BASIC FIBROBLAST GROWTH FACTOR (bFGF) GENE EXPRESSION IN RAT BRAIN DURING EARLY POSTNATAL DEVELOPMENT

By
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Abstract

Investigations *in vitro* have shown the importance of bFGF as a mitogen for astrocytes and as a neurotrophic factor for many neuronal populations in different regions of the brain. During postnatal growth and development of the rat brain, bFGF gene activity may be important for diversity of functions. To examine the pattern of bFGF gene expression, reverse transcription-polymerase chain reaction (RT-PCR) was used to quantitate the expression of bFGF messenger ribonucleic acid (mRNA) in various regions of the rat brain in early postnatal development. Basic FGF mRNA levels were determined relative to the level of mRNA for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the latter remains constant at the ages being studied. The levels of bFGF gene expression were compared in the cerebrum from male rats of ages one, three, seven, fourteen, twenty one, twenty eight days and one year. The brain bFGF mRNA level within the first postnatal week remained low and constant, followed by a sharp rise in the following two weeks and reached the adult level (5.9-fold over one day old) by 28 days. Analysis of different regions of 28-day-old rat brains revealed that the highest levels of bFGF mRNA occur in the hippocampus, followed closely by occipital cortex, cingulate cortex and inferior colliculus. The hypothalamus and combined pons-medulla showed intermediate levels of bFGF mRNA. The lowest levels of bFGF mRNA occur in cerebellum. When changes in bFGF mRNA levels in four brain regions were examined during development, different patterns of expression emerged. The combined pons-medulla exhibited little change in bFGF expression, but the occipital cortex and inferior colliculus achieved major increases (2.5-fold and 2.9-fold, respectively) of bFGF gene expression over the first four weeks of life. The cerebellum, however, possessed its highest levels of bFGF mRNA at one day, and then showed a 2.6-fold drop in bFGF mRNA during the next 28 days, such that by four weeks of age, the cerebellum has the lowest level of bFGF mRNA among many brain regions tested. The temporal and spatial changes in bFGF mRNA expression in various brain regions in early postnatal development suggest that bFGF may exert different physiological effects on different brain regions at different ages.

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List of Abbreviations

A	adenosine
aa	amino acids
aFGF	acidic FGF
ATP	Adenosine triphosphate
bFGF	basic FGF
bp	base pairs
BSA	bovine serum albumin
C	cytidine
^{0}C	degrees centigrade
cDNA	complementary DNA
CsCl	cesium chloride
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dUTP	deoxy-uridine triphosphate
EDTA	ethelynediaminetetraacetic acid
EtBr	ethidium bromide
FGF	fibroblast growth factor
FGFR	FGF receptor
g	gram
G	guanosine
GAGs	glycosoaminoglycan
GF/EGF/TGF/	growth factor/epidermal-/transforming-
PDGF/NGF	growth factor/platelet derived-/nerve-
GTP	guanosine triphosphate
HCl	hydrochloric acid
hr	hour

kb	kilobase
KCl	potassium chloride
KDa	kilodaltons
L	litre
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimolar
MMLV	Murine mouse leukaemia virus
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
nM	nanomolar
NTF/BDNF/CNTF	neurotrophic factor/brain-derived-/ciliary-
O/N	overnight
P	plasmid
PCR	polymerase chain reaction
RE	restriction endonuclease
RNA	ribonucleic acid
rpm	revolutions per minutes
RT	reverse transcription
RTase	reverse transcriptase
SDS	sodium doecyle sulfate
SSC	standard saline citrate
Taq	Thermus aquaticus
TBE	Tris borate EDTA buffer
TE	tris-EDTA
µg	microgram
µl	microlitre

µM	micromolar
µ Ci	microcurie
UT	untranslated
UV	ultraviolet
vol	volume
v/v	volume per volume
w/v	weight per volume
%	percent

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Introduction

I. Fibroblast growth factor family

Fibroblast growth factor (FGF) was first identified as an activity in extracts of pituitary (Hoffman 1940) and brain (Trowell 1939) that stimulated the growth of BALB/c 3T3 cells (see Gospodarowicz et al, 1987a,b for review). Two proteins were found to be responsible for such an effect, a basic protein (pI: 9.6) called basic fibroblast growth factor (bFGF, FGF-2), that was able to stimulate the proliferation and phenotypic transformation of BALB/c 3T3 fibroblasts, and an acidic one (pI: 5.6), acidic fibroblast growth factor (aFGF, FGF-1), that was able to cause proliferation and delay differentiation of myoblasts. Acidic FGF was later rediscovered on the basis of its ability to stimulate the proliferation of endothelial cells (reviewed in Baird and Bohlen et al, 1990 and Gospodarowicz et al 1987a,b). The similar biological effects of aFGF and bFGF is due to the fact that they are structurally related molecules. Significant portions of the FGF-like activities present in tumor extracts were found to bind to heparin (Shing et al, 1984). The feature that FGFs bind with high affinity to heparin helped in the isolation of highly purified native and recombinant FGFs (Gospodarowicz et al, 1984). Within the past six years, the FGF family expanded to include seven members after the characterization of another five novel factors, int-2 (FGF-3, Moor et al, 1986), hst/K-fgf (FGF-4, Sakamoto et al, 1986; Terada et al, 1986), FGF-5 (Zhan et al, 1987), FGF-6 (Marics et al, 1989) and KGF (FGF-7, Finch et al, 1989). Acidic and basic FGFs are the most characterized FGFs and are considered the prototype of the FGF family. FGF family members play important roles *in vivo* in different normal physiological processes such as embryonic development, angiogenesis,

nervous system differentiation and wound repair (Baird and Bohlen 1990). The amino acid-core homology between the 7 members of the FGF family ranges from 33 to 69%, and only 19% of residues within the core are invariant among all the seven members (for review see Goldfarb 1990). Basic FGF protein is expressed in a wide range of tissues of mesodermal and neuroectodermal origin. Basic FGF was detected in brain, retina, pituitary gland, macrophages, prostate, bone, cartilage, ovary, endothelial cells, myoblast, lens epithelial cells and in the developing embryo, a wide range of tissue specificity which suggests that bFGF is a multipotent growth factor (Goldfarb 1990). Acidic FGF shares 55% sequence identity with basic FGF and is 10-100 times less potent than bFGF, and its expression is mainly restricted to the nervous system (brain and photoreceptors; Baird and Bohlen 1990). Acidic and basic FGF have been detected in mouse embryo throughout gestation (Hebert et al, 1990). The expression of int-2, hst/K-fgf, FGF-5 and FGF-6 genes is mainly restricted to the developing embryo. The RNA of int-2 is expressed throughout embryogenesis and at birth but not in adult tissues (Goldfarb 1990; Benharroch and Birnbaum 1990). The temporal and spatial expression of int-2 suggests possible roles for int-2 in mesoderm migration, inner ear formation and development of cerebellar and retinal neurons (Wilkinson et al, 1988 and 1989). The expression of hst/K-fgf was found to be restricted to embryogenesis prior to gastrulation (Hebert et al, 1990). FGF-5 and FGF-6 genes are expressed throughout embryogenesis as well as in restricted sets of adult tissues (Hebert et al, 1991, De Lapeyriere et al,1990). Similar to the effect exerted by basic and acidic FGFs, the newer FGF members (int-2, hst/K-fgf, FGF-5 and FGF-6) stimulate the proliferation of

fibroblasts. FGF-5 and hst/K-fgf are endothelial cell mitogens and int-2 and hst/K-fgf act as mesoderm inducers (See Benharroch and Birnbaum 1990 for review). KGF, the seventh member of the FGF family is a potent mitogen for keratinocytes but not fibroblasts, and its expression is detected in dermis, kidney and gastrointestinal tract. Some endodermal cell types such as thyroid cells, prostatic cells and pancreatic cells were shown to respond to FGFs.

A possible role for FGFs in abnormal cellular processes such as cancer has become clear with the identification of oncogenes that belong to the FGF family. These oncogenes include int-2 (Peters et al, 1983), hst/K-fgf (Sakamoto et al, 1986) and FGF-5 (Zhan et al, 1987). Acidic and basic FGFs were detected in glioblastoma (Stefanik et al, 1990). Two of the high affinity FGF receptors (*bek* and *flg*) were coamplified in subsets of breast tumors (Adnane et al, 1991), suggesting the involvment of FGFs in the transformation of these cells.

II. Gene structure of bFGF

Basic FGF is the product of a single copy gene, located on chromosome 5 (Jaye et al, 1986) which spans at least 38 kb of genomic DNA and possesses 3 exons separated by two large introns. The first intron is at least 16 kb, and separates codons 60 and 61, while the second inton is 16 kb long separating 95 and 96 codons (Shibata et al,1991). The exon boundaries of bFGF, int-2, hst/ks and FGF-5 align perfectly, except the exon 1/intron boundary for bFGF is shifted by 3 nucleotides, suggesting that the exon structure did not change among FGF family members during evolution. Also, the high degree of similarity of bFGF and other members of the FGF family suggest that they are derived from a single ancestral gene, which became separate genes through the processes of duplication and evolutionary divergence (reviewed in Baird and

Bohlen 1990, Gospodarowicz et al, 1987a,b). At the amino acid level, human bFGF has 98.7% sequence homology with the bovine bFGF and 87% homology with the rat bFGF sequence (Kurokawa et al, 1987 and 1988). The high conservation of the coding region of bFGF gene among different species implies a strong selective evolutionary pressure for maintenance of function and structure. The open reading frame of the bFGF cDNA sequence suggests an AUG (Met) initiation of translation site that generates a 155 amino acids (a.a.; 18 KDa form), and the proteolytic cleavage of this form results in the 146 a.a (16 KDa) form. The absence of any stop signal in the nearby 5' upstream sequence of its cDNA suggests the possibility of the existence of higher molecular weight forms of bFGF (18 KDa to 29 KDa), which could be generated by two predicted leucine, (CUG) initiation sites in the nearby 5' upstream region (Florkiewicz and Sommer 1989; Sommer et al, 1989). *In vitro* and *in vivo* studies showed the existence of multiple molecular weight forms reported by Grothe et al 1990 {(18, 24, 30-33 and 46 Kilodaltons (KDa)}, Florkiewicz et al 1991 (18, 22, 23 and 24 KDa), Woodward et al, 1992 (18, 21.5 and 22.5 KDa), Giordano et al, 1991 (18, 21, 22 and 24 KDa) and Li and Shipley 1991 (18, 24 and 27 KDa). Various studies showed that multiple forms of the bFGF protein are temporally and spatially distributed and are highly regulated (Florkiewicz and Sommer 1989, Prats et al, 1989, Renko et al, 1990 and Bugler et al, 1991). The exact function of those multiple forms is not yet understood, but some studies suggest different functional importances of the multiple existing forms (Couderc et al, 1991 and Quarto et al, 1991). The cDNAs for bFGF and aFGF lack a classical signal peptide sequence (Jaye et al, 1986). Although aFGF and bFGF were localized in the basement membrane, the mode

of their release from cells is unclear. The lack of signal peptide sequence and the observation that little or no bFGF was detected in the conditioned media of endothelial cells (Schweigerer et al, 1987) led to the "conclusion" that no FGF is released from cells under normal conditions. Later studies argued against that conclusion as it was found that an astrocytoma cell line released 15 to 50 times more of bFGF compared to a corneal endothelial cell line (Sato et al, 1989). The stimulation of the bovine aortic endothelial cell proliferation and migration by the addition of exogenous FGF (Sato et al, 1989) suggested that extracellular FGF is important for normal cell function. Anti-bFGF antibodies inhibited the autocrine growth of endothelial cells (Schweigerer et al, 1987). Kimelman and Maas (1992) showed that injecting synthetic XbFGF mRNA into cells of early Xenopus embryos resulted in mesoderm induction. Also, Amaya et al, (1991) demonstrated that the extracellular domain of the Xenopus bFGF receptor (XbFGFR) was essential for mesoderm induction. These observations suggested that a classical signal peptide for FGF secretion was not required for normal FGF functioning. From the above mentioned observations, it seems that bFGF resembles interleukins and some other proteins that can be released from cells despite the lack of a signal peptide (reviewed in D'Amore et al, 1990; Baird and Bohlen 1990). One of the possible factors that may interfere with the detection of the released bFGF is its rapid association with some of the extracellular matrix components as well as its binding to the cell surface receptors. Other possible pathways were suggested for the release of FGF from cells. Angiogenesis is often observed in tissues where there are ischemic events, suggesting the release of angiogenic factors, such as bFGF, can take place due to cell death and lysis. The release of a

significant amount of FGF-like growth factor activity in transiently injured cells by scraping, demonstrates another possible way of FGF release without the need of cell death (reviewed in D'Amore 1990). FGF may be delivered locally by FGF-containing blood cells such as platelets and macrophages. The existence of a secreted form of the FGF receptor (Johnson et al, 1991), which can bind bFGF, suggests another possible mechanism for FGF transport outside the cell. A possible way of FGF release, which is consistent with the fact that FGF is associated with cell surfaces and extracellular matrix, was suggested by a novel mechanism reported for the secretion of lectin, a protein that lacks a signal peptide, from mouse muscle cells through plasma membrane evaginations (Cooper et al, 1990). The 37 residues upstream of the Met initiation site (present in the high molecular weight forms of bFGF), a glycine-rich sequence with interspaced arginines, is implicated for the presence of nuclear signal sequences that direct the internalized bFGF into the nucleus (Bugler et al, 1991; Amalric et al, 1991; Renko et al, 1990).

Multiple transcripts (7.0, 3.7 and 1.4 Kb) of the bFGF gene were detected in human tissues (Baird and Bohlen 1990), while a single major 6.0 Kb transcript for the rat bFGF mRNA (Emoto et al, 1989 and Shimasaki et al, 1988) and other smaller minor transcripts (3.0 and1.8 kb, Emoto et al, 1989) were reported. Ernfors et al, (1990) reported the presence of a major 3.7 Kb bFGF transcript as well as shorter 1.8 and 1.5 Kb mRNA species in the developing rat brain. Powell et al, (1990) detected 6.0, 3.7, 2.5, 1.8, 1.6, 1.4 and 1.0 Kb bFGF mRNA in the developing rat brain. The significance and functional importance of the existence of multiple bFGF transcripts is not yet clear.

III. FGF Receptor Family

Low and High affinity FGF binding sites

The biological response of cells to FGFs is mediated through specific high affinity cell surface receptors (kd=2-20x10^{-11}M) that possess intrinsic tyrosine kinase activity and are phosphorylated upon binding to FGFs (Coughlin et al, 1988). Low affinity FGF receptors (kd=2x10^{-9}M) have also been identified (Moscatelli et al, 1988) and were found to be heparin sulfate proteoglycans (HSPGs) that are present on the cell surface (Moscatelli et al, 1988) and in the extracelluar matrix (Voldavsky et al, 1987). Basic FGF could be specifically released from these low affinity binding sites by an excess of heparin or by enzymatic digestion with heparinases but not with closely related glycosaminoglycans (Moscatelli et al, 1988). The affinity of bFGF for heparin (Klagsbrun and Shing 1985) and along with the observation that various cell types express heparin-like molecules on their cell surfaces suggest a physiological function(s) for heparin-like, low affinity FGF receptors. Studies on chinese hamster ovary (CHO) mutant cells, expressing high affinity FGF receptors but lacking HSPGs, do not bind bFGF unless heparin or heparin sulfate is included in the binding medium (Yayon A. et al, 1991). These results suggest that HSPGs play a role not only in the stabilization of FGF but they are also crucial for FGF to bind to its high affinity receptors, probably through changes in the confirmation of FGF molecules. Syndecan, an integral membrane proteoglycan, contains both heparin sulfate and chondroitin sulfate glycosoaminoglycan (GAG) chains, (Rapraeger et al, 1985) originally isolated from mouse mammary epithelial cells. Syndecan binds to heparin-binding growth factors and acts as a low affinity FGF receptor (Kiefer et al, 1990).

High affinity FGF receptors (FGFRs) belong to the receptor tyrosine kinase (RTK) family (Ullrich and Schlessenger 1990). They show high degree of similarity to another member of the RTK family, the PDGF receptor family. The structural similarity of FGF receptors to other receptor-linked tyrosine kinases, has helped in predicting of the mode of signal transduction through these receptors (Ullrich and Schlessenger, 1990). On binding to FGF, the receptors form dimers, a process that is essential for signal transduction (Ullrich and Schlessenger, 1990). The formation of heterodimers with a mutant truncated high affinity FGF receptor that either lacked the first Ig-like domain (Ueno et al, 1992) or the tyrosine kinase domain (Amaya et al, 1991) lead to the inhibition of signal transduction by wild type FGF receptors. FGF receptors have been shown to activate tyrosine phosphorylation of several substrates in addition to phospholipase C-γ{(PLC-γ), Burgess et al, 1990}. A partial cDNA for human *flg* (fms-like gene), the first high affinity FGF receptor to be cloned, was isolated on the basis of its homology to the tyrosine kinase domain of the CSF-1 receptor (Ruta et al, 1989). The second high affinity FGF receptor to be cloned, *bek* (bacterially expressed kinase), was isolated by phosphotyrosine antibody screening of a mouse liver expression library (Kornbluth et al, 1988). The third member of FGF receptors , FGFR-3, can be activated by aFGF and bFGF (Keegan et al, 1991). The fourth member of the FGF receptor family, FGFR-4, is 55% identical with *flg* and *bek* and binds specifically with high affinity to aFGF but not bFGF (Partanen et al, 1991). Chick embryo kinase (CEK-3) as well as chicken flg and bek were also isolated by the use of phosphotyrosine antibody screening of a chicken embryo expression library (Pasquale et al 1990; Pasqual and Singer 1989). Each

receptor consists of an extracellular domain that contains 2 to 3 immunoglobulin (Ig) like domains, a transmembrane domain and an intracellular tyrosine kinase domain. The Ig-like domains indicate that FGF receptors belong to Ig superfamily (Williams and Barclay 1988). Between the first and the second Ig-like domains is a region with a high concentration of acid residues, acid box; it is especially noticeable in *flg*. The intracellular domain consists of a single chain, contains a relatively long juxtamembrane sequence and has an insertion of 14 amino acids in the conserved tyrosine kinase domain. Each FGF receptor was found to exist in multiple forms generated mainly by alternative splicing of the native mRNA of the receptor (Eisemann et al, 1991; Robinson 1991; Dionne et al, 1991). The multiple forms of each receptor mainly vary in the extracellular region. For example, one form of *flg* and *bek* is missing the first Ig-like domain. Another form of the *bek* gene product, the smallest extracellular domain of the FGF receptors yet reported, is missing both Ig-like domain and the acid box linker region. Other cDNAs predict soluble, secreted forms of the receptors (Johnson et al, 1991), the main function of which is still under investigation, but they may play a role in down regulation of FGF signalling or they may function as a carrier molecule to transport FGFs to their proper site of action. An alternative exon usage is a possible mechanism in producing the second half of the third Ig-like domain, which may be involved in the specificity of ligand-receptor binding. For example, usage of one exon shows high affinity binding to aFGF and bFGF (Dionne et al, 1990; Houssaint et al, 1990; Crumley et al, 1991), while usage of a different exon confers high affinity binding to aFGF and KGF but not bFGF (Miki et al, 1991). The diversity of FGF receptors produced by

alternative splicing, and the ability of these different forms to interact with more than one of the FGFs generated a high level of redunduncy in FGF receptor/ligand interactions. The spatial and temporal expression of specific forms of the receptors (Wanaka et al, 1990 and 1991) may be a factor in determining ligand specificity. In chick embryos, FGF receptor mRNAs were expressed abundantly in the germinal neuroepithelial layer and during late maturation of neuronal populations in the regions examined such as cholinergic nuclei of the basal forebrain, brain stem reticular and motor nuclei and cerebellar Purkinje and granule neurons (Heuer et al, 1990). This pattern of FGFR gene expression was generally reciprocal to that of NGF-R in the CNS and in some periods of development of the PNS (Heuer et al, 1990). These observations suggested sequential rather than simultaneous effects of FGF and NGF on neuronal development.

IV. Neurotrophic factors

Depending on their locations, 20 to 70% of the neurons of the developing nervous system will be eliminated through a process known as the naturally occurring neuronal programmed cell death, or apoptosis. This process is thought to be regulated by proteins called neurotrophic factors (NTFs). Nerve growth factor (NGF), for a long time, was the only known NTF (reviewed in Levi Montalcini 1987). Barde (1988) defined a NTF as a protein that is synthesized in the respective neuronal target tissue, present in a limited concentration, taken up by receptor-mediated retograde axonal transport, and able to prevent the ontogenetic neuron death. Only NGF completely fulfils Barde's criteria for defining a NTF. In general, development and maintenance of the vertebrate nervous system requires NTFs. Recent studies have shown that these factors can also stimulate neuronal sprouting in the brain (Ernfors et al, 1991). Studies on dorsal root ganglia (DRG) neurons suggest that NGF is produced by innervated target tissue, where it is taken up by nerve terminals to be transported to the neuronal soma to regulate gene transcription. At some developmental stages NGF is required for neuronal survival. Later on in the adult nervous system, it appears to regulate neurite outgrowth, synaptic connections and the quantity of neurotransmitter produced. However only a small minority of the neurons in the brain and spinal cord are responsive to NGF.

1. The neurotrophins

Although NGF was the only known NTF for several decades, the cloning of the brain-derived neurotrophic factor (BDNF, Leibrock et al, 1989) revealed

striking structural similarities between BDNF and NGF, suggesting the existence of a gene family of neurotrophic factors which allowed the characterization of three additional neurotrophins named: NT-3 (Maisonpierre et al, 1990a,b), NT-4 (Hallböök et al,1991), NT-5 (Berkemeier et al, 1991). All the above mentioned five neurotrophins are capable of promoting the survival and differentiation of many types of sensory neurons.

a. Nerve Growth Factor (NGF)

It is becoming clear that polypeptide growth factors play important roles in the development and maintenance of neural circuits (Snider and Johnson 1989). NGF is the most thoroughly studied neuronal growth factor that promotes the survival and outgrowth of peripheral sensory and sympathetic neurons (Levi-Montalcini and Calissana 1986). NGF also influences the development of certain groups of neurons including those of the basal forebrain-septum (Hartikka and Hefti 1988). NGF has a limited set of neural targets in comparison to other growth factors such as bFGF, and is believed to be active mainly in cholinergic neurons (Morrison et al 1988; Knusel et al 1990; Hefti et al, 1990). NGF interacts with several different types of receptors and a fast response involves the low affinity receptors (Radeke et al, 1987) while a slow response is mediated by the high affinity receptors such as NGF binding to the *trk* proto-oncogene product (Kaplan et al, 1991). *In vitro* studies on cultured neurons showed that NGF did not promote neuronal survival or neurite outgrowth in hippocampus or cortical neurons (Mattson et al, 1990). Low affinity NGF receptors are normally sparse or absent in hippocampal and cortical neurons (Koh et al, 1989) suggesting that NGF may not have biological activity in neurons at these brain sites. NGF can prevent

the damage of cholinergic neurons associated with brain lesions in animal models of neurodegenerative disorders (Hefti et al, 1989), and protect human and rat neurons against hypoglycaemic damage (Cheng and Mattson, 1991). NGF promotes the survival and differentiation of neural crest-derived sensory and sympathetic neurons during development and their maintenance in the adult (Barde 1989). Injury to the rat sciatic nerve leads to the induction of NGF receptors on the denervated Schwann cells and their disappearance on the regenerating axons of the axotomized, normally NGF n-sensitive sensory and sympathetic neurons (Raivich et al, 1991). These results suggest that while sensory and sympathetic neurons are primary targets of NGF, Schwann cells may become its primary target in the aftermath of nerve injury (Raivich et al, 1991).

b. Brain-derived neurotrophic factor (BDNF)

Originally characterized as a factor that increases the survival of DRG neurons, BDNF synthesis occurs mainly in the CNS, particularly in cortical and hippocampal neurons (Hofer et al, 1990). The *trk* B (Klein et al, 1990), the BDNF receptor, a tyrosine kinase, is also expressed in the same neuronal populations, which suggests an autocrine function for the BDNF in these tissues. The spatial and temporal distribution of BDNF mRNA (Maisonpierre et al, 1990b) suggests a possible role for BDNF in supporting the survival of CNS sensory neurons (Hofer and Barde 1988). BDNF promotes differentiation of basal forebrain cholinergic and mesencephalic neurons *in vitro* (Alderson et al, 1990, Hyman et al, 1991). All these observations have led to the hypothesis that BDNF is a specific regulator of CNS growth for a variety of peripheral sensory neurons. In the adult brain, BDNF mRNA concentrations increase as

a result of seizures (Isackson et al, 1991) and ischemia (Lindvall et al, 1992) indicating a role for BDNF in neurodegeneration-neuroprotection and neural plasticity. The highest levels of BDNF mRNA within the hippocampus are found in the pyramidal layer of the CA2 and CA3 regions as well as the dentate gyrus and hilus. BDNF has the most widespread distribution in comparison to the other neurotrophins in different regions of the brain (Ernfors et al, 1991) and its highest gene expression occurs two weeks after birth, a week before NGF reaches its peak (Ernfors et al, 1991)

c. Ciliary Neurotrophic factor (CNTF)

CNTF was originally identified and partially purified from avian ocular tissue and later isolated from the siatic nerve of rodents (Lin et al, 1990). Chemical studies showed that peripheral nerve is the richest tissue source of CNTF (Rende et al, 1992). CNTF was first demonstrated to support the survival of embryonic chick parasympathetic ciliary ganglion neurons *in vitro* and later was shown to support motor neurons of the spinal cord (Arakawa et al, 1990) and sympathetic neurons (Barbin et al, 1984). CNTF has been shown to rescue motor neurons from naturally occurring cell death during development of the chick embryo (Oppenheim et al, 1991). Recent *in vitro* studies have shown that CNTF promotes the survival of certain neuronal subpopulations of the hippocampus (Ip et al, 1991). An investigation by Hagg et al, (1992) on the effect of CNTF on axotomized neurons in the adult brain, showed that CNTF has a rescue effect on those neurons.

d. Epidermal growth factor (EGF)

EGF is a mitogenic peptide, first isolated from the male mouse submaxillary gland (Cohen 1962), influencing the growth and development of

a variety of tissues (for review see Carpenter and Wahl 1990). EGF has been shown to promote proliferation and differentiation of glia (Honegger and Guentert-Lauber 1983) and to enhance neuronal survival and process outgrowth (Morrison et al, 1988, Casper et al, 1991). EGF (Lazar and Blum 1992) and EGF receptors (Werner et al, 1988) are also detected in the developing and adult CNS. The level of expression of EGF mRNAs were shown to increase in the developing brain reaching its maximum level in the adult (Lazar and Blum, 1992). Also the EGF mRNAs were detected in all the different regions examined of the adult brain (Lazar and Blum 1992). These results suggested a role for EGF in the development of the mammalian CNS.

e. Platelet derived growth factor (PDGF)

Human PDGF is a major mitogen for cells of connective tissue origin and is involved in development and wound healing (Smits et al, 1991) and may be acting as a key growth regulator during normal development including neurodevelopment (Mercola et al, 1990). PDGF is disulphide linked homo- or hetero- dimer consisting of two related polypeptide chains, the A and B subunits, that are products of two different genes. In the embryo the PDGF A-chain mRNA was restricted to the PNS and CNS (Yeh et al, 1991). In adult mice, PDGF A-chain mRNA was abundant in neurons throughout the spinal cord, peripheral ganglia and brain, and the protein was present in both neurons and glial cells. PDGF-BB and PDGF-AB were present in neurons in many regions of the adult monkey brain (Sasahara et al, 1991). The presence of PDGF in axon terminals (Sasahara et al, 1991) raises the possibility that it may be involved in nerve-target interactions.

2. Basic FGF and its roles in the developing and adult nervous system

a. Basic FGF as a neurotrophic factor

Recently, bFGF has emerged as a growth factor that is produced in the nervous system and exerts a variety of *in vivo* and *in vitro* effects, stimulating mitogenesis, differentiation and maintenance of cells of neuroectodermal and mesodermal origin including neurons and glial cells suggesting a major role for bFGF in maintaining the integral function of various parts of the nervous system. Evidences for the direct action of bFGF on glial and neuronal cells were derived from various studies (Unsicker et al, 1990; Mattson and Rychlik 1990). Walicke and Baird (1991) showed that exogenous bFGF is internalized into vesicles in the cytoplasm of both astrocytes and hippocampal neurons, and then translocated to chromatin structures of the nucleus, suggesting a role for bFGF in gene regulation. The processed internalized bFGF fragments (15.5, 9 and 4 KDa) were similar in both the astrocytes and the neuroectodermal cells (neurons), suggesting a similar manner of metabolizing of bFGF in both major CNS cell types. Studies by Ferguson et al, (1990) showed that bFGF is transported anterogradely by retinal ganglion cells *in vivo*, an important step for a neurotrophic factor to interact with and exert its effects on the target neurons.

b. Basic FGF expression by neurons and glial cells

Pettmann et al, 1986 and Janet et al, 1988 detected bFGF immunoreactivity in neurons but not in glial cells in cultured brain and peripheral ganglia cells. Others reported the expression of bFGF mRNA only in cultured astrocytes (Emoto et al, 1989) and bFGF mRNA and protein in astrocytes from adult bovine corpus callosum (Ferrara et al, 1988). On the

other hand, *in situ* hybridization studies by Emoto et al, 1989 showed that neurons of several brain regions contained the bFGF mRNA. Gómez-Pinilla et al, (1992) showed that bFGF immunoreactivity was mainly localized in astrocytes and occasionally in microglial cells throughout the normal rat brain. Few neuronal populations in the septohippocampal nucleus, CA-2 field of the hippocampus, cingulate cortex, cerebellar Purkinje cells, deep nuclei, facial nerve nucleus and the motor and spinal subdivisions of the trigeminal nucleus and facial nerve nucleus were expressing bFGF mRNA (Gómez-Pinilla et al, 1992). Studies on the distribution of bFGF mRNA by Emoto et al, 1989, using in situ hybridization, showed similar distribution of bFGF mRNA compared to the distribution of the bFGF protein detected by Gómez-Pinilla et al, (1992), with a more restricted distribution in neuronal populations (CA-2 region of the hippocampus, layers 2 and 6 of the cingulate cortex, indusium griseum and fasciola cinereum). Put all the above mentioned observations together, it seems that bFGF is synthesized by both glial cells and specific neurons of the CNS, however, the exact neuronal cells synthesizing it and the amount produced by each neuronal population is still unclear.

c. *In vitro* effects of bFGF on

i. Neurons

One of the most important reported effects of bFGF on nervous system cells is the promotion of neuronal survival and neurite outgrowth. Basic FGF maintained the survival of many neurons of the CNS obtained from cerebral cortex, thalamus, striatum, septum (Walicke 1988), hippocampus (Walicke et al, 1986), mesencephalon (Ferrari et al, 1989) and spinal cord (Unsicker et al,

1987). In the PNS, application of bFGF to the proximal end of a severed sciatic nerve enhanced the remyelinization of its neuronal sheath and prevented the death of dorsal root ganglia (Baird and Boehlen 1990). Studies on the rat retina ganglion cells, showed that bFGF was able to enhance their survival and neurite outgrowth (Bahr et al, 1989). Neurons located in the subiculum (Walicke 1988), rat rondose and superior cervical ganglia as well as chick sympathetic and DRG (Unsicker et al, 1987) were not affected by bFGF addition. The mitogenic activity of bFGF was also exerted on neuroblast and neuroblastoma cells (Gensburger et al, 1987, Ludecke and Unsicker 1990). Other cellular changes following addition of bFGF were also reported such as the increase in the Choline-acetyltransferase activity in ciliary ganglion neurons (Unsicker et al, 1987), septal neurons (Grothe et al, 1989a,b) and spinal cord neurons (McManaman et al, 1989). Hippocampal neurons were rescued by bFGF from glutamate toxicity (Mattson et al, 1989).

ii. Glial cells

Basic FGF acts as a mitogen for astrocytes and oligodendrocytes (Delaunoy et al, 1988), stimulates the proliferation of different types of glial cells, and maturation of astrocytes, but as an inhibitor of the differentiation of glioblasts and maturation of oligodendrocytes (Westermann et al, 1990). Yong et al, 1988a,b found that bFGF is a mitogen for fetal, but not adult human astrocytes, oligodendrocytes and Schwann cells. *In vitro* studies showed that addition of bFGF to astrocytes (Perraud et al, 1990) and oligodendrocytes (Delaunoy et al, 1988) obtained from various regions of the brain resulted in changes of the morphology of these cells. Heparin and other glucosoaminoglycans are potent enhancers of the bFGF effects on glial cells

(Perraud et al, 1988a,b). In contrast, NGF does not exert any of the bFGF effects on the physiology and morphology of glial cells. Proliferation and neurite outgrowth induction by bFGF (Stemple et al, 1988) were observed on noradrenergic chromaphin cells (a type of the PNS neurons that contain bFGF) (Grothe and Unsicker 1990). Similar effects of bFGF on neurite outgrowth of a tumor cell line of the chromaffin cells,PC12, were reported (Neufeld et al, 1987).

d. *In vivo* effects of bFGF on neuroectodermal cells:

The naturally occurring death of the chick ciliary ganglion neurons between embryonic day 8 and day 14 (loss of 56% of the neurons), is almost completely prevented by the application of bFGF (Dreyer et al, 1989). Fimbria-fornix lesions and optic nerve transection were used to study the effect of bFGF on CNS injury. Fimbria-fornix transection results in the loss of about 87% of the neurons (Otto et al, 1989). About 60% of the cholinergic neurons in the medial septum (Anderson et al, 1988) dies after fimbria-fornix transection, 20% of those neurons were rescued from death by bFGF, and almost 80% of the dying (50%) cholinergic neurons in the diagonal band of Broca on the ipsilateral side (Anderson et al, 1988) survived after bFGF application. Twenty five percent of the retinal ganglion neurons were rescued from death by bFGF after optic nerve fiber transection (Sievers et al, 1987). Treatment with bFGF reduced the lesion-induced loss of choline acetyltransferase activity and increased the number of reactive astrocytes in the hipocampus (Barotte et al, 1989).

V. Angiogenesis

Proliferation of blood vessels (angiogenesis) is a process that is important for the normal growth and development of tissues (Folkman 1971). Blood vessel growth in early embryonic development and in the adult animals is tightly controlled. Some developing organs (like brain) are vascularized by sprouts branching from preexisting blood vessels (Klagsbrun and D'Amore 1991). In the adult, angiogenesis is not a common process except in few tissues such as female reproductive system, in the development of corpus luteum during ovulation and in the placenta after pregnancy. In normal adult tissues angiogenesis is induced in tissue repair processes such as wound healing (Broadley et al, 1989) and fractures (reviewed in Klagsbrun and D'Amore 1991).

In the brain, endothelial cells originate from outside the nervous system. Sprouts of endothelial cells form as compact masses with a lumen, which are produced as a result of secretion of extracellular matrix materials by endothelial cells (Gordon et al, 1985). Trophic factors produced by astrocytes play a role in the regulation of development of brain capillary endothelial cells. Angiogenesis increases within the period of glial cell proliferation, growth and differentiation of dendrites, synaptogenesis and myelination (within the first 3 weeks in postnatal rats). Capillaries formed in the brain are unique in that the capillary endothelial cells form continuous tight junctions, a feature that prevents molecules from passing between them, and form what is known as blood-brain barrier (reviewed in Jacobson et al, 1990). Abnormal blood vessel proliferation can lead to pathological conditions, as in the case of the growth of solid tumors that depend on the vascularity of those sites (Folkman et al,

1972). Angiogenesis is a multistep process that involves the degradation of the capillary basement membrane, the migration and proliferation of endothelial cells and tube formation. The search for candidate factors influencing angiogenesis was suggested by Folkman (1972) when he recognized that unless solid tumors become vascularized, they can remain embedded in tissues for a long period of time without noticeable tumor growth. Based on this observation, he suggested the presence of tumor angiogenesis factor (TAF). Soluble factors were identified that either stimulate or inhibit one step or more in the process of angiogenesis (reviewed in Klagsbrun and D'Amore 1991). FGF and TGF-α are growth factors that exert direct effect on endothelial cells, stimulating their proliferation and migration. Different studies reported the presence of a highly specific mitogen for vascular endothelial cells known as vascular endothelial growth factor (VEGF; Conn et al, 1990, Connolly et al, 1989). Besides being an endothelial cell mitogen and angiogenic factor, VEGF is a vascular permeability factor (Connoly et al, 1989). In contrast, angiotropin stimulates the migration but not the proliferation of endothelial cells, while angiogenin has no effect on their proliferation and migration (Klagsbrun and D'Amore, 1991). FGFs were identified on their ability to stimulate the proliferation of endothelial cells (reviewed in Folkman and Klagsbrun 1987) and their involvement in angiogenesis will be discussed in more details below.

1. FGF As an activator of angiogenesis

Endothelial cells synthesize large amounts of bFGF (Schweigerer et al, 1987) that remains associated with the cell and the subendothelial cell extracelluar matrix (ECM) (Baird and Ling 1987; Vlodavsky et al, 1987) and

released by the action of heparinase (Bashkin et al, 1989). The storage mechanism of FGF in the ECM may be an important process in the rapid response of FGF to stimulate angiogenesis and cell proliferation in wound healing. Basic FGF synthesis was also detected during the process of development of granulosa cells into the corpus luteum (the target of neovascularization in the ovary) (Neufeld et al, 1987). Basic FGF is a mitogen and is chemotactic for endothelial cells (Gospodarwicz et al, 1987a and b; Thomas et al, 1987 and Terranova et al, 1985). Basic FGF also stimulates the production of collagenase and plasminogen activator proteases that are capable of degrading the basement membrane (Mignatti et al, 1989). Basic FGF is an inducer of the migration of capillary endothelial cells to the collagen matrices and the formation of capillary-like tubes (Montesano et al, 1986). FGFs have been implicated in the process of formation of collateral vessels in ischemia from the observations that FGFs are localized in the heart and the brain and can stimulate vascularization when injected into the brain (Cuevas et al, 1988). FGF stimulates the production of new matrix components such as collagen (Davidson et al, 1985) and GAGs (Gordon et al 1985). FGF maintains the differentiated state of endothelial cells through delaying senescence (Gospodarwicz et al, 1986), stimulation of expression of nonthrombogenic apical cell surface (Vlodavsky et al, 1979) and increasing cell attachment to the substratum (Schubert et al, 1987). *In vitro* studies showed that bFGF can induce the rearrangement of endothelial cells to form tubular structures (Montesano et al, 1986). From all the above gathered information about the involvement of FGF in the process of angiogenesis, it seems that FGF directs endothelial cells at the site of neovascularization, breaks down the ECM in

that region to clear the path for the formation of new blood vessels and eventually, participates in the process of damage repair of the injured tissue.

2. FGF in Wound healing

FGF has been detected at the site of the focal brain wounds (Finkelstein et al, 1988). Acidic FGF is released early after rat brain injury (Neito-Sampedro and Cotman 1988). Addition of FGF to the injured corneal epithelium stimulated its healing (Muller et al, 1985). FGFs accelerate the wound healing process through triggering cellular responses that are important for the tissue repair such as attraction of important cells to the wound site (Sprugel et al, 1987) and the stimulation of fibroblast proliferation and extra cellular matrix components synthesis (Davidson et al, 1985, Sprugel et al, 1987, Buntrock et al, 1984).

VI. Factors involved in embryonic development

At the early blastula stage, the amphibian embryo (e.g Xenopus) consists of two cell types: presumptive ectoderm in the animal hemisphere, and presumptive endoderm in the vegetal hemisphere. Until fertilization, the egg is radially symmetrical around the animal-vegetal axis. Upon fertilization, sperm enters the ventral vegetal side of the egg causing a rotation of the cortex of the egg relative to its cytoplasmic core. This cortical rotation brings large yolk platelets and animal pole cytoplasm into close contact. The orientation of this rotation accurately predicts the future dorsoventral polarity in the vegetal hemisphere. Most of the dorsoventral patterning information is generated in the vegetal hemisphere (for review see Kay and Smith 1989, Hopwood 1990, Slack 1990, Slack et al 1990). Determination of both the dorsal-ventral and anterior-posterior axes are the most early major events in the development of an embyo. In the developing embryo cells are progressively specialized by a series of cellular interactions called inductions, in which a group of cells is affected by a signal from an adjacent group (Spemann et al, 1921). The first detected inductive signals during early amphibian development, produced by the endoderm of the blastula (vegetal hemisphere) result in the generation of mesoderm from ectoderm (the animal hemisphere of the embryo) around the blastula equator. Mesoderm, the middle germ layer, comprises the largest part of the body of vertebrates, such as muscles, connective tissues, most of the skeleton, the vascular system and most of urinary and genital systems. There are at least two types of signals that induce mesoderm formation. The first signal is generated from the dorsal vegetal hemisphere that induces dorsal mesoderm cell types (such as muscle

and notochord), and the second one derived from the ventral vegetal hemisphere of the embryo that induces mainly ventral tissue type (such as blood and mesothelium). A third interaction, known as dorsalization, takes place between the newly formed dorsal mesoderm and the nearby ventral mesoderm region and results in a further specialization to form other ventral mesodermal tissues. The furthermost dorsal mesodermal structure, the notochord, is the first to differentiate. Somites, lie on either side of the notochord, later differentiate to muscle, skeleton and connective tissue of the skin. The lateral plate mesoderm will give rise to heart, smooth muscle of the gut, the mesenteries and blood. The urinary and genital systems arise from the mesodermal region that connect lateral plate and somites. The second major induction interaction at the gastrula stage between the newly formed dorsal mesoderm and dorsal ectoderm leads the formation of neuroectoderm (Ruiz 1990).

1. Mesoderm inducing factors (MIF)

Factors from two growth factor families have been identified as the most potent mesoderm inducers in embryos. These include the dorsal mesoderm inducers, activin, XTC-MIF (the Xenopus homologue of the mammalian activin, Smith et al, 1990), and TGFβ2 from the TGFβ superfamily (Roberts et al, 1990), and ventral mesoderm inducers, members of the FGF family (aFGF, bFGF, hst-2/kFGF, FGF-5 and int-2, Slack 1990 and Paterno et al, 1989). Studies showed that low concentrations of activin induces the formation of ventral mesodermal tissues (mesenchyme and mesothelium), higher concentrations produces dorsal mesoderm (Muscle, Green et al, 1990a,b) and

highest concentrations induce notochord. On the other hand bFGF at both high and low concentrations only induces ventral mesoderm formation. The exact mechanism and the product of these induction processes are not clear yet, but these results suggest that each cell has a threshold corresponding to each response. Alternately, all cells would have one threshold and their response depends on the proportion of induced cells in each tissue. Xwnt-8, a member of the Wnt gene family that also has been hypothesized to play a role in embryonic patterning (Gavin et al, 1990) was localized to cells within the ventral vegetal region of the gastrula. Another member of the Wnt gene family, Wnt-1 is expressed in a temporally and spatially restricted fashion during murine embryogenesis (Shackleford and Varmus 1987). Both XTC-MIF and bFGF are activators of Xwnt-8 expression. FGFs and Wnt gene family members may cooperate to initially specify the complete dorsal-ventral axis of the mesoderm.

2. FGFs and their role in mesoderm induction

Many observations showed that FGF family members are the potential factors for the ventral mesoderm induction of embryonic ectoderm (reviewed in Smith et al, 1989). The detection of FGF or FGF-like molecules in the developing chick brain and in the kidney mesenchyme of the mouse embryo (Risau 1986; Risau et al, 1988; Risau and Ekblom 1986) as well as the presence of bFGF in the human placenta (Moscatelli et al, 1986) led to the hypothesis that FGFs could support the proliferation and differentiation of specific tissue cells during embryonic and fetal development. Later studies showed that FGF family members can induce all mesodermal derivatives

except notochord, and enhanced by TGF-β to induce mesoderm (Hopwood 1990). *In vitro* studies showed the response of ectoderm to FGF rises from about the 128-cell stage and falls again by the onset of gastrulation. The mRNAs and the protein for bFGF (Kimelman et al, 1988) and its receptor (Gillespie et al, 1989 and Musci et al, 1990) are present in the Xenopus embryos. Mesoderm induction in Xenopus embryos occurs over approximately a seven-hour time span from the 64-cell stage until the beginning of gastrulation (Woodland and Jones 1987), and the exposure of the animal cap cells to as little as 10 minutes for the added Xenopus bFGF (XbFGF) protein was sufficient to induce the formation of ventral mesoderm (Green et al 1990; Green 1990). Differential expression of two of the FGF receptors, flg and bek, in the mesenchyme and in the epithelium respectively in a variety of embryonic tissues including skin, limb, gut and respiratory tract (Peters et al, 1992) suggest the mediation of different functions of FGFs during development. A negative dominant mutant form of the FGF receptor failed to induce mesoderm in response to FGF in embryonic explants, causing specific defects in gastrulation and in posterior development in the whole embryo (Amaya et al, 1991). These defects were rescued by overexpression of the wild type of the FGF receptor, suggesting an important role for FGF in the formation of the posterior and lateral mesoderm in early embryogenesis. The low-affinity FGF receptor, syndecan, was detected at the 4-cell stage, and at cell-cell boundaries at the blastula stage. At the time of endoderm segregation, it become restricted to the interface between the endoderm and ectoderm (Sutherland et al, 1991) suggesting a role in mesoderm formation. In other studies, syndecan expression was found to undergo striking changes during epithelial-

mesenchymal transitions; it is turned on in mesenchymal cells that will become epithelial and turned off in epithelial cells that will become mesenchymal (Vainio et al, 1989 and Trautman et al, 1991). These observations suggest roles in stabilization of epithelia in the early pattern formation of embryonic mesoderm. A very recent study by Kimelman and Maas (1992) on Xenopus embryos, showed that XbFGF induces the formation of dorsal mesodermal structures when translated from injected synthetic RNA, suggesting a role for bFGF in establishing the dorsoventral axis of the embryonic mesoderm. The discrepency between this observation and the previous studies that showed bFGF as a ventral mesoderm inducer was explained by the difference in the way bFGF was delivered to the animal cap cells. Another member of the FGF family, int-2 was detected in the developing mouse rhombencephalon in a spatial and temporal pattern suggesting its importance in the normal development of the inner ear (Represa et al, 1991). The fourth member of the FGF family, hst/kFGF, was detected in the late blastocyst stage of the developing mouse embryo, in cells that give rise to all embryonic lineages, suggesting the maintenance of different cell types in that cell population. Later in development, hst/kFGF become restricted to the primitive streak and continues to be expressed at the gastrula stage in the distal two thirds of the primitive streak that will give rise to the future mesoderm and definitive endoderm. During subsequent development, its expression becomes localized in the branchial arch units, somatic myotome, the apical ectoderm ridge of the developing limb bud and tooth bud (Niswander et al, 1992), suggesting multiple roles for hst/kFGF during embryogenesis. The hst/kFGF is expressed in ectodermal cells near and in the primitive streak, while int-2 is expressed

at high levels in the mesoderm as cells leave the streak and move laterally (Niswander et al, 1992). These observations reveal a complementarity in the pattern of their mRNAs expression. FGF-5 mRNAs are detected in visceral endoderm surrounding the embryo, and in the ectoderm prior to the formation of the primitive streak (Hébert at al, 1991). A possible novel member of the FGF family that was cloned from Xenopus laevis embryo cDNA, eFGF, was shown to be expressed maternally and zygotically with a peak during the gastrula stage, and its expression was concentrated in the posterior of the body axis and later in the tailbud, suggesting its involvement in mesoderm induction at the blastula stage and in the formation of the anterior-posterior body axis at the gastrula stage (Isaacs et al, 1992). Taken together, the data suggest that different members of the FGF family are involved in a variety of processes such as a role in specific epithelial mesenchymal interactions, mesoderm formation, body plans formation and embryonic organogenesis.

3. Anterior-Posterior body axis formation

In the early amphibian gastrula, the vegetal side in contact with the animal hemisphere of the embryo releases growth factors that induce the activity of a region known as Spemann's organizer (Spemann et al, 1921) in overlying cells at the dorsal side of the embryo (Smith et al, 1989, Thomsen et al, 1990). Spemann's organizer induces the invagination of cells through the blastopore lip and consequently the extent of the future anterior-posterior body plan (A-P). There are at least forty homeobox (HOX) genes in the genome of the mouse and most other vertebrates that encode transcription activators, through protein-DNA interaction, and play a major role in the cell specification

along the A-P body axis of developing embryos. Those genes are located in four clusters, of about ten genes each, with genes located at the 5' end expressed in posterior regions of the embryo, and those in more 3' regions expressed in a more anterior parts of the embryo (reviewed in De-Robertis et al 1989). HOX gene expression is highly specific and show a gradient field of distribution of their mRNAs. One of the major sites of their expression is the nervous system, with distinct pattern of expression of each individual HOX gene, with distinct anterior boundaries mainly in the spinal cord and the hind brain (Hunt et al, 1991). Their distribution and pattern of expression suggest a fundamental role in the organization of the nervous system and the specification of the craniofacial and branchial structures. For example, the expression of the XlHbox 1 protein occurs in a narrow region in the anterior trunk of Xenopus embryo at the tail bud stage that includes mesoderm, anterior spinal cord and neural crest (De-Robertis et al, 1989). Three weeks later, XlHbox 1 protein was found to be distributed in a A-P gradient with maximal expression at the anterior. The field of XlHbox 1 expression shows its high specificity to the forelimb but not the hind limb (Hunt and Krumlauf 1991). The effect of growth factors in the A-P body plan formation may be through the activation of expression of certain HOX genes in specific regions of the developing embryos. FGF induces high levels of Xhox 3, a posterior member of the HOX gene family (Ruiz and Melton 1989), while activin induces the Xlhbox 6, an anterior marker (Cho and De Robertis 1990). these observations suggested the role of FGF as a posterior inducer, while activin is an anterior inducer.

VII. Research objectives

While bFGF protein is widely distributed in variety of adult tissues of mesodermal and neuroectodermal origin the expression of bFGF mRNA is only readily detectable in the brain (Shimasaki et al, 1988). Other studies showed that exogenous bFGF regulates the functions of cells in the nervous system (Walicke, 1988; Walicke and Baird, 1988). Those observations support the assumption that bFGF has important roles in the nervous system and may act as a neurotrophic factor. Whether endogenous bFGF exerts similar or identical effects on various parts of the developing CNS has to be determined. A first step toward understanding the endogenous effects exerted by bFGF on the developing nervous system, is to study its gene expression. The aim of this study is to reveal the regional distribution of bFGF mRNA in the developing rat brain in order to provide us with insight into the significance of bFGF in the development of the nervous system. RT-PCR provides a sensitive technique for detection of low abundance messages such as bFGF mRNA. To be able to determine the relative expression of bFGF mRNA in various regions of the developing rat brain, it was necessary to establish a quantitative RT-PCR protocol to be able to compare relative amounts of bFGF mRNA present on those tissues.

Materials and Methods

I. Tissue collection

Male Sprague-Dawley rats were anesthetized with isoflurane before their sacrifice. All the experimental procedures were performed in accordance with protocols authorized by the University of Manitoba Committee on animal care, which conforms to the procedures approved by the Canadian Council on animal care. With the brain still in the skull and after the removal of the superficial meninges, the following regions were dissected with a microknife and placed on dry ice; the same order was always used: occipital cortex, inferior colliculus and cerebellum, then with the brain removed and put on ice, the pons and medulla were taken as one piece. For one separate set of 28-day-old rats, in addition to the above regions, hypothalamus was separated at the optic tracts from one thick coronal cerebral slice, along with hippocampus and cingulate cortex (For diagrams showing positions of dissected regions see figures 17 and 18).

The cerebrum was used in an initial series and included cerebral hemispheres and most of the diencephalon except for part of hypothalamus. Five to ten minutes on average were required for dissection and collection of various brain structures. After collection, tissues were immediately stored at -70 °C. Information about animals used in this study is summarized in table 13.

II. Total RNA extraction

Total RNA was extracted either by the guanidine hydrochloride/cesium chloride method (for large amount of tissues, Chirgwin et al, 1979) or by the

fast single step method (for small amount of tissues, Chomczynski et al, 1987).

1. Guanidine hydrochloride/cesium chloride method

The same procedure described in Chirgwin et al, 1979 for RNA extraction using the guanidine hydrochloride/cesium chloride method was used.

2. Single step method for RNA extraction

A fast method for RNA extraction from small amount of tissues (50-200 mg), was adapted from the published protocol by Chomczynski et al, 1987. Tissue was homogenized with 1 ml of solution A (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7;0. 5% sarcosyl, 0.1 M 2-Mercaptoethanol) at room temperature. After tissue homogenization, 0.1 ml of 2 M sodium acetate, pH 4; 1 ml of phenol (water saturated) and 0.2 ml of chloroform/isoamyl alcohol mixture (49:1) were added to the homogenate, mixed thoroughly first by inversion then vigorously by vortexing for 10 sec.. The mixture was cooled on ice for 15 min., then centrifuged at 10000 rpm for 20 min. at 4 °C. After centrifugation, the aqueous phase (upper layer) was transferred to a fresh tube, mixed with 1 ml of isopropanol and placed at -70 °C for 1 hr. to precipitate RNA. Then the samples were centrifuged at 10000 rpm for 20 min. at 4 °C and the precipitated RNA pellet was dissolved in 0.3 ml of solution A, transferred to a 1.5 ml eppendorf tube that contains 1 volume of isopropanol and kept at -70 °C for at least 2 hrs.. The samples were centrifuged at 10000 rpm for 10 min. at 4 °C, and the RNA pellet was resuspended in 75% ethanol, sedemented, vacuum dried for 7 min. and dissolved in 50-200 µl of high performance liquid chromatography (HPLC) water.

III. Northern analysis

Northern analysis was carried out as described in Maniatis et al. (1982). Total RNA was thawed in a 65 °C water bath, and then kept on ice, denatured in 33% formamide and 2.2 M formaldehyde at 65 °C for 15 minutes. The RNA was then electrophresed in a 1% (w/v) agarose gel containing 2.2 M formaldehyde, 40 mM MOPS ph 7.0, 10 mM NaAc pH 5.2, 1 mM EDTA pH 8.0, and 0.06 µg/ml ethidium bromide (EtBr). The RNA was transferred to a nitroplus 2000 (Micon Separations Inc.) hybridization filter using 20 X SAC (1X SAC = 0.15 M NaCl, 0.015 M sodium citrate). The filter was baked for 2 hours under vacuum at 80 °C. The major, EtBr stained, total RNA bands (mainly the 28 S and the 18 S ribosomal RNA bands of rat total RNA) were visualized under ultraviolet (UV) light. DNA probes were labelled by random priming. Labelling was carried out using ^{32}P-dCTP (3000 Ci/mmol) by the use of a random priming kit (Amersham). Filters containing total RNA were placed into Seal-o-Meal plastic bags and prehybridized for at least 2 hours at 42 °C in hybridization solution containing 40% (v/v) formamide, 5 X Denhardt's solution (1X Denhardt's = 0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidine), 5X SSPE (1X SSPE = 1.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 250 µg/ml denatured salmon sperm DNA and 0.1% sodium dodecyl sulfate (SDS)]. After prehybridization, the prepared DNA probes were added to the bags using a 10 ml syringe. The bags were then resealed and placed in a 42 °C shaking water bath for approximately 16 hours. Following hybridization, the filters were washed 1 time in 2 X SAC/0.1% SDS at room temperature for 15 minutes, followed by 1 wash in 0.2 X SAC/0.1% SDS at 65 °C for 30 minutes. The filters were then autoradiographed using

Kodak XAR x-ray film and an intensifying screen at -70 °C.

IV. Reverse transcription (RT)

Total RNA (0.03-1.5 µg) was incubated with 200 units of MMLV reverse transcriptase (RTase; BRL) in a buffer containing a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 5% DMSO, 19 units of RNase Inhibitor (Pharmacia), 0.01% BSA, 0.25 µg of RT primer ($AGCTACAGCTGAGCTGAGCTCAGT_{20}$), and 0.5 mM of each dNTP (Pharmacia) in a final volume of 10 µl. The reaction mixture was incubated for 2 hours at 37 °C, and then stored at -20 °C.

V. Polymerase Chain Reaction (PCR)

1. Procedure

One twentieth of the RT reaction was used in the PCR reaction in a 50 µl final volume containing 200 µM of each dATP, dCTP, dGTP and 400 µM of dUTP, 50 pmol of each primer, 2 units of Taq Polymerase (BRL), 0.5 unit of uracil DNA glycosylase (UDG, BRL), 10 mM Tris (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.02% gelatin. The mixture was overlayed with two drops of mineral oil to avoid evaporation, and then incubated in a GTC-1 genetic thermal cycler (Scientific Precision) for 35 cycles (in case of bFGF amplification) or 22 cycles (in case of GAPDH and actin) using the following profile: an initial denaturation step at 94 °C for 7 minutes, then repeated cycles of 94 °C for 45 seconds (denaturation), 55 °C for 45 seconds (annealing), and 72 °C for 90 seconds (elongation). The samples were finally incubated at 72 °C for 7 minutes, and then stored at 4 °C. The samples were run on 1.2% agarose gels

and the PCR product was visualized under UV light and then photographed using black and white Polaroid films (high speed 4X5 in. instant sheet film). The actual size of PCR product was determined by the use of DNA marker (ϕX DNA digested with Hae III).

2. Primers used for the quantitative analysis of the levels of bFGF mRNA

a. Rat bFGF cDNA: Expected PCR product size = 372 bp

Predicted from the published rat bFGF cDNA sequence by Shimasaki et al, (1988)

Pf (forward primer): 5' AAGCGGCTCTACTGCAAG 3'; position: 617-634

Pr (reverse primer): 5' AGCCAGACATTGGAAGAAACA 3'; position: 969-988

b. Rat GAPDH cDNA: Expected PCR product size = 343 bp

Predicted from the published rat GAPDH cDNA sequence by Tso et al, (1985)

Pf (forward primer): 5' GCTGGGGCTCACCTGAAGGG 3'; position: 346-365

Pr (reverse primer): 5' GGATGACCTTGCCCACAGCC 3'; position: 669-688

c. Rat actin cDNA: Expected PCR product size = 291 bp

Predicted from the published rat β actin sequence by Nudel et al, (1983)

Pf (forward primer):5' TGAACCCTAAGGCCAACCGT 3'; position: 1657-1676

Pr (reverse primer): 5' CGCACGATTTCCCTCTCAGC 3'; position: 2402-2421

3. Primers used during the process of optimizing the amplification of bFGF

Different primers were tested for the amplification of a portion of the coding region of bFGF mRNA. Best primers (that gave strong signal and with minimum non-specific amplified products) were used for the quantitation of

bFGF mRNA levels in the rat brain (as described above). Other primers tested for the amplification of bFGF mRNA, but not used for quantitation are described below:

a. Expected PCR product size=201 bp

(Predicted from the published rat bFGF sequence by Shimasaki et al, 1988).

Pf (forward primer): $^{5'}$ CGGTACCTGGCTATGAAGGA $^{3'}$; position: 770-789

Pr (reverse primer): $^{5'}$ CAGTATGGCCTTCTGTCCAG $^{3'}$; position: 951-970

b. Expected PCR product size=301bp

(Predicted from the published rat bFGF sequence by Shimasaki et al, 1988).

Pf (forward primer): $^{5'}$GGAGAAGAGCGACCCACACGT$^{3'}$; position: 688-705

Pr (reverse primer): $^{5'}$ AGCAGACATTGGAAGAAACA $^{3'}$; position: 969-988

c. Expected PCR product size=354bp

(Predicted from the published rat bFGF sequence by Shimasaki et al, 1988).

Pf (forward primer): $^{5'}$AACGGCGGCTTCTTCCTG$^{3'}$; position: 635-652

Pr (reverse primer) $^{5'}$AGCAGACATTGGAAGAAACA$^{3'}$; position: 969-988

d. Expected PCR product size=415 bp

Pf (forward primer): $^{5'}$AACGGCGGCTTCTTCCTG$^{3'}$

Pr (reverse primer): $^{5'}$CTACAAGCTCTACCACAGGGGA$^{3'}$

VI. Detection of PCR product by the incorporation of ^{33}P-dATP

In addition to the PCR protocol described above, a 2 µci of α-^{33}P-dATP was added to each PCR reaction. The samples were amplified for 30 cycles in the case of bFGF or 20 cycles in the case of GAPDH. PCR products were separated using 6% polyacrylamide gels. The polyacrylamide gel used was prepared as 50ml of 6% polyacrylamide gel containing 5ml of 10 X TBE buffer,

8.33 ml of 29:1 acrylamide/bisacrylamide, 36.67 ml H2O, and 0.2 µg/ml EtBr. After degassing the gel mixture for few minutes, 25 µl of TEMED and 250 µl of ammonium persulfate were added to start polymerization. After preparing the polyacrylamide gel, one tenth of the PCR product was loaded into the gel, ran for 2-4 hours using 1 X TBE buffer at 100v. The gel was then transferred to a whatman paper, dried under vacuum and then exposed to XAR x-ray film O/N. The intensities of the signals were quantitated by densitometry.

VII. Southern analysis (for detection of non-radioactive PCR product)

Detection of DNA molecules were performed according to the method of Southern (1975). Fifteen microliters of the PCR reaction was run on a 1.2% agarose gel which was stained with 0.2 µg/ml ethidium bromide to visualize the PCR product bands under the UV light. The PCR products were then transferred to a nitrocellulose filter (Thomas 1980). The filter was baked for 2 hours under vacuum at 80 °C, and then prehybridized with 40% formamide prehybridization solution [40% (v/v) deionized formamide, 5X Denhardt's solution (1X Denhardt's = 0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidine), 5X SSPE (1X SSPE = 1.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 250 µg/ml denatured salmon sperm DNA and 0.1% sodium dodecyl sulfate] for at least 2 hours at 42 °C. A ^{32}P labeled rat bFGF cDNA and mouse GAPDH cDNA (prepared by using Amersham random priming kit) were hybridized with the filter at 42 °C for 24 h. The filter was washed first with 2X SAC (1X SAC = 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS at room temperature for 15 minutes, followed by one

wash in 0.2X SAC containing 0.1% SDS at 65 °C for 15 minutes. The filter was then exposed to X-ray film (XAR-5 Kodak) at -70 °C with intensifying screens.

VIII. Diagnostic digestion of PCR product or plasmids

PCR product or plasmid DNA was digested with restriction endonucleases (RE) obtained from either Pharmacia, Besthesda Research Laboratories (BRL), or Boehringer Mannheim at 37 °C for no longer than 3 hrs.. Either the buffer supplied with the enzyme, or the appropriate RE buffer as described in Maniatis et al (1982) was used in the digestion reactions. When reqiured, the RE were inactivated following digestion by heating at 65 °C or 85 °C for 20 minutes, or by addition of 80 µl of DDH_2O. The method of Andrews et al (1982) was used to separate the RE digested DNA fragments. The DNA was electrophoresed in 1.2% agarose gels stained with o.2 µg/ml EtBr. The gels were run at room temperature in TBE and the DNA bands were visualized under UV light.

IX. Small scale preparation of Plasmid DNA (Minipreps)

Minipreps were performed according to the method of Serghini et al (1989). Single colonies of bacteria, transformed with plasmid were picked from plates and grown O/N in 5 mls of LB broth, that contains 45 µg/ml ampicillin or 15 µg/ml of tetracyclin depending on the resistance of the plasmid, by shaking at 37 °C at 250 RPM. After O/N incubation, 1.5 mls of culture were transferred to an eppendorf tube and the bacteria were collected as a pellet by centrifugation. The bacterial pellet was resuspended in 50 µl of TE and equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) was added. The mixture

was vortexed and centrifuged and the aqueous layer was removed. To precipitate the DNA in the aqueous layer, a 2 M (final concentration) of ammonium acetate and 2 vol. of ethanol were added, and the mixture then kept at -70 °C. The sedimented DNA by centrifugation was then dried and resuspended in 20 µl of TE buffer. The DNA was analyzed by RE digestion and electrophoresis. DNase-free RNase (50µg/ml) was added to the RE buffer to degrade any RNA present in the samples.

X. Large scale preparation of plasmid DNA

Plasmids were amplified for use in experiments by the method outlined in Maniatis et al (1982). Bacteria, transformed with the plasmid of interest were grown O/N in 5 mls of LB broth containing ampicillin or tetracycline to select for those bacteria containing the plasmid. The 5 ml O/N cultures were added to 500 mls of fresh LB broth containing the appropriate antibiotic for plasmid selection and grown until the optical density (OD_{600}) was greater than 0.6. To stop bacterial growth and allow plasmid amplification, chloramphenicol was added to a final concentration of 10 µg/ml, and the culture was grown for a further 16 hours. The bacteria containing the amplified plasmids were collected by centrifugation at 6000 RPM for 10 minutes in a Beckman JA-10 rotor. The bacterial pellet was either stored at -70 °C or used immediately. The pellet was resuspended in 9.5 mls of lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose), transferred to sterile 50 ml Oakridge tubes (Nalgene), and 0.5 mls of freshly prepared lysozyme (20 mg/ml) was added. The tubes were placed sideway on ice and shaken for 30 minutes. Ten mls of a 0.2M NaOH/0.2% SDS solution was then added and the tubes were left to

shake for further 30 minutes. The mixture was centrifuged in a Beckman JA-20 rotor for 30 minutes at 17000 RPM at 4 °C and the supernatant was removed and extracted once with 20 mls of phenol/chloroform/isoamyl alcohol (25/24/1). The aqueous phase was seperated from the organic phase by centrifugation at 3000 RPM in 50 ml centrifuge tubes, removed, and the DNA in it was precipitated by the addition of 0.6 vol. of isopropanol. The DNA was pelleted by centrifugation in a JA-20 rotor at 15000 RPM for 10 minutes at 20 °C, and then resuspended in 6.0 mls of TE buffer. After DNA resuspension, 6.6 g of cesium chloride (CsCl) to yield 5.7 M was dissolved in the TE buffer and the mixture was transferred to a Beckman Quickseal centrifuge tube (16 x 76 mm). The TE buffer was overlayed with 0.2 ml of 10 mg/ml EtBr and the tube was filled to the top with mineral oil, balanced, sealed, and centrifuged in a Beckman Ti75 rotor at 55000 RPM for 16 hours at 20 °C. After 16 hours, the speed was reduced to 45000 RPM for 45 minutes. Following centrifugation, the UV light was used to differentiate between the plasmid and bacterial genomic DNA bands. The plasmid band was removed by a needle or syringe, and extracted several times with 2-3 volumes of isoamyl alcohol until the solution color is clear, as an indication of removal of the EtBr. The DNA was then ethanol precipitated and dried pellet redissolved in TE buffer pH 7.5.

XI. Sequence Analysis

The PCR product was purified from the gel using a 0.45 µM Ultrafree-MC polysulfone column (Millipore), made blunt ended with incubation with Klenow, and subcloned into EcoRI site in the plasmid pVZ I (Bluescribe vector modified by S. Henikoff, Fred Hutchinson Cancer Research Centre). The

subcloned PCR product was sequenced by the dideoxy termination method (Sanger et al, 1977). Upper and lower PCR primers and the universal primer for the PVZ sequence were used to initiate the sequencing reaction in the presence of T_7 DNA polymerase and the 4 dNTP's (the dCTP was labelled with ^{32}P). One µg/µl of the subcloned PCR product was annealed to 0.5 pmol/ µl of primer in the presence of 1 µl of 100 mM Tris, pH 8.0 and 1 µl of 50 mM $Mgcl_2$ buffer. After gentle mixing, the mixture was incubated at 65 °C for 5 min. to allow strand-primer annealing and then allowed to cool gradually at room temperature for 20 min..This mixture was divided into 4 tubes, (2 µl each), labelled G,A,T,C. To another 4 tubes, each containing 5 µl of ^{35}S-dATP, 0.5 mM of one type of dNTP was added followed by the addition of the corresponding ddNTP (0.25 mM) in a total volume of 10 µl. Those components were mixed by gentle pipetting and then 2 µl from each labelled tube was added to the labelled G,A,T,C tubes. The mixture was then incubated with 0.5 U/µl of T_7 DNA polymerase at room temperature for 17 min., then 2 µl of 2 mM of cold dNTP was added and the mixture was incubated again for 15 min.. To stop the reaction, 5 µl of formamide dye was added, boiled for 3 min, and then cooled quickly on ice before loading them into sequencing gel.

XII. Recombinant DNA probes

bFGF	Rat bFGF cDNA (0.8 kb) in PVZ1 (Kurokawa et al, 1988)
GAPDH	Mouse GAPDH cDNA (1 kb) in PVZ1 (Tso et al, 1985)
β actin	Mouse β actin cDNA (2 kb) in pBR322 (Nudel et al, 1983)
28S	28S ribosomal RNA cDNA (5 kb) in pBR322

XIII. Statistics

To test the significance of change in the levels of bFGF mRNA in different brain regions and different ages, the mean value for each brain region at one age was compared with that of the same brain region of a different age using ANOVA-Duncan's method. For brain regions of 28-day-old rats, a similar analysis was carried out to compare the mean value of each brain region with that of other brain regions at the same age.

Results

I. Optimization of cDNA amplifications by RT-PCR

During the course of optimizing the conditions of the PCR reaction, critical components for the efficiency of the RT-PCR reaction were investigated. The major components of the PCR reagents which dramatically influence the efficiency of the PCR reaction are discussed below.

1. $MgCl_2$

$MgCl_2$ is required for the functioning of the Taq polymerase enzyme, and its concentration can influence the efficiency and the accuracy of Taq polymerase in the polymerization step. Very low concentrations of $MgCl_2$ in the PCR reaction leads to poor amplification of the target sequence, while high concentrations of it affects the specificity of the Taq polymerase and can result in multiple nonspecific amplified PCR products. Consequently, inefficient target specific amplification is expected due to faster depletion of the PCR reagents and coamplification of more than one target simultaneously. PCR buffers with serial dilutions of $MgCl_2$ were tested, and a range of 0.5-1.5 mM of $MgCl_2$ (final), was found to be appropriate for amplification of bFGF cDNA and the other control gene products actin and GAPDH.

2. Taq polymerase

The number of units of Taq polymerase can affect (in a similar way to the effects of $MgCl_2$) the stringency of the PCR reaction. Taq polymerase (1.5 to 2.5 units; BRL) gave reproducible amplification of all cDNA sequences under study.

3. Primers

For specific and efficient amplification of cDNA or DNA sequences by PCR, selection of primers is a very critical step. To select suitable PCR primers, the following criteria should be applied as much as possible: upper and lower primers should match the target sequence (avoid any base mismatch at the 3' end of the primer sequence), similar annealing temperature required for the upper and lower primers to hybridize to the target sequence and G+C content of the primer should be close to the A+T content. RNA extracted from tissues is often contaminated with minute amounts of genomic DNA. Coamplification of the genomic DNA sequence with its corresponding RNA species is not desired. To decrease the chance of genomic DNA amplification, and to be able to differentiate it from its corresponding RNA sequence, primer pairs were designed such that the intervening sequence contained at least one intron. The selection of the PCR primers was carried out by using primer analysis software (Oligo; version 4.0; National Biosciences) that searches for best matched primers.

4. Annealing temperature

Although target specific primers were designed according to the criteria described above, it is useful to determine the actual optimum temperature required for the target cDNA amplification. This can be achieved by comparing the amplification of the target cDNA at different annealing temperatures. Varying the annealing temperature (50 °C, 56 °C, 60 °C and 65°C) to test the efficiency of amplification of bFGF, GAPDH and actin cDNAs revealed that 56 °C the optimum temperature for target specific amplification.

5. RT-PCR controls

To avoid PCR-product carryover or contamination (false positives), a major problem encountered by the use of the RT-PCR (Longo et al, 1990), clean environment and strict conditions were applied during reagents preparation. Tissue collection, RNA extraction and RT-PCR reactions were carried out in a physically distant laboratory from the site of PCR product isolation and storage. Gloves were changed 2-3 times while carrying out a PCR or an RT reaction. Separate pipetman and tips were devoted for the PCR reaction. Tips were changed whenever using a reagent or RNA/cDNA samples. Positive displacement PCR micropipets were used for aliquoting and addition of RNA/cDNA molecules to the RT/PCR reaction mixtures. Proper negative controls (blank) that contain all RT-PCR reagents, including enzymes and PCR primers but no RNA/cDNA were carried out. Other negative controls that contain all RT-PCR reagents including RNA, PCR primers and Taq polymerase but no reverse transcriptase were also examined. The substitution of dTTP with dUTP and the addition of uracil DNA glycosylase to the PCR master mixture was incorporated in the PCR protocol to eliminate product contaminant (Longo et al, 1990). Addition of UDG to the PCR reaction mixture had no effect on the PCR efficiency (Fig. 1a).

II. Identification of PCR product

1. Ethidium bromide (EtBr) stained gels: Agarose gels stained with EtBr were used to detect the amplified PCR product. The size of the PCR product was determined by the use of DNA markers (ΦX DNA digested with Hae III).

2. Southern analysis: The correct size bFGF or GAPDH PCR products were also tested by southern analysis using the appropriate ^{32}P-labeled cDNA probes.

3. Restriction enzyme digestion: The expected size bFGF PCR product was also confirmed by diagnostic cutting, using the appropriate restriction enzyme. Figure 1c shows the digestion of bFGF PCR product with Dde I results in two expected smaller fragments deduced from the published rat bFGF cDNA sequence (Kurokawa et al, 1988).

III. Parameters of quantitative RT-PCR

The goal of this study is to establish the relative expression of bFGF in different stages and regions of the rat brain in development. As with most growth factor genes, the half life of bFGF mRNA is short (Murphy, P.R. et al, 1990). The expected lower level of bFGF mRNA (Emoto et al, 1989, Riva and Mocchetti, 1991) and small size of the normal brain regions to be analyzed, necessitate the use of a highly sensitive technique for RNA detection. Northern blot technique was not used as a routine quantitative procedure because it requires 5 to 10 µg of poly $(A)^+$ RNA. Figure 2 shows that bFGF mRNA was barely detectable using 40 µg of total RNA obtained from adult rat brain. *In situ* hybridization, a more sensitive technique for RNA detection than Northern analysis, is not a suitable method for quantitation of gene expression, specially if the expression of the gene is very low and if cells expressing it are sparse (Emoto et al, 1989). The RT-PCR method was therefore employed in a quantitative manner (Murphy, L.D. et al, 1990, Becker-Andre and Hahlbrock, 1989, Gilliand et al, 1990, Horikoshi et al, 1992, Luqmani et al, 1992). A

method by Becker-Andre and Hahlbrock, (1989) and Gilliand et al, (1990) utilized cDNA standard templates, added in serial dilutions to aliquots of the cDNA mixture to be analyzed. However, such an approach does not control for variations in the quality and the quantity of RNA samples. Also, restriction enzyme digestions often used to separate the internal control PCR product from the target one are usually incomplete. To avoid these problems, an authentic internal standard which is expressed equally in the tissues to be studied was used. An internal standard cDNA ideally should be one whose expression is constant among different tissues. To test the approach for quantitation described by Becker-Andre and Hahlbrock, 1989, Gilliand et al, 1990, coamplification of bFGF and an internal control (actin) was examined. A dramatic reduction in the PCR efficiency for both the bFGF and actin was noticed (Fig.1B). The coamplification inhibition effect was also observed by Horikoshi et al, (1991). For the comparison of relative gene expression it is not necessary that the target and the internal control cDNA be amplified with the same efficiency, only that the efficiency of the PCR amplification of the same cDNA remains constant among different samples. Amplification of the target and the internal control gene products in separate tubes would be possible as long as their amplifications lie in the linear range. For that reason it was first necessary to establish the optimum number of PCR cycles and the RNA input that would give exponential amplification of both the target and the internal control cDNAs (Fig. 7,8). Genes like actin (Horikoshi et al, 1992) and GAPDH (Zentella et al, 1991; Bosma et al, 1991; Clontech 1992; Luqmani et al, 1992) have been reported to be equally expressed in different tissues and used as internal standards. GAPDH mRNA was less abundant than actin (Clontech

1992) and was chosen as the internal control for bFGF mRNA quantitation. The expression of GAPDH and actin detected by RT-PCR was similar in various regions of the brain of 28-day-old rats and in the rat cerebrum during the first month after birth (Fig. 3A and 3B). Also, Northern analysis confirmed that GAPDH expression was similar in various regions of the brain of 28-day-old rat (Fig. 4) and in the cerebrum of the developing rat (Fig. 5).

Previous quantitative studies using RT-PCR utilized radioactivity as a method for detection of the PCR product (Gilliand et al, 1990, Singer-Sam et al, 1990). A non-radioactive procedure in which PCR products were detected with ethidium bromide after gel electrophoresis was evaluated. Aliquots of the same RT reaction product (cDNA) were subjected to PCR amplification for bFGF and GAPDH using ^{33}P-labelled dATP. The bands of the photographs of the ethidium bromide stained gels and autoradiograms (Of ^{33}P-labelled PCR product) were quantitated by scanning densitometry. Results obtained by the two methods used for PCR product detection and quantitation were very similar (Fig. 6), confirming the reliability of using ethidium bromide stained gels as a method for detection of PCR product. This approach is fast, easier to perform and in turn, can be applied to compare large number of samples at the same time.

Relationship of PCR product as a function of PCR cycles for both bFGF and GAPDH cDNAs

The PCR reaction has two phases, an exponential phase and saturation phase. The length of the first phase depends on various factors mainly on the amount of cDNA template used, the amount of PCR product generated

(efficiency of amplification) and depletion of PCR reagents (during the PCR reaction) such as primers and dNTPs. Also, the exposure of Taq polymerase to high temperatures during each PCR cycle (94^0 C) reduces its activity. All those factors will affect the efficiency of the RT-PCR reaction and inturn quantitation by this method. Plotting the number of PCR cycles against the amount of PCR product obtained at each cycle, a graph can be obtained and the linear range of amplification can be determined (Fig. 7).

Relationship of the PCR product as a function of the amount of template RNA input

As the amount of RNA used will determine the amount of cDNA that will be generated in the RT reaction and later on, the quantity of the PCR product produced, serial dilutions of RNA were reverse transcribed and then amplified to determine the range of RNA concentration that results in a linear PCR product amplification. Results obtained from analyzing different RNA samples from different tissues showed that amplification of cDNAs under study was linear when 0.07 to 0.25 µg of total RNA was reverse transcribed and PCR amplified, (Fig. 8). Also, table 12A shows that results obtained from serial dilutions of RNA obtained from various brain regions were similar.

IV. PCR detection of a novel sequence in the 3' untranslated (UT) region of bFGF mRNA

In an effort to optimize the conditions for the amplification of bFGF cDNA by the reverse transcription-polymerase chain reaction (RT-PCR), three primers were designed (P_1,P_2,P_3) according to the published cDNA sequence for rat bFGF cDNA (Shimasaki et al, 1988; Fig. 11). P_1 (forward primer) and P_2

(reverse primer) correspond to positions 635 to 652, and 969 to 988 respectively, within the coding region. Also used was a second reverse primer P_3 which corresponds to position 1028 to 1049 in the 3' untranslated (UT) region of the published sequence (Shimasaki et al, 1988; see also Fig. 8). As expected, a 354 bp PCR product (lane 6, Fig. 9A) was obtained with the P_1 and P_2 primers. However, when primers P_1 and P_3 were used a single 722 bp PCR product (lane 1, Fig. 9) was obtained instead of a 415 bp product predicted by the published sequence (Shimasaki et al, 1988). Figure 9 also shows that no PCR product was detected when either RNA samples (lanes 3 and 5) or RTase (lanes 2 and 4) was omitted, a result indicating that the 722 bp PCR product was derived from authentic RNA transcript. The 722 bp PCR product was also obtained when RNA from adult rat ovary and 3 day and 21 day rat kidneys were reverse transcribed and amplified (Fig. 10). The same 722 bp PCR product was obtained from RNA extracted from brains of rats of four older ages: 21 days, 28 days, 4 months and 1 year old (results not shown). The 722 bp RT-PCR product from brain RNA, which we refer to as RATBFGF3, was subsequently sequenced (Fig. 11; El-Husseini et al, 1992). The sequence revealed an open reading frame identical to the coding region of the published rat ovary derived bFGF mRNA sequence (RATGFFO, Shimasaki et al, 1988) and rat brain derived bFGF mRNA sequence (RATGFBF, Kurokawa et al, 1988). These results revealed an additional 307 bp in the 3' UT region. This sequence (position 383 to 689 of RATBFGF3; Fig. 9) represents an insertion between positions 1016 and 1017 of the sequence published by Shimasaki et al, (1988; Fig. 12).

V. Basic FGF gene expression in the rat brain

1. Basic FGF mRNA levels in the rat cerebrum during early postnatal development

The levels of bFGF mRNA in the cerebrum obtained from male rats of ages 1 to 28 days and one year were first compared. For results obtained by RT-PCR, see tables 1A and 1B. Figure 13 shows a gradual increase in bFGF mRNA levels, reaching high levels at the end of the first month (about 5.9-fold of that of 1-day-old, Fig. 13). One year old rat cerebrum bFGF mRNA levels were as high as that from 28-day-old rats. Table 2A summarizes the results obtained from 3 independent sets of animals (see table 13). GAPDH mRNA levels remained relatively constant. Hence bFGF signals were equalized with respect to the GAPDH signal intensities for quantitation. Figure 13 shows that the greatest increase in the levels of bFGF mRNA occurred between the first and the second postnatal weeks (3.4 fold). The adult level of bFGF expression was reached by 3 weeks of age, its level being 4.8-fold over that of newborn rat cerebrum. For comparison, all the PCR results obtained were expressed relative to 21-day-old rat cerebrum set as 100%. Statistical analysis was carried out using ANOVA-Duncan's test (for summary of the analysis, see table 2B).

2. Regional distribution of bFGF mRNA in 28-day-old rat brain

The RT-PCR application revealed an uneven distribution of bFGF mRNA in several structures of 28-day-old rat brain (Fig. 14; also see tables 3, 4 and 5). The regional analysis of 28-day-old rat brain showed that the lowest levels of bFGF mRNA occur in the cerebellum while the highest levels are found in

the hippocampus (7 fold of that of cerebellum). Also the levels of bFGF gene expression were high in the occipital cortex and cingulate cortex (5 fold), followed by inferior colliculus (3.7 fold). Intermediate levels were observed in the hypothalamus and combined pons-medulla (2 fold). For relative comparison, all PCR results were expressed relative to the Inferior colliculus of 28-day-old rat set at 100%. Summary of PCR results obtained from different sets of animals is in table 6A. *In situ* hybridization studies by (Emoto et al, 1989) showed that bFGF gene transcripts are widely distributed in the cerebral cortex of the adult rat brain, and the highest levels were found in hippocampus and cingulate cortex. These results indicate that levels of bFGF gene expression are similar in the 28-day-old and the adult rat brain regions discussed above. Figure 6 (for PCR results see table 12B) shows that the results obtained by the non-radioactive PCR method were comparable to that obtained by the ^{33}P-dATP incorporation protocol, thus validating accuracy of the non-radioactive quantitative RT-PCR method. Statistical analysis was carried out using ANOVA-Duncan's test (for summary of the analysis, see table 6B).

3. Regional differences in bFGF gene expression during postnatal development

Four brain regions were selected to examine if different loci exhibit different time of bFGF expression during early postnatal development. In the occipital cortex (O.C.) and inferior colliculus (I.C.), a gradual increase in the levels of expression of bFGF within the first month of postnatal brain growth was observed, reaching high levels in 28 day [2.5 fold and 2.9 fold of the levels of bFGF mRNA in occipital cortex (Fig. 15; table 7A;B) and inferior colliculus

(Fig. 15; table 8A;B) respectively, when compared to the levels in 1-day-old]. For relative comparison, all the PCR results obtained were expressed relative to the inferior colliculus of 28-old-rat set at 100%. This increase of bFGF gene expression in early brain development correlates with the finding obtained by Riva and Mocchetti (1991), in various regions of the developing rat brain (cerebral cortex, hippocampus, striatum, hypothalamus and spinal cord). In the developing rat cerebellum (CB), in contrast, the highest levels of bFGF mRNA were at postnatal day one, which decreased by 1.8 fold at day seven and by 2.6 fold at days 14 and 28 (Fig. 15; table 9A;B). Expression of bFGF was the lowest in the cerebellum by two weeks of age. A third pattern of bFGF gene expression was observed for the pons-medulla (P&M) in which moderate levels of bFGF gene expression were detected in one day old rats, and this level of bFGF mRNA remained fairly constant throughout the active period of postnatal development (Fig. 15; table 10A and 10B). Results obtained by RT-PCR from different animal sets are summarized in table 11. Statistical analysis was carried out using ANOVA-Duncan's test (for summary of the analysis for O.C., I.C. CB and P&M see tables 7C, 8C, 9C and 10C respectively).

4. Basic FGF mRNA in non-neuronal elements

The levels of bFGF mRNA in the rat meninges (collected from 1 day and 7 days or 28-day-old rats) were much lower than the levels in 28-day cerebellum (Fig. 16). PCR product of the meninges was barely detectable by Southern blotting with a ^{32}P labelled bFGF cDNA probe. No PCR product was detected (using ethidium bromide stained gels) when RNA from blood (collected from 7-

day-old rats) was reverse transcribed and amplified (not shown). These observations indicate that contamination of brain tissues by blood or meninges would have negligible effect on changing the actual amount of bFGF detected in the cerebrum and other brain regions. The bFGF gene expression also detected in the kidney and liver of 7 days and the kidney of 21-day-old rats (data not shown).

Discussion

I. Distribution of bFGF in the rat brain

Neurotrophic factors are molecules that increase neuronal survival and stimulate neurite outgrowth. Theories concerning the regulation of brain growth during development frequently postulate a major role for neurotrophic factors (NTF). Basic FGF is produced in the nervous system and exerts a variety of *in vivo* and *in vitro* effects, stimulating mitogenesis, differentiation and maintenance of cells of neuroectodermal and mesodermal origin including neurons and glial cells (Walicke and Baird 1988, Delaunoy et al, 1988, Perraud et al, 1990).

Although bFGF protein is present in different tissues (Baird and Bohlen 1990, Gospodarowicz et al, 1987) its mRNA was only detectable in the brain (Emoto et al, 1989). In order to reveal the role of bFGF in the CNS, *in vitro* and *in vivo* models were designed to study the effect of bFGF on various cell types and tissues of the CNS (Westermann 1990, Walicke and Baird 1988). A direct effect of bFGF on glial and neuronal cell gene regulation was implicated by Walicke and Baird (1991). Basic FGF promotes neuronal survival and neurite outgrowth (Westermann 1990). Other cellular changes in certain metabolic compounds in neurons and glial cells after the application of FGF were also reported (Westermann 1991). The naturally occurring death of neurons was prevented by the application of bFGF (Dreyer et al, 1989). About 25% of the retinal ganglion neurons were rescued from death by bFGF after optic nerve fiber transection (Sievers et al, 1987).

Researchers investigated the importance of bFGF in the CNS through

studying its presence and distribution in different brain structures and cell types (Emoto et al, 1989, Caday et al, 1988, Riva and Mocchetti 1991, Grothe et al, 1991). The results reported about the cell type(s) and loci of bFGF gene transcription were not consistent. Some discrepancies in detecting bFGF transcripts were due to the technical difficulties in detecting the low quantities of bFGF mRNA and the number of cells expressing it. Is it glial cells or neuronal cells or both that express bFGF ? The answer is not clear. Pettman et al, (1987) and Janet et al, (1988) detected bFGF immunoreactivity in neurons but not in glial cells in cultured brain and peripheral ganglia cells. Others reported the expression of bFGF mRNA only in cultured astrocytes (Emoto et al, 1989). Also, bFGF mRNA and protein have been detected in astrocytes from adult bovine corpus callosum (Ferrara et al, 1988). *In situ* hybridization studies by Emoto et al, 1989 showed that neurons of several brain regions contained the bFGF mRNA. Gómez-Pinilla et al, (1992) points out that bFGF immunoreacitvity was mainly localized in astrocytes and occasionally in microglial cells throughout the normal rat brain and only detected in a few neuronal populations, specifically the septohippocampal nucleus, CA-2 field of the hippocampus, cingulate cortex, cerebellar Purkinje cells, deep nuclei, facial nerve nucleus and the motor and spinal subdivisions of the trigeminal nucleus and facial nerve nucleus. Studies on the distribution of bFGF mRNA by Emoto et al, 1989, using *in situ* hybridization, showed a similar distribution of bFGF mRNA when compared to the distribution of the bFGF protein detected by Gómez-Pinilla et al, 1992, only with a more restricted localization in neuronal populations (CA-2 region of the hippocampus, layers 2 and 6 of the cingulate cortex, indusium griseum and

fasciola cinereum).

II. Basic FGF gene expression in the rat brain during early postnatal development

Comparison of the levels of bFGF mRNA in the cerebrum obtained from male rats of ages 1 to 28 days and one year revealed a gradual increase in bFGF mRNA levels, reaching high levels at the end of the first month. The levels of bFGF mRNA of the third postnatal week were as high as that from one year old rat cerebrum (as shown in Fig. 13). Similar progression in both bioactive and immunoreactive bFGF levels in the rat brain in early postnatal ages that plateaued in the adult, was reported by Caday et al, (1990). Riva and Mocchetti (1991) used the RNase protection assay to study the level of bFGF mRNA in rat cerebral cortex, hippocampus, striatum and hypothalamus (all are regions that represent major part of the cerebrum) in early postnatal development. In agreement with the results obtained by RT-PCR used in the present study, Riva and Mocchetti found that the levels of bFGF mRNA in all the rat cerebrum regions mentioned above, were low in newborn rats and increased gradually to a peak of expression around the third postnatal week.

Much of neuronogenesis in the rat cerebrum takes place in the embryonic stage except few regions such as CA2 field and the granule cells of dentate gyrus of the hippocampus and granule cells of the olfactory bulbs (reviewed in Jacobson 1991). However, the maximum increase in brain angiogenesis, glia proliferation and maturation, axonal myelination and synaptogenesis occur mostly within the first 2 to 3 postnatal weeks in the brain of the developing rat (Fig. 19). As it was shown by several *in vitro*

studies, basic FGF plays important roles in angiogenesis, astroglial proliferation and in axonal and dendritic growth. The increase in bFGF gene expression in early postnatal ages in the rat cerebrum may reflect an important role of bFGF *in vivo* in regulating different processes occurring simultaneously in the rat brain such as glial proliferation, axonal myelination, synaptogenesis and brain angiogenesis .

The distribution of bFGF mRNA in several regions of 28-day-old rat brain (Fig. 14) correlate with the results obtained by *in situ* hybridization (Emoto et al, 1989) which showed that bFGF gene transcripts are widely distributed in the cerebral cortex of the adult rat brain, and the highest levels were found in hippocampus and cingulate cortex. Similar results obtained by Emoto et al, (1989) using Northern analysis to study the distribution of the 6 kb rat bFGF mRNA in various regions of the adult rat brain, suggesting that the single PCR product for bFGF obtained by RT-PCR of total RNA may represent the more abundant 6 kb bFGF transcript. Similarly, by RNase protection assay it was found that bFGF mRNA levels in the adult rat brain regions were highest in hippocampus and cerebral cortex, intermediate in hypothalamus and brain stem and lowest in cerebellum (10% of the cerebral cortex; Riva and Mocchetti, 1991).

Neurotrophic factors play an important role in preventing the naturally occurring neuronal cell death and maintaining their survival (Barde et al 1988). Different studies indicated that bFGF enhances the survival *in vitro* of neurons obtained from various regions of the brain (reviewed in Walicke and Baird 1988). Considering the fact that most of the postnatal developmental events in the rat brain (formation and closure of the blood brain barrier, glia

proliferation and axonal growth and myelination) occur within the first three weeks after birth (Jacobson 1991; see also Fig. 19), the high expression of bFGF in 28-day-old rat hippocampus, cingulate cortex, occipital cortex and inferior colliculus may suggest an important role for bFGF in maintaining the survival of neurons of those brain regions.

When levels of bFGF mRNA were examined in four structures of rat brain in early postnatal development, three different patterns of bFGF gene expression were observed: 1)steady increase in occipital cortex and inferior colliculus; 2)constant level in pons/medulla; 3)decrease in cerebellum.

The increase of bFGF gene expression in the occipital cortex and inferior colliculus within the first month of postnatal brain growth correlates with the finding obtained by Riva and Mocchetti (1991), in various regions of the developing rat brain (cerebral cortex, striatum, hypothalamus and spinal cord). In contrast, the variations in the expression of bFGF mRNA in the cerebellum (decreases with age in early postnatal rats) and pons/medulla (constant) in early postnatal ages (Fig. 15) differ from the general pattern (increases with age in early postnatal rats) observed in all the regions studied by Riva and Mocchetti (1991).

In the rat, the volume density of blood vessels in the inferior colliculus reaches maximum levels 24 days after birth (Andrew and Paterson 1989). Also vascularization of the cerebral cortex takes place within the first two postnatal weeks (Nieto-Sampedro and Cotman 1985). Thus, the increase in the bFGF mRNA levels within the first three postnatal weeks in those brain regions may suggest the involvement of bFGF (a potent angiogenic factor) in the formation and sprouting of those blood vessels. The maximal astroglial proliferation,

synaptogenesis and dendritic growth which take place in early postnatal development, suggest other possible physiological demands for bFGF in the occipital cortex and inferior colliculus for that period of time. During the first two postnatal weeks, major morphological and physiological changes occur in the rat inner ear and the onset of hearing happens between 9 and 12 days after birth (Uziel et al, 1981). The increase in the levels of bFGF gene expression in the inferior colliculus at this period of time may indicate the participation of bFGF in the development of auditory pathways. On the other hand, the bFGF mRNA increase could be a result of the development of those brain regions either because of increased number or increased differentiation of cells that make bFGF.

The increase of bFGF mRNA in the developing inferior colliculus does not correlate with the levels of the immunoreactive bFGF (bFGF-IR) detected in this region; bFGF-IR was detectable in postnatal days 8 and 11 but absent in postnatal day 28 and the adult (Grothe et al, 1991). The reason why the bFGF mRNA and not the protein was detected is not clear. Multiple molecular weight (MW) forms of bFGF were detectable in different studies (Baird and Bohlen 1990). The absence of the bFGF-IR in 28-day-old inferior colliculus may be due to the inability to detect the MW form produced from the bFGF RNA transcript at that age using the specific antibody (Grothe et al 1991).

In the developing rat cerebellum, the highest levels of bFGF mRNA were at postnatal day one, and they decreased by 1.8 fold at day seven and by 2.6 fold at days 14 and 28 (when compared to 1 day cerebellum; Fig. 15). Much of the neurogenesis in the rat brain takes place during embryonic development (Fig. 19, Jacobson 1991). The granule cells of the cerebellum constitute one of

only four brain loci that possesse active neurogenesis within the first two weeks after birth (other sites of active postnatal neurogenesis are hippocampus, olfactory bulb and some nuclei of the brain stem). The high level of bFGF mRNA at postnatal day one correlates with the period when postnatal neurogenesis is actively taking place in the cerebellum, suggesting a neurotrophic function for bFGF on cerebellar neurons in early postnatal development. The low level of bFGF gene expression in cerebellum in later stages of postnatal development may suggest less dependence of cerebellar cells on bFGF, and neurotrophic factors other than bFGF may be involved in maintaining the survival and function of cerebellar neurons.

Moderate levels of bFGF gene expression were detected in the combined pons-medulla in 1-day, 14-day and 28-day-old rats (shown in Fig. 15). The expression of bFGF gene in the pons-medulla remained fairly constant during the period between 1 day and 28 days. It is possible that the role played by bFGF in the pons-medulla in early postnatal development varies in different regions of the brain.

III. Kidney, liver, ovary and meninges express bFGF mRNA

Using the RT-PCR, the expression of bFGF was detected in the kidney and liver of 7 days, kidney of 21 days, meninges (collected from 1 day and 7 days or 28-day-old rats) and ovary of the adult rat. The detection of bFGF mRNA in the kidney, liver and ovary by RT-PCR and not by northern analysis (Emoto et al, 1989), demonstrate the sensitivity of this technique and the advantage of using it.

IV. PCR detection of the rat brain bFGF mRNA containing a unique 3'-untranslated (UT) region

When RT-PCR was employed to detect the presence of bFGF messenger RNA in rat brain, ovary and kidney, the use of P_1 and P_2 primers (Fig. 11) resulted in an unexpected PCR product (722 bp instead of 415 bp PCR band). The sequence of the 722 bp PCR product revealed an identical coding region to the published rat ovary derived bFGF mRNA sequence (RATGFFO, Shimasaki et al, 1988; see diagram in Fig. 8) and rat brain derived bFGF mRNA sequence (RATGFBF, Kurokawa et al, 1988). However, these results revealed an additional 307 bp in the 3' UT region. This sequence (position 383 to 689 of RATBFGF3, Fig. 12) represents an insertion between positions 1016 and 1017 of the sequence published by Shimasaki et al, 1988. Similar 722 bp PCR product was also obtained when total RNA from adult rat ovary, 3 days and 21-day-rat kidneys and rat brains of four older ages: 21 days, 28 days, 4 months and one year old (results not shown) were reverse transcribed and PCR amplified. The 722 bp PCR product was the single major band obtained (Fig. 9, 10) suggesting that the bFGF mRNA containing the novel 307 bp 3' UT sequence is the major transcript form of bFGF mRNA of rat tissues. In support of this finding, the 3' UT sequence from positions 383 to 458 of RATBFGF3 was identical to the sequence between positions 738 to 813 of the rat bFGF mRNA sequence, RATGFBF, reported by Kurokawa et al, (1988) (Fig. 11; Fig. 12). Also, the additional 307 bp 3' UT sequence shares 57% similarity to a 3' UT region in the human bFGF mRNA positions 873 to 1210 (Fig. 12; Kurokawa et al, 1987). Therefore, the ovarian bFGF mRNA species published by Shimasaki et al, 1988, which lacks the 307 bp 3' UT sequence,

may have been generated as a result of cDNA cloning artifact or may represent a minor bFGF transcript.

There is increasing evidence suggesting that regulation of many genes is accomplished through posttranscriptional mechanisms [Brawerman 1987]. A+T rich sequences conserved in the 3' UT region of mRNA's of growth factors, oncogenes and cytokines were shown to play a major role in the regulation of the stability of those mRNA's [Akashi et al, 1991]. Two such examples, $(ATTT)_n$ [Akashi et al, 1991] and $(TATT)_n$ [Reeves et al, 1987], have been demonstrated to affect mRNA stability. In the novel 3' UT region of bFGF mRNA , we have identified eight repeats of the (ATTT) motif and four of the (TATT) motif (Fig. 11). The presence of these motifs suggest a possible role for the 3' UT region as a determinant of bFGF mRNA stability (Brawerman 1987).

V. The use of RT-PCR as a method for quantitation of relative levels of expression of mRNAs of growth factors

The polymerase chain reaction (PCR) has enhanced the ability of detecting very low concentration of DNA molecules (Saiki 1985). As it was only possible to detect DNA and not RNA with PCR, a combination of PCR and another technique, reverse transcription (RT), can be applied to detect cDNA molecules synthesized from mRNAs under investigation (Rappolee et al, 1988). The RT-PCR technique was later used in a quantitative manner (Becker-Andre and Hahlbrock 1989). Amplification by PCR of cDNA obtained from RNA is theoretically suited to such situations. To control for errors that could arise from RNA preparation or from variation in the RT-PCR reaction efficiency, an authentic internal standard that is expressed equally in the tissues to be

studied was used. As the approach selected in this study does not employ the coamplification of the target cDNA and the internal control in the same tube, determination of the linearity of the PCR reaction was a critical step. Exploration of the linearity of the PCR reaction was shown in figures 6, 7 and table 12A (by varying the number of the PCR cycles, the input of RNA into the RT reaction and the input of cDNA into the PCR reaction). Because several tissues were to be compared, application of a simple method for such comparison is necessary. The use of non-radioactive method for detection of PCR product (using ethidium bromide stained PCR product) instead of the commonly used radioactive method (incorporation of radiolabelled deoxynucleotide into the PCR product) was possible. Results obtained from the two methods for the linearity of the PCR reaction and quantitation of the relative amounts of bFGF mRNA to GAPDH mRNA in various brain structures were similar (Fig. 6; table12B). This comparison indicated that the use of the non-radioactive method for PCR product detection and quantitation is as reliable as the radioactive one.

Similar results were obtained when the expression of bFGF mRNA in the occipital cortex of the rat in early postnatal ages were studied by quantitative RT-PCR (Fig. 15) or by RNase protection assay (Riva and Mocchetti 1991). Using quantitative RT-PCR, high levels of bFGF mRNA was detected in the rat brain in fourth week after birth and in the adult rat brain (Fig. 13). The same results were obtained using northern analysis by Emoto et al, 1989 and RNase protection assay by Riva and Mocchetti 1991. These results demonstrate the reliability of the RT-PCR as a tool for studying the relative expression of low abundance messages. Besides being a fast technique

(results can be obtained in one day), the advantage of using the RT-PCR is due to its high sensitivity for detection of very low amounts of messages and the use of nanograms of total RNA (instead of micrograms in case of Rnase protection assay or Northern analysis).

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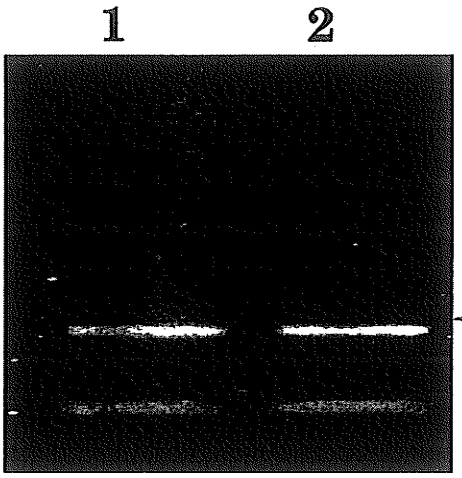
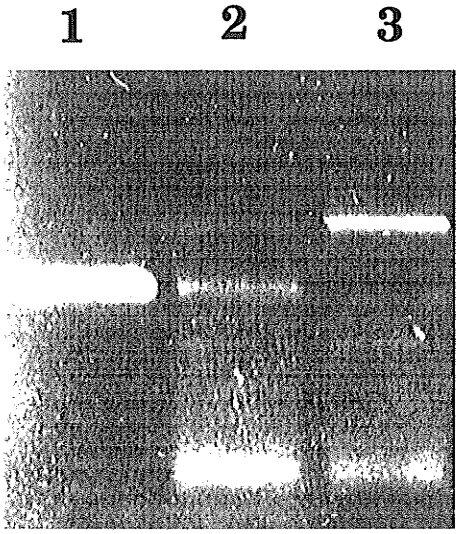
Fig. 1A Uracil DNA glycosylase (UDG) in the PCR reaction. Lanes 1 and 2 show bFGF PCR product obtained when UDG was added to the PCR mixture (lane 1) or eliminated (lane 2). **B.** RT-PCR coamplification of bFGF and actin mRNAs. PCR product obtained using actin primers only (lane 1), bFGF primers (lane 3) or both actin and bFGF primers (lane 2). **C.** Restriction enzyme analysis of bFGF PCR product. Southern analysis of bFGF PCR product using ^{32}P labelled rat bFGF cDNA probe was used. Lane 1 bFGF PCR product after digestion with Dde I enzyme. Lane 2 undigested bFGF PCR product. Positions of the expected size products are indicated by arrows.

A



B



C

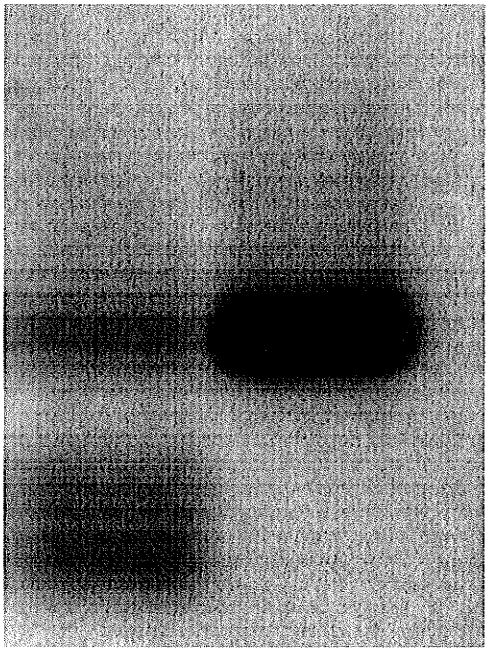


Fig. 2 Northern analysis of bFGF gene expression. ^{32}P labelled rat bFGF cDNA probe was used. Forty micrograms of total RNA obtained from a human astrocytoma cell line U-87 (lane 1), rat brain (lane 2) and rat kidney (lane 3). The size of bFGF mRNA bands and the 28S RNA band are indicated by arrows.

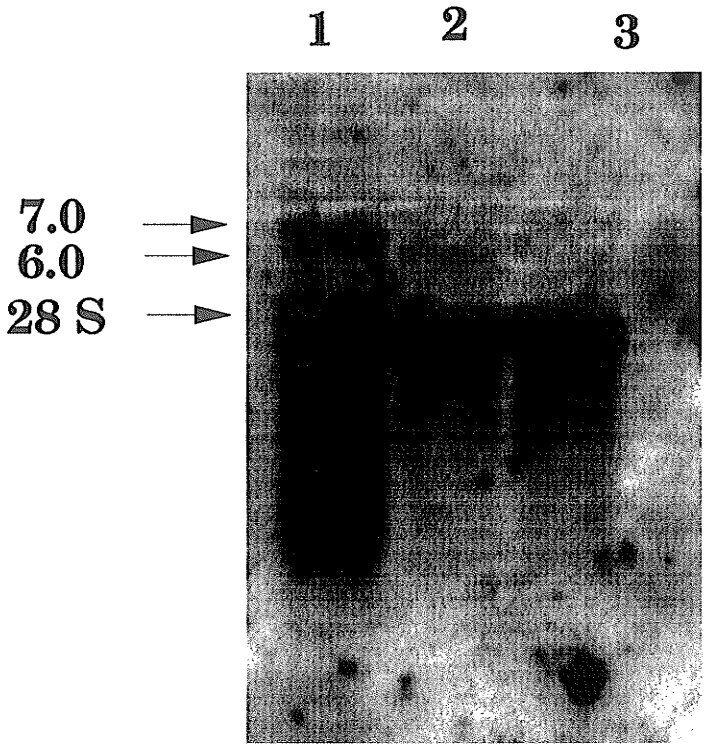
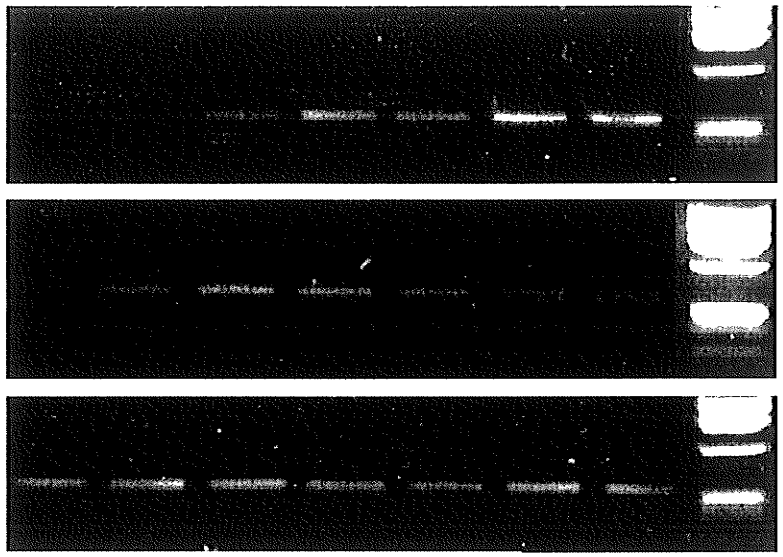

1
2
3
7.0
6.0
28 S

Fig. 3 RT-PCR Amplification of bFGF, GAPDH and actin cDNAs. **A. upper panel:** results obtained from postnatal rat cerebrum of: 1) 1 day, 2) 3 days, 3) 7 days, 4) 14 days, 5) 21 days, 6) 28 days and 7) one-year-old rats; **B. lower panel:** results obtained from a 28-day-old rat: occipital cortex (O.C.), inferior colliculus (I.C.), cerebellum (CB), pons-medulla (P&M) and hypothalamus (HYPO).

A.



B.

Fig. 4 Northern analysis of expression of the GAPDH gene in the brain of 28-day-old rat. ^{32}P labelled mouse GAPDH cDNA probe was used. Twenty micrograms of total RNA was used. Results obtained from a 28-day-old rat: 1) occipital cortex (O.C.), 2) inferior colliculus (I.C.), 3) cerebellum (CB), 4) pons-medulla (P&M), 5) hypothalamus (HYPO), 6) cingulate cortex (C.C.) and and 7) hippocampus (HIPPO). Results were expressed relative to the 28S signal and then expressed as a percentage of I.C. set at 100%.

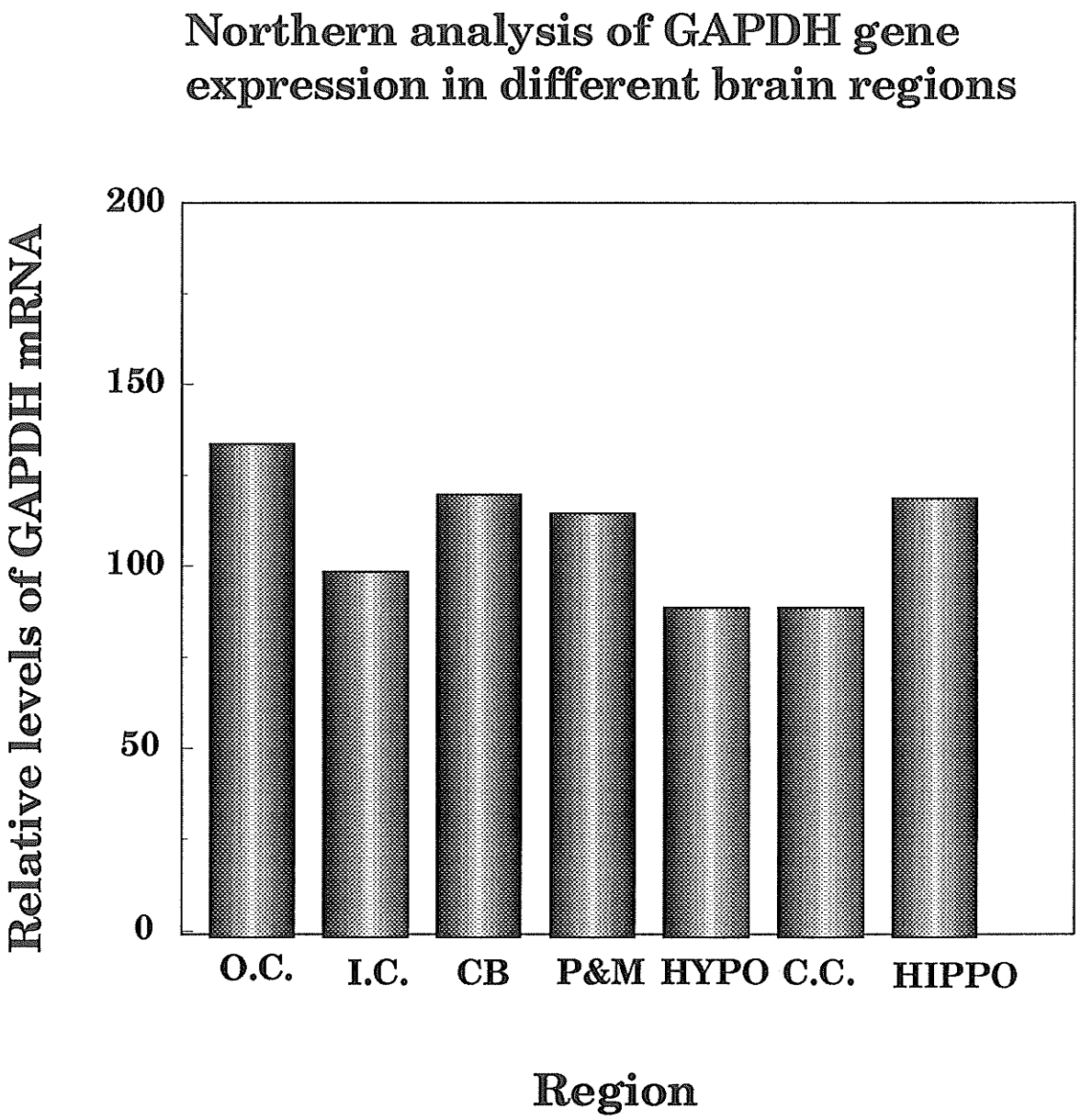

Northern analysis of GAPDH gene expression in different brain regions
Relative levels of GAPDH mRNA
200
150
100
50
0
O.C.
I.C.
CB
P&M
HYPO
C.C.
HIPPO
Region

Fig. 5 Northern analysis of expression of the GAPDH gene in the rat brain in early postnatal development. ^{32}P labelled mouse GAPDH cDNA probe was used. Results obtained from the rat cerebrum obtained from 1 day, 14 days, 21 days and 28-day-old rats using twenty micrograms of total RNA. Results were expressed relative to the 28S signal and then expressed as a percentage of 21-day-old cerebrum set at 100%.

Northern analysis of GAPDH gene expression in the rat cerebrum

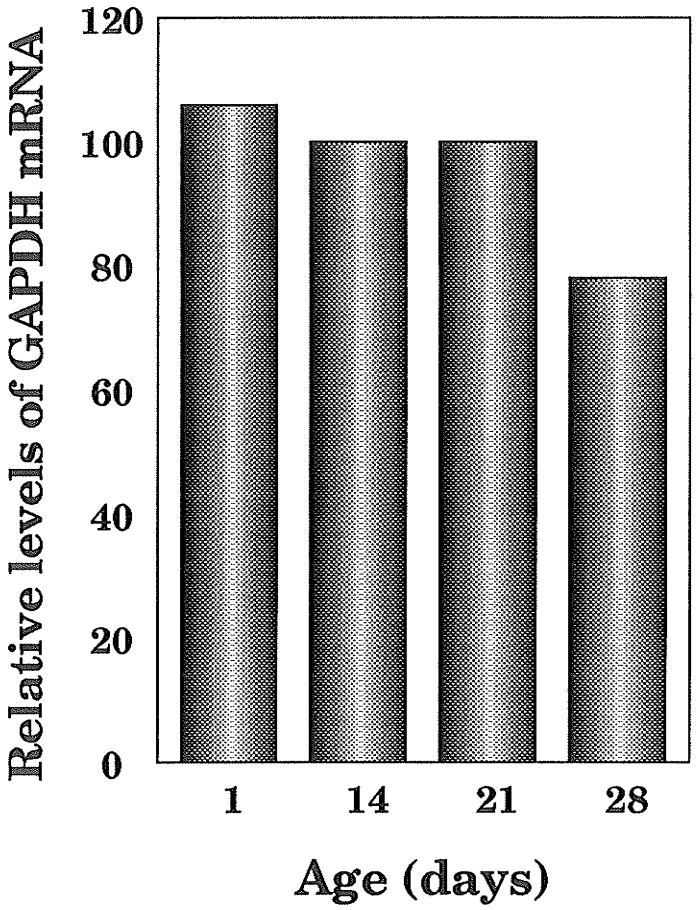
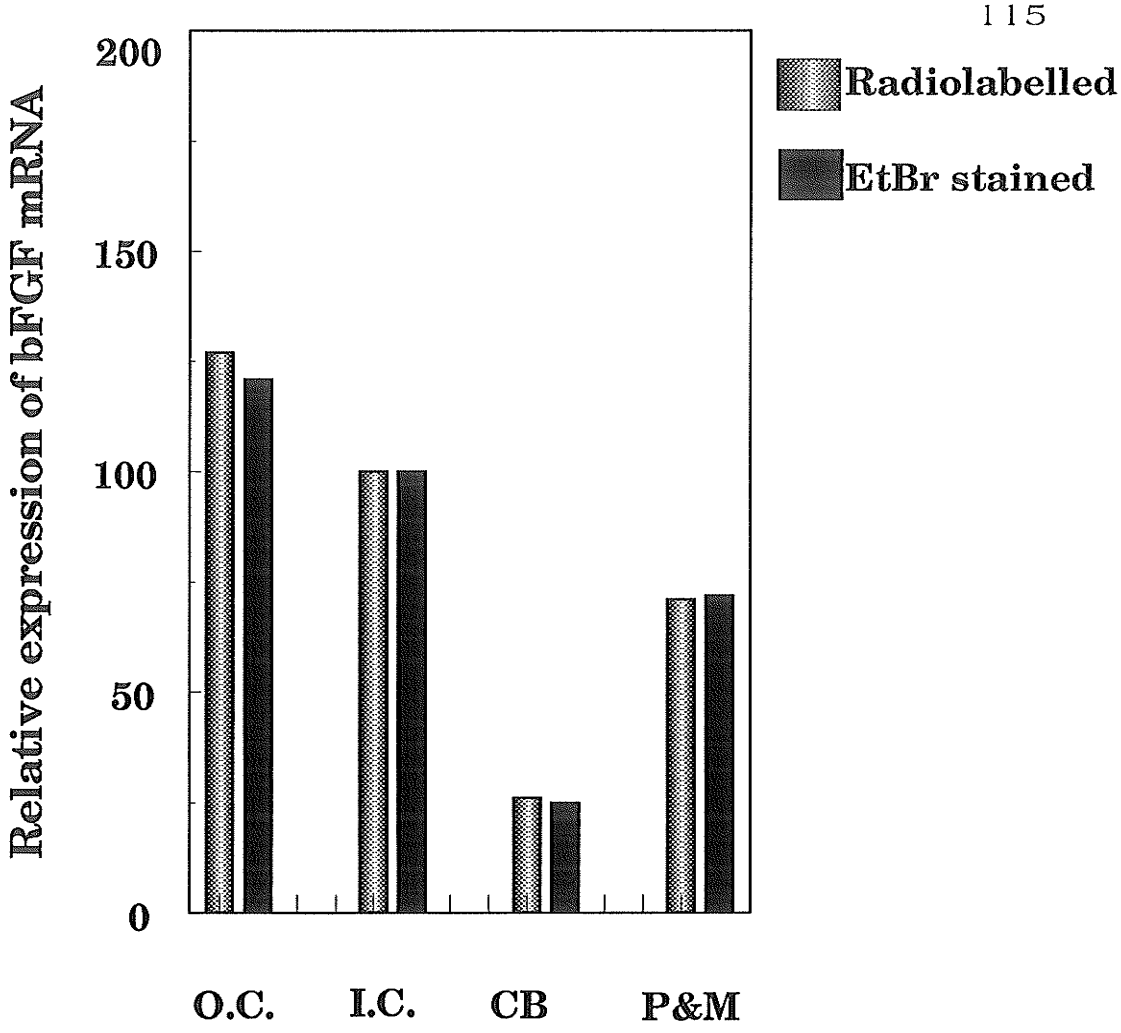


Fig. 6 Comparison between the non-radioactive and ^{33}P-labeling RT-PCR methods for the study of bFGF gene expression. Results obtained from ^{33}P-labelled (dotted bars) or ethidium bromide-stained (EtBr) PCR products (black bars) from 28-day-old rat: occipital cortex (O.C.), inferior colliculus (I.C.), cerebellum (CB), and pons-medulla (P&M). Results are expressed as percentage of Inferior colliculus of 28-day-old rat. Basic FGF and GAPDH cDNAs from the same RT sample were amplified in separate tubes. All bFGF PCR results were expressed relative to the internal control GAPDH. All subsequent quantitative analysis was carried out using ethidium bromide staining.


Relative expression of bFGF mRNA
200
150
100
50
0
Radiolabelled
EtBr stained
O.C.
I.C.
CB
P&M

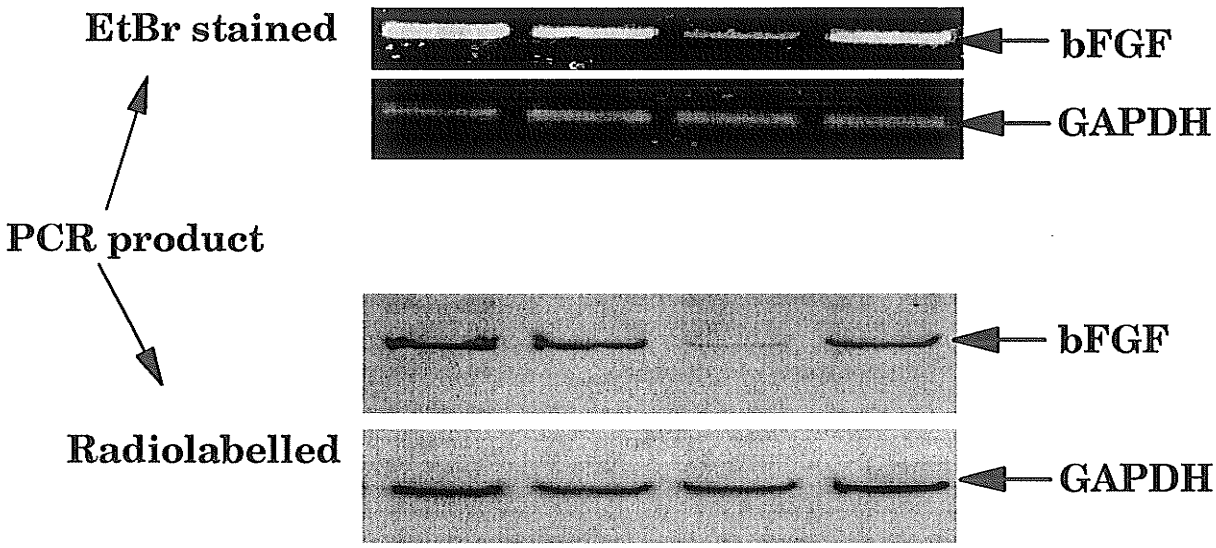
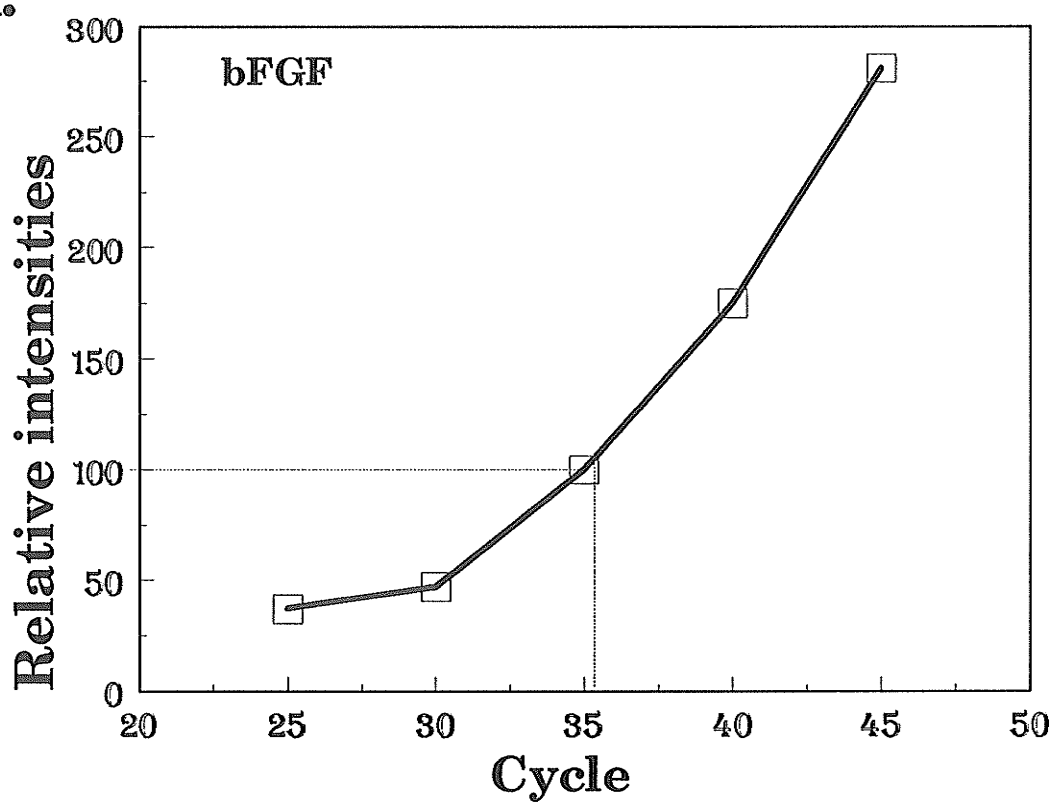

EtBr stained
bFGF
GAPDH
PCR product
bFGF
Radiolabelled
GAPDH

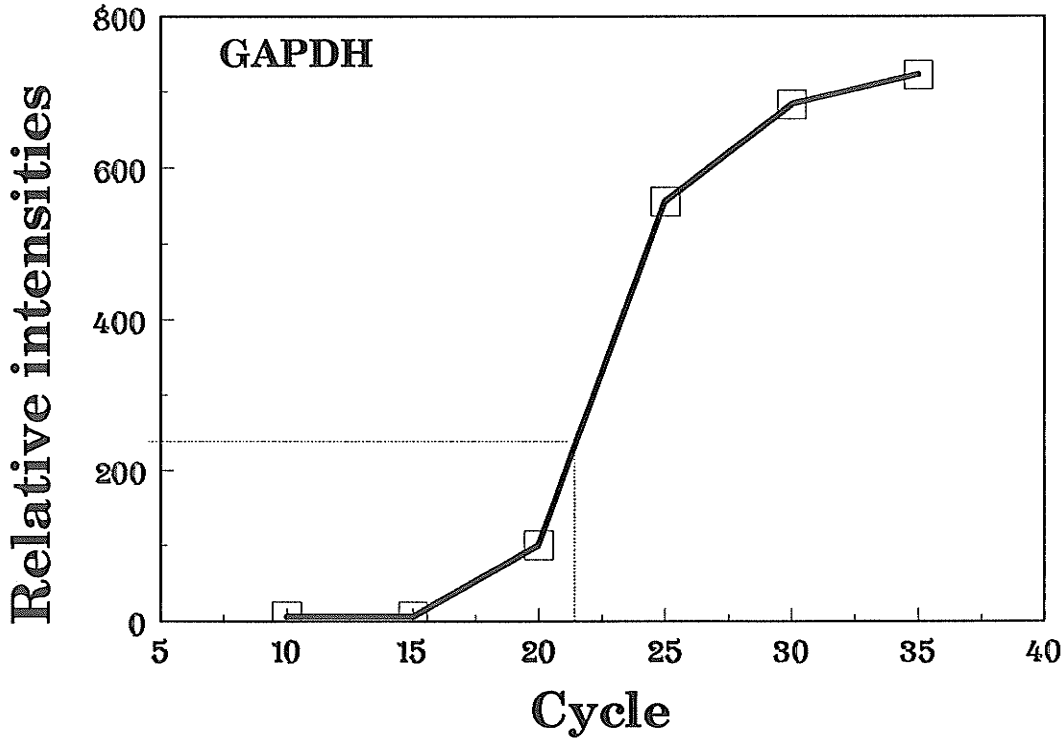
Fig. 7 A. Relationship of PCR products as a function of PCR cycles for both bFGF and GAPDH cDNAs. Total RNA (0.07 µg) from occipital cortex was reverse transcribed. The RT reaction was used to: **A.** amplify bFGF cDNA for 25 to 45 PCR cycles **B.** amplify GAPDH cDNA for 10 to 35 PCR cycles. Similar results were obtained when ^{33}P labelling was used as a method of PCR product detection (not shown). Results were expressed as percentage of the 35 PCR cycles in case of bFGF and 20 cycles in case of GAPDH.

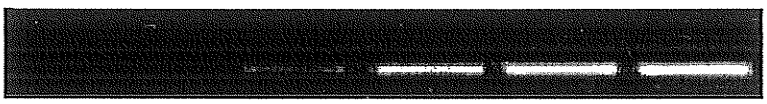
A.



bFGF

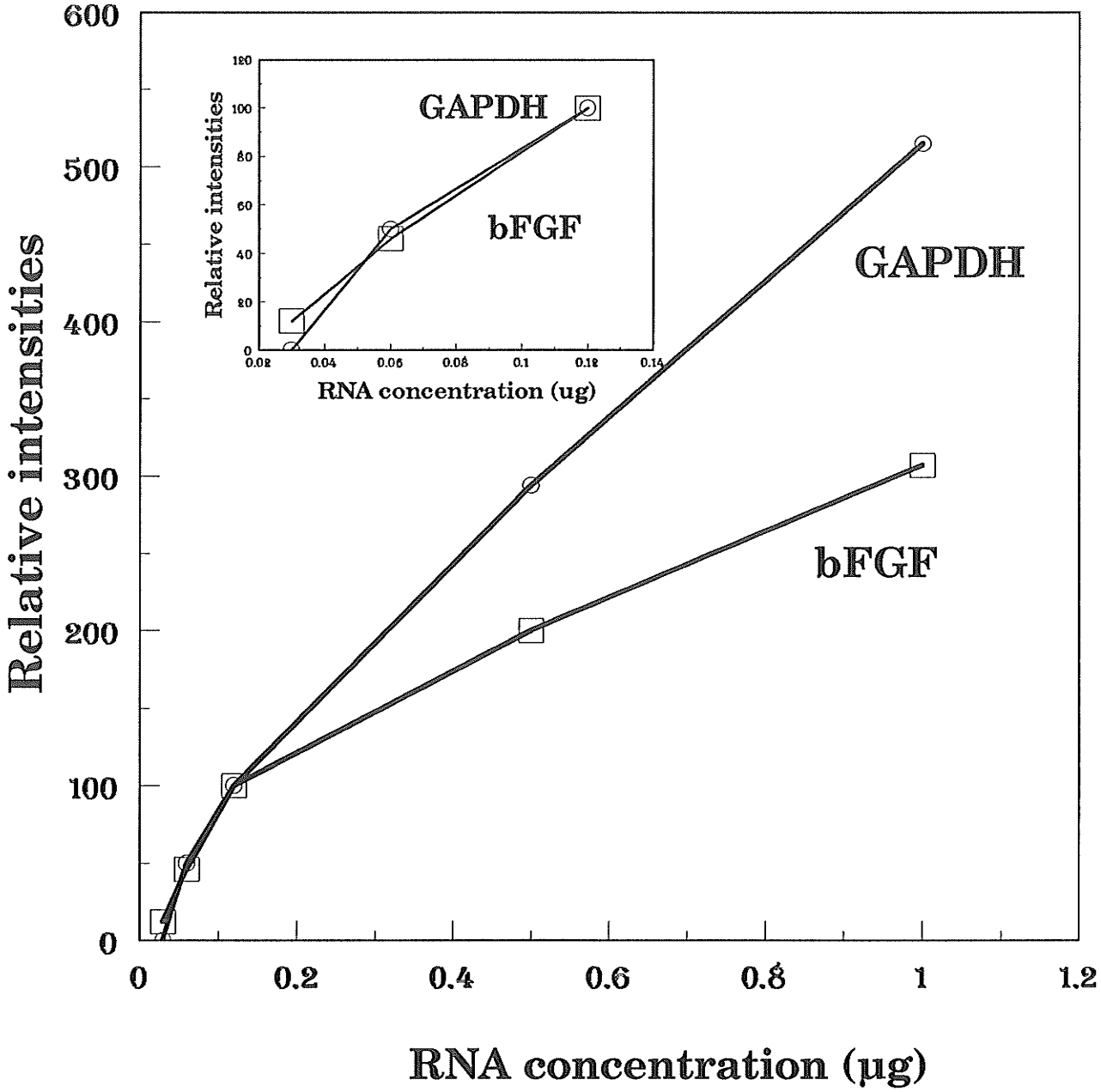
B.





GAPDH

Fig. 8 Relationship of the PCR products as a function of the amount of template RNA input. bFGF and GAPDH cDNAs were amplified in two separate reactions. Serial dilutions of RNA (0.03 µg to 1 µg of total RNA) were reverse transcribed (RT) and PCR amplified. GAPDH cDNA samples (bottom pannel) were amplified for 22 cycles while bFGF cDNAs (upper pannel) were amplified for 35 cycles. The PCR products from both bFGF and GAPDH respectively, were plotted as a function of total RNA used in the RT reaction. Results were expressed as percentage of the 0.12 µg RNA dilution. Inset: shows the range of total RNA concentrations that gives linear PCR product amplification for both bFGF and GAPDH.


Relative intensities
RNA concentration (μg)
GAPDH
bFGF
Relative intensities
RNA concentration (ug)
GAPDH
bFGF

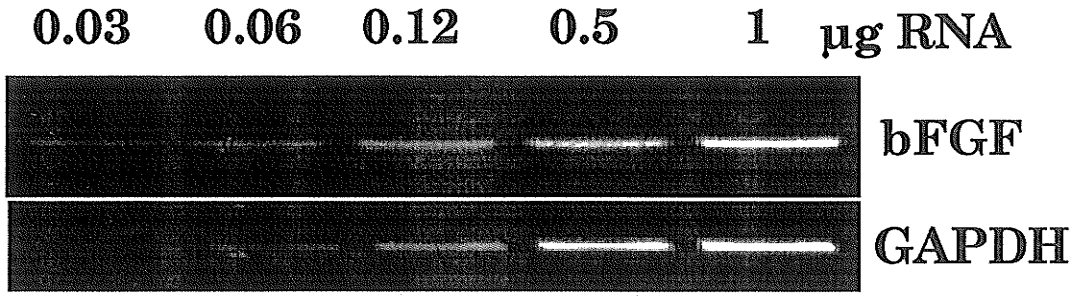
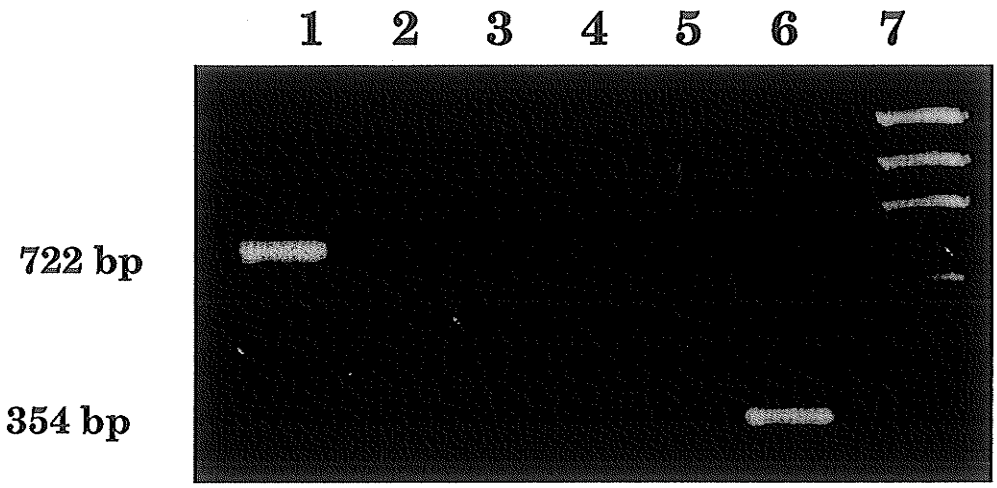

0.03
0.06
0.12
0.5
1
μg RNA
bFGF
GAPDH

Fig. 9 Ethidium bromide staining of RT-PCR products of rat brain total RNA. Primers used were: P1 (forward primer): $^{5'}$AACGGCGGCTTCTTCCTG$^{3'}$; P2 (reverse primer) $^{5'}$AGCAGACATTGGAAGAAACA$^{3'}$; P3 (reverse primer): $^{5'}$CTACAAGCTCTACCACAGGGGA$^{3'}$. Upper panel: lanes 1 - 3 : results obtained using P1/P3 primers. Lanes 4 - 6 : results obtained using P1/P2 primers.1) 722 bp product, 2) negative control (no RTase), 3) negative control (no RNA), 4) negative control (no RTase), 5) negative control (no RNA), 6) 354 bp product, 7) ϕx DNA digested digested Hae III. Fig. 9B is a diagram explaining the discrapncy between the expected size PCR product (415 bp) and the observed one (722bp). The positions of primers used are indicated by arrows. Lower panel: Diagram repesents an explanation for the discrepency between the expected and the observed PCR products.



	P1/P3	P1/P2
Observed	722 bp	354 bp
Expected	415 bp	354 bp

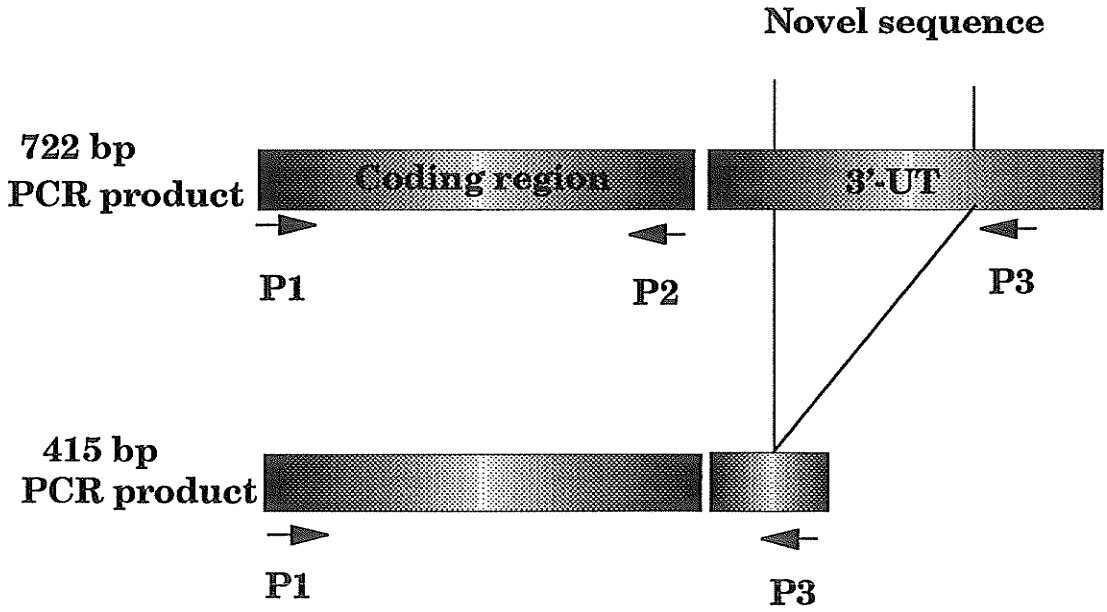


Fig. 10 RT-PCR product of bFGF mRNA from adult rat ovary and 3 day old rat brain revealed by Southern analysis; ^{32}P labelled rat bFGF cDNA (800 bp) was used as a probe. A 354 bp PCR product band was observed when P1/P2 primers were used (lanes 1, 3, 5), while a 722 bp PCR product band was observed when P1/P3 primers were used (lanes 2, 4, 6). The RT-PCR conditions were optimized for each tissue separately to provide a clearly visible signal, and therefore signal intensities are not quantitative.

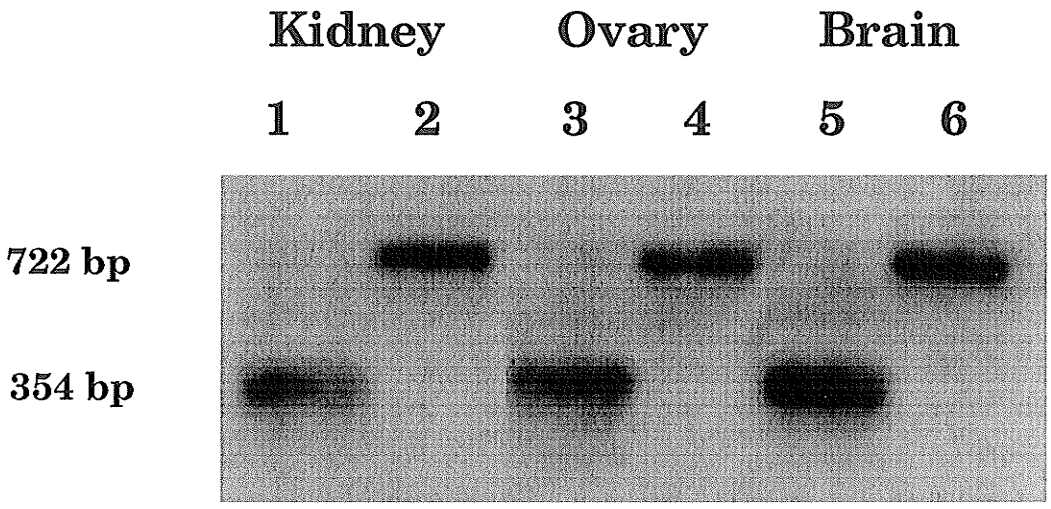

Kidney
Ovary
Brain
1
2
3
4
5
6
722 bp
354 bp

Fig. 11 Sequence comparison between the rat brain bFGF cDNA (RATBFGF3), rat brain bFGF cDNA (RATGFBF; Kurokawa et al, 1988) and rat ovary bFGF cDNA (RATGFFO; Shimasaki et al, 1988). The stop codon is shown in lower case letters. The novel sequence is from positions 383 to 689 of the RATBFGF3. The positions of the three primers used in this study are marked by arrows. The conserved A+T rich motifs, $(ATTT)_8$ and $(TATT)_4$ are shaded and overlined respectively.

```
RATGFFO    533 ATGGCTGCCGGCAGCATCACTTCGCTTCCCGCACTGCCGGAGGACGGCGGCGGCGCCTTCCCACC
RATGFBF    254 ATGGCTGCCGGCAGCATCACTTCGCTTCCCGCACTGCCGGAGGACGGCGGCGGCGCCTTCCCACC

RATGFFO    598 CGGCCACTTCAAGGATCCCAAGCGGCTCTACTGCAAGAACGGCGGCTTCTTCCTGCGCATCCATC
RATGFBF    319 CGGCCACTTCAAGGATCCCAAGCGGCTCTACTGCAAGAACGGCGGCTTCTTCCTGCGCATCCATC
RATBFGF3     1                                                AACGGCGGCTTCTTCCTGCGCATCCATC
                                                              ----------->
                                                                  P1

RATGFFO    663 CAGACGGCCGCGTGGACGGCGTCCGGGAGAAGAGCGACCCACACGTCAAACTACAGCTCCAAGCA
RATGFBF    384 CAGACGGCCGCGTGGACGGCGTCCGGGAGAAGAGCGACCCACACGTCAAACTACAGCTCCAAGCA
RATBFGF3    29 CAGACGGCCGCGTGGACGGCGTCCGGGAGAAGAGCGACCCACACGTCAAACTACAGCTCCAAGCA

RATGFFO    728 GAAGAGAGAGGAGTTGTGTCCATCAAGGGAGTGTGTGCGAACCGGTACCTGGCTATGAAGGAAGA
RATGFBF    449 GAAGAGAGAGGAGTTGTGTCCATCAAGGGAGTGTGTGCGAACCGGTACCTGGCTATGAAGGAAGA
RATBFGF3    94 GAAGAGAGAGGAGTTGTGTCCATCAAGGGAGTGTGTGCGAACCGGTACCTGGCTATGAAGGAAGA

RATGFFO    793 TGGACGGCTGCTGGCTTCTAAGTGTGTTACAGAAGAGTGTTTCTTCTTTGAACGCCTGGAGTCCA
RATGFBF    514 TGGACGGCTGCTGGCTTCTAAGTGTGTTACAGAAGAGTGTTTCTTCTTTGAACGCCTGGAGTCCA
RATBFGF3   159 TGGACGGCTGCTGGCTTCTAAGTGTGTTACAGAAGAGTGTTTCTTCTTTGAACGCCTGGAGTCCA

RATGFFO    858 ATAACTACAACACTTACCGGTCACGGAAATACTCCAGTTGGTATGTGGCACTGAAACGAACTGGG
RATGFBF    579 ATAACTACAACACTTACCGGTCACGGAAATACTCCAGTTGGTATGTGGCACTGAAACGAACTGGG
RATBFGF3   224 ATAACTACAACACTTACCGGTCACGGAAATACTCCAGTTGGTATGTGGCACTGAAACGAACTGGG

RATGFFO    923 CAGTATAAACTCGGATCCAAAACGGGGCCTGGACAGAAGGCCATACTGTTTCTTCCAATGTCTGC
RATGFBF    644 CAGTATAAACTCGGATCCAAAACGGGGCCTGGACAGAAGGCCATACTGTTTCTTCCAATGTCTGC
RATBFGF3   289 CAGTATAAACTCGGATCCAAAACGGGGCCTGGACAGAAGGCCATACTGTTTCTTCCAATGTCTGC
                                                              <-----------
RATGFFO    988 TAAGAGCtgaCTCTCTTTAGACACTGTCA                                P2
RATGFBF    709 TAAGAGCtgaCTCTCTTTAGACACTGTCACTGAGAGAAAAGAAAAGAATGTATACAGCTAAGTTT
RATBFGF3   354 TAAGAGCtgaCTCTCTTTAGACACTGTCACTGAGAGAAAAGAAAAGAATGTATACAGCTAAGTTT

RATGFBF    774 GGATGCCTTTTATGTAACAATAAGACACTTAGCCATTACC
RATBFGF3   419 GGATGCCTTTTATGTAACAATAAGACACTTAGCCATTACCTCAGTAAAGAAAAACAACAATTTTT

RATBFGF3   484 GGAAAATATTTGGACTTCCCCATTTTATATAGCTTTGGTTGTGACCCAGTGAAAATTCTAGCCAC

           549 AATCTTTGTCACGTATTTCCTTTATTTGAAAAGAGGATTTAAACATATATTCACAAAATTCACAT

           614 TTGTGAATTTCTACCCTGGAAACACAAGTATGTGAGGAAGGCTGGCATTCAATCTGTTCCCATTT


RATGFFO   1017              CTCTCAGGCAGTCCCCTGTGGTAGAGCTTGTAG
RATBFGF3   679 CAGCCTTCTACCTCTCAGGCAGTCCCCTGTGGTAGAGCTTGTAG
                             <-----------
                                  P3
```

Fig. 12 Diagram comparing the sequence of published bFGF cDNAs. The rat bFGF sequences are obtained from 3 day old rat brain (RATBFGF3; El-Husseini et al, 1992); adult ovary (RATGFBF; Kurokawa et al, 1988) and adult brain (RATGFFO; Shimasaki et al, 1988). HBFGF is the human bFGF cDNA sequence (Kurokawa et al, 1987). Position of the primers used to amplify the rat brain bFGF cDNA are indicated by arrows. For description of primers see Fig. 11. Unshaded areas represent the coding region and shaded areas represent the 3' UT region of the bFGF cDNA. Identical regions positions are shown above each sequence.

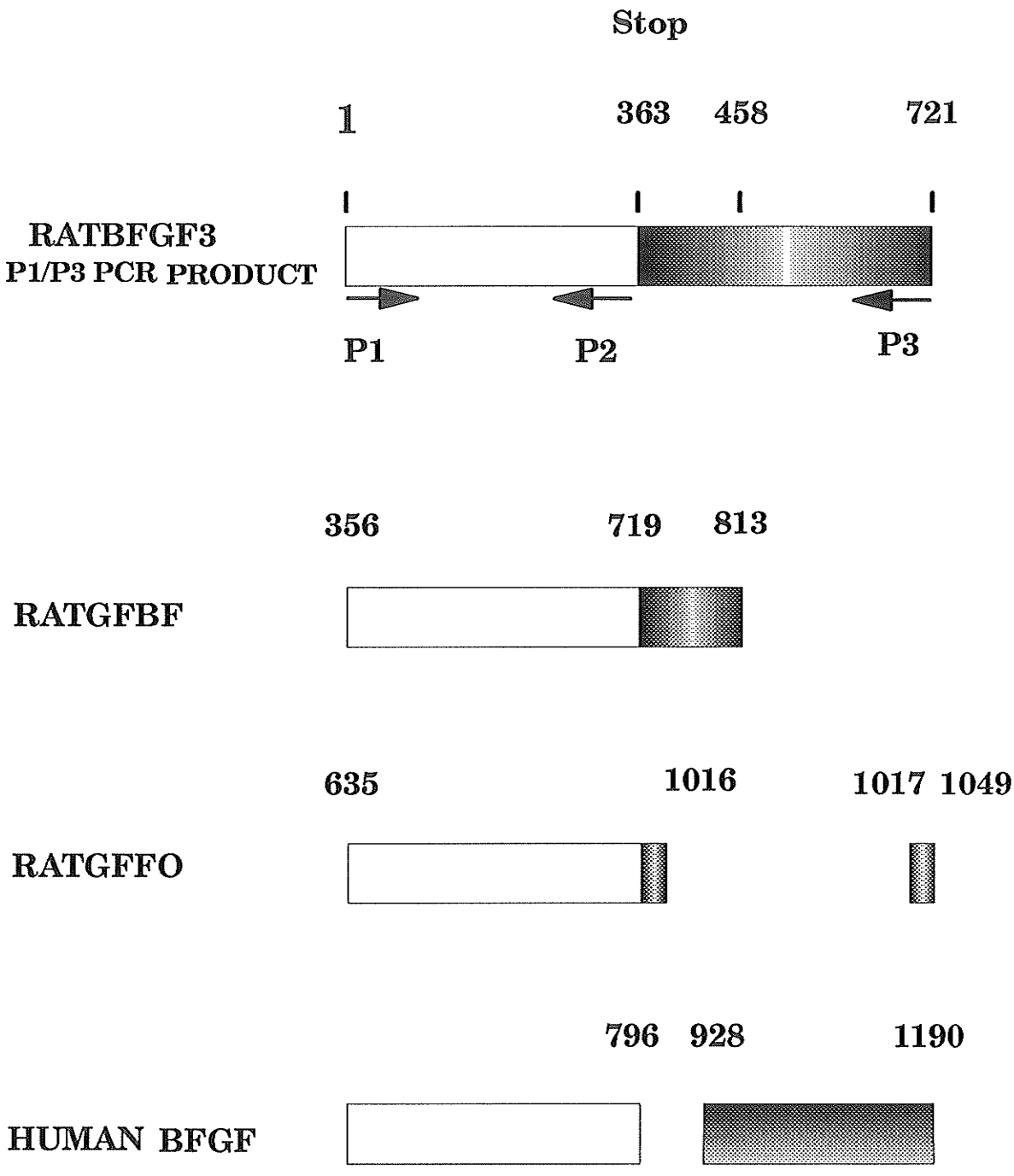

Stop
1
363
458
721
RATBFGF3
P1/P3 PCR PRODUCT
P1
P2
P3
356
719
813
RATGFBF
635
1016
1017
1049
RATGFFO
796
928
1190
HUMAN BFGF

Fig. 13 Basic FGF gene expression in the cerebrum of male rats in early postnatal development. Values were equalized with respect to GAPDH signal. Results are expressed as percentage of the cerebrum of 21-day-old rat.
This graph shows that levels of bFGF increased significantly between the first and the second weeks of postnatal brain growth.
(*)= Levels of bFGF mRNA in the cerebrum of 14, 21 and 28-day-old rats were significantly different from those from 1 day, 3 days and 7-day-old rats. Only 2 sets of one-year-old rats were tested. Statistical analysis was carried out using ANOVA-Duncan's analysis; $p<0.05$.

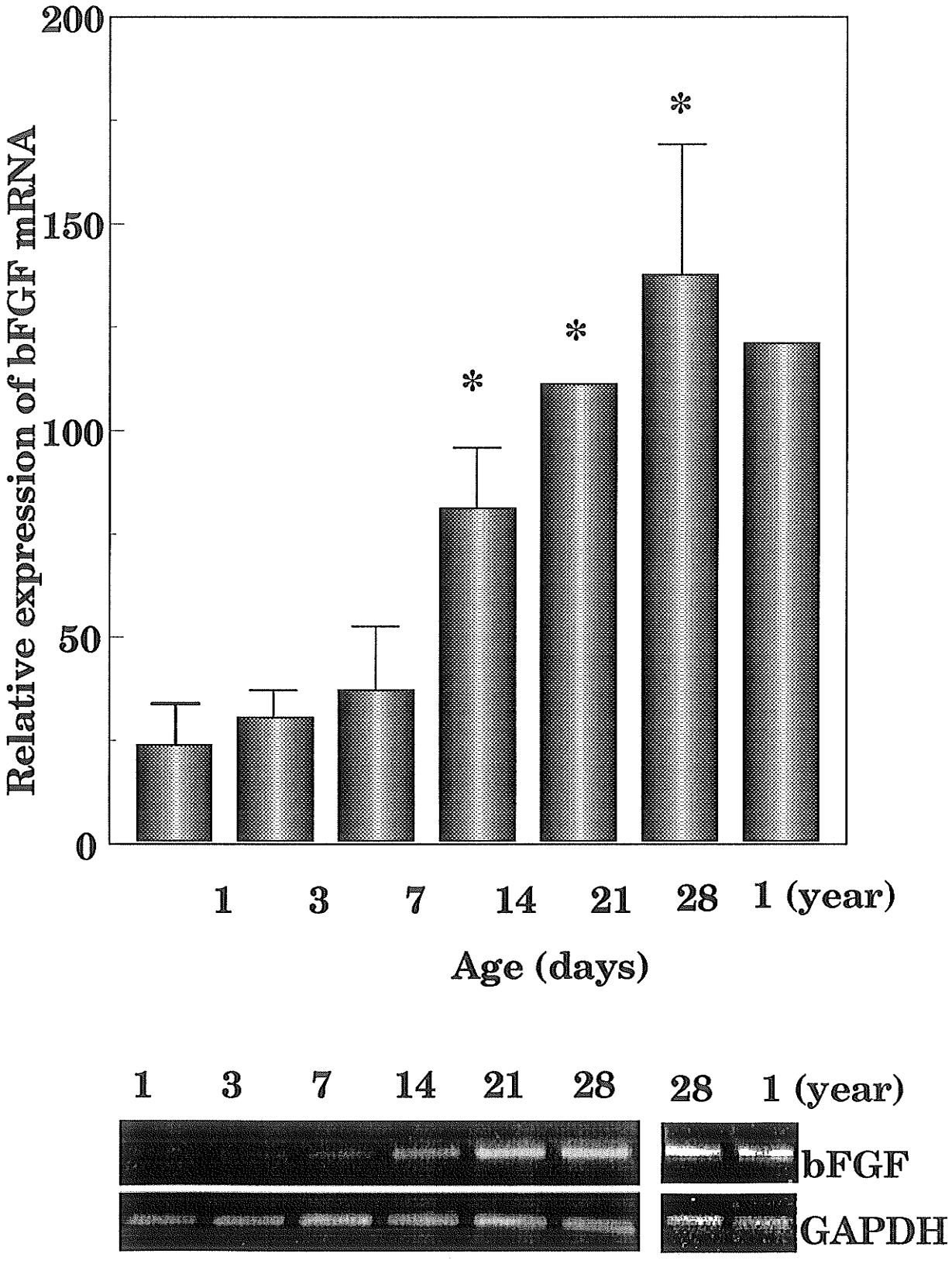

200
150
100
50
0
Relative expression of bFGF mRNA
*
*
*
1
3
7
14
21
28
1 (year)
Age (days)
1
3
7
14
21
28
28
1 (year)
bFGF
GAPDH

Fig. 14 Regional comparison of bFGF mRNA in the brain of 28-day-old male rats. Regions of the brain used are: O.C. (occipital cortex), I.C. (inferior colliculus), CB (cerebellum), P&M (combined pons-meduula), HYPO (hypothalamus), C.C. (cingulate cortex) and HIPPO (hippocampus). Levels of bFGF mRNA were highest in HIPPO followed closely by O.C., C.C. and I.C.. Moderate levels of bFGF mRNA were in HYPO and P&M, while lowest levels were in CB. (NB: For only one set of 28 day old rats, hypothalamus was separated at the optic tracts from one thick coronal cerebral slice, along with hippocampus and cingulate cortex). Results are expressed as percentage of Inferior colliculus of 28-day-old rat set at 100%.

Statistical analysis was carried out using ANOVA-Duncan's analysis; $p<0.05$.

(*) : level of bFGF mRNA in the CB was significantly different from O.C., I.C., and P&M.

level of bFGF mRNA in O.C. was significantly different from P&M and CB

level of bFGF mRNA in I.C. was significantly different from CB

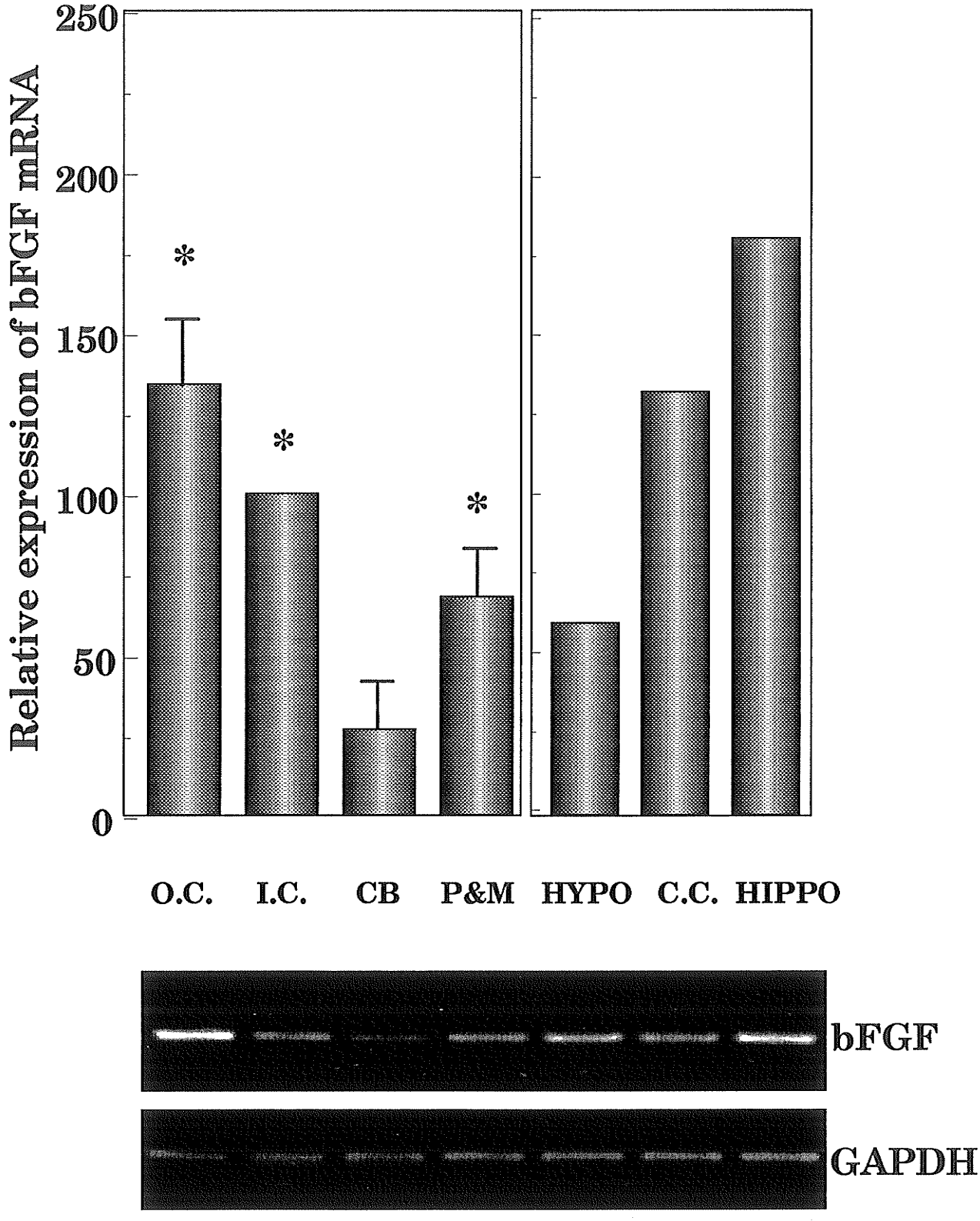

Relative expression of bFGF mRNA
250
200
150
100
50
0
*
*
*
O.C.
I.C.
CB
P&M
HYPO
C.C.
HIPPO
bFGF
GAPDH

Fig. 15 Regional comparison of bFGF mRNAs in early postnatal developmment Results are expressed as percentage of inferior colliculus of 28-day-old rat. This graph shows that bFGF mRNA expression is temporally and spatially regulated in various brain regions in early postnatal development: in occipital cortex and inferior colliculus: bFGF mRNA level increases during the first month after birth; in cerebellum: bFGF mRNA level was highest at postnatal day one; in pons-medulla: bFGF mRNA level was moderate during the first postnatal month.

(*) = Significantly different from day one for each region. Statistical analysis was carried out using ANOVA-Duncan's analysis; $p<0.05$.

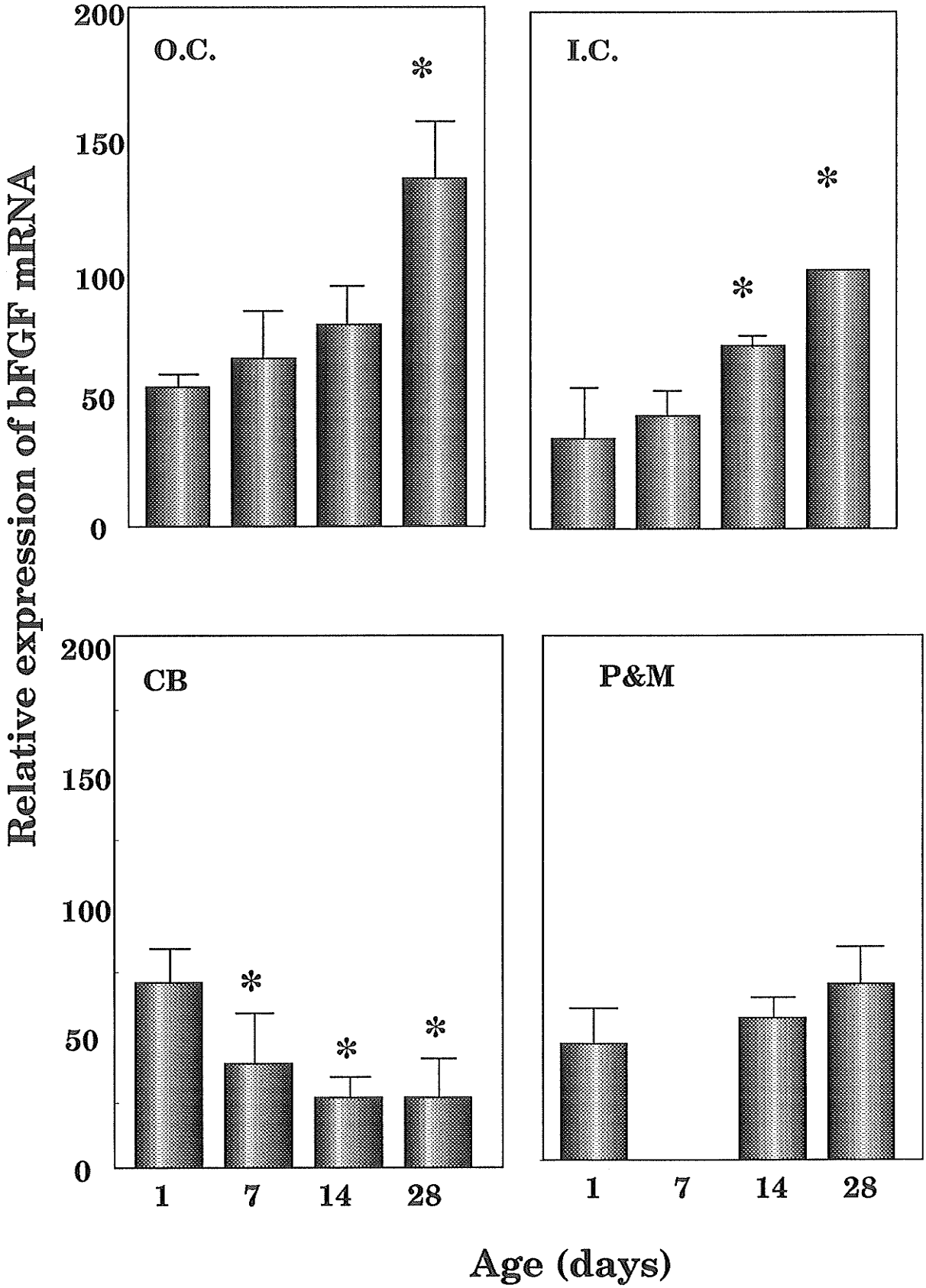

O.C.
I.C.
CB
P&M
200
150
100
50
0
1
7
14
28
*
Relative expression of bFGF mRNA
Age (days)

Fig. 16 Southern analysis of bFGF gene expression in the brain meninges of neonatal rats. a. The hybridization bands of the PCR products from rat cerebellum (28-day old; lane 1) and meninges (1 day and 7-day-old pool; lane 2) are compared. ^{32}P labelled bFGF cDNA probe was used as probe. b. ethidium bromide stained GAPDH and actin PCR products generated after the RT-PCR amplification of total RNA obtained from cerebellum and meninges.

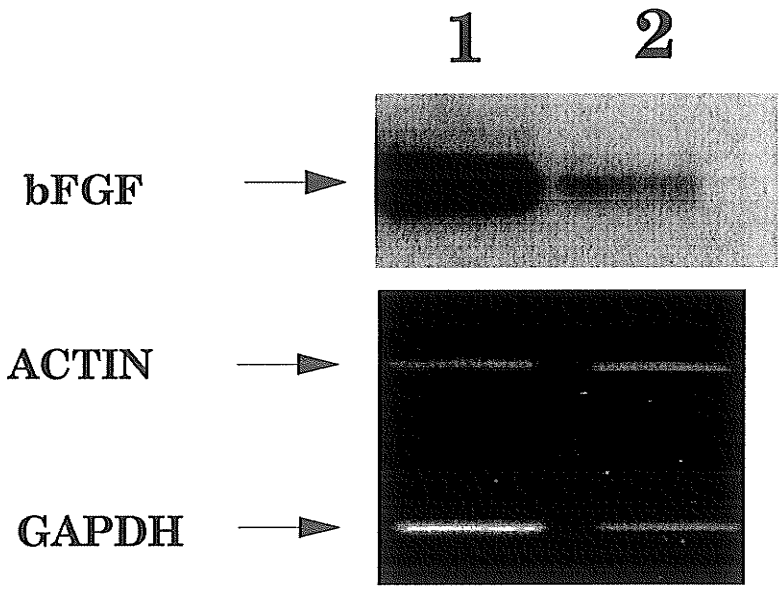
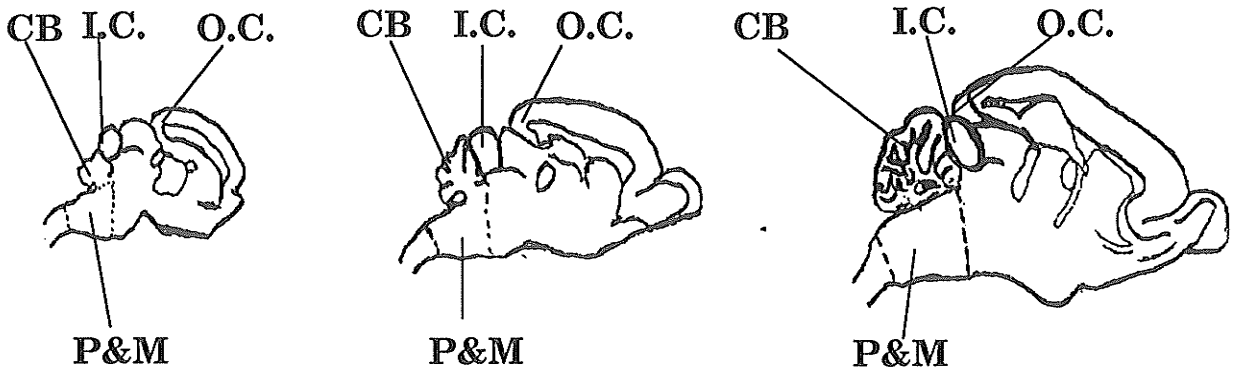

1
2
bFGF
ACTIN
GAPDH

Fig. 17 Diagram comparing the relative size and structure of the rat brain in early postnatal development. Saggital section in the brain to show regions dissected for the study of bFGF mRNA expression are indicated. O.C.=occipital cortex; I.C.=inferior colliculus; CB=cerebellum; P&M=pons-medulla.

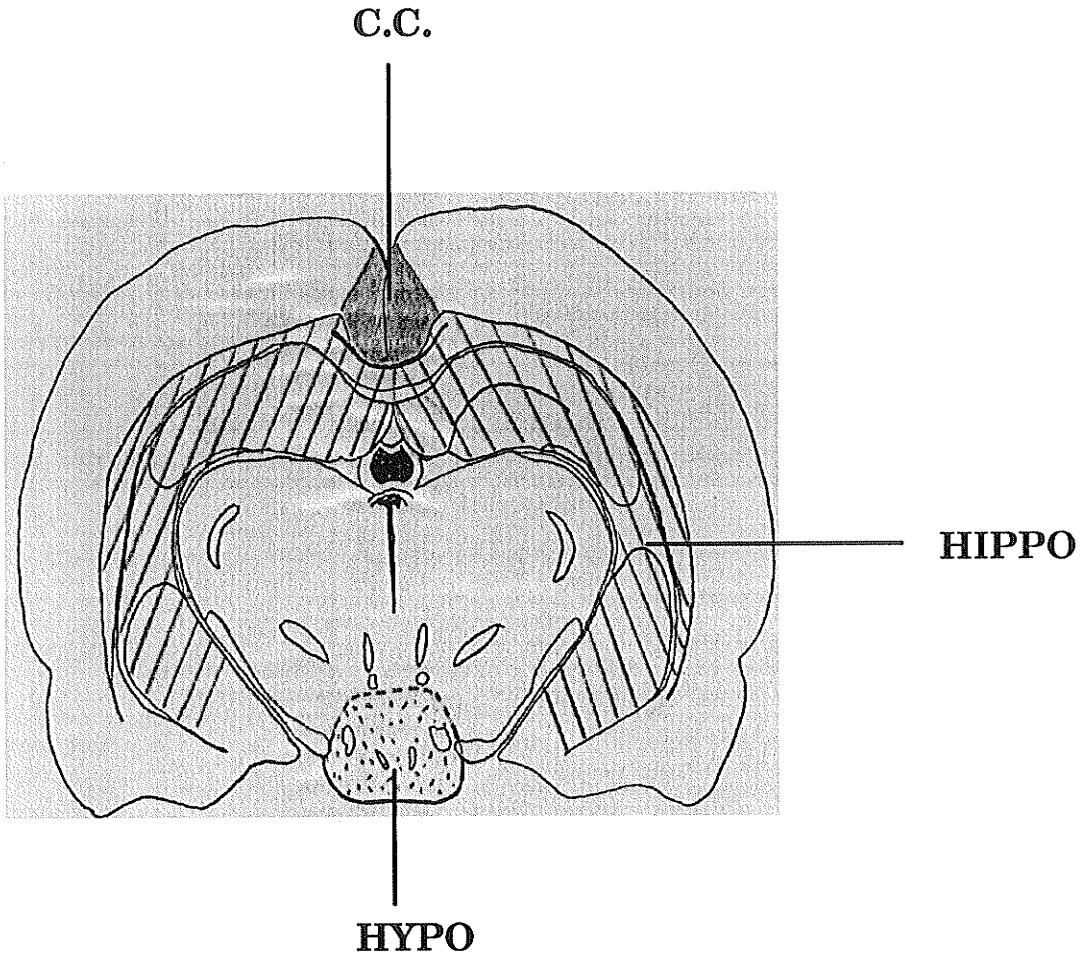


1 day **1 week** **3 weeks**

Saggital section

- **O.C.=occipital cortex**
- **CB= cerebellum**
- **I.C.=inferior colliculus**
- **P&M=pons-medulla**

Fig. 18 Coronol section of the brain of 28 old rat. This diagram shows positions of cingulate cortex (C.C.), Hippocampus (HIPPO) and hypothalamus (HYPO) dissected from 28 old rats.



4-week-old rat

Coronal section

HYPO=hypothalamus

C.C.=cingulate cortex

HIPPO=hippocampus

Fig. 19 Events taking place in most regions of the rat brain in early postnatal development.

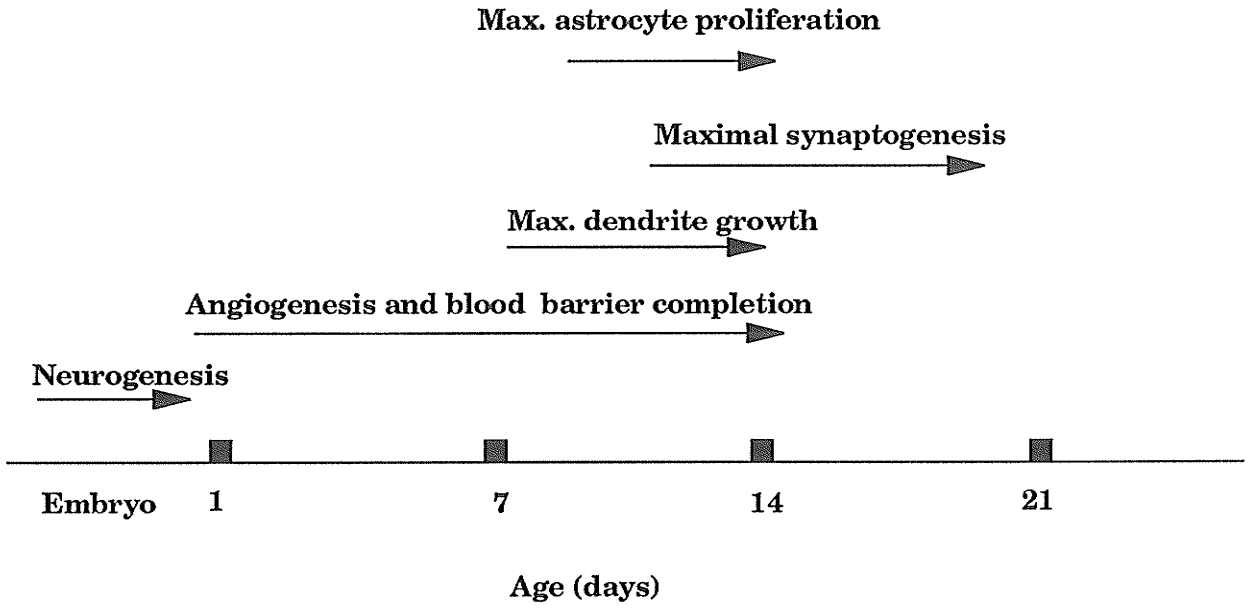

Max. astrocyte proliferation
Maximal synaptogenesis
Max. dendrite growth
Angiogenesis and blood barrier completion
Neurogenesis
Embryo
1
7
14
21
Age (days)

TABLE 1A

The RT-PCR analysis of bFGF gene expression in the cerebrum of 1 day, 3 days and 7-day-old rats

All numbers are relative to 21 day cerebrum set at 100%

M=Mean; SD= Standard deviation; d=Day;S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs.

Age	1 d	1 d	1 d	3 d	3 d	3 d	7 d	7 d	7 d
Set	s#1	s#2	s#3	s#1	s#2	s#3	s#1	s#2	s#3
PCR 1	4	20	6	16	12	49	12	19	27
2	29	26	7	25	26	16	24	25	33
3	16	37	24	40	19	16	22	32	27
4		24	33	52	33	33		64	86
5			15		14	15			56
6			42			39			46
7									32
8									
M±SD n^{RP}	16±13 n^{RP}=3	27±7 n^{RP}=4	21±14 n^{RP}=6	33±16 n^{RP}=4	21±9 n^{RP}=5	28±14 n^{RP}=6	19±6 n^{RP}=3	35±20 n^{RP}=4	44±21 n^{RP}=7
Total M±SD n		**21±6 n=3**			**27±6 n=3**		**33±13 n=3**		

TABLE 1B

The RT-PCR analysis of bFGF gene expression in the cerebrum of 14 days and 28-day-old rats

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; d=Day; y=Year; S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs.

Age	14 d	14 d	14 d	28 d	28 d	28 d	1 y	1 y
Set	s#1	s#2	s#3	s#1	s#2	s#3	s#1	s#2
PCR 1	44	33	125	155	58	107	129	176
2	36	100	129	225	121	124	63	111
3	55	104	63	97	90	223	133	59
4	88	90	111	134	105	86	85	113
5		53	48		104	48		
6			73			153		
7			64			131		
8								
M±SD n^{RP}	56±23 n^{RP}=4	76±31 n^{RP}=5	88±33 n^{RP}=7	153±54 n^{RP}=4	94±27 n^{RP}=5	125±55 n^{RP}=7	103±34 n^{RP}=4	115±48 n^{RP}=7
Total M±SD n		**73±16 n=3**			**124±30 n=3**			**109±9 n=2**

TABLE 2A

The RT-PCR analysis of the bFGF gene expression in the developing rat cerebrum

All numbers are relative to 21 day cerebrum set at 100%

M=Mean; SD= Standard deviation; d=Day; y=Year; S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}=number of repeated PCR runs.

Age	1 d	3 d	7 d	14 d	21 d	28 d	1 y
Set 1	16±13 n^{RP}=3	33±16 n^{RP}=4	19±6 n^{RP}=6	56±23 n^{RP}=4	100	153±54 n^{RP}=4	
Set 2	27±7 n^{RP}=4	21±9 n^{RP}=5	35±20 n^{RP}=4	76±31 n^{RP}=5	100	94±27 n^{RP}=5	
Set 3	21±14 n^{RP}=6	28±14 n^{RP}=6	44±21 n^{RP}=7	88±33 n^{RP}=7	100	125±55 n^{RP}=7	103±34 n^{RP}=4
Total M±SD n	**21±6 n=3**	**27±6 n=3**	**33±13 n=3**	**73±16 n=3**	**100 n=3**	**124±30 n=3**	**109±9 n=2**

Table 2B
Statistical analysis of bFGF gene expression in the cerebrum of the developing rat in early postnatal ages (using ANOVA-Duncan's test).

Tissue was pooled from individual animals to create sets for each day. Total RNA from each set-day was analyzed repeatedly by RT-PCR as described in tables 1A and 1B. To simplify comparisons of different tissues, results were expressed as a percentage of one of the brain regions selected as a reference point. Because of its intermediate band intensity, 21-day-old rat cerebrum was set at 100% and all other cerebrums obtained from other postnatal ages (see table 2A) were expressed relative to it. Data were averaged for all PCR runs to create a mean for each set-day.
Set-day means were averaged to create a mean for each age.

(Remarks: RT-PCR data obtained from all the other brain regions tested (tables 3 to 10) was treated similarly).

Age	Mean Average	SD	1 day	3 day	7 day	14 day	21 day	28 day
1 day	21	6	.	.	.	s	s	s
3 day	27	6	.	.	.	s	s	s
7 day	33	13	.	.	.	s	s	s
14 day	73	16	s	s	s	.	.	s
21 day	100	-*	s	s	s	.	.	.
28 day	124	30	s	s	s	s	.	.

* All numbers are relative to 21 day cerebrum set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 3

The RT-PCR Analysis of bFGF gene expression in 28 d rat O.C. and CB
All numbers are relative to 28 day inferior colliculus set at 100%
M=Mean; SD= Standard deviation; d=Day;S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs.
** The rest of RT-PCR results for this set are in table 7b

Region	O.C.	O.C.	O.C.	O.C.**	CB	CB	CB	CB
Set	s#1	s#2	s#3	s#4	s#1	s#2	s#3	s#4
PCR1	63	154	151	126	9	9	9	43
2	145	156	107	393	17	10	66	20
3	116	129	172	278	47	5	31	11
4	84		143	306	15		19	59
5			147	172	28		80	38
6				89				47
7				124				18
8				66				31
9				93				50
10				152				36
11				82				31
M±SD n^{RP}	102±36 n^{RP}=4	146±15 n^{RP}=3	144±24 n^{RP}=5	142±71 n^{RP}=32	23±15 n^{RP}=5	8±3 n^{RP}=3	41±31 n^{RP}=5	35±15 n^{RP}=11
Total M±SD n				134±21 n=3				27±15 n=4

TABLE 4

The RT-PCR Analysis of bFGF gene expression in 28 d rat P&M

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; d=Day;S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs.

Region	P&M	P&M	P&M	P&M
Set	s#1	s#2	s#3	s#4
PCR1	73	47	107	91
2	70	78	87	45
3	58	43	75	33
4	82		109	55
5			63	80
6				69
7				48
8				61
9				50
10				36
11				61
M±SD n^{RP}	71±10 n^{RP}=4	56 ±19 n^{RP}=3	88 ±20 n^{RP}=5	57 ±18 n^{RP}=11
Total Av±SD n				68 ±15 n=4

TABLE 5

The RT-PCR Analysis of bFGF gene expression in 28 d rat hypothalamus (HYPO), cingulate cortex (C.C.) and hippocampus (HIPPO).
All numbers are relative to 28 day inferior colliculus set at 100%.
M=Mean; SD= Standard deviation; d=Day; S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs.

Region	HYPO	C.C.	HIPPO
Set	s#1	s#1	s#1
PCR1	56	143	218
2	64	159	152
3	33	118	116
4	56	200	267
5	63	134	207
6	38	99	120
7	47	119	182
8	64	108	201
9	76	139	174
10	96	137	166
11	64	91	
M±SD n^{RP}	60±17 n^{RP}=11	132±30 n^{RP}=11	180±55 n^{RP}=10
Total M±SD n	60±17 n=1	`133±32 n=1	180±46 n=1

TABLE 6A

The RT-PCR analysis of bFGF gene expression in 28 d old rat brain

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; d=Day;S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}=number of repeated PCR runs. O.C.=occipital cortex; I.C.=inferior colliculus; CB=cerebellum; P&M=pons-medulla; HYPO=hypothalamus; C.C.=cingulate cortex; HIPPO= hippocampus

Region	O.C.	I.C.	CB	P&M	HYPO	C.C.	HIPPO
Set 1	102±36 n^{RP}=4	100	23±15 n^{RP}=5	71±10 n^{RP}=4			
Set 2	146±15 n^{RP}=3	100	8±3 n^{RP}=3	56±19 n^{RP}=3			
Set 3	144±24 n^{RP}=5	100	41±31 n^{RP}=5	88±20 n^{RP}=5			
Set 4	142±71 n^{RP}=32	100	35±15 n^{RP}=11	57±18 n^{RP}=11	60±17 n^{RP}=11	132±30 n^{RP}=11	180±55 n^{RP}=10
Total M±SD n	**134±21 n=4**	**100 n=4**	**27±15 n=4**	**68±15 n=4**	**60±17 n=1**	**132±30 n=1**	**180±55 n=1**

Table 6B
Statistical analysis of the significance of bFGF gene expression in regions of the brain of 28-day-old rat (using ANOVA-Duncan's test).

Because of its intermediate band intensity, 28-day-old-rat inferior colliculus (I.C.) was set at 100% and all the other brain regions examined from all postnatal ages (table 3 to 10) were expressed relative to it.
For explanation of RT-PCR data treatment for regional comparisons and statistical analysis, see table 2B (also see page 43).

Region	Mean Average	SD	O.C.	I.C.	CB	P&M
Occipital cortex (O.C)	134	21	.	.	s	s
Inferior colliculus (I.C.)	100	-*	.	.	.	s
Cerebellum (CB)	27	15	s	s	.	s
Pons-Medulla (P&M)	68	15	s	.	s	.

*All numbers are relative to 21 day inferior colliculus set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 7a

The RT-PCR Analysis of bFGF gene expression in the occipital cortex of the developing rat.

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; d=Day

S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs

Age	1 d	1 d	1 d	7 d	7 d	14 d	14 d	14 d
Set	s#1	s#2	s#3	s#1	s#2	s#1	s#2	s#3
PCR 1	43	89	45	91	66	97	84	102
2	70	76	77	70	43	122	76	78
3	47	30	47	55	54	86	64	93
4	58	50	29	68	57	84	65	97
5	73	45	45	87	42	100	76	89
6		21	63	103		39	45	
7						34	45	
M±SD n^{RP}	58±13 n^{RP}=5	52±26 n^{RP}=6	51±17 n^{RP}=6	79±18 n^{RP}=6	52±10 n^{RP}=5	80±32 n^{RP}=7	63±15 n^{RP}=7	92±9 n^{RP}=5
Total M±SD n		54±4 n=3			65±19 n=2		78±15 n=3	

TABLE 7b

Age	28 d	28 d	28 d
Set	s#4	s#4	s#4
PCR	70	216	94
	138	180	85
	134	124	123
	97	106	171
	101	122	106
	116	173	106
	182	114	106
M±SD n^{RP}		127±37 n^{RP}=21	

Table 7C

Statistical analysis of bFGF gene expression in the occipital cortex of the developing rat in early postnatal ages (using ANOVA-Duncan's test).

To relatively compare all RT-PCR data obtained from different brain regions, 28-day-old-rat inferior colliculus (I.C.) was set at 100% and all the other brain regions examined from all postnatal ages (table 3 to 10) were expressed relative to it.
For explanation of RT-PCR data treatment for regional comparisons and statistical analysis, see table 2B (also see page 43).

Age	Mean Average	SD	1 day	7 day	14 day	28 day
1 day	54	4	.	.	.	s
7 day	65	19	.	.	.	s
14 day	78	15	.	.	.	s
28 day	134	21	s	s	s	.

All numbers are relative to 21 day inferior colliculus set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 8a

The RT-PCR Analysis of bFGF gene expression in the inferior colliculus of the developing rat

*All numbers are relative to 28 day inferior colliculus set at 100%.

S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs

Age	1 d	1 d	1 d	7 d	7 d	14 d	14 d	14 d
Set	s#1	s#2	s#3	s#1	s#2	s#1	s#2	s#3
PCR 1	56	49	8	25	73	108	93	117
2	29	32	7	81	16	79	44	59
3	38	32	12	42	32	42	42	48
4	47	86	24	47	20	65	124	67
5	19	51	22	54	43	52	82	63
6		65					69	
7							54	
8								
M±SD n^{RP}	38±15 n^{RP}=5	53±21 n^{RP}=6	15±8 n^{RP}=5	50±20 n^{RP}=5	37±23 n^{RP}=5	69±26 n^{RP}=5	73±30 n^{RP}=7	71±27 n^{RP}=5
Total M±SD n		35±19 n=3			44±9 n=2		71±2 n=3	

TABLE 8b

Age	28 d	28 d	28 d
Set	s#4	s#4	s#4
PCR	100	100	100
	100	100	100
	100	100	100
	100	100	100
	100	100	100
	100	100	100
	100	100	100
M±SD n^{RP}		100±0 n^{RP}=21	
Total M±SD n		100±0 n=4	

Table 8C

Statistical analysis of bFGF gene expression in the inferior colliculus of the developing rat in early postnatal ages (using ANOVA-Duncan's test).

To relatively compare all RT-PCR data obtained from different brain regions, 28-day-old-rat inferior colliculus (I.C.) was set at 100% and all the other brain regions examined from all postnatal ages (table 3 to 10) were expressed relative to it.
For explanation of RT-PCR data treatment for regional comparisons and statistical analysis, see table 2B (also see page 43).

Age	Mean Average	SD	1 day	7 day	14 day	28 day
1 day	35	19	.	.	s	s
7 day	44	9	.	.	s	s
14 day	71	2	s	s	.	s
28 day	100	-*	s	s	s	.

*All numbers are relative to 21 day inferior colliculus set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 9a

The RT-PCR Analysis of bFGF gene expression in the cerebellum of the developing rat.

All numbers are relative to 28 day inferior colliculus set at 100%. M=Mean; SD=Standard deviation; d=Day; S=Set (one tissue pooled from various animals and used for subsequent analysis); n=number of independent sets; n^{RP}= number of repeated PCR runs

Age	1 d	1 d	1 d	7 d	7 d	14 d	14 d	14 d
Set	s#1	s#2	s#3	s#1	s#2	s#1	s#2	s#3
PCR 1	64	114	35	35	45	37	30	35
2	36	69	54	31	36	38	29	32
3	123	59	74	20	75	53	17	12
4	60	95	42	33	51	50	25	25
5	74		59	31	52	56	14	9
6			86	30		12	28	11
7			51			33	54	11
8			77					
M±SD n^{RP}	71±32 n^{RP}=5	84±25 n^{RP}=4	59±17 n^{RP}=8	30±5 n^{RP}=6	52±14 n^{RP}=5	34±14 n^{RP}=7	28±13 n^{RP}=7	19±11 n^{RP}=7
Total M±SD n		**71±13 n=3**			**40±18 n=2**		**27±8 n=3**	

TABLE 9b

Age	28 d	28 d	28 d
Set	s#4	s#4	s#4
PCR	37	30	35
	38	29	32
	8	17	12
	11	25	25
	11	14	9
	12	28	11
	33		
M±SD n^{RP}		26±14 n^{RP}=19	
M±SD n		**20±8 n=4**	

Table 9C

Statistical analysis of bFGF gene expression in the cerebellum of the developing rat in early postnatal ages (using ANOVA-Duncan's test).

To relatively compare all RT-PCR data obtained from different brain regions, 28-day-old-rat inferior colliculus (I.C.) was set at 100% and all the other brain regions examined from all postnatal ages (table 3 to 10) were expressed relative to it.
For explanation of RT-PCR data treatment for regional comparisons and statistical analysis, see table 2B (also see page 43).

Age	Mean Average	SD	1 day	7 day	14 day	28 day
1 day	71	13	s	s	s	.
7 day	40	18	.	.	.	s
14 day	27	8	.	.	.	s
28 day	27	15	.	.	.	s

All numbers are relative to 21 day inferior colliculus set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 10A

The RT-PCR Analysis of bFGF gene expression in the pons-medulla of the developing rat.

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; d=Day; S=Set (one tissue pooled from various animals and used for subsequent analysis); n= number of independent sets; n^{RP}= number of repeated PCR runs

Age	1 d	1 d	1 d	7 d	7 d	14 d	14 d	14 d
Set	s#1	s#2	s#3	s#1	s#2	s#1	s#2	s#3
PCR 1	83	43	38	79	71	63	59	46
2	58	56	58	300	36	68	75	66
3	73	59	16	278	75	91	59	48
4	36	79	15	327	51	118	40	51
5	47	37	20	268	52	21	31	68
6	17	43	31	117		20	39	27
7			26	214			36	57
8			27	186				64
9				95				
M±SD n^{RP}	52±24 n^{RP}=6	53±15 n^{RP}=6	29±14 n^{RP}=8	196±95 n^{RP}=9	57±16 n^{RP}=5	64±38 n^{RP}=6	48±16 n^{RP}=7	53±14 n^{RP}=8
Total M±SD n		**45±14 n=3**					**55±8 n=3**	

TABLE 10b

Age	28 d	28 d	28 d
Set	s#4	s#4	s#4
PCR	83	88	42
	35	70	91
	100	63	88
	70	46	108
	96	39	56
	39	55	96
	61	80	98
		47	
M±SD n^{RP}		71±23 n^{RP}=22	
Total M±SD n		70±13 n=4	

Table 10C

Statistical analysis of bFGF gene expression in combined pons-medulla of the developing rat in early postnatal ages (using ANOVA-Duncan's test).

To relatively compare all RT-PCR data obtained from different brain regions, 28-day-old-rat inferior colliculus (I.C.) was set at 100% and all the other brain regions examined from all postnatal ages (table 3 to 10) were expressed relative to it.
For explanation of RT-PCR data treatment for regional comparisons and statistical analysis, see table 2B (also see page 43).

Age	Mean Average	SD	1 day	14 day	28 day
1 day	45	14	.	.	.
14 day	55	8	.	.	.
28 day	68	15	.	.	.

All numbers are relative to 21 day inferior colliculus set as 100%
s = significantly different; $p<0.05$
. = not significantly different

TABLE 11

The RT-PCR analysis of bFGF gene expression in various regions of the developing rat brain

All numbers are relative to 28 day inferior colliculus set at 100%

M=Mean; SD= Standard deviation; S=Set; n=Number of sets; d=Day; n^{RP}=number of repeated PCR runs.

Age	1 d	1 d	1 d	1 d	7 d	7 d	7 d	7 d
Region	O.C.	I.C.	CB	P&M	O.C.	I.C.	CB	P&M
Set 1	58±13 n^{RP}=5	38±15 n^{RP}=5	71±32 n^{RP}=5	52±24 n^{RP}=6	79±18 n^{RP}=6	50±20 n^{RP}=5	30±5 n^{RP}=6	196±95 n^{RP}=9
Set 2	52±26 n^{RP}=6	53±21 n^{RP}=6	84±25 n^{RP}=4	53±15 n^{RP}=6	52±10 n^{RP}=5	37±23 n^{RP}=5	52±14 n^{RP}=5	57±16 n^{RP}=5
Set 3	51±17 n^{RP}=6	15±8 n^{RP}=5	59±17 n^{RP}=8	29±14 n^{RP}=8				
Total M±SD n	**54±4 n=3**	**35±19 n=3**	**71±13 n=3**	**45±14 n=3**	**65±19 n=2**	**44±9 n=2**	**40±18 n=2**	

Age	14d	14d	14d	14 d	28 d	28 d	28 d	28 d
Region	O.C.	I.C.	CB	P&M	O.C.	I.C.	CB	P&M
Set 1	80±32 n^{RP}=7	69±26 n^{RP}=5	34±14 n^{RP}=7	64±38 n^{RP}=6	102±36 n^{RP}=4	100	23±15 n^{RP}=5	71±10 n^{RP}=4
Set 2	63±15 n^{RP}=8	73±30 n^{RP}=7	28±13 n^{RP}=7	48±16 n^{RP}=7	146±15 n^{RP}=3	100	8±3 n^{RP}=3	56±19 n^{RP}=3
Set 3	92±9 n^{RP}=5	71±27 n^{RP}=5	19±11 n^{RP}=7	53±14 n^{RP}=8	144±24 n^{RP}=5	100	41±31 n^{RP}=5	88±20 n^{RP}=5
Set 4					142±71 n^{RP}=32	100	35±15 n^{RP}=11	57±18 n^{RP}=11
Total M±SD n	**78±15 n=3**	**71±2 n=3**	**27±8 n=3**	**55±8 n=3**	**134±21 n=4**	**100 n=4**	**27±15 n=4**	**68±15 n=4**

Table 12

Comparative analysis of bFGF gene expression in brain regions of 28 day old rat:

A. Using serial dilutions of RNA

All numbers are relative to 28 day inferior colliculus set at 100%. n^{RP}= number of repeated PCR runs.

RNA Conc. Method of detection	0.25 µg EtBr stained gels	0.15 µg EtBr stained gels	0.07 µg EtBr stained gels
O.C.	143±13 n^{RP}=2	135±66 n^{RP}=8	127±37 n^{RP}=21
I.C.	100 n^{RP}=2	100	100 n^{RP}=21
CB	39±16 n^{RP}=2	34±17 n^{RP}=10	26±14 n^{RP}=19
P&M	58±11 n^{RP}=2	59±18 n^{RP}=9	71±23 n^{RP}=22
HYPO	64±17 n^{RP}=2	58±6 n^{RP}=6	ND
C.C.	137±4 n^{RP}=2	135±33 n^{RP}=8	ND
HIPPO	145 n^{RP}=1	171±42 n^{RP}=7	ND

B. Non-radioactive and radioactive PCR product detection.

All numbers are relative to 28 day inferior colliculus set at 100%. n^{RP}= number of repeated PCR runs.

RNA Conc. Method of detection	0.07 µg EtBr stained gels	0.07 µg ^{32}P labeled PCR product
O.C.	127±37 n^{RP}=21	121± 9 n^{RP}=5
I.C.	100 n^{RP}=21	100 n^{RP}=5
CB	26±14 n^{RP}=19	25±8 n^{RP}=4
P&M	71±23 n^{RP}=22	72±14 n^{RP}=7

Table 13
Discription of Animals used

A. Animals used to study the bFGF gene expression in the cerebrum of the developing rat.
M=Mean; SD= Standard deviation; S=Set; n=Number of sets; g=gram; WT=weight

Age	1 day	3 day	7 day	14 day	21 day	28 day	1 year
S1 rat # rats Pool Rat Wt.	9042 1 5.5 g	9039 1 12.4 g	9035 1 14.5 g	9131 1 28.0 g	9040 1 50.0 g	9041 1 84.0 g	9048a 1 1000 g
S2 rat # rats Pool Rat Wt.	9158 1 5.5 g	9039 1 11.2 g	9035 1 14.0 g	9131 1 33.0 g	9040 1 52.0 g	9112 1 103.0 g	9048b 1 1000 g
S3 rat # rats Pool Rat Wt.	9158 1 6.0 g	9129 1 9.5 g	9159 1 14.0 g	9173 1 22.0 g	9111b 1 50.0 g	9041 1 92.0 g	

B. Animals used to study the bFGF gene expression in different regions of the brain of 28 day old rat.

O.C.=occipital cortex; I.C.=inferior colliculus; CB=cerebellum; P&M=pons-medulla; HYPO=hypothalamus; C.C.=cingulate cortex; HIPPO= hippocampus

Region	O.C.	I.C.	CB	P&M	HYPO	C.C.	HIPPO
S1 rat # rats Pool Rat Wt	9112a 2 96 g	9112a 2 96 g	9112a 2 96 g	9112a 2 96 g			
S2 rat # rats Pool Rat Wt	9043+911 2c 2 103 g	9134+911 2b 2 107 g	9043 2 108 g	9043 2 108 g			
S3 rat # rats Pool Rat Wt	9201 4 90 g	9201 4 90 g	9201 4 90 g	9201 4 90 g			
S4 rat # rats Pool Rat wt	9205 4 82 g	9205 4 82 g	9205 4 82 g	9205 4 82 g	9205 4 82 g	9205 4 82 g	9205 4 82 g

C. Animals used to study the bFGF gene expression in different regions of the brain of 1 day old rat.

Region	O.C.	I.C.	CB	P&M
S1 rat # rats Pool Rat Wt	9038 1 35 g	9038 1 35 g	9038 1 35 g	9038 1 35 g
S2 rat # rats Pool Rat Wt	9131 1 30 g	9131 1 30 g	9131 1 30 g	9131 1 30 g
S3 rat # rats Pool Rat wt	9173 1 22 g	9173 2 22 g	9173 2 22 g	9173 2 22 g

D. Animals used to study the bFGF gene expression in different regions of the brain of 7 day old rat.

Region	O.C.	I.C.	CB	P&M
S1 rat # rats Pool Rat Wt	9233 7 13 g	9233 7 13 g	9233 7 13 g	9233 7 13 g
S2 rat # rats Pool Rat Wt	9237 6 12 g	9237 6 12 g	9237 6 12 g	9237 6 12 g

E. Animals used to study the bFGF gene expression in different regions of the brain of 14 day old rat.

Region	O.C.	I.C.	CB	P&M
S1 rat # rats Pool Rat Wt	9038 1 35 g	9038 1 35 g	9038 1 35 g	9038 1 35 g
S2 rat # rats Pool Rat Wt	9131 1 30 g	9131 1 30 g	9131 1 30 g	9131 1 30 g
S3 rat # rats Pool Rat wt	9173 1 22 g	9173 2 22 g	9173 2 22 g	9173 2 22 g