



Role of fibroblast growth factor-2 in human brain: a focus on development

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Abstract

Trophic factors have gained a great degree of attention as regulators of neural cells proliferation and differentiation as well as of brain maturation. Very little is known, however, about their effects on human immature nervous system. In this paper, data on expression of fibroblast-growth factor-2 and its receptors are reviewed and discussed in the light of its possible role in human brain development. © 2000 ISDN. Published by Elsevier Science Ltd. All rights reserved.

1. Introduction

Even as the development of the brain has always been a central problem in neurobiology, the mechanism of its regulation is still poorly understood. There are many complex and interrelated events which embrace differentiation of neuroepithelial cells, their migration, and formation of interneuronal connections whose correct timing and spatial arrangement are crucial for normal development. This is particularly important in the human nervous system, whose maturation requires a longer time than in other species and whose complex organization is still beyond our understanding. However, considerable evidence is accumulating indicating that diffusible signals play an important role in several of these events, starting from the earliest stages (reviewed in [41]). Even though the actual nature of most of these signals is still unknown, it has been shown that trophic factors of diverse origins play a key role (reviewed in [89]). Among them,

fibroblast growth factor-2 (FGF-2) has recently gained attention for its numerous effects in the nervous system, which range from regulation of stem cell proliferation and differentiation to neuronal survival, to protection from neurotoxic agents, including alcohol. Thus, in this paper we will review the data presently available on distribution and effects of FGF-2 and its receptors in the immature brain in correlation with human CNS development.

2. FGF-2 and stem cell differentiation and proliferation

In the vertebrate nervous system, FGF-2 is a strong candidate as a regulator of neurogenesis. FGF-2 is present in the rat telencephalon as early as E9.5, and in the cerebral cortex also into adulthood (reviewed in [7,45]). Addition of FGF-2 stimulates proliferation of cortical neuroectoderm cells *in vitro*, leading to an increase in neuronal number [27,28,54]. *In vivo*, FGF-2 has been shown to stimulate proliferation of cells with characteristics of multipotential stem cells in rat embryonic telencephalon and E17 cortex [44]. Also, FGF-2 stimulates the division of committed neuronal progenitor cells derived from embryonic striatum [95] and olfactory epithelium [19]. However, the recent discovery that adult mouse forebrain contains multipo-

Abbreviations: bFGF, basic fibroblast growth factor; CNS, central nervous system; FGF-R, FGF receptors.

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tential (neuronal/glia) progenitor cells, which can be isolated and cloned *in vitro*, has opened a new field of investigation [72,73]. It has been found that FGF-2 stimulates both multipotential and committed glial precursors [44], acting more as a mitogenic agent than as a trigger of neuronal differentiation [9]. The FGF-2 dependent cells are mainly multipotential, rising cells displaying the antigenic properties of astrocytes, oligodendrocytes and neurons [33]. However, some regional difference seems to exist. MacDonald et al. [49], using explants of adult and embryonic mouse olfactory epithelium, has found that FGF-2 stimulates differentiation of neurons but not of glial cells. The FGF-2 regulation of neurogenesis has been further demonstrated *in vivo* by Tao et al. [88] who obtained a significant inhibition of granule cell precursor proliferation treating rat pups with antibodies against FGF-2, suggesting a specific effect on neuronal precursors. In the human brain, Buc-Caron [16] first was able to demonstrate that fetal neuroepithelial cells, isolated *in vitro*, could be induced to proliferate in response to FGF-2, which favored a neuroblastic differentiation. This finding was confirmed by Murray and Dubois-Dalcq [55] who expanded human embryonic neural multipotential precursors with FGF-2 and showed that cells were capable of differentiating not only into neurons, but also into oligodendrocyte precursors and GFA-P⁺ astrocytes. Since the presence of neuronal stem cells has been recently demonstrated in the adult human brain [21], a role in after birth regulation of neurogenesis by FGF-2 is likely.

Besides its involvement in neuroectodermal cell proliferation, FGF-2 has been implicated in neuronal differentiation and survival (reviewed in [7]). More specifically, Vicario-Abejn et al. [96] clearly showed that in cultures of E16 hippocampus there are two populations of cells, one responding to administration of FGF-2 with proliferation and the other with differentiation. Moreover, FGF-2 can initiate neuronal differentiation of sympathoadrenal precursors [2] and cooperate with NGF in promoting the differentiation and proliferation of embryonic striatal neurons [18]. On the other hand, Armstrong et al. [5] found that FGF-2 could influence differentiation of human pre-oligodendrocytes, but not their proliferation, as also confirmed by Gogate et al. [32].

In conclusion, the data demonstrate that FGF-2 can have a double role as mitogenic and/or differentiating agent in mammalian brain and its influence presumably depends upon the cell type and stage of differentiation.

3. FGF-2 and brain growth and maturation

FGF-2 has been shown to stimulate neurite out-

growth of hippocampal and cerebral cortical neurons [99] and to maintain the viability of neurons [53]. It exerts trophic functions on both cholinergic and non-cholinergic neurons *in vitro* and *in vivo* [3,62,94,100,101], so that its role as a neuroprotective/recovery agent has been hypothesized [70,75]. It modulates ion channels [70] of nervous cells as well as their *in vivo* electrical activity [38] and can protect rat cerebral granule cells against ethanol-induced cell death [48]. It also stimulates proliferation of differentiated glial cells [77,80], so that its key-role along the whole life span can be hypothesized.

However, FGFs are thought to exert many, if not all, of their biological effects through interactions with cell surface binding sites, both low- and high-affinity receptors. Thus, the analysis of the expression and localisation of FGF receptors is essential for identifying their target(s).

Thus, for a better understanding of the role of FGF-2 in the development of the human brain, we extensively investigated expression and localization of FGF-2 and its receptors in the immature human CNS both *in vivo* and *in vitro*.

4. FGF-2 expression and localisation

4.1. *In vivo* studies

Bohlen and associates [14] provided in 1985 the first demonstration about the presence and partial characterization of FGF-2 in human adult CNS. Even though no data were available, its synthesis during human brain development seemed highly probable after the description of FGF-2 expression in rat and mouse embryonic brain by several authors (reviewed in [7,84]). When human fetal brains at early stages of pregnancy were analyzed for their content in FGF-2, FGF-2-like activity was found in whole brain tissue extracts [20], further substantiating the hypothesis of its possible involvement in human CNS maturation. However, this work did not provide any evidence about the localisation of the trophic factor as well as the cell type/s responsible for its synthesis and secretion.

4.2. *In vitro* studies

Previous *in vitro* studies had suggested that both neurons and astrocytes could synthesize FGF-2. In particular, immunohistochemical and immunocytochemical observations demonstrated the presence of FGF-2 in neurons of prepuberal rat brain and cerebellum [66] and in cultured chick and rat neurons [40]. Moreover, newborn mouse cerebellar astroglial cells

and adult bovine corpus callosum astrocytes could synthesize FGF-2 [23,34].

Thus, the problem of the cellular origin of FGF-2 could be solved by the use of human fetal brain tissue cultures which were tested for FGF-2 content.

4.2.1. Production of FGF-2 by human fetal neuronal cultures

We had previously shown that these cultures contained 75–80% of neurons, accounting astrocytes for the remaining 20–25%, whereas contamination by endothelial cells or fibroblasts was absent [86]. Our data demonstrated the presence and the synthesis of a cell-associated Mr 18,000 FGF-2-like molecule which could be identified as FGF-2 on the basis of its cross-reactivity with highly specific anti-FGF-2 antibodies, its molecular weight, its affinity for heparin and its capacity to induce upregulation of urokinase-type plasminogen activator (uPA) production in endothelial cells [92]. Moreover, a strong immunoreactivity for FGF-2 exclusively observed in neuronal cells and immunoprecipitation assays added the important information that neurons were the only source of FGF-2 in our cultures [92]. This observation was in keeping with above-mentioned immunohistochemical and immunocytochemical findings [40,66]. With time in culture, the appearance of a weak reactivity for FGF-2 also in astrocytes was observed. In addition, when long-term human fetal astrocytic cultures were analyzed, a FGF-2-like molecule was identified (Presta et al., unpublished observations), suggesting a different timing in FGF-2 production between human neurons and astrocytes. This interpretation was supported by the findings of Pettman et al. [66] reporting the absence of FGF-2 immunoreactivity in immature astrocytes and of Hatten et al. [34] and of Ferrara et al. [23] who, on the contrary, found FGF-2 synthesis from newborn rat and adult bovine astroglia. Thus, since progressive modifications observed in fetal brain cells during aging in culture might reflect modifications occurring *in vivo* during development, our findings suggest that both human neuronal and glial cells are capable of synthesizing FGF-2 *in vivo*, but presumably at different times during embryogenesis.

In conclusion, FGF-2 is synthesized in the human fetal brain since early stages of development, but neurons are only responsible for this early production. Later, astrocytes could also develop the same capability. Such a difference in timing of FGF-2 expression suggested the possibility of a different influence of the growth factor depending upon stage of brain maturation as well as on the reciprocal influence between neurons and astrocytes. They also demonstrated that FGF-2 produced by embryonic brain cells could actively induce uPA production in endothelial cells, *i.e.* showing biochemical and functional properties identi-

cal to FGF-2 produced by other tissues and suggesting a possible effect of neural cells on brain vessels.

However, other studies had demonstrated that a third component of CNS cell population, microglia, was capable of FGF-2 synthesis [4].

Thus, we inquired about the capacity of human fetal microglial cells to produce FGF-2.

4.2.2. Production of FGF-2 by human fetal microglia cells

Microglia, the macrophages of the CNS, are cells involved in several immunological functions of the brain [4,24,25,36,74,83,91,104]. However, evidence supports the fact that they also contribute to histogenesis during the development of the CNS. In particular, microglia are responsible for the phagocytic removal of dead cells occurring through the routine cell death associated with development [39,47] and for selective elimination of certain axonal projections [22,43]. Moreover, they have been shown to secrete neurotoxic factors [30,90], thus inducing neural degeneration and/or death [31]. On the other hand, microglia produce trophic factors [4,29,36,85,104] thus likely contributing to neuronal survival. Consequently, it was important to know the possible involvement of human fetal microglia in the production of FGF-2, *i.e.* whether or not they could play a role in the normal histogenesis of the human brain.

When extracts of human fetal microglia cell cultures were assayed, results were similar to those obtained with neuronal cultures [69] regardless of the age of the embryo from which cells were obtained (11–15 weeks of gestation) and *in vitro* passages (from 5 to 40), showing that also human microglial cells were capable of FGF-2 synthesis. These observations were consistent with previous reports showing that cultured rat brain microglia produced FGF-2 [4,85]. Our data indicated that expression of FGF-2 in microglia represents an early event in the human brain which is maintained through CNS maturation. Thus, they support the hypothesis that microglia could be involved in histogenesis. For instance, microglial FGF-2 could prevent the naturally occurring neuronal death and/or exert a mitogenic effect on neuroblasts and glioblasts. Indeed, human recombinant FGF-2 can induce glioblast proliferation in human fetal brain cultures (Gremo et al., unpublished observations). Moreover, since microglia become activated as a result of pathological stimulation [91], they can be implicated in induction of neurite sprouting and other events such as gliosis following brain injuries.

5. Expression and localisation of FGF receptors in human brain cells

During the past several years, major advances have been made in the characterization and cloning of genes that code for FGF cell surface protein receptors. Two classes of FGF receptors have been identified. (1) Heparan sulphate proteoglycans present both on cell surface and in the extracellular matrix: they represent low-affinity, high capacity binding sites and are involved in modulating the interaction of FGF-2 with its tyrosine-kinase receptors [106]. Also, they may play a role in mediating internalization of FGF-2 within the cell [76,78] as well as in modulating its neurotrophic effect [57]. (2) Tyrosine-kinase transmembrane receptors (FGF-Rs) represent high-affinity, low capacity binding sites [11] and are responsible for intracellular signal transduction and for mediating the biological response of the cell to FGF-2 [52,64]. To date, at least five FGF receptor genes have been cloned and sequenced [35,63,79] even though some uncertainty still exists about the fifth receptor, called Flg-2, which might or might not be encoded by a separate gene. FGFRs are single membrane-spanning molecules with three extracellular immunoglobulin (Ig)-like domains, an acidic box located between the first and the second Ig-like loop, a transmembrane domain and an intracellular catalytic domain. Several RNA alternative spliced variants that structurally differ in the number of Ig-like loops and/or in the absence of the intracellular domain (soluble forms) were also described for FGFR-1 and FGFR-2 [14]. Interestingly, variation in the second half of the third Ig-like loop accounts for difference in binding to ligands [42,61], so that FGFRs differ not only in structure, but also in their ligand-binding properties, signal transduction pathways and their pattern of expression (for a review see Miller and Rizzino [51]).

Several studies support the notion that FGF-Rs are present in the brain of several species.

FGFR immunohistochemical activity has been described in the rat [50] and mouse [13] adult brain as well as in developing animals such as chicken [37], mouse [71] and rat [102]. Human pituitary gland [87] shows immunoreactivity for FGFR1 as well as human adenomas. Asai et al. [6] found that mRNAs for FGFR-1, -2 and 3 were widely distributed in the rat brain neurons, whereas FGFR-4 expression was restricted to the medial habenular nucleus. Yamaguchi et al. [105] found both FGFR-1 and FGFR-2 expression in human astrocytomas, even if a shift from -2 to -1 was likely to parallel the progression from benign to malignant phenotype. On the other hand, FGFR-1 mRNA and its product, and FGFR-3 mRNA were detected in neuroepithelial cells derived from embryonic mouse brain [15,95]. More recently,

FGFR-3 mRNA was detected in rat cochlea organ of Corti [68]. This finding was in accord with Peters et al. [65] who found FGFR-3 mRNA expression in the developing rat auditory sensory epithelium. Another type of sensory neuron, the dorsal root ganglion, expresses FGFR-1 and FGFR-2 [60]. On the contrary, FGFR-4 mRNA was found in low quantity in the developing human cerebellum [63]. Furthermore, studies on the expression of FGFRs during embryonic development argue that the regulation of FGFRs, in addition to the regulation of their ligands, is a critical mechanism that ensures the specificity of cell-cell signaling events in development [37].

5.1. Expression of FGFRs

Expression of FGFR-1, FGFR-2, FGFR-3 and FGFR-4 was assayed in human fetal neuronal cultures as well as pure astrocytic and microglial cultures obtained from 10 to 18 week old human fetal brains [8]. Results showed differential expression of FGFR mRNAs in the three types of cultures regardless of the age of the embryos and time in culture. In particular, neurons expressed high levels of all four members of the FGFR family while astrocytes expressed mainly FGFR-4 and lower levels of FGFR-1. FGFR-2 and -3 mRNAs were only barely detectable in astrocytes. In contrast, when compared to neurons, microglia expressed mainly FGFR-3 and lower levels of all the other mRNAs. Immunocytochemical localisation of FGFR-1 gene product showed association with the plasma membrane of all neurons. Labeling could be observed at the level of cell bodies, some processes and the axon hillock. In astrocytic cultures, GFA-P⁺ astrocytes showed weak labeling on the cell surface, whereas in microglial cultures only amoeboid cell membranes were strongly stained; flat, adherent ramified cells showed a much weaker labeling [8].

These data provided the first demonstration that all four members of the FGFR family were expressed by human fetal neurons. The presence of mRNA for FGFR-1, FGFR-2, FGFR-3 and, in very low amounts, for FGFR-4 has been previously reported in human fetal brain extract [63], but the source of each specific RNA was not identified. Our data also provided evidence on the differential expression of the members of FGFR family by brain cells during development. Human fetal astrocyte cultures expressed FGFR-1 and FGFR-4, but not FGFR-2 and FGFR-3. However, a developmental shift in the pattern of FGFR gene expression in macroglia derived from older fetuses could not be excluded. Indeed, as stated above, a shift from FGFR-2 toward FGFR-1 has been observed during progression of human astrocytomas from a benign to a malignant phenotype [105]. This suggests that FGFR-1 expression is related to low

differentiation and/or immaturity in glia. The presence of FGFR-1 mRNA and protein in undifferentiated, bipotent (neuronal/astroglial) cells derived from embryonic mouse brain [95] is in keeping with this hypothesis.

This observation raised the question of the biological significance of the presence of more than one type of FGFR in the same culture. Since cultures were highly pure [46,92,93], the possibility of contamination from cells of different type, also bearing FGFRs, could be excluded. However, at the present, we do not know whether different FGFRs are co-expressed in the same cell or subpopulations of cells, each expressing a single type of FGFR, are present within the culture. The physiological significance of a cell-specific differential expression of FGFRs also needs to be elucidated. Analogous forms from different FGFR genes exhibit different ligand binding specificity for the various members of the FGF family [51]. On the other hand, Wang et al. [103] recently found that different FGFRs have different signaling and mitogenic potential in non-neuronal cells. Thus, the hypothesis that cells achieve selective responsiveness to various FGFs by different FGFRs can be advanced [42]. Indeed, FGF-1, which binds FGFR-4 with high affinity, is more potent than FGF-2, which does not bind FGFR-4, to induce NGF synthesis and secretion by astrocytes [107]. On the other hand, neurons, which bear all FGFRs, have been shown to be influenced by both FGF-1 and FGF-2 [84,94]. Immunolocalisation experiments showed that FGFR-1 mRNA is actively translated into the mature protein in all the cell types tested. This indicated that human fetal neurons, amoeboid microglia and GFAP⁺ astrocytes are all potential targets for FGFs. As discussed, FGFs behave as neurotrophic factors [2,84,94,99] and the capacity of protoplasmic astrocytes to develop a fibrillar morphology in response to FGF-2 has been reported [67]. In particular, localisation of FGFR-1 on neuron hillock membrane and in processes is consistent with the well-known effect of FGF-2 in stimulating neurite outgrowth [99]. The presence of FGF-1 on the surface of neurites both in long-term cultures and during regeneration has been demonstrated [56], raising the hypothesis of an “in situ” accumulation of the growth factor on the neurite surface via receptor concentration. On the other hand, the presence of both FGF-2 and FGF receptors in neurons and microglia suggests an autocrine role of the endogenous FGF-2, which might be more effective in activated, amoeboid microglia.

5.2. Expression of low-affinity binding sites

We found the presence of low-affinity FGF-2 binding sites in human fetal microglia cultures [69], with no significant changes in the number and/or affinity con-

stant with respect to time in culture or fetal age. Their characteristics were quite similar to those found in other tissues [42] and their capacity to interact with FGF-2 could be inhibited by free heparin, thus confirming their identity as heparan sulphate like receptors.

Interestingly, modifications of the composition of cell surface heparan sulphate proteoglycans can regulate the sensitivity of neuronal cells to FGFs. This may be of particular relevance when the spatial and temporal control of the activity of different FGFs must be tightly enforced. This possibility is exemplified by the shift in cell-surface heparan sulphate proteoglycan properties from an FGF-1 to an FGF-2-binding phenotype in murine neuronal cells during embryonic development [58].

6. Conclusions

In conclusion, our data demonstrate that FGF-2 is synthesized in human brain since early stages of pregnancy. Even if neurons and microglia appear to be the major sources of FGF-2 within the first half of pregnancy, astrocytes might also be capable of synthesis at later stages. Most interestingly, all the three cell types can be the target of this growth factor and related peptides. The question further arises about the biological significance of the co-expression of FGF and FGFRs in immature brain cells. By now, it can only be a matter of speculation. The regulation of FGFs expression as well as of their receptors during development presumably plays a critical role in cell-cell signaling among neurons, astrocytes and microglia in the immature human brain (see Fig. 1). On the other hand, the wide range of effects of FGFs as well as their interaction with other growth factors suggest that FGF-2 influence might be quite complex and depend on the type of target cell and timing of exposure. For example, it could be speculated that FGF-2 might affect CNS neurogenesis during development and in adulthood, as already demonstrated in other species. On the other hand, the capacity of neurons and microglia to produce both FGF-2 and its receptors suggests an autocrine role for FGF-2 in these cells, as already demonstrated in melanoma [12], endothelial [82] and

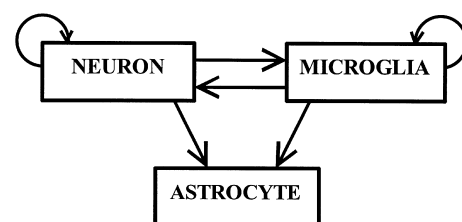


Fig. 1. FGFs/FGF-Rs related cross-talk between CNS cells.

glioma cells [59]. However, the biological significance of this autocrine mechanism remains to be elucidated.

Interestingly, we have observed the presence of low affinity, heparin-like binding sites on cell surface of microglial cells. These receptors have been implicated in accumulation of FGF-2 in the extracellular microenvironment [98]. Since stored FGF can be mobilized by the action of proteases and endoglycosidases [10,81], the hypothesis could be advanced that microglia represent a reservoir of trophic factors during normal development of the human brain. Moreover, heparan sulfates mediate the binding of FGF-2 to neural precursor cell surface receptor [15] and enhance its mitogenic effect [17]. On this basis, it seems possible to hypothesize a role for microglia in regulating neurogenesis. However, all these effects imply a secretion/release of FGF-2 by the cells. Even though FGF-2 lacks a classical signal for secretion [1], it is detectable in the extracellular matrix both in vivo and in vitro [98]. We had no evidence for FGF-2 release in our cultures. On the other hand, it has been suggested that FGF-2 may be released following cell injury or death [26] or through a specialized transport mechanism which leads to the binding of FGF-2 to the extracellular matrix [97]. Since neuronal death is a naturally occurring event in development, this could be the way for FGF-2 to be released by the cells and exert its action in the brain.

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