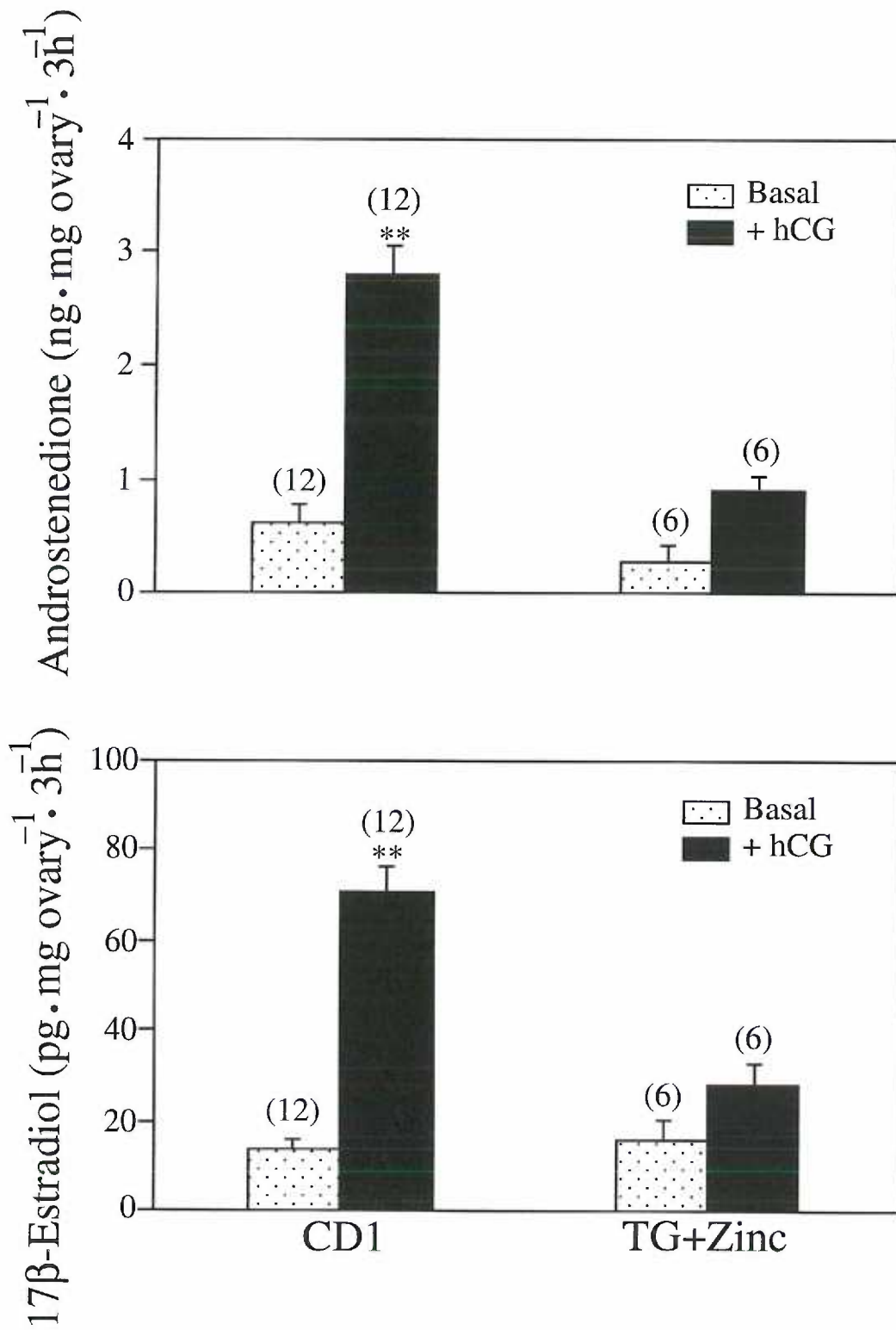
Figure 5. Inhibition of hCG-induced androstenedione (upper panel) and estradiol (lower panel) release <u>in vitro</u> from the ovary of TGFα transgenic mice after activation of hTGFα gene expression by a single s.c. injection of zinc chloride. The ovaries were collected for incubation 5 h after the injection.
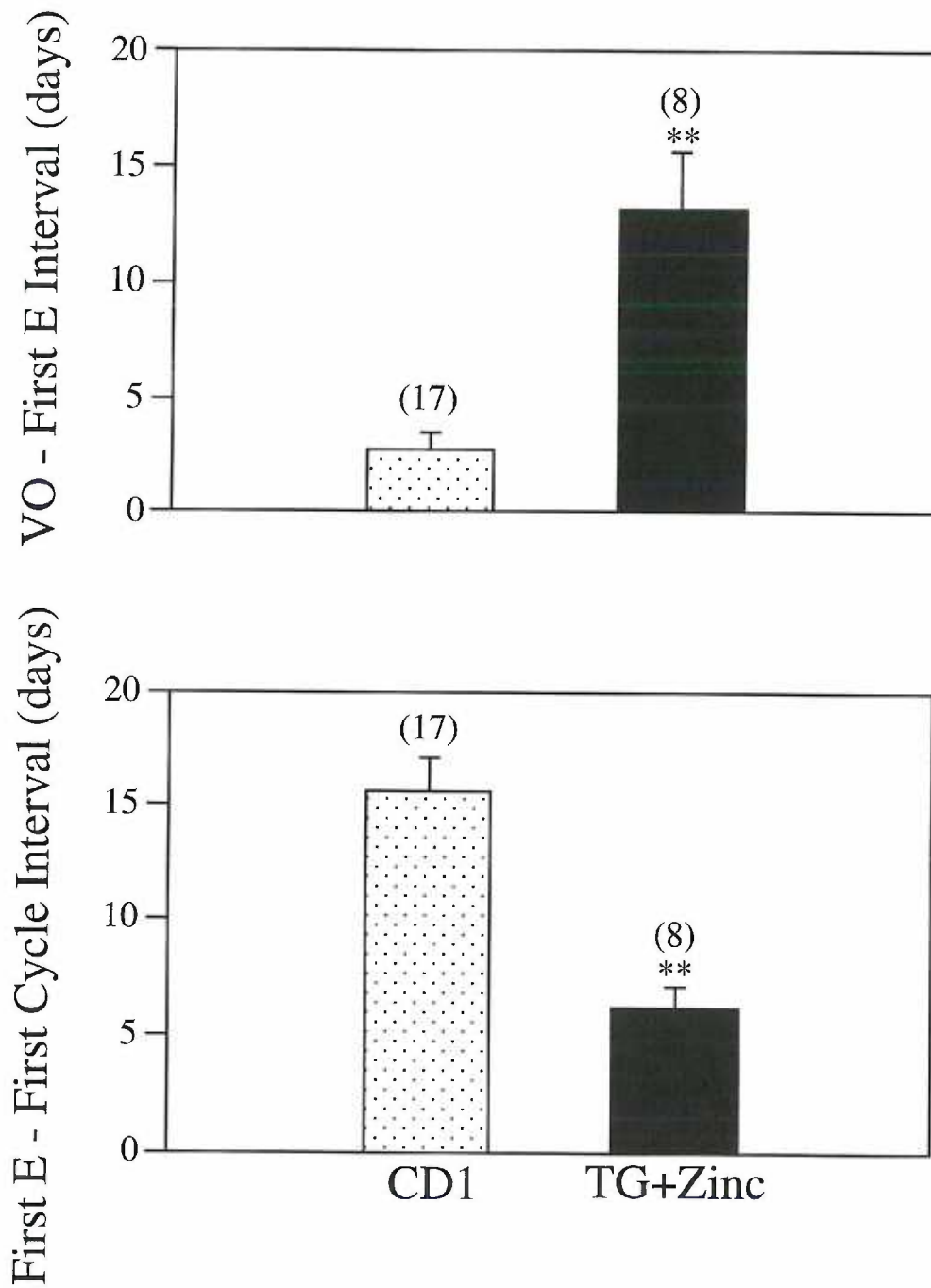
Figure 6. Effect of zinc-induced hTGFα overexpression on the onset of reproductive capacity of transgenic mice carrying a hTGFα fusion gene. Zinc chloride was administered in the drinking water to both controls and transgenic animals from postnatal day 22 onwards. VO = vaginal opening; E = estrus. ** = p < 0.01 vs. CD1 controls.
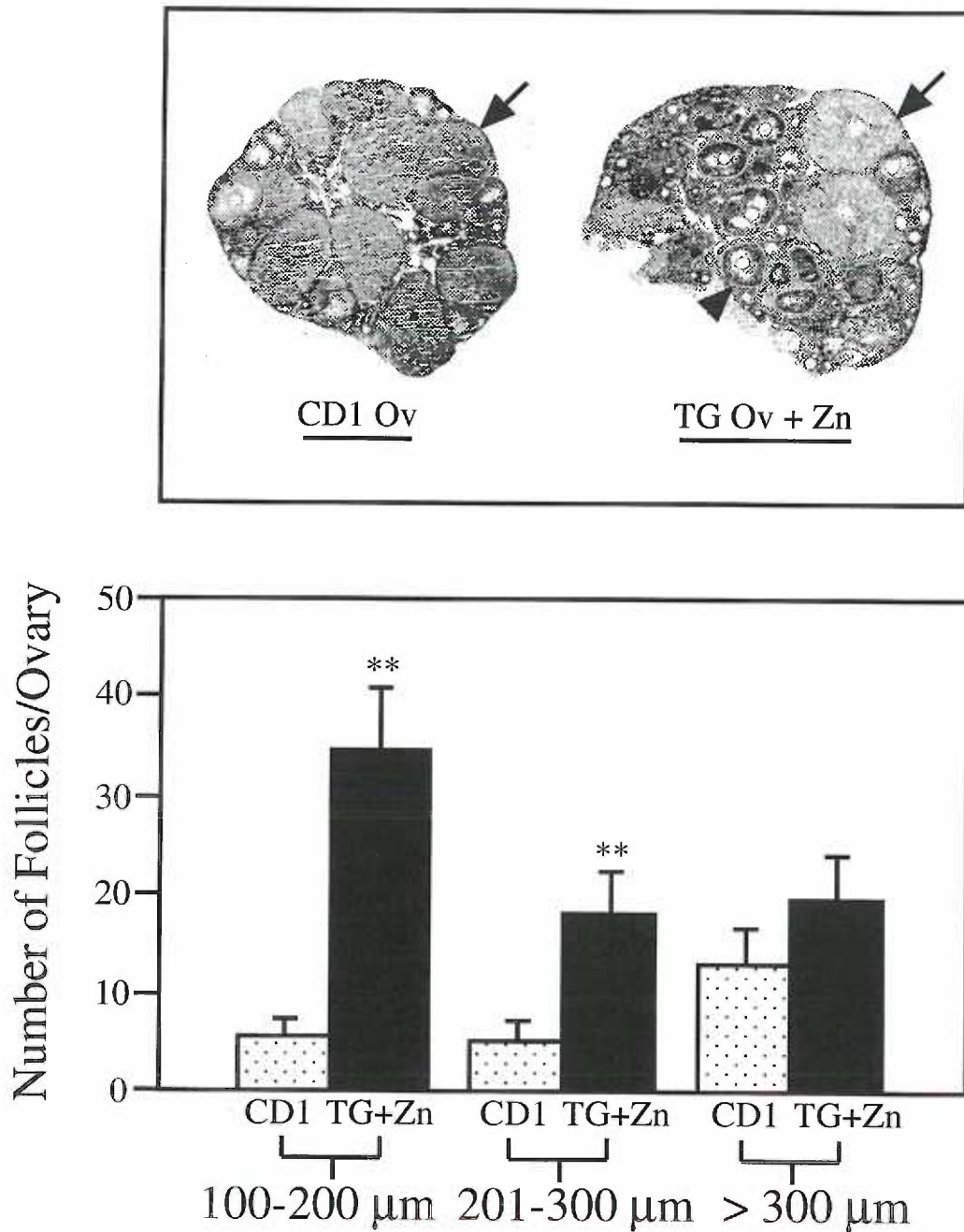
Figure 7. Upper Panel: Histological aspect of the ovaries from CD1 control animals (seven ovaries) and transgenic mice (six ovaries) in which TGFα overexpression was chronically activated by administration of zinc chloride in the drinking water. All the ovaries were collected on the first diestrus after occurrence of the first estrous cycle. Notice the paucity of corpora lutea (arrows) and the increased number of small antral follicles (arrowhead) in the ovary from a TGFα -overexpressing mouse (19x). Lower Panel: Increased number of small and medium-sized antral follicles in the ovaries of TGFα -overexpressing mice. ** = p < 0.01 vs. CD1 controls. The ovaries from transgenic mice not treated with zinc had a number of smaller- and medium-sized follicles not significantly different from that of zinc-treated transgenic ovaries.
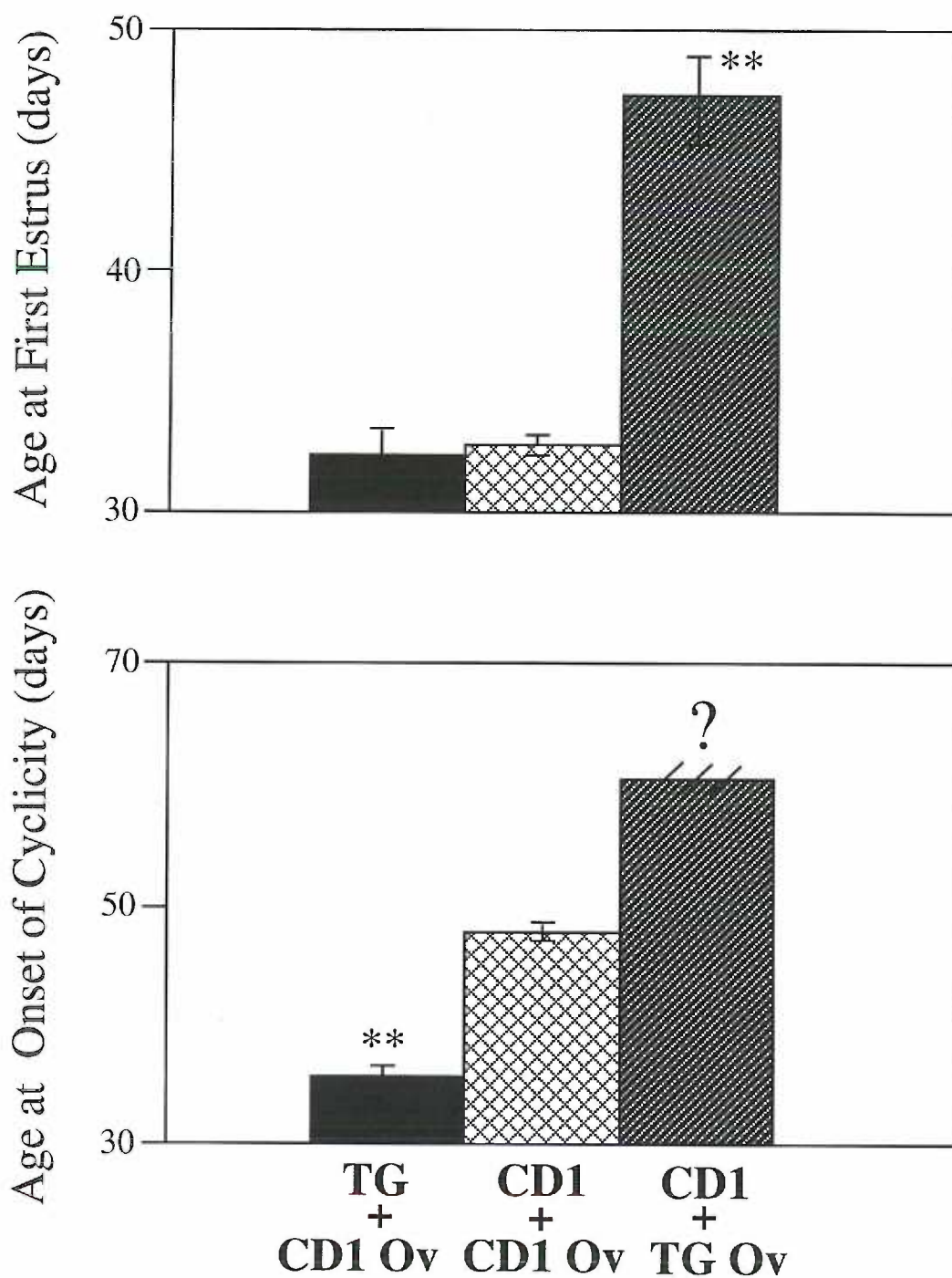
Figure 8. Age at first estrus (upper panel) and onset of estrous cyclicity (lower panel) in TGFα - overexpressing animals grafted with normal ovaries from CD1 controls (TG + CD1 Ov), CD1 control mice bearing autologous ovarian grafts (CD1 + CD1 Ov), and CD1 controls bearing transgenic ovarian grafts (CD1 + TG Ov). Starting 12 h after transplantation, all animals received zinc chloride in the drinking water. Notice the marked delay in age at first estrus and the lack of estrous cyclicity in normal mice grafted with TGFα -overexpressing ovaries, and the advancement of estrous cyclicity in TGFα -overexpressing mice grafted with normal ovaries. ** = p < 0.01 vs. all other groups.
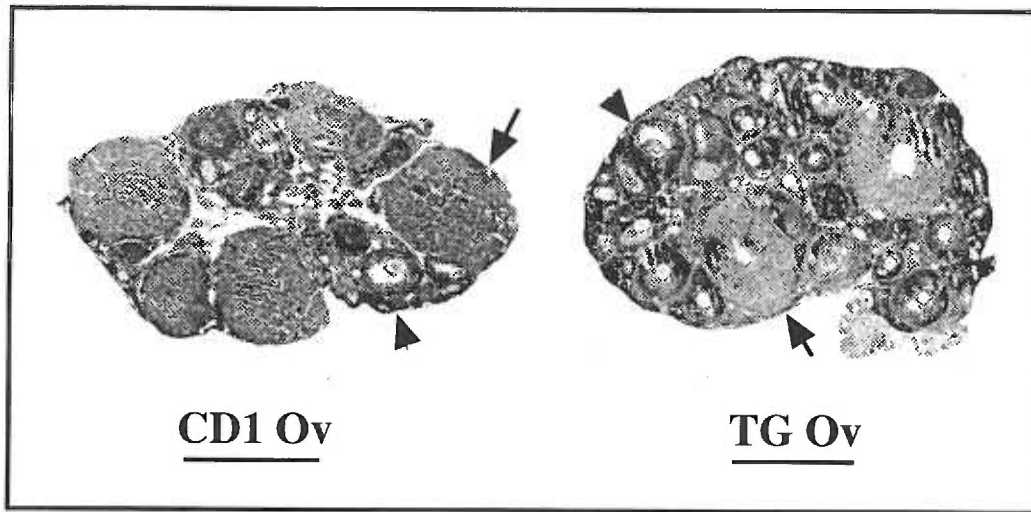
Figure 9. Histological aspect of ovaries from CD1 mice placed under the control of a TGFα-overexpressing hypothalamic-pituitary unit (CD1 Ov) and ovaries from TGFα transgenic mice placed under the control of a normal hypothalamic-pituitary unit. In both cases, hTGFα gene expression was chronically activated by administration of zinc chloride in the drinking water. Notice the abundance of corpora lutea (arrows) in the normal ovary controlled by a TGFα-overexpressing hypothalamus-pituitary unit, and the paucity of corpora lutea and abundance of small antral follicles (arrowheads) in TGFα-expressing ovaries controlled by a normal hypothalamic-pituitary unit (26x).

Developmental Expression of Transforming Growth Factor Alpha (TGFα)

mRNA in the Hypothalamus of Female Rhesus Macaques

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Abstract

Studies in female rats have shown that TGFα is able to stimulate luteinizing hormone-releasing hormone (LHRH) release from median eminence nerve terminals, and that expression of the TGFα gene in the hypothalamus increases during both the initiation of normal puberty and after hypothalamic lesions that result in sexual precocity. Since blockade of epidermal growth factor receptors, which mediate TGFα actions, delayed the normal timing of puberty, whereas TGFα overexpression in transgenic female mice accelerated the acquisition of reproductive capacity, we have suggested that TGFα is an integral component of the neuroendocrine process that underlies the initiation of normal female puberty. The present studies were undertaken to determine if hypothalamic expression of the TGFα gene changes during sexual development of non-human primates.

Oligodeoxynucleotides complementary to evolutionarily conserved DNA sequences of the TGFα gene were used to isolate from rhesus monkey brain RNA, using the reverse transcription-polymerase chain reaction, a 364 base pair cDNA fragment spanning the entire TGFα coding region. DNA sequencing revealed that monkey TGFα in this region has 97% sequence homology to human TGFα at the nucleotide level, and 98% homology at the protein level. The isolated TGFα cDNA served as a template for the synthesis of radiolabeled antisense RNA probes, which were then used to quantitate, by RNase protection assay, the changes in hypothalamic TGFα mRNA during sexual development and to localize, by hybridization histochemistry, the cells that produce TGFα in the hypothalamus.

TGFα mRNA levels were elevated during neonatal life (<6-months-old), when gonadotropin secretion is also increased, and low during juvenile development (1-2 years of age) at the time when LHRH secretion is minimal. Thereafter, they increased more than 10-fold at the expected time of puberty (about 3 years of age). Hybridization histochemistry demonstrated the presence of TGFα mRNA in cells scattered throughout the hypothalamus, but more predominantly in the median eminence, suprachiasmatic region, optic chiasm and cells along the wall of the third ventricle. The results suggest that an increase in hypothalamic TGFα gene expression may contribute to the activation of LHRH secretion leading to the initiation of female puberty in primates.

Abbreviated Title: Hypothalamic TGFα and primate puberty

Introduction

In higher primates, including humans, the onset of puberty is preceded by a protracted period of hypothalamic quiescence during which luteinizing hormone-releasing hormone (LHRH) output is minimal. That LHRH neurons themselves are not a limiting factor for puberty to occur is indicated by the ability of excitatory neurotransmitters functionally coupled to LHRH neurons to enhance gonadotropin secretion and elicit precocious puberty in immature rhesus monkeys (1). Although activation of these neurotransmitter systems may require synaptic inputs originated in brain regions outside the hypothalamus, surgical isolation of the primate hypothalamus does not prevent the acquisition of sexual maturation, but instead results in sexual precocity (2). Thus, it is clear that all the elements required for the peripubertal activation of the LHRH neuronal network are contained within the hypothalamus. Because the timing of puberty is highly reproducible within individuals of the same species, the mechanisms that set the process in motion are likely to involve genes whose transcriptional activation is under strict developmental control. Brain growth factors represent a class of such molecules, as their gene expression increases in specific developmental windows and they are able to promote not only neuronal survival and glial proliferation, but also affect differentiated neuronal and glial functions (3).

In experiments conducted in rodents, we have identified TGFα as a growth factor molecule involved in the etiology of normal female puberty and sexual

precocity induced by brain injury. Specifically, we showed that the genes encoding TGFα and its receptor, the epidermal growth factor receptor (EGFR) are expressed in the hypothalamus of developing female rats (4,5) and that TGFα-induced activation of EGFR results in LHRH release (4). This effect is mediated by prostaglandins (4) and does not require a change in LHRH gene expression (6). Both TGFα and its receptor were localized in glial cells of astrocytic lineage, as well as tanycytes of the third ventricle and median eminence (5,7). TGFα mRNA and its protein product were predominantly expressed in astrocytes of hypothalamic nuclei concerned with the control of LHRH secretion, and TGFα mRNA levels were found to increase markedly in these regions at the time of puberty (7). Moreover, TGFα gene expression was shown to be up-regulated by ovarian steroids, thus providing a link between the pubertal changes in hypothalamic TGFα mRNA and the increase in circulating steroid levels that accompanies the onset of puberty. Blockade of EGFR targeted to the region of the median eminence, where LHRH nerve terminals release LHRH into the portal vessels, delayed puberty (7) suggesting that a site-specific activation of EGFR is an essential component of the neuroendocrine process that leads to reproductive capacity. That a deranged expression of the genes encoding TGFα and its receptor is able to cause sexual precocity was demonstrated by the finding that lesions of the anterior hypothalamus, which advance puberty, are followed by increased levels of TGFα and EGFR mRNAs and their corresponding protein products in reactive astrocytes surrounding the lesion site (6,8). Pharmacological blockade of EGFR, this time targeted to the site of injury,

prevented the advancing effect of the lesion on puberty (8), demonstrating that -- at least in rodents -- activation of the hypothalamic TGFα ligand-EGF receptor system plays a pivotal role in the etiology of sexual precocity of cerebral origin. Most recent studies (Ma et al., 1993 in preparation, see Chapter 4) have shown that overexpression of TGFα gene in the hypothalamus of transgenic female mice, leads to enhanced LHRH release and hastens the acquisition of reproductive capacity.

These findings raise the intriguing possibility that primate puberty, which is governed mostly -- if not exclusively -- by brain-originated, gonadal-independent mechanisms, is under TGFα regulatory control. Identification of the brain factors that control sexual development in higher primates is a fundamental issue in neurobiology. It was the objective of this study to examine the hypothesis that TGFα is an integral component of the process by which the brain controls the initiation of puberty in primates. To accomplish this objective, we are using a highly sensitive technique to study the ontogeny of TGFα gene expression in the female rhesus monkey hypothalamus (present study), and a gene transfer-grafting methodology to accelerate the pubertal process via a focal, transcriptionally regulated increase in TGFα production near the area that contains neuroendocrine LHRH neurons (future studies).

Materials and Methods

Animals

Brain regions from female rhesus monkeys (*Macaca mulatta*) were obtained through the Tissue Distribution Program of the Oregon Regional Primate Research

Center. Thus far, a total of 13 animals have been used, three ranging from 125 days of gestation to six months of postnatal age (infantile group), two between 8 and 18 months of age (juveniles), three between 3 and 4 years of age (peripubertal) and five between 9 and 14 years of age (adults). Of these, two were used for hybridization histochemistry.

Tissue dissection

The brain regions collected were the medial basal hypothalamus (MBH), the preoptic area (POA), and samples of cerebral cortex (Cc) and cerebellum (Cb). The MBH was dissected by a rostral cut along the posterior border of the optic chiasm, a caudal cut immediately in front of the mammillary bodies and two lateral cuts along the hypothalamic sulci. The POA was dissected by two cuts converging from the lateral edges of the optic chiasm to a point rostral the decussation on the optic nerves. The thickness of both tissue fragments was about 4 mm. The collected tissues were immediately frozen on dry ice and stored at -85 C until RNA extraction.

Cloning of a rhesus monkey TGFα cDNA by reverse transcription polymerase chain reaction (RT-PCR)

RNA preparation: Total RNA was prepared by the acid-phenol method as reported (9,10).

Oligodeoxynucleotides: Oligodeoxynucleotides were synthesized in an Applied Biosystems 391 DNA synthesizer (Foster, CA). An oligonucleotide containing an 18-merpolydeoxythymidine [oligo(dT)] sequence, and Xhol and EcoRI restriction sites (5'-ACGTCTCGAGAATTCTTTTTTTTTTTTTTTTTT-3') was used for reverse

transcription to generate cDNAs from cellular polyadenylated mRNAs. Taking advantage of the sequence similarity between the human and rat TGFα mRNA (11,12), a set of gene-specific oligodeoxynucleotide primers was designed and synthesized. A 364 base pair cDNA fragment spanning the entire monkey TGFα coding region sequence was amplified using a sense oligonucleotide (5'-TGGAGAACAGCACGTCC-3') corresponding to nucleotides (nt) 102-118 in the human TGFα mRNA sequence, and an antisense oligonucleotide (5'-GCGCTGGG-CTTCTCGTG-3') complementary to the down-stream sequence of TGFα mRNA spanning nt 452 to 468.

RT-PCR procedures: Reverse transcription was carried out for 2 hr at 37 C in a 20 μl volume. The reaction mixture contained cerebral cortex RNA (5 μg), 1x RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 0.01 M dithiotreitol (DTT), 0.5 mM of each dNTPs, 20 U of RNasin, 25 pmol of oligo(dT) primer and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). PCR amplification was performed in a 100 μl final reaction volume containing the entire RT reaction mixture and a PCR cocktail consisting of 1x PCR buffer (50 mM KCl; 10 mM Tris-HCl; 0.1% Triton X-100), 1 mM $MgCl_2$, 0.2 mM of each dNTPs, 100 pmol of each 5'- and 3'-end TGFα gene specific primers, 20 U RNasin and 3 units of Taq polymerase (Promega Biotech, Madison, WI). Second DNA strand synthesis was carried out in a reaction that comprised a phase of denaturing (95 C, 1 min), one of annealing (50 C, 5 min) and one of extension (72 C, 40 min). Subsequent amplification of double stranded DNA was achieved by

35 cycles of denaturing (94 C, 1 min), annealing (55 C, 2 min) and extension (72 C, 3 min), followed by a final 15 min extension at 72 C.

Subcloning and sequencing of RT-PCR amplified monkey TGFα cDNA: The amplified and purified TGFα cDNA was subcloned into the SmaI site of the riboprobe vector plasmid pGEM-3Z (Promega). The cDNA was then sequenced by the dideoxynucleotide termination method of Sanger (13) using Sequenase T7 DNA polymerase and a kit (Sequenase Version 2.0) purchased from USB (Cleveland, Ohio, USA).

RNase protection assay

The RNase protection assay employed has been previously reported in detail (8,14). In brief, an antisense ^{32}P-CTP-labeled TGFα cRNA probe was synthesized by *in vitro* SP6 polymerase directed transcription, using the RT-PCR generated monkey (MK) TGFα cDNA as a template. The probe was gel-purified and hybridized (500,000 cpm/tube) to RNA samples (3 μg per tube) or different amounts (30 fg to 2 pg) of *in vitro* synthesized sense MK TGFα cRNA, which served as reference for the quantitation of cellular TGFα mRNA levels. Each sample tube also contained 1 pg of an *in vitro* synthesized fragment (132 nt) of sense MK TGFα RNA, which was utilized as an assay control to normalize for procedural losses. After 18-20 hr of hybridization at 45 C, single-stranded RNA was digested with ribonucleases A and T1, and the protected hybrids were isolated by polyacrylamide gel electrophoresis (5% acrylamide, 7 M urea) and visualized by autoradiography.

The quantitation procedure used was similar to that described earlier (7), the

only difference being that the TGFα mRNA values obtained were normalized to the internal TGFα sense RNA assay control instead of cyclophilin mRNA. This was done because at the time when these experiments were performed a monkey cyclophilin probe was not available.

Hybridization histochemistry

The procedure employed is based on that described by Simmons et al. (15) with modifications (8). Two monkeys were used for fixation of the brain. The monkeys were anesthetized with sodium pentobarbital, perfused with approximately 200 ml saline via an aortic cannula, followed by 6500 ml of 4% paraformaldehyde in borate buffer, pH 9.5. The brains were removed, dissected into blocks which were placed back into the fixative for 2 h. The blocks were then cryoprotected in 10% glycerol/2% DMSO dissolved in 0.1 M phosphate buffer and frozen in isopentane cooled in an ethanol-dry ice bath. Thirty μm sections were obtained with a sliding microtome, adhered to polylysine-coated slides, and dried under vacuum overnight before hybridization. The hybridization buffer was composed of 50% formamide, 0.25 M NaCl, 10 mM EDTA, 10 mM Tris (pH 8.0), and 2x Denhardt's solution; the sections were overlaid with 70 μl of this solution containing 1×10^7 cpm of probe per ml and hybridized for 18-20 hr at 55 C. Posthybridization washes were those recommended. All sections were dipped in NTB-2 emulsion and developed after 3 weeks of exposure.

Results

Comparison of monkey and human TGFα DNA sequences

Sequencing of the monkey TGFα cDNA obtained by RT-PCR showed that the coding region of monkey TGFα has a 97% sequence homology to human TGFα at the nucleotide level, and a 98% homology at the protein level (Fig. 1).

Changes in TGFα mRNA levels during postnatal sexual development

Quantitation of TGFα mRNA levels in the hypothalamus of developing monkeys by RNase protection assay revealed that the mRNA levels were elevated shortly after birth (when gonadotropin secretion is also increased), markedly decreased during juvenile development (12- to 18-month-old animals) at the time when hypothalamic LHRH secretion is minimal, and increased more than 10-fold at the expected time of puberty (Fig. 2). The "adult" group in Fig. 2 is comprised of three peripubertal animals and three adults. No differences in the TGFα mRNA levels were observed between these two developmental phases.

Sites of TGFα mRNA synthesis in the hypothalamus

Hybridization histochemistry using a ^{35}S-UTP radiolabeled antisense MK cRNA probe complementary to the coding region of the monkey TGFα mRNA showed that a major site of TGFα synthesis is the external layer of the median eminence (ME) (Fig. 3 A). Although TGFα mRNA was detected in cells scattered throughout the hypothalamus, intensively hybridizing cells were found immediately above the optic chiasm and in the suprachiasmatic nucleus (Fig. 3B), the area adjacent to the ventral aspect of the third ventricle (Fig. 3C) and along the lateral

wall of the third ventricle (Fig. 3D). Noteworthy, the optic chiasm was found to express high levels of TGFα mRNA in presumptive glial cells.

Discussion

Recent studies in rodents (7,8) have suggested that hypothalamic TGFα of glial origin contributes to the developmental process by which the hypothalamus controls the advent of normal female sexual maturation and by which hypothalamic lesions induce sexual precocity. These effects appear to be due to the ability of TGFα to enhance the secretory activity of neurons producing luteinizing hormone releasing hormone (LHRH) (4; Ma et al., in preparation, See Chapter 4).

Expression of the TGFα gene in the hypothalamus of developing rats was found to be maximal on the afternoon of the first proestrus (7), at the time of the first preovulatory surge of LHRH (16), suggesting that hypothalamic TGFα is involved in the process underlying the pubertal discharge of gonadotropins. Such a notion is supported by the ability of a blocker of EGFR to delay puberty when implanted directly into the median eminence of prepubertal rats (7).

Although many developmental processes described in the rat have been shown to also occur in primates, there are significant differences in the neuroendocrine control of sexual development between the two species. A major difference is that the activity of LHRH neurons in primates is greatly diminished during the juvenile phase of development, increasing in a gonadal-independent manner at the time of puberty, whereas the LHRH neuronal network in rats is capable of functioning at full capacity long before the initiation of puberty. It is then important to initiate studies

aimed at determining if TGFα is also involved in the neuroendocrine control of sexual development in primates. Although preliminary, the results of the present study suggest that this is the case, as TGFα gene expression in the hypothalamus of maturing female rhesus monkeys was found to vary in a pattern that closely matches the developmental pattern of gonadotropin secretion described to occur in these animals (17). The dramatic increase in hypothalamic TGFα mRNA levels observed between the juvenile period and adulthood suggest that TGFα may contribute to the activation of LHRH secretion that characterizes the initiation of puberty in higher primates.

The localization of TGFα mRNA in cells of the primate median eminence further supports this view. While the presence of TGFα mRNA in cells adjacent to the third ventricle and SCN is consistent with earlier findings in the rat, the abundance of TGFα mRNA expression in the optic chiasm and suprachiasmatic region is of special interest, as hypothalamic gliomas compromising this area have been found to result in precocious puberty in humans (18).

Experiments are now in progress to confirm these observations by increasing the number of animals studied in each developmental phase and to directly test the hypothesis that a selective increase in hypothalamic TGFα expression is able to accelerate the initiation of primate puberty.

These initial observations support the notion that TGFα mRNA may not only participate in the control of puberty in rodents, but may also play a significant role in regulating the initiation of this process in higher primates, including humans.

Acknowledgements

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| HUMAN TGFα | Leu Glu Asn Ser Thr Ser Pro Leu Ser Ala Pro Pro Val Ala Ala Ala <u>Val Val Ser His Phe Asn Asp Cys Pro Asp</u> |
| MONKEY TGFα | Leu Glu Asn Ser Thr Ser **Leu** Leu Ser --- Pro Pro Val Ala Ala Ala <u>Val Val Ser His Phe Asn Asp Cys Pro Asp</u> |

| HUMAN TGFα | <u>Ser His Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp Lys Pro Ala Cys Val Cys His Ser</u> |
| MONKEY TGFα | <u>Ser His Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp **Arg** Pro Ala Cys Val Cys His Ser</u> |

| HUMAN TGFα | <u>Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala</u> Val Val Ala Ala Ser Gln Lys Lys Gln Ala Ile Thr |
| MONKEY TGFα | <u>Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala</u> Val Val Ala Ala Ser Gln Lys Lys Gln Ala Ile Thr |

| HUMAN TGFα | Ala Leu Val Val Val Ser Ile Val Ala Leu Ala Val Leu Ile Ile Thr Cys Val Leu Ile His Cys Cys Gln Val Arg |
| MONKEY TGFα | Ala Leu Val Val Val Ser Ile Val Ala Leu Ala Val Leu Ile Ile Thr Cys Val Leu Ile His Cys Cys Gln Val Arg |

| HUMAN TGFα | Lys His Cys Glu Trp Cys Arg Ala Leu Ile Cys Arg His Glu Lys Pro Ser |
| MONKEY TGFα | Lys His Cys Glu Trp Cys Arg Ala Leu Ile Cys Arg His Glu Lys Pro Ser |

Figure 1. Deduced amino acid sequence of monkey TGFα and its similarity to that of human TGFα. The sequence of the mature peptide is underlined. Letters in bold denote amino acid substitutions in the monkey sequence.
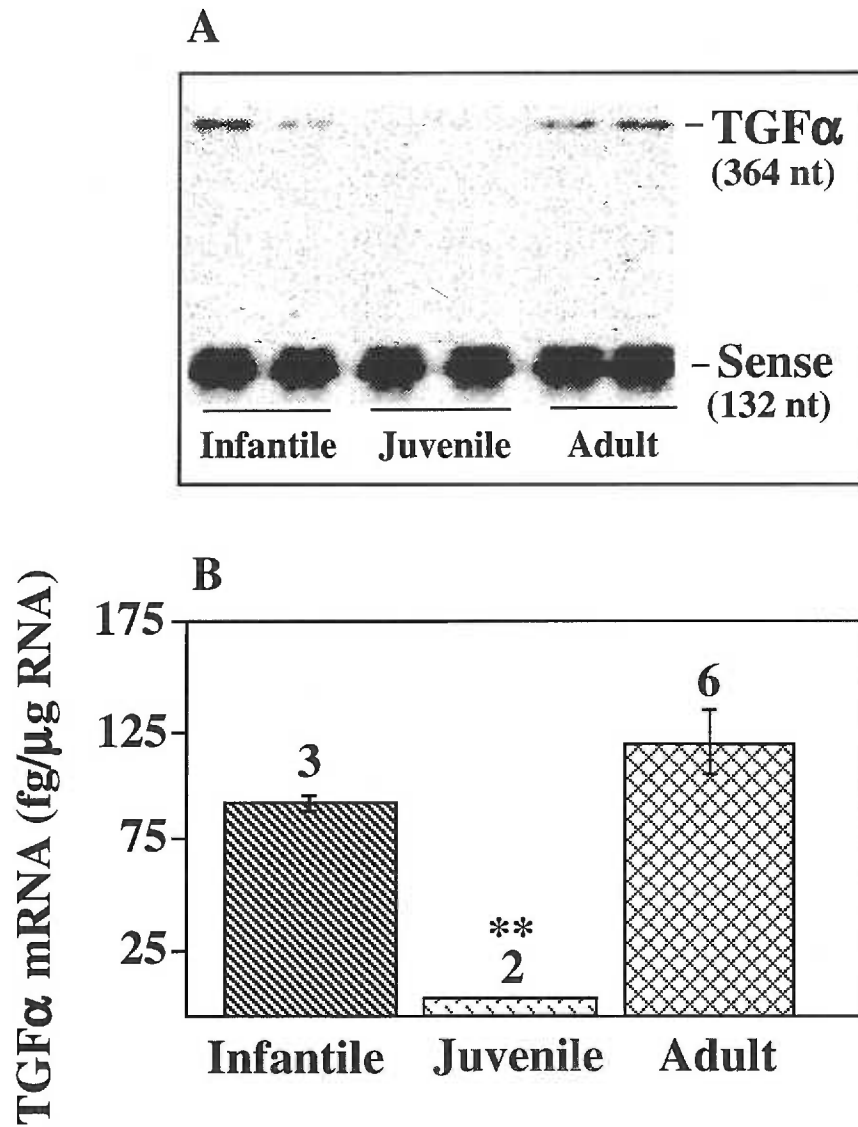
Figure 2 Changes in hypothalamic TGFα mRNA during postnatal development of female rhesus monkeys as determined by RNase protection assay. Panel A shows the developmental profile of hypothalamic TGFα mRNA, and Panel B depicts the quantitation of these changes. ** = $p < 0.01$ vs other developmental phases. Sense = short TGFα mRNA fragment used as an assay control to normalize TGFα mRNA values.
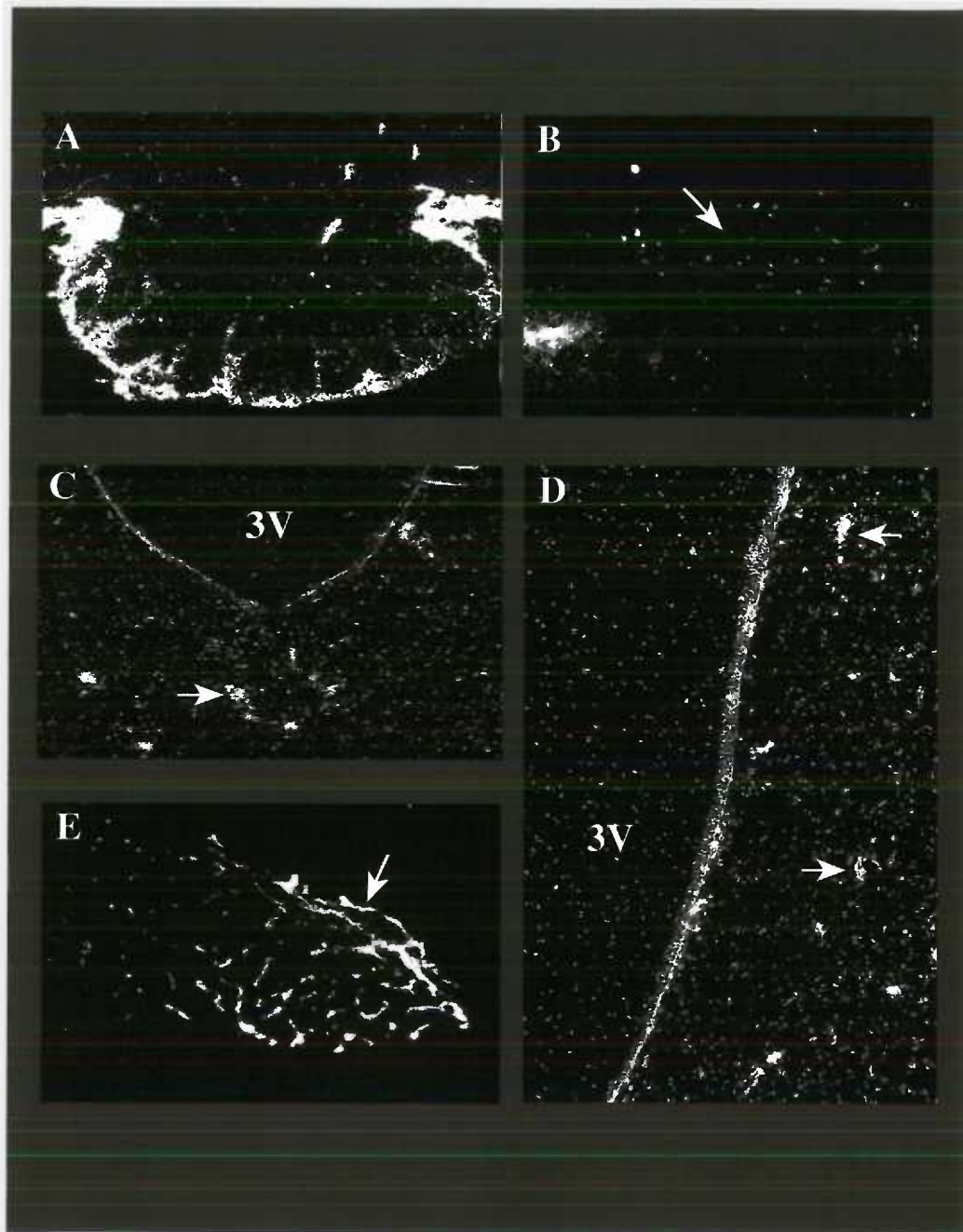
Figure 3. Localization of TGFα mRNA in the monkey hypothalamus by hybridization histochemistry. A = median eminence (30x); B = positive cells in the suprachiasmatic nucleus (30x); C = positive cells adjacent to the ventral aspect of the third ventricle immediately above the ME; D = positive cells scattered along the wall of the third ventricle; E = intense hybridization signal in cells (astrocytes?) of the optic chiasm (11x).

SUMMARY AND CONCLUSIONS

TGFα and Normal Sexual Development

Since earlier studies (Junier et al., 1991, see Chapter 1) showed that hypothalamic injury causes female sexual precocity by activating the secretory process of LHRH neurons and TGFα was implicated in this process, the studies in Chapter 1 were designed to address the issue of TGFα involvement in the normal process of sexual maturation. Chapter 1 (published in *Neuron* 9:657-670, 1992), describes experiments demonstrating that hypothalamic TGFα gene expression is developmentally regulated and involved in the regulation of normal sexual maturation. The results of this study show that TGFα mRNA and TGFα protein are found mostly in astroglia of hypothalamic regions concerned with LHRH control, thus suggesting that glia may contribute to regulating LHRH secretion. This is a finding of potential importance for neurobiology because the regulatory interaction between glial cells and LHRH nerve terminals in the median eminence via TGFα may represent an example of a common mechanism by which glial cells affect neuronal function. The levels of TGFα gene expression in the hypothalamus were dramatically increased at the time of puberty, but no such change was found in the cerebellum, a brain region irrelevant to neuroendocrine functions. Moreover, TGFα gene expression was increased by steroids involved in the regulation of LHRH release suggesting that TGFα is a molecule involved in the process by which estrogen stimulates LHRH release at puberty. The ability of a selective inhibitor of EGFR

tyrosine kinase activity to delay the normal onset of puberty when delivered to the median eminence, supports the hypothesis that TGFα of glial origin acts via EGFR-mediated processes to affect sexual development.

The results from the studies in Chapter 2 demonstrate that EGFR, the receptors for TGFα action, are expressed in hypothalamic glial cells, but not in LHRH neurons. This suggests that the stimulatory effect of TGFα/EGF on LHRH release (ref. 49, in Chapter 2) requires the participation of EGFR-bearing glial cells, and further strengthens the notion that activation of hypothalamic glial function contributes to the neuroendocrine mechanism that brings about the onset of puberty.

Autocrine/Paracrine Effect of TGFα in Astrocytes

The ability of astrocytes to produce TGFα and the presence of EGFR in glial cells, but not in LHRH neurons, had led to the hypothesis (in Chapter 3) that TGFα facilitates LHRH release indirectly via an autocrine/paracrine mediated activation of glial function. Experiments reported in Chapter 3 demonstrate that TGFα increases its own mRNA levels in isolated hypothalamic astrocytes, but, surprisingly, not in the cerebellar astrocytes, despite the fact that they express as much EGFR mRNA as hypothalamic astrocytes. This finding indicates that the hypothalamus, a major regulatory component of the neuroendocrine brain, contains a subset of astroglial cells molecularly and functionally different from astrocytes of a brain region not involved in neuroendocrine control.

Furthermore, the presence of estrogen receptor mRNA and the ability of 17ß-estradiol to increase TGFα mRNA levels in cultures of hypothalamic astrocytes

strongly suggests that glial cells of the neuroendocrine brain are targets for estrogen action. The presence of the estrogen receptors in hypothalamic astrocytes, but not in LHRH neurons (Shivers et al., 1983, see Chapter 1) supports the hypothesis that the effects of gonadal steroids on the release of LHRH are mediated, at least in part, by glial cells bearing the appropriate receptors.

An unexpected observation made in this study was that progesterone failed to increase the levels of TGFα gene expression in isolated hypothalamic astrocytes *in vitro* in contrast to its striking effectiveness *in vivo* (Ma et al., 1992, see Chapter 1 and 3). This is an issue that needs to be further pursued because of the possibility that progesterone may affect glial expression of TGFα *in vivo* indirectly by first affecting neuronal function.

Effect of Selective Activation of TGFα Gene Expression on Female Sexual Maturation

The results described in Chapter 1 to Chapter 3 strongly suggest that activation of the hypothalamic TGFα /EGFR system in glial cells contributes to the neuroendocrine process that underlies the initiation of normal female sexual maturation. They do not, however, demonstrate that selective activation of TGFα gene expression in developing animals actually leads to the precocious advance of puberty. The results of the studies from Chapter 4 provide direct evidence for this notion.

Taken altogether, the studies described in Chapter 1 through Chapter 4 demonstrate that hypothalamic TGFα /EGFR of glial origin is a physiological

component of the developmental process by which the hypothalamus controls the onset of female puberty. These studies firmly establish the novel concept that growth factors of glial origin participate in the process by which the brain controls the initiation of mammalian puberty (see Schematic Summary and Conclusion on page 195).

Finally, the findings from the experiments conducted in rodents (Chapter 1 - Chapter 4) raise the intriguing possibility that primate puberty is also under TGFα regulatory control. The preliminary results presented in Chapter 5 show a developmental profile of hypothalamic TGFα mRNA expression that closely matches the developmental pattern of gonadotropin secretion observed in these animals (ref. #17, see Chapter 5), thus suggesting that TGFα may also contribute to the pubertal activation of LHRH secretion in primates. The localization of TGFα mRNA in cells of the primate median eminence and regions of the hypothalamus that appear to participate in controlling LHRH secretion in primates further supports this view. Future studies will employ a gene transfer-grafting methodology to directly test the hypothesis that a selective increase in hypothalamic TGFα expression is able to accelerate the initiation of primate puberty via a focal, transcriptionally regulated increase in TGFα production near the area that contains neuroendocrine LHRH neurons. Successful completion of these experiments will provide new insights into the neuroendocrine mechanisms underlying primate puberty. Because of the striking similarity in the neuroendocrine control of sexual development between human and rhesus monkeys, it is anticipated that the results from these studies will further our

understanding of sexual precocity of normal puberty and cerebral origin in human. Importantly, the distinct possibility exists that the information provided by these experiments may be used to design new and more effective means to treat isosexual premature sexual development.
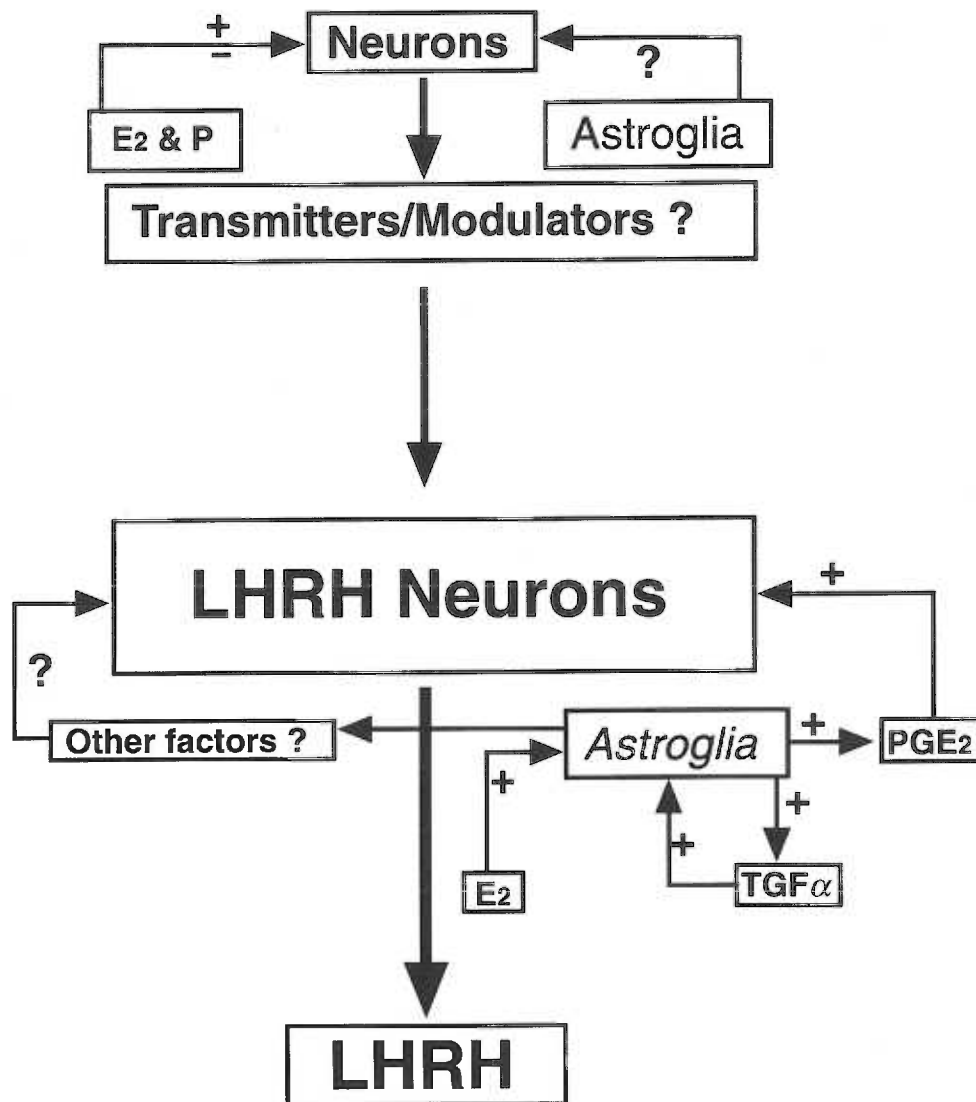
Schematic Summary and Conclusion

Diagrammatic representation of the glial-neuronal interactions that may participate in the control of LHRH neuronal function at puberty. It is postulated that the secretory activity of LHRH neurons is controlled trans-synaptically by neurotransmitters/neuromodulators, and in a paracrine manner by glial cells of the astrocytic lineage. While the glial control may be predominantly exerted at the level of LHRH nerve terminals in the median eminence, the neurotransmitters control may be exerted via synaptic contacts with LHRH neuronal processes and soma. It is envisioned that TGFα of glial origin contributes to the control of LHRH release by stimulating in a paracrine/autocrine manner, the secretion of neuroactive substance (prostaglandin E_2?) from glial cells adjacent to LHRH neurons. Ovarian steroids (estradiol and progesterone) may act on both neurons and/or glial cells functionally connected to LHRH neurons to affect LHRH production. Abbreviations: E_2 = 17β-estradiol; LHRH = luteinizing hormone releasing-hormone; P = progesterone; PGE_2 = prostaglandin E_2; TGFα = transforming growth factor alpha; + = stimulation; - = inhibition.

SCIENTIFIC CREDITS

Chapter #	Author's Name	Role of Author on the Project
1,2	Junier	Tissue collecting, scientific discussion
1,2,4,5	Costa	Immunohistochemistry, hybridization histochemistry
2	Felder	Provided RK-2 antibody against EGFR
2	Hill	Tyrosine kinase receptor assay
3	Berg-von der Emde	Collaboration on astrocyte cultures
3	Moholt-Siebert	Initial assistance in setting up astrocyte cultures
4	Coquelin	Measurement of mouse LH and FSH
4	Dissen	Ovarian transplantation
4	Merlino	Provided TGFα transgenic mice