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A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor

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Mice lacking the epidermal growth factor receptor (EGFR) exhibit strain-dependent phenotypes ranging from placental to postnatal skin, lung and brain defects. After birth, all mutant mice develop a progressive neurodegeneration in the frontal cortex, olfactory bulb and thalamus, characterized by massive apoptosis and upregulation of c-*fos*. These defects occur in a strain-independent manner, since neither rescue of the placental phenotype by aggregation of diploid 129/Sv EGFR mutant and tetraploid wild-type embryos, nor promotion of lung maturation by transplacental dexamethasone administration alters the course of neurodegeneration. VEGF is not induced during the degenerative process, excluding hypoxia and ischemia as causes of cell death. A migratory disorder is detected in the hippocampus with nests of ectopic neurons, which are also apoptotic. Cerebral cortices from EGFR mutants contain lower numbers of GFAP positive astrocytes, which display reduced proliferation *in vitro*. Since EGFR is expressed in the affected cell-types, these results define a specific function for EGFR in the proliferation and/or differentiation of astrocytes and in the survival of postmitotic neurons.

Keywords: astrocytes/EGF receptor/genetic background/knock-out mice/neurodegeneration

Introduction

The epidermal growth factor receptor (EGFR) is a member of a family of structurally related tyrosine kinase receptors that include erbB2/neu, erbB3 and erbB4 (Earp *et al.*, 1995). The EGFR binds and is activated by several polypeptide growth factors including epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF (HB-EGF), betacellulin and epiregulin (Prigent and Lemoine, 1992; Earp *et al.*, 1995). Ligand binding induces receptor dimerization and activation of the intrinsic tyrosine kinase with consequent autophosphorylation of key tyrosines located at the carboxy terminal tail of the receptor (Prigent and Lemoine, 1992; Earp *et al.*, 1995). Phosphorylated tyrosine residues act as binding sites for proteins containing Src-homology 2 domains (SH2) such as Grb2, SHC and PLCγ which, in turn, activate complex downstream signaling cascades, thus transducing extracellular stimuli to the nucleus (Lemmon and Schlessinger, 1994; Weiss *et al.*, 1997). The specificity of the cellular response is thought to be determined by the nature of the various signaling molecules recruited to the phosphorylated receptor (Earp *et al.*, 1995; Weiss *et al.*, 1997). EGFR dimerization can take place between two identical receptors (homodimerization) or with any of the three other members of the erbB family (heterodimerization), depending on which receptor proteins are expressed in a given cell (Lemmon and Schlessinger, 1994; Earp *et al.*, 1995). This increases the number of signaling pathways that can be activated after EGFR stimulation, thereby augmenting the signaling complexity needed to govern cell proliferation and differentiation (Lemmon and Schlessinger, 1994; Earp *et al.*, 1995; Weiss *et al.*, 1997).

During early mouse development, EGFR protein can be detected in the trophoectoderm of the blastocyst, which is the first epithelium that develops in mammalian embryos (Dardik *et al.*, 1992; Wiley *et al.*, 1992). From midgestation on, the EGFR is expressed in a variety of tissues and organs, but only few studies have determined which specific cell types express the receptor (Adamson, 1990; Partanen, 1990; Derynck, 1992). In adult mice, EGFR expression can be detected in all organs, particularly in the liver and in regenerating epithelia such as skin and gut (Adamson, 1990; Partanen, 1990; Derynck, 1992). Several studies have demonstrated the presence of immunoreactive EGFR in selected regions of the embryonic and adult brain, such as the frontal cortex, striatum, hippocampus and cerebellum (Gómez-Pinillla *et al.*, 1988; Werner *et al.*, 1988; Tucker *et al.*, 1993; Kornblum *et al.*, 1997). Furthermore, EGF and TGFα have also been detected in several brain regions including the striatum and hippocampus (Lazar and Blum, 1992; Seroogy *et al.*, 1993; Tucker *et al.*, 1993; Weickert and Blum, 1995; Kornblum *et al.*, 1997). Although the cellular distribution and developmental appearance of EGFR within these regions have been controversial, it seems that the receptor is present in certain specific neuronal cell populations and mature astrocytes (Gómez-Pinillla *et al.*, 1988; Nieto-Sampedro *et al.*, 1988; Topp *et al.*, 1989; Kornblum *et al.*, 1997). Particularly, EGFR is upregulated in reactive astrocytes after brain injury (Nieto-Sampedro *et al.*, 1988). This astrocytic response, also known as reactive astrogliosis, is characterized by the presence of astrocytes becoming hypertrophic, showing a great increase in glial fibrillary acidic protein (GFAP) expression and the capability to divide (Nieto-Sampedro *et al.*, 1988). EGF has also been shown to induce thymidine incorporation and proliferation in primary astrocytes cultured *in vitro* (Simpson *et al.*, 1982). Recently, it has been

shown that cells of the subependymal layer, a specialized zone of the striatum, are EGFR immunoreactive (for review see Weiss *et al.*, 1996). When cultured *in vitro*, these EGF responsive cells exhibit properties representative of stem cells, in that they are capable of maintaining and expanding themselves over extended periods of time and retain the ability to differentiate into neurons, astrocytes and oligodendrocytes (for review see Weiss *et al.*, 1996). EGF and TGFα have also been shown to increase the survival of cortical and midbrain neurons *in vitro* (Kornblum *et al.*, 1990; Casper *et al.*, 1991; Alexi and Hefti, 1993). These observations suggest that EGFR signaling may play an important role during brain development. In view of the expression pattern of EGFR and its ligands, it is intriguing that EGFR is amplified and overexpressed in human tumors of epithelial (carcinomas; Derynck *et al.*, 1987) and glial origin (glioblastomas; Wong *et al.*, 1992).

The observations that EGFR expression might be of physiological relevance during normal epithelial and neural development were confirmed by the analysis of mice that are defective in EGFR signaling. Mice deficient for the TGFα gene display mild phenotypes consisting of wavy coat, curly whiskers and sporadic eye defects (Luetteke *et al.*, 1993; Mann *et al.*, 1993). The phenotype of these mice is very similar to that of the naturally occurring mouse mutant strains waved-1 (wa-1) and waved-2 (wa-2) (Luetteke *et al.*, 1993, 1994; Mann *et al.*, 1993; Fowler *et al.*, 1995). The wa-1 mutation maps to the TGFα locus (Luetteke *et al.*, 1993; Mann *et al.*, 1993), whereas the wa-2 mutation is a hypomorphic EGFR allele that carries a point mutation in the kinase domain, resulting in a drastically reduced kinase activity (Luetteke *et al.*, 1994; Fowler *et al.*, 1995). This suggests that TGFα/EGFR signaling is critical for the development of normal hair follicles and skin. Besides a reduction in glial fibrillary acidic protein (GFAP) immunoreactivity in wa-1 mutant mice (Weickert and Blum, 1995), no obvious brain abnormalities were reported in any of these mice (Luetteke *et al.*, 1993; Mann *et al.*, 1993; Weickert and Blum, 1995).

Ourselves and others have generated mice which carry a null mutation in the EGFR gene (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). The analysis of EGFR mutant mice revealed a complex role for this receptor during embryonic and postnatal development. Mutant mice are growth-retarded and die at different stages of development depending on their genetic background (Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). In a 129/Sv genetic background, EGFR –/– embryos die around day 11.5 of gestation (E11.5), whereas in other backgrounds, mutant mice can survive until birth (C57BL/6) or to postnatal day 20 (MF1). Death *in utero* most likely results from a defect in the spongiotrophoblasts, a particular epithelial cell layer of the placenta (Sibilia and Wagner, 1995). While the size of the spongiotrophoblast layer is reduced to the same extent in mutant placentas of all genetic backgrounds, it remains to be clarified whether the absence of EGFR influences the severity of the placental defect or whether, in addition, it affects the development of EGFR mutant embryos in a strain-dependent manner. All surviving mutant mice show abnormalities in various epithelia such as skin, hair follicles, eyes and lungs, indicating that EGFR plays an essential role in epithelial cell proliferation and/or differentiation (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995). The lung immaturity is most probably responsible for the majority of mutant newborns being unable to initiate or sustain respiration (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995). We have observed brain defects in surviving EGFR mutant mice and an atrophy of the anterior cerebral cortex has previously been described (Threadgill *et al.*, 1995). The nature, extent and kinetics of the degenerative processes have not been studied and a strain-specific effect could not be excluded. In addition, these defects could have occurred as a consequence of impaired nutrition supply during embryogenesis, attributable to the placental defects or a reduced oxygen supply at birth caused by the lung immaturity. Here, we have characterized in detail the brain defects and demonstrate that EGFR signaling is involved in the proliferation and/or differentiation of astrocytes and survival of postmitotic neurons *in vivo*. We also show that the neurodegenerative disease is strain-independent and develops in all surviving mutant mice.

Results

CNS defects in EGFR mutant mice

EGFR mutant mice of MF1 and C3H background can survive up to postnatal day 20 (P20) with multiple epithelial defects, but develop severe brain abnormalities by P18 (Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). In order to investigate the onset and extent of neurodegeneration, brains of EGFR mutant mice of MF1 and C3H background were isolated at different stages of embryonic and postnatal development. During embryogenesis, mutant brains displayed no gross structural abnormalities when compared with controls (data not shown). At birth (P1) and during the following days (P2–3), the brains of all mutant mice were still comparable with the controls and histological examinations did not reveal any structural changes (data not shown). Between P4 and P6, leptomeningeal hemorrhages appeared on the surfaces of the forebrain and olfactory bulbs of all EGFR mutant brains, heralding the onset of neurodegeneration (Figure 1A and B). Initially, these leptomeningeal lesions were unilateral in some animals (Figure 1B), but later, both hemispheres were consistently affected (data not shown). There were differences in the extent of neurodegeneration between the affected and unaffected hemispheres (Figure 1B), but these usually correlated with the macroscopically visible hemorrhagic lesions. By P13, the forebrain size of the mutants was dramatically reduced (Figure 1D) with the loss of a major part of the frontal cortex and degeneration of the olfactory bulbs (Figure 1F). Conspicuously, the retrosplenial cortex (rc) was spared in all animals investigated (Figure 1F). At later timepoints, the degenerated tissue was replaced by large cystic cavities, covered only by a thin layer of subleptomeningeal cortical tissue (data not shown).

During embryogenesis and until P3, no signs of degeneration and no significant increase in TUNEL staining could be detected in EGFR –/– brains (data not shown). Between P4 and P5, when the leptomeningeal hemorrhages appeared, the first morphological changes were observed in neurons of EGFR –/– frontal cortex,

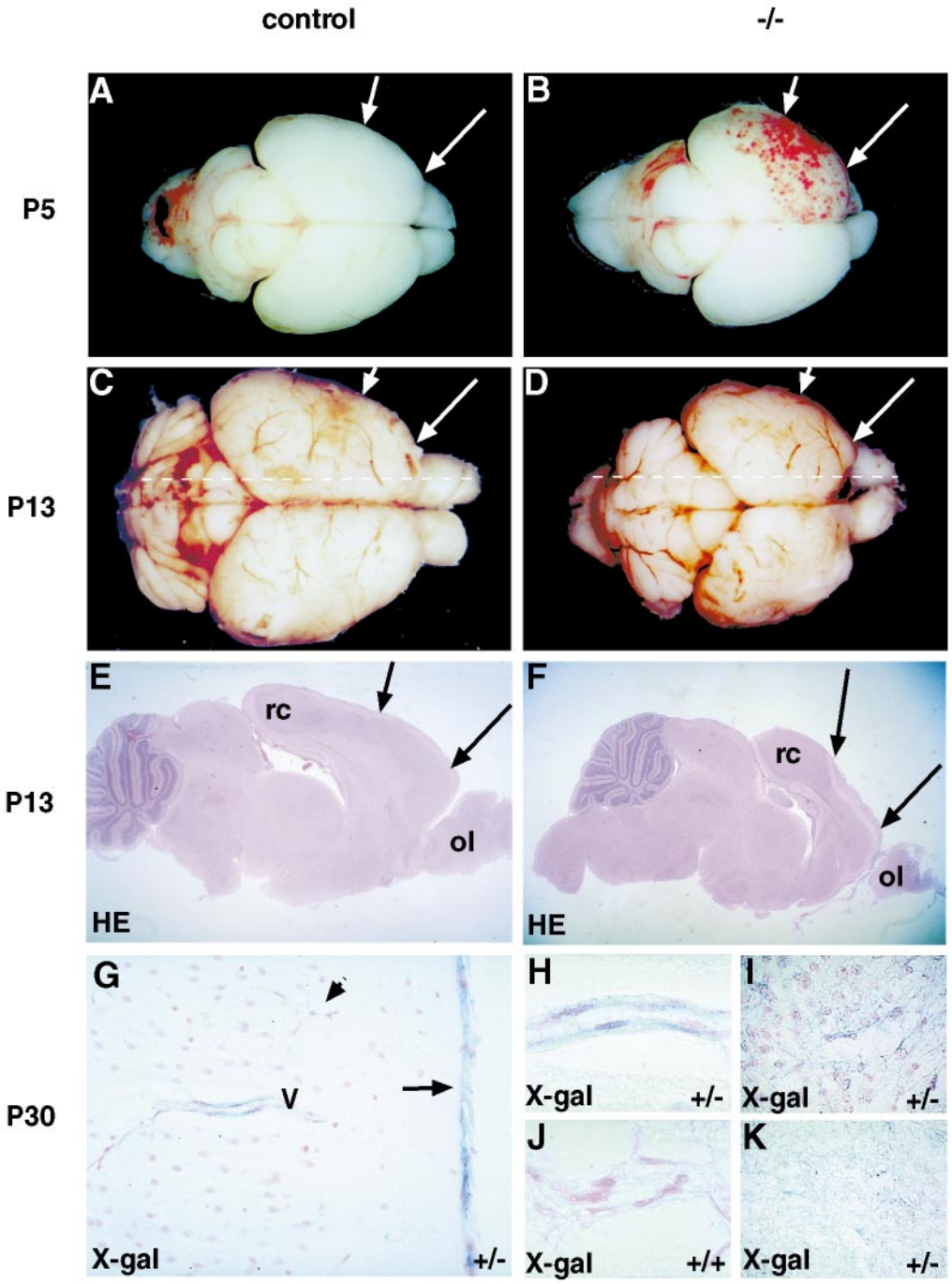

Fig. 1. Cortical degeneration and EGFR expression in mutant brains. Dorsal view of the whole brain of control (A and C) and EGFR –/– mice (B and D) isolated at postnatal day 5 (P5) and 13 (P13). Until P4 the brains of EGFR –/– mice look normal and the size is comparable to the control. (**A** and **B**) At day 5 hemorrhagic lesions appear on the surface of the anterior part of EGFR –/– forebrain (arrows) and olfactory bulbs. (**C** and **D**) By day 13, the forebrain of the –/– is dramatically reduced in size compared with the control. (**E** and **F**) Histological cross-sections at the level of the dotted lines of the P13 brains shown in (C) and (D), respectively. In correspondence to the leptomeningeal hemorrhages, the region of the cortex between the arrows and the olfactory bulbs (ol) is completely degenerated in the mutant brains, whereas the retrosplenial cortex (rc) is spared. A similar kinetic of cortical degeneration with the presence of uni- or bilateral leptomeningeal hemorrhages around P5 was observed in brains of tetraploid rescued and dexamethasone treated mice. (**G**–**K**) EGFR expression monitored by X-gal staining (blue) in P30 (G and H), P3 (I and K) EGFR +/– and P30 (J) EGFR +/+ brains. (G) Histological section through the cortex shows X-gal staining in the leptomeninges (arrow), vessels (V) and weak staining in neural cells (arrowhead). (H and J) Magnification of the vessel shown in (G) and of an EGFR +/+ control, respectively. (I and K) High magnifications of stained neurons in the cortex (I) and olfactory bulbs (K). HE, hematoxylin and eosin.

consisting of nuclear condensation and diminished cell density (Figure 2A and B), and TUNEL staining revealed abundant apoptosis (Figure 2C and D). The degenerative process extended rapidly, and by P6–8 there were virtually no viable neurons in the affected areas, as demonstrated by the complete loss of microtubule associated protein-2 (MAP-2) immunoreactivity (Figure 2E and F). No GFAP staining could be detected in the degenerating areas, indicating that the astrocytes were also affected. Only a thin ribbon of cortical tissue, localized below the leptomeninges, was spared (Figure 2F). The cellular debris was phagocytosed by accumulations of F4/80 positive

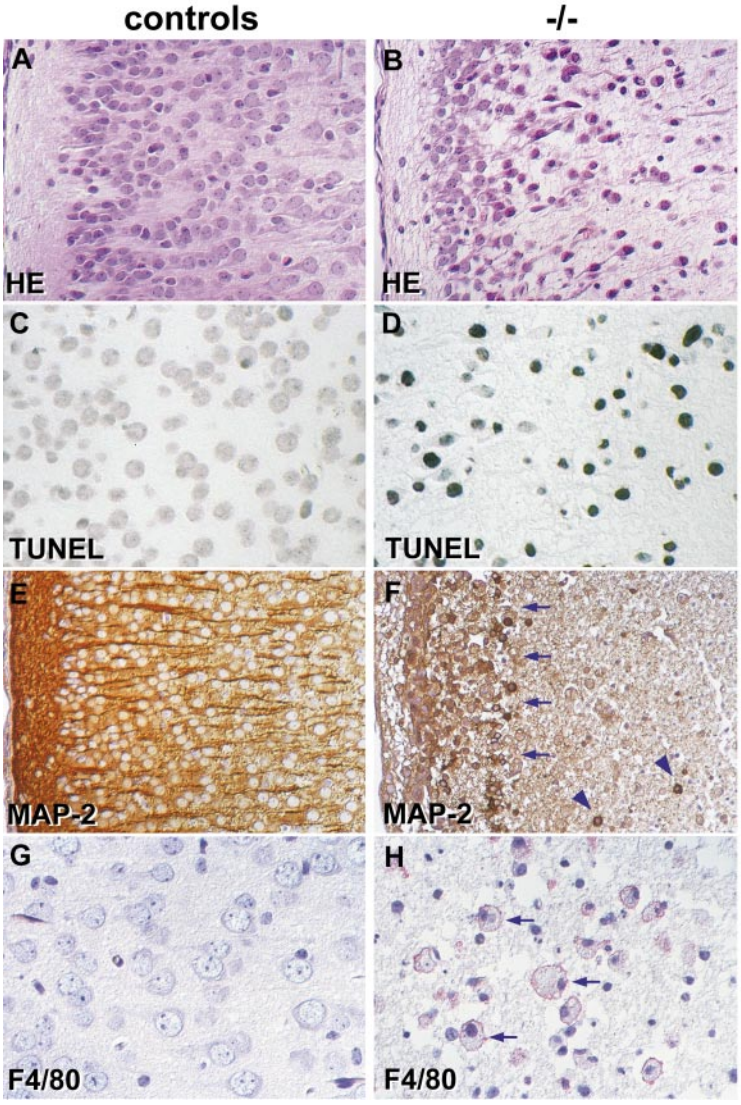

Fig. 2. Morphology of the degenerating frontal cortex. (A and B) Histo-architecture and morphology of P5 control (**A**) and EGFR –/– (**B**) frontal cortex seen in Figure 1A and B, showing neuronal degeneration with nuclear condensation and diminished cellular density in the mutants. Compared with control (**C**), abundant TUNEL staining indicative of apoptosis is observed in degenerating neurons at P5 (**D**). Compared with controls (**E**), MAP-2 immunostaining shows complete loss of neurons in a large part of P7 EGFR –/– frontal cortex (**F**) with only a thin ribbon of neurons in the sub-leptomeningeal layer being spared (F, arrows) The arrowheads point to residual apoptotic cells (F). (**G** and **H**) F4/80 staining of –/– and control sections reveal the presence of numerous macrophages in the degenerating cortical regions at P7 (H, arrows), whereas no macrophages are present in the control (G). Similar staining patterns were observed in EGFR –/– cortex of other surviving backgrounds and of tetraploid rescued and dexamethasone treated mice.

macrophages (Figure 2G and H), resulting in the formation of residual large cystic cavities (data not shown). In parallel, there was also massive degeneration in the olfactory bulbs. The mitral cells were most severely affected, while the glomerular layer was relatively spared. Increased apoptosis was also detectable in the granular cell layer (data not shown). Since neurogenesis apparently proceeds normally until birth, it seems that EGFR is dispensable for brain development during embryogenesis, but is absolutely required for preserving brain integrity after birth.

The targeted allele of EGFR mutant mice contains an *Escherichia coli LacZ* reporter gene with its own ATG downstream of the endogenous promoter (Sibilia and Wagner, 1995). Therefore, X-gal staining was used to monitor *Lac*Z expression, which reflects the endogenous EGFR promoter activity. To investigate whether there could be a cell-autonomous requirement for EGFR in the degenerating parts of the cortex and olfactory bulbs, we analyzed the expression profile of EGFR by X-gal staining of whole mount EGFR +/– and –/– brain slices from P1 to P20. Overall, the level of expression was low. The strongest staining was observed in the leptomeninges (Figure 1G), the choroid plexus and in the cells of the subependimal germinal layer (data not shown). There was also distinctive staining of endothelia of small brain vessels (Figure 1G and H), which could not be detected in EGFR +/+ controls (Figure 1J). In addition, *Lac*Z expression was recognizable in many cells from all parts of the cortex (Figure 1G and I) and olfactory bulbs (Figure 1K). Some neurons with marked staining were detected in the midbrain, hindbrain and cerebellum (data not shown). In

Table I.

	Crosses	Genotypes		
		–/–	+/–	+/+
+/–×+/–	control	0 (0%)	110 (58%)	77 (41%)
+/–×+/–	tetraploid (4N)	6 (12%)	46 (88%)	

Frequency of genotypes from F2 progeny of tetraploid aggregation chimeras and 129/Sv EGFR heterozygote (+/–) intercrosses at birth. Note that among the progeny of heterozygote intercrosses (control), no EGFR –/– fetuses were present at birth, whereas viable EGFR –/– pups were obtained from intercrosses rescued by tetraploid aggregation. One of the six tetraploid rescued pups was killed at P5 for analysis whereas the rest died between P16 and P25.

the cortex, *Lac*Z expression was essentially restricted to neurons, although some astrocytes were also positive, whereas oligodendrocytes consistently showed no staining (data not shown). No clear increase in the intensity of staining with age and no detectable differences in the levels of expression between EGFR +/– and –/– brains could be observed (data not shown). Immunohistochemistry with anti-β-galactosidase antibody qualitatively confirmed the specificity of the *lacZ* staining (data not shown).

The neurodegeneration occurs in a strain-independent manner and is not influenced by the placental and lung defects

The development of the brain phenotype could be restricted only to EGFR mutant mice of certain genetic backgrounds, such as MF1 and C3H, or it could be a more general phenotype occurring in all mouse strains, which would suggest that EGFR is essential for neural cell survival. Alternatively, neurodegeneration could be a consequence of impaired nutrition supply caused by other phenotypic alterations such as the placental defect or lung immaturity, since EGFR mutant mice of all backgrounds investigated (129/Sv, C57BL/6, CBA, MF1, C3H) showed placental and lung phenotypes (data not shown). In order to investigate whether the brain phenotype would also arise in the 129/Sv background, we rescued the placental defect by generating aggregation chimeras between tetraploid wild-type and diploid 129/Sv EGFR –/– embryos. Tetraploid cells, which can efficiently contribute to the development of all extra-embryonic tissues but not to the embryo itself (James *et al.*, 1995), should complement the placental defect of EGFR –/– embryos and allow us to study EGFR function in 129/Sv embryos. While no 129/Sv EGFR –/– pups were ever obtained from heterozygote intercrosses, six (12%) of the tetraploid aggregation chimeras were EGFR –/– at birth (Table I), confirming that the placental defects and no additional embryonic defects are responsible for the midgestation lethality.

Newborn tetraploid rescued 129/Sv EGFR –/– aggregation chimeras appeared similar to EGFR –/– pups of C57BL/6, C3H and MF1 background. They had open eyes and were slightly smaller than their control littermates (data not shown). Some of them survived until day 25 after birth (P25) and, similarly to EGFR mutant mice of C3H and MF1 backgrounds, the 129/Sv –/– aggregation chimeras were severely growth-retarded and exhibited eye

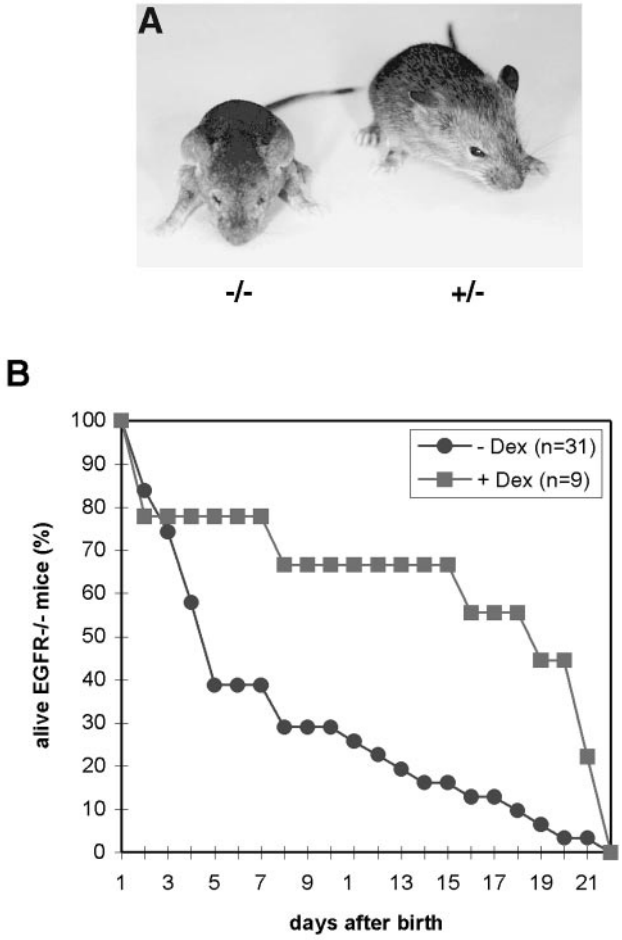

Fig. 3. (**A**) Analysis of EGFR mutant mice rescued by tetraploid aggregation and transplacental dexamethasone administration. External appearance of 129/Sv tetraploid aggregation chimeras at postnatal day 20 (P20). Like EGFR null mice of other backgrounds (Sibilia and Wagner, 1995), the 129/Sv EGFR –/– (–/–) tetraploid aggregation chimeras are severely growth retarded and exhibit eye and hair growth defects. (**B**) Viability of EGFR –/– mice after transplacental dexamethasone administration. The percentage of live EGFR –/– mice at various stages after birth is shown for dexamethasone treated (square) and untreated (circle) mice. Transplacental dexamethasone administration significantly increases the survival of EGFR –/– mice in the first 5 days after birth.

and hair defects (Figure 3A). Macroscopic and histological examinations of brains isolated from 129/Sv EGFR –/– tetraploid aggregation chimeras at different postnatal stages revealed exactly the same onset and kinetics of the neurodegenerative process as observed in spontaneously surviving MF1 and C3H EGFR mutant mice (data not shown). PCR analysis of genomic DNA isolated from placentas, yolk sacs and tails of 129/Sv EGFR –/– tetraploid aggregation chimeras confirmed that the placenta was derived from wild-type tetraploid cells and the fetus from –/– cells (data not shown). These results demonstrate that in EGFR mutant mice the postnatal epithelial and brain defects occur independently from the placental defect. Furthermore, they suggest that the genetic background influences mainly the development of extra-embryonic tissues, but not of the postnatal epithelial and brain defects.

At birth, EGFR mutant mice of all backgrounds have immature lungs and this defect is most probably

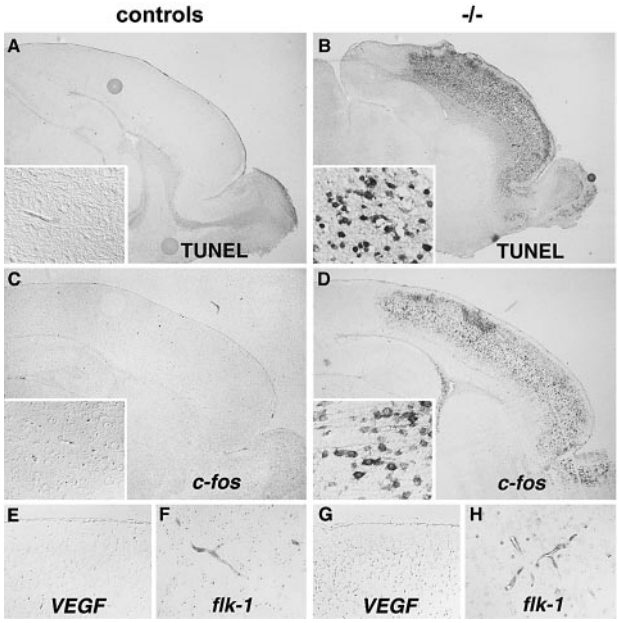

Fig. 4. Massive apoptosis and increased c-*fos* expression in the degenerating cortex of EGFR mutant brains at postnatal day 5 (P5). (A and B) TUNEL staining of EGFR –/– brain sections reveal massive apoptosis in the rostral part of the frontal cortex and olfactory bulb (**B**, inset) whereas no apoptotic cells can be detected in the control (**A**, inset). (**C**–**H**) *In situ* hybridization analysis showing the expression of the c-*fos* (C and D), VEGF (E and G) and *flk*-1 (F and H) genes in the cortex of EGFR –/– (D, G and H) and control (C, E and F) brains. Note that c-*fos* is upregulated in the degenerating cortical regions of EGFR –/– brains (D, inset), but it is not expressed at detectable levels in the controls (C, inset). There is no induction of VEGF mRNA in the degenerating cortex of EGFR mutants (G) compared with the controls (E). Normal levels of expression of *flk*-1 in –/– cortical blood vessels (H) and controls (F). Insets: magnifications of affected cortex in (A–D). *In situ* hybridization with sense control probes were consistently negative in degenerating areas (data not shown). Similar degenerative processes were observed in the cortex of surviving EGFR –/– of other backgrounds and of tetraploid rescued and dexamethasone treated mice.

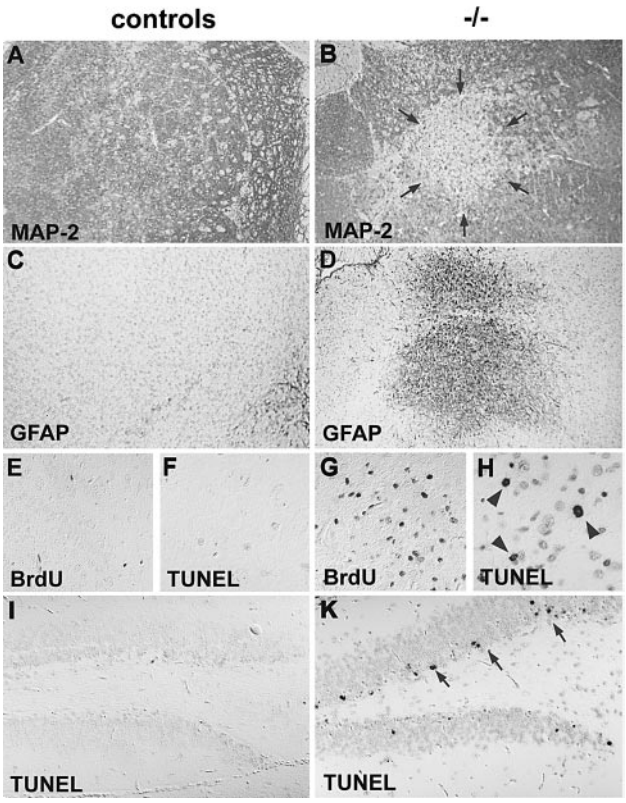

Fig. 5. Selective neuronal degeneration in thalamus and hippocampus of EGFR mutant brains. (**A** and **B**) Degenerating areas of the thalamus are revealed by the absence of immunohistochemical staining with MAP-2 antibodies in P7 –/– brains (arrows) (B). (**C** and **D**) GFAP staining shows a pattern of 'reactive astrogliosis' in the EGFR –/– thalamus (D), which is absent in the control (C). (**E**–**H**) BrdU and TUNEL staining showing high proliferative activity of mutant astrocytes (G) and increased neuronal apoptosis (H, arrowheads) in the degenerating thalamic regions compared with controls (E and F). Compared with controls (**I**), increased apoptosis in EGFR –/– granule cells of the dentate gyrus of the hippocampus at P19 (K, arrows). All these abnormalities were also observed in the brains of surviving EGFR –/– mice of other backgrounds and of tetraploid rescued and dexamethasone treated mice.

responsible for the inability of the majority of mutant newborns to initiate or sustain sufficient respiration (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995). Although EGFR mutant mice of C3H and MF1 background can survive for 3 weeks after birth, a high number of mutants (60%) die during the first 5 postnatal days (Figure 3B). To investigate whether this early postnatal mortality was attributable to lung immaturity and if persistent hypoxia during the first few days after birth could be responsible for the neurodegeneration, we attempted to ameliorate the lung phenotype by transplacental administration of dexamethasone, a glucocorticoid analogue. Administration of glucocorticoids to pregnant women is an established therapy for promoting lung maturation in human fetuses (Ballard, 1989). As shown in Figure 3B, dexamethasone treatment significantly increased the survival of EGFR –/– mice in the first days after birth, but there was no prolongation of the maximal lifespan. Corresponding to the improved viability, histological analysis of newborn dexamethasone-treated lungs revealed that the lung dysplasia of EGFR mutant offspring had been rescued (data not shown). However, in dexamethasone treated EGFR mutant mice, the onset and severity of the neurodegeneration were similar to spontaneously surviving mutants (data not shown). Therefore, it appears unlikely that the neurodegeneration is secondary to persistent perinatal hypoxia due to lung immaturity.

No evidence of hypoxia or pathological angiogenesis

Since the placental defect and lung immaturity do not seem to alter the course of neurodegeneration, we next investigated other possible mechanisms that could lead to the massive apoptotic cell death. Corresponding to the degenerating areas of the cortex and olfactory bulbs of EGFR mutant brains which were affected by apoptosis (Figure 4A and B), there was a remarkable induction of c-*fos*, which exactly mapped the extent of the degenerative process (Figure 4C and D). *In situ* hybridization revealed no differences in c-*fos* expression between mutants and controls at birth and up to P3, with most pronounced expression in the hippocampus, internal granule cell layer of the cerebellum and subependymal germinal layer (data not shown). The levels of c-*fos* gradually declined in controls, but in EGFR mutants there was strong expression of c-*fos* in neurons of the affected frontal cortex from P4

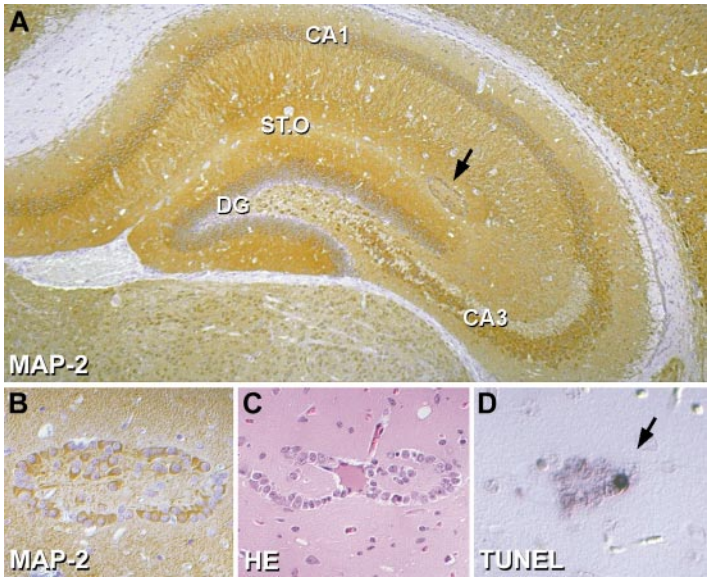

Fig. 6. Migration disorder with ectopic neurons in the hippocampus. (A) Histological sections through the hippocampus of P19 EGFR –/– brains stained with MAP-2 antibodies. The arrow points to a group of ectopic neurons in the white matter of the hippocampus. (B–D) Higher magnification of the heterotopias shown in (**A**) stained with MAP-2 (**B**), hematoxilin/eosin (HE) (**C**) and TUNEL (**D**). The ectopic neurons are also affected by apoptosis (arrow). These heterotopias were never observed in control mice, but were detected in all surviving mutant mice. DG, dentate gyrus; CA1/CA3, sublayers of the pyramidal cell layer of the hippocampus.

to P6 (Figure 4C and D). Concomitantly, in the olfactory bulbs there was strong expression in the mitral cells and also, to a lesser extent, in the granular cell layer (Figure 4C and D). With the loss of the neuronal population, the expression of c-*fos* diminished and by P8 to P9, only a few positive cells persisted (data not shown). In the olfactory bulb, expression was more sustained, with detectable expression up to P13 (data not shown). From the temporal and spatial pattern of induction, it can be speculated that c-*fos* is somehow involved in the molecular pathways leading to apoptotic cell death.

To exclude persistent hypoxia as the cause of neurodegeneration, *in situ* hybridization for VEGF, the best available marker for hypoxia *in vivo* and *in vitro (*Ikeda *et al.*, 1995), was performed. There was no enhanced induction of VEGF mRNA at any postnatal stage, including between P5 and P7, in the brains of EGFR mutants compared with controls (Figure 4E and G). The blood vessels in EGFR mutants appeared morphologically normal on HE and reticulin staining and there was no increase in the density of the capillary network (data not shown). *In situ* hybridization for Flk-1 and Flt-1 revealed moderate levels of expression in the endothelia of EGFR mutants, indistinguishable from controls (Figure 4F and H). Therefore, it is highly unlikely that disorders of angiogenesis or hypoxia/ischemia are responsible for neurodegeneration.

Defects in the thalamus and hippocampus

In contrast to the indiscriminate degeneration in the frontal cortex, which affected all neural cell types and ultimately led to severe structural defects, there was selective neuronal loss in other parts of the mutant brains. In the thalamus of EGFR mutants at P7, localized areas with a complete loss of neurons (Figure 5A and B) were detectable, whereas astrocytes were still able to respond to neuronal injury (Figure 5C and D). Compared with controls, reactive astrocytes, which were strongly positive for GFAP (Figure 5C and D), accumulated and showed a high proliferation index, as indicated by BrdU incorporation (Figure 5E and G). A moderate degree of reactive astrogliosis was also found in the cerebellum and in the corpus callosum, perhaps as evidence of subtle tissue injury (data not shown). TUNEL staining revealed apoptosis in the degenerating neurons of the affected thalamic regions (Figure 5F and H). In the mutant hippocampus at P19 there was an increased number of pyknotic neurons in the granule layer of the dentate gyrus which were also undergoing apoptosis (Figure 5I and K). However, there was not a general augmentation of apoptosis in all other structures of EGFR mutant brains. For instance, the amount of apoptosis in the external granular cell layer of the cerebellum and the subependymal germinal layer was essentially similar to that found in controls (data not shown). Normal oligodendrocyte density and distribution were detected by *in situ* hybridization for proteolipid mRNA, and Luxol-Nissl staining revealed normal myelin deposition (data not shown). Microglia were encountered in increased numbers only in degenerating areas (data not shown). These results indicate that, except for the frontal cortex, in other parts of the brain the neurons were the most affected cell type, whereas other neural cell types revealed no specific defects.

In addition to the degenerative processes, there was a striking structural abnormality in the brain of EGFR mutants, consisting of small nests of ectopic cells in the white matter of the hippocampus (Figure 6A–C). The cells formed oval- to ring-shaped groups and their neuronal identity was indicated by positive immunoreactivity for MAP-2 (Figure 6B) and synaptophysin (data not shown). Markers for glial cell types were negative. Interestingly, these cells were also affected by apoptosis (Figure 6D), but there was no evidence of continuous proliferation by

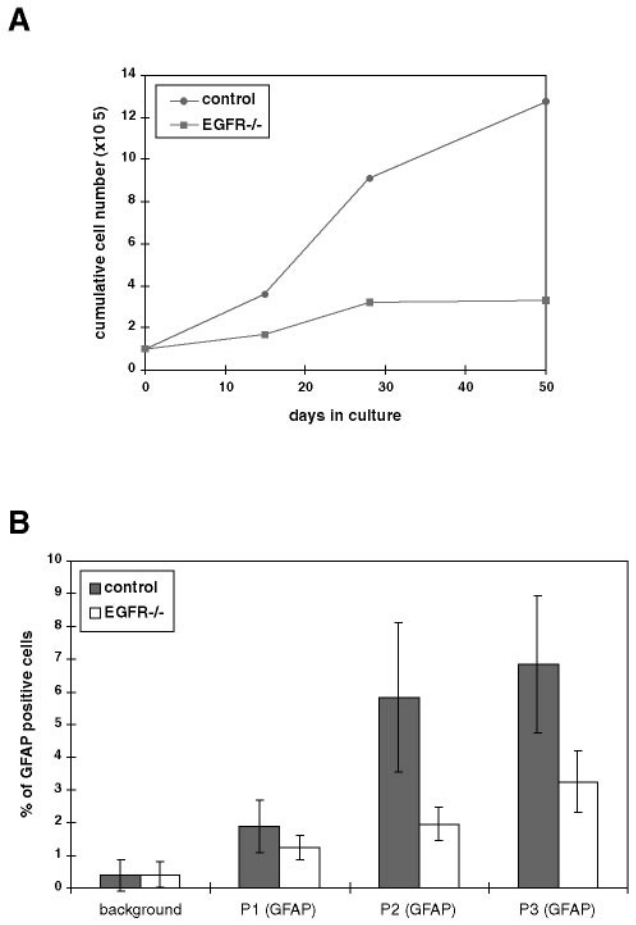

Fig. 7. Impaired proliferation of EGFR mutant astrocytes *in vitro* and reduced number of GFAP-positive astrocytes in mutant cortices after birth. (**A**) Cumulative cell number of control (circle) and EGFR –/– (square) astrocytes isolated from newborn (P1) cortices showing that mutant astrocytes display a significantly reduced proliferation capacity *in vitro*. Primary cell suspensions (day 0), were initially plated at 1×10^5 cells/cm^2 and afterwards cells were passaged and counted when approaching confluence at the indicated timepoints and replated at 1:2 or 1:3 split ratios. Isolates from ten controls and five EGFR –/– cortices were analysed and a representative growth curve from each group is shown. Based on the result of the quantitative FACS analysis, the initial cell population was assumed to contain $1–1.3\times10^4$ astrocytes. (**B**) Flow-cytometric analysis of primary cell suspensions prepared from P1, P2 and P3 EGFR –/– and littermate control cortices and stained for the astrocyte specific marker GFAP. The percentage of GFAP-positive astrocytes is significantly reduced in EGFR mutant P2 and P3 cortices. Data at each timepoint represent the mean ± SD of the analysis of three independent cortices of each genotype (EGFR –/– and control). Background control represents the mean ± SD of cells incubated with the same concentration of purified rabbit IgG. These analyses were performed in newborn mutant mice of C3H and MF1 genetic background and the size and weight of the brain of the mutant and control mice were comparable.

BrdU staining. These ectopic neurons were found in variable amounts in all EGFR mutants older than P10, but never in controls, and most probably represent a migratory disorder. In contrast, other migrating cell types appeared unaffected; for instance there was normal proliferation of the cells of the subependymal germinal cell layer, and the migration of these cells to the granule cell layer appeared uncompromised (data not shown). *In situ* hybridization for c-*fos* revealed continuous expression in the cells migrating into the olfactory bulbs, similar in EGFR mutants and controls (data not shown). This phenotype may suggest a previously unrecognized function of EGFR in the guidance of migrating hippocampal neurons.

Impaired proliferation of astrocytes in vitro and in vivo

Until birth, the neurons in the different cortical layers do not appear to be affected in the absence of EGFR and the cortical lamination is comparable to the controls. Neurodegeneration in the EGFR mutant cortex starts around P4 and therefore, the defect leading to massive apoptotic cell death is likely to occur during the first postnatal days. It is possible that neuronal cell death in the cortex might be the consequence of a local lack of trophic support by glial cells. Astrocytes are thought to be critical for the nutritional and structural support of neurons and most astrocyte differentiation or maturation seems to occur in the early postnatal period (Levison and Goldman, 1993). Since EGFR appears to play a role in the proliferation and differentiation of astrocytes (Simpson *et al.*, 1982), we investigated whether this cell type was affected in EGFR mutant cortices. We first examined whether the absence of EGFR influences the proliferation rate of primary astrocytes isolated from postnatal day 1 (P1) wild-type and mutant cortices. As shown in Figure 7A, EGFR –/– astrocytes show a significantly reduced proliferation rate compared with controls and after 50 days in culture, their cumulative cell number reached only 25% of the value obtained with the controls. Morphologically the cultured cells resembled type I astrocytes and immunofluorescence staining with antibodies against GFAP, vimentin and GalC confirmed that after the first passage, more than 95% of the cells were astrocytes (data not shown).

In vitro, astrocytes are known to proliferate in response to EGF. Therefore, the reduced proliferation observed in astrocytes lacking EGFR could be due to the fact that they are unable to respond to EGF present in the serum of the culture medium. In order to investigate whether the absence of EGFR also affected astrocyte proliferation *in vivo*, primary cell suspensions isolated from EGFR mutant and control cortices at P1, P2 and P3 were subjected to flow-cytometric analysis after staining with anti-GFAP antibodies. In control cortices, the percentage of GFAP-positive cells increased from ~2% (P1) to 7% (P3) and the major increment was observed between P1 and P2 (Figure 7B). GFAP staining in the mutants was comparable with the controls at P1, but at P2 and P3 the percentage of positive cells was significantly lower in the mutants, reaching only 30–50% of control levels. These results indicate that EGFR –/– astrocytes are impaired in their proliferation capacity *in vitro* as well as *in vivo*. Alternatively, since a reduced number of GFAP-positive cells was already observed at birth, the absence of EGFR might also affect either the survival or the ability of neural precursor cells to efficiently differentiate into astrocytes.

Discussion

Depending on the genetic background, EGFR mutant mice die at midgestation (129/Sv), at birth (C57BL/6) or can live up to postnatal day 20 (MF1 or C3H). The

multiple phenotypes are manifested in defects in the placenta, the skin, the lung and also in the brain. In this study, the brain lesions leading to a massive neurodegeneration were characterized in different mouse strains. Around P4, all mutant mice show a progressive neurodegeneration in the frontal cortex, olfactory bulb and thalamus leading to the loss of extensive parts of the brain by P8. This degeneration involves massive apoptosis and is accompanied by upregulation of c-*fos*. In addition, a migration disorder consisting of ectopic neurons is observed in the hippocampus of mutant brains. A reduced number of GFAP-positive astrocytes is present in mutant cortices and their proliferation capacity *in vitro* is also affected, suggesting that impaired proliferation and/or differentiation of astrocytes contribute to the brain phenotype.

When aggregation chimeras between 129/Sv EGFR mutant and tetraploid wild-type embryos were generated, viable EGFR mutant mice were obtained in a pure 129/Sv genetic background. These mice survived up to 3 weeks and developed similar defects as EGFR mutants in other backgrounds. In particular, they displayed the same pattern and degree of neurodegeneration, indicating that this degenerative process occurs in a strain-independent manner. Furthermore, the rescue of the lung immaturity by dexamethasone administration did not alter the course of neurodegeneration, excluding persistent hypoxia as the cause of the brain phenotype. There is no increase in the maximal lifespan in dexamethasone-treated mutant mice and it appears that the lung immaturity is not the limiting factor for survival once the first few days of life have passed. In contrast, the extensive neurodegeneration in the frontal cortex, thalamus and olfactory bulbs most probably adversely affects the viability of EGFR mutant mice. These defects do not influence the overall locomotor activity of EGFR mutant mice, but might interfere with their feeding behavior.

Regional distribution of the neurodegenerative lesions

It is unclear why only certain areas of the brain are affected and why others are spared. The observed distribution of lesions does not map to any vascular territory, nor is it compatible with the pattern of lesions found in anoxic encephalopathy, where neuronal death occurs predominantly in the pyramidal cell layer of the hippocampus (CA1) and in the cortex. In particular, there was a conspicuous sparing of the retrosplenial cortex, with sharp demarcation to the degenerating tissue of the neighboring frontal cortex. Leptomeningeal hemorrhages observed on the surface of the degenerating cortex always appeared concomitantly with the massive cell death and are likely to reflect a local inflammatory response which attracts monocytes/macrophages to the lesioned areas. Alternatively, there could be a primary defect in EGFR –/– blood vessels, which render them more likely to spontaneous ruptures, especially since we detected EGFR expression by X-gal staining in brain endothelia. EGFR expression had indeed been described previously on human brain endothelia (Styren *et al.*, 1993). However, neither morphological analysis by HE and reticulin staining nor molecular analysis by *in situ* hybridization for *flk*-1 and *flt*-1 revealed any vascular abnormalities such as hemangiomas or aneurysms in EGFR mutants and the hemorrhages were always observed in conjunction with degenerating areas in the brain.

Alternatively to a vascular defect, hypoxia or ischemia could be responsible for the neurodegeneration. Since VEGF expression was never enhanced in the brains of EGFR mutant mice, hypoxia and ischemia are unlikely to be involved in the neurodegeneration. In addition, VEGF causes upregulation of its cognate receptors, *flk*-1 and *flt*-1 on endothelia (Wilting *et al.*, 1996). However, normal levels of these receptors were detected in mutant brains. The late induction of c-*fos* just before the onset of the degenerative process is also a strong argument against a perinatal cause, since c-*fos* is rapidly and transiently induced by hypoxia and ischemia (Kinouchi *et al.*, 1994). In the nervous system, upregulation of c-*fos* has been found to precede apoptosis in many different models (Smeyne *et al.*, 1993), and there is increasing evidence that c-*fos* can act as a mediator of cell death *in vivo* (Preston *et al.*, 1996; Hafezi *et al.*, 1997). Therefore, our finding of high levels of c-*fos* expression concomitant with progressive cell death, suggests that c-Fos might be causally involved in the molecular pathways leading to apoptosis. We were unable to test the hypothesis that in the absence of c-Fos EGFR –/– brains would still be affected by apoptosis, since from intercrosses between EGFR +/– c-*fos* +/– mice, we could not obtain surviving mice lacking both EGFR and c-Fos (M.Sibilia and E.F.Wagner, unpublished results).

Migratory defects

Ectopic neurons were observed in the white matter of the hippocampus. The position of these ectopic neurons is in a region of the hippocampus, where EGFR has been shown to be expressed in rats (Tucker *et al.*, 1993). Possibly, the presence of EGFR on resident or migratory cells offers cues for the correct migration into the appropriate hippocampal structure. It appears likely that this phenotype is the consequence of a migration disturbance rather than aberrant proliferation, since no proliferative activity was detected in the ectopic cells by BrdU staining. EGFR has been shown to control cell migration, since EGF injection can induce subependymal cells to migrate from the lateral ventricle into the adjacent neural tissue (Craig *et al.*, 1996). Further, in TGFα –/– mice there is a deficit in the rostral migration of neuronal progenitor cells in the adult subependyma (V.Tropepe, personal communication). EGF-like motifs are common in many molecules with migratory function, such as astrotactin (Zheng *et al.*, 1996) and reelin (Hirotsune *et al.*, 1995), while EGFR has so far been mainly associated with proliferation and differentiation, but not neuronal migration.

Neuronal versus astrocyte defects

It seems unlikely that exogenous mechanisms are responsible for the degenerative process in the brain. A cell-autonomous defect appears to be the most likely reason for the massive apoptosis observed in various brain regions, suggesting that EGFR might be essential for neural cell survival. This is in agreement with our previously published data where we showed that in chimeras, EGFR –/– ES cell derivatives can efficiently contribute to the brain at E14.5. However, the EGFR –/– derivatives'

contribution to the brain decreases after birth, suggesting that neural cells lacking the EGFR might encounter a selective disadvantage compared with wild-type cells (Sibilia and Wagner, 1995). By X-gal staining, we detect EGFR expression in the subependymal layer, olfactory bulbs, cortex and in the basal forebrain. Our results are compatible with other reports detecting EGFR on both astrocytes and neurons (Gómez-Pinillla *et al.*, 1988; Nieto-Sampedro *et al.*, 1988; Topp *et al.*, 1989; Kornblum *et al.*, 1997). EGF and TGFα have been shown to increase the survival of cortical, mesencephalic and cerebellar neurons *in vitro* (Kornblum *et al.*, 1990; Casper *et al.*, 1991; Alexi and Hefti, 1993) and EGFR immunoreactivity has been demonstrated on neurons in the cortex (Tucker *et al.*, 1993). It is possible that EGFR stimulation is required for the survival of neurons in a cell-autonomous manner and that the lack of EGFR leads to apoptosis. Alternatively, in astrocytes, EGFR signaling may lead to the secretion of neurotrophic factors that are critical for the survival of neurons.

A reduced proliferation was indeed observed in EGFR mutant astrocytes, which is consistent with previous observations showing that EGF promotes the proliferation of primary astrocytes *in vitro* (Simpson *et al.*, 1982). We also detect reduced numbers of GFAP-positive astrocytes in mutant cortices at birth, and while the percentages of positive cells increase during the first postnatal days, they never exceed 50% of control levels. Therefore, it seems that also *in vivo* the proliferation of EGFR mutant astrocytes is impaired. In wa-1 mice, which are deficient in TGFα, reduced levels of GFAP mRNA have been observed but no obvious brain abnormalities were detected (Weickert and Blum, 1995). However, in this study the number of GFAP-positive cells was not directly assessed (Weickert and Blum, 1995). Our data suggest that EGFR signaling is important in regulating the proliferation of astrocytes *in vitro* and *in vivo.* The observation that the EGFR and TGFα genes are amplified in several malignant human gliomas further supports the hypothesis that EGFR signaling is important in glial cell proliferation (Yung *et al.*, 1990; Wong *et al.*, 1992).

It is very possible that the lack of EGFR could also affect the differentiation efficiency of neural progenitors into the astrocyte lineage. Pluripotent progenitors in the ventricular zone (VZ) of mouse embryonic forebrains and subependymal layer (SEL) of adult brains have been shown to proliferate in response to EGF and fibroblast growth factor 2 (FGF2) and differentiate into neurons, astrocytes or oligodendrocytes (for review see Weiss *et al.*, 1996). There is increasing evidence that different types of progenitor cells might exist in the VZ and SEL, which express either FGF receptor 1 (FGFR1), EGFR or both (V.Tropepe, personal communication). Therefore, EGFR expressing progenitors might be the ones that prevalently differentiate into astrocytes. This is substantiated by recent findings by Burrows and co-workers showing that increasing the level of EGFR signaling preferentially confers on progenitor cells the ability to differentiate into astrocytes (Burrows *et al.*, 1997). Alternatively, instead of directly inducing astrocyte differentiation, EGF might be essential for the expansion and/or survival of progenitors along the astrocyte differentiation lineage (Johe *et al.*, 1996) and, in the absence of EGFR, most of these progenitors would die. The fact that FGF2 alone can induce astrocyte differentiation of stem cells explains why in the absence of EGFR there is only a reduction in, rather than a complete block of, astrocyte differentiation in the forebrain.

It seems that in different degenerating parts of mutant brains, different cell types are affected. A complete structural disintegration was observed in the rostral part of the frontal cortex and in the olfactory bulbs, implying that neither neurons nor astrocytes may achieve sufficient support for survival in these areas. In contrast, in the retrosplenial cortex survival factors may diffuse from the neighboring midbrain and prevent both cell types from undergoing apoptosis. In the thalamic lesions, there was selective neuronal cell death with a vigorous 'reactive astrogliosis' indicating that astrocytes were initially not affected. These findings can possibly be explained by a different relative susceptibility of neurons and astrocytes to the loss of trophic support through EGFR signaling and by further quantitative differences in the vulnerability of both cell types in different regions of the mouse brain. Thus, in the thalamus, survival factors may suffice for astrocytes but not for neurons. Alternatively, neuronal death in the thalamus could be secondary to the cortical lesions and affect only the neurons that project to frontal and not to retrosplenial cortical regions.

Mechanism of neurodegeneration

Although a reduced number of GFAP-positive cells is observed in EGFR mutant cortices, we can not conclude that this defect is sufficient to cause massive death of neurons. Conditional ablation of astrocytes in postnatal transgenic mice has been shown to induce several cerebellar defects, including loss of granule neurons (Delaney *et al.*, 1996). Therefore, it is tempting to speculate that in EGFR mutant brains, a reduced number of astrocytes might not give sufficient support to the neurons and contribute in part to the neurodegeneration. This hypothesis is substantiated by studies where EGF- or TGFα-induced survival of dopaminergic neurons was shown to be mediated, in part, by the presence of astrocytes in the culture, which divided in response to EGF or TGFα (Casper *et al.*, 1991; Alexi and Hefti, 1993). Since EGFR is expressed in neurons as well as in astrocytes, it may be that in the absence of EGFR, the massive neurodegeneration is caused by a combination of two mechanisms, one acting directly on neurons, the other mediated by astrocytes. The phenotype is therefore far more dramatic than the changes described in other mouse models of neurodegeneration, such as in mice lacking various neurotrophic factors (Klein, 1994; Sendtner, 1995) or in mice with single or combined deficiencies in the corresponding receptors (Klein, 1994; Minichiello and Klein, 1996). Specific rescue experiments with conditional EGFR alleles or inactivating the EGFR in astrocytes or neurons will provide the definitive answer to which cell type causally contributes to the brain phenotype.

It would also be important to investigate whether related receptor tyrosine kinases such as the other erbB family members could functionally compensate for the lack of EGFR. These receptors are expressed in neuronal tissues and it has recently been shown that mice lacking either erbB2, erbB4 or neuregulin, the ligand for erbB3 and

erbB4, die at midgestation and show aberrant neural development (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). Variations in the expression pattern of the erbB receptors may even explain the regional distribution and the onset of the neurodegenerative processes. The fact that the degenerative process affects mature neurons suggests that EGFR signaling may be involved directly or indirectly in the maintenance of postmitotic neurons. If this can be substantiated, strategies that aim at enhancing EGFR signaling may be relevant for the treatment of neurodegenerative conditions such as Alzheimer's disease, where the balance between protective and noxious stimuli in neurons is disturbed.

Materials and methods

Mice

The generation of EGFR knock-out mice used in this study has been described in detail previously (Sibilia and Wagner, 1995). Briefly, the EGFR gene was inactivated in ES cells by replacing parts of the first exon with an *E.coli lacZ* reporter gene, which allows us to follow the stage- and tissue-specific expression pattern of EGFR during development. Correctly targeted ES cells were injected into C57BL/6 blastocysts and the resulting germline chimeras were mated to 129/Sv and C57BL/6 females to obtain EGFR +/– offspring of inbred 129/Sv and mixed 129/Sv×C57BL/6 genetic background, respectively. Starting from inbred 129/Sv EGFR +/– mice the mutant allele was subsequently bred into C57BL/6, MF1 and C3H strains and backcrossed for several generations. Heterozygous EGFR +/– mice of the respective genetic background were further intercrossed to generate homozygote EGFR –/– mice. Genotyping was performed by PCR as previously described (Sibilia and Wagner, 1995).

Generation of tetraploid (4N) embryos

B6CBAF1 (C57BL/6×CBA F1) female mice mated with males from the same F1 strain were used as donors of 2-cell stage embryos for the production of tetraploid embryos. Tetraploidy was achieved by electrofusion as described previously (Wang *et al.*, 1997). Briefly, the two blastomeres were fused in 0.3 M mannitol following a short electric pulse at 95 V for 30 ms in an effective field (2 V) using the CF-100 pulse generator (Biochemical Laboratory Service, Budapest). The fused embryos (~95%) were selected and further cultivated for 24–40 h to the 'non-compacted' morula stage (for tetraploid embryos: 4-cell-stage) in a microdrop of M16 medium under paraffin oil at 37°C in a 95% air/5% CO_2 incubator.

Morula aggregation

Non compacted 8-cell-stage morulae were isolated from oviducts of 2.5 p.c. inbred 129/Sv EGFR +/– females intercrossed with 129/Sv EGFR +/– males. Twenty-five per cent of these embryos are expected to be EGFR –/–. After removal of the zona pellucida by acid Tyrode's solution, diploid morulae from EGFR +/– intercrosses were aggregated at a 1:1 ratio with 4-cell-stage wild-type tetraploid morulae in M16 medium. The aggregates were cultured for further 20–30 h in M16 medium under paraffin oil at 37°C in a 95% air/5% CO_2 incubator. Under these conditions >95% of the embryos spontaneously aggregated overnight and formed blastocysts composed of diploid and tetraploid blastomeres. The chimeric blastocysts were transferred into the uterus of pseudopregnant recipients where the embryos developed to term. Pregnant recipients either delivered the fetuses spontaneously or were subjected to Cesarean section on day 18.5 of gestation in order to collect yolk sacs and placentas. EGFR –/– fetuses were recognized by the presence of open eyes at birth and their genotype was confirmed together with the genotype of yolk sac and placenta by PCR, as described previously (Sibilia and Wagner, 1995).

Transplacental dexamethasone administration

EGFR +/– females of C3H and MF1 genetic background were mated with EGFR +/– males of the respective genetic background and removed after they were plugged (day 0.5 of pregnancy). A stock concentration of dexamethasone (Dex; Sigma Immunochemicals) at 10 mg/ml was prepared in absolute ethanol and stored at –20°C. Before use, dexamethasone was diluted 1:100 in sterile phosphate-buffered saline (PBS) solution and injected intraperitoneally at day 15.5 of pregnancy (term of 19–20 days) at a concentration of 2 mg/g body weight. Control animals were injected with PBS containing 1% absolute ethanol.

In vivo BrdU labeling

A stock solution of BrdU was prepared in PBS at a concentration of 5 mg/ml, stored at 4°C and further diluted in PBS before injection. A final concentration of 50 mg/g body weight of BrdU was injected intraperitoneally into EGFR –/– mice and littermate controls 24 and 12 h before sacrificing.

Histological analysis and β-galactosidase staining

Mice were sacrificed and the isolated tissues were fixed immediately in 4% paraformaldehyde (PFA) in PBS at 4°C for 16–24 h. After fixation, the tissues were dehydrated through RNase-free graded alcohols and toluene and infiltrated with paraffin (Histowax, Reichert-Jung, Vienna) at 58°C overnight, under vacuum. Sections (2–4 mm) were cut, mounted on silanized slides and stained with hematoxylin and eosin (Sigma Immunochemicals). Brains used for X-gal staining were fixed for 10–20 min in 4% paraformaldehyde, cut into 2–3 mm slices and fixed for another 10 min in 4% PFA. X-gal staining was performed as previously described (Sibilia and Wagner, 1995).

Immunohistochemistry

Immunohistochemistry was performed using polyclonal antibodies to GFAP (DAKO, 1:300), MAP-2 (Boehringer Mannheim, 1:1000) F4/80 (1:80), Ki67 (Novocastra Laboratories, 1:1000), and monoclonal antibodies to BrdU (Caltag Laboratories, 1:50) and β-galactosidase (Cappel, 1:400). An ultrasensitive Avidin-Biotinylated Enzyme Complex (ABC) staining kit (Pierce Chemical Co., Rockford, IL) was used on paraffin sections according to the protocol specified by the suppliers. Positive staining was visualized by incubation with DAB (Vector Laboratories, Burlingame, CA). Control sections were treated with PBS instead of antibody. The APAAP-method, with neofuchsin as chromogen, was employed for F4/80 immunostaining (Dako). Microwave pretreatment in citric acid buffer was performed for MAP-2, β-galactosidase, BrdU and F4/80 immunostainings.

TUNEL

In situ nick end-labeling (TUNEL) was performed using the *in situ* cell death detection kit II (Boehringer Mannheim). Slides were deparaffinized, and sections were digested with proteinase K (20 mg/ml) for 15 min at 37°C, followed by incubation with terminal transferase for 1 h at 37°C in the presence of fluorescin-labeled dUTP. An alkaline phosphatase-coupled anti-fluorescin Fab fragment was used for detection, and 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride (Boehringer Mannheim) were employed as chromogens.

In situ hybridization

Sense and antisense RNA probes were transcribed *in vitro* with T3 and T7 RNA polymerase from linearized pBluescript vectors carrying c-*fos* (Hafezi *et al.*, 1997), VEGF (Breier *et al.*, 1995), flk-1 (Millauer *et al.*, 1993), flt-1 (Breier *et al.*, 1995) cDNAs in the presence of digoxygenin-11-UTP (Boehringer Mannheim). Fifty to 200 ng of labeled transcripts (0.7–1.2 kb) were hybridized to tissue sections at 65°C as described (Hafezi *et al.*, 1997). Digoxygenin was detected with alkaline phosphatase-labeled anti-DIG Fab fragments and 4-nitro blue tetrazolium chloride/5-bromo-4 chloro-3 inodyl phosphate (Boehringer Mannheim). Coverslips were mounted with glycerol gelatine.

Preparation of cortical astrocytes

Primary astrocyte cultures were prepared from brains of newborn EGFR –/– and littermate control pups using a modification of previously described procedures (Simpson *et al.*, 1982; Bambrick *et al.*, 1996). Cerebral cortices were dissected free of meninges and hippocampus and minced into small pieces. The cortical fragments were incubated at 37°C in 2× Trypsin–EDTA (0.1% trypsin, 0.04% EDTA; GIBCO), 0.001% DNase I (Sigma) in DMEM/F12 (1:1; GIBCO) for 30 min. Enzymatically softened tissues were titurated with a wide-bore pipette, resuspended into serum containing culture medium and passed through a 70 μm nylon cell strainer to obtain single cell suspensions. Cells were pelleted by centrifugation, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (PAA), Penicillin-Streptomycin (100 IU/ml to 100 μg/ml; GIBCO) and plated on poly-L-lysine (0.1 mg/ml; SIGMA) coated tissue culture dishes, initially at a concentration of 1×10^5 cells/cm^2. When approaching confluence, cells were trypsinized

and viable cell numbers determined using a Neubauer-type hemocytometer with trypan blue staining. Cells were then replated at 1:2 or 1:3 split ratio. Replated cultures were visually similar to long term primary cultures with respect to morphology and GFAP staining. Freshly prepared suspensions of mouse cortical cells were galactocerebroside- (Gal-C; Sigma) negative and contained <5% neurons. After the first passage, cultures consisted of virtually >95% GFAP- and vimentin- (Sigma) positive cells as determined by immunofluoresence.

Cytometric analysis of GFAP

Cell suspensions of cortices were fixed in 4% PFA at 4°C, washed with PBS, permeabilized in 0.2% Triton X-100 in PBS for 5 min at room temperature, rewashed with PBS and then incubated for 1 h with rabbit polyclonal antibodies to GFAP (DAKO; 1:100) in PBS + 1% bovine serum albumin (BSA; Sigma). As a negative control, the cells were incubated with the same concentration of purified rabbit IgG. Cells were washed twice with PBS + 0.1% Tween 20 (PBS-T; Fluka) and incubated with a 1:800 dilution of goat anti-rabbit IgG-Cy5 (Rockland). Cells were washed twice, resuspended in 0.5 ml PBS-T and analysed with FACS Vantage (Becton Dickinson).

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References

Adamson,E.D. (1990) Developmental activities of the epidermal growth factor receptor. *Curr. Top. Dev. Biol.*, **24**, 1–29.

Alexi,T. and Hefti,F. (1993) Trophic actions of transforming growth factor alpha on mesencephalic dopaminergic neurons developing in culture. *Neuroscience*, **55**, 903–918.

Ballard,P.L. (1989) Hormonal regulation of pulmunary surfactant. *Endocr. Rev.*, **10**, 165–181.

Bambrick,L.L., de Grip,A., Seenivasan,V., Krueger,B.K. and Yarowsky,P.J. (1996) Expression of glial antigens in mouse astrocytes: Species differences and regulation *in vitro*. *J. Neurosci. Res.*, **46**, 305–315.

Breier,G., Clauss,M. and Risau,W. (1995) Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev. Dyn.*, **204**, 228–239.

Burrows,R.C., Wancio,D., Levitt,P. and Lilien,L. (1997) Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron*, **19**, 251–267.

Casper,D., Mytilineou,C. and Blum,M. (1991) EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. *J. Neurosci. Res.*, **30**, 372–381.

Craig,C.G., Tropepe,V., Morshead,C.M., Reynolds,B.A., Weiss,S. and van der Kooy,D. (1996) In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J. Neurosci.*, **16**, 2649–2658.

Dardik,A., Smith,R.M. and Schultz,R.M. (1992) Colocalization of transforming growth factor-alpha and a functional epidermal growth factor receptor (EGFR) to the inner cell mass and preferential localization of the EGFR on the basolateral surface of the trophectoderm in the mouse blastocyst. *Dev. Biol.*, **154**, 396–409.

Delaney,C.L., Brenner,M. and Messing,A. (1996) Conditional ablation of cerebellar astrocytes in postnatal transgenic mice. *J. Neurosci.*, **16**, 6908–6918.

Derynck,R. (1992) The physiology of transforming growth factor-alpha. *Adv. Cancer Res.*, **58**, 27–52.

Derynck,R., Goeddel,D.V., Ullrich,A., Gutterman,J.U., Williams,R.D., Bringman,T.S. and Berger,W. (1987) Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. *Cancer Res.*, **47**, 707–712.

Earp,H.S., Dawson,T.L., Li,X. and Yu,H. (1995) Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res. Treat.*, **35**, 115–132.

Fowler,K.J. *et al.* (1995) A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. *Proc. Natl Acad. Sci. USA*, **92**, 1465–1469.

Gassmann,M., Casagranda,F., Orioli,D., Simon,H., Lai,C., Klein,R. and Lemke,G. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin recepto. *Nature*, **378**, 390–394.

Gómez-Pinillla,F., Knauer,D.J. and Nieto-Sampedro,M. (1988) Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res.*, **438**, 385–390.

Hafezi,F., Steinbach,J.P., Marti,A., Munz,K., Wang,Z.Q., Wagner,E.F., Aguzzi,A. and Remé,C.E. (1997) The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration *in vivo*. *Nature Med.*, **3**, 346–349.

Hirotsune,S. *et al.* (1995) The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. *Nat. Genet.*, **10**, 77–83.

Ikeda,E., Achen,M.G., Breier,G. and Risau,W. (1995) Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J. Biol. Chem.*, **270**, 19761–19766.

James,R.M., Klerkx,A.H., Keighren,M., Flockhart,J.H. and West,J.D. (1995) Restricted distribution of tetraploid cells in mouse tetraploid↔diploid chimeras. *Dev. Biol.*, **167**, 213–226.

Johe,K.K., Hazel,T.G., Muller,T., Dugich-Djordjevic,M.M. and McKay,R.D.G. (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.*, **10**, 3129–3140.

Kinouchi,H., Sharp,F.R., Chan,P.H., Koistinaho,J., Sagar,S.M. and Yoshimoto,T. (1994) Induction of c-fos, junB, c-jun, and hsp70 mRNA in cortex, thalamus, basal ganglia, and hippocampus following middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.*, **14**, 808–817.

Klein,R. (1994) Role of neurotrophins in mouse neuronal development. *FASEB J.*, **8**, 738–744.

Kornblum,H.I., Raymon,H.K., Morrison,R.S., Cavanaugh,K.P., Bradshaw,R.A. and Leslie,F.M. (1990) Epidermal growth factor and basic fibroblast growth factor: effects on an overlapping population of neocortical neurons *in vitro*. *Brain Res.*, **535**, 255–263.

Kornblum,H.I., Hussain,R.J., Bronstein,J.M., Gall,C.M., Lee,D.C. and Seroogy,K.B. (1997) Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J. Comp. Neurol.*, **380**, 243–261.

Lazar,L.M. and Blum,M. (1992) Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-alpha mRNA in mouse brain by a quantitative nuclease protection assay. *J. Neurosci.*, **12**, 1688–1697.

Lee,K.F., Simon,H., Chen,H., Bates,B., Hung,M.C. and Hauser,C. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac developmen. *Nature*, **378**, 394–398.

Lemmon,M.A. and Schlessinger,J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.*, **19**, 459–463.

Levison,S.W. and Goldman,J.E. (1993) Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. *Neuron*, **10**, 201–212.

Luetteke,N.C., Qiu,T.H., Peiffer,R.L., Oliver,P., Smithies,O. and Lee,D.C. (1993) TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell*, **73**, 263–278.

Luetteke,N.C., Phillips,H.K., Qiu,T.H., Copeland,N.G., Earp,H.S., Jenkins,N.A. and Lee,D.C. (1994) The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev.*, **8**, 399–413.

Mann,G.B., Fowler,K.J., Gabriel,A., Nice,E.C., Williams,R.L. and Dunn,A.R. (1993) Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell*, **73**, 249–261.

Meyer,D. and Birchmeier,C. (1995) Multiple essential functions of neuregulin in development. *Nature*, **378**, 386–390.

Miettinen,P.J., Berger,J.E., Meneses,J., Phung,Y., Pedersen,R.A., Werb,Z. and Derynck,R. (1995) Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*, **376**, 337–341.

Millauer,B., Wizigmann-Voos,S., Schnurch,H., Martinez,R., Moller,N.P., Risau,W. and Ullrich,A. (1993) High affinity VEGF binding and

developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*, **72**, 835–846.
Minichiello,L. and Klein,R. (1996) TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. *Genes Dev.*, **10**, 2849–2858.
Nieto-Sampedro,M., Gomez-Pinilla,F., Knauer,D.J. and Broderick,J.T. (1988) Epidermal growth factor receptor immunoreactivity in rat brain astrocytes. Response to injury. *Neurosci. Lett.*, **91**, 276–282.
Partanen,A.M. (1990) Epidermal growth factor and transforming growth factor-α in the development of epithelial-mesenchymal organs of the mouse. *Curr. Top. Dev. Biol.*, **24**, 31–55.
Preston,G.A., Lyon,T.T., Yin,Y., Lang,J.E., Solomon,G., Annab,L., Srinivasan,D.G., Alcorta,D.A. and Barrett,J.C. (1996) Induction of apoptosis by c-Fos protein. *Mol. Cell. Biol.*, **16**, 211–218.
Prigent,S.A. and Lemoine,N.R. (1992) The type 1 (EGFR-related) family of growth factor receptors and their ligands. *Prog. Growth Factor Res.*, **4**, 1–24.
Sendtner,M. (1995) Molecular biology of neurotrophic factors. *Baillieres Clin. Neurol.*, **4**, 575–591.
Seroogy,K.B., Lundgren,K.H., Lee,D.C., Guthrie,K.M. and Gall,C.M. (1993) Cellular localization of transforming growth factor-alpha mRNA in rat forebrain. *J. Neurochem.*, **60**, 1777–1782.
Sibilia,M. and Wagner,E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*, **269**, 234–238.
Simpson,D.L., Morrison,R., de Vellis,J. and Herschman,H.R. (1982) Epidermal growth factor binding and mitogenic activity on purified populations of cells from the central nervous system. *J. Neurosci. Res.*, **8**, 453–462.
Smeyne,R.J., Vendrell,M., Hayward,M., Baker,S.J., Miao,G.G., Schilling,K., Robertson,L.M., Curran,T. and Morgan,J.I. (1993) Continuous c-fos expression precedes programmed cell death *in vivo*. *Nature*, **363**, 166–169.
Styren,S.D., DeKosky,S.T., Rogers,J. and Mufson,E.J. (1993) Epidermal growth factor receptor expression in demented elderly: localization to vascular endothelial cells of brain, pituitary and skin. *Brain Res.*, **615**, 181–190.
Threadgill,D.W. *et al.* (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*, **269**, 230–234.
Topp,K.S., Faddis,B.T. and Vijayan,V.K. (1989) Trauma-induced proliferation of astrocytes in the brains of young and aged rats. *Glia*, **2**, 201–211.
Tucker,M.S., Khan,I., Fuchs Young,R., Price,S., Steininger,T.L., Greene,G., Wainer,B.H. and Rosner,M.R. (1993) Localization of immunoreactive epidermal growth factor receptor in neonatal and adult rat hippocampus. *Brain Res.*, **631**, 65–71.
Wang,Z.Q., Kiefer,F., Urbánek,P. and Wagner,E.F. (1997) Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection. *Mech. Dev.*, **62**, 137–145.
Weickert,C.S. and Blum,M. (1995) Striatal TGF-alpha: postnatal developmental expression and evidence for a role in the proliferation of subependymal cells. *Brain Res. Dev. Brain Res.*, **86**, 203–216.
Weiss,F.U., Daub,H. and Ullricj,A. (1997) Novel mechanisms of RTK signal generation. *Curr. Biol. Gen. Dev.*, **7**, 80–86.
Weiss,S., Reynolds,B.A., Vescovi,A.L., Morshead,C., Craig,C.G. and van der Kooy,D. (1996) Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci.*, **19**, 387–393.
Werner,M.H., Nanney,L.B., Stoscheck,C.M. and King,L.E. (1988) Localisation of immunoreactive epidermal growth factor receptors in human nervous system. *J. Histochem. Cytochem.*, **36**, 81–86.
Wiley,L.M., Wu,J.X., Harari,I. and Adamson,E.D. (1992) Epidermal growth factor receptor mRNA and protein increase after the four-cell preimplantation stage in murine development. *Dev. Biol.*, **149**, 247–260.
Wilting,J., Birkenhager,R., Eichmann,A., Kurz,H., Martiny-Baron,G., Marme,D., McCarty,J.E., Christ,B. and Weich,H.A. (1996) VEGF121 induces proliferation of vascular endothelial cells and expression of flk-1 without affecting lymphatic vessels of chorioallantoic membrane. *Dev. Biol.*, **176**, 76–85.
Wong,A.J., Ruppert,J.M., Bigner,S.H., Grzeschik,C.H., Humphrey,P.A., Bigner,D.S. and Vogelstein,B. (1992) Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc. Natl Acad. Sci. USA*, **89**, 2965–2969.
Yung,W.K.A., Zhang,X., Steck,P.A. and Hung,M.C. (1990) Differential amplification of the TGF-α gene in human gliomas. *Cancer Commun.*, **2**, 201–205.
Zheng,C., Heintz,N. and Hatten,M.E. (1996) CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. *Science*, **272**, 417–419.

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