

# Astrocyte Mitogen Inhibitor Related to Epidermal Growth Factor Receptor

MANUEL NIETO-SAMPEDRO

Epidermal growth factor (EGF) is a well-characterized polypeptide hormone with diverse biological activities, including stimulation of astrocyte division. A soluble astrocyte mitogen inhibitor, immunologically related to the EGF receptor, is present in rat brain. Injury to the brain causes a time-dependent reduction in the levels of this inhibitor and the concomitant appearance of EGF receptor on the astrocyte surface. Intracerebral injection of antibody capable of binding the inhibitor caused the appearance of numerous reactive astrocytes. EGF receptor-related inhibitors may play a key role in the control of glial cell division in both normal and injured brain.

IN THE MAMMALIAN CENTRAL NERVOUS system (CNS), the number of astroglial cells remains practically constant throughout adulthood, division remaining more a potentiality than a frequent event. It has long been suspected that mitogen inhibitors may be crucial to the control of CNS astrocyte populations but evidence of their presence has been lacking. After injury to neurons and neuronal death, astrocytes increase in size, number, and fibrous appearance. What is the signal that triggers the transition of astrocytes from the resting to the reactive state? The question has great importance from the clinical point of view because the formation of a "glial scar" after CNS injury may be the major obstacle to the regeneration of central nerve fibers (1).

Epidermal growth factor (EGF) and its receptor, EGFR, are one of the best characterized mitogen systems (2). In adult rat brain, EGFR immunoreactivity in astrocytes is very weak but increases in the proximity of a brain lesion (3), suggesting a possible role for the EGF-EGFR system in regulating the conversion of resting to reactive astrocytes. Accordingly, I tested the effect of antibody to EGFR (anti-EGFR) (3) on the mitogenic activity of extracts of normal and injured brain. Astrocyte incorporation of [<sup>3</sup>H]thymidine, promoted by brain extracts, was enhanced by antibodies to EGFR (Table 1). It is possible that anti-EGFR could have exerted its effect by mimicking the action of EGF, as reported for other antibodies to EGFR (2). However, anti-EGFR had a much smaller effect on [<sup>3</sup>H]thymidine incorporation in the absence of brain extract. Alternatively, anti-EGFR could have acted on an inhibitory molecule present in the brain extract. This hypothesis was tested by treating extracts of normal or injured brain with anti-EGFR immobilized on protein A-Sepharose. This treatment had the same effect as the direct addition of brain

extract plus antibody to the cultures (Table 1). Thus, anti-EGFR appeared to remove from the brain extracts a mitogen inhibitor with one or more epitopes in common with EGFR. This molecule will be called EGFR-related inhibitor or ERI.

If ERI is involved in the control of astrocyte division, its content ought to be affected by brain injury. This hypothesis was tested by using an enzyme-linked immunosorbent assay (ELISA) to compare the relative amounts of ERI in extracts from the brain of normal animals with those obtained at various periods after lesioning (4). Injury caused about 50% reduction in the levels of brain ERI (Table 2). Minimal levels were observed 6 days after injury and the values returned to normal 30 days after injury.

A reduction in ERI may be responsible for the transition from resting to reactive astrocytes after injury. If so, reduction of ERI by injection of anti-EGFR should cause the appearance of reactive astrocytes. Anti-EGFR was injected into the hippocampus of normal adult rats and nonimmune serum was injected in the contralateral side. The

animals were killed 1, 4, 7, and 20 days after the injection and immunostained with antibodies to EGFR and to glial fibrillary acidic protein (GFAP). One day after injection, a wide area around the anti-EGFR injection site was filled with GFAP-positive astrocytes that were weakly EGFR-immunoreactive. Astrocytes around the injection site became intensely EGFR-positive 4 days after injection, reaching maximal immunoreactivity between 7 and 20 days (Fig. 1A). Injection of normal serum caused the appearance of only a small number of EGFR-positive cells (Fig. 1B). The time course of astrocyte response to the injection of anti-EGFR was similar to the appearance of EGFR immunoreactivity (3) and astrogliosis (5) after injury, reinforcing the idea that brain ERI was involved in controlling the conversion of resting astrocytes into reactive astrocytes.

Injury caused membrane-bound astrocyte EGFR immunoreactivity to appear adjacent to the lesion (3). A similar effect was observed after intracerebral injection of anti-EGFR in uninjured animals. It was possible that membrane-bound EGFR appeared as a consequence of the removal of soluble ERI. The induction of EGFR by removal of ERI was also supported by *in vitro* data. Purified astrocytes were maintained for 3 days in confluent culture, without changing the medium, to permit accumulation of ERI. At this time, one group of cultures was treated for 24 hours with anti-EGFR, and another was kept as control. After 4 days in culture, control astrocytes stained very weakly with anti-EGFR (Fig. 1D) but the cells treated for 24 hours with anti-EGFR showed strong perinuclear and surface EGFR immunoreactivity (Fig. 1C).

The human carcinoma line A431 secretes a 105-kD glycoprotein whose amino termi-

**Table 1.** Effect of treatment of astrocytes with anti-EGFR, soluble and bound to protein A-Sepharose. Extract from injured brain (BE) was prepared and assayed for stimulation of [<sup>3</sup>H]thymidine incorporation as previously described (4). Monospecific rabbit anti-EGFR (3) was added to the culture medium to a final dilution of 1/100. ERI was adsorbed from brain extracts by using bead-bound antibody as follows: Protein A-Sepharose beads (15 mg) (Pharmacia, Piscataway, NJ) were washed with Dulbecco's modified Eagle's medium and Ham's F12 (DMEM and F12) and incubated for 1 hour at 25°C in the same medium (600  $\mu$ l) containing 12  $\mu$ l of anti-EGFR. After washing twice with medium, the Sepharose-protein A-antibody complex was treated with brain extract (2.4 mg protein in 600  $\mu$ l of a 1:1 mixture of DMEM and F12 for 1 hour at 25°C, separated by centrifugation and the supernatant used for mitogen assay after supplementation with fetal calf serum to a final concentration of 1%. Brain extract treatment with Sepharose-protein A alone or with bead-bound normal rabbit serum had no effect on its mitogenic activity. Abbreviations: BE, brain extract obtained 12 days after lesioning; Seph-PA, Sepharose-protein A. Values are means  $\pm$  SD.

Experimental conditions	<i>n</i>	[ <sup>3</sup> H]Thymidine incorporation (cpm/6 hours)
Medium (1% serum)	6	668 $\pm$ 37
+ anti-EGFR	6	2,548 $\pm$ 132
+ BE (2 mg/ml)	4	760 $\pm$ 45
+ BE (2 mg/ml) + anti-EGFR	4	13,183 $\pm$ 1,054
+ BE adsorbed with Seph-PA-anti-EGFR	4	23,640 $\pm$ 2,130

Department of Psychobiology, University of California, Irvine, CA 92717.

nal sequence, immunological properties, glycosylation, and EGF binding are identical or very similar to those of the extracellular domain of EGFR (6). It is generally believed that this glycoprotein is a truncated receptor peculiar to the transformed A431 line. Initial data show that brain EGFR immunoreactivity has a molecular size very different from that of the A431 protein. Protein immunoblot analysis of brain extracts with anti-EGFR showed a main band of apparent molecular size 41 kD (67%) and minor bands at 52 kD (25%) and 69 kD (8%), respectively. It is unlikely that ERI had arisen by nonspecific proteolysis of EGFR during extract preparation. EGFR and its extracellular receptor domain are very stable to proteases (2). Furthermore, EGFR cross-reacting material was unchanged in tissue extracts prepared in the presence of major protease inhibitors and under osmolarity conditions in which organelle disruption was minimized (4).

Thus, brain ERI may play a role in controlling astrogliosis. However, a more complete understanding of the brain EGF-EGFR-ERI system requires information about the nature of the molecules that are physiological carriers of the EGF domain, the cellular source of ERI, and the physiological mechanism of ERI removal after injury. Initial experiments suggest that astrocytes themselves are the probable source of ERI (7). As to the physiological carriers of the EGF domain, low levels of EGF precursor messenger RNA are present in brain (8), but the immunological characterization of brain EGF itself has given controversial results (9). The EGF domain has

been demonstrated in plasminogen activator (10), an enzyme, present in the brain, that is capable of causing astrocyte division (11) and is co-regulated with the EGFR (12). Molecules containing the EGF domain are probably capable of binding to the EGFR, since several examples of high-affinity recognition by the EGFR of molecules with less than 22% sequence homology with EGF have been described (2). Regarding the physiological mechanism of ERI removal after injury, macrophages or microglia (or both) may play an essential role. Implantation of peripheral macrophages in the hippocampus evokes a massive astrogliosis reaction around the implant. Blood-borne monocytes and macrophages invade the injury area soon after tissue damage and may be responsible for the specific removal of ERI. Macrophages and microglia carry on their surface low density lipoprotein receptor molecules (13); these receptors contain the EGF sequence (14) and may be capable of binding ERI. Microglia or macrophages, by removing extracellular ERI and causing the appearance of membrane astrocyte EGFR, may be key cells in the control of astrocyte division during development and after CNS injury.

The relation of ERI to EGFR suggests that the inhibitor may act by binding to mitogens containing the EGF domain. However, direct action on the target cells cannot be excluded at this time. The EGF-EGFR-ERI system, in addition to mediating astrocyte activation, seems to have other roles in brain. The presence of EGFR immunoreactivity in specific neuronal populations (3) supports the notion that EGF or an

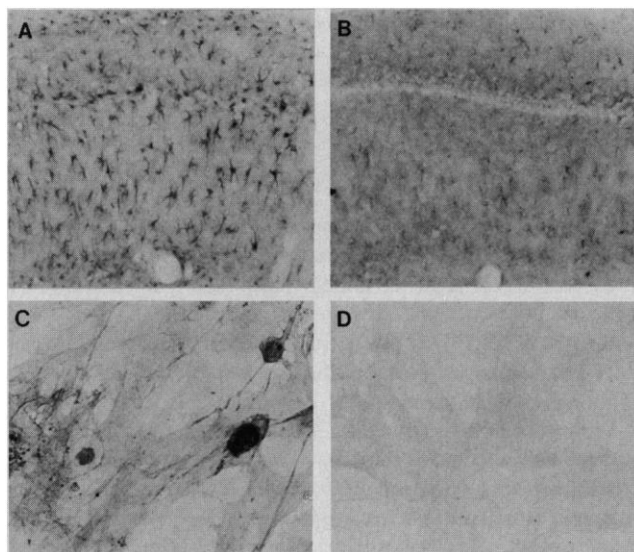
**Table 2.** Decrease in brain EGFR-cross-reacting material after injury. The relative concentration of this material in three extracts from brain tissue adjacent to cortical-hippocampal lesions was determined ( $n = 3$  determinations for each extract) by the ELISA dot-blot technique (4, 16). The concentration in injured tissue relative to that in uninjured brain (day 0) was determined from the linear portion of plots of specific absorbance versus protein concentration (1 to 10  $\mu$ g of extract protein). Results are means  $\pm$  SD.

Days after injury	Extract protein (mg/ml)	Relative concentration of ERI (%)
0	8.9	100
1	8.3	106 $\pm$ 15
3	11.7	56 $\pm$ 7
5	7.6	72 $\pm$ 14
12	8.2	60 $\pm$ 12
30	8.5	102 $\pm$ 6

EGF-like protein also has a neurotrophic role (15). If the biosynthesis of the EGF domain, EGFR, and ERI were regulated independently, such a mechanism would permit the same signal (the EGF domain) to have different biological meanings depending on the developmental state, physiological context, and anatomical location. From a practical point of view, the availability of astrocyte mitogen inhibitors and antibodies to them should offer the possibility of controlling glial scar formation.

I have observed EGFR cross-reactivity in soluble extracts from other tissues (liver, kidney, heart, lung, spleen, and testes). One of these extracts (liver) inhibits astrocyte mitogenic activity but most others do not, suggesting that the detailed structure of the ERI may be tissue-specific. Tissue-specific molecules similar to ERI may have a general role in the regulation of cell growth and differentiation.

**Fig. 1.** Increased number of brain EGFR-positive astrocytes after anti-EGFR injection. (A) Polyclonal anti-EGFR (2  $\mu$ l) was injected unilaterally in the hippocampus, and the appearance of EGFR immunoreactive cells was observed in 30- $\mu$ m sections 7 days after the injection by the peroxidase-antiperoxidase method (4). (B) As a control, the same volume of normal serum was injected in the contralateral side. The distribution and lifetime in the brain of the injected antibody were examined by treating adjacent tissue sections directly with goat antiserum to rabbit immunoglobulin G, without incubation with primary antibody. The antibody diffused in the first 2 days through a comparatively large area and was still detectable 7 days after the injection but not associated to cellular elements. In culture, (D) confluent purified astrocytes barely stained with anti-EGFR. However (C), after 24-hour treatment with anti-EGFR they expressed high EGFR immunoreactivity.



#### REFERENCES AND NOTES

1. P. J. Reier, L. J. Stensaas, L. Guth, in *Spinal Cord Reconstruction*, C. C. Kao, R. P. Bunge, P. J. Reier, Eds. (Raven, New York, 1983), p. 163.
2. G. Carpenter and S. Cohen, *Annu. Rev. Biochem.* **48**, 193 (1979); T. Hunter and J. A. Cooper, *ibid.* **54**, 897 (1985); G. Carpenter, *ibid.* **56**, 881 (1987).
3. M. Nieto-Sampedro, F. Gómez-Pinilla, D. J. Knauer, *Neuroscience* **22**, S279 (1987); F. Gómez-Pinilla, D. J. Knauer, M. Nieto-Sampedro, *Brain Res.* **438**, 385 (1988).
4. Extracts of uninjured and injured rat brain (adult Sprague-Dawley) were prepared and assayed for astrocyte mitogenic activity as previously described [M. Nieto-Sampedro, R. P. Saneto, J. de Vellis, C. W. Cotman, *Brain Res.* **343**, 320 (1985); M. Nieto-Sampedro, *Neurobiol. Aging* **8**, 249 (1987)]. Extracts were also prepared by gentle homogenization of the tissue under isotonic conditions (0.32M sucrose) in the presence of inhibitors of the more abundant protease types (5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, and  $10^{-7}$ M pepstatin). Under these conditions the total protein of the extract decreased by 53% but the ERI content, determined as described below, was not affected (that is, the specific content of ERI

increased). ELISA of EGFR-immunoreactive material was performed with a Biotod microfiltration apparatus (Bio-Rad, Richmond, CA). Serial dilutions in 20 mM HEPES-NaOH, pH 7.2, of pooled extracts from three to five animals, were applied to nitrocellulose paper, immunoreacted with rabbit anti-EGFR (dilution 1/1000) and developed by the immunoperoxidase method with the immunoblot assay kit provided by Bio-Rad. The relative concentration of ERI was determined from the linear portion of serial dilutions (1 to 10 µg of extract protein). ERI content was also determined by using <sup>125</sup>I-protein A binding after incubation with the antibody. Assuming a 1/1 stoichiometry for the binding of antibody to ERI attached to nitrocellulose and to protein A, the ERI content of adult brain extracts was estimated as about 15 ng per milligram of protein.

5. M. Nieto-Sampedro, in *Pharmacological Approaches to the Treatment of Brain and Spinal Cord Injury*, B. Sabel and D. G. Stein, Eds. (Plenum, New York, in press).
6. G. Carpenter, *Annu. Rev. Biochem.* **56**, 881 (1987); W. Weber *et al.*, *Science* **224**, 294 (1984).
7. Astrocytes in culture secrete an inhibitor of their own proliferation [F. Aloisi, C. Agresti, G. Levi, *Neurochem. Res.* **12**, 189 (1987); L. J. Kagen, S. L. Miller, M. Pedrotti, *Exp. Neurol.* **78**, 517 (1982)] and may be one of the ERI sources in rat brain. An ELISA (4) of astrocyte-conditioned medium showed that ERI reached maximal concentration after 48 hours. Furthermore, anti-EGFR potentiated [<sup>3</sup>H]thymidine incorporation promoted by EGF. When used alone, EGF (Collaborative Research, Bedford, MA 01730) at 100 ng/ml was weakly

mitogenic for confluent astrocytes [4087 ± 490 cpm (mean ± SD) incorporated in 6 hours; n = 6; controls identical to those in Table 1] but its mitogenic activity became comparable to that of brain extract in the presence of 1/100 dilution of anti-EGFR (12,264 ± 1,962 cpm in 6 hours; n = 6).

8. L. B. Rall *et al.*, *Nature* **313**, 228 (1985).
9. J. H. Fallon *et al.*, *Science* **224**, 1107 (1984); R. Probstmeier and M. Schachner, *Neurosci. Lett.* **63**, 290 (1986).
10. C. L. Young, W. C. Baker, C. M. Tomaselli, M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, M. O. Dayhoff, Ed. (National Biomedical Research Foundation, Silver Spring, MD, 1976), p. 73.
11. N. Kalderon, *J. Neurosci. Res.* **8**, 509 (1982); *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7216 (1984); G. Moonen *et al.*, *Dev. Brain Res.* **20**, 41 (1985).
12. J. L. Gross, M. N. Krupp, D. B. Rifkin, M. D. Lane, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2276 (1983).
13. D. Giulian and T. J. Baker, *J. Neurosci.* **6**, 2163 (1986).
14. D. W. Russell *et al.*, *Cell* **37**, 577 (1984).
15. R. S. Morrison, H. I. Kornblum, F. M. Leslie, R. A. Bradshaw, *Science* **238**, 72 (1987).
16. R. Hawkes, E. Niday, E. Gordon, *Anal. Biochem.* **119**, 142 (1982); R. Lim, D. J. Hicklin, T. C. Ryken, J. F. Miller, *Dev. Brain Res.* **33**, 49 (1987).
17. This work was supported by grants AG 00538-09A from the NIA and TC-87-01 from the American Paralysis Association. I thank J. T. Broderick for excellent technical assistance, D. J. Knauer for polyclonal antibody to EGFR, and J. Sabarro for invaluable help with immunoblot analysis.

11 January 1988; accepted 3 May 1988

## Coevolution of Sender and Receiver: Effect on Local Mate Preference in Cricket Frogs

MICHAEL J. RYAN AND WALTER WILCZYNSKI

Mate recognition in frogs requires congruence of call characters, such as dominant frequency, and properties of the auditory system, such as frequency sensitivity of inner ear organs. Two neighboring populations of cricket frogs (*Acris crepitans*) exhibit statistically significant differences in the dominant frequency of the advertisement call and the frequency to which the basilar papilla of the inner ear is most sensitive. Call frequency and frequency sensitivity are matched within but differ between populations. These characters usually are negatively correlated with body size, and thus their congruence and coevolution often is explained by pleiotropic effects of size. However, within this species call frequency and frequency sensitivity of the basilar papilla evolved independent of body size, yielding local mate preferences that could contribute to genetic differentiation among neighboring populations.

IN MANY ANIMALS, MALES PRODUCE signals that are used by females for recognition of conspecific mates. Mate recognition requires congruence between the structure of the signal and the response properties of the sensory system that decodes the signal. This occurs in visual, olfactory, and electrosensory modalities and has been especially well documented in acoustic mate recognition systems (1). This congruence is necessary for efficient communication, and during evolution must be maintained by correlated changes in the signal

and the receiver. By promoting assortative mating, these correlated differences in signal and receiver can restrict genetic exchange and promote genetic divergence among populations (2). Thus divergence in courtship signals can be an important component of the speciation process.

Studies of mate recognition systems usually are restricted to interspecific variation, but intraspecific variation in anuran advertisement calls has been investigated by Capranica *et al.* (3). Cricket frogs (*Acris crepitans*) from two localities 2500 km apart in the United States (New Jersey and South Dakota) showed correlated differences in both the dominant frequency of the adver-

tisement call and the frequency sensitivity of the central auditory system. Within one population, females discriminated among synthetic calls that differed in frequency alone. However, these populations were at opposite and extreme ends of a cline in body size. Differences in body size can account for differences in many traits among animals, such as brain size, metabolic rate, and territory size (4). Body size can also influence strongly both the dominant frequency of a frog's advertisement call (5) and the tuning of the peripheral auditory system, especially the best excitatory frequency (BEF) of the basilar papilla (6), the inner ear organ that is used in the reception of the calls produced by this species (7); both are correlated negatively with size.

Large size in cricket frogs is advantageous in arid western environments because it decreases desiccation, and pleiotropic effects of body size on the signal and receiver might be the major factor accounting for variation in the communication system that results in local mating call preferences (3). Such local preferences for natural calls have also been demonstrated for some distantly separated populations (3). Because of the gradual cline of body size variation across longitude in this species and the great distances separating the populations investigated, it is difficult to interpret the effect of any differences in the communication system on reproductive interactions.

We investigated correlated changes in the communication system of this species on a more fine-grained geographic scale. First, we determined whether there were differences in the call frequency and the tuning of the basilar papilla between two geographically proximate populations. Second, we examined whether pleiotropic effects of body size changes alone could generate differences between populations and preserve the match between sender and receiver within populations. Third, we determined whether differences in the mate recognition system between neighboring populations could result in female mate preferences for local calls and thus potentially contribute to intraspecific genetic divergence.

Within its geographic range in Texas, *A. crepitans* exhibits two areas in which there is a large change in dominant frequency of the advertisement call over a relatively short geographic distance. One is in East Texas in the zone of parapatry of the two subspecies of *A. crepitans* (*A. c. crepitans* and *A. c. blanchardi*), and another in Central Texas, near Bastrop, within what is thought to be the continuous distribution of *A. c. blanchardi* (3, 8). We examined 300 advertisement calls from ten cricket frogs each in Austin and Bastrop (9), which are within 65

Departments of Zoology and Psychology, University of Texas, Austin, TX 78712.