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Gonadotropin Releasing Hormone (GnRH) Neuron Migration: Initiation, Maintenance and Cessation as Critical Steps to Ensure Normal Reproductive Function

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Abstract

GnRH neurons follow a carefully orchestrated journey from their birth in the olfactory placode area. Initially, they migrate along with the vomeronasal nerve into the brain at the cribriform plate, then progress caudally to sites within the hypothalamus where they halt and send projections to the median eminence to activate pituitary gonadotropes. Many factors controlling this precise journey have been elucidated by the silencing or over expression of candidate genes in mouse models. Importantly, a number of these factors may not only play a role in normal physiology of the hypothalamic-pituitary-gonadal axis but also be mis-expressed to cause human disorders of GnRH deficiency, presenting as a failure to undergo normal pubertal development. This review outlines the current cadre of candidates thought to modulate GnRH neuronal migration. The further elucidation and characterization of these factors that impact GnRH neuron development may shed new light on human reproductive disorders and provide potential targets to develop new pro-fertility or contraceptive agents.

1.0 Introduction

Gonadotropin-releasing hormone (GnRH) is a hypothalamic releasing hormone that is synthesized in a small heterogenous neuronal population and secreted in an episodic fashion to control pituitary gonadotropin production and normal reproductive function(1-5). The GnRH neurons are unique among hypothalamic releasing factor neurons in that they originate in the olfactory placode/vomeronasal organ and migrate along vomeronasal nerves to the cribriform plate, the boundary between the peripheral olfactory system and the forebrain(1-5). Migrating GnRH neurons follow a branch of the vomeronasal nerve caudally into the hypothalamus(4). They then extend processes to the median eminence to release GnRH into the capillaries of the median eminence to modulate pituitary gonadotropin (luteinizing hormone, LH and follicle stimulating hormone, FSH) production and secretion. Loss of these neurons or misdirecting along the route results in failure of sexual maturation in mice and man. The underlying mechanisms that regulate the migration of GnRH neurons are incompletely understood. Recent studies have identified multiple factors extrinsic and

intrinsic to GnRH neurons that control specific steps along the migratory route (discussed in (6-9)). This review updates the list of candidate proteins and pathways involved in GnRH neuronal migration and highlights those shown to be involved in human disorders of GnRH deficiency.

2.0 Stages of GnRH neuron migration

The process of GnRH neuronal migration can be divided into four specific stages which is useful to define candidate proteins involved in the movement and appropriate targeting of the neuronal population(6,10). Analysis of this specific neuronal migratory process is limited by the fact that we have no marker of all GnRH neurons other than GnRH itself. No transcription factor or gene product has been demonstrated to mark all GnRH neurons early in development. The GnRH-GFP mice have green fluorescent protein expression driven by the GnRH promoter, but it is relatively weakly expressed early in development and while tracking GFP signal is useful, it does not reflect the total immunoreactive GnRH population(11). Since many external inputs inhibit GnRH gene and protein expression, the exact number of neurons and their location during development is not absolute but reflective of detected GnRH mRNA or protein. Future research is needed to identify markers of GnRH neurons. With this caveat, four steps of neuronal migration can be distinguished: 1)After their birth in the area of the olfactory placode in the mouse at approximately E10.5, GnRH neurons migrate together with vomeronasal axons across the nasal mesenchyme into the forebrain(1,5). This initial step requires both the movement of GnRH neurons and the specific factors that promote the adherence of the neurons to axons of the vomeronasal nerve. 2) At the level of the cribriform plate, specific cues are needed as the vomeronasal nerve (VNN) divides with a branch that guides GnRH neurons turning caudally into the forebrain. 3) After crossing the cribriform plate and movement towards the hypothalamus, specific factors promote the extension of long processes through the basal forebrain toward the median eminence. 4). Lastly, the neurons detach from their axonal guides and disperse further in the hypothalamus and stop migrating. The exact steps may differ slightly across species, but most agree that a similar set of mechanistic steps are critical to target GnRH neurons to their appropriate destination in the hypothalamus so that the connection to the pituitary and ultimately reproductive competence can be achieved.

3.0 Factors involved in the initiation of GnRH Neuron Migration

Investigators have examined potential factors that may modulate GnRH neuron migration (reviewed in (6-10). Many studies have focused on factors that modify the cell migration along the vomeronasal nerve (see Table 1).

3.1. Adhesion molecules tie the GnRH neurons to olfactory fibers

Molecules that are unique to the axon guides or to GnRH neurons would hypothetically restrict the neurons to follow these specific sets of axons into the forebrain. Identification of such factors has been has been complicated by the fact that GnRH neurons are phenotypically heterogeneous (12). The reason for this heterogeneity is not known, but it may be important for the regulation of the rate of migration, the ability to modulate migration at different times and locations along the route, and possibly to ensure that no single genetic mutation would prevent all or even most of these neurons from migrating to their intended destinations. The specific adhesion molecules elucidated to date include:

3.1.1. PSA-NCAM—GnRH neurons prefer to migrate along axons expressing a polysialic acid form of neural cell adhesion molecule (PSA-NCAM)(13,14). Removal of the PSA by enzymatic digestion blocked GnRH neuron migration. Evaluation of NCAM and NCAM-180 null mice, however, revealed no significant disruption of GnRH neuron

migration. These discrepant results may have been due to the redundancy of the NCAM subtype system, with GnRH neurons able to migrate instead with NCAM-140 positive axons (13,14). No human NCAM mutations have been reported to date in patients with hypogonadotropic hypogonadism.

3.1.2. Glycoconjugates including cell surface glycoproteins—Bless and coworkers(15) showed that a cell surface glycoconjugate, carrying a lactosamine moiety, influences GnRH neuron migration. A glycosyltransferase ß1,3-N-acetylglucosaminyltransferase-1 (ß3GnT1) helps synthesize lactosamine addition on a protein for a subset of GnRH neurons. Its expression peaks at E13 then decreases by E18.5 consistent with a potential role in GnRH neuron migration. Mice null for ß3GnT1 show GnRH neurons retained in the nasal compartment at E15 with fewer neurons detected in the forebrain and a tendency for those neurons to be displaced in the dorsal rather than the ventral forebrain(15), suggesting misdirection. No human mutations have been reported to date.

3.1.3. Anosmin—The product of the Kallmann-1 (KAL1) gene, anosmin, was the first protein shown to be involved in normal GnRH neuronal migration and as a cause of X-linked form of Kallmann syndrome in humans, a combination of hypogonadotropic hypogonadism and deficient smell(16-18). The human fetus evaluated with Kallmann syndrome was noted to have olfactory, vomeronasal and terminalis nerves tangled in a web near the cribriform plate with absent olfactory tracts and bulbs (1,19).

Anosmin is an extracellular matrix glycoprotein thought to be important in adhesion of the olfactory nerves along the initial migratory steps exiting the olfactory placode. It is a 680 amino acid secreted protein with motifs similar to those detected in axon guidance factors (20,21). Putative roles for anosmin include as a cell matrix guide for axons, a chemoattractant that is secreted important in olfactory axon pathfinding, or a protein secreted to digest matrix to allow the olfactory axons to crosstalk with the olfactory bulb (reviewed in (22-24). Therefore, the effects of KAL1 mutations on GnRH neuron migration are indirect. Recent studies suggest that anosmin may also be involved in fibroblast growth factor receptor signaling, a growth factor pathway important in GnRH neuron development discussed below (25).

3.2. Guidance cues

3.2.1. EphA5—The ephrins are a group of cell surface molecules that signal through membrane tyrosine kinase receptors (EphA and EphB) and play a major role in axon guidance in many areas of brain development (26). The potential importance of this signaling system in GnRH neuron development was implicated by the analysis of the GN23 mutant mouse in which a 67 kb deletion downstream of the subtype 5 EphA receptor (EphA5) gene resulted in over-expression in GnRH neurons. These mice displayed a failure of normal neuronal migration with disordered clumps of GnRH neurons along the olfactory neurons. It is hypothesized that over-expression of EphA5 triggered abnormal adhesion of the GnRH neurons along the initial migratory route. In the adult, only 12% of the GnRH neurons reached a normal destination in the hypothalamus, yet surprisingly, the onset of sexual maturation occurred normally in these mice, suggesting a few appropriately targeted GnRH neurons are sufficient to promote the onset of reproductive function. However, more careful analysis showed that females were infertile or subfertile due to an abnormal LH surge mechanism (27). These mice over-expressing EphA5 receptor support the importance of the ephrin system in the early migration of GnRH neurons and the critical timing of turning on and off specific signals to ensure proper targeting of the population to ultimately allow normal reproductive function. In addition, these data suggest that because of the

redundancy in the system, candidates which alter the number or location of GnRH neurons across development may result in subtle rather than absolute alterations in reproductive function. No human mutations in this system have been reported to date.

3.2.2. Nelf—Nasal embryonic LHRH factor (NELF) was isolated from expression profiling of migrating and non-migrating primary rodent GnRH neurons (28). Using an antiserum developed by the Wray group, studies suggested NELF was expressed at the membrane of GnRH and olfactory neurons during migration in the nasal region but then lost when they transitioned into the forebrain(28). Silencing of NELF transcripts by antisense oligomers in nasal explants resulted in a decrease in the number of GnRH neurons by 2/3 as well as the length and complexity of olfactory nerve fibers. Others have suggested NELF is a nuclear protein(29) whose role is implicated in neuronal migration. No knockout mouse model has been analyzed to date and the exact physiologic role of this protein awaits further studies. Recently human mutations in NELF have been identified in patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and KS(29-31).

3.3. Neurotransmitters that modulate GnRH neuronal migration

Slice cultures from transgenic mice expressing green fluorescent protein in GnRH neurons (GnRH-GFP mice)(11) showed that GnRH neurons move with greater frequency and with more changes in direction after they enter the brain. Perturbations of guiding fibers distal to moving GnRH neurons in the nasal compartment influenced movement without inducing detectable changes to the structure of the fibers in the immediate vicinity of moving GnRH neurons. These data suggest that the use of fibers by GnRH neurons for guidance may involve both specific signaling in addition to just mechanical guidance and supported the search for neurotransmitters involved in GnRH migration.

3.3.1. Gamma-aminobutyric acid (GABA—GABA may be prototypical for small secreted molecules that modulate GnRH neuronal migration (11,32,33). Consistent with the heterogeneity of the GnRH neuron population, only a subset of GnRH neurons (~30%) contain GABA during development (34). At the same time, almost all GnRH neurons contain GABAA receptors, but with heterogeneous complements of subunit composition (32,33). One of the ways GABA is produced is via the 67 kDa form of glutamic acid decarboxylase (GAD67) (34). Transgenic mice with the GABA synthetic enzyme glutamic acid decarboxylase-67 (GAD-67) selectively over-expressed in GnRH neurons increased the number inhibited by GABA, but still only influenced a percentage of the total population(35). Over-expression of GAD67 in GnRH neurons did not disrupt the onset of sexual maturation, but the female mice displayed altered estrus cyclicity and rates of pregnancy (35). In contrast, GAD67 null mice displayed increased numbers of GnRH neurons out of the nasal placode at E14.5 and E17.5, although the changes were not balanced along the migratory route making an assessment of cell migration difficult to evaluate(36). GABA may also play a role in normal reproductive function in the adult through other receptors since examination of $GABA_BR1$ subunit null mice demonstrated again a normal onset of sexual maturation, but abnormal estrus cyclicity and impaired fertility (37,38). No human mutations in the GABA system have been reported to date.

3.3.2. Cholecystokinin—Cholecystokinin (CCK)-8 is a widely distributed neuropeptide(39) that has been implicated in female reproductive behavior and modulation by sex steroids (40,41). CCK acts via a G –protein coupled receptor, CCK-1R, expressed in GnRH neurons(42) and modulates GnRH neuronal migration. Mice null for CCK-1R at E14.5 displayed an increased number of GnRH neurons in the brain, suggesting it serves as an inhibitory modulator of GnRH neuron movement. However, there was compensation for the loss because in the adult the number and placement of GnRH neurons was normal and

reproductive function was not impaired. Recent studies suggest that in the adult, CCK may also directly inhibit GnRH neuron firing (43). No human mutations have been detected.

3.4 Growth factors

3.4.1. FGF8/ FGFR1—Growth factors such as fibroblast growth factor 8 (FGF8) acting through fibroblast growth factor receptor 1 (FGFR1) play a role in GnRH neuron development and function (44). FGFs induce proliferation, differentiation and survival of many cell types. Mice null for FGFR1 are embryonic lethal at E9.5-12 related to altered early cell migration (45,46) Over-expression of a dominant negative FGFR1 targeted to GnRH neurons resulted in decreased total number of GnRH neurons and abnormal projections to the median eminence suggesting that FGFs impact on multiple stages of GnRH neuron migration and development (47,48). These mice had not only delayed puberty but with aging had premature ovarian failure (48,49).

Multiple putative FGF ligands are expressed in forebrain development, but FGF8 was a potential candidate ligand because of its role in olfactory system development (50). Mice homozygous for a hypomorphic Fgf8 allele (51) had absent GnRH neurons in the hypothalamus with heterozygote mice showing 40% of the normal complement (52). Heterozygote mice were fertile. Consistent with these mouse models, humans with both anosmic and normosmic hypogonadotropic hypogonadism have been shown to have mutations in FGFR1 and/or FGF8 (52). Using the human data concerning the location of mutations in FGF8 found to date, the cognate ligands for FGFR1 in GnRH neurons are most likely the FGF8e and FGF8f splice variants(52). Therefore, careful phenotyping and genotyping of human patients with failure of pubertal development has given new insights into the basic studies of FGF/FGFR signaling pathway in forebrain development. Downstream components of the FGFR signaling pathway are additional potential candidates for hypogonadotropic hypogonadism that remain to be explored.

Recently investigators have suggested a functional tie between anosmin and the FGF signaling pathway. Heparan sulfate proteoglycans (HSPGs) are cell membrane and matrix-associated proteoglycans required for both anosmin and FGF action(44) (see Figure 1). In FNCB4 human fetal olfactory neuroepithlial cells, anosmin-1 triggered cytoskeletal rearrangement and neurite outgrowth via crosstalk with FGF/FGFR1/HSPG complex (53). In other studies in C. elegans, however, Hudson and coworkers suggest that the anosmin-1 ortholog, KAL-1, does not modulate FGF signaling(54). They identified that the heparan sulfate cores that modulate GnRH neuron migration include both syndecan(SDN-1) and glypican (GPN-1) and suggest that these many HSPG complexes modulate multiple factors involved in GnRH neuron migration such as anosmin-1/KAL-1 and FGFs/FGFR1 (see Fig 1). Review of the literature suggests that PROK2, HGF, netrin and perhaps Axl and Tyro3 may be modulated by HSPGs (see below). This raises the intriguing possibility of specificity in the type of HSPG to restrict or amplify crosstalk between cell surface or extracellular matrix factors to modulate GnRH neuron migration. To date, no human mutations in HSPGs have been reported.

3.5. G-protein coupled receptors

3.5.1 PROK2/PROKR2—Prokineticin 2(PROK2) and its receptor (PROKR2) were recently shown to modulate GnRH neuron migration and reproductive function with the analysis of mice mutant for the ligand and/or receptor (55,56) and humans with absent or abnormal pubertal development (57,58). PROK2 is an 81 amino acid ligand for PROKR2, a 384 amino acid G protein-coupled receptor (56,59). Initial work focused on the role of this system in smooth muscle contraction of the gastrointestinal system, hematopoeisis, angiogenesis and circadian rhythms (59-61). However, mice null for PROKR2 were shown

to have a loss of most of the GnRH neurons in the forebrain in adults and at E13.5, had formed a tangled web of olfactory vomeronasal axons that would be expected to alter GnRH neuron movement(55). Mice deficient in PROK2 also likely have abnormal GnRH neuron development indirectly because of altered olfactory bulb neurogenesis. Few GnRH neurons were detected in adult forebrain of PROK2 null mice and at E13.5 GnRH neurons were trapped in the tangle of olfactory axons after crossing the cribriform plate (56). PROK2 and PROKR2 are not expressed in GnRH neurons(58). These data are consistent with the idea that factors that impact olfactory bulb development are likely to impact GnRH neuron development at least through indirect influences on the fibers that guide GnRH neuron migration into the forebrain. Recent studies suggest that PROK2 also has heparan sulfate binding characteristics (62), raising the possibility that this system may also crosstalk with anosmin and FGF signaling pathways (see Fig 1).

Humans with mutations in this ligand and/or receptor have a variable clinical phenotype presenting with Kallmann syndrome, normosmic hypogonadotropic hypogonadism and some family members detected as asymptomatic carriers (57,63,64). This lack of correlation of genotype to phenotype has supported the hypothesis that hypogonadotropic hypogonadism may often reflect digenicity with heterozygous mutations in more than one candidate gene involved in GnRH neuron development causing the ultimate phenotypic presentation (57,64).

3.6 Transcription factors: Ebf2

Ebf2 is a member of a helix loop helix transcription factor family implicated in neural development (65) and expressed in migrating GnRH neurons at E11 (66). Mice null for Ebf2 retained GnRH neurons clustered in the nasal mesenchyme(66). The effects appeared to be directly linked to GnRH neurons as the olfactory system development was not altered. The authors suggested a secondary loss of neurons although no studies of rates of apoptosis were shown. Since the only marker for GnRH neurons is either GnRH peptide or mRNA, failure to synthesize GnRH is difficult to differentiate from cell death and therefore many claims of cell loss remain ambiguous. At P0, the majority of detectable neurons were in the nasal mesenchyme or dorsal to the cribriform plate, but a small number of neurons was detected in the forebrain at the caudal edge of the olfactory bulb. The downstream effectors of the Ebf2 transcription factor in GnRH neurons are unknown. No mutations in Ebf2 were detected in a small number of KS and nIHH subjects analyzed (67).

4.0 Factors that guide the VNN and GnRH neurons toward the forebrain

4.1 Netrin 1/Deleted in colon cancer (DCC)

The turning of the caudal branch of the vomeronasal nerve toward the basal forebrain is regulated by netrin-1 chemoattraction (68). The caudal branch of the VNN expresses an Ig-superfamily protein, deleted in colon cancer (DCC). In other systems, DCC mediates netrin 1-dependent axon guidance. Analysis of the axon trajectories and position of GnRH neurons in DCC mutant mice showed that DCC negative c-VNN axons failed to turn ventrally in the forebrain, and instead were detoured into the cerebral cortex (68). The alterations in axon trajectories and cell migration seen in DCC and netrin-1 mutant mice are similar, suggesting that this receptor/ligand pairing is necessary and sufficient for regulating the guidance of the c-VNN towards the ventral forebrain (69). The loss of Unc5h3, the alternative netrin 1 receptor, did not affect the trajectory of DCC axons or GnRH neurons, showing the specificity of the ligand/receptor interaction. Netrin/DCC signaling is modulated by HSPG (70,71) (see Fig 1). No human mutations in this pathway have been reported.

4.2. Semaphorins and Plexins

Semaphorin 4D (Sema4D) is a membrane-bound semaphorin that is proteolytically cleaved to bind Plexin B1 in the olfactory placode and along the GnRH neuron migratory route (72). Plexin B1 and NCAM expression colocalized in the olfactory system at E12.5 but investigators were unable to detect specific immunoreactivity for Plexin B1 along the VNN or in GnRH neurons at 14.5 or 17.5. Consistent with a localized effect early in GnRH neuron migration, in primary nasal explants cultures, PlexinB1 was detected in early cell divisions, but not later(72). Plexin B1 null mice showed a defect in GnRH neuron migration with less GnRH neurons at E14.5 and 20% less at P3 with accumulation in the olfactory bulb region. In adults, decreased GnRH fibers in the median eminence were observed (72). Studies by Prevot and coworkers have also implicated multiple semaphorins in GnRH secretion by the remodeling of GnRH fibers in the median eminence across the estrus cycle in response to sex hormones (73,74). Together, these data suggest that the semaphorins may play specific roles in many aspects of GnRH neuron development and function.

To understand potential mechanisms of semphorin/plexin signaling in neuronal migration, investigators performed experiments in Gn11 GnRH neuronal cells. The GnRH neuronal cells migrated either towards hepatocyte growth factor (HGF) or Sema4D in a Boyden chamber model. These effects could be blocked with either an HGF inhibitor or siRNA silencing of the HGF receptor, Met, suggesting that these ligands crosstalk via a plexin/Met interaction to modulate GnRH neuron migration (see Fig 2). Since Sema4D null mice do not have a reproductive phenotype, however, there may be compensation by different semaphorins in the knockout mice or that semaphorins other than 4D may be more physiologically relevant. No human mutations in this pathway have been documented to date.

4.3. Semaphorins/Neuropilin2

In addition to semaphorin 4D/plexin/HGF/Met story, the Class 3 semaphorins may impact on GnRH neuron development (see Fig 2). The Class 3 semaphorins are a group of inhibitory secreted and membrane bound proteins that bind the receptor, neuropilin-2 (NPN-2) and were initially shown to modulate axonal growth cone guidance (75). Examination of the Npn2 null mice showed a 25% loss of GnRH neurons in the adult, with increased neurons detected along the nasal septum (76). The authors suggest the defect in GnRH neuron migration is due to the defasciculation of the vomeronasal axons in these mice which disrupts the normal path and trajectory of the GnRH neurons. The location of the defect suggests that this system acts at a similar time to that of netrin/DCC signaling in GnRH neuron development. In addition to this indirect effect, a direct effect of semaphorins via Npn2 on GnRH neurons could contribute since endogenous GnRH neurons might express both the Npn2 receptor and the ligand, semaphorin3a (76). No human mutations have been reported in this ligand/receptor pair.

4.4. Reelin

Reelin is an extracellular glycoprotein shown to be involved in neuronal migration in several brain regions (77). Cariboni et al (78) showed that despite only 5% of GnRH neurons expressing one of the reelin receptors (ApoER2/Lrp8), that Reeler mice that lack Reelin had a decrease in forebrain GnRH neurons associated with their previously known decreased fertility (77). Reelin is expressed by the vomeronasal neurons but not GnRH neurons, suggesting the defects in GnRH neuronal migration are indirect(78). No human mutations in this pathway have been reported.

5.0 Growth factor pathways that move GnRH neurons through the cribriform plate and to the hypothalamus

5.1 Hepatocyte growth factor (HGF)/cMET

Met is a membrane tyrosine kinase receptor activated by the cytokine, HGF, shown to induce mitogenic, migratory and chemoattractant activities in multiple neuronal populations (79-81). HGF is a member of the plasminogen regulated growth factor family where proHGF is cleaved by uroplasminogen (uPA), tissue plaminogen (tPA) or coagulation factors to activate cMet. Giacobini and colleagues (82,83) showed initially in GnRH neuronal cells, then in vivo, the potential importance of HGF/Met signaling to normal GnRH neuron development. In GnRH neuronal cells, HGF promoted cell migration and motility(82). HGF administration to embryonic nasal explants increased the distance that the cells migrated, whereas inhibition of HGF reduced both GnRH and olfactory axon outgrowth suggesting direct and indirect effects of this pathway. Since mice null for cMET are embryologically lethal, the investigators examined mice null for tPA/uPA (ie deficient in active HGF) and documented a 35% decrease in the number of GnRH neurons at 60-90d postnatally, associated with subfertility and decreased gonadotropin-induced frequency of ovulation(83). The exact timing of HGF/Met impact on GnRH neuron development awaits further study. The ability of Met to cross talk with other tyrosine kinases, G-protein coupled receptors and various docking proteins, however, suggests the potential interaction of this pathway with multiple other candidates discussed within this review (see Fig 1 and 2).

5.2. Axl and Tyro3

Axl, Tyro3 and Mer comprise the TAM family of receptor tyrosine kinases that play diverse roles in immune modulation, sexual function and tumorigenesis (84-86). We showed that Axl and Tyro3 were expressed in NLT cells as models for early migrating GnRH neurons while Tyro3 and Mer were expressed in GT1-7 cells as models for postmigratory GnRH neurons (87). In NLT GnRH neuronal cells, Growth arrest specific gene 6 (Gas6), the ligand for the TAM family, induced GnRH neuronal migration via a novel p38MAP kinase pathway(88) and protection from programmed cell death via ERK MAP kinase and PI3 kinase to Akt signaling(89), suggesting a potential role for Axl and Tyro3 in GnRH neuron development. Analysis of adult Axl and Tyro3 null mice showed a 25% decrease in the number of GnRH neurons overall, with a specific 34% loss in the preoptic area surrounding the OVLT coupled with a small increase in neuron numbers in rostral regions. Analysis of GnRH neuronal development in E15 embryos showed a 36% reduction in GnRH neurons reaching the ventral forebrain, whereas the population in the nose and dorsal forebrain were not altered. These data suggested a potential role for Axl and Tyro3 in GnRH neuron cell survival during the window of development where the neurons cross the cribriform plate region. Further studies showed increased incidence of apoptosis as indicated by activated caspase 3 amongst early migrating GnRH neurons (90). These migratory and survival defects are consistent with the mechanistic properties of this receptor kinase family in mediating movement and protection from programmed cell death(88,89). The functional importance of this selective loss of GnRH neurons in Axl/Tyro3 null mice was suggested by analysis of the reproductive function of mice null for Axl and Tyro3 showing delayed first estrus and persistent abnormal estrus cyclicity (90) and abnormal LH surge mechanism (91). Examination of mice null for the ligand, Gas6 is underway to dissect if ligand is needed for Axl and Tyro3 effects on GnRH neuronal migration and survival in vivo. Initial analysis of 96 KS and nIHH patients demonstrated several heterozygous Axl mutations, suggesting the potential importance of this pathway in human reproduction (92). Gas6 is also a heparan sulfate proteoglycans activated ligand similar to FGFs and HGF (see Fig 1). Whether TAM family members crosstalk with other growth factor receptor pathways or other steps in GnRH neuronal migration is under active investigation (Fig 1 and Fig2).

5.3. Chemokine attractants: SDF1/CXCR4

Based on the hypothesis that chemoattraction would be an important mechanism for guiding GnRH neurons during their migration into the forebrain(6), stromal cell derived factor 1 (SDF-1 renamed as CXCL12) was identified as another potential candidate. It had been previously shown to impact migration of sensory cell (93), cerebral (94,95) and cerebellar (96) precursors. CXCR4 is the G-protein coupled receptor for SDF-1(97). In the central nervous system, SDF-1 acts as a chemoattractant for granule cell precursors, and mice lacking either SDF-1 or CXCR4 show aberrant cerebellar development and neuronal proliferation(98-100). CXCR4 is also expressed by neurons in the embryonic olfactory system(101). SDF-1 is expressed in the nasal mesenchyme beginning at E10 in mice in a gradient spread across the caudal half of the nose(102), with highest levels in mesenchymal cells directly adjacent to the cribriform plate and forebrain where GnRH neurons migrate. CXCR4 is broadly expressed, and in many, but not all GnRH neurons by double-label in situ hybridization(102).

The migration of GnRH neurons was severely impaired in CXCR4 null mice (102). At E12, when 40% of GnRH neurons have migrated out of the VNO in control mice, almost all GnRH neurons in CXCR4 null mice still reside in the VNO. At E13, when about 50% of GnRH neurons have migrated into the forebrain in wild-type littermates, less than 3% of GnRH neurons migrated across the cribriform plate and none migrated caudally into the developing hypothalamus. There was also a significant increase in the number of TUNEL positive apoptotic cells in the VNO at E13, particularly along the rostral and ventral surface of the VNO. In CXCR4 null mice GnRH-1 neurons accumulated in the most rostral-ventral quadrant of the VNO. Likewise, $TUNEL^{+}$ cells were found in the same location. These data suggested the importance of SDF-1/ CXCR4 signaling to mediate GnRH-1 neuron migration from their birthplace in the VNO to their ultimate destination in the forebrain. The recent suggestion of HGF signaling to Met receptors as synergistic with SDF-1 signaling to CXCR4 receptors (83), (see Fig 2) provides another example of the complex interactions among signaling systems during GnRH-1 neuron development. No human mutations in this system have been reported.

5.4. Transcription factor: Nhlh2

Nhlh2 is a helix-loop-helix protein expressed in the arcuate and anteroventral periventricular regions of the hypothalamus as well as the pituitary during development and adulthood(103). It is a member of a family of proteins expressed in postmitotic neurons acting as differentiating effectors late in development. During embryonic development Nhlh2 is expressed in a subset of GnRH neurons but then is absent later during development. Later in embryogenesis it was detected in Kisspeptin containing neurons in the hypothalamus and in the pituitary (104). Mice null for Nhlh2 had a loss of GnRH neurons in adulthood that occurred sometime between birth and adulthood (loss of 60% in females and 30% in males). At P0 there was a mild potential migratory defect with 16% more neurons in the olfactory/preoptic area and loss of neurons in the OVLT/caudal hypothalamic areas. Although these mice had delayed first estrus and estrus abnormalities, exposure of the females to males induced ovulation. LH expression was also decreased in the pituitaries of Nhlh2 null mice. These data suggested a complex role of Nhlh2 in the central reproductive axis, perhaps via Kisspeptin signaling as well as direct effects on later steps in GnRH neuron function postnatally and in addition pituitary defects which require further analysis.

Intriguingly, one of the many actions of Nhlh2 may be through necdin, a downstream target of Nhlh2 that was shown to augment GnRH gene transcription by interrupting Msx repression(105). Analysis of mice null for necdin demonstrated decreased hypothalamic neurons including GnRH neurons (by 30%) in the adult (106), with a selective loss in

crossing the cribriform plate into the forebrain during embryogenesis (by 50% at E13.5 and by 30% at E 17.5), as well as a decrease in the extension of axons to the median eminence(105). No human mutations in Nhlh2 have been reported, but necdin has been implicated in Prader Willi, a syndrome of obesity and hypogonadotropic hypogonadism(107,108).

6.0. Factors that halt GnRH neuronal migration

6.1. Kisspeptin/KissR

While GABA is thought to slow GnRH-1 neuron migration in the nasal compartment (32,33) and it may function to alter GnRH-1 neuron associations with guiding fibers in the brain (11,33), little is known about the factors that may contribute to the cessation of GnRH neuron migration once they have achieved their proper location within the hypothalamus. Kisspeptin is a 154 amino acid peptide with many shorter proteolytic cleavage products that is the product of the *KiSS-1* gene, first shown to suppress metastasis of tumor cell lines (109). Kisspeptin binds to and activates GPR54(now called the Kiss receptor (KiSSR), a seven transmembrane, G-protein coupled receptor to mediate decreased cell motility(110-112). Although the majority of work in the field has focused on the role of kisspeptin/KiSSR signaling to promote GnRH secretion at the time of puberty (109,113-115), its embryonic expression and potential function(s) has only recently been investigated. Preliminary studies suggest that Kisspeptin is detectable by in situ hybridization in a small number of neurons in the fetal arcuate nucleus of mice (116). Thus, Kisspeptin/KiSSR signaling may also play a role in the embryonic development of GnRH neurons in addition to its role in the onset of puberty.

7.0 Candidate genes that are mutated in humans to alter pubertal development

Nine genes have been reported in association with human hypogonadotrophic hypogonadism do date(30,31,92,117-122). Table 2 illustrates the pattern of inheritance, the putative frequency of these genes found in patients with KS or nIHH and whether each has been noted to exhibit digenicity, ie., more than one mutation in an individual proband. The effects of KAL-1, FGF8, FGFR1, PROK2 and PROKR2 would be expected to alter the number and successful targeting of GnRH neurons, and the role of CHD7 in GnRH neuron development is not clear. Alterations in KiSSR would be expected to alter GnRH secretion at puberty but may have effects on early neuron migration or maturation since it is expressed in GnRH neurons across development, NELF and AXL have been recently described in a small number of patients. Phenotype, inheritance patterns and frequency of these genes are yet to be defined.

8.0 Summary

The number of factors involved in GnRH neuron migration and development continues to expand. Candidates range from transcription factors to multiple transmembrane tyrosine kinases or G-protein coupled receptors and their ligands to extracellular matrix proteins. Many of the factors identified to date influence GnRH neuron movement indirectly by altering the pace or targeting of the olfactory system. Candidates intrinsic to the GnRH neuron form a smaller subset of important guides. The inability to track endogenous GnRH neuron number independent of GnRH mRNA or protein remains a limitation to studies as to the effects of silencing of potential candidates in that one cannot distinguish between suppression of GnRH expression compared to loss of a subset of the neuronal population. Few of the candidates identified to date important in GnRH neuron development have been shown to be altered in patients with KS or nIHH. The lack of a definable molecular cause

for the majority of subjects with delayed or absent puberty suggests the field will expand further in its attempts to define the constellation of mechanisms that drive GnRH neuron development and migration to ensure normal reproductive development.

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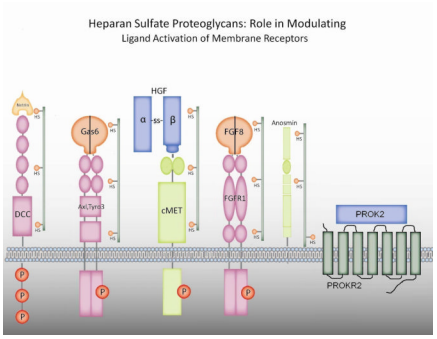

Figure 1.

Heparan sulfate proteoglycans: role in modulating ligand activation of membrane receptors. Expanding data suggests that certain ligands or their receptors with fibonectin domains or similar regions are modulated by classes of heparan sulfate proteoglycans (HSPGs) (eg. in c. elegans, syndecan-1 or glipican-1) with specific HS to control ligand activation. These include potential interactions with Gas6/Axl or Tyro3 and documented interactions with HGF/cMET, netrin/DCC, FGF8/FGFR1, anosmin and PROK2/PROKR2.

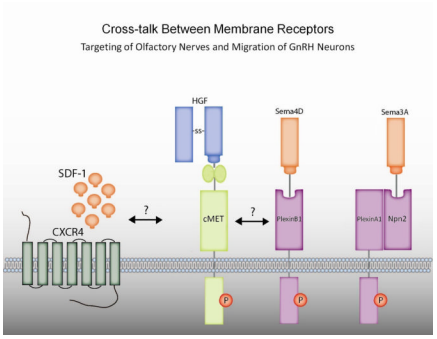

Figure 2.

Crosstalk between membrane receptors targeting of olfactory nerves and migration of GnRH neurons. HGF/Met, chemokine and Semaphorin pathways have been implicated in both olfactory nerve targeting and GnRH neuron migration. SDF-1 (CXCL12) chemokine and its receptor CXCR4 may interact with HGF (hepatocyte growth factor)/cMET which in turn may interact with semaphorins and their plexin receptors in the absence or presence of docking intermediates NP2 (neuropilin 2).

Table 1

Factors implicated in GnRH neuron migration. List of matrix proteins, secreted proteins, transcription factors, membrane receptors and ligands and transcription factors implicated in GnRH neuron development.

Cell Matrix/Adhesion	Neuro-transmitters	Growth Factors	G-Protein Coupled Receptors	Transcription Factors	Other
Neural cell adhesion molecule (NCAM)	GABA/GABA-A and GABA-B	FGF8/FGFR1	PROK2/PROKR2	Ebf2	NELF
Cell surface proteoglycans (eg. B3GNT1)	Cholecystokinin-8 (CCK8)/ CCK1R	Gas6/ Axl and Tyro3	SDF1/CXCR4	Nhlh2	
Anosmin (KAL1)		HGF/Met	Kiss/KissR		
Heparan sulfate Proteoglycans					
Ephrins/Ephrin receptors					
Semaphorin 4D/PlexinB1					
Semaphorin3a/Neuropilin2/PlexinA1					
Netrin/DCC					
Reelin/ApoER2/Lrp8					

B3GNT1- BetaGal beta-1,3-N-acetylglucosaminyltransferase 1; KAL 1 – Kallmann 1; DCC-Deleted in colorectal cancer gene; ApoER2- Apolipoprotein E receptor 2; Lrp8- Low density lipoprotein receptor-related protein 8; GABA- *Gamma*-Aminobutyric acid; CCK1R- Cholecystokinin-1 receptor; FGF8- Fibroblast growth factor 8; FGFR1- Fibroblast growth factor receptor 1; Gas6- Growth arrest specific gene 6; HGF- Hepatocyte growth factor; PROK2-Prokineticin 2; PROKR2-Prokineticin 2 receptor; SDF1 - Stromal cell derived factor 1; CXCR4- chemokine (C-X-C motif) receptor 4; Kiss-Kisspeptin; KissR- Kisspeptin receptor; Ebf2- Early B-cell factor 2; Nhlh2- Nescient helix loop helix 2; NELF- Nasal embryonic LHRH factor

Table 2

Candidates involved in GnRH neuron development mutated in human subjects. Names of genes shown to be mutated in probands with KS or nIHH with mode of inheritance, penetration, frequency of occurrence, phenotype and documentation of digenicity.

Candidate	Pattern of inheritance	Phenotype	Frequency in IHH patients	Digenicity
KAL-1 (Anosmin)	X-linked	Anosmic, complete penetrance	5-10%	Yes
FGF8 (KAL-6)	AD	Anosmic, normosmic NRP, FPP	2%	Yes
FGFR1 (KAL-2)	AD	Variable expressivity AP, DP, PP	10%	Yes
PROK2 (KAL-4)	AD, AR	Anosmic, normosmic Incomplete penetrance	2%	Yes
PROKR2 (KAL-3)	AD, AR	Anosmic, normosmic Incomplete penetrance	4%	Yes
CHD7 (KAL-5)	AD	Anosmic, normosmic CHARGE syndrome	5%	Not reported
NELF	AR	Hyposmic	<1%	Yes
KISSR (GPR54)	AR	Normosmic	2%	No
Axl	sporadic	Anosmic,normosmic	?	Yes

AD-autosomal dominant; AR-autosomal recessive; AP-absent puberty, DP-delayed puberty, DP-delayed pubery, NRP-normal reproductive phenotype, FPP-fully penetrant phenotype

CHARGE syndrome: coloboma, heart abnormalities, choanal atresia, retardation of growth and development, genital hypoplasia and ear abnormalities

KAL 1 – Kallmann 1; FGF8- Fibroblast growth factor 8; FGFR1- Fibroblast growth factor receptor 1; PROK2-Prokineticin 2; PROKR2-Prokineticin 2 receptor; CHD7- Chromodomain helicase DNA binding protein 7; NELF- Nasal embryonic LHRH factor; KISSR- Kisspeptin receptor