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The involvement of the Notch Pathway in the GnRH neurons development

PhD thesis of

Ludovica COTELLESSA

R11974

Tutors Prof Luca Persani and Prof Paolo Giacobini

Supervisor Prof Marco Bonomi, Dott.ssa Valeria Vezzoli

PhD coordinator Prof Massimo Locati

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I dedicated this work to my family for their love,
support and encouragement

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Il PhD è un percorso spesso difficile e pieno di salite. Oltre alle innumerevoli conoscenze teoriche e pratiche, uno dei doni più importanti che porto con me dopo il PhD è la "resilienza". La determinazione. Un qualcosa che non si impara sui libri, ma si può imparare facendo un PhD.

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***English version**:*

A *PhD is often a difficult route, full of climbs and hurdles. In addition to theoretical and practical knowledge, one of the most important traits I gained from the PhD is "resilience". The determination. Something that cannot be taught by books but can be learned by doing a PhD.*

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Index

MATERIAL AND METHODS

List of acronyms

CHH: Congenital Hypogonadotropic Hypogonadism

CP: Cribiform Plate

dpf: days post-fertilization

hpf: hours post-fertilization

HPG: Hypothalamus-Pituitary Gonadal axis

KS: Kallmann Syndrome

N.G.S.: Next Generation Sequencing

WES: Whole Exome Sequencing

OB: Olfactory Bulbs

OP: Olfactory Placodes

POA: Preoptic Area

OECs: Olfactory Ensheating Cells

OSNs: Olfactory Sensory Neurons

OE: Olfactory Epithelium

VNO: Vomeronasal Organ

WISH: Wholemount In Situ Hybridization

GW: Gestation Week

IHC: Immunohistochemical

MO: Morpholino

NICD: Notch Intra Cellular Domain

MAF: Minor Allele Frequency

KNDy: Kisspeptin-Neurokinin B-Dynorphin

NM: Nasal Mesenchyme

NP: Nasal Placode

NC: Nasal compartment

BF: Basal Forebrain

VNN: Vomeronasal Nerve

TN: Terminal nerve

LH: Luteinizing Hormone

FSH: Follicle Stimulating Hormone

AVPV: Anteroventral Periventricular nucleus

OP: Olfactory Placode

ME: Median Eminence

Hy: Hypothalamus

HS: Heparan Sulfate

VUS: Unknown clinical significance

AD: Autosomal Dominant

CNS: Central Nervous System

SP: Signal Peptide

ARC: Arcuate Nucleus

SNP: Single Nucleotide Polymorphism

MP: Medial Pallium

Hip: Hippocampus

DP: Dorsal Pallial

Ctx: isocortex

PirCtx: piriform Cortex

VP: Ventral Pallial

LP: lateral Pallial

BBB: Blood Brain Barrier

Zf: zebrafish

Hb: habenular nucleus

Abstract

The proper development of the hypothalamic-pituitary-gonadal (HPG) axis is essential for normal reproductive competence. Misfunctions in the axis that impairs GnRH synthesis or function result in GnRH deficiency. Idiopathic congenital hypogonadotropic hypogonadism (CHH) is a rare reproductive disorder, with significant heterogeneity of genetic inheritance, that is primarily caused by gonadotropin-releasing hormone (GnRH) deficiency. Clinically, the disorder is characterized by an absence of puberty and infertility. In approximately 50% of cases, CHH patients also suffer from a reduced or deficient sense of smell (hyposmia or anosmia, respectively). In this case, the disorder is termed Kallmann syndrome (KS) and results from a failure or incomplete embryonic migration of GnRH-producing neurons. The significance to elucidate the genetic causes of CHH is related to the relatively high percentage (about 50%) of patients that are still considered idiopathic. It is known from the literature that Notch signaling has a role in the migration of neurons in the developing cortex and that it is expressed in the olfactory system of many species, so we would like to understand if Notch signaling has a role in the migration of GnRH neurons in the olfactory system. Hence, the aim of my PhD was to understand: 1) if Notch signaling molecules are expressed along the GnRH migratory pathway in human fetal and adult post-mortem brain sections; 2) if Notch plays a role in the establishment of the correct GnRH migratory process or GnRH axonal targeting to the hypothalamic regions, using zebrafish as an *in vivo* model; 3) to address whether patients affected by CHH present mutations in the Notch signaling pathway; 4) to functionally validate the eventual mutations using an immortalized GnRH cell line (GN11).

We explored by multiplex fluorescent *in situ* hybridization and immunohistochemical assays the expression of both *JAG1*, *NOTCH1* and *NOTCH2* in coronal human fetal sections of the nasal compartment and nasal/forebrain junctions in early developmental stages. These experiments revealed that *JAG1*, *NOTCH1* and *NOTCH2* are expressed along the GnRH migratory pathway during human fetal development, suggesting a paracrine and/or autocrine

mechanism. We then used zebrafish as *in vivo* model to investigate the involvement of *jag1* in GnRH3 neurons development and migration. Firstly, we have demonstrated the expression of *jag1a*, *jag1b*, *notch1a* and *GnRH3* in the olfactory placode of zebrafish embryos, revealing a possible collaboration between these factors in the GnRH3 neurons development. Taking advantage of the zebrafish transgenic line tg(GnRH3:EGFP), we demonstrated that downregulation of *jag1b*, but not *jag1a*, strongly affects the development of the GnRH3 neurons at 48 and 72hpf. Treatment of *tg*(GnRH3:EGFP) embryos with the Notch inhibitor, DAPT (γ-secretase inhibitor), phenocopied the morpholino experiments, further supporting a role for *notch1/jag1* in the development of GnRH3 neurons. Additionally, we performed migration assay on immortalized GnRH cells, and we observe that JAG1 act as a repellent factor for the motility of these cells.

Based on the human and zebrafish data and considering the KS-like phenotype of our *jag1b* morphants, we next sought in our cohort of CHH/KS patients for possible mutation in *JAG1* gene and four rare missense variants in the *JAG1* gene were identified (R117G, F206Y, Y931I, 1160N). Overexpression of all JAG1 variants in GN11 cell line coupled with an immunofluorescence assay revealed that only the JAG1 variant D1160N was mislocalized and retained into the cytoplasm. The functionality was evaluated through the Luciferase assay, and D1160N variant did not activate the transcription of the Notch Responsive Element promoter. Combining morphological analysis *in vivo* (in human and zebrafish), together with genetic and pharmacological manipulation in zebrafish, and human genetic analysis, we provide compelling evidence that Notch1/Jag1 signaling has a role in the development of GnRH neurons/olfactory system and indicate that Notch1/Jag1 signaling insufficiency may contribute to the pathogenesis of CHH in humans.

Introduction

CHAPTER 1

PHYSIOLOGY OF HYPOTALAMUS-PITUITARY-GONADS (HPG) AXIS

The HPG axis plays a pivotal role in all vertebrates controlling many complex functions including growth, reproduction, osmoregulation, stress and metabolism. The physiological function of the HPG axis is based on the pulsatile release of the gonadotropin-releasing hormone (GnRH) by the GnRH-secreting neurons which are located in a specific area of the hypothalamus. Once secreted, GnRH arrives to the pituitary gland through the circulation of the hypophyseal portal system. There, GnRH hormone stimulate a specific compartment of the adenohypophysis represented by the gonadotropic cell via the interaction with its specific receptor, the GnRH receptor (GnRHR), which is expressed on the membrane surface of the gonadotropes. Stimulation of gonadotropin-secreting cells of the anterior pituitary, is resulting in the stimulation of both synthesis and secretion of the two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The two gonadotropins are then moved through the peripheral circulation to the gonads, where they interact with specific membrane G-protein coupled receptors, the LH receptor (LHR) and the FSH receptor (FSHR), which are expressed on the gonadal cells and they stimulate either the steroidogenesis (estrogen, progesterone, and androgens) or the gametogenesis (oocytes and spermatozoa). The gonadal steroids, in turn, through a negative feedback mechanism, auto-regulate their own secretion at the hypothalamus-pituitary level, decreasing the GnRH and gonadotropin secretion (Wierman, Kiseljak-Vassiliades et al. 2011). Hence, the HPG axis is controlled by homeostatic factors, like gonadal steroids and metabolic hormones, that act as a negative feedback that directly controls the GnRH neurons activity. Furthermore, in the last two decades it became evident the contribution of other neurons located in the hypothalamic arcuate nucleus that has been demonstrated to play a major role in the control of GnRH secretion (Fig. 2). In fact, it was demonstrated that the kisspeptin-Neurokinin B-Dynorphin (KNDy) neurons of the infundibular (in humans)/arcuate nucleus (in rodents) influence the

activity of GnRH making direct contact with GnRH cell bodies and dendrites in humans and projecting to the median eminence in rodents (Krajewski et al. 2005; Ciofi, Leroy, and Tramu 2006). KNDy cells act synergistically to produce coordinated and pulsatile GnRH secretion by controlling the neuroactivity of other KNDy cells. In fact, the expression of neurokinin B receptors and the receptor for dynorphin is evident within the KNDy cells, excepted for the kisspeptin receptor, which is only expressed by GnRH neurons (Krajewski et al. 2005; Navarro et al. 2009; Herbison et al. 2010).

This implies the stimulatory role of neurokinin B and the inhibitory action of dynorphin auto-synaptically coordinate the pulsatile release of kisspeptin, which in turn drives the pulsatile secretion of GnRH and LH (Navarro et al. 2009).

Kisspeptin-mediated GnRH stimulation is also regulated by the levels of circulating sex steroids, in fact estrogen and progesterone modulate kisspeptin activity at both the AVPV nucleus and the arcuate/infundibular nucleus through sex steroid receptors (Fig. 1).

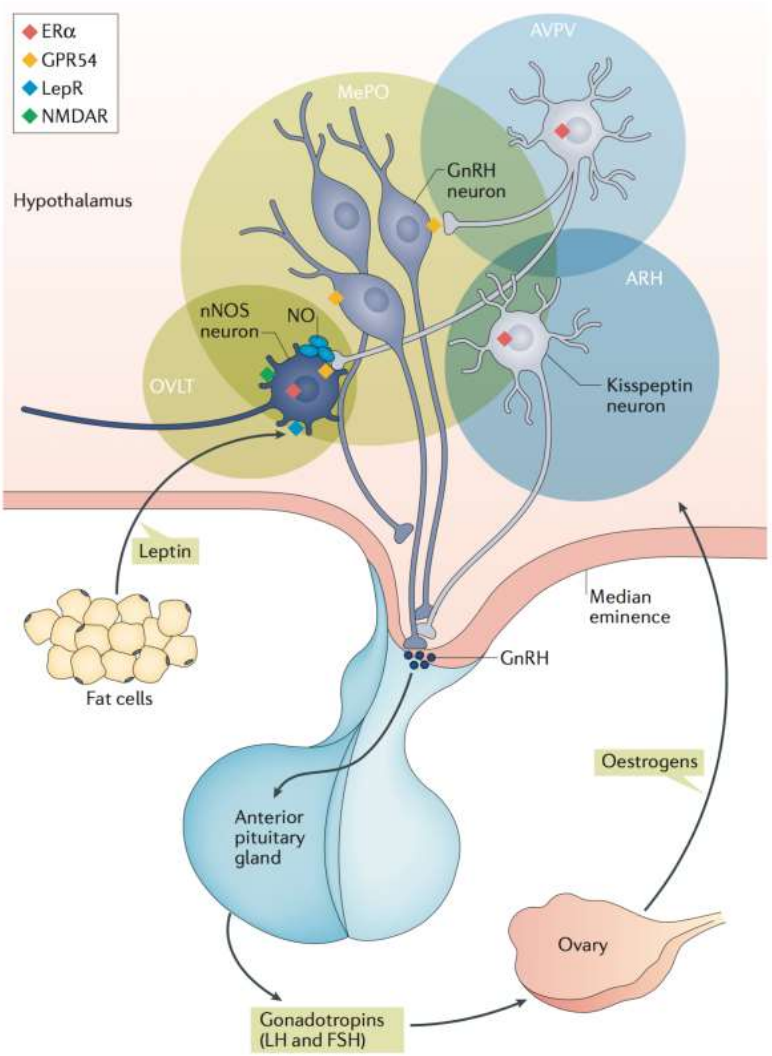

Fig. 1. *Regulation of the HPG axis by KNDy neurons action (Chachlaki, Garthwaite, and Prevot 2017).*

GONADOTROPIN RELEASING HORMONE

Gonadotropin-releasing hormone (GnRH) is a decapeptide neurohormone that plays a key role in the activation/regulation of the hypothalamus-pituitary gonadal axis and thus in the control of reproduction. Until now, 14 variants of GnRH have been described in all vertebrates. The GnRH sequence through vertebrates varies mainly at amino acid positions 5, 7 and 8, differently, the residues 1, 4, 9 and 10 are highly conserved (Lethimonier et al. 2004). In all vertebrates, two or three GnRH isoforms have been recognized, with different localizations: GnRH1 is primarily located in the Pre-Optic-Area (POA) and hypothalamus and it has the hypophysiotropic function, namely, it induces the releasing of gonadotrophins; GnRH2 is found in the midbrain and tegmentum and it is believed to have a role in the reproductive behavior and or control of appetite and metabolism (Temple, Millar, and Rissman 2003; Barnett et al. 2006); GnRH3 is located in the terminal nerve but its function is not clear yet, even if there are hypothesis suggesting its possible involvement in the reproductive behavior (Ogawa et al. 2006).

In the zebrafish animal model, only gnrh2 and GnRH3 were identified. Gnrh2 is observed in midbrain and tegmentum, while GnRH3 is detected in terminal verve, hypothalamus, POA, recapitulating the function of the mammalian GNRH1, probably missed during evolution (Kuo et al. 2005).

GnRH is secreted by specific hypothalamic neurons, called GnRH-secreting neurons, characterized by a unique morphology since they project one or both long dendrites to the median eminence, where they break up into short axon terminals to enable secretion of GnRH into the portal vasculature (Herde et al. 2013) (Fig. 2).

These projections are dendritic-like because they receive synaptic inputs, but also axonal-like because they conduct action potentials to the GnRH neurosecretory terminals in the median eminence, for these reasons they operate as ‘dendrons’ (Herde et al. 2013).

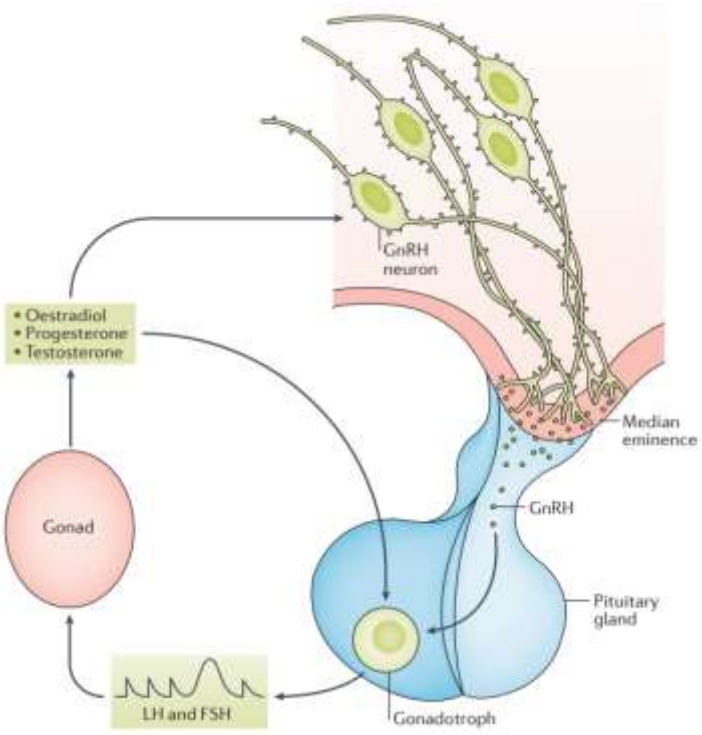

Fig. 2. *GnRH neurons are shown with their intertwined and back-projecting dendrons passing to the median eminence where they form many small axon terminals that release GnRH into the median eminence portal system (Herbison 2016).*

ORIGIN AND MIGRATION OF GnRH NEURONS IN VERTEBRATE:

THE DEVELOPMENT OF THE OLFACTORY PLACODE

To understand the development of hypothalamic, terminal nerve and midbrain GnRH cells is important to understand the complex movement of these cells during the development. The sensory structures in the head of vertebrate, including zebrafish, originates from the sensory placodes that is an ectodermal and neuro-ectodermal thickening that will give rise the olfactory and sensory epithelium, the adenohypophysis (also called anterior pituitary), lens of eye, part of cranial nerves and optic sensory epithelium. The vertebrate nervous system originates from the neural ectoderm that will develop a neural plate and subsequently the neural tube.

The cells that will develop the olfactory placodes (Fig. 3, in red) are flanked posteriorly by non-neural ectoderm (Fig. 3, in violet) and anteriorly by cells that will generate the adenohypophysis (anterior pituitary) (Fig. 3, in orange). Cells in violet will give rise to neural crest cells, that will move rostrally during the development (Fig. 3C) and finally converge to the neurohypophysis (posterior pituitary) (Fig. 3D) in close position to the hypothalamus. Additionally, neural crest cells will migrate with the olfactory/nasal placode cells, surrounding the OP (Fig. 3D), collaborating in the development of the olfactory placode (Fig. 3).

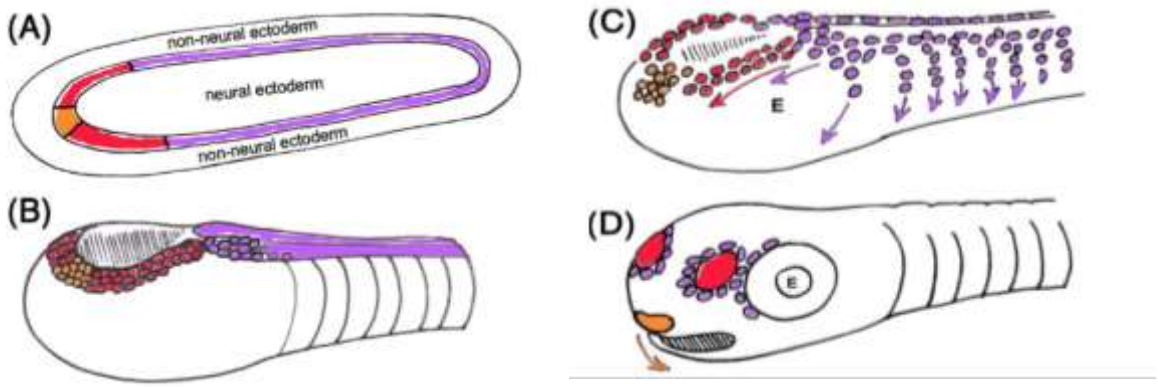

Fig. 3. *Development of the neural tube, olfactory placodes, pituitary and neural crest in the vertebrate embryo. Orange=anterior pituitary (adenohypophysis), red=olfactory placode cells, purple=neural crest cells. (A) Neural plate stage showing domain lying at the interface of neural and non-neural ectoderm that will give rise neural crest and sensory placodes. (B) The neural plate forms a neural tube and cells at the edge become localized to the dorsal side. (C) Cells that will form the olfactory placodes (red arrow) and cranial neural crest derivatives (purple arrows) migrate rostrally. E=eye. (D) Olfactory placodes are formed and surrounded by neural crest cells as the anterior pituitary (Rathkes pouch) migrates caudally (orange arrow) to fuse with the posterior pituitary (Whitlock et al. 2006).*

Hence, the olfactory organ is composed by the sensory epithelium surrounded by neural crest cell. The olfactory placode originates different cellular type including Olfactory Sensory Neurons (OSNs) (both sense odor neurons and sense pheromones neurons), GnRH neurons and Olfactory Ensheating Cells (OECs). During the development, the nasal placode invaginates creating the OE and VNO, from which GnRH neurons will give rise and migrate to the brain attached to the olfactory nerve fibers, surrounded by OECs (Wray 2002). In mice the development of the OP starts from the E9.5 when OP forms as an epithelial sheet. At E10.5 this epithelial sheet invaginate to form the nasal pit which is the beginning of the nasal cavity. At E11.5 the nasal pit deepens to finally form the nostrils. In the nasal pit both neural and non-neural structures are detected, hence, it will generate sensory and respiratory epithelium. The respiratory epithelium resides in the rostral region and its boundaries are

defined by the expression of BMP4 and Fgf8. The sensory epithelium differently is in the caudal region within the invaginating OP and contains sensory neurons, sustentacular supporting cells and mucus-producing Bowman's gland. OECs move from the lamina propria under the basal membrane and they start to surround olfactory axons. Following invagination, GnRH neurons delaminate from the OE and migrate with immature neurons attached to the olfactory nerve, supported in the migration by the OECs (Fig. 4).

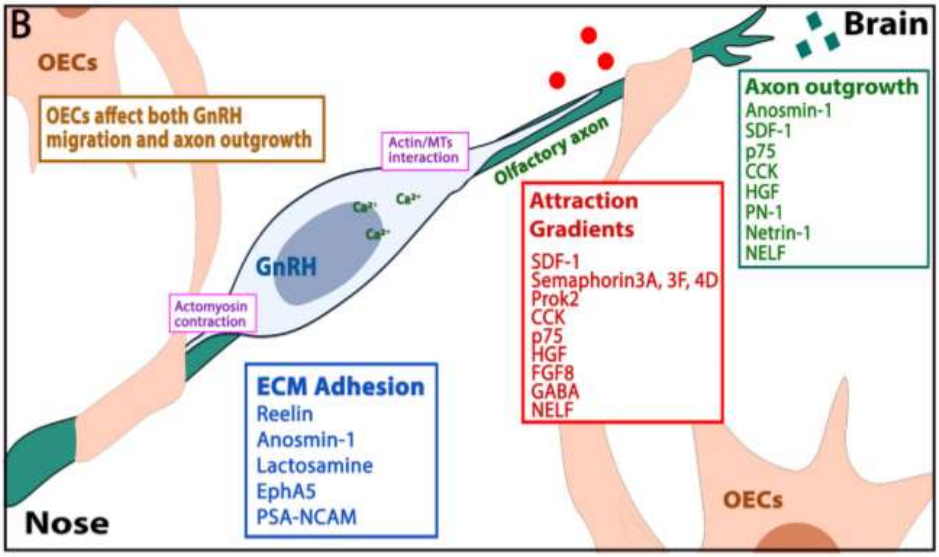

Fig. 4. *Schematic showing a GnRH neuron (blue) along the olfactory axon bundles (green), which are ensheathed by OECs (beige). Categories of axon outgrowth cues (green box), migration attraction gradients cues (red box) and ECM adhesion molecules (blue box) are indicated. Actin/microtubule interactions and actomyosin contractions (pink boxes) occur at the leading process and the trailing process of the migrating GnRH neuron (Cho et al. 2019).*

Proceeding with the development, the OE give rises the VNO from its ventral region, and from the VNO pheromone receptor cells project their axons to the accessory OB. The OE also originates the turbinate of the main OE (mOE) from which odor receptor project their axons to the main OB (Fig. 5). Modification in one of these steps could results in an altered GnRH

neurons specification and/or migration processes, with possible consequences on their correct function and, consequently, for the reasons above mentioned, lead to abnormalities in the reproduction of animals.

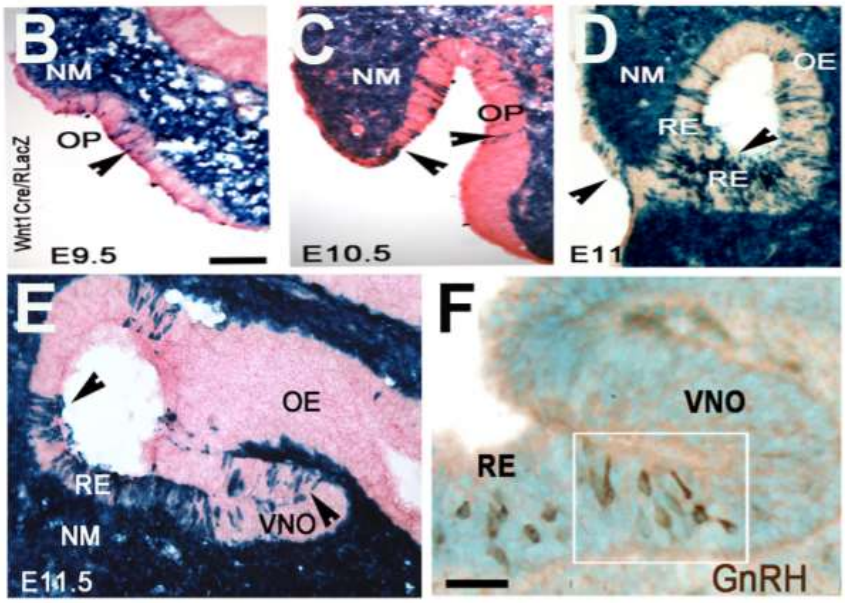

Fig. 5. *(B–E) X-Gal staining on nasal sections of E9.5–E11.5 neural crest reporter mouse (Wnt1Cre/RLacZ) showing Wnt1 expression (blue) in cells within the invaginating olfactory pit (OP, arrowheads), olfactory epithelium (OE), vomeronasal organ (VNO) and throughout the nasal mesenchyme (NM) and respiratory epithelium (RE). F Immunocytochemistry staining of E11.5 OE showing GnRH neurons (brown) located at the border between the RE and developing VNO (Cho et al. 2019).*

In the OE the stages of development can be identified looking at specific marker in the progenitors' cells. These progenitors' cells are initially located in both the basal and apical region of the epithelium, but later they are restricted only in its basal region in which it is possible to observe two different population of cells: the horizontal and the globose basal cells. From the horizontal basal cells derive neural and non-neural cells, differently, globose basal cells are actively dividing cells that originate neurons (Schwob et al. 2017). Several transcriptional factors have been identified as key regulator in this process. The first one is

PAX6 that is required for the placodal initial fate establishment. In fact, PAX6 and SOX2 are the main regulator in the olfactory neurogenesis holding the progenitors in an undifferentiated state and preventing the OSNs development (Packard, Lin, and Schwob 2016). Accordingly, the *Pax6*-null mice do not develop eyes or nasal placode, hence, this null mouse do not develop GnRH neurons. Another factor involved in the OE development is FOXG1 that is expressed in the ventral progenitor of the developing OE and its null mice results in a reduction of the OE without neuronal differentiation (Duggan et al. 2008). The intermediate neuronal progenitor of the OE, differently, expressed the transcriptional factor MASH1, also named ASCL1, that has a central role in the development of OSNs in both EO and VNO. MASH1 orchestrate the Notch pathway that is essential for the neuronal differentiation in the OE, and deletion of MASH1 results in an altered neurogenesis but normal OP or OE. The role of MASH1 in the neurogenesis is highlighted by its ability to regulate a huge number of downstream targets like Neurogenin-1 which is expressed in basal cells (Ma et al. 1997) that give rise daughter cells that exit from the cell cycle to become an immature OSNs. In fact, Neurogenin-1 activates the expression of NEUROD1 that is a marker for post-mitotic immature OSNs (Cau et al. 1997) (Fig. 6).

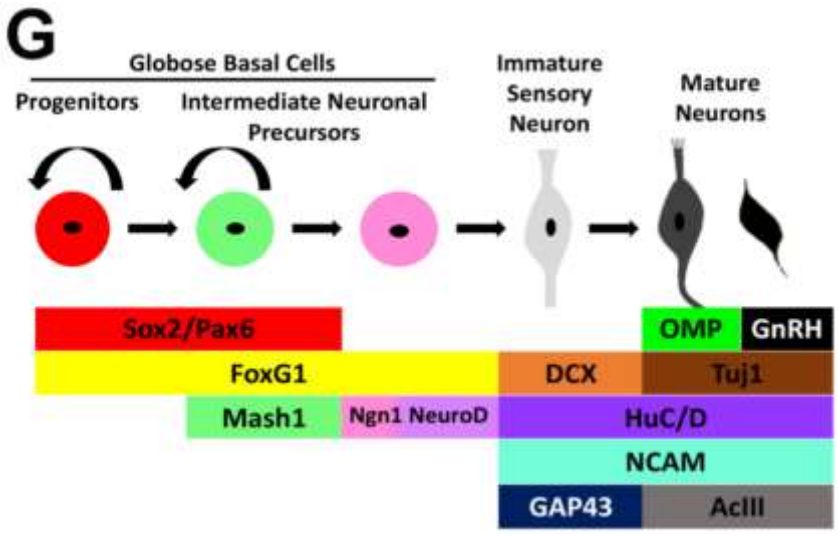

Fig. 6. *Schematic of olfactory neurogenesis. Cell types in the OE can be identified by expression of specific factors, which direct cells to remain as cycling progenitors or to undergo neuronal differentiation to form sensory and/or GnRH neurons (Cho et al. 2019).*

When the olfactory progenitors finally commit to the olfactory lineage, they differentiate becoming bipolar neurons and they start to migrate away from the basal layer expressing marker like NCAM (Calof and Chikaraishi 1989), DCX (Francis et al. 1999), HuC/D (Fornaro et al. 2001), GAP-43 (Pellier et al. 1994). When they reach maturation OSNs project their cilia into the nasal cavity reaching the apical surface in order to detect the odorants, and they extend their axons, wrapped by OECs, to the OB (Kawauchi et al. 2009). Mature OSNs express molecules such as TUJ1 (De Carlos, Lopez-Mascaraque, and Valverde 1996), ACIII (Wong et al. 2000) and OMP (Margolis 1972).

HYPOTHESIS ABOUT THE ORIGIN OF GnRH NEURONS

There are several hypotheses regarding the origin of GnRH neurons as we can see from the Figure below (Fig. 7).

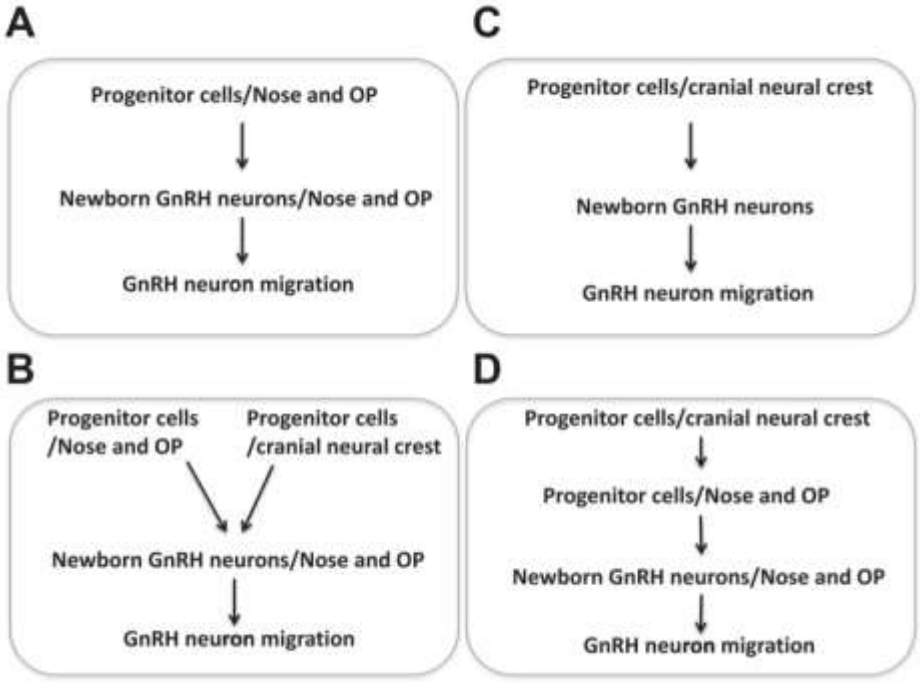

Fig. 7. *Hypotheses on the embryonic origin(s) of hypophysiotropic GnRH neurons (Duan and Allard 2020).*

The hypothesis A (Fig. 7A) support the idea that hypophysiotropic GnRH neurons are derived from progenitor cells in the nose-olfactory placode region. This idea is sustained by the use of mutant mice lacking olfactory placode (Dellovade, Pfaff, and Schwanzel-Fukuda 1998) and by ablation experiments in the newt (Murakami, Kikuyama, and Arai 1992), in which the decrease in GnRH neurons is just partial. These results have different interpretations. Firstly, the ablation may have left a few olfactory placode cells to survive, secondly, not all GnRH1 neurons are derived from olfactory placode in these species.

The hypothesis B (Fig. 7B) carries the idea that progenitor cells originate from both neural crest and olfactory placode in mice. In fact, ablation studies in mouse and chicken (Paolo E. Forni et al. 2011; Paolo E. Forni and Wray 2012) support the origin of GnRH precursor in the olfactory placode, even if, it is still possible that some GnRH neurons give rise to other regions. In particular, Whitlock and colleagues (Whitlock et al. 2005) demonstrate that GnRH3 cells derive from Sox2-dependent neural crest cells.

Studies in zebrafish have further complicated these hypotheses (Fig. 6C). In fact, from studies conducted by Abraham and colleagues in 2010 (Abraham et al. 2010) using the GnRH3:EGFP reporter line, the laser ablation of the GnRH3 soma in the nasal area in an early developmental stage resulted in a loss of olfactory, terminal nerve, preoptic area, and hypothalamic GnRH3, indicating that the GnRH3 neurons originate from the olfactory region and migrate to the brain in zebrafish. Besides that, Zhao and colleagues in 2013 (Zhao et al. 2013) using a different reporter line GnRH3:EMD demonstrated that GnRH3 neurons in the terminal nerve, which play a role in mating behavior in male zebrafish, may originate from cranial neural crest cells, while the GnRH3 neurons found in the hypothalamus and preoptic

area, with hypophysiotropic function, apparently originate from the adenohypophyseal placode, that is a non-neural ectoderm derived structure (Zhao et al. 2013). On the other hand, Saxena and colleagues in 2013 find an explanation that was able to fuse the previous hypothesis made by Withlock and Zhoa, demonstrating that some sox10-expressing cranial neural crest cells migrated to the olfactory placode and formed a capsule surrounding the placode during the first day of development. Some of these cranial neural crest cells may travel to the vicinity of the olfactory placode first and then give rise to GnRH neurons (Fig. 7D) (Saxena, Peng, and Bronner 2013).

Thus, several years after the identification of the nasal placode as source of GnRH neurons, their lineage of origins is still undetermined. Until now, the only way to define a GnRH neuron is the detected expression the GnRH mRNA/ peptide. For this reason, progenitor cells cannot be identified. It was recently shown by Aguillon and colleagues in 2018 that Islet1/2 monoclonal antibody shows immunoreactivity in the olfactory epithelium in the same temporal window in which GnRH3 promoter-driven GFP expression in transgenic zebrafish embryos. This open the possibility that the transcriptional factor Islet-1 or Islet-2 could have a role in regulating the differentiation of GnRH3 neurons, providing an earlier marker for these cells than GnRH expression itself (Aguillon et al. 2018).

MIGRATION OF GnRH NEURONS IN VERTEBTARE: NASAL COMPARTMENT, NASAL-FOREBRAIN JUNCTIONS AND WITHIN THE FOREBRAIN

As above mentioned, GnRH neurons originate outside the brain and migrate attached to olfactory axon to reach their destination: the forebrain. This migration starts in mice at E9.5 and is complete at E18.5 and it could be divided in three different steps: the migration from the nasal compartment to the Nasal-Forebrain Junction (NFJ); the cross of the Cribriform Plate (CP) penetrating in the brain in proximity to the OB; caudal turning to reach the forebrain, migrating attached to the VN and olfactory fibers. When they arrive to the hypothalamus in the ventral forebrain, GnRH neurons detached from their axonal guide and project their axons to the median eminence (Fig. 8).

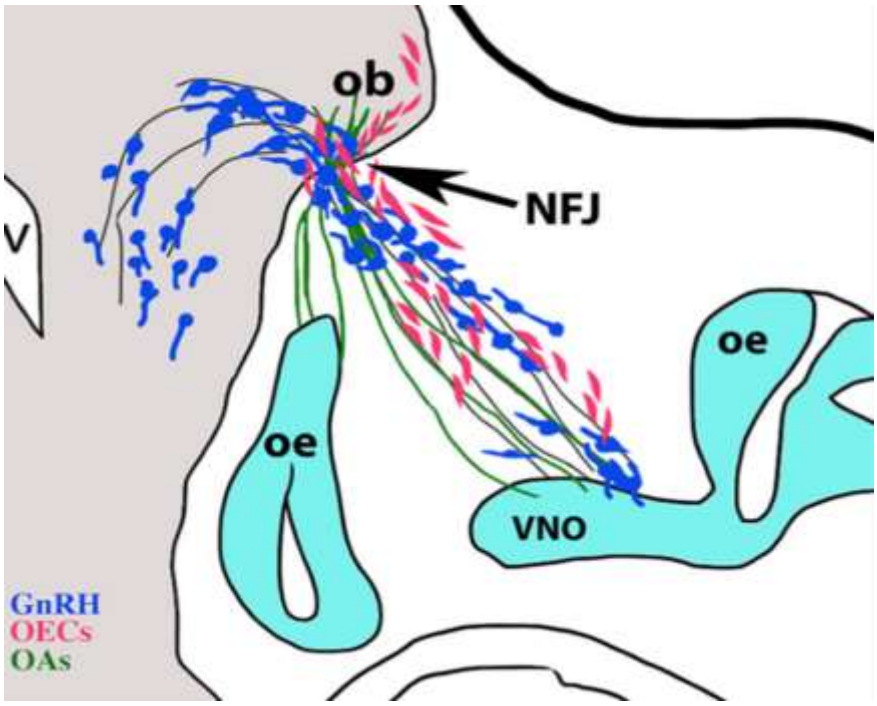

Fig. 8. *Schematic of multiple components forming the GnRH migratory bundle in mouse at E14.5. GnRH neurons (blue) migrate along VNO/terminal nerve axons (black lines) into the forebrain. Other VNO sensory axons bundle with olfactory sensory axons (green) and OECs (pink) enter the olfactory bulb (Cho et al. 2019).*

NASAL COMPARTMENT

The specific lineage from which GnRH cells give rise are still unknown and the factors involved in their migration are not completely understood. Studies in animal model reveal that the transcriptional factor Ebf2 has a role in the GnRH neuron migration in fact the *Ebf2*$^{-/-}$ mice show an accumulation of GnRH cells in the nasal compartment with a complete lack of GnRH cells reaching hypothalamus and projection in the median eminence. Bone Morphogenic Factor 4 (BMP4), regulated by its antagonist Noggin, are crucial in defining neuronal versus epidermal fates in the developing OP (Forni et al. 2013). Alteration in Noggin/BMP4 signaling alter the neurogenesis of the OE that results in a reduced number of GnRH neurons (Forni et al. 2013). Besides transcription factors, also other molecules have a role in the GnRH neuron migration. Cytokines and chemokines may act synergically to promote the exit from the nasal compartment and the navigation in the nasal mesenchyme. These soluble molecules usually have a pick in their expression at the time of GnRH cells migration and decrease when the process is ended. The Leukemia Inhibitory Factors (LIF) is a cytokine expressed in the nasal mesenchyme and it has been recently demonstrated that LIF induce chemotaxis in GN11, that are immortalized GnRH neurons cell line from mice, activating three different pathway that act on the cellular migration. Another cytokine is Hepatocyte Growth Factor (HGF) that binds its receptor c-met that has mitogenic, motogenic and chemoattractive effect on both neuronal and non-neuronal cells. HGF, like LIF, is expressed early in the nasal mesenchyme and studies on GN11 cell line and nasal explant highlight its importance in the GnRH neurons migration (Giacobini et al. 2007). The neutralization of HGF through an antibody implying in nasal explant, reduce the migration of GnRH cells and the olfactory axons outgrowth. Both these aspects are rescued treating with HGF. The chemokine Stromal Cell-Derived Factor 1 (SDF-1) has the same expression pattern, with low level of chemokine in the vomeronasal organ and high concentration in the

forebrain junctions, and it acts via its receptor CXCR4. This receptor has an important role in the GnRH neurons migration, in fact GnRH cells in the null mice for this receptor fail to exit from the vomeronasal organ.

Migrating GnRH neurons are attached to vomeronasal nerve that express several adhesion molecules like N-CAM, netrin-1 receptor (DCC), the nasal embryonic LHRH factor (NELF), and TAG-1 that are key regulator in the migration process. In 2000 the NELF expression was detected in the nasal epithelia of mouse but also in GnRH neurons and olfactory axons (Kramer and Wray 2000), in fact, *nelf* null mice reveals an impaired migration of GnRH neurons and fertility (Quaynor et al. 2015). In the same way, PSA-NCAM is expressed in GnRH neurons, vomeronasal axons and OECs and studies in chicken embryos demonstrate that the removal of PSA from PSA-NCAM molecule indices a significant reduction of caudal turning GnRH neurons into forebrain (Murakami et al. 2000). A relatively recent work also demonstrates the involvement of Eph5A in the axophilic migration of GnRH cells from the nasal compartment to the forebrain along the vomeronasal and terminal nerve axons (Gamble et al. 2005). The overexpression of *Eph5A* in mouse slow the migration of GnRH neurons that, as consequence, create clusters in the nasal compartment. Eph5A has a role in the adhesiveness between GnRH cells and olfactory axons that are the guide for the GnRH destination. GnRH clusters are also observed in the *Neurophilin-2* knock down mice (*Npn-2* -/-), in fact Npn-2 is involved in the axonal guidance acting as a co-receptor for class 3 semaphorins (Tamagnone and Comoglio 2004). This null mouse shows clear signs of infertility and defasciculation of vomeronasal nerve. Another recent finding highlights the role of cell surface glycoconjugate lactosamine in the GnRH migration, in fact GnRH cells strongly express lactosamine at E13.5 when the migration starts, and the expression decrease at E18.5 when the migration is ended. In addition, in mice lacking lactosamine

glycosyltransferase there is a significant reduction in the GnRH neurons migration and alteration in guidance axons (Bless et al. 2006).

All these findings indicate that the GnRH migration into the nasal compartment is affected by molecules that directly drive the migration and interaction with axons that guide neurons to their destination.

CRIBRIFORM PLATE/NASAL-FOREBRAIN JUCTIONS

From the nasal mesenchyme, GnRH neurons cross the cribriform plate (CP) to reach the forebrain. When they cross the CP most of them direct ventrally to the hypothalamus following the vomeronasal axons, while only a small number of cells follow axons projecting to the OB. It was proposed the presence of both factors to stimulate the migration through the hypothalamus and factors to induce a pause before entering the brain, probably because of maturation.

Histologic examination of human fetus with Kallmann syndrome (KS), which combine congenital GnRH deficiency and olfactory defects (see also Chapter3) harboring a mutation in *KAL1/ANOS1* gene reveal an arrest of GnRH neurons migration just before the CP. Anos-1 is the gene product of *KAL1/ANOS1*. Anos-1 is an extracellular matrix protein involved in the last steps of the olfactory axon guidance to the OB, promoting the axonal branching of OB neurons. In fact, mutations in *KAL1/ANOS1* are also responsible for OB hypoplasia. A major limitation in exploiting the role of *KAL1/ANOS1* in the GnRH neurons migration is the lack of a mice model because the *KAL1/ANOS1* murine homologue is still undetermined. There are evidences that neurotransmitter γ-aminobutyric acid (GABA) and neuropeptide Cholecystokinin (CCK) have a role in the crossing of the CP (Fueshko, Key, and Wray 1998; Giacobini et al. 2004). CCK is expressed in olfactory receptor cells and in fibers guiding the GnRH neurons migration; differently, CCK-1R are expressed on GnRH neurons and this receptor seems important in determining the correct timing for the GnRH crossing of the CP

(Giacobini et al. 2004). In addition, a subpopulation of GnRH cells (about 30%), olfactory axons and migrating cells express the neurotransmitters GABA and its receptor GABAAR has been found in the olfactory axons. Inhibition of its receptor using an agonist block the cell migration and GnRH projection so the GABA signaling tend to orientate the migration (Casoni et al. 2012).

Another important factor that regulates the caudal turning of the vomeronasal nerve to reach hypothalamus is netrin-1. As expected, the netrin-1 receptor (DCC) null mice reveal a failure in the caudal turn of branch and misrouted GnRH neurons to the cortex (Schwarting et al. 2001). Besides, it has been proposed that Reeling, an extracellular matrix protein involved in the lamination of cerebral and cerebellar cortex (Tissir and Goffinet 2003), could be involved in this process considering its expression in the olfactory system. *In vivo* and *in vitro* experiments reveal that it has a repulsive effect on the GnRH neurons migration, stimulating their migration caudally to the hypothalamus.

In conclusion, in this step of GnRH neurons migration, the caudal turning of the vomeronasal branch alone is not enough to ensure their correct migration, in fact, the presence of repulsive protein like Reelin prevent the invasion of the OB and cerebral cortex by GnRH neurons.

WITHIN THE FOREBRAIN

Once in the forebrain GnRH neurons detach from their peripherin-positive vomeronasal axonal guide and extend their projection to the median eminence. Recently, it has been proposed that the FGFR1 signaling is involved in the GnRH neurons projection to the median eminence, in fact the dominant negative *Fgfr1* mice display a reduction of the 30% in GnRH fibers in the forebrain with a significant reduction of projections toward the median eminence (Gill and Tsai 2006). In addition, Anos-1 enhances the FGFR1 signaling, hence, it has been proposed that Anos-1 finally regulates the FGFR1 signaling during the olfactory and GnRH neurons development (Cariboni, Maggi, and Parnavelas 2007).

The relevance of all these molecules has been confirmed from several null mice models that show an impaired GnRH neurons migration and fertility. The following image (Fig. 9) recapitulate all molecules described in this paragraph and the step promoted.

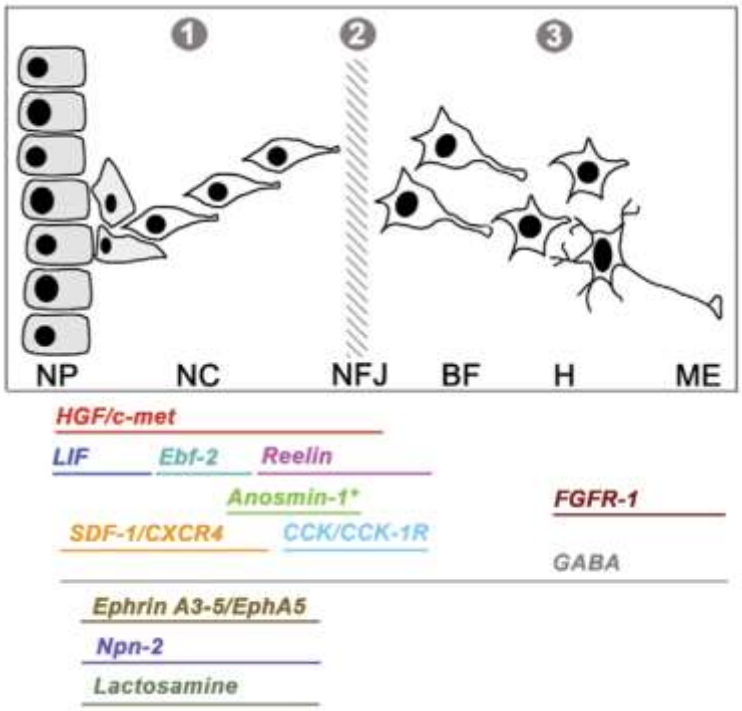

Fig. 9. *Factors that affect the migration of gonadotropin-releasing hormone (GnRH) neurons through the three compartments. Cartoon illustrating the movement of GnRH neurons from their origin in the nasal placode (NP), through the nasal compartment (NC), and their deflection at the level of the nasal-forebrain junction (NFJ) as they progress toward the basal forebrain (BF). Their migration finally terminates in the hypothalamus (Hy) from where they project to the median eminence (ME). Included is a list of factors that have recently been shown to affect GnRH neurons at different stages of their journey. Anosmin-1 is the only human factor included in this list (*) (Cariboni, Maggi, and Parnavelas 2007).*

Introduction

CHAPTER 2

NOTCH SIGNALING

The Notch pathway is the most well-conserved signaling pathway in animals and it acts during diverse developmental and physiological processes through juxtacrine cell-to-cell communication to coordinate development (Artavanis-Tsakonas, Rand, and Lake 1999). Mammals have four Notch receptors (Notch1-4) and many ligands, including JAGGED1 (JAG1) and JAGGED2 (JAG2) (homologues of serrate), and delta-like (DLL) proteins (Kopan and Ilagan 2009; D'Souza, Meloty-Kapella, and Weinmaster 2010). Notch and its ligands are single-pass transmembrane, hence, their binding create heterodimers (Fig. 10)

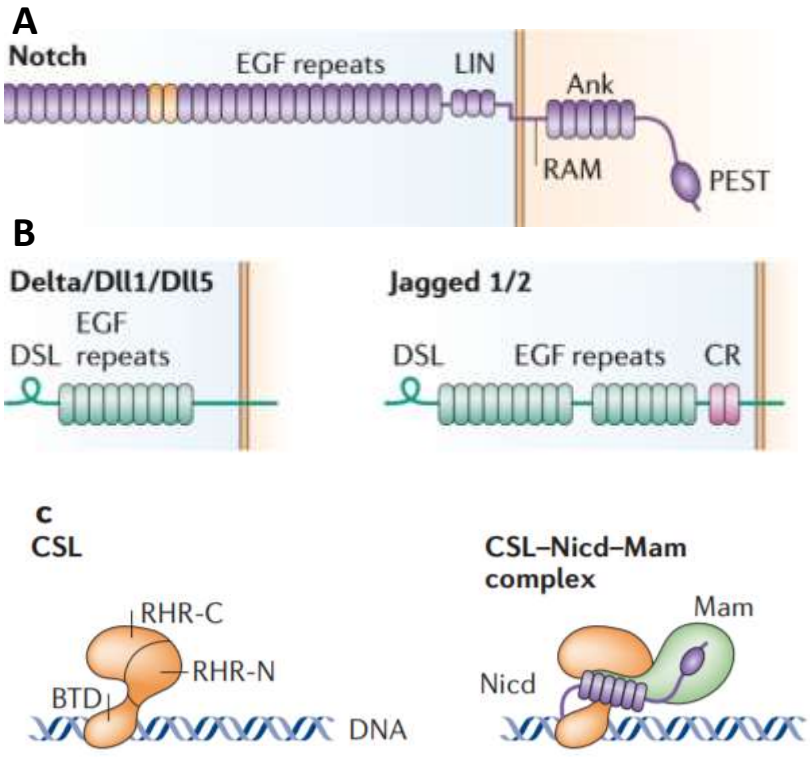

Fig. 10. *A. The mature Notch receptor with EGF-repeats 11 and 12 (orange) are essential for ligand binding. The intracellular portion consists of a RAM domain, six ankyrin (Ank) repeats and a C-terminal PEST domain. It also contains nuclear localization signals. B. Notch ligands are transmembrane proteins that are characterized by an N-terminal DSL (Delta, Serrate and LAG-2) domain that is essential for interactions with the Notch receptor. The extracellular domains of the ligands contain varying numbers of epidermal growth factor (EGF)repeats. The ligands are subdivided into two classes, Delta or Delta-like (Dll) and Serrate (Jagged in mammals), depending on the presence or absence of a cysteine rich (CR) domain (Bray 2006).*

The major players in the Notch signaling are Notch receptors (Notch1, Notch2 Notch3 and Notch4) (Fig. 10A), DLS ligands (Delta, Dll1, Dll2, Jagged1 and Jagged2) (Fig. 10B), and Nuclear factors (CSL DNA binding proteins) (Fig. 10C):

- Notch receptors also contain lots of domains that keep the receptor in an inactivated state in the absence of a ligand (Kopan and Ilagan 2009; Kovall and Blacklow 2010). The binding with the ligand results in a double cleavage from the metalloprotease ADAM10 and γ-secretase-mediated cleavage of the transmembrane domain of Notch, and releases of the Notch intracellular domain (NICD) into the cytoplasm. Ligands are also subject to γ-secretase-mediated cleavage, but the function of ligand cleavage is not completely understood (Yamamoto, Charng, and Bellen 2010). In the canonical Notch signaling pathway, NICD moves into the nucleus and, with mastermind-like protein 1 (MAML1), MAML2 or MAML3, converts the recombining binding protein suppressor of hairless (RBPJ) complex from a transcriptional inhibitor to a transcriptional activator (Fig. 11). The most studied Notch targets are the hairy and enhancer of split related (HESR) genes. NICD can also signal through a non-canonical pathway, presumably through protein–protein interactions and RBPJ-independent gene activation (Sanalkumar, Dhanesh, and James 2010).

- Notch ligands are transmembrane proteins with a huge number of epidermal growth factor (EGF)-Repeats in the N-terminus (extracellular region). The extracellular domain (C-terminus) of both Notch and its ligands is composed by multiple epidermal growth factor (EGF) repeats, DSL domain and cysteine rich domain in Jagged 1 and 2 ligands.

- The DNA binding protein CSL is similar to the Rel family of transcription factors. Excepted for the insertion of a central modified β-trefoil domain (BTD) between the two Rel-homology regions (RHR-N, RHR-C). RHR-N and BTD domains have a key

role in the DNA binding, additionally, the BTD domain contains a hydrophobic pocket that is thought to mediate the interaction with the Notch intracellular domain (NICD). To activate transcription only the DNA biding is not sufficient, in fact, to activate transcription is required the co-activator Mastermind (Mam). Mam proteins have an N-terminal region that forms an extended α-helical domain that contacts the RHR-N and RHR-C domains of CSL and the Ank domain of NICD in a trimeric complex

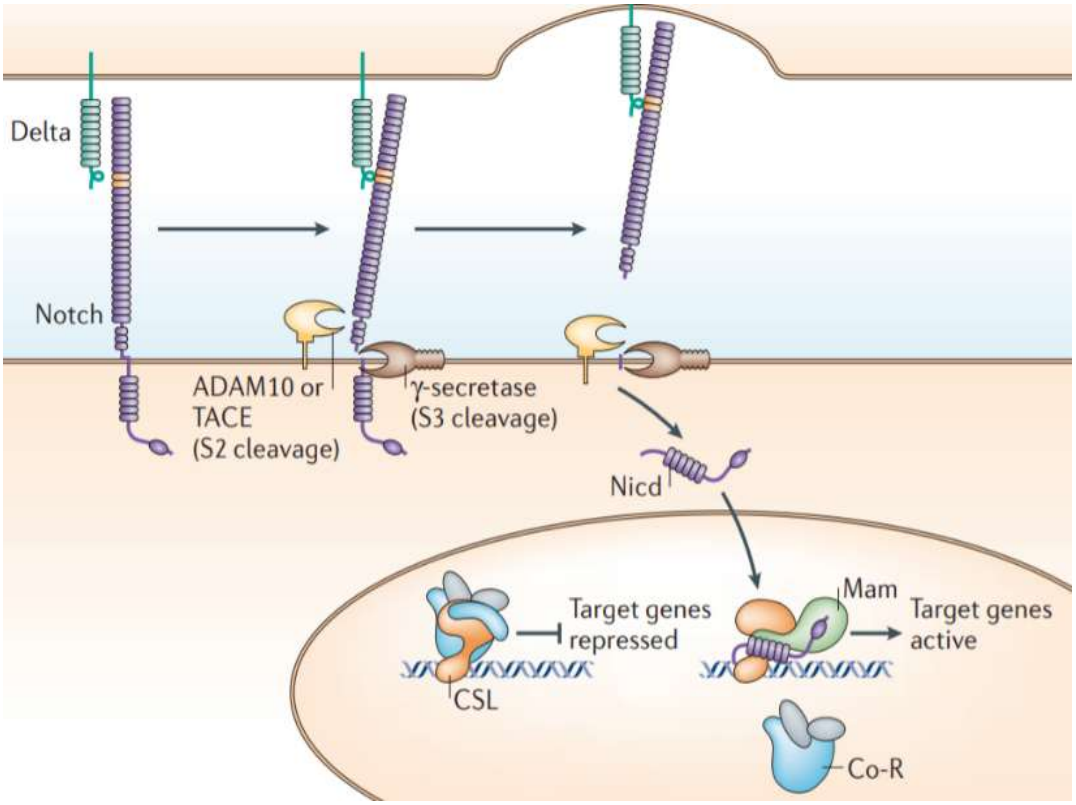

Fig. 11. *The Notch pathway. interaction of the Delta ligand (green) on one cell to the Notch receptor (purple) on another cell results in two proteolytic cleavages of the receptor made by the ADAM10 or TACE (TNF-α-converting enzyme; also known as ADAM17) metalloprotease (yellow) that catalyzes the S2 cleavage and generating a substrate for S3 cleavage by the γ-secretase complex (brown). This proteolytic cleavage mediates the releasing of the Notch intracellular domain (NICD) in the cytoplasm, which moves into the nucleus and bind with the DNA-binding CSL protein (orange). Co-activator Mastermind (Mam; green) and other transcription factors are recruited to the CSL complex, whereas co-repressors (Co-R; blue and grey) are released (Bray 2006).*

Additionally, the Notch signaling has three different modes of action: lateral inhibition, binary cell fate and lateral induction. In lateral inhibition, signaling between Notch ligand and Notch receptor on an adjacent cell inhibits ligand production in the receiving cell through a negative feedback loop (Bray 1998) (Fig. 12).

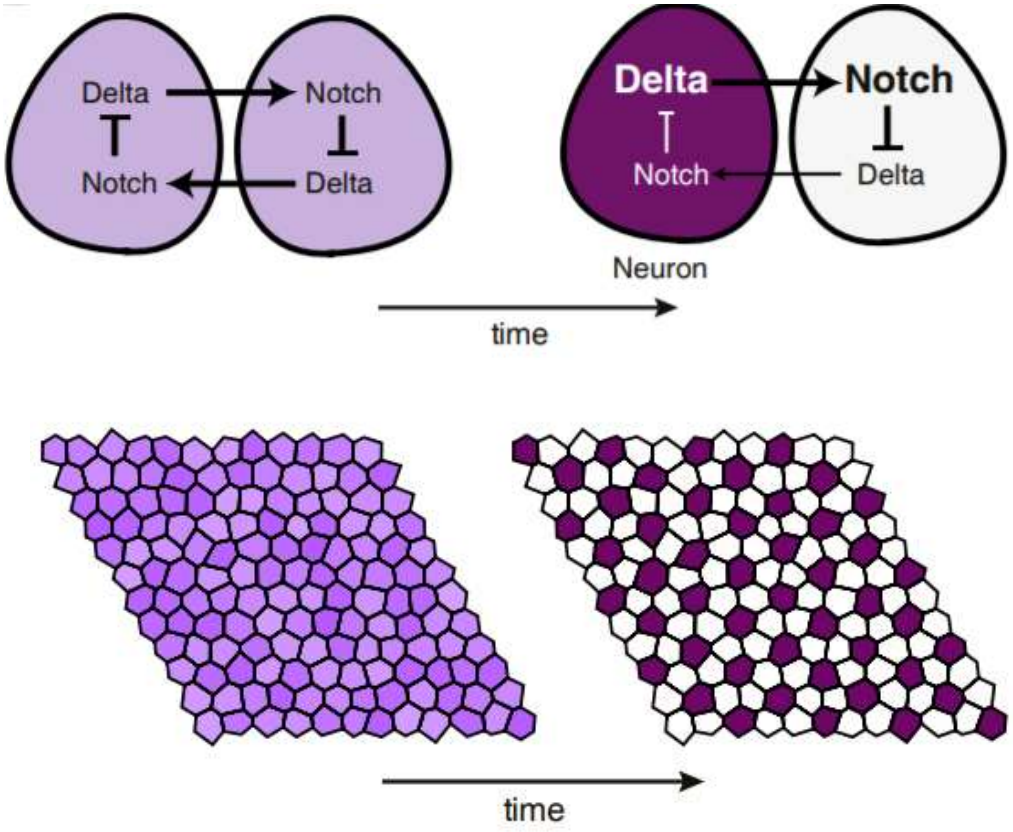

Fig. 12. *Schematic representation of lateral inhibition (Formosa-Jordan et al. 2013).*

During binary cell fate decisions, in contrast, distinct cell fates are determined by asymmetric distribution of Notch pathway components, such as the cytoplasmic Notch inhibitor Numb, resulting in different cell fate between neighbor cells (Saravanamuthu, Gao, and Zelenka 2009). Finally, in lateral induction, which is the least well described mode of Notch action, signaling between Notch ligand and Notch receptor on adjacent cells results in a positive feedback, which promotes ligand expression and activation of Notch on both cells. This

mechanism has been suggested to transmit Notch signals through a cell-to-cell relay mechanism (Ross and Kadesch 2004) (Fig. 13). These various modes of signaling allow Notch to perform different functions within the same tissue in a spatially and temporally regulated manner.

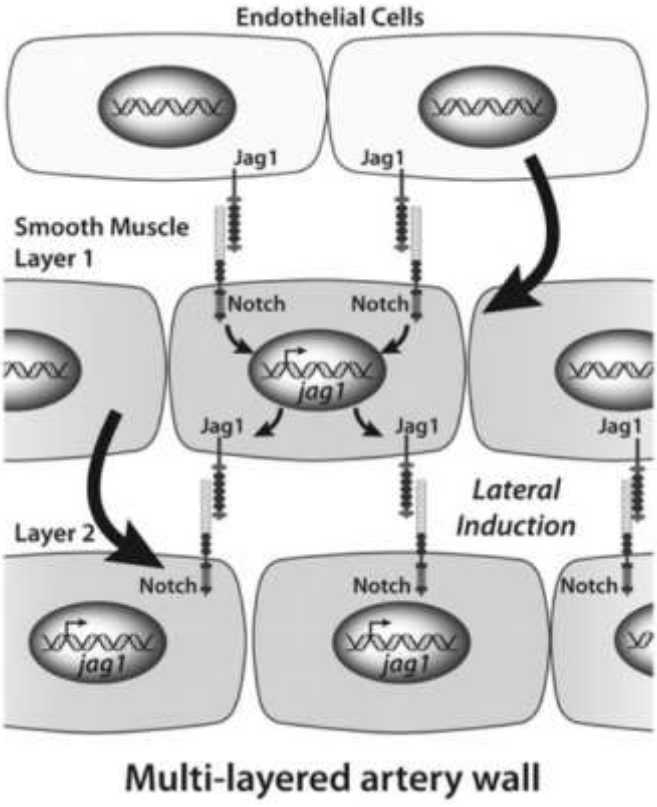

Fig. 13. *Model of later induction mediated by Notch signaling in the artery wall formation (Hoglund Virginia J. and Majesky Mark W. 2012).*

NOTCH SIGNALING IN NEURONAL MIGRATION

The neuronal migration is fundamental in the development of the brain and it was reported in several studies that the Notch signaling has a role in this migratory process. For example, Hashimoto-Torii and colleagues demonstrate that Notch activity plays a crucial role in the Reelin-signal-dependent neuronal migration. Reelin signal displays an important role in the cerebral cortex development, ensuring its correct lamination inside-out, with six layers distribution of neurons (Bar, Lambert de Rouvroit, and Goffinet 2000). Additionally, it has a repulsive effect on the GnRH neurons migration, stimulating their migration caudally, to the hypothalamus, preventing the invasion of the OB and cerebral cortex by GnRH neurons.

The activation of the Reelin receptor induces the release of the intracellular Dab1 that has an active role in preventing the NICD degradation FBW7-mediated (Suzuki et al. 2008). Increased or decreased degradation of NICD via proteasome corresponds to increased or decreased migration of post-mitotic cell in the developing cortex. In addition, alteration in the NICD levels result in an alteration of the shape of neurons (Suzuki et al. 2008), underlying the role of Notch Signaling in the neuronal migration for the correct lamination in the developing cerebral cortex. Another example is reported by Silbermann and colleagues, that elucidate the role of the Aspartyl-(Asparainyl)-β-hydroxylase (AAH) in Notch signaling regulation, and, by consequence, the effect on neuronal migration. AAH catalyzes the post-translational hydroxylation of aspartate and asparagine residues on EGF-like domain of protein such as Notch and Jagged (Monkovic et al. 1992; Dinchuk et al. 2000; 2002). Overexpression of AAH results in an enhanced translocation and accumulation NICD into the nucleus with consequent activation of downstream target genes like Hairly and Enhancer of Split 1 (HES1) resulting in an increased neuronal motility. In the same way, inhibition of AAH decrease the NICD nuclear translocation and signaling, reducing neuronal motility. Interestingly, AAH

induced-neuronal migration via Notch signaling is only able to affect the motility of neurons, without interfering with their ability to create synapsis (Silbermann et al. 2010).

NOTCH SIGNALING IN THE OLFACTORY SYSTEM

Until now, authors highlight an indirect involvement of the Notch signaling in GnRH neurons migration regulating the development of OECs, required for the correct GnRH neurons migration, or by the repulsive effect of Reeling in the targeting of GnRH neurons toward hypothalamus. Miller and colleagues demonstrated in 2016 that Notch-1 is necessary for the maturation of OECs, that ensheath bundles of unmyelinated olfactory axons from their peripheral origin in the olfactory epithelium to their target in the OB (Ekberg et al. 2012). Specifically, they demonstrate that Notch-1 is expressed in the olfactory axons, while Jag1 in expressed in sustentacular cells and olfactory receptor neurons in the OE in chicken and mouse embryos, supporting the idea that Jag1 is required on the olfactory epithelium to activate the Notch signaling in neighbor cells in the olfactory nerve during olfactory system development (Miller et al. 2016). The same author also demonstrated the role of Notch1 in the OECs maturation process from Mpz-positive OECs precursor to Sox10-positive immature OECs (more mature stage) regulating the migration of GnRH neurons (Miller et al. 2018). In fact, previously they demonstrate that Notch1 activates the expression of Sox10 inducing OECs maturation and, by consequence, promoting the GnRH neurons migration. Subsequently, they reported that mutation in Sox10 is found in about 30% of Kallmann syndrome patient, underling an effect of Sox10, Notch1/Rbpj-activated, in the migration of GnRH neurons hence in the etiopathology of Congenital Hypogonadotropic Hypogonadism (CHH) or KS. Specifically, Rbpj deletion in OECs using Mpz-Cre Driver line revealed no effect in the percentage of GnRH neurons entering in the forebrain, but lead a five-fold

increase in percentage of GnRH neurons located in the lateral region of the olfactory nerve and OB, rather than in the medial region of olfactory nerve and OB. This mis-localization of the GnRH neurons in the olfactory nerve and OB induces alteration in the olfactory sense and fertility.

Introduction

CHAPTER 3

CONGENITAL HYPOGONADOTROPIC HYPOGONADISM

Congenital hypogonadotropic hypogonadism (CHH) is caused by deficient production, secretion or action of gonadotropin-releasing hormone (GnRH) (Bianco and Kaiser 2009, Bohem et al 2015). The first reported case was in 1849 when Maestre de San Juan first documented the association of hypogonadism and absence of the olfactory system during the autopsy of a 40-yr-old man (Maestre de San Juan A 1856 Teratologia: Falta total de los nervios olfactorios con anosmia en un individuo en quien existia una atrofia conge´nita de los testículos y miembro viril. Siglo Medico 3:211–221). Approximately a century after Franz Josef Kallmann discovered the hereditary nature of this disease, Naftolin ascribed hypogonadism to a gonadotropin-releasing hormone (GnRH) deficiency (Naftolin, Harris, and Bobrow 1971). Thus, the association of CHH with olfactory defect is now named Kallmann syndrome (KS), while the form with a normal sense of smell normosmic CHH (nCHH or CHH).

Although widely considered to be rare diseases, ascertainment of CHH and KS true prevalence is influenced by the limited published literature. A French historic study on men called up for military service gave a male CHH prevalence of 1 in 4415 (Fromantin et al. 1973), but more recently, another retrospective study of hospital records in Finland gave a lower prevalence (Laitinen et al. 2011).

Historically CHH has been considered to have a male predominance (male-to-female ratio of 5:1) (Waldstreicher et al. 1996; Quinton et al. 2001), although male predominance may only reflect ascertainment bias, due to a greater likelihood of females being managed empirically as a functional hypothalamic amenorrhea by office- or community-based gynecologists or primary care physicians (Shaw et al. 2011; Hietamaki et al. 2017; Cangiano et al, 2020). Indeed, further analysis suggest that the real sex ratio is closer to 3:1 (Tang et al. 2017; Bonomi et al. 2018), even if more recent works demonstrate a sex ratio for CHH in families

with autosomal inheritance close to being equal between males and females (Francou et al. 2016; Maione et al. 2018).

The phenotype for CHH in adult males includes the absence of normal sex steroid levels that is evidenced by lack of virilization, i.e., poor muscle development, gynoid habitus, sparse body hair, high-pitched voice and undeveloped genitalia. Differently, in adult females, there are little to no secondary sexual characteristics, i.e., breast development, pubic hair and absent menses (primary amenorrhea).

Additionally, clinical presentation may be accompanied by a variety of highly variable non-reproductive phenotypes such as cleft lip or palate, dental agenesis, ear anomalies, congenital hearing impairment, renal agenesis/dysgenesis, bimanual synkinesis or skeletal anomalies (Costa-Barbosa et al. 2013; Boehm et al. 2015; Young et al 2019; Bonomi et al. 2018).

Phenotypes previously described can occur with highly variable rates, thus, patients can present a spectrum ranging from relatively milder forms (e.g., CHH and partial puberty) to more severe syndromic forms of CHH (e.g., KS with complete absence of puberty, unilateral renal agenesis and cleft lip/palate) (Bonomi et al, 2014).

In terms of basal hormone profile, CHH patients present low or undetectable concentrations of circulating sex steroids and low/normal LH and FSH serum levels, because of the impaired functionality of HPG axis due to the GnRH deficiency (see also Chapter 1).

Pathogenesis of CHH is so far still largely unknown although data from animal models and familial pedigrees in humans demonstrated a clear and strong genetic background (Cangiano et al, 2020). Since the discovery of *ANOS1* (formerly *KAL1*) in the pathogenesis of X-linked KS and thanking to the next generation sequencing techniques nowadays available, more genes have been evaluated in relation to CHH (Cangiano B et al, 2020)

GENETIC OF CONGENITAL HYPOGONADOTROPIC HYPOGONADISM

CAUSATIVE GENES

KS was originally described as caused by mutations in a specific X-chromosome gene, *KAL1* (Franco et al. 1991), that leads to a defected targeting of the olfactory axons and consequent alteration in the GnRH neurons migration. Nonetheless, in those years, it was possible to clearly identify a genetic cause only in a minority of CHH patients, thus in most cases their pathology was classified as "idiopathic". Moreover, the observation of familial cases soon indicated that CHH retains a highly heterogeneous genetic identity since not only the X-linked mode of inheritance was possible, but also both the autosomal dominant and autosomal recessive. Hence, the application of conventional linkage studies to investigate the genetic basis cannot correctly elucidate the basis of the inheritance in the CHH. In addition, most pedigrees tend to be of small size, considering that most patients remain infertile in the absence of therapeutic treatments.

However, in the last ten years, the knowledge on the pathogenesis of CHH has been profoundly deepened thanks to the use of animal and cellular models and the application of modern techniques of genetic investigation that brought evidence of previously unknown genetic determinants of CHH (both nCHH or KS).

These new findings played a significant role in disclosing the physiological complexities of the HPG axis, elucidating the physiopathology of CHH. Some genes are determinant for the proper embryonic development of the GnRH-secreting neurons or for encoding the signals essential for the correct migration of the GnRH neurons from their embryonic origin to the hypothalamus: the ANOS1/KAL1; the receptor-ligand pair fibroblast growth factor receptor 1 (Dode et al. 2003; Pitteloud et al. 2006) and fibroblast growth factor 8 (FGFR1/FGF8) (Falardeau et al. 2008; Trarbach et al. 2010); the nasal embryonic LH releasing hormone factor (NELF) (Xu, Kim et al. 2011); the heparan sulfate 6-O-sulfotransferase1 (HS6HST1)

(Tornberg et al. 2011); Anti-Mullerian hormone and its receptor (AMH/AMHR2) (Malone et al. 2019); the ligand–receptor complex prokineticin 2 and its receptor (PROK2/PROKR2) (Dode et al. 2006; Libri et al. 2014); the chromodomain helicase DNA binding protein 7 (CHD7) (Ogata et al. 2006; Balasubramanian et al. 2014); the class 3 and 7 semaphorins SEMA3A (Hanchate et al. 2012; Kansakoski et al. 2014), SEMA3E (Cariboni et al. 2015) and SEMA7A (Kansakoski et al. 2014); the Sry-related HMG box factor 10 (SOX10) (Pingault et al. 2013; Vaaralahti et al. 2014); the WD repeat-containing protein 11 (WDR11) (Kim et al. 2010; Quaynor et al. 2011), the fibroblast growth factors 17 (FGF17) (Miraoui et al. 2013), the interleukin-17 receptor D (IL17RD) (Oakley, Clifton, and Steiner 2009), the protein sprouty homolog 4 (SPRY4) (Xu, Kim et al. 2011), the dual specificity phosphatase 6 (DUSP6) (Oakley, Clifton, and Steiner 2009), the FEZ family zinc finger 1 (FEZF1) (Kotan et al. 2014) and Neuron-derived neurotrophic Factor (NELF) (Messina et al., 2020).

Other genes encode the elements of upstream signals for the activation of GnRH neurons, such as the two ligand–receptor couples formed either by the tachykinin 3 and its receptor also named neurokinin B, NKB, and/or neurokinin 3 receptor, NK3 (TAC3/TACR3) (Topaloglu et al. 2009; Gianetti et al. 2010) or the kisspeptin1 and its receptor, previously known as GPR54 (KISS1/KISS1R) (de Roux et al. 2003; Seminara et al. 2003).

Finally, candidate genes for CHH also include the GnRH gene itself (GnRH1) (Bouligand et al. 2009) and its receptor (GNRHR) (de Roux et al. 1997). From KAL1 in 1991 a growing number of genes have been demonstrated as involved in the pathogenesis of CHH, either in isolated or syndromic forms (Cangiano et al, 2020). Nonetheless, not all genes have an equal frequency of involvement in the pathogenesis of CHH, being some of them more commonly and importantly involved. Here, it was reported the list of all genes involved in the etiopathology of CHH and KS (Fig. 14).

More attention is given to the causative gene for CHH and KS, that are reported in this thesis given more explanations about their role in the development of olfactory/GnRH system and etiopathology for CHH or KS.

Table 1 | Genes implicated in CHH

Gene	OMIM	CTO	CHH phenotypes			Overlapping syndromes									
			KS	CHH	CHH reversal	CPHD	CPHD + SOD	WS	CHARGE	HS	SHFM	D-WS	MGS	PEPNS	GHS
KAL1 (ANOS1)	300836	✓	✓	×	✓	×	×	×	×	×	×	×	×	×	×
SEMA3A	614897	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×
SOX10	602229	×	✓	×	×	×	×	✓	×	×	×	×	×	×	×
OL14RD	606807	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×
HESX1	182230	×	✓	×	×	✓	✓	×	×	×	×	×	×	×	×
FEZF1	613301	×	✓	×	×	×	×	×	×	×	×	×	×	×	×
FGFR1	147950	✓	✓	✓	✓	✓	✓	×	×	✓	✓	×	×	×	×
FGF8	612702	✓	✓	✓	×	✓	×	×	×	×	×	×	×	×	×
CHD7	612370	×	✓	✓	✓	×	×	×	✓	×	×	×	×	×	×
FGF17	603725	✓	✓	✓	×	×	×	×	×	×	×	✓	×	×	×
HS6ST1	614880	✓	✓	✓	✓	×	×	×	×	×	×	×	×	×	×
PROK2	610628	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
PROKR2	147950	✓	✓	✓	✓	✓	×	×	×	×	×	×	✓	×	×
SEMA7A	607961	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
WDR11	614858	✓	✓	✓	×	✓	×	×	×	×	×	×	×	×	×
NSMF	614838	✓	✓	✓	✓	×	×	×	×	×	×	×	×	×	×
AXL	109135	×	✓	✓	×	×	×	×	×	×	×	×	×	×	×
GNRH1	614841	×	×	✓	×	×	×	×	×	×	×	×	×	×	×
GNRHR	146110	✓	×	✓	✓	×	×	×	×	×	×	×	×	×	×
KISS1	614842	×	×	✓	×	×	×	×	×	×	×	×	×	×	×
KISS1R	614837	✓	×	✓	×	×	×	×	×	×	×	×	×	×	×
TAC3	614839	✓	×	✓	✓	×	×	×	×	×	×	×	×	×	×
TACR3	614840	✓	×	✓	✓	×	×	×	×	×	×	×	×	×	×
LEP	614962	×	×	✓	×	×	×	×	×	×	×	×	×	×	×
LEPR	614963	×	×	✓	×	×	×	×	×	×	×	×	×	×	×
PCSK1	162150	×	×	✓	×	×	×	×	×	×	×	×	×	×	×
DMXL2	616113	×	×	✓	×	×	×	×	×	×	×	×	×	✓	×
RNF216	609948	×	×	✓	×	×	×	×	×	×	×	×	×	×	✓
OTUD4	611744	×	×	✓	×	×	×	×	×	×	×	×	×	×	✓
PNPLA6	603197	×	×	✓	×	×	×	×	×	×	×	×	×	×	✓
NR0B1	300200	×	×	✓	×	×	×	×	×	×	×	×	×	×	×

Abbreviations: CHH, congenital hypogonadotropic hypogonadism; CHARGE, coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital and/or urinary defects, ear anomalies or deafness; CPHD, combined pituitary hormone deficiency; CTO, contributes to oligogenicity; D-WS, Dandy-Walker syndrome; GHS, Gordon Holmes syndrome; HS, Hartsfield syndrome; KS, Kallmann syndrome; MGS, Morning Glory syndrome; OMIN, online Mendelian inheritance in man; PEPNS, polyendocrine deficiencies and polyneuropathies; SHFM, split-hand/foot malformation; SOD, septo-optic dysplasia; WS, Waardenburg syndrome.

Fig. 14. *List of causative genes for CHH/KS and possible association with other syndromes (Boehm et al. 2015).*

Causative genes for KS:

- *KAL1* or *ANOS1* was the first gene linked to Kallmann syndrome pathogenesis (Franco et al. 1991; Legouis et al. 1991). *ANOS1* encodes anosmin-1, a protein with an important role in the embryogenesis of brain, but also kidneys, respiratory and digestive systems (Tsai and Gill 2006). This extracellular matrix protein binds the cell membrane stimulating axonal outgrowth and acting as an axonal guidance molecule for the migration of GnRH neurons, olfactory cells and Purkinje cerebellum neurons (Soussi-Yanicostas et al. 2002) (Fig. 15).

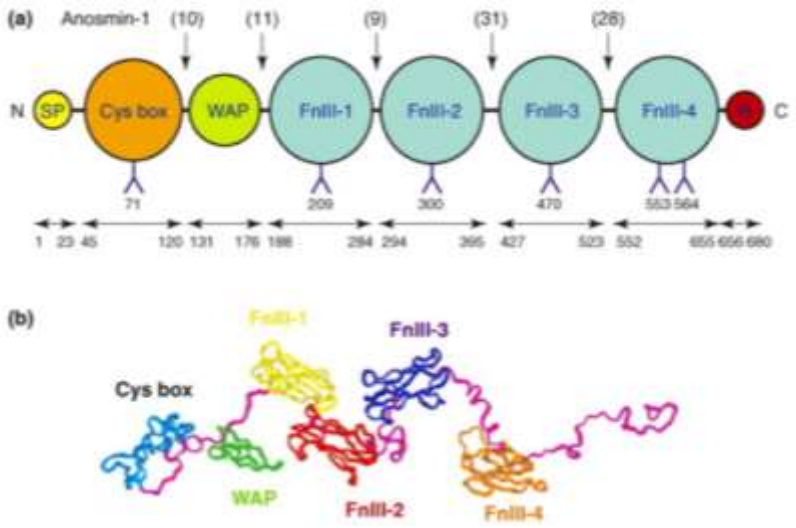

Fig. 15. *Anosmin-1 structure (from Hu Y. et al., Trends Endocrinol Metab. 2010).*

Anosmin-1 was detected in the olfactory system of human from week 5 onward restricted to the olfactory bulb assumed region and later to the primitive olfactory bulbs. Central insights into the pathology of X-linked KS came from the examination post-mortem male 19-weeks-old human fetus carrying a chromosomal deletion including the *KAL1* gene. In this study, researchers declared that olfactory,

vomeronasal and terminalis nerve fibers were not in contact with the brain neither were the GnRH neurons present in any portion of the brain. Further, they observed an abnormal accumulation of both nerve fibers and GnRH neurons in the upper nasal area, where GnRH neurons normally enter in the central nervous system (Schwanzel-Fukuda, Bick, and Pfaff 1989) (Fig. 16).

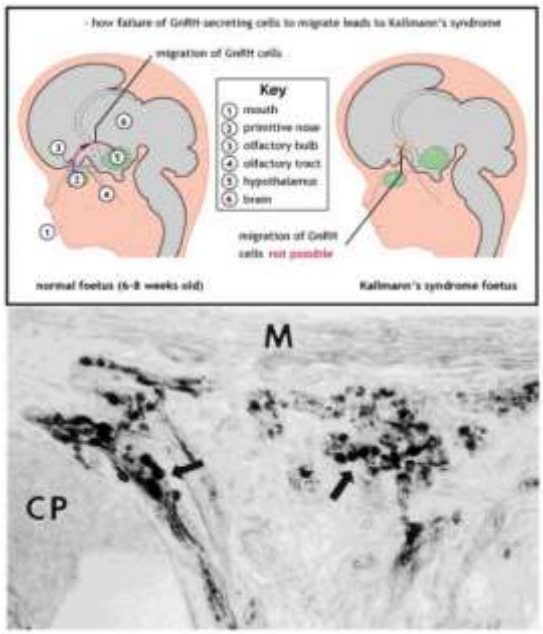

Fig. 16. *GnRH3 migration failure in a male fetus mutated for KAL1; CP= cribiform plate; M= meninges (Schwanzel-Fukuda, Bick, and Pfaff 1989).*

These results indicated that the causative defect primarily impairs the migration of early olfactory axons, which takes place from the beginning of week 5 (Bossy 1980). As consequence of the failed contact between the olfactory axons and the forebrain during weeks the olfactory bulb differentiation results abnormal (Gong and Shipley 1995).

Genetic studies on KS patients reported *KAL1* gene abnormalities including missense and nonsense mutations, splice site mutations, intragenic deletions and submicroscopic chromosomal deletions involving the entire *KAL1* gene (Bianco and

Kaiser 2009). The majority of patients harboring *KAL1* mutations present variable olfactory dysfunction and they often have a more complex phenotype (Trarbach, Silveira, and Latronico 2007) with a high incidence of renal agenesis and bimanual synkinesia defects that occurs in 30%–40% and 75% of the patients respectively (Dode et al. 2006). Analysis of familial cases of KS reveal that *KAL1* is mutated in almost the 60% of these patients, while only 10%–15% of males mutated for this gene present sporadic KS, suggesting an X-linked inheritance (Bianco and Kaiser 2009).

- *HS6ST1* gene is located on chromosome 2q21 and it is composed by only 2 exons, encoding for an enzyme made by 411 amino acids, whose function is to introduce a sulfate in the 6-O-position of the glucosamine sugar moiety within heparan sulfate (HS) (Fig. 17).

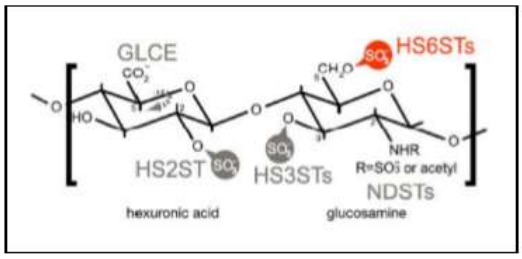

Fig. 17. *Chemical structure of HS. The red circle represents the sulfate residue introduced by HS6ST1 enzyme (Tornberg et al. 2011).*

HS polysaccharides are components of the extracellular matrix and they are important for neural development due to their control in cell-to-cell communications (Inatani et al. 2003), for the interaction and activation of the FGFR–FGF complex (Ibrahimi et al. 2004) and for the interaction between anosmin1 and the cell membrane (Gonzalez-Martinez et al. 2004). Bülow and coworkers, in using C. elegans model, demonstrated

that overexpression of human KAL1 orthologue in specific worm interneurons induces axonal-branching alterations, and that this *kal1* gain-of-function phenotype is extinguished by HS 6-O-sulfotransferase gene (Bulow et al. 2002) suggesting the role of *HS6ST1* in the pathogenesis of CHH/KS. Tornberg and colleagues support this hypothesis showing that all reported mutation in the coding exons and flanking splice sites of the gene *HS6ST1* in a cohort of 338 GnRH-deficient patients affect residues that are highly conserved in *HS6ST1*. In addition, they demonstrated that *HS6ST1* mutations recognized in patients with CHH reduce the enzyme activity *in vitro* and in *in vivo* (Tornberg et al. 2011). Clinically, patients carrying *HS6ST1* mutations could give a wide spectrum of severity of GnRH deficiency; this variability is also tangible in families that share the same genetic alteration (Tornberg et al. 2011). Because *HS6ST1* mutation associated with CHH has not been reported in the homozygous state and segregates as a complex inheritance patterns, it was suggested that the identified *HS6ST1* missense mutations might not be enough to cause disease. However, *HS6ST1* remains an important gene for the neuroendocrine control of human reproduction.

- *PROK2 and PROKR2*: PROK2 and PROK2R are respectively ligand and receptors belonging to the family of prokineticin, that is composed by two ligands PROK1 and PROK2 with similar affinities for their two receptors, namely PROKR1 and PROKR2. Receptors operate as molecular switches to relay extracellular ligand-activation to intracellular heterotrimeric G-proteins and present a high conservation in their sequences (about 85%). Meanwhile, whilst PROK1 and its receptor PROKR1 expressions are mainly confined to gastrointestinal system where they promote gut motility, PROK2 (locus 3p21.1) and PROKR2 (locus 20p13) reveal a more specific neuroendocrine profile (Lin et al. 2002; Soga et al. 2002). Indeed, they are in the

arcuate nucleus, olfactory track and suprachiasmatic nucleus. PROKR2, as a member of the GPCR family, has an extracellular N-terminal, an intracellular C-terminal domain and a central core formed by seven transmembrane α-helical segments (TM1–TM7) (Soga et al. 2002) (Fig. 18).

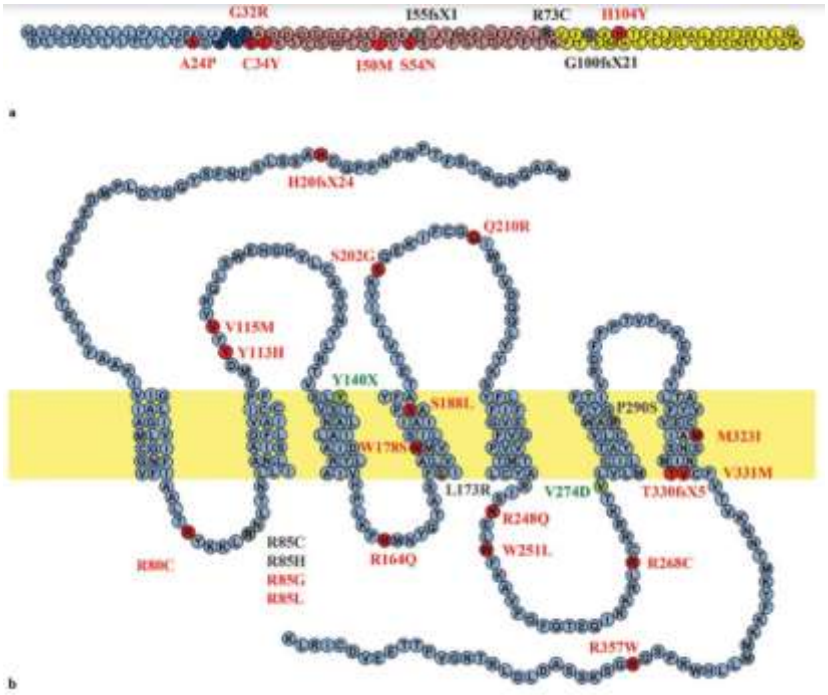

Fig. 18. *a. Peptide sequence of PROK2 (81 amino acids) with the signal peptide (27 amino acids, shown in light blue). Exon 1 includes the signal peptide and the amino acids shown in dark blue. Exon 2 is shown in pink and exon 4 in yellow. Exon 3 is not shown. No mutations have been identified in exon. b. Schematic representation of PROKR2(Abreu, Kaiser, and Latronico 2010).*

PROK2 gene encodes the protein Prokineticin 2, a peptide of 81 amino acids with pro-migratory activity that binds its cognate G protein-coupled receptor (GPCR) PROKR2. Their molecular interaction activates the PROKR2 downstream signal cascade via Gq and Gs (Chen et al. 2005). Prokineticins were demonstrated to be

involved in several physiological functions in neurogenesis, regulation of circadian rhythms, metabolism, angiogenesis, muscle contractility, pain perception, immune response, hematopoiesis, thermoregulation, and energy expenditure (Negri et al. 2007; Zhou et al. 2012). Alteration of this system are associated with several pathological conditions, including cancer (Shojaei et al. 2007; 2008), immunological response (J. Monnier et al. 2008), mood disorder (anxiety/depression) (Li, Hu, and Zhou 2009), and cardiomyopathy (Attramadal 2009). Analysis of knockout mice models for both ligand and receptor revealed the role of PROK2 signaling in olfactory bulb (OB) morphogenesis and sexual maturation, suggesting PROK2 and PROKR2 as strong candidates for human GnRH deficiency (Matsumoto S, et al. 2006). Indeed, the homozygous Prokr2 null mouse model results in KS phenotype, while the heterozygous mice do not show any important reproductive abnormality. The involvement of the PROKR2/PROK2 pathway in the pathogenesis of the CHH was first described in 2006 (Dode et al. 2006) and subsequently reported in several other patients' series (Martin et al. 2011; Bonomi et al. 2012). Despite the absence of a crystal structure, the knowledge about the prokineticin signaling pathway has been inferred through pharmacological and biochemical approaches, that demonstrate a variable impairment of their expression levels in heterologous cell system and a reduced activation of the Gq and Gs-dependent intracellular pathway (Cole et al. 2008; C. Monnier et al. 2009, 200; Libri et al. 2014). Genetic analysis of PROKR2 variants showed that most of them were found in heterozygous state, suggesting an autosomal dominant mode of inheritance due to either haploinsufficiency or a dominant negative effect. However, functional studies of selected PROKR2 mutants to date have failed to show a dominant-negative effect of these mutations (C. Monnier et al. 2009). Currently, oligogenic inheritance is the most plausible explanation for the phenotypes seen in patients with heterozygous mutations because interaction and

synergism between multiple genes causing GnRH deficiency has been demonstrated. Nevertheless, only the patients carrying homozygous mutations are displaying the most severe and penetrant clinical phenotypes (Dode et al. 2006; Sarfati, Dode, and Young 2010; Libri et al. 2014), while patients with heterozygous mutations are presenting a variable expressivity or incomplete penetrance of both the reproductive and olfactory phenotypes. Moreover, very recently, PROKR2 variants, but not PROK2, have been also described in patients with combined pituitary hormone deficits (Raivio et al. 2012; Reynaud et al. 2012). Altogether, these data suggest an extensive role of PROKR2 in the control of the entire neuroendocrine system. Notwithstanding knowledge about the precise molecular mechanisms of PROKR2 in the neuroendocrine axis remains largely unexplored, underlying that further investigations are necessary.

- *FGFR1* gene, also called *KAL2*, is a protein receptor that belong to the tyrosine kinase superfamily (Fig. 19).

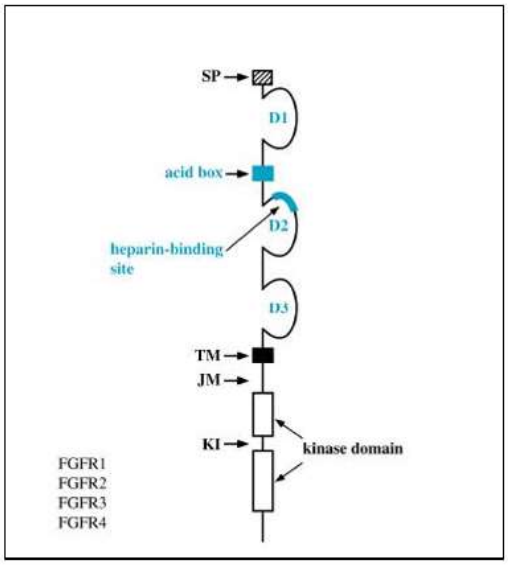

Fig. 19. *Scheme representing structural domains of FGFR1(Mohammadi et al. 1997).*

FGFR1 has been shown to play an essential role during embryogenesis, homeostasis, and wound healing. Fgf8 is expressed at the anterior end of the telencephalon. Using a CRE/loxP genetic approach to disrupt genes in the telencephalon of mice, morphological defects are observed at the anterior end of the telencephalon, most notably, the olfactory bulbs. Examination of the proliferation state of anterior telencephalic cells supports a model for olfactory bulb formation in which an FGF-dependent decrease in proliferation is required for initial bulb evagination. Moreover, $Fgfr1^{-/-}$ mice transfected with dominant negative FGFR1 mutations exhibited an early onset together with a 30% decrease of hypothalamic GnRH neurons (Hebert et al. 2003). In CHH dominant gain-of-function and loss-of-function mutations in FGFR1 sources different developmental disorders: premature fusion of skull bone sutures (craniosynostosis) and failed morphogenesis of the olfactory bulbs (Kallmann syndrome), respectively. Given the phenotype associated with gain-of-function mutation of FGFR1, it is remarkable that delayed closure of calvarial sutures has not been reported in KS. Heterozygous FGFR1 mutations are found in 10% of KS and in 6% of all CHH individuals (Bianco and Kaiser 2009). Pathogenic variants in FGFR1 include an high rate of different kind of mutations, like missense, nonsense, splice variants and in rare cases deletions and cause both KS and CHH with autosomal dominant mode of inheritance. This high rate of different mutations is linked to highly variable phenotypes, ranging from isolated hyposmia, delayed puberty to severe form of CHH with non-reproductive anomalies. The implication of FGFR1 and KAL1 in the same developmental disease underlines the possible functional interaction between these gene products, in fact, several arguments suggest that anosmin-1 is involved in FGF signaling through FGFR1. First, anosmin-1 binds to HSPGs (Soussi-Yanicostas et al. 1996), and HSPGs are important in the dimerization of the binary FGF–FGFR complex (Pellegrini 2001). Second, KAL1 and FGFR1 are co-expressed at different

sites during embryonic development. KAL1 is expressed in the presumptive olfactory bulbs (Hardelin et al. 1999), while FGFR1 expression in the rostral forebrain is required for initial olfactory bulb evagination in the mouse (Hebert et al. 2003).

- *FGF8:* FGF8 is a potent FGFR1 ligand. The FGF8 expression is observed in the diencephalon and prospective hypothalamus during embryonic development (McCabe et al. 2011). Specifically, Fgf8 is a key factor in the GnRH neurons specification and development of all olfactory system. Targeted transgenic mice lacking Fgf8 in the olfactory placode fail to develop GnRH neurons, underling its relevance in the GnRH neurons development. In humans, more than 1% of CHH cases has a mutation in Fgf8, with an apparently AD mode of inheritance. Other defects are usually associated with mutation in Fgf8 in CHH patients, like cleft lip or palate, but also holoprosencephaly, craniofacial defects and hypothalamic–pituitary dysfunction with a recessive inheritance (McCabe et al. 2011). Studies in mice revealed a reduction in 30–50% of total GnRH neurons in those animals with monogenic heterozygous Fgf8 mutations, whereas in mice with digenic Fgfr1/Fgf8 mutations the reduction in GnRH neurons numbers is more severe, suggesting a high rate of interaction between these factors in promoting tropic support for the emergence of GnRH neurons in the olfactory placode (Chung et al. 2010).

- *IL17RD:* IL17RD is a single transmembrane glycoprotein. Its sequence has a high rate of similarity with the intracellular domain of the interleukin-17 receptor. It works as antagonist of the FGF signaling pathway, at the level of the FGF receptors, including FGFR1 and FGFR2, but also at the level of downstream components of the Ras–ERK1/2 pathway by blocking active MEK and ERK complexes at the Golgi apparatus and inhibiting their dissociation. IL17RD expression is detected in the mice olfactory epithelium at E10.5, coinciding with the timeframe of GnRH neuronal fate

specification, which is dependent on FGF8, suggesting that it could has a role during the initial phase of GnRH neuron fate specification (Miraoui et al. 2013). Additionally, IL17RD appears to be important not only for GnRH neuron ontogeny but also for the correct development of the auditory system thanks to its ability to modulate (inhibit) FGF signaling. Phenotypic expression is likely only in the presence of biallelic IL17RD mutations or of oligogenicity, rather than from a single allelic defect (Miraoui et al. 2013).

- *SEMA3A:* SEMA3A encodes a class 3 (i.e., interacting with neuropilins as co-receptor for the ligand) semaphorin that is widely observed in the olfactory system as a secreted protein— that is important for guidance of vomeronasal (Cariboni et al. 2011) and olfactory (Pasterkamp et al. 1998) axons. Taking advantage from the *Sema3a* null mice, deletions, or missense mutations in the binding domain of NRP1 (encoding SEMA3A's co-receptor, Neuropilin-1), affect the migration of GnRH neuron and impair the development of the olfactory system (Hanchate et al. 2012). In CHH SEMA3A is found in missense or heterozygous state; at the beginning mutations in SEMA3A were considered with an autosomal dominant inheritance (Young et al. 2012; Hanchate et al. 2012). Today, the complex pattern of inheritance coupled with several evidence revealing associated variants in other CHH genes, the oligogenicity inheritance is easily accepted (Hanchate et al. 2012). Semaphorin-3A and neuropilin-1 activate the receptor Plexin-A1 that has a central role in neuronal development. Plexin-A1 is expressed in the olfactory system and the vomeronasal organ and nerve (Messina and Giacobini 2013). Experiments in mouse have shown that homozygous gene deletions induce a KS-like phenotype, albeit with incomplete penetrance (Marcos et al. 2017). In the last years, has been reported the synergistic action of another member of the PLXNA family. Thus, double *Plxna1* and *Plxna3* knockout mice

phenocopy the olfactory and GnRH neuron defects observed in *Sema3a* null mice (Oleari et al. 2019).

- *CHD7:* CHD7 encodes chromodomain helicase DNA-binding protein 7, which is widely expressed in fetal tissues, including the brain. CHD7 gene alterations are related with CHH and, most importantly, they are the leading cause for CHARGE syndrome. CHARGE syndrome is a rare developmental disorder with AD inheritances, characterized by iris coloboma, genital hypoplasia, choanal atresia, mental and growth retardation, congenital heart disease and ear malformations or deafness (Jongmans et al. 2006). CHH occurs in around 2/3 of patients carrying CHD7 mutations (Jongmans et al. 2006); on the other hand, only 6% of patients CHH (with or without anosmia) harbor CHD7 mutations (Kim et al. 2008). This is not surprising given the overlapping features of olfactory impairment and CHH observed in these two groups of patients (Jongmans et al. 2009). Therefore, while multisystem involvement and large de novo deletions characterize classical CHARGE syndrome, CHD7-associated CHH appears to result from inherited point mutations (Kim et al. 2008a; Balasubramanian et al. 2014).

- *WD11:* WD Repeat Domain 11 is a transcriptional factor hence it shuttles between nucleus and cytoplasm to activate transcription (Kim et al. 2010). WD11 is strongly expressed during the development of central nervous system (CNS), but its expression is remarkable in the region from which GnRH neurons will give rise (Kim et al. 2010). In later phases, WD11 is observed in olfactory structures, hippocampus, and cerebellum. In 2010, Kim and colleagues reported several missense heterozygous variants in WDR11. All these new variants occur in protein–protein interaction domain, and the clinical phenotype associate with both KS and CHH (Kim et al.

2010), alone or associated with mutations in other known genes (Kim and Layman 2011), indicating the chance of oligogenic or AD pattern of inheritance.

- *SOX10:* SOX10 is a transcription factor that has a role in the early development of neural crest cells, that are a population of multipotent precursor cells arising from the neural tube and that differentiate into several lineage of cells. Mutations in SOX10 are normally related with Waardenburg syndrome, that is a rare disorder characterized by sensorineural congenital hearing loss and altered pigmentation of the hair, eyes and skin (Pingault et al. 2010). Remarkably, loss-of-function mutations in SOX10 were additionally found in about 40% of KS patients in which the classical KS phenotype was associated with hearing defect (Pingault et al. 2013). SOX10 mutations were shown to affect olfactory ensheathing cells during early embryonic development of the peripheral olfactory system in mouse models, leading to developmental alteration in the olfactory neurons and migration of GnRH neurons, thus supporting the KS phenotype (Pingault et al. 2013).

Causative genes for CHH:

- *KISS1/KISS1R*: *KISS1* gene is located on chromosome 1q32 and encodes for a 145-amino acid kisspeptin 1. This precursor is cleaved in four products: kisspeptin 54 or metastatin the longest 54-amino acid peptide, and three minor products of 14, 13 and 10 residues called respectively kisspeptin 14, kisspeptin 13 and kisspeptin 10 (Fig. 20).

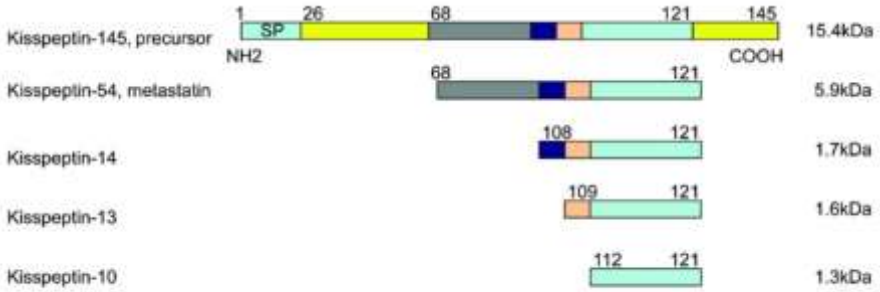

Fig. 20. *Relative size and cleavage points of Kisspeptin. Kisspeptin peptides of 145, 54, 14, 13, and 10 amino acids in length are resolved. SP: signal peptide within the Kp-145 precursor peptide Adapted from Fig.ure published by (Roseweir et al. 2009).*

All products showed the same affinity for receptor KISS1R; this leads to the hypothesis that is the C-terminal portion of the peptide the important region for binding and subsequent activation of the receptor. *KISS1R*, previously known as *GPR54*, was mapped to chromosome 19p13.3. Translation of its sequence generates a 398 amino acids G protein-coupled receptor of 75KDa (Kotani et al. 2001) (Fig. 21).

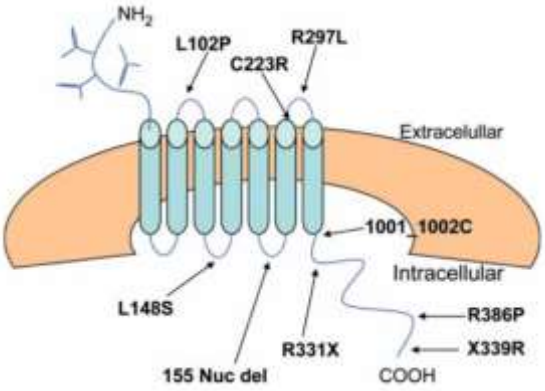

Fig. 21. *Structure of the GPR54 showing the location of the currently known mutations in human. The 'X' indicates the STOP codon (Gianetti and Seminara 2008).*

Expression analysis showed that while KISS1R is expressed in both the hypothalamus and pituitary, differently, KISS1 is restricted to hypothalamus (Kotani et al. 2001). Studies conducted on *Kiss1r* knockout mice demonstrate that Kissr1 only affects the releasing of GnRH hormone, but not the GnRH neuronal migration (Seminara et al. 2003; Messager et al. 2005). Both *Kiss1* and *GPR54*-deficient mouse models presented a similar CHH phenotype as observed in the human population. The male

mice had small testes, while the females had delayed vaginal opening and absence of follicular maturation. Both sexes showed responsiveness to exogenous gonadotrophins and GnRH administration (Messager et al. 2005). These findings support the role of KISS1 as a tiny regulator of puberty and HPG axis (Fig. 22). Indeed, its increased expression have been proposed to be one of the effector mechanisms at puberty (Rance 2009) while downregulation mediates the HPG suppression observed in severe nutritional deprivations (Castellano et al. 2009). Moreover, KISS1 neurons are highly responsive to estrogens, and were demonstrated to be implicated in both positive and negative central feedback of sex steroids to GnRH production (Castellano et al. 2009). The role of KISS1/KISS1R was supported by observations of GnRH deficiency and failure to initiate or progress through puberty in CHH patients, carrying mutations in these genes (Lippincott, True, and Seminara 2013). Inactivation of mutations in KISS1 and KISS1R shows an autosomal recessive pattern of inheritance.

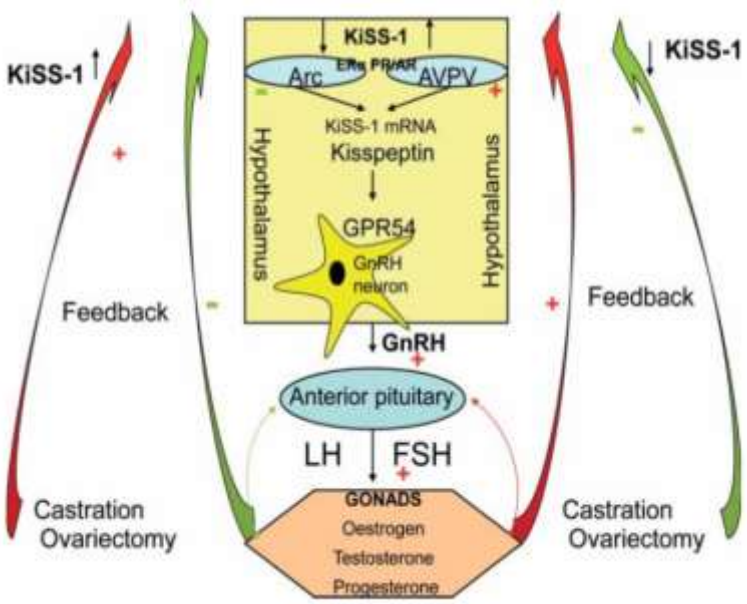

Fig. 22. *Positive and negative feedback of Kiss-1neurons. The positive (red) and negative (green) feedback loops of the Kiss-1 neurons on the AVPV and ARC are illustrated within the rodent hypothalamus with intact HPG axis. The effects of ovariectomy/castration are also*

depicted. GPR54: G protein coupled receptor 54; HPG: Hypothalamic Pituitary Gonadal; GnRH: Gonadotrophin Releasing Hormone; ARC: Arcuate nucleus; AVPV: Anteroventral Periventricular nucleus; FSH: follicle stimulating hormone; LH: luteinizing hormone (Dedes 2012).

- *TAC3/TAC3R*: *TAC3R* or tachykinin receptors 3 gene is located on chromosome 4q25. It encodes for NK3R, a GPCR primarily expressed in the central nervous system that is activated when bound with its ligand Neurokinin B (NKB). NKB is a member of the tachykinin superfamily of neuropeptides, located on chromosome 12q13–q2186. NKB expression is highest in the arcuate nucleus, where it co-localizes with estrogen receptor alpha (Era) and dynorphin, implicated in progesterone feedback to GnRH secretion, with some differences between humans and rodents (Skorupskaite, George, and Anderson 2014) (Fig. 23).

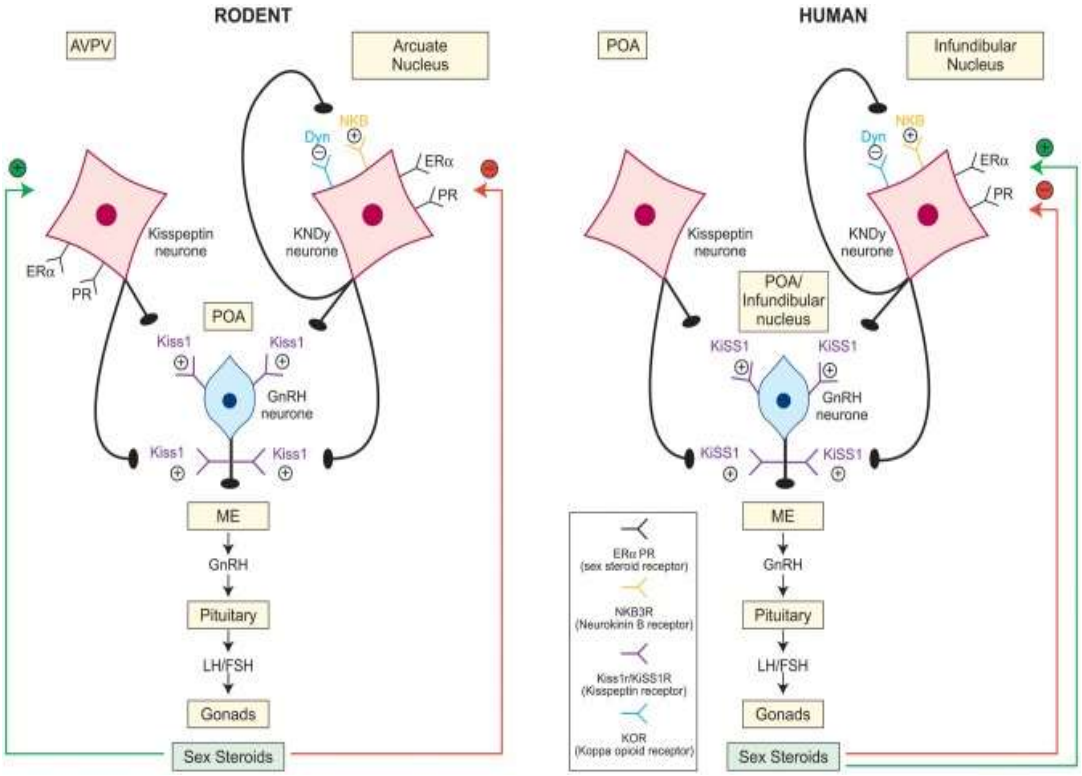

Fig. 23. *Schematic diagram showing the neuroanatomy of the kisspeptin-GnRH pathway and the relationship between KNDy neurons and GnRH neurons in humans and rodents. Kisspeptin signals directly to the GnRH neurons, which express kisspeptin receptor. The location of kisspeptin neuron populations within the hypothalamus is species specific, residing within the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus in rodents, and within the preoptic area (POA) and the infundibular nucleus in humans. Kisspeptin neurons in the infundibular (humans)/arcuate (rodents) nucleus co-express neurokinin B and dynorphin (KNDy neurones), which via neurokinin B receptor and kappa opioid peptide receptor auto-synaptically regulate pulsatile kisspeptin secretion, with neurokinin B being stimulatory and dynorphin inhibitory. Negative (red) and positive (green) sex steroid feedback is mediated via distinct kisspeptin populations in rodents, via the AVPV and the arcuate nucleus, respectively. In humans KNDy neurons in the infundibular nucleus relay both negative (red) and positive (green) feedback. The role of the POA kisspeptin population in mediating sex steroid feedback in humans is incompletely explored. ME, median eminence; +, stimulatory; −, inhibitory; ERα, estrogen receptor alpha; PR, progesterone receptor; Kiss1/KiSS1, kisspeptin; NKB, neurokinin B; Dyn, dynorphin (Skorupskaite, George, and Anderson 2014).*

Nevertheless, NK3R and NKB are also expressed in peripheral organs including ovaries and uterus (Patak et al. 2003), so additional peripheral effects cannot be formally excluded. Despite the overlapping expression of neurokinin B and kisspeptin in the arcuate nucleus in humans, studies conducted in animal models reveals different functions, in fact, experiments conducted in various species underline different ability between KISS and NKB in the stimulation of release of GnRH. Kisspeptin can stimulate GnRH release in almost all species, differently, NKB cannot. Moreover, observation of KO mice for *Nkb* or *Kiss* revealed distinct phenotypes, with *Nkb* KO mice with a normal fertility, whereas *Kiss* KO mice displayed a CHH classic phenotype (Chawla et al. 1997; Sandoval-Guzman and Rance 2004). Clinical studies on humans revealed a strictly association between mutation of TAC3R and microphallus and less association with cryptorchidism phenotypes in male patients, because phallic growth is strongly influenced by the integrity of the in utero activity of the HPG axis in late gestation (Herman et al. 2000), this consistent presence of microphallus suggests that NKB stimulated signaling plays an important role as a driver of GnRH secretory activity during this critical window of development, demonstrating the importance of NKB/NK3R system for normal fetal gonadotrophin secretion (Topaloglu et al. 2009).:

- *GNRH1*: Gonadotropin-releasing hormone (GnRH) is located on chromosome 8p21-11.2 and it is encoded by the *GNRH1* gene. Composed by four exons and three introns, the translation of GnRH1 sequence generates a 92 amino acids pre-pro-protein (pre-pro-GnRH), including 23 amino acids as signal sequence followed by two serine residues, the GnRH decapeptide, a GKR sequence and GAP 6 GnRH-associated peptide (Limonta and Manea 2013). It plays a crucial role in the HPG axis, regulating the reproductive physiology. GnRH was first identified as the hypothalamic

decapeptide (Fig. 24), that plays a key role in the control of the reproductive functions (Schally et al. 1971; Limonta et al. 2012).

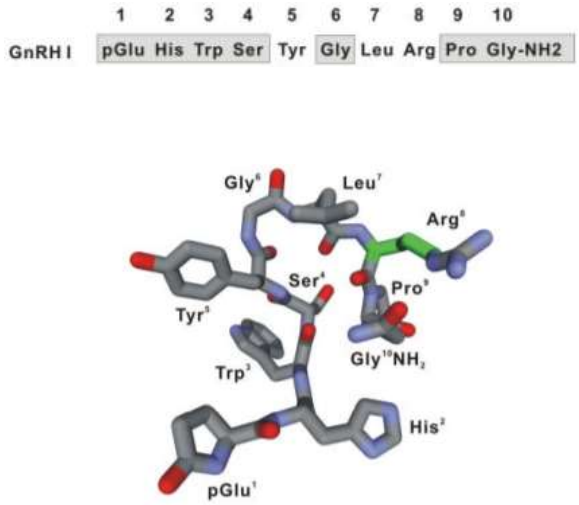

Fig. 24. *Structures of GnRH. The N-terminal amino acids (pGlu1 -His2 -Trp3 -Ser4) and C-terminal amino acids (Pro9 -Gly10-NH2) of the decapeptide ligands are highly conserved over 600 million years of the chordate evolution and are important for ligand binding and receptor activation.*

It is synthesized in a small number of hypothalamic neurons and released in a pulsatile manner into the hypophyseal portal circulation through which it reaches the anterior pituitary. By binding to specific receptors (GnRH-Rs) on pituitary gonadotropes, the decapeptide stimulates the synthesis and the release of the two gonadotropins, LH and FSH, that in turn, regulate gonadal steroidogenesis in both sexes. *Gnrh1* KO mice exhibited anomalous tooth maturation and mineralization and totally absent GnRH hormone that leads to infertility, failing in sexual maturation (Tiong, Locastro, and Wray 2007; Limonta et al. 2012). However, introduction of functional *GnRH* gene into the genome of mutant mice completely reversed the reproductive deficits (Mason et al. 1986). Same was observed in humans where pulsatile GnRH administration

reverted the delayed puberty phenotype in a male patient and his sister with homozygous mutation, affecting the peptide precursor pre-pro-GnRH (Bouligand et al. 2009).

- *GNRHR*: GNRHR is the receptor of GNRH1. Its sequence is mapped on chromosome 4q21.2 and generates a 328-amino acid GPCR of 60 KDa. This receptor belongs to the family of rhodopsin-like G protein coupled receptors (GPCR) containing seven transmembrane domains and an extracellular 35-amino acid amino-terminal domain with two putative glycosylation sites (Limonta and Manea 2013) (Fig. 25).

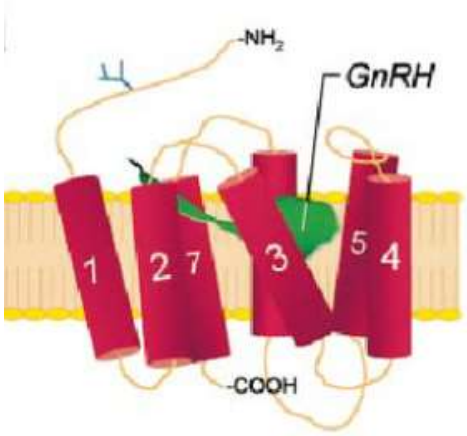

Fig. 25. *The human GnRH receptor. Counterclockwise orientation of a prototypic G protein-coupled receptor from transmembrane domains (Ulloa-Aguirre et al. 2004).*

After the binding with its ligand, GnRH1 stimulates in the pituitary gonadotrope cells the beta isoform of Phosphoinositide phospholipase C, which mobilizes calcium and protein kinase, resulting ultimately in the activation of proteins involved in the synthesis and secretion of the gonadotropins LH and FSH. The *Gnrhr* KO mouse model recapitulates clinical phenotype of CHH syndrome (Pask et al. 2005; Wu et al.

2010), displaying reduced size of sexual organs (penis, preputial gland, uterus, vagina, testis, and ovary), low gonadotropins and sex steroid levels, infertility, failure of sexual maturation and inability to respond to exogenous GnRH administration. Several human GNRHR loss of function mutations were described in CHH patients in the last 15 years (de Roux et al. 1997). Clinically, the phenotype of these individuals spans a wide spectrum of defects ranging from fertile eunuch syndrome to the complete form of GnRH resistance, characterized by micropenis, cryptorchidism, very low gonadotropins levels and the absence of pubertal development. GNRHR mutations have been described in about 40–50% of familial CHH cases, and in round 17% of sporadic CHH (Valdes-Socin et al. 2014).

CRITICAL ASPECTS IN CHH PHENOMICS AND GENOMICS

CHH was classically divided into two distinct clinical groups: KS and CHH. However, such separate classification has been questioned in recent years since these two clinical manifestations may exist in different relatives within unique familial settings, supporting the idea that they may represent variable phenotypic manifestations of a common genetic defects (Pitteloud et al. 2006; Bonomi et al, 2014). Thus, a novel vision about CHH considers this disease as a complex genetic disorder characterized by variable expressivity, penetrance, and modes of inheritance in which the inability to go through puberty represents the major manifestation. Many heterozygous variants were previously believed to have a dominant (or incompletely penetrant) transmission, however, only the most disrupting ones have a true AD inheritance and, in the majority of cases, CHH is more likely to arise from genetic hemizygosity (*ANOS1*), homozygosity, compound heterozygosity, or oligogenicity. Additionally, as in multifactorial complex diseases, the pathogenesis of CHH can be elucidated by environmental factors, but also the concurrent involvement of SNPs or other

genetic defects in two or multiple interacting genes challenging the long-held view of a strictly monogenic disorder. The overlap between KS, CHH, combined pituitary hormone deficits (CPHD), adult-onset hypogonadotropic hypogonadism (AHH) and hypothalamic amenorrhean (HA) in fact further complicates the scenario making it even more essential to deploy NGS analyses using ever-wider panels of candidate genes (Raivio et al. 2012; Jayakody et al. 2012). In conclusion, oligogenicity may partially account for the phenotypic variability of isolated GnRH deficiency. However, these advances in genomics come at a cost, with NGS allowing simultaneous screening of a vast number of genes, great numbers of variants of unknown clinical significance (VUS) are necessarily thrown up. The challenge now is to more cheaply, rapidly, and reliably identify those variants that are truly pathogenic.

OPEN ISSUES

The genetic analysis of all the so far known causative genes allows to identify a genetic cause in ~50% of the analyzed patients, letting half of them still "idiopathic" (Boehm et al. 2015; Cangiano et al, 2020). Thus, new strategical and methodological advances are needed to cover this gap. Moreover, the recent demonstration of oligogenicity as cause for the etiopathology of CHH open the question regarding how these genes interact determining this complex clinical phenotype. The use of specific animal models might be helpful in this respect.

Introduction

CHAPTER 4

ZEBRAFISH AS MODEL FOR GENETIC INVESTIGATION

Native to Southeast Asia, zebrafish (*Danio rerio*) has become a popular model in biomedical research due to his properties.

Advantages and limitations of zebrafish models in biomedical research and their relevance to neuroscience have been provided in recent literature (Kalueff, Stewart, and Gerlai 2014), and are briefly summarized.

Model advantages:

- An *in vivo* vertebrate model with common conserved cell types, organs, and physiological systems (e.g., stress endocrine axis Fig. 26)

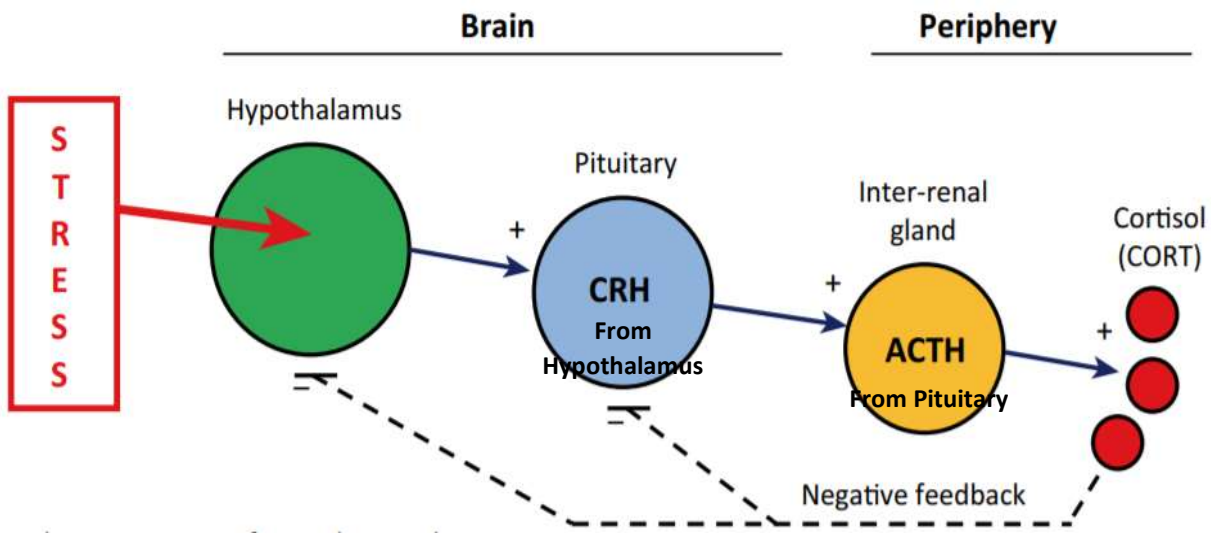

Fig. 26. *Acute stress evokes fast and robust cortisol responses with similar time dynamics in both humans and zebrafish (bottom row); also note that zebrafish and humans both use cortisol as their main stress hormone, unlike rodents which use corticosterone (Egan et al. 2009).*

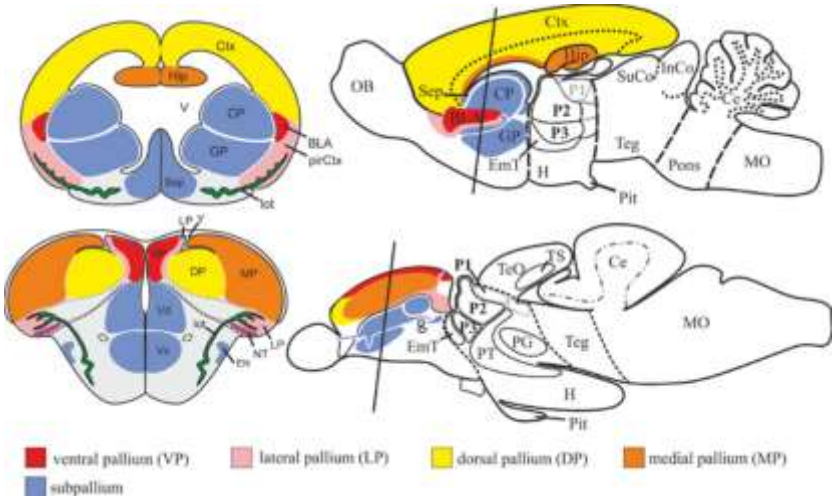

Fig. 27. *Schematic drawing illustrating topological correspondences between Teleostei (zebrafish) and Mammalian's (mouse) telencephalon. The zebrafish telencephalon is characterized by two massive lobes covered by a dorsally located T-shaped ventricle. The mouse telencephalon, in contrast, consists of two bilateral hemispheres surrounding centrally located ventricles. The topology of the zebrafish pallium is similar to the one in mouse and can be explained through topographical shifts of its constituting pallial divisions during a complex outward folding process. The zebrafish pallium, like its mammalian counterpart, consists mainly of four pallial divisions: a medial pallium (MP) homologous to the mammalian hippocampus (Hip), a dorsal pallial division (DP) topologically corresponding to the mammalian isocortex (Ctx), and ventral (VP) and lateral (LP) pallial divisions homologous to the mammalian pallial (basolateral) amygdala (BLA) and piriform cortex (pirCtx) respectively.*

- physiologically complex and with high homology to humans (Fig. 27)
- High genetic homology to humans; fully sequenced genome that can be easily manipulated with several genetic tools (i.e. Knock-Down gene expression through morpholino microinjection or Knock-Out gene expression through CrispR/Cas9 or TALENs) (Ata, Clark, and Ekker 2016)
- Quick and abundant eggs production per mating (e.g., a single female lays several hundred eggs each week)

- Rapid development: somitogenesis begins at about 9 hpf, at 24 hpf the zf embryo has already formed all the major tissues and many organ precursors, such as a beating heart at 23hpf, circulating blood, nervous system, eyes and ears, all of which can be readily observed under a simple dissecting microscope. Larvae hatch by about 2.5 dpf and they are swimming and feeding by 5–6 dpf (Kimmel et al. 1995; Lawson and Weinstein 2002). (Fig. 28).

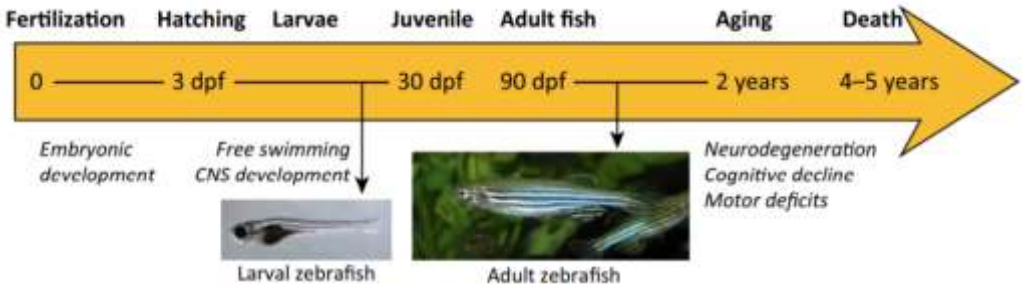

Fig. 28. *The zebrafish life cycle, from embryonic (0–72 hpf) stage to larval (3–29 dpf), juvenile (30–89 dpf), adult (90 dpf–2 years), and aged zebrafish (>2 years).*

- Embryos and larvae are optically clear, allowing for direct and non-invasive observation or experimental manipulation at all stages of their early development, such as whole mount in situ hybridization analysis (WISH) of gene expression patterns with extraordinarily high resolution (Vogel and Weinstein 2000)
- The externally developing embryos are readily accessible to experimental manipulation by techniques such as microinjection of biologically active molecules (RNA, DNA or antisense oligonucleotides), cell transplantation, fate mapping and cell lineage tracing (Holder and Xu 1999)
- External development of embryos that can be exposed to various environmental factors neonatally outside of the maternal organism, in a more controllable environment
- High space/cost-efficiency and excellent potential for high-throughput screens

- Availability of several zebrafish strains, with over 1000 transgenic and mutant zebrafish lines
- Adherence, as a lower vertebrate, to the 3R principles (replacement, refinement, reduction)
- Smaller brains which can be better assessed using the newest imaging techniques

Model limitations:

- Duplication of genome, in fact, some zebrafish genes have two copies instead of one as in mammals and often just one keep the ancestral function, while the other acquire a subfunction or new functions
- Not as many well-characterized inbred strains as mice have, in fact, must be note that zebrafish, differently from rodents, do not tolerate inbreeding and rapidly lose fertility with inbreeding
- The administration of not water-soluble drugs can be problematic, but the use of solvents as well as other routes can easily overcome this problem
- Species differences in blood–brain barrier (BBB): zebrafish develop the BBB like that of humans, however, species differences exist in the permeability for certain drugs
- Some complex behaviors are developed only in the adult stage (e.g. social behaviors are not prominent in larval fish)
- Parental care is not known/characterized
- Certain brain areas are not as developed as in mammals (i.e. cortex), and some CNS structures in zebrafish are still difficult to map to their mammalian counterparts (this knowledge gap may complicate the interpretation of circuitry–behavior interplay)

HPG AXIS IN ZEBRAFISH

HPG axis of zf exhibits an high similarity compared with more evolved vertebrates like mammals, preserving all the most central reproductive cells and hormones. In humans the HPG axis cascade take place from the hypothalamus through the GnRH (gonadotropin releasing hormone) and kisspeptin (Kiss1) secreting neurons (Vaudry and Seong 2014). Two forms of the kiss gene (*kiss1*, *kiss2*) and their respective receptor genes (*kiss1r*, *kiss2r)* have been identified in the nervous system of zf (Biran, Ben-Dor, and Levavi-Sivan 2008; Servili et al. 2011). Studies in adult zf showed that Kiss1 neurons are in the habenula, while Kiss2 neurons are in the dorsal and ventral hypothalamus (Ogawa and Parhar 2013) where it is possible to observe a huge number of GnRH3 neurons. Despite its location, it was demonstrated that treatment with kiss1, but not kiss2, increase the number of hypothalamic GnRH3 neurons, underling the relevance of kiss1 in the development of these neurons involved in the reproductive axis (Zhao et al. 2014).

Despite these differences through more and less evolved vertebrate, both in human and zf Kisspeptin and GnRH hormones produced by these two neuronal populations are essential for the release and fine regulation of the follicle stimulating hormone FSH (also called GTH-I in zf) and luteinizing hormone, LH (GTHII in zf) by gonadotropic cells in the pituitary (Liu, Lin, and Ge 2011). The zf gonadotropins GTH-I and GHT-II are produced in response to the stimulation of the GnRH hormone (Messager et al. 2005) and stimulate gametogenesis and steroidogenesis. FSH/GTH-I is responsible for spermatogenesis in male and folliculogenesis in female, while LH/GTH-II stimulate steroidogenesis in male acting on the Leydig cell in the testis and in oocyte maturation and growth (Howles 2000) (Fig. 29).

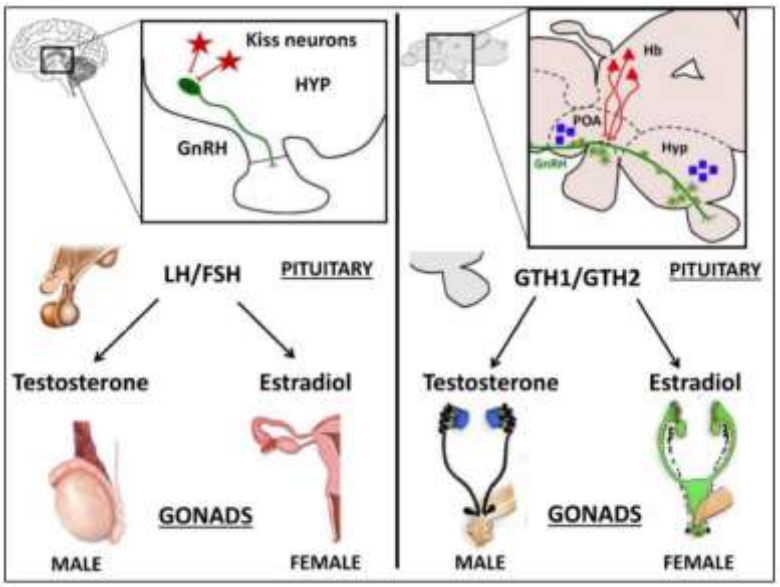

Fig. 29. *HPG axis in human (right) and zebrafish (left). In both organisms, Kiss neurons regulate GnRH neurons in the hypothalamus. In zf there are two populations of Kiss neurons: kiss1 neurons (triangle in the Fig.ure) are localized in the habenular nucleus (Hb) while kiss2 (square) are more widely scattered in the hypothalamus (Hy) and preoptic area (POA)*

The HPG axis architecture in zf revealed a different model for the delivery of GnRH3 hormone, in fact, in Teleostei, a true median eminence has not been identified; instead, direct innervation of the endocrine cells has been proposed as the predominant pathway by which the hypothalamus interacts with the gonadotropic cells. Nevertheless, taking advantage of FSH:EGFP and LH:mCherry zebrafish's reporter line (Golan, Biran, and Levavi-Sivan 2014) it was observed that GnRH3 fibers formed multiple boutons upon reaching the pituitary, but most of these structures were located in the neurohypophysis rather than adjacent to gonadotroph cells. These GnRH3 boutons are similar to the GnRH varicosities found in the mammalian median eminence from the structural point of view and are considered as the release sites of this peptide in mammals (Ojeda, Lomniczi, and Sandau 2008) and in fish (Oka and Ichikawa 1992). In addition, they found a close association between FSH cells and GnRH3 boutons, but only a fifth of the LH cells were in contact with GnRH3 axons. These data suggest a more direct regulation of FSH cells rather than LH cells and a combination of

neuroglandular and neurovascular delivery mode of GnRH3 hormone in zf (Golan et al. 2015).

THE OLFACTORY PLACODE DEVELOPMENT IN ZEBRAFISH AND HYPOTHESIS ABOUT THE NEUROGENESIS OF GnRH3 NEURONS IN ZEBRAFISH

In all vertebrate, including zf, the olfactory placode originates in the same way as it was previously described. In fact, also in zf, the olfactory placodes are composed by a mixture of sensory and neural crest cells deriving from the neural and non-neural ectoderm. Two different hypotheses about the origin of GnRH neurons are proposed, and both are reported and analyzed in this thesis. The first hypothesis supports the idea that olfactory placodes develop olfactory sensory neurons and their supporting cells, but also neuroendocrine GnRH neurons. In zebrafish, the development of *tg*(GnRH3:EGFP) transgenic line by Zohar group allowed to study in details the embryonic origins of GnRH3 neurons and their structures during zf embryogenesis. Using this transgenic reporter line, they observed that the first fluorescent signal is detectable at 24 hpf in olfactory regions (Abraham et al. 2008). Axons elongation begins at 26 hpf extending from GnRH3 perikarya clusters, located in olfactory placodes, towards the pallium. This process continues in the following and between 32–36 hpf these projections develop multiple additional branches and meet caudally at midline to form the first commissure in the subpallium. New fibers arise from the GnRH3 perikaria around 40 hpf and elongate caudoventrally along the anterior and the optic commissures, to innervate the eyes, in fact, it is known that GnRH3 has also a role in the development of eye, innervating the retina (Corchuelo et al. 2017). By 48 hpf dorso-caudally axons finish their route at the midline creating a second commissure in the pallium. Four distinct GnRH3 fibers tracts are notable at this developmental stage (48 hpf) in the brain: in the pallium, in the

subpallium, along the optic tract to the eyes and in the direction of the hypothalamus (Fig. 30).

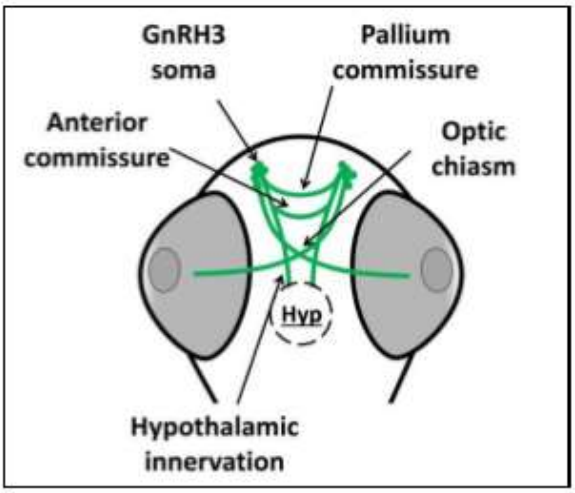

Fig. 30. *Representative illustration of zebrafish GnRH3 fibers at 48 hpf. Four structures can be recognized at this developmental stage: pallium commissure, anterior commissure, optic chiasm, and bilateral prolongations that reach hypothalamus (Hyp) (Abraham et al. 2008).*

Outside of the brain, GnRH3 fibers were observed in the retina, in the perikarya of the trigeminal ganglia and bilaterally to the trunk, along the spinal cord (Gopinath, Andrew Tseng, and Whitlock 2004). The hypothesis about the GnRH3 origin from the olfactory placodes is also supported by the observation on the perikaria migration from the olfactory region through the TN ganglion and from ventral telencephalon to the ventral hypothalamus among the preexisting vomeronasal-terminal nerves (VNN/TN) fibers as a scaffold. In fact, starting from 3dpf to 5dpf GnRH3 perikaria reaches the terminal nerve and the ventral hypothalamus at 7-12dpf. The pick in the perikaria migration is observed between 4 and 5dpf, reaching approximately 1µm/h (Abraham et al. 2008). Finally, these results are reinforced by studies on laser ablation of GnRH3 neurons soma in the nasal area during early development that resulted in a complete lack of olfactory, terminal nerve, preoptic area, and hypothalamic

GnRH3 neurons (Abraham et al. 2010). Taking together these findings, it was plausible to hypothesize a common embryonic origin for both olfactory bulb (OB)-terminal nerves (TN) and preoptic area (POA)–hypothalamic GnRH neuronal populations (Palevitch et al. 2007).
In 2016 Zohar's group generated a *GnRH3*$^{-/-}$ zebrafish line that harbors a deletion of 62 bp in the *GnRH3* gene (Spicer et al. 2016). Surprisingly, aside from an increasing of mRNA levels of pituitary gonadotropin genes (fshb, lhb, and cga) only during initial development, no GnRH3 neurogenesis or reproductive defects were observed in such GnRH3 knockout line in both sexes. Indeed, characterization of key components of the HPG axis downstream of GnRH3, performed in GnRH3-/$^{-}$ zf, indicates a normal gametogenesis and reproductive performance, also in this case, for both sexes (Spicer et al. 2016). These is in extreme contrast with previous experiments where GnRH3-expressing cells laser ablation in the early phase of zebrafish development (4–6 dpf) leads to infertility (Abraham et al. 2010), thus, the discussion about GnRH established role as key reproduction regulator in all species of vertebrates is still open, especially in zebrafish.

Nevertheless, data published in the past years (Abraham et al. 2008) demonstrate that the correct development and migration of the GnRH3 neurons remain fundamental to their appropriate function in zf, as previously demonstrated in humans. To explain the absence of phenotype observed in the GnRH3$^{-/-}$ zf model, they postulated that gene knockout could trigger a compensatory mechanism which is not activated by gene knockdown or cells ablation due to different timing or duration of the elimination (Spicer et al. 2016).

The second hypothesis about the origin of GnRH neurons is supported by Whitlock and coworkers that demonstrate in 2006 a multiple origin for GnRH3 neurons in zebrafish: GnRH3 neurons located in the terminal nerve originate from the neural crest cells, while the hypophysiotropic population give rise to the pituitary placode (Whitlock et al. 2006).

The region from which OPs give rise in zf surround the developing anterior pituitary placode. It has been demonstrated that the loss of the pituitary does not affect the GnRH3 in the terminal nerve area. In fact, the zebrafish mutant line you-too and detour does not develop the anterior pituitary (adenohypophysis), but OPs develop normally and, by consequence, also the GnRH3 development in terminal nerve area is not affected. The same result is also observed in mice, where you-too and detour homologue double mutant for gli2$^{-/-}$ and gli1$^{-/-}$ show a completely pituitary ablation, but normal GnRH1 neurons in terminal nerve area (Metz and Wray 2010). This theory is partially supported by the observation of a new zebrafish transgenic line GnRH3:EMD that allow the visualization of three different subpopulation of GnRH3 neurons during the zebrafish embryonic development. The first population appear at 17-25hpf, bilaterally, that are the trigeminal GnRH3 that project caudally to the spinal cord and anterior to the head. The second GnRH3 population arises at 20hpf in the terminal nerve area and they increase their number and project to the pallium and subpallium creating the anterior commissure. The last one is the hypothalamic GnRH3 population from which, starting from 30hpf, GnRH3 neurons start to migrate to the POA (Zhao et al. 2013) (Fig. 31).

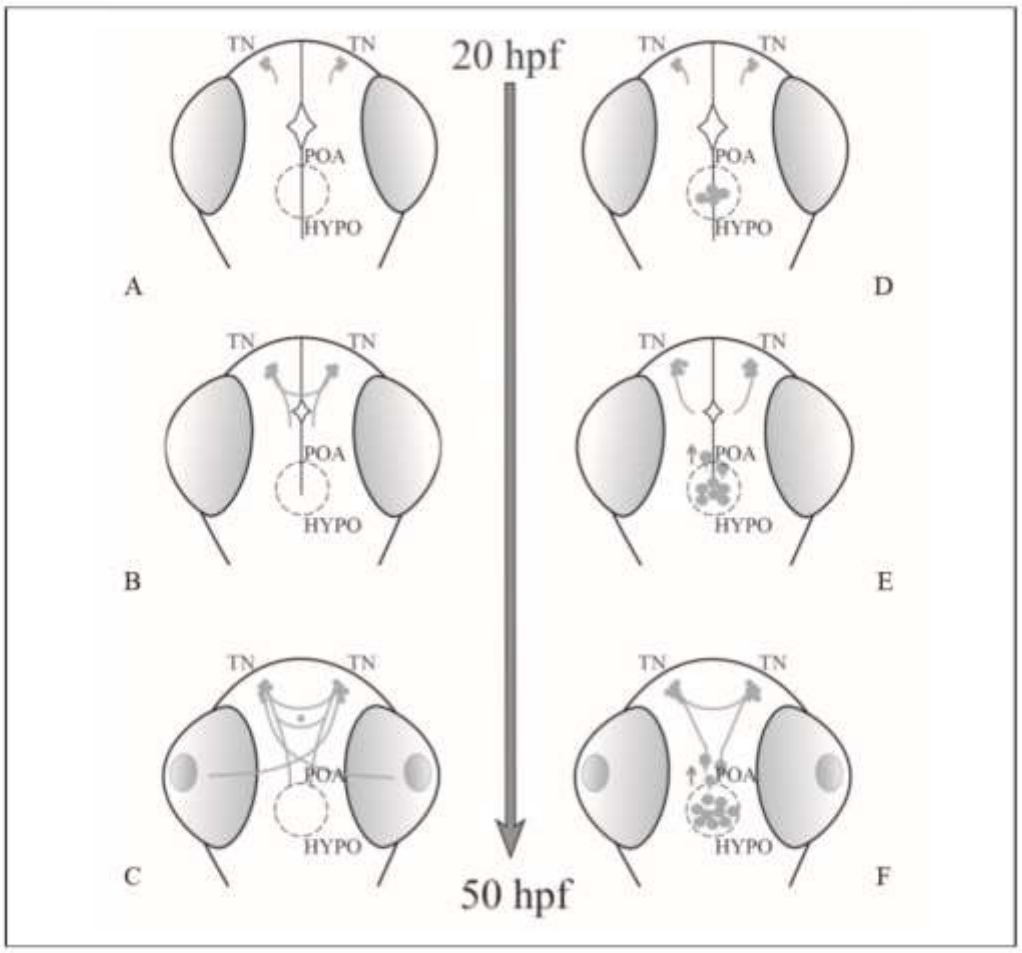

Fig. 31. *Comparison of the two hypotheses on the origin and migration of GnRH3 fibers during zebrafish early developmental stages. In Fig.ure 1A-C the source of hypophysiotropic GnRH3 neurons is the olfactory region. In tg(GnRH3:EGFP) zebrafish line, somas are observed only in terminal nerves regions (Tn) around 24 hpf where they send projection to form at 48 hpf the five distinctive networks described in the text. In Fig.ure 1D-F different GnRH3 somas populations arise during zebrafish development. Observations in Tg(GnRH3:EMD) line evidence the present at 20 hpf of two distinctive population of GnRH3 neurons, one in the terminal nerves (Tn), that begin to send projections, and one in the hypothalamus (HYPO). At later stages of development, a signal was also recognizable in the preoptic district, probably due to a migration process of hypothalamic GnRH3 somas to this region (Zhao et al. 2013).*

The lack of a unique hypothesis about the origin of GnRH3 neurons underline the importance to better elucidate the development of these neurons. In this regard, the transparency of the zebrafish embryos, external fertilization, the rapid development, and the disposal of several reporter line make zebrafish a good model for this investigation. On the other hand, the

difference in the fertility between the GNRH1 $^{-/-}$ mice model (infertile) and the GnRH3$^{-/-}$ zebrafish model (fertile) open several questions about the use of zebrafish as model to study the reproduction.

HYPOPHYSIOTROPIC FORM OF GnRH IN ZEBRAFISH

In vitro and *in vivo* experiments revealed how hypophysiotropic neurons are dispersed in a loose continuum from olfactory region to hypothalamus. In most vertebrate, GnRH1 is synthetized by a group of neurons located in the POA and hypothalamus, but zebrafish genome missed the mammalian orthologue *GnRH1* and possesses only two form of GNRH gene: *gnrh2* and *GnRH3*. Gnrh2 gene is expressed in midbrain tegmentum, like in human counterpart, while for *GnRH3* is more widely observed in forebrain areas of terminal nerve, ventral telencephalon, POA, and hypothalamus (Fig. 32). This expression in POA and hypothalamus, presenting the same expression pattern to the mammalian *GNRH1*, and the high sequence homology with human GNRH1 (80%) highlight that the zebrafish GnRH3 is the main regulator of gonadotropins releasing. This hypothesis was confirmed by its ability to increase the mRNA level of the luteinizing hormone beta (lhb) in cultured primary pituitary cells. To better reveal the hypophysiotropic role of the GnRH3 in zebrafish, it was tested their response to kisspeptin stimulation, considering that kisspeptin is a neuropeptide that stimulates puberty and fertility through its action of GnRH neurons. Treating embryos with 100nM of kisspeptin the GnRH3 in the terminal nerve develop an electrophysiological response (Zhao et al. 2013). Hence, it is possible to conclude that the zf GnRH3 neurons have an hypophysiotropic function.

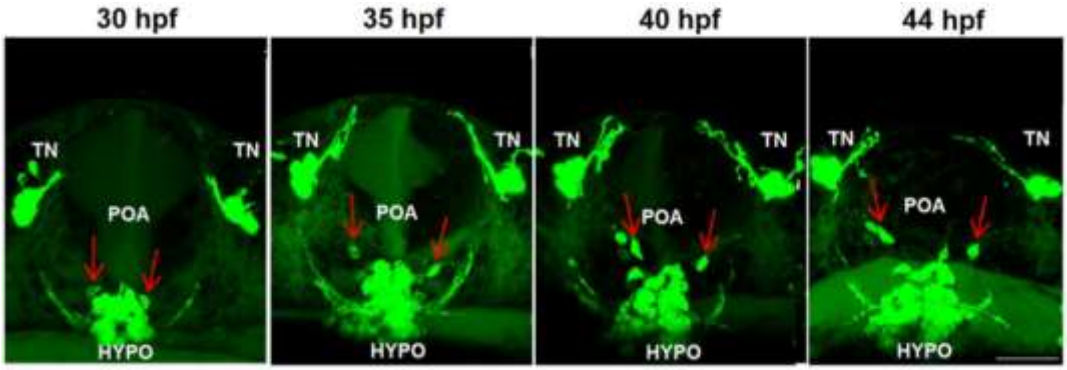

Fig. 32. *Representative confocal images (ventral view) of the Pre-Optic Area (POA) and hypothalamus (HYPO). Red arrows show GnRH3:EMD neurons migrating from hypothalamus toward the POA between 30 and 44 hpf. Scale bar: 50µm (Zhao et al. 2013).*

ZEBRAFISH AS MODEL FOR CHH

The sequencing of the zf genome has demonstrated a partial genome duplication occurred during evolution in the ray-fin fish lineage, prior to the teleost radiation. After this divergence, the zebrafish genome has been resolving back to a diploid state again, but this process was incomplete, and many orthologues are retained in a double copy (Meyer and Schartl 1999). As result, zf often presents two orthologues for the corresponding human gene. Despite this huge difference the analysis of the genome revealed that 71.4% of human genes has at least one zebrafish orthologue (Howe et al. 2013). Additionally, the 82% of human potential disease-related genes can be associated to at least one zebrafish orthologue, making zebrafish one of the most attractive models for understanding the biological activity of human disease-related genes (Ardouin et al. 2000). Due to this strong genome and, in this case, also a relevant anatomical conservation between structures, zf was used as a model for studying genes involved in CHH. For example, genes such as *KAL1, KISS1/KISS1R, FGF8, PROKR2* present two orthologues in zebrafish but just one of them keep the ancestral role, differently, the other one acquires new subfunctions.

kal1a/kal1b.

Two orthologs of human *KAL1* gene were identified in zf genome and called *kal1a* and *kal1b*. They present an homology in the amino acids sequences with human *KAL1* of 75.5% and 66.5%, respectively. Both proteins shared the same structural domain with human anosmin-1 that consist in a cysteine-rich region at N-terminal followed by a whey acidic protein-like (WAP), four fibronectin type III domains, and a C-terminal histidine-rich region excepted for anosmin-1b (*kal1b*), where the last domain is missing (Ardouin et al. 2000). From in situ hybridization experiments it was observed that the signal for kal1a transcript appears at 38 hpf in the olfactory bulbs, and with a lower expression, in midbrain, tectum, cerebellum, retina and spinal cord (Ardouin et al. 2000). Differently, *kal1b* mRNA seems to be expressed at later developmental stages, starting from 48 hpf, in the olfactory epithelium, in the rostral telencephalic area, the cerebral lobes and pronephric ducts. The downregulation of *kal1a* transcript affects proper axon fasciculation of olfactory neurons, and their terminal targeting at the olfactory bulbs. On GnRH system, *kal1a* knockdown abolishes in the hypothalamus GnRH-immunoreactive cells, while for *kal1b* only a limited reduction in GnRH3 neurons was observed (Ardouin et al. 2000), suggesting a more specific role for *kal1a* gene in the development of the GnRH system in zebrafish embryos.

kiss1/kiss2.

The neuropeptide Kisspeptin1 (product of the *KISS1* gene) is a key regulator on puberty onset and fertility by regulating the gonadotropin-releasing hormone (GnRH) neuronal system in mammals. Inactivating mutations in *Kiss1* and *Kiss1r* (also called *GPR54*) are associated with pubertal failure and infertility. In zf there are two kiss genes: *kiss1* and its paralogue *kiss2*. Using transgenic zebrafish model GnRH3:EGFP, it was investigated the role of kisspeptins on GnRH neuronal embryogenesis and on electrical activity during adulthood. Quantitative PCR revealed that both kiss1 and kiss2 mRNA are detectable starting from 24hpf, increasing

throughout embryonic and larval development. Early treatment with Kiss1 or Kiss2 showed that both treatments stimulated proliferation of trigeminal GnRH3 neurons located in the peripheral nervous system. However, only Kiss1, but not Kiss2, also stimulated the hypothalamic populations of GnRH3 neurons in the central nervous system (Zhao et al. 2013). In addition, immunohistochemical analysis of synaptic vesicle protein 2 (SV2) suggested that Kiss1, but not Kiss2, increased synaptic contacts on the cell body and along the terminal nerve-GnRH3 neuronal processes during embryogenesis. Finally, patch clamp analysis revealed that, while kiss1 increases spike frequency and depolarizes membrane potential, kiss2 exercises the opposite effect suppressing spike frequency and hyperpolarizing membrane potential, demonstrating a regulatory role of kiss1 on GnRH neurons. We conclude that in zebrafish, Kiss1 is the primary stimulator of GnRH3 neuronal development in the embryo and an activator of stimulating hypophysiotropic neuron activities in the adult, while Kiss2 plays an additional role in stimulating embryonic development of the trigeminal neuronal population (Ardouin et al. 2000).

fgf8a/fgf8b

the role of FGF signaling during formation of the forebrain commissures has also been conserved in the zebrafish. Zebrafish have two orthologues of FGFR1 (*fgfr1a* and *fgfr1b*) and two orthologues of FGF8 (*fgf8a* and *fgf8b*) (Reifers F et al., 2000) . For example, the *fgf8a* null mutant zebrafish (named “acerebellar”), display the conspicuous lack of a cerebellum together with several prominent forebrain defects such as fewer olfactory axonal condensations at the anterior telencephalon, disturbed ORNs differentiation in the olfactory epithelium, variable failure in the establishment of the anterior commissure (AC) that is interesting that some axonal projections from both olfactory and GnRH neurons projections follow the AC during their development (Whitlock KE et al., 2004). As consequence, these defects could contribute to a failure in coordination of the olfactory and GnRH systems.

prokr2

Prokineticin receptors (PROKR1 and PROKR2) are G protein-coupled receptors which control human central and peripheral reproductive processes. Importantly, allelic variants of *PROKR2* in humans are associated with altered migration of GnRH neurons, resulting in congenital hypogonadotropic hypogonadism (CHH (Seminata et al., 1998; Bohem et al., 2015). Although this association is established in humans, murine models failed to fully recapitulate the reproductive and olfactory phenotypes (in heterozygous state) observed in patients harboring PROKR2 mutations. Bassi and colleagues, taking advantage of zebrafish model demonstrated that *prokr1b* (ortholog of human *PROKR2*) spatial-temporal expression is consistent with GnRH3 (Bassi I et al., 2020). Moreover, knockdown and knockout of *prokr1b* altered the correct development of GnRH3 fibers, suggesting that *prokr1b* regulates the development of the GnRH3 system in zebrafish. However, analysis of gonads development and mating experiments suggest that *prokr1b* is not required for fertility in zebrafish supporting the thesis of a divergent evolution in the control of vertebrate reproduction (Bassi I et al., 2020).

Materials and Methods

PATIENTS

The largest biobank of Italian patients affected by CHH (n=846) was created thanks to the "Network Italiano Ipogonadismo Centrale" (NICe) supported by: Società Italiana di Endocrinologia (SIE), Società Italiana di Andrologia e Medicina della Sessualità (SIAMS) and Società Italiana di Endocrinologia e Diabetologia Pediatrica (SIEDP). The institutional Ethic Committee approved the study, and all patients gave their informed consent for the genetic investigations.

GENETIC ANALYSIS

DNA PURIFICATION FROM WHOLE BLOOD

3 ml of blood from CHH patients were processed to extract the Genomic DNA, after the addition of EDTA, according to Gentra® Puregene® Kit Qiagen® protocol. After extraction, the DNA was diluted to reach the final concentration of 25ng/µl and stored at -20°C until use.

NGS

The Ampliseq Custom DNA panel (Illumina) designed ad hoc included also all the coding exons and flanking splice sites of genes derived from microarray analysis (CHD7, LEP, NR0B1, DUSP6, LEPR, FSHB, FEZF1, NSMF, LHB, FGF17, PROK2, FGF8, PROKR2, FGFR1, SEMA3A, FLRT3, SEMA3E, GNRH1, SEMA7A, GNRH2, SOX10, GNRHR, SOX2, HS6ST1, SPRY4, IL17RD, TAC3, KAL1, TACR3, KISS1, WDR11, KISS1R, TBX3). Primers were designed using BaseSpace, DesignStudio Sequencing (Illumina). The total coverage of the target genes by the designed amplicons was 100%. Library was prepared using enzymatic DNA fragmentation, with 50 ng of total gDNA, quantified with Quant-iT PicoGreen (Thermo Fisher Scientific). We followed the Nextera Rapid Capture Enrichment

protocol (Illumina) to tagment gDNA, amplify tagmented gDNA, hybridize probes, capture hybridized probes, library capture and amplification. The library was then loaded onto the reagent cartridge (Illumina) and sequencing was performed on a NextSeq 500 (Illumina).

BIOINFORMATIC ANALYSIS

After sequencing, reads were analyzed to identify single nucleotide variants and small insertions/deletions. The first steps of the bioinformatic analysis (including base calling and demultiplexing) have been performed using MiSeq provided software (Real Time Analysis RTA v.1.18.54 and Casava v.1.8.2, Illumina). FastQ files for each sample, containing mate paired-end reads after demultiplexing and adapter removal, have been used as input for MiSeq pipeline. Briefly, FastQ files have been processed with MiSeq Reporter v2.0.26 using the Custom Amplicon workflow. This analytical method required FastQ files, a "Manifest file" containing information about the sequences of primer pairs, the expected sequence of the amplicons and the coordinates of the reference genome (Homo sapiens, hg19, build 37.2) as input. Each read pair has been aligned using the MEM algorithm of the BWA software. Local Indel realignment and base recalibration step were performed using the software GATK. The realigned and recalibrated BAM file were used as input to GATK Unified Genotyper thus generating a VCFv4.2 file for each sample. Quality control of sequencing data was performed directly on FastQ files using the FastQC software. Reads were also filtered based on quality mapping and reads with quality mapping < 20 were removed. Moreover, genetic variants showing a PHredScore lower than 20 were filtered. The genetic variants were finally annotated using Annovar software (Yang et al. 2015) and then classified as rare variants if resulting unknown or with a minor frequency allele (MAF) <0.01 in gnomAD database (ver. 2.1.1). Rare variants were also classified by interrogating the VarSome database (Kopanos et al. 2018). Identified rare variants were confirmed using Sanger sequencing.

SANGER SEQUENCING

Variants identified by N.G.S. analysis were confirmed by Sanger sequencing. This reaction consists in 4 steps:

1. Amplification of interested gene by PCR.

2. Electrophoresis on agarose gel loading amplified PCR fragment.

3. Purification of PCR products.

4. Sequencing reaction.

5. Analysis through automated DNA-sequencer.

The amplification of the gene by PCR for each gene was performed using the following the reaction mix and the following protocol:

Template DNA	100ng
Buffer 5X	5.0µl
Primer Forward (10pmol)	1.25µl
Primer Reverse (10pmol)	1.25µl
dNTPs mix (10Mm each)	0.5µl
Go Taq Promega™	0.25µl
DDW	Up to 25µl

Fragment were amplified in a Thermal Cycler.

ELECTROFORESIS ON AGAROSE GEL

PCR products were checked on 3% agarose gel to obtain a proper separation of DNA fragments between 100 and 1000 base pair. Gels were prepared with TAE 1X Buffer (0.04M Tris-acetate, 0.002M EDTA) and amended with the intercalating agent Midori Green (Bulldog Bio). 5µl of each sample were loaded onto the gel and the run was performed applying a 130mV voltage for around 30 minutes. In order to check PCR products, the gel is located into an UV transilluminator to make the bands visible; the molecular weight can be compared with molecular weight Marker 100bp DNA Ladder (Euroclone).

PURIFICATION OF AMPLIFIED FRAGMENTS

To purify enzymes, nucleotides, and salts from the PCR reaction, it was performed a purification of the remaining 20µl of volume of PCR by Amersham Biosciences, GFXTM PCR – DNA and Gel Band Purification Kit. Purification protocol was performed as suggested by the datasheet of the kit.

SEQUENCING REACTION

The Sanger sequencing is settled on the selective incorporation/addition of chain-terminating nucleotides by DNA polymerase during the DNA replication. The sequencing reaction requires a single strand DNA as template, just one DNA primer, and Big Dye Terminator mix 3.1 (Perkin Elmer) containing Amplitaq DNA Polymerase, MgCl2, dNTPs, and modified dideoxynucleotidetriphosphates (ddNTPs). The ddNTPs nucleotides terminate DNA amplification because they miss the 3'-OH group necessary for the formation of a phosphodiester bond between nucleotides; in addition, ddNTP are fluorescently labeled with different colors:

Adenine	HEK green
Cytosine	FAM blue
Guanine	NED black
Thymine	ROX red

Amplitaq DNA Polymerase is characterized by two mutations, the first one in the active domain, that enables it to incorporate ddNTP in the DNA structure, and the second one in the N-terminus domain, which eliminates 3'-5' exonuclease activity.

Sequencing reactions of gene fragments were performed with both sense and antisense primers, to ensure to sequence the full length of the fragments. Then, after the sequencing reaction, products are purified by ddNTPs and salts using CENTRI-SEP Spin Columns (Princeton Separations).

ANALYSIS BY AUTOMATED DNA-SEQUENCER

The principle of automated DNA-sequencer is based on capillary electrophoresis, where molecules of DNA with different size migrate in solution under the influence of an electric field. The shorter the fragments are the fastest one and they are absorbed on the chromatographic column; after, the fragments are hit by a laser that will excite the fluorescent molecule bound to the ddNTP. As result of the excitation a signal that will be detected by the analyzer according to the four different colors. The analysis of the signals detected by the analyzer was performed by the software ABI PRISM® 310 Genetic Analyzer (Perkin Elmer Applied Biosystem, Foster City, USA) and sequence analysis was done by Sequencing Analysis Navigator (Perkin Elmer Applied Biosystem, Foster City, USA). The output obtained is an electropherogram, a profile of peaks of different color and height and identify the four DNA bases.

JAG1 IN VITRO ANALYSIS

CREATION OF THE PLASMID

JAG1 variants (p.R117G, p.F206Y, p.T931I, p.D1160N) were obtained by mutagenesis of the Ultimate™ ORF Clone containing the human cDNA of JAG1 (ID IOH36569 Invitrogen, Life Technologies™) by using GeneArt® Site-Directed Mutagenesis PLUS System (Invitrogen, Life Technologies™) (T et al. 2016). These JAG1 clones were then sub-cloned into a mammalian expression vector of pcDNA3.2/V5-DEST plasmid (Invitrogen, Life Technologies™) by Gateway® LR Clonase® II enzyme mix (Invitrogen, Life Technologies™). Each variant was confirmed by direct sequencing.

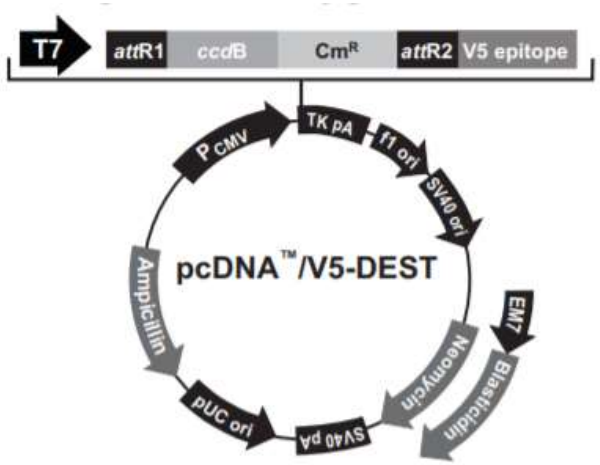

Fig. 33. *Jag1 plasmid (T et al. 2016)*

MUTAGENESIS PCR

Plasmids containing the 4 variants of JAG1 gene were generated by mutagenesis PCR of Ultimate™ ORF Clone containing the human cDNA of JAG1 (ID IOH36569 Invitrogen, Life Technologies™). DNA polymerase, which has a high proofreading activity, amplifies both

strands starting from synthetic oligonucleotides, of which one contains the mutation of interest. Primer pairs were 40 bp (table below) and designed to have same Tm. The sense primer carries the mutation at half of the total length and can bind specifically to the target sequence. The antisense primer is always WT and has a 20 bp-overlap with the coupled sense primer. The PCR products were checked by loading on 1% agarose gel with 1µl Gel Loading 6X and run applying a constant voltage of 130 Volt for 30'. Gel was then placed on a UV transilluminator and the interest band was identified by comparison with molecular weight 1kb DNA Ladder (Invitrogen).

R117G F	AGCCGCGGCAACGACGGCAACCGCATCGTGCTGC
R117G Rw	TGCCGTCGTTGCCGCGGCTGGCCTTGAGGTTGAAGG
F206Y F	TGCCGCCCCAGAGATGACTACTTTGGACACTATGCCTGTGACC
F206Y Rw	AGTAGTCATCTCTGGGGCGGCAGAACTTATTGCAGCCAAAGCC
T931I F	AGTGCTTCGTCCACCCCTGCATTGGTGTGGGCGAGTGTCGG
T931I Rw	ACCAATGCAGGGGTGGACGAAGCACTGGTCGTCCAGGATGG
D1160N F	TGAAGTAGAAGAGGACAACATGGACAAACACCAGCAGAAAGC
D1160N Rw	TGTTGTCCTCTTCTACTTCAGAATTGTGTGTCCTTATTTTAGAC

ENZYMATIC DIGESTION

PCR products were then digested with Dpn I (New England Biolabs) which, binding methylated sequence Gm6ATC, degrades WT templates in the reaction (E. coli DNA is indeed methylated) and leaves neo-synthetized DNA that contain the mutation. Seventeen µl of PCR products are digested for 2 hours at 37° C, with 10 units of Dpn I (1µl) and 2µl of NEB 4 buffer (50mM Potassium acetate; 20mM Tris-acetate; 10mM Magnesium acetate; 1mM DTT pH=7,9 at 25 °C) (New England Biolabs).

TRANSFORMATION OF BACTERIAL CELLS

One Shot® TOP10 (Invitrogen), E. coli competent cells, were transformed as recommended by the manufacturer.

PCR ON COLONIES FROM PLATES

At least 10 colonies were selected from LB agar plates. The growth occurs in LB plates with ampicillin 50mg/ml for 5 hours at 37°C, at 170 rpm. The clones were then identified by sequencing to verify if further mutations were inserted by random mutagenesis in the DNA.

Primers used for the different reactions are the following:

hJAG1_check_1Fw	CTGCGAGCCAAGGTGTGT
hJAG1_check_2Fw	ATGGGCCCCGAATGTAACAG
hJAG1_check_3Fw	GAGTGTGAGTGTTCCCCAGG
hJAG1_check_4Fw	CGCTGTATCTGTCCACCTGG
hJAG1_check_5Fw	AAGTGCAAGAGTCAGTCGGG
hJAG1_check_6Fw	AATGACTGCAGCCCTCATCC
hJAG1_check_7Fw	CTGAATGGACGGATCGCCTG
hJAG1_check_8Fw	TGTTCCCTTGCTGAGCTCTG
hJAG1_check_1Rev	CGCGGGACTGATACTCCTTG
hJAG1_check_2Rev	GGCACACACACTTAAATCCGT
hJAG1_check_3Rev	TGGAAGCCATGGCCACTGTG
hJAG1_check_4Rev	TCCTTGATCGGGTTCCCATC
hJAG1_check_5Rev	CCTCTTCTACTTCAGAATTGTG

MAXI PREP

To obtain the highest yield of plasmidic DNA of the selected clone, it was performed a plasmidic DNA extraction using PureYield^{TM} Plasmid Maxiprep System (Promega), following the manufacturer's datasheet. Plasmidic DNA obtained by the extraction was then quantified by spectrophotometer.

CELL COLTURE

GN11, GT1-7 and Cos-7 cells were grown in monolayer at 37°C under 5% CO_2 in DMEM (Invitrogen, Carlsbad CA, USA) containing 1mM Pyruvate, 2mM L-Glutamine (Invitrogen, Carlsbad CA, USA), 100 µg/ml streptomycin, 100 U/ml penicillin and 9 mg/ml glucose (MP Biomedicals, Santa Ana CA, USA), supplemented with 10% foetal bovine serum (complete medium). Cells were maintained below full confluence by trypsinization and seeding onto 10 cm^2 dishes.

TRASFECTION PROTOCOL

Protocol for 6 well plates:

Plate $2{,}5x10^5$ cell/well and transfect 24h later. Cells were transfected with Lipofectamine 3000 Reagent (Thermo Scientific, L3000015) according to the datasheet, using 1 µg of plasmid. Before the transfection replace the growing medium with the Serum Free medium and replace it with growing medium 5h after transfection.

Protocol for 10cm dish:

Plate cells and transfect 24h after with at least a 70% of confluency. Cells were transfected with Lipofectamine 3000 Reagent (Thermo Scientific, L3000015) according to the datasheet, using 5 µg of plasmid. Before the transfection replace the growing medium with the Serum Free medium and replace it with growing medium 5h after transfection.

WESTERN BLOT

Western Blot is used to detect specific proteins in a sample of tissue homogenate or extract. Proteins were extracted from cells using the RIPA buffer (10mM Tris-HCl pH 7.5, 500 mM NaCl, 0,1% SDS, 1% NP40, 1% sodium deoxycholate, 2 mM EDTA, 2 mM Na2VO4, 2 mM Na4P2O7, 2mM NaF). GN11 cells were plated (1×10^5) in 35 mm Petri dishes and transfected with 1 µg of DNA. Forty-eight hours after transfection, cells were washed with PBS and suspended in 200 µl of RIPA Buffer (10 mM Tris–HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) supplemented with protease inhibitors and lysed using a syringe with a small gauge needle. Homogenates were centrifuged at 4000 × g for 10 min at 4°C to remove cell debris. Protein content was assayed by the BCA protein Assay Kit (Pierce, Rockford, IL 61101 USA). Total proteins were fractionated by SDS electrophoresis on NuPage 4–12% Bis-Tris gel (Life Technologies) and electrotransferred onto nitrocellulose membranes (Hybond-C super, Amersham Biosciences, Bucks HP97 9NA, UK). After blocking with TBS supplemented with 5% non-fat dry milk and 0.1% Tween 20, membranes were incubated overnight at 4°C with mAb Jagged1 (28H8) Rabbit (cell signaling #2620), and anti-actine (#A5313 AB_476743, Sigma-Aldrich) as internal control. After three washings in TBS-0.1% Tween solution, membranes were incubated for 1 h at room temperature with a 1:10000 dilution of peroxidase-coupled goat anti-mouse antibody. Antibody–protein complexes were then detected using the Novex ECL Chemiluminescent substrate reagent kit (Life Technologies, Carlsbad, CA, USA) followed by autoradiography. For deglycosylation reactions with N-glycosidase F (PNGaseF), the manufacturer's instructions were followed (New England Biolabs, Westburg, Leusden, The Netherlands).

QUANTITATIVE PCR ON GN11 and GT1-7 CELL LINES

RNA extraction on GN11 and GT1-7 cell lines was performed using the E.Z.N.A. Total Rna Kit II (Omega Bio-tek, R6934-02) according to the protocol and the cDNA were reverse transcribed using SuperScript ® (Life technologies, Carlsbad CA, USA) following the recommended procedures. Real-Time OCR was carried out on Applied Biosystem 7900HT FasT Real Time PCR System using exon-span-specific TaqMan® Gene Expression Assay (Applied Biosystems, Carlsbad CA, USA). Following probes are used: Notch1 (Mm00627185_m1); Jag1 (Mm00496202_m1); Actb (Mm00607939) as housekeeping. Amperase activation was achieved by heating at 50°C for 2 minutes, before denaturing at 95°C for 20 seconds, followed by 40 cycles of 1second 95°C with 20 seconds of extension time at 60°C. Gene expression data were analyzed using SDS 2.4.1 and Data Assist 3.0.1 software (Applied Biosystems, Carlsbad CA, USA).

LUCIFERASE ASSAY

For the luciferase assay, GN11 cells were plated (1×10^5) on 35 mm Petri dishes and co-transfected with: 1ug of the hJAG1 constructs (hJAG1_WT or hJAG1_muts) or pcDNA3.2/V5-DEST empty vector as negative control; 0.5 ug of the firefly luciferase construct driven by six tandem copies of the RBP-Jk-response element (pGa981-6) and 20 ug of the Renilla pRL-TK Vector (Promega) as an internal control. Forty-eight hours after transfection Luciferases activity was measured with Dual-Glo® Luciferase Assay System (Promega) following manufacturer's instruction. Samples' luminescence was measured with the Fluoroskan Ascent FL multiplate reader. Six independent experiments were performed.

IMMUNOFLUORESCENCE IN VITRO ASSAY

IF was performed on two different samples: GN11 cells were growth in 6-wells plates and transfected according to transfection protocols previous described and human fetus section, obtained by cry-sectioning 9.5 gestation weeks old embryos.

For immunofluorescence assay on GN11 cells were used the anti-Jag1 1C4 Rabbit mAb (Cell Signaling) as primary antibody and Anti-Mouse IgG whole molecule–FITC produced in goat as secondary antibody. Pictures of microscope slides were taken using Nikon C2+®.

GN11 cells were plated (1×10^5) on sterile coverslips placed in 35 mm Petri dishes and transfected with 1 µg of DNA. Twenty-four hours after transfection, cells were washed with PBS, fixed with PBS containing 3% paraformaldehyde (Sigma-Aldrich St. Louis, MO, USA) for 10 min at room temperature and rinsed twice with PBS. Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), blocked with 5% goat serum (Invitrogen, Auckland, NZ), incubated with primary antibody Jagged1 (1C4) (Rabbit mAb #2155, ell Signaling) 1:100 and in goat serum (4G2) overnight at 4°C and incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, Life Technologies, Carlsbad, CA, USA) 1:1000 for 1 h at room temperature and a Phalloidin conjugated (Sigma-Aldrich, P2141, 50 µg/ml) was used to stain filopodia incubating 40 mins room temperature. Finally, cells were mounted with SlowFade Gold antifade reagent with Dapi (Life Technologies, Carlsbad, CA, USA). Images were acquired by using a laser scanning confocal system installed on a Nikon Eclipse Ti microscope with a 60× oil immersion objective. Alexa Fluor 488 was excited with a 488-nm argon laser and detected with a 525–550-nm band pass filter.

MIGRATION ASSAY

Transwell chambers were used according to manufacturer's instructions (Falcon). In brief, GN11 cells grown in complete medium until sub-confluence and re-suspended in SFM. Cells were seeded (50.000 cells/transwell) on the upper side of 8 mm pore membranes and incubated for 12 hrs with SFM, SFM plus human recombinant JAG1 (rhJagged1 Chimera, Cat Num 1277-JG, R&D system) or with DMEM supplemented with 10% fetal bovine serum for chemo-attractive assay. Differently, JAG1 was placed on the upper chamber of the transwell with GN11 cells, in SFM, to check its possible chemo-repulsive effect on GN11 cells migration. GN11 cells were incubated in both cases in presence of recombinant JAG1 (50 ng/ml and 100ng/ml). 12 hrs after cells on the upper side of the filters were mechanically removed and cells on the lower side fixed in methanol for 30 mins at 4°C before nuclei labelling with DAPI. Four non-overlapping regions were imaged per membrane using a Zeiss 20x objective (N.A. 0.8) mounted on a Axio Imager Z2 light microscope (Zeiss), with nuclei counted using an ImageJ plugin (National Institute of Health, Bethseda) and averaged to produce an average per well. The n for each experiment is detailed in the Fig.ure legends.

EXPRESSIONAL ASSAY ON HUMAN TISSUE

IMMUNOFLUORESCECE ASSAY

Human tissues were cryo-sectioned (Leica Cryostat) at 18 µm. Sections were thawed at room temperature and boiled at 80-90°C in the Citrate buffer (9ml Citric Acid buffer 0.1M + 41ml Sodium Citrate buffer 0.1M + water to 1L) for the antigen retrieval until the temperature reached 40°C. Slides were washed 3 times in PBS1X and incubated for three days in the primary antibody solution (PBS1X, 0.3% Tryton X-100, 2% Normal Donkey Serum) at 4°C.

slides were rinsed 3 times in PBS1X and incubated in the secondary antibody solution (PBS1X, 0.3% Tryton X-100, 2% Normal Donkey Serum) 1h at room temperature.

Primary antibody	Secondary antibody
Anti DLL1 Rabbit (abcam)	Alexa-fluor™ 568 Donkey anti-Rabbit IgG (Invitrogen)
Anti Jag1 Rabbit (Cell Signaling)	Alexa-fluor™ 568 Donkey anti-Rabbit IgG (Invitrogen)
Anti TAG1 Goat (R&D SYSTEMS)	Alexa-fluor™ 488 Donkey anti-Goat IgG (Invitrogen)
Anti GnRH Guinea Pig #1018 (created by Erik Hrabovszky)	Alexa-Fluor™ 647-conjugated AffiniPure Donkey Anti-Guinea Pig IgG (Jackson immune research)

After 3 additional rinsing the counterstain nuclei Hoescht 33528 was performed. It was diluted 1:1000 in H_2O, for 5 min, room temperature, protected from light. Slides were rinsed again before mounting with Mowiol. Pictures of the slides were taken using ZEISS LSM 710 AiryScan Confocal microscope.

RNA SCOPE

The RNAScope® Assays use a novel and proprietary method of in situ hybridization (ISH) to visualize single RNA molecules per cell in samples mounted on slides. The assays are based on the patented signal amplification and background suppression technology. Proprietary RNA-specific probes (RNAScope® Probes; available separately) are hybridized to target RNA and are then bound to a cascade of signal amplification molecules culminating in signal detection (Fig. 34).

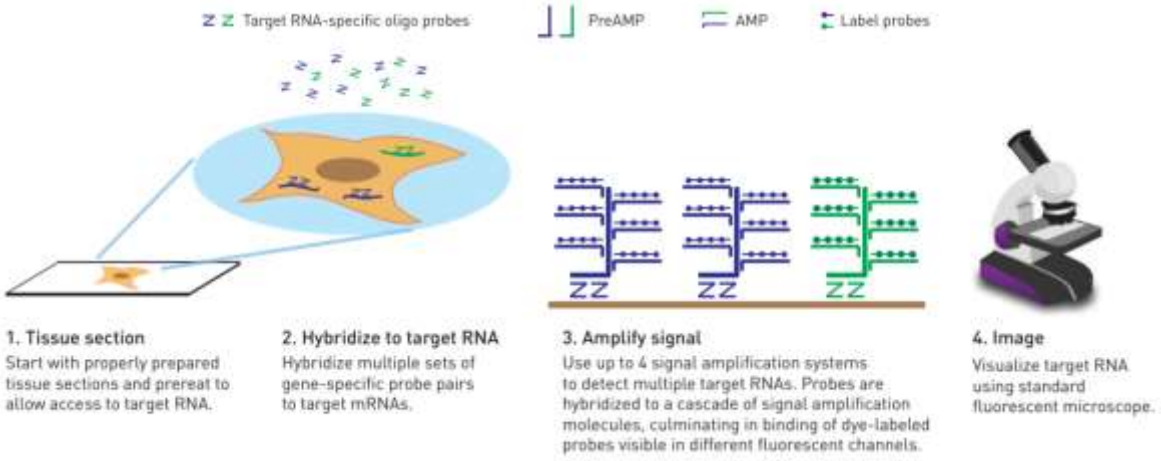

Fig. 34. *Four fundamental steps in RNAScope® technology.*

Single molecule fluorescent *in situ* hybridization was performed on frozen human embryonic head (GW 10) using the RNAScope® Multiplex Fluorescent Kit-v2 according to manufacturer's protocol. Specific probes were used to detect Jag1 (#546181), GnRH1 (#562591), Notch1 (#311861-C2), and 3-Plex Positive (#320861) as human positive control.

JAG1 IN VIVO ANALYSIS

FISH MAINTENANCE AND BREEDING

Zebrafish (Danio rerio) embryos obtained from natural spawning were raised and maintained following established techniques (Westerfiled, The Zebrafish Book, 2007), according to EU regulations on laboratory animals (Directive 2010/63/EU). All experimental protocols were carried out in accordance with relevant guidelines and regulations of Good Animal Practice approved by the institutional and licensing committee IACUC (Institutional Animal Care and Use Committee) and University of Milan by the Italian Decree of March 4th, 2014, n.26. Embryos were staged according to morphological criteria (Kimmel et al., 1995). From epiboly

stage (around the 6 hours-post fertilization, hpf) embryos were cultured in fish water containing 0.003% PTU (1-phenyl-2-thiourea; Sigma–Aldrich, Saint Louis, MO) to prevent pigmentation and 0.01% methylene blue to prevent fungal growth (Westerfiled, The Zebrafish Book, 2007).

The zebrafish *tg*(GnRH3:EGFP) line used in the present study was obtained by the Gothilf Lab (Tel Aviv University, Israel). As previously described, the *tg*(GnRH3:EGFP) line, in which GnRH3 neurons express the fluorescent reporter, allows *in vivo* study of the development and function of the forebrain gonadotropin-releasing hormone neuronal system in fish (Abraham, 2009). The zebrafish *tg*(GnRH3:EGFP/12xnre:mCherry) is a double reporter line obtain crossing the *tg*(GnRH3:EGFP) and the *tg*(12xnre:mCherry) gently provided by the Prof. Tiso Natascia (University of Padua, Italy). This reporter line was firstly generated as *tg*(12xNRE:EGFP) created by injection of a construct composed of six copies of the Epstein-Barr Virus Tp1 enhancer, each containing two Rbp-Jk binding sites, for a total of 12 Notch-responsive elements, placed in front of a murine beta-globin basal promoter driving EGFP (Parsons et al., 2009). This reporter, which consists of 12 repeats of Notch-responsive elements driving EGFP expression (Moro et al., 2013), is specifically responsive to Notch signaling, as manifested by its dose-dependent downregulation in response to DAPT treatment. Subsequently, the same group created the *tg*(12xnre:mCherry) but the data have not been published yet.

QUANTITATIVE PCR

Quantitative Polymerase Chain Reaction (qPCR) was performed on total RNA prepared from 20 zebrafish embryos at different developmental stages using TRIzol^{TM} (Thermo Fisher Scientific), and treated with DNaseI-RNase free (Roche, Basel, Switzerland) to avoid possible contamination from genomic DNA. RNA concentrations and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA).

Total RNA (1 µg) was reverse transcribed to produce cDNA using GoScriptTM Reverse Transcription System (Promega), following the manufacturer's instructions. In all cases, a reverse transcriptase negative control was used to test for genomic DNA contamination.

Quantitative Real Time PCR (qPCR) analysis was performed by ABI PRISMTM 7900HT Fast Real-Time PCR System) using SYBRGreen MasterMix (Invitrogen). The PCR reaction comprised an initial denaturation step at 95°C for 30s, followed by 40 cycles at 95°C for 5s, 60°C for 15s, and 72°C for 45s. The dissociation curve was used to check the specificity of PCR products.

For each developmental stage and for each tissue 50ng of cDNA were used. The elongation factor1-alpha1 (eEF1a1) gene was used as endogenous control. The quantification of target genes was normalized using the comparative CT method (also known as the ΔΔCT method), after ensuring that the targets and endogenous control had similar or relatively equivalent PCR efficiencies. Each experiment was conducted as a triplicate and repeated three times.

List of qPCR primers:

eef1a-Fw	5' CTGGTGTCCTCAAGCCTGGTA 3'
eef1a-Rw	5' ACTTGACCTCAGTGGTTACATTGG 3'
hoxA7a-Fw	5' GCTGGCGATCTCTGTAAAGC 3'
hoxA7a-Rw	5' TTTTGATGGTAGCCCCTCTG 3'
hoxA10b-Rw	5' GAGCTAAGGGGGTCCACTG 3'
hoxA10b-Rw	5' CACTTTGGAATCTCCTGCTTT 3'

CLONING ANTISENSE PROBES

To produce antisense probes used in this study, a fragment of *GnRH3, jag1a, jag1b, notch1a, notch1b, notch2,* and *notch3* has been generated by PCR starting from a sample of zebrafish genomic DNA, using the following primer sequences:

GnRH3 Fw	5’ AGCATGGAGTGGAAAGGAAG 3’
GnRH3 Rw	5’ AGCCCATCTGTTCCTTCAGT 3’
Jag1a Fw	5’ ATGGGAAGGAGCAACGTGTA 3’
Jag1a Rw	5’ AAGGTGAAGGTGACGTTTGC 3’
Jag1b Fw	5’ GAGTGTCAGTCTTCTCCCTG 3’
Jag1b Rw	5’ TCAGTTTTGGGGCTTGGTAT 3’
Notch1a Fw	5’ AGTAACGGCGGCGTGTGTCA-3’
Notch1a Rw	5’-TGATGCCACTGAAGCCCGCA-3’
Notch1b Fw	5’- ATCACGCCGGCTCCTAAGGT-3’
Notch1b Rw	5’- TGCAGAGCCGCCGAACAGTT-3’
Notch2 Fw	5’- ATAACGGCCGCTGTGACCCA-3’
Notch2 Rw	5’- TCCGTGTTGCAGCCCTGGTT-3’
Notch3 Fw	5’- AGCCCATCCTGCAGCCAACA-3’
Notch3 Rw	5’ TGGCTGTGAAGTTGCTGCGG-3’

The PCR products were cloned into the pGEM®-T Easy Vector (Promega), following the manufacturers’ instructions. The pGEM®-T cloning system provides linearized plasmid with covalently attached topoisomerase I for the fast insertion and ligation of any PCR product (Fig. 35). Vectors containing the different DNA of interest were introduced in bacterial *E.coli* cells (One Shot® TOP10 Chemically Competent E.coli, Life Technologies) using the heat

shock protocol, as previously described. Extraction and purification of plasmid DNA were performed using commercial kits provided by Promega (PureYeldTM Plasmid Maxiprep System), based on the alkaline lysis of bacterial cells to release plasmid DNA, followed by DNA binding end elution from silica matrix columns. High-quality plasmid DNAs were eluted form the column with 50-100 µl of nuclease-free water.

As the cloning system allow the desired PCR fragment to be inserted in the backbone without a preferential orientation, all clones have been sequenced with both T7 and SP6 universal primers.

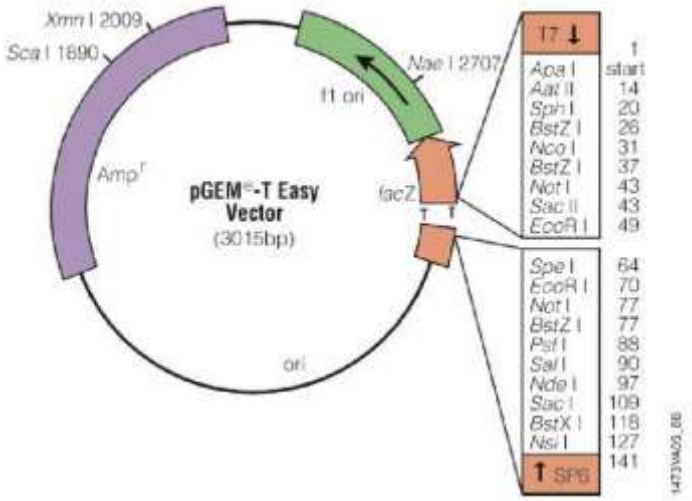

Fig. 35. *pGEM®-T Vector Map*

ANTI-SENSE PROBE SYNTHESIS

To generate an epitope-tagged antisense RNA probe against the targeted mRNAs, the plasmid DNAs were first linearized, using suitable restriction enzymes, a the 5’ end of the inserted PCR fragments. Linearized plasmids were then purified and the antisense RNAs were transcribed starting from the phage promoter (T7 or SP6) of the pGEM-T vector flanking the 3’end of the gene of interest. The nucleotide mix contained a part of digoxigenin-labelled

UTP, which has been incorporated by RNA polymerases to obtain the labeled antisense probes. Synthesis of mRNA probes were performed in a 20ul reaction volumes at a 37°C for 4 hours:

-linearized plasmid DNA (1 µg) Xµl

-10X transcription buffer (Roche) 2µl

-DIG-RNA Labeling Mix (Roche) 2µl

-RNAsin® Ribonuclease Inhibitor (Promega) 1µl

-(T7, or SP6) RNA Polymerase (Roche) 2µl

The purification of antisense RNA probes is performed using a commercial kit (MEGAclear™ provided by Ambion®). Once purified, the RNA probe can be quantified and stored at -80°C.

WHOLE MOUNT IN SITU HYBRIDIZATION (WISH)

Whole mount in situ hybridization is a technique largely used to study gene expression patterns. The method uses RNA complementarity to the endogenous RNA to generate an "antisense-RNA" labeled with a particular antigenic group, as previously described. These probes were hybridized to the embryo and visualized using anti-digoxygenin antibody conjugated to alkaline phosphatase. Various chromogenic substrates for alkaline phosphatase are commercially available and we routinely used NBT/BCIP (blue precipitate). In brief, the method used for in situ hybridization (Thisse and Thisse 2008) involved fixing the embryos with 4% paraformaldehyde (PFA)/ phosphate buffered saline (PBS), digesting with proteinase K to partially free the target mRNA from proteins, pre-hybridizing to block non-specific binding of probe to the fixed material, hybridizing with the probe and detect the probe enzymatically.

MORPHOLINO-BASED KNOCKDOWN EXPERIMENTS

In order to generate a loss-of-function phenotype for a known zebrafish gene during development it is possible to transiently inhibit the protein function by injecting antisense-oligos, so called morpholinos, into fertilized eggs (Nasevicius and Ekker 2000). The morpholinos are 20-25 bases chemical modified oligos that bind specifically to the 5'UTR region or early coding sequences of a given mRNA and block the translation. Morpholinos can also be designed to overlap splice sites and to block splicing (Draper, Morcos, and Kimmel 2001; Nasevicius and Ekker 2000).

Morpholinos used during this work were directed against zebrafish jagged1a (GenBank accession number: NM_131861) and zebrafish jagged1b (GenBank accession number: NM_131863) exon/intron boundaries. MO oligos were designed and synthesized by Gene Tools, Philomath, USA (www.gene-tools.com).

The sequences of MOs used in this work are as follows:

jag1a_MO-spl_E2i2: 5'-AAACAGCCTCTGAAACTCACCGGCC-3';

jag1b_MO-spl_E3i3: 5′-AATCCTGCTACTCACTTTCACTGGC-3′;

As a negative control we injected standard control morpholino (std ctrl-MO) that targets human β-globin gene, that has no target in zebrafish:

- std ctrl_MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

The stock solution (8 mg/ml) was prepared with nuclease-free water, as advised by Genetool's protocols, and kept at RT. Morpholino working solutions were prepared by diluting stock solutions in Danieau Buffer 1X (58 Mm NaCl; 0.7 mM KCl; 0.4 mM MgSO4, 0.6 mM (CaNO3)2, 5 mM HEPES, pH 7.6), adding 0.5 % Phenol Red (Sigma), to make the solutions visible during microinjection, and 0.5% rhodamin-dextran (Molecular Probes).

Rhodamin-dextran enables the selection of the embryos efficiently injected that can be scored for red fluorescence during gastrulation. Microinjection was performed using a micromanipulator (Micromanipulator 5171; Eppendorf), and a microinjector (Femtojet; Eppendorf). Different MO concentrations were tested in a range between 0.2 and 1 μg/ml.

From epiboly stage, the injected embryos were treated with 1X PTU to inhibit pigment formation. At 24 hpf, controls and morphants were anaesthetized using 1X Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt, SIGMA; stock tricaine 25X 0.08 g in 20 ml of distilled H2O) and placed under the microscope to analyze the MO-associated phenotypes. The confirm efficiency of MOs to affect the splicing of *jag1a*- and *jag1b* pre-mRNAs, the total RNA was extracted from pools of 30-50 embryos at 48hpf, retrotranscribed into cDNA and subjected to PCR and sequencing using the following primers:

jag1a_FOR	5'-TGTGGATGCGGGTATGTGAG-3'
jag1a_RW	5'-ATGCTGCCAGTGCTTGAGTC-3'
jag1b_FOR	5'-CCGAGGTCCTACACGTTGAT-3'
jag1b_RW	5'-GCAGATCGCTGTGTTGGCAGT-3'

Once chosen the desired MOs concentration, the injected embryos were raised to the desired stages for observations or collected and fixed in 4%PFA (4% paraformaldehyde in 1X PBS, Sigma), for further analysis.

WHOLE MOUNT IMMUNOHISTOCHEMISTRY (IHC)

To quantify the number of GnRH3-positive cells, embryos at 48 hpf were fixed in 4% PFA at 4°overnight, rinsed in 1X PBT (1X PBS, 0.1% Tween-20), and subjected to immunofluorescence according to a published protocol (Santos et al, Methods in Molecular

Biology, 2018) using the mouse anti-GFP (1:200; Origene) and the anti-mouse IgG-AlexaFluor 488 as secondary antibody (1:500, Life Technologies).

DAPT TREATMENT

For the inhibition of the Notch pathway, from 50% epiboly to 48 or 72 hpf, uninjected embryos were treated with 100µM DAPT (N-[N(3,5difluorophenacetyl)-l-alanyl]-S-phennylglycine t-butyl ester, Sigma) (Geling et al. 2002), or 1% DMSO as a control vehicle.

MOUNTING AND IMAGING

WISH embryos were mounted in 85% glycerol on a depression slide and acquired with a Leica M205FA equipped with Leica DFC450FC digital camera. Confocal acquisitions were performed using Nikon Eclipse Ti microscope equipped with a 20x objective. For IHC acquisitions, the embryos were flat mounted on slides provided with small chambers cut into several layers of adhesive tape (2 layers for 48 hpf and 3 layers for 72 hpf). The embryos were then placed in the desirable position and covered with glycerol and a cover slip. For the *in vivo* acquisition of GnRH3 fibers, the *tg*(GnRH3:EGFP) embryos were placed in a 35 mm imaging dish with an ibidi Polymer Coverslip Bottom (Ibidi) and covered with 1% low melting agarose gel (Sigma).

Digitalized pictures are saved as TIFF files, then adjusted for contrast, color balance and brightness using a Photoshop C2 Software.

STATISTICAL ANALYSIS

All data were shown as means ± standard error (SEM) or percentages. The t-student test was used to comparison of differences between groups. The $p<0.05$ was considered statistically significant (*$p<0.05$; ** $p<0.01$; ***$p<0.001$). All analyses were conducted with the software package GraphPad Prism 4.0 (GraphPad, San Diego, CA).

Results

IN SITU HYBRIDIZATION REVEALS CO-ESPRESSION OF JAG1, NOTCH1 AND NOTCH2 IN MIGRATING GNRH CELLS IN HUMAN FETUSES.

The expression of *Jag1* was previously reported in the OE of mouse at E12.5 by Cau et al in 2002 (Cau, Casarosa, and Guillemot 2002). In this study we have explored by multiplex fluorescent *in situ* hybridization the expression of *JAG1* (Fig. 35 B), *NOTCH1* (Fig. 35 C) and *NOTCH2* (Fig. 35 D) transcripts in coronal human fetal sections of the nasal compartment of a 8.5 and a 10 gestational week (GW) fetuses. *JAG1* expression was robustly detected in the apical layer of the olfactory epithelium, in a complementary pattern as compared to *NOTCH1*, which is expressed the basal layer (Fig. 35 E, F), while *NOTCH2* is predominantly expressed in the nasal mesenchyme (Fig. 35 G). *JAG1* and *NOTCH1* transcripts were detected also in the lumen of the vomeronasal organ (Fig. 35 B, C, F) and in cells belonging to the migratory mass (Fig. 35 D, E arrows). To verify if *JAG1* and *NOTCH1* expressing cells of the migratory mass were GnRH cells we have coupled the fluorescent *in situ* hybridization for *JAG1*, *NOTCH1* and *NOTCH2* with immunofluorescence experiments for GnRH. These experiments revealed that *JAG1*, *NOTCH1* and *NOTCH2* are expressed by GnRH neurons migrating across the nasal septum (Fig. 35 I, J). The expression of *JAG1* and *NOTCH1* was not only restricted to the GnRH cells but prominent *JAG1* and *NOTCH1* mRNAs was evident in other cellular types that coalesce with GnRH neurons (Fig. 35 E, F).

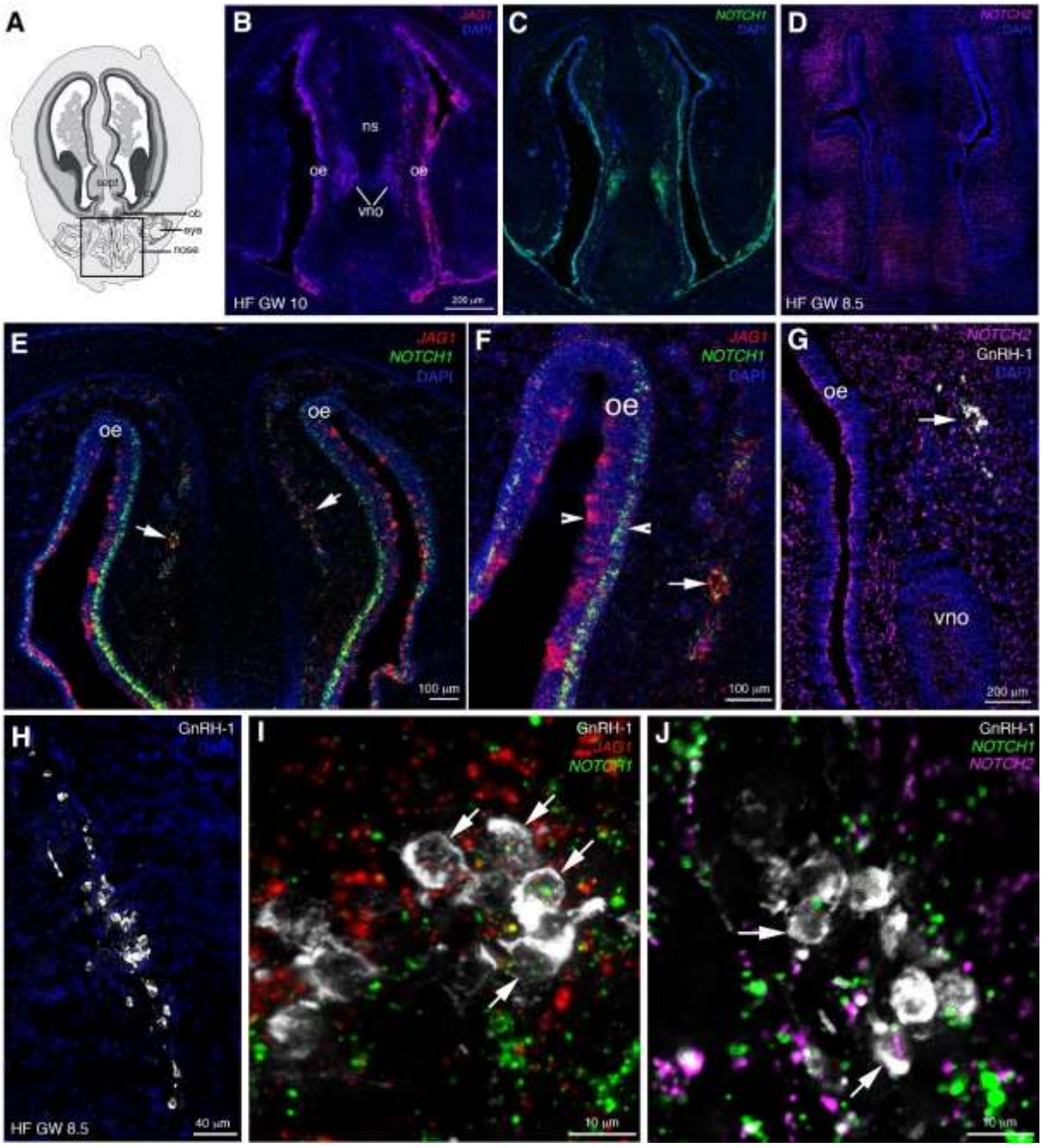

Fig. 35. ***JAG, NOTCH1 and NOTCH2 are expressed by GnRH cells and in the olfactory system of human fetuses***. ***A***. *Schematic representation of a GW10 human fetus head (coronal view) illustrating the nasal area (box) used for fluorescent in situ hybridization.* ***B-D*** *JAG1 (red), NOTCH1 (green) and NOTCH2 (purple) expression in a coronal section of a GW10 and 8.5 fetuses.* ***E-G*** *expression of JAG1(red), NOTCH1 (green) and NOTCH2 (purple) in the olfactory epithelium (OE). Arrowheads indicate the expression of JAG1 (red) in the apical layer of cells and NOTCH1 (green) in the basal layer of the OE. Arrows indicate JAG1 and NOTCH1 positive cells along the migratory mass.* ***H***. *GnRH (white) expression in the nasal septum in coronal section of a GW8.5 fetus.* ***I-J.*** *High magnification of GnRH (white) positive cells in the nasal septum co-expressing JAG1 (red) and*

NOTCH1 (green) and NOTCH2 (purple). VNO: vomeronasal organ; OE: olfactory epithelium; OB: olfactory bulb; NS: nasal septum; CX: cortex. Scale bar B, C, D, G 200 µm. scale bar E, F 100 µm. Scale bar H 40 µm. Scale bar I, J 10 µm.

JAG1 AND NOTCH1 PROTEINS ARE EXPRESSED IN GNRH CELLS AND IN THE MIGRATORY MASS IN EARLY HUMAN FETUSES.

We next performed immunofluorescence experiments for JAG1 and TAG-1 (transient axonal glycoprotein), which is a marker of the olfactory projections (Yoshida et al., 1995) and that was used to highlight the GnRH migratory scaffold. JAG1 protein was expressed in migratory GnRH cells (Fig. 36 A, D, arrows) as well as in other cells belonging to the migratory mass (Fig. 36 A, D, arrowheads).

Since there are no available specific antibodies against human NOTCH1 for immunohistochemical detection, we used an antibody against DLL1 (Delta-Like 1 ligand), a known ligand of NOTCH1 (Blaumueller et al. 1997). This antibody has been validated in a previous study (Greene et al. 2016).

DLL1 was observed in the migratory mass (Fig. 36 E, H, arrowheads), including GnRH cells in nasal region (Fig. 36 E, H, arrows) and in GnRH cells that migrated into the brain at GW11.5 (Fig. 36 J, L). Finally, to verify whether JAG1 was expressed by GnRH cells also in post-natal brains we performed a IHC (JAG1/GnRH) in coronal hypothalamic sections of a post-mortem brain from a 50 years old man (Fig. 36 N, P). We detected GnRH positive neurons in the infundibular region (Fig. 35 N), consistently with the literature (Skrapits et al. 2015). However, no JAG1 immunoreactivity was detected in those sections (Fig. 35 P) suggesting that JAG1 expression in human GnRH neurons is restricted to fetal development.

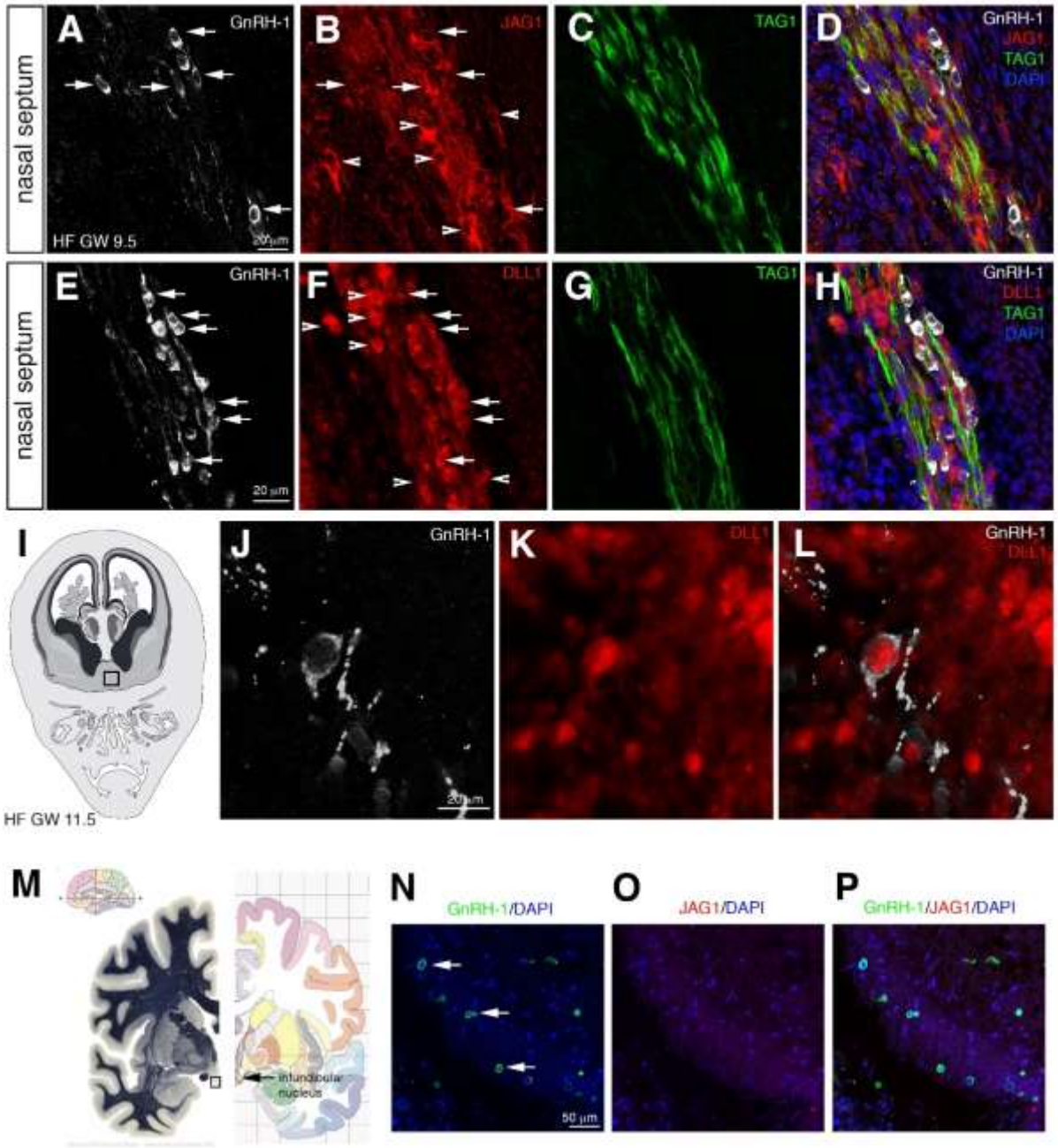

Fig. 36. ***JAG1 and DLL1 are expressed in GnRH neurons and in the olfactory system of human fetus***. ***A-C.*** *GnRH (white), JAG1 (red) and TAG-1 (green) expression in a coronal section of the nasal septum of a GW 9.5 human fetus. Arrows indicate GnRH cells (white) co-expressing JAG1 (red) and arrowheads indicate JAG1 positive cells in the migratory mass, co-expressing TAG-1(green).* ***D.*** *Triple immunolabeling for GnRH (white), JAG1 (red) and TAG-1 (green).* ***E-G.*** *GnRH (white), JAG1 (red) and TAG-1 (green) expression in a coronal section of the nasal septum of a GW 9.5 human fetus. Arrows indicate GnRH cells (white) co-expressing DLL1 (red) and arrowheads indicate DLL1 positive cells in the migratory mass.* ***H****. Triple immunolabeling for GnRH (white), DLL1 (red) and TAG-1 (green)* ***I.*** *Schematic representation of a GW11 human fetus head (coronal view) illustrating the forebrain (black box) used for immunofluorescent assay.* ***J-L.*** *High magnification of coronal section of the forebrain of a GW 11 human fetuses immunostained for GnRH (white) and JAG1 (red).* ***M****.*

*Schematic representation of an adult human brain coronal section. The black box highlights the infundibular nucleus, immunostained in **N-P** for GnRH (green) and JAG1 (red). **A-H, J-L** Scale bar 20 µm. **N-P** Scale bar 50 µm.*

JAG1A, *JAG1B*, *NOTCH1A* AND *GNRH3* mRNA ARE EXPRESSED IN THE OLFACTORY PLACODE OF ZEBRAFISH EMBRYOS AT 48HPF.

To evaluate the functionality of Jag1 *in vivo*, we took advantage of the zebrafish model, since Jag1 deficiency in mice results in embryonic lethality (Xue Y. et al., 1999). We first explored the expression of *notch* and *jag1* transcripts in zebrafish embryos. In zebrafish, there are two homologues of *JAG1* (*jag1a* and *jag1b*) (Elisabetta Zecchin et al. 2005) and 4 Notch receptors (*notch1a, notch1b, notch2* and *notch3*).

We found by whole mount *in situ* hybridization (WISH) that *jag1a* and *jag1b* were expressed in the olfactory placode in similar, but not identical patterns (Fig. 37 A). *Jag1a* was observed in the olfactory placode at 32 and 48 hpf, while *jag1b* transcript was detected in the same region at 48 hpf. Additionally, we found that *notch1a* transcript was also expressed, similarly to *jag1a* and *jag1b*, in the olfactory placode at 48 hpf (Fig. 37 B). Interestingly, the spatio-temporal expression of *jag1b* and *notch1a* seem to overlap with the documented expression of *GnRH3* mRNA (Bassi et al. 2020) (Fig. 37 C).

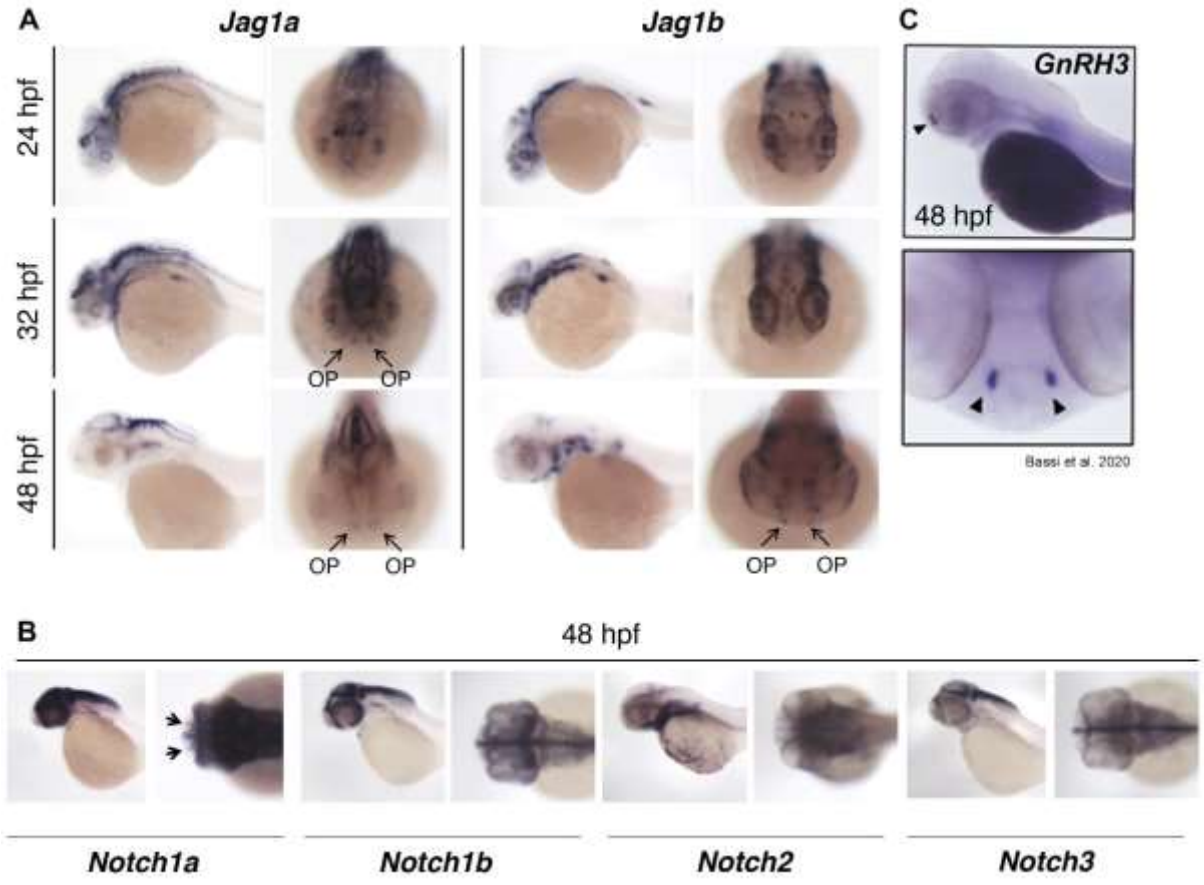

Fig. 37. ***Jag1a, jag1b, notch1a and GnRH3 mRNA was detected in the OP of zebrafish embryos at 48 hpf***. ***A.*** *Whole mount In Situ Hybridization (WISH) on zebrafish embryos at 24hpf, 32hpf and 48hpf showing the expression of jag1a and jag1b mRNAs in the OP (arrows).* ***B****. WISH on zebrafish embryos at 48hpf showing notch1a, notch1b, nootch2 and notch3 mRNAs expression. Arrows indicate the expression of notch1a in the OP.* ***C.*** *WISH on zebrafish embryos at 48hpf showing the GnRH3 mRNA localization in the OP (arrows). OP: olfactory placode.*

The Notch signaling expression in GnRH3 was also confirmed taking advantage of two zebrafish reporter lines: *tg*(GnRH3:EGFP) and *tg*(12xnre:mCherry). Crossing these two reporter lines it was possible to follow the expression of *GnRH3* and of the *notch responsive element* during early developmental stages. Analysis of the double reporter *tg*(GnRH3:EGFP x 12xnre:mCherry) showed that at 32 hpf there was a co-expression of EGFP and mCherry in the olfactory placode (Fig. 38 B, C).

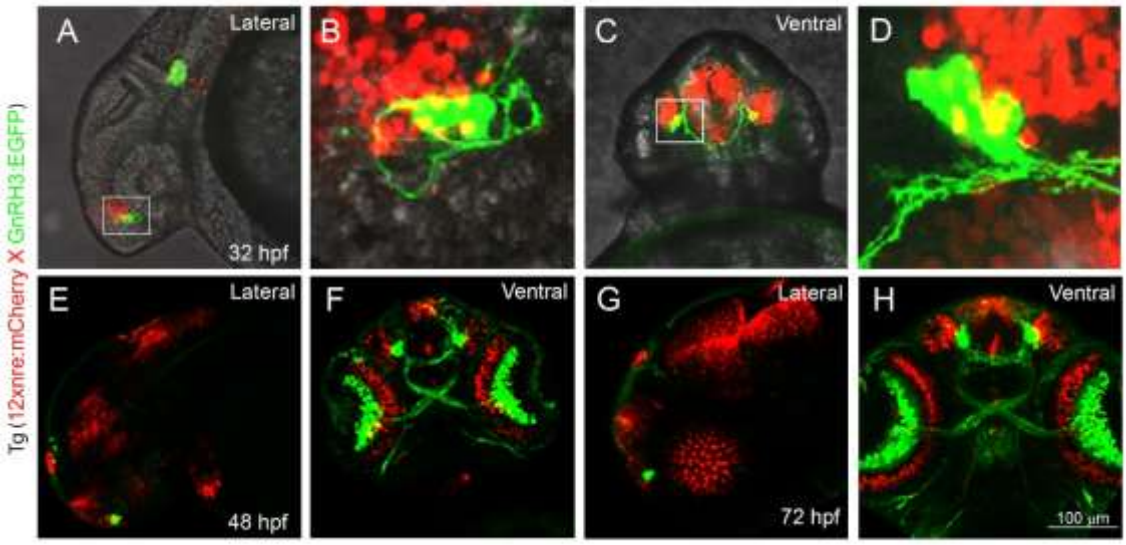

Fig. 38. ***tg(GnRH3:EGFP x 12xnre:mCherry) showed that at 32 hpf there was a co-expression of GnRH3 and notch responsive elements. A-C.*** *Lateral and ventral views of the tg(GnRH3:EGFP x 12xnre:mCherry) double reporter line at 32 hpf. The area depicted in the white boxes in* ***A*** *and* ***C*** *are shown at higher magnification in Fig.ures* ***B*** *and* ***D****, respectively.* ***E-F*** *and* ***G-H****, Lateral and ventral view of the tg(GnRH3:EGFP x 12xnre:mCherry) double reporter line at 48hpf and 72hpf, respectively. Scale bar 100 µm.*

JAG1A AND *JAG1B* SILENCING IMPAIR THE DEVELOPMENT OF GNRH3 NEURONS.

To define the function of *jag1a* and *jag1b* during the development of GnRH3 neurons, we used two antisense morpholino oligos (MOs, http://www.gene-tools.com/) to knock-down the expression of *jag1a* and *jag1b* (T et al. 2016). The *jag1a* and *jag1b* MOs were targeted against the splice donor sites of the second and third exon-intron boundary, which results in the loss of the majority of the exon 2 and the whole exon 3 (Fig. 39 A). The sequencing of these products revealed that these splice-blocking MOs activated cryptic splice donor sites and produced premature truncated jag1a and jag1b proteins (Fig. 39 B). The efficiency of both MOs was verified by PCR showing that increasing amount of MO induced a dose-related decrease of the WT product for both proteins (Fig. 39 C).

Silencing of *jag1a* and *jag1b* genes was evaluated taking advantage of the *tg*(GnRH3:EGFP) zebrafish transgenic line (gently provided by prof. Yoav Gothilf, Tel-Aviv University, Israel) and previously used in our Lab by Bassi et al (Sci Reports, 2020). The phenotype was evaluated by confocal analysis of alive *tg*(GnRH3:EGFP) embryos at 48 and 72 hpf, according to the temporal development of GnRH3 neurons in the reporter line (Zhao et al. 2013). In control animals at 48 hpf GnRH3 cells are located in proximity of the olfactory placodes (Fig. 39 D). Moreover, branches of GnRH3 axons depart from these perikarya and project to the anterior commissure and to the optic chiasm (Fig. 39 D). Injected embryos with *jag1a* and *jag1b* MOs showed profound alterations in the GnRH3 system (Fig. 39 D). GnRH3 fibers appeared highly disorganized as compared to WT animals, especially at level of anterior commissure and optic chiasm, where they were defasciculated. Additionally, GnRH3 expression in the retina and hypothalamus of *jag1a* and *jag1b* morphants was dramatically reduced compared to controls (Fig. 39 D). The migration of GnRH3 somata from the olfactory region is known to end around 72 hpf when GnRH3 neurons project to the final

hypothalamic compartment (Zhao et al. 2013). In agreement with those studies, we found that in control embryos at 72 hpf, several GnRH3 fibers innervate the hypothalamus (Fig. 39 D), while in the double morphants no GnRH3 fibers projected to the hypothalamus (Fig. 39 D). These data further suggest an involvement of jag1/notch signaling in the proper development and fiber projections of GnRH3 neurons.

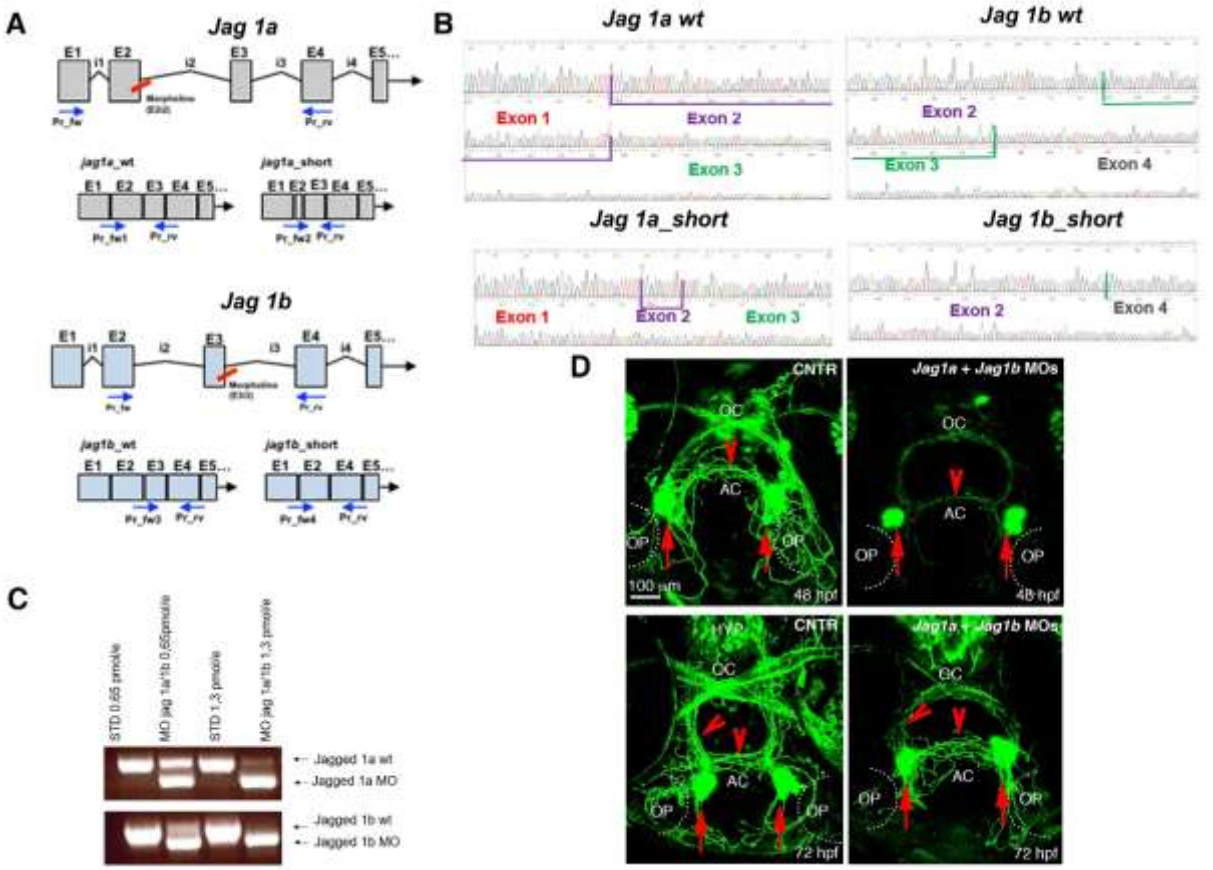

Fig. 39. ***Knock down of jag1a and jag1b affects the development of GnRH3 neurons. A****. The jag1a and jag1b premRNA and mRNA structures resulted from co-injection of jag1a (E2i2) and jag1b (E3i3) morpholinos were designed. Squared boxes indicate exon, and the black lines correspond to introns. The position of the primers used to analyze the altered splicing by RT-PCR or qRT-PCR is indicated by blue arrow.* ***B****. Sequences obtained from these primers have shown the effect for both morpholinos .* ***C.*** *PCR showing a dose-dependent reduction of the WT product and a dose-dependent increase of the short form of the proteins, according to injected dose of jag1a and jag1b morpholino.* ***D****. Confocal analysis of tg(GnRH3:EGFP) WT and jag1a and jag1b double morphant embryos at 48hpf and 72hpd, respectively. OP, olfactory placode; OB, olfactory bulb; AC, anterior commissure; OC, optic chiasma; Hyp, hypothalamus. Scale bar 100µm.*

SILENCING OF *JAG1B* BUT NOT *JAG1A* IMPACTS THE DEVELOPMENT OF GNRH3 NEURONS.

In the subsequent set of experiments, we aimed at understanding the possible contribution of *jag1a* and *jag1b* respectively in the development of GnRH3 neurons. To do that, we knocked down the expression of *jag1a* and *jag1b* in a single-MO silencing approach and we investigated the phenotype of these morphants by confocal analysis at 48 and 72 hpf. At 48hpf, control embryos (Fig. 40 A) displayed a normal development of GnRH3 system, as compared with *jag1a* morphants at 48hpf (Fig. 40 B). Inversely, *jag1b* morphants displayed severe anomalies of GnRH3 cell number, GnRH3 localization and GnRH3 fiber organization (Fig. 40 C). In particular, *jag1b* silencing induced a significant increase in the number of GnRH3+ neurons located in the OP as opposed to WT animals at 48 hpf (Fig. 40 D, E), a severely mis-positioned or scattered GnRH3+ cells in the same regions (Fig. 40 F), and a severe defasciculation of the GnRH3+ anterior commissure fiber network (Fig. 40 G).

We next investigated the development of GnRH3 neurons at 72 hpf. Control embryos displayed a normal GnRH3 neurons migration and GnRH3 fibers projections to the hypothalamus (Fig. 40 H). Similarly, to what we observed at 48 hpf, at 72hpf, silencing of *jag1a* did not result in any morphological changes of GnRH3 system development (Fig. 40 I). However, the *jag1b* morphants showed a disorganization of GnRH3 cell bodies' distribution in the olfactory compartment, and a defasciculation of GnRH3+ fibers in the anterior commissure and optic chiasm (Fig. 40 J). Moreover, no GnRH3 fibers innervated the hypothalamus in *jag1b* morphants (Fig. 40 J).

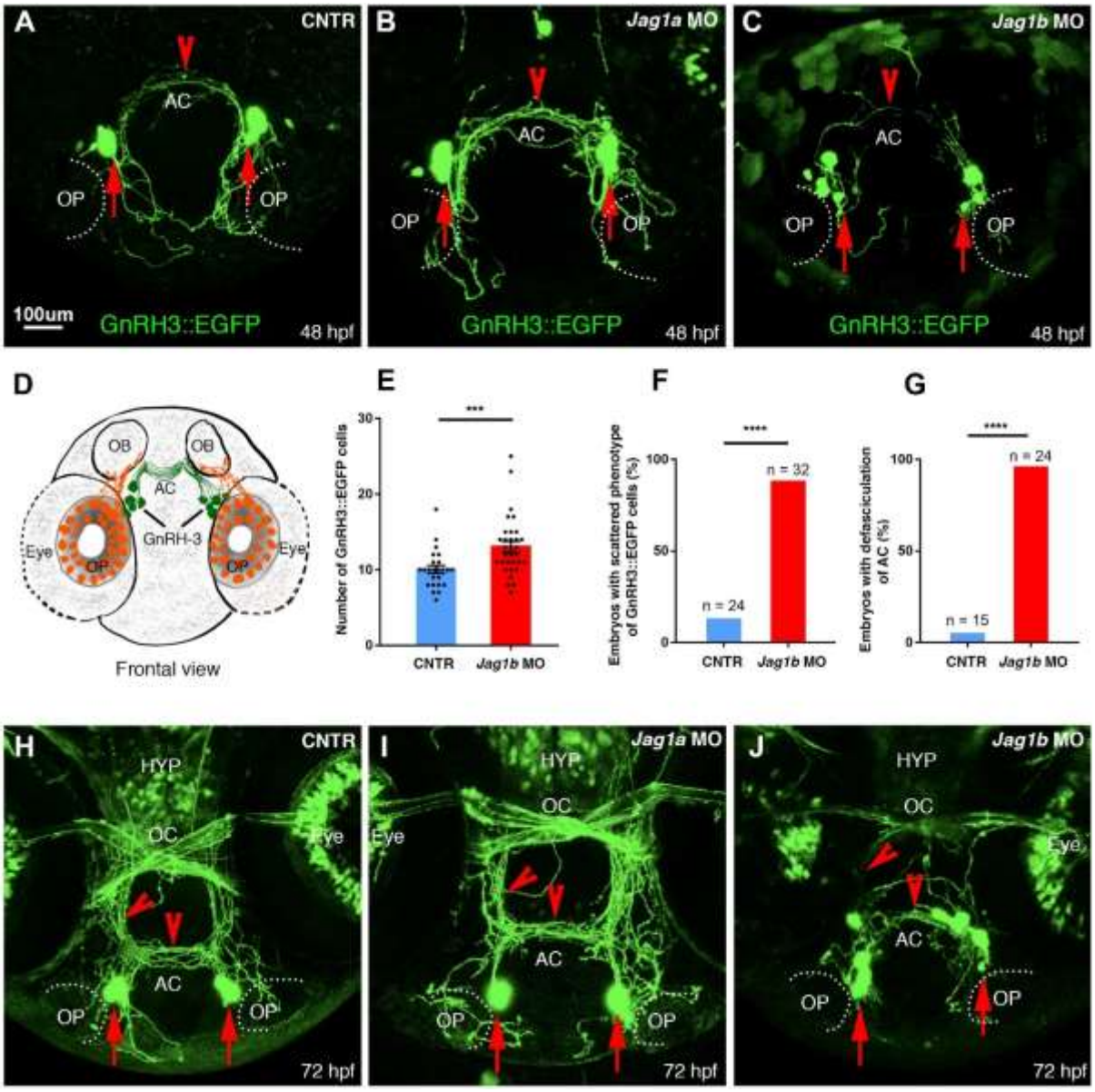

Fig. 40. ***Jag1b, but not jag1a, knock-down affects the GnRH3 neurons development inducing a reduction of the fluorescent signal in both embryonic stages***. ***A-C.*** *Confocal analysis of tg(GnRH3:EGFP) control embryos, jag1a morphants and jag1b morphants at 48hpf, respectively*. ***D.*** *Schematic picture showing the frontal view position of GnRH3 neurons (green) relative to the OP and OB in zebrafish embryos at 48hpf.* ***E-G.*** *Quantification of GnRH3+ cells number between control and jag1b morphants; percentage of embryos showing a scattered GnRH3+ cells phenotype between controls and jag1b morphants at 48hpf; percentage of embryos showing a defasciculation of the anterior commissure between controls and jag1b morphants at 48hpf.* ***H-J***. *Confocal analysis of tg(GnRH3:EGFP) control embryos, jag1a morphants and jag1b morphants at 72hpf, respectively . Arrows indicate GnRH3 positive cells in the OP; arrowheads indicate GnRH3 fibers in the AC and innervating hypothalamus. OP, olfactory placode; OB, olfactory bulb; AC, anterior commissure; OC, optic chiasma; Hyp, hypothalamus. Scale bar 100µm. Red arrows indicate the somata of GnRH3+*

*cells. Red arrow heads highlight the GnRH3+ fiber in the anterior commissure or innervating hypothalamus at 72hpf. Scale bar 100 µm. Mann Whitneytest and Fisher's exact. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$,*

Since notch signaling is known to properly shape embryonic development in several species including zebrafish (Dutta et al. 2008; Golson et al. 2009; High and Epstein 2008; Latimer and Appel 2006; Yamamoto, Charng, and Bellen 2010; E. Zecchin et al. 2007), we next evaluated whether impairment of notch/jag signaling pathway may result in delays of embryonic developmental stages. The morphology of embryos is an important indicator to properly assess the developmental stage. Morphological analysis of these embryos revealed no differences between morphants and controls at 24 (Fig. 41 A, B) and 48 hpf (Fig. 41 D, E) respectively. In agreement with previous studies (T et al. 2016), the *jag1b* morphants at 48 hpf had cardiac and brain edema (Fig. 41 E). Since, the most accurate strategy to check the stadiation of embryos is to measure the level of *hoxA7a* and *hoxA10b* genes, (Garaffo et al. 2015), we checked by q-PCR the expression of these genes in control and *jag1b* morphant at 24 (Fig. 41 C) and 48 hpf (Fig. 41 F). This analysis revealed no differences in the expression levels of *hoxA7a* and *hoxA10b* between control and morphant in both stages (Fig. 41 C, F). Taking together the morphological analysis and the qPCR data we have concluded that the phenotype observed in *jag1b* morphant is not resulting from a delay in the embryonic development.

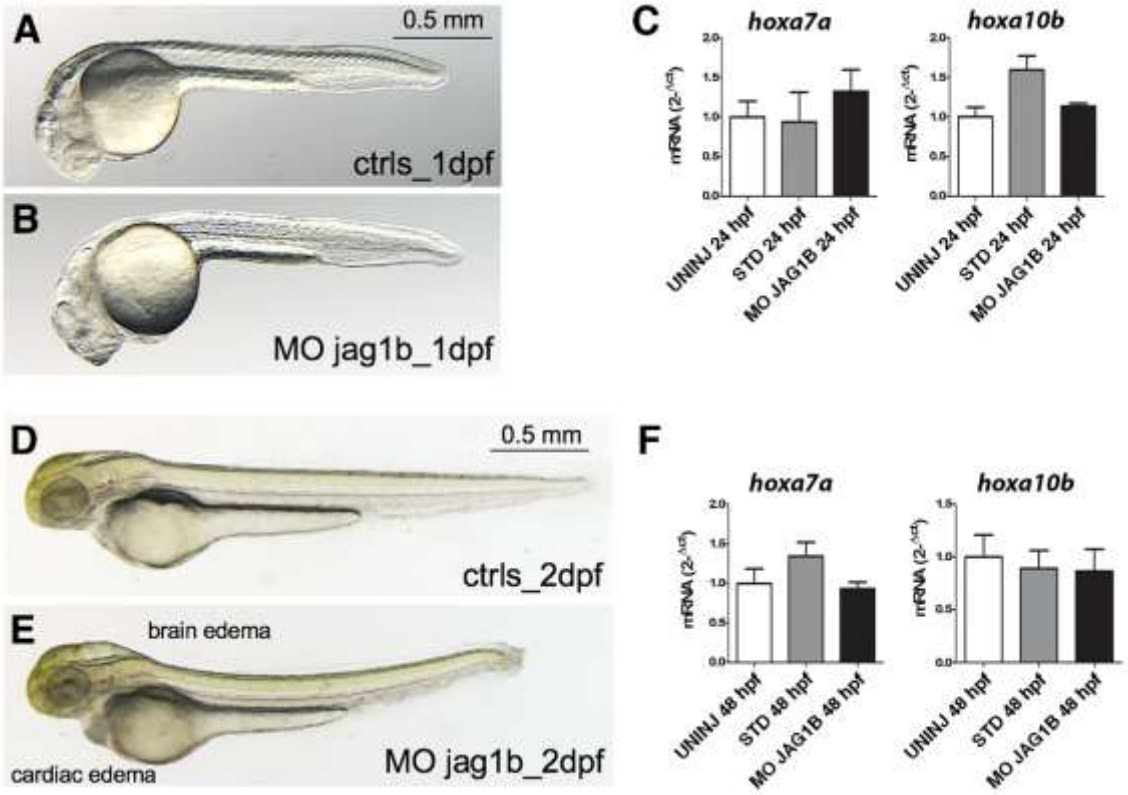

Fig. 41. ***The phenotype of jag1b morphants was specific and not related with an embryonic delay***. ***A, B -D, E.*** *Whole-mount bright-field image of control and injected embryos at 48hpf and 72hpf.* ***C, F.*** *Q PCR to measure the relative abundance of hoxA7a and relative abundance of hoxA10b mRNAs in whole embryos injected with jag1b MO, to monitor developmental progression and exclude a generalized delay. Scale bar 0.5 mm.*

PHARMACOLOGICAL INHIBITION OF NOTCH SIGNALING MIMICS THE ALTERATION IN THE GNRH3 DEVELOPMENT OBSERVED IN JAG1B MORPHANT.

The chemical compound (2S)-N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine 1,1-dimethylethyl ester (DAPT) is widely used inhibitor of Notch signaling since it blocks γ-secretase, preventing the release of NICD (Geling et al. 2002). Firstly, we used the *tg*(12xnre:mCherry) to establish the optimal doses at which DAPT silences Notch signaling. Control embryos (not-treated and DMSO-treated) displayed normal expression of the 12xrne reporter (Fig. 42 A, B). Differently, we observed that 50mM of DAPT induced a significant inhibition of the notch responsive elements as compared with untreated and DMSO treated embryos (Fig. 42 C). However, 100mM DAPT achieved total silencing of the signaling (Fig. 42 D) as previously reported in the literature (Geling et al. 2002). Using both doses the survive ratio was not affected, and the morphological phenotype with a bended tail observed was in perfect concordance with the data in the literature (Geling et al. 2002). To further investigate the role of Notch signaling in the development of GnRH3 neurons, we treated *tg*(GnRH3:EGFP) zebrafish embryos with the defined dose of DAPT (100mM). Controls embryos revealed normal development of the GnRH3+ fibers network (Fig. 42 E, H and F, I) (Zhao et al. 2013). Interestingly, the phenotype of treated embryos at 48 and 72 hpf phenocopies some aspects of the *jag1b* morphant (Fig. 42 G, J). Specifically, at 48 hpf DAPT-treated embryos displayed a disorganization of the GnRH3 somata in the olfactory placode area, a reduced number of fibers, a defasciculation of the anterior commissure, and absence of fibers in the optic chiasm and in the retina (Fig. 42 G). At 72 hpf the DAPT-treated embryos showed a severe phenotype (Fig. 42 J). The disorganization of the GnRH3 somata in the olfactory placode area, defasciculation of the anterior commissure were observed also in this stage. The optic chiasm and the signal from the retina were present, but the expression

pattern resulted strongly impaired (Fig. 42 J). Furthermore, we observed a dramatic impairment of GnRH3 fiber projections to the hypothalamus of DAPT treated embryos (Fig. 42 J) as compared to control groups (Fig. 42 H, I). These experiments confirmed the involvement of Notch signaling in the correct development of GnRH3 neurons.

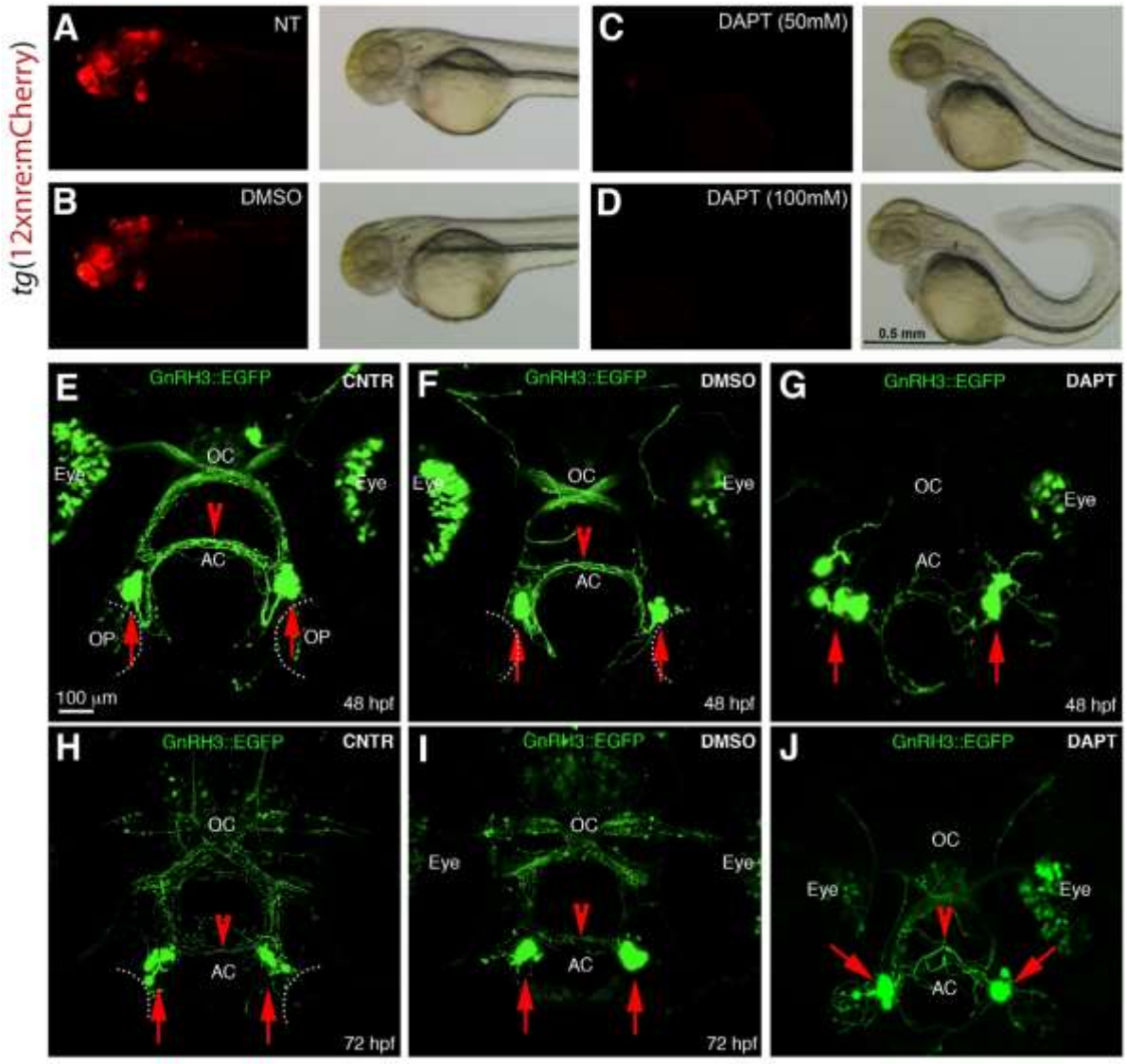

Fig. 42. ***DAPT treated embryos mimic the GnRH3 developmental defects of the jag1b morphant****. **A-D.** Confocal analysis of non-treated, DMSO-treated and DAPT-treated tg(12xnre:mCherry) embryos at 48 hpf. At 100mM the DAPT achieves complete inhibition of the Notch responsive element red signal.* ***E-G****. Confocal analysis of non-treated, DMSO-treated and DAPT-treated tg(GnRH3:EGFP) embryos at 48 hpf and 72 hpf. Arrows indicate GnRH+ cells in the OP, and arrowheads indicate the anterior commissure. OP, olfactory placode; OB, olfactory bulb; AC, anterior commissure; OC, optic chiasma. Scale bars 0,5 mm and 100 µm.*

JAG1 REPULSES GNRH MIGRATING NEURONS *IN VITRO*

Since JAG1 is highly expressed along the GnRH migratory pathway and NOTCH1 and NOTCH2 are expressed by GnRH migratory neurons in human fetuses and because *jag1b* silencing results in severe alterations of the GnRH3 system, we hypothesized that this molecule could affect GnRH directional migration.

To evaluate the functionality of JAG1 *in vitro*, we used two immortalized neuronal cell lines, GN11 and GT1-7 cells (Mellon et al. 1990; Radovick et al. 1991), which represent respectively migratory and post-migratory GnRH neurons. We first analyzed by qPCR the expression of *Jag1*, *Notch1*, *Notch2*, *Notch3* and *Notch4* mRNAs in these cell lines. Interestingly, GN11 cells express *Jag1*, *Noch1* and *Notch2* transcripts, consistent with our findings in human fetuses (Fig. 44 A). GT1-7 cells strongly express *Jag1*, *Notch3* and *Notch4*, whereas they do not express *Notch1* and *Notch2* (Fig. 44 B). Since GN11 express *Jag1* receptors, we took advantage of this cell line for subsequent functional studies.

We performed transwell migration assays and evaluated whether JAG1 promoted chemotaxis (chemoattraction or chemo-repulsion) of GN11 cells. Exposure to increasing doses of JAG1 (50 ng/ml, 100 ng/ml) plated in the lower chambers did not affect GN11 cell motility as compared to control conditions (serum free medium, SFM) (Fig. 44 B, C). Stimulation of GN11 cells with 10% FBS (fetal bovine serum, positive control) significantly induced the migration of GN11 to the lower chamber (Fig. 44 B, C).

To test whether JAG1 could potentially act as a repulsive factor for GN11 cell motility, we performed transwell migration assays by plating the same doses of JAG1 in only the upper chamber in serum-free conditions (SFM). JAG1 produced a significant increase in migration as compared to that in control conditions (Fig. 44 B, C). In combination with the Jag1b

morphants phenotype, these results suggest that JAG1 acts as a chemorepulsive molecule on GnRH cell migration.

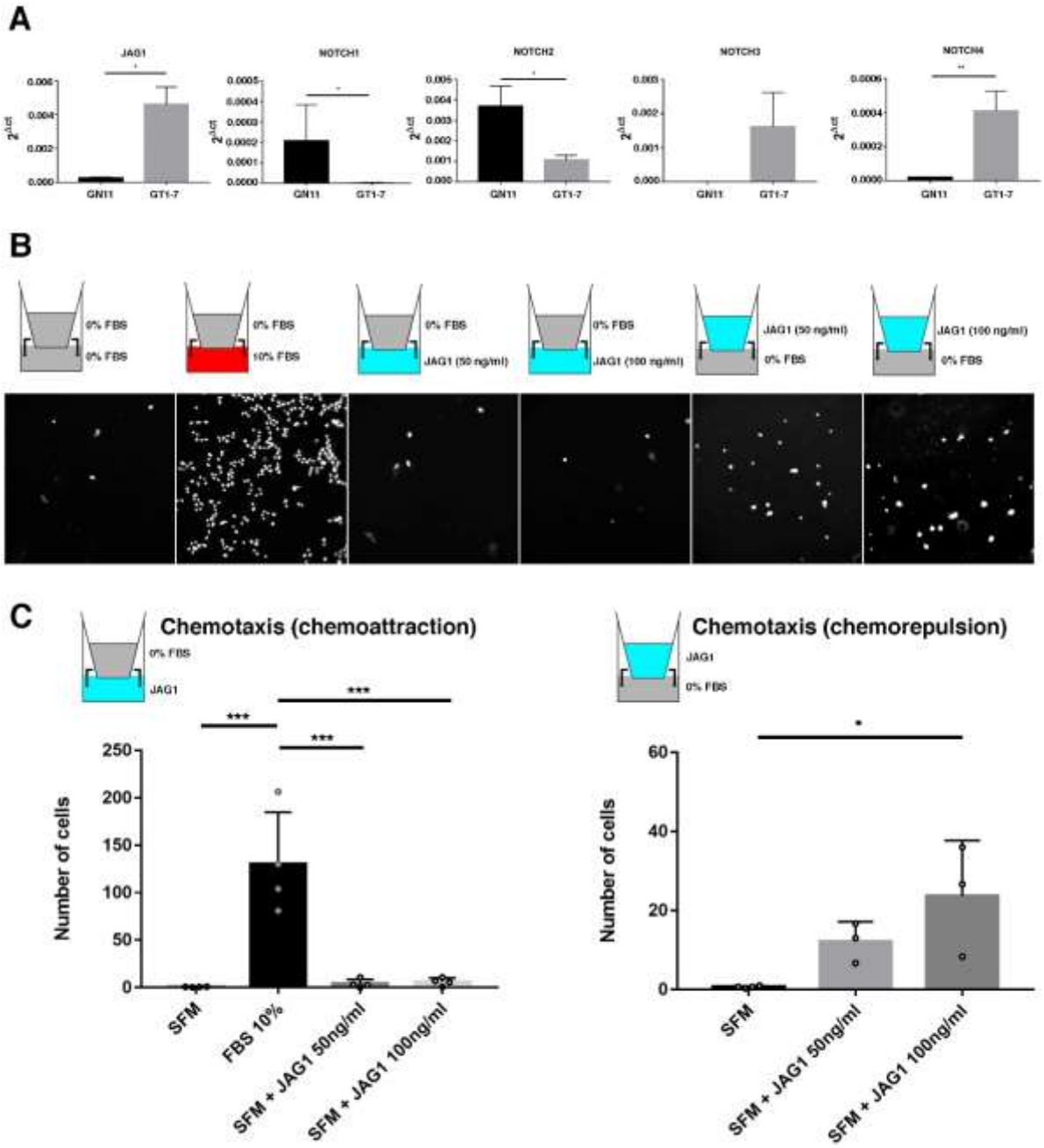

Fig. 44. JAG1 is has a chemorepulsive effect in the migration of GN11 cells. A. *QPCR in GN11 and GT1-7 cell lines showing the expressional levels of Jag1, Notch1, Notch2, Notch3 and Notch4 genes.* ***B, C****. Migration analysis on GN11 cells to verify the chemotaxis (chemo-attractive or chemo-repulsive) role of JAG1. *: p < 0.05, **: p < 0.01, ***: p < 0.001, one-way ANOVA test.*

INDIVIDUALS WITH CONGENITAL HYPOGONADOTROPIC HYPOGONDISM HARBOR JAG1 MUTATIONS

Interestingly one polymorphism in the *JAG1* gene, ligand of the Notch pathways, has been recently reported in one normosmic patient with CHH (Quaynor et al. 2016), suggesting a possible role of this gene in the development of GnRH neurons. Consistently, the NGS analyses conducted in our cohort of 127 CHH/KS cases revealed the presence of four rare (MAF<0,01) missense variants in the *JAG1* gene (p.R117G/c.C349G, p.F206Y/c.T617A, p.Y931I/c.C2792T, p.D1160N/c.G3478A). The clinical profile of male patients is reported in the Figure below (Fig. 44). The allelic variants identified were present in compound heterozygosity with other variants in candidate genes for CHH (Fig. 44). The missense variants identified in our patients were already reported in Ensembl (https://www.ensembl.org/index.html) where we verified that 3 out of 4 variants of JAG1 were already identified (p.R117G rs *not available*, p.F206Y rs1440630344, p.Y931I rs368032094, p.D1160N rs755047447) but never functionally characterized. The *in silico* analysis performed revealed that all the missense variants identified in our cohort impact on highly conserved residues and were predicted to be deleterious from at least 3 out of 6 bioinformatic tools (Fig. 44). Thus, we decided to proceed with the functional characterization of the above-described gene allelic variants.

ID	1	2	3	4
Diagnosis	nIHH	KS	nIHH	nIHH
Sex	M	M	M	M
Disease onset	PPO	PPO	AO	PPO
Associated phenotypes	Dental agenesis	Dental agenesis	none	Ogival palate and gynecomastia
JAG1 variants	p.D1160N	p.T931I	p.R117G	p.F206Y
Oligogenicity	SEMA3E (P701I)	SEMA7A (D514Y)	SEMA7A (R622H) SEMA3E (N432S)	IL17RD (P566S)
SIFT	T	T	T	T
Polyphen2	B	B	B	D
LRT	D	D	D	D
MT	D	D	D	D
MA	N	L	L	L
FATHM	D	D	D	T

Fig. 46. *Phenotype and genotype of four nCHH patients with heterozygous JAG1 mutations. N: neutral; L: low impact; T: tolerate; D: damage; B: benign; PPO: pubertal onset; AO: adult onset. M: male.*

THE D1160N VARIANT MISLOCALIZED INTO THE CYTOPLASM AND IS NOT FUNCTIONAL

To better assess the expression of JAG1 variants we have performed an immunofluorescence assay to investigate the cellular localization of the JAG1 variants overexpressed in GN11 cells (Fig. 44 A-F). The IHC experiment showed that the JAG1 WT (Fig. 46 A) and the variants R117G (Fig. 46 B), F206Y (Fig. 46 C) and Y931I (Fig. 46 D) are observed in the cell membrane of GN11 cells, as expected considering the canonical localization of transmembrane proteins. On the contrary, the D1160N variant of JAG1 is mislocalized (Fig. 44 E), and it is retained into the cytoplasm. Then, we performed a western blotting assay on total lysates from GN11 transfected cells, over-expressing the JAG1 variants: R117G, F206Y, T931I and D1160N. The WB showed that all JAG1 variants were correctly translated as compared with the WT JAG1 (Fig. 44 G).

In accordance with the IHC experiment, the luciferase functional assay (Fig. 46 H) revealed that JAG1 variants activate the notch responsive element in a comparable manner with the WT protein with the exception of p.D1160N, according to its cytoplasmic retention (Fig. 44 H). Combining the *in silico* analysis of this mutant, which predicts a damaging effect, with the *in vitro* functional analysis, we can conclude that the D1160N variant is likely pathogenic in the context of CHH.

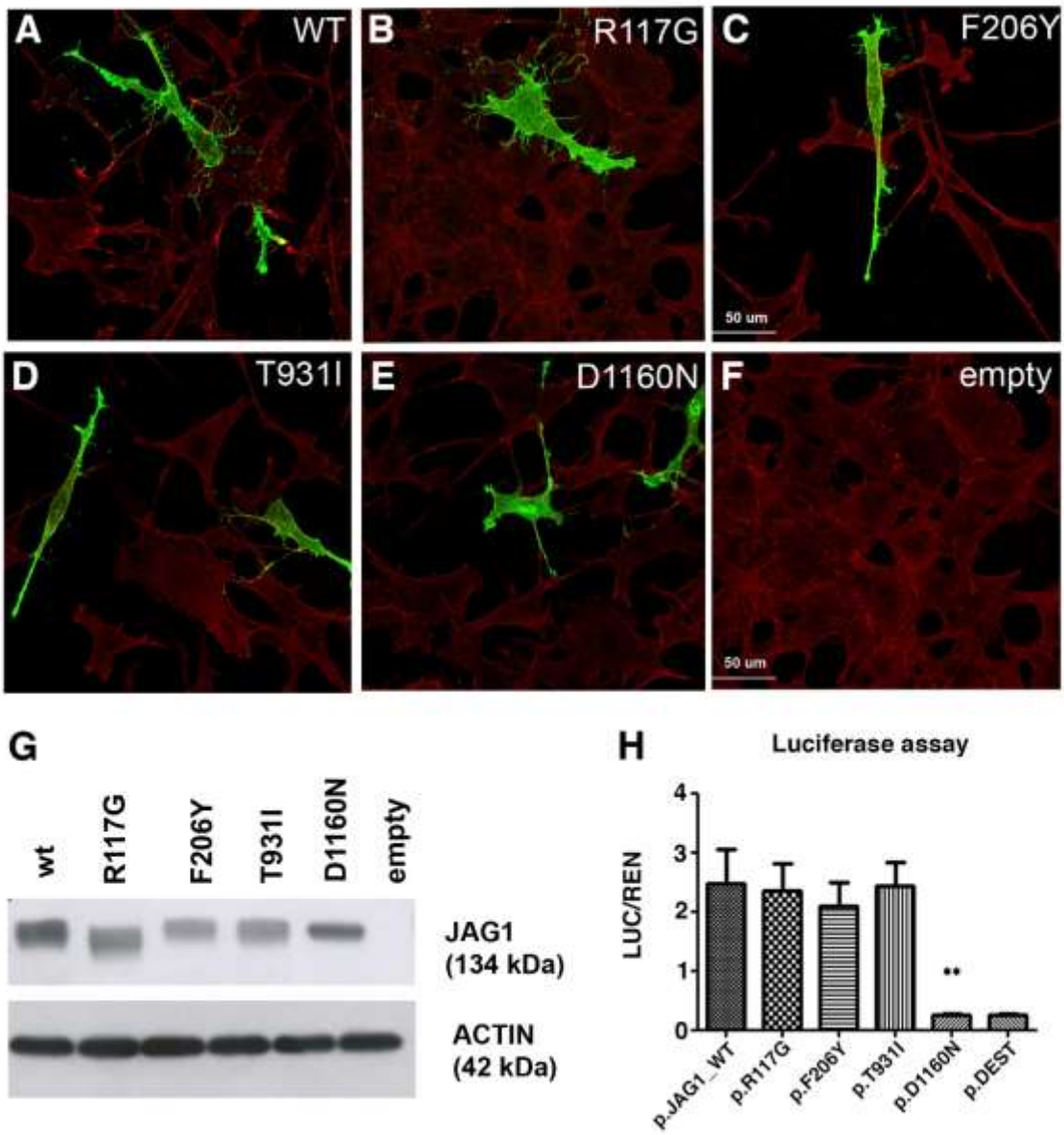

Fig. 46. ***Analysis of new JAG1 variants found in the cohort of CHH patients****. **A-F**. Immunofluorescence assay on GN11 cell line transfected with JAG1 WT and R117G, F206Y, T931I and D1160N variants. **G**. Western Blot of JAG1 and variants. On the bottom, ACTIN levels were measured and used to normalize JAG1 protein levels. **H**. Luciferase assay on WT and variants of JAG1, where it was evident that the D1160N variant is not able to activate Notch responsive elements, acting like the empty plasmid. One-way ANOVA *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.00$. Scale bar 50 µm.*

Discussion

Notch signaling pathway is one of the major regulators of cell fate, cell proliferation and cell death during development (Bray 2006). Interestingly enough, Hashimoto-Torii and colleagues demonstrated that Notch1/Jag1 signal works in synergy with Reelin to regulate neural migration and stratification of the cerebral cortex (Hashimoto-Torii et al. 2008), highlighting an additional role of Notch signaling in cortical neuronal migration. However, whether Notch signaling may regulate the migration of other brain cell populations during development remains unknown.

The aim of this thesis was to elucidate the possible role of Notch signaling in the development of the GnRH/olfactory system and to get insights into whether this is required for the proper migratory process of GnRH neurons from nose to brain and establishment of reproductive functions.

The expression of Notch1/Jag1 in the developing olfactory epithelium (OE) has been reported in several species, including mouse and chicken (Miller et al. 2016) and functional studies have shown that Notch1 and Jag1 play a role in the development of the olfactory ensheathing cells (OECs) (Miller et al. 2018). Interestingly, these cells are known to mingle with GnRH migratory neurons, as previous rodent studies demonstrated that OECs envelop GnRH neurons in the nasal compartment and that defects in OECs development result in an accumulation of GnRH cells in the nasal region (Barraud et al. 2013). It was also reported that interactions between Mash1 and Notch signaling are important for the specification of adult Olfactory Sensory Neurons (OSN) (Cau, Casarosa, and Guillemot 2002), supporting the relevance of Notch signaling in the development and the maintenance of the olfactory system of mice during both embryonic development and adulthood. In the studies described above, the expression of Notch1/Jag1 in the OE of mice parallels the expression pattern that we observed in fetal human noses at different early GW, with JAG1 being expressed in the apical region of the OE, in the lumen of VNO, in GnRH cells, and in cells in cells belonging to the

migratory scaffold its expression is significantly high. NOTCH1 is expressed in the basal layer of the OE, in the lumen of VNO, in GnRH cells and in cells belonging to the migratory mass. Moreover, NOTCH2 is expressed by GNRH cells and in fibroblasts of the nasal mesenchyme. These data highlight a possible paracrine and or autocrine function of the Notch signaling in the development of GnRH cells, either affecting the migration or axon elongation. The expression of JAG1/ NOTCH1/NOTCH2 in the different cellular components of the migratory mass requires further investigation. For instance, we do not know whether the human OECs express those molecules and immunohistochemical experiments using OECs markers, such as SOX10, are granted. It is also important to underline that for our NOTCH1 expression experiments we could not find commercially available anti-NOTCH1 antibodies specific for human tissue and compatible with immunofluorescence experiments. Therefore, a human DLL1 (Delta-like 1 ligand of Notch receptor) antibody was used as a surrogate marker of NOTCH1 expression (Blaumueller et al. 1997). From this first part of experiments, it was possible to conclude that NOTCH1, NOTCH2 and JAG1 mRNAs and proteins are expressed by GnRH cells, OE, VNO and cells belonging to the migratory mass of human embryos and that they could act in a paracrine and/or autocrine manner in the regulation of the early stages of GnRH neuronal development, comprising either cell migration or GnRH axonal elongation and targeting, or both.

The absence of JAG1 expression in a human postmortem brain (obtained from the CHU Hospital in Lille, France) further suggest that the role of Notch signaling on GnRH neurons could be restricted during developmental stages.

Jag1 knockout mice show vascular defects that result in early embryonic lethality, happening before E11.5 (Xue et al. 1999), when GnRH neurons are known to begin their migratory process (Wray et al., 1989; Schwanzel-Fukuda and Pfaff, 1989)Thereby, these animals cannot be used to address the function of Jag1 in GnRH neuronal system development. To

overcome this issue, we first analyzed the expression for *jag1* and *notch* receptors in zebrafish embryos. We document that *Notch1a, Jag* genes and *GnRH3* were expressed in the OP of zebrafish embryos at 48 hpf suggesting a possible interaction between these molecules in the GnRH3 neuronal development. This pattern of expression was also confirmed using the double reporter line *tg*(GnRH3:EGFP x 12xnre:mCherry) that showed the EGFP and mCherry reporters are both activated in the OP area at 32 hpf. We then silenced *jag1a* and *jag1b* in zebrafish and we provided evidence that *jag1b* knock-down results in strong alterations in the GnRH3 neuronal development. Specifically, *jag1b* morphants presented a higher number of GnRH3+ cells in proximity of the OP, a scattered distribution of these cells and a profound disorganization of the GnRH3 fibers network. These data were confirmed by a pharmacological treatment using the well-characterized Notch-inhibitor, DAPT (γ-secretase inhibitor). Interestingly, pharmacological inhibition in *tg*(GnRH3:EGFP) embryos mimicked several aspects of the *jag1b* morphants, confirming the relevance of the Notch signaling in the development of GnRH3 cells. Altogether, these results suggest a role for Jag1b in GnRH3 cell fate specification, GnRH3 cell migration and axonal elongation.

Herein, we show that Jag1 and Notch receptors are expressed in the nasal region after formation of the olfactory placode in humans and zebrafish. Furthermore, their spatio-temporal expression pattern overlaps the GnRH neuron migratory route. In addition to the Jag1 expression pattern, the effect of recombinant JAG1 on GnRH neuronal motility *in vitro* and the migratory defect of GnRH3 neurons in *jag1b* morphants provide strong evidence for a role of JAG1 as a chemo-repulsive molecule for GnRH neuron migration. Here we speculate that JAG1 maybe secreted by different cell types of the GnRH migratory route (i.e. OECs, ORNs, GnRH cells) and it could play an important role in initiating GnRH neuron migration by pushing these cells from the nasal region toward the forebrain.

The target sequencing analysis for the known CHH/KS candidate genes by the TruSeq Custom Amplicon assay (Illumina) in our cohort of patients (n=127) identified four rare (MAF<0.01) allelic variants, all in the heterozygous state, in the *JAG1* gene (p.R117G, p.F206Y, p.Y931I, p.D1160N). These *JAG1* variants were already reported in the database of Ensembl (https://www.ensembl.org/index.html), excepted for the R117G. These *JAG1* heterozygous allelic variants are found in 3,15% of CHH probands in our cohort (4 out of 127). This is consistent with the genetic feature of CHH in which the majority of known CHH genes have a low mutational prevalence (<5%) (Boehm et al., 2015; Cangiano et al, 2020). Over the past few years, the traditional Mendelian view of CHH as a monogenic disorder has been revised following the identification of oligogenic forms of CHH (Sykiotis et al. 2010). CHH is genetically heterogeneous, with both sporadic and familial cases and several modes of inheritance have been identified, including X chromosome-linked recessive, autosomal recessive and dominant (Boehm et al. 2015; Cangiano et al, 2020). To date, around 60 (Cangiano et al., 2020) associated loci have been implicated in Kallmann syndrome and/or CHH etiopathology (Fig. 14 in the Introduction), accounting for ~50% of cases (Boehm et al. 2015; Cangiano et al, 2020). This implies that other possible candidate genes are yet to be discovered. Consistently with the oligogenic basis of CHH, our mutations are associated with mutations in *SEMA3E, SEMA7A and IL17DR* (Fig. 43 in the Results). The D1160N variant of *JAG1* and heterozygous mutations in *SEMA3E* (P701I) were found mutated in one patient. *SEMA3E* was already reported in the literature (Cariboni et al. 2015) as causative gene for KS and this paper authors identified a different mutation in the *SEMA3E* (p.R619C) gene than the one that we found in our cohort. Additionally, *SEMA3E* and *CHD7* are related with CHH and CHARGE syndrome, this latter being considered as a severe form of KS, revealing a cross talk between these two syndromes (Lalani et al. 2004). Differently, the p.T931I variant of *JAG1* is associated with a heterozygous gene variant in *SEMA7A* (p.D514Y). Sema7a ortholog is involved in GnRH neuron migration in mouse and *in vitro* models, being widely

expressed both in the nasal placode and along the olfactory/vomeronasal axonal guide and having a central role in the regulation of GnRH cell motility (Messina et al. 2011). SEMA7A binds Plexin-C1 and induces a decrease in the integrin-mediated cell attachment and spreading (Messina and Giacobini 2013). In a second study, the authors found two *SEMA7A* heterozygous rare missense variants (p.R148W and p.R474Q) in CHH patients, coupled with mutation in other genes, confirming the possible known oligogenic feature of CHH (Kansakoski et al. 2014). The p.R117G variant of *JAG1*, the unique variant absent in Ensembl database showed a segregation with both *SEMA3E* (p.R622H) and *SEMA7A* (p.N432S). Finally, the p.F206Y variant of *JAG1* is associated with another allelic variant in *IL17RD* (p.P566S). A previous study identified *IL17RD* mutations in KS individuals and they were strongly linked to hearing loss (6/8 individuals), confirming a possible association between CHH and hearing defects (Miraoui et al. 2013).

Among the four identified, only the p.D1160N gene variant was found to be retained into the intracellular compartments of GN11 cells, suggesting that it might affect the maturation process of JAG1. However, confirmatory experiments are planned to determine whether JAG1 is accumulated in endoplasmic reticulum or in other intracellular organelles. Even more interestingly, the luciferase assays showed that the p.D1160N variant loses the ability to activate Notch responsive elements, further supporting the notion that this gene variants behaves indeed as loss-of-function. Our findings are in agreement with the previous above-mentioned study reporting *JAG1* variants in CHH/KS patients (Quaynor et al. 2016). In this work DNAs from CHH/KS patients (17 males and 31 females) were analysed by targeted next generation sequencing (NGS) of 261 genes involved in hypothalamic, pituitary, and/or olfactory pathways, or suggested by chromosome rearrangements. In particular, the researchers found a mutation in *JAG1* c.323A > T, p.N108I, which we did not identify in our cohort of patients. Interestingly, in the same paper, the authors also found a mutation in

NOTCH1 c.2333C > T, p.T778I in a CHH patient. However, no functional experiments were performed in this paper to validate the relevance of those gene variants.

As the current study is limited by the small number of probands harbouring *JAG1* variants, confirmation in larger CHH cohorts will be necessary to establish the specific contributions of *JAG1*, and possibly of *NOTCH* receptors genes, in the pathogenesis of CHH and possibly associated them to specific sub-phenotypes of CHH. Currently, in collaboration with Prof. Nelly Pitteloud (University of Lausanne, Switzerland), we are performing Whole Exome Sequence (WES) analysis in 340 of CHH/KS patients and we identified n=10 new *JAG1* variants, different from the ones identified in our laboratory. Functional *in vitro* studies are currently ongoing to validate the potential relevance of these mutations and to assess whether some of these variants are loss-of-function in different experimental paradigms (i.e. GnRH cell motility, GnRH secretion, protein processing and maturation).

In conclusion, we have identified JAG1 as a novel factor involved in GnRH neuronal system development in humans and zebrafish. Identifying the molecular signaling pathway used by JAG1 in GnRH neuron ontogeny (cell fate specification) and migration, along with the which particular receptors and downstream targets are involved, will be critical for future investigation. Furthermore, it will also be crucial to study the role of JAG1 in GnRH axonal elongation to establish whether this molecule could also be involved in the correct targeting of GnRH axons and/or in GnRH secretion.

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