FGF8 Signaling In Brain Development: Ex Uno, Plures

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Abstract

A steady stream of studies solidifies fibroblast-growth-factor-8 (fgf8) as a crucial mediator of regionalization in the developing vertebrate brain. Secreted from secondary organizers within the forebrain, midbrain and hindbrain, FGF8 is implicated in patterning, proliferation, specification, migration, differentiation and axon guidance. How can a single molecule drive this diverse array of developmental processes in tight or overlapping spatial and temporal contexts? Signal diversification arises from isoform-specific deployment of FGF8 and its receptors which in turn activate several intracellular pathways each subject to negative feedback modulation. This review will summarize the multiple roles of FGF8 and begin to pull together two decades of work on these diversifying mechanisms. In place of a more conventional understanding of FGF8 as a broadly acting morphogen, this body of work motivates a new conceptualization of FGF8 as a nimble component of several isoform-specific ligand-receptor-pathway axes which guide different aspects of vertebrate brain development.

Keywords

Body patterning
Brain development
FGF8
Alternative splicing
CNS

Introduction

Fibroblast growth factors (FGFs) are a class of growth factor proteins which play an integral role in vertebrate brain development. Of the many members of this large family, FGF8 has been a focus for its role in directing regionalization of neuroepithelium and subsequent specification of neural territories. FGF8 exerts its developmental influence as the primary ligand secreted from a handful of "secondary organizers" within the central nervous system (CNS) during and after neural tube closure[1]. The role of the secondary organizers is to define each brain region – forebrain, midbrain and hindbrain - laying a groundwork for the more particular developmental programs of each region. The two best characterized organizers are the anterior neural ridge (ANR) along the most rostral aspect of the forebrain and the midbrain-hindbrain boundary (MHB) between the developing midbrain and hindbrain. There are additional foci of expression in the dorsal diencephalon (DD) and ventral diencephalon (hypothalamus) (Figure 1A).

It has become clear that FGF8 regulates a wide variety of developmental programs in neural tissue. Newly identified roles for FGF8 are strikingly diverse and include regulation of anterior-posterior patterning, cell proliferation, cell specification, cell survival, axon guidance and hormone production (Figure 1B). These results challenge a classical conception of FGF8, and FGFs more broadly, as rather blunt and broadly acting mitogenic and morphogenic molecules and begs the question of how the FGF8 ligand can guide a remarkable variety of cellular programs in small subsets of cells within the developing CNS. What is the molecular basis for its signaling diversity?

There are clear signs that FGF8 employs a variety of methods to generate its multiplicity of functions including alternative splicing of *fgf8* and three of the four vertebrate FGF receptors (*fgfr*s)[2-6]. In addition, FGFs are capable of activating several different intracellular signaling cascades which in turn can induce a growing list of feedback inhibitors[7-8]. This paper will be concerned with reviewing and organizing the many known roles FGF8 signaling plays in CNS development. I will then discuss what is known about the diversification methods just mentioned. Ultimately, I will suggest that this exciting body of literature requires a reformulation for how we view FGF8 signaling in development – not as the effect of a master ligand, but as the function of specific ligand-receptor-pathway axes. While a modest reformulation, this mode of thought can more effectively guide future experimentation.

Many FGF8 functions

FGF8, like most FGFs, is an ER-Golgi secreted protein with strong affinity to heparin and heparan-like glycosaminoglycans (HLGAGs) of the ECM. FGF8 was first identified as a secreted molecule from an androgen--dependent mouse mammary carcinoma cell line[9]. Vertebrate species generate multiple isoforms through alternative splicing of four 5' exons (exons 1A, 1B, 1C and 1D). Exons 2 and 3 are conserved across all isoforms. To date, there are four human,

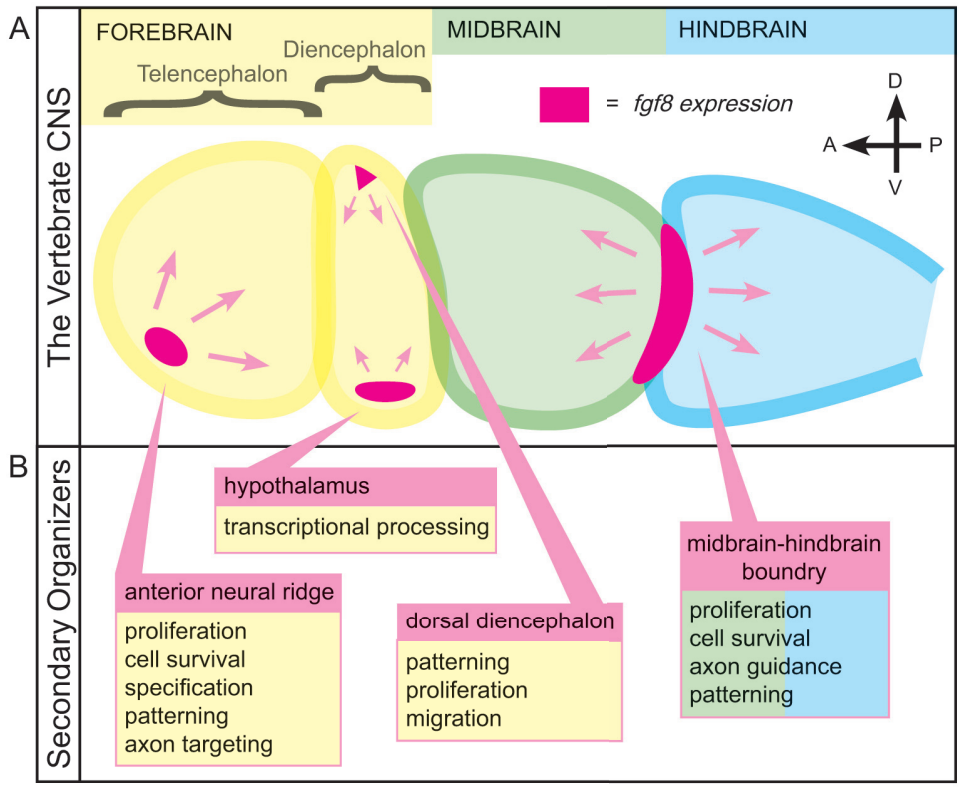

Figure 1. A schematic of the vertebrate CNS showing the three major divisions as well as the subdivisions of the forebrain. The four main secondary ogranizers expressing fgf8 are shown in pink. These expression domains arise during neurulation and persist for some time after that (A). Each organizer is labeled and the known functions of FGF8 for that organizer are listed below (B). Axes give anterior and posterior to the left and right respectively; dorsal and ventral are up and down respectively.

eight mouse, two chick and two fish isoforms[3,10-12] (Figure 2A). Once secreted, FGF8 binds to cellular membranes via a coordinated interaction between heparin/HLGAGs and one of four vertebrate FGFRs[13]. The binding interaction results in receptor dimerization and cross-phosphorylation of their intracellular domains initiating signal transduction[14].

The anterior neural ridge. In the mouse, the generation of an allelogenic mouse series has been incredibly illuminating to the study of FGF8[16].[a] Null alleles reveal the absolute requirement for FGF8 for proliferation as well as cell survival in the forebrain[7,17]. However, hypomorphs show normal proliferation and no ectopic cell death, thus no reduction in the size of the telencephalon[7]. Instead, there is a rostralization of expression of neocortical transcription factors suggesting a shift in the cortical identities of subregions[18]. The functional implications of the territorial shift within the telencephalon were explored further by *in utero* electroporation studies in mouse brain. Overexpression of *fgf8* in the ANR shifts neocortical subregional boundaries posteriorly. The addition of *fgf8* caudally leads to an ectopic S1 barrel field – an area of cortex which processes somatosensory information from the whiskers of the mouse[19]. Barrel fields normally receive thalamic inputs from the ventrobasal thalamic nuclei. In the case of *fgf8* overexpression, both endogenous and ectopic fields received thalamic innervation[20]. Together these results suggest proliferation and cell survival depend on basal levels of FGF8 while patterning events of the cortex are more dosage--dependent. How do the cells of the telencephalon as well as axon growth cones of the thalamic inputs interpret precise levels of FGF8 signal? The answer is still largely obscure.

FGF8's ability to drive specification is also evident in the ANR. The gonadotropin-releasing hormone (GnRH) neurons which drive sexual development derive from the ANR as a part of the olfactory placode. These endocrine cells then migrate to the hypothalamus. Without FGF8, GnRH precursors are not specified and no GnRH neurons populate the hypothalamus[15]. This crucial function of FGF8 underlies the pathophysiology of Kallman Syndrome – a combination of idiopathic hypogonadotropic hypogondism

a. This approach allows for the generation of an allelic series from a founder line. In this case, the founder contains an fgf8 knock-in with an intronic neomycin cassette which renders the allele hypomorphic. The construct also takes advantage of both cre and flp recombinase systems. Exons 2 and 3 are floxed and the neo cassette is frted allowing null mutations and wildtype rescues respectively to be deployed in a tissue specific manner by crossing the founder with desirable cre- or flp- transgeneic lines16.

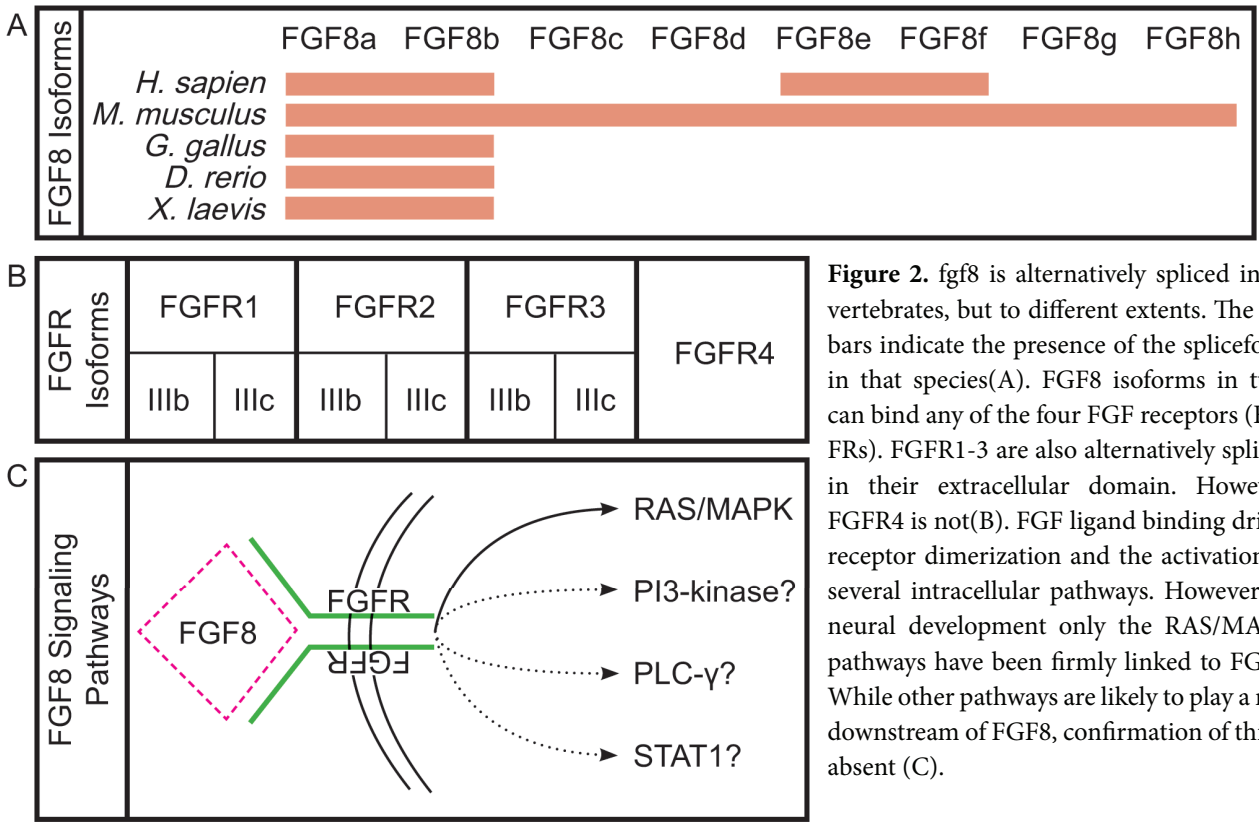

Figure 2. fgf8 is alternatively spliced in all vertebrates, but to different extents. The red bars indicate the presence of the spliceform in that species(A). FGF8 isoforms in turn can bind any of the four FGF receptors (FGFRs). FGFR1-3 are also alternatively spliced in their extracellular domain. However, FGFR4 is not(B). FGF ligand binding drives receptor dimerization and the activation of several intracellular pathways. However, in neural development only the RAS/MAPK pathways have been firmly linked to FGF8. While other pathways are likely to play a role downstream of FGF8, confirmation of this is absent (C).

and anosmia – the only known clinical outcome of human *fgf8* mutations.

The diencephalon. The multiple roles of FGF8 seen in the ANR are similar to the findings of Martinez-Ferre *et al* in the DD of mice[21]. Allelogenic analysis suggests FGF8 acts as "the master gene" for the DD. Formation of the dorsal structures (the pineal gland and habenular nuclei) relies on FGF8 in a dose-dependent manner. In this context, FGF8 regionalizes the DD by inhibiting posteriorizing Wnts, activating dorsalizing Wnts and stimulating proliferation. In contrast to the ANR, FGF8 does not contribute to cell survival, but does guide migration of some epithalamic neurons into the more ventral thalamus. So while some effects of FGF8 are the same as in the telencephalon, others are not.

To complicate the matter, the role of FGF8 in the DD of zebrafish is strikingly different. *fgf8* nulls retain intact DDs with a prominent pineal gland[22]. Martinez-Ferre *et al.* hypothesize that "master gene" *fgf8* expression in the DD began *de novo* in the vertebrate lineage allowing for the development of DD structures[21]. The presence of a pineal gland in zebrafish lacking FGF8 challenges this assertion. Instead, in the zebrafish, FGF8 is indispensable for the asymmetric migration of the parapineal, an accessory organ to the pineal[22]. This tension highlights the question of how FGF8 can direct proliferative and migratory cues differently across species or, in the case of mouse, simultaneously in tight spatial proximity.

More ventrally, oxytocin-producing cells derive from the ventricular zone before migrating to the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus[23]. Mice hypomorphic for FGF8 show a reduced number of cells in the PVN and SON positive for mature oxytocin yet a wildtype level of oxyphysin transcript, the oxytocin prohormone. In the oxytocin system, FGF8 takes on a role in regulating processing of the prohormone into the mature oxytocin molecule. This is a novel function for FGF8, but not unprecedented among other FGFs[24].

The midbrain-hindbrain boundary (MHB). The MHB is the most extensively characterized FGF8 signaling center. Also known as the isthmic organizer, it is crucial for the development of both the midbrain and hindbrain and sits along the border of these two regions (Figure 1A). As in the telencephalon, MHB-specific deletion of FGF8 leads quickly to increased cell death in both the midbrain and hindbrain

deleting the entire midbrain, the MHB and the cerebellum within the hindbrain. Interestingly, the cell death which produces the deletion occurs a full half day earlier in the midbrain than in the cerebellum[25].

Proliferation along the MHB is also FGF8 dependent. In exquisite work, high resolution microscopy reveals that FGF8 acts in a thin band along the basal aspect of the ventricular zone[26]. Here FGF8 signals through the basal processes of the neural progenitors to maintain proliferative divisions among the dividing progenitors. In the absence of FGF8, cells undergo neurogenic divisions and exit the cell cycle prematurely reducing the progenitor population.
Complementing axon targeting in the telencephalon, FGF8 directs axon outgrowth in the MHB. Midbrain dopaminergic neurons arise near the MHB and extend axons to innervate diencephalic and telencephalic targets. *In vitro* implantation of FGF8-soaked beads into whole-mouse embryo cultures formed ectopic MHBs and perturbed axon outgrowth of dopaminergic neurons leaving the endogenous MHB[27]. In this context, FGF8 was found to induce expression of the axon guidance cue *semaphorin3f* throughout the MHB. The semaphorin is then interpreted as a short-range chemorepellant by *neuropilin2* receptors on the dopaminergic axons. This result is of particular interest as the understanding of rostral-caudal axon guidance lags far behind that of dorsal-ventral guidance. It will be interesting to determine the mechanism by which FGF8 influences axons in the forebrain.

Generating signal diversity

We have seen that a single ligand, FGF8, expressed in a few secondary organizers in the developing CNS is able to execute a variety of cellular programs (Figure 1B). Work in the FGF8 field is uncovering how we go from the vague notion of a secondary organizer to a more nuanced understanding of FGF8 signaling in brain development.

Ligand splicing. fgf8 has multiple spliceforms across vertebrate species[3] (Figure 2A). These different isoforms have different transforming potentials on tumors, suggesting that if different isoforms are expressed in developing tissues they may have different effects[28]. Indeed, *in ovo* electroporation of *fgf8a* or *fgf8b* reveal that *fgf8a* transforms diencephalon into midbrain and expands midbrain, but only fgf8b can induce cerebellum in these tissues. Significantly, weaker overexpression of *fgf8b* yields an *fgf8a*-like phenotype suggesting that different effects of the isoforms are due to dosage as opposed to different molecular mechanisms[11]. However, more recent work challenges this conclusion. Overexpression of *fgf8a* via electroporation in chick does not phenocopy *fgf8b*'s transformative activity[14]. Complementing these overexpression studies, Guo *et al.* have built a genetic mouse model containing isoform-specific knockouts[29]. They find that only loss of FGF8b has any discernible effect on midbrain and cerebellar formation. Loss of FGF8a leads to no gross effect on these brain regions. This may warrant a closer look at FGF8a knockouts, but preliminarily reveals what overexpression experiments could not, fgf8a is dispensable for the bulk of MHB organizer activity.

This raises the question; can spliceforms ever play a simultaneous role? The possibility remains as crystal structure and biochemical analysis reveal a mechanism to explain the above described overexpression studies. A single phenylalanine at position 32 of the FGF8b isoform confers a significant difference in receptor binding[4]. Replacing the phenylalanine with an alanine converts the transformative ability of FGF8b to that of FGF8a, when electroporated into chick midbrains and murine midbrain explants. It will be very interesting to see if isoform-specific knockouts reveal simultaneous but unique requirements in other brain regions.

Receptor diversity. Another mechanism diversifying FGF8 signaling is the four FGF receptors and the alternative splicing of three of them[30] (Figure 2B). Indeed, FGFR1 alone mediates some FGF8 functions already discussed. FGFR1 is the FGF8 receptor for GnRH neuron specification and is crucial for some, but not all, MHB function in both fish and mouse[15, 31-32]. This indicates that various downstream effects of FGF8 at the MHB are mediated by different FGFRs. While investigating the differences between FGF8a and FGF8b, Olsen *et al.* also tested the association of FGF8 isoforms with the many receptor isoforms using *in vitro* surface plasmon resonance (SPR) to determine dissociation constants[4]. An alternative splicing event in the third immunoglobulin domain generates either a "b" or "c" isoform of FGFR1, 2 and 3. In all cases, the "c" isoform confers a greater affinity for FGF8b. This is due to a hydrophobic groove, exposed in the "c" isoform, that can more directly interact with the ligand phenylalanine at position 32 previously discussed[4]. These structural and *in vitro* results are not true in *in vivo* conditions, but nonetheless provide a possibility of alternative receptor splicing as a method to regulate ligand specificity. It remains to be seen if FGFR1 activity in the MHB is isoform-specific. It is exciting to imagine a suite of experiments combining isoform-specific knockouts of both ligand and receptors.

Signaling Pathways & negative feedback. Two additional layers of diversification in FGF8 signaling have become apparent recently, signaling cascade selectivity and negative-feedback modulators. FGFs have at least four separate signaling cascades they can activate; RAS/MAPK, PI3 kinase, PLC- and STAT1[7] (Figure 2C). There is an FGF8 isoform-specific relationship with some pathways. For example, FGF8b, but neither FGF8a nor low doses of FGF8b, activates the RAS/MAPK pathways along the MHB[33]. However, in ANR signaling, RAS/MAPK signaling persists in the absence of all FGF8 indicating that other signaling cascades are activated by FGF8 in the forebrain of zebrafish; which cascades is unclear[34].

Various cascades lead to activation of negative regulators of the FGF8 pathways; *sprouty*, *sef* and *mkp3* are principle among these[8]. Very recently, the negative regulators *Sprouty1* and *Sprouty2* have been shown to inhibit FGF8 rostralization in early cortical patterning, however, later only Sprouty2 shows a role by inhibiting the RAS/MAPK pathway in the telencephalic ventricular zone[35]. The mechanism and dependence on FGF8 of this switch are uncertain. Much more work must be done to understand which intracellular pathways are used to effect different FGF8 functions.

Conclusions and Future Directions.

We have seen studies connecting ligand isoforms to receptor isoforms, receptor isoforms to signaling pathways and signaling pathways to negative feedback regulators[4,33,35]. A clear direction forward is to begin to piece together these links to form a chain of developmental signaling. We must continue to find endogenous isoform-specific ligand-receptor pairs and begin to pare out which intracellular pathways as well as negative regulators are subsequently activated. In looking forward it may be helpful to begin to construct ligand-receptor-pathway axes as opposed to listing broad effects downstream of FGF8. The broad view does not reflect the evolutionary diversification of the components of FGF8 signaling so central to neurodevelopment. As work incrementally enriches our understanding of isoforms, cascades and feedback mechanisms, simpler axes may immerge from what otherwise appears to be a multifarious interaction network. More than just a tool to clear our heads, these hypothetical axes can guide experiments taking advantage of isoform-specific knockouts as well as signaling cascade and feedback regulator mutants.

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Acknowldegments. I would like to thank Dr. Josh Gamse and Josh Clanton for helpful conversation. This work was funded by NIH grant HD054534 to Dr. Gamse. The author was supported by the Vanderbilt Medical-Scientist Training Program (T32 GM07347 from the NIH).

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