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GENERATION OF GONADOTROPIN-RELEASING HORMONE NEURONS FROM HUMAN PLURIPOTENT STEM CELLS

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ABSTRACT

The onset of puberty and sexual development as well as normal reproductive function are dependent on pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH is secreted from the GnRH neuron nerve terminals in the hypothalamic median eminence into the portal vessels that lead to the anterior pituitary. The pulsatile secretion of GnRH stimulates the release of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), which, in turn, regulate various gonadal functions. In rare occasions, the onset of puberty is delayed or completely absent. This can be caused by disrupted development, migration, or function of GnRH neurons, resulting in defects in sexual development and infertility. Congenital GnRH deficiency is termed congenital hypogonadotropic hypogonadism (CHH), and CHH combined with hyposmia or anosmia (reduced or absent sense of smell) is known as Kallmann syndrome (KS). CHH and KS are genetically heterogeneous diseases, with over 30 genes reported in association with KS and CHH to date. How mutations in these genes cause GnRH deficiency is not yet comprehensively understood, but several are postulated to affect GnRH neuron development.

Human pluripotent stem cells (hPSCs) are the equivalent of undifferentiated cells in the early embryo, and able to give rise to all tissues and cell types in the human body. Thus, hPSCs have become a widely used tool for studying the differentiation of specialized cell types and the causes for human diseases *in vitro*.

Developing methods for directed differentiation of hPSCs into GnRH neurons requires insight into the events which lead to the specification of GnRH neurons during embryonic development. GnRH neurons are born in the olfactory placodes in the nasal area of the developing embryo. After their delamination from the olfactory neuroepithelium, the differentiated postmitotic GnRH neurons take an upward migratory route along the axon fibers of the terminal nerve around the olfactory bulb, cross the cribriform plate to the forebrain, and finally make a ventral turn into to the preoptic area of the hypothalamus. The exact cell type within the olfactory placodes that gives rise to GnRH neurons is not entirely known. Its precursors have been proposed to be of both preplacodal ectoderm and neural crest origin.

The aim of this work was to create a model in which to study the molecular mechanism of GnRH neuron differentiation and the mechanisms of CHH-associated genetic mutations on GnRH neurogenesis in humans. The literature review of this thesis addresses the relevant background in the field of GnRH neuron development; from neural crest and preplacodal development at gastrulation stages, to ontogeny, migration, and maturation of GnRH neurons at puberty. This thesis presents experimental validation of the methods for *in vitro* generation of GnRH neurons from human pluripotent stem cells, and the findings include the discovery of several genes and proteins expressed during GnRH neuron differentiation.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications (I-III):

I Noisa P, Lund C, Kanduri K, Lund R, Lähdesmäki H, Lahesmaa R, Lundin K, Chokechuwattanalert H, Otonkoski T, Tuuri T, Raivio T. “Notch signaling regulates neural crest differentiation from human pluripotent stem cells.” *Journal of cell science*, 2014 May 1; 127(Pt 9):2083-94

II Lund C, Pulli K, Yellapragada V, Giacobini P, Lundin K, Vuoristo S, Tuuri T, Noisa P, Raivio T. ”Development of gonadotropin-releasing hormone-secreting neurons from human pluripotent stem cells.” *Stem cell reports*, 2016 Aug 9;7(2):149-57

III Lund C, Yellapragada V, Vuoristo S, Balboa D, Trova S, Allet C, Eskici N, Pulli K, Giacobini P, Tuuri T, Raivio T. “Characterization of the human GnRH neuron developmental transcriptome using a GNRH1-TdTomato reporter line in human pluripotent stem cells. “ *Disease models & mechanisms,* 2020 Mar 13;13(3): 10.1242/dmm.040105

In addition, some unpublished data are presented.

ABBREVIATIONS

AMH = anti-Müllerian hormone

APC = adenomatous polyposis coli (gene)

ARN = arcuate nucleus

BIO = 6-bromoindirubin-3'-oxime, GSK3 inhibitor

BMP = bone morphogenetic protein

CDGP = constitutional delay of growth and puberty

CHARGE = coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities

CHH = congenital hypogonadotropic hypogonadism

ChIP = chromosome immunoprecipitation

CNS = central nervous system

CRISPR = clustered regularly interspaced short palindromic repeats

CS = Carnegie stage, human embryo development

DAPT = N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DCC = Deleted in colorectal cancer (gene), Netrin-1 receptor

DKK1 = Dickkopf WNT signaling pathway inhibitor 1

DM = Dorsomorphin

DISCO = 3D imaging of solvent-cleared organs

DCX = Doublecortin

ECC = embryonic carcinoma cell

EDTA = ethylenediamine tetraacetic acid

E (nn) = embryonic days, mouse development

EMT = epithelial-to-mesenchymal transition

ESC = embryonic stem cell

FACS = fluorescence-activated cell sorting

FBS = fetal bovine serum

FGF = fibroblast growth factor

FSH = follicle-stimulating hormone

GnRH = gonadotropin-releasing hormone

GSIs = gamma-secretase inhibitors

GSK3-β = glycogen synthase kinase 3β

GW = gestational week (of pregnancy)

hCG = human chorionic gonadotropin

HGF = hepatocyte growth factor

HPG = hypothalamus-pituitary-gonad (axis)

hPSC = human pluripotent stem cell

iPSC = induced pluripotent stem cell

IVF= *in vitro* fertilization

KEGG = Kyoto encyclopedia of genes and genomes

KS = Kallmann syndrome

LH = luteinizing hormone

LIM = Lin11/Isl1/Mec3 domain proteins

mESC= mouse embryonic stem cell

NC = neural crest

NICD = Notch intracellular domain

OE = olfactory epithelium

OP = olfactory placode

ORN = olfactory receptor neuron

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PFA = paraformaldehyde

pNCC = premigratory neural crest cells

qPCR = quantitative real-time polymerase chain reaction

RE = respiratory epithelium

ROCK = rho-associated protein kinase

RP3V = rostral periventricular nucleus of the third ventricle

SDF1 = stromal cell-derived factor 1

TGF-β = transforming growth factor β

TN = terminal nerve

VNO = vomeronasal organ

Wnt = wingless-related integration site

WT = wild type

INTRODUCTION

Embryonic GnRH neurons were first described as a postmitotic, migratory group of cells travelling from the olfactory placodes towards the forebrain (Wray, Grant & Gainer, 1989a, Wray et al., 1994). Their precursors are thought to arise in the neuroepithelium of the developing olfactory organ in the developing face. We still to this day do not know the previous events leading up to the delamination of postmitotic GnRH neurons from the neuroepithelium, and no definitive, selective GnRH neuron progenitor markers have been discovered. Therefore, the precursor subtype that gives rise to GnRH neurons has never been precisely defined. Several theories of their origin and development have emerged in a few models of different animal species.

A stem cell has a capacity to give rise to new, more specialized cells, or self-renew, and retain differentiation potential. Their fate depends on the time and place, the signals received from the environment, and intrinsic genetic and epigenetic mechanisms, leading to combinations of signal interpretations inside the cells. Therefore, when kept in appropriate culture conditions, pluripotent stem cells retain the potential to differentiate into any specialized cell type. This feature can be utilized in research, for modelling development and disease of specialised tissues.

REVIEW OF THE LITERATURE

1. Human pluripotent stem cells as a research tool

1.1 Blastocyst stage embryos

The human embryo reaches the blastocyst stage in five to six days after fertilization. At this stage the embryo is a ball-shaped hollow cellular structure with a single layer of outer cells (trophectoderm) and a small cluster of inner cell mass (ICM) cells inside the fluid filled sphere. Further in the development, the outer trophectodermal cells contribute only to extraembryonic tissues such as placenta, while ICM cells are pluripotent and serve as starting material for the entire embryo. As development proceeds to gastrulation the ICM goes on to form three germ layers, which give rise to all the tissues and organs in the body (Hill, 2020) (Figure 1 A).

1.2 Embryonic stem cells *in vitro*

The derivation of pluripotent stem cells into laboratory culture was inspired by studies of teratomas in the 1950s. Teratomas are extraordinary tumors that can contain different types of tissue, such as hair, teeth, and bone. These tumors are otherwise rare but were found to be more common in a specific inbred mouse strain, ‘129’ (Stevens, Little, 1954). The tumor cells from the 129 mouse line could be easily grown and expanded *in vitro* as immortal cell

lines, called embryonic carcinoma cells (ECCs) (Kleinsmith, Pierce, 1964). The ECCs were undifferentiated *in vitro*, and when transplanted into new mice, they would give rise to new teratomas. These findings led to further interest toward potential methods to capture cells from an early stage embryo to utilize their innate embryonic properties of pluripotency also *in vitro*. In 1981, Gail R. Martin described the isolation of mouse blastocyst ICM cells and their subsequent culture *in vitro* as mouse embryonic stem cells (mESC). The cells were maintained and expanded in culture as pluripotent stem cell colonies and like ECCs, the undifferentiated mESC were capable of giving rise to teratomas when transplanted into extrauterine tissues. The term 'embryonic stem cell' was thus introduced for the first time, and the authors made certain predictions into the future of this scientific field; *"Given these results, it seems likely that there will soon be available pluripotent, embryo-derived cell lines with specific genetic alterations that should make possible a variety of new approaches to the study of early mammalian development"* (Martin, 1981).

After injection into blastocyst embryos mouse ECS were able to contribute to multiple adult tissues of chimeric mice (Bradley et al., 1984), which thereby became a golden standard test for assessing stem cell pluripotency. In the 1990's, research proceeded into primate embryonic stem cells, which further confirmed and generalized the ability of ESCs to retain their pluripotency during *in vitro* cultures (Thomson et al., 1995).

Targeted gene editing of mouse ES cells, and transfer to the inner cell mass to create a mosaic embryo, was first shown by Schwartzberg and colleagues, who reported germline transmission of the genetic mutation in the mice chimeras by homologous recombination (Smithies et al., 1985, Capecchi, 1989, Schwartzberg, Goff & Robertson, 1989). Mario Capecchi, Sir Martin J. Evans, and Oliver Smithies were awarded the Nobel Prize in Physiology or Medicine 2007 because of their research in the principles of homologous recombination, which made it possible to produce mice with modified genomes using ES cells. These advances played a key role for researcher's ability to study the function of genes, and to model genetic diseases in mice. Since then, new genome editing techniques have emerged, up to the latest discovery of CRISPR-Cas9 gene editing-based on the defense system of bacteria against viruses, allowing for faster, cheaper, efficient, and precise gene editing, also in human pluripotent stem cells (Ran et al., 2013).

1.3 Human embryonic stem cells and induced pluripotent stem cells

In 1998, the first human pluripotent stem cells were isolated and cultured from blastocyst embryos donated from a fertility clinic. The cell lines described in this report would later become the source of the most widely used human embryonic stem cell lines grown *in vitro* (Thomson et al., 1998).

A new significant break-through in the field of pluripotent stem cells took place in 2006, when the first induced pluripotent stem (iPS) cells were generated from mouse fibroblast cells (Takahashi, Kazutoshi, Yamanaka, 2006). It was found that by the ectopic expression of only four factors, *Oct3/4, Sox2, Klf-4*, and *c-Myc*, the somatic cells could be reprogrammed into a stem cell stage and thus reverted back to pluripotency. The cells were consequently capable of teratoma formation, and they contributed to a chimeric fetus after injection into blastocyst embryos. Not long after, the same team succeeded also in the generation of human iPS (hiPSC) cells from skin fibroblasts (Takahashi, K. et al., 2007). This allows the use of patient-specific stem cells to study diseases, and potentially develop new treatments. Today hiPSCs are widely used to model the molecular mechanisms of disease-associated genetic mutations, by differentiation of hiPSCs to specific cell types of interest. Also, hiPSCs have potential for future use in regenerative medicine and tissue replacement therapy. With current genome editing tools, a patient's genetic mutation can be corrected in hiPSCs (Gravesteijn et al., 2020). In the future, this could be used to create autologous tissue replacements from the patient's own cells, to overcome immune rejection that can arise in organ transplantations.

1.4 Development of culture conditions and differentiation techniques

After the discovery of hESCs and hIPSCs, the cells were typically cultured on mouse, or later human, fibroblast feeder cells (Hovatta et al., 2003), and cell culture media contained fetal bovine serum (Eguizabal et al., 2019). This was later found to be a cause of variability between experiments. In order to develop more defined culture conditions that reduce the risk of infection during potential clinical use, a few important steps were taken; I) by moving to feeder-free cultures, II) the use of serum free media and, III) animal free (xenofree) products (Ludwig et al., 2006, Chen et al., 2011, Nakagawa et al., 2014). Gradual improvements to culture conditions has thus allowed for a new field in stem cell research; the directed differentiation into specialized human cell types *in vitro*. For example, the reporting of BMP pathway inhibition using small molecules during neuronal lineage differentiation has greatly improved differentiation efficiencies from human pluripotent stem cells to neurons (Chambers et al., 2009).

The future for hPSCs now lies in creating new innovative treatments and personalized medicine based on what we have learned from stem cell research during the last decades. Progress in the field of biotechnology has led to development of microfluidic devises that can be integrated with cells in 3D culture, referred to as 'organ-on-chip' technology. This technology allows mimicking of tissue environments and physiological characteristics on a micro-scale *in vitro*. This way we can limit the need for animal models, simultaneously allowing for personalized disease modelling in human-specific settings (Kimura, Sakai & Fujii, 2018). In the field of neuroscience, brain organoids or so called 'mini brains' are 3D cultures

generated from hPSC-derived neuronal cells. Brain organoids are cell aggregates that integrate several cell types and can be used to study aspects such as neuronal connections, electrical activity, and cell patterning, which holds great potential for modelling neuropsychological disorders, epilepsy, stroke etc. in a patient-specific context (Marton, Pasca, 2019).

1.5 Directing neuronal differentiation

The events leading to development of the embryonic nervous system (Figure 1 A) are essentially similar in all vertebrates, and have been extensively studied in amphibian and avian embryos since the early 1900s. In the 1920's, Hilde Mangold and Hans Spemann introduced the term 'embryonic induction', describing how the development of neural ectoderm of an amphibian embryo becomes induced, by a cluster of cells referred to as the 'organizer' ((Spemann, Mangold, 2001) (Orig. publ. 1923)). Later, the two Finnish researchers Lauri Saxén and Sulo Toivonen made important contributions to the field of embryology by presenting the two-gradient hypothesis for induction of the nervous system. Their model described the requirement of gradients of two opposing chemical signals, derived from nearby tissues, to induce and pattern the structures of the nervous system (Saxén, Toivonen, 1961). Today we know that induction is achieved by secreted signalling molecules, which form morphogen gradients that instruct and pattern the tissue as it develops. During late gastrulation, the primitive node and the notochordal process act as the primary inductors of the nervous system (Figure 1 A). The primitive node forms from cells at the tip of the primitive streak, and during primitive streak elongation, a transient tissue condensation forms the notochord at the axial midline. The notochord induces neural plate formation in the overlying ectoderm by expression of signalling molecules noggin and chordin. The induction and patterning of the developing nervous system is thus achieved by the activation and repression of the main inductive signalling pathways, BMP (bone morphogenic protein), FGF (fibroblast growth factor), SHH (sonic hedgehog), and Wnt (Figure 1 B). The signalling molecules and their receptors, in turn, activate or inhibit the expression of cell type-specific transcription factors that regulate the determination and specification of a cell, from a multipotent progenitor, to a lineage-restricted cell type (Carlson, 2019).

Bone morphogenic proteins (BMPs) are secreted peptide hormones that induce growth of bone (osteogenesis), as the name implies. In surface ectoderm of the gastrula embryo, BMP signalling induces the specification of the epidermis. This also means that it is necessary to locally repress BMP in the neural plate for the neuroectoderm to form. Neural induction is achieved by secreted proteins that bind to and antagonize the effect of BMP signalling, such as noggin, chordin and follistatin (Hawley et al., 1995, Fainsod et al., 1997, McMahon et al.,

1998). Neuralization of the surface ectoderm is thus the in essence the ‘default state’, unless epidermis becomes induced by BMP (Hemmati-Brivanlou, Melton, 1997).

For directing neuronal specification from pluripotent stem cells *in vitro*, Noggin has been found to be sufficient for neuronal conversion. This was first reported using embryoid body (EB)-based neuronal differentiation method (Elkabetz et al., 2008). The small molecule inhibitor SB43152 was later reported to be stable and efficient in neural conversion of pluripotent stem cells (Smith et al., 2008). Small molecule SB43152 antagonizes Activin/Nodal signalling through the Nodal receptor. Nodal is a member of TGFβ superfamily, and like BMP, it represses neuronal cell fates.

A more efficient and reproducible means of neuronal specification was enabled by the combination of dual SMAD inhibition and feeder-free monolayer culture (Chambers et al., 2009) (Figure 1 C). Synergistic inhibition of endogenous SMAD signalling by noggin and SB43152 in stem cells plated in high density 1) efficiently suppressed BMP signalling in order to repress pluripotency and trophoblast cell type differentiation and 2) directed differentiation toward neurectoderm expressing neuronal stem cell markers *SOX1* and *PAX6*. The advantages of this method include low levels of neural crest or mesodermal differentiation, and thus, higher specificity is achieved. High cell density in the monolayer cultures also led to a higher overall number of neuronal stem cells than previously reported (Chambers et al., 2009). This robust method of dual SMAD inhibition has become the basis for multiple differentiation protocols towards specialized neuronal subtypes.

After the neural tube has developed, the neuronal stem cells need patterning to acquire regional subtype identities. In the developing brain, the establishment of the apical-caudal, dorsal-ventral, and medial-lateral axes are dependent on locally secreted growth factors; SHH, retinoic acid, FGFs, and Wnt family proteins. In the human foetal brain, transcription factor profiles differ between brain regions, and these regionally specific combinations of markers can be used to monitor the regionalization of neuronal progenitors *in vitro*. Regional specification is important in order to be able to efficiently and reliably produce selected cell types (Tao, Zhang, 2016). Advances in regional subtype patterning *in vitro* have relied on clues gathered from classical developmental biology experiments as well as rare human embryo material (for instance in (Kirkeby et al., 2012)).

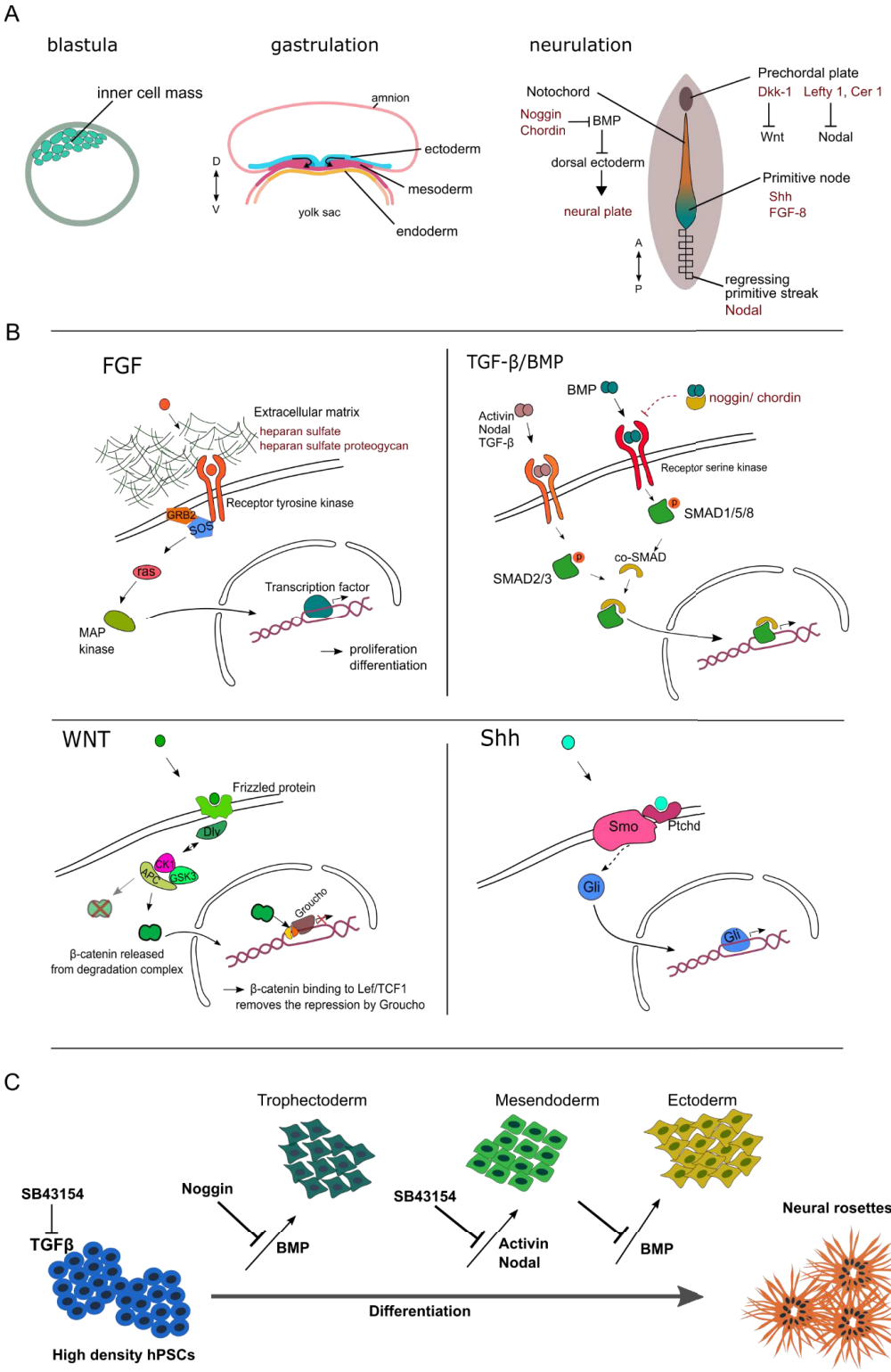

A
blastula
gastrulation
neurulation
inner cell mass
amnion
ectoderm
mesoderm
endoderm
yolk sac
D
V
Notochord
Noggin
Chordin
BMP
dorsal ectoderm
neural plate
Prechordal plate
Dkk-1
Lefty 1, Cer 1
Wnt
Nodal
Primitive node
Shh
FGF-8
A
P
regressing
primitive streak
Nodal
B
FGF
Extracellular matrix
heparan sulfate
heparan sulfate proteogycan
Receptor tyrosine kinase
GRB2
SOS
ras
MAP
kinase
Transcription factor
proliferation
differentiation
TGF-β/BMP
BMP
noggin/ chordin
Activin
Nodal
TGF-β
Receptor serine kinase
SMAD1/5/8
SMAD2/3
co-SMAD
WNT
Frizzled protein
Dlv
CK1
APC
GSK3
Groucho
β-catenin released
from degradation complex
β-catenin binding to Lef/TCF1
removes the repression by Groucho
Shh
Smo
Ptchd
Gli
C
Trophectoderm
Mesendoderm
Ectoderm
SB43154
TGFβ
Noggin
BMP
SB43154
Activin
Nodal
BMP
Neural rosettes
Differentiation
High density hPSCs

Figure 1. Signalling pathways directing neural induction *in vivo* and *in vitro*.
A) Embryonic development from a blastula to neuronal induction in late gastrula stages. The inner cell mass (ICM) of the blastula-stage embryo contains pluripotent cells that will give rise to all the embryonic tissues. In the gastrulation-stage embryo, the three germ layers are formed by convergent-extension movement of cells through and around the primitive streak, forming embryonic mesoderm, endoderm, and ectoderm. Gastrulation also leads to formation of transient signalling centers that are important for neural induction. The primitive node, (an accumulation of cells at the anterior tip of the primitive streak, also called 'Hensen's node'), the prechordal plate, (mesoderm anterior to the notochord, also called the 'head organizer'), and the notochord, function as sources for the signalling molecules that induce central neural system development. BMP inhibitors Noggin and Chordin, secreted from the primitive node and the notochord, are important neuronal inducers (Carlson, 2019). B) Simplified summary of the major growth factor signalling pathways that are responsible for the differentiation and patterning of cells and tissues during development. C) Directed neuronal induction from human pluripotent stem cell *in vitro* by inhibition the TGF-β, Activin/Nodal and BMP signalling pathways. Image adapted based on (Borooah et al., 2013, Chambers et al., 2009). D = dorsal, V= ventral, A = anterior, P = posterior.

2. The human reproductive axis

2.1 Hormonal regulation of puberty and fertility

GnRH neurons are essential regulators of reproduction and a part of the hypothalamic-pituitary-gonadal axis. The onset of puberty and sexual development rely on pulsatile secretion of GnRH from the nerve terminals of the hypothalamic medial eminence into the portal vessels leading to the anterior pituitary. GnRH secretion stimulates endocrine release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) form pituitary gonadotropes, which leads to increased secretion of the gonadal hormones, testosterone, and estrogen (Charlton, 2008). The GnRH signal is received through the G-protein-coupled receptor (GnRH-R) in pituitary gonadotropes, and LH and FSH is released into systemic circulation. LH stimulates testosterone release from Leydig cells in the testes in males, which, in turn, stimulates growth and development of the secondary sexual characteristics (Nassar, Raudales & Leslie, 2019). In females, FSH promotes follicular maturation in the ovary, and theca cells in the follicles respond to LH by secretion of testosterone, which is converted into estrogen via aromatase from the FSH-responsive granula cells. During the follicular phase of the menstrual cycle, FSH stimulates a gradual rise in estrogen, and when a critical level is reached, a rise in LH release is stimulated. This is refreed to as the 'LH surge' which triggers ovulation. LH stimulates the release of progesterone from the corpus luteum during the luteal phase of the menstrual cycle. Progresterone together with estrogen stimulates the endometrium to prepare for embryo implantation. Negative feedback from estrogen, as well as inhibin from corpus luteum, downregulates FSH during the luteal phase. Progesterone is stimulated by human chorionic gonadotropin if pregnancy occurs, and if not, the corpus luteum will degrade and allow for a new cycle to begin, by stimulation of GnRH, and FSH (Rosner, Samardzic & Sarao, 2019).

The HPG axis is briefly activated during foetal development, and again during the first 3-6 months after birth, which is referred to as minipuberty (Lanciotti et al., 2018, Waldhauser et al., 1981). At birth, the drastic decrease in placental hormones leads to the reduction of the

negative feedback loop that regulates GnRH secretion. Therefore, gonadotropin and sex steroid levels begin to increase during the first 6-10 days after birth (Schmidt, Schwarz, 2000). Minipuberty is thought to be essential for the development of the genital organs and fertility. The HPG axis then remains relatively quiescent until puberty onset (Lanciotti et al., 2018).

2.2 Regulation of GnRH secretion in the hypothalamus

At puberty, the frequency and amplitude of GnRH secretion increase. This pulsatility is a crucial feature that allows for activation of gonadotrope stimulation as a response (Belchetz et al., 1978, Wildt et al., 1981). GnRH neurons are able to synchronize their electrical activity and generate an episodic pattern of hormone secretion (Herbison, 2018).

GnRH neurons send single neuronal projections into the median eminence from the cell bodies in the hypothalamus. In contrast to classical axonal or dendritic properties, these single projections have both axonal and dendritic properties; they can receive and integrate synaptic inputs, in addition to containing a spike initiation site that triggers action potentials, thereby termed ‘dendrons’ (Herde et al., 2013). The GnRH neuron dendrons become densely concentrated just outside median eminence, where dendrons split into short nerve terminals that innervate the median eminence. The dense concentration of dendrons in this area suggests that synaptic input to dendrons, and dendron intercommunication outside the median eminences, may be involved in synchronizing GnRH neuron activity to form pulses (Herbison, 2018, Moore et al., 2018).

Kisspeptin is a potent activator of GnRH neurons in the hypothalamus and crucial for normal puberty development (de Roux et al., 2003, Seminara et al., 2003). The Kisspeptin receptor (KISS1R, formerly known as GPR54) is expressed in GnRH neurons. Kisspeptin signalling induces depolarization of GnRH neurons, and responsiveness to Kisspeptin is increased before puberty onset (Han et al., 2005, Leon et al., 2016, Herbison, 2018). Kisspeptin is secreted by neurons located in the arcuate nucleus (ARN) in the mediobasal hypothalamus, and the rostral periventricular nucleus of the third ventricle (RP3V). The ARN Kisspeptin neurons also secrete Neurokinin B and Dynorphin A, and are referred to as KNDy neurons (Yip et al., 2015). These peptides are suggested to autoregulate the secretion of Kisspeptin by autosynaptically conveying stimulatory (Neurokinin B) and inhibitory (Dynorphin) signals that lead to synchronized Kisspeptin neuron activity (Navarro, 2012, Lehman, Coolen & Goodman, 2010). The ARN Kisspeptin neurons have been denoted as the “pulse generators” of GnRH secretion, as they exhibit synchronized calcium activity episodes that coincide with LH pulsatility (Clarkson et al., 2017). GnRH neuron projections are present in the ARN and exhibit contact with KNDy neurons, as well as in the areas proximal to the median eminences. Furthermore, complex interconnections have been observed between the GnRH neurons, the

ARN, and the RP3V Kisspeptin neurons, and feedback mechanisms relay upstream input from local and periferal cues (Yip et al., 2015, Moore, Coolen & Lehman, 2019). Indeed, metabolic signals, chronic malnutrition, stress, and reproductive diseases, for instance, are known to suppress the secretion of GnRH (Tsutsumi, Webster, 2009).

3. Puberty

The neurobiological aspect of puberty initiation can be characterized as an increase in frequency and amplitude of GnRH secretion. Evidence suggests that this occurs as a result of lifted repression by neurobiological and epigenetic factors that have been set in place during the quiescent period of adolescence (Livadas, Chrousos, 2019, Heras et al., 2019). The timing of puberty is variable and influenced by genetic and epigenetic mechanisms, nutritional state, adipose tissue, environmental factors such as chemicals that are endocrine disruptors, as well as psychological stress (Parent et al., 2003, Sisk, Foster, 2004, Gajdos et al., 2010, Messina et al., 2016). A high number of genes are associated with the timing of puberty onset, and, therefore, it is difficult to pinpoint the exact function of these genes (Perry et al., 2014, Day et al., 2017). The collective hypothesis is that the state of the body is relayed by an “intrinsic developmental clock” consisting of signals that can convey the stage of sufficient (neurobiological) maturation, growth, and metabolic status, before puberty is initiated (Herbison, 2016).

3.1 Congenital Hypogonadotropic Hypogonadism and Kallmann Syndrome

Disrupted development, migration, or function of GnRH neurons lead to defects in reproduction and sexual development. Rare genetic diseases that cause GnRH deficiency and lead to absent puberty and infertility are termed congenital hypogonadotropic hypogonadism (CHH). CHH combined with hyposmia or anosmia (reduced or absent sense of smell) is known as Kallmann syndrome (KS) and draws a link between GnRH neuron development and the olfactory system. CHH and KS are genetically heterogeneous developmental disorders, and KS accounts for around 50% of CHH cases. To date, over 30 CHH-associated genes have been identified (Boehm et al., 2015, Young et al., 2019). The mutations associated with CHH and their molecular mechanisms are not yet comprehensively characterized but several of these genes are postulated to affect GnRH neuron development, migration, or function.

3.2 Foetal development of the HPG axis

During human foetal development, GnRH neurons arise at gestational week (GW) 5, and pituitary LH is detectable during GW 9. Human chorionic gonadotropin (hCG) is provided by

the placenta during the first trimester. The exact timing when pituitary gonadotropes become under hypothalamic control is not known, but foetuses lacking a hypothalamus due to anencephaly show normal development of pituitary up to GW 17 or 18, and CHH patients have normally differentiated genitalia. Pituitary LH levels first peak at midgestation and decrease near term, which is likely due to the increase in placental estrogen and progesterone, gonadal feedback, and maturation of gonadotropes by acquisition of relevant cell surface receptors (Young et al., 2019).

In male foetuses, the gonadal organ differentiation into testicles starts at GW 5-7. Sex determination is initiated by the SRY gene from the Y-chromosome, and is followed by sex differentiation in the bipotential gonads, derived from intermediate mesoderm in the early embryo. Sex differentiation leads to specification of the Sertoli and Leydig cells, followed by development of the external genitalia which is driven by testosterone. The foetal Leydig cells synthesize testosterone, which stabilizes the Wolffian (male urogenital) structures. Sertoli cells produce anti-Müllerian hormone (AMH), which has an important role in regression of the source for the reproductive tissues in the female, the Müllerian ducts (Makela et al., 2019). GnRH-driven LH from the pituitary is needed for normal penile growth and testicular decent during the third gestational period. CHH patients have higher prevalence of micropenis and cryptorchidism (Bouvattier et al., 2011).

In female foetuses, the ovary develops in the absence of Y-chromosome, and the fallopian tubes, uterus and vagina start to form in the Müllerian ducts in the absence of AMH and testosterone. Furthermore, local signals including sutained expression of Wnt-4 is required for repression of Leydig cell development and maintencance of female germ line cells (Vainio et al., 1999). Primoridal foetal ovarian follicles develop during GW 15 independent of gonadotropin. Estrogen is produced at this stage by the placenta and foetal ovaries have low levels of hormone production (Kurilo, 1981).

3.3 Minipuberty

Postnatal activation of GnRH secretion is initiated after the decline in placental-derived estrogen levels during the first weeks after birth (Waldhauser et al., 1981). In boys this leads to a peak in testosterone levels during 1-3 months of age, stimulation of Inhibin B in the Sertoli cells of the testis, and increase in testicular volume. The Sertoli cell population increases during minipuberty, which is critical for normal sperm production in adulthood. Spermatogenesis is not yet initiated since the Sertoli cells exhibit low levels of androgen receptor. In girls, estradiol levels increase at minipuberty, and remain upregulated up for approximately six months (Young et al., 2019). The dectectable levels of AMH at minipuberty in female infants suggests a mechanism for keeping primordial follicles at a resting state during

minipubertal HPG axis activity. AMH acts negatively on the FSH sensitivity of primordial follicles, and the estrogen biosynthesis in granulosa cells of the ovary (Muratoglu Sahin et al., 2019).

Minipuberty can be a critical time-window for early diagnosis of CHH in males, since micropenis and cyptorchidism are seen in higher frequencies in CHH and KS patients. Hormonal testing is not routinely prescribed, but hormonal evaluations can be performed at minipuberty for children born from one parent with CHH. Female infants lack the clinical features, and diagnosis during minipuberty is therefore rare (Young et al., 2019). Low levels of AMH in female infants are associated with CPP, and premature thelarche (PT) which is a milder form of premature activation of HPG axis that leads to premature breast development. The underlying cause for lower levels of AMH has, however, not been resolved (Muratoglu Sahin et al., 2019)

3.4 Delayed puberty

Delayed puberty is defined as 2 or 2.5 years later onset of puberty compared to the population mean. This is traditionally reported after the age of 14 for boys and 13 for girls. Low testis volume in boys and absence of breast development in girls are general criteria (Young et al., 2019). It is still difficult to distinguish between CHH and constitutional delay of growth and puberty (CDGP). CDGP is related to transient GnRH deficiency that is more common in the general population. This diagnosis is done by exclusion of permanent forms of HH, and can be managed by short-term hormonal treatment or expectant observation (Palmert, Dunkel, 2012).

The main characteristics of CHH in females is primary amenorrhea in nearly 90% of patients, and in 10% only one or two episodes of bleeding occurred during adolescence. Absent breast development before hormone therapy seems to be the case only for a minority of patients. For males, absent or minimal virilisation, erectile dysfunction, low testicular volume due to absence of puberty, absence of secondary sexual characteristics, in addition to previously mentioned cryptorchidism and micropenis are common problems. In 75% of CHH patients, puberty never occurs spontaneously (Young et al., 2019).

3.5 Associated phenotypes and genetics of CHH

Anosmia has been reported in around 50% of CHH cases defined as KS. Other phenotypes occur at lower prevalence, such as mirror movements (synkinesia), unilateral renal genesis, eye movement disorders, sensorineural hearing loss, midline brain defects, cleft lip and palate, dental agenesis, and skeletal and cardiovascular defects (Young et al., 2019).

Some syndromic forms of CHH are associated with specific combination of phenotypes. Approximately 46 syndromes have reported CHH traits, some of which are known to exhibit also genetic overlap with CHH, including CHARGE syndrome (*CHD7*, *SEMA3E*), Waardenburg syndrome (*SOX10*), and Hartsfield syndrome (*FGFR1*) (Marcos et al., 2014, Lalani et al., 2004, Cariboni et al., 2015, Pingault et al., 2013, Miraoui, Dwyer & Pitteloud, 2011).

To date, over 30 CHH-associated genes have been discovered (Listed in: Article III, Fig. 4 A). The development in sequencing methods and genetic techniques will hopefully lead to the discovery of further genes, which would be very important for achieving more efficient genetic diagnoses, as around 50% of cases lack molecular diagnosis. The heterogeneous genetic background of CHH mirrors the complex development of GnRH neurons. GnRH deficiency can be the result of defects in different mechanisms during the different stages of development, including GnRH neuron fate specification, GnRH neuron migration, axon guidance, abnormal neuroendocrine secretion and homeostasis, and gonadotrope defects (Young et al., 2019).

In the general population, the variation of age of puberty is largely genetically influenced (50-80%). Genome-wide association studies have recently reported ca 400 independent loci associated with variance in the timing of puberty (age at menarche, first occurrence of menstruation) in ~370000 women of European ancestry (Day et al., 2017). Several of the genes associated with age at menarche in this study where implicated in neuronal tissues based on transcriptome-wide association. One molecule involved in neurobiological repression of HPG axis before puberty is the ubiquitin ligase makorin ring finger protein 3 (MKRN3), which is expressed in the ARN before puberty onset. MKRN3 levels decrease during puberty (Busch et al., 2016), and loss-of-function mutations of *MKRN3* are implicated in CPP (Abreu et al., 2015, Heras et al., 2019).

4. Development and origin of GnRH neurons

GnRH neurons are born in the nasal area, emerging from the olfactory placodes during early embryogenesis. From the olfactory placodes, GnRH neurons take a migratory route intertwined with the axon fibers of the terminal nerve (TN), travelling around the olfactory bulb, crossing the cribriform plate to the forebrain, and making a ventral turn into to the preoptic area of the hypothalamus (Wray, Grant & Gainer, 1989b, Schwanzel-Fukuda, Pfaff, 1989, Casoni et al., 2016). The development and migration of GnRH neurons is highly conserved across evolution (Abitua et al., 2015, Casoni et al., 2016). FGF8 is strongly implicated as a crucial signalling molecule for GnRH neuron specification and proper development (Falardeau

et al., 2008, Chung, Moyle & Tsai, 2008). The most important embryonic tissues and events that precede GnRH neurogenesis and migration will be discussed in this section.

4.1 Craniofacial development

The embryonic face is developed by morphological movements, tissue bending, and growth (Helms, Cordero & Tapadia, 2005). The cranial neural crest cells migrate from the neural folds to form the majority of the connective tissues, bones, and peripheral neurons and glial cells in the face. The cranial placodes form the sensory organs; the eyes, ears, and nose all contain sensory neurons (Schlosser, 2006).

4.2 Neural crest

The neural crest (NC) is a transient embryonic population of cells unique in their multipotency and migratory properties, which has evolved as a trait specific to vertebrate development. NC cells hold a potential to give rise to specialized cell types contributing to diverse tissues and biological functions in the body, from peripheral neurons and glia to cranial cartilage, dentin, skin melanocytes, and the septum of the heart (Dupin, Le Douarin, 2014). The NC thereby has been referred to as the “the fourth germ layer” by developmental biologists (Shyamala et al., 2015). The NC cells derive from the neural plate border region, located in the dorsal-most parts of the gastrula stage embryo, from where they migrate into their peripheral locations to contribute to organ and tissue development (Dupin, Le Douarin, 2014).

Based on their anterio-posterior position along the neural tube, the NC can be subdivided into cranial, cardiac, vagal and sacral, and trunk neural crest:

The cranial neural crest cells are the source of most of the mesenchymal tissue that forms the facial structures, including cartilage, bone, cranial neurons, glia, as well as odontoblasts of the tooth primordia, and bones in middle ear and jaw.

Cardiac neural crest gives rise to musculoconnective tissue that forms walls of large arteries from the heart and the aorticopulmonary septum.

Vagal and sacral neural crest generate enteric ganglia in the gut.

Trunk neural crest gives rise to a substantial part of the neurons and glia forming in the peripheral nervous system, and, in addition, secretory endocrine cells, and pigment cells of the skin (Bhatt, Diaz & Trainor, 2013, Shyamala et al., 2015).

4.3 Neural crest induction at the neural plate border

The neural plate border region, which later segregates into progenitors of NC and cranial placodes, arises by interaction between the neural plate and epidermis. FGF, Wnt, and attenuated BMP are involved in the border region establishment at late blastula to gastrula stages (Schille, Schambony, 2017). The positioning and maintenance of the neural plate border region is regulated by the expression of the neural plate border specifier genes; the Zic, Pax, Dlx, and Msx families of transcription factors. Wnt signalling is needed to activate the expression of *Pax3* and *Msx1/2*, and other neural plate border specifiers (*Zic1, Zic3, Dlx5, Msx1/2*) are responsive to BMP inhibition. After the establishment of the neural plate border region, these specifiers induce formation of NC by activating the expression of the neural crest specifier genes (Meulemans, Bronner-Fraser, 2004, Simoes-Costa, Bronner, 2015). The presumptive NC lies between the epidermis exhibiting high BMP signalling activity, and the neural ectoderm with strong BMP antagonistic activity (Figure 2) (Selleck, Bronner-Fraser, 1996). The overlying epidermis has the capacity to ectopically induce NC in underlying neural plate, as shown originally by Dickinson and colleagues (Dickinson et al., 1995). BMP antagonists are also expressed in the underlying dorsolateral mesoderm (Steventon et al., 2009). Together, this balanced inhibitory and activating regulation is thought to result in a precise level of BMP activity, generating an area permissive for NC induction (Stuhlmiller, Garcia-Castro, 2012) (Figure 2).

The NC fate becomes restricted in the neural plate border by expression of neural crest specifier genes including *Snail* (also known as *Snai1*), *Slug* (also known as *Snai2*), *Pax3, Twist, Tfap2 (AP-2α), Foxd3,* and *Sox10* (Simoes-Costa, Bronner, 2015). NC specifier genes regulate the specification and the maintenance of multipotent NC progenitors. The NC specifiers also regulate downstream neural crest effector genes, which further regulate migration and progressive differentiation into neural crest derivatives. The progression from premigratory progenitor cells to migratory neural crest is enabled by the process of epithelial to mesenchymal transition (EMT). EMT requires altering of cell-cell adhesion properties and remodelling of the actin cytoskeleton to reduce cell polarity, which thereby allows changes in overall shape and adhesiveness of the cells (Cheung et al., 2005, Simoes-Costa, Bronner, 2015, Werner et al., 2007). HNK-1 is a known marker for migratory human neural crest (Tucker, G. C. et al., 1988). In addition, protein expression of PAX3, SOX9, and SOX10 in the premigratory, and SOX9, SOX10, P75, and AP-2α in rostral migratory NC, have been reported in human embryo samples (Betters et al., 2010).

4.3.1 BMP signalling pathway

Animal models of NC induction have demonstrated the importance of BMP regulation (Prasad, Charney & Garcia-Castro, 2019). Modulation and magnification of the BMP signals are reinforced by other signalling pathways, such as FGF and Wnt signals derived from the mesoderm (Stuhlmiller, Garcia-Castro, 2012). To define the level of BMP activation or inhibition required for NC induction has been a challenge. This is seen especially when modelling NC induction *in vitro* using hPSCs. Based on reported protocols for NC generation, at least temporary BMP inhibition is required during NC induction (Menendez et al., 2011, Mica et al., 2013, Hackland et al., 2017).

4.3.2 Wnt signalling pathway

Canonical Wnt signalling is an intracellular pathway that function by activating the β-catenin signal transduction, to modulate transcription of the target cell. The Wnt signalling pathway is activated by secreted Wnt proteins acting as ligands to seven-transmembrane receptor Frizzled, and by activating its co-factors lipoprotein-receptor-related protein (LRP) 5/6 and Disheveled (Dvl) proteins. In the absence of Wnt ligands, β-catenin is degraded in the cytoplasm by a destruction complex consisting of the negative Wnt regulator Axin, glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), and casein kinase 1 (CK1). In brief, Wnt activation causes translocation of Axin and the destruction complex to the plasma membrane, which allows for increased free intercellular β-catenin to enter the nucleus and induce transcription of Wnt target genes (Clevers, 2006) (Figure 1 B).

Animal models have demonstrated a role for secreted Wnts derived from the adjacent mesoderm in the induction of NC (Garcia-Castro, Marcelle & Bronner-Fraser, 2002, Steventon et al., 2009, Ji et al., 2019). Studies in *Xenopus* showed that the activation of Wnt pathway remains critical from gastrula to neurula stages, whereas the requirements for BMP signalling varies (Steventon et al., 2009, Prasad, Charney & Garcia-Castro, 2019). The importance of Wnt pathway activation in the induction of mammalian NC was later confirmed in several *in vitro* studies using human pluripotent stem cell differentiation (Menendez et al., 2011, Mica et al., 2013, Leung, A. W. et al., 2016). In this type differentiation experiments, small molecule Wnt agonists can be used for Wnt activation during differentiation, such as BIO and 1-azakenpaullone, which act as inhibitors of GSK-3β (Sato, N. et al., 2004, Prasad, Charney & Garcia-Castro, 2019).

4.4 Neural crest cell multipotency

Retained developmental plasticity of NC progenitors is an important characteristic feature, and the gene expression profile of NC cells share common features with pluripotent blastocysts

cells (Thomas et al., 2008), including the expression of transcription factors. But as embryonic development progresses, the NC cells become integrated into developing tissues and differentiate terminally. Several reports suggest that neural crest stem cells are multipotent, and their fate depends on the environment (Bronner-Fraser, Fraser, 1989, Frank, Sanes, 1991, Fraser, Bronner-Fraser, 1991). Lineage-tracing studies in chick revealed that individual premigratory cells are multipotent (Frank, Sanes, 1991). The progeny of one cell could give rise to neurons with distinct localization, shape, innervation, and lineage subtypes of neurons and glia. This has also been observed *in vitro* by showing that the progeny of a single NC generated both melanocytes and neuronal cells (Sieber-Blum, Cohen, 1980).

Yet, it has been observed that the cranial NC contributes to different cell lineages somewhat differently depending on the timing of migration. Early-migrating cranial NC cells contribute to both dorsal (melanocytes, dorsal dermis, and neurons of the ciliary ganglion) and ventral (Schwann cells and cartilage, bone and dermis of the jaw) cell types. The late-migrating cells show a lesser tendency toward bone and cartilage differentiation. When early-migrating NC cells from the chick were grafted into late-migratory-stage of the quail, the grafted cells contributed to bone and cartilage formation normally. Conversely, when substituting early-migrating cells with late, the cells no longer contributed equally to jaw skeleton and only formed dorsal derivatives. When late-migrating cells were grafted into a late-stage embryo lacking both late and early NC cells, the grafted population did contribute to jaw bone and cartilage, which suggests that the presence of early-migrating cells actually restricts the later-migrating cell differentiation (Baker et al., 1997). This illustrates the innate plasticity of the neural crest cells, and advocates for environmental regulation during and after migration to gradually restrict multipotency.

The multipotency of NC is well recapitulated *in vitro*, where cells are both spontaneously and by directed differentiation able to generate a multitude of NC derivatives, as well as retain the capacity of self-renewal for several generations (Stemple, Anderson, 1992, Calloni, Le Douarin & Dupin, 2009, Kerosuo et al., 2015). However, *in vivo* the multipotency of NC cells is still a transient phenomenon, and researchers are continuing investigations into the routes and mechanisms of lineage restriction (Kalcheim, Kumar, 2017).

4.5 Notch signalling and neural crest development

Notch signalling acts as an important regulatory feature during tissue morphogenesis, cell fate decisions and differentiation, and cell-cell communication. Notch signalling is responsive to other external signals, as well as intrinsic signalling (Sjoqvist, Andersson, 2019).

4.5.1 Notch signalling pathway

Notch signalling is based upon cell-cell communication between ligand and receptor-expressing cells. The mammalian Notch pathway consists of Notch receptor paralogs (Notch1-4) and five DLS family members of Notch ligands; Delta-like (Dll1, Dll3, and Dll4) and Jagged (Jagged1/Jag1 and Jagged2/Jag2). When ligands bind to Notch receptor, the Notch intracellular domain (NICD) is proteolytically cleaved by alpha-secretase and gamma-secretase complexes, and translocated to the nucleus. In the nucleus NICD interacts with RBP-J (Recombination Signal Binding Protein, also known as CSL (CBF-1-suppressor of hairless-LAG-1)) and the MAML1 coactivator, and forms an active signalling complex (Borggrefe et al., 2016) (Figure 2 A).

The outcome of Notch signalling depends on the intercellular and intracellular context. The initial signal is transferred by trans-activation between the Notch ligand-presenting and receptor-presenting cell. The directionality of the Notch signal has traditionally been explained based on the models of lateral inhibition and lateral induction. Lateral inhibition produces bias between two equipotent cells after trans-activation, which is reinforced by downregulation of ligand in the signal-receiving cell, thereby inhibiting Notch activation in its contacting cells. In other contexts lateral induction can be employed, for example in boundary formation, where a positive feedback loop enhances the expression of Notch in contacting cells, leading to a lateral spreading of Notch activation (Sjoqvist, Andersson, 2019). However, evidence supports more complex spatial and temporal regulation to determine the signalling outcome. Distribution of ligands and receptors is more seldom equal between cells. Thus, the signal progression within a tissue can be regulated in several ways, including cell polarity and asymmetrical expression of ligands or receptors within a cell, and cell motility (Bray, 2016).

Different ligands and receptors also produce different strength or duration of the Notch signal. For example, Jag1 has lower affinities for Notch1 compared to Dll4, which causes lower signal strength and a specific signalling outcome (Gama-Norton et al., 2015). In the case of receptors, Notch1 and Notch2 show variance in signal outcome even in the same pattern of expression. This is thought to be based on differences in stability and amount of NICD moieties and their availability of proteases for activating cleavage (Fan et al., 2004, Liu et al., 2013).

In addition to trans-activation, ligand and receptor expression on the same cell can also have a cis-inhibitory effect, by inhibiting a cell surface receptor in the same cell. Cis-inhibition by ligand presenting cells reduces the levels of receptor on the cell surface available for trans-activation. Furthermore, the Notch ligand Dll3 lacks trans-activation features, therefore having an inhibitory role, most likely by acting only through cis-inhibition (Bray, 2016, Ladi et al., 2005).

Among common targets of the NICD-RBP-J complex that act as effectors of Notch signalling are the HES family genes. HES genes are basic helix-loop-helix (bHLH) transcription factors which act as repressors of several downstream targets that include tissue-specific transcription factors (Iso, Kedes & Hamamori, 2003). Events in the cell nucleus further regulate the effect on transcription of effector genes. RBP-J functions in a cell-specific manner to activate or inhibit different transcriptional programmes depending on the cell type and tissue. This can be partially explained by cooperation with tissue specific transcription factors, but also depends on accessible chromatin, levels of inhibitory transcription factors, and co-repressors and co-activators (Bray, 2016).

The Notch signalling pathway plays important roles in stem cell maintenance as well as in the tissue differentiation. During neural crest specification, Notch signalling has been implicated in the regulation of the expansion vs. restriction of the NC territory at the neural plate border region (Mead, Yutzey, 2012) (Figure 2 B). During cortical neurogenesis, active Notch signalling is required for maintenance of the proliferative state of neuronal stem cells to allow for tissue expansion, in a process that lies downstream of FGF signalling (Rash et al., 2011). Notch pathway can be inhibited by gamma-secretase inhibitors (GSIs) that block the cleavage of Notch, for example to induce differentiation in neuronal progenitor cells. The most commonly used gamma-secretase inhibitor is DAPT (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-Butyl ester) (Purow, 2012).

4.6 Specification of preplacodal ectoderm

The sensory organs of the face are formed by the ectodermal thickenings called cranial placodes, which constitute a large part of the embryonic cranial ectoderm. The cranial placodes are derived from the pre-placodal ectoderm (PPE), a “pan-placodal primordium” which consists of a crescent-shaped area around the anterior neural plate (Sclosser 2014) (Figure 2 C). The PPE is specified by the expression of transcription factors of the Six family, and their co-factors, the Eyas. Eyas bind to other transcription factors, (c-terminal Eya-domain) and have transactivation properties (Zou et al., 2004, Laclef et al., 2003). They are transported to the nucleus by binding to SIX, where they act as transcriptional co-activators (Ohto et al., 1999). *Six* family genes are homeodomain transcription factors, out of which *Six 1/2* and *Six 4/5* are known to have panplacodal expression. In vertebrates, at least one *Eya* gene is expressed in all cranial placodes, with varying distribution of paralogues depending on species (Schlosser, 2006). The *Six* genes can act as either transcriptional repressors or activators depending on their interaction with co-factors. In addition to Eyas, Six can bind to co-activators and co-repressors such as Dach and Groucho, thereby modulating their activity (Brugmann et al., 2004). The expression of *Six* and *Eya* in cranial placodes is required for the induction of

neurogenic transcription factors *NeuroD* and *Neurogenin2*. In addition to the *Six* and *Eya* genes, transcription factors such as *Dlx* homeobox and *Gata* class zinkfinger transcription factors are needed to acquire placodal competence. For neurogenesis, *Sox* genes and *Irx* (*Xiro* in *Xenopus*) are important inducers (Schlosser, 2006).

Although *Six* and *Eya* expression is found in the PPE and remain expressed in the ready formed placodes, it is not enough to promote full development of the placodes, and co-operation with other factors during placode development is needed (Brugmann et al., 2004, Christophorou et al., 2009). The area of *Six* and *Eya* expression is defined during gastrulation together with the other domains within the neural plate border region; the neural crest, epidermis, and the neural plate (Figure 2 C). Signals from these areas and the underlying mesoderm, such as FGFs, BMP inhibitors, and Wnt inhibitors induce and restrict the PPE territory (Litsiou, Hanson & Streit, 2005).

Further on in the development, the preplacodal ectoderm will become subdivided into distinct cranial placodes. Rostrally the adenohypophyseal placode, the only single placode in contrast to the other paired cranial placodes, produces neuroendocrine cells of the anterior pituitary, including ACTH, PRL, TSH, FSH, GH producing cells (Asa, Ezzat, 2004, Sanchez-Arrones et al., 2017). Other anterior placodes include the paired olfactory and lens placodes. The lens placode gives rise to the lens of the eye. The olfactory placode (OP) gives rise to sensory neurons and epithelia that form the sense of smell, as well as the pheromone-sensing vomeronasal epithelia of the vomeronasal organ (VNO). The otic placode gives rise to sensory hair cells, supporting cells, and endolymph producing cells of the inner ear (Whitfield, 2015). The epibranchial, profundal, trigeminal, hypobranchial and the paratympanic placodes, are neurogenic placodes which generate various sensory and somatosensory neurons of the face (Schlosser, 2014).

4.7 Development of the olfactory placodes

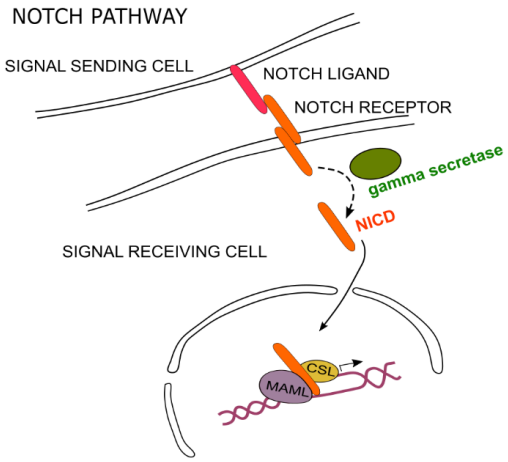
Placodal ectoderm becomes regionally specified by the expression of various homeobox genes that roughly define the anterioposterior and dorsoventral subregions of the placodal ectoderm. In the anterior placodal domain, where the OPs reside, there is expression of *Otx2, Emx2, Six3/6, ANF, Pitx*, and *Pax6* (Simeone et al., 1992, Oliver et al., 1995, Grindley, Davidson & Hill, 1995, Lagutin et al., 2003, Dutta et al., 2005, Schlosser, 2006).

It has been suggested that a lens-identity bias is initially established for the anterior placodes by the expression of *Dlx5* and *Pax6*, and OP identity is acquired by cells sustaining *Dlx5*, whereas it is downregulated in the lens placode (Bailey, A. P. et al., 2006, Bhattacharyya et al., 2004, Long et al., 2003). Fgf8 in the anterior neural ridge promotes specification of the olfactory placode, while supressing lens placode (Bailey, A. P. et al., 2006).

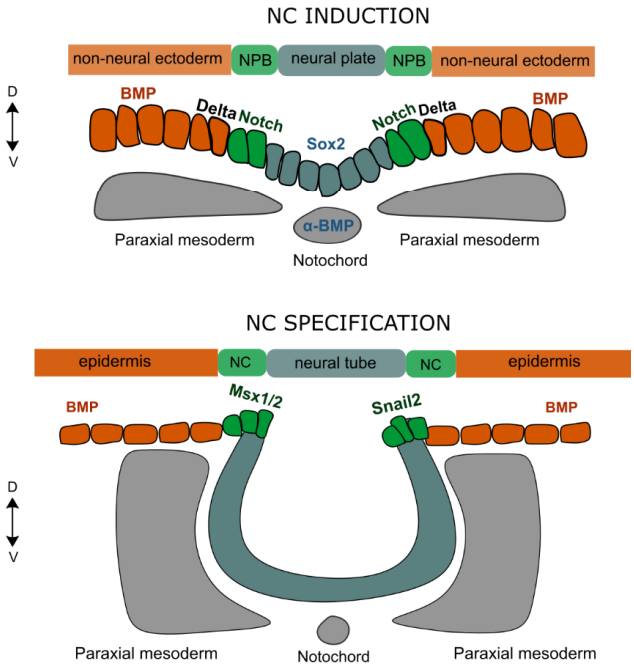
Studies from zebrafish have demonstrated that OP structures are established by morphological movements at the end of gastrulation (Whitlock, Westerfield, 2000, Harden et al., 2012). From the anterior neural plate border region, the presumptive OP cell population migrates anteriorly away from the neural plate, and becomes gradually separated from the forebrain by migrating neural crest cells (Torres-Paz, Whitlock, 2014). Once established, the ectoderm thickens and invaginates to form the olfactory pit and olfactory epithelia, which will comprise respiratory and sensory (olfactory receptor neurons) epithelium. Here, neuroepithelial induction versus respiratory fate is regulated by opposing action of BMP and FGF signalling (Maier et al., 2010).

Figure 2. **Notch signalling, neural crest, and preplacodal ectoderm induction.**
A) Notch signalling is based on cell-cell signalling between a signal sending and signal-receiving cell. Ligand binding to the Notch receptor induces the cleavage of the Notch intracellular domain (NICD), which forms a complex with CLS and MAML1 in the nucleus. B) During the induction of neural crest in the neural plate border area, BMP and Notch pathways are involved in patterning and restriction of the presumptive NC during development, by activation of NC specifier genes (Rogers et al., 2012). C) The preplacodal ectoderm form in a crescent-shaped area in the NPB of the anterior embryonic ectoderm, and becomes separated from the presumptive NC by expression of PPE-specific transcription factors. NBP = neural plate border, NT = neural tube, NC = neural crest, PPE = Preplacodal ectoderm, D = dorsal, V = ventral, A = anterior, P = posterior.

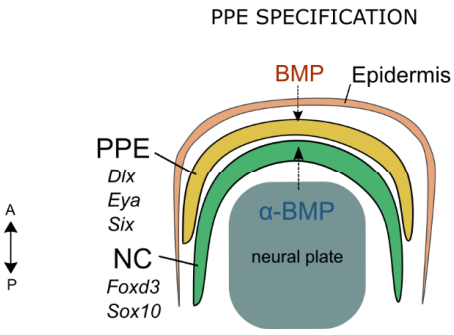
A


NOTCH PATHWAY
SIGNAL SENDING CELL
NOTCH LIGAND
NOTCH RECEPTOR
gamma secretase
NICD
SIGNAL RECEIVING CELL
CSL
MAML

B


NC INDUCTION
non-neural ectoderm
NPB
neural plate
NPB
non-neural ectoderm
D
V
BMP
Delta Notch
Sox2
Notch Delta
BMP
Paraxial mesoderm
α-BMP
Notochord
Paraxial mesoderm
NC SPECIFICATION
epidermis
NC
neural tube
NC
epidermis
BMP
Msx1/2
Snail2
BMP
D
V
Paraxial mesoderm
Notochord
Paraxial mesoderm

C


PPE SPECIFICATION
BMP
Epidermis
PPE
Dlx
Eya
Six
α-BMP
neural plate
NC
Foxd3
Sox10
A
P

4.8 Neurogenesis in the olfactory placode

FGF8 is an important morphogen during OE neurogenesis. Mutations in FGF8 signalling in mice cause defects in olfactory neurogenesis and GnRH neuron specification (Chung, Moyle & Tsai, 2008, Falardeau et al., 2008, Chung et al., 2010, Sabado et al., 2012). During the invagination and formation of the murine nasal pit, *Fgf8* is expressed by ectodermal cells around the outside border of the forming pit (Figure 3 A), (Kawauchi et al., 2005). The entire epithelium inside the pit expresses stem cell marker *Sox2*. Alignment of neuronal cell types lies in an outside-in organization, with the youngest cells at the outer rims, next to the *Fgf8*-expressing cells, expressing *Ascl1* (formerly known as *Mash1*). *Ngn1*-expressing intermediate neuronal progenitors lie next to the Ascl1 region, and in the center of the pit Ncam-expressing postmitotic neurons are becoming olfactory receptor neurons (ORNs) (Kawauchi et al., 2005). *Foxg1* is also expressed by the neuronal progenitors of the OE, and Fgf8 exhibits a paracrine/autocrine role in their survival by regulating the normal expansion of the epithelium required for differentiation of all neuronal cell types. Conditional knockout of *Fgf8* in the *Foxg1*-expressing cells leads to apoptosis of the early Sox2-positive cells at the outside rim of the olfactory pit, and eventually halts neurogenesis in the whole OE (Kawauchi et al., 2005). Fgf8 regulates the dosage of *Sox2*, so that a ventro-medial to dorso-medial gradient of *Sox2* is generated proximal to the source of secreted Fgf8. At the same time, an opposite ventro-lateral gradient of *Meis1*, expressed by slow dividing multipotent stem cells, is formed in cells exhibiting low *Sox2* expression. Meis1 represses Ascl1, while Sox2 promotes Ascl1 expression in rapidly dividing neuronal progenitors. Fgf8 is thought to promote the generation of transit amplifying *Ascl1*-expressing progenitors, responsible for expansion of the intermediate neuronal progenitors that give rise to differentiated neurons. Thus, Fgf8 creates a transcriptional network that modulates the proliferative status of the cells in the OE (Tucker, E. S. et al., 2010).

GnRH neurons express Fgfr1 and Fgfr3, and both act as receptors for Fgf8, but Fgfr1 has been primarily implicated in olfactory neurogenesis and GnRH neuron differentiation (Mott et al., 2010). *Fgf8* and *Fgfr1* hypomorphic mice show craniofacial anomalies during embryogenesis, including a reduction in the number of GnRH neurons (Meyers, Lewandoski & Martin, 1998, Chung, Moyle & Tsai, 2008). Fgf8 and Fgfr1 homozygous hypomorphs retained small OP-like thickenings and partial invagination of the pit. At E11.5, when GnRH neurons have normally appeared, GnRH was not detected in homozygous *Fgf8* hypomorphs, and was completely absent at birth. *Fgfr1* hypomorphs also presented a significantly lower number of GnRH-positive neurons at E11.5. The Fgfr3 KO mice did not present any significant change in GnRH neuron numbers, which suggest that FGF8 is acting on GnRH neurogenesis mainly through FGFR1, but, however, small compensation through FGFR3 cannot be ruled out (Chung, Moyle & Tsai, 2008, Gill, Moenter & Tsai, 2004).

Forni and colleagues further described the developing OE and nasal pit, and found that the Fgf8-expressing cells reside in an area containing AP2α- and MSX1/2-positive cells, which mark respiratory epithelia (RE) in the rostral part of the nasal pit in E11.5 mice embryos (Forni et al., 2013). This Fgf8-positive area in the RE coincided with low levels of BMP4, but not with the neuronal progenitor marker HuC/D, which is found only distally form the Fgf8 source. High levels of BMP4 expression, and active SMAD 1,5,8 surrounded the rostral nasal pit and epithelial rims, and high expression of Noggin was detected in the mesenchyme. Neurons in the OE always appeared proximal to the high Noggin area. GnRH neurons appeared in the area juxtaposed to the Noggin source in the ventromedial OP, and GnRH neurons were found in increasing number from the rostral to caudal midface (Forni et al., 2013). Noggin expression can be triggered by active BMP, and is crucial for neurogenesis in the OE (Maier et al., 2010). This suggests that the ventromedial mesenchymal source of Noggin, proximal to the Fgf8 source in the RE, attributes a GnRH neurogenic niche (Figure 3 B). Decrease in *Fgf8* expression led to considerable changes in the areas of Bmp4 and Noggin expression in the OP, which severely disrupted neurogenesis and morphogenesis in and around the OP area. Thereby, the effect of *Fgf8* (and *Fgfr1*) mutations on loss of GnRH neurons may be partially secondary to the changes caused by dysregulation of Bmp signalling (Forni et al., 2013). That does, however, not provide comprehensive evidence to completely rule out a role for Fgf8 in neuronal linage specification, as was previously suggested (Kawauchi et al., 2005, Tucker, E. S. et al., 2010). Fgf8 has been implicated in survival and sustained proliferation, (inhibiting neuronal differentiation) as well as positional patterning of neuronal stem/progenitor cells (Lahti et al., 2011, Crespo-Enriquez et al., 2012, Storm, Rubenstein & Martin, 2003). So far, the exact mechanism of FGF8 on GnRH neuron progenitor cells has not been resolved.

4.9 Origin of GnRH neurons?

After the discovery of GnRH neurons arising within the olfactory placodes and migrating to the hypothalamus in association with the terminal nerve, the dogma of OP neuronal progenitor origin was challenged with the hypothesis that the NC could contribute to the OP, giving rise to a subset of GnRH neurons (Whitlock, Wolf & Boyce, 2003, Forni et al., 2011). A study conducted in zebrafish proposed that the neurons expressing GnRH near the terminal nerve in the frontonasal area and the hypothalamic GnRH neurons would be derived from separate progenitor populations (Whitlock, Wolf & Boyce, 2003). The NC would give rise to the TN population and the adenohypophysis would give rise to the hypothalamic endocrine cells.

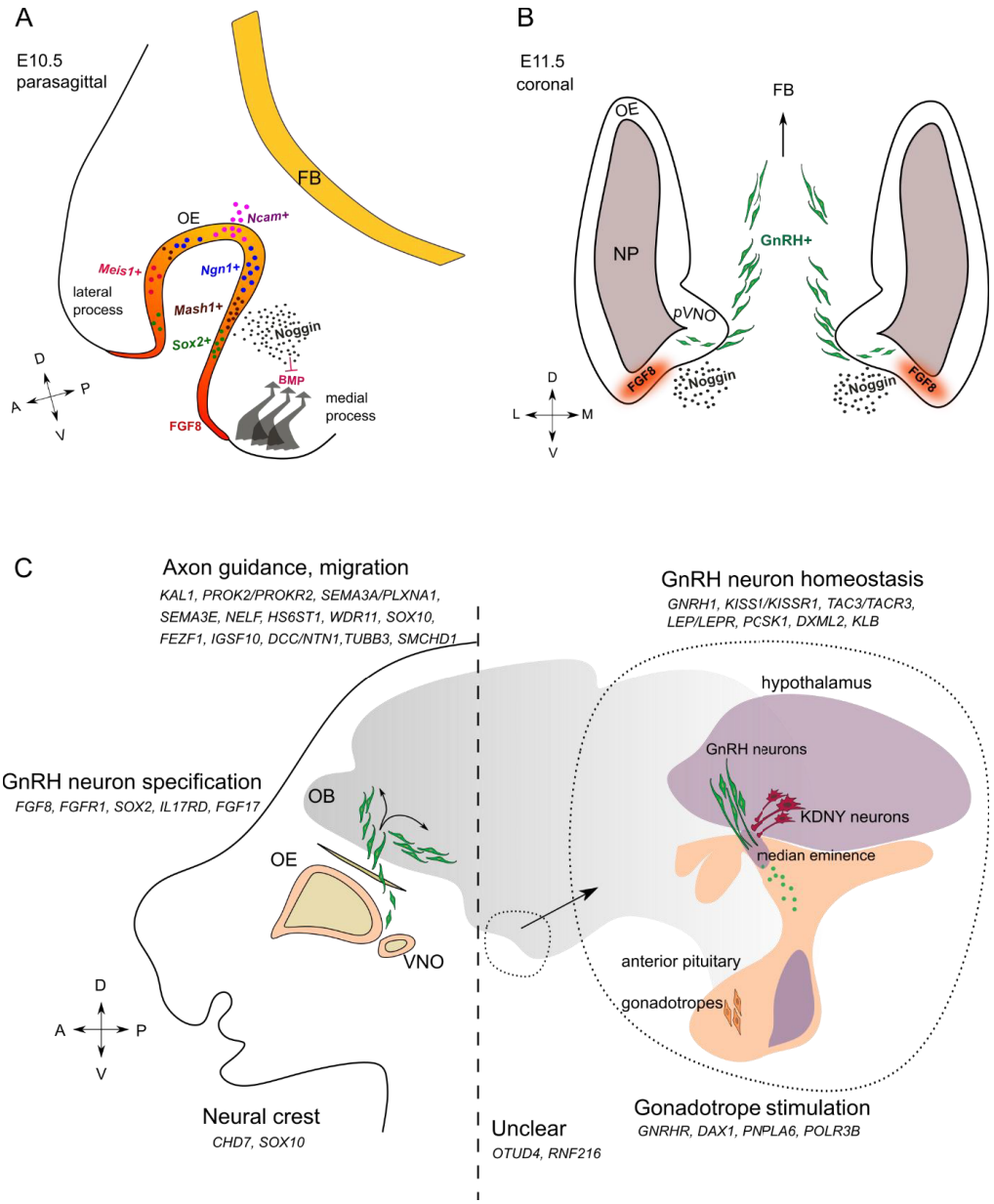


Figure 3. Neurogenesis in the olfactory epithelium and emergence of GnRH neurons in mice. A) In the developing nasal pit, Fgf8 is expressed by cells at the rostral tip of the invaginating epithelium. Figure drawn based on (Kawauchi et al., 2005, Tucker, E. S. et al., 2010, Forni et al., 2013). B) Emergence of GnRH neurons in the frontonasal mesenchyme, proximal to the areas expressing Fgf8 and Noggin, at the border of the presumptive VNO. Postmitotic GnRH neurons migrate toward the forebrain, in groups of cells called the migratory mass. C) As they enter the brain, GnRH neurons diverge as a dorsal route to the forebrain, and a ventral route to the hypothalamus (left panel). In the adult (right), hypothalamic GnRH neurons send their dendrons, which split into axons as they enter into the median eminence. KDNY neurons modulate GnRH secretion to the gonadotropes in the anterior pituitary, CHH-associated genes are listed under their suggested roles in GnRH neuron biology (Stamou, Cox & Crowley, 2015, Young et al., 2019).

The hypothesis about adenohypophyseal origin is, however, conflicted by the fact that GnRH neurons are normal in mice lacking the anterior pituitary (Metz, Wray, 2010).

The *Cre-loxP* system allows for conditional genetic recombination, that has been a ground-breaking technique for mammalian cell-lineage tracing. Expression of a reporter allele combined with a Cre recombinase-expressing line leads to tissue-specific reporter expression, which can be used to trace stem cells and their progeny. The *Wnt1-Cre* transgenic mouse line (Danielian et al., 1998) expresses Cre in premigratory NC cells in the dorsal neural tube, and in combination with the *ROSA26 (R26R)* Cre-reporter line, recombination can be detected by reporter expression in approximately 96% of migratory NC cells (Debbache, Parfejevs & Sommer, 2018). In the study by Forni and colleagues, the transgenic mouse line *Wnt1-Cre;R6RYFP+* was used to label NC cells, and some of the reporter labelling was found within the OP, overlapping with GnRH+ immunolabeling (Forni et al., 2011). They thereby proposed that both the OP and NC would be able to contribute to the GnRH neuron population.

Shortly thereafter, conflicting evidence arose from the chick embryo (Sabado et al., 2012). By grafting of the neural folds (the source tissue of NC), from transgenic GFP-chick embryos to WT hosts, Sabado and colleagues found that GFP+ cells enter the frontonasal mass and surround the olfactory nerve but do not integrate to the OE, and no GnRH1+ cells were found positive for GFP. By labelling using focal injections of lipophilic dyes in either OP or adenohypophyseal placode, they discovered that GnRH+ cells are born within the OP, and not from the anterior pituitary progenitors. Nasal explant cultures from different developmental stages showed that OSNs begin to be specified before GnRH neurons, which are specified in a short time window at HH16-17, and have already specified terminally by HH20. The authors, furthermore, express concerns about the reliability of mouse Wnt1-Cre;R6RYFP+ which was used by Forni and colleagues, as it had shown ectopic labelling of cells in telencephalon and retina. Moreover, Barraud and colleagues had previously shown that NC cells contribute to the olfactory ensheating cells (OECs), glial cells that surround the olfactory nerve, but no contribution to olfactory neuronal cell types have been reported since (Barraud et al., 2010).

Another study conducted in zebrafish also showed that NC cells do not mix with OP cells during OE development, but that NC cells remain in close association with the OP, giving rise to OECs that surround the olfactory nerve (Harden et al., 2012). This was later supported by studies using a *Sox10*-mutant model, which disrupted development of many cranial NC lineages including OECs, but did not affect GnRH neurons (Aguillon et al., 2018). This study also showed backtracking of anterior PPE and *in vivo*, which revealed that precursors of GnRH+ cells were backtracked to the anterior PPE which lies close to the progenitor region of the adenohypophyseal placode (Dutta et al., 2005). Neuronal progenitors were not backtracked to the NC, which further argues against NC contribution to the OP, and supports the hypothesis that *"cell type heterogeneity in the zebrafish olfactory epithelium is generated*

from progenitors within the PPE" (Aguillon et al., 2018). In human foetuses, SOX10-immunolabeling has not been reported to co-localize with GnRH neurons, albeit they were detected in close association with SOX10-positive ensheating cells (Casoni et al., 2016).
So far, all these data point that GnRH neurons are being specified in the OP from neuronal stem cells of preplacodal origin, and they acquire the required linage competence under the paracrine influence by FGF8 and Noggin. Six1-positive cells in the non-neuronal ectoderm, the PPE, become further restricted by local signals to give rise to the olfactory placode. OPs are either assembled separately from rostrally migrating cells or as a two gradually segregated groups of the rostral tip of the telencephalon, which converge rostrally from lateral populations of the neural tube, and eventually become separated from the forebrain by groups of NC cells during facial morphogenesis. Further support on NC origin has not been acquired in the last ~10 years. With increasing development in the field by single-cell sequencing and CRISPR-based genome editing, new data on this matter are expected to take us forward during the following 10 years, to hopefully get an answer to the question of GnRH neuron ontology.

4.10 Emergence and migratory path of GnRH neurons during embryogenesis

The first report on GnRH neurons in the mouse nasal region dated the emergence of GnRH neurons to embryonic day 11 (E11). Cytoplasm GnRH-immunoreactivity (Then called LHRH) was detected from E11 forward in the medial OP in cells with axons that appeared to be coming out from the epithelium of the VNO. As a sign of migration, GnRH-positive cells were detected entering the brain at E14, and at E16 in the septal preoptic area (Schwanzel-Fukuda, Pfaff, 1989) (Figure 3C). Further characterization revealed increased labelling of GnRH mRNA and peptide at E11.5- E12.5 in the nasal mesenchyme (Wray, Grant & Gainer, 1989b). At E12.5, a total number of approximately 800 GnRH-expressing neurons were counted, and 90% of these were found in the nasal area. The GnRH neurons that were detectable by immunohistochemistry also expressed markers for postmitotic neurons. To approximate the birthdate of the GnRH neurons, immunohistochemistry was combined with 3H-thymidine autoradiography, which suggested that most of the GnRH neuron precursors underwent their final mitotic division between E10.5-E12.5, with the highest 3H-thymidin labelling in GnRH neuron at E11.5.
In a human foetus, GnRH immunoreactivity was discovered at the ~42nd day of gestation, but not at the earlier stages examined (days 28-32 of gestation) (Schwanzel-Fukuda et al., 1996). At day 42, the TN was observed as axon fibers spanning from the medial nasal mesenchyme, and entering the forebrain medially to the olfactory bulbs, in the direction of the ventral septal regions of the brain. GnRH-expressing cells were observed as a continuous stream from the nose to the preoptic area, always in contact with N-CAM expressing nerve fibers. A highly

sialated form of N-CAM was also detected by using PSA-NCAM antibodies in certain parts of the migratory pathway in close association with GnRH neurons. The polysialic forms of N-CAM have been implicated in lower cell adhesiveness in contrast to the stable, highly adhesive N-CAM (Rutishauser, 1996, Johnson et al., 2005) which is abundant along the terminal and vomeronasal nerves. This suggested that contact with the less adhesive PSA-NCAM may facilitate acceleration of movement away from the olfactory epithelium, and that the stable connection to less sialated NCAM fibers may serve as guides for along the migratory stream (Schwanzel-Fukuda et al., 1996, Yoshida et al., 1999). More recently, a subset of human foetuses were investigated using immunohistochemistry and DISCO tissue-clearing techniques, for further characterization GnRH early emergence and migratory pathway (Casoni et al., 2016). In the human foetus, the OPs have invaginated and formed olfactory pits by Carnegie stage (CS) 16, around 39 days of gestation. In the medial part of the OP, a small loop of the invaginating epithelia forms the presumptive vomeronasal organ (pVNO). At CS 16, a small number of GnRH neurons have emerged in the medial nasal mesenchyme proximal to the pVNO basal lamina, seen as GnRH-immunopositive cells that co-express the immature neuron marker Doublecortin (DCX) (Gleeson et al., 1999). Thus, the point of GnRH neuron specification likely occurs at 5-6 weeks of gestation. As shown in the mouse, the emerging GnRH neurons co-express markers for postmitotic neurons, which suggests that they have terminally differentiated at the stage when GnRH expression is detectable (Casoni et al., 2016). In total, Casoni and colleagues reported approximately 10 000 GnRH-expressing neurons in human foetuses from CS16-21.

At the onset of their migration, GnRH neurons converge into a group of migratory cells in the nasal mesenchyme, called the migratory mass. The migratory mass contains also other cells expressing DCX and TUJ1 in addition to GnRH-expressing neurons. The migratory mass moves along VNO and TN axon bundles in the nasal septum during CS 18-20. At CS 18 the first GnRH neurons have already entered the brain. Detected in a CS 21 embryo, Casoni and colleagues describe GnRH neurons migrating in a chain-like organization along Peripherin-expressing sensory and motoneuron axon fibers that project into the forebrain. Surprisingly, the GnRH neurons take two different streams; the first one takes a loop that turns ventrally towards the hypothalamus, similarly to previous reports (Schwanzel-Fukuda et al., 1996), and another migratory stream appears to be directed toward the pallial and subpallial telencephalon (Figure 3 C). GnRH immunopositive cells were also found around the OBs, cerebral cortex, hippocampus, piriform cortex, amygdala and habenula (Casoni et al., 2016). The putative functions of the extrahypothalamic GnRH-expressing neurons are not known.

4.11 How is GnRH neuron migration regulated?

Many guidance molecules and their receptors have been implicated in the regulation of GnRH neurons' migration from the nose to the brain, and are associated with GnRH deficiency in developmental disorders (Cariboni, Maggi & Parnavelas, 2007, Wierman, Kiseljak-Vassiliades & Tobet, 2011). In addition to the previously mentioned N-CAM, other reports include:

Netrin-1 and its receptor DCC, expressed in GnRH neurons (Schwarting et al., 2001, Schwarting et al., 2004),

Reelin, the ECM protein, which repulses of GnRH migration (Dairaghi et al., 2018),

Nelf, the nasal embryonic LHRH factor (Kramer, Wray, 2000, Xu et al., 2010),

Ephrins, the contact-dependent signalling molecules and their receptor tyrosine kinases (Gamble et al., 2005),

Semaphorins (SEMAs), **their Neuropilin receptors (NRPs), and the Plexin co-receptors** (Cariboni et al., 2007, Messina et al., 2011, Hanchate et al., 2012, Messina, Giacobini, 2013, Oleari et al., 2019),

SDF1, the chemokine attractant, and its receptor CXCR4 (Schwarting et al., 2006),

HGF and its receptor Met (expressed in early migrating GnRH neurons (Giacobini et al., 2007)).

In mice, the depletion of *ARX1*, a homeodomain transcription factor expressed by neuronal progenitors of the OB, causes severe tangling of the olfactory and vomeronasal nerves. A recent study using *ARX1* (null) mutant mice showed that the development of the olfactory bulbs, and their olfactory and vomeronasal axonal connections are not crucial factors for GnRH migration into the brain (Taroc et al., 2017). Instead, the terminal nerve is the most prominent scaffold for GnRH neuron migration. Taroc and colleagues detected different guidance receptors for the TN compared to vomeronasal and olfactory axon fibers; the vomeronasal and olfactory nerves, but not the GnRH neurons nor the TN, exhibit strong expression of Neuropilin 1 & 2, receptors for the Sema3 family of secreted guidance molecules (Schwarting et al., 2000, Cariboni et al., 2007). Sema3A expression was seen in the forebrain behind the olfactory bulb, and was thereby suggested to prevent the vomeronasal and olfactory axons from entering the brain, and staying in the olfactory bulbs, whereas the GnRH neurons were found to enter the Sema3A-expressing area, along with the TN.

Other guidance molecules, Slit1, Slit2, and the Robo receptors, also showed similar differences. Strong cortical and forebrain expression of Slit1 repels Robo1- and Robo2-expressing olfactory and vomeronasal fibers from entering the brain (Nguyen-Ba-Charvet et al., 2008). GnRH neurons and TN do not express Robo1 or 2, but instead they do express Robo3, which silences Slit-mediated repulsion (Taroc et al., 2017).

The neural crest-derived OECs are closely associated with GnRH neurons during their migration along the TN (Geller et al., 2013). OECs have been implicated in migratory guidance, and survival of olfactory axons and GnRH neurons during their migration to the brain (Barraud et al., 2013). The Notch pathway is involved in regulating the differentiation and maturation of OECs. Activated Notch/RBP-J suppresses neuronal differentiation, and promotes glial cell types in the cranial neural crest cells that inhabit the olfactory nerves (Miller et al., 2018).

AIMS

To recapitulate and study the embryonic development of GnRH neurons *in vitro,* by using human pluripotent stem cells:

I. To derive multipotent neural crest progenitor cells and cranial neural crest by differentiation of human pluripotent stem cells, in order to investigate the disease mechanisms of Kallmann Syndrome

II. To generate a protocol for differentiation of GnRH neurons from human pluripotent stem cells *in vitro*.

III. To isolate and characterize the developmental transcriptome of hPSC-derived neuronal progenitors and early postmitotic GnRH neurons.

4.12 Ethical consideration

No new hIPS cell lines were generated. Human pluripotent stem cell lines used in this work were existing cell lines acquired from WiCell (H9 hESCs), and the Biomedicum Stem Cell Center, University of Helsinki. No patient cell lines were used in this work.

Human foetal work was performed at INSERM, Lille, France, in the laboratory of Paolo Giacobini in accordance with French bylaw (Good practice concerning the conservation, transformation and transportation of human tissue to be used therapeutically, December 29, 1998). The permission to use non-pathological human foetal tissues was granted by French Agency for Biomedical Research (Saint-Denis la Plaine, France, protocol no. PFS16-002).

5. Article I

5.1 Culture and maintenance of human pluripotent stem cells and DNA transfections

We cultured H9 hESCs (WiCell) and Hel11.4 iPSCs (Mikkola et al., 2013) on Geltrex™- (Life technologies) coated plates with StemPro (Invitrogen). We passaged cells using collagenase IV (Invitrogen), and scraped the bottom of the wells using a cell scraper to detach cells from the culture dish, so that cells remained in small colonies in the suspension. We performed transfection with Lipofectamine 2000 (Invitrogen) as described in (Noisa et al., 2010). We split cells when confluent in 1:2 ratio using 0.02% EDTA (Sigma) into a Geltrex-coated culture dish 24 hours before transfection. We initiated selection (G418) 48 h after transfection, at 200 mg/ml for 3 weeks. We manually picked surviving colonies to separate wells in 24-well plate for expansion.

5.2 shRNA-mediated knockdown of JAG1 and overexpression of human NICD1

We used previously described siRNA for JAG1 (Choi et al., 2008), with the target sequence for JAG1: 59-AGGATAACTGTGCGAACATC-39, and scrambled siRNA: and 59-GGGCGTCGATCCTAACCGG-39. We annealed and cloned the sequences to pSuperior-Neo plasmid, driven by the H1 promoter (OligoEngine). We obtained the human NICD1 sequence from Addgene [plasmid 17623 (Yu, X. et al., 2008)] and subcloned it into a vector containing the CAG promoter and a G418 resistance cassette.

5.3 Neural crest, neural lineage and mesenchymal derivative differentiation

For neural crest cell differentiation, we optimized a previously reported protocol in our lab (Menendez et al., 2013). We passaged confluent hESCs with EDTA 0.02% in PBS on Geltrex-coated dishes with StemPro. After 24 hours (or at approx. 75% confluency) we added N2B27

medium supplemented with 2 μM Dorsomorphin (Sigma) and 5 μM 1-azakenpaullone (GSK3-β inhibitor, Sigma). We changed medium every other day, and passaged cells with TrypLE (Invitrogen). For neural differentiation after pNCCs establishment, we added 20 μM DAPT (Sigma) to the same culture medium. We used StemPro Osteogenesis Kit, StemPro Chondrogenesis Differentiation Kit and StemPro Adipogenesis Differentiation Kit (Invitrogen) for osteocyte, adipocyte, and chondrocyte lineage differentiation of pNCCs. For neural differentiation of hPSCs, we used 2μM Dorsomorphin without adding other factors.

5.4 Immunocytochemistry

We fixed cells using 4% paraformaldehyde (10'), and blocked unspecific proteins with 10% foetal bovine serum and 0.1% Triton X-100 in PBS (1 h), then incubated the primary antibody over night at 4˚C, washed with PBS, and then we applied secondary antibodies (45', RT). We mounted coverslips onto microscope slides using Vectashield mounting medium with DAPI (Vectorlabs).

5.5 Flow cytometry

We detached the cells with trypsin-EDTA and washed with PBS. We stained cells for antibodies against cell-surface antigens on ice and we analysed the samples with FACScan (BD Biosciences) using CELLQUEST software (BD Biosciences), using 10,000-20,000 cells per sample. For antibodies against nuclear proteins, we fixed the cells with 4% paraformaldehyde (15'), permeabilized with 100% ethanol for (2') and treated with 10% goat serum (15') after washing with PBS. We incubated samples with primary antibodies (30'), and secondary antibodies (30') before the analysis.

5.6 Migration assay

We used wound-healing assay to measure migration. We cultured cells until confluent and made a scratch using a 10 μl pipette tip. We then observed cell migration after 24 and 48 hours under a light microscope.

5.7 Microarray transcription profiles

We extracted total RNAs from the samples using the All Prep DNA/ RNA/Protein Mini kit (Qiagen), and assessed the quality of the RNA using a 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent). We performed transcriptome analysis with Illumina Human HT-12 Expression BeadChip according to the manual provided by Illumina. We pre-processed the raw data using the lumi package in R (Du, Kibbe & Lin, 2008), including background correction, variance stabilization, and quantile normalization. We removed duplicate probe sets with genefilter package in R by retaining the probe set with the highest interquartile range. Present and

absent calls for the probe sets were obtained by using the detection P-values. We performed differential expression analysis using an unpaired moderated t-test as implemented in limma in R (Smyth, 2004). Threshold for differentially expressed genes according to Benjamini-Hochberg adjusted P-value as 0.05 and the log fold change < -1, or > 1. The GEO accession number for data deposition is GSE53203.

5.8 Reverse transcription and real-time PCR

We used RNA Spin II kit (Macherey-Nagel) for RNA extraction according to the manufacturer's instructions. We synthesized first-strand cDNA using SuperScript III reverse transcriptase (Invitrogen) with oligo dT primer (Invitrogen). We included 1% of the resulting cDNA in a 20 µl mixture containing 10 µl of SYBR Green Taq solution (Sigma) and 5 µl of 2 µM primer mix. We performed PCR reactions using Corbette thermal cycler (Qiagen) for 40 cycles of 95˚C for 15 s, 60˚C for 30 s and 72˚C for 30 s. We calculated relative expression by calibrating their CT values to that of the housekeeping gene (cyclophillin G, also known as PPIG) and normalized to the expression level in hESCs (day 0) by ΔCT method.

5.9 Chromatin immunoprecipitation

We performed Chromatin immunoprecipitation and real-time PCR with Agarose ChIP Kit as described in the manufacturer's protocol (Thermo Scientific, Pierce). We had 26106 cells crosslinked by the addition of formaldehyde into the medium to a final concentration of 1% for 10‘ at 37˚C, and quenching by glycine to the final concentration of 0.125 M for 5’ (RT). We digested cell extracts with micrococcal nuclease (MNase) for 15‘ at 37˚C. We mixed fragmented chromatic with 5mg of anti-NICD1 antibody (Cell Signaling Technology, #4147) and 5 mg of normal rabbit IgG (provided with the kit), and left on rotating wheel o/n at 4˚C. We added 20 ml of Protein-A/G-plus–agarose beads and incubated for 3 h at 4˚C. After the elution of the immune complexes, we extracted the DNA and performed real-time PCR with specific primers. We used fold enrichment method to determine the level of enrichment of NICD1 at a particular region of DNA, taking into account the total chromatin (input) and any non-specific binding (normal rabbit IgG).

6. Article II

6.1 Cell lines

For differentiation experiments, we used the hESC line H9 ((Thomson et al., 1998); Wicell) and two hiPSC lines HEL11.4 (Mikkola et al., 2013) and HEL24.3 (Trokovic et al., 2015), provided by the Biomedicum Stem Cell Centre, University of Helsinki Finland. Cells were cultured on Geltrex-coated plates with StemPro (Thermo Scientific) or Essential 8 (Thermo

scientific) mediums. We always switched to StemPro before starting a differentiation experiment, in order to acquire a confluent plate of cells, which is not possible in E8 medium.

6.2 Differentiation procedure

For GnRH neuron differentiation, we used N2B27 medium. On days 0-10 the medium contained 2 μM dorsomorphin, and 10 μM SB431542 (Sigma). We passaged the cells in 1:2 ratio on day 10, with collagenase IV (Invitrogen) and supplemented medium with 10 μM Rock inhibitor Y37632 (Sellekchem) over night. On days 11-20 medium containing 100 ng/ml FGF8 (Peprotech) we refreshed daily. Another split was performed at day 20, in ratio 1:6-1:8 using 0.5 mM EDTA in PBS (Invitrogen). On days 21-27 (and onwards) we supplemented the medium with FGF8 and 20 μM Notch inhibitor DAPT* (Sigma). Control cells did not receive FGF8 supplementation. After day 21 we refreshed medium every other day.

*After this article we have started using DAPT provided by Selleckchem, as the product was no longer available from Sigma.

6.3 Analysis of GnRH Expression and Secretion

We extracted RNA from cell material using Nucleospin RNA kit (M&N), and reverse transcription using Superscript III (Thermo Fischer). We performed qPCR using HOTFIREpol SolisGreen qPCR mix (SolisBioDyne), and ran samples in LightCycler 480 (Roche). We normalized ct values to cyclophilin G, and compared with undifferentiated hPSCs (Relative expression, ΔCT method). For immunocytochemistry, we fixed cells by 4% paraformaldehyde, and immunostainings were performed using standard protocols (described in detail in Supplementary methods, article II and III). FOXG1 and GnRH immunopositive cells were quantified by manually counting the cells in images taken with a 40x objective (n=3 independent experiments). We quantified secreted GnRH from cell culture medium by a competitive fluorescent enzyme immunoassay (Phoenix Pharmaceuticals).

6.4 Human samples immunohistochemistry

Validation immunohistochemistry was performed at INSERM in Lille, France. One human foetus (8 gestational weeks) was obtained from a voluntarily terminated pregnancy with the parent's written informed consent. The permission to utilize human foetal tissues was obtained from the French agency for biomedical research (Agene de la Biomédecine, Saint-Denis la Plaine, France, protocol no. PFS16-002). Tissues were made available in accordance with French bylaws. Tissues were fixed with 4% paraformaldehyde and immunostaining was performed on 20 μm cryosections. Immunostaining was performed according to standard procedures (P.G. INSERM, France).

Statistical Analyses

In the text and figures, “n” stands for number of independent experiments. We performed statistical comparisons using paired ratio t-test in Prism 5.0 (GraphPad). $p < 0.05$ was accepted to indicate statistical significance.

7. Article III

7.1 Generation of GNRH1-TdTomato reporter cell line

We generated the reporter cell line using CRISPR-Cas9. Our strategy was to target the stop codon at the *GNRH1* gene for insertion of donor template GNRH1_T2A_NLS_TdT_PGK_puro. The donor template contained homologous arms of 577bp (5p) and 599 bp (3p) for homologous recombination into genomic DNA in hPSC lines H9 and HEL11.4. We cloned homologous arms by PCR from the genomic DNA of the human embryonic stem cell line H9 with primers containing BamH1 and Nhe1 (5p homologous arm) and Ascl1 and Xba1 (3p homologous arm) restriction sites for cloning, and PCR purified (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel) and digested the fragments. We then purified them from the agarose gel and ligated to the donor template vector T2A-2xNLS-TdTomato-PGK-Puro. We designed Guide RNA sequences using [crispr.mit.edu/Guides] and amplified and purified guide cassettes as described previously (Balboa et al., 2017).

7.2 Electroporations and expansion of the reporter clones

After culturing hPCS lines HEL11.4 and H9 in StemPro (Thermo Fisher Scientific), we dissociated the cells with Accutase (Life Technologies) and resuspended them in cold 5% FBS/PBS. We performed three electroporations using Neon Transfection system (Life Technologies) according to manufacturer’s instructions, with a total of 6x10E6 cells with 18 µg CAG-Cas9 (Addgene plasmid ID 89995), 6 µg pUC-GNRH1-T2A-NLS-TdT-PGKPuro, and 1.5 ug guide RNA using a pre-optimized program (1100V, 20 ms, 2 pulses). We then plated cells onto Matrigel matrix-coated (Corning) dishes with 10 µM ROCK inhibitor (Y-27632 2HCl, Selleckchem) in StemPro, and changed to StemPro with 5 µM ROCK inhibitor on the following day. We removed ROCK inhibitor after 48h, and started selection using 0.15 µg/ml Puromycin (Sigma) after 72h. We picked surviving colonies onto Matrigel coated 96-well plates in StemPro + 5 µM Rock inhibitor around 7-10 days after electroporation.

7.3 Screening for reporter integration

For DNA isolation, we treated the cell colonies with 0.5 mM EDTA (Invitrogen) for 4 min, and replaced EDTA with 100 µl StemPro + 10 µM ROCK inhibitor, and detached cells by scraping with a 10 µl pipette tip. We took half of the suspension to a V-bottom plate for lysis, and left

the other half for continued culture. We centrifuged the V-bottom plate to remove supernatant and lysed cells in 60 µl PCR Direct lysis buffer (Viagen Biotech) and 5 µl Proteinase K, and incubated the cells at 55°C/2h, and 85°C/1h, and then froze at -20°C until use. We performed touchdown PCR for detection of integration of the 5' and 3' homology arms using primers and conditions listed in Supplemental Text 1 of article III, Lund et al 2020.

7.4 Differentiation

After screening of the insertion sites into the correct genomic location, we thawed and expanded three clones per cell line for differentiation into GnRH neurons, as previously described in article II, Lund et al., 2016, and selected the most efficient TdTomato-expressing clones for further experiments. We isolated TdTomato-positive cells by FACS on days 25 and 27 of the differentiation protocol for RNA sequencing.

7.5 Fluorescence-activated cell sorting

We prepared single cell suspensions of the differentiated neurons by incubation with Accutase 5 min 37°C, then added 3 volumes of 10% FBS (Life Technologies) in PBS and centrifuged 3 min at 200 g. We resuspended cells in FACS buffer (10% FBS, 2 µM EDTA, 0.625 mM HEPES buffer (Sigma), and 10 µM ROCK inhibitor, in HBSS (Life Technologies)) at approx. 1.5-2 million cells/ml, and transferred them to 5 ml Falcon tube with cell strainer snap cap (Falcon). We performed FACS sorting using SH800 cell sorter (Sony Biotech), and collected TdTomato-expressing cells into Eppendorf tubes (at least >2000 cells per sample for RNA isolation).

7.6 Quantitative polymerase chain reaction

We extracted RNA using RNAqueous micro Total RNA isolation kit (Thermo Scientific), and reverse transcribed the mRNA using iScriptTM cDNA Synthesis Kit (BIO-RAD, 170-8891) according to manufacturer's instructions. We performed qPCR as described in article II Lund et al 2016.

7.7 Immunocytochemistry

We plated cells on Geltrex-coated glass coverslips until pre-fixed them by applying 4% PFA in 1:1 ratio into the cell culture medium for 5 min, then washed carefully with PBA and continued with 4% PFA for 17 min and washed 3-4 times in PBS. We permeabilized cells with 0.05% Triton-X100 (Sigma) in PBS for 7 min, then blocked with Ultra Vision Protein Block (Thermo Scientific) for 10 min, and applied primary antibodies diluted in 0.1% Tween (Sigma) in PBS overnight at +4°C. After three washes with 0.1% Tween + PBS we applied secondary antibodies for 35 min-1h at room temperature. We captured immunofluoresence images by Zeiss Axio Imager Z1 upright epifluorescence microscope and Zen Blue (Zeiss) at Biomedicum Imaging Unit and processed images using ImageJ.

7.8 Human tissue collection and immunohistrochemistry

Authors P.G., S.T. and C.A at INSERM (France) collected and processed the human fetus samples which where obtained with the parent's written informed consent (Gynaecology Hospital Jeanne de Flandres, Lille, France). We obtained samples from 2 foetuses 10.5 weeks post amenorrhea, immersion-fixed in 4% paraformaldehyde in 0.1M phosphate-buffer saline (PBS) (pH 7.4) for 1 weeks at 4 °C, cryoprotected in 30% sucrose in PBS for 48 h, embedded in Tissue Tek (Miles), and frozen in liquid nitrogen, then cryosectioned samples at 18 µm on a CM3050 Leica Cryostat. Protocols for indirect immunofluorescence have been described in detail by Casoni et al 2016, and in Lund et al, 2016 and 2020.

7.9 RNA sequencing

After RNA isolation, we measured concentration, integrity and quality using Qubit Fluorometer and TapeStation 4200 (Agilent), and performed sequencing runs with Illumina NextSeq500 sequencer (Illumina) at FUGU (University of Helsinki) with NEBNext Ultra Directional polyA capture method as paired-end sequencing for read length 75 bp. We did the first run with day D25 FACS sorted TdTomato +/- samples (n=3), and the second run with D20 FGF8-treated cells, D20 w.o. FGF8 treatment, as well as D27 TdTomato + cells (n=4/group) in order to study differences in expression between progenitors and GnRH neurons, and the effect of FGF8 treatment in the progenitor cells (Article III, Fig. 2 A, B). D stands for day of differentiation.

7.10 RNA sequencing data analyses

We analysed quality of raw sequencing data in FASTQ format using FASTQC (Simon Andrews Babraham Bioinformatics), trimmed reads using Trimmomatic (Bolger, Lohse & Usadel, 2014), and aligned the reads against GENCODE GRCh38 reference (Harrow et al., 2012) using STAR (Dobin et al., 2013). We assessed quality of the sequencing by Qualimap (Garcia-Alcalde et al., 2012), generated read counts from bam files by FeatureCounts (Liao, Smyth & Shi, 2013), and annotated with ensemble release 87 using BioMart package in R (Yates et al., 2016, Durinck et al., 2009). We normalized and perfomed differential expression analysis using DESeq package in R (Anders, Huber, 2010). We compared the differentially expressed genes with absolute log-fold change over 1 and under the p-value of 0.05 (Benjamini-Hochberg) as criteria. We performed over-representation analysis using Genetrail 2.0 (Stockel et al., 2016). We used Graphpad Prism 7 for drawing bar charts of differentially expressed genes, and Ingenuity Pathway Analys (IPA, Qiagen Inc.) for drawing gene interaction pathways. We have deposited full RNA sequencing data to ArrayExpress (E-MTAB-7938, -7956, -8519).

RESULTS

8. Article I: Notch signalling during the differentiation of neural crest from human pluripotent stem cells

8.1 Differentiation of premigratory neural crest cells from human pluripotent stem cells

For the differentiation of NC cells from hPSC, we based our protocol on a previously reported method by Menendez et al. (2011). This protocol produces neural crest-like stem cells, and was based on dual SMAD inhibition with Noggin and SB43521 for Activin A/Nodal and BMP signalling inhibition with simultaneous activation of the canonical Wnt pathway with BIO ((2′Z,3′E)-6-bromoindirubin-3′-oxime). BIO is a small molecule inhibitor of GSK3 (glycogen synthase kinase 3). This method was first created by Menedez et al., in order to induce the formation of ectoderm lineage, while concurrently directing cells away from a neuronal fate and toward the NC by using Wnt activation (Garcia-Castro, Marcelle & Bronner-Fraser, 2002, Patthey, Edlund & Gunhaga, 2009, Menendez et al., 2011) In our case, instead of Noggin we used Dorsomorphin, which is an effective small molecule inhibitor of BMP pathway (Yu, P. B. et al., 2008). For Wnt pathway activation, instead of BIO, we used 1-azakenpaullone as GSK3-β inhibitor, since it has been reported to have higher selectivity towards GSK3-β (Kunick et al., 2004).

After culturing cells in these conditions for 10 days (hereafter referred to as D10), we detected an increase in NC specifier genes; *PAX3, TWIST1, SLUG, MSX2, HAND2*, and *SOX10* by qPCR (Article I, Fig. 1 A). Neuronal markers *PAX6* and *NGN2* were low after D10 (Article I, Fig. S1). Immunocytochemistry detected protein level expression of NC markers PAX3, Nestin, TWIST, SLUG and SOX10, as well as a few interspersed AP-2α-positive cells (Article I, Fig. 1 A). Flow cytometry showed an abundance of PAX3, SLUG, and SOX10 positive cells. We saw a relatively low number of cells positive for AP-2α, which expressed in NC cells and their derivatives (Mitchell et al., 1991), and was described in human migratory NC cells (Betters et al., 2010). This suggested that the cells were not yet in the migratory stage of differentiation. Furthermore, with this protocol premigratory NC cells (pNCCs) could be maintained for up to 20 passages with the same morphology, while retaining NC marker expression.

In order to ensure differentiation potential, we performed clonal expansion from single cells at the pNCCs stage in the same medium conditions and differentiated the clones into NC derivatives by directed differentiation; to adipocytes, chondrocytes, osteocytes, and neurons (Article I, Fig. 1 D-E). Neurons formed when pNCC stage cells were cultured in N2B27 without added factors (Article I, Fig. 1 F).

An increase in NICD1 was detected by Western Blot, whereas no considerable change in the NOTCH1 receptor was seen (Article I Fig.1 G). The relative mRNA expression of the Notch ligand *JAG1* was significantly increased, as were the Notch downstream effector genes *HES1* and *HES5*, but this was not seen for *NOTCH1*, *NOTCH2*, and *JAG2* (Article I Fig.1 H). These data suggest that an upregulation of Notch pathway occurs early in NC differentiation, which is likely caused in part by the upregulation of *JAG1* expression.

8.2 Notch signalling during specification of neural crest

To investigate the role of Notch signalling during NC specification from pluripotent stem cells, we used small molecule inhibition of the Notch pathway by DAPT. In addition, we used shRNA to reduce endogenous expression of Notch ligand *JAGGED1* (*JAG1*) in the hPSC cell pool. By examining the mRNA expression levels of NC marker genes at D10 of the differentiation, we found that inhibition of Notch by DAPT in two concentrations (10 μM and 20 μM) had decreased the level of NC marker induction (Article I Fig. 2 A). Knockdown of *JAG1* in hPSCs caused a ~60% reduction in detected *JAG1* mRNA, and after 10 days of neural crest differentiation, the NC marker expression was, similarly, decreased (Article I Fig. 2 D).

In small molecule inhibition (DAPT) experiments, we observed an increase in *NGN2* and *NGN3* at D10 with DAPT, but not without DAPT (Article I Fig. 2 A). Similarly, in JAG1 knockdown we observed an increase in *NGN2* (Article I Fig. 2 E). The upregulation of *NGN2* was also detected by RNA microarray between the DAPT-treated and non-treated pNCCs at D10, in addition to the neuronal genes *NGN1, NGN2, MASH1, NEUROD1,* and *PAX6* (Article I Fig. 3 A). Simultaneously, the NC specification markers *TWIST1*, *PAX3*, *WNT3a*, and *HAND1* were downregulated in the dataset. These genes were also detected showing a similar pattern by qPCR (Article I Fig. 3 A, C). To further analyze the potential effect of Notch inhibition on gene networks, we performed pathway analysis of the microarray data to identify putative interactions between the differentially expressed genes and Notch signalling. The pathway analysis suggests target genes such as *CER1, FOSB, FOXC1,* and *IGFBP4* to be regulated by Notch during NC specification (Article I Fig. 3 D). These data imply that active Notch signalling is required to sustain the expression of a large network of NC specifier genes, and their downstream targets.

We next asked how Notch regulates neural crest specifier genes expression. We studied the effect of activating Notch signalling by overexpression of NICD1 in HEK293 cells and in hPSCs. In HEK293 cells, the transfection of the expression vector lead to an increase of HES1 promoter and CBF1RE-luciferase activity. The successful overexpression of NICD1 in hPCS was confirmed by Western Blot, and pluripotency markers and morphology of the cells were monitored before differentiation (Article I Fig. 4 A-D). NICD1 overexpression in hPCSs and

subsequent differentiation into NC followed the same pattern in gradual upregulation of NC specifier genes *PAX3, TWIST, SLUG,* and *DLX5* during the 10 first days of differentiation, but fold changes in relative mRNA expression were magnified (Article I Fig. 4 E). This suggests that higher NICD1 availability in the cells during the early differentiation led to a more effective induction of transcription factors regulating NC specification. Immunocytochemistry showed that TWIST1, SOX10, PAX3, and SLUG were detectable at D5, which was not the case for the control (non-transfected) cells (Article I Fig. 4 F). We next used chromatin immunoprecipitation (ChIP) to address whether NICD1 itself directly binds to 5' promoter regions of NC specifier genes. ChIP showed that the recognition sequence of NICD1-CBF1 complex was binding to DNA fragments corresponding to *PAX3, DLX5, SLUG*, and *TWIST1* (Article I Fig. 4 G, H). This suggests that NICD acts as an activator of NC specifier genes, thereby promoting NC differentiation.

8.3 Notch signalling and patterning of premigratory neural crest

Notch signalling has been shown to retain cells in an undifferentiated state, whereas FGF8 has been shown to act as a chemotactic during cardiac NC migration (Sato, A. et al., 2011). We next asked how FGF8 treatment and Notch signalling inhibition affect the migration of NCCs. Using wound healing assay, we found that FGF8 treatment alone did not induce migration within 24 h, but migration was induced by the combination of FGF8 and DAPT (Article I Fig. 5 A). An increase in markers that are seen in migratory cells (*AP-2 a, HNK1, CDH6, ERBB6, ITGA4* (Tucker, G. C. et al., 1988, Betters et al., 2010) was seen by qPCR (Article I Fig. 5 B). There was an increase in the number of AP-2α -positive cells detected with immunocytochemistry, and flow cytometry showed increase in the cell surface markers p75 and HNK1 (Article I Fig. 5 C-E). This suggests that Notch inhibition combined with FGF8 can induce migratory NCCs.

8.4 Neuronal differentiation of pNCC occurs after Notch inhibition

After 3 days of treatment with DAPT alone, the pNCCs appeared to have neuronal morphology and expression of neuronal markers *DBH, PHOX2B, TUJ1, MASH1, NEUROD1, NGN2* were increased (Article I Fig. 6 A, B). Immunostainings showed positive cells for BRN3A and Peripherin (peripheral neurons), PHOXB1, and TUJ1 (autonomic neuron) (Article I Fig. 6 C, D). These data suggest that inhibition of the Notch pathway, after the initial pNCC establishment, promotes neuronal differentiation when no further factors are added to instruct and support differentiation into other lineages.

GnRH neuron differentiation was also attempted from NC culture, but no increase in *GNRH1* expression was detected in these cells (data not shown).

9. Article II: Generation of GnRH-expressing and -secreting neurons from human pluripotent stem cells

9.1 Differentiation of anteriorly patterned neuronal rosettes

The protocol for neuronal ectodermal induction from hPSCs was based on previously reported inhibition of BMP and TGF-β/Activin as a fast efficient method in monolayer culture, which produces anteriorly primed neuronal progenitors in the absence of caudalizing factors (Chambers et al., 2009). After employing small molecular inhibition using Dorsomorphin (hereafter referred to as DM) and SB431542 (hereafter referred to as SB), we acquired similar results, including increased mRNA expression of neuroectodermal markers *SOX1* and *PAX6* (Article II Fig. 1 A, B). Transcription factors *PAX6, EMX2, FOXG1*, *DLX2*, and *DLX5* are expressed in anterior neuronal progenitors, including the olfactory neuroepithelium and rostral forebrain (Simeone et al., 1994, Merlo et al., 2007). We found that after D10 of DM+SB, the cells resemble neuroectodermal cells with an increased mRNA expression of anterior markers, *PAX6*, *EMX2* and *FOXG1*, but not preplacodal markers *SIX1* and *EYA* (Article II Fig. 1 B), which suggested that cells did not get patterned toward a preplacodal state. As FGF8 is strongly implicated in GnRH neuron development, we next treated the cells with FGF8 until D20, and the dish became filled with neuronal rosette structures that were positive for SOX2 and FOXG1 antibodies. FGF8 treatment had further increased the mRNA expression of *EMX2* and *FOXG1*, and gradually a rise in neuronal progenitor markers associated with olfactory neurogenesis; *DLX2* and *DLX5* (Article II Fig. 1 C, D). In conclusion, neuronal induction of hPSCs in DM+SB produces neuroectodermal cells, which form neuronal rosettes that are patterned to anterior neuronal fate after treatment with FGF8.

9.2 Notch inhibition accelerates neuronal differentiation of GnRH-expressing cells

After FGF8-treatment, we inhibited Notch using DAPT, which lead to an increase in *GNRH1* expression after 4-8 days (Article II Fig. 2 A). We have, however, more recently observed that the *GNRH1* mRNA peaks in most experiments approximately at D25, which is 4 days after the addition of DAPT (Yellapragada et al., 2019). We detected α-GnRH-positive cells by immunocytochemistry in bipolar cells that were also positive for neuronal-specific TUJ1 and α-MAP2 antibodies (Article II Fig. 2 B-D). FOXG1 transcription factor was seen also as cytoplasmic in D27 immunostainings, compared to mainly nuclear before the addition of DAPT. In accordance, the subcellular localization of FOXG1 has been reported to shift from nuclear in proliferating, to cytoplasmic in differentiating cells (Regad et al., 2007). We detected cytoplasmic FOXG1 in the GnRH-positive neurons at D27, which suggests that the FOXG1-

positive cells in the neuronal rosettes at D21 are the source of GnRH-expressing cells (Article II Fig. 2 E).

After DAPT addition, we measured the secretion of the GnRH decapeptide using EIA, which showed gradual rise with the first detectable level at D25. KCl-induced polarization increased secreted decapeptide medium levels by 1.97 fold (95% confidence interval, 1.62–2.31) (Article II Fig. 3 A-C). Furthermore, cells were observed to be highly migratory at the end of the differentiation. $GABA_A$ receptor stimulation has been reported to inhibit migration of GnRH neurons (Casoni et al., 2012). Using a gap-closure assay we studied if $GABA_A$ receptor agonist muscimol shows any effect on migration. We found that cell number invading the gap space after 50 h treatment was reduced with muscimol ($n = 4$; $p < 0.05$), suggesting that muscimol reduces migration.

According to these results, we concluded that GnRH-expressing and -secreting postmitotic neurons differentiate from anteriorly patterned, highly proliferative neuronal progenitors expressing FOXG1. The co-staining of with α-FOXG1 in GnRH neurons was validated in the 8th gestational week of the human foetus. FOXG1-positive cells were seen in the migratory mass of cells exiting from the olfactory epithelium, including the migrating GnRH-positive neurons (Article II Fig. 4).

10. Article III: Transcriptome of hPSC-derived GnRH neurons and FGF8-treated progenitor cells

10.1 Generation of GNRH1-Tdtomato reporter cell line and transcriptome characterization

We used CRISPR-Cas9 to generate a reporter hPSC line for detection of *GnRH1*-expressing neurons by fluorescent protein TdTomato. The reporter cell line was generated by knockin of a sequence containing 2A self-cleaving peptide followed by nuclear signal-tagged TdTomato at the stop-codon of the third exon of *GNRH1* (Article III Fig. 1 A). As a result, cells which have integrated the reporter sequence by homology-directed repair, will express nuclear TdTomato signal as a result of *GNRH1* transcription. We performed differentiation according to the protocol described in article II (Lund et al., 2016). GnRH and the TdTomato signal coincided in the same cells with high specificity, although the sensitivity was not 100% (Article III Fig. 1 C-E). We tested the detection efficiency by performing FACS isolation of TdTomato positive and negative cell pools at D25 of the neuronal differentiation protocol, and performed RNA sequencing and differential expression analysis (Article III Fig. 1 F). *GNRH1* was significantly upregulated in TdTomato positive cells *versus* negative cells. In addition, we found upregulation of several KS/HH associated genes including *ANOS1* (Hardelin et al., 1999), as well as *CAMK2A, GAD1, GRIA1, GRIA2, GRIA4*, and *SEMA6D*, whose expression has been previously reported in GnRH neurons of animal models (Vastagh et al., 2016, Heger et al.,

2003, Di Giorgio et al., 2013, Spergel et al., 1999, Bailey, J. D., Centers & Jennes, 2006, Ebert et al., 2012). These data support the specificity of the *GNRH1*-Tdtomato reporter cell line, and provides a useful tool for GnRH neuron isolation and transcriptome analyses.

10.2 RNA sequencing reveals differences between two stages of differentiation

D20 FGF8-treated progenitors contain neuronal rosettes with anterior patterning that have not yet undergone terminal differentiation, nor do they contain GnRH-expressing cells (Article II, Fig. 1 C-D). After this stage, the cells are treated with Notch inhibitor DAPT, and GnRH neurons start to emerge. By D27 most of the cells have become postmitotic (Article II, Fig. 2), and we have not observed any considerable increase in the number of GnRH neurons after this point. Therefore we chose D27 as the time point to collect newly formed, postmitotic GnRH neurons, and we performed RNA sequencing in D20 FGF8-treated progenitor pool, and, D27 TdTomato-positive neurons after FACS (Article III Fig. 2 A-C). Differential expression analysis revealed over 6000 significantly differentially expressed genes. Out of the top 50 upregulated genes in TdTomato positive cells, *GNRH1,* as well as *RELN, SEMA3C, RBFOX1, PLXNA2, SCN2A, TAC1, GNRH1, MYT1L, BCL11B,* and *ISL1* were validated by qPCR, and the protein expression of RBFOX1, SEMA3C, DSCAM, SCN2A, PLXNA2, Substance P (*TAC1*), and PTPRN2 were validated by antibody staining (Article III Fig. 2 C, S2-5).

ISL1 is a LIM/homeodomain family transcription factor with a known role as a specifying transcription factor in early spinal motor neurons (Rhee et al., 2016, Cave, Sockanathan, 2018). Using pathway analysis based on reported interactions between genes and proteins that were represented in the top 500 upregulated genes, we observed a high number of interaction with *ISL1* (Article III Fig. 2 G). The transcription factor ISL1 was also immunostained and found in the nuclei of α-GnRH-positive cells at D27, but was not detected in progenitor stage cells (Article III Fig. 3 A). The result was validated in a human foetus (GW 10.5), which showed ISL1 in all GnRH-positive cells of the migratory mass, throughout the migratory path (Article III Fig. 3 B-G).

10.3 CHH and KS-associated genes are differentially expressed during GnRH neuron differentiation

Out of the concurrently reported list of 37 CHH-associated genes, we found 15 within the differentially expressed genes between D20 FGF8-treated progenitors and D27 TdTomato-positive cells (Article III Fig. 4 A, B). In addition of *GNRH1* itself, *DCC, PLXNA1, PCSK1, FGF17, NTN1, POLR3B* were upregulated in TdTomato-positive cells. Roles in GnRH neuron migration have been previously implicated in Netrin (*NTN1*), DCC, and Plexin-A1 (*PLXNA1*) (Low et al., 2012, Bouilly et al., 2018, Schwarting et al., 2004, Marcos et al., 2017). *PCSK1, FGF17,* and *POLR3B* have not, to our knowledge, been reported in GnRH neurons. Out of

these, FGF17 is a KS-associated gene, implying a possible role in GnRH neuron development (Valdes-Socin et al., 2014).

In addition, we found eight CHH-associated genes downregulated in TdTomato-positive cells, when compared to the D20 FGF8-treated progenitors; *AXL, FEZF1, ANOS1, TAC3, TACR3, FGFR1, PROK*2, and *HESX1* (Article III, Fig 4 B). Out of these, *HESX1*, *FGFR1*, *ANOS1* and *FEZF1*, are reportedly expressed during olfactory placode development (Carvalho et al., 2003, Hirata et al., 2006, Kotan et al., 2014). *PROK2* and *AXL1* are reportedly associated with the GnRH neuron migratory route (Pitteloud et al., 2007, Allen et al., 2002, Pierce et al., 2008), and *TAC3* and *TACR3* are expressed in hypothalamic Kisspeptin/Neurokinin B/Dynorphin-expressing neurons (Navarro, Tena-Sempere, 2011). This data shows that some of the CHH-associated genes are indeed expressed during GnRH neurons differentiation, and that the hPSC-based model for GnRH neuron development is a promising tool that can be utilized in the investigation of the disease mechanisms.

10.4 Overlapping genes in FGF8-treated progenitors and TdTomato-positive neurons

In addition to changes in transcriptome between progenitor and terminally differentiated neuron, we were interested in finding genes that are common and putatively specific progenitor markers for GnRH neurons. Since the FGF8-containing conditions produce GnRH neurons but non-treated conditions do not (article II), we compared FGF8-treated progenitors and TdTomato-positive neurons to non-treated cells from the same protocol at day 20. We asked; which genes are upregulated in both FGF8-treated progenitors and TdTomato-positive neurons, compared to “unpatterned” neuronal cells? We found, perhaps surprisingly, 37 genes with these criteria (Article III, Fig. 5 C), which suggests that the differences may be larger than the similarities and most genes are dependent on the stage (or timing) of neuronal maturation. Out of these, the most highly upregulated gene in TdTomato-positive cells was *DUSP4,* which has a role in negative feedback loop in FGF signalling pathway by ERK dephosphorylation that reportedly increases concomitantly with Fgf stimulation (Peng, Zhou & Wu, 2010, van Boxtel et al., 2018). Interestingly, DUSP6 has been implicated as CHH candidate gene, which shows association with FGF8-expression *in vivo*, in the so-called ‘FGF8 synexpression group’ (Miraoui et al., 2013). The FGF8 synexpression group, gathered from the literature by Miraoui and colleagues, is a cluster of genes which shows spatiotemporally similar expression patterns to *FGF8* across several organisms, and modulate signalling of FGF8 through FGFR1 as enhancers or inhibitors. The synexpression group also includes another gene in our list, *FGF17.* Also, one of the upregulated genes in this analysis was the previously mentioned *FOXG1*. Together, these data supports that *FOXG1,* and *DUSP4* together with *FGF17,* upregulated by FGF8, are interesting candidates for important roles in GnRH neuron

specification and differentiation. Lastly, previously reported genes with direct or indirect interaction to GnRH; *TLE4, SOX11, MEF2C, GAL, GAD1, CPE*, and *GPR173* were upregulated in TdTomato-expressing cells only (Article III, Fig. 5 E). Interestingly*, SOX11* has been implicated in the activation of *GNRH1* transcription in murine cells (Kim, H. D. et al., 2011).

11. Previously unpublished results

During the protocol planning of GnRH neuron differentiation, we considered the possibility that a preplacodal ectodermal stage, including upregulation of placodal markers such as *SIX1* and *EYA1*, would be required prior to neuroectoderm establishment for the correct (precursor) cell fate to be generated. For this purpose, we tested two different options. 1) outlining a new protocol based on developmental signals implicated during establishment of the cranial placodes, and 2) testing a published protocol for the generation of SIX1-positive cells (Leung, Alan W., Kent Morest & Li, 2013), and continuing differentiation from these progenitors.

11.1 The olfactory placode protocol

Since BMP and Wnt inhibitors are present during preplacodal ectoderm fate specification, and FGF8 is expressed in the developing olfactory placode, we hypothesized that a combination of BMP and Wnt inhibitors and FGF8 treatment could be needed for olfactory placode differentiation. We started differentiation using these factors in N2B27 medium, passaging cells weekly and acquiring samples on D7, 14, and 21, to detect expression of olfactory placode-associated genes (Figure 4 A). We found a modest increase of the preplacodal markers *SIX1* and *EYA1,* and the OP-associated *DLX5,* and *FOXG1* expression progressively increased until D21 (Figure 4 B). Immunocytochemistry at D21 showed PAX6-reactivity in most of the cells, and EYA1, SIX1 and DLX5 in a subset of cells (Figure 4 C). However, the reactivity for SIX1 and DLX5 did not seem to co-localize to the same cells, which suggests the presence of two developmentally distinct populations of cells, either by subtype (neuron vs. non-neuron) or different developmental stage. The neuron progenitor marker ASCL1 (‘Mash1’ in Figure 4 C) and neuronal marker TUJ1 were also detected, which suggests that neuronal differentiation had occurred. On D21 onward, we applied DAPT to inhibit the Notch pathway and accelerate neuronal differentiation, and cultured cells until D28. *GNRH1* expression remained low until D21, but was upregulated at D28. *FOXG1* expression showed a peak at D21, and was slightly reduced after Notch inhibition on D28, while the expression of *EMX2,* expressed in OPs and forebrain *in vivo*, had increased (Mallamaci et al., 1998) (Figure 4 D).

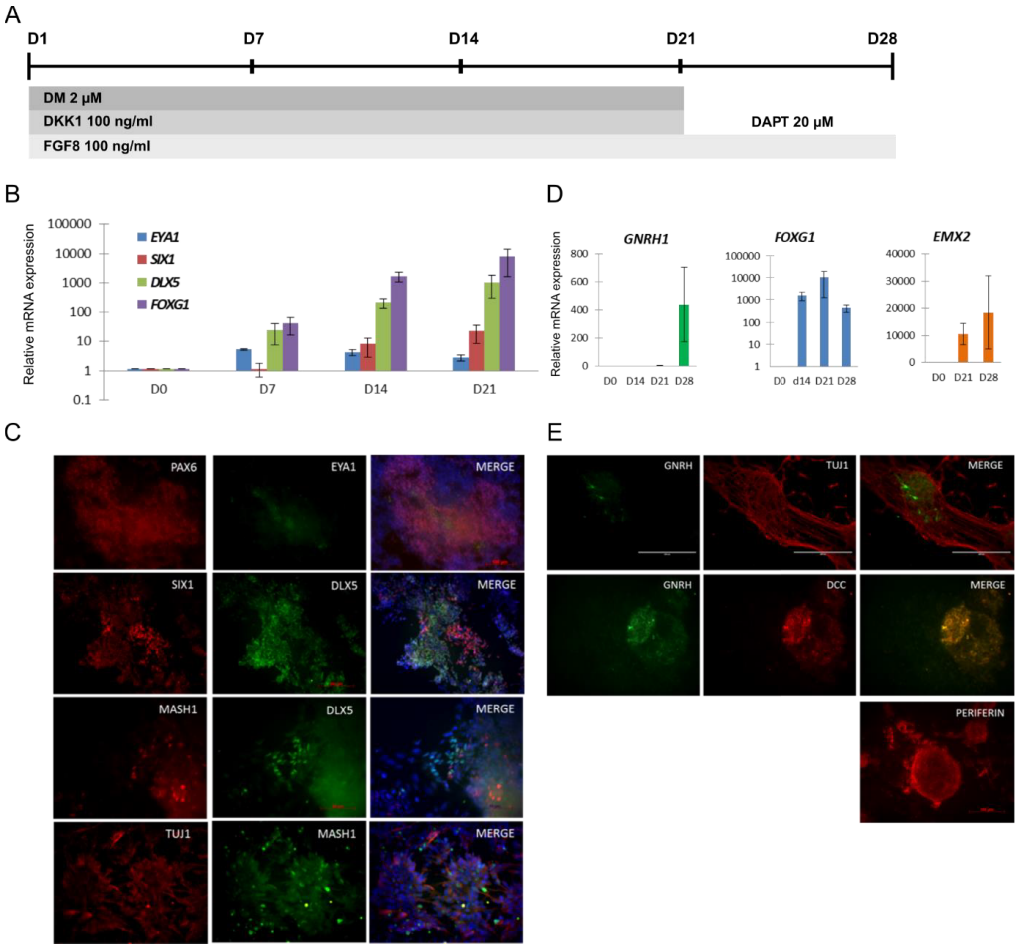


Figure 4. Protocol for differentiation of GnRH neurons based on the 'olfactory placode differentiation' protocol. A) Protocol timeline and added factors during differentiation. D stands for days of differentiation. Cells were passaged on D7, 14, and 21. DAPT was added on D22. B) Relative expression to D0 hPSCs of the placodal genes *EYA1, SIX1*, and OP genes *DLX5, FOXG1* during protocol detected by qPCR. C) Immunocytochemistry at D21 of the differentiation. D) Relative expression of *GNRH1, FOXG1,* and *EMX2* during differentiation detected by qPCR. E) Immunocytochemistry at D28 of differentiation, scale bar indicates 100 µm. In panels B and D, n = 4, error bars indicate standard error of the mean. Images in C captured using EVOS FL inverted epifluorescence wide-field microscope (ThermoFisher), and in E using Axio Imager Z1 upright epifluorescence microscope (Zeiss) (Lund 2014, unpublished data).

The cell morphology was heterogeneous. GnRH-immunopositive cells were detected in clusters that were positive for TUJ1, DCC and the peripheral neuron marker Peripherin (Figure 4 E). This suggests that only a subset of cells were able to undergo full neuronal differentiation, and albeit GnRH-expressing neurons were successfully obtained, the overall efficiency was relatively low. However, we saw that *FOXG1* upregulation preceded GnRH neuron differentiation, and we continued our work with the hypothesis that *FOXG1*-expression provided a marker for successful differentiation toward GnRH neurons.

11.2 Preplacodal ectoderm protocol

The preplacodal ectoderm cell differentiation was based on a report published by Leung and colleugues (Leung, Alan W., Kent Morest & Li, 2013), where a serum-free medium recipe containing N2 and B27 supplements (without addition of growth factors or Neurobasal media) induced PPE in roughly 6 days. This protocol reported the highest expression of *SIX1* and *EYA2* in the absence of extrinsic noggin or BMP, followed by late inhibition of BMP on D6-9, which induced expression of anterior placode markers *PAX6* and *PAX3* (Leung, Alan W., Kent Morest & Li, 2013). We repeated this protocol and continued with FGF8 and DAPT treatment from D9-28. *SIX1, EYA1, PAX6*, and *AP2* were initially induced but no considerable rise in *GNRH1* expression was observed (Figure 5). This result suggested that differentiation through a PPE-like stage with upregulation of *SIX1* and *EYA1* was not an effective strategy for GnRH neuron differentiation *in vitro*. Possibly, the SIX1- and EYA1-positive cells represented a population that had already been patterned toward other types of cells, and were therefore not competent to produce GnRH neurons in this protocol.

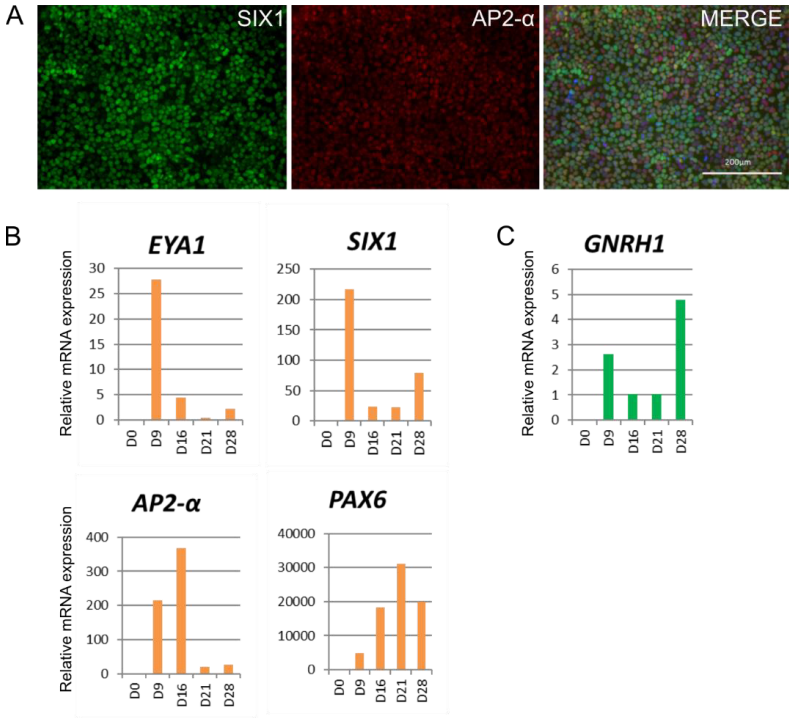


Figure 5. Attempt to differentiate GnRH neurons via preplacodal ectodermal stage.
A) Preplacodal ectoderm differentiation at D9, containing SIX1 and AP-2α immunopositive cells. Imaged using EVOS FL inverted epifluorescence wide-field microscope (ThermoFisher). Scale bar indicates 200 µm. B) Relative expression to D0 hPSCs of *EYA1*, *SIX1*, *AP-2α* and *PAX6* during differentiation performed by qPCR. D stands for days of differentiation. C) Relative expression of *GNRH1* during differentiation. Experiments were repeated three times until D21, and two times until D28. Data from one representative experiment is shown. (Lund 2014, unpublished data).

11.3 Expression of FGF13 in neuronal progenitors and GnRH-expressing neurons

After the RNA sequencing described in article III, we found that *FGF13* was one of the genes upregulated in both the FGF8-treated progenitor pool and TdTomato-expressing GnRH neurons. We performed immunocytochemistry for FGF13 at both stages. In the progenitors at D21, FGF13 was localized inside neuronal rosette cells, polarized apically toward the luminal center of the rosette (Figure 6 A). In GnRH-immunopositive neurons at D27, FGF13 was detected in the neuronal projections (Figure 6 B).

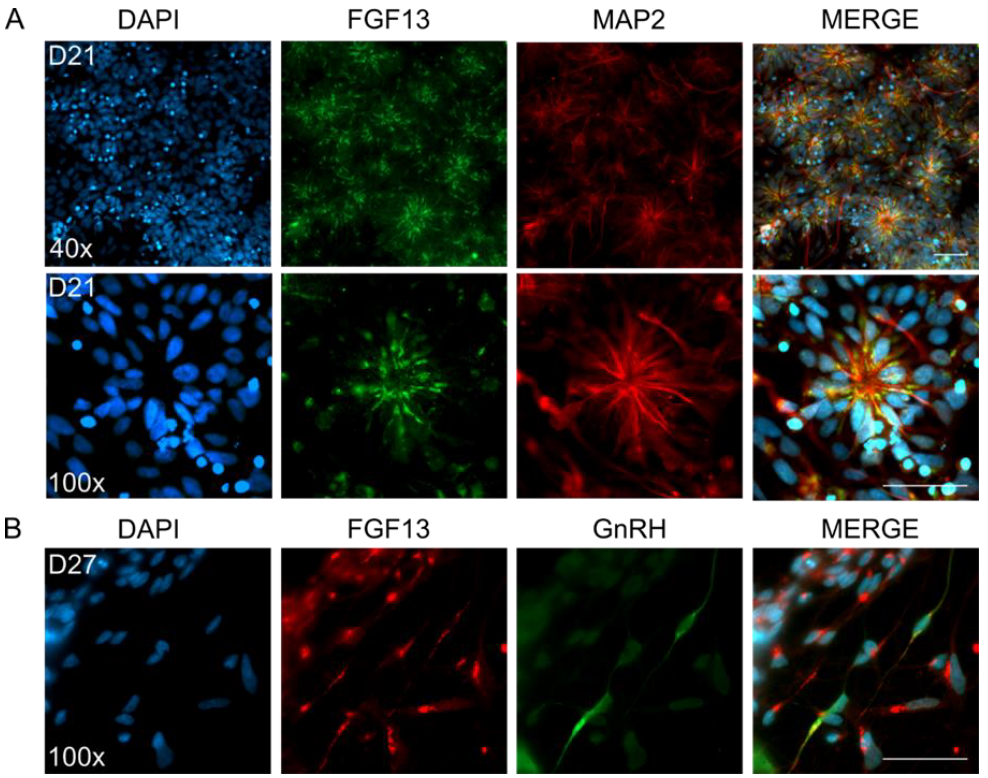


Figure 6. FGF13 immunocytochemistry in FGF8-treated neuronal rosettes, and GnRH-positive neurons, derived from human pluripotent stem cells. A) FGF13 and MAP2 in neuronal rosettes at D21 of the GnRH neuron differentiation protocol presented in article II (Lund et al., 2016), and B) FGF13 and GnRH at D27 of differentiation. Imaged using Axio Imager Z1 upright epifluorescence microscope (Zeiss). Scale bars indicate 50 µm, D stands for days of differentiation (Lund 2019, unpublished data).

DISCUSSION

12. Neural crest differentiation *in vitro*

Neural crest cells are a transient stem cell-like population of cells with extraordinary potential for migration and developmental plasticity. The transient nature of the population *in vivo* has prompted research to include *in vitro* modelling of neural crest multipotency and differentiation. Our first aim was to develop an effective and reproducible protocol for differentiation of neural crest and their derivatives, in order to, in the future, study certain cell types affected by a disease. Before the publication of our manuscript, a few articles had recently presented important data on directed differentiation of neural crest-like cells from hPSCs (Menendez et al., 2011, Mica et al., 2013). These authors had already established the effectiveness of BMP /Nodal/ TGF-β pathways' modulation and activation of Wnt signalling to induce differentiation through activating a combination of critical transcription factors marking NC specification, including *AP-2α, PAX3, SNAIL*, and *SOX10*. We found that this was achieved using Dorsomorphin, a small molecule inhibitor of ALK2, 4, 6, which blocks BMP-mediated SMAD 1/5/8 phosphorylation, and 1-azakenpaullone, small molecule inhibitor of GSK3-β, efficiently activating canonical Wnt signalling. This proved to be a simple way to mimic NC differentiation *in vitro*, resulting in a population of approximately 70-80% efficiency in conditions that support retainment of their developmental potential for up to 20 passages. Because of the ability to induce migratory behaviour and directed differentiation of these cells, we considered the cells as an early stage cell type of premigratory NC-like cells (pNCCs). More recently, the generation of non-adherent cultures of human premigratory NC-like cells was reported (Kerosuo et al., 2015). Kerosuo and colleagues found that a non-adhesive substrate favoured multipotency and thereby performed neurosphere-like culture condition to generate crestosphere cultures of NC-like stem cells with high expression of premigratory NC markers *PAX7, FOXD3*, and *SOX10*, and moderate expression of stem cell marker *SOX2*, mimicking avian premigratory NC *in vitro*. In these conditions, cells retain multipotency and self-renewal capacity up to 7 weeks, (*in vivo* estimate is around 5 hours). Proliferation gradually decreased during this time, resulting in loss of transit-amplifying progenitors at the end of 7 weeks of culture. *In vivo*, NC formation is achieved in the context of neural plate and epidermal induction. The 3D-organization reported by Kerosuo et al. 2015 possibly mimics more closely the epithelial organization and resembles the *in vivo*-like premigratory stage of development. However, if looking to study specific derivative cell types of NC for instance for disease modelling purpose, a more homogeneous monolayer can prove to be a more efficient, and pragmatic approach. For instance, during our differentiation protocol from hPSCs *in vitro*, the emergence of epidermis is purposely blocked by BMP inhibition. Thus, the interaction between these tissues cannot be observed, but it is easier to detect and study NCCs, specifically. That

said, NC cells being a highly plastic kind of progenitor cell type, the definitive NC specification can be difficult to define, and the combination of markers used to define premigratory and migratory neural crest cells might currently be too limited. For example, during *in vitro* culture the NC-progenitor cells can retain multipotency for long periods when provided with right conditions. How do the culture conditions affect the transition between premigratory, migratory, and postmigratory NC cell types generated in the dish, when proliferation is sustained? At what stage does priming to downstream lineages occur, and is cell potential biased by the culture method? Very recently, new methods in single-cell RNA sequencing have recovered new insight into the gene expression associated with that transient state of premigratory neural crest cells, and cell fate decision from neural tube stages to migration and differentiation (Soldatov et al., 2019). The study was performed in mice neural crest by combined linage tracing and functional perturbations to study the effect of transcriptional changes on cell fate decisions. Results indicated that cell fate choices take place as a sequence of binary decisions, which are gradually acquired. The first binary decision to arise is between neuro-glial and mesenchymal cell fates at early NC migratory stages, followed by autonomic and mesenchymal lineages. Transcription associated with the cell decision is sequential, and occurs through initial co-activation, gradual biasing, and commitment phases. In the premigratory state, early genes of competing cell fate programs become co-activated in the same cell. Gradually, cells come to a bifurcation point, where activation and repression further increase and lead to a cell fate bias. Commitment occurs by activation of mutually exclusive cell fate genes. In cranial neural crest, transcriptional changes suggesting cell fate bias for neuronal vs. mesenchymal fate were present already at the delamination phase (Soldatov et al., 2019). Therefore, it is possible that long-term culture of NC cells *in vitro* affects multipotency by gradual enrichment of “biased” cells in these cultures. Single-cell analyses will most likely be a useful tool for developing effective differentiation protocols for specific cell lineages *in vitro*.

13. Notch signalling and neural crest

Since Notch signalling modulation had been previously utilized successfully in neuronal differentiation protocols to affect differentiation and maturation of neurons, we were interested in the effect of Notch during NC cell differentiation. During neural crest specification *in vivo*, Notch signalling has been reported to contribute in the establishment of the border between neural and non-neural territories in the neural plate border region. In the *Xenopus* embyo, the activation of Notch pathway leads to neural crest territory expansion, and the blocking of Notch signalling leads to inhibition of NC marker expression (Glavic et al., 2004). Since it is known that BMP signalling regulates NC and the overlying epidermis expresses BMP during NC

induction, Notch pathway was proposed to regulate the level of BMP activation. Specifically, by Notch ligand Delta 1 maintaining BMP4 expression in the neighbouring epidermal tissue (Endo, Osumi & Wakamatsu, 2002, Glavic et al., 2004). The homeoprotein transcription factor *Xiro* is expressed in the neural plate border in *Xenopus* embryos during neural crest specification (Mayor, Aybar, 2001). Glavic and colleagues observed that the activation of *Xiro1* and Notch repressed the expression of BMP4 and the Notch effector *Hairy2* during neural crest specification, which led to the hypothesis that Xiro1 lies upstream of Delta in the regulation of BMP4 expression at the border between the NC and the epidermis. This way, Notch pathway restricts the activation of NC specifying factors outside the prospective NC territory, as well as induces, or re-enforces appropriate transcription factor expression within the NC territory. Our results are in agreement with these findings and suggest that Notch activation is important for proper neural crest specification. This is seen by the effect of blocking of Notch during the first 10 days of the protocol, which inhibits formation of NC cells by not allowing for proper activation of neural crest specifier genes. Together, this might suggest that regulation of NC development by the Notch pathway could act by 1) expansion of NC progenitors in the NC territory, or 2) allowing for progression of differentiation from neural plate border-like ectodermal cells, to neural crest definitive specification, or perhaps both.

In our results, overexpression of NICD resulted in increased expression of NC specifiers, and accelerated NCC specification by several days. Supporting evidence in chick exists, stating that an appropriate level of Notch activation leads to NC induction (and EMT) (Gouti, Briscoe & Gavalas, 2011). We found that applying Notch signalling inhibition after pNCC specification initiated cell migration, and accelerated differentiation towards neuronal derivatives. This kind of role for Notch signalling during murine neural crest differentiation has been studied using Cre-mediated conditional knockout and overexpression of Notch in NC cells (Mead, Yutzey, 2012), which revealed that Notch affected cell migration, proliferation, differentiation, and cell fate choices of NC derivatives in cranial, cardiac, and trunk NC. Gain of Notch signalling resulted in increased proliferation and deficient migration of NCCs.

Because of a broad array of derivatives, and the complexity of NC cells, in order to reveal the mechanisms of neurocristopathy-associated genes and their developmental processes, further research endeavours focusing more closely on the effect of Notch pathway (and other signalling pathways) in cell fate decision would be highly useful. Such an example that would benefit the field of puberty research and GnRH biology are the ontogeny and differentiation of NC-derived olfactory ensheating cells, which have been affirmed to support the development of GnRH neurons (Barraud et al., 2013, Geller et al., 2017).

14. Neural crest contribution to GnRH neuron cell population

NC lineage tracing experiments in mice have presented evidence that, perhaps surprisingly, suggest contribution of neural crest cells to the olfactory placode (Forni et al., 2011), which was not in accordance with the contemporary dogma of GnRH neuron origin. This led us to hypothesize that *in vitro* generation of GnRH neurons might be also possible from neural crest progenitor cells, but these results could not be obtained at the time. Neural crest contribution has been since disputed in other species, but the question of GnRH neuron ontogeny has not to this day been fully resolved. The lack of known progenitor markers specific for GnRH neurons causes considerable difficulty. To this day, no reports exist where hPSC-derived NC could give rise to GnRH neurons, which could support their NC origin, even though the amount of research on NC differentiation has increased rapidly. Characterization of human foetal GnRH neurons has not been able to identify any SOX10 expression in early developing GnRH neurons in the nose (Casoni et al., 2016), which argues against the hypothesis of dual origin (the neural crest and olfactory placode) of GnRH neurons.

15. Olfactory placode and GnRH neuron specification

Less efforts had been made at the time when we started this work to generate cranial placode cells and derivatives from human pluripotent stem cells. The first protocols for placode differentiation were published in 2013 (Leung, Alan W., Kent Morest & Li, 2013, Dincer et al., 2013). These protocols produced placode-derived trigeminal ganglia cells, lens fibres, anterior pituitary hormone-producing cells (Dincer et al., 2013), lens placode, and oral ectoderm (pituitary precursor cells) (Leung, Alan W., Kent Morest & Li, 2013). Specific differentiation into olfactory placode or differentiated cell types of the main olfactory system have not been reported so far. The difficulty in defining successfulness of OP patterning in these protocols may be due to the fact that most markers that represent early OP and OE in the frontonasal area are also found in the forebrain, such as *DLX2, DLX5, PAX6*, and *FOXG1* (Duggan et al., 2008, Merlo et al., 2007, Simeone et al., 1994).

Six1 and *Eya1* are the only markers that are specific for placodal ectoderm and not expressed in the neural tube and neural crest. The *Six* and *Eya* family genes are, however, thought to be required for development of all the cranial placodes (Schlosser, 2006). Thus, OP being closely associated to the anterior-most neural tube might be an important clue into finding differences specific between the distinct placodes. Defining such differences at early time points in development would be one of the possible prerequisites to finding out the true origin of GnRH neurons. A recent report in zebrafish traced back the progenitors of GnRH-expressing neurons to cells migrating out of the most anterior part of the PPE at the neural plate border (Aguillon

et al., 2018). The same population gave rise also to olfactory microvillus neurons, and considerable overlap to adenohypophysis progenitors was observed. The latter has been described in zebrafish previously (Toro, Varga, 2007), but in the chick, these progenitors were described as non-overlapping distinct populations (Sabado et al., 2012). Overlap of this sort has so far not been described in mice. With development of better tools for genetic tracing, it would be interesting to study at which point the definitive specification of separate olfactory and adenohypophyseal placodal progenitors occur, how the PPE cells adopt differing fate based on position and patterning signals, and how this is translated during *in vitro* specification of GnRH neurons in our differentiation protocol.

16. Differentiation of GnRH-expressing neurons

High confluency monolayer culture promotes neuroectoderm induction from hPSC (Chambers et al., 2009) and we observed that this method, beginning from over 90% confluent dishes, produced more homogeneous neuronal cultures than lower cell densities. Already at D10 of differentiation, neuronal rosettes started to appear. Neuronal rosettes are radial structures that form spontaneously during neuronal induction *in vitro*, initiated by cell polarisation and asymmetric distribution of proteins, such as tight junction markers ZO-1 and N-cadherin, leading to formation of a central lumen toward which cells are attaching their apical membranes, connected by adherence junctions (Elkabetz et al., 2008). Elkabetz and colleagues first describe the rosette stage cells to have broad differentiation potential. Expression of high *FOXG1* was observed in spontaneously forming rosettes, which suggests that early rosettes adopt an anterior CNS patterning by default but can be patterned to caudal and ventral fates by the addition of extrinsic factors such as SHH and Wnt.

We also observed *FOXG1* upregulation already at D10, which was further upregulated during FGF8 treatment until D20. FGF8 has been shown to promote neurogenesis and transient self-renewal of neuronal rosettes derived from mouse ES cells (Sterneckert et al., 2010).

DAPT treatment has been found to disrupt the rosette morphology and induce neural differentiation (Elkabetz et al., 2008). Another study found that Presenilin2, a component of the gamma-secretase complex, is distributed apically in the rosettes, co-localizing to adherence junctions at the central lumen (Banda et al., 2015). This may be related to high Notch activity in rosette-stage cells, by gamma-secretase cleavage of Notch to release NICD in the cells.

Our observation during the protocol is that FGF8 treatment results in a higher number of cells number at D20, increases the overall number of rosettes, and keeps cells in the rosette stage (clear rosette organization) for a longer time compared to FGF8 non-treated controls. *FOXG1* expression is high, whereas *NKX2.1*, expressed in ventral forebrain and diencephalon, is kept

low. Other hypothalamic neuroendocrine cell types, such as POMC-expressing neurons can be generated by SHH treatment during differentiation. This supresses *FOXG1* and promotes ventralization by *NKX2.1* expression (Merkle et al., 2015), but this protocol does not produce GnRH neurons. Therefore, these data suggest that FGF8 promotes survival of immature, but anteriorly patterned, neuronal progenitor cells *in vitro*, as long as the rosette structure is retained. This may be in part regulated by high Notch activity. Furthermore, our data suggest that these FOXG1-expressing progenitor cells give rise to GnRH-expressing neurons, supported by retained cytoplasmic FOXG1 in early postmitotic GnRH-positive cells, and co-expression also in human foetal GnRH neurons.

17. Transcriptomic analysis during in vitro differentiation revealed a high number of genes upregulated in newly emerged GnRH- expressing neurons

The GnRH neuron early transcriptome is an important step towards understanding human puberty development and rare diseases where GnRH neuron development is disrupted. Our transcriptome analyses gave a large amount of information that could generate lots of interesting ideas and hypotheses to discuss. The data was therefore somewhat challenging to summarize. We chose to describe the transcriptomic data as an independent entity to bring forward and support the development in the field using previously unreported methods, and in hope that hPSC modelling might become more readily used in the future for dissecting molecular mechanisms of GnRH neuron biology, together with animal models. Many new interesting hypotheses can be drawn. The following sections briefly discuss our findings and their potential implications.

17.1 Major differences between FGF8-treated and non-treated neuronal progenitor cells

The sample group used for differential expression analyses are summarized in article III, Fig. 2 A. Differential expression analysis revealed 511 upregulated and 275 downregulated genes in the FGF8 treated *versus* non-treated cells at D20. Over-representation analysis of genes (genetrail) upregulated or downregulated showed that: 1) Cell component gene groups showed some differences related to cell shape and attachment. For instance, in FGF8-treated cells, the enriched terms included *contractile fibers, microtubule associated complex, extracellular matrix component, apical junction complex, cell cell contact zone,* and *apical plasma membrane.* These terms are well in line with the highly abundant rosette structures in FGF8-treated culture. In the non-treated cultures, the rosettes do appear after D10, but are generally larger, and fewer in number. The enriched cellular components included more terms associated with neuronal differentiation, such as *postsynaptic membrane, axon terminus, neuron projection terminus, axon growth cone,* and *synaptic membrane.* This supports the

previously discussed role of FGF8 in promoting the rosette organization of the cells, which keeps them in undifferentiated state. 2) In the FGF8-treated cells, KEGG pathways showed enrichment of endocrine cell type-related terms, such as *insulin secretion, pancreatic secretion, thyroid hormone synthesis,* and *endocrine and other factor-regulated calcium reabsorption.* It is interesting to speculate that this could be related to the development of GnRH neuron precursor cells. 3) Enriched KEGG pathway terms in FGF8-treated cells also included the signalling pathways *Rap1 signalling, HIF-1 signalling, Pi3K-Akt signalling,* and *MAPK signalling* pathway, whereas non-treated cells showed enrichment of *Hippo signalling pathway*.

Intracellular signalling pathways HIF-1, Pi3K-Akt, and MAPK pathway are associated with many processes including, proliferation, differentiation, and survival (Xie, Lowry, 2018, Yu, J. S., Cui, 2016, Sun et al., 2015), and Rap1 is implicated in cell adhesion (Kooistra, Dube & Bos, 2007), while the Hippo pathway is known for its role in limiting organ size by modulating cell proliferation (Ehmer, Sage, 2016).

17.2 Major differences between FGF8-treated progenitors and TdTomato-positive neurons

A long list of differentially expressed genes (over 6000) was found between these two sample groups, suggesting that the differentiation and specialization that has occurred between the rosette-stage progenitor and postmitotic GnRH-expressing cell is extensive.

Over-representation analysis showed that the progenitor-enriched pathways where high in terms related to proliferation, and, for instance, the Notch pathway was indeed represented in the top 50 most (D27 Tdt+ versus D20FGF8) downregulated genes. Interestingly, the gene with the highest fold change score was *DLK1. DLK1* (Delta-like non-canonical Notch ligand 1) *DLK1* has been described in patient with paternally inherited deletion associated with familial central precocious puberty (Dauber et al., 2017) and has been suggested as potential regulator of puberty timing (Day et al., 2017). DLK1 is a transmembrane protein with an extracellular domain containing six epidermal growth factor repeats. DLK1 has sequence homology with Delta, but lacks the DSL-motif that can activate Notch. DLK1 marks embryonic tissues and immature cells, implicated in tissue growth and development and regenerative potential (Floridon et al., 2000). The exact function is not known. DLK1 has been implicated in Notch inhibition, but has also been found highly expressed in several embryonic tissues where Notch signalling is high (Falix et al., 2012). *DLK1* downregulation is required for the initiation of final maturation is some tissues, including pancreatic β-cells. The embryonic and foetal expressions of DLK1 in several endocrine tissues have suggested a role for DLK-1 in maturation of endocrine/neuroendocrine cells. *DLK1* expression is restricted to certain populations of cells during lung and pancreas morphogenesis, which could imply a role of

grading of Notch activity (or other factors) to regulate cell fate (Floridon et al., 2000). Furthermore, DLK1 has been implicated in regulation of other pathways, such as FGF (Miyaoka et al., 2010). *DLK1* expression is lost in adrenal capsule of FgfrIIIb knockout mice, which exhibit hypoplastic adrenals with impaired steroidogenic differentiation (Guasti et al., 2013).

Perhaps one of the most interesting findings of upregulated genes in TdTomato-positive cells was *ISL1*, which was immunostained also in human foetal GnRH neurons. ISL1 is a LIM factor that forms heterodimers with other LIM factor family transcription factors, such as *LHX* genes, and promotes differentiation of specialized cell types including motor neurons and forebrain cholinergic neurons by regulating the expression of cell type specific genes (Cho et al., 2014). With interest to predict interaction partners of *ISL1* in our protocol, we looked at the gene interaction network represented in the data from this analysis. The network contained 13 previously reported protein-protein interactions, including the LIM-domain containing genes *LMO2, LDB2,* and *AJUBA*. Since *LMO2* was upregulated and *LDB2* and *AJUBA* were downregulated, it would be interesting to study whether ISL1 interaction between them is involved in regulation of terminal differentiation of GnRH neurons.

17.3 Kallmann Syndome and CHH genes represented in the data

In the same differential expression analysis described in the previous section, the D20FGF8-treated vs GnRH neurons, we found the highest number of KS and CHH genes (Article III, Fig 4A). Seven genes upregulated in TdTomato-positive cells, and eight in the progenitor pool. The list contained genes related to FGF signalling: *FGFR1, ANOS1,* and *FGF17* (Miraoui et al., 2013), neuropeptide regulation; *TAC3* and *TACR3* (Francou et al., 2011, Yang et al., 2012), and migration-associated genes *FEZF1, DCC, PLXNA1*, and *NTN1* (Kotan et al., 2014, Bouilly et al., 2018, Marcos et al., 2017). All 15 genes are interesting candidates to study further their involvement in GnRH neuron specification and differentiation. It is possible that genes needed for specification have been upregulated earlier, but future methods such as single-cell RNA sequencing at different stages of differentiation might be helpful to find out such associations.

17.4 FGF8-treated progenitors and TdTomato positive neurons, overlapping gene expression

We used the FGF8 non-treated D20 neuronal cultures as controls in comparison with both D20FGF8 and TdTomato, to find possible overlaps in the upregulated genes. The question would be whether there is any putative markers that become expressed already before terminal differentiation in FGF8-treated cells that can still be detected in GnRH neurons, similar to *FOXG1* shown in article II. The overlap was quite moderate, with 37 genes (Article

III, Fig 5 A), however not surprising, as the samples did show considerable divergence in the previously described analyses. Among these 37 was indeed *FOXG1*.

DUSP4 was the gene with highest ranked expression in TdTomato positive cells at D27, which was also found upregulated in the progenitor stage cells (Article III, Fig 5 A). *DUSP4* has been implicated in regulation of neuronal differentiation and calcium homeostasis by modulating ERK1/2 phosphorylation (Kim, S. Y. et al., 2015).

We tested some of these markers using antibody staining, and found that FGF13 was, similarly to FOXG1, stained both in the rosette stage progenitors and in the postmitotic GnRH neurons (Figure 6). Interestingly, *FGF13* has been recently implicated as a KS candidate gene (Quaynor et al., 2016), and mRNA expression was reported in mouse GnRH neurons in a recent study (Burger et al., 2018). FGF13 is an intracellular non-secretory protein that functions as a microtubule-stabilizing protein during cerebral cortex development (Wu et al., 2012). Our immunostainings in the previously unpublished data (Figure 5) indeed showed that FGF13 is localized to the apical side of the rosette cells, clearly toward the luminal center of the rosettes, and more broadly and predominantly one-sidedly in the delaminating neuronal cells just outside the rosettes. An additional observation was that at D27 there are some GnRH-negative cells with shorter projections, which have more local expression of FGF13 next to the nucleus, and appear to be shorter in shape. These may be less mature cells that have not yet differentiated to neurons, or represent another cell type with distinct morphology.

18. Conclusions and future perspective

GnRH neuron differentiation from human pluripotent stem cells *in vitro* is efficiently and in a consistently repeatable manner achieved by dual SMAD inhibition, FGF8 treatment and subsequent Notch inhibition. The protocol for *in vitro* differentiation is a promising tool for studying molecular mechanisms of KS and CHH-associated genes in the future. The transcriptomic characterization has brought better understanding in how the differentiation might be regulated, and basis for many new hypotheses to help approach the questions that remain about GnRH neuron specification and developmental specialization.

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