Semaphorin Signaling in GnRH Neurons: From Development to Disease

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Abstract

In mammals, fertility critically depends on the pulsatile secretion of gonadotropin-releasing hormone (GnRH) by scattered hypothalamic neurons (GnRH neurons). During development, GnRH neurons originate in the nasal placode and migrate first into the nasal compartment and then through the nasal/forebrain junction, before they reach their final position in the hypothalamus. This neurodevelopmental process, which has been extensively studied in mouse models, is regulated by a plethora of factors that might control GnRH neuron migration or survival as well as the fasciculation/targeting of the olfactory/vomeroneuronal axons along which the GnRH neurons migrate. Defects in GnRH neuron development or release can lead to isolated GnRH deficiency, with the underlying genetic causes still being partially unknown. Recently, semaphorins and their receptors neuropilins and plexins, a large family of molecules implicated in neuronal development and plasticity, are emerging as key regulators of GnRH neuron biology and deficiency. Specifically, semaphorins have been shown to play different roles in GnRH neuron biology by regulating migration and survival during embryonic development as well as secretion in adulthood.

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Gonadotropin-Releasing Hormone Neurons

Gonadotropin-releasing hormone (GnRH) neurons are key neuroendocrine cells in the establishment and control of the hypothalamic-pituitary-gonadal axis. In mammals, GnRH neurons mainly reside in the adult hypothalamus of the brain, where they synthesize and secrete the neurohormone GnRH in a pulsatile manner. The neurohormone is released at the level of the median eminence (ME) into a system of portal blood vessels to reach the pituitary gland where it stimulates the release of the gonadotropins LH and FSH. Via the general circulation, in turn, FSH and LH reach the gonads where they act directly and stimulate the synthesis of sex steroids (androgens and estrogens), which represent the main hormones involved in sexual maturation and in the regulation of fertility [1].

Unusual aspects characterize the GnRH neuronal system. First, despite their central role in controlling the reproductive axis, GnRH neurons are relatively few and scattered in the brain, where their cell bodies can be found between the olfactory bulbs and the medial preoptic area (MPOA). Second, GnRH neuron development is unique as they originate outside the brain and need to migrate for long distances and through different environments before reaching the brain. This peculiar development has

been extensively studied in mice, where GnRH neurons are first detected in the nasal placode (NP) around embryonic day (E) 10.5 [2] and then migrate through the nasal compartment into the basal forebrain before continuing their migration within the brain to attain their final positions in the hypothalamus [3, 4]. Recent lineage tracing studies on tissue-specific conditional mouse lines have helped to clarify their embryonic origin, which is now considered to be of placodal and neural crest mixed derivation [5].

Ex vivo nasal explants from embryonic mouse tissues have helped to better clarify some of the features of migrating GnRH neurons, including their saltatory movement [6] and their axonophilic migration [7]. Thus, GnRH neurons migrate throughout the nasal mesenchyme along a neural scaffold composed of axons originating from cell bodies that reside in the developing nose. According to the current working model, the axonal scaffold followed by GnRH neurons is formed by intermingled axons extending from olfactory/vomerolateral (OLF/VN) neuron soma. These neurons are located in the NP-derived olfactory epithelium and vomeronasal organ in the nose and project to the main and accessory bulbs, respectively. Then, at the junction between the nose and forebrain, GnRH neurons enter the basal forebrain following a subset of peripherin-expressing axons that turn caudally and that are known as the caudal branch of the vomeronasal nerve (cVNN) [8]. Recently, Taroc et al. [9] demonstrated that GnRH neuron migration is independent of the correct formation of OLF/VN nerve scaffolds, since arhinencephalic *Arx1*-null mice displayed normal GnRH neuron development. Instead, they showed that GnRH neurons appear to migrate along the axons of the putative terminal nerve (TN) or cranial nerve 0 to access the brain. These novel findings are changing the previous view rendering the nature of the axonal scaffold followed by GnRH neurons under debate.

Recently, the study of GnRH neuron migration has been extended to humans thanks to the availability of human fetuses and tissue-clearing technologies, providing evidence that GnRH neuron development is conserved between rodents and primates [10].

Isolated GnRH Deficiency

Defects in GnRH neuron development or function can lead to a pathological condition known as isolated GnRH deficiency (IGD). IGD is characterized by low serum concentrations of LH and FSH in the presence of low circu-

lating concentrations of sex steroids that lead to the absence of puberty and, if not treated, to reproductive failure [11].

Clinically, IGD could be present solely as GnRH deficiency, in association with absent (anosmia) or diminished (hyposmia) sense of smell, or in multisystemic syndromes. When associated with hyposmia/anosmia, IGD is referred to as the Kallmann syndrome (KS) and represents 50% of overall IGD cases. Instead, when the sense of smell is intact, IGD is referred to as normosmic hypogonadotropic hypogonadism (nHH). Finally, IGD may be present as a clinical trait in multisystemic syndromes or may overlap with multisystemic syndromes sharing only some clinical features, such as the CHARGE syndrome, which is sharing olfactory and reproductive defects with IGD [12–14].

IGD is genetically heterogeneous, with both sporadic and familial cases. Several modes of inheritance have been identified, including X-chromosome-linked recessive, autosomal-recessive, and dominant inheritance [13]. However, IGD is not solely a monogenic disorder as several IGD genes are shown to genetically interact modulating the severity of GnRH deficiency, strengthening the idea that IGD can also be considered a digenic/oligogenic disorder [15, 16]. To date, up to 30 genes have been demonstrated to lead to IGD, but they account only for the 50% of overall IGD patients; genetic causes for the remaining cases are so far still unknown [13]. IGD causative genes can be divided into two groups as they disrupt either the development of GnRH neurons (neurodevelopmental genes) or the action/signaling of GnRH in normally developed GnRH neurons (neuroendocrine genes). Mutations in neurodevelopmental genes usually lead to KS, whereas defective neuroendocrine genes usually underlie nHH. Several genes have a dual role both during development and function of GnRH neurons and, therefore, can cause either KS or nHH. In addition, some genes have a pleiotropic role, controlling many different developmental processes, so mutations lying on these genes can lead to either KS/nHH or syndromic IGD, depending on the nature of the mutation [13, 17].

The high degree of phenotypic and genetic variability, in addition to the low penetrance observed for some genes, makes the identification of novel genes implicated in the pathogenesis of IGD difficult. In addition, canonical genetic investigations in affected families are limited by the negative effect of IGD on fertility so that screened patients are few in numbers; finally, our biological knowledge on the hypothalamic-pituitary-gonadal axis is still limited. However, in recent years, several new IGD causative genes have been discovered following two main ap-

Fig. 1. Schematic drawing showing ligand-receptor complexes and related biological activity of the identified semaphorin signaling pathways in GnRH neuron biology. ME, median eminence; cVN, caudal vomeronasal.

proaches: (i) exome sequencing-based identification of novel mutated genes in affected individuals and functional validation with in silico, in vitro, and/or in vivo experimental models; (ii) basic science-driven identification of candidate genes involved in GnRH neuron physiology and targeted sequencing of the candidate genes in affected individuals [15, 17].

Role of Semaphorins and Their Plexin/Neuropilin Receptors in the GnRH Neuronal System

Over the past few years, work from several laboratories including ours have highlighted the importance of semaphorins and their receptors/coreceptors in the control of the GnRH neuronal system and predicated or validated mutations of such genes in human patients by using different experimental approaches [18–20]. To date, mutations in 3 semaphorins (*SEMA3A*, *SEMA3E*, *SEMA7A*) and in their receptors (*PLXNA1*, *NRP1*, *NRP2*) have been found in IGD patients [19–23] strongly supporting the involvement of these genetic pathways in the control of GnRH neuron physiology.

In this minireview, we will examine what is known for each molecule identified from a biological and genetic/clinical point of view. The roles of semaphorins and their receptors in GnRH neuron biology are depicted in the schematic drawing in Figure 1.

Semaphorins constitute one of the largest families of guidance cues, are highly conserved across species, and grouped into 8 classes. Semaphorins were first discovered as growth cone collapsing factors and, therefore, as repulsive neuronal guidance cues, but depending on their receptors they can also promote chemoattraction. The expression patterns of semaphorins have been best characterized in the nervous system, where they are dynamically expressed in specific areas during development, while their expression often decreases with maturity [24]. All semaphorins exhibit redundant structural features such as a distinctive SEMA domain, which allows both the dimerization between 2 semaphorins and the binding to receptors, 2 essential molecular events for semaphorin function. The major signal-transducing receptors are the plexins, a large family of single transmembrane-spanning cell surface receptors [25]. In addition, class 3 semaphorins bind to neuropilins (NRP1 and NRP2) which serve

as coreceptors in concert with A-type plexins (PLXNAs), with the exception for SEMA3E, which directly binds to PLXND1.

Other transmembrane molecules are functionally coupled to semaphorin receptors, including cell adhesion molecule L1, and receptor tyrosine kinases, such as off-track kinase (OTK), scatter factor receptors (SFRs), and vascular endothelial growth factor receptors (VEGFRs) [26]. The possibility to form receptor complexes on the membrane allows for cross talk between the different signaling pathways.

Semaphorin 3A and 3F, and Receptors

Previous expression studies have highlighted the expression of semaphorins and neuropilins in the olfactory structures [27]. Further analysis of *Nrp2*-knockout (KO) mice has revealed that *Nrp2* is required for axonal fasciculation/zonal segregation of vomeronasal neurons into the accessory olfactory bulb glomeruli [28]. Thus, the SEMA3F coreceptor NRP2 was then suggested to be involved in GnRH neuron migration based on in vitro and in vivo studies from our laboratory. Specifically, we found that *Nrp2* is expressed by nasal axons that form the axonal scaffold followed by GnRH neurons to migrate into the nasal compartment. Accordingly, *Nrp2*-KO mice display defasciculation of nasal axons, with a consequent reduction in the number of hypothalamic GnRH neurons [29] and reduced fertility. The lack of a strong phenotype in the *Nrp2*-null mice analyzed in this study suggested that other member(s) of the class 3 semaphorin-signaling pathway might also be involved in this process and prompted us to analyze different single and compound KO mouse models. Specifically, we found that *Sema3f*, which is the canonical *Nrp2* ligand, is dispensable for GnRH neuron migration, with a normal migration and number of GnRH neurons in mice lacking *Sema3f*. Instead, we found that *Sema3a*-null mice show a defective GnRH neuron migration, with clusters of cells accumulated in the nasal compartment and unable to reach the forebrain. This is due to the aberrant targeting of the cVNN which forms tangles of axons in the nasal compartment and fails to penetrate the brain [18]. Unexpectedly, but in agreement with a possible role of both *Nrp1* and *Nrp2* in mediating *Sema3a* signaling, we found that single *Nrp1* mutants lacking the *Sema* binding domain only partially phenocopy the defects of *Sema3a*-null mice, whereas the combined loss of both *Nrp1* and *Nrp2* fully phenocopied *Sema3a*-null mice. These results, together with binding assays on knockout tissues, demonstrated that *Sema3a* via *Nrp1* and *Nrp2* controls targeting of the

cVNN and indirectly regulates the migration of GnRH neurons towards the brain. In agreement, subsequent mutational screening on IGD patients revealed mutations in the *SEMA3A* gene in several IGD patients [19, 21, 22], and expression studies on human fetuses confirmed the expression of NRP1 on the cVNN [19]. Recently, it has been shown that *Sema3a* is highly expressed at the nasal-forebrain junction level, where it may play a dual role: on the one hand, it prevents OLF/VNN from invading the forebrain, and, on the other hand, it attracts both GnRH neurons and the peripherin-expressing axons (cVNN according to a previous model or TN in agreement with more recent findings) that are followed by GnRH neurons [9]. This double role of *Sema3a* might be explained by the presence of different sets of receptors and coreceptors on each cell population: according to this working hypothesis, cVNN/TN, as well as GnRH neurons, which express *Nrp1*, are attracted by *Sema3a*, whereas OLF/VNN, which express both *Nrp1* and *Nrp2*, are repelled by *Sema3a*, preventing brain invasion [9].

In addition to differential expression of NRPs, different combinations of PLXNAs, which are obligatory transducing receptors of SEMA3A, may contribute to the diverse response to SEMA3A signaling. Thus, heterozygous *PLXNA1* mutations along with mutations in *SEMA3A*, *NRP1*, and *NRP2* have also been reported in KS patients recently [23]. In this paper, mice with a homozygous *Plxna1*-null mutation showed, although with low penetrance, a reproductive phenotype, characterized by a low fertility index on postnatal day 40 and by less GnRH neuron-innervated ME [23]. The GnRH neuron defects observed in the *Plxna1*-KO mutant embryos are also not fully penetrant, with only one-third embryos showing defects in cVNN targeting. These observations raise the possibility that other members of the *Plxnas* family may additionally contribute to mediate the *Sema3a* effects on the targeting of the cVNN.

Further studies demonstrated that *Sema3a* exerts a crucial role also in adulthood by remodeling GnRH fibers that project to the ME during the estrus cycle. Specifically, brain endothelial cells released *Sema3a* that promotes GnRH neuron axonal sprouting through *Nrp1* expressed by GnRH neuron axon terminals; in addition, the amount of *Sema3a* released increases dramatically during the proestrus, when GnRH is released in massive surges to sustain LH surge [30].

Overall, these pieces of evidence strongly support the existence of a dual role for *Sema3a* signaling in the control of reproduction via effects on prenatal GnRH neuron development and adult GnRH release.

Semaphorin 4D

Semaphorin 4D (SEMA4D) is a membrane-bound semaphorin that is proteolytically cleaved to be able to bind PLXNB1 and Met tyrosine kinase receptor. Thus, *Sema4d* induces growth cone collapse by interacting with Plxnb1 [31], while it promotes angiogenesis via the combined interaction with Plxnb1 and Met [32]. In the developing olfactory system, the *Sema4d* receptors Plxnb1 and Plxnb2 are highly expressed [33]. Further, work from Giacobini et al. [34] demonstrated that Plxnb1 and Ncam expression colocalized in the nasal mesenchyme only at early stages of development (E12.5 but neither E14.5 nor E17.5) and that *Sema4d* promotes the chemomigration of immortalized GnRH neurons via the activation of Plxnb and Met signaling. Consistent with a cell-autonomous effect of *Sema4d* on migrating GnRH neurons in the nose, *Plxnb1*-null mice showed a reduced number of GnRH neurons reaching the forebrain with accumulation in the olfactory bulb region. A decrease in GnRH neurons in the adult brains resulted also in a decrease in GnRH fibers at the ME. Instead, while this study reported a normal number of GnRH neurons in adult *Sema4d*-KO brains, a more recent work has shown that female *Sema4d*-KO mice exhibit decreased levels of GnRH mRNA in the hypothalamus and reduced ovarian follicle maturation [35]. However, despite the presence of reproductive defects of both *Plxnb1*- and *Sema4d*-null mice, no human mutations in this pathway have been documented so far.

Semaphorin 7A

SEMA7A is the only glycosylphosphatidylinositol-anchored semaphorin, and it can act either as a membrane-bound protein or secreted factor after proteolytic cleavage. Although originally identified in immune cells, SEMA7A displays widespread expression and function in a wide variety of biological processes in different organs. Well-characterized biological effects of SEMA7A include bone and immune cell regulation, neuron migration, and neurite growth. These effects are mediated by PLXNC1 and integrin receptors [36]. Specifically, SEMA7A binds with equal affinity to PLXNC1, reducing the integrin-mediated cell adhesion, and to β_1 -integrin (ITG β_1), to promote integrin clustering and, therefore, cell attachment.

Since *Sema7a* is expressed in the NP and in the olfactory bulb, a role for *Sema7a* was postulated. Indeed, in 2011, Messina et al. [37] demonstrated that *Sema7a* promotes GnRH neuron migration in the nasal compartment, whereas its expression is turned off in the MPOA. *Sema7a* is expressed by OLF/VN nerves during the embryonic development whilst the expression pattern of the

2 *Sema7a* receptors on GnRH neurons is strictly temporally and spatially regulated: in the nasal mesenchyme, GnRH neurons express Itg β_1 and migrate along the *Sema7a*⁺ axonal scaffold, and instead of once crossing the brain, they begin to express Plxnc1 to cease migration. Consistently, *Sema7a*-null mice display defective GnRH migration and, as a consequence, a less innervated ME and dysfunctional gonads.

Further, conditional inactivation of the *Sema7a* receptor Itg β_1 in GnRH neurons alters migration of GnRH neurons and targeting of their axons to the ME, resulting in suboptimal maturation of the GnRH system in the adult brain. Examination of fertility revealed that lack of β_1 -integrin in GnRH neurons caused delayed pubertal onset and disruption of estrous cyclicity in female GnRH-*Itgb1*^{-/-} mice, which greatly impaired reproduction. These findings demonstrated a critical role for β_1 -integrin in GnRH neuronal function and mammalian reproduction [38].

Similarly to *Sema3a*, *Sema7a* exerts an additional function in ME plasticity, where it is expressed by tanycytes, specialized glial cells lining the third ventricle. There, *Sema7a* induces retraction of GnRH fiber terminals and promotes the spread of tanycyte end feet by acting through Plxnc1 and Itg β_1 , respectively [39]. In agreement with the essential role exerted by *Sema7A* in GnRH neuron development and plasticity in mice, mutations in *SEMA7A* have been found both in KS and nHH patients [22].

Semaphorin 3E

SEMA3E has previously been shown to play different roles during development, such as neuron migration, axon guidance, and vascular system formation, as well as in cancer progression, through the interaction with different receptors and complexes. Via Plxnd1, *Sema3e* controls, for example, the mitogenic potential of Cajal-Retzius cells in the developing cortex [40], and it regulates VEGF function in developmental angiogenesis via a feedback mechanism [41]. Further, *Sema3e* mediates axon growth interacting with Vegfr2, Plxnd1, and Nrp1 [42]. In cancer cells, SEMA3E promotes cell invasiveness and metastatic spread by transactivating the oncogenic tyrosine kinase ErbB2 that associates with PLXND1 [43], whereas it suppresses tumor cell death triggered by the PLXND1 dependence receptor in metastatic breast cancers [44].

In our recent work, we combined exome sequencing with in vitro and in vivo functional assays to identify *Sema3e* as an essential gene for GnRH neuron development. First, we found that the *Sema3e* receptor Plxnd1 is

expressed during GnRH neuron development, with a peculiar spatial and temporal expression pattern. Specifically, we found *Plxnd1* expression on blood vessels and OLF/VN axons but not on migrating GnRH neurons in the nose. Instead, when GnRH neurons enter the forebrain to scatter in the MPOA, *Plxnd1* is highly expressed by GnRH neurons. In agreement with a possible neuroprotective role of *Sema3e* in the MPOA, mice lacking *Plxnd1* have a reduced number of GnRH neurons in this region, and *Sema3e* prevents the cell death of immortalized postmigratory GnRH neurons (GT1–7 cells) [45].

Clinically, *SEMA3E* has been linked to the CHARGE syndrome [46], a complex syndrome whose phenotypic spectrum also includes syndromic IGD, which is in the majority of cases caused by mutations in the *CHD7* gene. Interestingly, by further interrogating the exome of the 2 IGD-affected patients carrying heterozygous mutations of *SEMA3E* studied in this work, we found the coexistence of a heterozygous *CHD7* mutation, suggestive of a possible interaction between the 2 genetic pathways. The analysis of compound heterozygous mice for neuronal *Plxnd1* and *CHD7* revealed a marked reduction in both GnRH neuron ME innervation and gonadal size compared to single *Chd7* heterozygous mice, used as a mouse model for the CHARGE syndrome [47]. Interestingly, recent reports have documented that *Chd7*, a chromatin-remodeling protein that controls gene expression, regulates the expression of other members of the semaphorin family using a *Chd7*-deficient mouse model [48], supporting the possibility of *CHD7* being an upstream regulator of semaphorin expression.

Conclusions

In conclusion, the possibility to interrogate exome sequencing data from affected individuals combined with in vitro and in vivo models aimed at validating the functional relevance of the mutated genes is likely to uncover the remaining causes of IGD. Given the complex roles of semaphorins in axon guidance, neuronal migration, as well as in neuronal plasticity and the unique development and physiology of GnRH neurons, it is not surprising if additional members of the semaphoring-plexin signaling pathways will be found implicated. Further, given the important dual role of semaphorin signaling in regulating neuronal and vascular patterning, future studies will be needed to prove their reciprocal influence in the GnRH system.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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