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Fibroblast Growth Factor Signaling in the Developing Neuroendocrine Hypothalamus

Pei-San Tsai^{*}, Leah R. Brooks, Johanna R. Rochester, Scott I. Kavanaugh, and Wilson C. J. Chung

Department of Integrative Physiology and Center for Neuroscience, University of Colorado, Boulder, CO 80309-0354

Abstract

Fibroblast growth factor (FGF) signaling is pivotal to the formation of numerous central regions. Increasing evidence suggests FGF signaling also directs the development of the neuroendocrine hypothalamus, a collection of neuroendocrine neurons originating primarily within the nose and the ventricular zone of the diencephalon. This review outlines evidence for a role of FGF signaling in the prenatal and postnatal development of several hypothalamic neuroendocrine systems. The emphasis is placed on the nasally derived gonadotropin-releasing hormone neurons, which depend on neurotrophic cues from FGF signaling throughout the neurons' lifetime. Although less is known about neuroendocrine neurons derived from the diencephalon, recent studies suggest they also exhibit variable levels of dependence on FGF signaling. Overall, FGF signaling provides a broad spectrum of cues that ranges from genesis, cell survival/death, migration, morphological changes, to hormone synthesis in the neuroendocrine hypothalamus. Abnormal FGF signaling will deleteriously impact multiple hypothalamic neuroendocrine systems, resulting in the disruption of diverse physiological functions.

Keywords

FGF signaling; GnRH neurons; olfactory placode; ventricular zone; diencephalon; oxytocin; vasopressin; hypothalamic nuclei

1. Introduction

The family of fibroblast growth factors (FGFs) is among the most dominant of signaling molecules involved in the temporally and spatially specific orchestration of vertebrate brain development. The FGF family consists of 22 members of closely related peptides that are secreted through the canonical or non-canonical pathway, or act in an intracrine fashion within the nucleus or cytoplasm [70]. Numerous studies have shown that they are involved in a wide range of central nervous system (CNS) functions including neural induction, patterning, cell fate specification, neuronal survival, axon outgrowth, and cell migration [27]. The biological effects of the secreted FGFs are mediated primarily via four tyrosine kinase FGF receptors (FGFR1-4) that activate several signal transduction cascades [70]. The

^{*}Address Correspondence to: Dr. Pei-San Tsai, Department of Integrative Physiology, 114 Clare Small, University of Colorado, Boulder, CO 80309-0354, Phone: +1 303 735-1877, Fax: +1 303 492-0811, pei-san.tsai@colorado.edu.

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significance of secreted FGFs and their receptors in the CNS development is further underscored by the severe degeneration of brain regions in transgenic mice unable to produce them [57].

To date, an indispensable involvement of FGFR signaling has been well-documented in the formation of the telencephalon, dorsal diencephalon, midbrain-hindbrain junction, and cerebellum [70]. One brain region with relatively less information on an FGF involvement is the ventral diencephalon. The ventral diencephalon houses the neuroendocrine hypothalamus, which includes a collection of critical neurosecretory neurons. Collectively, these neurosecretory cells project to the median eminence (ME), neurohypophysis, and in some cases adenohypophysis for hormone release. Neurohormones secreted by the neuroendocrine hypothalamus regulate diverse physiological functions including reproduction, growth, metabolism, feeding, to stress. Developmental disruption of the neuroendocrine hypothalamus will likely impact physiology, homeostasis and, secondarily, cognitive function and emotional state of the organism.

At present, studies on factors that pattern and specify the hypothalamus are largely focused on secreted morphogens such as Sonic hedgehog (Shh) [47;67;80;111] and bone morphogenic proteins (BMP) [67;79;80], and diverse families of transcription factors [72;103;112;113]. We will not review the roles of Shh and BMP since this information can be found in several excellent reviews [11;49]. Some transcription factors have been linked to FGF signaling in the developing neuroendocrine hypothalamus and are discussed in Section 6. Relatively more evidence for an FGF involvement exists for a population of hypothalamic neuroendocrine neurons that secretes gonadotropin-releasing hormone (GnRH). An interesting aspect of GnRH neurons is that although they spend much of their embryonic and the entire postnatal life within the neuroendocrine hypothalamus, they are not derived within the ventral diencephalon. There is a wealth of information on what signaling factors regulate the development of GnRH neurons [132], but most of these factors participate in only a discrete developmental window of GnRH neurons' relatively long lifespan. In contrast, FGF signaling influences GnRH neurons at every stage of their prenatal and postnatal development. This raises the exciting possibility that FGF signaling may also be involved in the genesis and maintenance of other neuroendocrine neurons in the hypothalamus.

In this review, we will discuss the developmental roles of FGF signaling in the formation of the neuroendocrine hypothalamus. The majority of information is derived from the rodent literature, but other vertebrate models such as zebrafish, human and chick are included. In addition to discussing how FGF signaling influences multiple stages of GnRH neurons' life history, we also present new findings from our and other groups that strongly suggest an emerging role of FGF signaling in the development of several populations of neuroendocrine neurons that arise within the diencephalon. Lastly, since the neurohypophysis is derived from the ventral diencephalon and infundibulum, we also discuss the role of FGF signaling in mediating the formation of this endocrine structure. We hope to convey that FGF signaling deficiency impacts not only a specific population of neuroendocrine neurons, but also leads to an overall disorganization of the neuroendocrine hypothalamus. These developmental anomalies may be translated into diverse disease states in humans harboring loss-of-function mutations in FGF signaling genes.

2. Structure and function of FGF ligands and receptors

Currently, there are 22 known members of the FGF ligands found in humans and mice, with human *Fgf19* being the ortholog of mouse *Fgf15* [91]. Homology between FGFs is mainly based on the presence of a highly conserved 120 amino acid core region and the 30–60% overall amino acid identity across all FGFs [91] (Fig. 1). Analysis of the N-terminal amino

acid sequence reveals that the majority of FGF ligands (FGF3-8, 10, 17–19, 21 and 23) possess a signal peptide and are known to be secreted into the extracellular milieu [56;91]. FGF9, 16, and 20 lack an obvious N-terminal signal sequence but are also secreted [56;91]. Similarly, FGF1 and FGF2 lack a signal peptide but are thought to be released through pathways independent of the endoplasmic reticulum-Golgi route or by damaged cells. FGF22 has a putative N-terminal signaling peptide but remains attached to the cell membrane. The remaining four FGFs (FGF11–14) lack a signal peptide and are localized to the cell nucleus or cytoplasm [56;91]. These FGFs have been grouped into seven subfamilies based on amino acid sequence homology and expression pattern [56;91].

FGF signaling is mediated through membrane-bound tyrosine kinase receptors that contain an extracellular ligand-binding domain, a transmembrane domain and a split intracellular tyrosine kinase domain (Fig. 1). Although FGFRs are classical membrane receptors, in some instances FGFRs have been shown to translocate to or reside in the cell nucleus [33;96;109;140]. Currently, 4 membrane FGF receptors (FGFR1-4) have been identified in humans and mice. The extracellular domain of all full-length FGFRs contains three immunoglobulin (Ig)-like domains, within which the FGF and heparin sulfate proteoglycan (HSPG)-binding sites are localized (Fig.1). In the presence of HSPG, stable binding of FGFs to FGFRs causes FGFR dimerization and phosphorylation of cytoplasmic tyrosine residues in the tyrosine kinase domains. This in turn initiates a myriad of intracellular signal transduction pathways including the phosphorylation of SH2 domain of phospholipase C γ and the hydrolysis of phosphatidylinositol biphosphate. FGFRs also activate the extracellular signal-regulated kinase (ERK) pathway, specifically ERK1 and ERK2 [8]. In addition, FGF signaling can activate the phosphoinositide 3-kinase (PI3K)/Akt pathway, which in turn protects against apoptotic cell death by inhibiting caspase-dependent cell death [12;127].

Our understanding of FGF signaling is in part complicated by the presence of multiple splice variants for FGFR1-3. Specifically, the second half of the third Ig-like domain can be alternatively spliced to give rise to IIIa, IIIb or IIIc variants that are expressed in a tissue-specific manner. The IIIa variant is secreted into extracellular environment, whereas the IIIb and IIIc variants are preferentially produced in epithelial cells and mesenchymal cells, respectively [143]. Binding affinity studies based on mitogenic activity show that each FGFR binds to a variety of FGF ligands, indicating functional redundancy. For example, one study shows that the FGF8 subfamily (consisting of FGF8, 17, and 18) acts preferentially through $FGFR3c < FGFR4c < FGFR2c < FGFR1c < FGFR3b$ [143]. On the other hand, a more recent study using surface plasmon resonance analysis demonstrates that binding affinities of the FGF8b splice variant are similar for FGFR1, 2 and 3, suggesting that FGF8 signaling is mediated equally by all three FGFRs [83]. The complexity and redundancy in FGF ligands and receptors demonstrate the inherent robustness of FGF signaling during embryonic development.

3. Expression of FGF signaling genes during the development of neuroendocrine hypothalamus

3.1. Introduction to neuroendocrine hypothalamus development

The neuroendocrine hypothalamus consists of clusters of magnocellular and parvocellular neuroendocrine neurons originating primarily within the proliferative ventricular zone (VZ) of the diencephalon [3;68]. In the developing rat, the majority of the neuroendocrine neurons become post-mitotic (birthdated) between embryonic day (E) 12–17 [4;69]. After a brief lag, they begin to produce their respective hallmark neurohormones, migrate from the germinal VZ to their final destinations in the ventral diencephalon to form discrete nuclei, and

elaborate projections to form synaptic connections or allow hormone release [72]. Unlike cortical development, the rat hypothalamic nuclei are formed in a lateral to medial fashion, with neuroendocrine neurons that are birthdated early populating the more lateral zones, and the neurons birthdated later populating the more medial zones [55]. During this time, multiple hypothalamic cell types emerge within the heterogeneous VZ either simultaneously or in a staggered fashion. The general pattern of hypothalamic development is thus characterized by the simultaneous birth, migration, and axon outgrowth of a large number of neuroendocrine neuronal cell types [3;68;69]. Generated in this fashion are the neuroendocrine neurons of two magnocellular nuclei secreting arginine vasopressin (AVP) and oxytocin (OT), and five major parvicellular nuclei secreting corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GRH), dopamine (DA), and somatostatin (SST). Additional parvicellular neuroendocrine neurons with less defined hypophysiotropic roles appear to emerge in a similar fashion [6].

One notable exception to the aforementioned pattern of hypothalamic neuroendocrine neuron development is a population of neurons that secretes GnRH. GnRH neurons differ from other hypothalamic neuroendocrine neurons in several respects. In most vertebrates including the rodents, GnRH neurons do not cluster into identifiable hypothalamic nuclei [52]. Rather, they are found scattered across a rostral-caudal continuum that ranges from the olfactory bulbs to the posterior hypothalamus, with the majority of GnRH neurons concentrated in the preoptic area (POA) [52]. Further, GnRH neurons are born within a peripheral tissue called the olfactory placode, instead of the VZ [99;135], before they undergo an *en masse* migration through the telencephalon to reach their final destination. Despite the inherent difference in the final anatomical location and birthplace, the developmental transitions into fate specification, migration and axon outgrowth undertaken by GnRH neurons [19] are similar to neuroendocrine neurons of hypothalamic origin.

Based on anatomical features and demarcation by POU-III homeobox expression, it is generally agreed that the POA, which houses the majority of GnRH neurons, is diencephalic in origin [5]. Therefore, three zones in the developing embryonic heads appear particularly relevant to the formation of a functional neuroendocrine hypothalamus: (1) the olfactory placode, which gives rise to GnRH neurons, (2) the telencephalon, which houses the migratory trajectory of GnRH neurons, and (3) the ventral diencephalon, which gives rise to and supports the maturation of most hypothalamic neuroendocrine neurons. Abundant evidence points towards the presence of FGF signaling in these three zones during the developmentally relevant periods. The expression of multiple FGF signaling components may occur sequentially or simultaneously, suggesting a complex and sometimes redundant regulatory system.

3.2. Expression of Fgfrs and secreted Fgfs in regions relevant to GnRH neuronal development

In the murine olfactory placode, at least 5 FGF ligands are produced at E10.5 when GnRH neurons are undergoing fate specification [7;41;59]. Transcripts for *Fgf3*, 8, 9, 10, and 17 have all been found in the olfactory placode at different levels and with slightly varied spatial distributions [7;63]. *Fgf8* and *Fgf9* are expressed both in the medial and lateral rims of the olfactory placode [7;59], whereas *Fgf10* is largely limited to the lateral, and *Fgf3* and *Fgf17* to the medial, rims of the placode [7] (Fig. 2). Of these, *Fgf8* has the highest and broadest expression pattern, extending from the medial nasal process to the lateral rim bordering the respiratory epithelium (except the center of the invaginating placode), and encompassing the entire dorsal to ventral circumference of the placodal epithelium (Fig. 2). In fact, only *Fgf8* expression extends medially to the ventral-most subdomain of the placode, where GnRH neurons emerge [7] (Fig. 2). Considering FGFs are bound tightly by HSPG in the extracellular matrix and do not diffuse far after they are secreted, FGF production sites

and target sites are likely within close proximity of each other [110]. Overall, despite the simultaneous expression of 5 FGF ligands in the olfactory placode, it appears that FGF8 is the one positioned for the most direct control of GnRH neuronal fate specification. This notion is supported by the developmental studies showing GnRH neurons cannot emerge under reduced FGF8 signaling (see Section 5.2).

Three FGF receptors, FGFR1-3, have been detected in the developing olfactory placode at the time of GnRH neuron fate specification [7;22;41]. High levels of FGFR1-2 proteins and transcripts are seen in both the placodal epithelium and the adjacent nasal mesenchyme of the developing mouse and chick noses [7;35;41]. In contrast, the level of FGFR3 protein in the olfactory region is very low and restricted to the placodal epithelium [41]. The inability of some to detect the *Fgfr3* transcript in either mouse or chick olfactory placode [7;35] suggests a low presence and unlikely involvement of FGFR3 in the fate specification process of GnRH neurons. Embryonic GnRH neurons, after fate specification, produce FGFR1 and FGFR3 proteins [41] and can respond directly to diverse FGF ligands in the surrounding milieu as they develop.

From E12.5 to E14.5 of mouse development, during the period of massive GnRH neuronal migration into the forebrain, several studies suggest abundant FGF ligands are present along this migratory route. First, robust expression of *Fgf15* is observed in the septal region of the telencephalon and differentiating olfactory neuroepithelium [37;43;44]. Second, *Fgf1* expression is found in the olfactory nerve ensheathing cells that surround the olfactory axons [61], suggesting a striking proximity between the FGF1 source and GnRH neurons that migrate along the olfactory nerves. Lastly, the rostral midline of the presumptive olfactory bulbs, a site of initial contact between GnRH neurons and the forebrain, expresses high levels of *Fgf8*, *Fgf17* and *Fgf18* [22;23]. Once GnRH neurons cease migration, their axons extend towards the ME to terminate upon the fenestrated capillaries, which together, with adjacent fibers and glial cells, provide a rich source of FGF2 [46;100]. Overall, two general conclusions can be made with regard to the source and the type of FGFs critical for GnRH neuronal development. First, multiple forms of FGFs are readily available to GnRH neurons in the nasal and forebrain regions between E10.5 and E15.5, a period encompassing three major stages of GnRH neuronal development. Second, given the transient and dynamic nature of FGF expression during development, it is unlikely that a single FGF is responsible for all stages of GnRH neuronal development.

3.3. Expression of *Fgfrs* and secreted *Fgfs* in the developing ventral diencephalon

There is less information on the presence of FGF signaling components in the developing ventral diencephalon compared to the more anterior head regions mentioned above. Most of *Fgf* expression studies in the ventral diencephalon are conducted on postnatal animals [10;30;34;36;92;128;133] and suggest a wide presence of FGF1, 2, and FGFR1-3 in this region after birth. Only a few studies examine the availability of FGF signaling to the developing neuroendocrine neurons originating within the ventral diencephalon (see below).

On E13.5 and E17.5 of mouse development, *Fgfr1*, *Fgfr2* and *Fgfr3* are expressed along the linings of the third ventricle [9], where heterogeneous cell populations including neuroendocrine neurons are born. Levels of *Fgfr1* and *Fgfr3* expression in the ventral VZ surpass that of *Fgfr2* [9]. Outside the ventricular linings, *Fgfr1* and *Fgfr2* are also expressed in the presumptive hypothalamus on E12.5-13.5 and later in the hypothalamic parenchyma during rodent development [9;84;128], but at levels lower than those observed along the VZ. The expression of *Fgfr3* does not occur in ventral diencephalon until four days later [9]. There is also a robust expression of *Fgf8* along the ventral midline in the anlage of infundibular hypothalamus and neurohypophysis, as well as optic stalks, nerves, and chiasmatic area that persists until E14.5 [22]. It is important to note that the majority of the

expression studies reveal general patterns of FGF signaling expression but lacks temporal and spatial resolution for specific hypothalamic structures. However, three conclusions can be drawn. First, the strong presence of *Fgfr1* and *Fgfr3* along the ventral aspect of the VZ suggests these two receptors may be critical for the genesis of diverse neuroendocrine neurons. Second, the strong presence of *Fgf8* in the infundibular hypothalamus could reflect a role of FGF8 in the evagination of neurohypophysis and possibly the axon targeting of magnocellular neuroendocrine neurons. Lastly, *Fgf8* presence in regions surrounding the optic chiasm implicates it in the development of adjacent hypothalamic nuclei such as the suprachiasmatic nucleus (SCN) and supraoptic nucleus (SON).

3.4. Expression of intracellular Fgfs in the developing neuroendocrine regions

The discussion on FGF expression thus far focuses on secreted FGF ligands and their receptors. Worth mentioning is the expression pattern of *Fgf11-14*, which are localized to the cell nucleus or cytoplasm and do not bind classical FGFRs [82;107;129]. The actions of these intracellular FGFs are not well understood but are thought to involve interactions with functional proteins such as MAP kinase-scaffold protein [97] and voltage-gated sodium channels [45;64;65] (Fig. 1). *Fgf12* is robustly expressed in the murine olfactory placode at E12.5 [48], a period coinciding with the migration of many newly specified GnRH neurons out of the olfactory placode, raising the possibility of FGF12 involvement in GnRH neuronal development immediately post-fate specification. Further, *Fgf11-14* are all highly expressed in the developing ventral diencephalon during the period consistent with hypothalamic formation [48;107;129]. The expression of *Fgf11-14* in the developing ventral diencephalon appears stronger and more widely distributed than most secreted FGFs during this period. These findings suggest FGF signaling independent of FGFR activation may also contribute to development of the neuroendocrine hypothalamus.

4. *In vitro* effects of FGF signaling on hypothalamic neuroendocrine cells

Global and conditional deletions and hypomorphs of FGF signaling genes have revealed their involvement in the development of many CNS structures [57]. However, the functional and structural integrity of the neuroendocrine hypothalamus in these transgenic animals has rarely been characterized. Studies using cultured neuroendocrine cells have been useful in filling some gaps in our knowledge by uncovering the *in vitro* biological effects of FGF signaling on hypothalamic neuroendocrine cells and providing an impetus for pursuing these studies in the whole animal. Even in rare cases when animal studies have already shown a dependence of a developing neuroendocrine system for a specific FGF signaling component, these *in vitro* studies remain useful in revealing the molecular mechanisms and cell-specific effects of FGF signaling.

Early evidence that FGF signaling is neurotrophic in hypothalamic neuroendocrine neurons comes from studies using GT1 GnRH neuronal cell lines. Consistent with endogenous embryonic and postnatal GnRH neurons [41], GT1 cells express both *Fgfr1* and *Fgfr3*, but the expression level of *Fgfr1* is much higher than *Fgfr3* [124]. Treatment of GT1 cells with FGF2 (a prototypic FGF) activates ERKs 1 and 2, enhances cell survival in serum-free condition and promotes neurite outgrowth, but has no effect on *Gnrh* transcript levels [78;124;126]. FGF2 treatment increases transcript levels of prohormone convertase 2 (*PC2*), an endopeptidase required for GnRH prohormone processing, and enhances the preferential secretion of C-terminal-extended GnRH [126;131]. These early studies suggest FGF signaling acts not only on developmental events such as cell survival and process extension, but also hormone synthesis and release, which could impact postnatal neuroendocrine functions. Of interest is the recent finding that FGF8b, a FGF8 splice variant, differentially autoregulates the expression of its cognate receptors, *Fgfr1* and *Fgfr3*, in GT1 cells, raising

the possibility that FGF ligands can influence the function of their own receptors in a modular fashion to enhance the diversity of cellular responses [76].

Several neurotrophic effects of FGF2 observed in GT1 cells are recapitulated in primary GnRH neuronal and hypothalamic cell cultures. As in GT1 cells, FGF2 promotes neurite outgrowth in primary cultures of dissociated embryonic rat and mouse GnRH neurons [41;126] and activates a number of signaling pathways, including ERKs, CREB1 and cFos in the latter [42]. An interesting effect of FGF2 on primary embryonic rat hypothalamic cultures is its ability to stimulate insulin-like growth factor-1 (IGF-1) release and IGF-1 receptor [88]. Although the cell types affected by FGF2 in these mixed cultures are unclear, the results raise the possibility that FGF signaling may exert neurotrophic effects on hypothalamic neurons through the autocrine/paracrine upregulation of IGF-1 and by synergizing with IGF-1 signaling. Studies on GT1 cells corroborate these data by demonstrating that a suboptimal dose of FGF2 potentially enhances the responsiveness of these cells to the neurotrophic effect of subsequent IGF-1 exposures [39]. This sensitizing effect could be due to the upregulation of IGF-1 receptor by FGF2 [88].

Compared to GnRH neuronal cell lines or primary cultures of GnRH neurons, virtually no *in vitro* studies have examined other hypothalamic neuroendocrine cell types for their responsiveness to FGF signaling. That said, FGF signaling elicits diverse effects on neuroendocrine cell lines of non-hypothalamic origins, such as PC12 cells of adrenal chromaffin origin [20], 44-2C cells of thyroid C-cell origin [141;142], and BON cells of pancreatic origin [118]. These effects range from stimulation of cell survival, neurite outgrowth, to hormone production. Although these studies are not directly applicable to hypothalamic neuroendocrine neurons, they demonstrate the ability of FGF signaling to affect neurons with neurosecretory phenotypes. Of interest is the ability of FGF2 to induce prominent neuroendocrine phenotype in cultured progenitor cells isolated from the embryonic rat hypothalamus [95]. This observation, in combination with the strong expression of *Fgfr1* and *Fgfr3* in the embryonic VZ (see Section 3.3), supports a role of FGF signaling in directing the neuroendocrine fate of stem cells in the germinal VZ.

5. FGF signaling in the development of GnRH system

5.1. Introduction to GnRH system development

In all vertebrates except jawless fishes, hypophysiotropic GnRH neurons originate in the olfactory placode of the nose [117]. Because the developmental pattern and timing of GnRH neurons vary among major taxa, we will focus primarily on the mouse, a model in which most of the developmental studies on GnRH neurons have been conducted.

The olfactory placode in mice develops from the anterior neural ridge [37] as a ventrolateral ectodermal thickening, which on E10 begins to invaginate to form the olfactory pit, which invaginates again to form a secondary ventro-medial recess after 12 hours. In mice, the majority of GnRH neurons (defined by the production of GnRH) are first seen in this recess at about E11.0-E11.5 [99;137]. After fate specification, GnRH neurons begin their migration (~E11.5) to the forebrain. This is a process guided by the peripherin-positive tracts of the vomeronasal, terminal, and olfactory nerves [134]. By E14.5, the majority of GnRH neurons have crossed the nasal-forebrain junction, and are following a subset of peripherin-positive fibers to reach the POA and hypothalamus [136]. At this time, a portion of GnRH neurons begins to extend their axons to the ME [66;73;104]. By E16, a diffuse network of GnRH neurons can be observed in a bilateral continuum from the base of the olfactory bulbs to the ME, and the formation of the GnRH neuronal system is largely complete. There is now ample evidence to suggest FGF signaling orchestrates all phases of GnRH neuronal development, including fate specification, migration, and axon targeting.

5.2. FGF signaling in the fate specification of GnRH neurons

The vast majority (~80%) of mouse GnRH neurons undergo their last division in the olfactory placode between E9.5 and E10.5 [135]. Like hypothalamic neuroendocrine neurons, these post-mitotic GnRH progenitor cells do not express detectable levels of the hallmark neuropeptide immediately. A lag of 24–48 hours is required for GnRH peptide to become detectable at E11.5 [99;135]. The absence of measurable GnRH peptide prior to E11.5 is a major hurdle for studying the molecular processes that regulate GnRH neuronal fate specification, because at present the only marker for GnRH neurons is GnRH itself. Therefore, post-mitotic olfactory placode progenitor cells that are destined to become GnRH neurons cannot be identified during a portion of fate specification before they express measurable GnRH levels. Notwithstanding this major caveat, there is increasing evidence that the activation of FGF signaling is important for the fate specification of the mouse GnRH neuronal system.

As mentioned earlier in Section 3.2, the murine olfactory placode produces FGFR1-3 on E10.5, a period consistent with the specification of GnRH neuronal fate [41]. The earliest data indicating that FGF signaling drives GnRH neuronal fate specification are derived from *in vitro* nasal explants isolated from E10.5 mouse embryos. In this experimental paradigm, the number of GnRH neurons in the nasal explants increases after two or three days *in vitro* [41]. This increase is potently inhibited by the addition of a FGFR antagonist SU5402 [41], suggesting the activation of FGFRs is required for GnRH neuron fate specification.

After fate specification, GnRH neurons continue to produce FGFR1 and FGFR3 [41]. Based on this, it is hypothesized that the activation of either FGFR1 or FGFR3 is needed for mouse GnRH neuronal fate specification. This hypothesis is tested using the global *Fgfr1* hypomorphic or *Fgfr3* knockout (KO) mice, which confer a major advantage. Unlike tissue-specific KO animals that require the GnRH promoter to be active before disruption can occur, the global disruption of genes guarantees FGFR1 or FGFR3 deficiency at the time of GnRH neuronal fate specification, prior to GnRH promoter activation. In newborn *Fgfr1* hypomorphs, the number of GnRH neurons in homozygous *Fgfr1* hypomorphs is about 88% lower than their wildtype littermates [18]. On the other hand, the number of GnRH neurons is unaffected in newborn *Fgfr3* KO mice [18]. These whole animal studies corroborate the nasal explant studies and support the idea that the activation of FGFR function is a key pathway required for the emergence of GnRH neurons [41]. Further, comparison of GnRH neuronal system between *Fgfr1* hypomorphic and *Fgfr3* KO mice suggests the developmental activation of FGFR1, not FGFR3, is required for the normal number of GnRH neurons in the newborn mouse head. It is interesting to note that although GnRH neuron numbers are normal in newborn *Fgfr3* KO mice, they become reduced in adults, suggesting the role of FGFR3 in GnRH neurons is postnatal rather than prenatal [17].

Of the 22 identified FGF ligands, evidence suggests FGF8 is the most likely FGF candidate involved in the early development of GnRH neurons. First, *Fgf8* mRNA levels is expressed the ectodermal region ventral/lateral of the commissural plate on E9.5, which gives rise to the olfactory placode [22;59], and later in the ventro-medial olfactory placode, where GnRH emerge [7]. Second, a partial loss of FGF8 function results in the absence of the olfactory bulbs and vomeronasal organs (VNO) [18;74]. The absence of the VNO has a special relevance given that the VNO also originates in the olfactory placode [135]. Therefore, it is likely that the disruption of FGF8 signaling could severely impair the genesis of the GnRH neuronal system. This hypothesis is tested by examining the GnRH neuronal system in newborn mice globally hypomorphic for *Fgf8*, which in the homozygous state shows a ~54% reduction of *Fgf8* mRNA [74]. Surprisingly, the GnRH neuronal system in newborn homozygous *Fgf8* hypomorphs is not just reduced, but eliminated in its entirety [18;32]. An equally striking observation is that newborn heterozygous *Fgf8* hypomorphs have

approximately 50% fewer GnRH neurons compared to wildtype littermates [18;32]. The loss of GnRH neurons is attributed to defective fate specification, since GnRH neurons are already missing or reduced in the E11.5 olfactory placode and continue to be missing or reduced thereafter [18]. Overall, these results lead to the conclusion that the fate specification of the mouse GnRH neuronal system is extremely dependent on the presence of FGF8 signaling acting through FGFR1.

The exact mechanism by which FGF8 supports specification of GnRH neuronal fate is unclear, but evidence suggests FGF8 deficiency leads to an aberrantly formed olfactory placode, which may secondarily impact GnRH progenitor cells emerging from this region. As mentioned earlier, GnRH neurons emerge from a secondary recess in the ventro-medial olfactory placode. This recess is virtually absent in the E11.5 olfactory placode of homozygous *Fgf8* hypomorphs [18]. The dysgenesis of this secondary recess is likely one of the causes underlying the failure of GnRH neurons to emerge,

The molecular factors downstream of FGF8/FGFR1 signaling responsible for the specification of GnRH neuronal fate remain unclear. Recent studies show that the epithelium of the olfactory placode comprises specific transcription factor domains [125]. For instance, during mid-gestational development (~E11.5) the medial olfactory placode expresses primarily *Sox2* and *Ascl1*, which are associated with the rapid terminal division of neurogenic olfactory precursor cells. In contrast, *Meis1* is primarily detected in slowly dividing and self-renewing non-neurogenic cells (i.e., negative for the neuronal marker TuJ1) in the lateral olfactory placode [125]. FGF8 signaling is involved in establishing these specific transcriptional zones. Indeed, both *Sox2* and *Ascl1* expression is diminished in the medial olfactory placode in homozygous *Fgf8* hypomorphic embryos. These changes coincide with a marked reduction of medial olfactory precursor TuJ1 expressing cells from which GnRH neurons are thought to emerge [125]. In contrast, the *Meis1* expression domain is expanded ectopically to the medial olfactory placode and diminished in its normal expression domain, the lateral olfactory placode [125]. Currently, it is unknown how these transcriptional domains in the olfactory placode contribute to the emergence of GnRH neurons. A very relevant observation is that mice lacking *Sox2*, *Ascl1* or *Meis1* expression still possess GnRH neurons, although a substantial reduction in GnRH neurons is observed in *Sox2*-deficient mice [125]. Regardless, this partial reduction of GnRH neurons still differs from the complete elimination of GnRH neurons in *Fgf8* hypomorphic mice [18]. It is surmised that FGF8 signaling induces the spatially specific expression of complex downstream effector genes that come together to drive the formation of the olfactory placode, therefore GnRH neurons. There appears to be no single downstream substitute for FGF8 that can direct this process.

At the cellular level, a major consequence of reduced FGF8 function in the olfactory placode is reduced cell survival. In *Fgf8* conditional KO and hypomorphic mice, abnormally high levels of apoptosis in the olfactory epithelium are seen between E10.5-E12.5 [18;59]. Moreover, the incidence of apoptosis is much higher in the medial olfactory placode, where GnRH neurons emerge, than the lateral olfactory placode (W.C. Chung, unpublished data). This may lead to the elimination of the precursor cells that give rise to GnRH neurons. Stimulation of cell proliferation is another important FGF8 function. Recent studies showed that FGF8 promotes neurogenesis in the medial olfactory placode by enhancing the expression of *Sox2*, while at the same time suppressing *Meis1* expression [125]. Based on our current understanding, FGF8 may act to both prevent the death and stimulate the genesis of the GnRH precursor cells in the medial olfactory placode, thereby promoting the emergence of GnRH neurons.

Overall, there is now abundant evidence to suggest FGF8 is a seminal ligand without which GnRH neurons cannot emerge. FGF8 acts through FGFR1 during early development to induce region-specific expression of transcription factors in the olfactory placode. Through these downstream genes, FGF8 promotes the morphogenesis of the olfactory placode, and the survival and/or proliferation of precursors that eventually give rise to GnRH neurons. Of all stages of GnRH neuronal development, fate specification is perhaps the stage most dependent on FGF signaling.

5.3. FGF signaling in the migration of GnRH neurons

Numerous studies showed that GnRH neurons, once out of the olfactory placode, migrate along the pathways established by the axons of the olfactory, vomeronasal, and terminal nerves to reach their destinations in the forebrain [134]. Cues from the forebrain are not required for neuronal migration, since GnRH neurons in the nasal explant retain the normal migratory characteristics in the absence of the brain tissue [38]. Thus, cues for GnRH neuronal migration appear to reside within the actual guiding axonal pathways. Since the discovery that *Fgfr1* mutations cause an autosomal dominant form of Kallmann syndrome (KS) [26], there is a great deal of interest in understanding how FGF signaling regulates the migration of GnRH neurons.

KS is a disease characterized by hypogonadotropic hypogonadism (HH) coupled with anosmia (inability to smell) and is presumably caused by developmental anomalies in the olfactory region that simultaneously disrupt the formation of olfactory and GnRH systems. The interest in how FGF signaling regulates the migration of GnRH neurons stems from the notion that HH in KS is caused by the inability of developing GnRH neurons to migrate from the nose to the forebrain [15]. This notion is traced back to a study demonstrating the failure of olfacto-terminal nerves to contact the olfactory bulbs in a 19-week old fetus with X-linked KS, a form of KS caused by mutations in *anosmin-1*, an extracellular adhesive protein [98]. As a result, these nerves are arrested in a tangle within the meninges. GnRH neurons that normally migrate along the scaffold established by these nerves are also trapped within the meninges. To date, this is the only study examining the developmental anomalies in the KS head. From this single snapshot, it is surmised that *anosmin-1* deficiency leads to abnormal olfacto-terminal nerve extension and contact with the olfactory bulbs.

Consequently, GnRH neurons lose their guidance cues and fail to enter the brain. While this assumption may very well be true for KS patients with *anosmin-1* mutations, it has not been proven in KS patients with *Fgfr1* mutations.

At present, data from transgenic mice do not fully support the view that defects in FGF signaling seriously impair GnRH neuronal migration. One of these transgenic mice, called dnFGFR mice, expresses a mutant dominant-negative FGFR (dnFGFR) in their GnRH neurons to disrupt the function of FGFR1-3 in a tissue-specific manner [123]. Adult dnFGFR mice show normal patterns of GnRH neuronal distribution in the forebrain, indicating disruption of FGF signaling specifically in GnRH neurons does not grossly impact migration [123]. That said, a subtle problem with migration is noted in dnFGFR mice [122]. A small fraction of GnRH neurons (~10%) in dnFGFR mice encounters difficulty penetrating into the forebrain and accumulates in the inter-hemispheric area (IHA) outside the brain [122]. Nevertheless, the delayed GnRH neurons in dnFGFR mice do eventually “catch up”, enter the brain, and assume their normal distribution. In fact, there is adequate arrival of GnRH neurons in the forebrain of dnFGFR mice to support reproduction [123]. These data suggest that the disruption of FGFR function directly at the level of GnRH neurons plays only a minor role in their migration.

One might argue that perhaps the role of FGF signaling is to indirectly support GnRH neuronal migration by allowing guiding axons from the nose to grow and make proper

contact with the olfactory bulbs. It is interesting to note that peripherin-positive fibers of the olfacto-vomer nasal nerves in homozygous *Fgf8* hypomorphs have in fact failed to enter the anterior telencephalon [18]. Previous studies show that the elongation of olfactory fibers requires the presence of olfactory ensheathing cells, which are glia that originate from the olfactory epithelium [115;116]. These olfactory ensheathing cells express high levels of *Fgfr1* and are permissive for the elongation and growth of olfactory axons [16;54]. It is plausible that *Fgf8* hypomorphs suffer an attenuation of FGF8-dependent FGFR1 activation of olfactory ensheathing cells, which impairs the growth and targeting of olfacto-vomer nasal nerves. Consequently, this may indirectly impact the migration of GnRH neurons. However, it is important to reiterate that GnRH neurons are already missing in *Fgf8* hypomorphs prior to migration, thus the cause of GnRH system defect in these mice is failure to emerge, not failure to migrate. Further, in heterozygous *Fgf8* hypomorphs with 50% of GnRH neurons still remaining, all GnRH neurons are normal in their distribution. There is no evidence of abnormal accumulation of GnRH neurons along the migratory pathway prior to entering the brain [18]. A similar observation is made for homozygous *Fgfr1* hypomorphs [18].

Overall, it remains possible that FGF signaling affects GnRH neuronal migration indirectly by supporting the growth and targeting of guiding axons of the olfactory, vomeronasal and terminal nerves. However, data from mice suggest that FGF signaling plays a much more significant role in GnRH neuronal fate specification than migration. When FGF signaling is deficient, failure of GnRH neurons to emerge will be the first to occur, thereby masking any disruption of the subsequent migratory process.

5.4. FGF signaling in axon targeting of GnRH neurons

During axon targeting, GnRH fibers extend ventrally in a directional manner to terminate upon the fenestrated capillaries within the external zone of the ME. In order for GnRH axons to target the ME properly, two events must occur: 1) the extension of the axons, and 2) the directional growth of axons to the target. There is some evidence suggesting FGF signaling regulates both of these events in mice.

Regarding the extension of axons, *in vitro* data showed that this process is modulated by FGF signaling [41;42]. The addition of FGF2, a prototypic FGF, significantly stimulates neurite outgrowth in cultured E15.5 GnRH neurons [41]. GnRH neurons isolated from dnFGFR mouse embryos exhibit attenuated neurite extension in culture [42], suggesting FGF signaling is an endogenous cue that maintains GnRH axon growth. Regarding the directional axonal growth to the proper target, Silverman *et al.* [105] show that GnRH neurons grafted into the third ventricle target axons to the ME, and this directional axonal growth is due to the presence of unknown diffusible chemoattractive signal(s) from the target [40;94]. Our laboratory has demonstrated, in cultured embryonic GnRH neurons, the directional outgrowth of neurites toward the co-cultured ME explant is significantly reduced in GnRH neurons from dnFGFR mice [42]. Thus, FGFs are very likely one of the endogenous chemoattractive signals emanating from the ME.

Overall, *in vitro* results have shown that attenuated FGF signaling disrupts both the extension and directional growth of GnRH neurites. Do these *in vitro* alterations translate into GnRH system defects in the whole animal? Evidence suggests they do, but these *in vivo* defects are somewhat less striking. GnRH neurons in dnFGFR mice show a significant but small reduction (15%) in the uptake of peripherally injected fluorogold, a retrograde tracer, indicating compromised contact between GnRH axons and circumventricular organs such as the ME [42]. That GnRH axon targeting is only modestly disrupted in dnFGFR mice argues for the presence of other compensatory mechanisms guiding GnRH axons *in vivo*. Since GnRH axon targeting is a highly axophilic event in which GnRH axons travel with other

growing axons [94], these guiding axons can drive the extension and directional growth of GnRH axons via physical or other chemical means not involving FGFs.

Overall, GnRH axon targeting in the whole animal does not appear to be a process that critically requires FGF signaling. Despite significant defects observed *in vitro*, adequate GnRH fibers have targeted to the ME of dnFGFR mice to support reproduction [123]. However, it is important to note that when compounded with other defects such as those of fate specification and migration, targeting defects could further reduce the efficacy of a GnRH system that is already suboptimal due to developmental disruption at earlier stages.

5.5. FGF signaling in postnatal development of GnRH neurons

The term “development” can also be applied to postnatal periods marked by elevated functional and structural changes, such as puberty and senescence. These two postnatal developmental periods are characterized by the acceleration and decline of GnRH neuronal function, respectively, and define the reproductive lifespan available for offspring propagation. At present, there is evidence for a role of FGF signaling in the onset and termination of the mouse GnRH system function, thereby determining the duration of the reproductive lifespan.

The number of GnRH neurons in the lifetime of a rodent appears relatively constant after reaching adulthood. Wu *et al.* reports that there are approximately 1100 GnRH neurons in neonatal mice, and this number declines to approximately 800 in adult mice [138]. From then on, the number of GnRH neurons remains either unchanged [51] or declines only very modestly by 18% in aging mice [75]. In this respect, GnRH neurons do not appear to undergo substantial dedifferentiation or cell death as they age. Instead, the aging GnRH system is more likely to undergo functional declines in GnRH synthesis and secretion [139].

Contrary to the steady presence of GnRH neurons in normal adult rodents, a marked age-related decline in GnRH neurons is observed in the dnFGFR [77] (Fig. 3). At 30 days of age, dnFGFR mice exhibit only a 30% reduction in the number of GnRH neurons [123]. By 1-2 years of age, dnFGFR mice lose approximately 70% of their GnRH neurons [77] (Fig. 3). This marked age-related GnRH neuronal loss is likely responsible for the premature reproductive senescence observed in females [123]. To our knowledge, dramatic age-related loss of GnRH neuron number has only been reported in one other disease state. R6/2 mice, a Huntington's disease model, also undergo a 33% to 90% reduction in GnRH neuron number between 5–9 weeks of age [85]. These mice also suffer an early loss of male fertility by 8 weeks of age [85], supporting the notion that the failure to maintain postnatal GnRH neurons results in the premature termination of reproductive function.

Besides early reproductive senescence, dnFGFR mice also exhibit delayed puberty. The establishment of a mature GnRH neuronal network at puberty is complex and requires the expression of proper neurotransmitter receptors, ion channels, and the receipt of various forms of stimulatory inputs [81]. The delay in pubertal onset of dnFGFR mice may be attributable to the 30% reduction in GnRH neurons observed at puberty [123], but this is unlikely since a mere 12% of GnRH neurons have been shown to be sufficient for normal pubertal onset in female mice [50]. A more likely explanation is that GnRH neurons in dnFGFR mice undergo delayed or reduced functional maturation. The precise mechanism underlying this dysfunction is unclear, but the deficit must be initiated within GnRH neurons, since the disrupting dnFGFR is targeted only to GnRH neurons [123]. The most parsimonious explanation is that FGF signaling deficiency interferes with the ability of GnRH neurons to undergo functional or morphological changes necessary for pubertal onset. During the pubertal transition, mouse GnRH neurons transform from having a complex dendritic tree bearing a few spines to having a simple dendritic structure bearing a

large number of spines [21]. Consequently, the remodeled GnRH neurons in adults possess high dendritic spine densities, indicating the acquisition of abundant excitatory synaptic inputs that could drive puberty [14]. We have shown that FGF signaling alters *in vitro* neurite branching of postnatal, but not embryonic, GnRH neurons [41], implicating FGF signaling in the postnatal remodeling of GnRH neuronal morphology. We are currently pursuing direct evidence for this hypothesis.

Overall, emerging data suggest FGF signaling is critical for the maintenance of the postnatal GnRH system, and therefore a normal reproductive lifespan. This notion is supported by the clinical studies showing patients harboring either *Fgfr1* or *Fgf8* mutations manifest delayed puberty as a less serious form of reproductive phenotype [32;86;87;90;119]. However, early reproductive senescence in patients harboring FGF signaling gene mutations has not yet been studied. Such studies should lend greater clinical relevance to the role of FGF signaling in the postnatal development of GnRH neurons. In a broader sense, GnRH neurons are not unlike other neuronal systems such as motor neurons that continue to rely heavily upon neurotrophic support for postnatal maintenance [53;101].

6. FGF signaling in the presumptive hypothalamus

Numerous transcription factors are expressed during the development of the neuroendocrine hypothalamus and mediate pathways required for the formation of this structure [72;103;112;113]. There is increasing evidence that some of these critical transcription factors act upstream or downstream of FGF signaling in the developing neuroendocrine hypothalamus. Below, we will discuss how FGF signaling interacts with these transcription factors, and how FGF deficiencies impact the development of a number of hypothalamic neuroendocrine nuclei in the whole animal.

The morphogenesis of the CNS involves the precise coordination of numerous signaling molecules and genes. During gastrulation, the ectoderm is induced to become neural tissue. Subsequently, the neural tube becomes segmented along the anterior/posterior axis to form the forebrain, midbrain and hindbrain. As neurogenesis proceeds, the temporal and spatial presence of transcription factors and signaling molecules like FGFs drives the specialization of these regions. The hypothalamus originates from the most rostral portion of the neural tube [89]. Studies in zebrafish and rodents have shown that not only are *Fgfs* expressed in the hypothalamus, they also interact with important transcription factors. Some prominent examples of transcription factors important for hypothalamic and/or pituitary development that also interact with FGF signaling are: *Orthopedia (Otp)*, *Lhx2* of the LIM homeodomain transcription factor family, the homeobox *Nkx2.1*, and the HMG box family member *Sox3* [25;62;114;144;145].

6.1. FGF signaling in developing hypothalamic neuroendocrine neurons and related structures

A highly conserved transcription factor important for the developing neuroendocrine hypothalamus is the homeobox gene *Otp* [24;29]. *Otp* is first expressed in the mouse forebrain at E9.5 and becomes regionally restricted to the presumptive neuroendocrine hypothalamus by E12.5 [1;106]. These regions develop into the paraventricular nucleus (PVN), SON, anterior periventricular and arcuate nuclei [1;106]. In *Otp* KO mice, OT-, AVP-, TRH-, CRH-, and SST- producing neurons are eliminated; however, the AVP neurons in the SCN are unaffected. This suggests that the AVP neurons in the SCN are derived from a different region or are modulated by a different transcriptional network [2;28;29;130]. *Otp* is also involved in the terminal differentiation of OT-, AVP-, TRH-, CRH- and ST- neurons and the lateral migration of these post-mitotic neuroendocrine neurons from the VZ [1;58]. In the zebrafish, FGF8 inhibits *Otp1* expression in the rostral

POA (anterior alar diencephalon) from which the neuroendocrine system arises [25], making *Otp* one of the downstream effectors of FGF8 in this region.

Another transcription factor family important for the development of the diencephalon is the LIM homeodomain transcription factors. These factors play a role in CNS patterning, cell fate determination and axon pathfinding [108]. Most studies on a LIM factor, *Lhx2*, have focused on its role in the development of the telencephalon and the eye. Recently, *Lhx2* has been shown to be essential for diencephalon development in both the zebrafish and mice [102;144]. In zebrafish, *Lhx2* expression is found in the embryonic POA, a region that is diencephalic in origin [102]. An interesting connection is that FGF signaling is required for the onset and maintenance of *Lhx2* expression in zebrafish [102]. Application of FGFR antagonist SU5402 to zebrafish embryos reduces or eliminates *Lhx2* expression in the diencephalon [102]. These zebrafish studies establish a clear link between FGF signaling and the development of the diencephalon. *Lhx2* has a similar expression pattern in mice, and the targeted deletion of *Lhx2* gene leads to the malformation of the ventral hypothalamus, including the absence of the ME [144]. However, the control of *Lhx2* expression by FGF signaling in regions relevant to the developing neuroendocrine hypothalamus has not yet been established in mammals.

The most direct evidence implicating a role of FGF signaling in the development of the mammalian neuroendocrine hypothalamus comes from a study on *Fgf8* hypomorphic mice [13]. In comparison to wild type littermates, the magnocellular OT neurons in both the PVN and SON are significantly reduced in newborn homozygous *Fgf8* hypomorphic mice [13] (Fig. 4A-D). This reduction in the OT neurons is accompanied by a significant reduction in oxyphysin prohormone-positive neurons in the SON but not the PVN of *Fgf8* hypomorphs [13]. These observations are indicative of (1) a greater involvement of FGF8 signaling in the development of SON compared to PVN, and (2) a reduced ability of PVN of *Fgf8* hypomorphs to process oxyphysin prohormone into OT. Overall, these data suggest a global FGF8 deficiency leads to an overall reduction of mature OT that may have resulted from either a reduction in prohormone production or processing. These data are consistent with studies on GT1 cells showing an alteration of prohormone processing and *PC2* expression by FGF signaling (Section 4). Since proper hormone synthesis is a hallmark of mature neuroendocrine neurons, this study also suggests that FGF8 signaling may contribute to the phenotypic maturation of a neuroendocrine system that originates within the diencephalon.

In addition to a reduction in magnocellular OT/oxyphysin-positive neurons, the magnocellular AVP neurons in the PVN and the parvicellular AVP neurons in the SCN are also reduced in *Fgf8* hypomorphs (Fig. 4E-H). Of note, the reduction appears more severe in the SCN, with virtually no AVP neurons remaining in the SCN of *Fgf8* hypomorphs (Fig. 4E-H). In conjunction with data on OT neurons, a general pattern is emerging to suggest that the negative impact of *Fgf8* deficiency is more pronounced in the ventral SCN and SON compared to the more dorsal PVN [13] (Fig. 4). SCN neurons arise from the preoptic recess of the VZ, which is likely regulated by a different transcriptional network than the AVP neurons in the PVN and SON [29]. In contrast, magnocellular neurons in PVN and SON appear to share the same VZ origin [4]. These observations suggest that the reduction of *Fgf8* has a greater impact on the ventral hypothalamic structures (SON, SCN) than the more dorsal PVN regardless of their origin within the VZ. In rats, neurons begin to form in the SON and SCN around E13-15 and E14-17, respectively. However, they do not form discrete structures until around E17 which coincides with the arrival (SON) or decussation (SCN) of the optic tracts. These optic tracts, along with the chiasmatic area and presumptive infundibulum, appear to be the primary sources of FGF8 in the developing hypothalamus, since no other structures in the presumptive hypothalamus express detectable *Fgf8* [22]. Thus, high concentrations of FGF8 emanating from these primary sources may locally

modulate the development of the more ventral and nearby SON and SCN. In contrast, the more dorsal PVN is likely exposed to much lower levels of FGF8 diffused from these primary sources. In this regard, the development of the PVN may not be as dependent on FGF8 signaling as SON and SCN.

In general, although data are still relatively sparse, there is now evidence to implicate FGF signaling in the development of neuroendocrine neurons that originate within the diencephalon. Studies from *Fgf8* hypomorphic mice also suggest that not all hypothalamic neuroendocrine neurons are equally sensitive to FGF8 signaling deficiency, which is not surprising considering the heterogeneity of these neurons. Worth noting is that although both SON and SCN appear to require high levels of FGF8 signaling for development, only SON is dependent on downstream *Otp1* [2;28;29;130]. This suggests the downstream transcription networks activated by FGF8 are likely diverse and region-specific.

6.2. FGF signaling in the developing neurohypophysis

The hypothalamic infundibular region is anatomically and functionally associated with the hypothalamic-hypophyseal system [120]. At E10.5 of murine development, an evagination of the neuroectoderm in the ventral diencephalon gives rise to the infundibulum, from which the neurohypophysis is derived [60;144;146]. By E13.5, the neurohypophysis is evident [145]. It is well documented that FGF signaling in this region is necessary for the induction of the Rathke's pouch, an adjacent structure that becomes the adenohypophysis [31;121], but less is known about how FGF signaling affects the formation of neurohypophysis.

As previously noted, FGF signaling is important for the expression and maintenance of the LIM homeobox gene, *Lhx2*, in the zebrafish diencephalon, but infundibular development has not been examined in zebrafish lacking functional *Lhx2* [102]. In contrast, *Lhx2* mutant mice lack proper infundibular evagination, leading to defective infundibular and neurohypophysis formation. Interestingly, the *Fgf8* expression domain that demarcates the boundary of infundibular evagination is rostrally expanded in these mutants, implicating *Fgf8* as a downstream mediator of *Lhx2* action.

Similarly, the homeobox transcription factor *Nkx2.1* (T/ebp Ttf1, Titf1) influences the *Fgf8* ventral diencephalon-expression domain [114]. Murine *Nkx2.1* is expressed from E11-13 in the ventral diencephalon [62]. In addition to extensive abnormalities in ventro-medial hypothalamic structures, homozygous *Nkx2.1* KO mice lack both adenohypophysis and neurohypophysis [62]. The ventral *Fgf8* expression domain that demarcates the boundary of neurohypophysis evagination is completely eliminated in *Nkx2.1* KO mice as well [114].

Another transcription factor important for murine neurohypophyseal development is *Sox3* [93]. *Sox3* is expressed in the ventral diencephalon and is required for morphogenesis and differentiation of the infundibulum [93]. In embryonic *Sox3*-null mutant mice, the infundibulum fails to evaginate fully and the ventral diencephalon is flattened and stretched [93]. Similar to *Lhx2* mutants, the domain of *Fgf8* expression in *Sox3* mutant mice is extended rostrally along the neuroectoderm. The expanded rostral *Fgf8* border may be a cause for the irregular infundibular evagination, as there is a negative correlation between the size of the ventral *Fgf8* expansion and the extent of the infundibular evagination [93].

Overall, the aforementioned studies demonstrate that a reduction in *Lhx2*, *Nkx2.1* and *Sox3* redefines *Fgf8* expression domains and deleteriously impacts the evagination of the neurohypophysis. This places FGF8 signaling downstream of all three major transcription factors known to orchestrate the formation of the neurohypophysis. Additional studies that manipulate *Fgf8* expression in the presence or absence of these transcription factors are needed to establish FGF8 signaling as a direct mediator of neurohypophyseal formation.

7. Conclusions and Perspectives

Evidence is emerging that FGF signaling drives the development of multiple hypothalamic neuroendocrine systems and associated structures (Table 1). Animals harboring FGF signaling deficiencies manifest significant hypothalamic neuroendocrine defects (Table 1). For the GnRH system, FGF signaling provides sustained neurotrophic support throughout its entire developmental history, from the neurons' birth to death. How FGF signaling regulates neuroendocrine systems originating within the diencephalon is less studied, but we now have unambiguous evidence showing that the formation of multiple hypothalamic neuroendocrine nuclei requires FGF8. This evidence lays the groundwork and provides a strong impetus for further investigation.

FGF signaling, consisting of 22 ligands, 4 receptors, and their splice variants, appears inherently robust in its built-in redundancy. It is curious that this redundancy often fails to guarantee functional compensation by other members when one is reduced or missing, such as in the case of reduced FGF8. This is a strong testament to the extraordinary temporal and spatial precision under which many FGF ligands and receptors operate. In this regard, one must caution against the general assumption that FGF ligands can easily cross-rescue one another simply because they appear to be expressed within a broadly defined developmental window or anatomical zone. In other words, FGF signaling components may be much more specific than they once appeared and deserve to be studied with greater temporal and spatial resolution. We would also like to emphasize that the intracellular FGF subfamily (FGF11-14), which is expressed extremely robustly in the presumptive hypothalamus (Section 3.4) and grossly understudied, deserves special attention for its involvement in the development of the neuroendocrine hypothalamus.

Another phenomenon worth noting is the presence of humans harboring loss-of-function mutations in *Fgfr1* and *Fgf8* [26;32]. These patients have been diagnosed with and treated mostly for HH due to insufficient GnRH, but other defects in the neuroendocrine system such as diabetes insipidus and absent infundibular stalk are beginning to surface [71]. This lends great clinical relevance to studying the actions of FGF signaling in the neuroendocrine hypothalamus in general, not just the GnRH system. With the impetus and knowledge from animal and clinical studies, we are now on the cusp of discovering a significant and possibly indispensable involvement of FGF signaling in the formation of the neuroendocrine hypothalamus.

Research Highlights

- FGF signaling is most critical during the fate specification of GnRH neurons.
- FGF signaling maintains the postnatal GnRH system.
- Deficiency in FGF8 signaling deleteriously impacts the neuroendocrine hypothalamus.

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Fig. 1.

Schematic representation of FGF signaling in vertebrates. In mice and humans, there are 22 FGF ligands, including secreted FGFs (FGF-s; blue spheres) and intracellular FGFs (FGF-i; brown spheres). Homology between FGFs is based on the presence of a highly conserved ~120 amino acid (aa) core region and the 30–60% overall amino acid identity across all FGFs (see inset). Secreted FGFs bind to one of the four classical FGFRs. The extracellular region of the full-length FGFR is characterized by the presence of three immunoglobulin (Ig)-like domains within which the FGF- and heparin sulfate proteoglycan (HSPG)-binding sites are localized. Stable binding of secreted FGFs in the presence of HSPG causes dimerization of FGFRs and phosphorylation of cytoplasmic tyrosine residues in the tyrosine kinase (Tk) domains, which in turn initiates a myriad of intracellular signal transduction pathways. Intracellular FGFs do not bind to the classical FGFR. Instead, they alter cellular physiology by interacting with proteins such as MAP kinase-scaffold protein (MAPK-SP) and voltage-gated sodium channel.

Fig. 2. Spatial distribution of 5 *Fgf* transcripts in the murine olfactory placode at E10.5, a period consistent with GnRH neuron fate specification. The image represents the coronal view from the anterior tip of the developing nasal prominence. Each *Fgf* transcript is represented by a different color. CP, commissural plate; NP, nasal pit. Asterisks denote the location of the presumptive ventro-medial secondary recess, where GnRH neurons emerge. The drawing is based on a previously published study [7].

Fig. 3. Representative photomicrographs of neurons immunoreactive for mature GnRH in 100-day-old (A, B) and 550-day-old (C, D) wildtype (WT; A, C), and homozygous dnFGFR mice (B, D) at the plane of the organum vasculosum of lamina terminalis, the area of greatest GnRH neuron concentration. Note the significant reduction in the number of GnRH neurons (brown staining) in 550-day old dnFGFR mice compared to the younger dnFGFR mice, and absence of such an age-dependent reduction in the WT mice. Scale bar = 100 μ m.

Fig. 4.

Representative photomicrographs of neurons immunoreactive for mature OT (A-D) and AVP (E-H) in a newborn wildtype (WT; A, C, E, G) and homozygous *Fgf8* hypomorph (HOMO; B, D, F, H) in the PVN, SON and SCN. Note the visual reduction of OT and AVP neurons in WT compared to *Fgf8* hypomorph in the PVN (A, B, E, F), SON (C, D) and SCN (G, H). The reduction is more severe in the SON and SCN of the *Fgf8* hypomorph, with essentially no immunoreactive neurons remaining. Brown color represents specific immunostaining. Green color represents methyl green nuclear counterstain. The boundary of SON is delineated by the dotted line. Inset in C is a low-magnification photomicrograph of the same section, with the SON delineated by the dotted line. Arrow points to the third ventricle. Scale bar = 100 μ m.

Table 1

Summary of the effects of disrupted FGF signaling on hypothalamic neuroendocrine systems.

Type of Disruption	FGF Signaling Disrupted	Affected Neuron Type and Location	Phenotype
Global	FGF8 and FGFR1	GnRH neurons in olfactory placode	Elimination/reduction of GnRH neurons [17;18;32]
Global	FGF8	Olfactory receptor neurons in nose	Olfactory axons fail to enter anterior telencephalon; potential disruption of GnRH neuronal migration [18]
GnRH neuron- specific	FGFR1 and/or 3	GnRH neurons	Small reduction in targeting efficiency of GnRH axons [42]
GnRH neuron- specific	FGFR1 and/or 3	GnRH neurons	Small difficulty in penetrating the forebrain [122]
GnRH neuron- specific	FGFR1 and/or 3	GnRH neurons	Significant postnatal loss of GnRH neurons [77] (Fig. 3)
Global	FGF8	OT and AVP neurons in PVN	Reduced OT & AVP neurons [13] (Fig. 4)
Global	FGF8	OT neurons in SON	Reduced OT neurons [13] (Fig. 4)
Global	FGF8	AVP neurons in SCN	Reduced AVP neurons [13] (Fig. 4)