Role of Sema3A/Neuropilin1 signaling in GnRH system development and study of the involvement of NO-synthesizing neurons in the kisspeptin-dependent preovulatory activation of adult GnRH neurons

Naresh Kumar Hanchate

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UNIVERSITE DROIT ET SANTE DE LILLE II

Ecole Doctorale Biologie - Santé

These

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Spécialité : Neuroscience

Presentée par

Naresh Kumar HANCHATE

Role of Sema3A/Neuropilin1 signaling in GnRH system development and Study of the involvement of NO-synthesizing neurons in the kisspeptin-dependent preovulatory activation of adult GnRH neurons

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Reproduction in mammals is regulated by neurons that synthesize and secrete gonadotropin-releasing hormone (GnRH) and across the species these neurons are present in few numbers scattered in the hypothalamus. Due to limited neurogenesis of these neuronal cell types outside the brain in the olfactory placode, these neurons are subjected to tight regulation during embryonic development to reach their final targets in the hypothalamus, from birth until puberty for minimal secretion of hormone and during adults to achieve pulsatile secretion of the hormone. Deregulation in any of these mechanisms may lead to deleterious effects on adult reproduction and clinical pathologies like absence of puberty, hypogonadism, sterility, amenorrhea, etc. Kallmann syndrome (KS), one of these severe reproductive pathologies is an inherited disorder and patients affected with this syndrome display anosmia (inability to smell) and hypogonadotropic hypogonadism (HH). Genetic screening of molecules in these patients lead to identification of genes like *KAL1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *WDR11* and *CHD7* encoding proteins that play an important role in migration and targeting of olfactory system during embryonic development however these genes account only for 30% of KS cases emphasizing the need for further characterization and identification of other genes. While these proteins are involved in ontogenesis olfactory and GnRH system, genetic screening of molecules in patients suffering from normosmic idiopathic HH lead to identification of genes encoding for Kisspeptin receptor-*GPR54*, *TAC-TACR3*, *LEP-LEPR, PCSK-1*, GnRH receptor-*GnRHR* and *GnRH-1* itself that play a crucial role in occurrence of puberty or adult reproduction.

Here, for my PhD thesis, we focused on studying the role of guidance molecule Semaphorin3A (Sema3A)-Neuropilin1 (Nrp1) interactions in ontogenesis of GnRH neurons during embryonic development while in adults we first addressed the question if hypothalamic Kisspeptin neurons interact with neurons containing neuronal nitric oxide synthase (nNOS), the mutation of which causes HH in mice, and physiological significance of this interactions in regulation of GnRH neurons and neuroendocrine control of female reproduction.

Semaphorin3A, that belongs to highly conserved semaphorin family of guidance molecules has been known to be implicated in ontogenesis and targeting of olfactory system

through its receptor Neuropilin1 however its role in development of GnRH neurons and adult reproduction is not known. Using genetic mouse model *Nrp1*$^{sema/sema}$ that has a mutated Neuropilin1 receptor lacking functional semaphorin binding domain, we identified the crucial role of these interactions implied in ontogenesis of GnRH neurons. These mice had disrupted olfactory and vomeronasal projections to the olfactory bulb and to the ventral forebrain respectively. Disrupted olfactory projections lead to suckling dysfunction and early postnatal death while the disrupted vomeronasal projections lead to defective migratory behavior of GnRH neurons. The few mutant mice that grow until adults display reproductive deficits and KS like phenotype and interestingly genetic screening of human patients suffering from KS by our collaborators lead to the identification of inactivating mutations of *SEMA3A* gene in these patients.

Hypothalamic Kisspeptin and Nitric oxide synthesizing neurons have been recognized as key regulators of GnRH neurons modulating adult reproductive function. However, no efforts have been made to study if there is any cross-talk existing between the 2 neuronal cell types. Here, we show that kisspeptin neurons project to specific population of nNOS neurons in the preoptic region and the nNOS neurons in these region express kisspeptin receptor-Gpr54. Intraperitoneal injections of kisspeptin-10 readily induced posttranslational modification of nNOS protein in these neurons and these modifications by kisspeptin-10 require Gpr54 receptor and is mediated through PI3K-AKT pathway. Interestingly, we demonstrate that during the gonadal steroid negative feedback, a constitutive basal level of NO released by neurons in the preoptic region maintain tonic inhibition on GnRH neurons resulting in nadir levels of gonadotropin release whereas high activation of NO synthesizing neurons in the preoptic region induced by increased estrogen-kisspeptin signaling during the gonadal steroid positive feedback leads to high amount of NO release that eventually set GnRH neurons to release peak levels of GnRH hormone resulting in a surge of gonadotropins necessary to trigger ovulation.

Finally our results demonstrate that Sema3A-Nrp1 interactions are implicated in ontogenesis of olfactory and GnRH neurons during embryonic development and nNOS neurons are important mediators of peripheral estrogens-kisspeptin signaling onto GnRH neurons and adult reproduction and propose to further study the implication of nNOS neurons in reproductive pathologies.

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Introduction

1. The GnRH system

Mammalian reproduction is controlled by integrated sets of interactions between the hypothalamus, pituitary gland and gonads. Each component of the reproductive system is regulated by feedback mechanisms that coordinate the processes leading to gonadotropin secretion, gamete production and maintenance of the species (Conn and Crowley, 1994; S.R. Ojeda, 2006). In most mammalian species, GnRH neurons are distributed in the preoptic area and adjacent sites in the rostral region of the hypothalamus, rather than concentrated in a discrete nucleus. These scattered neurons are believed to form a diffuse neural network that functions coordinately as a GnRH pulse generator (Knobil, 1990). The generation of pulsatile GnRH release at the median eminence is the central and essential element governing reproductive function, and depends on the coordinated activities of the 1500 or so GnRH neurons that are located in the hypothalamus (Herbison et al., 2008; Wray, 2001; Wray, 2010). Studies using intrahypothalamic injection of immortalized GnRH neurons (GT1–7 cells) in hypogonadal mice revealed that regardless of the number of neurons injected, normal reproductive function could be restored (Silverman et al., 1992).

1.1. Brief History

The GnRH decapeptide from the hypothalamus of pigs and sheep was first isolated and characterized in the 1970s (Burgus et al., 1972; Matsuo et al., 1971). In the early 1980s, a second GnRH isoform from chickens was isolated and described (chicken GnRH-II) (Miyamoto et al., 1984) and a third isoform was found in fish (salmon GnRH) (Sherwood et al., 1983). As more forms of GnRH were isolated, initially they were named after the animals in which they were first found. Since the initial localization of GnRH1 to the hypothalamus, other forms of GnRH have been isolated and localized to the nervus terminalis of the forebrain (GnRH3) and to the midbrain (GnRH2) **(Figure. 1)**. GnRH3 neurons of the nervus terminalis (a cranial nerve) have a neuromodulatory role in the forebrain and olfactory epithelium (Abe and Oka, 2002; Eisthen et al., 2000), and also act as modulators of olfactory mediated behaviors (Wirsig-Wiechmann et al., 2002). Similarly, the GnRH2 cells of the midbrain also appear to modulate sexual behaviors (Millar, 2003). Therefore, GnRH-containing cells of the nervus terminalis (GnRH3) and midbrain (GnRH2) appear to mediate

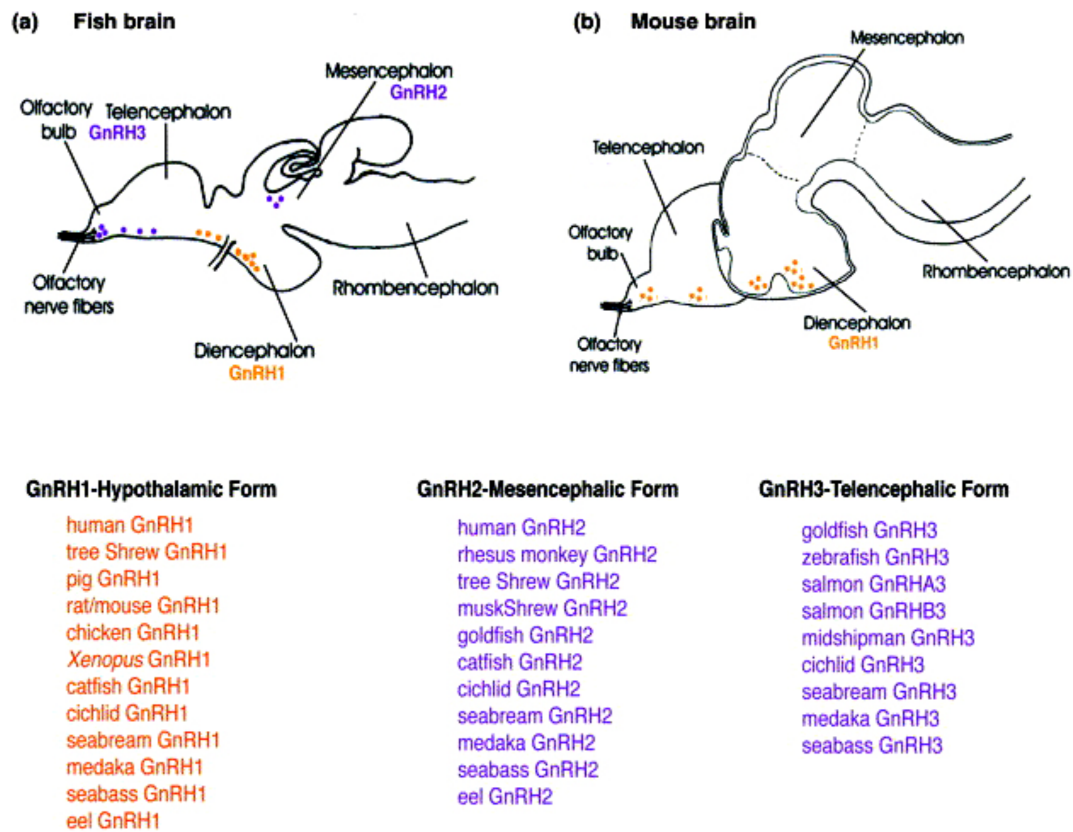

Figure 1. **Location and forms of GnRH in representative brains of fish and mammals.** Sagittal sections of fish **(a)** and mouse **(b)** brain, anterior to the left. GnRH1 cells are depicted in orange, GnRH2 and GnRH3 cells are depicted in purple because they are proposed to have the same developmental origin. These are generalized locations in the brains and can vary among vertebrates. **(c)** Forms of GnRH for which cDNA has been sequenced and grouped, showing that representatives of all vertebrates have at least two forms of GnRH (GnRH1 and GnRH2), whereas forms having the structure of GnRH3 are so far specific to fishes. [Adapted from (Whitlock, 2005)]

reproductive behaviors, whereas GnRH cells of the hypothalamus (GnRH1) play an endocrine role.

1.2. Embryonic origin of GnRH cells

In 1989, two separate laboratories reported that the GnRH cells of the ventral forebrain of the mouse appeared to arise from the olfactory placode. Based on observations of staged fixed tissue using immunocytochemistry and *in situ* hybridization, GnRH cells appeared to migrate through the nasal septum into the forebrain along the terminal nerve–vomeronasal nerves (Schwanzel-Fukuda et al., 1989; Wray et al., 1989a; Wray et al., 1989b). In these studies, GnRH-positive cells were first seen in association with the olfactory placode, and then appeared in association with the vomeronasal nerve (VNN) and nervus terminalis at intermediate locations between the olfactory placode and forebrain. Once the GnRH cells entered the forebrain, they left the VNN and entered the hypothalamus, following no anatomically defined pathway. Thus, it was proposed that the olfactory placode generated not only olfactory sensory neurons and support cells, but also neuroendocrine cells containing GnRH.

1.3. The GnRH neurons migratory pathway

The GnRH neuronal migratory route can be divided into four specific stages **(Figure. 2)** (Tobet et al., 2001; Tobet and Schwarting, 2006; Wray, 2010). Analysis of this specific neuronal migratory process is limited by the fact that there is 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 (Spergel et al., 1999; Suter et al., 2000). 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 (Schwanzel-Fukuda et al., 1989; Wray, 2010; Wray et al., 1989a). This initial step requires both the movement of GnRH

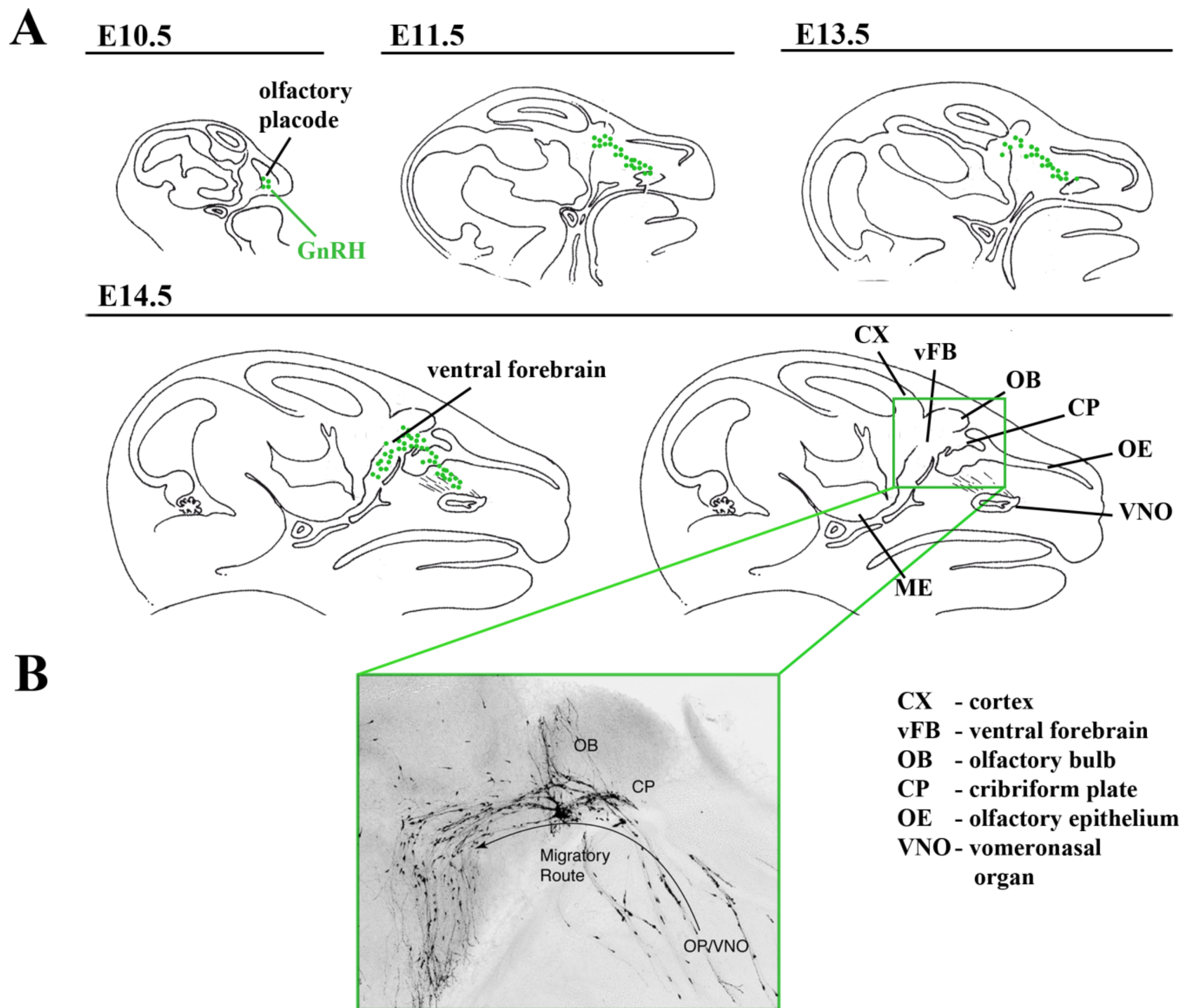

Figure 2. Scheme illustrating the embryonic origin and migratory pathway of GnRH neurons. GnRH neurons first appear in the olfactory placode at E10.5. (A) Series of sagittal sections from mouse head at Embryonic day (E) 10.5, 11.5, 13.5 and 14.5 illustrating the migratory route of GnRH neurons from the olfactory placode to ventral forebrain areas. (B) Microphotograph of a mouse embryo at E15 in sagittal plane illustraing the migration of GnRH neurons from the olfactory placode (OP/VNO) to the olfactory bulb that eventually enter the ventral forebrain towards the preoptic / Hypothalamic areas. (Adapted from Wiermann et al., 2004)

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 ventral forebrain, specific factors promote the extension of long processes through the basal forebrain toward the hypothalamus. (4) Lastly, the neurons detach from their axonal guides and disperse further in the hypothalamus and stop migrating. The migratory process is usually completed by birth and the GnRH neurons as they reach the preoptic areas send their axons to the median eminence where they secrete and release the hormone. 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.

1.4. Puberty

Puberty is the transition to adulthood that culminates in the production of mature gametes and the initiation of reproductive activity (Ojeda, 2006). The process begins within the central nervous system, where gonadotropin-releasing hormone (GnRH) neurons are activated to release high-frequency pulses of the neurohormone, stimulating pituitary gonadotropic hormone secretions that in turn direct gonadal steroid hormone production and maturation of gametes (Plant, 2006; S.R. Ojeda, 2002; Sisk and Foster, 2004; Terasawa and Fernandez, 2001). Though GnRH neuronal axons achieve formation of neurovascular junctions at the median eminence by birth, GnRH neurons are maintained under continous inhibitory synaptic inputs to release minimum amounts of the hormone into the portal blood until puberty (Terasawa and Fernandez, 2001). An ill-defined developmental clock, as well as permissive somatic and environmental signals, govern the onset, progression, and completion of the pubertal acceleration of GnRH release.

1.5. Pulsatile and cyclic secretion of GnRH

GnRH neurons are distributed in the preoptic area and adjacent sites in the rostral region of the hypothalamus, rather than concentrated in a discrete nucleus. These scattered neurons are believed to form a diffuse neural network that functions coordinately as a GnRH pulse generator (Moenter et al., 2003; Van Goor et al., 1999a; Van Goor et al., 1999b). In mammals, the pattern of gonadotropin secretion includes both pulse and surge phases, which

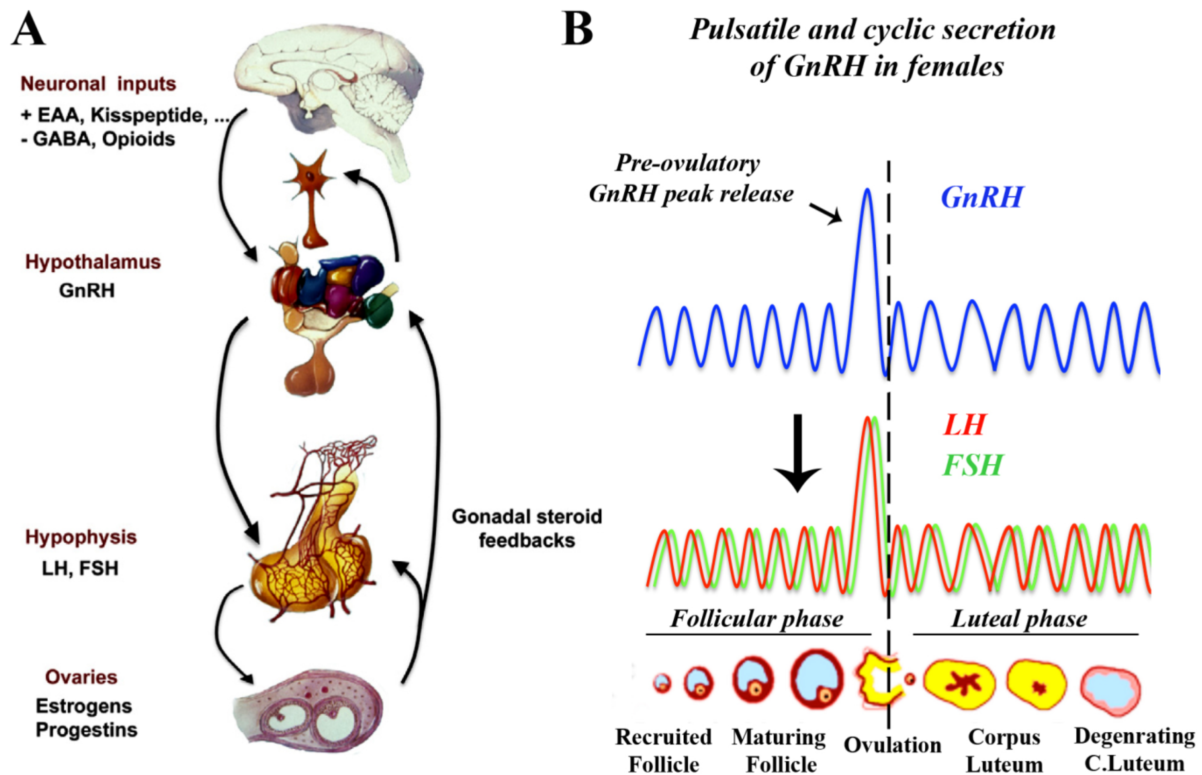

Figure 3. (A) **The Hypothalamic-pituitary-gonadal axis.** The hypothalamic GnRH neurons are the final common pathway for central control of gonatropin secretion are subjected to a complex array of excitatory and inhibitory transsynaptic inputs that modulate their activity. GnRH neuroendocrine neurons project to the median eminence where they make contact with basal lamina and open into the pericapillary space of the primary hypophyseal portal plexus. Upon reaching the pituitary portal system, GnRH travels to the pituitary to stimulate the synthesis and secretion of pituitary gonadotropins: luteinizing hormone (LH) and follicle stimulating homone (FSH). Blood-borne LH and FSH act on target cells in the gonads (here the ovaries) to direct production of gametes, as well as the secretion of steroid hormones. Within the brain, gonadal steroids influence GnRH secretion via neuroendocrine feedback loops. EAA, excitatory amino acids and GABA, gammaaminobutyric acid. (Adapted from Prevot et al 2010). (B) In females, the GnRH is secreted in a pulsatile and cyclic manner characterized by the pre-ovulatory peak release that is necessary to induce peak secretion of gonadotropins - LH and FSH and trigger ovulation. During the ovarian cycle, follicular phase denotes the period during which the follicules mature, followed by ovulation (release of oocyte or female gamete). Luteal phase begins with the formation of corpus luteum that may end into pregnancy upon successful mating or luteolysis or degeneration of corpus luteum. In humans, the ovarian cycle is called menstrual cycle that lasts for 28 days while in rodents is called the estrous cycle that lasts for 4-5 days.

are regulated independently **(Figure. 3)** (Tsutsumi and Webster, 2009). Both the frequency and amplitude of the GnRH release in pulsatile manner are important for the development of sex function. GnRH secretion in pulsatile fashion is important in order to avoid the down regulation and sensitivity of GnRH receptor in the pituitary gonadotrophs (Belchetz et al., 1978; Schang et al., 2011). Many of the stimulatory and inhibitory signals may influence the pulse-generator by acting on secondary neurons.

In most of mammalian females, GnRH/LH is secreted in a cyclic manner i.e, called ovarian cycle or precisely estrous cycle in rodents and menstrual cycle in humans that has a duration of 4-5 days in rodents and 28 days in humans necessary for successful ovulation (Bakker and Baum, 2000; Bronson and Vom Saal, 1979; Christian and Moenter, 2010). In rats, the estrous cycle that has a duration of 4-5 days can be monitored by observing the cellular morphology of vaginal lavages that contain round lymphocytes during the diestrous phase I and II (low gonadotropins), round nucleated cells during the proestrous (LH surge) followed by cornified cells during the estrous phase (Freeman.M.E., 2006). Unlike rats, the estrous cycle in mice may vary between 4-7 days and usually do not follow a regular pattern of cycle. Throughout the estrous/menstrual cycle, GnRH and gonadotropin secretion are regulated by the negative feedback of ovarian steroids resulting in low level of secretion, but rising estrogens released by the developing follicles reach at a peak level that at acts a positive feedback resulting in a preovulatory GnRH/LH surge triggering ovulation.

2. Hypogonadotropic hypogonadism

Idiopathic hypogonadotropic hypogonadism (IHH) is characterized by delayed or absent sexual development associated with inappropriately low gonadotropin and sex steroid levels in the absence of anatomical or functional abnormalities of the hypothalamic–pituitary–gonadal axis (Layman, 2007). The major underlying cause of IHH is failure to activate pulsatile secretion of gonadotropin-releasing hormone (GnRH) during puberty, a developmental stage characterized by a substantial increase in the frequency and amplitude of pulses of this hormone. The development of GnRH neurons is unusual in that they originate outside the brain. Similar to the development of olfactory fibers, GnRH neurons are formed in the nasal placode, from which they migrate by use of the olfactory pathway to help guide them to their ultimate destination in the hypothalamus (Tobet et al., 2001). Other abnormal phenotypes that are commonly associated with and segregate with hypogonadism in affected families is anosmia, an inability to perceive smells, which is explained by the common

Table 1. Genes identified to cause Kallmann syndrome / normosmic HH in humans.

Gene Name	Chromosomal locus	Gene product	OMIM no.
KAL1	Xp22.3	Anosmin-1	300836
FGFR1	8p12	Fibroblast growth factor receptor-1	136350
FGF8	10q.24	Fibroblast growth factor 8	600483
PROKR2	20p12.3	Prokineticin Receptor 2	607123
PROK2	3p13	Prokineticin 2	607002
CHD7	8q12.2	Chromodomain helicase DNA-binding protein 7	608892
NELF	9q34.3	Nasal embryonic LHRH factor	608137
WDR11	10q26	WD repeat-containing protein 11	606417
KISS1R	19p13.3	KISS1 receptor	604161
TAC3	12q13–q21	Tachykinin 3	162330
TACR3	4q25	Tachykinin receptor 3	162332
LEP	7q31.3	Leptin	164160
LEPR	1p31	Leptin Receptor	601007
GNRH1	8p21–p11.2	Gonadotropin-releasing hormone	152760
GNRHR	4q21.2	Gonadotropin-releasing hormone receptor	138850

Table 2. Prevalence of known genetic defects in hypogonadotropic hypogonadism

Kallmann syndrome		Normosmic HH	
KAL1	10-20 %	*GNRHR*	10-40 %
FGFR1	10 %	*GNRH1*	< 1 %
FGF8	< 2 %	*TACR3*	? *
PROKR2	5 %	*TAC3*	? *
PROK2	2-5 %	*KISS1R*	< 3 %
CHD7	5 % #	*PROKR2*	1 %
NELF	1 %	*PROK2*	1 %
		FGFR1	3 %
		FGF8	< 2 %
		CHD7	5 % #
Unkown HH (KS + nIHH) 70 %			

* small studies reported to date found mutations in *TACR3* in 40% and in *TAC3* in 10% if familial cases of nIHH, but further reports are awaited .
# May usually be identified by accesory features including semicircular can hypoplasia.
[Adapted from (Semple and Topaloglu, 2010)]

embryonic origins and developmental pathways of GnRH and olfactory neurons. Therefore HH can be divided into kallmann syndrome (IHH with anosmia) and normosmic IHH.

2.1 Kallmann syndrome

More than 150 years ago, Aureliano Maestre de San Juan described an adult male with testes of prepubertal size and absent olfactory bulbs (Maestre, 1856). In the 1940s, Franz Kallmann added to this by documenting hypogonadism co-segregating with anosmia in two families (Kallmann.F.J, 1944), convincingly establishing a genetic basis for the condition, while de Morsier later added neuropathological detail (de morsier G, 1963). The discovery of GnRH in the early 1970s (Guillemin, 1977; Guillemin, 2005) led quickly to the demonstration that the hypogonadism of Kallmann Syndrome (KS) – as the combination of HH and anosmia has come to be labelled in the English speaking world – was central in origin (Lieblich et al., 1982).

Kallmann Syndrome has a prevalence of around 1 in 8000, and is five times more common in men than women. X-linked recessive, autosomal dominant (AD) and autosomal recessive (AR) patterns of inheritance are observed; however, many cases are sporadic or do not appear to show a Mendelian inheritance pattern (Dode and Hardelin, 2009). Other associated neurological and somatic abnormalities, such as synkinesia (Conrad et al., 1978; Quinton et al., 1996), agenesis of the corpus callosum (Dode et al., 2003; Dode et al., 2006), sensorineural deafness (Coatesworth and Woodhead, 2002; Hill et al., 1992), abnormal eye movements (Schwankhaus et al., 1989; Soderlund et al., 2002), unilateral renal agenesis (Kirk et al., 1994; Wegenke et al., 1975) agenesis of one or several teeth (hypdontia) (de Zegher et al., 1995; Dode et al., 2003; Hardelin et al., 1993b; Molsted et al., 1997) and cleft palate (Molsted et al., 1997; Santen and Paulsen, 1973), may segregate with the Kallmann syndrome phenotype, which suggests a common genetic origin of these abnormalities (Tsai and Gill, 2006). The first underlying genetic defect was identified 47 years after Kallmann's report (Ballabio et al., 1986), later there has been a dramatic increase in the rate of identification of other genes **(Table. 1)**. described below that play an important role in the pathogenesis of KS.

2.1.1. Genes implied in the aetiology of Kallmann syndrome

2.1.2. KAL1

KAL1 gene encodes for anosmin-1, the first protein shown to be involved in X-linked form of kallmann syndrome in humans (Franco et al., 1991; Legouis et al., 1991). The *KAL1* gene comprises 14 exons and encodes an extracellular 680-amino-acid protein with complex structure that includes an N-terminal signal peptide followed by a cysteine-rich region, a whey acidic protein-like domain, four fibronectin-like type III repeats that are homologous to cell adhesion molecules, and several predicted heparin sulfate proteoglycan (HSPG) binding regions (Franco et al., 1991; Legouis et al., 1991). During the development, anosmin-1 is expressed in basement membranes of developing olfactory bulb, retina and kidney (Bribian et al., 2006; Hardelin et al., 1999; Lutz et al., 1994; Lutz et al., 1993; Rugarli et al., 1993). Anosmin-1 is involved in the control of different cell functions, including cell adhesion, neurite/axonal elongation and fasciculation, epithelial morphogenesis as well as in the migratory activity of GnRH neurons (Bulow et al., 2002; Cariboni et al., 2004; Hu et al., 2004; Schwanzel-Fukuda et al., 1989). Various types of *KAL1* abnormalities have been reported distributed throughout the entire gene including missense, nonsense and splice site mutations, intragenic deletions and submicroscopic chromosomal deletions (Albuisson et al., 2005; Balasubramanian et al., 2010; Bianco and Kaiser, 2009; Bouloux et al., 1991; Hardelin, 1997; Hardelin et al., 1993a; Hardelin et al., 1992; Hardelin et al., 1993b; Izumi et al., 2001; Kim et al., 2008a; Legouis et al., 1991; Massin et al., 2003; Matsuo et al., 2000). Patients with *KAL1* mutations usually exhibit an almost uniformly severe and highly penetrant reproductive phenotype. Most patients with X-linked Kallmann syndrome have micropenis and bilaterally undescended testes at birth, reflecting severe congenital GnRH and gonadotropin insufficiency. Bimanual synkinesia, characterized by involuntary upper body mirror movements, may be caused by an abnormal projection of the corticospinal tract connecting the motor cortex with the primary anterior motor neurons in the spinal cord (Conrad et al., 1978; Quinton et al., 1996; Schwankhaus et al., 1989). These *KAL1* abnormalities have been identified in approximately 8–11% of the sporadic and in 14–50% of the familial cases of X-linked Kallmann (Bhagavath et al., 2007) however recently mutations in this gene have been identified along with mutations in other genes supporting the digenic/oligogenic pattern of inheritance (Albuisson et al., 2005; Crowley et al., 2008; Dode and Hardelin, 2009; Hardelin and Dode, 2008; Pitteloud et al., 2010; Pitteloud et al., 2007a; Salenave et al., 2008; Sykiotis et al., 2010a; Sykiotis et al., 2010b). Since rodent homologue of *KAL1* gene has not been identified $Kal1^{-/-}$ mice does not exist to unravel exact mechanisms of this protein, however studies have shown that ansomin-1 co-localizes with fibroblast growth factor receptor-1, in the olfactory bulb during development indicating that it involves in the FGFR1 signaling

cascades (Bribian et al., 2006; Gonzalez-Martinez et al., 2004).

2.1.3. FGFR1 and FGF8

FGFR1 gene containing 21 exons encodes for fibroblast growth factor-1 protein. Human FGFR1 is a member of the receptor tyrosine kinase superfamily (Mason, 2007; Tsai et al., 2011). The prototypical FGFR comprises three extracellular Ig-like domains (D1, D2 and D3), one acid box domain, one transmembrane helix domain, and two intracellular tyrosine-kinase domains (Beenken and Mohammadi, 2009). FGFR1 signaling is achieved by receptor conformational changes upon ligand binding in presence of heparan or heparan sulfate proteoglycan (HSPG), leading to dimerization and subsequent activation by autophosphorylation of the tyrosine-kinase intracellular domains. FGFR1 signaling majorly activates MAP Kinase pathway and regulates cell proliferation, migration, differentiation, and survival essential for various stages of development (Mason, 2007; Tsai et al., 2011). It is expressed in multiple embryonic tissues and organs such as skeletal tissues, inner ear, and rostral forebrain (Bachler and Neubuser, 2001; Ford-Perriss et al., 2001). Several lines of evidence hypothesized that FGF signaling is critical for the development, proper formation and maintenance of a functional GnRH system (Chung et al., 2008; Chung and Tsai, 2010; Falardeau et al., 2008; Gill et al., 2004; Gill and Tsai, 2006; Miraoui et al., 2011; Tsai et al., 2005; Tsai et al., 1995), and it can be modulated by anosmin-1 (Hu et al., 2009). The FGFR1/anosmin-1 connection is supported by the shared clinical findings of patients carrying *KAL1* and *FGFR1* mutations (Pitteloud et al., 2007a).

Of the 22 identified FGF ligands, evidence from mouse studies indicate FGF8 is the most likely FGF candidate for FGFR1 involved in the early development of olfactory/vomeronasal and GnRH systems (Falardeau et al., 2008; Meyers et al., 1998). *Fgf8* mRNA is found as early as E9.5 in the ectodermal region of ventral/lateral of the commisural plate and later in the ventro-medial olfactory placode (Kawauchi et al., 2005). Reduced mRNA levels (54%) in the homozygous hypomorphic Fgf8 mice leads to absence of olfactory bulb, vomeronasal organs and the GnRH neuronal system is completely eliminated in the newborn mutant mice while the heterozygous mice contains approximately 50 % fewer GnRH neurons (Falardeau et al., 2008). In addition, mutations in *FGF8* gene were identified human patients suffering from rare adult onset hypogonadism strongly suggest involvement of FGF8-FGFR1 signaling in kallmann syndrome (Falardeau et al., 2008; Trarbach et al., 2010).

2.1.4. PROKR2 and PROK2

PROK2 gene encodes for 81 amino acid ligand prokineticin 2 (PK2) (also called Bv8), is a secreted bioactive protein possessing 10 conserved cysteines that form five disulfide bonds. *PROKR2* encodes a 384 amino acid G-protein-coupled receptor, prokineticin receptor 2 (PK-R2) (Bullock et al., 2004; Martin et al., 2010). While other *PROKR1* encodes prokineticin receptor 1 is preferentially expressed in the peripheral tissues, PROKR2 is predominantly expressed in the CNS (Masuda et al., 2002; Negri et al., 2007). The binding of PK2 to PK-R2 leads to their coupling to Gq protein, promoting intracellular Ca^{2+} mobilization (Lin et al., 2002; Martucci et al., 2006) and activation of signaling cascades that are important for development of olfactory system and GnRH neuronal progenitors (Martin et al., 2010). Initial studies on knockout mice lacking *Pk2* (*Pk2*$^{-/-}$ mice) (Pitteloud et al., 2007b) and *Prokr2* (*Prokr2*$^{-/-}$) (Matsumoto et al., 2006; Ng et al., 2005) shed lights on their roles in the development of olfactory/GnRH neuronal systems while in contrast the knockout mice lacking *Pkr1* (*Pkr1*$^{-/-}$) (Matsumoto et al., 2006) had no adverse affects on the development of olfactory bulb and GnRH neurons. Both the null mice exhibit olfactory bulb hypoplasia due to altered neurogenesis and decreased GnRH neuron migration to the hypothalamus resulting in adult hypogonadotropic hypogonadism however it has been shown that PROKR2 is not expressed in GnRH neurons suggesting the effect is indirect (Pitteloud et al., 2007b). Later, several mutations in *PROK2* and *PROKR2* genes have been found in heterozygous, homozygous or compound heterozygous state in humans suffering from either kallmann syndrome or normosmic IHH (Balasubramanian et al., 2010; Bhagavath and Layman, 2007; Bhangoo and Jacobson-Dickman, 2009; Bianco and Kaiser, 2009; Cole et al., 2008; Dode et al., 2006; Hardelin and Dode, 2008; Martin et al., 2010; Monnier et al., 2009; Pitteloud et al., 2007b).

2.1.5. CHD7

CHD7 gene encodes the chromodomain helicase DNA-binding protein 7, belonging to a family which shares a unique combination of functional domains consisting of two N-terminal chromodomains, followed by a SWI2/SNF2-like ATPase/-helicase domain and a DNA binding domain (Marfella and Imbalzano, 2007). *CHD7* protein complexes that affect chromatin structure and gene expression is expressed in the olfactory epithelium, the hypothalamus and the pituitary gland, which suggests its important roles development of olfactory bulb and GnRH system during embryonic development (Bosman et al., 2005; Hurd et al., 2007; Sanlaville et al., 2006). Originally mutations in *CHD7* were identified in patients

with CHARGE syndrome, a developmental multisystem autosomal-dominant disorder consisiting of eye *c*oloboma, *h*eart defects, choanal *a*tresia, *r*etardation of growth and development, *g*enito-urinary anomalies, and *e*ar abnormalities (Blustajn et al., 2008; Cortez et al., 1993; Lalani et al., 2006; Pinto et al., 2005; Tellier et al., 1998), but recently mutations in this gene were identified in KS/IHH patients without CHARGE syndrome thus representing a milder allelic variant of CHARGE syndrome (Bianco and Kaiser, 2009; Dode and Hardelin, 2009; Kim et al., 2008b).

2.1.6. WDR11

WDR11 gens encodes for 1,224 amino acid WD repeat-containing protein 11 that belongs to the WD repeat-containing protein family. WD repeats that typically occur multiple times within a protein are approximately 30- to 40-amino acid domains containing several conserved residues, including a trp-asp at the C-terminal end (Chernova et al., 2001; Philipps et al., 2008). These domains are involved in protein-protein interactions and often WD repeats are found in heterotrimeric G proteins and regulatory proteins, such as those involved in cell division, cell-fate determination, gene transcription, mRNA modification, transmembrane signaling, and vesicle fusion (Stirnimann et al., 2010). Recently, (Kim et al., 2010) have found 6 different missense mutations in WDR11 in KS/IHH patients by deletion and linkage mapping of genes near the 10q26 chromosomal breakpoint. Characterization of *Wdr11* gene expression in rats revealed that its highly expressed in the ovary, olfactory bulb, hypothalamic preoptic area and other regions of brain. Yeast two-hybrid screening to identify its putative binding partners revealed that it interacts with EMX1, a homeodomain transcription factor that participates in the development of olfactory neurons and in situ hybridization analysis in mouse embryo revealed a strong expression of *Wdr11* throughout the developing central nervous system right from E10.5 - E14.5 when the GnRH neurons begin to originate and migrate from the olfactory placode. Though the exact function of this protein is unknown, the mutations identified in these patients lead to reduced binding of WDR11 to EMX1 and authors suggested it could be probably be involved in the Shh signaling pathway that terminates with Emx protein (Kim et al., 2010).

2.1.7. NELF

Nasal embryonic LHRH factor (NELF) was isolated from expression profiling of migrating and non-migrating primary rodent GnRH neurons (Kramer and Wray, 2000). High levels of mRNA and protein expression were found in the forebrain, olfactory epithelium, and olfactory

pit of embryos with maximal expression between E12.5 and E14.5 in the olfactory epithelium and olfactory pit. NELF protein expression was demonstrated in the cell soma and processes and appeared to be on the surface of the neurons. Since NELF is present on both olfactory and GnRH neurons, these authors suggested that NELF may serve as a common guidance cue for olfactory axon projections and subsequent migration of GnRH neurons. When nasal explants were treated with antisense oligonucleotides, NELF protein decreased by 60%, GnRH neuron fiber complexity and length decreased, and there was a reduction in the number of GnRH neurons in the periphery of the explant. These findings suggested that NELF may play an important role in GnRH neuron migration. The human ortholog was cloned by (Miura et al., 2004) and, although they identified one heterozygous missense mutation, no functional analysis was performed. Recently, the coexistence of a NELF mutation and an FGFR1 mutation was reported, which together produced IHH (Pitteloud et al., 2007a). No knockout mouse model has been analyzed to date and the exact physiologic role of this protein awaits further studies.

2.2. Normosmic idiopathic hypogonadotropic hypogonadism (nIHH)

While mutations in genes playing an important role in ontogenesis of olfactory / GnRH system leads to kallmann syndrome, mutations in genes playing an important role in pubertal activation, GnRH activation/secretion lead to hypogonadotropic hypogonadism leaving the olfactory system intact. First identification of loss-of-function mutations in GnRH receptor in 1997 by (de Roux et al., 1997) responsible for complete or partial gonodotropic deficiency lead to series of discovery of genes involved in normosmic IHH listed in table 1.

2.2.1. GNRHR

The human GnRH receptor (*GNRHR*) gene maps to chromosome 4q13.2–13.3 and is comprised of three exons that encode a 328-amino acid protein, with >85% homology within mammalian species, with near identity in the transmembrane domains (Cheng and Leung, 2005; Cheng and Leung, 2000). The GnRHR contains seven transmembrane (TM) domains, six of which alternate extra- and intracellular loops with an extracellular amino terminus. However, GnRHR is unique among the rhodopsin family of GPCRs in its lack of an intracellular carboxy terminus. The extracellular domains and superficial regions of the TMs are involved in binding of GnRH, and the TMs are believed to be involved in receptor

configuration and conformational change associated with signal propagation (receptor activation) (Millar et al., 2004; Stojilkovic et al., 1994). These changes are thought to propagate into conformational changes in the intracellular domains involved in interacting with G proteins and other proteins for intracellular signal transduction. GnRH binds to the GnRHR in a hairpin structure with the amino- and carboxy-terminal domains contributing mainly to receptor binding and activation (Karten and Rivier, 1986). Since the original reports of *GNRHR* mutations causing nIHH (de Roux et al., 1997; Karges et al., 2003), a variety of inactivating mutations have been described (Bedecarrats and Kaiser, 2007). Most are missense mutations and a significant number of compound heterozygous changes are seen. The most common mutations occur in the first extracellular and third intracellular loops, although they span across the receptor. The first extracellular loop mutations reduce ligand affinity and the third intracellular loop mutations reduce signal transduction. Recently, a cell-permeant small molecule that was a GnRH antagonist was shown to rescue most of the naturally occurring mutants by increasing their expression, presumably by stabilizing their intracellular processing and transport (Leanos-Miranda et al., 2002). These small molecular 'chaperones' offer future therapeutic options for patients with *GNRHR* and other G-protein coupled receptor mutations. *GNRHR* mutations can account for up to 40% of familial cases of nIHH and perhaps up to 17% of sporadic cases of nIHH (Balasubramanian et al., 2010). Patients with *GNRHR* mutations present with a wide spectrum of reproductive symptoms ranging from severe hypogonadotropism including microphallus and undescended testes in males at birth to failure of pubertal development in adolescence (Beranova et al., 2001) as well as infertility in adults (Balasubramanian et al., 2010; Beranova et al., 2001; Cerrato et al., 2006; de Roux, 2006; de Roux et al., 1997; Karges et al., 2003; Nimri et al.). However, partial defects are also seen with significant variations in phenotypes despite similar genetic functional defects. This variability of clinical phenotypes presumably reflects several issues including the severity of intrinsic disruption of the GNRHR processing and/or function, the dosing of genes involved (heterozygous, biallelic/homozygous or compound heterozygous mutations), the coexistence of mutations in other GnRH deficiency causing genes (oligogenicity) (Pitteloud et al., 2007a), and as yet unapparent epigenetic and environmental factors.

2.2.2. GNRH1

The human *GNRH1* gene is located at 8p21–8p11.2, consists of 4 exons and encodes the preprohormone that is ultimately processed to produce GnRH decapeptide (Cheng and Leung,

2005). Mutation in *GNRH1* is an obvious candidate as an etiology for human GnRH deficiency and in keeping with this, in the hypogonadal (*hpg*) mouse model that competely lacks detectable levels of GnRH leading to low levels of gonadotropins (LH and FSH) and failure of development of testes and ovaries (Cattanach et al., 1977; Mason et al., 1986a; Mason et al., 1986b). In situ hybridization studies show detectable mRNA transcripts of GnRH but immuncytochemical analysis fail to detect any GnRH antigen in these mice. Detailed analysis of the GnRH gene locus revealed a deletion of 33.5 kilobases encompassing the distal half of the *Gnrh1* gene encoding the common biosynthetic precursor of GnRH and GnRH-associated peptide that results in active transcription of the gene but translationally incompetent mRNA resulting in the absence of decapeptide (Cattanach et al., 1977; Mason et al., 1986a; Mason et al., 1986b). Recent studies on homozygous *hpg*/ GFP-positive mice have shown normal distribution of GnRH neurons in the septopreoptic area, projections of GnRH neurosecretory axons to the median eminence and normal electrophysiological properties of GnRH-GFP neurons establishing that autocrine-paracrine GnRH-signaling is not required to the developmental migration of GnRH neurons into the brain and GnRH peptide itself is mandatory for gonadotropins releasing function and absence of GnRH as in the *hpg* mice results in abnormal reproductive function leading to hypogonadotropic hypogonadism (Gill et al., 2008). Although human *GNRH1* mutations have been elusive for many years, two independent groups have recently described homozygous frameshift *GNRH1* mutations in patients with GnRH deficiency (Bouligand et al., 2009a; Bouligand et al., 2009b; Chan et al., 2009b). In both studies, consistent with the critical role played by GnRH, subjects with homozygous mutations in *GNRH1* showed severe hypogonadism with affected males having microphallus. In addition to homozygous frameshift mutations, heterozygous rare sequence variants in *GNRH1* have also been described in one of these studies. As seen with other genes implicated in GnRH neuronal ontogeny, it is likely that oligogenicity and genotypic synergy with known/unknown genes may operate to produce the phenotype. No non-reproductive features were reported in these patients.

2.2.3. KISS1R / GPR54

In 2003, using linkage analysis in inbred families, two groups independently identified *GPR54*, a G protein-coupled receptor, and its cognate ligand, kisspeptin, to be upstream gatekeepers of GnRH neurons (de Roux et al., 2003; Seminara et al., 2003). Coupled with complementary mouse genetics and in vitro confirmation of loss of biological activity of the mutant receptor protein, *GPR54* was implicated as a key regulator of puberty and a number of

mutations in this receptor have now been described in nIHH patients (Balasubramanian et al., 2010). Review of human and mouse *KISS1R* investigations have provided fascinating insights into the role of *KISS1/ KISS1R* biology in human reproduction that is described later in this section. Both *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ mice are phenocopies of nIHH and interestingly show normal GnRH content in the hypothalamus, providing the first indication that mutations in *KISS1R* do not affect GnRH neuronal migration or GnRH synthesis but rather GnRH release (d'Anglemont de Tassigny et al., 2007b; Seminara et al., 2003). Neuroendocrine profiling of probands with *KISS1R* mutations has generally shown dampened but present low amplitude LH pulses suggesting some degree of endogenous GnRH secretion that is synchronized but reduced in pulse amplitude (Chan et al., 2009a; Tenenbaum-Rakover et al., 2007). An African-American proband with a compound heterozygous mutation in *KISS1R* also showed a striking leftward shift in his LH dose-response relationship to exogenous GnRH. This observation suggests some degree of endogenous pituitary priming by intact but dampened GnRH pulsatility. In one published report of a male with *KISS1R* mutation who presented with cryptorchidism and micropenis in infancy, neuroendocrine evaluation at 2 months of age revealed undetectable gonadotropins also suggesting a role for the *KISS1/KISS1R* system in the ‘mini-puberty’ of infancy (Semple et al., 2005). Some patients with *KISS1R* mutations are able to undergo folliculogenesis, spermatogenesis and successful pregnancies following therapy with exogenous GnRH, suggesting some degree of intact pituitary gonoadotropin function with no significant primary gonadal defects or defects in placentation in these individuals (Pallais et al., 2006). Although a variety of diverse phenotypes is often seen in human GnRH-deficient subjects, nonreproductive phentoypes have yet to be described in subjects with *KISS1R* mutations.

Although mutations in the *KISS1/KISS1R* pathway do not seem to be a significant contributor to human GnRH deficiency (<5%), their relative rarity probably suggests an evolutionarily critical role in reproduction and species propagation that might well have undergone negative selection. The recently reported documentation of a ligand independent gain-of-function mutation in GPR54 in a girl with idiopathic central precocious puberty demonstrates the crucial role of this system in the maturation of the reproductive axis (Teles et al., 2008). Thus, the discovery of this previously unsuspected pathway in human reproduction that has now been discovered to represent a key control point of GnRH at sexual maturation in all mammals studied to date has ignited a burst of basic and clinical research into the *KISS1/KISS1R* axis in mammalian physiology. This system offers a further approach to

therapeutically modulate human GnRH function and potentially translate the biology of this pathway into wider clinical applications across diverse reproductive phenotypes.

2.2.4. TAC3 and TACR3

TAC3 gene encodes for neurokinin B and *TACR3* encodes for G protein-coupled receptor, neurokinin B receptor (NK3R). The tachykinins (TAC) are a family of evolutionarily conserved peptides that are widely distributed within the mammalian peripheral and central nervous systems and play a well-recognized role as excitatory neurotransmitters (Almeida et al., 2004). The tachykinins are also present in non-neuronal cells and in non-innervated tissues, and they have been implicated in the regulation of many physiological and pathological processes. Neurokinin B belongs to a phylogenetically conserved family of proteins which also includes substance P, neurokinin A and hemokinin-1 (Pennefather et al., 2004). Tachykinins mediate their biological actions through three distinct G-protein-coupled receptors, denoted NK1, NK2 and NK3 (Almeida et al., 2004). The NK3R, mainly expressed in the CNS (central nervous system), is the most selective of the tachykinin receptors, with highly preferential binding and activation by NKB. Recently (Topaloglu et al., 2009) using autozygosity mapping through use of genome-wide SNP genotyping in familes with affected individuals with nIHH identified homozygous loss-of-function mutations in either *TAC3* or *TACR3*. This human genetic investigation highlights a hitherto unrecognized role of the TAC3/TACR3 pathway in regulation of the GnRH pulse generator. Male patients with *TACR3* mutations characteristically have micropenis and fail to enter puberty during adolescence. These reproductive phenotypes strongly suggest an important role of the TAC3/TACR3 pathway in both the 'mini-puberty' of infancy and gonadotropin activation at puberty. None of the patients reported in the first human study had KS, suggesting a primary role of this TAC3/TACR3 pathway in functional integrity of the GnRH pulse generator. The receptor TACR3 is expressed on the GnRH axons and the KNDy (kisspeptin, neurokinin and dynorphin) neuronal population and neurons coexpressing KISS1 and TAC3 have been described in the arcuate nucleus and postulated to be the primary pathway mediating the sex-steroid feedback to the GnRH neurons (Ciofi et al., 1994; Ciofi et al., 2007; Goodman et al., 2007; Goubillon et al., 2000). However, murine deletion of the murine ortholog of *TACR3* has not been associated with reproductive abnormalities as seen in humans (Kung et al., 2004). Recently, Gianetti et al. have reported several *TAC3/TACR3* mutations in a large outbred cohort of patients with nIHH (Gianetti et al., 2010). In this detailed report, micropenis was observed in a majority of male subjects. In addition, evidence of neuroendocrine recovery of

the hypothalamo-pituitary-gonadal axis was observed in a significant number of male and female subjects during adulthood. These observations strongly suggest that the TAC3/TACR3 signaling pathway is critical during the neonatal period and puberty, but is relatively less-critical in adulthood . No nonreproductive features have been reported in patients with *TACR3* mutations, but *TAC3* mutation have been associated with learning disabilities. In addition, the potential oligogenic interactions of the *TAC3/TACR3* pathway with the other genes related to GnRH ontogeny will need to be determined. The hypothalamic interplay between KISS1 and NKB is already well recognized and this interaction is likely to be a subject of intense study in the coming years (Rance et al., 2010).

2.2.5. LEP and LEPR

Leptin, encoded by *LEP*, is a fat-derived hormone that regulates food intake, energy expenditure and reproduction at the hypothalamic level. Exogenous administration of leptin accelerates puberty in mice and normalizes reproductive deficiencies in leptin-null (*ob/ob*) mice, which suggests that leptin may be a link between body fat and reproductive capability (Chehab, 1996; Chehab et al., 1996). Accordingly, loss of body fat owing to starvation or excessive exercise is known to suppress reproduction and results in amenorrhea and infertility (Licinio, 1997). Likewise, inactivating mutations in *LEP* (Strobel et al., 1998) or the gene encoding its receptor, *LEPR* (Clement et al., 1998), have been found in patients with severe obesity and hypogonadotropic hypogonadism. These inactivating mutations account for less than 5% of normosmic IHH and have an autosomal recessive pattern of transmission (Table 1). The central role of leptin in these cases is highlighted by the recovery of gonadotropin secretion and menstrual cycles after treatment with recombinant leptin in females with amenorrhea due to congenital leptin deficiency (Licinio et al., 2004) or hypothalamic amenorrhea (Welt et al., 2004).

In humans, leptin has a permissive role in the control of reproduction, being necessary but not sufficient for the onset of puberty and maintenance of fertility. Evidence from studies of animal models points that GnRH neurons themselves do not express receptor for action of leptin and hence require other cell types to mediate the reproductive effects of leptin (Quennell et al., 2011); (Donato et al., 2011a; Donato et al., 2011b).

3. Emerging concept of Digenic / Oligogenic inheritance involved in pathogenecity of KS. More KS/nIHH genes to be discovered.

IHH has been classically considered a monogenic disorder with Mendelian inheritance pattern. In light of the report by Pitteloud et al., 2007, where two IHH families were described, one with Kallmann syndrome with FGFR1 and NELF mutations and another with normosmic IHH with GNRHR and FGFR1 mutations, a possible digenic model causing the IHH phenotype was proposed (Crowley et al., 2008; Pitteloud et al., 2010; Pitteloud et al., 2007a; Sykiotis et al., 2010b). Coexistence of mutations in PROKR2 and KAL1 genes and in PROKR2 and PROK2 genes has also been described in cases of Kallmann syndrome (Dode et al., 2006; Guimiot et al.; Hardelin and Dode, 2008; Leroy et al., 2008). Defects in different genes appear to act synergistically to modify the severity of the GnRH deficiency, partially explaining the wide phenotypic variability observed within and across families with IHH and Kallmann syndrome. Moreover, the genes identified so far account only for 30% of the KS/nIHH case indicating other genes could be involved.

Table 3. Examples of digenic interactions in hypogonadotrophic hypogonadism

Gene 1	Gene 2	Reference
PROK2	*PROKR2*	Cole et al.35
FGFR1	*NELF*	Pitteloud et al.40
GNRHR	*FGFR1*	Pitteloud et al.40
FGF8	*FGFR1*	Falardeau et al.25
KAL1	*PROKR2*	Canto et al.

[Adapted from (Semple and Topaloglu, 2010)]

Here we list probable candidate genes that have shown to play an important role in ontogenesis of olfactory / GnRH system or in regulation of HPG axis in adults in rodents.

4. Factors involved in ontogenesis of olfactory/GnRH system during embryonic development

4.1. PSA-NCAM

NCAM (Neural cell adhesion molecule) and is a glycoprotein of Immunoglobulin (Ig) superfamily. PSA-NCAM with its long α 2,8-linked sialic acid polymer (PSA), has been shown to serve as an overall negative regulator of cell interactions (Brennaman and Maness, 2010; Panicker et al., 2003), probably through the ability of this large carbohydrate to interfere with intermolecular and/or membrane–membrane contacts. PSA-NCAM is abundantly expressed in the developing embryo and plays a key role in both regulation of axon tract formation and migration of cells (Fryer and Hockfield, 1996). Enzymatic removal of PSA with PSA-specific endoneuraminidase N (endo N) or mutant NCAM, inhibit migration of neurons from the subverntricular zone of the lateral ventricle to the olfactory bulb (Yoshida et al., 1999). PSA-NCAM is highly expressed by the olfactory axons and VNN exiting the VNO. Acute removal of the PSA by enzymatic digestion blocked GnRH neuron migration in vivo however, evaluation of NCAM and NCAM-180 null mice revealed no significant disruption of GnRH neuron migration. These discrepant results may have been due to the redundancy of the NCAM subtype system. These findings shed light on the role of NCAM on migration of GnRH neurons.

4.2. Hepatocyte Growth Factor (HGF) / Met signaling

Met is a membrane tyrosine kinase receptor activated by the cytokine, HGF, shown to induce mitogenic, migratory and chemoattractant activities in multiple neuronal populations (Ebens et al., 1996; Maina et al., 1997; Yamamoto et al., 1997). 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 showed initially that HGF promotes cell migration and motility in GnRH neuronal cells (GN11) (Giacobini et al., 2002), then in vivo, the potential importance of HGF/Met signaling to normal GnRH neuron development (Giacobini et al., 2007). 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 (i.e. deficient in active HGF) and documented a 35% decrease in the number of GnRH neurons at 60–90 d postnatally, associated with subfertility and decreased gonadotropin-induced frequency of ovulation. 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 below. No human mutations in this gene/pathway has been detected so far.

4.3. Cholecystokinin-8 / CCK-1R

Cholecystokinin (CCK), originally discovered in the gastro-intestinal tract is one of the most abundant neuropeptides in the brain (Vanderhaeghen et al., 1981; Vanderhaeghen et al., 1975). Recently Giacobini et al., 2004 have characterized the role of this peptide in the migratory behavior of GnRH neurons during embryonic development (Giacobini et al., 2004). They have reported that during embryonic development, CCK ligand and its receptors CCK-1R and CCK-2R were expressed by the developing olfactory/vomeronasal neurons and along the fibers projecting to the presumptive olfactory bulb. The GnRH neurons that expressed only CCK-1R but not CCK ligand or CCK-2R were found migrating along the CCK immunoreactive fibers. Functional analysis using embryonic nasal explants revealed that CCK inhibited both olfactory axons outgrowth and GnRH neurons migration and this was mediated specifically throught CCK-1R and not CCK-2R. Studies on mutant mice at E14.5 showed that while the mice lacking CCK-2R receptor (*Cck-2r*$^{-/-}$) had normal development like the wild type mice, mutant mice lacking CCK-1R receptor (*Cck-1r*$^{-/-}$) had accelerated GnRH neurons migration into the forebrain areas suggesting CCK/CCK-1R interactions are important modulators of GnRH neuron migration during development. However, in the adult mutant mice 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. (Giacobini and Wray, 2007). No human mutations in this gene/pathway has been detected so far.

4.4. SDF-1 / CXCR4

Stromal cell derived factor-1 (SDF-1 renamed as CXCL12) has been known to act as a chemoattractant for granule cell precursors and regulates migration of sensory cell, cerebral and cerebellar precursors (Belmadani et al., 2005; Peng et al., 2004). Mice lacking either

SDF-1 or CXCR4 show aberrant cerebellar development and neuronal proliferation (Klein et al., 2001; Ma et al., 1998). CXCR4 is also expressed by neurons in the embryonic olfactory system (Tissir et al., 2004). SDF-1 is expressed in the nasal mesenchyme beginning at E10 in mice in a gradient spread across the caudal half of the nose , 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 as studied by double-label in situ hybridization (Schwarting et al., 2006). The migration of GnRH neurons was severely impaired in CXCR4 null mice. 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 (Giacobini et al., 2007), provides another example of the complex interactions among signaling systems during GnRH-1 neuron development. No human mutations in this system have been reported.

4.5. GABA

The 67 kDa isoform of glutamic acid decarboxylase (GAD 67), encoded by Gad1, accounts for around 90% of γ-aminobutyric acid (GABA) synthesis in the mouse brain (El Mestikawy et al., 2011). Consistent with the heterogeneity of GnRH neuronal population, only a subset of GnRH neurons (~ 30%) contain GABA during development (Tobet et al., 1996). In addition to its role as an inhibitory neurotransmitter, GABA can also inhibit the migration of GnRH neurons from olfactory placode explants (Fueshko et al., 1998). Transgenic mice have been used to study the significance of this observation in vivo. Over-expression of a glutamic acid decarboxylase (GAD-67) transgene in GnRH neurons gives a more dispersed distribution of GnRH neurons in the forebrain but does not prevent puberty although estrous cyclicity and female fecundity are compromised (Heger et al., 2003). GAD67 knock out mice show faster GnRH neuronal migration from the nasal placode at E14.5 but effects on puberty cannot be

studied as the mice die as neonates (Lee et al., 2008). These results show that GABA production within the GnRH neuronal network can alter the migratory fate of these cells in vivo.

4.6. OTX2

Orthodenticle homeobox 2 (OTX2) is a homeodomaain protein necessary for early development of the head during gastrulation, and later, specifies anterior head structures particularly forebrain. Loss-of-function of *Otx2* results in malformation of the head and eyes (Hide et al., 2002; Wyatt et al., 2008). Later in development, OTX2 has been localized to migrating neuronal populations in the thalamus (Inverardi et al., 2007) and plays a continuing role in eye development and plasticity (Rebsam and Mason, 2008; Sugiyama et al., 2008). OTX2 is among the regulatory proteins that have been shown to be expressed in GnRH neurons, control expression of murine GnRH by binding to the conserved Otx2 binding sites in the proximal promoter of GnRH both *in vitro* and *in vivo* (Kelley et al., 2000; Kim et al., 2007; Larder and Mellon, 2009). Generation of conditional knockout mice with *Otx2* specifically in GnRH neurons (GnRH-*Otx2* KO) shed more lights on the regulatory role of this gene in GnRH neurons development and adult reproductive function (Diaczok et al., 2011). Due to programmed cell death of GnRH neurons these mice had fewer GnRH neurons crossing forebrain and entering into the ventral hypothalamus at E14.5, E15.5 and Postnatal day 1 resulting in total decreased numbers in the adult brains. Females mice displayed delayed puberty onset, decrease in GnRH and LHβ mRNA levels as well as a reduction in sex steroid hormone levels associated with persistent estrus, failure to generate a LH surge, and absence of the corpora lutea. These results along with recent identification of mutations in *Otx2* found in patients with anophthalmia, pituitary hypoplasia, and a combined pituitary hormone deficiency, including disorders of puberty and hypogonadism demonstrate the critical role that *Otx2* plays in GnRH neuronal function and mammalian reproduction (Dateki et al., 2008; Dateki et al., 2010; Diaczok et al., 2008).

4.7. Axl

Axl belongs to the TAM (Tyro3, Axl and Mer) family of receptor tyrosine kinases that are comprised of an extracellular domain characteristics of neural cell adhesion molecules, containing fibronectin and Ig repeats as well as an intracellular kinase (Lai and Lemke, 1991; Lemke and Rothlin, 2008; Linger et al., 2008). Growth arrest specific gene 6 (Gas6), specific

ligands induce the formation of hetero as well as homodimers of TAM family of receptors for receptors and these interactions have been implicated in immune modulation, sexual function and tumorigenesis (Hafizi and Dahlback, 2006). Recently, (Pierce et al., 2008) showed that Axl and Tyro3 were expressed in early migratory NLT GnRH neuronal cells, while Tyro3 and Mer were expressed in the postmigratory GT1-7 GnRH cells. In NLT cells, Growth arrest specific gene 6 (Gas6), induced migration via a novel p38 MAP kinase pathway and also increased their survival from programmed cell death via ERK/MAP kinase and PI3-kinase/Akt signaling pathway, 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 overall number of GnRH neurons, 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. 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. 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 and abnormal LH surge mechanism. Examination of mice null for the ligand, Gas6 can allow to gain further insights if it is necessary 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 (X.M. Knox Aj, 2009). Gas6 is also a heparan sulfate proteoglycans activated ligand similar to FGFs and HGF. Whether TAM family members crosstalk with other growth factor receptor pathways or other steps in GnRH neuronal migration is under active investigation.

4.8. Reelin / ApoER2 / Lrp8

Reelin is an extracellular glycoprotein shown to be involved in neuronal migration in several brain regions (Bernier et al., 2000; Caviness and Sidman, 1972; Caviness et al., 1972; D'Arcangelo, 2005; D'Arcangelo et al., 1995). Reelin is abundantly expressed in the rodent

olfactory system during development in the main and accessory olfactory bulb, vomeronasal organ and in VNN prompting its probable role in migratory events of GnRH neurons (Ikeda and Terashima, 1997; Teillon et al., 2003). Recent studies by Cariboni et al., showed that only a small population (5%) of GnRH neurons expressed only one of the reelin receptors (ApoER2/Lrp8) but not Vldlr and neither its intracellular adaptor protein Dab1, however the GnRH cell lines GN11 showed expression of Vldlr mRNA (Cariboni et al., 2005). Using the mutant *reeler* mice that lack Reelin they have shown a decrease in forebrain GnRH neurons associated with their previously known decreased fertility and mutant mice lacking other receptors ApoER2/Vldlr (*ApoER2*$^{-/-}$/*Vldlr*$^{-/-}$) or intracellular adpaptor protein Dab1 (*Dab1*$^{-/-}$) had normal distribution of GnRH neurons in adult brains and fertility. Finally, they concluded that effect of Reelin on GnRH neurons migration could be indirect and independent of Dab1. No human mutations in this pathway have been reported.

4.9. Ephrin / Ephrin receptor

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 (Flanagan and Vanderhaeghen, 1998). The potential importance of this signaling system in GnRH neuron development was implicated by the analysis of the GNR23 mutant mouse in which a 67 kb deletion downstream of the subtype 5 EphA receptor (EphA5) gene resulted in overexpression of EphA5 (Gamble et al., 2005). Analysis of these mutant mice revealed deficit in migratory behavior of GnRH neurons. Abnormal clusters of GnRH neurons were found along the olfactory axons in their initial migratory route and only 12% of GnRH neurons reached their final destinations in the hypothalamus. The authors hypothesize that the over-expression of EphA5 results in abnormal increased adhesion of GnRH neurons to the olfactory fibers that express the Ephrin ligands. Despite a few numbers reach hypothalamus in these mice the male mice exhibited normal fertility. In contrast, female mutant mice were markedly subfertile with irregular estrous cycles, failing to ovulate and produce normal litters despite normal occurence of first estrous cycle at puberty. 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.

4.10. Netrin / DCC

Netrins are versatile guidance molecues that act as chemoattractant or chemorepellant in different aspects of developing nervous system (Killeen and Sybingco, 2008). Two mammalian netrin-1 receptors have been identified: Deleted in colon cancer (DCC), a transmembrane protein belonging to the Ig superfamily and Unc5h3 (Rajasekharan and Kennedy, 2009). DCC protein is heavily expressed in the olfactory system as early as E14 in rats but declines by E18 in the lateral olfactory tract (Livesey and Hunt, 1997). DCC mRNA is also detected in the rat olfactory epithelium and precurors and in addition netrin-1 is expressed in the hypothalamus and median eminence suggesting a probable role in GnRH neurons development (Deiner and Sretavan, 1999). Recently, Schwarting et al., 2000 have shown that DCC is expressed by the cells in the VNO, along the VNN and also in subset of GnRH neurons present in the nose (Schwarting et al., 2001; Schwarting et al., 2004). Analysis of the VNN axon trajectories and position of GnRH neurons in DCC mutant mice (*Dcc*$^{-/-}$) showed that DCC negative c-VNN axons failed to turn ventrally in the forebrain, and instead were detoured into the cerebral cortex. This altered the migration of GnRH neurons into the cerebral cortex that followed the misguided VNN instead of reaching ventral forebrain areas. The alterations in axon trajectories and cell migration seen in *Dcc*$^{-/-}$ and Netrin-1 (*Ntn*$^{-/-}$) 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. This effect was not oberved in the mice lacking Unc5h3 (*Unc5h3*$^{-/-}$), the alternative netrin-1 receptor, showing the specificity of the ligand/receptor interaction (Schwarting et al., 2004). It could have been worth to sudy the adult reproductive behavior of these mutant mice but unfortunately they die soon after birth. No human mutations in this pathway have been reported.

4.11. Semaphorins

Semaphorins are secreted or membrane associated glycoproteins that have been grouped into eight classes on the basis of their structural elements and amino acid sequence similarity **(Figure. 4)** (Capparuccia and Tamagnone, 2009; Derijck et al., 2010; Pasterkamp and Giger, 2009; Zhou et al., 2008). The semaphorins found in invertebrates are grouped in classes 1 and 2; classes 3–7 are vertebrate semaphorins; and the final group is encoded by viruses.

Individual proteins are designated by a letter code - for example, Sema3A and Sema4D. There are currently descriptions of more than 20 types of semaphorin. All semaphorins contain a conserved ~400 amino acid ‘Sema’ domain. The central feature of this structure is a seven-blade β-propeller fold with overall structural similarity to the β-propeller repeats of α integrins. The Sema domain is also found in plexins and the receptor tyrosine kinases Met and Ron. The semaphorins range in size from 400 to 1000 amino acids, depending on what other domains they have in addition to the Sema domain and a PSI (plexins, semaphorins and integrins) domain. C-terminal to the Sema and PSI domains, a single immunoglobulin (Ig)-like domain is found in semaphorin classes 2, 3, 4 and 7, whereas class 5 semaphorins have seven thrombospondin domains. Proteins in semaphorin classes 1, 4, 5, 6 and 7 are membrane-associated, whereas those in classes 2 and 3 and the viral semaphorins are secreted. Of the transmembrane semaphorins, class 6 proteins have the largest intracellular domains by far (~400 amino acids), all of which contain proline-rich motifs. Class 4 semaphorins frequently have PDZ-domain binding motifs at their C termini.

4.11.1. Semaphorin and their receptors

Semaphorins were initially characterized for their role in mediating axon guidance during development but recent tremendous amount of work lead to identification of semaphorins in in wide range of processes like regulation of cell adhesion and motility, angiogenesis, immune responses and tumor progression (Casazza et al., 2007; Comoglio et al., 2004; Kruger et al., 2005; Mann et al., 2007; Mann and Rougon, 2007; Neufeld and Kessler, 2008; Pasterkamp and Giger, 2009). Being a large family of secreted and membrane bound molecule, semaphorins mediate their action through multiple receptors/receptor complexes of which plexins and neuropilins are the most prominent high affinity receptors **(Figure. 4)**. Plexins are large, phylogenetically conserved, type 1 transmembrane proteins. While invertebrate genome contain only 2 plexin genes, nine plexin genes divided into four classes Plexin-A (1-4), Plexin-B (1-3), Plexin-C1 and Plexin-D1 are identified in vertebrates. Plexins function as both ligand-binding and as signaling receptors for semaphorins. Similar to their ligands, plexins contain a sema-like domain in their extracellular portion that mediate most of their interactions with their ligands; in addtion, they have two to three repeated PSI domains (**P**lexins, **S**emaphorins and **I**ntegrins) and three IPT domains (**I**g-like fold shared by **p**lexins and **t**ranscription factors).

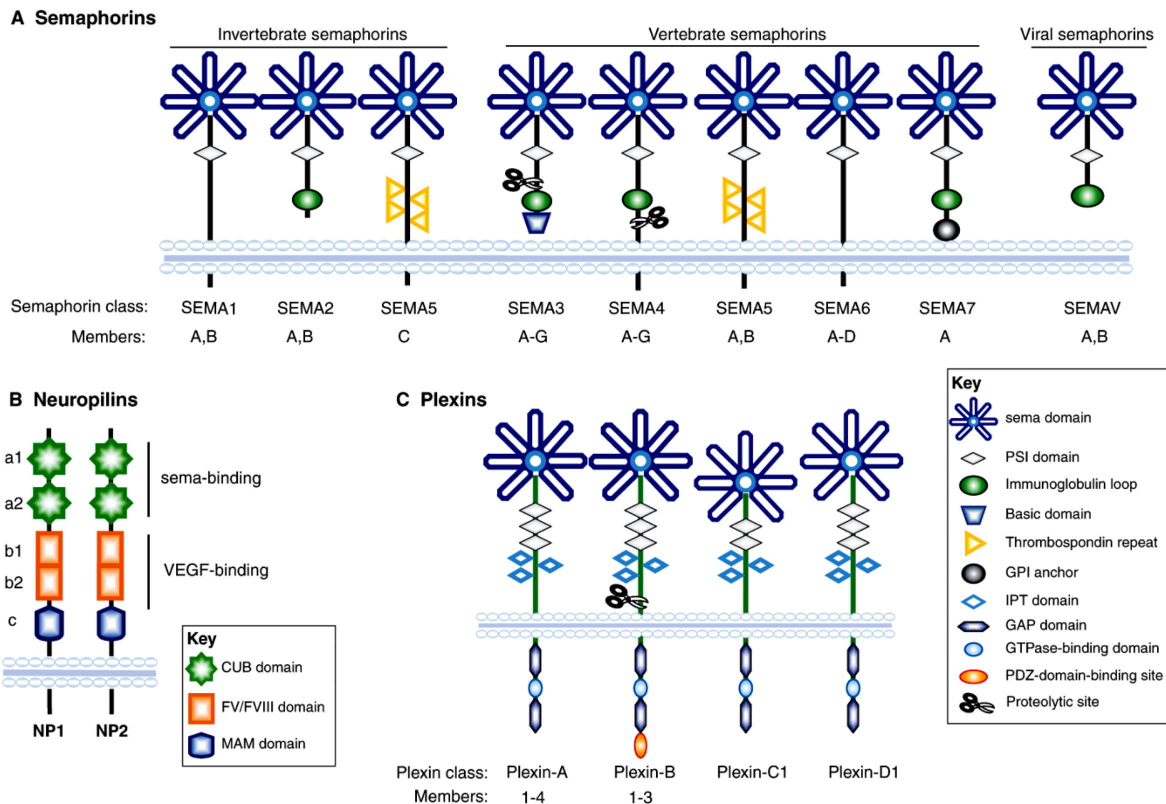

Figure 4. Schematic representation of semaphorins and their receptors (neuropilins and plexins), divided into subfamilies on the basis of sequence similarity and structural features. (A) All semaphorins are characterized by a large sema domain and one PSI domain (a cysteine-rich motif). Of the vertebrate semaphorins, those in classes 4, 5 and 6 are transmembrane proteins, whereas those in class 7 are membrane-anchored via glycophosphatidylinositol (GPI). Class-3 secreted semaphorins have C-terminal basic-charged sequences, which are required for binding to neuropilins. Several class-3 semaphorins and SEMA4D have been shown to undergo regulatory cleavage by furins or metalloproteases. Class-5 semaphorins are distinguished by thrombospondin repeats. Several semaphorins also contain immunoglobulin-like domains. (B) Neuropilins are transmembrane receptors that are characterized by two complement-like (CUB) domains (also called the a1 and a2 domains), two FV/FVIII coagulation factor-like domains (also called the b1 and b2 domains) and a meprin-like MAM domain (also called the c domain). (C) Plexins contain one sema domain, two to three PSI domains and three IPT (Ig-like fold shared by plexins and transcription factors) domains. The cytoplasmic domain of plexins contains two amino acid stretches that are weakly similar to GTPase-activating proteins (GAPs). In addition, B-subfamily plexins contain a C-terminal binding site for PDZ (PSD-95/Dlg/ZO-1) domains. A cleavage site for furin-like pro-protein convertases is also present in the extracellular domain of plexin-B1 and B2, generating heterodimeric receptors (Artigiani et al., 2003). [Adapted from (Capparuccia and Tamagnone, 2009)]

Neuropilins (Nrp1 and Nrp2), which are found only in vertebrates, are single-span type I transmembrane glycoproteins that share a similar domain structure act as obligatory co-receptors along with plexins for the secreted semaphorins (Sema3s) of class 3 and for the members of vascular endothelial growth factor (VEGF) family (Appleton et al., 2007; Cheng et al., 2001; Giger et al., 1998; Gu et al., 2003; Kolodkin et al., 1997; Maier et al.; Polleux et al., 1998; Schwarz et al., 2004; Tamagnone et al., 1999; Wu et al.; Yaron et al., 2005) . For example, Nrp1-Plexin-A1/A4 complexes are principal Sema3A receptors while Nrp2-Plexin-A3 complexes mediate the action of Sema3F (Yaron et al., 2005). Sema3E, in contrast to the other Sema3s, binds directly only to Plexin-D1 and is involved in guidance of growing axons and migrating endothelial cells. In addition to neuropilins, a number of co-receptors have been described that do not directly support semaphorin binding but are part of a functional semaphorin holoreceptor complex. Additional transmembrane molecules are found in semaphorin-receptor complexes in association with plexins and Nrps, including cell-adhesion molecule L1 (Castellani et al., 2002) and the receptor tyrosine kinases (RTKs) VEGFR2 (Toyofuku et al., 2004), erythroblastic leukemia viral oncogene homolog 2 (ErbB2) (Swiercz et al., 2004), off-track kinase (OTK) (Winberg et al., 2001) and Met (also known as HGFR) (Giordano et al., 2002). Recently, other ligands for Nrps have also been described, including fibroblast growth factor 2 (FGF2) (West et al., 2005), hepatocyte growth factor (HGF) (Sulpice et al., 2008) and galectin-1 (Gal-1) (Hsieh et al., 2008). In addition, Nrps were recently observed to form complexes with additional cell-surface receptors, including Met (Matsushita et al., 2007), β1 integrin (Fukasawa et al., 2007) and transforming growth factor-β1 (TGF-β1) (Glinka and Prud'homme, 2008).

Action of class 4 Semaphorins (Sema4A-4D) is mediated by the PlexinBs. PlexinBs are known to be associated with RTKs like Met, ErbB2 and OTK. For example, Sema4D-plexinB1 binding stimulates the tyrosine kinase activity of Met or ErbB2 required for activation of downstream signaling pathways. In the immune system, CD72 and Tim2 (T-cell immunoglobulin and mucin domain-containing protein 2) were found to interact functionally (although with low affinity) with the transmembrane semaphorins Sema4D and Sema4A, respectively (Suzuki et al., 2008).

Biological function and signaling of class 5 semaphorins (Sema5A and Sema5B) is rudimentary. Sema5A mediate its action through plexin-B3-Met receptor complex signaling (Artigiani et al., 2004). In addition, heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) can modulate the action of Sema5A to attraction or repulsion,

respectively (Kantor et al., 2004).

Unlike class 3 semaphorins, membrane bound class 6 semaphorins bind to plexinAs in an Nrp-independent manner involving a unique bidirectional signalling mechanism i.e, they can not only act as ligands and also as receptors themselves. For example, Sema6D can act as ligand and receptor to plexin-A1 during the chick cardiac development (Toyofuku et al., 2004).

The only GPI-linked semaphorin, Sema7A, uses integrin receptors (specifically integrin β1) to exert its neurite growth promoting and branching effects (Pasterkamp et al., 2003; Suzuki et al., 2007). In addition to integrins, Sema7A also binds plexin-C1 (Walzer et al., 2005).

The repertoire of semaphorin plexin signaling may be further expanded by the ability of plexins belonging to different subclasses to form heterodimers. In addition, transmembrane semaphorins and plexins may interact in cis, leading to altered downstream signaling events. While the functional significance of plexin–plexin interactions and semaphorin–plexin cis associations needs further characterization, they provide important clues about the diversity of interactions in which semaphorins and their receptors may participate (Derijck et al., 2010; Pasterkamp and Giger, 2009).

4.11.2. Sema3A signaling

Class 3 semaphorins (Sema3A–Sema3G) have been identified as axon repellents and, in a few cases, axon attractants that control the formation of neuronal connections in vivo. Accumulating evidence now demonstrate that Sema3s and their receptors plexins are also expressed in non-neuronal tissues and regulate cardiac morphogenesis, angiogenesis, organogenesis and cancer through their effects on cell growth, survival, migration and proliferation (Yazdani and Terman, 2006). Sema3s do not interact directly with plexinA receptors but, instead, bind Nrp1 or Nrp2 to assemble and activate Nrp–plexinA holoreceptor complexes. In this complex, plexinAs serve as signal-transducing subunits. Several molecules in different pathways have been characterised and identified that are downstream signaling units to the Nrp-Plexin receptor complexes.

One of the best characterized intracellular signaling events downstream of repulsive semaphorins is ligand binding-induced R-Ras inactivation mediated by the plexin RasGAP (GTPase-activating protein) domain **(Figure. 5)** (Pasterkamp, 2005). PlexinA GAP activity is regulated by FARP2-mediated Rac1 activation [FARP2 (or FERM, RhoGEF and pleckstrin domain protein 2) is a RacGEF (guanine nucleotide exchange factor)]. In presence of Nrp1,

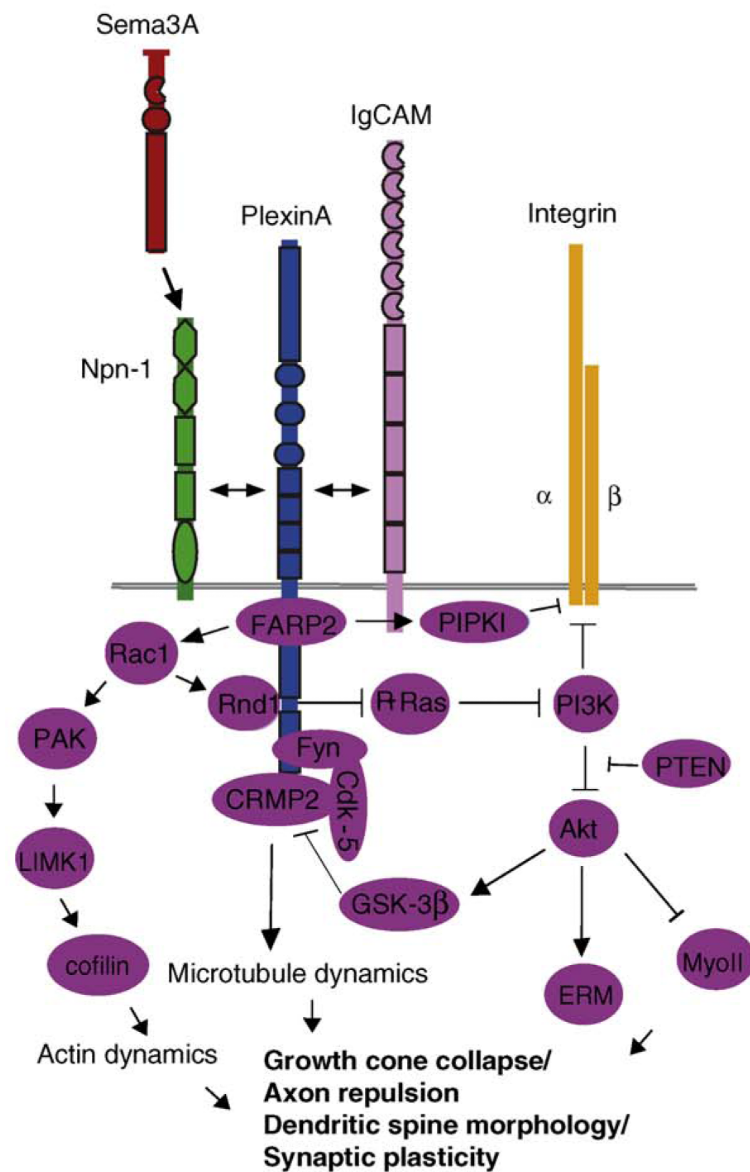

Figure 5. Sema3A binding to the Npn–plexinA complex promotes FARP2 dissociation from plexinA. Dissociated FARP2 activates Rac1, which facilitates Rnd1–plexinA associations and drives PIPKIg661-mediated inhibition of integrin function. Active Rac1 also controls actin dynamics through a PAK-LIMK1-cofilin pathway. Rnd1–plexinA interactions stimulate plexinA RasGAP activity that suppresses R-Ras and inactivates PI3K–Akt signaling. Interestingly, Sema3s also regulate PI3K–Akt through PTEN. Downregulation of PI3K–Akt signaling leads to the inhibition of integrin-mediated adhesion, activation of myosin II (MyoII), and reduced phosphorylation of ERM and GSK-3b. Phosphorylation of CRMP2 by GSK-3b inactivates CRMP2 and relies on a Cdk5-dependent and Fyn-dependent priming phopshorylation. CRMP2 regulates microtubule dynamics. [Adapted from (Zhou et al., 2008)]

FARP2 associates directly with Plexin-A1, after Sema3A exposure, FARP2 dissociates from plexinA1 and activates Rac1 in neuronal growth cones. Active Rac1 facilitates the association of Rnd1, a small GTPase, with plexinA1 and might modulate actin dynamics through the sequential activation of p21-activated kinase (PAK), LIM kinase 1 (LIMK1) and cofilin (Pasterkamp and Kolodkin, 2003; Toyofuku et al., 2005). Rnd1–plexinA1 interactions stimulate plexinA1 GAP activity toward RRas by releasing inhibitory interactions within the plexin cytoplasmic region (Puschel, 2007). The subsequent decrease in the amount of active R-Ras triggers the inactivation of phosphatidylinositol-3-OH kinase (PI3K) to inhibit β1 integrin signaling. This is probably not the sole mechanism by which semaphorins regulate integrin function because after its release from plexinA1, FARP2 can inhibit type 1 phosphatidylinositol phosphate kinase (PIPKIg661) and consequently suppress integrin activation (Toyofuku et al., 2005).

Another consequence of neuronal plexin-induced PI3K inactivation is the inactivation of Akt (v-akt murine thymoma viral oncogene homolog). Interestingly, plexins employ different strategies to suppress PI3K–Akt signaling; Sema3A repulsive signaling in neurons also requires the downregulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10), a PIP3 phosphatase and a negative regulator of the PI3K–Akt pathway (Chadborn et al., 2006). Plexin-induced inhibition of PI3K–Akt signaling prevents the inactivation of the serine/threonine kinase glycogen synthase kinase-3b (GSK-3b), thus promoting the phosphorylation and inactivation of Collapsin response mediator protein 2 (CRMP2).

CRMPs comprise a small family of plexinA-interacting phosphoproteins that contribute to Sema3A-induced growth-cone collapse (Schmidt and Strittmatter, 2007). GSK-3b-mediated CRMP2 phosphorylation is dependent on a Cyclin-dependent kinase 5 (Cdk5)-mediated CRMP2-priming phosphorylation event. Sema3A-dependent Cdk5 activation relies on the recruitment of a Fyn–Cdk5 complex to the plexinA cytoplasmic domain and subsequent Fyn-mediated Cdk5 tyrosine phosphorylation (Ahmed and Eickholt, 2007). Other CRMP-associated proteins that have been implicated in Sema3 signaling include the tyrosine kinase Fes (also called Fps), a2-chimaerin and phospholipase D2 (Pasterkamp and Kolodkin, 2003). Further work is necessary to determine how these different cues cooperate to regulate CRMP function. In addition to the aforementioned proteins, several other intracellular proteins have been implicated in Sema3 signaling, including cell division cycle 42 (Cdc42), the Ras family members RhoD and RhoA, 12/15 lipoxygenase, Ranbinding protein

(RanBPM) and mitogen-activated protein kinases (MAPKs) (Kruger et al., 2005). If or how these signaling molecules function in the pathways detailed above should be a focus of much future research.

4.11.3. Semaphorins identified to play an important role in ontogogenesis of GnRH/olfactory system

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4.11.4. Sema3F / Neuropilin2

The Class 3 semaphorins are a group of inhibitory secreted and membrane bound proteins that bind the receptor, neuropilin-2 (Nrp2) and were initially shown to modulate axonal growth cone guidance. Cariboni et al 2007 have recently in order to explain reduced fertility of mice lacking Npn 2 (*Nrp2*$^{-/-}$), characterised the role of Neuropilin-2 in GnRH neuron migration (Cariboni et al., 2007). Examination of the *Nrp2*$^{-/-}$ null mice showed a 25% loss of GnRH neurons in the adult, with increased neurons detected along the nasal septum [14]. 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 Nrp2 on GnRH neurons could contribute since endogenous GnRH neurons might express both the Nrp2 receptor and the ligand, sema3A. No human mutations have been reported in this ligand/receptor pair.

4.11.5. Sema4D / Plexin B1 / Met

Semaphorin 4D (Sema4D) is a membrane-bound semaphorin that is proteolytically cleaved to bind Plexin B1 with Met tyrosine kinase receptor (Conrotto et al., 2005). These interactions are generally known to mediate growth cone collapse of developing axons, induce chemotaxis of epithelial and endothelial cells (Derijck et al.; Derijck et al., 2010; Zhou et al., 2008). Moreover high affinity receptors of Sema4D, Plexin B1 and Plexin B2 are highly expressed in the developing olfactory structures (Perala et al., 2005). Recently, Giacobini et al have shown that Sema4D regulates GnRH neuronal migration through PlexinB1-Met receptor complex (Giacobini et al., 2008). 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, Plexin B1 was detected in early

cell divisions, but not later. Plexin B1 null mice showed a reduced number of GnRH neurons migrating to the brain at E14.5 and 20% less migrating into the ventral forebrain areas at P3 with accumulation in the olfactory bulb region. Decreased GnRH neurons in the adult brains resulted in decreased GnRH fibers in the median eminence. 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. The Sema4D null mice do not have a reproductive phenotype with normal migration of GnRH neurons suggesting 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.

4.11.6. Sema3A / Neuropilin1 / Neuropilin2

Earlier studies by Cariboni et al have determined the role of Neuropilin2 in GnRH neruons migration and in the same study in vitro experiments suggested that Sema3A and Sema3F could be involved in this process (Cariboni et al., 2007). In order to unravel the exact mechanisms involving class 3 semaphorins and their corresponding Neuropilin receptors in GnRH migration, they have made extensive studies using various genetic mouse models (Cariboni et al., 2010). Mice lacking *Sema3a*$^{-/-}$ show severe defects in vomeronasal projections resulting in almost complete absence of GnRH neurons in the forebrain areas during development at E14.5 and E18.5. On the other hand, mice lacking semaphorin signaling through neuropilin1 receptor (*Nrp1*$^{sema/sema}$) or the neuropilin2 (*Nrp2*$^{-/-}$) receptor only partially phenocopy the defects observed in *Sema3a*$^{-/-}$ mice, while the compound mutant mice (*Nrp1*$^{sema/sema}$ *Nrp2*$^{-/-}$) showed similar phenotype as observed in *Sema3a*$^{-/-}$ mice. Mice lacking Sema3F ligand (*Sema3f*$^{/-}$) had no effect on the vomeronasal axons or the GnRH neuron migration suggesting Sema3F ligand is dispensible for GnRH development. Double ICC showed that both Nrp1 and Nrp2 are expressed on the vomeronasal axons and finally the authors conclude that Sema3A ligand unconventionally signals through Nrp1 and Nrp2 to control vomeronasal axons targeting to the brain that guide GnRH neurons into the brain. No human mutations have been yet reported in any of these signaling pathways.

Studies by Campagne C et al 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 (Campagne, 2008).

Together, these data suggest that the semaphorins may play specific roles in many aspects of GnRH neuron development and function.

5. Factors involved in maturation and function of GnRH system

5.1. Kisspeptin-Gpr54 signaling in Reproduction

5.1.1. Brief history

Originally discovered as a metastasis-suppressor gene in 1996 (Lee et al., 1996), KISS1 was named for its role as a suppressor sequence (ss); the letters "KI" were appended to the prefix "SS" to form "KISS" in homage to the location of its discovery, Hershey, Pennsylvania, home of the famous "Hershey Chocolate Kiss." Although the term metastin had been coined for the 54-amino acid product of the *Kiss1* gene, another research group named the family of neuropeptides coded by the *Kiss1* gene, kisspeptins (Kotani et al., 2001). Use of both terms continues to this day, with cancer biologists largely preferring the term metastin, whereas investigators in other fields have favored the term kisspeptin. In 2001, four independent groups identified kisspeptin as a high-affinity RFamide (Arg-Phe-NH_2) peptide ligand for a then orphan G protein-coupled membrane receptor, GPR54 (Clements et al., 2001; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). GPR54, now termed "Kiss1r" for its role as a kisspeptin receptor, was initially described in the rat in 1999 (Lee et al., 1999), and shortly thereafter, the human homolog of GPR54 (KISS1R; then referred to as AXOR12 or hOT7T175) was identified (Kotani et al., 2001; Lee et al., 1996). In 2003, kisspeptin-KISS1R signaling piqued the interest of reproductive physiologists when two independent research groups nearly simultaneously reported that mutations in *KISS1R* were associated with the idiopathic hypothalamic hypogonadism and impaired pubertal maturation found in their patients (de Roux et al., 2003; Seminara et al., 2003). Moreover, studies of mice bearing targeted deletions of *Kiss1r* produced the same phenotypic anomaly of reproductive dysfunction (Seminara et al., 2003). Thus emerged the idea that kisspeptin-KISS1R signaling plays a vital role in reproduction.

5.1.2. Nomenclature

In order to maintain consistency among the various fields working on kisspeptin-Gpr54 signaling and to eliminate confusion among different studies international committees

established to standardize nomenclature like the Human Genome Nomenclature Committee recomended established to standardize nomenclature (http://www.informatics. jax.org/mgihome/nomen/gene.shtml) recomended, *KISS1* and *Kiss1* should be used to represent the human and nonhuman kisspeptin genes, respectively while the nonitalicized versions of the gene, KISS1 and Kiss1 for the gene products. Similar convention applies to the receptor, *KISS1R* and *Kiss1r* denote the receptor geens for human and nonhuman species, respectively while the nonitalicized versions of the gene, KISS1R and Kiss1r for the gene products (Gottsch et al., 2009).

5.1.3. Kisspeptin gene products and its cleaved forms

The initial product of the *Kiss1* gene is a 145-amino acid peptide, from which is cleaved a 54-amino acid protein known as kisspeptin-54 **(Figure. 6)** (West et al., 1998). In the full-length protein, the sequence of kisspeptin-54 is surrounded by pairs of basic residues, where furin or prohormone convertases are thought to proteolytically cleave (Kotani et al., 2001). There are also shorter peptides (kisspeptin-10, -13, and -14) that share a common RF-amidated motif with kisspeptin-54; collectively, they are termed kisspeptins. Although no obvious cleavage sites have been identified that would result in these shorter peptides, it has been suggested that kisspeptin-54 is unstable and may be proteolytically cleaved into the shorter products. All four peptides (kisspeptin-10, -13, -14, and -54) exhibit the same affinity and efficacy for the Kiss1r, indicating that the C-terminal end of the peptide is responsible for the binding and activation of the receptor, Kiss1r. Although all four kisspeptin products are biologically active (Muir et al., 2001), the *in vivo* relevance of the shorter peptides is as yet unknown. Because members of the RFamide family of peptides often share several identical C-terminal amino acid residues, the generation of specific antisera has been a technical challenge. For example, many of the available KISS1 antisera raised against the shortened C-terminal human peptide cross-react with other members of the RFRPs (*i.e*., RFRP-1 and RFRP-3) (Fukusumi et al., 2001). RFRP-1 and RFRP-3 have been shown to stain strongly in the dorsomedial hypothalamus (DMH; an area that is not known to express *Kiss1* mRNA) with marked fiber projections in the arcuate nucleus (ARC) (Fukusumi et al., 2001; Ukena et al., 2002; Ukena and Tsutsui, 2001), which raises the possibility that previous kisspeptin antibodies demonstrating similar staining patterns may exhibit cross-reactivity with these related RFRPs.

5.1.4. Gpr54 receptor signaling

G protein-coupled receptors (GPCRs) transduce a variety of inputs to activate signaling

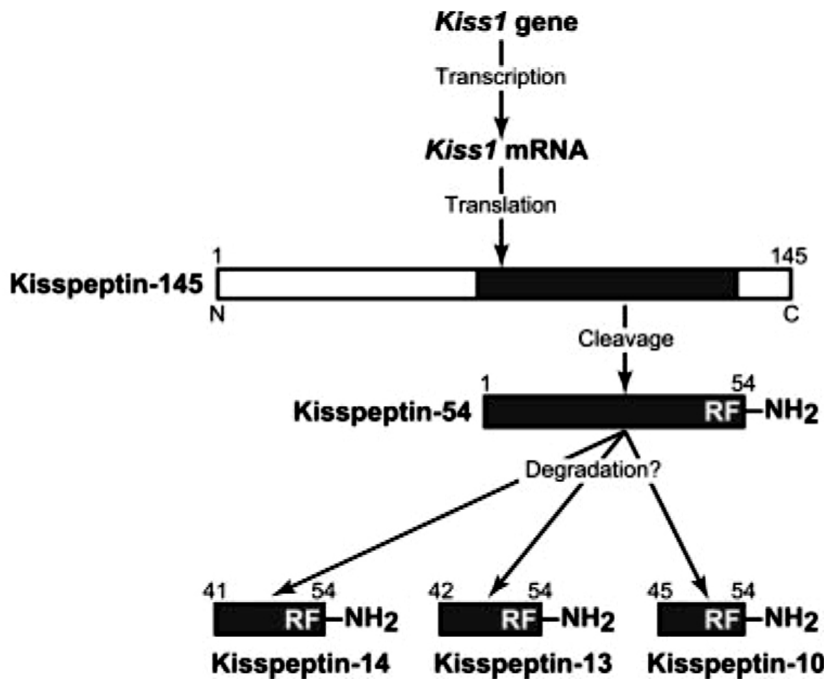


Figure 6. Products of the *Kiss1* gene. *Kiss1* mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are cleavage sites on the propeptide that lead to the production of the RF-amidated kisspeptin-54. Shorter peptides (such as kisspeptin-10, -13, and -14) share a common C terminus and RF-amidated motif with kisspeptin-54. Because no putative cleavage sites have been identified on the propeptide that would lead to synthesis of the shorter peptides, such peptides may be degradation products of kisspeptin-54. [Adapted from (Popa et al., 2008)]

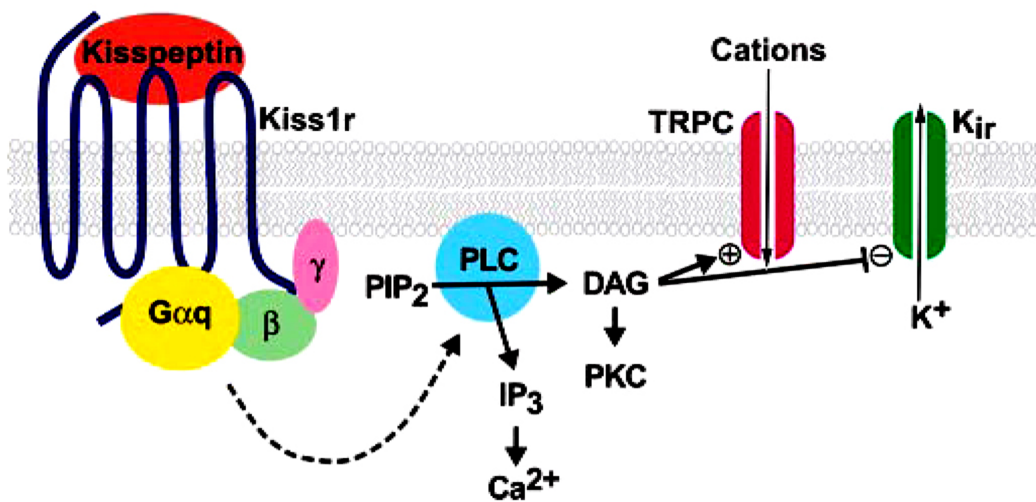


Figure 7. Proposed mechanism of neuronal depolarization by kisspeptin binding to its receptor, Kiss1r. Kisspeptin binding to its GPCR, Kiss1r, activates the G protein, $G\alpha_q$, and PLC to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and DAG. DAG activates a signal cascade by activating PKC, whereas IP3 mobilizes calcium ions (Ca^{2+}), which participate in the cascade by activating other proteins. Membrane depolarization is caused by activation (+) of nonselective TRPC cation channels and inhibition (-) of inwardly rectifying potassium channels (K_{ir}), possibly through involvement of DAG. [Adapted from (Oakley et al., 2009)]

pathways involved in diverse functions such as cell growth, proliferation, and migration. The GPCR superfamily can be classified into three subdivisions, *e.g.*, rhodopsin-, secretin-, and metabotropic glutamate receptor-like families (Marchese et al., 1999). Typical of the rhodopsin family of GPCRs, Kiss1r contains seven transmembrane domains, with three glycosylation sites at the N terminus (Clements et al., 2001). Kiss1r is most similar to the galanin receptor family (~45% homologous), although it does not bind either galanin or galanin-like peptide (Lee et al., 1999). Screens for agonists that bind Kiss1r identified several neuropeptides of the RFamide and RWamide family. The RMRFamide (Phe-Met-Arg-Phe-NH_2)-related peptides (RFRPs), of which Kiss1 is a member, constitute a superfamily of neuropeptides that terminate with the sequence Arg-Phe-NH_2 and exist in all phyla (Greenberg and Price, 1992).

The binding of Kiss1r by Kiss1 peptide leads to the activation of G protein-activated phospholipase C (PLCβ), suggesting a $G\alpha_{q/11}$-mediated signaling pathway **(Figure. 7)** (Constantin et al., 2009; Kotani et al., 2001; Liu et al., 2008; Stafford et al., 2002). PLCβ activation leads to the generation of the intracellular second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG); these signaling molecules in turn mediate intracellular Ca2+ release and activation of protein kinase C, respectively. Kisspeptin is thought to stimulate GnRH secretion by activating transient receptor potential canonical (TRPC)-like channels and inhibiting inwardly rectifying potassium channels (Zhang et al., 2008), likely mediated by DAG and/or Ca2+. Additionally, Kiss1r has been shown to stimulate arachidonic acid release and ERK1/2 and p38 activation, as well as Rho activation, which causes stress fiber formation (Castellano et al., 2006). Endogenous kisspeptin may activate Kiss1r via a ligand transportation pathway, in which initial binding of a ligand to the membrane is followed by lateral diffusion to the receptor (Lee et al., 2009). Current efforts are aimed at understanding more about the coupling of Kiss1r and G proteins. By examining the efficacy of signaling in various models of Kiss1r mutations, one recent report has identified the IL2-10 residue as a key player in the structural rearrangement of Kiss1r upon binding of the ligand, kisspeptin (Wacker et al., 2008). This approach may shed new light on fundamental concepts regarding GPCR/G protein signaling of kisspeptin and other ligand-receptor interactions.

5.1.5. Distribution of kisspeptin

Using a polyclonal rabbit antibody AC566, directed towards the final ten C-terminal amino acids of murine kisspeptin, Clarkson et al have mapped the distribution of kisspeptin in mouse

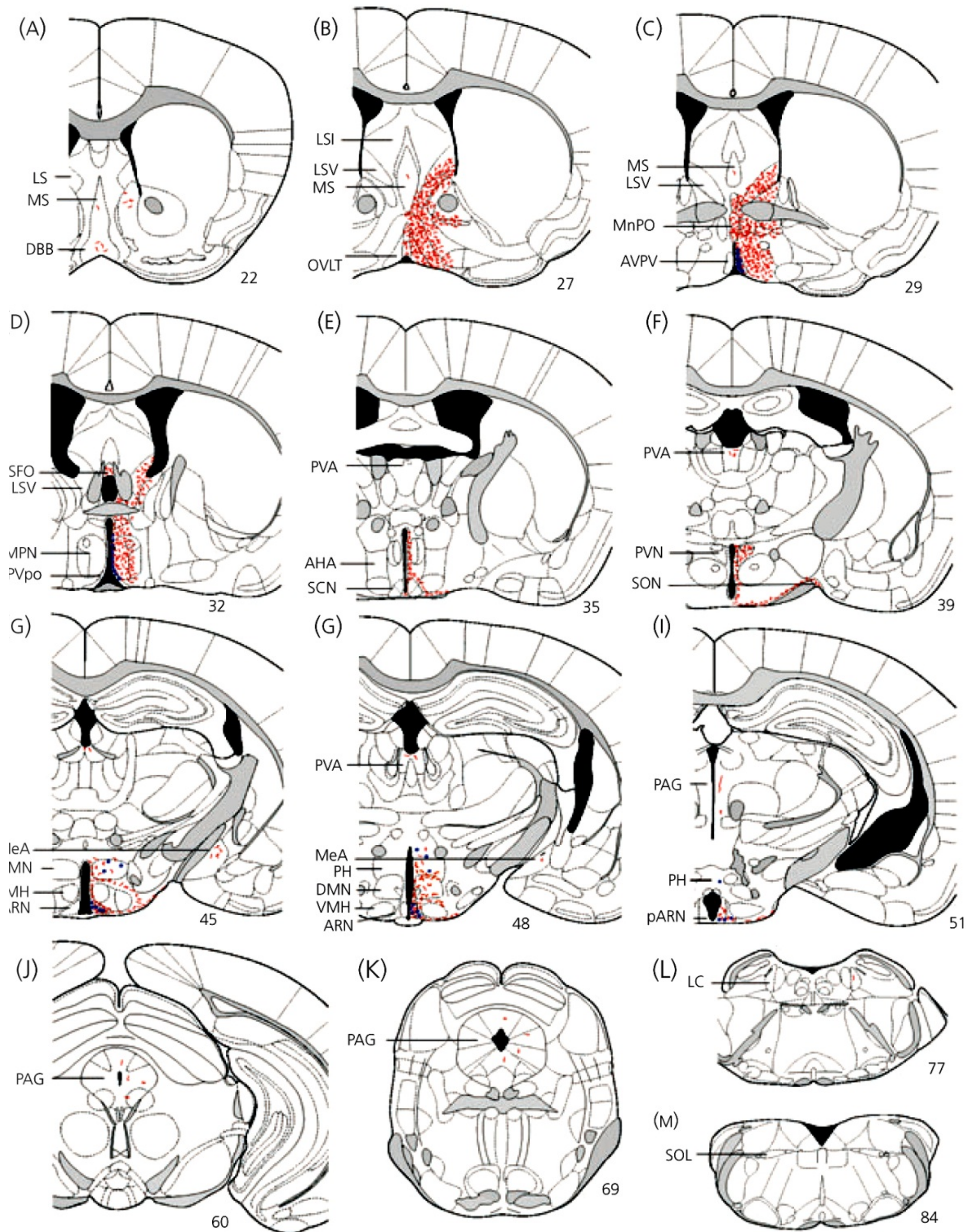


Figure 8. **The distribution of kisspeptin-immunoreactivity (ir) in the adult female mouse brain.** Kisspeptin-ir is depicted on this series of brain maps; modified with permission from Paxinos and Franklin. Kisspeptin-ir cell bodies, blue circles; kisspeptin-ir fibres, red lines. LS, lateral septum; MS, medial septum; DBB, diagonal band of Broca; LSI, lateral septum intermediate part; LSV, lateral septum ventral part; OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic area; AVPV, anteroventral periventricular nucleus; SFO, subfornical organ; MPO, medial preoptic area; PVpo, preoptic periventricular nucleus; PVA, paraventricular thalamic nucleus anterior part; AHA, anterior hypothalamus; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; MeA, medial amygdala; DMN, dorsomedial nucleus; VMN, ventromedial nucleus; ARN, arcuate nucleus; PAG, periaqueductal grey; pARN, posterior arcuate nucleus; LC, locus coeruleus; SOL, solitary tract. The numbers under each coronal section indicate the respective Paxinos & Franklin plate number. [Adapted from (Clarkson et al., 2009b)]

brain **(Figure. 8)** (Clarkson et al., 2009b). Three populations of kisspeptin-expressing cell bodies were identified in the adult female mouse brain. One exists as a dense periventricular continuum of cells within the rostral part of the third ventricle (AVPV-PeN), another is found within the arcuate nucleus (ARC), and another is identified as a low-density group of scattered cells within the dorsomedial nucleus and posterior hypothalamus. Kisspeptin-immunoreactive fibres were abundant within the ventral aspect of the lateral septum and within the hypothalamus running in periventricular and ventral retrochiasmatic pathways. Notable exclusions from the kisspeptin fibre innervation were the suprachiasmatic and ventromedial nuclei. Outside of the hypothalamus, a small number of kisspeptin fibres were identified in the bed nucleus of the stria terminalis, subfornical organ, medial amygdala, paraventricular thalamus, periaqueductal grey and locus coerulus. All kisspeptin cell body and fibre immunoreactivity was absent in brain tissue from *Kiss1*$^{-/-}$mice. These observations provide a map of kisspeptin neurones in the mouse brain and indicate that a limited number of mostly medial hypothalamic and lateral septal brain regions are innervated by the three hypothalamic kisspeptin cell populations; the functions of these projections remain to be established.

5.1.6. Distribution of Gpr54 expressing cells

The expression of Gpr54 at the cellular level is low. Studies using in situ hybridiztion of Gpr54 mRNA were performed to identify its presence in GnRH neurons (Han et al., 2005; Irwig et al., 2004) and few other studies report its expression in limbic brain regions including the hypothalamus, hippocampus and periaqueductal gray (PAG) (Lee et al., 1999). Recently using a transgenic Gpr54 LacZ knock-in mouse model, Herbison *et al* described a detailed map of cells expressing Gpr54 in the mouse brain and also analysed expression of Gpr54 in GnRH neurons across postnatal development **(Figure. 9)** (Herbison et al., 2010). The highest density of Gpr54-expressing cells in the mouse central nervous system was found in the dentate gyrus of the hippocampus beginning on postnatal d 6 (P6). Abundant Gpr54 expression was also noted in the septum, rostral preoptic area (rPOA), anteroventral nucleus of the thalamus, posterior hypothalamus, periaqueductal grey, supramammillary and pontine nuclei, and dorsal cochlear nucleus. No Gpr54 expression was detected in the arcuate and rostral periventricular nuclei of the hypothalamus. Dual-labeling experiments showed that essentially all Gpr54-expressing cells in the rPOA were GnRH neurons. Analyses of mice at birth, P1, P5, P20, and P30 and as adults revealed a gradual increase in the percentage of GnRH neurons expressing Gpr54 from approximately 40% at birth through to approximately

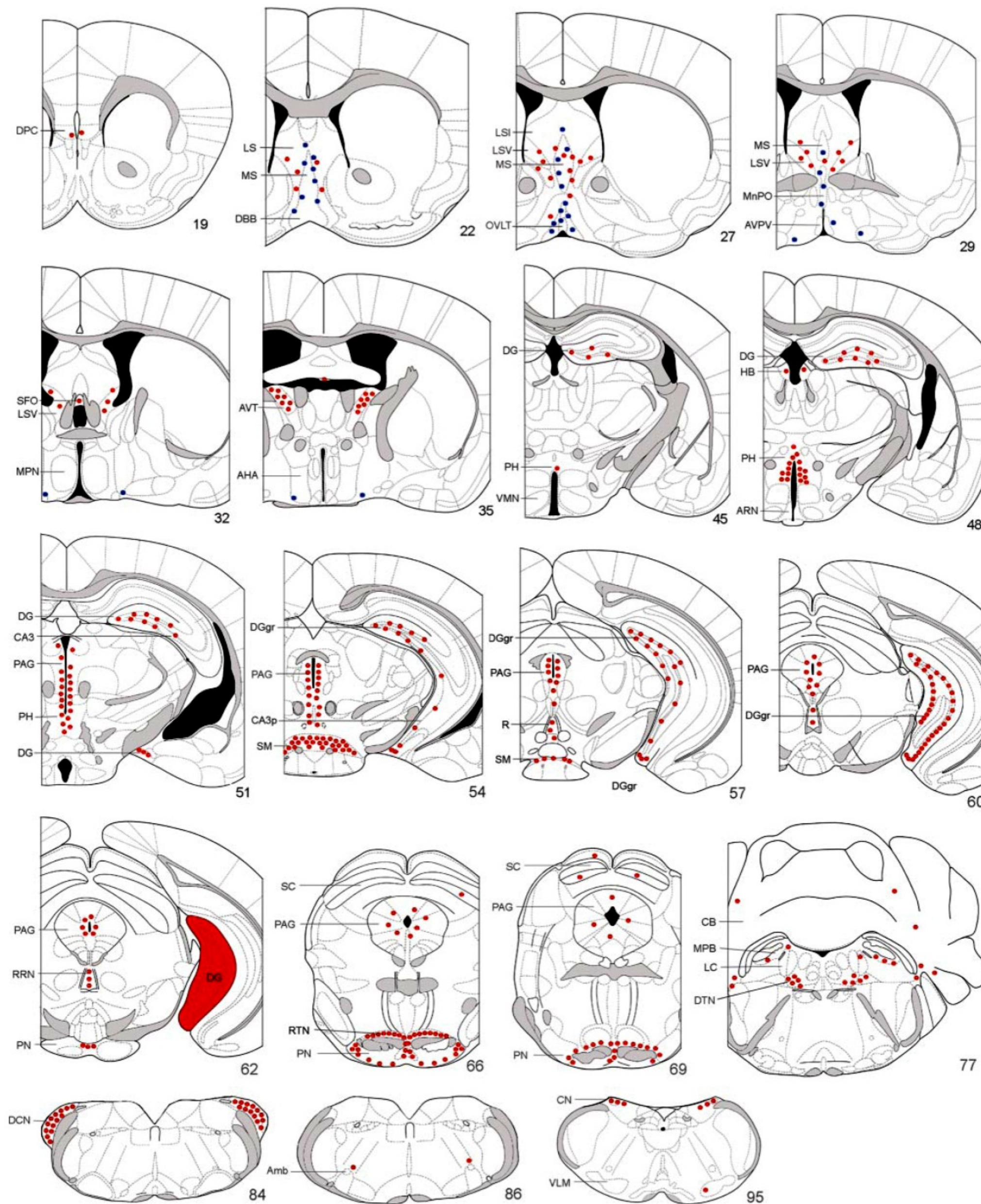


Figure 9. Schematic diagrams showing distribution of Gpr54-expressing cells in adult female mouse brain. *Red dots*, Xgal-containing cells. *Blue dots*, Xgal-containing GnRH neurons. The high density of Xgal cells in the dentate gyrus is represented by *solid red*. Diagrams are adapted from Paxinos and Franklin (41) with permission (plate numbers at *bottom right* for each schematic). Amb, Nucleus ambiguus; ARN, arcuate nucleus; AVT, anteroventral nucleus of thalamus; AVPV, anteroventral periventricular nucleus; CA3p, pyramidal cell layer CA3 hippocampus; CB, cerebellum; CN, cuneate nucleus; DBB, diagonal band of Broca; DCN, dorsal cochlear nucleus; DG, dentate gyrus; DGgr, granule cell layer of dentate gyrus; DPC, dorsal peduncular nucleus of cortex; DTN, dorsal tegmental nucleus; HB, habenula; LC, locus coereulus; LSI, intermediate division of LS; LSV, ventral division of LS; MPB, medial parabrachial nucleus; MPN, medial preoptic nucleus; MnPO, median preoptic nucleus; OVLT, organum vasculosum of lamina terminalis; PH, posterior hypothalamus; PN, pontine nucleus; R, raphe; RRN, rostral raphe nuclei; RTN, reticulotegmental nuclei; SC, superior colliculus; SFO, subfornical organ; SM, supramammillary nuclei; VMN, ventromedial nucleus. [Adapted from (Herbison et al., 2010)]

70% from P20 onward. Whereas GnRH neurons located in the septum displayed a consistent increase across this time, GnRH neurons in the rPOA showed a sharp reduction in Gpr54 expression after birth (to ~10% at P5) before increasing to the 70% expression levels by P20. Together these findings provide an anatomical basis for the exploration of Gpr54 actions outside the reproductive axis and reveal a complex temporal and spatial pattern of Gpr54 gene expression in developing GnRH neurons.

5.1.7. Direct and indirect effects of kisspeptin on GnRH neurons

Several major lines of evidence suggest that kisspeptin signals directly to GnRH neurons **(Figure. 10)** (Colledge, 2009). First, the majority of GnRH neurons express *Kiss1r* (Han et al., 2005; Herbison et al., 2010; Parhar et al., 2004). Second, kisspeptin-ir fibers are found in close association with GnRH neurons (Clarkson and Herbison, 2006; Roseweir et al., 2009). Third, kisspeptin can act directly to depolarize and increase firing rates of GnRH neurons *in vitro* (Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008). It should be noted that although kisspeptin may act through traditional synaptic mechanisms to stimulate GnRH secretion, it may also act directly in a nonsynaptic manner, particularly in the ME (d'Anglemont de Tassigny et al., 2008; Franceschini et al., 2006; Ramaswamy et al., 2008). In addition to acting directly on GnRH neurons, there is growing evidence to suggest that kisspeptin also acts on intermediary neurons, such as GABAergic cells, to regulate GnRH secretion (Pielecka-Fortuna et al., 2008).

5.1.8. Kisspeptin - Gpr54 signaling in Puberty

Since the discovery of inactivating mutations in human *KISS1R* gene linked to hypogonadotropic hypogonadism and absence of puberty by two independent groups in 2003, several groups have focused on the role of kisspeptin in puberty (de Roux et al., 2003; Seminara et al., 2003). Many species exhibit a marked increase in Kiss1 and/or Kiss1r expression in association with the onset of puberty, suggesting that kisspeptin acts as gatekeeper for puberty (Filby et al., 2008; Han et al., 2005; Martinez-Chavez et al., 2008; Navarro et al., 2004; Shahab et al., 2005).

In rodents, sheep, and other animals, a “gonadostat” hypothesis has held that the pace of puberty is a function of changing sensitivity of the GnRH neurosecretory system to the inhibitory feedback actions of gonadal steroids. In the mouse, the distribution of Kiss1 neurons and expression of *Kiss1* mRNA changes over development. The number of kiss1 neurons in the AVPV/PeN increases exponentially from postnatal day 10 through puberty and

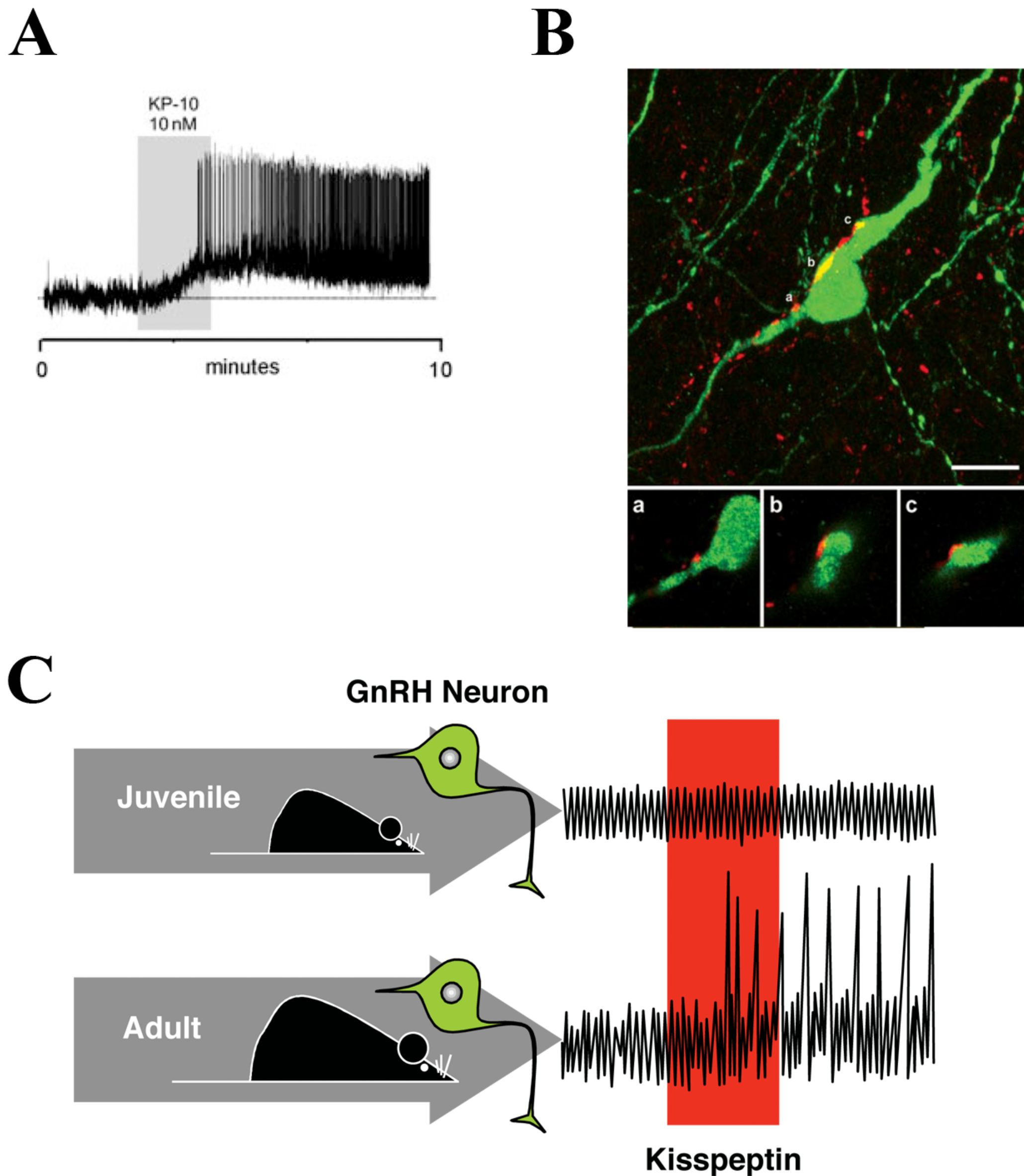


Figure 11. **Kisspeptin exerts a direct potent activational effect on GnRH neurons.** (A). Perforated-patch, voltage recordings from proestrous female GnRH-GFP neurons (resting membrane potential = - 68 mV) in the acute brain slice demonstrate a remarkably intense and prolonged activation by 10 nM kisspeptin (KP-10). [Adapted from (Han et al., 2005)]. B. Kisspeptin projections to GnRH neurons in adult female mice. Confocal stack of 75 images showing a single GnRH neuron (*green*) with kisspeptin (*red*) fibers surrounding and apposed to it. Single 370-nm-thick optical sections through the three regions indicated by a, b, and c of the GnRH neuron are given below to demonstrate the close apposition between kisspeptin fibers and GnRH neuron elements. Scale bar, 10 μm. [Adapted from (Clarkson et al., 2009a)]. C. Possible role of kisspeptin in the onset of puberty. Recent observations suggest that GnRH neurons become increasingly responsive to kisspeptin as a function of pubertal maturation in the mouse. In this example, gramicidin-perforated patch recordings were used to assess the electrophysiologic response of GnRH neurons to central kisspeptin administration (red bar).

in addition the proximity of appositions between kisspeptin fibers and GnRH neuronal somata increases at the time of puberty (Clarkson et al., 2009a), however, this change is not associated with an increase in GPR54 mRNA expression in GnRH neurons. Moreover, the percentage of GnRH neurons electrical responsiveness to kisspeptin increases from approximately 25% in juveniles to approximately 45% in prepubertal mice to more than 90% in adults (Han et al., 2005), suggesting that GnRH neurons become more sensitive to kisspeptin throughout postnatal development—without altering *Kiss1r* gene expression. Furthermore, central administration of submaximal doses of kisspeptin stimulates LH secretion in adult, but not prepubertal male mice. Central and peripheral administration of kisspeptin to juvenile female rats also stimulates LH release and ovulation and advances the timing of vaginal opening (Matsui et al., 2004; Navarro et al., 2004).

Based on these observations and the fact that estradiol increases the number of kiss1 cells in the AVPV/PeN, Clarkson and Herbison have proposed a model to explain the pubertal activation of gonadotropin secretion (Clarkson et al., 2009a). According to their model, estradiol in the prepubertal period stimulates Kiss1 neurons in the AVPV/PeN that activate GnRH neurons. Increased GnRH secretion then stimulates gonadotropin release, which subsequently drives further estradiol production from the ovary—thus producing a feed-forward activational loop. This putative mechanism would cause AVPV/PeN neurons to act as "estradiol-dependent 'amplifiers' of GnRH neuron activity." In support of this hypothesis, these investigators demonstrate that Kiss1 neurons are virtually absent in estradiol-deficient, aromatase knockout (ARKO) mice—which is perhaps not surprising because the expression of *Kiss1* in the AVPV/PeN has been shown to be estrogen/ERα/ERE-dependent (Smith et al., 2005). In support to these hypothesis, and to further gain insights in role of ERα signaling in kisspeptin neurons, Mayer C *et al* have recently generated a conditional knockout [Kisspeptin-IRES-Cre/ERα$^{lox/lox}$ (KissIC^{+}/ERα$^{lox/lox}$) mice)] of ERα (*Esr1*) gene in kisspeptin neurons and assessed the tempo and completeness of their reproductive maturation (Mayer et al., 2010). Suprisingly, these mice exhibit a premature activation of reproductive axis as observed by vaginal opening and elevated (six fold) serum LH levels at P15 compared to their wild-type littermates, but do not attain normal reproductive maturation as observed by abnormal estrous cyclicity and lowered serum LH levels in adults compared to the wild-type littermates. Neuroanatomical studies in these mice reveal diminished kisspeptin expression in the AVPV and while in contrast the kisspeptin expression is increased at the ARC nucleus.

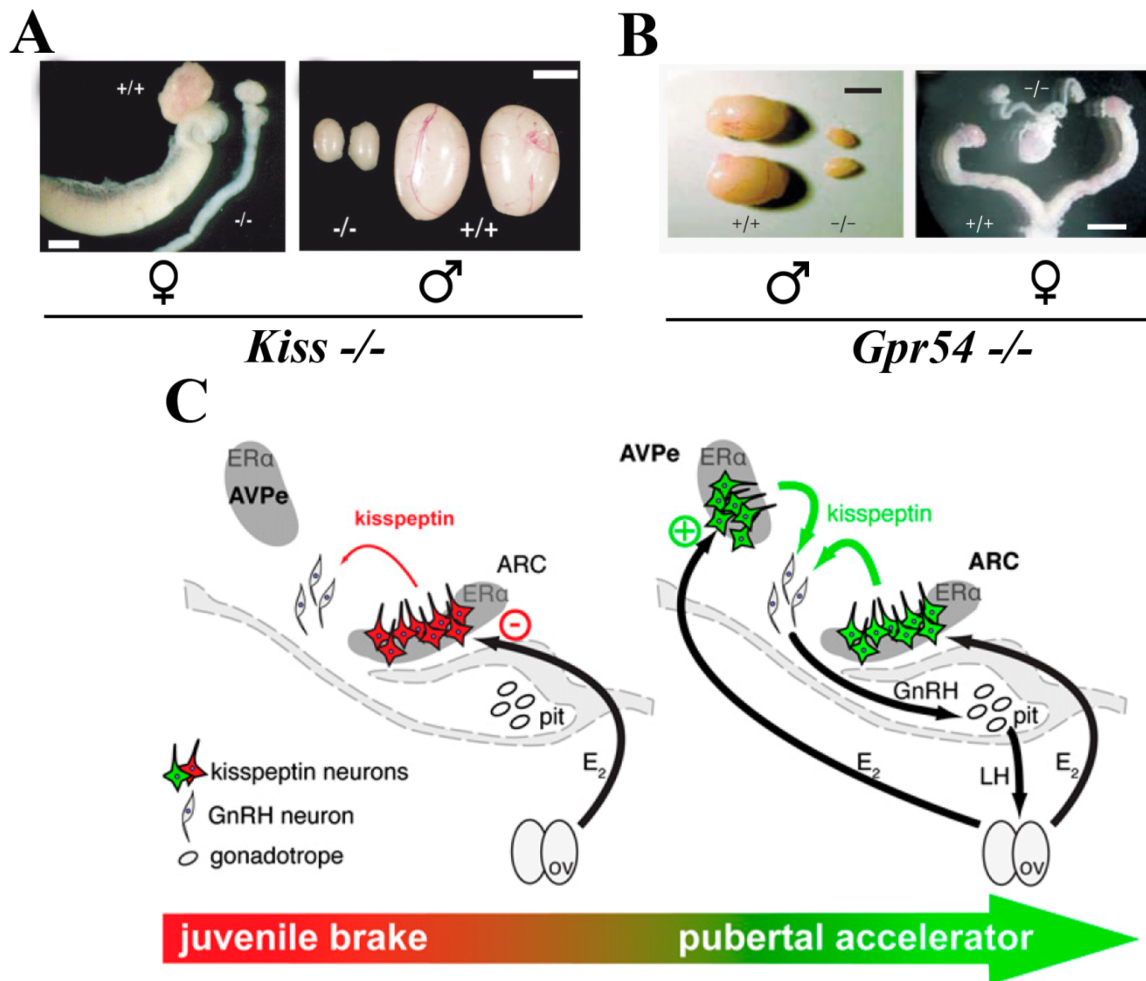


Figure 12. Deletion of *kiss1* gene (A) [Adapted from (d'Anglemont de Tassigny et al., 2007b)] or *Gpr54* gene (B) [Adapted from (Seminara et al., 2003)] in mouse leads to absence of puberty onset and hypogonadotropic hypogonadism. (C) Schematic representation of the hypothetical mechanisms of the regulation of pubertal onset and progression by ERα signaling in kisspeptin neurons. During the prepubertal period, ERα signaling mediates inhibition of kisspeptin expression in ARC and hence restraint of GnRH release. Pubertal onset is associated with decreased sensitivity to this suppressive mechanism in the ARC, as well as increased activation of ERα in AVPe kisspeptin neurons; the latter process stimulates kisspeptin expression in AVPe cells and increases GnRH release rates throughout the completion of puberty. [Adapted from (Mayer et al., 2010)]

These studies reveal that two ERα-dependent mechanisms—one a brake and the other an accelerator— are sequentially operated in two different populations of kisspeptin neurons during pubertal development of female mice to gate, and then to activate, GnRH release **(Figure. 11).** A juvenile restraint of GnRH release appears to be mediated by ERα activation in ARC kisspeptin neurons, whereas the subsequent pubertal stimulation of GnRH release depends upon ERα signaling in AVPe kisspeptin neurons. This appears to ensure coordinated progression of neuroendocrine events culminating in increased basal reproductive hormone secretions and the capacity to sustain ovulatory cyclicity, hallmarks of the fully mature adult reproductive state.

Given the importance of kisspeptin-GPR54 signaling in pubertal onset and sexual maturation, a recent phenomenal study by Mayer C and Boehm U determined that female mice can undergo normal reproductive maturation in the absence of kisspeptin / GPR54 expressing neurons (Mayer and Boehm, 2011). Using 'toxin strategy' that allows the ablation of specific cell population, these authors have specifically ablated kisspeptin and GPR54 expressing cells in the whole mice. This technology involves transcriptionally silenced 'Diphtheria toxin A' (*DTA*) gene that can be induced to synthesize the toxin using cre recombinase only in specific population of cells. KissIC mice described above, GPR54-IRES-Cre (GPIC) have been crossed to ROSA26-DTA (R26-DTA) mice that results in genetic ablation of kisspeptin and GPR54 expressing neurons respectively in littermate mice. Pubertal onset as observed by vaginal opening were normal in these mice. Though the adult mutant mice had low ovarian weight and abnormal estrous cyclicity yet displayed all stages of estrous cycle with presence of copura lutea in the ovaries and gave birth to normal size litters. Interestingly, induced ablation of kisspeptin neurons in adults resulted in acyclicity and none of female produced offsprings while the ablation of GPR54 expressing neurons in adults had no affect on the estrous cycle and the 50% of female mice gave birth to offspring.

Taken together, these findings indicate that puberty onset is precisely timed in the absence of kisspeptin/GPR54 signaling. However, if the GnRH pulse generator can be activated in the absence of kisspeptin and GPR54 neurons, what could be the physiological function of kisspeptin neurons and GPR54 receptors on GnRH neurons? On the basis of comparison of the different mouse strains with the complementary knockout mice, it can be proposed that kisspeptin neurons integrate various cues and relay this information to GnRH neurons via kisspeptin/GPR54 signaling to optimize reproductive success in wild-type mice while Kisspeptin/GPR54 signaling, however, is neither essential for activating GnRH

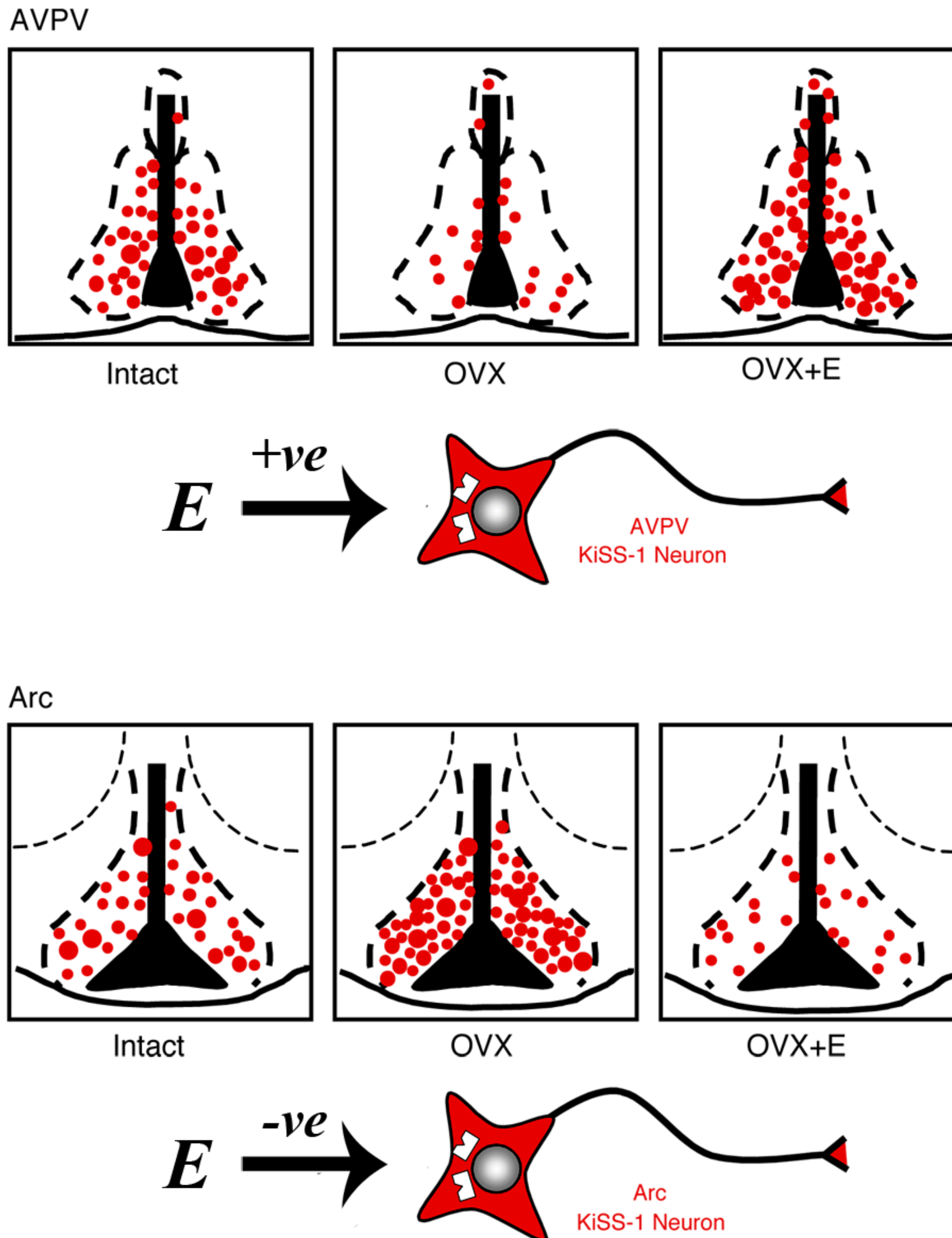


Figure 10. KiSS-1 mRNA is differentially regulated by estrogen in the forebrain of the mouse. The upper panel depicts the expression of KiSS-1 mRNA (red dots) in the anteroventral periventricular nucleus (AVPV) of the female diestrus (intact), ovariectomized (OVX), and OVX-estradiol replaced (+E) mouse. The diagram illustrates the apparently positive (+ve) regulation of KiSS-1 mRNA by E in the AVPV. The lower panel depicts the expression of KiSS-1 mRNA in the arcuate nucleus (Arc) of the female diestrus (intact), OVX and OVX + E mouse. The diagram illustrates the apparently negative (-ve) regulation of KiSS-1 mRNA by E in the Arc. [Adapted from (Smith et al., 2005)]

neurosecretion at puberty onset nor for becoming fertile in female mice.

5.1.9. Differential regulation of 2 kisspeptin population by estrogens

5.1.10. Negative feedback action of sex steroids on Kiss1 gene expression in ARC

In the female mammal, during most days of the estrous (and menstrual) cycle, the negative feedback control of gonadotropin secretion predominates, and a relatively low plasma level of gonadal steroids restrains GnRH and LH secretion. Kisspeptin neurons appear to play a key role in the negative feedback action of estradiol in the female, as they do for testosterone in the male. The expression of *Kiss1* mRNA in the ARC changes as a function of the estrous cycle in the rat, with levels reaching nadirs at or around the time when estradiol levels are highest **(Figure. 12)** (Smith et al., 2006). Ovariectomy causes an increase in hypothalamic expression of *Kiss1* mRNA in the ARC of rodents, sheep, and monkeys (Rometo et al., 2007; Smith et al., 2007; Smith et al., 2005). The increase in expression of *Kiss1* is reversible upon treatment with estradiol (Oakley et al., 2009; Smith et al., 2006). Finally, female mice bearing targeted deletions of *Kiss1r* do not show a postcastration rise in LH levels despite exhibiting a dramatic increase in the expression of *Kiss1* mRNA (Dungan et al., 2007). These observations suggest that kisspeptin neurons in the ARC of both the male and female provide tonic drive to GnRH neuronal activity, which is modulated by the negative feedback effects of gonadal steroids (testosterone in the male and estradiol in the female). However, the situation with kisspeptin neurons in the AVPV (of the rodent) is different.

5.1.11. Positive feedback action of estradiol on Kiss1 gene expression in AVPV

Early on proestrus in rodents (or late in the follicular phase of the menstrual cycle in primates), the rising tide of estradiol in the plasma triggers a surge of GnRH and LH secretion, which induces ovulation. In rodents, this so-called positive feedback effect of estradiol appears to involve estradiol-sensitive neurons in the AVPV, which act directly on GnRH neurons to stimulate the preovulatory surge of GnRH and thus LH (Clarkson et al., 2008; Wintermantel et al., 2006). Nearly all kisspeptin cells in the AVPV of the female rodent express ERα (Clarkson et al., 2008); moreover, the AVPV is a sexually dimorphic nucleus, with sexually differentiated expression of tyrosine hydroxylase (Simerly, 2002), *Kiss1* (Kauffman, 2009), neurotensin, and other genes. Kisspeptin neurons in the AVPV of rodents appear to play a central role in relaying the positive feedback effects of estradiol to GnRH neurons. First, treatment with a kisspeptin antiserum to block kisspeptin signaling completely abolishes the LH surge in female rats (Adachi et al., 2007; Kinoshita et al., 2005). Second, in

the mouse, the expression of *Kiss1* mRNA in the AVPV is dramatically induced by estradiol (Clarkson and Herbison, 2009). Third, in the rat, the expression of *Kiss1* mRNA in the AVPV peaks at a time coincident with the GnRH/LH surge, and Kiss1 neurons in the AVPV show Fos induction at precisely this time (Clarkson et al., 2008; Smith et al., 2006). Fourth, a population of rodent ERα-positive neurons makes direct synaptic contact with GnRH neurons (Wintermantel et al., 2006) and these neurons are likely to be Kiss1 neurons. Finally, a report by Clarkson *et al.* showed that whereas normal wild-type mice that have been ovariectomized and treated with both estradiol and progesterone show a clear LH surge, mice bearing targeted deletions in *Kiss1r* appear to lack this capacity (Clarkson et al., 2008). Moreover, in this same study, approximately 50% of GnRH neurons in wild-type mice showed Fos expression coincident with the LH surge, whereas none of the mutant animals showed evidence of Fos expression at this same time. Together, these observations imply that activation of kisspeptin neurons and their signaling to GnRH neurons is a prerequisite for generating the estradiol/progesterone-induced GnRH/LH surge in the female mouse.

5.1.12. Kisspeptin signaling in seasonality

The role of kisspeptin in the photoperiodic control of reproduction has been examined in several recent reviews (Clarke et al., 2009; Simonneaux et al., 2009). Seasonal breeders, such as hamsters and sheep, restrict fertility to a particular time of year to ensure the birth of offspring during favorable environmental conditions. Photoperiod is a predominant environmental cue that governs the pattern of melatonin secretion from the pineal gland, which helps the animal determine season. For example, reproductive activity of the Syrian hamster is promoted by long summer days and inhibited by short winter days. Levels of *Kiss1* mRNA in the ARC are reduced in male Syrian hamsters after transfer from longday to short-day conditions, which leads to reproductive quiescence (Revel et al., 2006). This seasonal change appears to be melatonin- dependent because pineal gland ablation prevents this short-day induced down-regulation of *Kiss1* expression; however, it is unclear whether melatonin acts directly on Kiss1 neurons. Remarkably, chronic infusion of kisspeptin restores testicular activity in Syrian hamsters despite persisting photoinhibitory conditions. Both male and female Siberian hamsters held in short-day conditions exhibit a reduced response to exogenous kisspeptin treatment and show negligible kisspeptin expression in the AVPV and high expression in the ARC (Mason et al., 2007). In longday conditions, however, this expression is reversed, with marked kisspeptin staining in the AVPV and only minor expression in the ARC (Greives et al., 2007; Mason et al., 2007). It should be noted that some

studies performed in the hamster have been confounded by a lack of specificity (and proper validation) of the antibodies used to detect kisspeptin by immunocytochemistry. Furthermore, interpreting the results of semiquantitative immunocytochemistry can be challenging. For example, when there is little apparent expression of kisspeptin, this could mean either that little kisspeptin is being made (thus none appears) or that whatever is being made (perhaps even in great abundance) is rapidly released. Thus, analysis of staining intensity by immunocytochemistry should be interpreted with caution. Nevertheless, it does appear that low levels of kisspeptin and a reduced sensitivity to the hormone may contribute to the reproductive quiescence induced by short-day photoperiods. Investigations in the hamster are complicated by the fact that kisspeptin activity in the two species (Syrian and Siberian) appears to respond differently to short days, which makes generalizations difficult. The sheep, another seasonally breeding species, becomes reproductively active as the days become shorter in autumn and becomes quiescent as the days become longer. The expression of kisspeptin also varies with season in the sheep. For example, Kiss1 expression is lower and there are fewer kisspeptin terminal contacts ontoGnRHneurons during the nonbreeding period (long days) compared with the breeding period (short days) (Smith et al., 2007; Smith et al., 2008). Moreover, during anestrus (non breeding) season, infusion of kisspeptin for several days can induce ovulation (Caraty et al., 2007). Hence, there appears to be a fundamental contribution made by kisspeptin signaling to regulate seasonal breeding in a variety of species.

5.2. Nitric oxide system

5.2.1. Introduction

Nitric oxide (NO) is a physiological intercellular/intracellular messenger in central and peripheral nervous sytem (Bredt and Snyder, 1990; Vincent and Hope, 1992). It is the intercellular signal that controls vascular tone (hence blood pressure), insulin secretion, airway tone, and peristalsis, and is involved in angiogenesis (growth of new blood vessels) and in the development of nervous system. In the CNS, NO is an important messenger molecule, which is involved in various cellular functions in the brain such as differentiation, development, synaptic plasticity and neurosecretion (Garthwaite, 2008). NO has also been implicated in reproductive functions and behaviours, including ovulation, oocyte meiotic maturation (Brann et al., 1997; Jablonka-Shariff and Olson, 1998), estradiol synthesis (Olsen et al., 1996) lorodosis and penile erection (Mani et al., 1994). It is believed to function as a

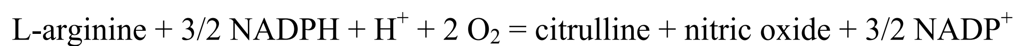
retrograde neurotransmitter and hence is likely to be important in learning. For their discovery of involvement of nitric oxide signaling in cardiovascular development, R. Furchgott, L, Ignarro and F, Murrad were awarded a nobel prize in physiology and medicine in 1998 (Furchgott, 1991; Furchgott and Jothianandan, 1991).

Three isoforms of Nitric oxide synthases (NOS) are known that generate NO by oxidizing a guanidino nitrogen group from L-arginine using nicotinaminde adenine dinucleotide phosphate (NADPH) as an electron donor,

1. neuronal NO synthase (nNOS)
2. endothelial NO synthase (eNOS) and
3. inducible NO synthase (iNOS)

nNOS and eNOS are constitutively expressed while iNOS is an inducible enzyme whose baseline expression is very low but its expression can be induced in numerous cell types, when subjected to immunological challenges (for review see (Alderton et al., 2001) (Stuehr, 2004; Stuehr et al., 2004)

The canonical reaction catalyzed by NOS is:



L-arginine + 3/2 NADPH + H^+ + 2 O_2 = citrulline + nitric oxide + 3/2 $NADP^+$

Mapping of the constitutive forms of NOS, through specific antibodies and NADPH-diaphorase histochemistry, show both nNOS and eNOS are expressed in the brain (Huang et al., 1993) (Keilhoff et al., 2001; Topel et al., 1998) . While eNOS is mainly found in the vasculature in both the periphery and the brain, it has also been documented in neurons of the hippocampus (Stanarius et al., 1997). The nNOS isoform is located in several populations of neuronal cells in the cerebellum, olfactory bulb, hippocampus, and is also highly expressed within neurons of the hypothalamus (Bredt et al., 1991a; Dawson et al., 1991; Herbison et al., 1996).

While the activity of the inducible isoform is Ca^{2+} independent (Cho et al., 1992), the constitutive NOS isoforms activities are dependent on both Ca^{2+} and calmodulin (Bredt and Snyder, 1990; Forstermann et al., 1991a; Forstermann et al., 1991b; Garthwaite et al., 1988; Pollock et al., 1991). In the central nervous system, it has been well described that the activation of NMDA receptors (NMDA-R) by glutamate provides the necessary increase in intracellular calcium required for activation of nNOS, whereas other pathways that increase intracellular Ca^{2+} are much less efficient in eliciting nNOS activity (Bredt et al., 1991a; Bredt et al., 1991b; Bredt and Snyder, 1990; Garthwaite et al., 1988; Kiedrowski et al., 1992). In

endothelial cells, the abundance of the principal resident coat protein of caveolae, caveolin-1, appears to be an important regulator of eNOS activity (Feron et al., 2001; Feron and Kelly, 2001). Caveolin-1 interacts with eNOS (Feron et al., 1996) and leads to its inhibition in a reversible process modulated by Ca^{2+}- calmodulin (Michel et al., 1997). Both nNOS and eNOS must be bound to the membrane, and require further phosphorylation of 1412 and 1179 serine residues, respectively, through the PI3K/AKT signaling pathway, for full activation (Fulton et al., 1999; Rameau et al., 2007). The eNOS isoform is a dually acylated peripheral membrane protein that targets into the Golgi region and plasma membrane of endothelial cells and is, itself, compartmentalized to the membrane. This stands in contrast to the nNOS isoform that requires a scaffolding protein to couple itself to the membrane (Aarts et al., 2002; Christopherson et al., 1999; Sattler et al., 1999). Recent studies suggested that nNOS might also be subjected to repressive sets of posttranslational modifications in vivo, such as phosphorylation at the Ser847 site by calcium-calmodulin-dependent kinase II (Hayashi et al., 1999; Rameau et al., 2004; Rameau et al., 2007). Thus, the regulation of the activity of nNOS appears to be mainly dependent on three factors: sufficient Ca^{2+} influx through activation of NMDA-R, subcellular localization via a scaffolding protein, and phosphorylation of specific serine residues. Regarding eNOS, the most important mechanism for sustaining its activity is phosphorylation on serine-1179, enabling the enzyme to function at resting Ca^{2+} cytosolic concentrations (see for review (Dudzinski et al., 2006)) and interaction of eNOS with caveolin is used to prevent inadequate NO production under basal conditions (Dessy et al., 2010).

Since NO is not stored in vesicles, following its synthesis, NO diffuses across the biological membranes to produce its effect. However, the ability of NO to exert an action is delineated by its half-life and the proximity of NO-containing cells to their target, which must be within 200 μm (Garthwaite and Boulton, 1995; Hall and Garthwaite, 2009; Prast and Philippu, 2001). This distance indeed corresponds to the physiological sphere of influence from a single point source of NO that emits for 1-10 sec (Garthwaite and Boulton, 1995). In the brain, NO is typically considered to be a retrograde neurotransmitter (i.e. increasing release of other neurotransmitters, such as GABA and glutamate from presynaptic sites), however, it also acts at post-synaptic sites (see for review (Garthwaite, 2008)). The most well documented target or “receptor” of NO is the soluble guanylate cyclase (sGC), in which the activation by NO results in formation of cGMP to mediate intracellular effects (Arnold et al., 1977; Knowles et al., 1989). NO binds to the ferrous heme of sGC with high affinity to change the conformation of sGC and therefore dramatically increase its catalytic activity (Garthwaite, 2010). Following treatment of NO, the synthesis of prostaglandins (PGs) has

also been documented (Rettori et al., 1992). The conversion of arachidonic acid to PGs requires cyclooxygenase (COX), thus COX may also be considered as a downstream target of NO. Similar to the sGC enzyme, COX contains an iron-heme group suggesting a direct interaction between NO and COX (Salvemini et al., 1993; Savchenko et al., 1997). Indeed, it was demonstrated that COX is a direct target for NO, and the increase of PGs was independent of cGMP (Salvemini et al., 1993; Savchenko et al., 1997). Of note, the formation of PGE_2 by COX is considered to be critical to the reproductive axis (Ojeda et al., 1975; White and Ojeda, 1982).

5.2.2. Modulation of GnRH neuronal activity by neuronal nitric oxide during the ovarian cycle.

Since the early 1990's, NO has been known to be a regulator of luteinizing hormone (LH) secretion (see for review (Brann et al., 1997; Kalra et al., 1997; McCann et al., 2003; Prevot et al., 2000)). Indeed, several pharmacological studies suggested NO as a key modulator of GnRH secretion, and a critical contributor in the onset of the preovulatory surge of GnRH/LH (Aguan et al., 1996; Bonavera et al., 1994; Moretto et al., 1993; Rettori et al., 1993). Intracerebroventricular injection of NOS antisense oligodeoxynucleotides was shown to block the LH surge in steroid-primed ovariectomized rats (Aguan et al., 1996), whereas intracerebral infusion of N (G)-nitro-L- arginine methyl ester (L-NAME, a NOS inhibitor) either within the preoptic region or the median eminence caused a marked disruption of rat estrous cyclicity (d'Anglemont de Tassigny et al., 2007a; De Seranno et al., 2004). Intriguingly, in mutant mice, the first targeted disruption of the nNOS gene in exon 3 did not alter fertility (Huang et al., 1993). However these mutant mice retained residual nNOS activity (Huang et al., 1993). In contrast, deletion of exon 6, which harbors the catalytic domain of nNOS, was shown to cause hypogonadotropic hypogonadism and infertility in mutant mice (Gyurko et al., 2002).

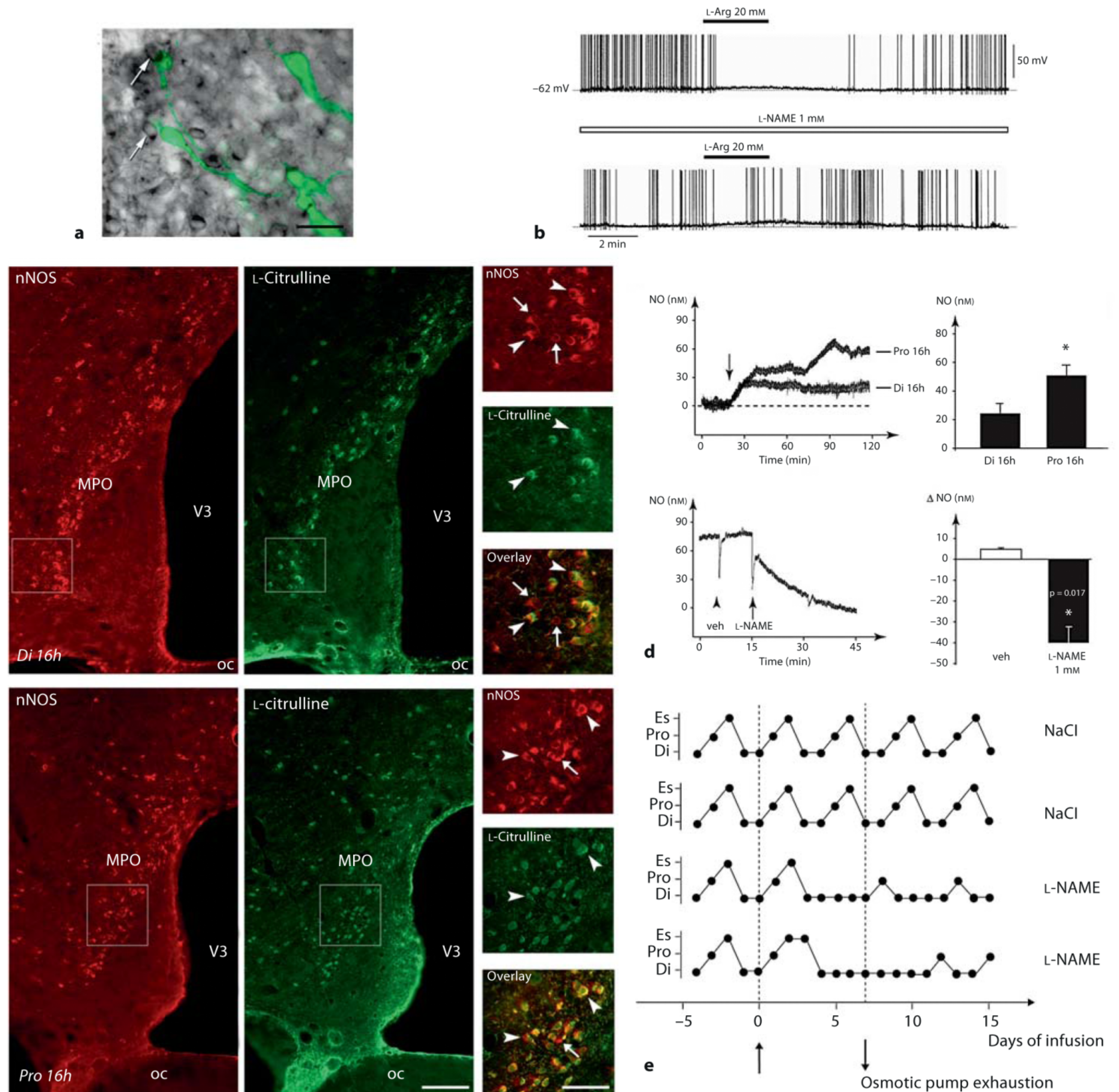


Figure 13. Within the preoptic region, NO release modulates GnRH neuronal activity and is required for estrous cyclicity. (**a**) The relationship of NO-producing neurons stained with NADPH-diaphorase to GnRH neurons in GnRH-GFP transgenic mice. NOS and neuronal NADPH diaphorase are identical in brain and peripheral tissues (Dawson et al., 1991). Arrows show close relationships between NADPH diaphorase-containing neurons (dark precipitate) and GnRH-GFP neurons (green). Scale bar: 40 μM. (**b**) Whole-cell patch-clamp recordings show that L-arginine (L-Arg, the precursor of NO) alters spontaneous firing in GnRH neurons (top panel). Application of L-NAME, a NOS inhibitor, prevented L-Arg-mediated inhibition of spontaneous discharges in L-Arg-responsive GnRH neurons (bottom panel) (Clasadonte et al., 2008). **c** Microscopic visualization of nNOS catalytic activity using immunofluorescence to localize L-citrulline, a byproduct of NO synthesis. Representative illustration of nNOS-expressing neurons (red) and L-citrulline detection (green) by fluorescent immunocytochemistry in coronal brain sections from diestrous (Di 16h) and proestrous (Pro 16h) rats. V3 = Third ventricle; oc = optic chiasm. Right panels: high magnifications of the areas delineated by rectangles. Arrowheads indicate double-labeled neurons expressing nNOS and L-citrulline. Arrows indicate nNOS single- labeled neurons. Low magnification scale bar: 210 μM; high

magnification scale bar: 60 μM. (**d)** Top panel: representative profiles of spontaneous NO release from preoptic region explants at two different stages of the estrous cycle in the female rat. Differential current measured by the self-referencing probe, converted to flux (vertical axis), increased after the addition of single preoptic explants in survival medium at the time indicated by arrow. NO effluxes recorded from each explant during the 90-min data acquisition phase were averaged and plotted on the bar graph. Comparisons between stages of the estrous cycle indicated that preoptic explants produced significantly more NO on the afternoon of proestrus than on the afternoon of diestrus (* p = 0.014). Bottom panel: representative response of a proestrous rat preoptic explant to vehicle (veh, arrowhead) or to 1 mM L-NAME (arrow). Comparisons between treatments indicated that L-NAME, but not vehicle, significantly reduced NO production by the preoptic region during proestrus (* p = 0.017). The transient drops in current upon substance application (arrowhead, arrow) are artifacts. **e** Disruption of estrous cyclicity in young adult rats by the pharmacological blockade of NO synthesis targeted to the preoptic region of the hypothalamus. L-NAME (5 mM) or vehicle (NaCl 0.9%) delivered via a stereotaxically implanted stainless steel cannula connected to a subcutaneously placed osmotic pump delivering its content at a rate of 0.5 μl/h for 7 days. Infusion starts at P0 (arrow pointing up) and ends 7 days later (arrow pointing down), when the pump content is exhausted. Di = Diestrus; Pro = proestrus; Es = estrus. [Adapted from (Bellefontaine et al., 2011)]

5.2.4. Neuronal NO exerts an acute postsynaptic action on GnRH neurons

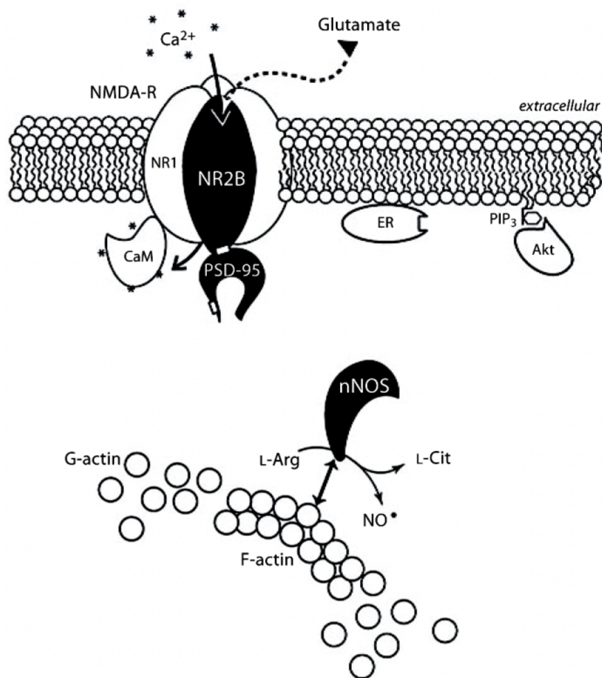
Early studies using immortalized GnRH cell lines suggested that NO acts directly at the level of the cell body to either inhibit (Sortino et al., 1994) or stimulate GnRH release and synchronize pulsatile GnRH secretion (Lopez et al., 1997; Moretto et al., 1993; Sortino et al., 1994; Westel, 1995). In contrast to these in vitro models where GnRH-secreting cells express NO producing enzymes (Belsham and Mellon, 2000; Lopez et al., 1997; Mahachoklertwattana et al., 1994), consistent mapping of the nNOS isoform within the hypothalamus demonstrated that GnRH perikarya are surrounded by nNOS neurons, but do not express nNOS themselves (Bhat et al., 1995; Clasadonte et al., 2008; Grossman et al., 1994; Herbison et al., 1996). Due to the scattered nature of GnRH neurons across the preoptic region, the putative action of NO at the level of the GnRH cell body was difficult to assess. With the advancement in genetic techniques and the engineering of mice expressing green fluorescent protein (GFP) in GnRH neurons (Han et al., 2004; Spergel et al., 1999; Suter et al., 2000), GnRH neuronal activity can now be readily examined in brain slice preparations. Using patch-clamp recordings from GnRH-GFP mouse brain slices, our laboratory has provided the first electrophysiological evidence that NO is a direct modulator of native GnRH neuron excitability (Clasadonte et al., 2008). Both endogenous and exogenous sources of NO were shown to cause acute inhibition of spontaneous firing in GnRH neurons (Clasadonte et al., 2008). Importantly, L-arginine (the natural substrate for NOS-mediated NO production) inhibitory effects on GnRH bursting activity were abolished when brain slices were incubated with L-NAME, a broad spectrum NOS inhibitor, or (4S)-N-(4-amino-5-[aminoakyl]aminopentyl)-N'-nitroguanidines (AAANG), a selective inhibitor of nNOS (Clasadonte et al., 2008; Hah et al., 2001). The ability of NO to inhibit GnRH neuron firing and induce GnRH neuron hyperpolarization is retained during synaptic uncoupling with a medium containing low-Ca^{2+} and high-Mg^{2+}, and TTX, a voltage-gated sodium channel blocker. This indicates a direct action of NO at a postsynaptic site to change membrane properties in GnRH neurons (Clasadonte et al., 2008). Notably, NO appears to require the sGC-cGMP signaling cascade to modulate neuronal excitability in GnRH neurons. The presence of a sGC antagonist blocked the inhibition of firing promoted by NO in GnRH neurons, while an exogenous cGMP analog mimicked the action of NO (Clasadonte et al., 2008). By revealing that NO is a direct modulator of GnRH neuronal activity, these data introduce the intriguing possibility that the neuronal release of this highly diffusible gaseous neurotransmitter with a short half-life (< 1 sec) may be a key mechanism used by the neuroendocrine brain to both modulate bursting firing patterns, and set into phase the bursting activity of GnRH neurons (Fig. 1). Because NO production is key

to the occurrence of the preovulatory surge of GnRH (Aguan et al., 1996; Bonavera et al., 1993; Bonavera et al., 1994; Moretto et al., 1993; Rettori et al., 1993) , it is tempting to speculate that NO may serve as a transitory switch between pulse and peak release of GnRH in proestrus (Moenter et al., 1991; Sarkar et al., 1976; Sarkar and Minami, 1995).

5.2.5. Regulation of neuronal nitric oxide synthase by ovarian steroids

Estrogens exert a wide range of effects on brain function such as the hypothalamic-pituitary-gonadal axis regulation, but also the control of dynamic changes in spine density in hippocampus (Frankfurt et al., 1990; Simerly, 2005; Woolley et al., 1990). Estrogens have also been shown to play a role in controlling nitric oxide (NO) production (d'Anglemont de Tassigny et al., 2007a; d'Anglemont de Tassigny et al., 2009; Lamar et al., 1999). Short-term variations in circulating levels of estrogens during the reproductive cycle may result in neuronal connectivity coupled with a remodelling in protein-protein interactions at the post synaptic density thereby constituting a potential mechanism to control NO production within the brain (Weiner et al., 1994; Pu et al., 1996).

In previous studies, our lab demonstrated that cyclic variations in estrogen levels regulate the state of activation of nNOS through changes in NMDA receptor/PSD-95/nNOS association in the hypothalamic preoptic region (d'Anglemont de Tassigny et al., 2007a; d'Anglemont de Tassigny et al., 2009). Amperometric measurements performed with a NO-specific probe on rat hypothalamus explants showed that NO production varies during the estrous cycle in the preoptic region and that the amplitude of NO effluxes is significantly higher in proestrus (when estrogen levels are highest) than in diestrus . Intriguingly, in contrast with previous studies suggesting that estrogen could modulate hypothalamic nNOS gene expression (Lamar et al., 1999; Okamura et al., 1994; Rachman et al., 1998; Sica et al., 2009), these changes in NO secretion seen during the estrous cycle are not associated with changes in nNOS protein synthesis but rather with changes in nNOS activity (Parkash et al., 2010). Estradiol induces the formation of a NMDA receptor-nNOS complex in neurons of the preoptic region (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010), thus enhancing NO secretion (d'Anglemont de Tassigny et al., 2009) by coupling nNOS to its main stimulatory calcium influx pathway (Bredt and Snyder, 1990; Garthwaite et al., 1988). This differential coupling of nNOS with NMDA receptors during the estrous cycle was shown to



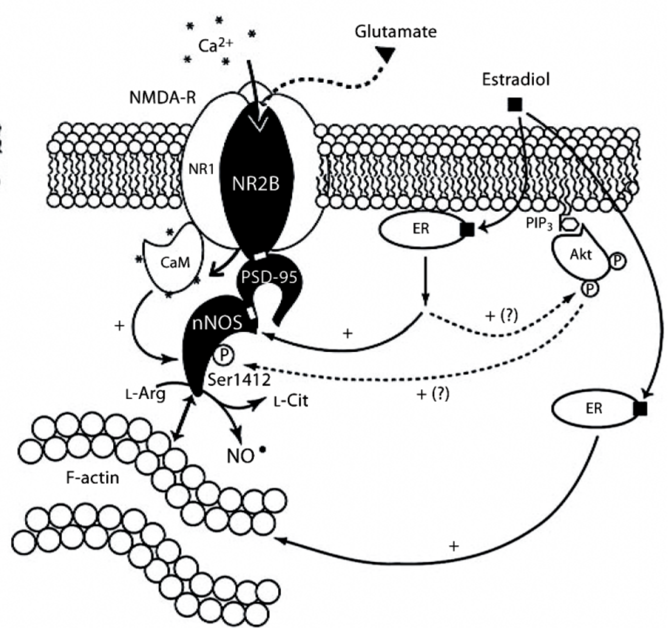


Figure 14. Schematic representation of the possible estradiol-mediated changes in protein-protein interactions involved in the control of nNOS activity in the preoptic region of the hypothalamus during the ovarian cycle. nNOS activity is primarily regulated by increases in the local intracellular [Ca $^{2+}$] (*), which activates nNOS through calmodulin (CaM) binding (Bredt and Snyder, 1990). The physical interaction of nNOS with NMDA-Rs involves the PSD-95 scaffolding protein and the assembly of a ternary complex (Christopherson et al., 1999). Only Ca 2+ influx through the NMDA-R promotes an efficient NO production (Garthwaite et al., 1988). In parallel, nNOS is also subjected to posttranscriptional modifications (such as phoshorylation) that modulates its catalytic activity (Rameau et al., 2007). Natural fluctuations of estrogen levels across the ovarian cycle, i.e. low in diestrus and high in proestrus, regulate the activation state of nNOS by modulating its coupling with NR2B-containing NMDA-Rs by the PSD-95 scaffolding protein. In turn, this regulation, that could be mediated by estrogen- dependent ER activation (d'Anglemont de Tassigny et al., 2009) results in the phosphorylation of nNOS, an effect known to increase nNOS enzymatic activity (Rameau et al., 2007). Estrogens may induce nNOS phosphorylation through the activation of the Src/PI3K/Akt pathway (Gingerich and Krukoff, 2008). The mechanism by which estrogen promotes nNOS anchoring to the PSD-95/ NMDA-R complex remains unknown. Estrogen exhibits widespread effects throughout the brain and its actions are intensively investigated, but very few studies have considered the relationship between estrogen and PSD-95. In vitro estrogen upregulates the synthesis of PSD-95 via the Akt/protein kinase B pathway and alters the shape of hippocampal dendritic spine where PSD-95 is present in high amounts. Perhaps the nNOS association with PSD-95 is enhanced at synaptic sites in response to dynamic events at the postsynaptic density. One plausible mechanism for this would be that the estrogen signalling mediates the coalescence of cytoskeleton-tethered nNOS to PSD-95 through spine formation, which would require remodeling of the actin cytoskeleton (Hering and Sheng, 2001) [Adapted from (Bellefontaine et al., 2011)]

involve the scaffolding protein post-synaptic density- 95 (PSD-95) (d'Anglemont de Tassigny et al., 2007a; d'Anglemont de Tassigny et al., 2009) and to require estrogen receptor activity (d'Anglemont de Tassigny et al., 2009) (Fig. 2). PSD-95 knock-down via the administration of antisense PSD-95 oligodeoxynucleotides strikingly impaired ovarian cyclicity (d'Anglemont de Tassigny et al., 2007a) and nNOS activity in preoptic neurons both in vitro (d'Anglemont de Tassigny et al., 2009) and in vivo (d'Anglemont de Tassigny et al., 2007a). Importantly, inhibition of estradiol-induced NO release by selective NMDA receptor blockers demonstrated that estrogen actually promotes the coupling of glutamatergic fluxes for NO production in preoptic neurons (d'Anglemont de Tassigny et al., 2009) (Fig. 2). The evidence that NO-producing neurons in the hypothalamus could be targets of glutamate was demonstrated by neuroanatomical studies showing that virtually all preoptic nNOS neurons, also known to express estrogen receptor-α (Sato et al., 2005; Scordalakes et al., 2002), express NMDA receptors (Bhat et al., 1995; d'Anglemont de Tassigny et al., 2007a). Interestingly, other studies have shown that most NMDA receptor-expressing neurons of the preoptic region also contain estrogen receptor-α (Chakraborty et al., 2003), which can be visualized in cell nuclei, perikaryal cytoplasm, and dendrites (Blaustein, 1992). In parallel of promoting changes in protein-protein interactions, natural fluctuations of estrogens across the ovarian cycle were also recently shown to regulate the state of activation of nNOS through changes in nNOS stimulatory phosphorylation levels in the preoptic region (Parkash et al., 2010). Phosphorylation of nNOS at Ser1412 was shown to be maximal on the afternoon of proestrus and this phosphorylation-activated nNOS isoform was seen to physically interact with the PSD-95/NMDA receptor complex at the plasma membrane (Parkash et al., 2010) (Fig. 3). In vitro experiments performed in primary culture of hypothalamic neurons showed that estradiol promotes phosphorylation of nNOS at Ser1412 via a Src/PI3K/Akt-dependent pathway (Gingerich and Krukoff, 2008).

The above findings suggest a direct action of estrogens on nNOS neurons of the preoptic region. This would couple NO production to glutamate fluxes that exert a well-known stimulatory influence on GnRH secretion during their positive feedback on the reproductive brain (Brann and Mahesh, 1991; Urbanski and Ojeda, 1990). The resulting production of NO may then act on GnRH neurons to synchronize their activity and adjust their firing behavior to enable peak release of GnRH.

5.3. Jak2

The cytokine family of hormones, growth factors, and chemokines like leptin, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) interact with cytokine class of receptors expressed in hypothalamic areas and have been implicated in the central regulation of reproduction (Dozio et al., 2005; Magni et al., 2005), although the precise mechanism of action are still being explored. One of these pathways that are involved is the Janus-activated kinase 2 (Jak2) - signal transducers and activators of transcription 3 (STAT3), and it has been shown that these are expressed in GnRH neuronal cell lines, GN11 and GT1-7 (Argetsinger et al., 1993). Knockout of *Jak2* gene in mice results in embryonic lethality (Parganas et al., 1998), hence in order to understand the role of this pathway in reproduction, recently Wu et al have generated conditional knockout mice Jak2 G $^{-/-}$ using LoxP-Cre recombinase system, that has Jak2 gene knocked out only in GnRH neurons (Wu et al., 2011). These mice had a normal number of GnRH neurons reaching their final destinations indicating a normal migratory behavior but they had reduced GnRH gene expression reduced LH levels. The female mice had delayed puberty and first estrus cycle, abnormal cyclicity, blunted preovulatory LH surge and impaired fertility. These observations indicate that Jak2 signaling in GnRH neurons is essential for normal female reproductive behavior.

5.4. Six6

It is well known that very few number of GnRH neurons are present in the mammalian brain, as in mice ~800-1000 GnRH neurons are dispersed throughout the septohypothalamic region. What factors dictate the differentiation of their precursors into such limited number of GnRH neurons. Efforts are being made to identify the transcription factors that are active and direct the expression of GnRH. Generation of immortalized cell lines like GT1-7 cells that represent a fully differentiated GnRH neurons and secretes high levels of GnRH in a pulsatile manner and in contrast the GN11 cells that represent the developmental, migratory GnRH neurons that express low levels of GnRH and respond to migratory cues has been useful in analyzing the GnRH transcritional regulation (Radovick et al., 1991; Wetsel et al., 1991; Wetsel et al., 1992). Using these two cell lines, recently Larder *et al* have identified sine oculis related homeobox 6 (Six6), a homeodomain protein that is dramatically increased during GnRH maturation and that overexpression of Six6 in neuroanal cell lines induces GnRH transcription (Larder et al., 2011). This induction of GnRH expreession is mediated via binding of Six6 to evoluationary conserved ATTA sites located within the GnRH proximal promoter. Mutant mice lacking Six6 homeodomain leads to reduced GnRH neurons resulting in infertility in

both females and males. The transcriptional regulation of GnRH neurons during migration and maturation is complex and deregulation in any of these factors can lead to adult reproductive disorders.

5.5. TTF1

Thyroid transcription factor 1 (TTF1) [also known as Nkx2.1 (related to the NK-2 class of homeobox genes) and T/ebp (thyroid-specific enhancer-binding protein)], a homeodomain gene plays an important role in the morphogenesis of basal forebrain (Kimura, 1996; Sussel et al., 1999). In the *Ttf1* knockout mice, the pallidum is absent, cholinergic neurons of basal forebrain fail to develop, migration of GABAergic neurons from the pallidum to the striatum and cortex is impaired, the development of hypothalamus is severely impaired and the mice are born dead. In the absence of one allele, the basal ganglia functions are impaired. In normal mice TTF1 expression persists in some postmitotic basal forebrain neurons, suggesting a role for TTF1 in maintaining neuronal differentiated functions (Marin et al., 2000; Marin et al., 2002; Sussel et al., 1999). Such a role might extend to postnatal life, because TTF1 remains expressed after birth in selected striatal/pallidum interneurons, as well as in defined glial and neuronal subsets of the hypothalamus and the POA (Lee et al., 2001). Specifically, TTF1 is detected in POA neurons that control reproductive function, in preproenkephalinergic neurons of the lateral ventromedial nucleus (Davis et al., 2004), which restrain the initiation of puberty by transsynaptically inhibiting LHRH neurons (Ojeda et al., 2006a; Ojeda et al., 2006b), in unidentified neurons of the arcuate nucleus, and in ependymoglial cells of the third ventricle and median eminence (ME). In female rats, TTF1 mRNA abundance increases in the hypothalamus preceding the natural onset of puberty suggesting an involvement of TTF1 in the control of female puberty. Gene expression profiling of the nonhuman primate hypothalamus revealed that TTF1 expression increases at puberty. In order to gain insights in the role in TTF1 in puberty and reproduction, using $Ttf1^{SynCre}$, mutant mice that has *Ttf1* gene deleted only in postmitotic terminally differentiated neurons Mastronardi *et al* have demonstrated the critical role played by TTF1 in female reproduction without impairing the basal ganglia function (Mastronardi et al., 2006). $Ttf1^{SynCre}$ mice had signficiantly reduced Kiss1 mRNA and increased preproenkephalin mRNA levels in the MBH suggesting TTF1 spcifically enhances the *Kiss1* gene expression via specific recognition motifs present in the Kiss1 promoter and it represses the preproenkephalin gene, which restrains the pubertal process. Consistent with the above observations the female $Ttf1^{SynCre}$ mice had delayed onset of puberty and diminished reproductive capacity. Mutations in the human *TTF1* gene result in

neurological symptoms that include hypotonia, dyskinesia, and choreoathetosis (Krude et al., 2002; Pohlenz et al., 2002), a condition consisting of rapid involuntary and slow writhing movements however these symptoms are caused by impairments in basal ganglia function that are also observed in mice carrying only one *Ttf1* allele.

5.6. EAP1

The initiation of mammalian puberty involves controlled coordinated regulation of interactions and changes in neuronal and glial inputs to GnRH neurons (Ojeda et al., 2010; Ojeda et al., 2009), however the precise hierarchy of transcription of genes involved in the central control of puberty and reproduction is not known. Recently Heger S *et al* performed global analysis of gene expression in the developing female monkey and found a gene with unknown function called C14ORF4, now named as *EAP1* (enhanced at puberty 1) whose gene expression increased consistently at the time of puberty (Heger et al., 2007). In situ hybridization studies detected abundant expression of EAP1 mRNA in arcuate of female primate hypothalamus and immunocytochemical analysis detected EAP1 expression in the nuclei of cells in the medial preoptic area, arcuate and ventromedial nucleus of female rat hypothalamus. EAP1 expression was found in the nuclei of GnRH neurons, required for initiation of puberty and also in preproenkephalinergic neurons which are involved in the inhibitory control of GnRH secretion. Consistent with the presence of nuclear localisation signal in its sequence, using functional promoter assays EAP1 has been shown to transactivate the GnRH promoter in GT1-7 cells while it represses the preproenkephalin promoter in the same cells. In order to determine the functional importance of EAP1 of female reproduction, lentiviral-mediated delivery of EAP1 siRNA to rat POA, that readily decreased the *EAP1* expression resulted in delayed puberty, disruption of estrous cyclicity, reduced plasma gonadotropins (LH and FSH) and estradiol levels that lead to abnormal follicular development compared to normal development in lentiviral eGFP-injected control rats. The authors finally conclude EAP1 to be considered as as one of the upper-echelon genes that is necessary for initiation of puberty and female reproductive cycles. *Eap1*-null mice that doesnt exist yet can allow the researchers to further determine the precise roles played by EAP1 in reproduction. Human *EAP1* gene is mapped to the chromosome 14q24.3 and earlier it has been shown that abnormalities in this chromosomal region (14q23-14q32) is associated with precocious puberty in humans (Sutton and Shaffer, 2000) (Martin et al., 1999) suggesting that *EAP1* gene could be involved in any of these process related to puberty or adult reproduction in humans.

5.7. Glial factors

5.7.1. ErbB receptor signaling

In the hypothalamus, glial cells are now considered as an important regulatory component of the GnRH neuronal network (Ojeda et al., 2003). Hypothalamic astrocytes express only erbB2 and erbB4 receptors but not erbB3 receptors (Ma et al., 1999) and ligand dependent activation of this astroglial erbB4/2 complex sets in motion a signaling cascade that results in prostaglandin E2 (PGE2) production (Ma et al., 1999; Rage et al., 1997). PGE2 then stimulates secretion of GnRH, the neuropeptide controlling sexual development, from hypothalamic neurons. Transgenic mice have helped to define the role of glial derived growth factors such as the epidermal growth factor (EGF) family and their astrocytic erbB receptor in activating GnRH neurons at puberty. Mice expressing a dominant-negative erbB4 (DN-erbB4) receptor specifically in astrocytes, show delayed sexual maturation (Prevot et al., 2005; Prevot et al., 2003). DN-erbB4 mice exhibit impaired release of GnRH due to the inability of the hypothalamus to respond to the neuregulin ligand with production of prostaglandin E2 (PGE2). PGE2 elicits GnRH release in normal animals . Waved 2 (Wa2) mice have a point mutation in the Erbb1 gene encoding the ErbB1 receptor which normally binds transforming growth factor alpha (TGFα) and these mice show delayed puberty (Prevot et al., 2005). Cells isolated from Wa2 mice do not release PGE2 in response to TGF α and therefore do not induce GnRH release from immortalized GnRH GT1-7 cell line. Inducible disruption of erbB1 receptor signalling by expressing mutant erbB1 receptors in astrocytes also leads to compromised reproduction due to alteration in GnRH secretion. Recently, studies by Clasadonte *et al* (described in detail in the annexe of thesis) using patch clamp recordings have provided electrophysiological evidence of role of astrocytes in GnRH electrical activity (unpublished data). Bath application of PGE_2 readily exerts a dose-dependent postsynaptic excitatory effect of GnRH neurons. Immunocytochemical analysis revealed abundant expression of EP2 receptors in GnRH neurons present scattered in the preoptic region and excitatory of PGE_2 were mimicked specifically by EP2 receptor agonist, butaprost through cAMP-PKA pathway and not by EP1 or EP1/EP3 receptor agonists, 17-PT PGE_2 or sulprostone respectively. Using GnRH-GFP or mutant GnRH-GFP/ GFAP-DN-erbB4 mice, we have also shown that astrocytes are the sources of PGE_2, as inhibition of cycloxygenase (COX) enzyme activity by bath application of indomethacin or pre-treatment of hypothalamic brain slices with glial toxin, fluoroacetate or expression of DN-erbB4 in astrocytes resulted in striking reduction of spontaneous electrical of GnRH neurons. These results indicate that

astocyte-to-neuron communication in hypothalamus may be essential for GnRH neuron activity and deregulation in their activity can lead to adult reproductive disorders. No human mutations in these pathways have been reported yet.

5.7.1. SynCAM1

Since female sexual maturation requires ErbB4 receptor signaling in the hypothalamic astrocytes and in order to dissect the downstream signaling pathways involved, recently Sandau et al., have identified Synaptic adhesion molecule 1 (SynCAM1) and its crucial role played in female sexual development (Sandau et al., 2011a; Sandau et al., 2011b). SynCAM1 also named as tumor suppressor of lung cancer-1 (Tslc1) or Nectin-like protein 2 (Necl2) is encoded by the gene *Cadm1* (Biederer, 2006). SynCAM1 is a synaptic adhesion molecule with signaling capabilities is usually expressed in neurons and these studies indicate SynCAM1 is also expressed in hypothalamic astrocytes and is functionally associated with erbB4 receptor activity. SynCAM1 was identified using isotope-coded affinity tags (ICAT) (Gygi et al., 1999), a proteomics approach that identifies and quantifies individual components of highly heterogeneous protein mixtures (Aebersold and Goodlett, 2001; Tao and Aebersold, 2003), as the protein affected by the loss of astrocytic erbB4 function by comparing the hypothalamic proteome of wild-type mice to GFAP-DNerbB4 mutant mice. Indeed, ligand-dependent activation of astroglial erbB4 receptors results in rapid association of erbB4 with SynCAM1 and activation of SynCAM1 gene transcription. In order to determine whether astrocytic SynCAM1-dependent intracellular signaling is required for normal female reproductive function, the investigators have generated a transgenic mice that expresses a dominant-negative form of SynCAM1 lacking the intracellular domain in an astrocyte-specific manner (GFAP-DN SynCAM1). The mutant protein was correctly targeted to the cell membrane and was functionally viable as shown by its ability to block intracellular calcium/calmodulin-dependent serine protein kinase redistribution, a major SynCAM1-mediated event. DN-SynCAM1 female mice had a delayed onset of puberty, disrupted estrous cyclicity, and reduced fecundity. These deficits were associated with a reduced capacity of neuregulin-dependent erbB4 receptor activation to elicit prostaglandin E2 release from astrocytes and GnRH release from the hypothalamus. These observations conclude that one of the mechanisms underlying erbB4 receptor mediated facilitation of glial-neuronal interactions in the neuroendocrine brain involves Syn-CAM1-dependent signaling and that this interaction is required for normal female reproductive function.

In a separate study, the same group had identified SynCAM1, also as a factor involved

in astrocyte-to-astrocyte and astrocyte-to-GnRH neuron adhesiveness in the mouse hypothalamus (Sandau et al., 2011b). SynCAM1 contains an extracellular domain with three Ig-like domains, an extracellular juxtamembranous region subjected to alternative splicing, a single transmembrane domain, and a short intracellular domain endowed with two protein-protein interaction motifs. Alternative splicing in the extracellular juxtamembrane region of SynCAM1 generates five isoforms (SynCAM1 1–5) (Biederer, 2006) with different molecular properties (Biederer et al., 2002). Four of them are membrane-spanning (isoforms 1– 4), and one corresponds to a secreted protein (isoform 5). In this study, investigators show that, in addition to expression of SynCAM1 in hypothalamic astrocytes, it is also expressed in GnRH neurons,. Cell adhesion assays indicated that SynCAM is recognized by both GnRH neurons and astrocytes as an adhesive partner and promotes cell-cell adhesiveness via homophilic, extracellular domain mediated interactions. Hypothalamic astrocytes and GnRH-producing GT1-7 cells express mainly isoform 4 mRNA, and sequential N- and O-deglycosylation of proteins extracted from these cells yields progressively smaller SynCAM1 species, indicating that isoform 4 is the predominant SynCAM1 variant expressed in astrocytes and GT1-7 cells. Neither cell type expresses the products of two other SynCAM genes (*SynCAM2* and *SynCAM3*), suggesting that SynCAM-mediated astrocyte-astrocyte and astrocyte-GnRH neuron adhesiveness is mostly mediated by SynCAM1 homophilic interactions.

Altogether, these results suggest that SynCAM1 is an important component of adhesive communication between astrocytes and GnRH neurons in the neuroendocrine brain however the contribution of SynCAM1-mediated adhesiveness to the reproductive phenotype seen in mutant mice with disrupted astrocytic SynCAM1 function remains to be elucidated. No human mutations have been identified related to these pathways yet.

Objectives

This bibliographical study helped us to conclude that GnRH deficiency in human patients suffering from hypogonadotropic hypogonadism involves complex genetics and multiple disease loci could be responsible for the varying heterogenic phenotypes among the subjects. Genes identified underlying GnRH deficiency play an important role either in specification or proliferation of GnRH neurons, their migration from the olfactory placode to their final destinations in the hypothalamus during embryonic development, in triggering their activation to initiate puberty, in regulating their pattern of secretion or in the response of pituitary gonadotropes to GnRH stimulations. GnRH deficiency in human subjects can be found either in isolated conditions with normal ability to smell (normosmic IHH) or along with the inability to smell (anosmia; KS) and variably with non-reproductive phenotypes suggesting the putative mutations could be involved either in genes necessary for normal development of olfactory / GnRH system or in genes necessary for initiating puberty / GnRH secretion.

Therefore, here we propose the following objectives to study during the course of PhD -

1. To evaluate the role of the guidance molecule Semaphorin3A and its obligatory co-receptor Neuropilin1 in the development of the GnRH system in mice and humans and determine whether Semaphorin 3A insufficiency is associated with Kallmann syndrome.

2. To study the role of the kisspeptin-GPR54 signaling in NO producing neurons in the neuroendocrine control of reproduction in mice.

PART I.

To evaluate the role of the guidance molecule Semaphorin3A and its obligatory co-receptor Neuropilin1 in the development of the GnRH system in mice and humans and determine whether Semaphorin 3A insufficiency is associated with Kallman syndrome.

Specific aims

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1. To study the expression pattern of Neuropilin1 receptor in migrating GnRH neurons and olfactory/vomeronasal axons during embryonic development of mice and humans.

2. To study the migratory behavior of GnRH neurons in *Nrp1*$^{sema/sema}$ knock-in mice (that harbors a mutation in the semaphorin binding domain of Neuropilin1 receptor) during embryonic development, at birth and in adults.

3. To study the olfactory and vomeronasal projections in *Nrp1*$^{sema/sema}$ mice during development.

4. To study the adult reproductive phenotype of *Nrp1*$^{sema/sema}$ mice

5. To sequence and identify putative mutations in *SEMA3A* and *NRP1* gene of human subjects suffering from Kallmann syndrome.

Summary

Here, we show that migratory GnRH neurons strongly express Neuropilin1 receptor during their early migratory phase in the nasal compartments and at the nasal-forebrain junction during embryonic development of human and mice embryos. Using the *Nrp1*$^{sema/sema}$ mice, we show that these interactions are necessary for the projections of olfactory axons from the olfactory epithelium to the olfactory bulb and formation of glomeruli. Disruption of these projections and absence of glomeruli in the *Nrp1*$^{sema/sema}$ mice leads to anosmic condition leading to their early postnatal death due to suckling disorders. *Nrp1*$^{sema/sema}$ mice that survive into adults have reduced fertility.

These interactions play an important role in the projections of ventral branch of vomeronasal nerves to the ventral forebrain. Disruption of these fibers that provide guidance to the migrating GnRH neurons leads to decreased numbers of GnRH neurons to their final targets in the hypothalamus.

Altogether these results reflect kallmann syndrome like phenotype in the *Nrp1*$^{sema/sema}$ mice. Finally sequencing of *SEMA3A* in human subjects suffering form kallmann syndrome lead to identification of 6 mutations thus identifying *SEMA3A*, as a new disease locus responsible for Kallmann syndrome. These mutations in *SEMA3A* have been identified along with earlier known KS genes supporting the digenic or oligogenic mode of inheritance is involved in the pathogenesis of this disease.

Semaphorin-3A signaling insufficiency in humans affected by Kallmann syndrome

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Abstract

Kallmann syndrome (KS) combines hypogonadism due to gonadotropin-releasing hormone (GnRH) deficiency, with anosmia. The genetics of KS involves various modes of transmission, including oligogenic inheritance. Here, we report that *Nrp1*$^{sema/sema}$ mutant mice that lack a functional semaphorin-binding domain in neuropilin-1, the obligatory semaphorin-3A coreceptor, have a KS-like phenotype. Pathohistological analysis of these mice indeed showed abnormal development of the peripheral olfactory system and defective embryonic migration of the neuroendocrine GnRH cells to the basal forebrain, which results in increased mortality of newborn mice and reduced fertility in adults, respectively. We thus screened 196 KS patients for the presence of mutations in *SEMA3A*, and identified presumably deleterious mutations in 11 patients, specifically, five missense mutations (R66W, I400V, V435I, T688A, R730Q) and a frame-shifting small deletion (D538fsX31). Four of the missense mutations resulted in reduced semaphorin-3A signaling activity in the GN11 cell line derived from embryonic GnRH cells. All the mutations were found in heterozygous state, and five patients harbored an additional pathogenic mutation in another KS gene. Our findings indicate that semaphorin-3A signaling insufficiency contributes to the KS phenotype in humans, and further substantiate the oligogenic pattern of inheritance in this developmental disorder.

Introduction

Kallmann syndrome (KS) is an inherited neurodevelopmental disorder defined as the association of hypogonadotropic hypogonadism, due to gonadotropin-releasing hormone (GnRH) deficiency, and the inability to smell (anosmia or hyposmia), related to abnormal development of the peripheral olfactory system (olfactory nerves and olfactory bulbs) (1). Neuroanatomical studies of KS fetuses have shown that the olfactory and reproductive phenotypes result from a pathological sequence in embryonic life, whereby premature interruption of the olfactory, vomeronasal and terminal nerve fibers in the fronto-nasal region disrupts the migration of neuroendocrine GnRH cells, which normally migrate from the nose to the brain along these nerve fibers (2, 3). What causes the primary failure of these fibers to establish proper contact with the rostral forebrain is, however, still unknown. Since KS is genetically heterogeneous, identification of the various genes involved and the study of appropriate animal models are expected to provide valuable clues. Barely 30% of the KS patients have mutations in any of the seven genes known so far, specifically, *KAL1*, *FGFR1*, *FGF8*, *PROKR2*, *PROK2*, *WDR11*, *CHD7*, and current efforts thus concentrate on the identification of other genes that contribute to this disorder (4). One possible strategy is based on close pathohistological examination of targeted mutant mice that may reproduce the human KS phenotype. Here, we report on *Nrp1*$^{sema/sema}$ mutant mice, which are defective for the semaphorin-binding domain of the membrane coreceptor neuropilin-1. We show that these mice have a KS-like phenotype, and provide genetic evidence that decreased signaling through the secreted protein semaphorin-3A causes KS in humans.

Neuropilin-1 expression delineates the migratory route of embryonic GnRH cells in mice and humans

In the mouse, GnRH cells begin to leave the epithelium of the medial olfactory pit around embryonic day 11.5 (E11.5). They migrate in the fronto-nasal region in close association with fibers of the developing vomeronasal and terminal nerves, then penetrate into the rostral forebrain together with the central processes of these nerves, and continue their migration towards the hypothalamic region along a branch of the vomeronasal nerve that projects to the basal forebrain or along fibers of the terminal nerve itself (5-7).

Proper navigation of growing axons depends on guidance cues. Semaphorin-3A (Sema3A) is a secreted protein which has repulsive effects on axons that express its coreceptor neuropilin-1 (Nrp1) (8, 9). Sema3A is produced in the olfactory epithelium (10), by ensheathing cells in the olfactory bulb nerve layer (10, 11), and by subsets of glomerular cells in the anterior olfactory bulb (10, 12) during development in the mouse. Sema3A is involved in the navigation of primary olfactory axons (9-11, 13, 14). It orients axon bundles, pushing Nrp1-positive axons along the correct axis for projection onto the olfactory bulb and proper olfactory map formation (10). The role played by Sema3A signaling in the navigation of vomeronasal/terminal axons and embryonic GnRH cells is still unclear, but previous studies in rodents have shown that migrating GnRH cells are morphologically associated with Nrp1-immunoreactive axons (13). Indeed, we were able to confirm these findings in E14.5 mouse embryos, and extend them to 9-week old human fetuses (Figure 1), using specific antibodies to Nrp1 (Supplementary Figure 1). Notably, the caudal branch of the vomeronasal nerve that accompanies GnRH cells in their intracerebral path was also Nrp1-immunoreactive in the mouse embryos (data not shown). In addition, we found that migrating GnRH cells

themselves are Nrp1-immunoreactive (Figure 1A, B). These observations indicate that semaphorin signaling through Nrp1 imparts guidance information to axons of the vomeronasal neurons and migrating GnRH cells.

Defective migration of GnRH cells in *Nrp1*$^{sema/sema}$ mice

Unlike *Nrp1* knockout mice, which die around E12.5 (15), *Nrp1*$^{sema/sema}$ mutant mice harboring inactivating aminoacid substitutions in the semaphorin-binding domain of Nrp1 survive until birth (16). In *Nrp1*$^{sema/sema}$ newborn mice (n=4), many axons of olfactory receptor neurons were stuck at the dorsal aspect of the cribriform plate and did not project to the olfactory bulb glomeruli (Figure 2A). Olfactory cues are thought to play an important role in suckling behavior (17). Analysis of six litters at postnatal day 1 (P1) indeed showed that 7 out of 8 *Nrp1*$^{sema/sema}$ pups had little or no milk in their stomachs, whereas most *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/+}$ littermates (18 out of 21) had full stomachs. These findings account for the decreased survival rate of *Nrp1*$^{sema/sema}$ pups (16), and strongly suggest that the sense of smell is affected in *Nrp1*$^{sema/sema}$ mice.

In the mouse embryo, the vomeronasal nerve extends across the medial aspect of the olfactory bulb and projects both dorsally, to the accessory olfactory bulb, and caudally, to the ventral forebrain (5). In *Nrp1*$^{sema/sema}$ mutant mice (n = 4), DiI axonal labeling at E14.5 showed abnormal projection of this nerve to the ventral forebrain (Figure 2B). Since the caudal branch of the vomeronasal nerve forms the axonal scaffold for the intracerebral migration of GnRH cells (5, 18), we analyzed the distribution of these cells in E14.5 and newborn *Nrp1*$^{sema/sema}$ mice. At E14.5, a significant accumulation of GnRH cells in the nasal compartment and concomitant decreased cell number within the brain already indicated abnormal cell migration (Figure 2E). In addition, while GnRH cells normally turn ventrally towards the basal forebrain, in *Nrp1*$^{sema/sema}$ embryos, many GnRH cells were found to migrate dorsally and

medially towards the cortex and the thalamus, respectively (Figure 2C). The migratory defect was still conspicuous at birth, a time when neuroendocrine GnRH cells have completed their migration in normal mice (2). Brains of *Nrp1*$^{sema/sema}$ newborn mice indeed contained 38% fewer GnRH cells (n = 4), which were dispersed in the ventral forebrain, while there was a 36% increase in the number of GnRH cells detected in the rostral forebrain compared with *Nrp1*$^{+/+}$ littermates (Figure 2E) (n=5, $p<0.01$ for both comparisons). This GnRH-cell migration defect in *Nrp1*$^{sema/sema}$ animals resulted in decreased GnRH immunoreactivity in the median eminence of the hypothalamus (Figure 2D), which is the projection field of neuroendocrine GnRH cells.

Of the *Nrp1*$^{sema/sema}$ newborn mice, only four males and two females survived into adulthood. Both females had delayed pubertal activation, specifically, the first ovulation occurred more than 10 days later than in *Nrp1*$^{sema/+}$ littermates, and monitoring of the ovarian cycle from P60 showed that one female stayed in the diestrous stage (a stage with low gonadotropin outputs) throughout the 3-week study period, while the other female had disrupted ovarian cyclicity (data not shown). Male reproductive capacity was assessed by breeding the young adult (P90) *Nrp1*$^{sema/sema}$ males with confirmed wild-type dams, and monitoring the occurrence of litters over 10-13 months. While *Nrp1*$^{sema/+}$ males (n=4) produced about one litter per month, the fertility index (number of litters per month) was markedly reduced in the *Nrp1*$^{sema/sema}$ males, which only gave birth to 2 to 4 litters (fertility index: 0.29±0.04 *vs.* 1.08±0.12 in *Nrp1*$^{sema/+}$, Student's t-test, $p<0.001$). Moreover, neuroanatomical analysis of *Nrp1*$^{sema/sema}$ adult brains showed significantly reduced GnRH cell populations in the preoptic and hypothalamic regions (384±33 cells, n=4) compared to *Nrp1*$^{sema/+}$ littermates (767±25 cells, n=4; Student's t-test, $p<0.001$). Therefore, the defective GnRH cell migration found in *Nrp1*$^{sema/sema}$ mouse embryos was not corrected during later development and caused subfertility in adult mutants.

Mutations in *SEMA3A* cause Kallmann syndrome in humans

The KS-like phenotype of *Nrp1*$^{sema/sema}$ mice and *Sema3a*$^{-/-}$ mice ((13) and this study) prompted us to ask whether insufficient Sema3A signaling through Nrp1 might also be involved in the human disorder. We sought mutations in the 17 coding exons of *SEMA3A* in 196 unrelated KS patients, of whom 61 already harbored a mutation in one of the known KS genes, specifically, *KAL1* (11 patients), *FGFR1* (19 patients), *FGF8* (2 patients), *PROKR2* (24 patients), or *PROK2* (5 patients). Three missense mutations (R66W, T688A, R730Q) and a frame-shifting deletion of 14 nucleotides (c.del1613_1626; p.D538fsX31) were detected, all in heterozygous state, in 4 patients, none of which were found in 400 control alleles. In addition, two missense mutations also found in some control individuals, I400V and V435I, were found in one and six patients, respectively (Table 1). The deleterious effect of the five missense mutations was tested *in vitro* on the signaling activity of Sema3A, using the GN11 cell line (19, 20) and conditioned media from transfected COS-7 cells producing Sema3A either from the wild-type *SEMA3A* cDNA or from cDNAs harboring the mutations. We found that the conditioned medium from COS-7 cells transfected with the wild-type *SEMA3A* cDNA was as potent at inducing phosphorylation of FAK (focal adhesion kinase) and ERK1/2 (extracellular signal-regulated kinases 1 and 2) in GN11 cells as was the purified recombinant human Sema3A. By contrast, all Sema3As harboring the missense mutations, except the R730Q mutant, were inefficient, although they were apparently produced and secreted in normal amounts (Figure 3 and data not shown). We thus conclude that these are loss-of-function pathogenic mutations that affect the signaling activity of Sema3A, and surmise that the R730Q mutation, which removes an evolutionarily conserved basic residue in the C-terminal basic motif of the protein (8), has a different, as yet uncharacterized pathogenic effect. Notably, the patients carrying the T688A and I400V mutations, and three patients carrying the V435I mutation also carry Y217D, R268C (two patients), H70fsX5, and G687N

pathogenic mutations in *KAL1*, *PROKR2*, *PROK2*, and *FGFR1*, respectively (Table 1). Together with the normal phenotype of *Nrp1*$^{sema/+}$ heterozygous mice, this indicates that monoallelic mutations in *SEMA3A* are not sufficient to induce the KS phenotype, and further substantiates the digenic/oligogenic mode of inheritance of KS (21). Accordingly, the other patients who carry monoallelic mutations in *SEMA3A* are expected to carry at least one additional mutation in another, still unknown KS gene. Although *NRP1* might be viewed as a good candidate, we did not find a mutation within the 17 coding exons of *NRP1* in any of these patients, nor did we in a group of 100 KS patients without *SEMA3A* mutations.

Materials and Methods

The study was approved by the national research ethics committee (agence de biomédecine, Paris, France).

Patients

Informed consent was obtained from all individuals analyzed. Clinical data regarding the patients were obtained from the referring endocrinologists. All patients had hypogonadotropic hypogonadism and anosmia or hyposmia. Genomic DNAs were prepared from white blood cells using a standard procedure.

Human fetuses

Human fetuses were obtained from voluntary terminated pregnancies, with parent's written informed consent. Gestational age was established by crown-rump length measurement. Fetuses were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, for 3 weeks at 4°C, and then immersed in 0.1 M PBS containing 30% sucrose for 2 days

at 4°C. The heads were embedded in OCT embedding medium (Tissue-Tek®), frozen, and sagittal cryosections (20 µm) were taken and processed for immunohistofluorescence.

Animals

All experiments were carried out in accordance with the European Communites Council Directive of November 24, 1986 (86/609/EEC) regarding the mammalian research and French bylaw. *Nrp1*$^{sema/sema}$ mice (B6.129(C)-Nrp1tm1Ddg/J) were purchased from the Jackson laboratory (Maine, USA), maintained on a controlled 12h:12h light cycle, provided with food and water ad libitum, and genotyped as described earlier (16). E14.5 embryos (plug day, E0.5), postnatal day 0 (P0), and adult wild-type and *Nrp1*$^{sema/sema}$ mice were obtained (19) and processed for immunofluorescence as described (19).

Immunohistofluorescence

Immunohistofluorescence experiments were carried out as described (19). Primary antibodies were: rabbit anti-GnRH (dilution 1:3000), a gift from G. Tramu (University of Bordeaux, France); goat anti-neuropilin1 (dilution 1:400), AF566 (R & D systems); goat anti-OMP (dilution 1:6000), a gift from F. L. Margolis (University of Maryland, Baltimore, USA).

DiI labeling of vomeronasal nerve fibers

Vomeronasal nerve fibers were traced anterogradely with the lipophilic fluorescent dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) as described (5). After diffusion of the tracer, serial sagittal sections (100 µm thick) were taken through the forebrain, and analyzed using a LSM 710 confocal microscope (Zeiss) and the ImageJ analysis software (NIH, Bethesda, USA).

Cell cultures

COS-7 cells and GN11 cells were grown in monolayers in 5% CO_2 at 37 °C, in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 1 mM sodium pyruvate, 2 mM glutamine, 9 mg/ml glucose and supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml streptomycin and 100 U/ml penicillin.

Signaling activity of wild-type and mutant Sema3A in GN11 cells

A cDNA containing the entire coding region of the human *SEMA3A* (GenBank NM_006080) was inserted into a pRK5 plasmid expression vector. Recombinant plasmids containing *SEMA3A* cDNAs harboring each of the five missense mutations identified in the KS patients were then engineered by using the QuickChange mutagenesis protocol (Stratagene). COS-7 cells were transiently transfected using fast-forward protocol (Lipofectamine 2000, Invitrogen) (19). Conditioned medium was collected 48 h after transfection, tested for the presence of Sema3A by western blot using an anti-Sema3A antibody (Santa Cruz, sc-10720, dilution 1:100), and then processed for signaling activity experiments in the GN11 cell line. Briefly, subconfluent GN11 cells were grown overnight in serum-free medium, and then stimulated for 20 min with human recombinant Sema3A (R&D systems) at 100 ng/ml, or with concentrated conditioned media from transfected COS-7 cells. Western blot experiments were (19) carried out on cell lysates using antibodies to P-ERK (#9101L) and ERK (#9102L) from Cell Signaling (dilution 1:1000), or P-FAK (sc56901) and FAK (sc81493) from Santa Cruz (dilution 1:500).

DNA sequencing

Each of the *SEMA3A* and *NRP1* coding exons and flanking splice sites was PCR-amplified from genomic DNA using a specific primer set (see supplementary materials for primer

sequences), and sequenced using either PCR oligonucleotide as sequencing primer. The mutations were confirmed by sequencing two independent PCR products on both DNA strands. Exons 2, 11, 14, and 17 of *SEMA3A*, in which mutations were identified in some patients, were analyzed by denaturing high performance liquid chromatography (DHPLC) scanning on an automated HPLC instrument (Wave technology) in 200 unrelated Caucasian controls.

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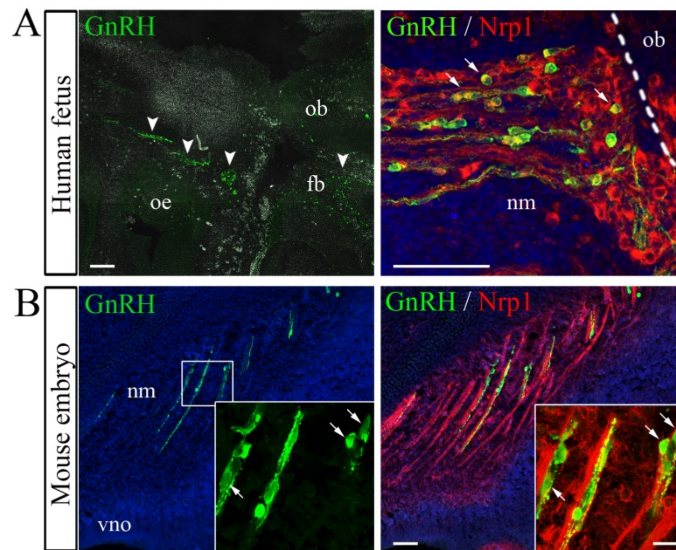


Figure 1. Expression of the Sema3A coreceptor Nrp1 by vomeronasal / terminal nerve fibers and migrating GnRH cells in human and mouse embryos.

(A) Sagittal section of the olfactory epithelium (oe) and olfactory bulb (ob) regions (left panel) and detail of the fronto-nasal region (right panel) in a 9 week-old human fetus. Clusters of GnRH-immunoreactive cells (green, arrowheads) are visible in the fronto-nasal mesenchyme and the rostral forebrain (fb). In the fronto-nasal region, these cells migrate in close contact with Nrp1-immunoreactive axons (red). Note that migrating GnRH cells are also Nrp1-immunoreactive, as shown by their yellow (green+red) staining in the right panel (arrows). (B) Sagittal section of the nasal region in an E14.5 mouse embryo. In the nasal mesenchyme (nm), migrating GnRH-immunoreactive cells (green) are morphologically associated with Nrp1-immunoreactive nerve fibers (red) originating in the vomero-nasal organ (vno). Single plane confocal images at higher magnification (insets) show that GnRH cells are Nrp1-immunoreactive (arrows). Scale bars: 100 μm (25 μm in inset).

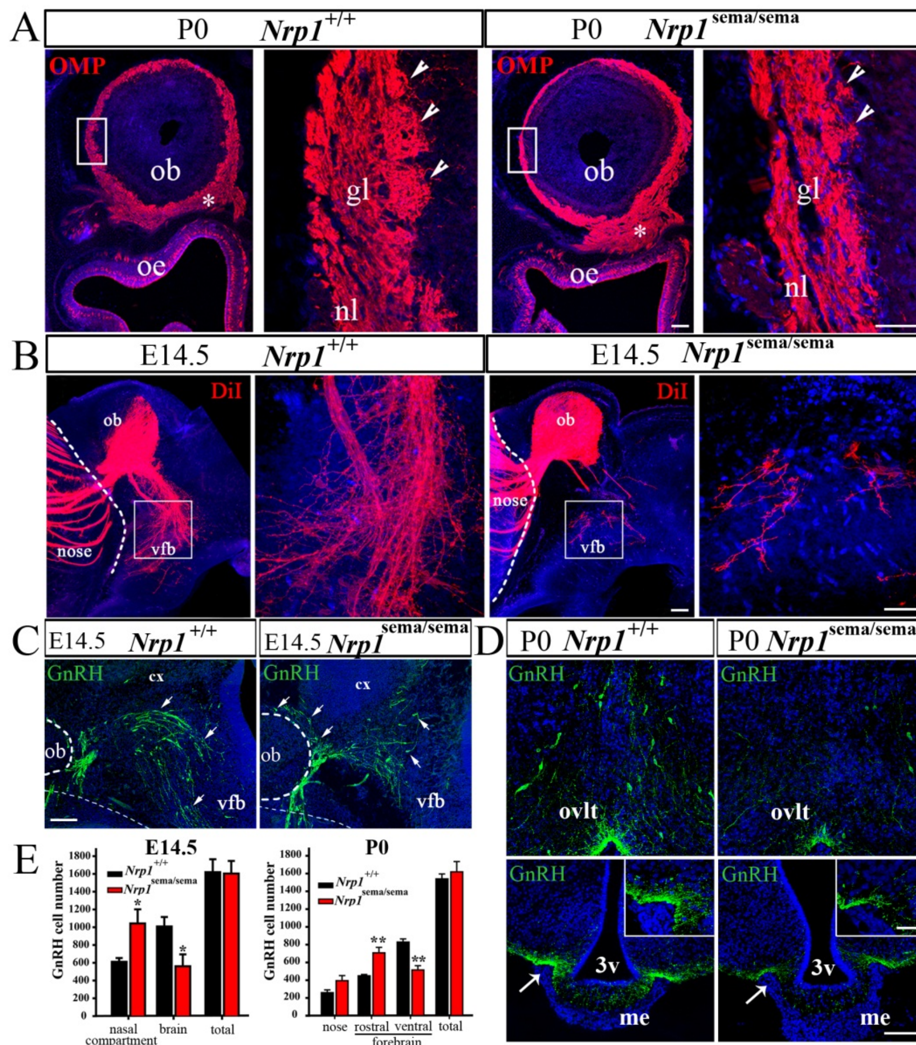


Figure 2. Defects in olfactory and vomeronasal axons, and GnRH cell migration in *Nrp1*$^{sema/sema}$ mutant mice. (A) Coronal sections of the right olfactory epithelium (oe) and olfactory bulb (ob) regions (left panels), and detail of the olfactory bulb showing the olfactory nerve layer (nl) and glomerular layer (gl) (right panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ P0 mice. Axons of the olfactory receptor neurons are immunostained (red) for the olfactory marker protein (OMP). In the *Nrp1*^{semasema} mouse, the immunostaining is both enlarged below the olfactory bulb ventro-medial aspect (asterisks) and markedly reduced in the glomerular layer (arrowheads) compared to wild-type. (B) Sagittal sections of the rostral and ventral forebrain regions (left panels), and detail of the caudal branch of the vomeronasal nerve (right panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ E14.5 mice. A crystal of the DiI lipophilic fluorescent dye has been placed in the vomeronasal organ lumen to anterogradely label vomeronasal axons. In the mutant mouse, fibers in the caudal branch of the vomeronasal nerve are scarce compared to wild-type. (C) Sagittal sections of the rostral and ventral forebrain regions at E14.5, immunostained for GnRH (green). Note the abnormal distribution of GnRH-immunoreactive cells in the *Nrp1*$^{sema/sema}$ mouse (arrowheads). (D) Coronal sections of the preoptic region (upper panels) showing GnRH neuroendocrine cells (green) and their projections in the median eminence (me, arrows) (lower panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ newborn (P0) mice. The immunostaining is reduced in the *Nrp1*$^{sema/sema}$ mouse. (E) Quantitative analysis (mean ± s.e.m.) of GnRH cell distributions at E14.5 and P0. * and ** denote statistically significant differences, using Student's t test with $p<0.05$ and $p<0.01$, respectively. Other abbreviations: cx, cerebral cortex; ovlt, organum vasculosum of lamina terminalis; 3v, third ventricle. Scale bars: 100 µm (50 µm in inset).

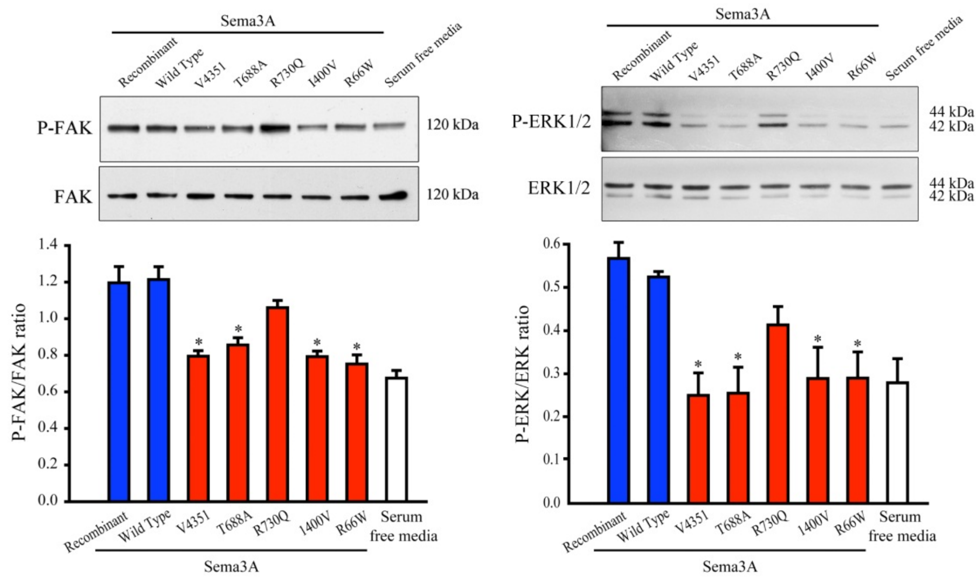


Figure 3. Defective signaling activity of Sema3A proteins harboring the missense mutations identified in Kallmann syndrome patients.

Upper panels: Representative western blots for the phosphorylated and total forms of FAK (left-panel) and ERK1/2 (right-panel) in GN11 cells following the indicated stimulations. Bar graphs in lower panels illustrate the mean ratio (± s.e.m.) of the western blot signal obtained for phosphorylated FAK (P-FAK) or ERK1/2 (P-ERK1/2) to that of total FAK or ERK1/2, respectively. * denotes statistically significant difference with wild-type Sema3A (Student's t test, $p<0.05$).

Nucleotide mutation	Exon	Aminoacid change	Protein domain	Allele frequency in controls	Nb of patients	Additional	
c.197C>T	2	R66W	sema	0/400	1	-	
c.1198A>G	11	I400V	sema	§	1	*PROKR2*	R268C
c.1303G>A	11	V435I	sema	6/400	3	-	
“	“	“	“	“	1	*PROKR2*	R268C
“	“	“	“	“	1	*PROK2*	H70fsX5
“	“	“	“	“	1	*FGFR1*	G687F
c.del1613_1626	14	D538fsX31	PSI	0/408	1	-	
c.2062A>G	17	T688A	interdomain	0/446	1	*KAL1*	Y217D
c.2189G>A	17	R730Q	basic motif	0/446	1	-	

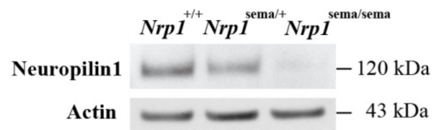
Table 1: *SEMA3A* mutations in Kallmann syndrome patients

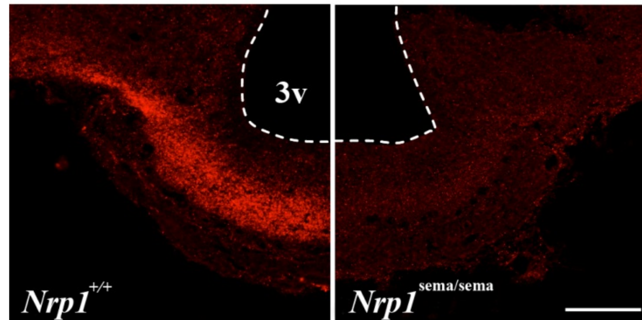
Supplementary information

Expression of Neuropilin1 receptor

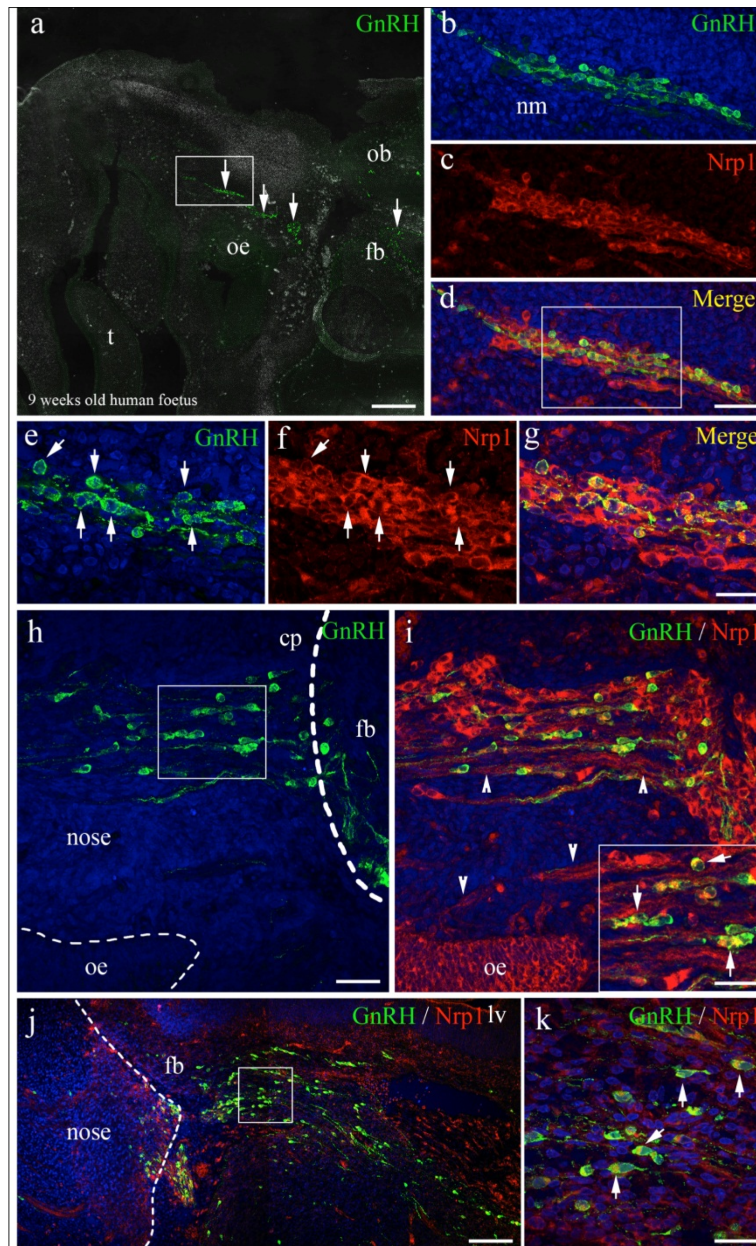
Specificity of neutralizing Neuropilin1 antibody that recognizes specifically the semaphorin binding domain was tested using *Nrp1*$^{sema/sema}$ homozygous mice (**Supplementary Figure 1**).

Neuropilin1 receptor is expressed by the migratory GnRH neurons and olfactory/vomeronasal systems both in mice (**Supplementary Figure 3 and 4**) and human embryos (**Supplementary Figure 2**) during embryonic development.

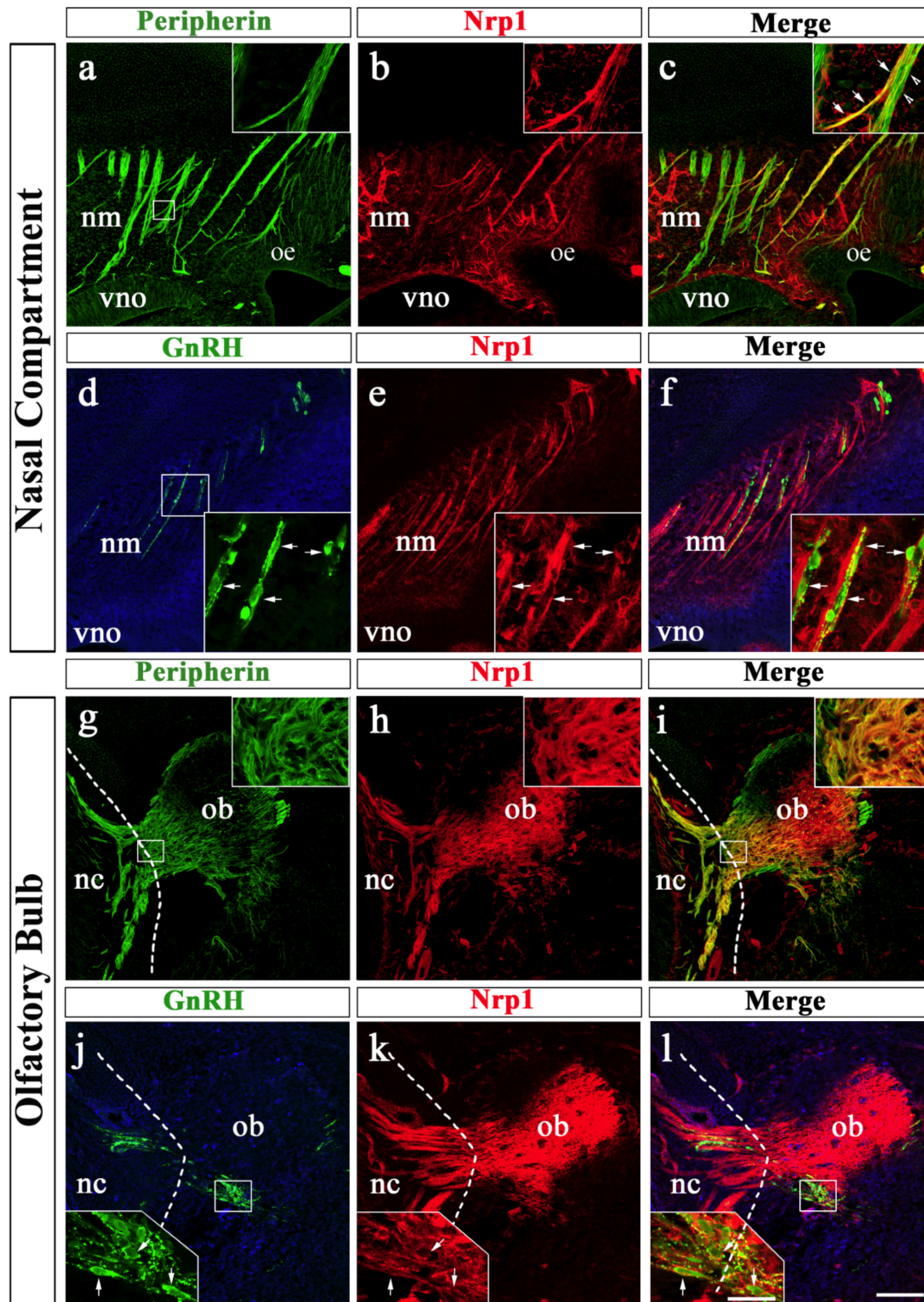




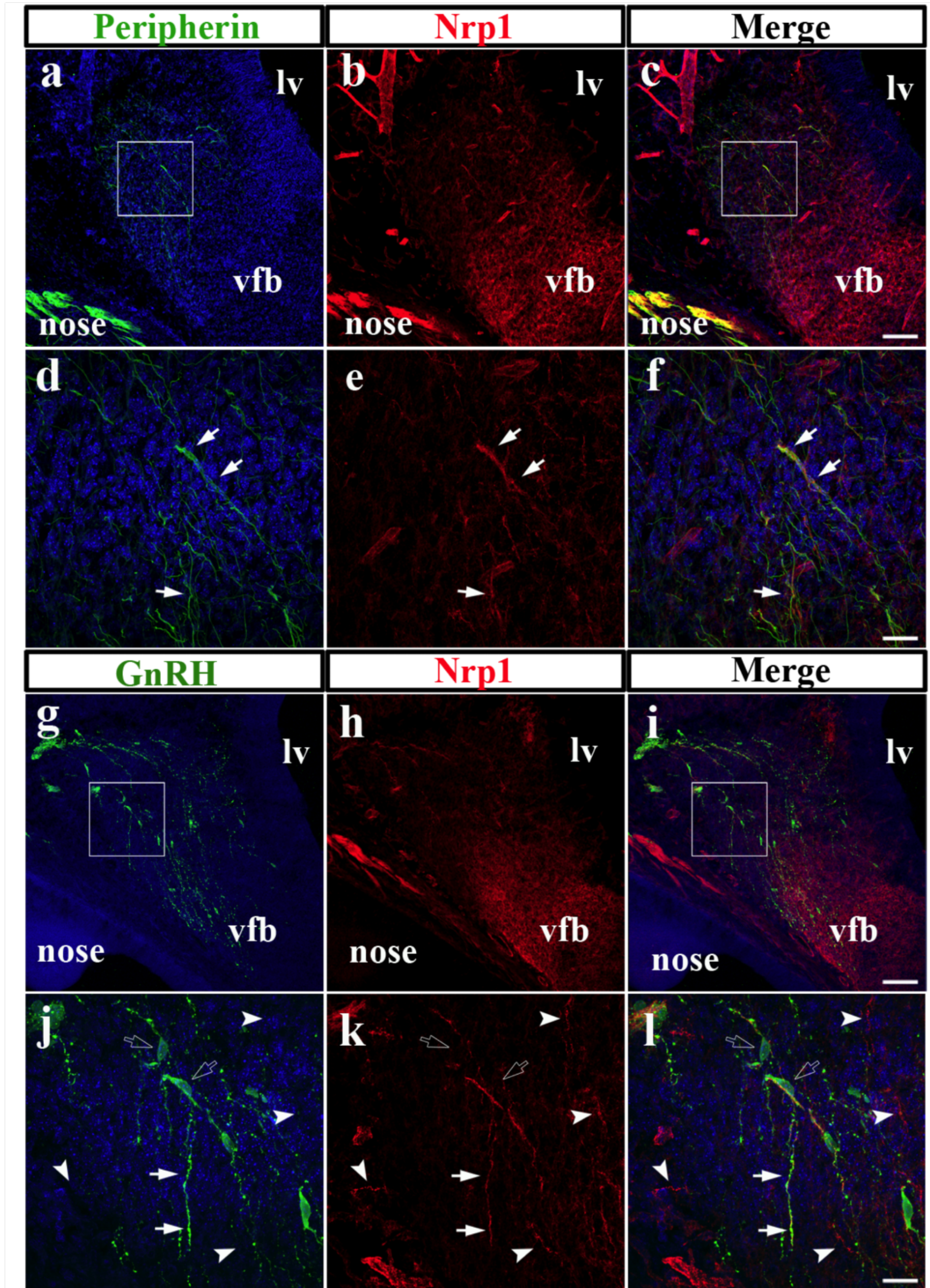
Supplementary Figure 1. The anti-neuropilin1 (Nrp1) polyclonal antibody AF566 (R & D systems) selectively recognizes the semaphorin-binding domain of the protein. Top panel: western blot analysis of Nrp1 in protein extracts from the hypothalamus of *Nrp1*$^{+/+}$, *Nrp1*$^{sema/+}$ and *Nrp1*$^{sema/sema}$ mice (antibody used at 1:1000 dilution). Bottom panel: immunohistofluorescence analysis of Nrp1 in the median eminence of *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ newborn mice (antibody used at 1:400 dilution). Scale bar: 200 μm.



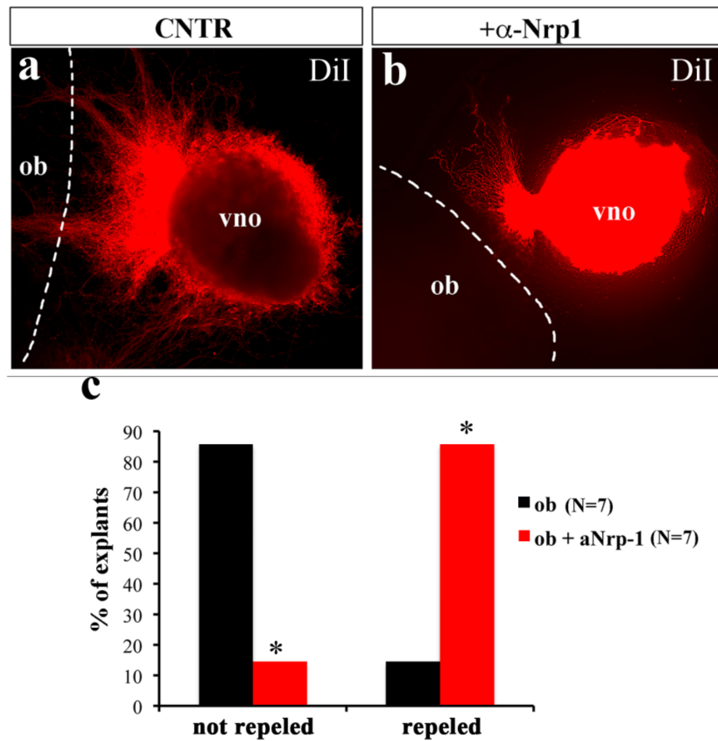
Supplementary Figure 2. Expression of Nrp1 in olfactory axons and migrating GnRH neurons in human 9 week-old fetus. (a) Low magnification microphotographs showing distribution of GnRH-immunoreactive (-ir) cells in a sagittal section of a human fetus. GnRH-ir cells (arrows) are found migrating in the nasal mesenchyme towards the olfactory bulb (ob) and eventually entering the forebrain (fb). A phase-contrast image is used to visualize the morphological limits of each anatomical structure (grey). (b-d) High magnification image of the zone delineated in a. In the nasal compartment, robust expression of Nrp1-ir is found in a migratory mass of cells (c,d) including GnRH neurons (a,d). (e-g) Higher magnification images showing colocalisation of Nrp1 in GnRH-ir cells (arrows). (h,i) At the nasal forebrain junction, GnRH neurons are seen morphologically associated with Nrp1-ir olfactory axons (i, arrowheads) and Nrp1 is also expressed in the olfactory epithelium (i, oe). (j,k) In the forebrain, Nrp1-ir markedly decreases in GnRH cell bodies (k, arrows). In b-k sections are counterstained with Hoechst to visualize cell nuclei. t, tongue; oe, olfactory epithelium; ob, olfactory bulb; fb, forebrain; nm, nasal mesenchyme; cp, cribriform plate; lv, lateral ventricle. Scale bar = 100 µm applies to a,h,j; 50 µm in d applies to b-d; 25 µm in d applies to e-g i inset and k.



Supplementary Figure 3. Expression of Nrp1, in olfactory/vomeronasal axons and migrating GnRH neurons in the mouse 14.5 day-old embryo. (a-c) Representative microphotographs showing distribution of peripherin and Nrp1 immunoreactivities (-ir) in sagittal sections an E14.5 embryo. Note that while all Nrp1-ir axons are also +ve for perpherin (inset), many peripherin-positive fibers do not colocalize Nrp1 (arrowheads). (d-e) In the nasal mesenchyme, GnRH-ir neurons are morphologically associated with Nrp1-ir fibers. Single plane confocal images showing Nrp1-ir in GnRH neurons (insets). (g-i) At the nose-brain junction, peripherin and/or Nrp1-ir nerve fascicules converge before crossing the cribriform plate and project into the rostral forebrain. Note that the ventral branch of vnn express Nrp1 (h,i; arrow). (j-l) GnRH neurons migrating along with Nrp1-positive (red) fibers into the rostral forebrain. nm: nasal mesenchyme; oe, olfactory epithelium; vno, vomeronasal organ; ob, olfactory bulb; vfb, ventral forebrain; vnn, vomeronasal nerve. Scale bar: 200 μm and 50 μm in a and in inset, respectively (applies to b-l).



Supplementary Figure 4. In the ventral forebrain, Nrp1 is expressed in axons of the caudal branch of the vomeronasal nerve and in GnRH neurites but not in GnRH cell bodies. (a-f) Representative microphotographs showing the distribution of peripherin and Nrp1 (red) immunoreactivities (-ir) in sagittal sections of the ventral forebrain region in a E14.5 mouse embryo. Note that all Nrp1-ir axons are also immunoreactive for peripherin (d,f). (g-l) In the forebrain, GnRH-ir neurons are morphologically associated with Nrp1-ir fibers. Single plane confocal images show expression of Nrp1 in GnRH neuronal processes (j,k; arrows), but not GnRH cell bodies. In (j-l) arrowheads show GnRH negative Nrp1-ir fibers. vfb, ventral forebrain. Scale bar: 200 μM in a - c and g - i; 50 μM in d - f and j - l.



Supplementary Figure 5. Vomeronasal fibers sense Sema3A released from olfactory bulb. Representative images of DiI labelled Vomeronasal explants co-cultured with repective olfactory bulb explants at E13.5 in presence (b) or absence (a) of blocking-function Neuropilin1 antibodies. c. Graph illustrating the percentage of explants not replelled or repelled in presence of absence of blocking function Neuropilin1 antibodies. *< 0.05

Supplementary materials: primer sequences

SEMA3A

SEMA3A-1A: GAAACTGACCTAAATCACCTG
SEMA3A-1B: GTTGGGAGGGAGTTCAAGG

SEMA3A-2A: ACAGCAGTCAATATAGTCAGG
SEMA3A-2B: TATCTATAACTTGAGATGCTTC

SEMA3A-3A: CAGTTGCCCCAATGTCATCT
SEMA3A-3B: GAATGAAGGATACTCAACCTG

SEMA3A-4A: ATCTGTTCCAGCATGCCTAG
SEMA3A-4B: TGAATAGAAAGGGGTCATGGA

SEMA3A-5A: TCTCTGATTAACTGATGTGTTG
SEMA3A-5B: AAAGAACATACAACCTGTTTGT

SEMA3A-6C: ATGGTCATAACATGAAACTTGC
SEMA3A-6B: TCAAGTCATATTGCATGTACTG

SEMA3A-7A: AATGGACTGTTCAGAATGGTAT
SEMA3A-7B: TTGTATATGCACACAGGTAGAA

SEMA3A-8A: TTGAGGGAACGATTCGACC
SEMA3A-8B: TCTATACATAAACACTAGCTTC

SEMA3A-9A: AACTGCGTAAGAAGCTAGTGT
SEMA3A-9B: TATGAGTACTTGGATAGCACC

SEMA3A-10A: CTCAGTATCAATATTTCTCTTAC
SEMA3A-10B: TATCTGTCTGTAGCTGCATTG

SEMA3A-11A: AGAACCATTGAGGCCATGTG
SEMA3A-11B: CATCCCAACCCCTGAGATG

SEMA3A-12A: GGAAGACCGATATCAAAGGTT
SEMA3A-12B: GGAAAGACGTACAACTGAACT

SEMA3A-13A: ACTAATGGCAGTGCCTTGAG
SEMA3A-13B: CTAATCTACTAGCTTATTGTAAG

SEMA3A-14A: GAACAAGAGATTTAAAAGACAAG
SEMA3A-14B: ACTTGGAATCAGATAGGATAAC

SEMA3A-15D: TTGGAGACTGCTCTTACAGG
SEMA3A-15B: CTTTAAGTATTCTGAGAGATGC

SEMA3A-16A: TTGGCAATAACTTGTCTCCTG
SEMA3A-16B: AGGATAAGCATTCTCAGTGCT

SEMA3A-17A: ACACGGAGTTTCAGAGCTTC
SEMA3A-17B: GGAACTCAGCTGAATTTCCC

NRP1

NRP1-1A:	GAGGATTGTACAGCTCTAGG
NRP1-1B:	TCGGTTGTTCCCGGCTGAT
NRP1-2A:	GCTAGATTACCTAACAGGTTG
NRP1-2B:	AGACAGGCGTGACCACTAG
NRP1-3A:	AAGGGCATTTCTCACCAACC
NRP1-3B:	GCCACCACACTCGGCCTA
NRP1-4A:	ATGTTCTGTCTTTACCCAGGT
NRP1-4B:	GATTCATGTATCATGAGACTTG
NRP1-5A:	TGCTAATTCTTGCATCTGCTG
NRP1-5B:	CATGTGGCCGCAGGTGTG
NRP1-6A:	TCCAAGTATCAGTGCTATATTCC
NRP1-6B:	TCAACAACCTCTCTAGATGGT
NRP1-7A:	CCTGTTGATCCCAGGTGGA
NRP1-7B:	GGCCAGACAGAAAGCTACC
NRP1-8A:	ATCTCATCCTTGATCGACAAC
NRP1-8B:	CATTTACAAACTTATTTACCCTG
NRP1-9A:	TGAGGGAGGAAAGATTTACTG
NRP1-9B:	TCCTCTAATGTCATGGCTGG
NRP1-10A:	ACAAAGTGGTAGAATGAAGCC
NRP1-10B:	TTTGCAAAGGCACCATCAGG
NRP1-11A:	TCCAACAGCCAGAGGCTTG
NRP1-11B:	GTTAGCTGGTCCCAACTAAG
NRP1-12A:	CTCTGATAGACATTTGTAAGCA
NRP1-12B:	CCAGATGCTGTGTGGCATC
NRP1-13A:	TTCCATGAGTGAACCAAGGG
NRP1-13B:	ATTCACAGACATTAGAAACCCT
NRP1-14A:	GTGGTAATCCTGGCCACTC
NRP1-14B:	TCAGCATTCCTGCAGCCAC
NRP1-15A:	TAGCTGGAGCTGAACAAGCA
NRP1-15B:	AGACCATCATATTGGCAAGAG
NRP1-16A:	ATGTACCTAAAGGTTTGTGTAG
NRP1-16B:	ACCAGTGTATTAGAGTTCCAC
NRP1-17A:	GTGCTAAGCAGTCTGTCAGA
NRP1-17B:	AAGATCAACAGCTCCCCCAGC

PART II.

To study the role of the kisspeptin-GPR54 signaling in NO producing neurons in the neuroendocrine control of reproduction in mice.

Specific aims

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1. To study morphological interactions between NO producing neurons and kisspeptin producing neurons in the preoptic region where GnRH neurons are present scattered.

2. To study if the NO producing neurons in the preoptic region express kisspeptin receptor Gpr54 using the LacZ-Gpr54 transgenic mice.

3. To study if kisspeptin induces any posttranslational modifications in the neuronal nitric oxide synthase enzyme.

4. To study if Gpr54 receptor is required for kisspeptin induced posttranslational modifications in nNOS and the downstream signaling pathways involved.

5. To study if ovarian steroids modulate nNOS neurons through kisspeptin-Gpr54 signaling.

6. To study the role of kisspeptin-nNOS neurons interactions in GnRH release during ovarian cycle.

These studies demonstrate a crucial morphological and functional interaction between two neuronal populations in the preoptic region necessary for the ovulation in mice. While kisspeptin neurons have a direct action on GnRH neurons, several recent reports indicate kisspeptin neurons can also have indirect actions on GnRH neurons. These studies recognize nNOS in the preoptic region as one of these cell populations that kisspeptin cells interact to modulate the activity of GnRH neurons and GnRH/LH release.

Kisspeptin-immunoreactive fibers were found in close apposition on/around nNOS neurons specifically in the OVLT and MePO while no inputs were found on/around the nNOS neurons present in the medial septum or in the AVP nuclei. Studies using the Gpr54-LacZ mice demonstrate that along with the expression of Gpr54 in GnRH neurons, the nNOS neurons present in the OVLT and the MePO also expressed Gpr54 receptor.

Peripheral injections of Kisspeptin-10 readily induced posttranslational modifications of nNOS protein resulting in increased phosphorylation levels at the Ser1412 residue specifically in the OVLT and the MePO. These effects were mediated by the only known kisspeptin receptor, Gpr54 and PI3K-AKT signaling pathway is involved in the kisspeptin mediated induction in posttranslational modifications of nNOS protein. Estrogens that were earlier known to strongly modulate the activity of nNOS in the preoptic region during the ovarian cycle by inducing increased phosporylation at the Ser1412 residue and results that are shown in this study suggest that estrogens require kisspeptin-Gpr54 signaling to mediate their effect on the nNOS neurons in the preoptic region.

Finally, NO released by the nNOS neurons have different effects on the GnRH/LH release during the ovarian cycle. During the diestrus stage NO is released in minimal amounts and this acts as inhibitory on the GnRH/LH release and in the absence of NO, kisspeptin readily induces high amounts of GnRH/LH whereas during the proestrus stage, NO that is released in higher amounts is required for kisspeptin mediated GnRH/LH surge.

These findings suggests that nNOS neurons once known for its important role in GnRH regulation is well integrated in the neural circuits regulating GnRH neurons.

Kisspeptin-GPR54 signaling in mouse NO-synthesizing neurons participates in the hypothalamic control of ovulation

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Abstract

Reproduction is controlled in the brain by a neural network that drives the secretion of gonadotropin-releasing hormone (GnRH). Various permissive homeostatic signals must be integrated to achieve ovulation in mammals. However, the neural events controlling the timely activation of GnRH neurons are not completely understood. Here we show that kisspeptin, a potent activator of GnRH neuronal activity, directly communicates with neurons that synthesize the gaseous transmitter nitric oxide (NO) in the preoptic region to coordinate the progression of the ovarian cycle. Using a transgenic *Gpr54*-null IRES-LacZ knock-in mouse model, we demonstrate that neurons containing neuronal NO synthase (nNOS), which are morphologically associated with kisspeptin fibers, express the kisspeptin receptor GPR54 in the preoptic region, but not in the tuberal region of the hypothalamus. The activation of kisspeptin signaling in preoptic neurons promotes the activation of nNOS through its phosphorylation on serine 1412 via the AKT pathway, and mimics the positive feedback effects of estrogens. Finally, we show that while NO release restrains the reproductive axis at stages of the ovarian cycle during which estrogens exert their inhibitory feedback, it is required for the kisspeptin-dependent preovulatory activation of GnRH neurons. Thus, interactions between kisspeptin and nNOS neurons may play a central role in regulating the hypothalamic-pituitary-gonadal axis *in vivo*.

Key words: LHRH, nitric oxide synthase, KISS1R, luteinizing hormone, hypothalamus

Introduction

The survival of a mammalian species depends upon the ability of its individuals to quickly, effectively, and reproducibly transmit homeostatic signals to the hypothalamic neuronal population that releases gonadotropin-releasing hormone (GnRH) and controls reproduction. This requires the mediation of neural networks that sense moment-to-moment changes in physiological inputs throughout life (Wintermantel et al., 2006; Mayer et al., 2010; Donato et al., 2011; Mayer and Boehm, 2011). We hypothesized that interactions between hypothalamic neurons expressing kisspeptin and those expressing neuronal nitric oxide synthase (nNOS) could be of particular importance in regulating the neuroendocrine output of GnRH neurons during the ovarian cycle, as the mutation of either of these causes infertility in mice (Gyurko et al., 2002; d'Anglemont de Tassigny et al., 2007b; Lapatto et al., 2007).

In rodents, GnRH neurons are predominantly located in the preoptic region of the ventral forebrain. They project to the median eminence of the hypothalamus, where GnRH is released into portal blood vessels for delivery to the anterior pituitary. In the pituitary, GnRH elicits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone, which stimulate gametogenesis and gonadal hormone secretion and thus support reproductive function. In recent years, kisspeptin and its receptor GPR54 (also known as KISS1R) have emerged as key players in the regulation of GnRH/LH release (Oakley et al., 2009; d'Anglemont de Tassigny and Colledge, 2010; Navarro and Tena-Sempere, 2011). Kisspeptin-expressing neurons, which are found primarily in the hypothalamus, directly innervate and stimulate the electrical activity of GnRH neurons, which express the kisspeptin receptor GPR54 (Clarkson and Herbison, 2009). Mice with a targeted deletion of *Gpr54* are sterile (Funes et al., 2003; Seminara et al., 2003; Dungan et al., 2007; Kauffman et al., 2007; Lapatto et al., 2007), and kisspeptin-GPR54 signaling appears essential for the GnRH neuronal activation that initiates ovulation (Clarkson et al., 2008). In addition to acting directly on GnRH neurons, an

increasing body of evidence suggests that kisspeptin also operates on unidentified neurons to modulate the strength of synaptic afferents and regulate GnRH secretion (Pielecka-Fortuna et al., 2008). Nitric oxide (NO), a gaseous neurotransmitter known for its important role in regulating neuronal transmission (Garthwaite, 2008) and the neuroendocrine control of reproduction (Bellefontaine et al., 2011), has recently been implicated in the regulation of GnRH neuronal activity (Clasadonte et al., 2008). This NO is produced in the vicinity of GnRH-containing perikarya by neurons expressing nNOS (Clasadonte et al., 2008), an enzyme whose activity has been shown to be tightly regulated by estrogens during the ovarian cycle in rats (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010).

We investigated whether kisspeptin-positive neurons projected onto nNOS neurons, and whether kisspeptin signaling in these cells could contribute to the hypothalamic control of reproduction. Using genetic mouse models, we demonstrate that kisspeptin neurons directly act on NO-synthesizing neurons to promote preovulatory nNOS activation and that these interactions modulate LH secretion.

Material and Methods

Animals

Experiments were performed on adult (2-3 months old) female C57Bl/6J (Charles River Laboratories) and *nNOS* null mice (Huang et al., 1993) (Jax mice, Jackson Laboratory, Bar Harbor, ME), *Gpr54*-null mice (Seminara et al., 2003), *Kiss1*-null mice (d'Anglemont de Tassigny et al., 2007b) and their wild-type littermates. Genotypes were determined by PCR as reported previously (Huang et al., 1993; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007b). Mice were housed under a 12h:12h lighting schedule with *ad libitum* access to food and water. For most of the experiments, vaginal smears were taken daily to identify mice in a specific day of the estrous cycle. All experiments were performed in accordance with the

guidelines for animal use specified by the European Communities Council Directive of November 24th, 1986 (86/609/EEC) regarding mammalian research and were approved by the University of Lille 2 Animal Use Committee, or following the United Kingdom Home Office Project Licence and were approved by the Cambridge Animal Ethics Committee.

Drugs

Synthetic Kisspeptin-10 [Rodent Metastin (45-54) amide; YY-10-NH_2] was purchased from GeneCust (Luxembourg). The NOS inhibitor N G-Nitro-L-arginine Methyl Ester, HCL (L-NAME), the PI3-kinase inhibitor, LY294002 (440202) the MEK inhibitor, U0126 (662005) and DMSO (317275) were purchased from Calbiochem, Meudon, France. 17β-estradiol 3-benzoate (E8515), 17β-Estradiol (E8875), progesterone (P0130) and sesame oil (S3547) were purchased from Sigma.

Antibodies

Immunofluorescence. The sheep polyclonal anti-nNOS antibody (1:3000) was a generous gift from Dr. P. C. Emson (Medical Research Council, Laboratory for Molecular Biology, Cambridge, UK) (Herbison et al., 1996). The rabbit polyclonal anti-kisspeptin antibody (1:5000; AC-566) was a generous gift from Dr. Alain Caraty (INRA, Tours, France) (Franceschini et al., 2006). The rabbit polyclonal anti- GnRH (1:3000) was a generous gift from Prof. G. Tramu (Centre Nationale de la Recherche Scientifique, URA 339, Université Bordeaux I, Talence, France) (Beauvillain and Tramu, 1980). The rabbit polyclonal antiphosphorylated nNOS (Ser^{1412}) antibody (71-8600; 1:500) was purchased from Affinity BioReagents. The Alexa Fluor 568-conjugated donkey anti-sheep secondary antibody (A-21099; 1:500) used for nNOS immunolabeling, the Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (A21206; 1:500) used for GnRH and P-nNOS immunolabeling, and

the streptavidin-conjugated Alexa Fluor 488 (S32354; 1:500) used for kisspeptin detection were purchased from Invitrogen (Eugene, OR). A biotinylated donkey anti-rabbit secondary antibody (711-065-152; 1:500) was purchased from Jackson ImmunoResearch (West Grove, PA). *Western Blotting***.** The rabbit polyclonal anti-nNOS (sc-8309; 1:500) and goat polyclonal anti-actin (sc-1616; 1:1000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-phospho-nNOS antibody (Ser^{1412}; PA1-032; 1:1000) was purchased from Affinity BioReagents (Golden, CO, USA). The rabbit monoclonal anti-phospho-Akt (Ser^{473}; #4060S; 1:1000), rabbit monoclonal Akt (#4691S; 1:1000), rabbit polyclonal anti-phospho-p44/42 MAPK (p-Erk1/2) (Thr^{202}/Tyr^{204}; #9101L; 1:1000) and rabbit polyclonal anti-p44/42 MAPK (Erk1/2) (#9102L; 1:1000) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated secondary antibodies (1:10,000) were purchased from Sigma (Saint-Quentin Fallavier, France).

Immunohistofluorescence

Animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Brains were removed and immersed in the same fixative for 2 h at 4°C and stored in PB until slicing. Free-floating coronal sections (60 μM thick) containing the preoptic region were cut on a Vibratome (VT1000S; Leica, Wetzlar, Germany), collected in ice-cold PB, incubated in blocking buffer [PBS containing 5% normal donkey serum (D9663; Sigma) and 0.3% Triton X-100 (Sigma)] for 1 h at RT and then incubated with sheep polyclonal anti-nNOS (1:1000) and rabbit polyclonal anti-kisspeptin (1:5000), or rabbit polyclonal anti-P-nNOS (Ser^{1412}; 1:500) primary antibodies prepared in blocking solution for 48 h at 4°C. Sections were rinsed thoroughly 4-5 times in PBS and exposed to secondary antibodies: first with Alexa Fluor 568-conjugated donkey anti-sheep and biotinylated donkey anti-rabbit

antibodies in blocking solution for 1 h at room temperature (RT) and then with streptavidin-conjugate Alexa Fluor 488. Kisspeptin immunoreactivity was localized with a biotinylated donkey antirabbit IgG (1:500, Vector Laboratories). Tyramide signal amplification was accomplished by placing the sections in an avidin-biotin solution (Vectastain) for 1 h, followed by incubation in tyramide signal amplification solution for 20 min, according to the manufacturer's instruction (TSA-Indirect kit, New England Nuclear Life Science). Deposited biotin was detected with Alexa 488-conjugated streptavidin (1:500, Invitrogen). After washing, sections were mounted on glass slides and coverslipped with Permafluor medium (434990; Immunon, Pittsburgh, PA). Double-immunofluorescent images were acquired using an Axio Imager.Z1 ApoTome microscope (AxioCam MRm camera; AxioVision 4.6 software system; Zeiss, Germany). For illustration purposes, photomontages of the preoptic region were prepared with the help of Photoshop CS4 software (Adobe System, San Jose, CA) using digitalized images acquired with a 20X objective (NA 0.5). Single-labeled nNOS neurons and dual labeled P-nNOS / nNOS double-labeled neurons were quantified in the upper focal plane of sections (60 μM thick) in different parts of the preoptic region using digitalized images. For confocal observation and analyses, an inverted laser scanning Axio observer microscope (LSM 710, Zeiss) with an EC Plan NeoFluor 100x/1.4 NA oil-immersion objective (Zeiss) was used (Imaging Core Facility of IFR114, of the University of Lille 2, France).

X-gal histochemistry coupled with GnRH/nNOS double-immunocytochemistry

Free-floating 40 uM-thick sections were cut on a cryostat from adult *Gpr54*-null mouse brains processed as described above. A series of sections containing the preoptic and tuberal region of the hypothalamus was washed thoroughly with 0.1 M PBS (pH 7.4) and placed in 2% 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) solution [2 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, and 4 mg/ml X-gal] overnight at RT. After PBS washes and

preincubation in blocking buffer (PBS containing 5% donkey serum and 0.05% Triton X-100) for 1 hr at RT, sections were incubated with rabbit anti-GnRH (1:3000) and sheep anti-nNOS (1:3000) primary antibodies diluted in blocking buffer at 4°C overnight. After PBS rinses, sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit (1:500) and Alexa Fluor 568-conjugated donkey anti-sheep (1:500) antibodies diluted in blocking buffer for 2 h at RT. Sections were rinsed thoroughly, mounted on glass slides and coverslipped with Permafluor medium (434990; Immunon, Pittsburgh, PA).

Sections were examined using an Axio Imager.Z1 ApoTome microscope (Carl Zeiss, Germany) equipped with a motorized stage and an AxioCam MRm camera (Zeiss). Specific filter cubes were used for the visualization of GnRH immunoreactivity in green (excitation filter: 475/40 nm; dichroic mirror: 500 nm; barrier filter: 530/50 nm) and nNOS immunoreactivity in red (excitation filter: 550/25 nm; dichroic mirror: 570 nm; barrier filter: 605/70 nm), and X-gal staining was visualized simultaneously by switching to brightfield. GnRH or nNOS cells were considered X-gal-positive if the X-gal reaction product was found within the cytoplasm of the soma or proximal dendrite. X-gal/GnRH double-labeled cells, X-gal/nNOS double-labeled cells, X-gal-labeled unidentified cells and single-labeled GnRH or nNOS cells were counted. Analysis was performed by counting all single-labeled and double-labeled cells in 2 sections from each animals corresponding to plates 17-21 of the Swanson brain atlas focusing on the medial septal nucleus (MS), the organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MePO), the anteroventral preoptic nucleus (AVP), in which nNOS neurons are known to interact morphologically with GnRH neurons and the arcuate nucleus of the hypothalamus (ARH), a kisspeptin neuron-containing structure (Oakley et al., 2009; Navarro and Tena-Sempere, 2011) adjacent to the median eminence, to which GnRH neurons project. Values from each mouse were used to determine mean counts and data are represented as means ± SEM.

For illustration purposes, photomontages of different parts of the preoptic region containing GnRH/nNOS immunoreactive neurons along with X-gal staining in brightfield were prepared with the help of Photoshop CS4 software (Adobe System, San Jose, CA), using digitalized images acquired with a 20X objective (NA 0.5) (Zeiss).

Protein extraction

The preoptic region was dissected from each animal and protein extracted for western blotting as described previously (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010). Briefly, mice were killed by decapitation following treatment. After the rapid removal of the brain, the meninges and optic chiasm were removed and the preoptic region was dissected under a binocular magnifying glass with the Wecker scissors (Moria, France). The external limits for this dissection were: laterally, the external border of the medial preoptic area and dorsally, the internal border of the anterior commissures. Anteroposteriorly, the dissected region was comprised between the atlas level 16 and 20 of the Swanson Atlas (Swanson, 2004). The tuberal region of the hypothalamus was similarly dissected; the external limits for this dissection were laterally, the external border of the lateral hypothalamic area and dorsally, the roof of the third ventricle. Anteroposteriorly, the dissected region was comprised between atlas levels 26 and 31 of the Swanson Atlas. After dissection, each fragment was placed in a microcentrifuge tube, snap frozen in liquid nitrogen and stored at -80°C.

Protein extracts were prepared from each preoptic region sample in 200 µl of lysis buffer (25 mM Tris, pH 7.4, β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin A, 10 µg/ml apoprotinin, 100 µg/ml PMSF, and 1% Triton-X100) by homogenization of the fragments through 22 and 26 gauge needles in succession. Tissue lysates were cleared by centrifugation at 14000 rpm for 15 min at 4°C. Protein content was determined using the Bradford method

(Bio-Rad, Hercules, CA) and equal amount of protein were mixed with 4X sample buffer (Invitrogen). Samples were boiled for 5 min and stored at -80°C until use.

Western blot analyses

Samples were reboiled for 5 min after thawing and electrophoresed for 75 min at 150 V in 7% Tris-acetate, or for 50 min at 200 V in precast 4–12% MES SDS-polyacrylamide gels according to the protocol supplied with the NuPAGE system (Invitrogen). After size fractionation, the proteins were transferred onto 0.2 µm pore-size polyvinylidene difluoride membranes (LC2002; Invitrogen) in the blot module of the NuPAGE sytem (Invitrogen) for 75 min at RT. Membranes were blocked for 1 h in blocking buffer [TBS with 0.05% Tween 20 (TBST) and 5% nonfat milk] at RT, and incubated overnight at 4°C with the appropriate primary antibody diluted in blocking buffer. Membranes were washed four times with TBST the following day before being exposed to HRP-conjugated secondary antibodies diluted in blocking buffer for 1 h at RT. Immunoreactions were visualized using the ECL detection kit (NEL101; PerkinElmer, Boston, MA). When stripping and reprobing was required, membranes were incubated in a stripping solution (62.5 mM Tris-HCl, 2% SDS, pH 6.7, and 100 mM β-mercaptoethanol) for 30 min with gentle rocking at 65°C. An HRP-conjugated secondary antibody was used to verify that all former immunoreactivity had been successfully stripped away. Immunoblots were scanned using a desktop scanner (Epson Expression 1680 PRO) and Adobe Photoshop, and band intensities were determined using ImageJ software (NIH, Bethesda).

Kisspeptin-10 treatment

Diestrous mice received a single intraperitoneal (i.p.) injection of 100 µl of PBS containing 1nmol Kp10 or PBS only. After 30 min, mice were either killed by decapitation and the

hypothalamic preoptic region dissected and processed for western blotting or deeply anesthetized and processed for immunofluorescence analyses.

Mouse treatment with PI3 kinase, MAPK, and NOS inhibitors

Ovariectomized mice received a single injection (i.p.) of LY294002 (100 mg/kg) or U0126 (30 mg/kg) or DMSO. After 30 min, animals received a single injection (i.p.) of 100 μl PBS containing 1 nmol Kp10 or PBS only. After an additional 30 min, mice were killed by decapitation and the preoptic region of each brain dissected from each brain and processed for Western blotting. For L-NAME treatment, mice first received a single injection (i.p.) of the NOS inhibitor (50 mg/kg) or PBS; 3 hours later, they were given a second injection of either Kp10 or PBS, and killed after 30 min by decapitation. Trunk blood was collected in tubes containing EDTA (0.2 M), centrifuged at 6500 rpm for 15 min at 4°C and the supernatant obtained (plasma) was stored at -80°C until ELISA for LH.

Estradiol treatment in ovariectomized mice

Gpr54-null mice and their wild-type littermates were bilaterally ovariectomized (OVX; day 0) under anesthesia by intraperitoneal injection of 60 mg/kg ketamine and 10 mg/kg xylazine. On the 15th day at 9 a.m, mice received either a single subcutaneous injection of estradiol benzoate (E2; 1 μg/20 g body weight in sesame oil) or vehicle alone and on the 17th day between 6:30 – 7:30 pm, mice were killed by decapitation and hypothalamic preoptic region dissected and processed for western blotting. This protocol has previously been used to assess the effect of preovulatory rise of estrogens on the phosphorylation of nNOS protein in preoptic region (Parkash et al., 2010).

Steroid-induced LH surge protocol

Mice were bilaterally ovariectomized (OVX) as described in the previous paragraph, and

implanted subcutaneously with Silastic capsules containing 17-β-estradiol (E_2; 1 μg/20 g body weight). The Silastic capsules were prepared as follows: crystalline E_2 was dissolved in absolute ethanol, mixed with Silastic medical adhesive (Type A) (Dow Corning) at a concentration of 0.1 mg/ml adhesive and injected into Silastic tubing (Dow Corning; internal diameter, 1mm; external diameter, 2.125 mm) (Bronson, 1981; Clarkson et al., 2008). Six days after OVX, mice received a single injection (s.c.) of 17β-estradiol 3-benzoate (1 μg/20 g of body weight in sesame oil) at 9 a.m. On the following day, animals received another injection (s.c.) of progesterone (500 μg/20 g body weight in sesame oil) at 9 a.m. Between 7:30 - 8:30 p.m (lights off at 8 p.m.) on the same day, mice were anesthetized with an overdose of chloral hydrate (400 mg/kg; i.p.) and trunk blood was collected for LH assay.

Luteinizing hormone assay

Plasma LH was measured using Rodent LH ELISA kit (ERKR7010-A; Endocrine Technologies, Newark, CA) with a sensitivity of 0.3 ng/ml and 7% intra-assay and 10% inter-assay coefficients of variation.

Ovarian histology

Dissected ovaries were fixed in 4% paraformaldehyde, washed in PBS, wax embedded in paraffin, sectioned at 5 μm intervals and stained with hematoxylin and eosin.

Estrous cyclicity

To examine the possible effects of mutations on estrous cyclicity, vaginal lavages from adult (> 2 months old) female *nNOS*-null mice and their wild type littermates were performed everyday in the morning (10 a.m. to 1 p.m.) using 0.9 % saline. The smears were observed under the microscope and the phase identified as diestrus (L) if they predominantly contained

leukocytes, as proestrus (N) if they predominantly contained basal and cornified nucleated cells and as estrus (C) if they predominantly contained cornified epithelial cells. An estrous cycle was considered normal when the vaginal lavage had leukocytes for 2 d followed by 1 d of nucleated and 1-2 d of cornified cells.

Statistics

Differences between two groups were analyzed by Student's t-test. Differences between several groups were analyzed using one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test for unequal replication. The level of significance was set at $p < 0.05$.

Results

Subsets of NO-synthesizing neurons are abundantly surrounded by kisspeptin-immunoreactive fibers in the preoptic region

To determine whether kisspeptin neurons morphologically interact with nNOS neurons in the preoptic region, we performed dual immunofluorescent-labeling studies using well-characterized antibodies to kisspeptin (Clarkson et al., 2009) (Fig. 1A) and to nNOS (Herbison et al., 1996). Fluorescence microscopic examinations were conducted in forebrain areas in which nNOS and GnRH neurons are known to interact morphologically and functionally in mice (Clasadonte et al., 2008) (n = 4 mice). They clearly establish the existence of distinct populations of NO-synthesizing neurons that are morphologically associated with kisspeptin-immunoreactive fibers (Fig. 1B). Notably, in the median preoptic nucleus (MePO) and the organum vasculosum of the lamina terminalis (OVLT), kisspeptin-

immunoreactive fibers were found to be abundantly apposed to nNOS-containing cell bodies (Fig. 2), whereas in the medial septum (MS) and the anteroventral preoptic nucleus (AVP), virtually no apposition was seen between kisspeptin-immunoreactive axons and the cell bodies of nNOS neurons (Fig. 2B and 2F).

NO-synthesizing neurons in the OVLT and MePO express GPR54

Using a transgenic GPR54-LacZ knock-in mouse model, we next sought to test the intriguing possibility that nNOS neurons in the preoptic region could express GPR54. Xgal histochemistry coupled with double-immunofluorescence labeling revealed that in the OVLT and MePO, LacZ, and by extension, GPR54, was not only contained in GnRH neurons as previously described (Messager et al., 2005; Herbison et al., 2010), but also in nNOS neurons (Fig. 3). Quantitative analyses showed that 25 ± 5 % and 41 ± 4 % of Xgal-positive cells in the OVLT and 23 ± 2 % and 57 ± 6 % of Xgal-positive cells in the MePO were GnRH and nNOS neurons, respectively (n = 4 female mice). In contrast, Xgal staining in the MS was restricted to GnRH neurons (9 ± 2 % of Xgal-positive cells) and a population of nNOS-immunonegative cells (Fig. 3A,B); in the AVP, GnRH neurons were the only Xgal-positive cells (Fig. 3E,H). Together with our data showing that kisspeptin-containing fibers are closely apposed to nNOS neurons in the OVLT-MePO, these results strongly suggest that kisspeptin, the ligand for GPR54, may act directly on these neurons.

NO-synthesizing neurons are surrounded by kisspeptin-immunoreactive fibers in the arcuate nucleus of the hypothalamus, but do not express the GPR54 kisspeptin receptor

In the mouse brain, nNOS neurons are also found in the tuberal region of the hypothalamus and, in particular, within the arcuate nucleus of the hypothalamus (ARH) (Sica et al., 2009), a kisspeptin neuron-containing structure (Oakley et al., 2009; Navarro and Tena-Sempere,

2011) that is adjacent to the median eminence, to which GnRH neurons project. To determine whether Kisspeptin neurons also interact morphologically and functionally with nNOS neurons in this hypothalamic region, we carried out additional neuroanatomical studies. While only a few kisspeptin-immunoreactive fibers were found in apposition to nNOS neurons in the posterior (PH; Fig. 4A,D) and dorsomedial hypothalamus (DMH; Fig. 4B,D), numerous kisspeptin fibers were apposed to nNOS neurons in the ARH (Fig. 4C,D). Intriguingly, no GPR54 expression was seen in the ARH (Fig. 4E,H) or in the DMH (Fig. 4E,G), as revealed by XGal staining in GPR54-LacZ knock-in mice. In contrast, Xgal staining was found to be abundant in the PH and was mostly seen in nNOS-immunoreactive neurons (Fig. 4E,F).

Kisspeptin promotes the phosphorylation of nNOS on its Ser1412 activation site in the preoptic region, but not in the tuberal region of the hypothalamus

The catalytic activity of nNOS is modulated during the ovarian cycle by posttranslational modifications of the enzyme, such as phosphorylation of the Ser1412 residue (Parkash et al., 2010), which increases its catalytic activity (Rameau et al., 2007). Ser1412 phosphorylation of nNOS is maximal in the preoptic region on the afternoon of proestrus, when estrogens exert their positive feedback effects on the GnRH system (Parkash et al., 2010). To determine whether kisspeptin neurons could play a role in the regulation of these posttranslational modifications, we investigated the effects of an intraperitoneal (i.p.) injection of kisspeptin-10 (1 nmol, 30 min) on nNOS phosphorylation, using a phospho-specific antibody directed against Ser1412-phosphorylated nNOS (P-nNOS). In diestrous mice, in which circulating estrogen levels are low, immunofluorescence analysis demonstrated a striking increase in the proportion of nNOS neurons immunoreactive for P-nNOS following kisspeptin-10 injection (36.8 ± 6.0 % in vehicle-injected *vs.* 74.3 ± 4.0 % in kisspeptin-10-injected mice, n = 4;

Student's t-test, $p < 0.01$); this effect was restricted to the OVLT-MePO region (Fig. 5A). Indeed, no induction of nNOS phosphorylation was seen in nNOS neurons in the MS or the AVP (Fig. 6). Western blot analyses revealed that P-nNOS expression was easily detected as a single band of 165 kDa in protein extracts from the mouse preoptic region (Fig. 5B), and confirmed the potent stimulatory effect of kisspeptin-10 on nNOS phosphorylation in diestrous mice as seen using immunohistochemistry (Fig. 5A). Notably, P-nNOS levels induced by kisspeptin-10 treatment in diestrous mice were comparable to those seen in proestrus (Fig. 5B). In contrast, similar analyses performed in the tuberal region of the hypothalamus clearly showed that kisspeptin-10 failed to stimulate nNOS phosphorylation in this hypothalamic region in diestrous mice (Fig. 7).

Estradiol-promoted phosphorylation of nNOS in preoptic neurons requires kisspeptin-GPR54 signaling

To further explore the role of estrogens and kisspeptin in triggering nNOS phosphorylation in preoptic neurons, we performed experiments in ovariectomized (OVX) mice. As shown in Figure 5C, kisspeptin-10 (1 nmol, 30 min i.p.) was as potent at inducing Ser1412 phosphorylation of nNOS in OVX mice, when compared to controls, as a subcutaneous injection of sesame oil containing 17β-estradiol 3-benzoate, which mimics the preovulatory increase in plasma estrogens that occurs at proestrus ($n = 5$ animals per group). To examine whether kisspeptin signaling is necessary for the estrogen-mediated activation of nNOS, we then tested the ability of estradiol to promote nNOS phosphorylation in *Gpr54*-null mice (Seminara et al., 2003). Western blot analyses clearly showed that estradiol-treatment did not induce any change in nNOS phosphorylation in *Gpr54*-null mice (Fig. 5C; $p > 0.05$). Taken together, our results strongly suggest that kisspeptin neurons act directly on NO-synthesizing neurons to promote preovulatory nNOS activation in the preoptic region.

Kisspeptin promotes nNOS phosphorylation via the activation of the PI3K/AKT signaling pathway

To gain further insight into the downstream pathways used by kisspeptin-GPR54 signaling to promote nNOS phosphorylation in preoptic neurons, we investigated whether kisspeptin activation of its cognate receptor is coupled with the activation of the phosphatidylinositol-3 kinase (PI3K)/AKT signaling pathway, which is thought to mediate the phosphorylation of Ser1412 in nNOS (Rameau et al., 2007), and has been shown to be stimulated by GPR54 signaling (Stathatos et al., 2005; Novaira et al., 2009). We also explored the putative activation of the ERK1/2 signaling pathway, which has also been shown to be stimulated by the activation of GPR54 (Castellano et al., 2006). Immunoblot analysis revealed that kisspeptin-10 (1 nmol, 30 min) stimulated the phosphorylation of nNOS (Fig. 8A) and AKT (Fig. 8B), but not ERK1/2 (Fig. 8C) in wild-type diestrous mice, and that these effects of kisspeptin were absent in *Gpr54*-null littermates (Fig. 8 A,B ; n = 4-5 per group). To determine whether AKT activation is actually required for kisspeptin to exert its stimulatory effects on nNOS phosphorylation, we treated wild-type OVX mice with the PI3K inhibitor LY294002 (100 mg/kg, i.p.) for 30 min before exposing them to kisspeptin-10. As shown in Figure 8D and E, the inhibition of PI3K activity abrogated the ability of kisspeptin to induce the phosphorylation both of nNOS and of AKT. In contrast, the treatment of mice with the MAPK inhibitor U0126 (30 mg/kg, i.p.) affected neither nNOS nor AKT phosphorylation (Fig. 8D,E), whereas it significantly inhibited the basal phosphorylation of ERK1/2 (Fig. 8F). Thus, the activation of the PI3K/AKT signaling pathway is likely to underlie the phosphorylation of nNOS induced by kisspeptin in neurons of the preoptic region.

NO release imposes a tonic brake on LH secretion during the estrogen-mediated

negative feedback phase

Having shown that kisspeptin and nNOS neurons morphologically and functionally interact in specific regions of the ventral forebrain in which a subset of GnRH neurons reside, we next sought to determine whether this kisspeptin-nNOS neuronal interaction could play a role in the control of LH release. In diestrous mice, a treatment with kisspeptin-10 (n=10) resulted in a robust and significant increase in plasma LH when compared to vehicle-treated animals (n=9) (Fig. 9A). Inhibiting NOS activity via the intraperitoneal administration of the NOS inhibitor L-NAME (50 mg/kg, i.p.) resulted in a comparable increase in LH release (n=6) (Fig. 9A). Kisspeptin-10 treatment of L-NAME-treated animals (n=10) triggered the release of LH at levels comparable to those seen in proestrus (n=4) (Fig. 9A). Correspondingly, the treatment of *Gpr54*-null mice with L-NAME (n=4) and of *nNos*-null mice with kisspeptin-10 (n=4) resulted in the release of similar proestrus-like levels of LH (Fig. 9A). Altogether, these data reveal that NO exerts a tonic inhibitory effect on GnRH/LH release at stages of the ovarian cycle in which gonadal steroids exert their negative feedback effects.

Neuronal NOS activity is essential for the LH surge

Daily inspection of vaginal cytology in *nNos*-null mice (n=7) in which no residual nNOS expression was seen in the preoptic region (Fig. 9B), revealed an absence of normal estrous cyclicity (Fig. 9C, upper panels). *nNos*-null mice exhibited an alternation of persistent periods of vaginal cornification and a persistent diestrous state with the sporadic occurrence of complete 4-5-d ovarian cycles, whereas wild-type littermates showed regular estrous cyclicity. In contrast to wild-type mouse ovaries (n=4), which contain large Graafian follicles and corpora lutea, histological inspection of adult *nNos*-null mouse ovaries (n=4) revealed the presence of primary and secondary follicles and occasionally an early Graafian follicle, as well as numerous large follicular cysts, but few or no corpora lutea (Fig. 9C, lower panels).

These findings show that *nNos*-null mice display ovarian cyclicity deficits and may present impaired ovulation, as previously suggested (Klein et al., 1998). The ability of the hypothalamic-pituitary axis to respond to positive feedback by gonadal steroids was tested in mutant mice. While OVX wild-type mice (n = 7) exhibited an LH surge in response to gonadal steroid treatment, only 1 of 5 *nNos*-null littermates exhibited an LH surge ($p < 0.05$) (Fig. 9D). Concordantly, L-NAME treatment to inhibit NOS activity (n = 10) mimicked the effects of the null mutation on the gonadal steroid-induced LH surge in wild-type mice (Fig. 9D, $p = 0.003$).

Discussion

We report here that NO-synthesizing neurons are important components of the neural circuit used by estrogens and kisspeptin neurons to trigger the onset of the preovulatory GnRH/LH surge in mice. The use of a genetic strategy to visualize the GPR54-expressing cells targeted by kisspeptin demonstrates that, in addition to GnRH neurons themselves, discrete populations of nNOS neurons abundantly surrounded by kisspeptin fibers also express the kisspeptin receptor GPR54 in the preoptic region. We found that kisspeptin selectively promotes the phosphorylation of the Ser1412 nNOS activatory site in these neurons by activating the PI3K/AKT signaling pathway. A null mutation of *Gpr54* prevents both kisspeptin and estradiol effects on nNOS phosphorylation in ovariectomized mice, showing that estrogens require kisspeptin-GPR54 signaling to influence nNOS activity during the ovarian cycle, an effect that is maximal on the day of proestrus when positive feedback comes into play ((d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010), present study). Correspondingly, mice harboring a null mutation of the *nNos* gene exhibit a markedly impaired ability to generate an LH surge in response to gonadal steroids, and a conspicuous ovulation deficiency. Together, these observations provide a number of new insights that

expand our current understanding of kisspeptin- and NO-mediated regulation of the hypothalamic-pituitary-gonadal axis in adult mammals.

NO, which travels readily across cellular membranes and enters presynaptic and postsynaptic sites, is capable of coordinating neuronal inputs in a restricted brain volume defined by its half-life and diffusion constant (Gally et al., 1990). Since NO cannot be stored in synaptic vesicles, unlike other neurotransmitters, mechanisms that regulate its synthesis in time and space are crucial in determining its biological effect (Garthwaite, 2008). In the mouse ((Clasadonte et al., 2008), present study), as in the rat (Grossman et al., 1994; Herbison et al., 1996), GnRH neurons are surrounded by NO-synthesizing neurons in the basal forebrain, one of the major sites of NOS expression in the central nervous system (Bredt et al., 1991; Yamada et al., 1996; Edelmann et al., 2007). The production of neuronal NO in the vicinity of GnRH neurons is tightly regulated by estrogens (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010) and has been shown to directly modulate GnRH neuronal activity (Clasadonte et al., 2008). The present findings reveal, intriguingly, that NO has a dual effect on GnRH/LH release during the ovarian cycle. Pharmacological and genetic inhibition of nNOS activity significantly increase basal and kisspeptin-stimulated LH release, respectively, in diestrous mice, suggesting that during the negative-feedback phase of estrogen action, a constitutive basal level of NO release maintains the tonic inhibition of GnRH neurons, keeping LH levels at their nadir. These results are in good agreement with data collected in ewes suggesting a role for nitric oxide in the preoptic region in the suppression of GnRH/LH secretion during estrogen negative feedback in seasonal anoestrus (Stefanovic et al., 2000; McManus et al., 2007), but stand out from those of a previous study performed in rats, in which the pharmacological inhibition of nNOS failed to alter basal or kisspeptin-evoked LH release (Navarro et al., 2005). However, sex differences may account for this discrepancy as that particular study was performed using males, and a recent human study has demonstrated

that kisspeptin-stimulated LH release is sexually dimorphic (Jayasena et al., 2011). In contrast, we also show that the blockade of NO synthesis blunts the LH surge in steroid-primed mice, known to involve the activation of the kisspeptin-GPR54 signaling (Clarkson et al., 2008) initiated by the kisspeptin neuronal population residing in the anteroventral periventricular nucleus (AVPV) (Clarkson et al., 2008; Gottsch et al., 2009; Mayer et al., 2010). Together, these results suggest that NO- and kisspeptin-synthesizing neurons interact in synergy to coordinate the progression of the ovarian cycle, and thus reconcile the recent evidence that kisspeptin signaling plays a key role in the neuroendocrine control of reproduction (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007b; Dungan et al., 2007; Kauffman et al., 2007; Lapatto et al., 2007; Clarkson et al., 2008; Mayer et al., 2010; Mayer and Boehm, 2011) with the originally postulated role for NO in controlling GnRH secretion (Rettori et al., 1993; Mahachoklertwattana et al., 1994), the onset of the preovulatory GnRH/LH surge (Bonavera et al., 1993; Aguan et al., 1996; d'Anglemont de Tassigny et al., 2007a) and fertility (Gyurko et al., 2002). These findings also raise the exciting possibility that the estrogen-evoked kisspeptin-mediated activation of nNOS neurons in proestrus serves as an intermediate synchronizing ‘switch’ for the GnRH system that enables the transition between pulsatile and peak release of GnRH (Christian and Moenter, 2010). NO could operate such a switch by acting either directly on GnRH neurons (Clasadonte et al., 2008) or on its trans-synaptic inputs (Pielecka-Fortuna et al., 2008; Zhang et al., 2009; Pielecka-Fortuna and Moenter, 2010), or both.

A recent study has demonstrated, provocatively, that kisspeptin neurons and GPR54-expressing cells are not indispensable for reproductive function when ablated during a certain window early in development (Mayer and Boehm, 2011), suggesting that the GnRH system can be turned on independently of kisspeptin neurons and kisspeptin/GPR54 signaling. Accordingly, another recent study has demonstrated that an agonist of the NMDA-type

glutamate receptor, which triggers puberty, ovulation and GnRH secretion in both rodents (Urbanski and Ojeda, 1990; Brann and Mahesh, 1991) and primates (Plant et al., 1989; Claypool et al., 2000), is capable of activating the reproductive axis in both *Kiss*- and *Gpr54*-null mice, and that these effects may involve nNOS neurons (d'Anglemont de Tassigny et al., 2010). Since most nNOS neurons of the preoptic region express the NMDA receptor (d'Anglemont de Tassigny et al., 2007a) and estrogen receptor α (Scordalakes et al., 2002; Sato et al., 2005), both of which are critical for the estrogen positive-feedback effect on GnRH neurons (Brann and Mahesh, 1991; Wintermantel et al., 2006) and nNOS activity (d'Anglemont de Tassigny et al., 2007a; d'Anglemont de Tassigny et al., 2009), it is tempting to speculate that nNOS neurons may be involved in the cellular mechanisms underlying compensation in animals in which neurons mediating kisspeptin/GPR54 signaling are absent (Mayer and Boehm, 2011). Estrogens may indeed directly regulate nNOS activity via several modes of redundancy, which would not be exclusive of one other. For instance, in parallel to promote phosporylation-dependent activation of nNOS (Parkash et al., 2010), estrogens have been shown to stimulate nNOS activity by modulating the physical and functional coupling of nNOS to the glutamate NMDA receptors *in vivo* (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010), a phenomenon also observed *in vitro* in primary hypothalamic neurons in the absence of kisspeptin signaling (d'Anglemont de Tassigny et al., 2009).

Intriguingly, our immunofluorescent experiments demonstrated that the systemic administration of kisspeptin selectively induces nNOS phophorylation in an area proximal to the OVLT, which is a brain area devoid of the blood brain barrier (Broadwell and Brightman, 1976; Ciofi et al., 2009) containing numerous kisspeptin fibers and nNOS neurons (present paper), and where GnRH neurons have recently been shown to extend dendrites (Herde et al., 2011; Prevot, 2011). Even though peripheral injections of kisspeptin-10 are shown to be efficient in promoting GnRH/LH release, these data suggest that this form of kisspeptin

peptide does not readily cross the blood brain barrier and thus fails to target all hypothalamic GPR54-expressing neurons in our experimental paradigm. This important caveat notwithstanding, our studies raise the interesting possibility that the OVLT may be a privileged site of interactions between kisspeptin, nNOS, and GnRH neurons in the neuroendocrine brain.

In addition to modulating GnRH neuronal activity within the preoptic region, accumulating evidence suggest that NO is involved in the control of GnRH release within the median eminence, which is the termination field of GnRH neuroendocrine neurons (Rettori et al., 1993; Knauf et al., 2001; De Seranno et al., 2004; de Seranno et al., 2010) and another brain site devoid of the blood brain barrier (Ciofi et al., 2009; Mullier et al., 2010). Because kisspeptin has also been shown to stimulate GnRH release from hypothalamic explants containing both the median eminence and the ARH (d'Anglemont de Tassigny et al., 2008), and because arcuate nNOS neurons reside adjacent to the median eminence (Sica et al., 2009), one could argue that a part of the effects observed on LH secretion after the systemic administration of kisspeptin and L-NAME in wild-type and *nNos*-null animals could be due to an alteration of nNOS activity in these arcuate neurons. However, by showing both that kisspeptin fails to modulate nNOS phosphorylation in the tuberal region of the hypothalamus and, in accord with a previous study (Herbison et al., 2010), that nNOS neurons of the ARH do not express any detectable GPR54 promoter activity, our data clearly suggest that this population of nNOS neurons is unable to respond directly to kisspeptin. Yet the possibility still remains that the kisspeptin neurons intimately associated with nNOS neurons in the ARH communicate through other signaling pathways, such as the ones involving dynorphin and neurokinin B, two transmitters coexpressed with kisspeptin (Ciofi et al., 2006; Ciofi et al., 2007; Goodman et al., 2007; Hrabovszky et al., 2010), which have been postulated to drive short feedback loops within the tuberal region of the hypothalamus to control the rhythmic

discharge of kisspeptin and drive the release of GnRH from fibers in the median eminence (Navarro et al., 2009; Wakabayashi et al., 2010).

Together, the results presented here identify preoptic nNOS neurons as an integral component of the neural network controlling ovarian cyclicity and ovulation, and indicate that the production of NO may play an important and hitherto unappreciated role in the kisspeptin-dependent preovulatory activation of GnRH neurons. NO could potentially be used by kisspeptin neurons to facilitate synchronous activity among GnRH neurons that were previously operating independently, and ultimately to maximize the release of this neurohormone into the blood.

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Figure legends

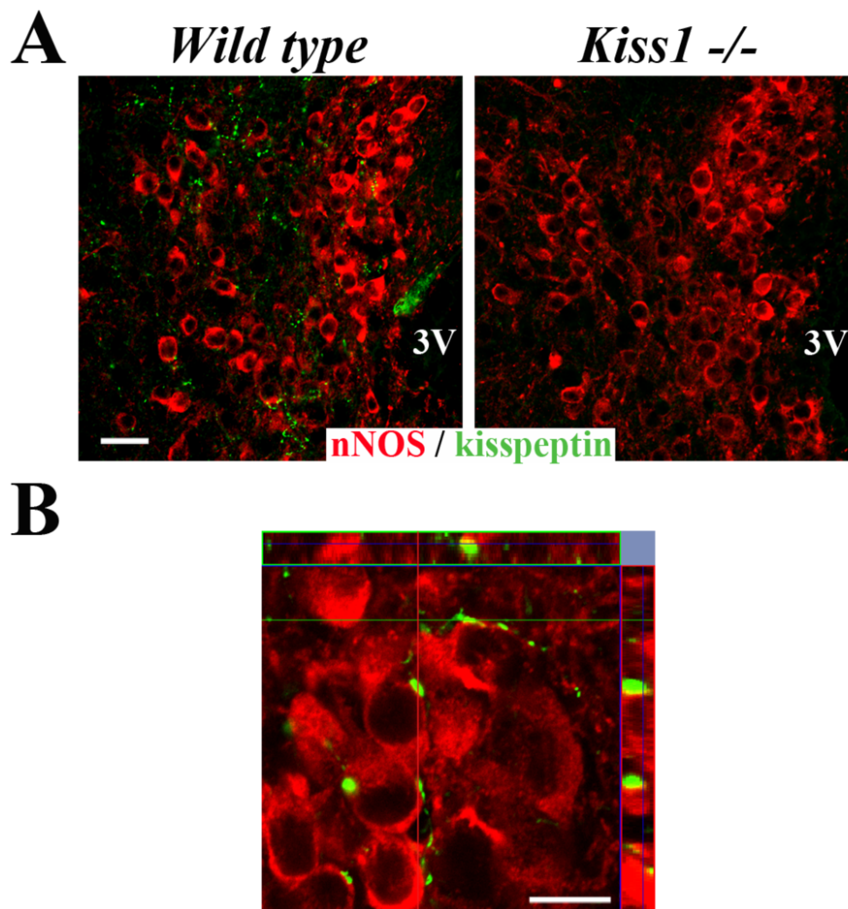


Figure 1. Morphological appositions between kisspeptin fibers and hypothalamic nNOS neurons in the hypothalamic preoptic region. **A.** Kisspeptin-immunoreactivity (green, arrows) localized in fibers apposed to nNOS neurons (red) is present in wild-type mice but not in *Kiss1*-null littermates. **B.** Confocal image obtained using a 100X objective showing the close apposition between kisspeptin-immunoreactive fibers (green) and nNOS-immunoreactive neuronal cell bodies (red). The illustration is an overlay and maximum projection of the z-stack files of a color-combined image of four single consecutive single confocal planes separated from each other by 400 nm. All images were acquired at the level of the organum vasculosum of the lamina terminalis (OVLT) in the basal forebrain. 3V: third ventricle. Scale bars = 50 µm in A and 10 µm in B and C.

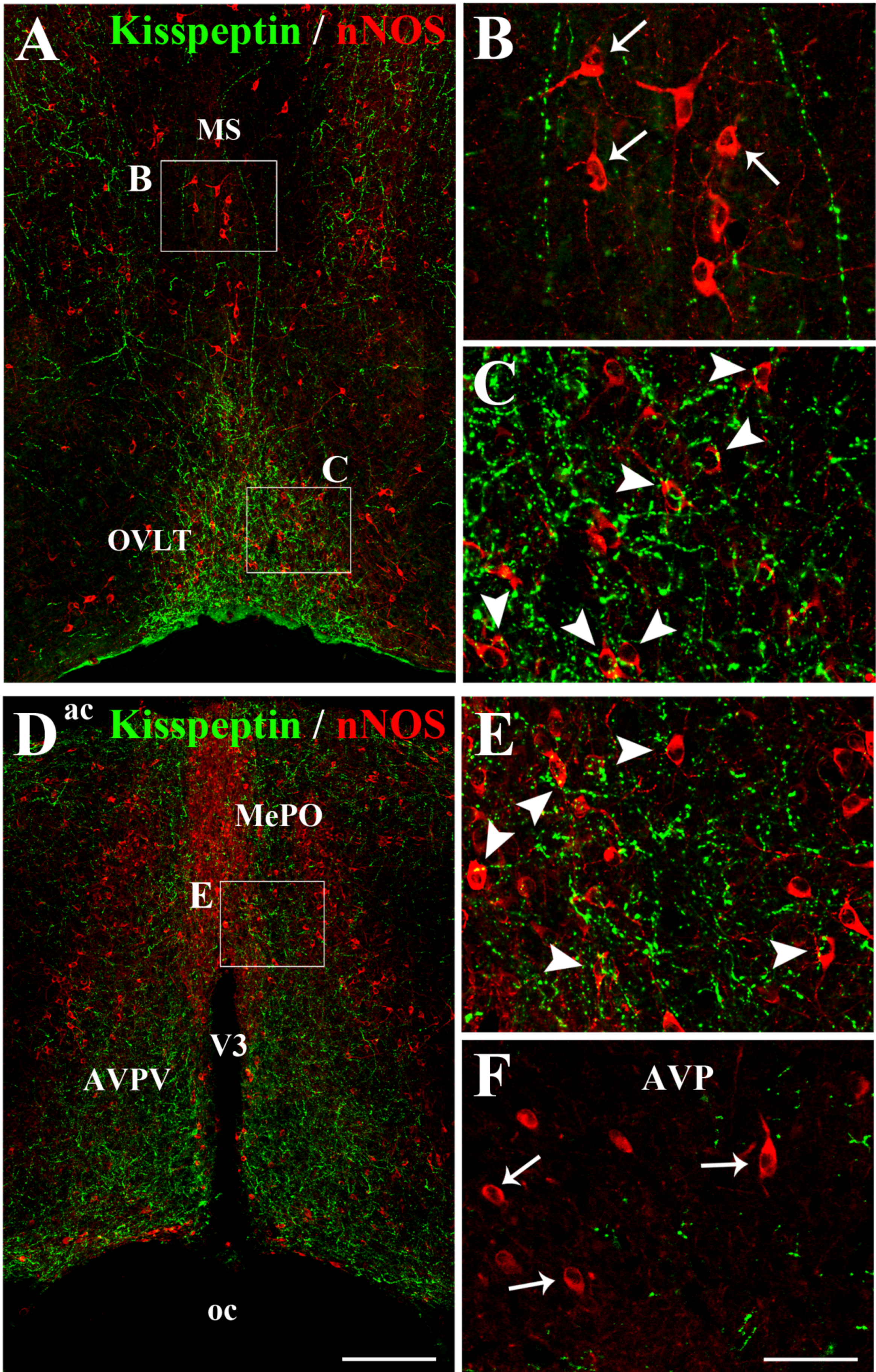
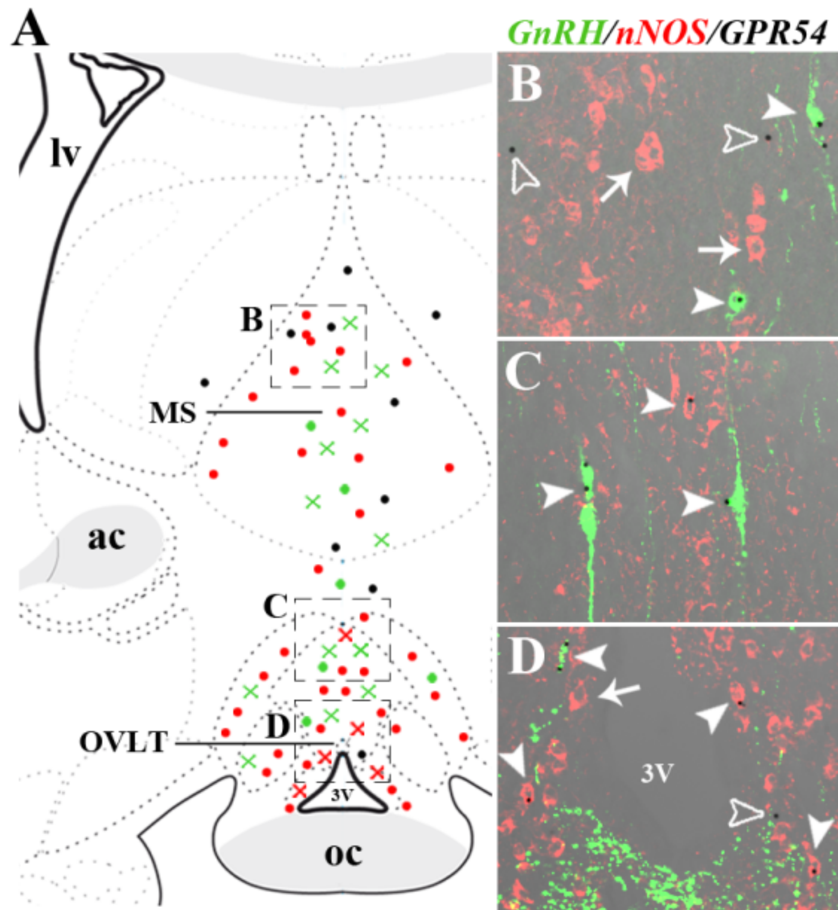

A
Kisspeptin / nNOS
MS
B
C
OVLT
B
C
D
ac
Kisspeptin / nNOS
MePO
E
V3
AVPV
oc
E
F
AVP

Figure 2. Kisspeptin-neurons project onto nNOS neurons in the preoptic region. **A**. Low-magnification photomontage of kisspeptin (green) and nNOS (red) immunofluorescence in the hypothalamic preoptic region at the level of the organum vasculosum of the lamina terminalis (OVLT) and the medial septum (MS). **B,C.** High-magnification images showing kisspeptin immunoreactive fibers (green) and nNOS immunoreactive cell bodies (red) in the MS (B) and the OVLT (C). Note that abundant kisspeptin fibers are apposed to nNOS neurons in the OVLT (arrowheads, C), but not in the MS (arrows, B). **D**. Low-magnification photomontage of kisspeptin (green) and nNOS (red) immunofluorescence in the hypothalamic preoptic region at the level of the median preoptic nucleus (MePO) and the anteroventral periventricular nucleus (AVPV). **E,F.** High-magnification images showing kisspeptin immunoreactive fibers (green) and nNOS immunoreactive cell bodies (red) in the MePO (E) and the anteroventral preoptic nucleus (AVP) (F). Note that abundant kisspeptin fibers are apposed to nNOS neurons in the MePO (arrowheads, E), but not in the AVP (arrows, F). V3, third ventricle; ac, anterior commissure; oc, optic chiasm. Scale bars: 200 μm in A and D; 50 μm in B, C, E and F.


A
GnRH/nNOS/GPR54
lv
B
MS
ac
C
OVLT
D
3V
oc
B
C
D
3V

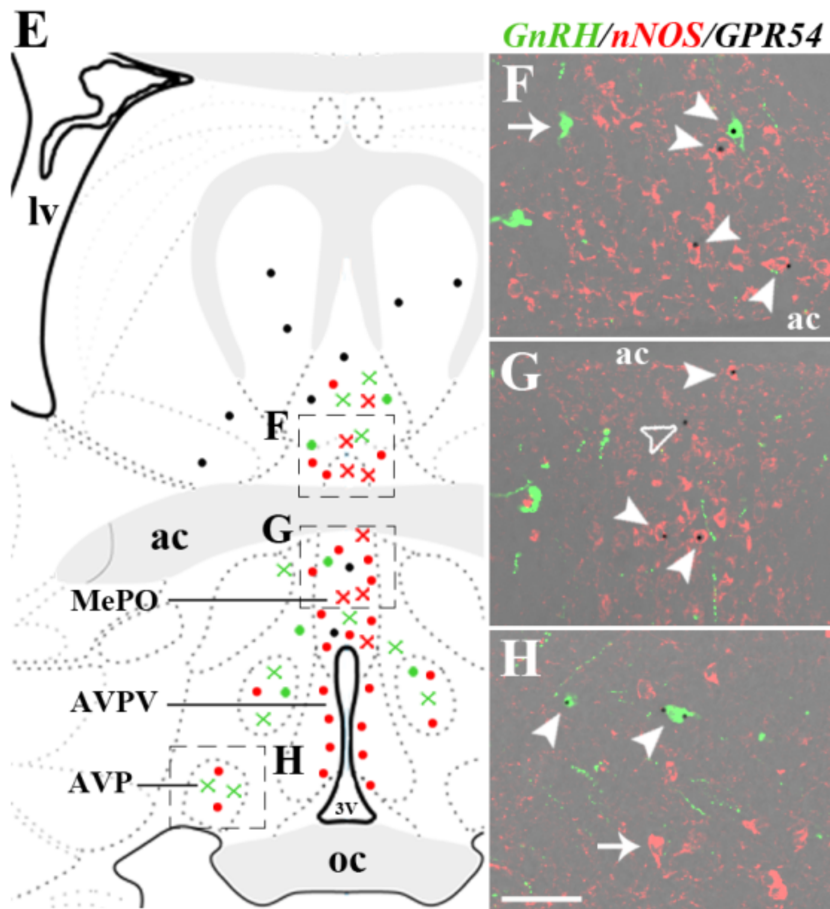

E
GnRH/nNOS/GPR54
lv
F
ac
G
MePO
AVPV
H
AVP
3V
oc
F
ac
G
ac
H

Figure 3. GPR54 is expressed both in nNOS and GnRH neurons in the preoptic region. **A,E.** Schematic brain maps demonstrating the distribution of GnRH (green) and nNOS (red) neurons expressing (crosses) or not expressing (circles) GPR54 in the preoptic region. Black circles indicate GPR54-expressing cells of unidentified phenotype. **B-D,F-H.** X-gal histochemistry (dark dots) revealing GPR54 expression both in GnRH (green, white arrowheads) and nNOS (red, white arrowheads) neurons in the OVLT (D) and MePO (F,G), but not in the MS (B) and AVP (H) where GPR54 is expressed in GnRH neurons and cells of unidentified phenotype (arrowhead outlines). OVLT, organum vasculosum of the lamina terminalis; MePO, median preoptic nucleus; MS, medial septal nucleus; AVPV, anteroventral periventricular nucleus; AVP, anteroventral preoptic nucleus; lv, lateral ventricle; ac, anterior commissure; V3, third ventricle; oc, optic chiasm. Scale bar: 50 μm.

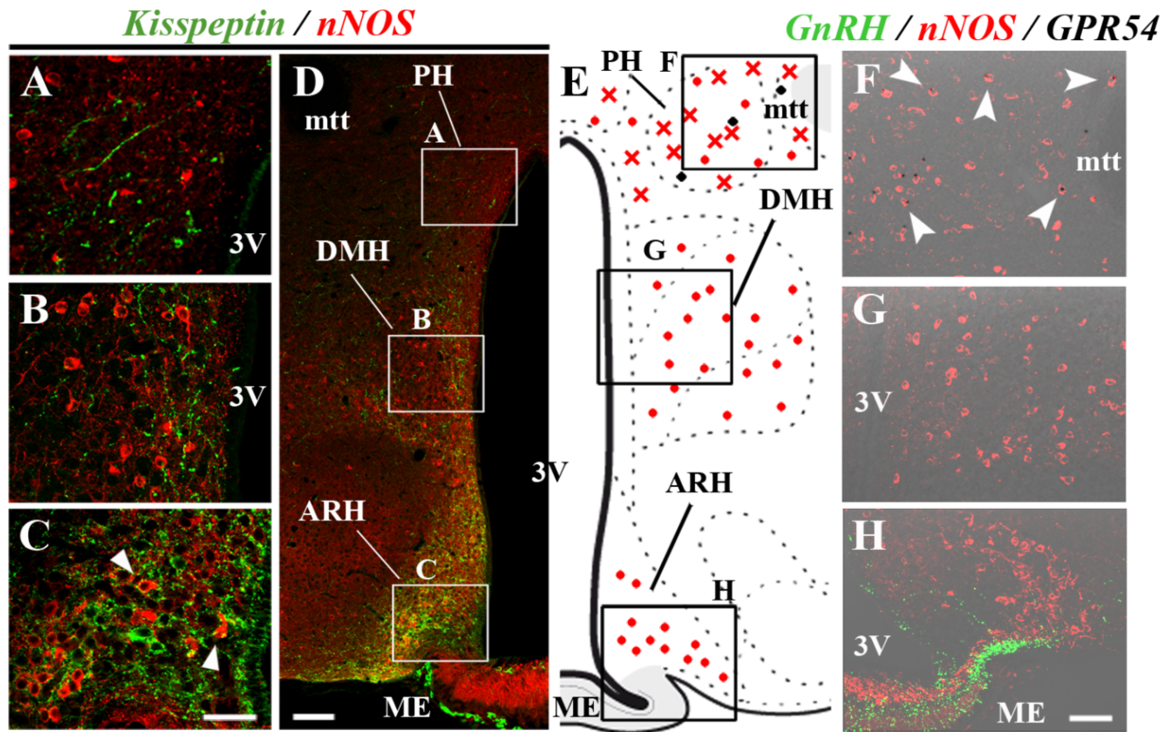
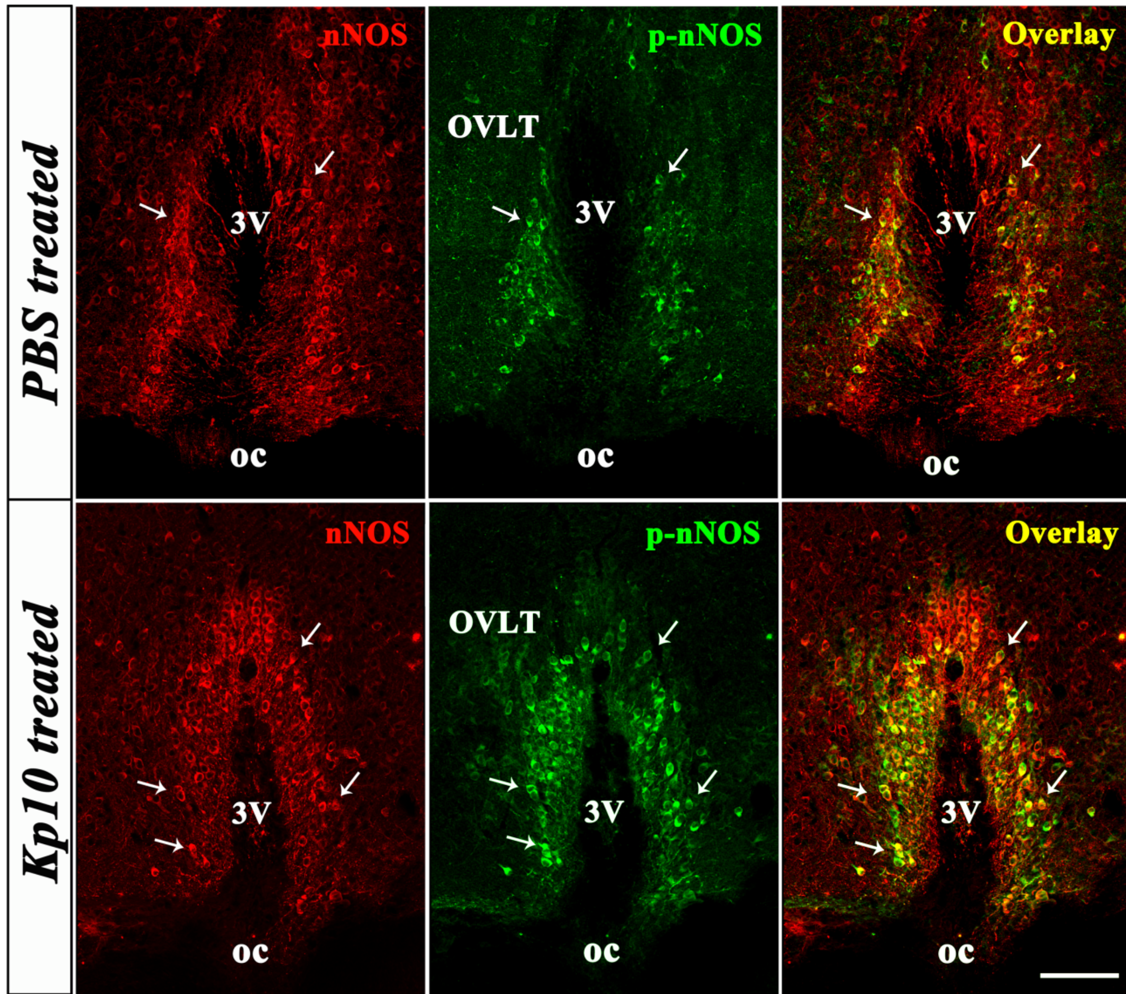
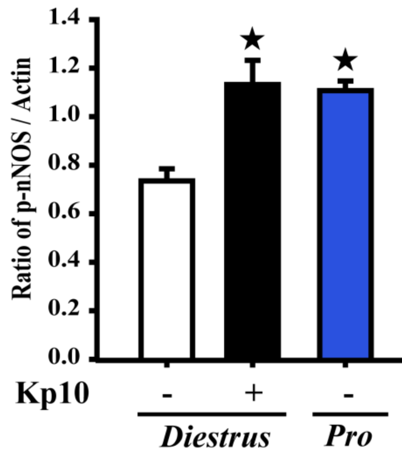
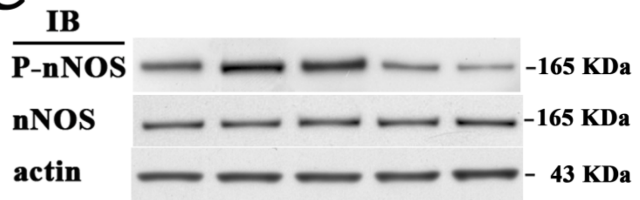


Figure 4. Kisspeptin-neurons project onto nNOS neurons that do not express GPR54 in the ARH. **A-D.** Immunofluorescence labeling showing abundant kisspeptin fibers (green) apposed to nNOS neurons (red) in the ARH (arrowheads, C), whereas Kisspeptin-nNOS morphological interactions are less conspicuous in the DMH and the PH (A, B). **E.** Schematic brain map demonstrating the distribution of nNOS neurons (red) expressing (crosses) or not expressing (circles) GPR54 in the tuberal region of the hypothalamus. Black circles indicate GPR54-expressing cells of unidentified phenotype. **F-H.** X-gal histochemistry (dark dots) revealing GPR54 expression in nNOS neurons (red, arrowheads) in the PH (F), but not in the DMH (G) nor in the ARH (H), in which no GPR54 expression is detected. Note in **H** that GnRH neuroendocrine axons (green) residing in the ME lie ventral to arcuate nNOS neurons. ARH, arcuate nucleus of the hypothalamus, DMH, dorsomedial nucleus of the hypothalamus; PH, posterior hypothalamus; ME, median eminence; mtt, mammillothalamic tract; 3V, third ventricle. Scale bars: 40 µm in A-C; 100 µm in D; 50 µm in F-H.


A
PBS treated
Kp10 treated
nNOS
p-nNOS
Overlay
OVLT
3V
oc

B
IB
P-nNOS
nNOS
actin
-165 KDa
-165 KDa
- 43 KDa


Ratio of p-nNOS / Actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Kp10
-
+
-
Diestrus
Pro


C
IB
P-nNOS
nNOS
actin
-165 KDa
-165 KDa
- 43 KDa

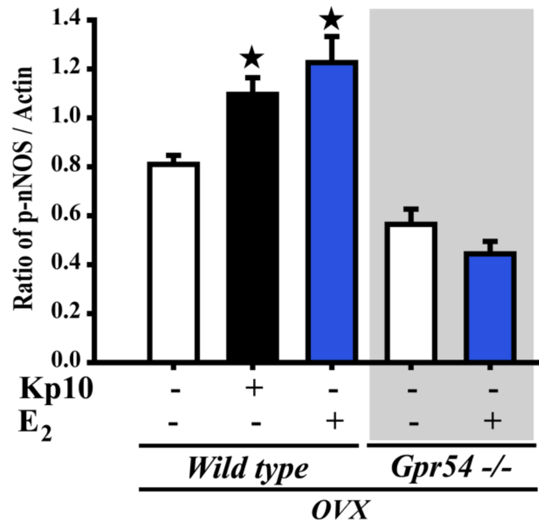

Ratio of p-nNOS / Actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Kp10
E2
Wild type
Gpr54 -/-
OVX

Figure 5. Kisspeptin and estradiol promote phosphorylation of nNOS via GPR54 activation in neurons of the preoptic region. **A.** nNOS (red) and P-nNOS (green) immunoreactivity in forebrain coronal sections passing through the OVLT of vehicle (PBS)- and kisspeptin (Kp10)-treated mice. Arrows show double-labeled neurons. oc, optic chiasm. Scale bar:100 μm. **B-C.** Immunoblotting (IB) of hypothalamic preoptic area protein extracts reveals that kisspeptin mimics both the effects of the ovarian cycle (B) and of estradiol (C) in promoting the phosphorylation of nNOS. Estradiol requires GPR54 signaling to exert its effects on nNOS phosphorylation (C). Bar graphs illustrate the mean ratio of the signal obtained for P-nNOS to that of actin in blots from 5 independent experiments (n = 5 animals). Error bars indicate SEM. OVLT, organum vasculosum of the lamina terminalis. *, $p < 0.05$; experimental groups versus untreated controls. OVX, ovariectomized animals.

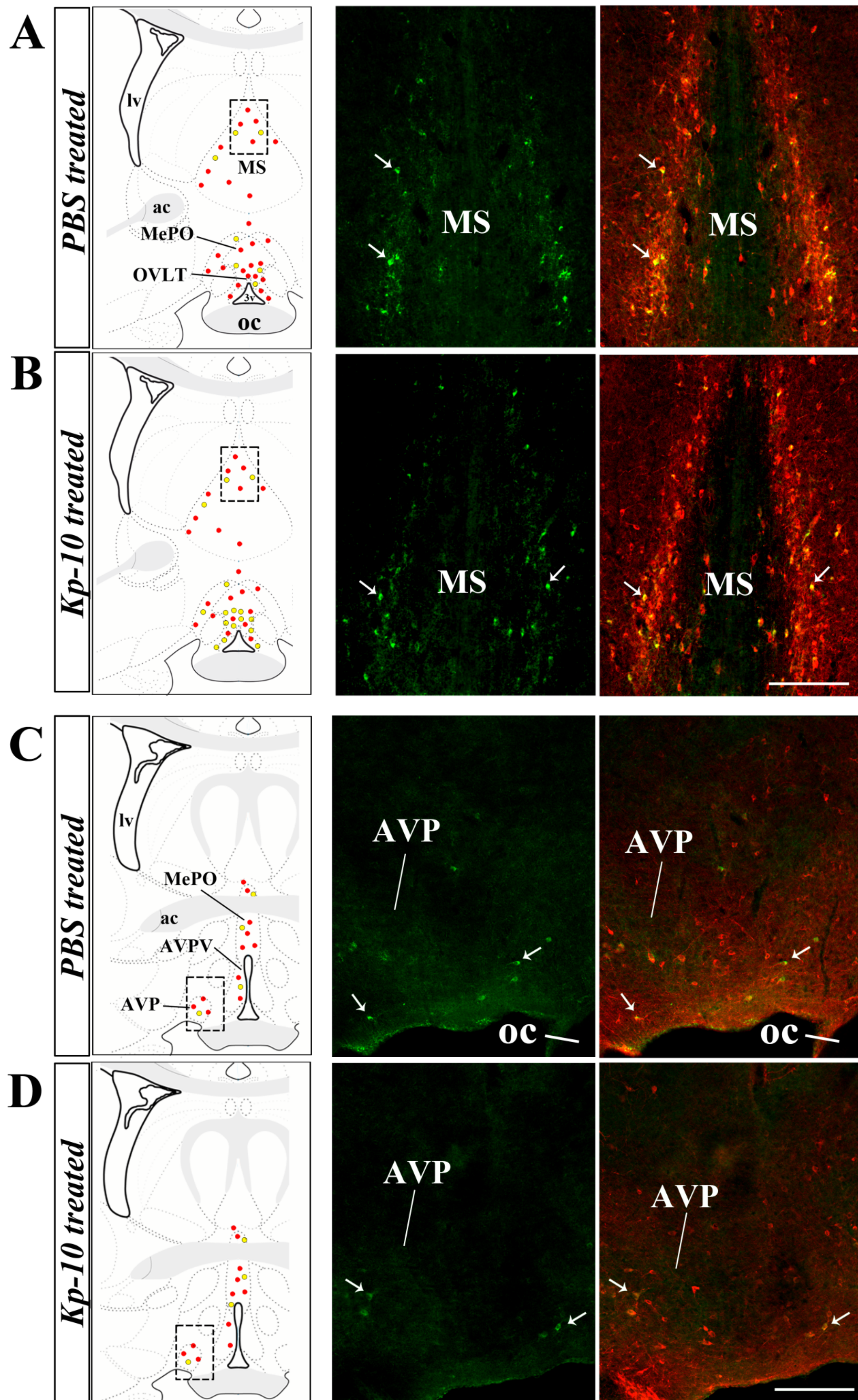

A
PBS treated
lv
MS
ac
MePO
OVLT
3v
oc
MS
MS
B
Kp-10 treated
MS
MS
C
PBS treated
lv
MePO
ac
AVPV
AVP
AVP
oc
AVP
oc
D
Kp-10 treated
AVP
AVP

Figure 6. Kisspeptin promotes the phosphorylation of nNOS in the OVLT, but not in the MS or the AVP. **A-D.** *Left-hand panels.* Schematic brain maps demonstrating the distribution of P-nNOS-immunoreactivity in nNOS neurons of the OVLT, MePO, MS, AVP and AVPV after kisspeptin (Kp-10) or vehicle (PBS) treatment (B,D and A,C, respectively). (●), *Red dots*: single-labeled nNOS neurons; (○), *yellow dots*: P-nNOS/nNOS double-labeled neurons. *Rigth-hand panels;* are found representative fluorescent photomicrographs showing P-nNOS (green) and nNOS (red) immunoreactivity in the MS (A,B) and AVP (C,D) after the aforementioned treatments. OVLT, organum vasculosum of the lamina terminalis; MePO, median preoptic nucleus; MS, medial septal nucleus; AVP, anteroventral preoptic nucleus; AVPV, anteroventral periventricular nucleus; oc, optic chiasm; lv, lateral ventricle, ac, anterior commissure. Scale bar: 200 µM.

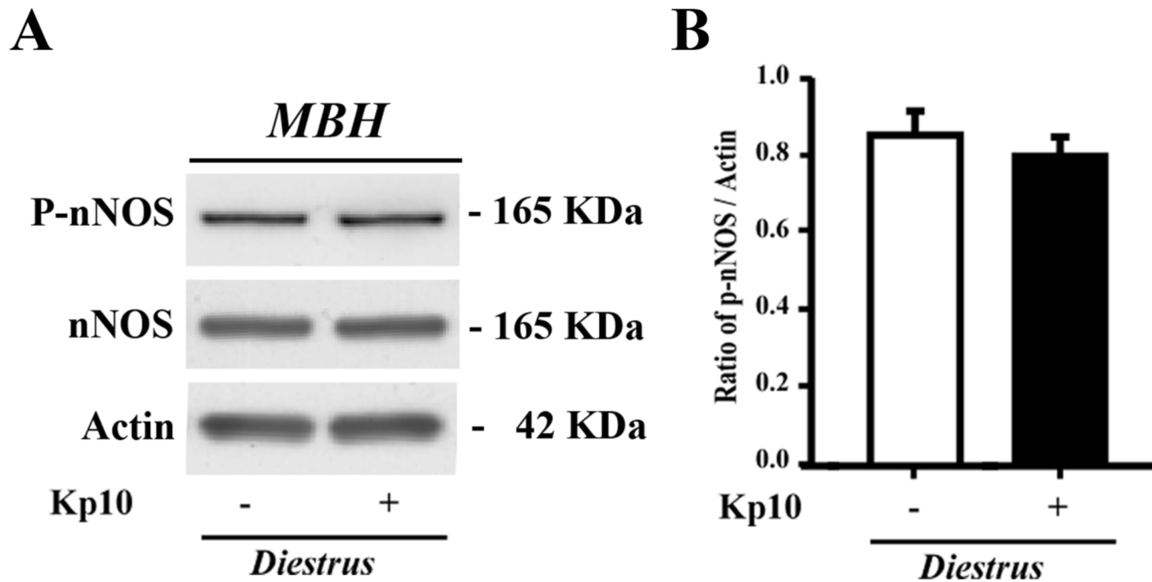
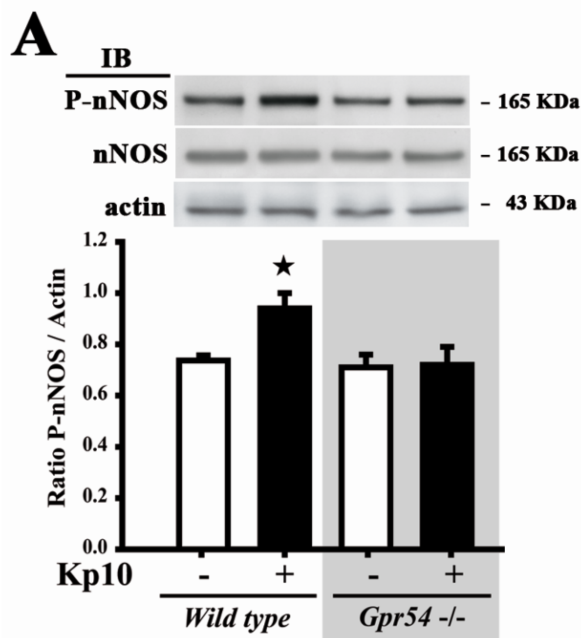


Figure 7. Kisspeptin fails to promote the phosphorylation of nNOS in the tuberal region of the hypothalamus. **A**. Immunoblotting (IB) demonstrating that kisspeptin-treatment in diestrous mice has no effect on nNOS phosphorylation in the hypothalamic tuberal region. **B.** Bar graph illustrating the mean ratio of the signal obtained for P-nNOS to that of actin in blots from 5 independent experiments (n = 5 animals). MBH, mediobasal hypothalamus. Error bars indicate the SEM.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IB
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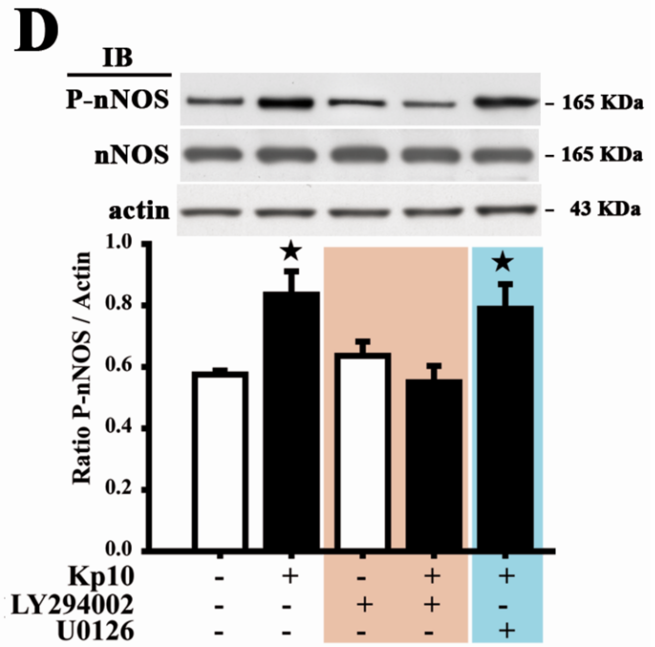
nNOS
actin
165 KDa
165 KDa
43 KDa
Ratio P-nNOS / Actin
Kp10
Wild type
Gpr54 -/-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165 KDa
165 KDa
43 KDa
Ratio P-nNOS / Actin
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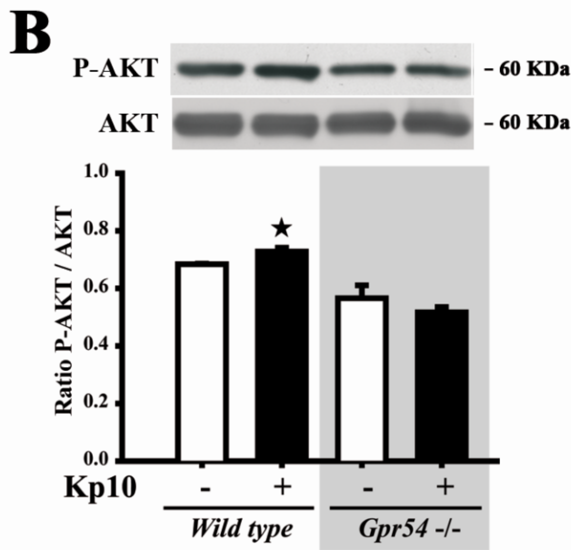
U0126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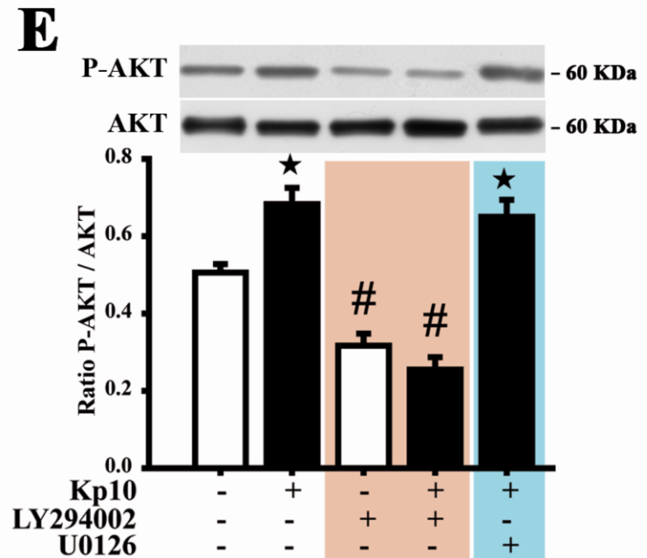
P-AKT
AKT
60 KDa
60 KDa
Ratio P-AKT / AKT
Kp10
Wild type
Gpr54 -/-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AKT
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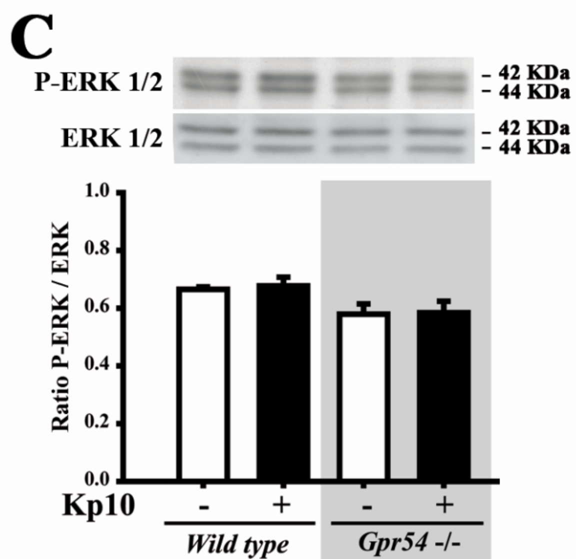
60 KDa
Ratio P-AKT / AKT
Kp10
LY294002
U0126


C
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ERK 1/2
42 KDa
44 KDa
Ratio P-ERK / ERK
Kp10
Wild type
Gpr54 -/-

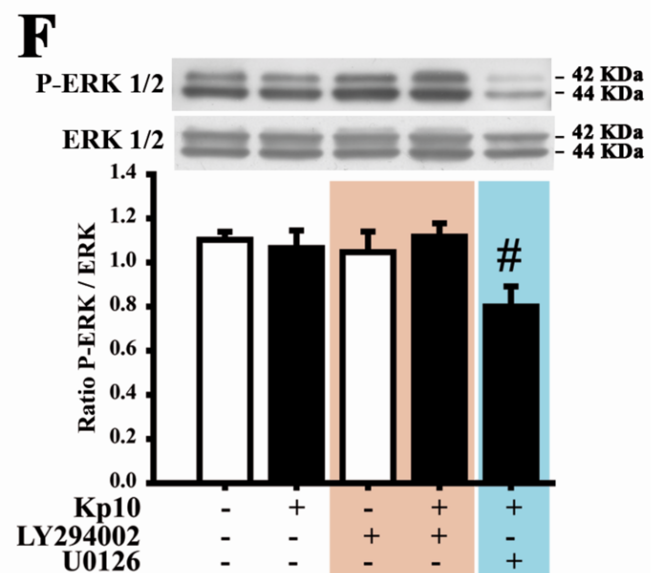
F
P-ERK 1/2
ERK 1/2
42 KDa
44 KDa
Ratio P-ERK / ERK
Kp10
LY294002
U0126

Figure 8. The activation of the GPR54 and PI3K-AKT pathways are necessary for the kisspeptin-induced increase in nNOS phosphorylation. Representative immunoblots showing that kisspeptin promotes nNOS (A,D) and AKT (B,E), but not ERK (C,F) phosphorylation in a GPR54-dependent manner (A,B) in diestrous (A-C) and ovariectomized (D-F) mice; the effects of kisspeptin are inhibited by the PI3-Kinase inhibitor LY294002 but not the MEK inhibitor U0126 (D-F). Bar graphs illustrate the mean ratio of the signal obtained for P-nNOS to that of actin (*D*), the ratio of P-AKT to that of AKT (*E*) and of P-ERK and that of ERK (*F*) from 4 independent experiments. Error bars indicate SEM. * and #, $p < 0.05$, experimental groups versus untreated controls.

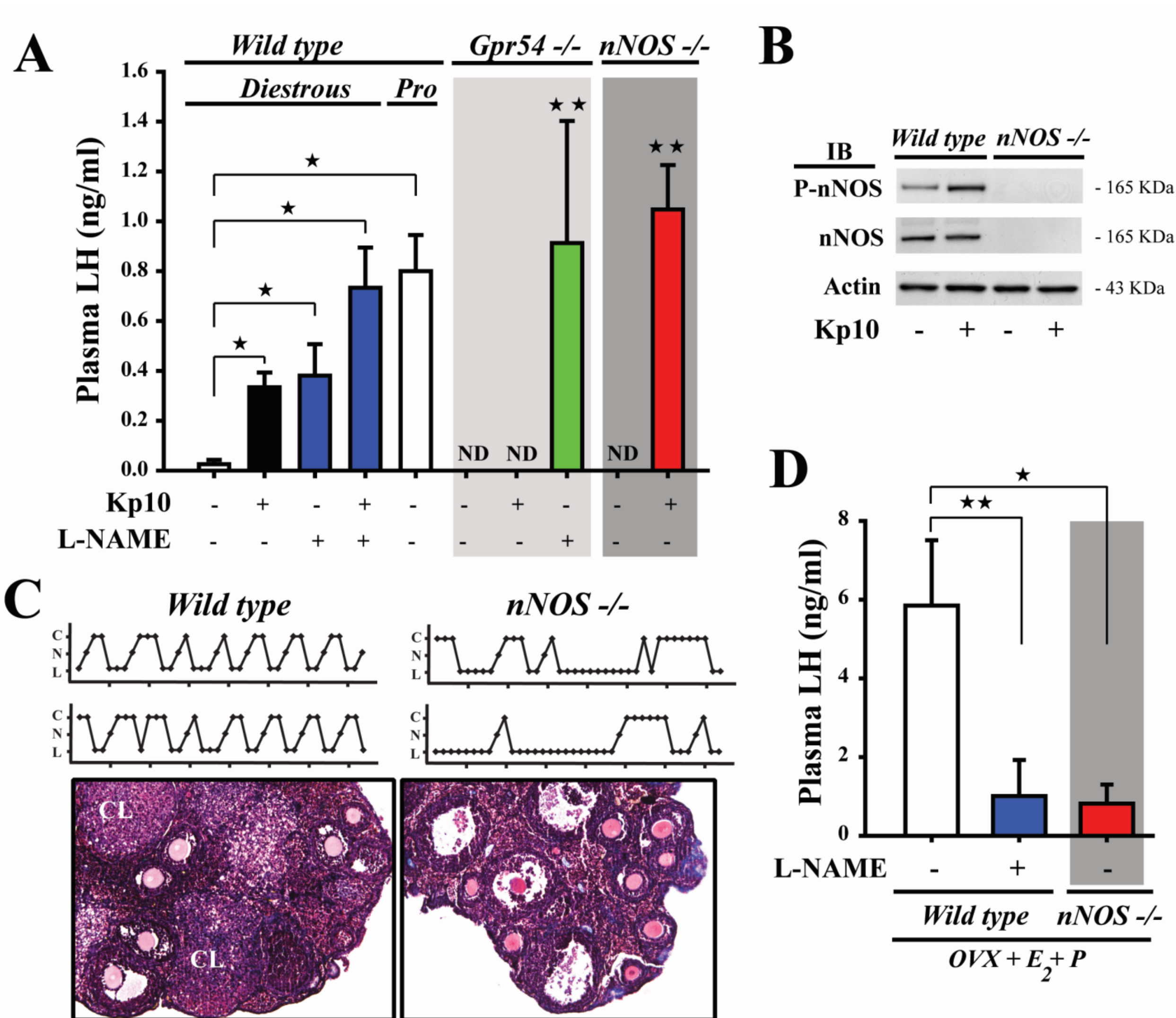

Figure 9. Kisspeptin-nNOS interactions are required for the neuroendocrine control of reproduction. **A.** Basal nNOS activity maintains the tonic inhibition on the GnRH system during the estrogen-mediated negative feedback phase, as assessed by LH release in wild-type and *Gpr54*- and *nNOS*- null mice in the presence or absence on the NOS inhibitor L-NAME. Values represent means ± SEM. *, $p < 0.05$; **, $p < 0.005$, experimental groups versus untreated controls. **B.** Western blot analysis showing the absence of nNOS protein expression in *nNOS*-null mice. **C.** *top panels*, *nNOS*-null mice do not exhibit normal estrous cyclicity as do wild-type mice, but instead enter prolonged periods of diestrus. *nNOS*-null mice sometimes display isolated complete cycles; however, these are often followed by prolonged periods of acyclicity. C, cornified (estrus); N, nucleated (proestrus); L, lymphocytic (diestrus). *Bottom panels*, histology of ovaries from *nNOS*-null mice reveals a greater number of atretic follicles and the relative paucity or absence of corpora lutea (CL) when compared to the ovaries of wild-type littermates in which follicular development is normal. **D.** nNOS activity is required for the onset of the gonadal steroid-evoked LH surge in ovariectomized animals.

Discussion

SEMA3A gene : A new disease locus for Kallmann syndrome

Here we found loss-of-function mutations in *SEMA3A* gene that encodes Sema3A, a chemotrophic ligand for Neuropilin1 receptor in subjects affected by Kallmann Syndrome that were not found in normal individuals and therefore we believe *SEMA3A* to be a novel locus for human GnRH deficiency. Further support for a critical role of Sema3A in GnRH neuron development was provided by our studies using $Nrp1^{sema/sema}$ transgenic mice that harbors a mutation on the semaphorin binding domain of the Neuropilin1 receptor with intact VEGF-Nrp1 signaling (Gu et al., 2003). These mice show defects in both developing olfactory/vomeronasal and GnRH system strongly supporting our findings in humans.

We demonstrated that Nrp1 is robustly expressed along the GnRH migratory route during mouse and human embryonic development, with higher expression levels in the nasal compartment and the rostral forebrain, the GnRH neuron early migratory path. It is well known that GnRH neuron migration is intimately linked to the olfactory system during development (Schwanzel-Fukuda et al., 1989; Wray et al., 1989). GnRH neurons originate in the nasal placode and migrate along their olfactory/ vomeronasal guiding fibers to reach their final targets in preoptic region in the ventral forebrain. Herein, we demonstrate that $Nrp1^{sema-}$ mutation in mice results in an aberrant migration of GnRH neurons in the dorsal forebrain after crossing the cribiform plate and in the loss of the caudal branch of the vomeronasal nerve, which supports GnRH neuron migration from the rostral forebrain to the preoptic region (Schwarting et al., 2001; Yoshida et al., 1995). As a result, only few GnRH neurons reach their final target area in the hypothalamus. These migratory defects are associated with a marked bundling of olfactory fibers that fail to contact their proper glomerular layers in the olfactory bulb and remain clustered at the cribriform plate thus causing anosmia in these mutants. Anosmia dramatically increases infantile death due to nipple-feeding defects and

animals that grow into adulthood are subfertile. Similar defects in suckling behavior leading to early postnatal death has been observed in the arhinencephaly mouse (*Pdn/Pdn*) that have complete developmental failure in the olfactory nerve projection to the central nervous system and absence of the olfactory bulb architecture (Hongo et al., 2000). These defects in *Nrp1*$^{sema/sema}$ recapitulate the anatomical features of Kallmann syndrome patients (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010). Previous studies have shown that Sema3A is expressed in the developing olfactory bulb (Cloutier et al., 2002; Imai et al., 2009; Schwarting et al., 2000), and that Sema3A-Nrp-1 signaling is critical for olfactory map formation (Sakano, 2010).

Secreted semaphorins and their receptors have been implicated in the development of the nervous system through their chemorepulsive effect on a wide variety of neuronal projections (Castellani and Rougon, 2002; Chedotal, 2010; Derijck et al., 2010). Here, we demonstrate that Sema3A-Nrp-1 signaling is also required for the formation of the caudal branch of the vomeronasal nerve. Sema3A indeed appears to serve as a guidance cue that repels Nrp1-expressing vomeronasal fibers ventrally for projection into the hypothalamus when they reach the rostral forebrain. Loss of the caudal branch of the vomeronasal nerve in *Nrp1*$^{sema/sema}$ mice diverts GnRH neurons away from their normal path to the hypothalamus, a phenotype that strikingly resembles the one described for mice harboring mutations in the netrin-1/DCC signaling pathway where the caudal vomeronasal nerve extends into the medial wall of the cerebral cortex and misguiding the GnRH neurons into the cortex. (Schwarting et al., 2001).

Our data stand in striking contrast to prior works suggesting that Sema3A-Nrp-1 signaling is either not or only mildly involved in vomeronasal axon guidance (Cariboni et al., 2011; Cloutier et al., 2002) and GnRH neuron migration. Previous studies analyzing axon growth

from E15 rat vomeronasal epithelia indeed showed that Sema3A, in contrast to Nrp-2 ligands, was unable to repel vomeronasal axons. Interestingly, developmental studies of the vomeronasal organ projections demonstrated that while vomeronasal fibers projecting dorsally to the accessory olfactory bulb form between E15 and E16, the caudal projections are fully developed by E15 in the rat (Yoshida et al., 1995). In addition, co-culture of vno with respective olfactory bulbs showed that Sema3A repels vomeronasal fibers from mouse explants at E13.5, when caudal vomeronasal organ projections are being established (Schwarting et al., 2001; Yoshida et al., 1995). These apparent discrepancies thus raise the intriguing possibility that Sema3A-Nrp1 signaling action on vomeronasal projections is restricted to the embryonic critical period when caudal vomeronasal axons are guided to their target in the ventral forebrain. Others claim that mutation of the semaphorin domain in Nrp1 Sema3A receptors only partially affects caudal vomeronasal nerve formation and GnRH neuron migration (Cariboni et al., 2011). In the present study, analysis of the vomeronasal nerve in *Nrp1*$^{sema/sema}$ mice using a fluorescent lipophilic tracer provided direct evidence that semaphorin signaling through Nrp1 receptors functions as an essential pathway for vomeronasal system development, promoting formation of caudal vomeronasal axonal projections that latter guide GnRH neuron migration towards the hypothalamus during embryogenesis. Because Sema3A expression is known to be complementary to Nrp1 expression in the olfactory system (Imai et al., 2009), Sema3A likely constitutes the Nrp1 ligand capable of directing the development of the axonal scaffold for GnRH neuron migration during embryonic life. Consistent with this idea, Sema3A repels vomeronasal fibers in a Nrp1-dependent fashion *in vitro*, and mutations of Sema3A in human patients causes GnRH secretion deficiencies known to be linked to altered GnRH neuronal migration during fetal life (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010).

In summary, Sema3A-Nrp-1 signaling is critical for olfactory axon path finding, formation of the caudal branch of the vomeronasal nerve and GnRH neuron migration. Defects in Sema3A-Nrp1 signaling are involved, therefore, in neurodevelopmental disorders combining anosmia and GnRH secretion deficiency, i.e., Kallmann syndrome.

Kallmann syndrome was initially considered as a monogenic disorder, however, several recent findings and the present study reinforce the emerging concept of oligogenic / digenic mode of inheritance is involved in KS patients. Heterozygous *Nrp1*^{sema} mice have a normal phenotype indicating the monoallelic mutation is not sufficient to induce KS. Normal phenotype is also observed in other heterozygous mice like *Prokr2*, while the homozygous mutants show clear absence of olfactory bulbs and defects in GnRH neurons migration (Matsumoto et al., 2006). Although several mutations have been found in several genes, these account only to 30 % of cases and varying phenotype among the KS patients signify yet more disease loci to be unearthed. Recently, heterozygous mutations in genes related to idiopathic hypogonadotropic hypogonadism / KS like *FGFR1, PROKR2, KAL1* and *GNRHR* have been identified in women with hypothalamic amenorrhea (Caronia et al., 2011). This raises the speculation that decreased signaling in any of these pathways may lead to normal development of olfaction but a partially compromised GnRH neuronal network owing to less number of GnRH neurons successfully migrating to the hypothalamus and undergoing a suboptimal maturation during puberty. However, the patients may be predisposed to abnormal GnRH secretion under the influence of external environmental factors like stress, excessive exercise, etc., in turn leading to hypothalamic amenorrhea.

Taken together, these findings suggest that GnRH-deficiency in humans involves complex genetics and identification of more genes in either sporadic or familial cases can put new insights in the pathogenesis of this disease.

GnRH-deficiency in humans may be due to defects in ontogenesis of GnRH/olfactory system or defects in initiating puberty or regulation of GnRH secretion during adulthood. While studies in the part I of this thesis identifies the *SEMA3A* as a new gene involved in kallmann syndrome the studies in part II of this thesis identifies the crucial role played by nitric oxide neurons in the neuroendocrine progression of ovulation in mice and speculate that *nNOS* gene in humans could be a new disease locus involved in iHH.

In the part II studies we demonstrate that nitric oxide neurons are integral part of neural circuits that convey peripheral feedbacks especially estrogens to GnRH neurons. Using GPR54-LacZ mice that had been previously used to study expression of Gpr54 in GnRH neurons, we demonstrate that in addition to the GnRH neurons a discrete population of hypothalamic nitric oxide containing neurons receiving abundant kisspeptin fiber appositions also expresses kisspeptin receptor Gpr54. We found that Gpr54 receptor expressed in these neurons functionally contributes to the kisspeptin mediated phosphorylation-dependent activation of nNOS through the PI3K-AKT signaling pathway. Absence of similar activation of nNOS and PI3K-AKT signaling pathway by kisspeptin and estradiol in overiectomized *Gpr54*-null mice suggests that estrogen requires kisspeptin-Gpr54 signaling to impact maximal nNOS activity to exert their positive feedback on the day of proestrus. In support to these findings, *nNOS*-null mice exhibited a markedly impaired ability to generate an LH surge in response to gonadal steroids and conspicuous ovulation deficiency. Taken together these observations put new insights in unknown interaction between kisspeptin-nNOS neurons and their role in regulating the hypothalamic-pituitary-gonadal axis in adult mammals.

NO, like other gaseous neurotransmitters cannot be stored in synaptic vesicles, can readily travel across cell membranes to act on both presynaptic and postsynaptic sites and coordinate neuronal inputs in a restricted brain volume (Gally et al., 1990). However, delimited its half-life and diffusion constant, the mechanisms that regulate their synthesis with respect to time and and space are crucial in determining its biological effect (Garthwaite, 2008). Nitric oxide containing neurons in the preoptic region are well placed in the vicinity of GnRH neurons to regulate their neuronal activity (Grossman et al., 1994; Herbison et al., 1996). Previous studies from our laboratory have demonstrated that NO can directly modulate the electrical activity of GnRH neurons (Clasadonte et al., 2008). While the production and amount of NO is tightly regulated by estrogens (d'Anglemont de Tassigny et al., 2007; Parkash et al., 2010), the present study intriguingly revealed that a dual effect of NO on GnRH/LH release during the ovarian cycle. During the diestrous stage, when the estrogens exert their negative feedback, inhibition of nNOS activity resulted in increase in basal and kisspeptin-stimulated LH release, respectively suggesting basal constitutive level of NO release maintains tonic inhibition of GnRH neurons, keeping LH level at their nadir. In contrast to our findings, a previous study in performed in rats showed that pharmacological inhibition of nNOS activity failed to inhibit both basal and kisspeptin-stimulated LH release (Navarro et al., 2005). However, as these studies were performed in prepubertal male rats, the indifference in the effect of kisspeptin could be related to differences in species and sex. In addition, the kisspeptin-stimulated LH release before puberty may not be the same keeping in (into ?) account that the neuroendocrine circuits regulating puberty or GnRH secretion may be in a maturing phase. In contrast to these studies, we also show that blockade of NO synthesis blunts the LH surge in steroid-primed mice, which is known to require activation of kisspeptin neurons (Clarkson et al., 2008).

Altogether, these results suggest that NO- and kisspeptin-synthesizing neurons synergistically interact to coordinate the progression of the ovarian cycle and hence reconcile the recent evidence that kisspeptin-Gpr54 signaling plays a key role in the neuroendocrine control of reproduction with the role of NO that has originally postulated as important in the control of the onset of the preovulatory GnRH/LH surge and fertility.

GnRH deficiency can be caused either due to deficits in ontogenesis of GnRH/olfactory system or in initiating puberty or in regulation GnRH secretion in adults. Deregulation in pathways inolving development of GnRH/olfactory system are usually associated with deficits in sense of smell and reproduction, together called the Kallmann syndrome while deficits in activation of pubertal onset or regulation of GnRH secretion in adults leads to isolated idiopathic hypogonadotropic hypogonadism. Here, we identify Sema3A, a chomotrophic factor involved in the ontogenesis of olfactory/vomeronasal and GnRH systems and on the other hand we identify a crucial interaction between hypothalamic kisspeptin and Nitric oxide containing neurons involved in the neuroendocrine control of reproduction.

Perspectives

1. *To investigate the role of Sema3A-Nrp1 interactions specifically in the ontogenesis of GnRH neurons using GnRHKONrp1 mice.*

Transgenic mice

Having obtained the results in Part I of this thesis showing that GnRH neurons express Nrp1, we then asked the question whether, in addition to play a role in olfactory/vomeronasal fiber projections, Sema3A-Nrp1 signaling also exerts a cell autonomous effect on GnRH neuronal migration. To answer this, we generated a conditional knockout mice (GnRHKONrp1) by crossing the GnRH-Cre recombinase mice (that has Cre-Recombinase cloned under the GnRH promoter) with LoxP-Nrp1 mice (that has LoxP sites flanking the neuropilin1 gene). This cross results in deletion of Nrp1 receptor specifically in GnRH neurons.

Preliminary results

Quantitative analysis of GnRH neurons showed that the homozygous GnRHKONrp1 mice have increased total number of GnRH neurons during development at the E14.5 compared to the wild type littermates and more number of GnRH neurons are present scattered in the nasal compartment of homozygous embryos compared to the wild-type littermates (**Figure. 1**). The homozygous mice survived normally into adults that allowed to study the adult reproductive phenotype. These mice had advanced pubertal onset marked by earlier vaginal opening and first estrous than their wild type littermates **(Figure. 2)**. Interestingly, these mice also had increase in body weight compared to wild type littermates during the period of puberty.

These preliminary results show Nrp1-Sema3A signaling plays an unidentified role in the ontogeneis of GnRH neurons that eventually results in advanced activation of the HPG axis. Increased GnRH neurons could be either due to increased neurogenesis or increased survival of GnRH neurons. These observations also suggest the possibility that Nrp1-Sema3A could be involved in the formation of synaptic inputs implied in puberty, as shown in other neuronal systems (ref). These mutant mice can also be used to study the interplay between GnRH neurons and growth as they show an earlier increase in body weight associated with a precocious onset of puberty, when compared to their littermates.

Specific deletion of Neuropilin1 receptor only in GnRH neurons leads to increased GnRH neurons during development

Distribution of GnRH neurons @E14.5

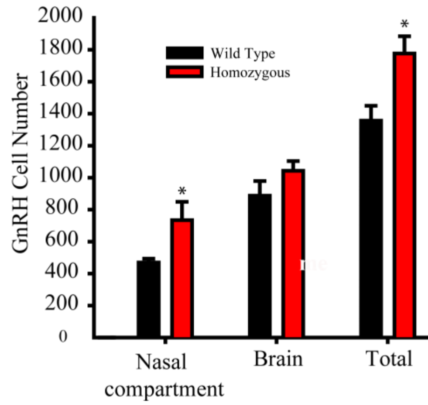

Figure 1. **Increased number of GnRH neurons in the GnRHKONrp1 mice**. Quantification and distribution of GnRH cells in Wild type and Homozygous mice at E14.5 embryos. * < 0.05

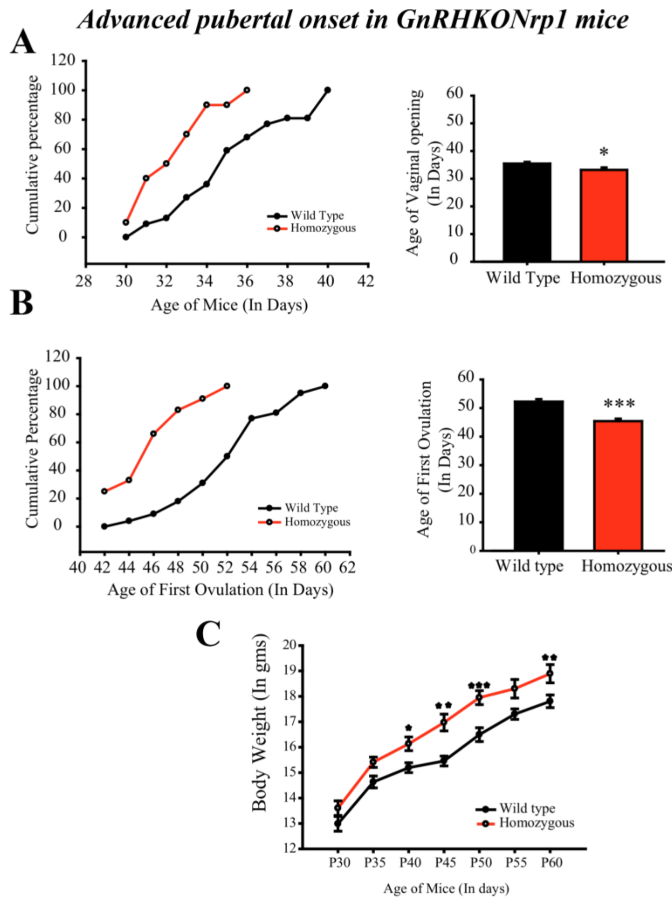

Figure 2. **GnRHKONrp1 have advance pubertal onset**. A.B. Graphs illustrating the cummulative percentages and means, resepevtively, of the age appearance of vaginal opening and first ovulation in the wild type and homozygous GnRHKONrp1 mice. C. Graph representing the body weights of wild and Homozygous GnRHKONrp1 mice during the puberty. * < 0.05; ** < 0.01; *** < 0.005

2. *Mapping of Gpr54 expressing-nNOS neurons in mouse brain using the Gpr54-Lac Z mice*

The studies performed in Part II of this thesis lead to identification of kisspeptin receptor-Gpr54 in nNOS neurons. Although it becomes increasingly clear that kisspeptin neurons directly communicate with GnRH neurons to regulate their activity, emerging evidence suggest that kisspeptin neurons may also interact with other neuronal populations. Our work that identifies for the first time the presence of Gpr54 receptor in non-GnRH cells in the hypothalamus strengthens this hypothesis. During the course of this study, we observed that Gpr54 expressing nNOS neurons are not only present in the preoptic region but also in other areas of the hypothalamus like the paraventricular hypothalamus (PVH), the posterior hypothalamus and in extra-hypothalamic areas such as the amygdala, etc emphasizing the need to map the Gpr54 expressing-nNOS neurons in the brain.

While the Part II study of the thesis shows that the kisspeptin-nNOS neuron interactions in the preoptic region may be involved in the regulation of GnRH neuronal activity during the ovarian cycle, the presence of Gpr54 expressing-nNOS cells in other regions suggest other unidentified roles for these interactions. Additional studies are thus required to identify these new functions.

3. *Possible cross-talk between nNOS neurons and Kisspeptin neurons in the preoptic region.*

In the preoptic region, cell bodies of Kisspeptin neurons are localised in the AVPV. While the studies in Part II of this thesis show that kisspeptin fibers morphologically interact with nNOS neurons in the preoptic region, our earlier careful observations show that kisspeptin and nNOS neuronal cell bodies are present in close appositions with each other in the AVPV. Because NO can travel up to 100 uM from its source, AVPV nNOS neurons may play a role in regulating the neuronal activity of kisspeptin neurons. This awaits further study.

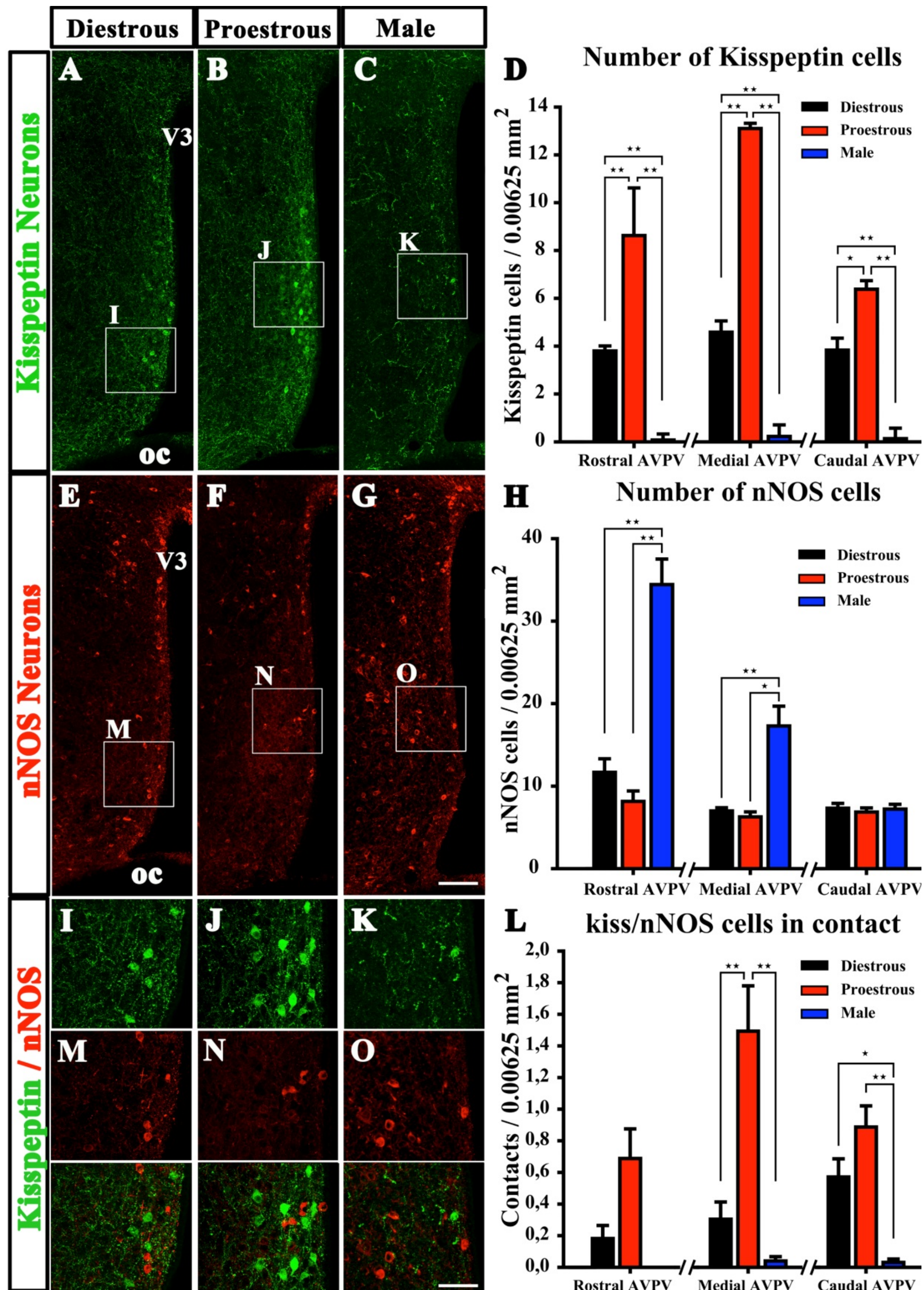

Figure 2. Distribution of kisspeptin and nNOS neurons in adult female and male mice at the AVPV. Representative low magnfication microphotgraphs illustrating distribution of kisspeptin (A-C) immunoreactive cells, nNOS immunoreactive cells (E-G) and high magnification images of boxes delineated illustrating the kisspeptin/nNOS cells (I-K; M-O) in close contact in the AVPV in adult female mice in diestrous (A,E,I,M) , proestrous (B,F,J,N) stage and male mice (C,G,K,O). Representative graphs illustrating number of kisspeptin (D), nNOS (H) and kisspeptin/nNOS cells in contact (L) in the AVPV in diestrous and proestrous female and male mice. *< 0.05; ** < 0.01; ***< 0.005

Studies in the part I of this thesis reinforce the emerging concept of digenic or oligogenic pattern of inheritance involved in the pathogenesis of Kallmann syndrome. Here, we identified *SEMA3A* gene as a new KS gene and along with earlier known KS genes (*KAL1, FGFR1, PROK2, PROKR2*).

GnRH deficiency in humans can be due to defects in GnRH neuronal migration (usually associated with KS), initiation of puberty, in GnRH secretion or in responsiveness of the pituitary gonadotropes to GnRH stimulations. Studies in the part II of this thesis suggest that nNOS neurons are integral components of the neural circuits that convey peripheral inputs like the estrogen feedbacks on GnRH neurons. Two mutant mice of nNOS gene have been generated till date - one with a deletion in the exon 3 and the second with a deletion in the exon 6. While the first mutant mice only had a mild reproductive phenotype, likely due to the presence of residual nNOS activity, the second mice generated were sterile and showed hypogonadotropic hypogonadism. These observations suggest that nNOS neurons play an important role in the neuroendocrine control of reproduction and hereby propose to sequence the *nNOS* gene in human subjects suffering from reproductive disorders like hypothalamic amenorrhea and nIHH.

Annexe

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1. Clasadonte J, Poulain P, **Hanchate NK**, Corfas G, Ojeda SR, Prevot V. Prostaglandin E2 release from astrocytes triggers gonadotropin-releasing hormone (GnRH) neuron firing via EP2 receptor activation. Proc Natl Acad Sci U S A. 2011 Sep 20; 108(38): 16104-9.

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3. Prevot V, **Hanchate NK**, Bellefontaine N, Sharif A, Parkash J, Estrella C, Allet C, de Seranno S, Campagne C, de Tassigny X, Baroncini M. Function-related structural plasticity of the GnRH system: a role for neuronal-glial-endothelial interactions. Front Neuroendocrinol. 2010 Jul; 31(3): 241-58.

4. Prevot V, Bellefontaine N, Baroncini M, Sharif A, **Hanchate NK**, Parkash J, Campagne C, de Seranno S. Gonadotrophin-releasing hormone nerve terminals, tanycytes and neurohaemal junction remodelling in the adult median eminence: functional consequences for reproduction and dynamic role of vascular endothelial cells. J Neuroendocrinol. 2010 Jul; 22(7): 639-49.

Abbreviations

°C	degree Celsius
5-HT	Serotonin
Ac	Anterior commissure
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
AGRP	Agouti-related protein
AMCA	Aminomethylcoumarin acetate
AMP	Adenosine mono phosphate
ADP	Adenosine di phosphate
ATP	Adenosine tri phosphate
ANOVA	Analysis of variance
Arc	Arcuate nucleus
BSA	Bovine serum albumin
BW	Body weight
CCK	Cholecystokinin
cDNA	Complementary DNA
ChAT	Choline acetyltransferase
CNS	Central nervous system
Cre	Cre recombinase
CRH	Corticotropin-releasing hormone
cRNA	Complementary RNA:
Cy3	Cyanine dye 3
DAB	Diaminobenzidine
DBH	Dopamine-β-hydroxylase
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
DSIP	Delta sleep-inducing peptide
EDTA	Ethylenediamine tetra-acetate
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-regulated kinase
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
gDNA	Genomic DNA
GMP	Guanosine mono phosphate
GFP	Green fluorescent protein
GH	Growth hormone
GHRH	GH-releasing hormone
GLU	Glutamate
GLUT	Glutamate transporters
GnRH	Gonadotrophic releasing Hormone
GnRHR	GnRH receptor
HDB	Horizontal limb of the diagonal band
HEPES	4-(2-Hydroxyethyl)-piperazin-1- ethansulfonic acid
HPG	Hypothalamic-pituitary-gonadal
IF	Immunofluorescence
Ig	Immunoglobulin
IGF	Insulin-like growth factor

IHC	Immunohistochemistry
IHH	idiopathic hypogonadotropic hypogonadism
IL	Interleukin
INF	Infundibular stalk
ISH	In situ hybridization histochemistry
KS	Kallmann syndrome
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
ME	Median eminence
MPN	Median preoptic nucleus
MPOA	Medial preoptic area
MSG	Monosodium glutamate
NCAM	Neural cell Adhesion Molecule
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NT	Neurotensin
OD	Optical density
OVLT	Organum vasculosum of lamina terminalis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCS	Pericapillary space
POA	Preoptic area
POMC	Pro-opiomelanocortin
PSA	Polysialic Acid
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
sc	Subcutaneous(-ly)
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SSC	Standard saline citrate
T	Testosterone
TBS	Tris-buffered saline
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
Tris	Tris (hydroxymethyl)-aminomethane
UV	Ultraviolet
MSH	α-melanocyte-stimulating
β-END	β-endorphin
sema	semaphorin
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar

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