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The role of GnRH in the age-related cognitive decline in some disorders including Down syndrome

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Composition du jury :

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To my parents,

to bomma and bompa,

for their endless support

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AUEC: Comportement adapté aux modèles physiopathologiques

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COMMUNICATIONS

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PUBLICATIONS

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ABSTRACT

The role of gonadotropin-releasing hormone (GnRH) neurons as the master regulators of a complex neural network, mainly in the hypothalamus, controlling the onset of puberty and regulation of fertility is well established. Nonetheless, it is becoming apparent that GnRH may be involved in a variety of non-reproductive functions. Here, we show that GnRH neurons are critical for maintaining cognitive function and olfaction. A first study demonstrates a dysregulated microRNA (miR)-transcription network underlying a progressive loss of GnRH expression in the Ts65Dn mice, a mouse model of Down syndrome. This GnRH loss is concomitant with a cognitive and olfactory decline. Intriguingly, these deficits are reversible by viral-vector-mediated miR-200b overexpression, the restoration of GnRH functionality by grafting wild-type GnRH neurons, or simply delivering pulsatile GnRH. A second study demonstrates mutations in the neuronal nitric oxide (NO) synthase (*NOS1*) gene as a causal factor of congenital hypogonadotropic hypogonadism and Kallmann syndrome, two genetic disorders rooted in a GnRH deficiency. Similar to human, *Nos1* deficient mice do not only show a delayed puberty onset and infertility, but also cognitive and olfactory impairments. Moreover, the administration of inhaled NO during the late infantile period can restore sexual maturation, cognition and olfaction in this mouse model. Overall, the results of this Ph.D. thesis provide evidence that GnRH plays an unexpected yet critical role in non-reproductive functions such as cognitive function and olfaction and hold novel opportunities for therapeutic strategies against various neurodevelopmental and neurodegenerative disorders.

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ABBREVIATIONS

α-MSH	α-melanocyte-stimulating hormone
aa	amino acids
Aβ	amyloid β
ABR	auditory brainstem-evoked response
ac	anterior commissure
ACTH	adrenocorticotropic hormone
AD	Alzheimer's disease
ADH	antidiuretic hormone
Ago	argonaute protein
AgRP	agouti-related protein
AHA	anterior hypothalamic area
APP	amyloid precursor protein
AR	androgen receptor
ARH	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BBB	blood-brain-barrier
BoNT	botulinum neurotoxins
BoNT/B	botulinum neurotoxin serotype B
bp	base pairs
Ca_2	calcium
cAMP	cyclic adenosine monophosphate
CAPS	calcium-dependent activator proteins for secretion
cc	corpus callosum
CDGP	constitutional delay of growth and puberty
cGMP	cyclic guanosine monophosphate
CHH	congenital hypogonadotropic hypogonadism
CNS	central nervous system
CRH	corticotropin-releasing hormone
CTF	C-terminal fragment
CVO	circumventricular organ
DGCR8	DiGeorge syndrome chromosomal region 8
DS	Down syndrome
DSCR	Down syndrome critical region
DYRK1A	dual specificity tyrosine-phosphorylation-regulated kinase 1A
E	embryonic day
E_2	estradiol

EDC	endocrine disrupting chemical
ER	estrogen receptor
ERT	estrogen replacement therapy
FAS	free α-subunit
FSH	follicle stimulating hormone
FSH-R	FSH receptor
fx	fornix
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAP	GnRH-associated protein
GC	glucocorticoid
Gfp	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GPCR	G-protein coupled receptor
hCG	human chorionic gonadotropin
HH	hypogonadotropic hypogonadism
HPA	hypothalamic-pituitary-adrenal
HPD	hypothalamic-pituitary-disconnected
HPG	hypothalamic-pituitary-gonadal
HPS	hypothalamic-pituitary-somatotropic
hsa	human chromosome
iDISCO	immunolabeling-enabled imaging of solvent-cleared organs
IHC	immunohistochemistry
IQ	intellectual quotient
ISH	*in situ* hybridization
Kiss1	kisspeptin
Kiss1-R	kisspeptin receptor
KP	kisspeptin
KNDy	kisspeptin, dynorphyn, neurokinin B
KO	knockout
KS	Kallmann syndrome
LDCV	large dense core vesicle
LH	luteinizing hormone
LH-R	luteinizing hormone receptor
LHRH	luteinizing hormone secreting hormone

L-NAME	N(ω)-nitro-L-arginine methyl ester
LTD	long-term depression
LTP	long-term potentiation
MBH	mediobasal hypothalamus
ME	median eminence
MEAad	anteriordorsal amygdala
miRISC	micro ribonucleic acid-induced silencing complex
miRNA	micro ribonucleic acid
mmu	murine chromosome
mPOA	medial preoptic area
mRNA	messenger ribonucleic acid
MS	medial septum
NC	neural crest
nCHH	normosmic congenital hypogonadotropic hypogonadism
NFT	neurofibrillary tangles
NKB	neurokinin B
NKCC1	Na-K-Cl cotransporter
NMDA	N-methyl-D-aspartate
nNOS	neural nitric oxide synthase
NO	nitric oxide
NOR	novel object recognition
NOS	nitric oxide synthase
NPY	neuropeptide Y
NSC	neural stem cell
NT	nervus terminalis
nt	nucleotides
OB	olfactory bulb
OE	olfactory epithelium
OP	olfactory placode
ORX	orchidectomy
OVLT	organum vasculosum of the lamina terminalis
OVX	ovariectomy
P	postnatal day
PDE	phosphodiesterase
PIN	inhibiting protein Nos1
PIP2	phosphatidylinositol diphosphate
piRNAs	PIWI-interacting RNAs
PKA	protein kinase A

PKC	protein kinase C
PLC	phospholipase C
POA	preoptic area
Pol II	ribonucleic acid polymerase II
POMC	proopomelanocortin
PR	progesterone receptor
pre-miRNA	precursor micro ribonucleic acid
pri-miRNA	primary micro ribonucleic acid
PVN	paraventricular nucleus
PVT	paraventricular thalamus
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
rPOA	rostral preoptic area
RSV	rare sequence variants
SCN	suprachiasmatic nucleus
SHFM	split-hand foot malformation
SNAP	soluble N-ethylmaleimide sensitive factor attachment protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SON	supraoptic nucleus
T	testosterone
Tau-Cter	Tau C-terminal
TRBP	Tar ribonucleic acid binding protein
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
$t_{1/2}$	half-life
UTR	untranslated region
UV	ultraviolet
VAMP	vesicle-associated membrane protein
VNN	vomeronasal nerve
VNO	vomeronasal organ
wt	wild-type
3v	third ventricle

INTRODUCTION

CHAPTER 1

The hypothalamic-gonadal-pituitary axis in reproduction

1.1 History of hormones and the endocrine role of the hypothalamus

The communication within the body has attracted the interest of scientists since a very long time now. Back in the 1800's, little was known about the body's internal communication until the work of pioneers, such as Arnold Adolphe Berthold and Claude Bernard, led to the concept that different organs in an animal are communicating in a chemical way. The present word 'hormone' was not used until June 1905, when Ernest Starling coined it and defined it as "*the chemical messengers which speeding from cell to cell along the blood stream, may coordinate the activities and growth of different parts of the body*" (Starling, 1905). The introduction of the term 'hormone' initiated a cascade of research that focused on identifying the source and chemical nature of these chemical messengers or so-called hormones. It also led to one of the most important principles of endocrinology, the one stating that hormones are playing a major role in the interplay and feedback between the central nervous and endocrine systems. This research has been of indescribable value for today's knowledge about the catalytic role of hormones in the regulation of the organism's development, homeostasis, behavior, reproduction and physiologic processes.

The hypothalamus, located at the base of the brain, is one of the most important brain regions as it directly or indirectly regulates all functions of the body. It integrates both vegetative and endocrine regulations controlling the most important body processes, including cardiovascular function, metabolism, thermoregulation, homeostasis, stress, circadian rhythms and reproduction. The hypothalamus contains specialized neuronal cell populations that sense circulating hormones levels and nutrients, and as a response secrete regulatory factors. Some of these regulatory factors, on their turn, exert a stimulatory effect on the secretion of hormones by the pituitary gland that will control the peripheral endocrine glands (Clarke, 2015). The ability to act as a chemosensory organ and neuroendocrine gland at the same time contributes to the hypothalamus' regulatory role in all vital body processes.

1.2 The hypothalamic-pituitary-gonadal axis

In mammals, three main components form the hypothalamic-pituitary-gonadal axis (HPG-axis), respectively the hypothalamus, pituitary gland and gonads. These three structures interact and depend on one another so closely that they are often referred to as a single system. Together, they create an important connection between the central nervous system (CNS) and periphery that is essential for different body processes, including the function of the immune system, ageing, sexual development, fertility and reproductive function, but also plays a role in many other associated behaviors such as sexual behaviors, territoriality and aggression. Within the interest of this manuscript, the reproductive aspect of the HPG-axis, as well as a plausible role in ageing and age-related cognitive decline will be further discussed.

The key players of the HPG-axis, the gonadotropin-releasing hormone (GnRH) neurons, are mainly located within the hypothalamus. At the level of the median eminence (ME), the interface between the neural and peripheral endocrine systems, GnRH is released in a pulsatile manner into the hypophyseal portal blood to target gonadotropic cells in the pituitary gland. In this way, GnRH regulates the synthesis and secretion of the gonadotropins, specifically luteinizing hormone (LH) and follicle stimulating hormone (FSH), that will mainly act on the gonads. At the level of the gonads, the gonadotropins regulate gonadal maturation, steroidogenesis and the synthesis and release of peripheral sex hormones. These hormones act back on the hypothalamus and pituitary gland, and close the feedback loops to regulate the secretion of GnRH and gonadotropins, respectively (**Figure 1**).

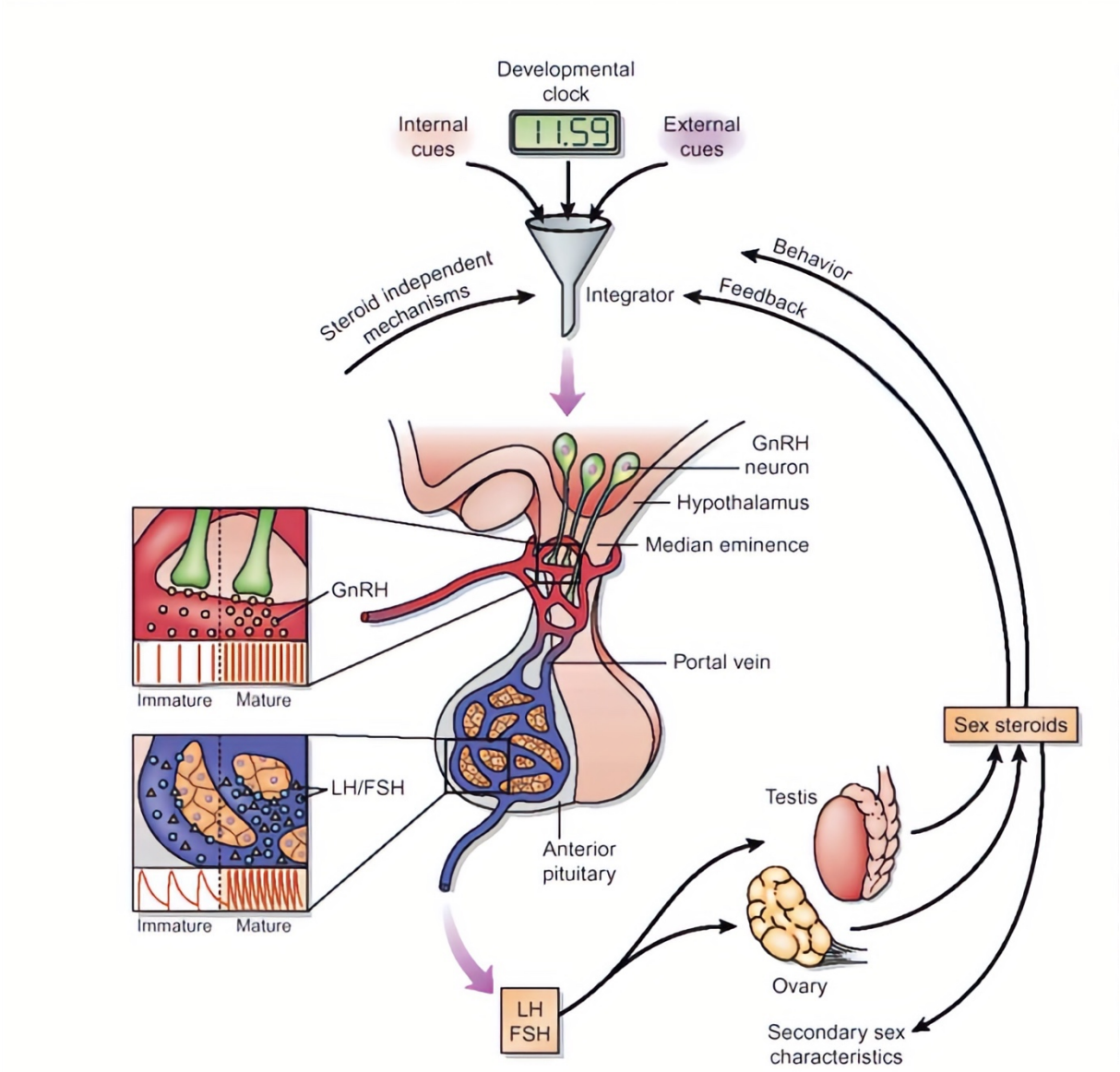


Figure 1. The hypothalamic-pituitary-gonadal axis. Schematic representation of the HPG-axis regulating reproduction in mammals. At the level of the median eminence, hypothalamic GnRH neurons release their neurohormone in the portal circulation. From there, GnRH targets the gonadotropic cells of the anterior pituitary gland and regulates the synthesis of LH and FSH, and their release in the systemic circulation. LH and FSH will regulate the maturation and function of the gonads, that secrete sex steroid hormones. These steroid hormones act back on the central nervous system, and close the essential feedback loop to regulate the secretion of GnRH from the hypothalamus and secretion of gonadotropins from the pituitary gland. Figure taken from (Sisk et al., 2004).

1.2.1 The hypothalamus

The hypothalamus is a relatively small region in the brain that is located between the thalamus and the third ventricle (3v), at the base of the diencephalon. It orchestrates its control over the endocrine glands via the secretion of multiple neurotransmitters and neuropeptides. It comprises different specialized neuronal populations that are able to sense the levels of certain hormones and nutrients, and as a response secrete specific hormones in the hypophyseal portal system. This portal vascular system is constituted by fenestrated capillaries, forming two important plexuses. The primary plexus lays within the ME, the level where GnRH neurons and other neurosecretory releasing hormone neurons, such as corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH) and thyrotropin-releasing hormone (TRH) neurons, release their neurohormone into the portal vasculature. A secondary plexus can be found within the adenohypophysis, where the neurohormones will target the pituitary gland and control the synthesis and release of different pituitary hormones (Pantic, 1995).

The different hypothalamic cell populations are grouped within specific nuclei, which on their turn, are located in three hypothalamic regions:

(1) The rostral or supraoptic region of the hypothalamus includes the supraoptic nucleus (SON), paraventricular nucleus (PVN), the anterior hypothalamic area (AHA) and the preoptic area (POA). Neurons located in the SON and PVN are mainly projecting towards the posterior pituitary or neurohypophysis (Everitt et al., 1990). Containing the majority of GnRH neurons, the POA constitutes a crucial region in the control of the reproductive axis.

(2) The medial or tuberal region of the hypothalamus includes the lateral hypothalamic area, arcuate nucleus (ARH), ventromedial nucleus and dorsomedial nucleus. Neurons located within this region project to the level of the ME, where they release their hormones in the hypophyseal portal system and target the anterior pituitary or adenohypophysis (Everitt et al., 1990).

(3) The caudal region of the hypothalamus that includes the mammillary bodies, that form a part of the limbic system.

The ME lies within the center of the tuber cinereum, and functionally links the hypothalamus and anterior pituitary gland. As already mentioned above, a primary plexus of fenestrated vessels is located within the ME and connects the hypothalamus with the pituitary gland through the pituitary stalk, a very thin tissue layer. The ME is considered as one of the circumventricular organs (CVOs), brain structures characterized by high permeable capillaries and the absence of the blood-brain-barrier (BBB). Its advantageous location and the presence of special ependymal glial cells lining the wall of the 3v support the role of the ME in the communication of the peripheral signals with the hypothalamus and maintain homeostasis (Langlet et al., 2013).

1.2.2 The pituitary gland and gonadotropins

1.2.2.1 The pituitary gland

The pituitary gland or hypophysis is a small pea-sized endocrine gland located at the base of the brain and protected by the sella turcica of the sphenoid bone. The pituitary gland can be divided in two embryologically and functionally distinct structures, the adenohypophysis and the neurohypophysis (Nussey et al., 2001). The anterior part of the pituitary gland, the adenohypophysis, consists of many endocrine cells and non-endocrine cells. Immunohistochemical techniques enabled the classification of these endocrine cells as somatotropes (producing growth hormone (GH)), lactotropes (producing prolactin), corticotropes (producing adrenocorticotropic hormone (ACTH)), gonadotropes (producing LH and FSH) and thyrotropes (producing thyroid-stimulating hormone (TSH)). The synthesis and secretion of hormones produced by the aforementioned cells are controlled by hypothalamic hormones that are released in the hypophyseal portal system, the functional link between the hypothalamus and the adenohypophysis. In contrast to the anterior part of the pituitary gland, the posterior part or neurohypophysis is anatomically connected with the hypothalamus. Some hypothalamic neurons located in the SON and PVN directly project to the neurohypophysis, which is constituted of the hypothalamic axonal processes and their nerve terminals, as well as modified astrocytes. Oxytocin and the antidiuretic hormone (ADH) are synthesized within the SON and PVN and stored within the nerve terminals at the level of the neurohypophysis until electrical excitation triggers their secretion into the general circulation.

1.2.2.2 The gonadotropins

The gonadotropins LH, FSH and human chorionic gonadotropin (hCG) are glycoprotein hormones (Themmen et al., 2000) secreted by the gonadotropes of the adenohypophysis and placenta respectively. The heterodimers LH and FSH consist of two non-covalently bound subunits: an identical α-subunit, and a distinct β-subunit that confers their biological specificity. Their function is exerted by binding to G-protein coupled receptors (GPCRs) (Ascoli et al., 2002) embedded in the cell membrane of the target cells, mainly located at the level of the gonads. The activation of the gonadotropin receptors, the LH receptor (LH-R) and FSH receptor (FSH-R), induces a rise of intracellular cyclic adenosine monophosphate (cAMP) levels via the activation of adenylate cyclase, and results in the activation of protein kinase A (PKA) (Szczypka et al., 2001). It has been demonstrated that the activation of the gonadotropin receptors can additionally activate the phospholipase C (PLC) pathway and induce a rise in intracellular calcium (Ca_2) levels (Herrlich et al., 1996). A prolonged stimulation of the LH-R and FSH-R has been reported to desensitize the receptors via the downregulation and functional uncoupling of the G-protein (Ferguson, 2001, Rivero-Muller et al., 2013).

1.2.3 The gonads

The gonads comprise the ovaries in females and the testes in males. Being the primary reproductive organs, the ovaries and testes have two important functions: the synthesis of mature gametes (gametogenesis; oogenesis and spermatogenesis) and the synthesis and secretion of the peptidergic and steroid hormones. These steroid hormones exert a regulatory role on the secretion of GnRH and gonadotropins at the level of the hypothalamus and pituitary gland, respectively, besides exerting their peripheral effects.

In women, the ovaries are responsible for the differentiation and maturation of the fertilizable oocytes, as well as the synthesis and secretion of several sex steroids such as estrogens and progesterone. These processes are controlled by LH and FSH, binding to their respective receptors. LH-Rs are expressed in the ovarian theca cells, interstitial cells, differentiated granulosa cells and luteal cells (Ascoli et al., 2002), while FSH-Rs are expressed in the granulosa cells of developing follicles (Minegishi et al., 1997). During oogenesis, the oocyte increases its size, interstitial cell layer and granulosa cell layer, which is the main source of 17β-estradiol (E_2) in females. Accordingly, steroidal hormonal levels change during this maturation process under the changing levels of LH, FSH and other paracrine factors such as insulin-like growth

factors and gonadal peptides (inhibins, activins and follistatins), and lead to a surge of LH, triggering the rupture of the ovarian follicle or ovulation (Everett et al., 1949, Everett, 1964). Ovulation happens approximatively every 28 days in women, and every 4 to 6 days in female mice depending on the strain. Cyclic variations in the reproductive activity of rodents can be followed by changes in the vaginal epithelium, which happen as a consequence of changes in the secretion of ovarian steroids.

In men, the testes are responsible for spermatogenesis, the maturation of spermatozoa, and the synthesis and release of androgens. They comprise two distinct compartments, the interstitial and tubular compartment (Kerr, 1992, Kerr et al., 2006), whose interaction is crucial for proper gonadal functioning. The interstitial compartment comprises different cell types, including Leydig cells, immune cells, fibroblasts and endothelial cells (Kerr, 1992, Kerr et al., 2006). The Leydig cells synthesize and release androgens, such as testosterone (T), in the presence of LH binding to its receptor. Intratesticular T levels play an important role in the stimulation of spermatogenesis, while it needs to be metabolized into dihydrotestosterone or E_2 to exert functions at the level of the periphery and CNS respectively. The tubular compartment comprises both Sertoli cells, expressing FSH-Rs, and germ cells (Kerr, 1992, Kerr et al., 2006). Germ cells will develop into mature spermatozoa under the influence of FSH and T, while Sertoli cells support the development of germ cells (Griswold et al., 2006) and synthesize inhibins and activins under the influence of FSH.

1.3 The GnRH-system

1.3.1 Distinct forms of GnRH

GnRH was first discovered and isolated from the hypothalamus of mammals in 1971 (Baba et al., 1971, Matsuo et al., 1971, Schally et al., 1971). In the following decades, it was also identified in Chordata and invertebrates (Millar, 2005, Hasunuma et al., 2013). As it was believed that distinct hypothalamic hormones were responsible for the secretion of LH and FSH, GnRH was first called luteinizing hormone-releasing hormone (LHRH). This first form of GnRH was found to have an important role in reproduction, and was given the name ‘GnRH1’ when two paralogous GnRH genes were discovered. GnRH1 and its function will not be reviewed in this paragraph, as it will be extensively described in this manuscript later. The second form of GnRH, named GnRH2, was first found in chickens (Miyamoto et al., 1984).

The GnRH2-synthesizing neurons are located in the midbrain tegmentum, close to the 3v. Their projections can be found within the whole brain, though they are mainly focused on the hind- and midbrain with axon terminals ending at the 3v (Gonzalez-Martinez et al., 2002). Both GnRH1 and GnRH2 act at the level of the pituitary gland, but only GnRH1 is thought to evoke the secretion of gonadotropins (Kobayashi et al., 1994, Montaner et al., 2001, Mongiat et al., 2006). GnRH2, which is absent in mice and rats, is suggested to play a regulatory role in food intake, energy homeostasis and the balance between survival and reproduction in musk shrews (Temple et al., 2003, Kauffman et al., 2005). A third form of GnRH, GnRH3, was first discovered in lamprey, and nowadays known to be only present in a part of Actinopterygii. Their neurons are localized near the olfactory bulb (OB), more specifically in the terminal nerve ganglion (Chiba et al., 1996), and play a modulatory role on neurons in the forebrain, olfactory epithelium (OE), and modulate olfactory mediated behaviors (Wirsig-Wiechmann et al., 2002). To summarize, one could state that GnRH1 is the key player in reproduction, whilst GnRH2 and GnRH3 regulate sexual activity-related behaviors. From this point, GnRH mentioned in this manuscript refers to GnRH1.

1.3.2 GnRH : from gene to protein secretion

The *GnRH1* gene is located on the murine chromosome (mmu) 14 and human chromosome (hsa) 8, and the primary ribonucleic acid (RNA) transcript comprises four exons and three introns (**Figure 2**). Exon 1 encodes the 5' untranslated region (UTR), while exon 2 encodes the GnRH decapeptide, the amidation/proteolytic processing signal and N-terminus of the GAP (GnRH associated protein) sequence. Exons 3 and 4 encode the central part and C-terminus of GAP and the 3' UTR. (Selmanoff et al., 1991, Gaillard et al., 2018).

As many other neuropeptides, the GnRH neurohormone, which consists of 10 high conserved amino acids (aa), is synthesized as a part of a precursor protein. This precursor protein, prepro-GnRH, is composed of 92 aa and undergoes subsequent steps of enzymatic cleavage to form: (i) the GnRH decapepide, (ii) the N-terminus that consists of 23 aa and regulates intracellular packaging and secretion, (iii) a Gly-Lys-Arg proteolytic processing site, and (iiii) the C-terminus that consists of 56 aa and encodes for GAP, which is secreted together with GnRH from the vesicles. The function of GAP has not completely been determined yet, but it has been thought to regulate both gonadotropin and prolactin secretion from the hypophysis (Adelman et al., 1986, Wetsel et al., 2002).

The amount of biologically available GnRH is controlled at a multitude of biosynthetic stages. GnRH gene expression is determined by the rate of transcription of its precursor (pro-GnRH), polyadenylation and capping of the RNA, processing of the primary GnRH transcript into the mature messenger RNA (mRNA), and its transport from the nucleus into the cytoplasm. Subsequently, the rate of mRNA translation into pro-GnRH and further processing into mature GnRH, and further GnRH degradation determine the GnRH levels that will be packed into the large dense core vesicles (LDCVs) of the GnRH neurons that will be secreted in the portal circulation and evoke gonadotropin secretion (King et al., 1983, Gore et al., 1997, Gore et al., 2002). All these consecutive regulating steps contribute to the idea that biosynthesis and secretion of GnRH are not closely linked to each other, and that GnRH neurons possess a cytoplasmic reserve of GnRH mRNA. The 22h-30h half-life ($t_{1/2}$) of GnRH mRNA enables the GnRH neurons to keep a GnRH mRNA pool. This pool could be used for replenishing peptide levels very quickly, as Wang and colleagues suggest happens before a pre-ovulatory surge (Wang et al., 1995). As a result of this whole process starting with the transcription of the GnRH gene and ending with posttranslational modifications, the mature GnRH neurohormone is formed. It is a decapeptide that consists of a pyroglutamate N-terminal residue and a glycine-amide C-terminus, which are thought to play a role in the stabilization of the structure and to slow down the blood degradation (Chertow, 1981).

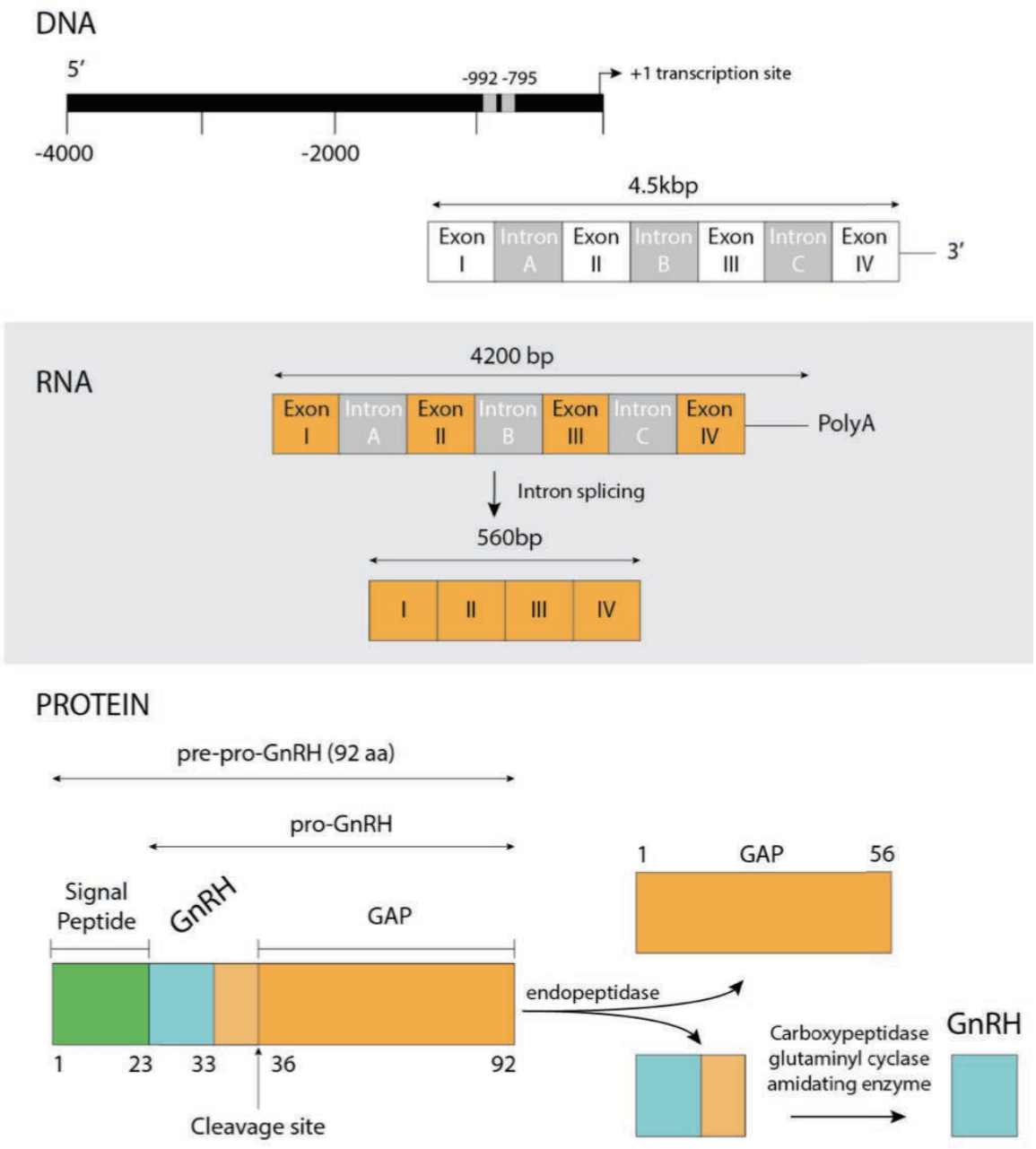


Figure 2. GnRH and GAP biosynthesis. (DNA) The mouse, rat and human structure of the GnRH gene. (RNA) GnRH RNA, splicing and processing intermediates. (Protein) Final GnRH decapeptide after translation and posttranslational modifications. Figure taken with the permission of (Malone, 2017).

As demonstrated by electron microscopy, GnRH is stored in LDCVs that can be found within the soma, dendrites, axons and axonal terminals of GnRH neurons (Lehman et al., 1988, Prevot et al., 1998, Prevot et al., 1999). These GnRH-containing LDCVs are stored at the level of the axonal terminals and attached to the plasma membrane at the site of exocytosis. Their priming, which may be Ca_2 dependent, is controlled by cytoplasmic proteins that regulate the assembling of proteins located on the plasma membrane, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). Three SNARE proteins, respectively vesicle-associated membrane protein (VAMP), syntaxin and SNAP-25, form a stable complex (Weimbs et al., 1998) that induces a rearrangement of membranous phospholipids. The reorganization of the membranes results in the creation of a pore, allowing an exchange between the vesicle and the external environment of the neuron (**Figure 3**). The most abundant expressed VAMP protein in neurons is the VAMP-2 isoform, that is highly expressed in the POA (Trimble et al., 1990) and endocrine cells (Kasai et al., 2012).

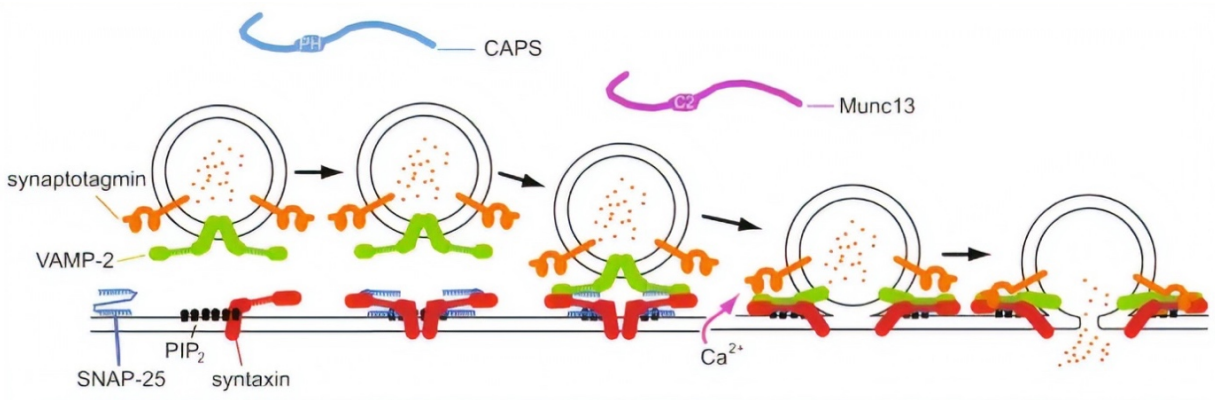


Figure 3. Calcium-triggered exocytosis. Schematic showing the different steps of Ca_2-dependent vesicular exocytosis. The vesicular SNARE, represented here by VAMP-2, interacts with the SNAREs of the plasma membrane, respectively SNAP-25 and syntaxin. The Ca_2 signal, and more specifically the fixation of the Ca_2/calmodulin at the level of the VAMP-2, induces a conformation of the proteins and the recruitment of synaptotagmin. This conformation allows the fusion of the vesicular membrane and the plasma membrane, and therefore the release of the neurotransmitter or peptide in the synapse. This process requires other molecules as well, for instance Munc13 and phosphatidylinositol diphosphate (PIP2).; CAPS, Ca_2-dependent activator protein for secretion; SNAP-25, soluble N-ethylmaleimide-sensitive factor attachment protein receptor 25; PIP2, phosphatidylinositol diphosphate. Picture taken from (Martin, 2012).

Clostridium botulinum neurotoxins (BoNTs) are frequently used within the research of SNARE proteins. They are produced by *Clostridium botulinum* bacteria and cause the fatal disease

botulism, inducing a paralysis of the muscles (Montecucco et al., 2005). These toxins are known to recognize and enzymatically cleave the SNARE proteins (Montecucco et al., 1994). It has been demonstrated that the BoNT serotype B (BoNT/B) specifically cleaves the VAMP2 protein, and therefore inhibits the secretion of neurotransmitters and other peptides in the brain (Schiavo et al., 1992).

1.3.3 The GnRH receptor

Reproductive function is controlled by GnRH receptors (GnRH-Rs) that are predominantly expressed on the membrane of the pituitary gonadotropic cells (Childs et al., 1997). Multiple studies suggested the expression of the GnRH-R gene in other tissues, showing their ability to bind a radioactive GnRH agonist. These tissues comprise specific regions of the CNS, including the hippocampus, the ARH and ventromedial nuclei of the hypothalamus (Jennes et al., 1997). The GnRH-R is a member of the GPCRs (Stojilkovic et al., 1994). The binding of GnRH to the extracellular domain and associated changes in its conformation activate the $G_{q/11}$ protein and PLC (Millar et al., 2004), resulting in an increase of intracellular Ca_2 levels and activation of the protein kinase C (PKC), which are essential for the synthesis and secretion of the gonadotropins (Conn et al., 1987). Inactivating mutations in the gene encoding the GnRH-R are known to result hypogonadotropic hypogonadism (HH) (de Roux et al., 1997).

The GnRH-R expression determines the ability of the gonadotropic cells of the pituitary gland to respond to GnRH (Wise et al., 1984), and is regulated by a multitude of factors including gonadal steroid hormones (Reeves et al., 1971, Laws et al., 1990, Yasin et al., 1995), activins (Braden et al., 1992) and inhibins (Wang et al., 1988). Besides these regulatory components, one of the most important regulators of GnRH-R expression is GnRH itself. A disconnection of the hypothalamus from the anterior pituitary gland, removing GnRH input, decreases *GnRH-R* mRNA and the total number of GnRH-Rs in the pituitary gland of ovariectomized (OVX) ewes (Clarke et al., 1987, Gregg et al., 1989). Moreover, the administration of GnRH in a pulsatile manner restores the number of GnRH-Rs in hypothalamic-pituitary-disconnected (HPD) ewes (Clarke et al., 1987, Hamernik et al., 1988), while a continuous exposure decreases *GnRH-R* mRNA and numbers of GnRH-Rs in the pituitary gland (Nett et al., 1981, Turzillo et al., 1995, Turzillo et al., 1995, Turzillo et al., 1995). Depending on the concentrations of a wide variety of regulating components, the density of GnRH-Rs varies during the estrous cycle, and is highest prior to ovulation (Nett et al., 1981).

1.3.4 The origin and migration of GnRH neurons

1.3.4.1 Origin of GnRH neurons

GnRH neurons as well as other neuronal populations of the hypothalamus, such as CRH-, GHRH- and TRH-expressing neurons, secrete their neuropeptide into the capillary system and initiate a feedback loop. By using *in situ* hybridization (ISH), Morales-Delgado and colleagues determined that CRH-, GHRH- and TRH-expressing neurons find their embryonic origin within the peduncular paraventricular domain of the hypothalamus (Markakis, 2002, Morales-Delgado et al., 2014). Unlike these neurons, GnRH neurons do not originate from the embryonic hypothalamus but originate from outside the brain, more specifically from the olfactory placodes (OPs).

The OPs first arise as cell thickenings by the convergence of cellular fields at the border of the anterior neural plate (Farbman, 1992), a structure that forms simultaneously with the primitive placodal thickening (Brunjes et al., 1986). As the neural plate starts folding, the primitive placodes separate from the neural crest (NC) and developing CNS. During further embryonic development, cell movements will drive the placodes to invaginate and form the nasal pits. The central part of the nasal pit will give rise to the OE, important for chemosensory olfaction, whilst the medial walls will form the vomeronasal organ (VNO), important for pheromone detection.

The finding that GnRH neurons arise from the OPs dates back to 1989. Using immunohistochemistry (IHC) and ISH, two separate laboratories discovered that the OPs are not only generating olfactory sensory neurons and other support cells, but also GnRH-expressing neurons. These GnRH-expressing neurons were first observed in association with the vomeronasal nerve (VNN) and the nervus terminalis (NT), along whom they migrate through the nasal septum into the forebrain. From there, GnRH-expressing neurons enter the developing hypothalamus following a pathway that is not unravelled yet (Schwanzel-Fukuda et al., 1989, Wray et al., 1989, Wray et al., 1989).

Although decades of research have proven the link between the development of the olfactory and GnRH system, the exact embryonic precursor lineage of the GnRH neurons has yet not been identified due to technical limitations. Further research in different animal models have brought up evidence for the origin laying within the NC, placodal and non-placodal ectoderm

(Daikoku et al., 1993, Kramer et al., 2000). As it is assumed that GnRH pre-dates the appearance of jawed vertebrates, it is very unlikely that different groups of animals (having a pituitary and hypothalamus) developed different mechanisms for integrating GnRH in the hypothalamus. It is more likely that this mechanism is well-conserved in different species, however, more research is needed to firmly delineate the embryonic origin of these neuroendocrine GnRH neurons.

1.3.4.2 Migration of GnRH neurons

The migration of GnRH neurons from the OPs into the hypothalamic system is a precise orchestrated journey through different molecular environments, that is completed by birth. Much research has been conducted to determine the migratory pathway of GnRH neurons, and has led to the characterization of four specific stages of migration (Tobet et al., 2001, Tobet et al., 2006). There may be some species-specific properties, but a similar set of stages are indispensable for GnRH neurons to reach their final destination in the brain and to regulate reproduction via the adenohypophysis.

1) GnRH neurons that arise at the OP from embryonic day (E) 10,5 in the mouse, together with axons of the VNN and/or NT, start migrating across the nasal mesenchyme to the cribriform plate. This first step depends upon the movement of the GnRH neurons and factors that stimulate the adherence of the GnRH neurons to the axons of the VNN and/or NT. The first GnRH neurons begin migrating from the OP at E11,5.
2) At the cribriform plate the VNN divides and forms a branch that turns caudally towards the ventral forebrain, and that is using specific factors to guide the GnRH neurons along. The splitting of the VNN takes place at E12,5, at the point that already more than 50% of the GnRH neurons reached the nasal septum (Tobet et al., 1996).
3) GnRH neurons are guided towards the hypothalamus by the extension of long processes through the basal forebrain. Between E13,5 and E14,5 in mice, GnRH neurons enter the forebrain.
4) After detaching from the axonal guides, the neurons will disperse throughout the hypothalamus to settle in their final location and send projections to the ME. The first GnRH neurons reach the POA of the hypothalamus at E14,5 and detach from the axonal support, before they send out projections towards the ME (**Figure 4**).

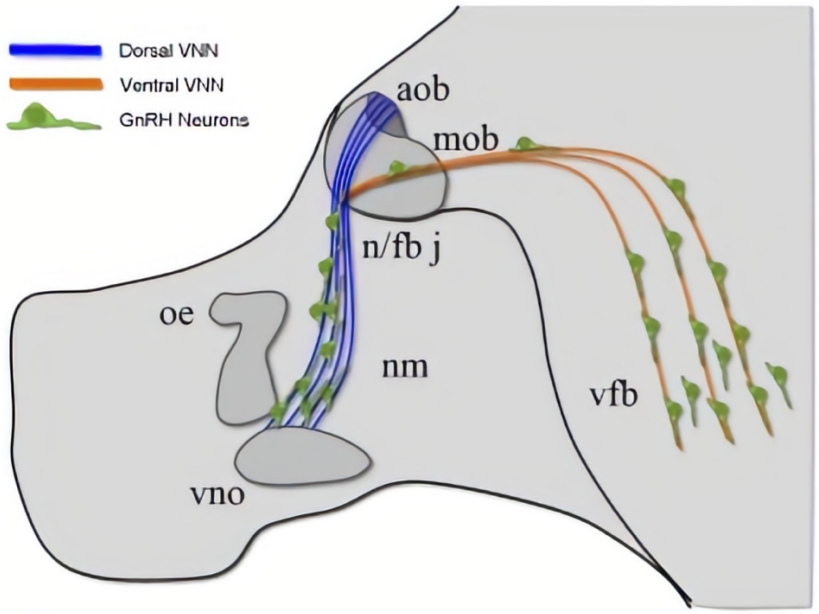


Figure 4. The GnRH migratory pathway. Illustration of the head of a mouse embryo at E14.5, representing the migration of GnRH neurons from the VNO into the forebrain, along the vomeronasal VNN and/or terminal nerve fibers. VNN, vomeronasal nerve; Vno, vomeronasal organ; oe, olfactory epithelium; nm, nasal mesenchyme; n/fb j, nasal/forebrain junction; aob, accessory olfactory bulb; mob, main olfactory bulb; vfb, ventral forebrain. Figure taken from (Messina et al., 2013).

1.3.5 The onset of puberty

Puberty is the transition period between childhood and adulthood, a period that is hallmarked by physiological and psychological changes to attain sexual maturation and fertility. This transitional period is highly dependent on the HPG-axis, which on its turn is tightly regulated by a complex variety of inhibitory and excitatory factors, both neuronal and non-neuronal. Puberty is not just depending on chronological age, but requires a complex variety of factors to be adjusted to each other. The GnRH secretory system needs to receive inputs from many different processes, such as growth, the metabolic state and energy balance, and peripheral hormone levels and its regulation can be altered by many factors, including environmental factors (Parent et al., 2003) and endocrine-disrupting chemicals (EDCs)(Pinson et al., 2017). The main regulatory components of the GnRH neuronal circuit will be discussed in section 1.3.8. Changes or failure of this complex network to synchronize lead to a different timing or absence of puberty onset, and associated disorders (**Figure 5**).

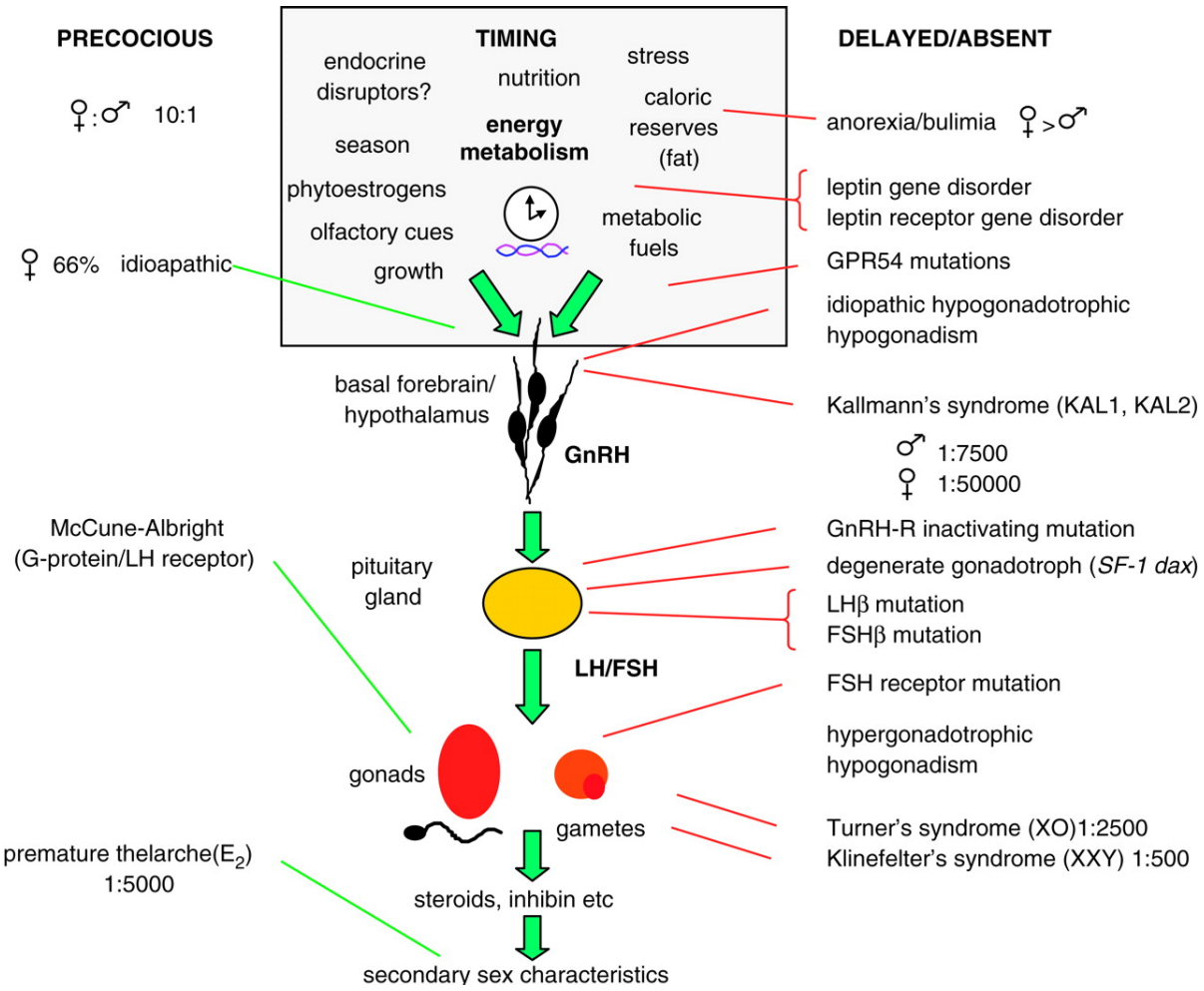


Figure 5. A complex network of inputs regulating puberty onset and fertility. GnRH neurons, integrating a variety of internal and external cues, in the central control of puberty onset by driving the HPG-axis. Different disorders in the timing of puberty onset are indicated on this scheme, reflecting dysfunction at the different levels of the HPG-axis. Figure taken from (Ebling, 2005).

1.3.5.1 Stages of puberty

The inhibitory and activatory inputs that regulate the activity of the HPG-axis change during the different stages of life (**Figure 6**). The axis gets activated during the embryonic and early postnatal stages, after which its activity gets quenched. Along childhood until puberty, the GnRH neurons are held under the input of inhibitory signals and release a minimal amount of GnRH in the portal blood. At puberty, changes in the regulating network lead to the increase in the pulsatile GnRH secretion from the GnRH neurons of the hypothalamus, essential for an individual to undergo puberty (Ojeda et al., 2006).

The postnatal development of the HPG-axis can be divided into four stages (Ojeda et al., 1980):

1) The neonatal period; from birth to postnatal day 7 (P7)
2) The infantile period; from P8 to P21 (minipuberty) (Kuiri-Hanninen et al., 2011, Kuiri-Hanninen et al., 2014)
3) The juvenile period; from P21 to P30 (approximately)
4) The peripubertal period; from P31 (approximately) until the onset of puberty

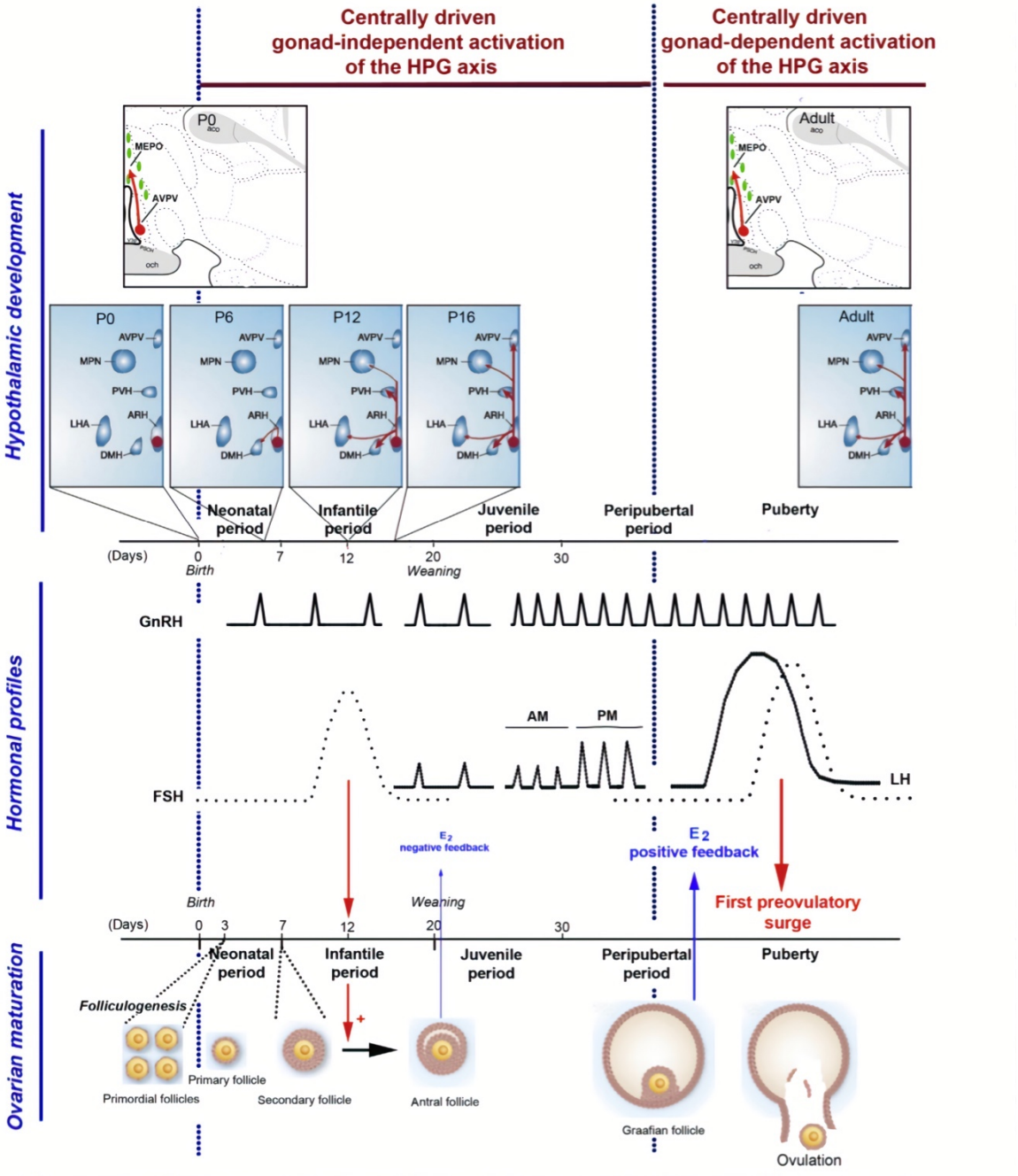


Figure 6. The activation of the female HPG-axis during development. *(Hypothalamic development)* The morphological development of the hypothalamic anteroventral periventricular nucleus (AVPV), important for the E_2-mediated positive feedback on the GnRH neurons, is already completed at birth. However, the axons of the ARH neurons, important for the estrogen-mediated negative feedback, first appear in the POA during the infantile period and are mature by P16. *(Hormonal profiles)* Schematic representation of the centrally driven gonad-independent and gonad-dependent hormonal changes during postnatal development. *(Ovarian maturation)* Schematic representation how the aforementioned changes interfere in follicular maturation, and how follicular maturation affects the hypothalamus and/or pituitary gland by the secretion of E_2. AVPV, anteroventral periventricular nucleus; ARH, arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; MEPO, median preoptic nucleus; MPN, medial preoptic nucleus; PVH, paraventricular nucleus. Figure taken from (Prevot, 2015).

1.3.6 The GnRH system at adulthood

The GnRH neuron population is relatively small, ranging from 800 neurons in rodents to 1000-2000 neurons in human. In rodent species, they can be found scattered all along their migratory path into the brain (Wray et al., 1986), but most of the cell bodies are detected at the level of the medial septum and around the organum vasculosum of the lamina terminalis (OVLT), one of the CVOs of the brain. Few neurons can be found in the OBs or on the sides of the 3v at the level of the ME (**Figure 7**).

As mentioned before, GnRH neurons send out fiber projections towards the ME and release their neurohormone in the fenestrated portal vasculature to target the pituitary gland. At the level of the adenohypophysis, GnRH regulates the synthesis and secretion of LH and FSH, and their secretion in conducted in two distinct manners. It has been demonstrated that a low GnRH pulse frequency favours the secretion of FSH, while a higher GnRH pulse frequency favours the secretion of LH (reviewed by (Constantin, 2011)). The balance of secretory modes of the GnRH neurons emphasizes the importance of correct GnRH functioning for the release of gonadotropins and functioning of the gonads.

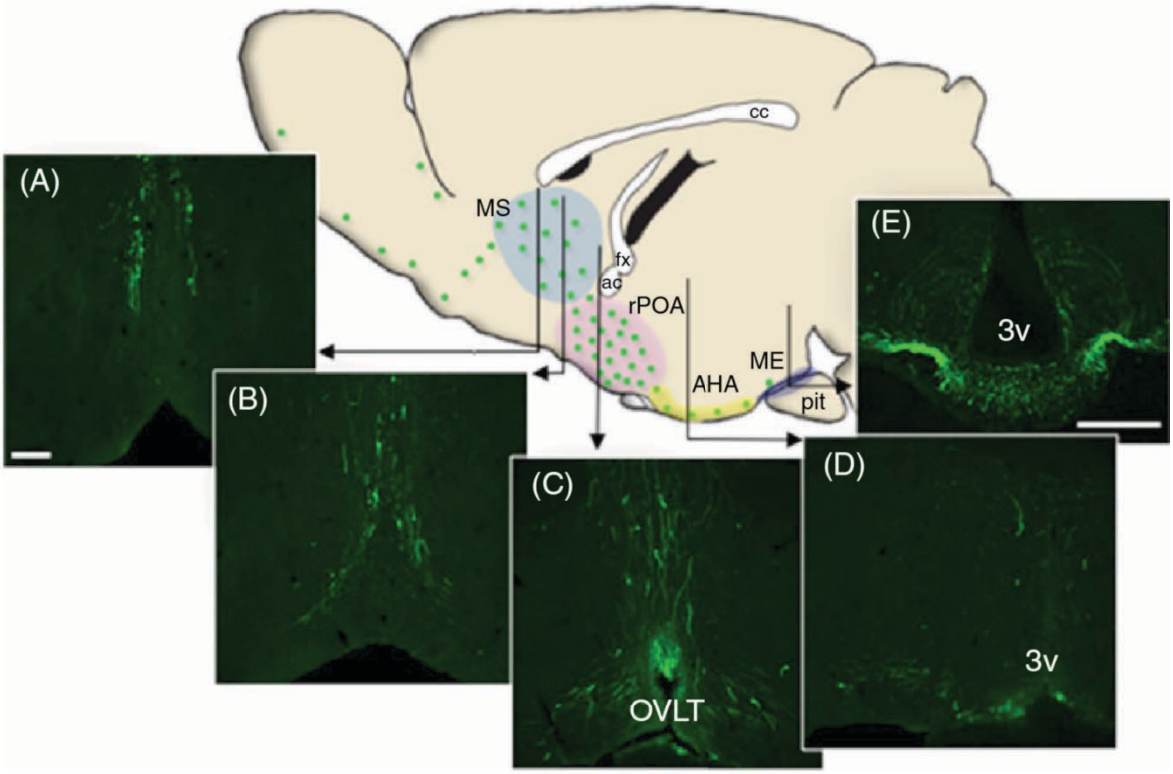


Figure 7. Distribution of GnRH neurons in the rodent brain. Schematic representation of a rodent brain, with the anatomical locations of GnRH neurons highlighted; the medial septal region (MS, blue), the rostral POA (rPOA, pink), the AHA (yellow) and the ME (purple). Coronal sections of a GnRH-green fluorescent protein (Gfp) transgenic mouse brain are represented and connected to their representative location on the schematic via a vertical line. Green cell bodies can be detected at (A) the MS area, (B) the rPOA, (C) the OVLT and (D) the AHA. (E) Projections of the GnRH neurons can be seen as green fibers at the level of the ME. MS, medial septum; cc, corpus callosum; fx, fornix; ac, anterior commissure; rPOA, rostral preoptic area; OVLT, organum vasculosum of the lamina terminalis; AHA, anterior hypothalamic area; ME, median eminence; pit, pituitary gland; 3v, third ventricle. Figure taken from (Campbell et al., 2018).

Menopause is the time in a woman's life that marks the end of the natural fertile period. It is a natural occurring biological process that happens as a result of follicular depletion, and is accompanied by a subsequent loss of estrogen and progesterone levels. Although the key role of follicular depletion in menopause is not questioned, recent studies brought up evidence for the association between ageing and gonadal-independent changes in hypothalamic and pituitary components of the HPG-axis (Wise et al., 1997), suggesting a new role for hypothalamic and/or pituitary changes in ending the fertile period and entering menopause.

The depletion of primary ovarian follicles discharges the hypothalamic and pituitary components of the HPG-axis from the negative feedback loop induced by the ovarian steroids, and evokes an immediate augmentation of LH and FSH levels (Yen et al., 1971, Monroe et al., 1972, Studd et al., 1978). Whether the gonadotropins and the gonadotropin free α-subunit (FAS) increase or decline as a function of age after the onset of menopause remains a point of controversy in the field (Chakravarti et al., 1976, Scaglia et al., 1976, Alexander et al., 1990, Kwekkeboom et al., 1990). Answering to this controversy, Hall and colleagues stated that the capability to estimate GnRH pulse frequency in the absence of negative feedback from the sex steroids, and plausible age-related changes, may have been hampered by methodological limitations in previous studies (Hall et al., 2000). Refining the tools that are used to assess the characteristics of GnRH pulsatility, they demonstrated an age-related decline in LH, FSH and FAS levels and a decrease in the frequency of GnRH pulses after menopause onset (Hall et al., 2000).

1.3.7 The GnRH pulse generator

Over decades of time, independent investigations have demonstrated that GnRH is secreted in a pulsatile manner into the portal vasculature, and thereby evoking a pulsatile release of the gonadotropin hormone LH. In follicular-phase females, a pulse of LH is observed approximatively every hour. In luteal-phase females and males, a peak of LH is observed every 2 to 3 hours. Altogether, this work led to the concept of a 'GnRH pulse generator', a neural orchestra that regulates the episodic GnRH secretion responsible for the pulsatile character of LH release (Goodman et al., 1981, Pohl et al., 1982).

The distinct patterns of pulsatile GnRH release observed at the different stages of the ovarian cycle, and especially changes in GnRH pulse frequency, differentially regulate the synthesis and secretion of the pituitary gonadotropins (Thompson et al., 2014). It has been shown that a constant activation of the GnRH-R has been shown to suppress the HPG-axis (Belchetz et al., 1978), and that an impaired LH pulsatile character contributes to infertility (Jayasena et al., 2014). Moreover, fertility in patients with HH (discussed in section 1.4) can be rescued administering GnRH in a pulsatile manner (Martin et al., 1990). These findings highlight the importance of the pulsatile character of GnRH secretion. Surprisingly, to maintain a sufficient LH secretion and normal fertility, only 10% of the GnRH population is required (Kokoris et al., 1988, Czieselsky et al., 2016). Until today, little is known about the nature and functioning of

the GnRH pulse generator. Two main modes of operation have been proposed; an intrinsic or extrinsic GnRH pulse generator, as reviewed by (Herbison, 2018).

The idea of an intrinsic GnRH pulse generator arose in 1992, when Weiner and colleagues discovered that GnRH-secreting GT1 cells in culture had the ability to secrete GnRH in a pulsatile manner (Weiner et al., 1992). This phenomenon was further supported by similar findings in cultured embryonic GnRH neurons of different origins. From then, it was thought that GnRH neurons could be interconnected and synchronize their own activity with a pulsatile character of GnRH secretion as a result. The translation of the *in vitro* findings to the *in vivo* situation is challenging, since GnRH neurons are dispersed and cannot take advantage of gap junctions to intercommunicate. Though it is demonstrated that GnRH and co-expressed factors glutamate and galanin regulate the electrical activity of GnRH neurons (Herbison, 2006), a lack of sufficient synaptic intercommunication prevents their synchronization. Notably, GnRH neuron processes intertwined among themselves, sharing efferent synaptic inputs; therefore, the synchronisation of the GnRH neuron population may be controlled by upstream efferents (Campbell et al., 2009). Indeed, no evidence has been found for the synchronization of GnRH neuron firing on brain slice preparations (Constantin et al., 2012). Moreover, it has been demonstrated that hypothalamic tissue preparations without GnRH cell bodies exhibit a pulsatile secretion of GnRH for multiple hours (Rasmussen, 1993, Bourguignon et al., 1997, Purnelle et al., 1997). The only imaginable possibility of an intrinsic GnRH pulse generator lays within the dendron that GnRH neurons project towards the ME. A dendron is a process of the neuron with characteristics of both the dendrite and axon (Herde et al., 2013), and can both conduct action potentials and receive synaptic inputs. These dendrons are known to be regulated by various neurotransmitters, and could be the potential location where GnRH neurons synchronize their activity.

The idea of the extrinsic pulse generation, the most plausible, starts from the idea that GnRH neurons receive synchronizing influence from a separate group of neurons. As GnRH neurons receive inputs from at least 17 different brain regions (Wintermantel et al., 2006), the pulse generating neurons may possible be found in this pool of neuronal populations. Although independent studies demonstrated that the pulse-generating neurons were located within the ARH (Blake et al., 1974, Soper et al., 1980, Wilson et al., 1984), the nature of these neurons targeting the distal dendrons of GnRH neurons remains unclear. New studies suggest

kisspeptin (Kp) neurons of the ARH as being the GnRH pulse generator, as Kp signalling is critical for puberty and fertility (de Roux et al., 2003, Seminara et al., 2003, Gianetti et al., 2010) and Kp receptor (*KISS1-R)* mutations are associated with an abnormal character of LH pulsatility (Tenenbaum-Rakover et al., 2007). Recent studies in the mouse have suggested a subpopulation of *Kiss1* neurons of the ARH as being the GnRH pulse generator in rodents (Clarkson et al., 2017). Despite this ground-breaking work, many mechanistic questions regarding the function of this pulse generator remain unanswered.

1.3.8 The GnRH neuronal circuit

The GnRH neurons are the major players in the neuroendocrine control of puberty onset and reproduction. However, the proper functioning of the HPG-axis depends on the functioning of GnRH neurons, that are controlled by a dynamic network of internal and external signals. In this section, the main components of the GnRH neuronal circuit will be described.

1.3.8.1 Central regulators

1.3.8.1.1 Kisspeptin

Neuropeptides play an important role in the regulation of puberty onset. A neuropeptide that has been found to be crucial for pubertal activation of the HPG-axis are Kps, encoded by the *Kiss1* gene. Multiple studies have shown the importance of Kp in the excitatory network regulating GnRH secretion (Dhillo et al., 2005, Jayasena et al., 2015) and its role in the regulation of puberty and reproductive function has been extensively described in literature (de Roux et al., 2003, Seminara et al., 2003, Clarkson et al., 2009, Tena-Sempere, 2013). Some researchers even suggest “puberty starts with a *Kiss*” (Dungan et al., 2006). Recent research proposes that a surge in leptin, observed close to the timing of puberty onset, may promote Kp secretion and therefore GnRH secretion (Holmes, 2014). Moreover, it has been shown that GnRH neurons express *Kiss1R* and that their responsiveness to Kp increases at the time of sexual maturation (Han et al., 2005). At the same time, an increase in *Kiss1* mRNA is observed in the hypothalamus (Han et al., 2005, Clarkson et al., 2009). Supporting the role of Kp and the Kiss1R in sexual maturation and fertility, *Kiss1R* knockout (KO) mice present an absence of puberty onset and severe dysfunction of the reproductive function at adulthood (Teles et al., 2011).

Two important *Kiss*1 neuronal populations for the control of reproduction are located within the hypothalamus. The first group is located in the ARH and thought to act as the extrinsic GnRH pulse generator and modulate GnRH pulsatility at the level of the ME (Popolow et al., 1981). A second group of neurons is located in the AVPV and send projections to the medial POA (mPOA) to regulate the preovulatory GnRH surge (Gu et al., 1997, Wang et al., 2019). ARH *Kiss1* neurons were initially thought to mediate the steroid ovarian hormone negative feedback, whereas AVPV *Kiss1* neurons were thought to regulate the positive feedback (Oakley et al., 2009, Lehman et al., 2010). More recent studies suggest that the ARH *Kiss1* neuronal population that co-expresses neurokinin B and dynorphin, being redefined as KNDy (Kp, Dynorphin, Neurokinin B (NKB)) neurons (Navarro et al., 2009, Navarro et al., 2011), controls GnRH pulsatile release through stimulating actions of NKB onto *Kiss1* neurons and inhibitory actions of dynorphin to terminate the pulse.

1.3.8.1.2 Glutamate and ɣ-aminobutyric acid

Glutamate is one of the most abundant excitatory neurotransmitters in the CNS and widely accepted to play a role in the control of GnRH neuron excitability. It is described that GnRH neurons are responsive to glutamatergic inputs, and that their number gradually increases during the maturation of the HPG-axis and reaches a maximum after puberty onset (Goroll et al., 1994). It is believed that glutamatergic signalling is involved in the regulation of GnRH expression, and thereby puberty onset and reproduction (Urbanski et al., 1990).

ɣ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter of the CNS and requires the decarboxylation of glutamate by the glutamic acid decarboxylase (GAD). A gradual increase of *GAD* mRNA can be observed in the POA during the maturation of the HPG-axis, similar as the observed increase in glutamate content (Davis et al., 1996). GnRH neurons are known to express both types of GABA receptors, and receive inputs from estrogen-sensitive GABA neurons of the POA. These preoptic GABA neurons are thus thought to be mediators of estrogens to GnRH neurons (Curran-Rauhut et al., 2002). Multiple studies have shown the importance of this inhibitory neurotransmitter in various processes including GnRH neuronal migration, the puberty of onset and controlling the pulsatile release of LH (Christian et al., 2007, Casoni et al., 2012). It should be noted that GABA acts as an excitatory factor on the HPG-axis during early stages, and switches to exerting an inhibitory role around the

pubertal period (Moguilevsky et al., 1991, Szwarcfarb et al., 1994). Directly onto GnRH neurons, the effect of GABA remains excitatory (Herbison et al., 2011).

1.3.8.1.3 Nitric oxide

Nitric oxide (NO) is an important gaseous neurotransmitter, synthesized by the NO synthase (NOS) enzyme, for the regulation of neuronal transmission. The expression of the neuronal isoform of NOS (nNOS) has been described to be in close proximity to GnRH neurons in the POA (Herbison et al., 1996, Clasadonte et al., 2008) and more distant from GnRH fibers at the level of the ME (Herbison et al., 1996). Indeed, the synthesis and secretion of NO is required for proper GnRH/LH secretion (Moretto et al., 1993, Bonavera et al., 1996). Moreover, research conducted by the laboratory of Dr. Prevot demonstrated that NO exerts a direct inhibitory effect on the GnRH neuronal activity (Clasadonte et al., 2008) and that inhibiting NO activity results in increasing LH levels (Moretto et al., 1993, Bonavera et al., 1996, Hanchate et al., 2012). It should also be mentioned that nNOS neurons are directly regulated by various hormones, for instance leptin (Bellefontaine et al., 2014) and E_2 (d'Anglemont de Tassigny et al., 2007), and that they may be important for the integration of signals required for the maturation and functioning of the HPG-axis. This is supported by the fact that nNOS KO mice present a hypogonadic phenotype (Gyurko et al., 2002, Hanchate et al., 2012).

1.3.8.1.4 Proopomelanocortin and neuropeptideY/agouti-related peptide

The anorexigenic proopomelanocortin (POMC)-expressing neurons are located within the hypothalamus and are known to modulate GnRH neuronal activity and the reproductive function. POMC neurons located in the ARH co-express the estrogen receptor α (ERα) and interact with the preoptic GnRH neurons, suggesting a role in mediating the negative feedback action of estrogens (Xu et al., 2011). Two products of the POMC neurons, the anorectic α-melanocyte-stimulating hormone (α-MSH) and the opioid β-endorphin, are known to interact with GnRH neurons. α-MSH has been shown to promote firing of preoptic GnRH neurons (Roa et al., 2012) and suggested to be involved in the transmission of the permissive effect of leptin on puberty onset (Manfredi-Lozano et al., 2016), while it has been described that the receptors for the opioid β-endorphin are expressed on GnRH neurons (Lagrange et al., 1995).

The interaction of GnRH neurons with the orexigenic neuropeptide Y/agouti-related peptide (NPY/AgRP)-expressing neurons is similar as the interaction with POMC neurons. Like POMC neurons, estrogen-responsive NPY/AgRP neurons (i) send projections to morphologically interact with GnRH neurons at the level of the POA (Simonian et al., 1999) and (ii) modulate the neuronal activity of the GnRH neurons. The orexigenic peptide NPY can exert both inhibitory and excitatory effects on GnRH neurons, depending on the receptor (Roa et al., 2012). In addition, it has been demonstrated that NPY exerts a direct effect on the neuronal activity of GnRH neurons and thereby puberty onset and fertility (Minami et al., 1990).

1.3.8.2 Peripheral and environmental regulators

1.3.8.2.1 Metabolic signals

The metabolic state and energy balance of an individual are of tremendous importance in the control of puberty onset and fertility, as these processes require appropriate energy levels. Different studies performed in the 1960s and 1970s showed that nutritional and metabolic factors are playing an important role in the functioning of the HPG-axis (Frisch et al., 1970, Frisch et al., 1974). In conditions of undernutrition or obesity, this disrupted equilibrium of nutritional and metabolic factors causes an accelerated or delayed puberty onset and impaired fertility. One of the first peripheral factors that linked metabolism with the HPG-axis was leptin (Ahima et al., 2000), an adipocyte-derived hormone that is proportionally secreted with the reserve of body energy or fat mass (Considine et al., 1996). Studies have shown that circulating leptin levels are essential for the maturation of the HPG-axis, puberty and fertility (Farooqi et al., 2014). Mutations of this hormone or its receptor are known to be implicated in altered puberty onset and infertility, as reviewed by (Elias et al., 2013). Another peripheral metabolic hormone that affects puberty onset and fertility is ghrelin, an orexigenic hormone mainly secreted by the stomach. It does not only regulate food intake and the secretion of GH, but also exerts an inhibitory effect on GnRH secretion in the prepubertal period (Lebrethon et al., 2007). Other metabolic hormones, such as insulin and peptide YY, are involved in the control of puberty as well, but not will not be further discussed in this manuscript.

1.3.8.2.2 Environmental signals

Not only metabolic cues, but also environmental factors are implicated in the timing of sexual maturation and puberty onset (Parent et al., 2003). Human studies have demonstrated a link between the hours of daylight and the timing of puberty onset in girls (Albright et al., 1990).

Also, endocrine disrupting chemicals (EDCs) have been shown to influence the age of puberty onset (Rasier et al., 2006, Lopez Rodriguez et al., 2019) and reproductive health (Rehman et al., 2018).

1.3.8.2.3 Gonadal steroid hormones

Sexual steroids comprise estrogens, androgens and progestins that are mainly synthesized and released by the gonads. These hormones mediate their effect by binding on intranuclear receptors, respectively estrogen receptors α and β (ER-α, ER-β), androgen receptor (AR) and progesterone receptors A and B (PR-A, PR-B) and thereby activate intracellular signalling cascades. Gonadal steroids are known to be implicated in various processes in the CNS such as mood, affective and cognitive functions, but exert their main function on the HPG-axis and its key components through feedback loops. Sexual steroids are not only implicated in the sexual differentiation of the brain, but also modulate the secretion of GnRH/LH and activate neural circuits involved in reproductive behaviors (Young et al., 1964, Herbison et al., 2001).

Estrogens have a dual effect on the hypothalamus, exerting both inhibitory and stimulatory influences on the secretion of GnRH and gonadotropins, depending on the anatomical location within the hypothalamus (Radovick et al., 2012). Estradiol exerts negative feedback upon the pulsatile release of GnRH and LH, and negative feedback upon the preovulatory surges of GnRH and LH. These effects are thought to be mediated via the kisspeptin neurons in the ARH and AVPV, respectively (Moenter et al., 2019). The suggested biological substrate for the dual effect of estrogens is Kp, as it is demonstrated that estrogen decrease *Kiss1* in the ARH and increase *Kiss1* in the AVPV (reviewed by (Radovick et al., 2012)). Moreover, E_2 can exert both negative or positive feedback actions on the *Nos1* neurons in the POA (Chachlaki et al., 2017).

1.3.8.3 Another regulatory mechanism: microRNAs

1.3.8.3.1 MicroRNAs

MicroRNAs (miRNAs) define a class of small non-coding RNA molecules, with a length of 18 to 28 nucleotides (nt), that regulate gene expression at posttranscriptional level by degrading the target mRNA or inhibiting the mRNA translation. Initially, it was believed that miRNAs only exert a negative regulating effect on gene expression by binding to the 3’ UTR region of its target mRNA. Nowadays, it is known that miRNAs can not only decrease, but also increase gene expression by binding to its target mRNA. The binding sites can vary over the 3’ UTR, as

well as the promotor or coding regions of the mRNA (Hausser et al., 2009, Hendrickson et al., 2009, Breving et al., 2010). It should be mentioned that a single miRNA can regulate multiple mRNAs, and that one mRNA can be regulated by multiple miRNAs (Friedman et al., 2009).

1.3.8.3.2 Synthesis of microRNAs

The first step in miRNA synthesis comprises the transcription of the miRNA encoding genes in the nucleus of the cell, and requires the cooperation of the RNA polymerase II (Pol II) and multiple transcriptional factors, for instance c-Myc or p53. This process results in 100 to 1000 nt long molecules, the primary miRNA (pri-miRNA). The pri-miRNA is then cleaved by a microprocessor complex that includes the ribonuclease (RNase) III endonuclease Drosha and partner DiGeorge syndrome chromosomal region 8 (DGCR8), to form a 70 nt precursor miRNA (pre-miRNA). After this conversion, the exportin-5/Ran-GTP complex transports the pre-miRNA into the cytoplasm, where it undergoes further processing by RNase III Dicer, cofactor Tar RNA binding protein (TRBP) and argonaute protein (Ago). This step on conversion results in a 22 nt miRNA/miRNA duplex. Generally, one miRNA strand is preserved and integrated with the miRNA-induced silencing complex (miRISC) to become functional. Once functional, they bind the target mRNA and promote its cleavage, degradation or translational suppression (**Figure 8**).

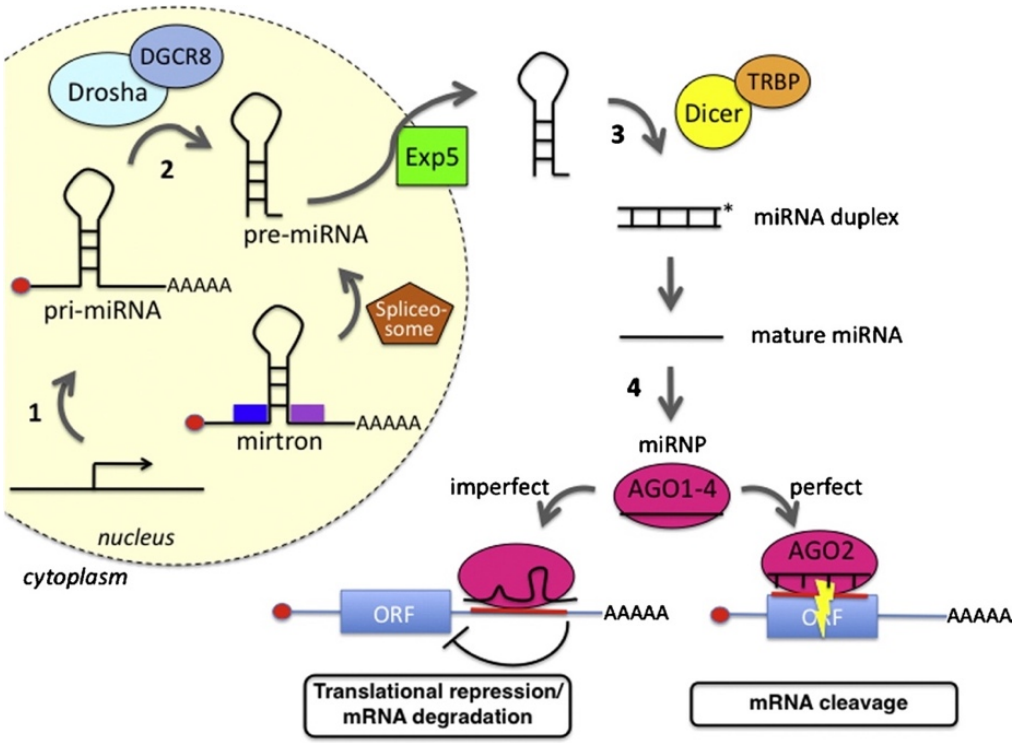


Figure 8. Schematic representation of the synthesis of miRNAs. The synthesis of miRNAs is an extremely complex process that requires the RNase-III enzymes Drosha and Dicer, together with multiple cofactors. The control of the miRNA maturation and their biological activity are regulated on different levels. Figure taken from (Breving et al., 2010).

1.3.8.3.3 The role of microRNAs on GnRH expression

Independent studies already suggested the involvement of the Lin28/let-7 miRNA system in normal development, including (mechanisms leading to) puberty onset (Lettre et al., 2008, He et al., 2009, Sangiao-Alvarellos et al., 2013). Despite these fundamental works, it was only in 2016 that Messina and colleagues demonstrated the modulatory role of miRNAs on the balance between inductive and repressive signals in the GnRH gene network during the infantile period, and that it is essential for the maturation of the GnRH neurons that control puberty onset and fertility (Messina et al., 2016). This critical infantile period coincides with the so-called minipuberty, the first centrally driven, gonad-independent activation of the HPG-axis. During this short time period, changes in the expression of certain miRNAs switches an array of GnRH repressors (e.g. *Zeb1* and *Cebpb*) and activators (e.g. *Otx2*), and in this way permits the increase of the GnRH neurohormone that is required for sexual maturation of the HPG-axis. The two most important miRNAs that regulate *Zeb1* and *Cebpb* expression are respectively (i) the miR-200 family, including miR-200a-c, miR-141 and miR-429, that is upregulated and selectively enhanced in GnRH neurons, and (ii) miR-155, upregulated in GnRH neurons and also acts on other hypothalamic cells. *In vivo* dysregulation of the miR-200/429-transcription factor micro network resulted in an impaired puberty onset and fertility (Messina et al., 2016).

1.4 Reproductive syndromes

The embryonic and postnatal development of GnRH system is a tightly regulated process, that is controlled by a complex variety of factors, and eventually leads to the onset of puberty and maturation of the HPG-axis. Accordingly, aberrations at many levels in the development of the GnRH system lead to infertility syndromes. Two examples of genetic disorders rooting in a GnRH deficiency will be described in this manuscript, respectively idiopathic congenital HH (CHH) and Kallmann syndrome (KS).

1.4.1 Congenital hypogonadotropic hypogonadism

Idiopathic CHH is a rare reproductive disorder that originates from GnRH deficiency. Clinically, GnRH deficiency is associated with low levels of the gonadotropins LH and FSH, and therefore very low or even undetectable levels of sex steroids. As one could imagine, these altered hormone levels prevent puberty onset and normal reproductive function, leading to infertility. Diagnosis with CHH might be challenging due to the heterogeneity and large spectrum of the phenotypic presentation (Boehm et al., 2015). The prevalence is likely to be around 1 per 5,000 individuals. Most patients benefit from specialized treatments, for instance programmable minipumps administering gonadorelin acetate, recovering fertility.

1.4.2 Kallmann syndrome

The term ‘Kallmann syndrome’ comprises the presentation of CHH in association with a reduced or absent sense of smell, also called hyposmia or anosmia and is associated with impairments in the GnRH neural migration during embryonic development (Seminara et al., 1998). The first observations of KS were made in 1856, when Maestre de San Juan observed patients with a micropenis and altered olfactory structures (de San Juan, 1856). The hereditary nature of this disorder was discovered a few years later by Kallmann and colleagues and the (partial) absence of OBs and its axons in this disorder were further described by De Morsier and Gauthier in the 1950s (De Morsier et al., 1963). KS is often associated with multiple anomalies, for instance cleft lip and/or palate (Waldstreicher et al., 1996). KS has a prevalence of 1/8,000 in male, and 1/40,000 in female.

CHAPTER 2

Down syndrome

2.1 Generalities of Down syndrome

Down syndrome (DS), also known as trisomy 21, is a genetic disorder that is caused by the presence of an extra copy or major portion of chromosome 21. It is the most common and complex human autosomal aneuploidy that is compatible with postnatal survival. It occurs in approximatively 10 to 14 per 10,000 live births (Parker et al., 2010, Khoshnood et al., 2011). Like many other disorders characterized by chromosomal perturbations, the risk of DS is known to increase with maternal age (Reeves et al., 2001).

Although some aspects of DS were described before by Jean Etienne Dominique Esquirol, DS is named after the British doctor John Langdon Down that fully described the syndrome in 1866. Almost 100 years later, in 1959, Jérôme Lejeune discovered the underlying cause of DS (Lejeune et al., 1959). Because of this breakthrough in DS research and Lejeune's dedication to improving patients' life, the French society that focuses on patients suffering from intellectual disability with a genetic origin was named after him. Lejeune's dedication to finding a cure and thereby improving so many patients' lives is represented in a famous quote about his high hopes and expectations for finding a treatment for DS.

"Nous trouverons, il est impossible que nous ne trouvions pas. C'est un effort intellectuel beaucoup moins important que d'envoyer un homme sur la lune."

- Professor Jérôme Lejeune

Most DS patients are characterized by distinctive physical characteristics (**Table 1**), and have a high risk of developing other potential health and medical problems. Some of the most noticeable characteristics are related to the morphology of the face and eyes, short stature and intellectual disability. In addition to some typical noticeable characteristics, some other important comorbidities are very frequently observed in DS patients. These include, for instance, congenital heart diseases and defects of the cardiovascular system (Freeman et al., 1998) and Alzheimer's disease (AD) (Wisniewski et al., 1985, Mann et al., 1989).

At birth	Infancy and childhood	Adulthood
Structural		
Dysmorphic features[a]	Growth retardation and obesity	
Congenital heart disease		
Duodenal stenosis or atresia		
Imperforate anus		
Hirschprung disease		
Central nervous system		
Hypotonia	Developmental and mental retardation	Decrease in cognitive function
	Decreased sensitivity to pain	Alzheimer disease
Immune and hematopoietic systems		
Transient myeloproliferative disorder	Leukemia	
	Immune defects and/or infection	
Other	Thyroid dysfunction	Male sterility
		Reduced longevity

Table 1. Chronology of the major components observed in the Down syndrome phenotype. Table summarizing the most frequently observed phenotypic characterizations of DS at different life stages, including intellectual disability at infancy and childhood, a decrease in cognitive function, AD and male sterility at adulthood. Dysmorphic features include many characteristics, for instance upslanting palpebral fissures, loose skin on the neck, a short neck and a flat nasal bridge. Table taken from (Antonarakis et al., 2006).

2.2 Etiology of Down syndrome

Trisomy 21 can present in three different types:

1) **Free trisomy 21** (90-95% of the cases). Free trisomy 21 is the most common type of DS, characterized by three complete copies of hsa 21 in all cells (Ahmed et al., 2005, Azman et al., 2007). Approximatively 90% of the cases result from a chromosomal nondisjunction of maternal origin, mainly happening in meiosis I, while the other cases result from an additional paternal chromosome or post-zygotic mitotic non-disjunction (Vranekovic et al., 2012).
2) **Partial trisomy 21** (2-4% of the cases). Some DS patients do not possess a complete third copy of chromosome 21, but have a partial trisomy. In this case, an additional part of chromosome 21 is attached to any other chromosome by translocation, the phenomenon characterized by different chromosomes breaking and rejoining each other (Kondo et al., 2006).
3) **Mosaicism** (2-4 % of the cases). Rarely, a third copy of chromosome 21 can only be detected in some cells of the body. An individual with trisomy 21 mosaicism has both trisomic and euploid cell lines. Mosaicism results either from euploid cells that duplicate one of the hsa 21 during cell division, either from trisomic cells that lose one copy because of a mitotic error (Pangalos et al., 1994).

2.3 Characteristics of Down syndrome

The presence of an additional copy of chromosome 21 result in either increased transcript and protein levels, either in the repression of transcripts (Epstein, 1990). The mechanisms and assumptions made about this so-called gene dosage imbalance will be discussed in section 2.4. What should already be mentioned is that this gene dosage imbalance possibly affects different aspects of development and thereby causes a wide variability of characteristics observed in DS patients (Reeves et al., 2001).

2.3.1 Intellectual disability and Alzheimer's disease

2.3.1.1 Intellectual disability

Intellectual disability is one of the main characteristics observed in all DS patients, although the severity varies between different individuals. It includes developmental delays, language and memory impairments, as well as many other cognitive abnormalities (Epstein, 1986, Haxby, 1989, Schapiro et al., 1989). Several findings reported the growth of cognitive abilities through childhood, adolescence and early adulthood (Carr, 2005, Couzens et al., 2012), however, the intellectual quotient (IQ) and cognitive abilities are known to decline during adolescence and further adulthood respectively (Carr, 1994, Carr, 2005). These findings are supported by multiple longitudinal studies (Hauser-Cram et al., 1999, Carr, 2005, Couzens et al., 2011). Although neurodegenerative changes even occur in the absence of clinical signs of dementia, cognitive decline during adulthood is thought to be associated with dementia representative of AD (Ballard et al., 2016, Lott et al., 2019). The exact causes underlying intellectual disability and cognitive impairment are still unknown, and more research is required to answer this question. One of the genes on which research is focused nowadays is the dual specificity tyrosine phosphorylation kinase 1A (*DYRK1A*) gene. This gene is very active during the development of the fetal brain and known to be implicated in cell proliferation (Ionescu et al., 2012) and neuronal development (Tejedor et al., 2011), as well as many signalling pathways. *DYRK1A* disruption is associated with learning difficulties in trisomic mice (Van Dyke, 2003).

2.3.1.2 Alzheimer's disease

AD is a neurodegenerative disorder that is very closely connected to DS. The first reports about AD in DS were made in 1948, when G.A. Jervis described three DS patients with a similar neuropathological profile as non-DS patients described by Alois Alzheimer in 1906 (Jervis, 1948). Further analysis revealed that brains of DS patients present neurofibrillary tangles (NFT) and a large-scale neuronal loss (Struwe, 1929, Bertrand et al., 1946). Multiple publications confirmed the association between AD and DS. Extensive research over the years led to the knowledge that neuropathological changes of AD can be found in virtually all DS adults around the age of 40 years (Wisniewski et al., 1985, Mann et al., 1989). Although amyloid β (Aβ) depositions and NFTs can be found in almost all individuals with DS, only 20-25% develop a dementia or cognitive decline that is characteristic for AD (Mehta et al., 1998, Mehta et al., 1999, Ghezzo et al., 2014).

The observed association between DS and AD can be partially addressed to the overexpression of the of the amyloid precursor protein (APP). The *APP* gene that encodes the APP protein is located on hsa 21, and therefore triplicated in individuals with DS. This discovery played a key role in the development of the 'amyloid cascade' hypothesis, that states that an accumulation of Aβ plaques evokes a pathological cascade including neuritic injury and the formation of NFT via tau protein that results in neuronal dysfunction and cell death (Hardy et al., 1992, Dickson, 1997, Selkoe, 1999). However, the pathophysiology of AD is not only explained by APP and requires more contributing factors including apolipoprotein E (Huynh et al., 2017), neuroinflammation (Di Bona et al., 2008, Karch et al., 2015) and changes in reproductive hormones (Zhao et al., 2015).

Over the past decades, strong evidence for the role of reproductive hormones in the vulnerability to AD has been provided. This is reflected by the fact that nearly two-third of AD patients are women (Brookmeyer et al., 2011), a disproportion that could be attributed to the associated loss of ovarian hormones during menopause (Zhao et al., 2009). Recent studies have demonstrated that AD pathology is more likely expressed as dementia in women than men (Barnes et al., 2005), and that the spectrum of dementia-related behavioral symptoms and the cognitive deterioration tend to be more severe in AD women (Schmidt et al., 2008, Chapman et al., 2011, Hall, 2012, Irvine et al., 2012). Not only the pathophysiological presentation of AD, but also the response effect to treatment are different in women

compared to men (Mielke et al., 2012, Claxton et al., 2013). These observed differences may be partially addressed to the changes in estrogens and therefore other reproductive hormones that women undergo during menopause, as reproductive hormones regulate a wide range of brain processes. This hypothesis is supported by the finding that estrogen replacement therapy (ERT) decreases the risk of developing AD in women when started early after menopause onset.

2.3.2 Olfaction

Olfactory regions are among the earliest and most extensively damaged brain regions in AD physiopathology (Esiri et al., 1984, Ohm et al., 1987), and impairments of olfactory functioning are one of the earliest clinical symptoms observed in AD (reviewed by (Zou et al., 2016)). Human studies demonstrated that also individuals with DS present impairments in olfactory function, more specifically odor threshold and identification (Murphy et al., 1996). Despite previous reports of normal olfactory function in DS (McKeown et al., 1996), a recent study demonstrated that olfaction may already be impaired at relatively young age (Cecchini et al., 2016). These findings are supported by other studies that show the presence of olfactory impairments in children with DS, and that these impairments worsen with ageing (Nijjar et al., 2002).

2.3.3 Reproduction

Impaired reproductive function is observed in women and men with DS. Despite a normal evolution of puberty, men with DS are generally considered infertile (Stefanidis et al., 2011). In literature, only three confirmed cases of spontaneous conception by fathers with DS are reported (Sheridan et al., 1989, Zuhlke et al., 1994, Pradhan et al., 2006). The first signs of reproductive impairments are observed after puberty onset, for instance a decrease in testicular volume, decreased production of anti-Müllerian hormone (AMH) and increased production of gonadotropins (Campbell et al., 1982). These changes in AMH and gonadotropin levels evidence a gonadal dysfunction (Hsiang et al., 1987), that is known to worsen with age. Moreover, oligospermia and azoospermia have been observed in men with DS and suggest an impaired spermatogenesis (Stearns et al., 1960). The mechanisms underlying the abnormal low sperm count remain unclear, although it may be an effect of DYRK1A (Liu et al., 2017). Similar to men with DS, women with DS go through a normal puberty. Many cases of

pregnancy in DS women have been reported (Bovicelli et al., 1982), however only fifteen to thirty percent of DS women are fertile. This lower fertility rate may be associated with the reduced ovarian reserve (Hojager et al., 1978), leading to early menopause in DS women. It has been described that estrogen deficiency as a result of menopause is associated with the onset of AD in DS women (Coppus et al., 2010).

2.4 Gene dosage hypothesis and beyond

Hsa 21 is the smallest human chromosome, comprising approximatively 48 M basepairs (bp). Sequencing of the long arm (21q) determined that it contains 696 genes, including at least 235 protein-coding genes and 142 pseudogenes (Sturgeon et al., 2011). It did not take researchers too long to define the DS critical region (DSCR), a 5,4 Mbp region that contains approximatively 50 genes and was thought to be responsible for the DS phenotype (Delabar et al., 1993). Whether an additional copy of the DSCR can explain the majority of symptoms seen in DS, including intellectual ability, remains the subject of intense debate. Recently, more-detailed genetic mapping of partial trisomy 21 demonstrated multiple regions of the hsa 21 contribute to the DS phenotype (Korbel et al., 2009, Lyle et al., 2009). Moreover, the fact that major characteristics of DS are observed in patients with a partial trisomy 21 without a third DSCR copy suggest that the DSCR is a susceptibility region modified by other loci on, for instance, hsa 21 (Lyle et al., 2009). These findings led to two hypotheses trying to explain trisomy 21-linked phenotypes, respectively the ‘gene dosage hypothesis’ and ‘amplified developmental instability hypothesis’ (Pritchard et al., 1999, Dierssen et al., 2001).

The ‘gene dosage hypothesis’ is based upon the idea that the DS phenotype directly results from the overexpression of individual causative genes located on chromosome 21. The triplication of the causative genes is hereby called a gene dosage imbalance. The criteria that have been set to identify plausible candidate genes are (i) their location within the DSCR, (ii) their expression pattern in the brain or (iii) the function of the encoded protein. Following these criteria, several genes have been identified to be possibly involved in the DS-associated phenotype. One of these genes is *DYRK1A* that encodes an enzyme that is associated with cell proliferation and brain development (reviewed in (Dierssen et al., 2009, Wiseman et al., 2009)). It is essential to understand that the effect of overexpressing genes on DS phenotypes can not only derive from the direct regulation of neural functions, but also from the indirect

regulation. The effect of genetic imbalance may be exerted through gene-specific transcriptional and/or post-translational regulations, or via feedback mechanisms in complex expression networks.

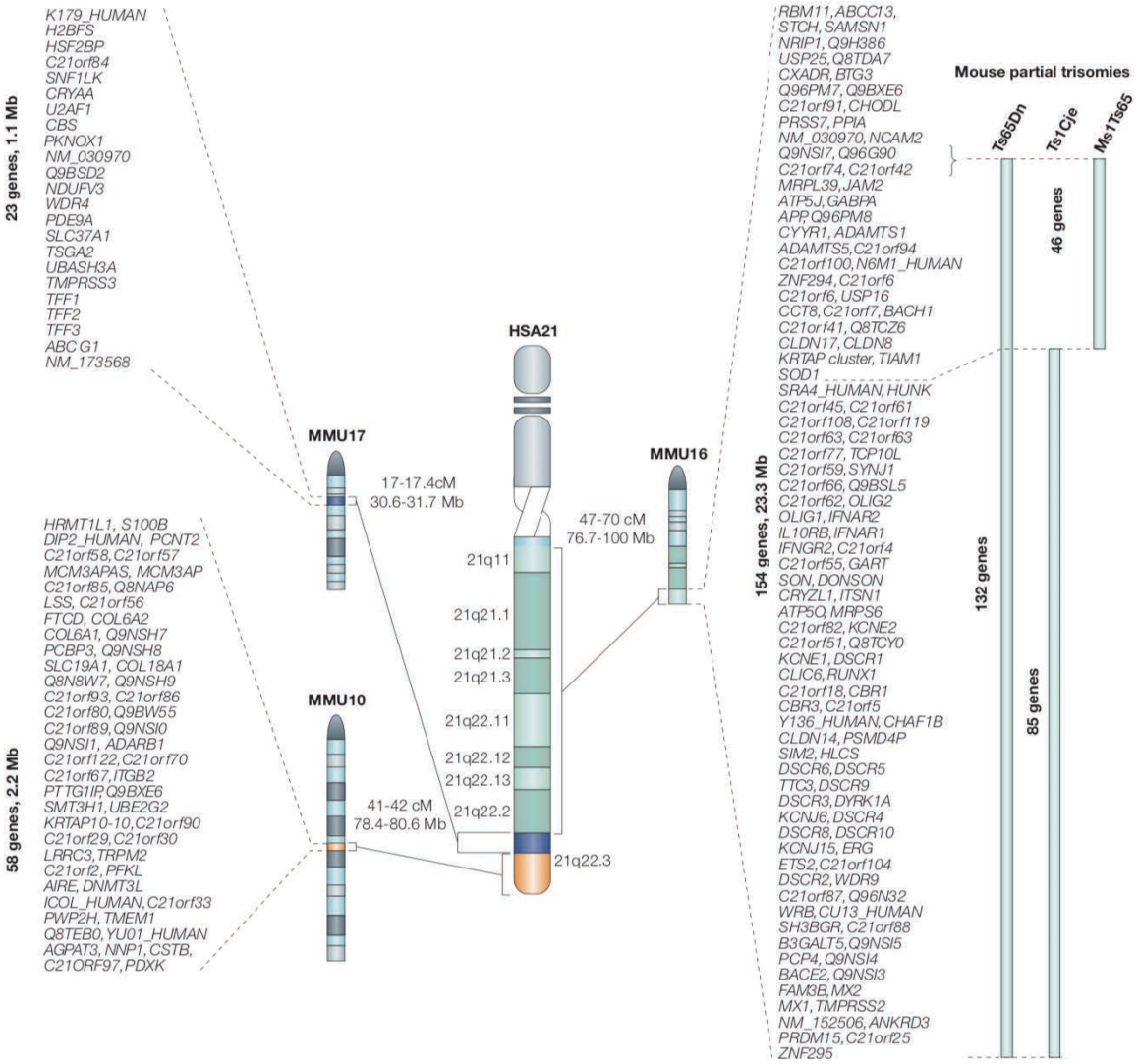
The 'amplified developmental instability hypothesis' states that the dosage imbalance of hsa 21 results in an impaired cellular homeostasis, and that the severity of the DS phenotype is correlated with the size of the triplicated genetic region (Shapiro, 2001). This idea has been supported by certain animal studies (Olson et al., 2004), although it is controversial to certain human cases reported with full trisomy and mild intellectual disability (Korbel et al., 2009).

However, over the past decade, the understanding and view of the mechanisms underlying DS have dramatically changed. Dosage imbalance of individual genes located on hsa 21 has been proven to contribute to the variable DS-linked phenotype. More global mechanisms are required to fully understand and explain the full complexity of DS. A recent proposed mechanism suggests that the imbalance of copy number alterations of functional, non-traditional genomic elements, and their indirect and direct effects on gene expression of both hsa 21 and other chromosomes, contribute to DS. These elements include miRNAs, PIWI-interacting RNAs (piRNAs) and endogenous small interfering RNAs. Recently, it has been demonstrated that at least five miRNA genes, respectively *miR-99a*, *miR-125b-2*, *miR-802*, *miR-155* and *let-7c*, are overexpressed in the DS brain (Elton et al., 2010). The dosage imbalance of these miRNAs may influence the expression of specific target proteins, and in their way, contribute to the DS phenotype (Kuhn et al., 2010). For instance, it has been shown that miRNAs are essential for the conversion of short-term to long-term memory (Ryan et al., 2015). Not only miRNAs, but also other RNA species, and epigenetic mechanisms could contribute to the DS pathophysiology (Senti et al., 2010). These findings led to a new 'genome instability hypothesis'. This hypothesis states that dosage imbalance affects multiple hsa 21 and other chromosomal genes, together with alterations in the functional regulation of mRNA, regulatory non-coding elements and epigenetic regulation lead to the pathophysiology of DS (Dierssen, 2012).

2.5 Mouse models

The use of mouse models has played a prominent role in the study of DS, as with many other human diseases. However, DS is one of the more complicated human conditions to adequately model in mice and still challenges researchers nowadays. This can be explained by the fact that several genes lying in close proximity to one another on hsa 21 are duplicated, but that their orthologs are located on segments of three murine chromosomes, respectively mmu 16, mmu 17 and mmu 10 (Davisson et al., 2001). Ideally, from a genetic point of view, a mouse model for DS must be trisomic for the three involved segments. Nowadays, none of all constructed mouse models is trisomic for all hsa 21 orthologs (**Figure 9**) and breeding a complete trisomic mouse model remains very challenging.

The oldest, first viable and most frequently used segmental trisomy mouse model is the Ts65Dn model (Davisson et al., 1990, Davisson et al., 1993). Other mouse models, such as the Ts1Cje and Ts1Rhr, are trisomic for only a subset of genes trisomic in the Ts65Dn model (Sago et al., 1998) and present with other phenotypic characteristics (**Table 2,3**). Among many other developed mouse models, the Tc1 model is worth mentioning as it carries a hsa 21.



7, and 10. In the mouse, orthologs of human chromosome 21 are distributed over chromosomes 16, 17 and 10. The mouse models Ts65Dn, Ts1Cje and Ms1Ts65 are trisomic for a variable part of the mmu 16, as presented on the right. Figure taken from (Antonarakis et al., 2004).

Trisomy	Hsa21			Segment of Mmu16				Segment of Mmu17		Segment of Mmu10	Segment of Mmu16, Mmu17, and Mmu10	Hsa21
	DS	Dp(16)1Yey/+	Ts65Dn	Ts2Cje	Ts1Cje	Ms1Ts65	Ts1Rhr	Dep(17)1Yey/+	Ts1Yah	Dp(10)1Yey/+	Dp(10)1Yey/+; Dp(16)1Yey/+; Dep(17)1Yey/+ Yu et al., 2010	Tc1
Brain volume	Reduced		Reduced	Reduced during the embryonic period	Reduced during the embryonic period	Reduced	Reduced at 4 months of age					
Neuronal density	Reduced		Reduced	Reduced	Not affected							
Cerebellar volume	Reduced		Reduced		Reduced	Not affected	Reduced					Reduced
Cerebellar neuronal density			Reduced		Reduced	Reduced						Reduced
Neurogenesis	(i) Impaired neural precursor proliferation (ii) Slowing of the cell cycle (iii) Impaired neurodifferen-tiation (iv) Impaired cerebellar neurogenesis		(i) Impaired neural precursor proliferation (ii) Slowing of the cell cycle (iii) Impaired neurodiffer-entiation (iv) Impaired cerebellar neurogenesis	(i) Impaired neural precursor proliferation	(i) Impaired neural precursor proliferation (ii) Impaired neurodiffer-entiation (iii) Impaired cerebellar neurogenesis							
Dendrites and dendritic spines	(i) Impaired morphology (ii) Reduced		(i) Impaired morphology (ii) Reduced	(i) Impaired morphology (ii) Reduced	(i) Impaired morphology (ii) Reduced		(i) Impaired morphology (ii) Reduced					

Table 2. Neuromorphological alterations in Down syndrome and Down syndrome mouse models. Taken from (Rueda et al., 2012).

Trisomy	Hsa21		Segment of Mmu16					Segment of Mmu17		Segment of Mmu10	Segment of Mmu16, Mmu17, and Mmu10	Hsa21
	DS	Dp(16)1Yey/+	Ts65Dn	Ts2Cje	Ts1Cje	Ms1Ts65	Ts1Rhr	Dep(17)1Yey/+	Ts1Yah	Dp(10)1Yey/+	Dp(10)1Yey/+; Dp(16)1Yey/+; Dep(17)1Yey/+ Yu et al., 2010	Tc1
Motor skills	Delayed acquisition		Delayed acquisition									
Motor coordination	Impaired		Impaired									Impaired
Activity and attention	Reduced attention		Hyperactivity and reduced attention		Normal activity	Normal activity	Normal activity					Increased spontaneou activity
Context discrimination		Impaired	Impaired					Unchanged		Unchanged	Impaired	
Spatial learning and memory	Impaired	Impaired	Impaired		Impaired	Impaired		Unchanged	Enhanced	Unchanged	Impaired	Impaired
Working and reference memory	Impaired		Impaired									Impaired
Novel object recognition			Impaired				Impaired		Impaired			
Operant conditioning			Impaired									

Table 3. Behavioral and cognitive alterations in Down syndrome and Down syndrome mouse models. Taken from (Rueda et al., 2012).

2.5.1 The Ts65Dn mouse model

The Ts65Dn mouse model is a widely-used mouse model of DS that was formed from the translocation of the distal region of mmu 16 onto the centromere of mmu 17 (Davisson et al., 1993, Reeves et al., 1995) and extends a region of approximatively 23.3 Mbp; from the *Mrpl39* to the *Znf295* genes (Kahlem et al., 2004). This region contains approximatively 132 genes orthologous to hsa 21 genes (Antonarakis et al., 2001), of which 55% are protein coding genes (Duchon et al., 2011).

Ts65Dn mice present a broad variety of phenotypic abnormalities, including a developmental delay that already presents at birth by a reduced body weight, and muscular trembling (Davisson et al., 1993). Male mice are known to be sterile, while females are fertile but have small litters and provide poor care to their offspring (Moore et al., 2010). Many alterations affecting the brain can be detected in this mouse model, for instance a delayed brain development, significant decrease in the cerebellar and hippocampal volume and impaired neurogenesis (Bianchi et al., 2014). Similar to the physiopathology of AD in humans, Ts65Dn mice show an age-related atrophy of the brain and degeneration of the basal forebrain cholinergic regions, as well as extensive astrocyte hypertrophy and alterations in different neurotransmitters. These observed changes are associated with learning and behavioral deficits (Reeves et al., 1995, Antonarakis, 1998). It has been reported that the Ts65Dn model is unable to discriminate between familiar and novel objects in the novel object recognition (NOR) paradigm at adult ages. (Fernandez et al., 2007). No reports have been made about the presence of skeletal or cardiac anomalies or leukaemia in this mouse model (Reeves et al., 1995), which are frequent comorbidities of DS.

Despite the olfactory impairments observed in individuals with DS and the importance of sensory modality in rodents, only a few studies investigated the olfactory performance in trisomic Ts65Dn mice. One study reports that the ability to detect odors of Ts65Dn mice is indistinguishable from controls at young ages, and seems to be impaired at mid-age (Bianchi et al., 2014). These findings are supported by a second, independent study that shows no differences in a peanut butter finding test. Nevertheless, this study demonstrates an olfactory associative learning deficit (de Souza et al., 2011), which is also observed in humans (Sliger et al., 2004). It needs to be mentioned that nor olfactory discrimination, neither olfactory processing has been investigated in this DS mouse model yet.

2.5.2 The Ts1Cje mouse model

Together with the Ts65Dn mouse model, the Ts1Cje model is one of the most-well studied mouse models of DS. It is characterized by a monosomy of the distal part of mmu 12 and a reciprocal translocation of 77 genes of mmu 16, thus containing a smaller trisomic region than the Ts65Dn model. The behavioral and cognitive phenotype of these mice is comparable with Ts65Dn mice, although cognitive and learning deficits present milder (Sago et al., 1998, Sago et al., 2000, Duchon et al., 2011, Guedj et al., 2015). This is concomitant with the fact that an age-related degeneration of basal forebrain cholinergic neurons has not been observed in these mice. Regarding the reproductive phenotype, many studies report both male and female Ts1Cje mice to be fertile. However, one study has shown male Ts1Cje mice to be subfertile, and present a lower testis weight and sperm concentration (Davisson et al., 2007).

2.6 Therapies

Despite a lot of research, no single, standard treatment for DS have been forthcoming. Eventual treatments are adapted to the physical and intellectual needs, as well as to the strengths and challenges of an individual. Certain comorbidities may require specific therapeutic interventions, for instance heart defects. Improved personalised medical care does not only improve life quality for DS patients, but also increases their life expectancy. A variety of therapeutically approaches are provided to individuals with DS to encourage their maximal development and optimize life quality. These approaches include (i) physical therapy to improve motor skills, muscle strength, posture and balance, (ii) speech-language therapy to improve communication skills, (iii) occupational therapy to adjust to daily tasks and (iiii) emotional and behavioral therapies. However, cognitive impairments still exert a big influence on the life of an individual with DS. The focus of current therapies can be divided in two main streams, focusing on respectively drugs used for the treatment of AD and miscellaneous drugs.

Considering the neurochemical and neuropathological similarities between DS and AD, it is not surprising that drugs used for the treatment of AD are examined as potential therapeutics for DS as well. One common characteristic is the loss of cholinergic inputs to limbic and cortical regions, which in AD is associated with cognitive impairments. Many studies have investigated the cholinergic neurotransmission in DS, but no clear improvement of cognitive performance and functioning was found using for example acetyl-L-carnitine (De Falco et al., 1994,

Pueschel, 2006), nicotine (Seidl et al., 2000, Bernert et al., 2001) or acetylcholinesterase inhibitors (Kishnani et al., 2010). The same applies to the use of N-methyl-D-aspartate (NMDA) antagonists like memantine. Even though the first indications looked very promising, memantine does not seem to be an effective treatment in DS (Hanney et al., 2012).

As none of the investigated potential therapies resulted in a clear improvement of cognitive function in individuals with DS, it has been considered a hopeless disease from a therapeutic point of view since a long time. Nevertheless, recent research provided evidence that treatments focusing on normalizing the expression levels or the function of specific candidate molecules may hold a therapeutic window to some DS phenotypes (de la Torre et al., 2012). A potential therapeutic technology is RNA interference (RNAi), a process in which RNA molecules inhibit the expression or translation specific target genes. In the Ts65Dn mouse model, RNAi has been demonstrated successfully in knocking down the chloride importer Na-K-Cl cotransporter (NKCC1) and restoring behavioral performance (Contestabile et al., 2016). These findings in the Ts65Dn mouse model and multiple other studies (Makimura et al., 2002, Shishkina et al., 2004) support the idea of RNAi as a powerful therapeutic method to target specific genes that are associated with CNS pathology, such as DS (Dierssen et al., 2006)

CHAPTER 3

Ageing and cognitive decline:

a possible role for the hypothalamic-pituitary-gonadal axis

3.1 Ageing

Ageing is a biological process defined as "*a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration*" in the *Evolutionary Biology of Aging* (Rose, 1991). Others define it as a progressive decline in the physiological function, resulting in decreased reproduction and survival rates with increasing age and finally leading to death (Flatt et al., 2009). Many definitions of ageing exist, and all conclude that, in humans, it is an inevitable change in physiological function that happens over time and ultimately leads to death (Park et al., 2013).

3.1.1 Ageing-associated physiological changes

The progress of ageing has been generally thought to start from birth, together with growth, and to include social, psychological and many physiological changes, such as tissue atrophy, a reduction in metabolic rate and abnormalities in Ca_2 metabolism. These changes eventually lead to impairments of vital systems, including neurological and neuroendocrine function (further discussed below), and a higher vulnerability to the development of pathological conditions. Protecting against the ageing process can be considered a method of protection against ageing-related diseases, as it acts as a major risk factor for certain pathological conditions.

3.1.1.1 Changes of the central nervous system

Along with ageing, the cell count of neurons and supporting glial cells in the CNS gradually declines. An approximate loss of 0.1% neurons per year is observed between the age of 20 and 60, after which the process only accelerates (Esiri, 2007). Reaching the age of 90, the brain will have lost around 11% of its total weight compared to a 50-year old individual (Wyss-Coray, 2016). The cellular loss is accompanied by increased concentrations of potentially harmful components in the remaining tissues, including free radicals and pigmentation depositions (Ottis et al., 2012).

Neuronal loss is the most abundant in the cerebral cortex, presenting deeper sulci with advancing age. It was originally believed that specific brain regions as the frontal lobes are more likely to be affected by age-dependent neuronal loss (Robbins et al., 1998, Rabbitt, 2005). Nevertheless, other cortical structures such as the parietal lobes show similar losses as

the frontal lobes (Fjell et al., 2014). Frontal and parietal brain lobes are known for their implication in memory functions, and structural changes in these regions contribute to memory impairment. Another critical brain region for memory function and the ability to learn new skills that loses neural tissue during ageing is the hippocampus. Indeed, learning, memory and executive functions that rely on the different brain regions affected by a loss of neural tissue during ageing, show an important age-related decline (as reviewed by (Burke et al., 2006)). Not only memory-related brain regions are affected by age-related changes, also other brain regions including the somatic motor cortex show atrophic signs from middle age onwards (Manini et al., 2013).

The ageing process of the brain does not only include a loss of neural tissue. Many other changes can be observed with advancing age, such as an increase in the size of the ventricles (Raz et al., 2006) and a decline in the cerebral blood flow (Chen et al., 2011). The integrity of the BBB has been demonstrated to diminish with age, and thereby allows the access of pro-inflammatory mediators and harmful substances into the brain. The weakening of the BBB is first observed in the hippocampus and may play a role in hippocampal shrinkage, and associated cognitive decline (Montagne et al., 2015). Moreover, a decline in the production of neurotransmitters such as dopamine, serotonin, noradrenaline and glutamate could contribute to the age-related cognitive decline as well (Chowdhury et al., 2013).

3.1.1.2 Changes in the neuroendocrine system

The endocrine systems also undergo an ageing process. Starting from middle age, multiple hypophyseal-peripheral axes decrease their activity and function. Many of these endocrine disturbances find their origin in neuroendocrine changes, and may have many peripheral consequences.

One of the neuroendocrine systems that is most affected by ageing is the HPG-axis. Around the age of 50 years, the female reproductive period halts abruptly due to the arrest of estrogen and progesterone production. The production of these ovarian steroids is determined by the gonadotropin secretion by the pituitary gland, which in turn is regulated by the GnRH system. Accordingly, an age-related decline in the frequency of GnRH pulses and LH, FSH and FAS levels are observed after the onset of menopause (Hall et al., 2000). Similar to observations in women, levels of the major steroid sex hormone in men, T, decrease with

age and are associated with lower cognitive performance (Moffat et al., 2002). The primary mechanisms mediating this age-related decline in T are still unknown, but is thought to find its origin within a multisite impairment; a reduction in hypothalamic GnRH release and abating testicular function and therefore androgenic negative feedback (Keenan et al., 2004, Keenan et al., 2006). Although available data point towards an important role of hypothalamic GnRH release, the primary mechanisms remain unknown.

Changes in the hypothalamic-pituitary-somatotropic axis (HPS-axis) and more specifically the age-related decline of GH secretion (Corpas et al., 1993, Veldhuis, 2008) are considered as a symptom of neuroendocrine ageing, or as a protection mechanism from cancer and other diseases. A dysfunction of the HPS-axis caused by the absence of GH signals slows down the natural ageing process and extends life span in mice (Koopman et al., 2016). This impact on longevity is not observed in corresponding endocrine syndromes affecting the HPS-axis in humans, but these patients appear to be less sensitive to age-related diseases (Laron, 2008, Guevara-Aguirre et al., 2011, Aguiar-Oliveira et al., 2017).

Ageing is also associated by changes in the regulation and activity of the hypothalamic-pituitary-adrenal axis (HPA-axis), the main axis regulating endocrine stress in humans. The HPA-system induces a hormonal cascade via CRH and ACTH, resulting in the secretion of glucocorticoids (GCs), including cortisol. Through a negative feedback system, GCs downregulate the secretion of CRH and ACTH. Limited but suggestive evidence points towards higher age-related levels of cortisol and ACTH, a decreased GC negative feedback at the level of the hypothalamic PVN, the hippocampus and prefrontal cortex (as reviewed in (Gupta et al., 2014)).

3.1.2 Mechanisms of ageing

The exact mechanisms underlying the biological ageing profile are, despite numerous studies, not unraveled yet. Although there are many theories about the causes of ageing, none of them can explain the whole process. This suggests that a combination of different mechanisms may contribute to the process of ageing. Therefore, several theories that have gained attention over the years will be shortly introduced in this manuscript.

3.1.2.1 DNA damage and repair

Since a very long time, the accumulation of damaged DNA has been proposed to be a major causal factor of the ageing process (as reviewed in (Vijg et al., 2013)). DNA is vulnerable to damage as a result of extrinsic stress factors such as irradiation and ultraviolet (UV) light, intrinsic stress factors such as reactive oxygen species (ROS) and replication errors. The majority of DNA damage is detected and repaired, however, some lesions remain unrepaired and accumulate. The accumulation of such lesions is harmful for the individual, leading to changes in gene expression, as well as to cellular senescence, apoptosis or even cancer. The ability to repair damaged DNA has been shown to be directly proportional to a species' lifespan, and is impaired due to the ageing process (Hart et al., 1974, Bohr et al., 1995). Moreover, human studies have demonstrated that genetic defects in the repair enzymes can lead to syndromes of premature ageing (Yu et al., 1996, Sun et al., 1998).

3.1.2.2 Free radicals

The free radical theory of ageing was originally described in 1956 by Denham Harman (Harman, 1956). It proposes ageing to be a cumulative result of oxidative damage, introduced by ROS that may be incidentally produced in normal aerobic metabolic processes. For many years, this theory has been the most popular theory within the field of ageing. Numerous studies demonstrated the increase in ROS and oxidative damage with advancing age (reviewed in (Stadtman, 1992)). Thus, an increased production of ROS has been associated with a shortened life span, and reduced oxidative stress in various model organisms, such as fruit flies (Fleming et al., 1992) and mice (Dai et al., 2014), extended their life span (Kirkwood et al., 2012).

However, an increasing number of studies contradicts the free radical theory (Koc et al., 2004, Blagosklonny, 2008, Mockett et al., 2010, Van Raamsdonk et al., 2012). Antioxidant proteins were believed to extend lifespan, but overexpressing antioxidants has been found to be ineffective in some experimental systems (Mockett et al., 2010). Increased levels of antioxidants may lead decrease an organisms lifespan, whilst a decreased function of antioxidants may prolong it (Van Raamsdonk et al., 2009). The crucial role of ROS in the ageing process is questioned as ageing even occurs under anaerobic conditions, in very low concentrations of ROS. For example, anaerobically grown yeast cells present a shorter lifespan compared to aerobically grown cells, which is not regulated by antioxidants and seems to be

even shortening under conditions of caloric restriction (Koc et al., 2004). Nowadays, it is believed that ROS contributes in many signalling functions, and that it is important to consider positional effects of ROS generation and their primary targets (Kirkwood et al., 2012).

3.1.2.3 Mitochondrial damage

From the moment Harman included mitochondria within his free radical theory (Harman, 1972), many studies arose providing evidence for the association between increased ROS levels, mitochondrial dysfunction and age(-related diseases) (Barja, 2013, Vina et al., 2013, Lane et al., 2015). Mitochondrial DNA is very susceptible to damage from external factors, as it is not optimally protected and no DNA repair enzymes are present in the mitochondria. Damaged mitochondrial DNA on its turn leads to a vicious circle of a lower energy production and increase in the production of free radicals, inducing more oxidative stress. Indeed, deletions of mitochondrial DNA polymerase lead to a shortened life span in mice (Trifunovic et al., 2004, Balaban et al., 2005).

3.1.2.4 Telomere shortening

Telomeres are repetitive nucleotide sequences located at the end of all linear chromosomes. Due to end-replication problems during DNA replication, telomeres shorten with each cell division. Telomere shortening has been suggested the reason why human diploid fibroblasts only divide a certain number of times before undergoing replicative senescence, also known as the Hayflick limit (Hayflick, 1991). This limitation can be overcome by making these cells express telomerase, an enzyme that adds telomere repeat sequences to the 3' end of telomeres (Bodnar et al., 1998, Vaziri et al., 1998). Until now, however, no correlation between telomere length and life span of an individual or species has been demonstrated yet (Cristofalo et al., 1998). For example, no thorough ageing defects can be found in telomerase-deficient mice (Rudolph et al., 1999). In 2002, von Zglinicki suggested that oxidative stress might be an important regulator of telomere loss, as it accelerates the telomere shortening rate (von Zglinicki, 2002). A recent study indeed showed that the telomere shortening rate is strongly correlated with the life span of a species (Whittemore et al., 2019), suggesting that not the length of telomeres but the shortening rate determines ageing.

3.1.2.5 The neuroendocrine theory

According to the neuroendocrine theory, the ageing process results of changes in the neural and endocrine functions crucial for the coordination and programming of the communication and responsiveness of the organism and its environments, as well as maintaining an optimal status for reproduction and survival. Changes in these functions have detrimental effects and do not only affect the neurons (and therefore hormones) regulating evolutionarily significant functions as development, growth and reproduction, but also neurons critical for survival by adapting to stress factors and situations. Life span, that is regulated by "biological clocks", would go through a continuum of stages that are driven by nervous and endocrine signals. Even the smallest changes of the biological clock would disrupt its function and corresponding adjustments (Finch, 1976, Timiras, 1978, Weinert et al., 2003).

The ageing process is characterized by many changes in the (neuro)endocrine system and hormonal levels, such as a decrease in the levels of GH, T, estrogen and dehydroepiandrosterone. Some hormone replacement strategies have been tested and found to modify some attributes of the ageing process, however, negative side effects often occur, as was the case for a replacement therapy of GH (Bouillanne et al., 1996). One of the hormone replacement strategies that was welcomed very positively is replacing estrogen. ERT has a long clinical history and has been demonstrated to increase the quality of life in postmenopausal women (Wiklund et al., 1993). Although it has been called the first true anti-ageing therapy because of its preventive effect on osteoporosis (Richman et al., 2006) and reducing the risk of dementia (LeBlanc et al., 2001), controversy is existing about the efficacy of ERT for dementia (Espeland et al., 2004).

It should be noted that throughout the sequential stages of life, the interaction and integration of the neuroendocrine and immune systems play a crucial role. The interaction between these two systems happens in different ways (Tosato et al., 2007): (i) neuropeptides and cytokines of the immune system (ii) hormones from the pituitary gland, and (iii) a reciprocal action of cytokines on the neuroendocrine system. As an example of interaction of the immune system on the neuroendocrine system, Zhang and colleagues recently demonstrated that the *GnRH* mRNA levels depend on the IkB kinase (IKKβ)/nuclear factor kB (NF-kB) pathway, involved in immune and inflammatory responses (Zhang et al., 2013).

3.1.2.5.1 The hypothalamus and GnRH: a new ageing mechanism?

In addition to its involvement in a wide variety of life-supporting functions, a crucial role for the hypothalamus in ageing development and lifespan control has recently been reported (Zhang et al., 2013). Zhang and colleagues showed that the NF-kB-directed innate immune pathway is chronically activated in the murine hypothalamus during ageing development. Overactivation of the NF-kB pathway in the mediobasal hypothalamus (MBH) leads to muscle weakness, poor cognitive performance and a shortened lifespan, while inhibiting this pathway reverses the phenotype and improves the aforementioned characteristics compared to control mice. This effect could be mediated via hypothalamic glia, as the microglia-specific ablation of IKKβ, the upstream kinase of NF-kB, results in improvements in age-related muscular weakness and cognitive impairment. Furthermore, they revealed that ageing is associated with reduced levels of hypothalamic *GnRH* mRNA and that the inhibition of the IKKβ/NF-kB pathway increases *GnRH* levels in ageing mice. To investigate whether GnRH could affect the ageing process, old constitutively-active IKKβ mice were subjected to daily GnRH injections. Peripheral administered GnRH abrogated the pro-ageing phenotype and induced an amelioration of the ageing-related cognitive decline in these mice (Zhang et al., 2013).

Ageing-associated disorders are often correlated with a decline in adult neurogenesis (Greenberg et al., 2006, Molofsky et al., 2006, Encinas et al., 2011, Villeda et al., 2011). Moreover, neural stem cells (NSCs) are present in the hypothalamus (Lee et al., 2012, Li et al., 2012, McNay et al., 2012), and more specifically in the MBH, a crucial region for the organism's neuroendocrine regulation. Whether NSCs are implicated in the ageing process, had not been reported until 2017. Zhang and colleagues demonstrated an age-dependent loss of hypothalamic NSCs, especially in the 3v wall, which was reflected by an age-related physiological decline. Restoring the number of cells, neuronal grafting with newborn mice-derived hypothalamic NSC in which the inflammatory response is inhibited resulted in an increased life span (Zhang et al., 2017). However, this study suggests that the effect results from the endocrine character of the NSCs, secreting miRNAs, as the modulation of ageing was relatively short to originate from neurogenesis. A fraction of the implanted hypothalamic NSCs differentiated into GnRH-expressing cells, however, the neuropeptide secretion by the hypothalamic NSCs was not addressed in this study (Zhang et al., 2017). This is very interesting,

as their previous study showed that GnRH is implicated in the hypothalamic control of ageing (Zhang et al., 2013).

3.2 Ageing-related cognitive decline

Despite the encounter of certain limitations in the studies concerning cognition and ageing (as reviewed by (Salthouse, 2010, Harada et al., 2013)), there is evidence for cognitive changes during the healthy ageing process. The term cognition can be divided into specific cognitive subdomains such as language, attention, executive function, visuospatial abilities and memory, and all of them decline with age (Lezak et al., 2012). It should be noted that the speed of processing information is slower in older compared to younger adults (Salthouse, 2010). This could possibly interfere with the cognitive performance in each of the domains, as they all require the perception of the stimulus and processing of the information before the individual is ready to respond accordingly.

Complex attentional tasks that require selective or divided attention, i.e. that require a focus on something while ignoring irrelevant information and a focus on multiple tasks simultaneously, decline progressively with age (Carlson et al., 1995, Salthouse et al., 1995). Other cognitive subdomains such as executive function, visuoperceptual judgment and visuospatial orientation, and memory are all characterized by an age-related decline. (Howieson et al., 1993, Lezak et al., 2012, Salthouse, 2012, Bendayan et al., 2017). Executive function implicates planning and coordination actions for which a standard response is not the most appropriate and to which an individual should adapt its behavior. This is critically controlled by the prefrontal cortex, and demonstrated to decline during the ageing process (Lezak et al., 2012). Although some aspects of memory are maintained stable, many of them are affected and declining with advancing age, including new learning abilities, and recalling detailed episodic memories (as reviewed in (Murman, 2015)).

3.2.1 Role of reproductive hormones and regulating factors in cognitive decline

3.2.1.1 GnRH and its receptor

It has been known for a long time that GnRH does not only affect the pituitary gland, but also affects other parts of the CNS, such as hypothalamic (Herbison et al., 1984), hippocampal (Yang et al., 1999), cerebellar (Renaud et al., 1975) and cortical neurons (Renaud et al., 1975, Renaud et al., 1976). Moreover, it has been demonstrated that GnRH cell bodies as well as fibers are widely distributed in non-reproductive brain areas such as the OBs, hippocampus, piriform cortex and amygdala (Casoni et al., 2016). Similarly, the GnRH-R is expressed in the the OBs, piriform cortex, hippocampus and amygdala (Jennes et al., 1988, Albertson et al., 2008). These findings suggest that GnRH may be involved in yet unexplored non-reproductive functions, such as its recently proposed role in the regulation of systemic ageing (Zhang et al., 2013). The presence of GnRH in the cerebrospinal fluid (CSF) at concentrations proportional to concentrations detected in the portal blood vessels (Van Vugt et al., 1985, Skinner et al., 2002, Caraty et al., 2008) could represent another way in which GnRH can potentially target and signal to multiple brain regions.

3.2.1.2 Luteinizing hormone and its receptor

The abundant presence of the LH-R in the CNS, more specifically in regions associated with learning and memory, the choroid plexus and ependymal cells of the ventricles, is well described in literature (Emanuele et al., 1983, Lei et al., 1993, Hamalainen et al., 1999, Apaja et al., 2004). This also counts for the cerebral expression of LH itself, and the ability to exert an influence on many brain processes including synaptic function (Gallo et al., 1972, AA et al., 1997, AA et al., 1997) and behavior (Lukacs et al., 1995, Palm et al., 2014).

The LHR is a GPCR that is involved in the production of sex steroids by the gonads, and its activation results in the activation of downstream signalling pathways that are known to be associated with hallmarks of synaptic plasticity and memory formation (English et al., 1997, Atkins et al., 1998), and lead to the induction of long term potentiation (LTP) (Roche et al., 1996, Barria et al., 1997, Mammen et al., 1997) and synaptic remodelling. These findings suggest that LHR activation may exert a positive action on memory formation and cognition,

which is supported by a study showing an impairment in cognitive functioning in young male HH patients compared to healthy age-matched controls (Lasaite et al., 2014).

However, detrimental effects of LH on the working memory have been demonstrated in rodents, more specifically a mouse model overexpressing LH (Casadesus et al., 2007) and guinea pigs receiving an exogenous administration of human LH (Wahjoepramono et al., 2011). These findings are supported by studies that demonstrate weakening of the working memory after the chronical administration of hCG, an LH analog, in a mouse model of AD (Berry et al., 2008, Barron et al., 2010). Moreover, estrogen associated benefits in spatial memory can be reversed by elevating the hCG levels at the peripheral level (Berry et al., 2008, Barron et al., 2010). These findings suggesting a role for LH in cognitive functioning are supported by multiple studies pharmacologically downregulating peripheral LH levels after ovariectomy (OVX) using GnRH super-agonists or competitive antagonists of the GnRHR (Casadesus et al., 2007, Telegdy et al., 2009, Bryan et al., 2010, Telegdy et al., 2010, Ziegler et al., 2010, Palm et al., 2014, Blair et al., 2016). A crucial finding was that the cognitive benefits resulting from pharmacologically reducing peripheral LH levels were not dependent on the timing of treatment onset (Blair et al., 2016).

Accumulating research provides evidence that the levels of LH in the brain are inversely correlated to the levels observed at the periphery. A study in cycling adult female rats showed a 10-fold increase in the pituitary gland and periphery, while hypothalamic levels were decreased (Emanuele et al., 1981). Additionally, the administration of both LH and hCG into the ME significantly lowered the LH levels at the level of the pituitary gland and periphery in both intact and castrated male and female rats (David et al., 1966, Hirono et al., 1972). These findings substantiate the idea of an inverse relationship between the central and peripheral levels of LH, and suggest that hCG and LH possibly self-regulate their levels through a feedback system into the brain (David et al., 1966, Molitch et al., 1976, Patritti-Laborde et al., 1979). The sensitivity of this short-loop has been thought to change with the time after castration (Molitch et al., 1976) and raises the question whether self-regulation of the hypothalamic LH production is more relevant than gonadal loss. The loss of the short-loop with time after gonadectomy possibly underlies the sensitivity to estrogens during a critical period after menopause onset, and could be due to the rapid internalization of the LHR in the presence of its ligands (Segaloff et al., 1990, Peegel et al., 1994, Min et al., 1998, Kishi et al., 2001). As

many studies used supraphysiologic doses of exogenous hCG, the internalization of the LHR might explain the deleterious effects of LHR activation to cognition that were observed in these studies (Lukacs et al., 1995, Ziegler et al., 2010, Burnham et al., 2017). The inverse relationship between the observed LH levels in the CNS and periphery provide a plausible explanation for the paradox concerning the LHR; notably that the LHR activates signal cascades associated with learning and memory, and the beneficial effects of LHR downregulation on these aspects.

3.2.1.3 Estrogen depletion and replacement therapy

As mentioned before, the increased risk of AD in menopausal women has been attributed to the loss of estrogen (Andersen et al., 1999, Launer et al., 1999), making it a considerable and promising therapeutic avenue to tackle the increased risk of AD. Initially, the replacement therapy showed promising results in postmenopausal women with and without AD, reducing multiple types of memory decline (Hogervorst et al., 2009, Rocca et al., 2014). However, no overall cognitive improvements were found in a randomized, double-blind placebo-controlled trial. It was demonstrated that ERT was beneficial when started at the moment of menopause or shortly after, while it slightly increased the risk of dementia when started 15 years after the onset of menopause (Shumaker et al., 2003, Espeland et al., 2004). These results led researchers to believe the existence of a "critical period" after the onset of menopause during which estrogen can confer benefits, and after which it becomes ineffective and possibly exert harmful effects (Resnick et al., 2002, Henderson, 2004, Shao et al., 2012, Singh et al., 2013). This idea is supported by studies which demonstrate the beneficial effect of estrogen on cognitive improvement in rodents (Gibbs, 2000) and primates (Lacreuse et al., 2002). Yielded knowledge about estrogen and ERT make researchers believe that estrogen plays an important role for the function and structure of the CNS during the reproductive period, but afterwards possibly contributes to age-related menopausal dysfunction.

3.2.1.4 Kisspeptin and its receptor

Both Kp and the Kiss1R are widely distributed in the CNS (Fukusumi et al., 2006) including learning- and memory-associated brain regions such as the hippocampus (Arai, 2009). This suggests that Kp may play a role in the regulation of learning and memory processes. Little is known about the role of Kp and its receptor in recognition processes, and no firm conclusions can be drawn from the very limited amount of studies. Jiang and colleagues demonstrated

that KP-13, one of the Kp isoforms, possibly facilitates memory formation and retention through Kiss1Rs and GnRH-Rs (Jiang et al., 2015). However, Delmas and colleagues demonstrated that deleting Kiss1R, and therefore Kiss1R signalling, in mice does not hamper spatial learning and memory (Delmas et al., 2018).

3.2.1.5 Nitric oxide and nitric oxide synthase

Within the hippocampus, interneurons expressing nNOS represent one of the most widespread interneuron subpopulations (Fuentealba et al., 2008). Multiple studies examined the role of NO on synaptic plasticity, and demonstrated NO to be implicated in the induction of LTP and long-term depression (LTD) (Zhuo et al., 1995). Hippocampal LTP is a frequently used model to investigate the synaptic basis of learning processes and memory formation in vertebrates (Bliss et al., 1993), while hippocampal LTD-like synaptic plasticity has been associated with visual recognition memory (Griffiths et al., 2008). Moreover, a spatial memory task has been found to be accompanied by the increase of hippocampal NOS activity in rats (Bernabeu et al., 1995), and a correlation between learning processes and nNOS activity has been suggested by studies in mice (Okere et al., 2000) and sheep (Kendrick et al., 1997). These results are supported by the finding that multiple NO donors facilitated learning and memory tasks, while multiple inhibitors of NO synthesis impaired learning and memory formation (Paul et al., 2011).

AIMS AND OBJECTIVES

The role of GnRH neurons as the master regulators of a complex neural network, mainly in the hypothalamus, controlling the onset of puberty and regulation of fertility is well established. Nonetheless, it is becoming apparent that GnRH may be involved in a variety of non-reproductive functions.

Chapter 4. Progressive GnRH insufficiency underlies olfactory and cognitive dysfunction in Down syndrome. DS patients who show an intellectual disability and age-related cognitive decline also present acquired anosmia, i.e. the loss of odor detection and/or discrimination. Additionally, the ability to reach puberty and become fertile is also affected in these patients. An intriguing candidate, whose absence could mediate most of these phenotypes, is GnRH. This led us to hypothesize that hypothalamic GnRH plays a crucial role in odor perception and cognitive performance during postnatal development. Building on this hypothesis, we sought to examine the role of acquired GnRH deficiency in age-dependent olfactory and cognitive decline using a DS mouse model. Moreover, we aimed to study whether GnRH replacement therapies would hold therapeutic potential.

Chapter 5. Defects in NOS1 activity cause GnRH deficiency in human and mice: evidence for reversal after NO treatment in infantile mice. The NO signalling pathway plays a key role in the regulation of GnRH, that is crucial for sexual maturation and fertility. We hypothesized that a disruption of neuronal NO synthase (NOS1) activity underlies GnRH deficiency by affecting key hypothalamic neuronal circuits controlling fertility. Building on this hypothesis, we sought to examine whether *NOS1* mutations can be identified in patients with CHH or KS, two genetic disorders rooted in a GnRH deficiency. Furthermore, we aimed to investigate whether *Nos1* deficient mice exhibit a similar phenotype compared to human patients, including non-reproductive comorbidities such as olfactory and cognitive impairments, and whether restoration the NO activity could rescue the reproductive and non-reproductive phenotype.

RESULTS

CHAPTER 4

Progressive GnRH insufficiency underlies olfactory and cognitive dysfunction in Down syndrome

4.1 Introduction

DS, also known as trisomy 21, is the most common genetic form of intellectual disability, with a prevalence of 10 to 14 per 10,000 live births worldwide, and is associated with accelerated ageing and multiple age-dependent comorbidities that often manifest by the age of 40 (Dierssen, 2012, Antonarakis, 2017, Bayen et al., 2018). In particular, adults with DS are at a very high risk of developing AD, partly due to the overexpression of APP, encoded by the APP gene located on chromosome 21. Interestingly, progressive anosmia or the lack of olfactory capacity is a hallmark of both DS (Nijjar et al., 2002) and dementia (Doty, 2012). While postmortem studies have revealed that adults with DS exhibit AD histopathology by the age of 40 years (Editorial, 2013, Lott et al., 2019), clinical studies suggest that the overall rate of dementia in younger individuals (< 40 years) is low despite lifelong intellectual impairment. However, the clinical symptoms of dementia rapidly emerge from the age of 40 onwards (Ballard et al., 2016).

One factor that has consistently been associated with poor cognitive performance and the increased risk of AD in the wider population with age is the decrease in sex steroid levels (Yaffe et al., 2002, Moffat et al., 2004). The natural ageing process alters the HPG (HPG) axis, leading to a rapid decline in estrogen in women (Studd et al., 1978), and a gradual reduction in T in men (Deslypere et al., 1987). In women, the dramatic decline in gonadal steroids is due to the loss of the ovarian reserve at menopause, and the consequent lack of the negative feedback of estrogens on the hypothalamic neurons releasing GnRH, the neurohormone controlling reproduction. This triggers a marked and permanent increase in the activity of GnRH neurons and a pronounced increase in circulating levels of the gonadotropins released by the pituitary, including LH (Yen et al., 1971, Studd et al., 1978). With age, postmenopausal women also show a marked decrease in GnRH pulsatility (Hall et al., 2000). In older men, the decrease in circulating T levels is associated with an alteration of hypothalamic-pituitary function, which may result in part from a gradual decrease in GnRH secretion with age (Deslypere et al., 1987). Consistent with this, recent studies in mice have also suggested a role for the GnRH system in systemic ageing (Zhang et al., 2013).

Here we analyzed the role of acquired GnRH deficiency in age-dependent cognitive decline using a murine DS model (Schapiro et al., 1987). We demonstrate that both the acquisition of

cognitive defects as well as a decline in olfactory perception are accompanied by a gradual loss of GnRH expression in the brain during postnatal development, related to upstream miRNA-transcription factor network alterations, and are associated with altered LH secretion in adulthood. Overexpressing a key miRNA or restoring physiological GnRH levels attenuate these pathological changes. Our data raise the possibility that pulsatile GnRH treatment, typically administered to patients with GnRH deficiency to manage their infertility (Boehm et al., 2015, Gordon et al., 2017), may offer a hitherto unappreciated therapy to enhance intellectual abilities and therefore well-being in DS patients.

Progressive GnRH insufficiency underlies olfactory and cognitive dysfunction in Down syndrome

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4.2 Results

4.2.1 Delayed sexual maturation, hypogonadism and infertility in Ts65Dn mice

Abnormalities in sexual development and infertility have been described in DS patients (Hsiang et al., 1987) as well as Ts65Dn mice; an established murine DS model carrying a partial trisomy of chromosome 16 (the orthologous region of hsa 21) (Reeves et al., 1995). Whereas Ts65Dn males are infertile, Ts65Dn females are subfertile (Moore et al., 2010). Characterizing reproductive maturation in Ts65Dn mice (**Figure 1A**), we found that males were smaller and weighed less when compared to wild-type (wt) littermates during development (**Figure 1B**). We also observed delayed balanopreputial separation (**Figure 1C**), a smaller penis, undescended testes (**Figure 1D**), irregular expression of major urinary proteins (markers of murine sexual maturation) (**Figure 1E**) and severe hypogonadism (**Figure 1F,G**), indicating profound deficits in the sexual maturation of Ts65Dn males that are comparable with the deficits in sexual maturation seen in DS patients (Hsiang et al., 1987).

Ts65Dn females (**Supplemental Figure 1A**) also showed reduced body weight gain during postnatal development (**Supplemental Figure 1B**), but exhibited normal vaginal opening (**Supplemental Figure 1C**) (an external marker of sexual maturation in female mice) and first estrus (**Supplemental Figure 1D**), which is strictly correlated with the acquisition of reproductive capacity, i.e., puberty (Prevot, 2015). While Ts65Dn females featured normal reproductive cycles at 3 months, these mice were subfertile; producing fewer litters over a 120-day period and fewer pups per litter (**Supplemental Figure 1E,G,H**). By 12 months, these mice were anovulatory (**Supplemental Figure 1F**). Both male (**Figure 1H-J**) and female (**Supplemental Figure 1I-K**) Ts65Dn mice showed normal LH pulse frequency but decreased LH pulse amplitudes.

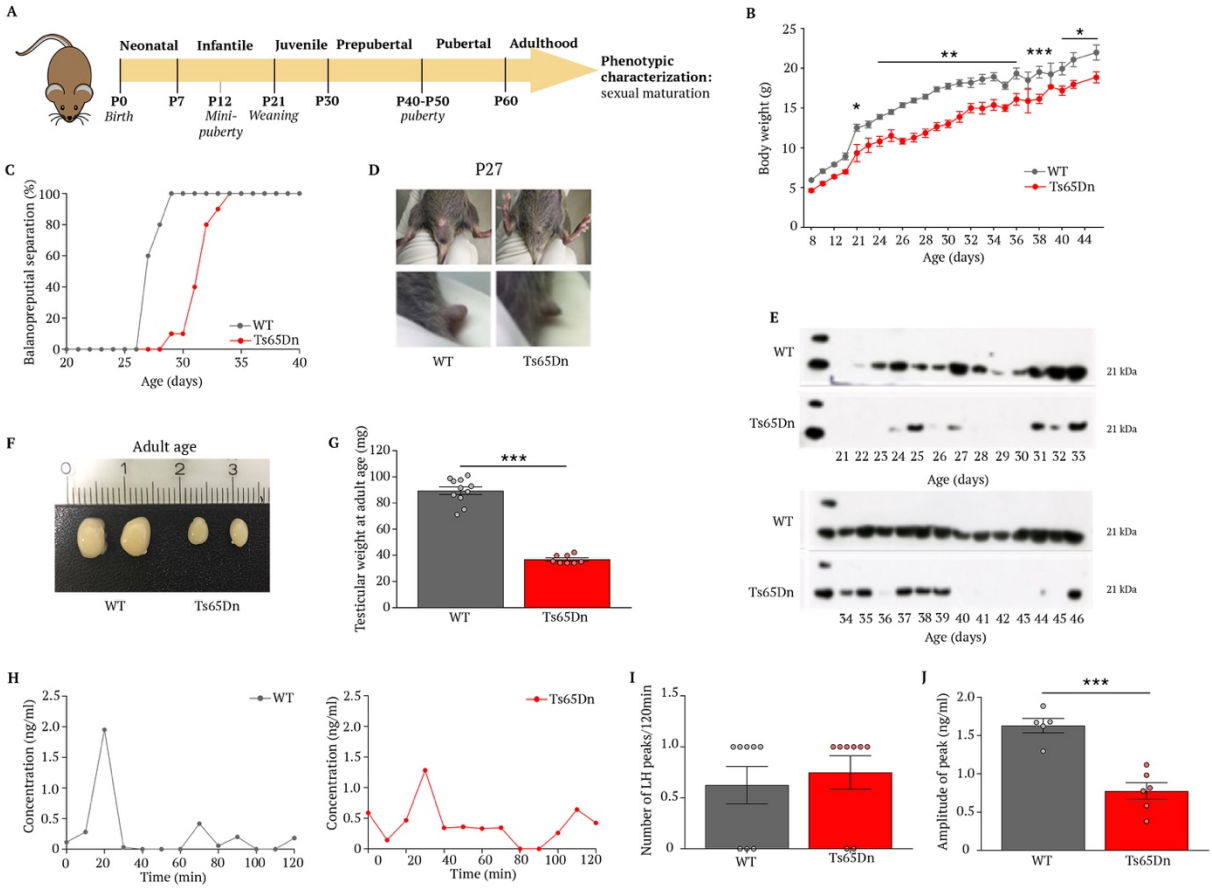


Figure 1. Ts65Dn male mice show delayed sexual maturation, hypogonadism and infertility. (**A**) Schematic representation illustrating the phenotypic characterization of reproductive maturation performed in Ts65Dn mice from birth to the adulthood. (**B**) Ts65Dn males presented significantly lower body weight gain during postnatal development. (**C-E**) A marked delay in sexual maturation was observed in male Ts65Dn mice compared with wt littermates. Ts65Dn males exhibited delayed balanopreputial separation day (****$p < 0.0001$) (**C**), a smaller penis and undescended testes (**D**); all external signs used to follow sexual maturation. (**E**) Ts65Dn mice showed an irregular expression profile of major urinary proteins. (**F,G**) Adult Ts65Dn males revealed severe hypogonadism, exhibiting smaller testes and lower testicular weight. (**H**) Representative graphs for LH pulsatility assessment by serial blood sampling. Ts65Dn mice had a normal LH pulse frequency (**I**), but a significantly decreased LH pulse amplitude (**J**) compared to wt littermates. Statistical differences were evaluated using two-way repeated measures ANOVA (**B**); Gehan-Breslow-Wilcoxon test (**C**); Mann-Whitney (**G,I**); Unpaired t-test (**J**). *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.2 Age-dependent loss of GnRH expression in Ts65Dn mice

We next evaluated the distribution of GnRH neurons in the brain of Ts65Dn mice by iDISCO neonatal (P0) and adult (P90) brains immunolabeled for GnRH (Casoni et al., 2016, Belle et al., 2017) and conventional immunohistofluorescence. We found a profound loss of GnRH-immunoreactive somata and fibers (**Figure 2A-C**) starting after puberty onset in Ts65Dn mice (**Figure 2B, Figure S2**) compared to wt controls. Our recent studies in human fetuses have identified GnRH somata and fibers in several extrahypothalamic sites (Casoni et al., 2016). Consistent with this, 3D analyses of the GnRH fiber network in adult wt mice also revealed numerous extrahypothalamic GnRH projections (**Figure 2D**), which were often seen in close apposition to the walls of the lateral ventricles. Stereotaxic injections of adeno-associated viral vectors (AAV) driving the expression of YFP (AAV9-eYFP) into the ME of adult wt *Gnrh::Cre* mice demonstrated that at least part of these extrahypothalamic GnRH projections actually came from neuroendocrine GnRH neurons (**Supplemental Figure 3A**). We observed YFP expression not only in GnRH neuron cell bodies and processes located in the POA (**Supplemental Figure 3B,C**), but also in processes located in extrahypothalamic sites such as the cortex (**Supplemental Figure 3D,E**), the hippocampus (**Supplemental Figure 3D,F**) and the paraventricular thalamus (**Supplemental Figure 3D,F**) after unilateral AAV9-eYFP injection into the dorsolateral ME. These results demonstrate that hypophysiotropic GnRH neurons project to brain areas controlling cognitive and social behaviors. Furthermore, the potential role of GnRH in the regulation of non-reproductive processes is also consistent with our iDISCO analyses identifying GFP-labeled GnRH Receptor (GnRHR) neurons in the cortex and the hippocampus in the murine brain (**Figure 2E-L**).

Similar anatomical observations conducted in trisomic mice revealed that although GnRH fibers were visible in the ME (**Figure 2C**), the expansive GnRH projections typically observed in wt controls were absent in adult Ts65Dn mice (**Figure 2D**). Taken together, our data raise the possibility that the deficiency of extrahypothalamic GnRH fibers in Ts65Dn mice acquired during postnatal development may contribute to their cognitive phenotype.

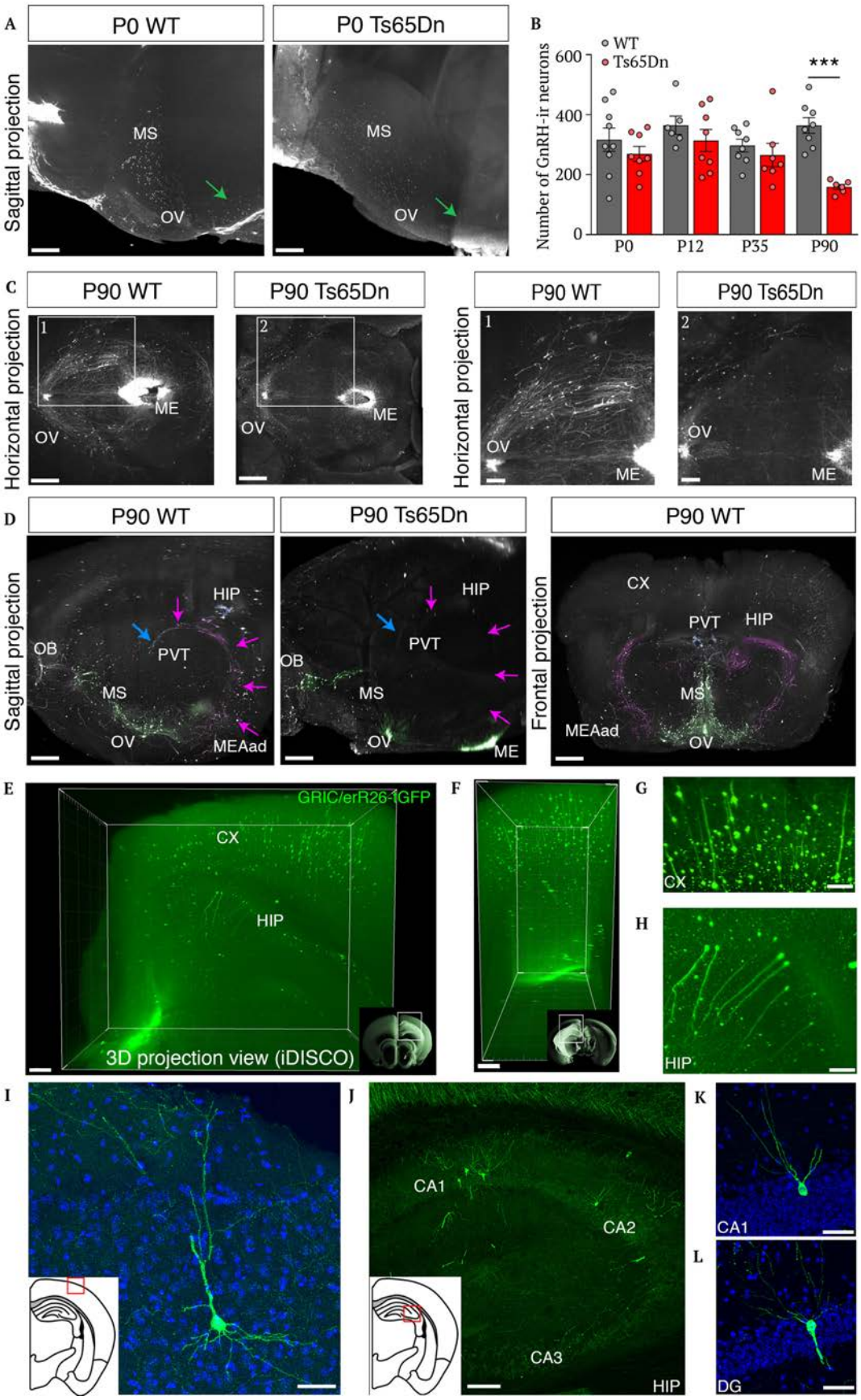

A
P0 WT
P0 Ts65Dn
Sagittal projection
MS
OV
B
WT
Ts65Dn
Number of GnRH-ir neurons
600
400
200
0
***
P0
P12
P35
P90
C
P90 WT
P90 Ts65Dn
Horizontal projection
1
2
ME
OV
D
Sagittal projection
HIP
PVT
OB
MS
OV
MEAad
ME
Frontal projection
CX
E
GRIC/erR26-1GFP
3D projection view (iDISCO)
F
G
H
I
J
CA1
CA2
CA3
HIP
K
L
DG

Figure 2. Ts65Dn male mice show an age-dependent loss of GnRH immunoreactivity. (**A-C**) GnRH-immunoreactivity in Ts65Dn mice decreases during postnatal development compared to wt littermates. Whole-mount of neonatal (P0) (**A**) and adult (P90) (**C**) brains immunolabelled for GnRH followed by iDISCO. (**B**) In parallel, conventional neuroanatomical analyses conducted at different stages of postnatal development revealed a loss of GnRH-immunoreactive cell bodies in the preoptic region of adult Ts65Dn mice. This phenotype is intriguingly reminiscent to the one seen in mice in which we had selectively knocked out Dicer in GnRH neurons (Messina et al., 2016). (**D**) Neuronal fiber tracing shows numerous GnRH-immunoreactive projections to extrahypothalamic regions in adult wt mice that are absent in Ts65Dn male littermates. The different colors are projections of GnRH neurons that have been pseudocolored based on their pathway. Arrowheads show pathway of GnRH-immunoreactive neurons. We identified three different pathways: green, GnRH population that projects to ME; magenta, neurons that project to the MEAad; blue, neuron projections to the PVT. (**E-H**) iDISCO analyses showing Cre-dependent expression of Tau-GFP in neurons in the cortex (CX) and the hippocampus (HIP) of mice expressing Cre under the control of the GnRH Receptor (GnRHR) promoter. (**I-L**) Confocal microscopy of GFP-labelled GnRHR–expressing cells in the cortex (**I**) and in the dentate gyrus (DG) and CA1-3 layers of the hippocampus (**J-L**). CX, cortex. CA, cornu ammonis. DB, diagonal band of Broca. DG, dentate gyrus. HIP, hippocampus. ME, median eminence. MEAad, anteriordorsal amygdala. MS, medial septal nucleus. OB, olfactory bulb. OV, organum vasculosum of the lamina terminalis. PVT, paraventricular thalamus. Scale bars: **A**, 300 µm; **C**, 600 µm (1 and 2 panels, 300 µm) ; **D**, horizontal 350 µm and frontal 500 µm; **E,F**, 200 µm; **J**, 80 µm; **G,H**, 50 µm; **K,L**, 10 µm; **I**, 30 µm. Statistical differences were tested using unpaired t-test. *** $p < 0.001$.

4.2.3 Age-dependent loss of olfactory and cognitive function in Ts65Dn mice

DS patients and Ts65Dn mice not only present with intellectual/cognitive disability (Epstein et al., 1991, Reeves et al., 1995), but also with an age-related impairment of olfaction (Nijjar et al., 2002, Bianchi et al., 2014). Intriguingly, the inability to perceive odors is also associated with GnRH deficiency in patients with KS (Boehm et al., 2015). In order to test whether the loss of GnRH expression seen in Ts65Dn mice is associated with olfactory and cognitive deficits, we next performed habituation/dishabituation tests to assess the ability to discriminate between different odors (Breton-Provencher et al., 2009), and a NOR test (a test also used in patients with DS (Nelson et al., 2005)) to assess recognition memory (**Figure 3A**). Wt control prepubertal animals showed reduced sniffing time when an odor was re-introduced (habituation), with the reinstatement of sniffing when a novel odor was presented (dishabituation). However, both male and female prepubertal Ts65Dn mice, once habituated, were unable to distinguish a novel odor from known ones (**Figure 3B**), revealing an olfactory

deficit that occurs between birth and the juvenile period in these animals. Ts65Dn mice thus phenocopy patients with DS who exhibit odor identification deficits starting as early as in preadolescence (Nijjar et al., 2002). In contrast, Ts65Dn mice at P35 were as capable as their wt littermates of recognizing new objects in their environment (**Figure 3D**). However, when we performed these tests in young adults, we found that both olfactory perception and recognition memory were impaired in Ts65Dn mice (**Figure 3C,E**). These results reveal age-dependent cognitive decline in both male and female Ts65Dn mice, temporally orchestrated to coincide with the loss of GnRH expression in the brain (**Figure 2B).** The olfactory and cognitive impairment seen in Ts65Dn mice were likely not due to gonadal deficits or altered communication between the brain and the gonads, as orchidectomized (ORX) animals behaved similarly (**Figure 3F,G**) to intact controls (**Figure 3C,E**).

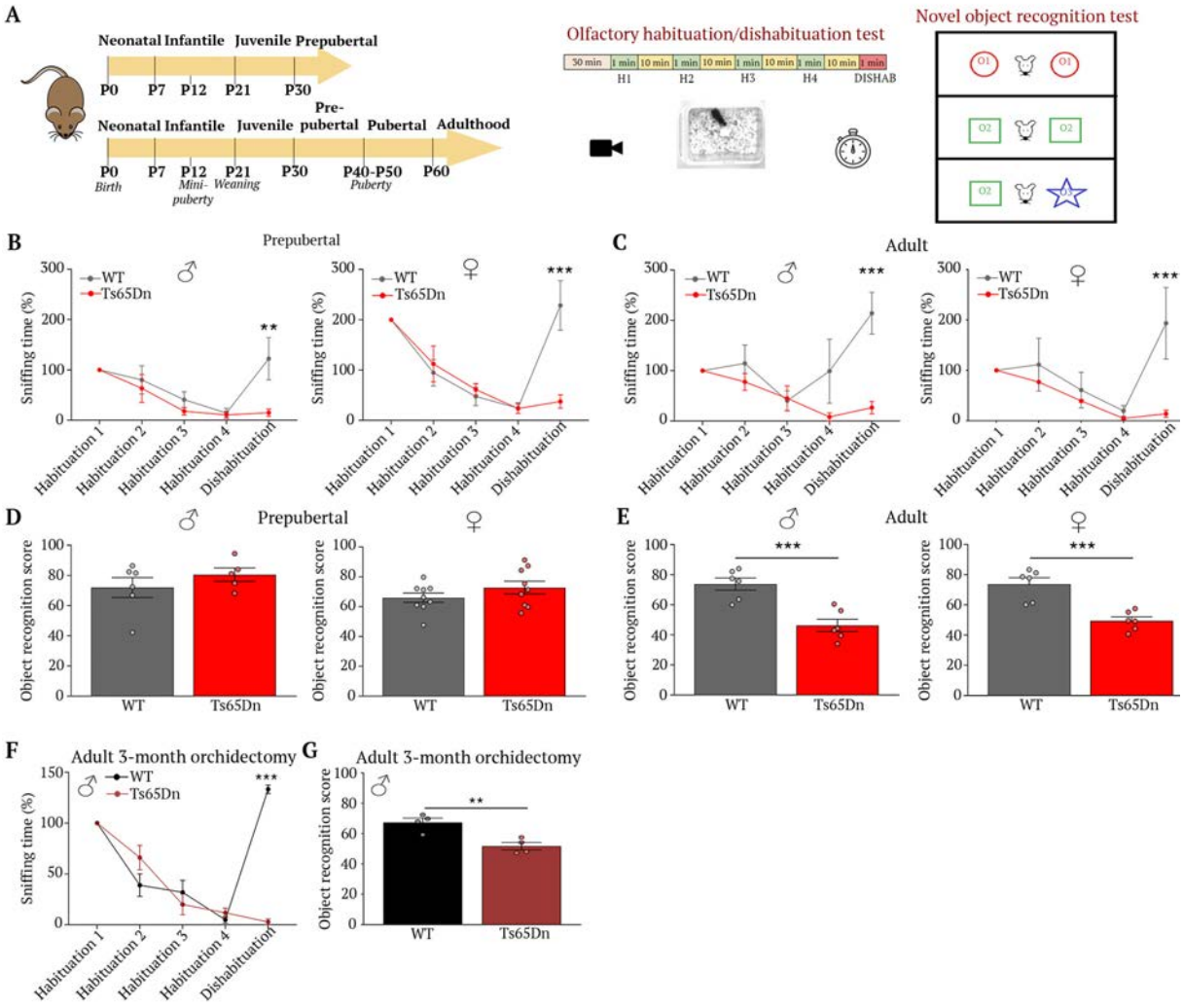


Figure 3. Ts65Dn mice show an age-dependent loss of the ability to recognize new odors and objects. (**A**) Schematic of experimental design to evaluate the ability of mice to discriminate olfactory and visual cues during postnatal development. Habituation/dishabituation test was used to assess the ability to differentiate between different odors and a novel object recognition test was used to evaluate recognition memory. (**B,D**) At P35, Ts65Dn mice were unable to differentiate between two distinct odors (**B**) while they were equally able to recognize the introduction of new objects in their environment (**D**) when compared to wt littermates. (**C,E**) In adulthood, Ts65Dn mice showed a loss of the capacity to differentiate both different odors (**C**) and objects (**E**) when compared to wt littermates. (**F,G**) Both wt and Ts65Dn littermates behaved similarly to intact animals (**C,E**) 3 months after orchidectomy. Statistical differences were tested using two-way repeated measures ANOVA (**B,C,F**) and unpaired t-test (**D**,**E,G**). * $p < 0.05$; ** $p < 0.01$; ** $p < 0.001$

Age-acquired cognitive decline has previously been linked to an increase in inflammatory processes in the hypothalamus (Zhang et al., 2013). Our qPCR analyses showed an increase in the expression of Nf-κB1 transcripts in the POA of middle-aged mice when compared to young adults, but no difference in Nf-κB1 mRNA expression when comparing Ts65Dn mice with wt littermates (**Supplemental Figure 4A**). We also did not find Nf-κB activation in the hippocampus of 3- and 8-month old wt and Ts65Dn mice (**Supplemental Figure 4B**). Exacerbated neuroinflammation is thus unlikely to explain the accelerated cognitive decline in Ts65Dn animals.

The *App* gene triplication present in both DS patients and mice (Reeves et al., 1995) has been linked to the early-onset AD phenotype in DS. To determine whether the acquired deficits in Ts65Dn mice occur in parallel with the development of AD pathology in DS, we next analyzed the expression of AD-related proteins (APP, its C-terminal fragment (CTF) and the Tau C-terminal (Tau-Cter)). We found increased APP expression in the hippocampus (**Supplemental Figure 5A,B**) of middle-aged, but not in young adult (3-month) Ts65Dn males. In contrast, we did not detect any change in the expression of CTF and Tau-Cter in the hippocampus of Ts65Dn males (**Supplemental Figure 5A,B**). In the cortex, the expression of AD-related proteins remained unaltered in Ts65Dn males (**Supplemental Figure 5C,D**). In 12-month old Ts65Dn females, we found a significant increase in APP and CTF expression in the hippocampus (**Supplemental Figure 5E,F**) and cortex (**Supplemental Figure 5G,H**), although there was no difference in Tau-Cter expression either in the hippocampus or in the cortex (**Supplemental Figure 5E-H**). In summary, we show that the expression of APP is unaffected in 3-month old Ts65Dn mice (**Supplemental Figure 5A-D**). Taken together, these data demonstrate that the

decline in olfactory perception and cognition in Ts65Dn mice occurs before any obvious change in APP expression, and may thus have other proximal causative factors.

4.2.4 Ts65Dn mice show an imbalance in the miRNA-gene network controlling Gnrh expression

Hsa 21 and mmu 16, which has been duplicated to generate the Ts65Dn mouse strain (Reeves et al., 1995), code for several miRNAs (miR-99a, let-7c, miR-125b-2 and miR-155) that are expressed in GnRH neurons and influence the expression of the miRNA-200 family (Messina et al., 2016). Members of this family regulate Zeb1, which is an important repressor of *Gnrh* promoter activity (Messina et al., 2016). To investigate the molecular mechanism underlying the postnatal loss of GnRH-immunoreactivity in Ts65Dn mice, we first analyzed global miRNA and gene expression in the POA, which contains the main population of GnRH neuronal cell bodies. We found a downregulation of most miRNA-200 family members accompanied by an upregulation of *Zeb1* mRNA in the adult Ts65Dn POA, accompanied by a marked decrease in *Gnrh* expression (**Figure 4A,B**). Real-time PCR analyses of cell-sorted GFP-labeled GnRH neurons from *Gnrh*::*gfp*;Ts65Dn mice confirmed that, as soon as during the infantile period, *Gnrh* mRNA expression was decreased, accompanied by an increase in *Zeb1* expression and by a downregulation of transcripts coding for the *Gnrh* promoter activators *Otx2* and *Kiss1r* (**Supplemental Figure 6A; Figure 4C,D**). Taken together, these data suggest that the altered miR-200 expression in Ts65Dn mice causes an imbalance in the miRNA-gene network controlling *Gnrh* promoter activity, in turn causing the gradual loss of GnRH expression in these animals (**Figure 4E**). Consistent with this mechanism, the selective overexpression of miR-200b in the POA of adult Ts65Dn males using stereotaxic injections of AAV (**Figure 4F**) rescued both the capacity to differentiate odors (**Figure 4G**) and recognize novel objects (**Figure 4H**), while Ts65Dn mice injected with control AAV continued to show olfactory and memory deficits.

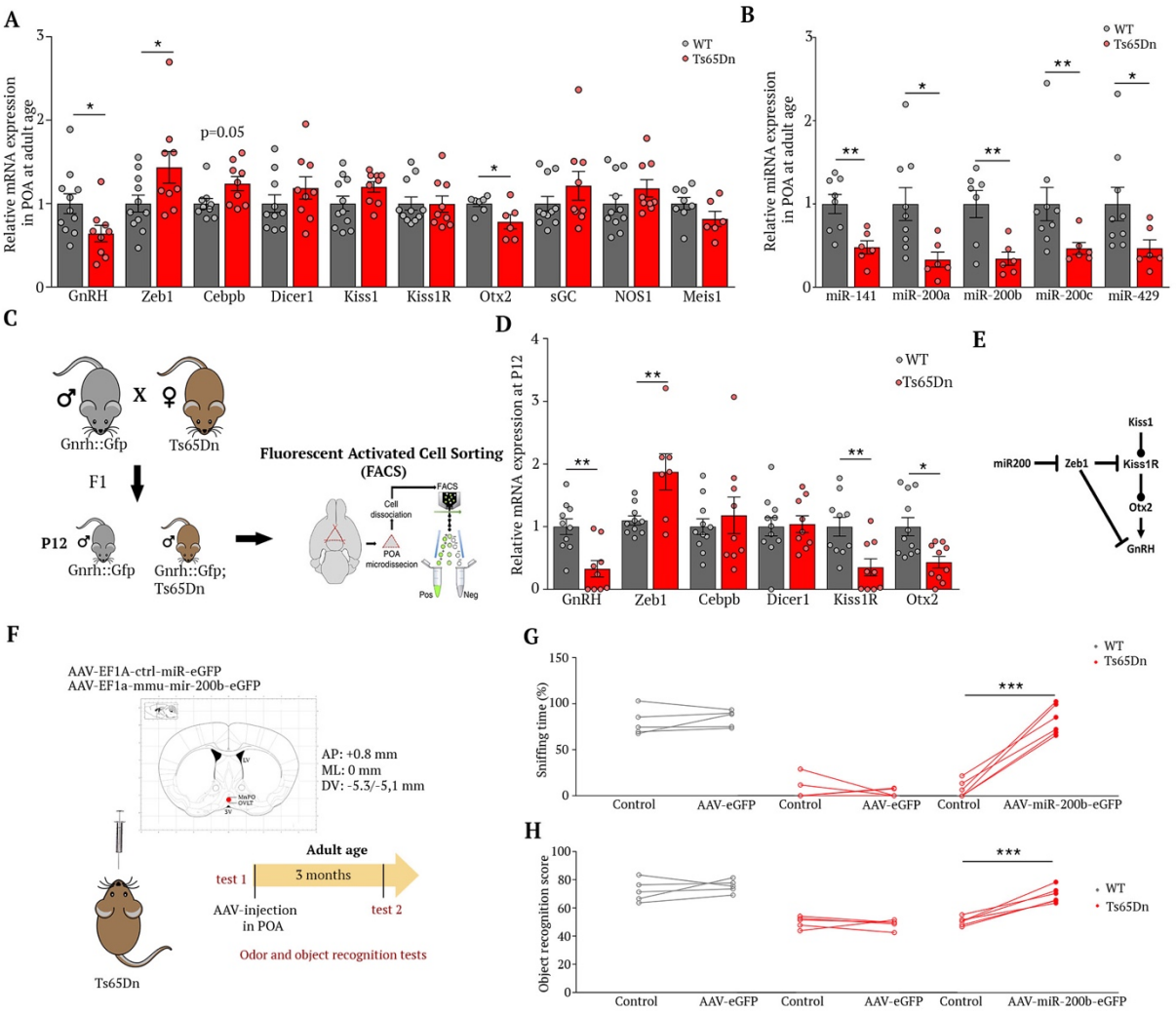


Figure 4. The age-dependent loss of cognitive performances is associated with a switch in the miRNA-gene network controlling age-dependent *Gnrh* expression in Ts65Dn mice. (**A,B**) RT-PCR analysis of the expression of different genes (**A**) and miRNAs (**B**) in the POA of adult wt and Ts65Dn littermates. (**C**) Schematic diagram illustrating the generation of *Gnrh::Gfp;Ts65Dn* reporter mice, which express GFP under an ectopic *Gnrh* promoter. GnRH-GFP neurons have been isolated by FACS from the preoptic region of *Gnrh::Gfp* and *Gnrh::Gfp*;Ts65Dn littermates at P12. (**D**) RT-PCR analysis of the expression of different genes in FACS-sorted GnRH-GFP cells. (**E**) Putative miRNA-gene network illustrating the convergent contributions of miR-200 and *Zeb1* in the control of GnRH expression via the repression of the Kp receptor (Kiss1R)/Otx2-signaling pathway. (**F**) Schematic of experimental design performed to evaluate the functional involvement of the miR-200 family members in odor and object recognition tasks in Ts65Dn mice. The red dot indicates the injection site of the viruses; OVLT, organum vasculosum of the lamina terminalis; MePO, median preoptic nucleus; LV, lateral ventricle. (**G,H**) The overexpression of miR-200b in the POA using adeno-associated viral vectors resulted in a rescue of both the capacity to differentiate odors (**G**) and recognize novel objects (**H**) in Ts65Dn mice. Statistical differences were tested using unpaired t-test or Mann-Whitney (**A,B,D**) and paired t-test or Wilcoxon matched-pairs test (**G,H**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.5 GnRH replacement therapies reverse olfactory and cognitive impairments in Ts65Dn mice

We next tried to rescue the olfactory and cognitive deficits exhibited by adult Ts65Dn mice by GnRH replacement by first grafting neonatal POA transplants into the 3v of adult mice (Charlton et al., 1987). Enzymatically dissociated neonatal cells (Messina et al., 2016) from the POA of wt pups (WT-POA) were stereotaxically injected into the 3V of adult male Ts65Dn mice (**Figure 5A**). Remarkably, this procedure reversed both olfactory and cognitive impairments (**Figure 5B**). We also evaluated short-term visuospatial memory in these animals and found that the grafted Ts65Dn animals performed similar to wt controls in a Y-maze test (**Figure 5D-F**). Similar results were obtained in adult Ts65Dn females, with cognition recovery after the implantation of neonatal WT-POA (**Figure 5C**). However, grafted Ts65Dn females did not show full rescue of their olfactory capacity (**Figure 5C**). Here, in contrast to hypogonadal adult mice (Charlton et al., 1987), WT-POA transplantation did not restore fertility in either Ts65Dn males or females (**Supplemental Figure 1F**), demonstrating that the rescue of olfactory capacity and cognition is not coupled to the restoration of gonadal function. APP and CTF expression levels were unchanged in the cortex and the hippocampus of Ts65Dn males after transplantation (**Supplemental Figure 5A-D**). However, Tau-Cter expression was decreased in the cortex of grafted Ts65Dn mice, when compared to controls (**Supplemental Figure 5D**).

To selectively silence GnRH neurons, we next generated *Gnrh::Cre*; *BoNTB*$^{loxP\text{-}STOP\text{-}loxP}$ double transgenic mice, in which the vesicular release in GnRH neurons is blunted by the cell-specific expression of the botulinum neurotoxin B (*BoNTB*^{Gnrh}). We then injected neonatal POA cells from these animals into the 3V of adult Ts65Dn males (*BoNTB*^{Gnrh} POA) (**Figure 5G**). No olfactory or cognitive rescue was observed in Ts65Dn mice grafted with *BoNTB*^{Gnrh}-POA cells (**Figure 5H,I**). Importantly, an acute intraperitoneal GnRH injection (50 μg/kg of body weight 2h before behavioral tests) 6 months post-graft rescued olfactory and cognitive deficiencies both in sham (**Figure 5B**) and *BoNTB*^{Gnrh} POA-grafted (**Figure 5H,I**) Ts65Dn mice.

To determine whether genetic activation of endogenous GnRH neuronal activity in Ts65Dn mice is sufficient to rescue olfactory and cognitive function in these mice, adult *Gnrh::Cre* and Ts65Dn; *Gnrh::Cre* mice received bilateral injections of a Cre-dependent activating hM3Dq DREADD viral vector into the POA (**Figure 5J**). Both *Gnrh::Cre* and Ts65Dn;*Gnrh::Cre* males

responded to intraperitoneal clozapine N-oxide (CNO) injections by an increase in LH release (**Figure 5L,N**), while vehicle injection had no effect (**Figure 5K,M**). The CNO-mediated activation of GnRH neurons in trisomic mice acutely restored the capacity to discriminate between different odors (**Figure 5O**) and corrected cognitive deficits (**Figure 5P**) to wt levels.

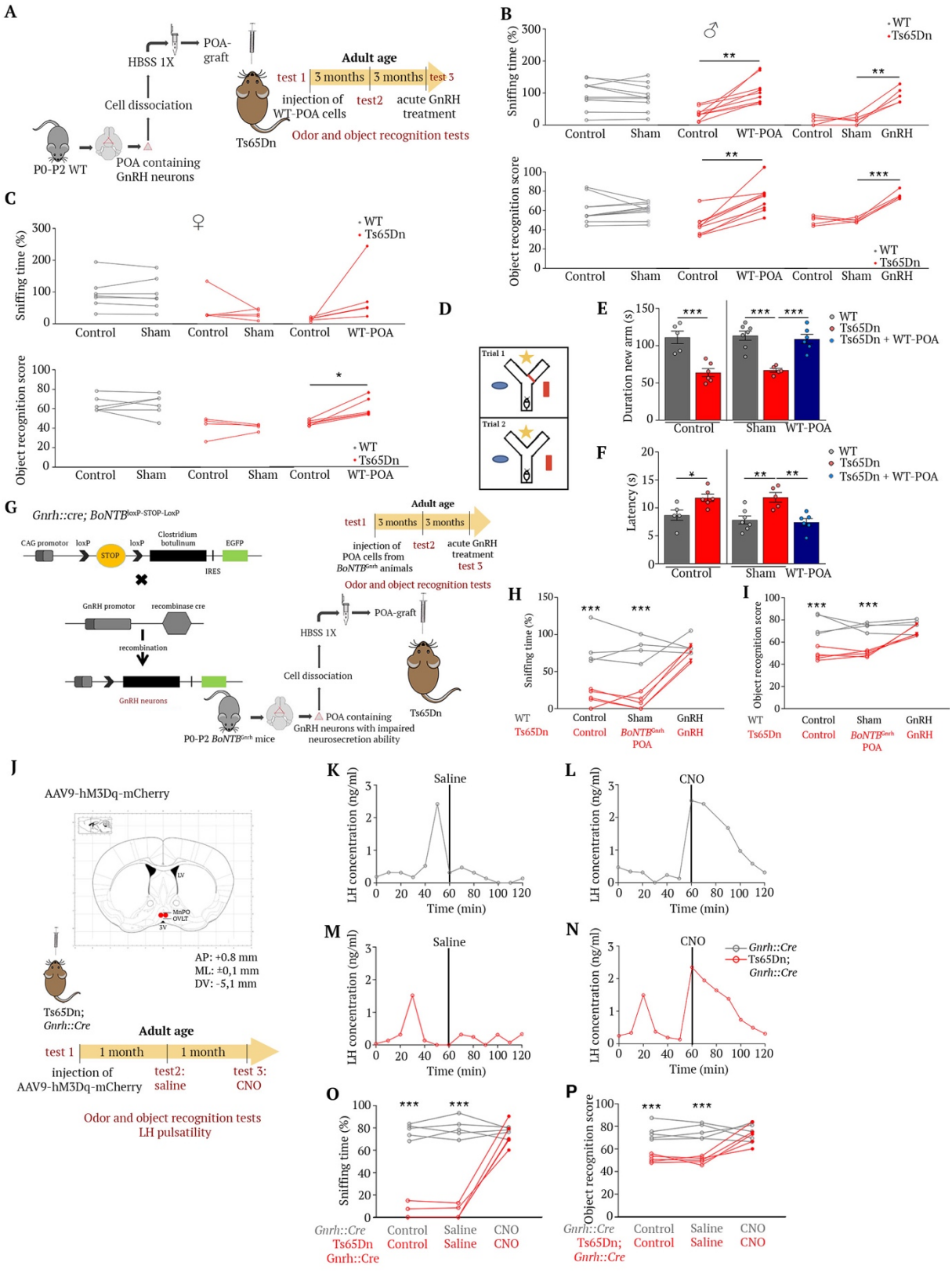

A
HBSS 1X
POA-graft
Cell dissociation
P0-P2 WT
POA containing GnRH neurons
Ts65Dn
Adult age
test 1
3 months
3 months
test 3
injection of WT-POA cells
test2
acute GnRH treatment
Odor and object recognition tests
B
♂
WT
Ts65Dn
Sniffing time (%)
Object recognition score
Control
Sham
Control
WT-POA
Control
Sham
GnRH
C
♀
Sniffing time (%)
Object recognition score
Control
Sham
Control
Sham
Control
WT-POA
D
Trial 1
Trial 2
E
Duration new arm (s)
WT
Ts65Dn
Ts65Dn + WT-POA
Control
Sham
WT-POA
F
Latency (s)
G
Gnrh::cre; BoNTB loxP-STOP-LoxP
CAG promotor
loxP
STOP
loxP
Clostridium botulinum
EGFP
IRES
GnRH promotor
recombinase cre
recombination
GnRH neurons
P0-P2 BoNTB Gnrh mice
HBSS 1X
POA-graft
Cell dissociation
POA containing GnRH neurons with impaired neurosecretion ability
Ts65Dn
Adult age
test1
3 months
3 months
injection of POA cells from BoNTB Gnrh animals
test2
acute GnRH treatment
test 3
Odor and object recognition tests
H
Sniffing time (%)
WT
Ts65Dn
Control
Sham
GnRH
Control
BoNTB Gnrh
POA
GnRH
I
Object recognition score
WT
Ts65Dn
Control
Sham
GnRH
Control
BoNTB Gnrh
POA
GnRH
J
AAV9-hM3Dq-mCherry
LV
MnPO
OVLT
3V
AP: +0.8 mm
ML: ±0,1 mm
DV: -5,1 mm
Ts65Dn; Gnrh::Cre
Adult age
test 1
1 month
1 month
injection of AAV9-hM3Dq-mCherry
test2: saline
test 3: CNO
Odor and object recognition tests
LH pulsatility
K
Saline
LH concentration (ng/ml)
Time (min)
L
CNO
LH concentration (ng/ml)
Time (min)
M
Saline
LH concentration (ng/ml)
Time (min)
N
CNO
Gnrh::Cre
Ts65Dn; Gnrh::Cre
LH concentration (ng/ml)
Time (min)
O
Sniffing time (%)
Gnrh::Cre
Ts65Dn
Gnrh::Cre
Control
Control
Saline
Saline
CNO
CNO
P
Object recognition score
Gnrh::Cre
Ts65Dn; Gnrh::Cre
Control
Saline
CNO

Figure 5. GnRH replacement reverses olfactory- and cognitive-associated impairments in Ts65Dn mice. (**A**) Schematic diagram illustrating the cell therapy strategy performed in adult Ts65Dn mice. Neonatal cells from the POA of wt mice (P0-P2) were enzymatically dissociated and stereotaxically injected into the third ventricle of adult Ts65Dn mice. (**B**) After a 3-month recovery period, implantation of neonatal cells from brain tissue containing wt GnRH neurons in Ts65Dn males (wt-POA) rescued both the capacity to differentiate between odors and recognition memory compared to non-grafted Ts65Dn mice (Sham: injected with vehicle solution). (**C**) Ts65Dn females recovered cognition after the implantation of neonatal POA from wt pups. (**D-F**) The Y-maze test was performed to evaluate the short-term visuospatial memory of the animals (**D**). Ts65Dn grafted males (wt-POA) spent the same time in the new arm than wt littermates compared to non-grafted Ts65Dn mice (Sham) (**E**) and needed less time to first enter the new arm (**F**)**.** (**G**) Schematic of the experimental design used to determine whether GnRH neurons were playing a role in the rescue of the cognitive phenotypes in Ts65Dn mice by transplanting enzymatically-dissociated POA from mice with exocytotically-silenced GnRH neurons (*Gnrh::Cre*; *BoNTB*$^{loxP\text{-}STOP\text{-}loxP}$). (**H-I**) After a 3-month recovery period, Ts65Dn mice grafted with *BoNTB*^{Gnrh}-POA cells recovered neither the ability to discriminate odors (**H**) nor improved their performances in the object recognition task (**I**). However, acute GnRH intraperitoneal injection restored both of these functions in Sham- (**B**) and *BoNTB*^{Gnrh}-cells grafted (**H-I**) Ts65Dn mice. (**J**) Schematic diagram illustrating the protocol performed to study the effect of chemogenetic activation of GnRH neurons on LH pulsatility profile, cognitive and olfactory performance. Adult *Gnrh::Cre* and Ts65Dn; *Gnrh::Cre* mice were tested before and after injection with a hM3Dq DREADD viral vector. Red dots indicate the injection sites of the virus; OVLT, organum vasculosum of the lamina terminalis; MePO, median preoptic nucleus; LV, lateral ventricle. (**K-N**) Representative graphs for LH pulsatility assessment after viral transfection and intraperitoneal injection of vehicle (saline) or clozapine N-oxide (CNO) solution (1 mg/kg of body weight). The chemogenetic activation of GnRH neurons induced by CNO rescued the capacity to discriminate between different odors (**O**) and cognitive deficits (**P**) in Ts65Dn; *Gnrh::Cre*-DREADD injected mice, while saline injection had no effect. Statistical differences were tested using unpaired t-test and one-way ANOVA in animals not subjected and subjected to surgery, respectively (**E**,**F**), Wilcoxon matched-pairs test or paired t-test (**B**, **C**) and repeated-measures one-way ANOVA when comparing three conditions (**B**). Two-way repeated measures ANOVA was used in **H**,**I**,**O**,**P**. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Finally, we tested whether restoring GnRH pulsatility in Ts65Dn mice rescues cognitive performance in these animals. Adult Ts65Dn males were implanted either with a subcutaneous osmotic pump to receive a continuous infusion of Lutrelef (0.25 μg per 3h), a GnRH peptide used clinically to restore fertility in patients with HH (Boehm et al., 2015); or with a subcutaneous programmable mini-pump, to receive pulsatile Lutrelef infusions (0.25

µg delivered during 10 min every 3h) for 15 days (**Figure 6A**), mimicking GnRH/LH pulsatility reported in wt mice (Czieselsky et al., 2016). The pulsatile infusion of Lutrelef increased LH pulse frequency and amplitude to wt levels in Ts65Dn males whereas continuous infusion blunted LH pulsatility (**Figure 6B-G**). Pulsatile Lutrelef infusion also rescued both the capacity to discriminate between different odors (**Figure 6H**) and cognitive deficits (**Figure 6I**) in these animals. However, while continuous Lutrelef infusion had no effect on olfactory and cognitive performance in Ts65Dn mice, it appeared to have markedly deleterious effects on these tasks in wt mice (**Figure 6H,I**). To determine whether the phenotypic rescue in Ts65Dn mice was due to the restoration of HPG-axis function, we repeated these experiments in 3-month ORX wt and Ts65Dn mice; bilateral ORX did not affect the rescue of olfaction (**Figure 6J**) or recognition memory (**Figure 6K**) by pulsatile Lutrelef infusion. Taken together, our data demonstrate the hitherto unsuspected importance of the pulsatile nature of GnRH secretion for olfactory perception and cognition.

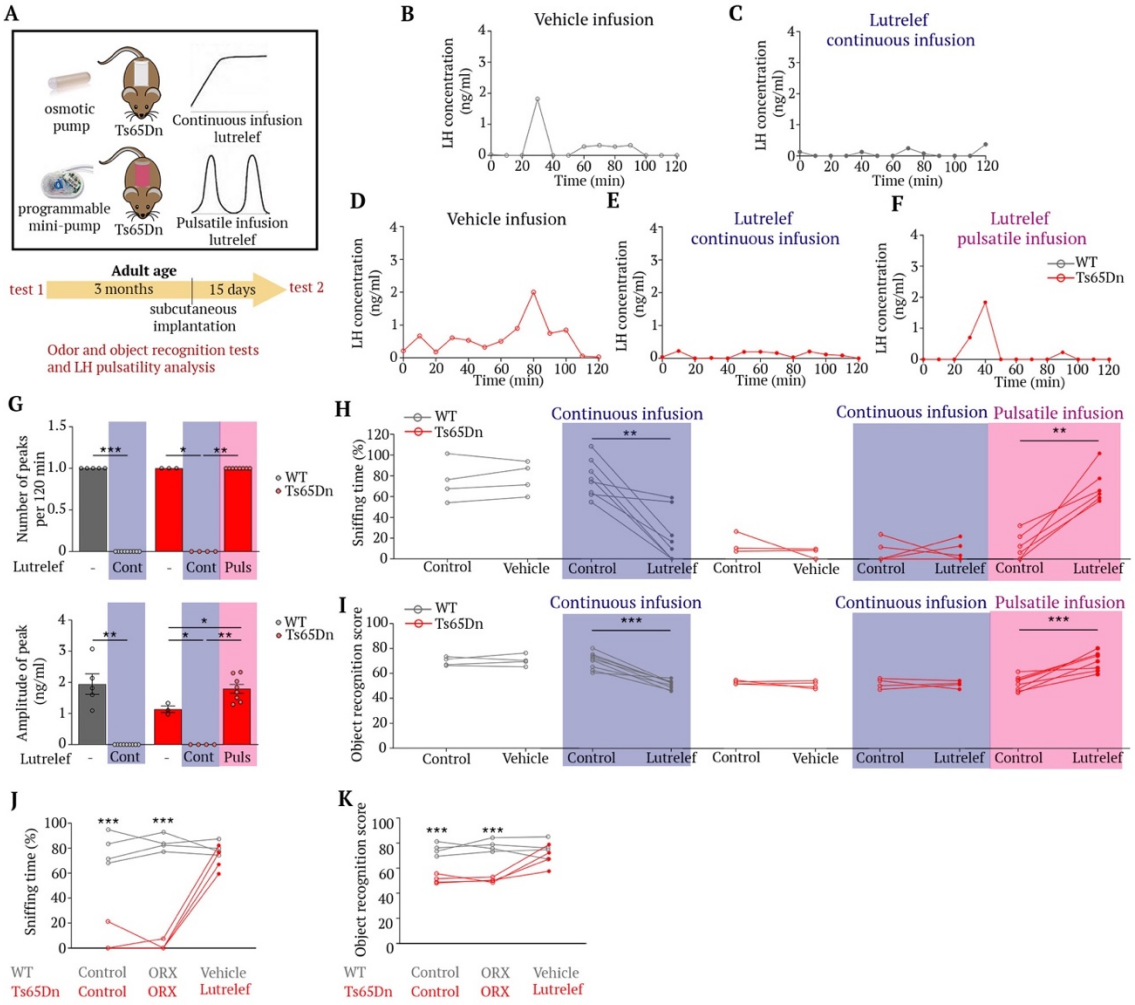


Figure 6. Pulsatile GnRH infusion reverses both olfactory- and cognitive-related impairments in Ts65Dn mice. (**A**) Schematic diagram illustrating the pharmacological therapy performed in adult Ts65Dn mice with Lutrelef, a GnRH peptide used clinically. Mice were implanted with osmotic pump, to receive continuous infusion of vehicle or Lutrelef (0.25 μg/ 3h during 2 weeks); or with a programmable mini-pump (iPRECIO), to receive pulsatile Lutrelef infusion (0.25 μg/10 min every 3 hours during 2 weeks). (**B-F**) Representative graphs for LH pulsatility assessment after 15 days of vehicle or Lutrelef subcutaneous administration. (**G**) Lutrelef pulsatile infusion in Ts65Dn males significantly increased LH pulse frequency and LH pulse amplitude compared to Lutrelef continuous infusion which blunted both LH pulse frequency and LH pulse amplitude both in wt and Ts65Dn mice**.** (**H**,**I**) Lutrelef pulsatile infusion rescued the capacity to discriminate between different odors (**H**) and cognitive deficits (**I**) in Ts65Dn mice**.** (**J-K**) Both orchidectomized (ORX) wt and Ts65Dn littermates behaved similarly to intact animals (controls), while Lutrelef pulsatile infusion rescued the capacity to discriminate between different odors (**J**) and cognitive deficits **(K**) in ORX Ts65Dn mice. Statistical differences were tested using Mann-Whitney and unpaired t-test (**G**), Wilcoxon matched-pairs test (**H**), paired t-test (**H**,**I**) and two-way repeated measures ANOVA (**J**,**K**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Our study paves the way for the development of new treatment strategies to prevent age-dependent cognitive decline, mobilize the cognitive reserve, and thus improve well-being in patients with neurodevelopmental (e.g., DS) and neurodegenerative disorders (e.g., DS and AD).

4.3 Material and methods

4.3.1 Animals

All mice were housed under specific pathogen-free conditions in a temperature-controlled room (21-22°C) with a 12h light/dark cycle. The day the litters were born was considered as day 0 of age (P0). Animals were weaned at P21 and were provided with *ad libitum* access to food and water.

Ts65Dn *(B6EiC3Sn.BLiA-Ts(1716)65Dn/DnJ; Stock no. 005252*) mice (Reeves et al., 1995, Reinholdt et al., 2011, Ahmed et al., 2012) carrying a partial trisomy of chromosome 16, the orthologous region of hsa 21, were purchased from Jackson Laboratories (New Harbor, ME, USA). As the Ts65Dn line has a genetic background wt for the Pde6b gene, the line was maintained by crossing Ts65Dn trisomic females to Pde6b+ (C57BL/6JEiJ x C3Sn.BLiA-Pde6b+/DnJ)F1/J; Stock no 003647) males. This mating system results in wt and Ts65Dn

animals. *Gnrh::Cre* (Tg(Gnrh1::Cre)1Dlc) and *Gnrh::Gfp* were a generous gift from Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA) (Yoon et al., 2005) and Dr. Daniel J. Spergel (Section of Endocrinology, Department of Medicine, University of Chicago, IL)(Spergel et al., 1999), respectively. Tg(CAG-BoNT/B,EGFP)U75-56wp/J (*BoNTB*$^{loxP\text{-}STOP\text{-}loxP}$) mice and *GnRHR::Cre*;*Tau-Gfp*$^{LoxP\text{-}STOP\text{-}LoxP}$ have been generated as described before (Wen et al., 2010, Slezak et al., 2012). Mice were genotyped by PCR using the primers listed in **Supplemental Table S1.**

Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the University of Lille; all experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU). The sex of the animals used is specified in the text and/or figure legends. The genotype or/and treatment group of animals was blinded for the study except when the morphological or physiological differences were too obvious to be ignored.

4.3.2 Drugs

Clozapine N-oxide hydrochloride (SML2304, Sigma) was dissolved in sterile saline (0.9% NaCl) and stored at 4°C. On the day injections were administered, CNO stock was brought to room temperature and diluted in sterile saline (0.9% NaCl). Lutrelef (Ferring Pharmaceuticals, Switzerland) was dissolved in sterile 0.1M PBS and stored at 4°C. The day before osmotic minipumps and programmable micro infusion pumps were implanted, Lutrelef stock was taken to room temperature and diluted in sterile 0.1M PBS. GnRH-1 peptide (Genecust) was dissolved in sterile H_2O and stored in aliquots at -80°C. On the day injections were administered, GnRH-1 stock was thawed and diluted in a solution of sterile saline.

4.3.3 Physiological measurements

Pubertal studies. Males were checked daily for balanopreputial separation and urine samples were collected from weaning to P45. Weaned female mice were checked daily for vaginal opening. After vaginal opening, vaginal smears were performed daily and analyzed under an inverted microscope to identify the specific day of estrous cycle.

Fertility index. Female fertility indices were calculated from the number of litters per female during a 120-day long mating.

4.3.4 Urine collection and protein analysis

To assess major urinary protein (MUP) profile diversity, urine was collected from weaning to P45 in male mice following either spontaneous urination when handled, or provoked after exerting a gentle pressure on the mouse bladder. The urine was collected in microcentrifuge tubes kept on ice during the collection procedure. All samples were initially frozen at −20°C then kept at −80°C until further processing. For protein analysis, 1 µl of urine was mixed with 1X sample buffer (Invitrogen) and 1X reducing agent (Invitrogen). Samples were boiled for 5 min and electrophoresed for 75 min at 150 V in 4–12% MES SDS-polyacrylamide gels according to the protocol supplied with the NuPAGE system (Invitrogen). After the migration, the proteins were transferred onto 0.2 µm nitrocellulose membrane (Invitrogen) in the blot module of the NuPAGE system (Invitrogen) for 90 min at 30V in cold conditions. Membranes were then blocked for 1 h in blocking buffer [(TBS with 0.05% Tween 20 (TBST) and 5% non-fat dry milk] at room temperature, and incubated for 48 h at 4°C with the primary antibody (rabbit polyclonal anti-MUP1, 1:200 dilution, sc-66976, Santa Cruz Biotechnology, INC) diluted in blocking buffer. Following this, membranes were washed three times with 1X TBST before incubation with the secondary antibody (peroxidase anti-Rabbit IgG (H+L), 1:2000 dilution, PI-1000, Vector Laboratories) diluted in blocking buffer for 1 h at room temperature. After incubation with secondary antibody, the membranes were washed three times with 1X TBST. Immunoreactions were developed using the ECL detection kit (NEL101; PerkinElmer, Boston, MA) and scanned using a desktop scanner (Epson Expression 1680 PRO).

4.3.5 Pulsatile LH measurements

Adult mice were habituated with daily handling. Blood samples (5 µL) were taken from the tail at 10 min intervals during a period of 2 h (between 10:00 and 12:00) and were diluted in 45 µL of 1X PBS-T (0,05%) and immediately frozen and stored at -80°C. LH levels were determined using a previously described sensitive LH sandwich ELISA (Steyn et al., 2013). A 96-well high-affinity binding microplate (Corning) was coated with 50 µL of capture antibody (monoclonal antibody, anti-bovine LHβ subunit, 518B7; L. Sibley; University of California, UC Davis) at a final dilution of 1:1,000 (in 0.1M Na_2CO_3/$NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Wells were incubated with 200 µL blocking buffer (5% (w/v) skimmed milk powder in 1X PBS-T pH 7.4 (0.1 M PBS, 0.05% Tween 20 (Sigma #P9416)) for 2 h at room temperature. A standard curve was generated using a twofold serial dilution of mouse LH (reference preparation, AFP-

5306A; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program (NIDDK-NHPP)) in 1% (w/v) BSA (Sigma, A9418) in 1X PBS-T. The LH standards and blood samples were incubated with 50 µL of detection antibody (rabbit LH antiserum, AFP240580Rb; NIDDK- NHPP) at a final dilution of 1:10,000 for 1.5 h at room temperature. Each well containing bound substrate was incubated with 50 µL of horseradish peroxidase-conjugated antibody (goat anti-rabbit; Vector Laboratories, PI-1000) at a final dilution of 1:1,000. After a 1.5 h incubation, 100 µL of 1-Step Ultra TMB-Elisa Substrate Solution (ThermoFisher Scientific, cat. #34028) was added to each well and left at room temperature for 10 min. The reaction was stopped by the addition of 50 µL of 3M HCl to each well, and the absorbance was measured at 450 nm. Pulses were confirmed using DynPeak (Vidal et al., 2012).

4.3.6 Behavioral studies

4.3.6.1 Habituation/dishabituation test

The habituation/dishabituation test was used to assess the ability to differentiate between different odors (Breton-Provencher et al., 2009). Mice were single-housed for 8 days prior to testing. This olfactory test included a presentation of acetophenone (00790, Sigma) for habituation and octantal (05608, Sigma) for dishabituation, or vice versa. Before the test, mice were allowed to explore the open-field area and an empty odor box for 30 min. After this habituation period, mice were sequentially presented with one odor for four consecutive trials for a duration of 1 min, and an inter-trial interval of 10 min was maintained to ensure the replacement of the odor. After four consecutive trials, a second odor was presented during a 1 min trial. Odors (20µl of 1:1000 dilution) were administered on a filter paper and placed in a perforated plastic box to avoid direct contact with the odor stimulus. The measurement consisted of recording the total amount of time the mouse spent sniffing the object during different trials.

4.3.6.2 Novel object recognition test

Recognition memory was assessed using the NOR test (Leger et al., 2013). Mice were single-housed for 5 days prior to testing. On day 1, two identical objects (A+A) were placed within the open-field arena on opposite sides of the cage, equidistant from the cage walls. Each mouse was placed within the two objects and allowed to explore them for 15 min. Day 2

consisted of two phases, a familiarization and a test phase. During the familiarization phase (trial 1) that lasted 15 min, mice explored two other identical objects (B+B). After this phase, mice were placed back in its home cage for 1 hour before starting the test phase. During the test phase, one object from trial 1 and a completely new object (B+C) were placed within the open-field area and mice were allowed to explore them for 5 min (trial 2). The object recognition score was calculated as the time spent exploring the new object (trial 2) over the total exploration time, and is used to represent recognition memory function.

4.3.6.3 Y-maze test

Natural spontaneous exploratory behavior and visuospatial short-term memory were tested using the Y-maze (Dellu et al., 2000, Bridoux et al., 2013). The Y-maze consisted of three white wooden arms (24.0 cm x 6.5 cm x 15 cm), elevated to a height of 41.0 cm above the floor and was surrounded with visual cues on the wall. Mice were placed in the start arm, facing the end of this arm, and were allowed to explore the maze for 10 min while one arm was blocked (novel arm). Consequently, mice were placed in their home cage for 1 h before being allowed to explore all three arms for 5 min. Trajectories of the mice were recorded using EthoVision video tracking equipment and software (Noldus Bv, Wageningen, The Netherlands). The time spent in the novel arm and latency to enter the novel arm were compared between mice.

4.3.7 Brain tissue dissection

Mice were euthanized by decapitation. The hippocampus, cortex and POA of the hypothalamus were dissected using Wecker scissors (Moria, France) under a binocular magnifying glass, placed in dry ice immediately and stored at − 80°C until further processing and assays.

4.3.8 Tissue protein extraction and western blot analyses

Both hippocampus and cortex from adult Ts65Dn and wt mice were sonicated in 400 µL (for hippocampus) or 800 µL (for cortex) of lysis buffer (10 mM Tris pH 7.4, 10% sucrose and proteases inhibitors (1 pellet for 10mL Complete; Roche Diagnostics GmbH)) and stored at −80°C until use. Protein concentration was determined using the BCA assay (Pierce), subsequently diluted with 2X LDS (Life) and supplemented with reducing agent (Life). Samples were boiled for 10min at 100°C. Proteins were separated onto precast 12% Criterion XT Bis-Tris polyacrylamide gels (Bio-Rad) using 1X MOPS SDS running buffer. Subsequently, proteins

were transferred onto a 0.4 µm nitrocellulose membrane (G&E Healthcare). For low molecular weight proteins, such as carboxy-terminal fragments of APP (CTFs), 16,5% Criterion XT Tris-Tricine polyacrylamide gels (Bio- Rad) in 1X Tris-Tricine SDS running buffer were used. These were transferred onto a 0.2 µm nitrocellulose membrane (G&E Healthcare). For estimation of molecular weights, a molecular weight marker (Novex and Magic Marks, Life Technologies) was used. Membranes were incubated in blocking buffer [TNT (Tris 15 mM pH 8, NaCl 140 mM, 0.05% Tween) and 5% non-fat dry milk or 5% bovine serum albumin (BSA)] at RT and incubated overnight at 4°C with the appropriate primary antibody (**Supplemental Table 2**) diluted in blocking buffer (TNT with 5% Milk or BSA). Following this, membranes were incubated with corresponding secondary antibodies (**Supplemental Table 2**). Immunoreactions were developed using using chemiluminescence kits (ECL™, Amersham Bioscience) and visualized using a LAS3000 imaging system (Fujifilm). Results were normalized to GAPDH and quantification was performed using ImageJ software (Scion Software).

4.3.9 RNA isolation from POA and quantitative RT-PCR analyses

Total RNA, containing mRNA and miRNA, was extracted with the Ambion mirVana™ miRNA Isolation Kit (Ambion, Inc; CA, USA) by trituration of the fragments through 22 and 26 gauge needles in succession. Quality and concentration of RNAs were determined by spectrophotometer ND-1000 NANODROP 385 (Thermo-scientific). For gene expression analyses, mRNAs were reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies). Real-time PCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System using exon-boundary-specific TaqMan® Gene Expression Assays (Applied Biosystems) (**Supplemental Table 3**).

MicroRNA expression analyses were performed using TaqMan specific RT primers and the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems). Thereafter, quantitative real-time PCRs were performed using predesigned assays for miRNAs (Applied Biosystems) (**Supplemental Table 3**) on an Applied Biosystems 7900HT thermocycler using the manufacturer's recommended cycling conditions. Gene and miRNA expression data were analyzed using SDS 2.4.1 and Data Assist 3.0.1 software (Applied Biosystems).

4.3.10 Isolation of hypothalamic GnRH neurons using Fluorescence-Activated Cell Sorting and quantitative RT-PCR analyses

The preoptic regions of *Gnrh::Gfp* and *Gnrh::Gfp;Ts65Dn* mice were microdissected and enzymatically dissociated using a Papain Dissociation System (Worthington, Lakewood, NJ) to obtain single-cell suspensions. FACS was performed using a FACS ARIA SORP (BD Bioscience). The sort decision was based on measurements of GFP fluorescence (excitation: 488nm, 50 mW; detection: GFP bandpass 530/30 nm, autofluorescence bandpass 695/40nm) (**Supplemental Figure 6)**. For each animal, GFP positive and negative cells were sorted directly into 10 µl extraction buffer [0.1% Triton® X-100 (Sigma-Aldrich) and 0.4 U/µl RNaseOUT™ (Life Technologies)].

In order to analyze gene expression, mRNAs obtained from FACS-sorted GnRH neurons were reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies) and a linear pre-amplification step was performed using the TaqMan® PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems). Real-time PCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System as described previously using specific TaqMan® Gene Expression Assays (Applied Biosystems) (**Supplemental Table 3**).

4.3.11 Orchidectomy

Adult males were subjected to bilateral gonadectomy under isoflurane anesthesia through a small ventral midline incision in the scrotum and subsequently sealed.

4.3.12 Acute GnRH injections

In order to study the effect of GnRH on cognitive and olfactory performance, both adult male Ts65Dn mice sham and grafted with a POA explant from *Gnrh::Cre*; *BoNTB*$^{loxP\text{-}STOP\text{-}loxP}$ double transgenic mice (*BoNTB*^{Gnrh}) were treated with GnRH-1 peptide (Genecust), in a dose of 50 µg/kg of body weight, or vehicle (saline 0.9%).

To test the olfactory discrimination capacity, the animals received the one single intraperitoneal injection of GnRH-1 or vehicle 2 h before the habituation phase. In the case of NOR test, on day 1, the animals received two ip injections of GnRH-1 peptide or vehicle. The first injection was given 2 h before the start of the trial, and a second one 12 hours after the

first injection to promote memory consolidation. On day 2, the animals received the intraperitoneal injection 2 h before the start of the first trial.

4.3.13 Preparation of donor tissues and neuronal grafting

Tissue donors for the POA grafts were obtained from P2 WT mice, that contained GnRH neurons which release GnRH (WT-POA), and *Gnrh::Cre*; *BoNTB*$^{loxP\text{-}STOP\text{-}loxP}$ mice, that contain GnRH neurons which do not release GnRH (*BoNTB*^{Gnrh}-POA). The tissues were microdissected and enzymatically dissociated using a Papain Dissociation System (Worthington, Lakewood, NJ) to obtain a cell suspension in 5 µl 1X HBSS solution. Two preoptic tissues were used by implant. Adult Ts65Dn mice were placed in a stereotaxic frame (Kopf® Instruments, California) under anesthesia (isoflurane), and a burr hole was drilled -1.7 mm from Bregma at the midline, according to a mouse brain atlas (Paxinos et al., 2004). A 25 µl Hamilton syringe (22-gauge needle) was slowly inserted into the 3v (5.6 mm deep relative to the dura), and 5 µl of the different solutions which contain the WT-POA or *BoNTB*^{Gnrh}-POA explants were injected using an infusion pump (KD Scientific, Holliston, MA) over 10 min. Under the same conditions, adult Ts65dn and wt mice were injected with 5µl of vehicle solution (HBSS 1X) (sham groups).

4.3.14 Viruses and stereotactic injections

For the viral tracing of hypophysiotropic GnRH neurons, adult *Gnrh::Cre* mice received a unilateral injection of an adeno-associated virus (AAV) (300 nl; AAV9.EF1a.DIO.eYFP.WPRE.hGH, 1×10^{13} vg/ml) (Addgene plasmid # 27056) into the ME (AP:-1.0 mm, ML:-0.2 mm, DV: −6.1 mm).

To study the effect of GnRH neurons activation, Cre-dependent DREADDs (Designer Receptor Exclusively Activated by Designer Drugs) were used. pAAV-EF1a-DIO-hM3D(Gq)-mCherry was a gift from Bryan Roth (Addgene plasmid # 50460). Viral preparations were performed at the Molecular Biotechnology Center (MBC; Prof. Emilia Turco Lab), University of Turin, Italy. The viral construct AAV9.EF1a.DIO.hM3D(Gq)-mCherry was injected bilaterally (250 nl or 500 nl total; 2×10^{13} gc/ml) in the rPOA (AP:+0.8 mm, ML:±0.1 mm, DV: −5.1 mm) of adult *Gnrh::Cre* and Ts65Dn; *Gnrh::Cre* mice.

For the selective overexpression of miR-200 family, particularly the member miR-200b, a unilateral injection, in two sequential steps, (150 nl or 300 nl total) of scAAV9-EF1a-mmu-miR-200b-eGFP (AAV-miR200b, 2.1×10^{13} gc/ml) or scAAV9-EF1a-ctrl-miR-eGFP (AAV-GFP,

2.2×10^{13} gc/ml) (Vector Biolabs) was administrated into the rPOA (AP:+0.8 mm, ML:0 mm, DV: -5.3/−5.1 mm) of adult Ts65Dn mice.

In brief, mice were anaesthetized (isoflurane/O_2) and placed on stereotaxic frame (Kopf® Instruments, California). The injection was carried out using a 2 µl Hamilton syringe at a rate of ~40 nl/min, and the needle was kept in place for 5 min before and 7 min after the injection. Injection coordinates were based on the Paxinos mouse brain atlas (Paxinos et al., 2004).

4.3.15 Chemogenetic activation of GnRH neurons

The effect of chemogenetic activation of GnRH neurons on cognitive and olfactory performance, and LH pulsatility profile was studied according to the following procedure. Adult *Gnrh::Cre* and Ts65Dn; *Gnrh::Cre* mice were tested one month after the hM3Dq DREADD injection. For the habituation/dishabituation test, the animals received an intraperitoneal injection of 200µl of vehicle solution 30 min before the habituation phase. For the NOR test, the animals received the same vehicle injection 30 min before the start of the trial of the first day, and 30 min before the start of the first trial of the second day. Blood sampling for LH pulsatility analysis was performed every 10 min over 120 min, 60 min before and 60 min after the injection with the vehicle solution. After a 1-month recovery period, animals were tested following the same protocol and receiving an intraperitoneal injection of clozapine N-oxide (CNO) (SML2304, Sigma), which activates the hM3Dq DREADD. For the behavioral tests, CNO was administered in a dose of 2 mg/kg of body weight (Tuscher et al., 2018). For the LH pulsatility analysis, CNO was administered in a dose of 1 mg/kg of body weight.

4.3.16 Continuous and pulsatile subcutaneous infusion

Adult mice were implanted with osmotic minipumps (1002, Alzet, USA) receiving continuous infusion of vehicle (sterile 0.1M PBS) or Lutrelef (0.25 µg/ 3 h) (Ferring Pharmaceuticals, Switzerland); or with a programmable micro infusion pump (SMP-300, iPRECIO, Japan) receiving pulsatile infusion of vehicle or Lutrelef (0.25 µg delivered over 10 min, every 3 h), mimicking GnRH/LH pulsatility reported in wt mice (Czieselsky et al., 2016), and a basal infusion with a low dose (0.0025 µg/10 min) for the rest of the time. The pumps were placed under the skin on the back of the mouse. Both olfactory and cognitive deficiencies were confirmed before in these animals. One week after the surgery, mice were retested to evaluate their olfactory and cognitive performance. Two weeks after the surgery, repetitive

tail-tip blood sampling was undertaken (as described elsewhere) to assess the LH pulsatility profile.

4.3.17 Brain preparation for immunohistochemical analysis

Neonatal (P0) mice, anesthetized on ice, and infantile (P12), prepubertal (P35) and adult mice, anesthetized with 50-100 mg/kg of Ketamine-HCl and 5-10mg/kg Xylazine-HCl, were perfused transcardially with 10-100 ml of saline, followed by 10-100 ml of 4% paraformaldehyde (PFA, pH7.4). Brains were collected and fixed with the same fixative for 2h at 4°C, embedded in optical cutting temperature (OCT) embedding medium (Tissue-Tek), frozen on dry ice, and stored at −80°C until cryosectioning.

4.3.18 Immunohistochemistry and quantification

Tissues were cryosectioned (Leica cryostat) at 16 µm for P0 and at 35 µm (free-floating sections) for P12, P35 and adult brains, unless otherwise indicated.

4.3.18.1 Assessment of GnRH protein expression

Immunohistofluorescence experiments were carried out as previously reported (Messina et al., 2011, Hanchate et al., 2012). Coronal sections were then washed in 0.1M PBS, and incubated in blocking solution (2% goat serum + 0.5% Triton X-100) in PBS 0.1M for 60 min. Subsequently, sections were incubated in guinea pig anti-GnRH (1:10000) raised by Dr. Erik Hrabovszky, (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) (Hrabovszky et al., 2011) in the case of P0 brains; and in rabbit anti-GnRH (1:3000), a generous gift from Prof. G. Tramu (Centre Nationale de la Recherche Scientifique, URA 339, Université Bordeaux I, Talence, France) (Beauvillain et al., 1980), in the case of P12, P35 and adult brains, in blocking solution for 48 h at 4°C. After incubation in the primary antibody, sections were rinsed with 0.1M PBS three times for 10 min each and then incubated Alexa fluor 568 conjugated anti-guinea pig (Life Technologies, Molecular Probes, Invitrogen, A-11075) or anti-rabbit (Invitrogen, A11077) secondary antibodies both at 1:500 for 90 min at room temperature. Sections were then washed, counterstained with Hoechst (1:10,000; ThermoFisher Sci #H3569, RRID:AB_2651133) for 3 min, rinsed with 0.1M PBS three times for 10 min and mounted with coverslips using Mowiol coverslip mounting solution. Because the GnRH neuronal population is very limited in the mouse brain, all neurons were counted by eye under the microscope in

alternate series of brain (P12, P35 and adult) or head (P0) sections. Images were acquired using a Zeiss Axio Imager Z2 ApoTome microscope (Zeiss, Germany), with detailed information in Fluorescence Microscopy section below.

4.3.18.2 Conventional immunolabelling of GnRH-R::GFP neurons

Coronal sections were then washed in 0.1M PBS, and incubated in blocking solution (2% goat serum + 0.3% Triton X-100) in PBS 0.1M for 60 min. Subsequently, sections were incubated with chicken anti-GFP (1:1000; Aves Labs, Inc GFP-1020) in 2% normal donkey serum / 0.25% BSA / 0.3% Triton X-100 incubation solution for 48 h at 4°C. After incubation in the primary antibody, sections were rinsed with 0.1M PBS three times for 10 min each and incubated Alexa Fluor 488-AffiniPure donkey anti-chicken IgY (IgG) (1:400; Jackson ImmunoResearch Ltd.; code # 703-545-155).

4.3.18.3 Assessment of adeno-associated viral vector

Coronal sections were then washed with 0.1M PBS, and incubated with blocking solution [(5% donkey serum + 0.5% Triton X-100) in 0.1M PBS] for 60 min. The sections were then incubated with chicken anti-GFP (1:500; Aves Labs, Inc GFP-1020) and rabbit anti-GnRH (1:3000), a generous gift from Prof. G. Tramu (Centre Nationale de la Recherche Scientifique, URA 339, Université Bordeaux I, Talence, France), in blocking solution for 48 h at 4°C. Following this, sections were rinsed with 0.1M PBS three times for 10 min each and then incubated with the secondary antibody Alexa fluor 488 conjugated donkey anti-chicken (1:500; Jackson Immuno Research 703-545-155) and Alexa 568 conjugated donkey anti-rabbit (1:500; Invitrogen A10042) for 90 min at room temperature. Sections were then washed, counterstained with Hoechst (1:10,000; Thermo Fisher Scientific Cat# H3569, RRID:AB_2651133) for 3 min, rinsed with 0.1M PBS three times for 10 min and mounted with coverslips using Mowiol coverslip mounting solution. Images were acquired using a LSM 710 Zeiss upright confocal laser-scanning microscope equipped with LSM 710 software (Zeiss, Germany).

4.3.19 iDISCO

iDisco is a solvent-based clearing method that renders brain tissue transparent while preserving fluorescence (Erturk et al., 2012, Erturk et al., 2013).

4.3.19.1 Sample pre-treatment with methanol

Samples were washed in PBS (twice for 1 hour), followed by incubation in 50% methanol in 0.1M PBS (once for 1 h), 80% methanol (once for 1 h) and 100% methanol (twice for 1 h). Next, samples were bleached in 5% H_2O_2 in 20% DMSO/methanol (2ml 30% H_2O_2/2ml DMSO/8ml methanol, ice cold) at 4°C overnight. Following this, samples were washed in methanol (twice for 1 h), in 20% DMSO/methanol (twice for 1 h), 80% methanol (once for 1 h), 50% methanol (once for 1 h), PBS (twice for 1 h), and finally, PBS/0.2% TritonX-100 (twice for 1 h) before proceeding to the staining procedures.

4.3.19.2 Whole-mount immunostaining

Samples were incubated at 37°C on an adjustable rotator in 10 ml of blocking solution (PBSGNaT) [1X PBS containing 0.2% gelatin (Sigma), 0.5% Triton X-100 (Sigma-Aldrich) and 0.01% NaAzide (Casoni et al., 2016)] for 3 nights. Samples were then placed at 37°C in rotation for 7 days in 10 ml of PBSGNaT containing the following primary antibodies: rabbit anti-GFP (A6455, Thermofisher, 1:10,000), rabbit anti-GnRH (a generous gift from Prof. G. Tramu, University of Bordeaux, 1:3,000). This was followed by six washes of 30 min in PBSGNaT at room temperature and a final wash in PBSGNaT overnight at 4°C. Next, samples were incubated with secondary antibodies: donkey anti-rabbit Alexa Fluor 647 (1:500, Jackson IP,706-605-152) and donkey anti-rabbit Alexa Fluor 647 (1:400; Jackson IR, 706-605-152) diluted in 10 ml PBSGNaT for 2 days at 37°C in a rotating tube. After six 30 min washes in PBSGNaT at RT, the samples were stored in PBS at 4°C in the dark until clearing.

4.3.19.3 Tissue clearing

All incubation steps were performed at RT in a fume hood, on a tube rotator at 14 rpm covered with aluminum foil to avoid contact with light. Samples were dehydrated in a graded series (20%, 40%, 60%, 80% and 100%) of Methanol (Sigma-Aldrich) diluted in H_2O for 1 h. This was followed by a delipidation step in 66% dichloromethane / 33% methanol (DCM; Sigma-Aldrich). Methanol was then washed out in 100 % DCM for 15 to 30 min. Samples were cleared in dibenzylether (DBE; Sigma-Aldrich) for 2h at room temperature in rotation and in the dark. Finally, samples were moved into fresh DBE and stored in glass tube in the dark at room temperature until imaging. We were able to image samples, as described below, without any significant fluorescence loss for up to 6 months.

4.3.20 Digital image acquisition

The different immunohistofluorescence experiments described before were analyzed using one of the microscopes mentioned below and Adobe Photoshop (Adobe Systems, San Jose, CA, RRID:SCR_014199) was used to process the images.

4.3.20.1 Fluorescence microscopy

Unless otherwise indicated, sections were analysed using a *Zeiss Axio Imager Z2 ApoTome microscope (Zeiss, Germany*, equipped with a motorized stage and an AxioCam MRm camera (Zeiss, Germany). Specific beam splitter (BS), excitation (Ex) and emission (Em) wavelengths were used for the visualization of green (Alexa 488-BS: 495 nm, Ex: 450/490 nm, Em: 500/550 nm), red (Alexa 688-BS: 570 nm, Ex: 538/562 nm, Em: 570/640 nm), far red (Alexa 647-BS: 660 nm, Ex: 625/655 nm, Em: 665/715 nm) and nuclear staining (Hoechst-BS: 395 nm, Ex: 335/383 nm, Em: 420/470 nm).To create photomontages, single-plane images were captured sequentially for each fluorophore using the MosaiX module of the AxioVision 4.6 system (Zeiss, Germany) and a Zeiss 20x objective (numerical aperture NA=0.80). High magnification photomicrographs represent maximal intensity projections derived from a series of triple-ApoTome adjacent images collected using the Z-stack module of the AxioVision 4.6 system. All images were captured in a stepwise fashion over a defined z-focus range corresponding to all visible staining within the section and consistent with the optimum step size for the corresponding objective and the wavelength.

4.3.20.2 Light sheet imaging and 3D visualization

3D imaging was performed as previously described (Belle et al., 2014). An Ultramicroscope I (LaVision BioTec) using ImspectorPro software (LaVision BioTec) was used to perform imaging. The light sheet was generated by a laser (wavelength 488 or 561 nm, Coherent Sapphire Laser, LaVision BioTec) and two cylindrical lenses. A binocular stereomicroscope (MXV10, Olympus) with a 2x objective (MVPLAPO, Olympus) was used at different magnifications (1.6x, 4x, 5x, and 6.3x). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated from the side by the laser light with maximum sheet width. A PCO Edge SCMOS CCD camera (2,560 × 2,160 pixel size, LaVision BioTec) was used to acquire images. The step size between each image was fixed at 2 µm. The resulting tiff series was

converted to Imaris file format (Imaris FileConverter, Bitplane) for 3D reconstruction, and imported in Imaris (Bitplane) for visualisation, snapshots and animation.

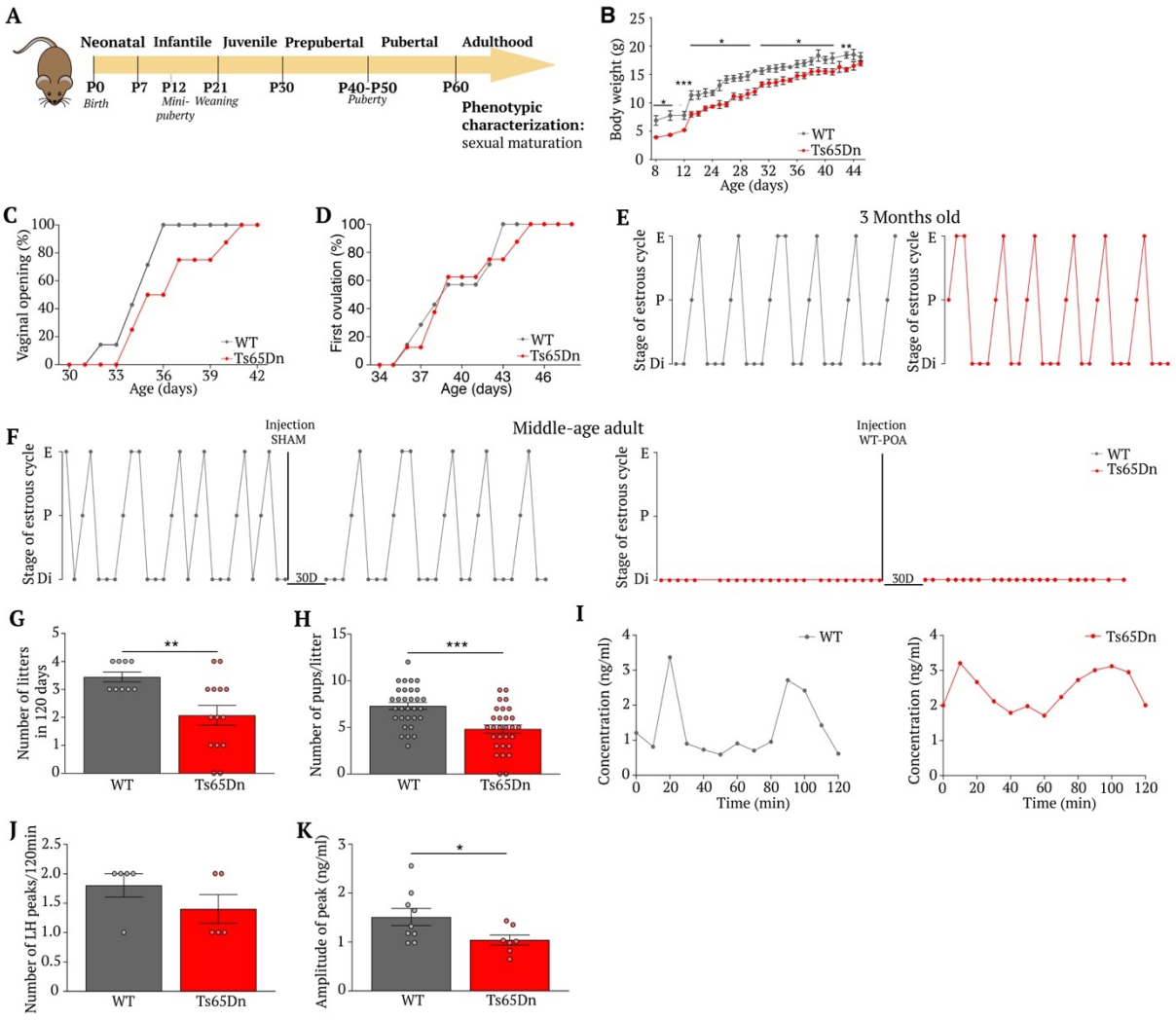
4.3.21 Sample size and randomization statement

Sample sizes for physiological and neuroanatomical studies and for miRNA and gene expression analyses were estimated based on past experience and those presented in the literature. Mice from at least three different litters from each group were used to study sexual maturation, fertility and used to perform quantitative RT-PCR analyses in cells isolated by FACS, anatomy and immunostaining. No randomization method was used to assign subjects in the experimental groups or to collect and process data.

4.3.22 Presentation of data and statistics

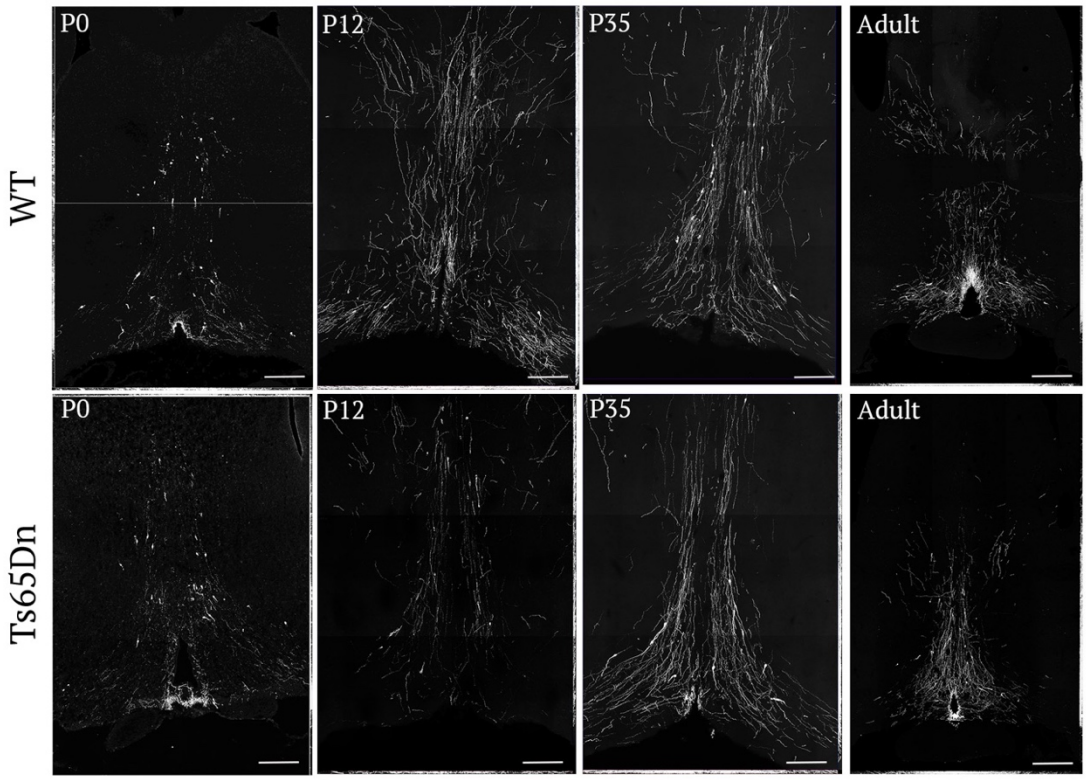
All statistical analyses were performed using Prism 8 (Graphpad software) and assessed for normality (Shapiro-Wilk test) and variance, when appropriate. Sample sizes were chosen according to standard practice in the field. Statistical differences were evaluated using unpaired/paired two-sided Student's *t*-tests for comparison of two groups and one- or two-way analysis of variance (ANOVA) with Tukey's post hoc tests for comparison of more than two groups. When the criterion for normality or equal variances was not met, the Mann-Whitney or Wilcoxon matched-pair test were used for comparison of two groups and the Kruskal-Wallis was used to compare more than two groups. For balanopreputial separation, vaginal opening and first ovulation, comparisons between groups were carried out using Gehan-Breslow-Wilcoxon matched-pairs test. The significance level was set at $p < 0.05$. The data are presented as means ± S.E.M. The *P* values are indicated in the figure legends.

4.4 Supplemental Figures and Tables

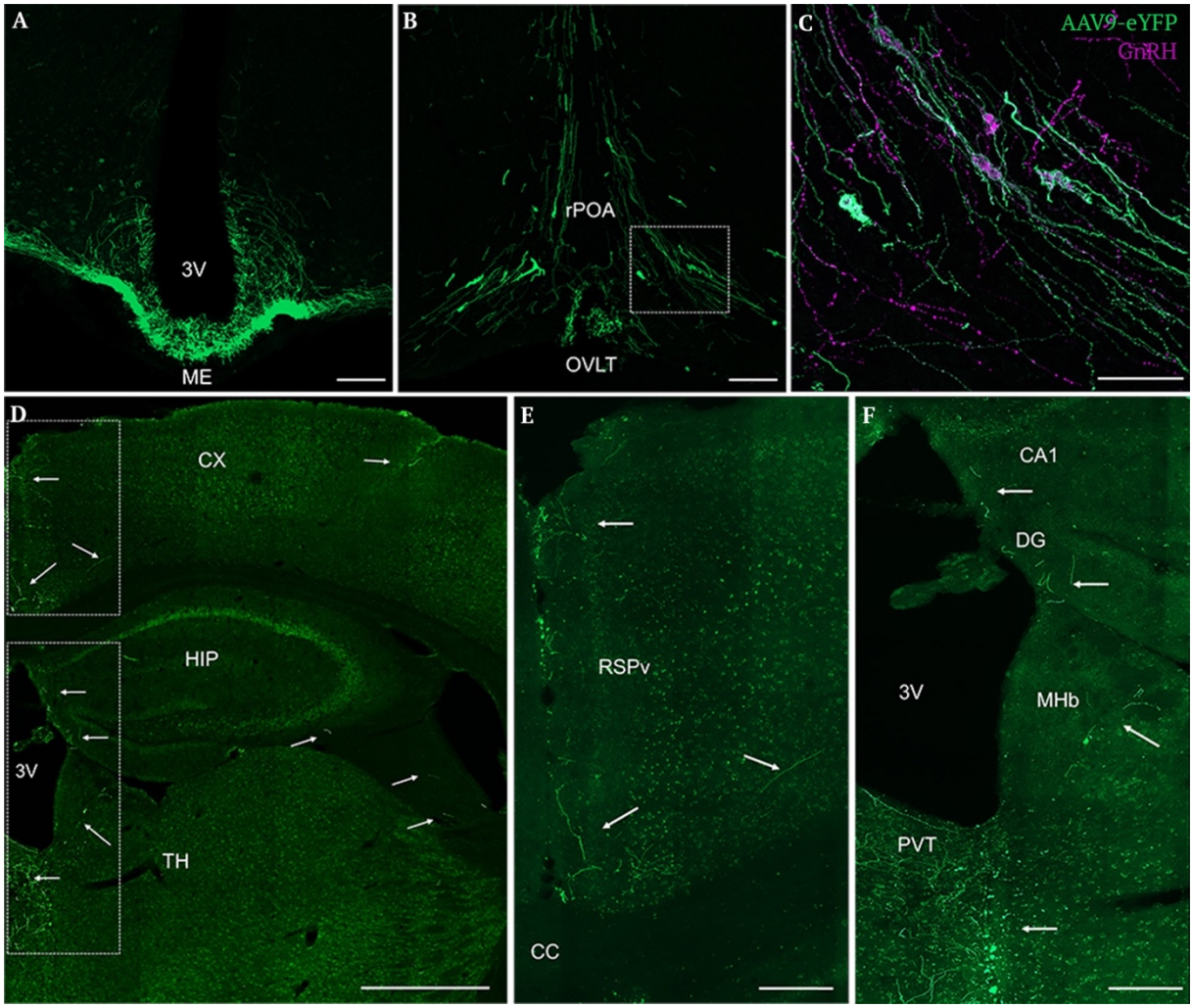


Supplemental Figure 1. Ts65Dn female mice show normal sexual maturation but subfertility and precocious ovarian failure. (**A**) Schematic representation illustrating the phenotypic characterization of reproductive maturation performed in Ts65Dn mice from the birth to adulthood. (**B**) Ts65Dn females presented significantly lower body weight gain during postnatal maturation and the pubertal transition. (**C,D**) A normal sexual maturation was observed in female Ts65Dn mice compared with wt littermates. Ts65Dn females exhibited a delay in vaginal opening (**C**), but not in the first estrus day (**D**). (**E**) Representative estrous cyclicity of 3-month old wt and Ts65Dn females during 28 consecutive days. (**F**) Representative estrous cyclicity of middle-age adult (12-month old) before and after the implantation of neonatal cells from brain tissue containing wt GnRH neurons in Ts65Dn females (WT-POA). Di, diestrus, P, proestrus; E, estrus. (**G,H**) Adult Ts65Dn females showed subfertility, showing fewer litters produced over 120-days period with fewer pups per litter. (**I**) Representative graphs for LH pulsatility assessment by serial blood sampling. Ts65Dn mice showed normal LH pulse frequency (**J**) and decreased LH pulse amplitude (**K**).

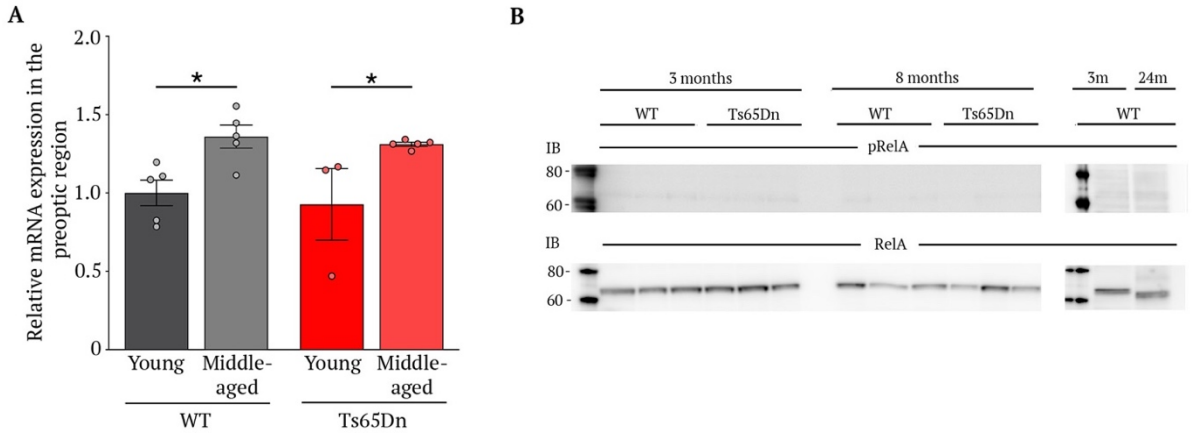
Significance was tested using multiple t-tests (**B**); Mann-Whitney (**G,J**); Unpaired t-test (**H,K**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



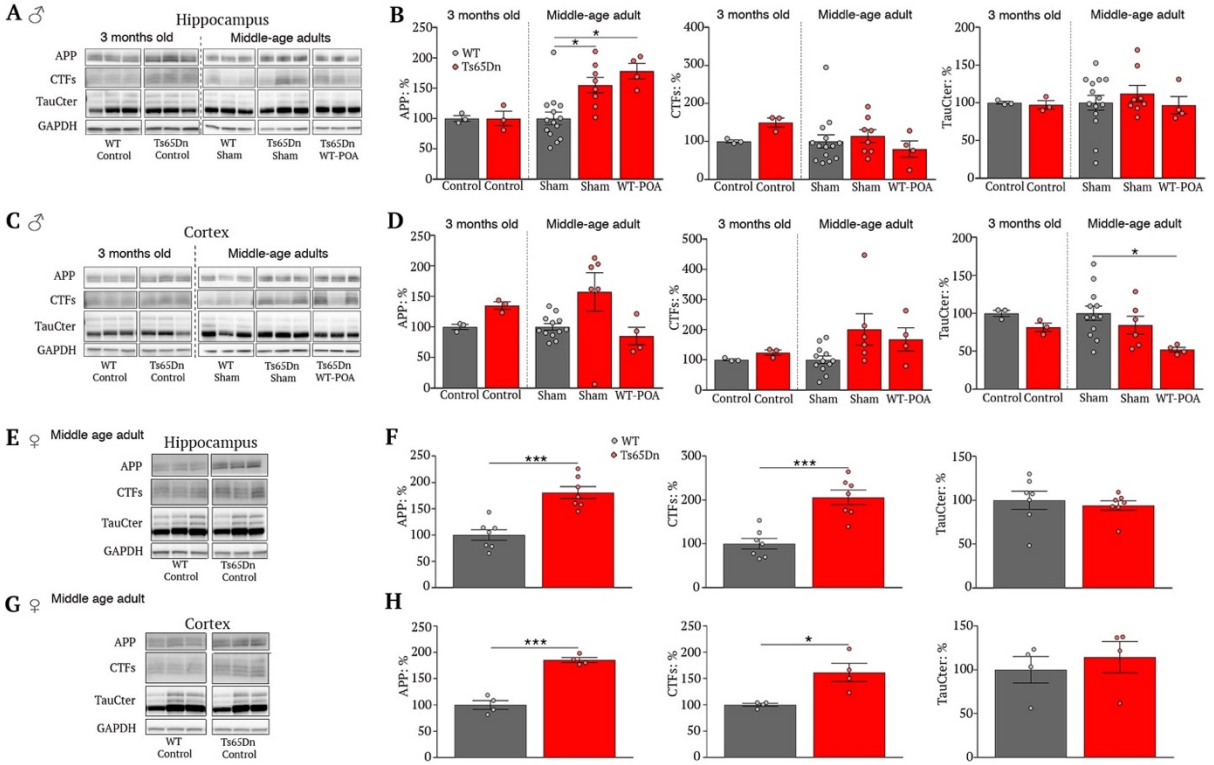
Supplemental Figure 2. Ts65Dn mice show an age-dependent loss of GnRH immunoreactivity. Representative photomicrographs of GnRH immunoreactivity taken from male Ts65Dn and wt mice littermates at different ages of the postnatal development. Neonatal (P0), minipuberty (P12), prepubertal (P35) and adult ages. Scale bars: 200 µm.



Supplemental Figure 3. Innervation in the extrahypothalamic regions by hypophysiotropic GnRH neurons in adult mice. (**A**) Cre-dependent viral tracing from the median eminence (ME) targeting the expression of eYFP (green) in hypophysiotropic GnRH neurons of *GnRH::Cre* mice. (**B**) Single injection of AAV9-eYFP induces the expression of eYFP in GnRH neurons located in the rostral preoptic area (rPOA) and in the organum vasculosum of the lamina terminalis (OVLT). (**C**) High-magnification of the frame in **B** showing colocalization between virally induced eYFP expression and immunodetection of GnRH (magenta) in the rPOA. (**D**) Visualization of AAV9-eYFP-expressing GnRH neuron fibers in the cortex (CX), hippocampus (HIP) and thalamus (TH). (**E**) High-magnification image shows the presence of AAV9-eYFP-transduced GnRH neuron fibers reaching the ventral layer of the retrosplenial (RSPv) region of the cortex. (**F**) High-magnification image shows the AAV9-eYFP-transduced GnRH neuron fibers in the dentate gyrus (DG) and CA1 layers of the HIP, in the medial habenula (MHb) and paraventricular thalamus (PVT). 3V, third ventricle. CX, cortex. CA, cornu ammonis. CC, corpus callosum. DG, dentate gyrus. HIP, hippocampus. ME, median eminence. MHb, medial habenula. OVLT, organum vasculosum laminae terminalis. PVT, paraventricular thalamus. rPOA, rostral preoptic area. TH, thalamus Scale bars: **D**, 500 µm; **A-B,E-F**, 100 µm; **C**, 50 µm.

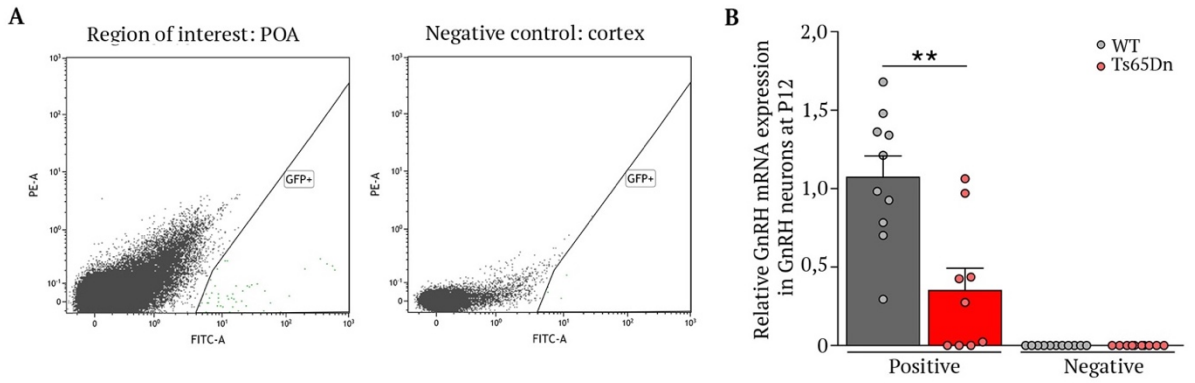


Supplemental Figure 4. Age-related expression of NF-kB in wt and trisomic littermates. (**A**) Real time PCR analysis showed an increase in the expression of Nf-κB1 transcripts in the preoptic area of the hypothalamus in middle-aged mice (8-12 months of age) when compared to young adults (3 months of age). (**B**) Protein analyses revealed an absence of Nf-κB activation both in the hippocampus of 3- and 8-months wt and Ts65Dn mice. To modulate gene expression, Nf-κB forms a dimeric transcription factor complex with RelA, the activity of which is regulated by phosphorylation. Significance was tested using Unpaired t-test or Mann-Whitney. * $p < 0.05$.



Supplemental Figure 5. Hippocampal and cortical APP, CTF and Tau-Cter expression levels in Ts65Dn mice. (**A,C**) Representative western blots showing hippocampal and cortical APP, CTF and Tau-Cter levels in 3-month old and middle age adults (8-12-months), grafted with POA from wt mice (wt-POA) or ungrafted (Sham), male Ts65Dn mice and wt. (**B,D**) Quantification of protein levels of APP, CTF and Tau-Cter in

hippocampus (**B**) and cortex (**D**) of males. (**E,G**) Representative WB showing hippocampal and cortical APP, CTF and Tau-Cter levels in 3-month old and middle age adult (12-months) female Ts65Dn mice. (**F,H**) Quantification of protein levels of APP, CTF and Tau-Cter in hippocampus (**F**) and cortex (**H**) of females. GAPDH was used as a loading control. Significance was tested using Kruskal Wallis or Mann-Whitney tests (**B**); Kruskal Wallis, Mann-Whitney or One-way ANOVA (**D**); Unpaired t-test (**F,H**). $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Supplemental Figure 6. Isolation of hypothalamic GnRH neurons in postnatal mice. (**A**) GnRH-GFP positive neuron isolation by FACS from preoptic area (POA). Cortex was used as negative control. (**B**) Real-time PCR analysis of GnRH mRNA expression in GFP-positive (POS) and negative (NEG) cells. performed when appropriate. Significance was tested using Mann-Whitney test. ** $p < 0.01$.

Primer	Sequence 5' → 3'	Genotype
65Dn-F	GTG GCA AGA GAC TCA AAT TCA AC	Ts65Dn
65Dn-R	TGG CTT ATT ATT ATC AGG GCA TTT	
OIMR7338	CTA GGC CAC AGA ATT GAA AGA TCT	
OIMR7339	GTA GGT GGA AAT TCT AGC ATC C	
GnRHCre-F	CTG GTG TAG CTG ATG ATC CG	*Gnrh::Cre*
GnRHCre-R	ATG GCT AAT CGC CAT CTT CC	
GnGFP-F1	GAA GTA CTC AAC CTA CCA ACG GAA G	*Gnrh::Gfp*
GnGFP-R1	GCC ATC CAG TTC CAC GAG AAT TGG	
Tox-F	CGT GTT CCA CTC GAA GAG TT	iBot
Tox-R	GGC AAA ACT TCA TTT GCA TT	

Supplemental Table 1. Primers used for genotyping.

Primary antibody	Animal	saturation	Secondary antibody
APPCter-C17 (1/10000)	Rabbit, polyclonal	Milk 5%	Rabbit (Sergeant et al., 2002)
TauCter 994 S4 (1/10 000)	Rabbit, polyclonal	Milk 5%	Rabbit (Buée-Scherrer et al., 1996)
GAPDH (1/50000) (Sigma)	Rabbit, polyclonal	Milk 5%	Rabbit (Vector)
ReIA, pRelA (1/500) (Cell Signaling, #8214)	Rabbit, polyclonal	BSA 5%	Rabbit (Buée-Scherrer et al., 1996)

Supplemental Table 2. Antibodies used for western blot analysis

Gene	TaqMan® Gene Expression Assays
Actb	Actb-Mm00607939_s1
Cebpb	Cebpb-Mm00843434_s1
Dicer1	Dicer1-Mm00521722_m1
Gnrh1	Gnrh1-Mm01315605_m1
Kiss1	*Kiss1*-Mm03058560_m1
Kiss1-r	*Kiss1r*-Mm00475046_m1
Nos1	*Nos1*-Mm01208059_m1
Nf-κB1	*Nfkb1*-Mm004763361_m1
Otx2	Otx2-Mm00446859_m1
Meis1	Meis1-Mm00487664_m1
sGC	Gucy1b3-Mm00516926_m1
Zeb1	Zeb1-Mm00495564_m1

miRNA	TaqMan® Gene Expression Assays
miR-141	mmu-miR-141-00463
miR-200a	mmu-miR-200a-000502
miR-200b	mmu-miR-200b-002251
miR-200c	mmu-miR-200c-002300
miR-429	mmu-miR-429-001077

Supplemental Table 3. Primers used for real-time PCR

CHAPTER 5

Defects in NOS1 activity cause GnRH deficiency in human and mice: evidence for reversal after NO treatment in infantile mice

5.1 Introduction

Pulsatile secretion of GnRH is critical for the activation of the HPG-axis, which controls pubertal onset and fertility. The HPG-axis is transiently activated during late fetal development and early infancy, dormant during childhood, and reactivated during the onset of puberty.

CHH is a rare genetic form of GnRH deficiency characterized by a failure of puberty and infertility. Notably, the transient activation of the HPG-axis during minipuberty is thought to be altered in CHH, although it has rarely been studied (Boehm et al., 2015). The consequences of altered minipuberty are largely unknown beyond defects in testicular descent and penis growth. The alteration of minipuberty could impact the timing of puberty and reproductive fitness (Kuiri-Hanninen et al., 2014), in the context of the early programming theory (Barker et al., 1989).

CHH is often associated with anosmia in approximately 50% of cases and is termed KS (Boehm et al., 2015). Other phenotypes such as sensorineural hearing loss (Pingault et al., 2013), skeletal defects (e.g. cleft palate or split-hand foot malformation [SHFM]) and cognitive or mental disorders (Aydogan et al., 2012, Lasaite et al., 2014) are also reported in CHH with variable frequencies.

The genetics of CHH is heterogeneous. Mutations in more than 30 genes, acting either alone or in combination, have been identified in 50% of cases (Cassatella et al., 2018). Inactivating mutations in GnRH (*GNRH1)* (Bouligand et al., 2009) or GnRH receptor (*GNRHR*) (de Roux et al., 1997) confirm the essential role of GnRH in reproduction. Further, mutations in other CHH genes (Boehm et al., 2015) have been critical to unraveling the complex biological processes affecting GnRH neuronal fate specification, migration during embryonic development and/or GnRH secretion/action in adulthood (Boehm et al., 2015).

The identification of inactivating mutations in genes encoding KP-1 (*KISS1*) (Topaloglu et al., 2012) and its receptor (*KISS1R*) (de Roux et al., 2003, Seminara et al., 2003) in CHH led to the breakthrough discovery of the KP system as a potent upstream activator of GnRH neurons (Herbison, 2016). Kisspeptin neurons are critical E_2-sensitive hypothalamic neurons that convey feedback loop from the gonads to GnRH neurons. Kisspeptin also directly acts on NO

synthase (*Nos1*) neurons, another estrogen-responsive neuron population regulating GnRH neurons through NO production (Hanchate et al., 2012, Chachlaki et al., 2017).

NO is involved in a wide range of biological processes in both humans and mice, including neuronal development and plasticity (Charriaut-Marlangue et al., 2013, Mandal et al., 2013). It exerts its effects by stimulating the production of cyclic guanosine monophosphate (cGMP) by binding to soluble guanylate cyclase. The duration and intensity of NO signaling are modulated by the activity of phosphodiesterases (PDE), which hydrolyze cGMP (Chachlaki et al., 2017). In the hypothalamus, neuronal NO acts on GnRH neurons as a strong inhibitory signal that integrates both metabolic and gonadal information (Chachlaki et al., 2017). In addition, *Nos1*-deficient mice exhibit infertility (Gyurko et al., 2002). We thus hypothesized that loss-of-function mutations in *NOS1* underlie GnRH deficiency by affecting key hypothalamic neuronal circuits controlling fertility.

Defects in NOS1 activity cause GnRH deficiency in human and mice: evidence for reversal after NO treatment in infantile mice

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5.2 Results

5.2.1 NOS1 distribution in the adult human hypothalamus

NOS1-expressing neurons were distributed widely in the adult human hypothalamus (**Supplemental Figure 1**). They intermingled and interacted morphologically with GnRH neurons at various sites, including the infundibulum (**Supplemental Figure 1a, Supplemental Figure 1e**). However, GnRH neurons consistently did not co-express NOS1. Both NOS1 and GnRH neurons received input from Kp neurons (**Supplemental Figure 1a-c**). Unexpectedly, a subset (11.4±3.0%) of Kp neurons was positive for NOS1 (**Supplemental Figure 1c**), a phenomenon not reported in rodents (**Supplemental Figure 1f**).

5.2.2 CHH patients harbor heterozygous NOS1 mutations

Based on the known biology of NOS1, we hypothesized that *NOS1* mutations are implicated in CHH pathogenesis. *NOS1* is a 29-exon gene encoding NOS1α, a 150 kDa protein consisting of 1434 aaa (RefSeq ID: NM_000620.4). NOS1α is the most commonly occurring isoform in the nervous system (Chachlaki et al., 2017). Eight heterozygous missense rare sequence variants (RSV), mapping to highly conserved amino-acids of *NOS1* and predicted to be "likely damaging", were identified in 11 (8%) unrelated probands through exome sequencing (**Supplemental Table 1; Figure 1, Supplemental Figure 2**). These variants span the oxygenase domain, as well as the C-terminal reductase domains including the flavodoxin-like and ferrodoxin reductase-type FAD-binding domains (**Figure 1a**) but spare the N-terminal PDZ domain critical for protein-protein interactions. p.Asp241Asn lies within the PIN (inhibiting protein NOS1)-binding region, which is involved the dimerization of NOS1. p.Arg260Gln, p.Phe262Val, p.Gly595Ser, p.Met619Leu are all located on the oxygenase domain, part of the catalytic domain of the enzyme. Substitution of Gly595, by a polar serine is predicted to affect the particular folding of NOS1. p.Gly864Asp, p.Glu1124Lys and p.Ile1223Met lie within the reductase domains; p.Gly864Asp is located on the binding site for the flavin mononucleotide (FMN) within the flavodoxin-like domain, while both p.Glu1124Lys and p.Ile1223Met are located on the FAD-binding domain of the protein (**Figure 1a,b**).

Two *NOS1* mutations were identified in multiple unrelated probands: the heterozygous p.Gly595Ser in two KS and one normosmic CHH (nCHH) and p.Asp241Asn in two KS patients. The other 6 mutations were found in 3 KS and 3 nCHH probands (**Table 1**). The clinical

characteristics of affected patients are illustrated in **Table 1**. As expected, the majority of probands were male (8/11). While one patient exhibited partial puberty and subsequent CHH reversal (H:II-1), the remaining patients had absent puberty. In addition, 4 out of 8 males also reported a history of cryptorchidism and/or micropenis, suggesting severe GnRH deficiency with an altered minipuberty. All probands inherited their mutations from unaffected or partially affected parents (**Supplemental Figure 2b**). Interestingly, the proband harboring the p.Met619Leu *NOS1* mutation (A:II-1) exhibits nCHH and inherited the mutation from his father with constitutional delay of growth and puberty (CDGP), a transient form of GnRH deficiency (I-1). The mutation was also found in his two brothers (II-2 and II-4) with CDGP. Notably, his mother (I-2) and his older brother (II-1) also have CDGP but are not carriers, suggesting oligogenic inheritance in this pedigree. In addition, family H includes monozygotic twin sisters with KS carrying the p.Gly864Asp mutation inherited from their father with CDGP. The two sisters also exhibited neuro-developmental defects (mental retardation and speech delay, and neuro-sensoral hearing loss). In total, 3 probands (Family A, B, and G) exhibited neuro-sensoral hearing loss and three probands (Family B, C, and K) had mental retardation. Detailed pedigrees and clinical summaries of all probands are described in the **Supplemental Figure 2b**.

Family	Subject N°	Diagnosis	Heterozygous NOS1 mutations	Sex	Inheritance	Puberty	Associated phenotypes
A	II-3	nCHH	p.Met619Leu	M	F	A	**sensorineural hearing loss** cleft lip/palate oligodontia
B	II-2 II-3 (monozygotic twins)	KS	p.Gly864Asp	F	F	A	**sensorineural hearing loss** **intellectual disability** tooth agenesis speech delay heart defects
C	II-1	KS	p.Asp241Asn	M	S	A	**intellectual disability**
D	II-1	KS	p.Asp241Asn	M	S	A	synkinesia marfan's syndrome clinodactyly
E	II-1	KS	p.Arg260Gln	M	F	A	supernumerary tooth
F	II-1	nCHH	p.Phe262Val	M	S	A	none
G	II-1	KS	p.Gly595Ser	M	S	A	**sensorineural hearing loss** bilateral short clavicule
H	II-1	nCHH*	p.Gly595Ser	M	F	P	none
I	II-1	KS	p.Gly595Ser	M	S	A	none
J	II-1	KS	p.Glu1124Lys	F	F	A	scoliosis
K	II-1	nCHH	p.Ile1223Met	F	S	A	**intellectual disability**

Table 1. Clinical phenotype of CHH probands harbouring heterozygous *NOS1* mutations. CHH, Congenital hypogonadotropic hypogonadism; nCHH, normosmic CHH; KS, Kallmann syndrome; nCHH*, nCHH with

reversal. Amino acid changes are based on reference cDNA sequence NM_000620.4; Sex: M, male; F, female; Inheritance; S, sporadic; F, familial; Puberty: A, absent puberty; P, partial puberty. Associated phenotypes highlighted in bold correspond to the hearing and cognitive defect seen in *Nos1* knock-out mice.

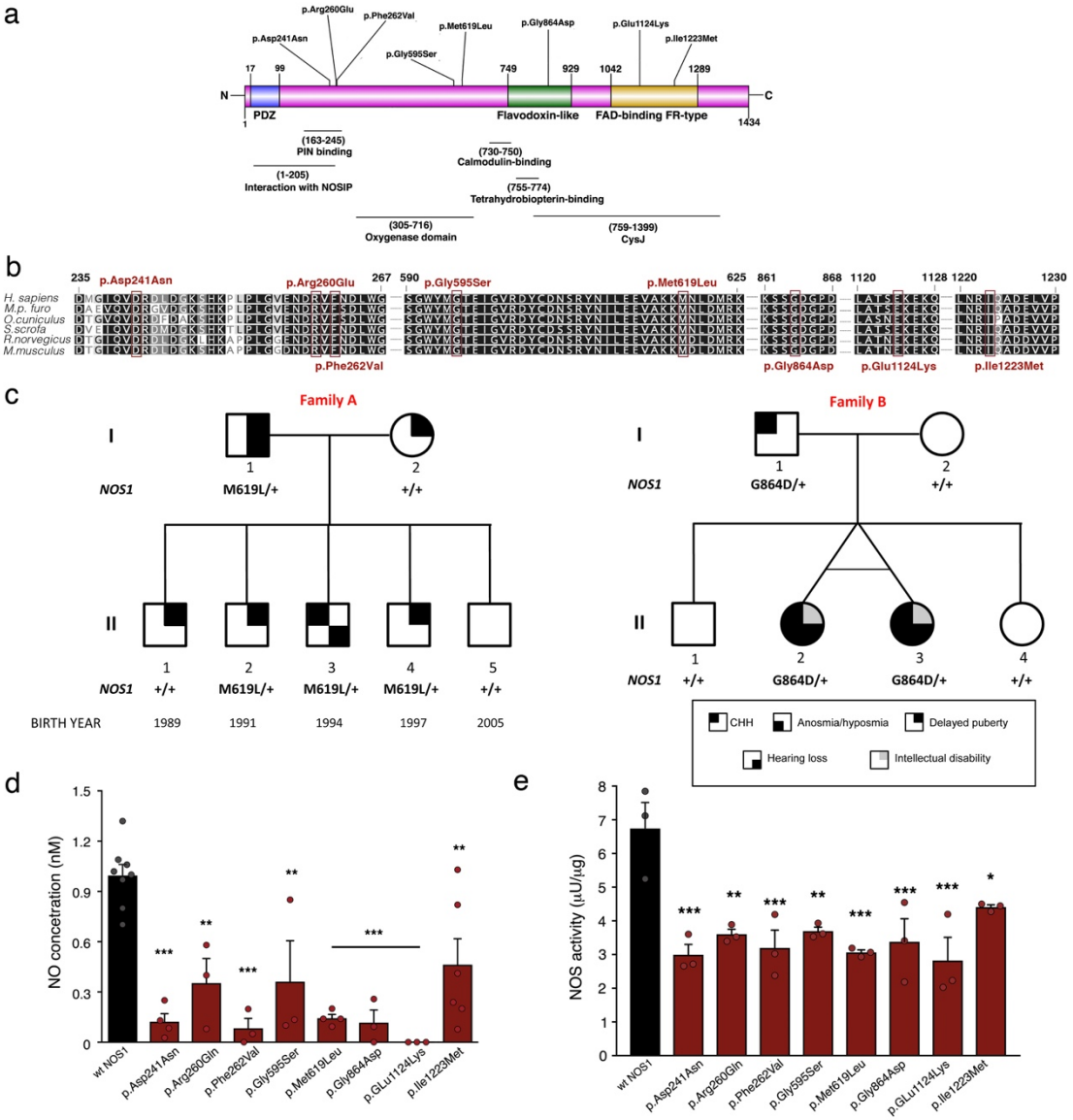


Figure 1. Identification of *NOS1* mutations in CHH and KS probands (**a**) Schematic figure showing the position of the identified mutations on the human neuronal nitric oxide synthase (NOS1). *NOS1* mutations are mostly found localized on the oxygenase (p.Gly595Ser, p.Met619Leu) and the reductase domains (CysJ, flavodoxinJ, ferrodoxin; pGly864Asp, p.Glu1124Lys and p.Ile1223Met) of the protein. One of the mutations is located on the region permitting interaction with the PIN protein family (p.Asp241Asn). (**b**) Conservation of the *NOS1* domains among human (*H.sapiens, P29475*), ferret (*M.p.furo, M3XUN6*), rabbit (*O.cuniculus, O19132*), pig (*S.scrofa, F1RKF2*), rat (*R.novegicus, D3ZEW7*) and mouse (*M. musculus, Q9Z0J4*) as well as the localization of the mutated aaa on the protein. (**c**) Pedigrees of CHH probands harboring *NOS1* mutations. (**d**) Measurement of the [NO] upon endogenous stimulation of the NO signaling pathway in NO-detector

cells (FlincG3 -transfected HEKGC/PDE5) expressing the wt or mutated NOS1 protein. Mutants are compared to wt values (one-way ANOVA with Dunnett's post-hoc test). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Values indicate means ± SEM. (**e**) Measurement of the enzymatic activity (μU/μg) of the NOS1 protein as this was detected in HEK GC/PDE5 cells expressing the wt or the mutated NOS1 protein, using a commercially available kit (each dot represents an independent experiment). Mutants are compared to wt values (one-way ANOVA with Dunnett's post-hoc test). Values indicate means ± SEM.

5.2.3 NOS1 mutations are loss-of-function

The maximal NO release by cells transiently expressing mutated *NOS1* was significantly attenuated compared to cells transfected with the wt plasmid for all reported mutations (**Figure 1d, Supplemental Figure 3**), suggesting decreased NOS1 activity. Additionally, an alternative method using a fluorescent probe to measure the concentration of nitrates confirmed that all mutants exhibited decreased enzymatic activity (**Figure 1e**).

5.2.4 NOS1 facilitates GnRH neuronal migration

NOS1 mutations underlie nCHH and KS. Further, NO is implicated in regulating neuronal migration in the brain during mouse embryogenesis (Mandal et al., 2013). We thus explored a possible involvement of NOS1 in GnRH neuronal migration. First, we examined NOS1 expression during prenatal development using IHC. We observed, unexpectedly, that NOS1 and GnRH were co-expressed in migrating GnRH neurons in the nose of human fetuses (**Supplemental Figure 4a**) and mouse embryos (**Supplemental Figure 4b**)**.** However, GnRH neurons were devoid of NOS1 expression once they reached the forebrain region in both developing humans and mice (**Supplemental Figure 4a,b**), consistent with a tightly regulated spatio-temporal action of NO along the migratory path of GnRH neurons. Second, to investigate the putative role of NOS activity in GnRH neuronal migration, we induced a transient and site-specific inhibition of NO production by infusing the NOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) locally into the nasal region of wt mouse embryos on E12.5, the time when GnRH cells are just starting to enter the rostral forebrain (**Supplemental Figure 4c**). In contrast to the vehicle-treated group (**Supplemental Figure 4d,e**), blunting NO production at E12.5 resulted in a major defect in the migratory process (**Supplemental Figure S4d,e**); at E14.5, the vast majority of GnRH neurons, which formed part of Nos1-immunolabeled aggregates, were backed up in the region of the nose where GnRH neurons

temporarily stop before entering into the brain during normal migration (**Supplemental Figure 4d, e).**

5.2.5 Altered sensory and cognitive performance in NOS1-deficient mice

The presence of associated phenotypes like anosmia, hearing loss and mental retardation in CHH patients harboring heterozygous *NOS1* mutations (**Table 1**) led us to evaluate these traits in a mouse model lacking exon 2 of *Nos1* (*Nos1*$^{-/-}$ mice) (Huang et al., 1993), in which Nos1 activity is markedly impaired yet retains some residual activity.

Olfaction. We evaluated the ability of *Nos1*$^{-/-}$ mice to detect and differentiate various social and non-social odors. During the social odor discrimination test, both *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ mice failed to be attracted by volatile urine odors of the opposite sex (**Figure 2a**). During the habituation/dishabituation test, *Nos1*$^{-/-}$, but not *Nos1*$^{+/-}$, mice were able to discriminate novel non-social odors (**Figure 2b**). However, *Nos1*$^{-/-}$ mice exhibited a discrete hyper-reactivity to new stimulus (**Figure 2b**), suggesting a decreased effectiveness in encoding olfactory information, similar to what has been shown in premature infants during a visual habituation-dishabituation task (Kaplan et al., 1986, Kavsek et al., 2010). In summary, the alteration of sense of smell in *Nos1*-deficient mice consists of impairment in olfactory processing without gross defects in odor detection.

***Cognition*.** Both *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ mice demonstrated defective cognitive performance compared to wt littermates in the NOR test (**Figure 2c**).

***Hearing*.** We studied hearing in *Nos1*-deficient mice by measuring distortion-product otoacoustic emissions. Male, but not female, *Nos1*$^{-/-}$ mice exhibited defects in the auditory pathway at the level of the cochlear-nucleus as shown by an increased latency in the auditory brainstem-evoked response (ABR) wave II (**Figure 2d**), and the significant mean threshold elevations of 18.9 dB at 40 kHz (**Figure 2e**).

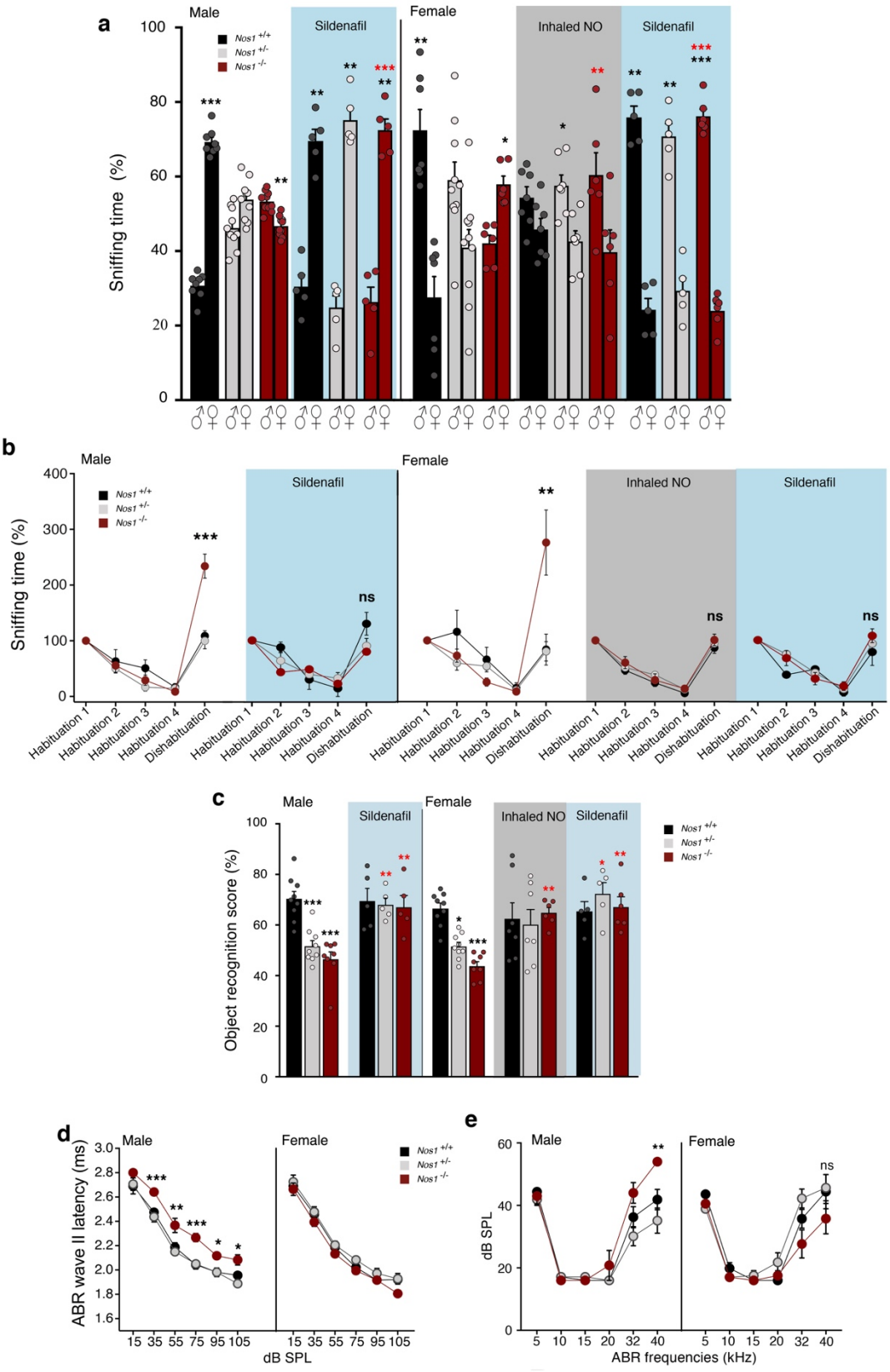

a
Male
Female
Sildenafil
Inhaled NO
Sildenafil
Nos1 +/+
Nos1 +/-
Nos1 -/-
Sniffing time (%)
b
Male
Female
Sildenafil
Inhaled NO
Sildenafil
Sniffing time (%)
ns
Habituation 1
Habituation 2
Habituation 3
Habituation 4
Dishabituation
c
Male
Female
Sildenafil
Inhaled NO
Sildenafil
Object recognition score (%)
d
Male
Female
ABR wave II latency (ms)
dB SPL
e
Male
Female
dB SPL
ns
ABR frequencies (kHz)

Figure 2. Behavioral tests in *Nos1*-deficient mice: olfaction, cognition and hearing. (**a**) During the social olfactory preference test *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ male and female mice were exposed to urine samples from an adult C57BL6/J WT stud male and estrous female for 30 min. *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ male and female mice failed to discriminate between volatile urine odors of the same or the opposite sex in contrast to the *Nos1*$^{+/+}$ mice tested. Interestingly, inhaled NO treatment (grey shaded area), and Sildenafil treatment (blue shaded area) during the infantile period from P10 to P23 rescued the olfactory deficit in the *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ female mice. Black stars indicate the preference of each group for male versus female odor, (paired t-test). Red stars indicate a comparison between mice of the same sex and genotype using as a changing parameter the treatment that was performed (unpaired t-test for males and one-way ANOVA with Dunnett's post-hoc test for females). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Values indicate means ± SEM. (**b**) During the habituation/dishabituation test using non-social odors (acetophenone and octanal) we assessed the ability of *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ male and female mice to differentiate between different odors. *Nos1*$^{-/-}$ mice seemed capable of discriminating the novel odorant, yet they showed a discrete hyper-reactivity to the new stimulus. Inhaled NO treatment (grey shaded area), and Sildenafil treatment (blue shaded area) during the infantile period from P10 to P23 normalized the dishabituation levels in the *Nos1*$^{-/-}$ male and female mice. *Nos1*$^{+/+}$ values during the stage of dishabituation are compared to the ones of *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice for each group of measurements (one-way ANOVA with Dunnett's post-hoc test) * $p < 0.05$; *** $p < 0.001$; ns $p > 0.05$. Values indicate means ± SEM. (**c**) Recognition memory was assessed using the novel object recognition test. *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ male and female mice showed a defective cognitive performance when compared to the *Nos1*$^{+/+}$ mice tested. Inhaled NO (grey shaded area) or Sildenafil (blue shaded area) treatment during the infantile period rescued the cognitive performance in the female *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ mice. *Nos1*$^{+/+}$ values are compared to the ones of *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice for each group of measurements (Kruskal-Wallis test with Dunn's post-hoc test). Red stars indicate a comparison between mice of the same genotype using as a changing parameter the treatment that was performed (Mann-Whitney test for males and Kruskal-Wallis test with Dunn's post-hoc test for females)* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *Nos1*$^{-/-}$, or *Nos1*$^{+/-}$ versus *Nos1*$^{+/+}$ mice and between indicated groups. (**d, e**) We examined the involvement of Nos1 in hearing by measuring (d) latencies at the cochlear-nucleus level (distortion-product otoacoustic emmissions were identical in all mice), as well as (e) the auditory brainstem-evoked response (ABR) thresholds in *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ male and female mice. Note that *Nos1*$^{-/-}$ male mice exhibited increased latency (d), in contrast to the *Nos1*$^{-/-}$ females who seemed to be "protected" (**e**). At 40 kHz the ABR recordings from the *Nos1*-/- male mice presented significant mean threshold shifts, in contrast to their female littermates who presented negative shifts that however did not reach significance. *Nos1*$^{+/+}$ values are compared to the ones of *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice for each group of measurements (two-way ANOVA with Dunnett's post-hoc test). * $p < 0.05$; *** $p < 0.001$; ns $p > 0.05$ *Nos1*$^{-/-}$ versus *Nos1*$^{+/+}$ mice and between indicated groups. Values indicate means ± SEM.

5.2.6 Infantile NOS1 activity shapes minipuberty

While it is known that *Nos1*$^{-/-}$ mice exhibit central reproductive defects and infertility (Gyurko et al., 2002), the underlying mechanisms are largely unknown. In a recent study, we demonstrated that NOS1 activity significantly increases in the preoptic region including the OVLT during the infantile period (Messina et al., 2016), a time known to be crucial for the establishment of the GnRH neural network (Prevot, 2015, Chachlaki et al., 2017). The majority of hypothalamic Nos1-expressing neurons in mice lie in the OVLT region (Chachlaki et al., 2017) where GnRH neuronal cell bodies and dendrites also reside (Chachlaki et al., 2017). Herein, we measured the levels of immunoreactivity for phosphorylation-activated Nos1 (P-Nos1) in the OVLT during several different developmental stages: neonatal P7), infantile (P10 and P12) and post-weaning (P23) (**Figure 3a, Supplemental Figure 5**). The proportion of Nos1 neurons expressing P-Nos1 increased significantly at P12 (**Figure 3a**), the day of the infantile period on which circulating FSH levels culminate in minipuberty in mice (Messina et al., 2016). Thereafter, P-Nos1 remained expressed in the OVLT (**Figure 3a**). To determine whether this infantile increase in P-Nos1 expression could be linked to the FSH-induced estrogen output from the ovaries (Kumar et al., 1997), we analyzed P-Nos1 expression in P23 wt mice after OVX at P12 and found a striking loss of P-Nos1 immunoreactivity in the OVLT (**Figure 3b, Supplemental Figure 5a**) as well as in the hippocampus (36.2±5.9% *vs.* 10.4±2.4% P-Nos1-immunoreactive Nos1 neurons, n=3 and 4 per group, respectively, *P*=0.006; Figure S5c). The fact that the increased activity of Nos1 coincides with the awakening of the GnRH axis points toward a putative role of NO in shaping minipuberty.

Recent data implicated infantile NO in the transcriptional regulation of GnRH expression at the level of the promoter during minipuberty (Messina et al., 2016). We thus studied GnRH expression in *Nos1*-deficient mice at P12 using real-time PCR analyses on GnRH neurons after fluorescent-activated-cell-sorting and found a 4-fold increase in GnRH transcripts when compared to wt littermates (**Figure 3b**). This increase was associated with increased spontaneous firing by infantile GnRH neurons during the third week of life (**Figure 3c,d, Supplemental Figure 6**) consistent with an increased release of GnRH, as shown by the elevated FSH levels in *Nos1*-deficient female mice at P12 (**Figure 3e**). Strikingly, while post-weaning levels of FSH reached their nadir in wts at P23, they remained abnormally high in *Nos1*-deficient mice (**Figure 3e**). Combined, these results suggest that increased Nos1 activity

during minipuberty is required for the onset of gonadal steroid negative feedback loop and the repression of the HPG-axis at the end of minipuberty.

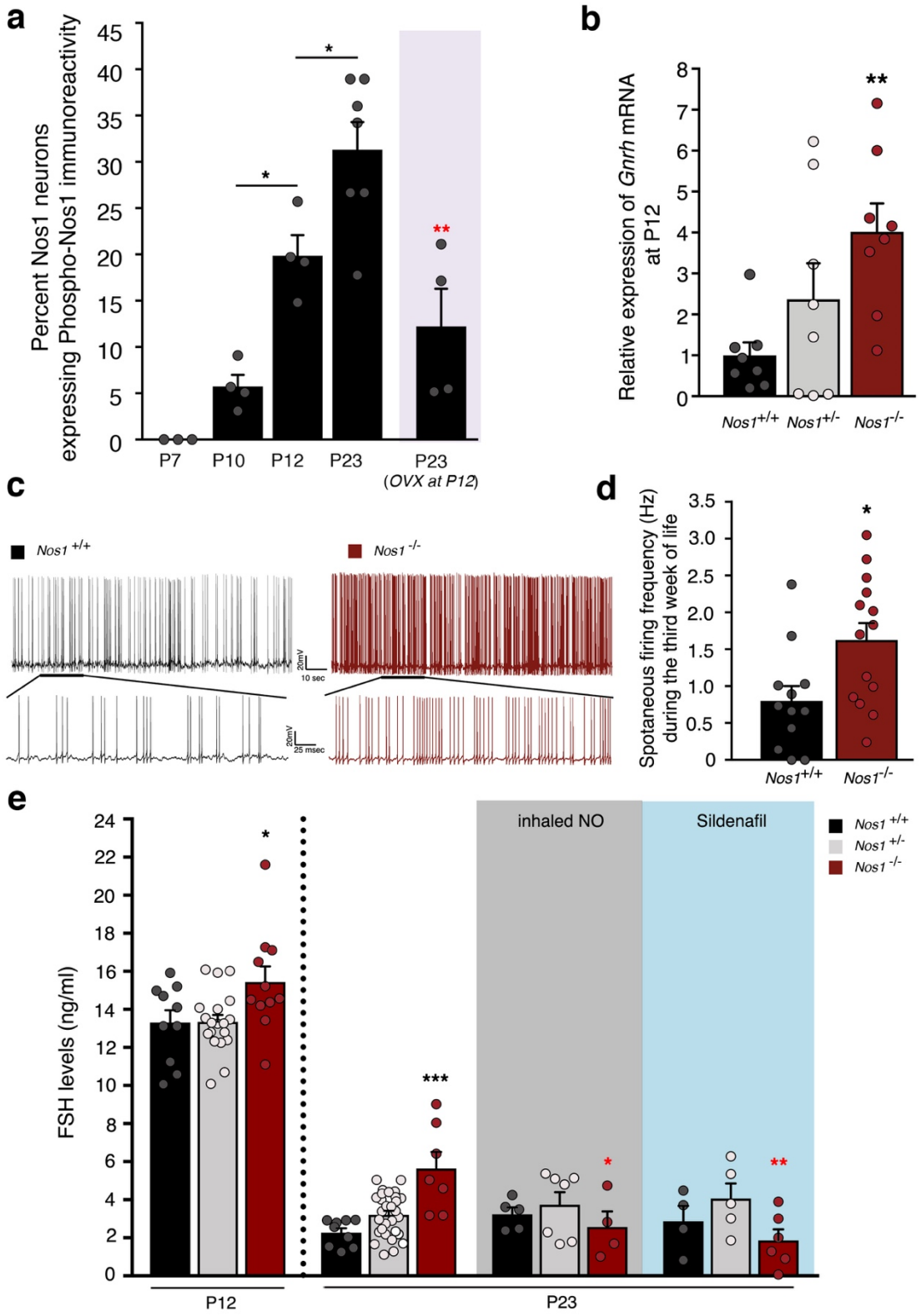


Figure 3. Nos1 activity controls infantile GnRH neuronal function. (**a**) Progressive phosphorylation of Nos1 during postnatal development in the region of the organum vasculosum laminae terminalis (OVLT). Ovariectomy (OVX) at P12 (P12) leads to significantly decreased phosphorylation levels of Nos1 at P23. Bar graphs illustrate the mean ratio of the Nos1-immunoreactive positive pixels to that of p-Nos1-immunoreactive positive pixels. The pNos1 levels are compared across all developmental stages (one way ANOVA with Tukey's post-hoc test). The values after ovariectomy at P12 are independently compared to the P23 values (unpaired t-test). * $p < 0.05$; ** $p < 0.01$; ns $p > 0.05$. Values indicate means ± SEM. (**b**) RT-PCR analysis of the expression of *Gnrh* in FACS-isolated GnRH-GFP neurons from *Gnrh::Gfp; Nos1*$^{+/+}$, *Gnrh::Gfp; Nos1*$^{+/-}$ and *Gnrh::Gfp; Nos1*$^{-/-}$ bigenic mice at P12. In the absence of Nos1 there is a significant increase in the *Gnrh* mRNA content. *Gnrh::Gfp; Nos1*$^{+/+}$ values are compared to the ones of *Gnrh::Gfp; Nos1*$^{+/-}$ and *Gnrh::Gfp; Nos1*$^{-/-}$ mice (one-way ANOVA with Dunnett's post-hoc test) * $p < 0.05$; ** $p < 0.01$; ns $p > 0.05$. Values indicate means ± SEM. (**c, d**) Electrophysiological recordings of the spontaneous activity of preoptic area GnRH neurons in late infantile *Gnrh::Gfp; Nos1*$^{+/+}$ and *Gnrh::Gfp; Nos1*$^{-/-}$ bigenic mice (P14-P21). (c) Representative trace of spontaneous firing in a GnRH neuron from a *Nos1*$^{+/+}$ (left panel) and *Nos1*$^{-/-}$ (right panel) animal. The lower trace shows a small region of the top trace at an expanded time scale. (d) Quantitation of spontaneous firing frequency in GnRH neurons from *Gnrh::Gfp; Nos1*$^{+/+}$ and *Gnrh::Gfp; Nos1*$^{-/-}$ mice. GnRH neurons demonstrate increased firing frequency in the absence of NOS1 activity. *Gnrh::Gfp; Nos1*$^{+/+}$ values are compared to the ones of *Gnrh::Gfp; Nos1*$^{-/-}$ mice (unpaired t-test). * $p < 0.05$; ** $p < 0.01$; ns $p > 0.05$. Values indicate means ± SEM. (**e**) FSH levels at P12 and P23 in *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ female mice. *Nos1*$^{-/-}$ female mice show increased FSH values comparing to the *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ mice at P12 and P23. Inhaled NO (grey shaded area) or Sildenafil (blue shaded area) treatment during the infantile period lead to the normalization of the *Nos1*$^{-/-}$ FSH levels at P23. FSH *Nos1*$^{+/+}$ values are compared to the ones of *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice for each group of measurements (one-way ANOVA with Dunnett's post-hoc test). Red stars indicate a comparison between mice of the genotype using as a changing parameter the treatment that was performed (one-way ANOVA with Dunnett's post-hoc test).* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Values indicate means ± SEM.

5.2.7 Inhaled NO rescues infertility

Because pharmacologically-induced NO deficiency in wt mice between P10 and P21 (**Figure 4a-d, Supplemental Figure 7a**), but not between P7 and P12 (**Supplemental Figure 7b**), mimics the reproductive phenotype of *Nos1*-deficient mice (**Figure 6e,f,h**), we next investigated whether inhaled NO during P10-P23 critical period could improve the reproductive phenotypes of the *Nos1*-deficient mice. Infantile inhaled NO not only rescued vaginal opening (**Figure 4e**) and pubertal onset (**Figure 4f**), but also normalized estrous cyclicity in adulthood in *Nos1*$^{-/-}$ females (**Figure 4h**). Because the *Nos1*$^{-/-}$ mouse model used in this study exhibits

some residual Nos1 activity (Huang et al., 1993), we next determined whether treating these mice with Sildenafil, a selective inhibitor of cGMP-specific PDE5 commonly used in neonates as an alternative to inhaled NO (Lakshminrusimha et al., 2016), could also rescue at least part of the phenotype of these mice. Daily Sildenafil injections between P10 and P23 significantly improved sexual maturation in *Nos1*$^{-/-}$ mice as shown by the assessment of balano-preputial separation in males (**Figure 4g**) and sexual maturation and estrous cyclicity in females (**Figure 4e,f,h**).

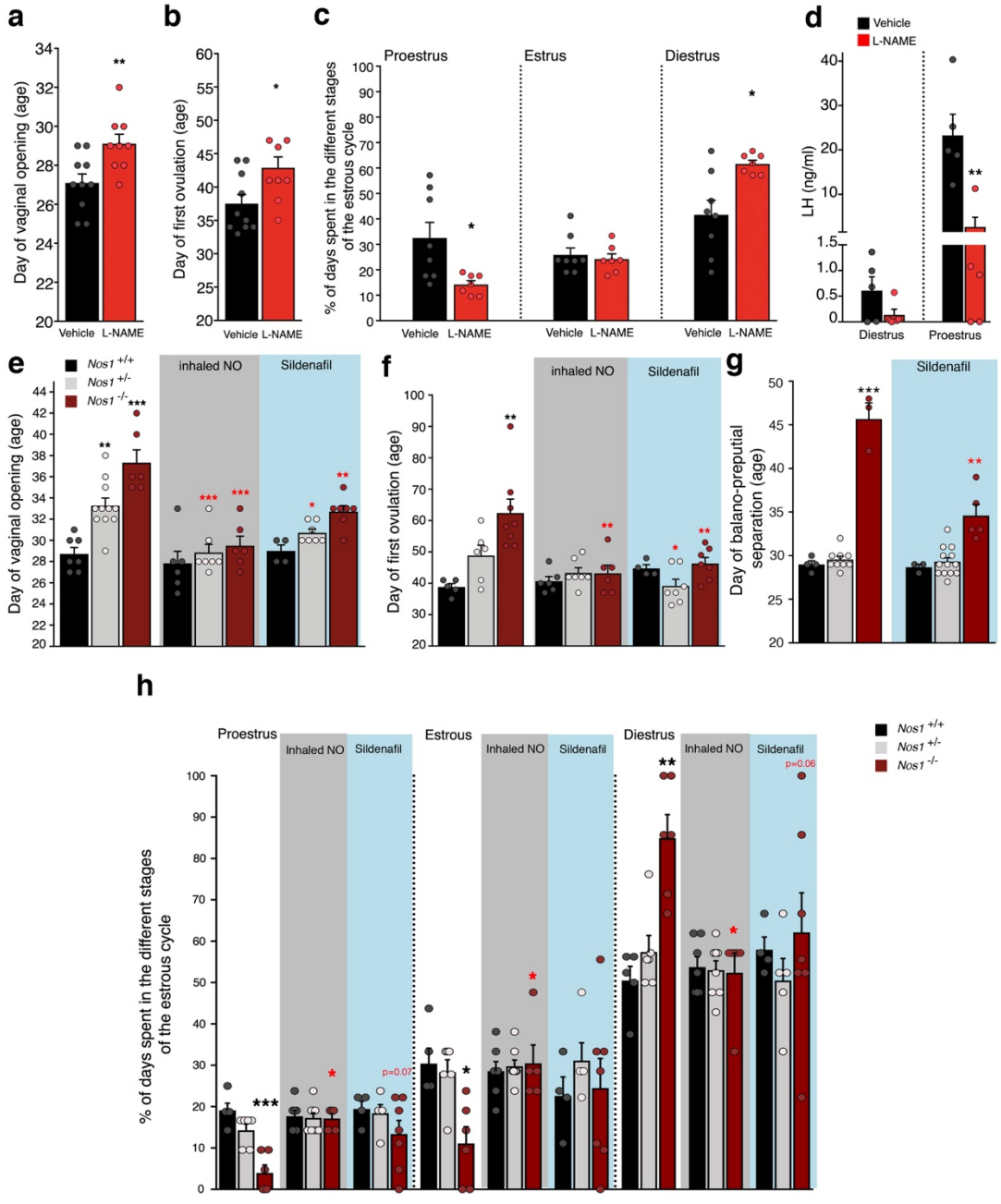


Figure 4. The action of NO during the critical infantile period is required for establishing a sexually mature phenotype. (**a-d**) Inhibition of NO synthesis by the daily injection of the NOS inhibitor L-NAME during the infantile period (P10-P21) resulted in a delay of (**a**) vaginal opening, (**b**) age at puberty, as well as abnormal (**c**) adult estrous cyclicity and (**d**) LH levels. Vehicle-treated animals are compared to L-NAME injected mice (unpaired t-test).* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Values indicate means ± SEM. (**e-h**) Inhaled NO (grey shaded area) and Sildenafil (blue shaded area) treatment during the infantile period (P10-P23) rescue the delay of the *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice in (**e**) vaginal opening and (**f**) age at puberty and (**g**) balano-preputial separation in males, while it normalizes the (**h**) adult estrous cyclicity in females. *Nos1*$^{+/+}$ values are compared to the ones of *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice for each group of measurements (one-way ANOVA with Dunnett's post-hoc test). Red stars indicate a comparison between mice of the same genotype using as a changing parameter the treatment that was performed (unpaired t-test for males and one-way ANOVA with Dunnett's post-hoc test for females) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Values indicate means ± SEM.

5.2.8 Inhaled NO rescues olfaction and cognition

Administration of inhaled NO during infancy in *Nos1*-deficient mice also restored olfaction (**Figure 2a,b**) and cognition in adulthood (**Figure 2c**), demonstrating that these neuro-developmental impairments are at least partially caused by Nos1 deficiency. Interestingly, PDE5 inhibitor treatment during the infantile period also enabled the rescue of the olfactory and cognitive phenotypes in *Nos1*$^{-/-}$ and *Nos1*$^{+/-}$ mice (**Figure 2a-c**).

5.3 Material and methods

5.3.1 Patients

The CHH cohort included 180 probands (105 KS and 75 nCHH). The diagnosis of CHH was made on the basis of: i) absent or incomplete puberty by 17 years of age; ii) low/normal gonadotropin levels in the setting of low serum T/E_2 levels; and iii) otherwise normal anterior pituitary function and normal imaging of the hypothalamic-pituitary region (Boehm et al., 2015). Olfaction was assessed by self-report and/or formal testing (Lewkowitz-Shpuntoff et al., 2012). When available, family members were included for genetic studies. This study was approved by the Institutional Review Board of Partners Healthcare and the ethics committee of the University of Lausanne. All participants provided written informed consent prior to study participation.

5.3.2 Genetic analyses

Genomic DNA was extracted from peripheral-blood samples using the Puregene Blood Kit (Qiagen), following the manufacturer's protocol. Exome capture was performed using the SureSelect All Exon capture v2 and v5 (Agilent Technologies, Santa Clara, CA USA) and sequenced on the HiSeq2500 (Illumina, San Diego CA USA) at BGI (BGI, Shenzen, PRC). Raw sequences (fastq files) were analysed using an in-house pipeline that utilizes the Burrows-Wheeler Alignment algorithm (BWA) (Li et al., 2009) for mapping the reads to the human reference sequence (GRCh37), and the Genome Analysis Toolkit (GATK) (DePristo et al., 2011) for the detection of single nucleotide variants (SNVs) and insertion/deletions (Indels). The resulting variants were annotated using SnpEff version 4.0 (Cingolani et al., 2012) and dbNSFP version 2.9 (Liu et al., 2013) to calculate minor allele frequency (MAF). Only variants with MAF<0.5% were used for subsequent analysis. Variants were confirmed by Sanger sequencing on both strands with duplicate PCR reactions, and are described according to HGVS nomenclature (den Dunnen et al., 2000).

Forty-four probands out of a cohort of 180 CHH harbored pathogenic or likely pathogenic variants in the known CHH genes (according to ACMG guidelines (Richards et al., 2015), see **Supplemental Appendix**), and were excluded from subsequent analyses. The remaining 136 probands were evaluated for RSVs in *NOS1*. Position-specific evolutionary preservation tool (PANTHER-PSEP) (Tang et al., 2016) was used to determine whether the identified missense variants were at sites conserved among species (see **Supplemental Appendix**) and to predict their putative damaging effect.

5.3.3 Studies of NOS1 expression and signaling

NOS1 expression was studied by IHC in fetal and adult human hypothalamic tissues as described in the Supplementary Appendix.

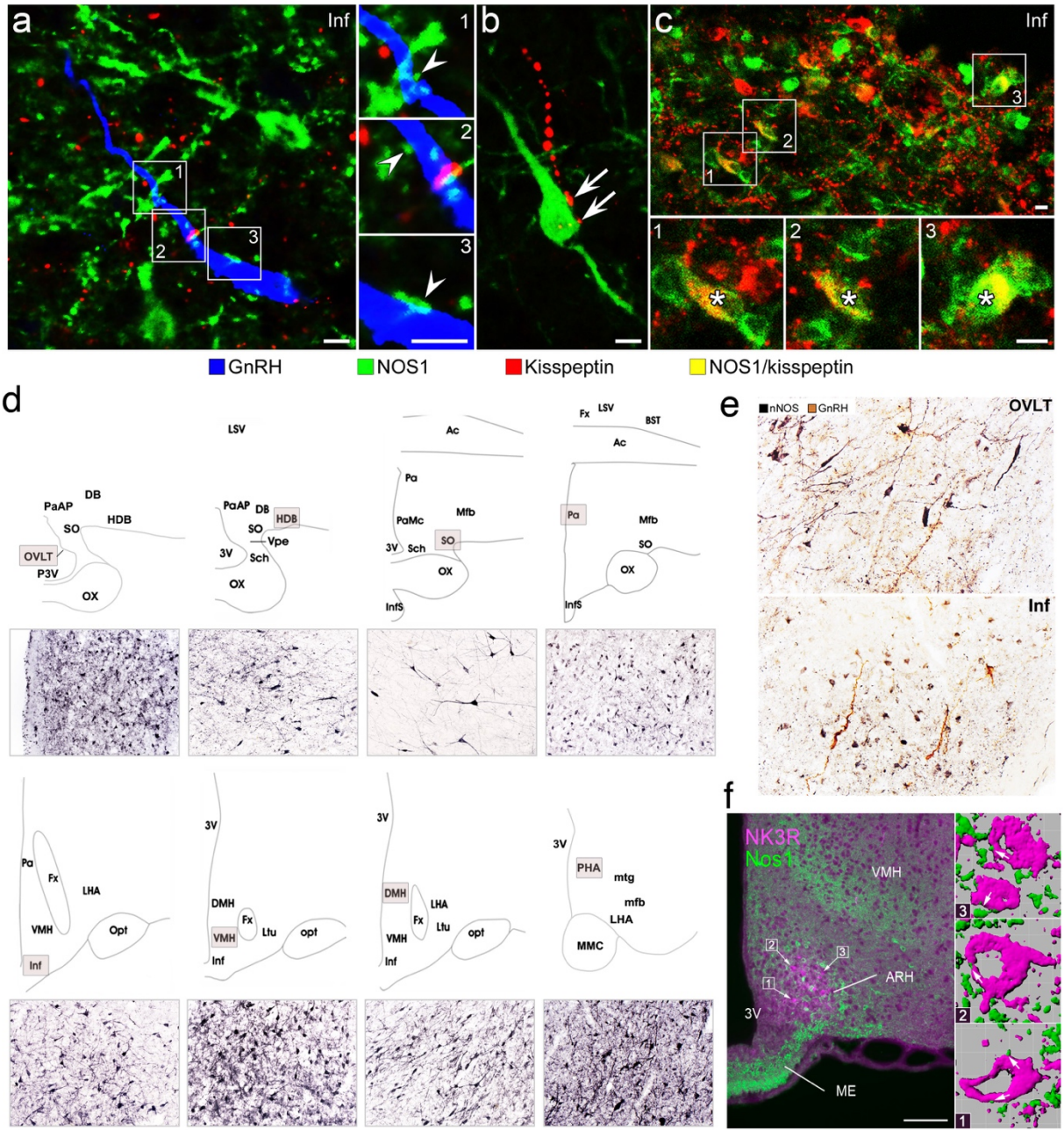
Kidney cells (HEK 293T) from human embryo were transiently co-transfected with each NOS1 mutant and the FlincG3 NO-detector plasmid (pTriEx4-H6-FGAm) (Batchelor et al., 2010, Bhargava et al., 2013) and subjected to live imaging to assess concentrations of NO release upon application of the Ca_2 ionophore A23187 as described in the **Supplemental Appendix**. NOS1 activity was also assessed using an alternative method (see **Supplemental Appendix**).

5.3.4 Assays in mice

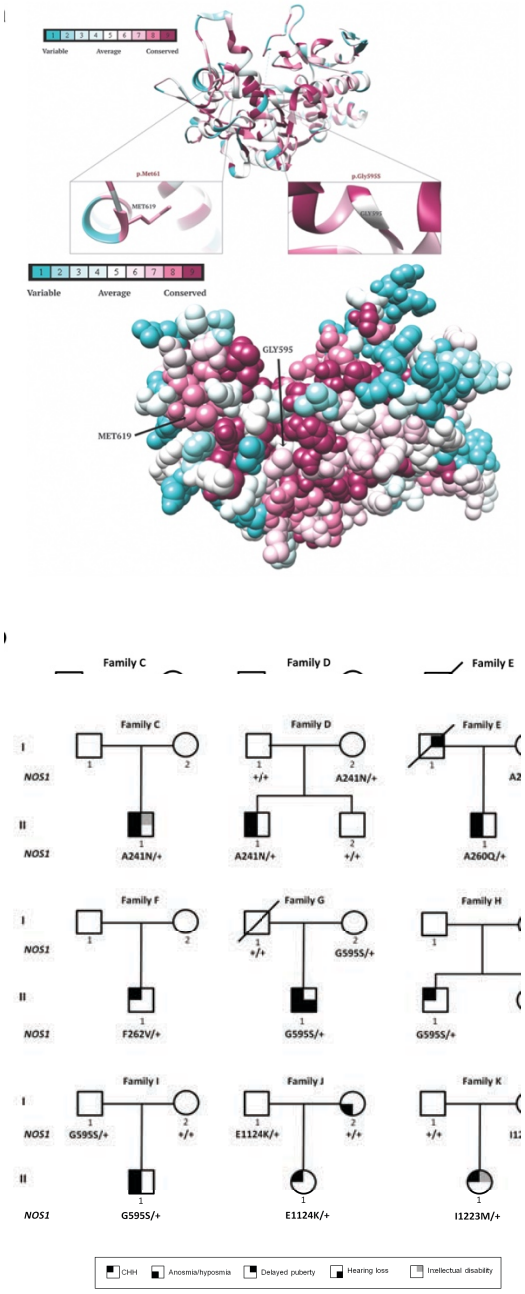
Neuroanatomical analyses, electrophysiological recordings and gene expression in GnRH neurons, examination of reproductive physiology and behavioral testing have been performed in male and female *Nos1*$^{-/-}$ mice and their *Nos1*$^{+/-}$ and *Nos1*$^{+/+}$ littermates subjected or not to inhaled NO (20 ppm) and Sildenafil (15 mg/kg, intraperitoneally) treatment during the infantile period (see the **Supplemental Appendix**).

5.4 Supplemental Appendix

5.4.1 Supplemental Figures and Tables



Supplemental Figure 1. NOS1 expression in the GnRH neuronal system in humans. (**a**) Results of triple-immunofluorescent experiments on adult human hypothalami reveal an abundant expression of NOS1 (green) in the region of the infundibulum (Inf). NOS1-immunoreactive processes form contacts (white arrowheads) with GnRH neurons (blue). (**b**) Kisspeptin fibers (red) innervate (white arrows) NOS1 cells, in addition to their well-established connections with GnRH neurons. (**c**) Colocalization analysis reveal that a subpopulation of the Kp neurons (asterisks) co-express the NOS1 signal in the infundibulum. Scale bars: 15 µm. (**d**) Distribution of NOS1-immunoreactive neurons (purple labeling) throughout the adult hypothalamus in human. (**e**) Anatomical relationship between NOS1 neurons (purple) and GnRH neurons (light brown) in the region of the organum vasculosum laminae terminalis (OVLT, upper panel) and the infundibulum (Inf, lower panel) in adult human hypothalamus. (**f**) Localization of Nos1 immunoreactivity with respect to the NK3R-immunoreactive Kp neurons in the ARH of the hypothalamus in mice. Separate neurons display labeling for Nos1 (green) and NK3R (magenta), but their interaction is visible under the form of putative Nos1-contacts onto NK3R-somatodendritic domains (arrows in insets numbered 1-3). *Left*, videocamera image of a 12 □m-thick section. *Right*, surface renderings of confocal optical sections, 1□m-thick over a 10□m-grid. Ac, anterior commissure; BST, bed of the stria terminalis; DB, diagonal band of Broca; DMH, dorsomedial hypothalamic nucleus; fx, fornix; HDB, horizontal limb of the diagonal band; LHA, lateral hypothalamic area; LSV, lateral septal nucleus mfb, medial forebrain bundle; LTu, lateral tuberal nucleus; MMC, medial mammillary nucleus; mtg, mammillo-tegmental tract; opt, optic tract; ox, optic chiasma; Pa, paraventricular nucleus; PaAP, paraventricular nucleus anterior parvicellular; PaMC, paraventricular nucleus magnocellular part; PHA, posterior hypothalamic area; Sch, suprachiasmatic nucleus; SO, supraoptic nucleus; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle. Bar, 100 □m in (**a**) and (**c**), and 50 □m in (**b**).



Supplemental Figure 2. ConSurf analysis and pedigrees of CHH probands harboring *NOS1* mutations. (**a**) ConSurf analysis for NOS1. The ribbon (top panel) and surface (bottom panel) representation of Nos1 (aa region) is coloured by the conservation grade of the aaa using the colour-coding panel. Colour grade is assigned as follows: 9 (maroon) contains the most conserved positions and 1 (turquoise) contains the most variable consitions. The aaa Gly595 and Met619 are zoomed and annotated in the index squares. (**b**) pedigrees of CHH probands harboring *NOS1* mutations.

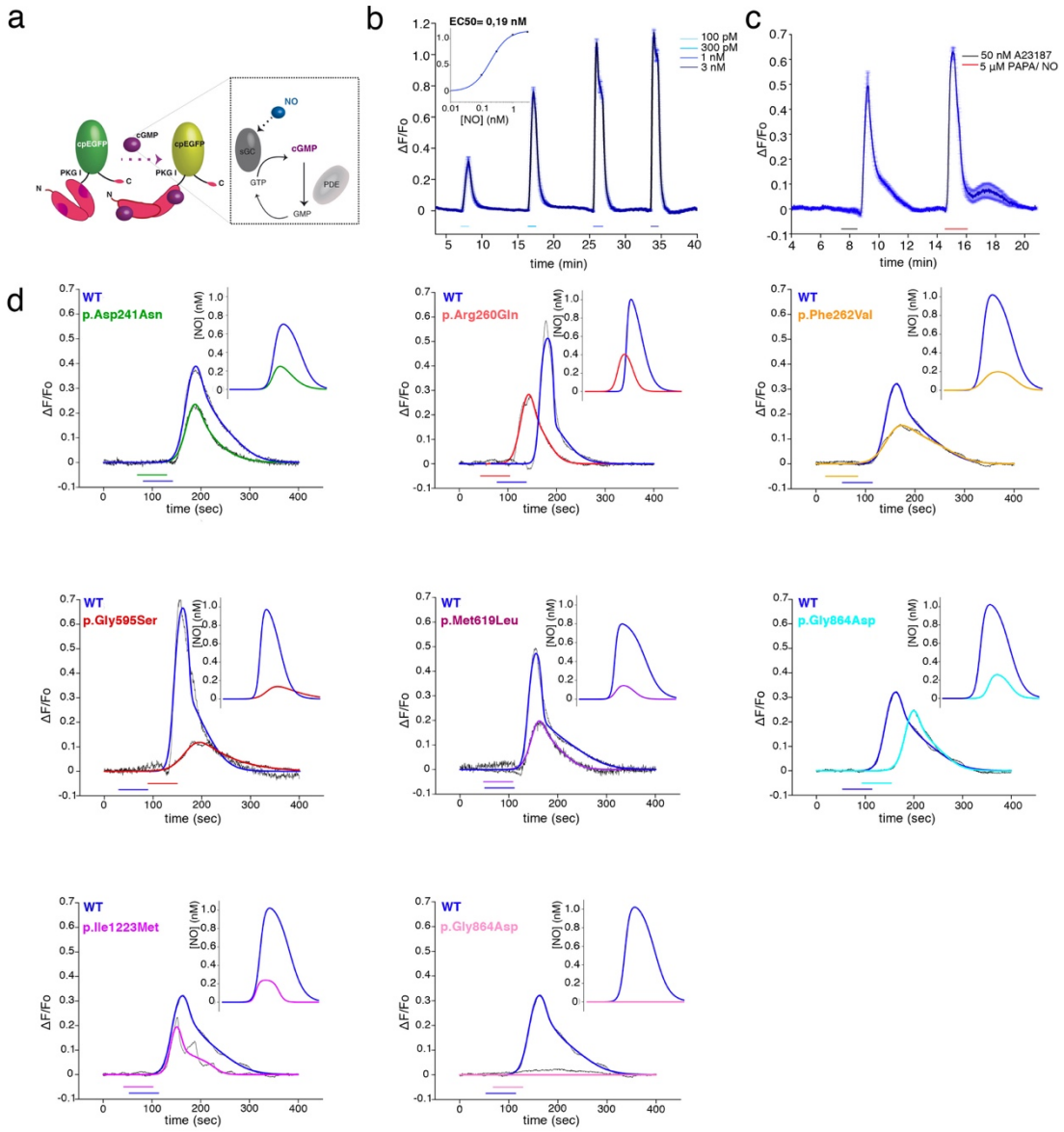


Figure S3. Functional assays of the NOS1 mutants *in vitro*. (**a**) Graphic represents the flincg3 construct and its mode of action: the regulatory domain of PKG I is fused to a cpEGFP fragment containing cGMP binding sites. The presence of NO promotes the activation of guanylate cyclase (GC) and the subsequent production of cGMP, inducing cGMP-dependent increase in the intensity of fluorescence. The decay of the response to baseline levels occurs after NO washout and reflects the action of the PDE5, expressed by the population of HEK cells. (**b**) Response to increasing clamped NO concentrations (100 pM, 300 pM, 1nM, 3 nM) applied at the horizontal bars. The inset illustrates the peak flincG3 response according to the different NO applications. The EC_{50} value and the concentration-response curve to the clamped NO applications corresponds to the one previously published (EC_{50}= 0.19 nM, s.e.m= ± 0.007). (**c**) Demonstration of the experimental protocol routinely used for testing the different NOS1 variants: Superfusion of the Ca_2 ionophore A23187 (50 nM) elicited a seemingly rapid fluorescence response that reached a peak within the first minute of the application in cells expressing the wt plasmid before recovering to baseline values on washout. Superfusion of a high concentration of the NO donor PAPA/NO (5 uM) allowed us to estimate the

peak of the A23187-evoked increase in fluorescence using the published concentration-response curve parameters in FlincG3-transfected HEKGC/PDE5 cells. Data are means of different cells recorded from the same coverslip (n>20 for each experiment). (**d**) Behaviour of FlincG3-transfected HEKGC/PDE5 cells co-transfected with the wt or mutated NOS1 in response to application of the A23187. Mutants are illustrated in comparison to wildype cells from the same experiment (transfection and imaging). The colour-coded line (wt NOS1 is represented in blue and mutants are each in a different colour) fits the data to the GC/PDE5 model previously published. The inset illustrates the attempts to describe NO generation using the Mathcad model, which are in good agreement with those calculated using the Hill equation (data not shown). The similarities in the shapes of the derived NO concentration profiles indicate that the time-courses for the mutants are similar to wt, suggesting reduced net NO synthesis as opposed to altered activation kinetics. Note that NO release was not observed in cells not containing any NOS1 construct, while it was abolished in the presence of the guanylate cyclase inhibitor ODQ (1 µM) (data not shown).

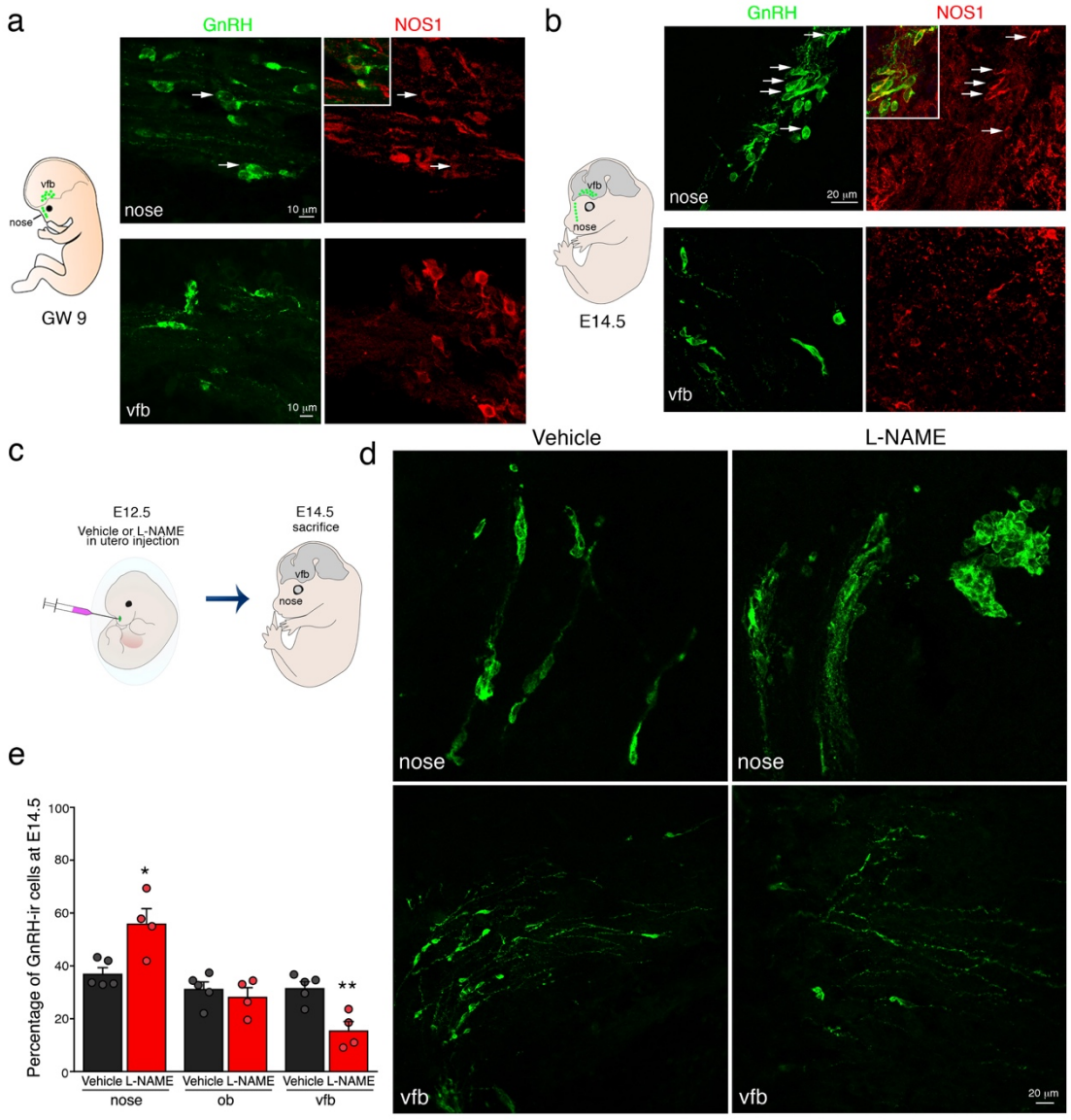


Figure S4. Migrating GnRH neurons co-express NOS1 in the nose. (**a**) 8-week old human fetus immunostaining showing GnRH neurons (in green) coexpressing the NOS1 protein (in red) in the region of the nose (upper panels), but no in the ventral forebrain (vbf; lower panels) during neuronal migration of the GnRH population. (**b**) Immunostaining of a mouse embryo during embryonic day (E) 14.5 showing migrating GnRH neurons (in green) coexpressing the NOS1 protein (in red) in the region of the nose (upper panels). Like in humans (a), mouse GnRH cells are seen to no longer co-express NOS1 once they have migrated into the vfb (**lower panels**). (**c-e**) Local inhibition of NO production in the nose *in utero* results in a failure of GnRH neurons to properly migrate in the forebrain region. (**f, e**) At E14.5, half of the migrating GnRH neurons are still in the nose (**d, green, upper left panel**), while the rest are found in the olfactory bulb and the forebrain region (**d, bottom left panel**) in embryos injected with saline in the nasal compartment at E12.5 (**c**). (**d, e**) LNAME injection causes a major perturbation in the migratory process of GnRH cells, resulting in incomplete migration into the forebrain region (**d, bottom right panel**) and arrestment of the majority of the cells in the region of the nose (**d, top right panel**). (**e**) Quantification of the distribution of the GnRH neurons at E14.5 in vehicle (black)- and L-NAME (red)- treated embryos. * $p < 0.05$; ** $p < 0.01$ L-NAME versus vehicle. Values indicate means ± SEM.

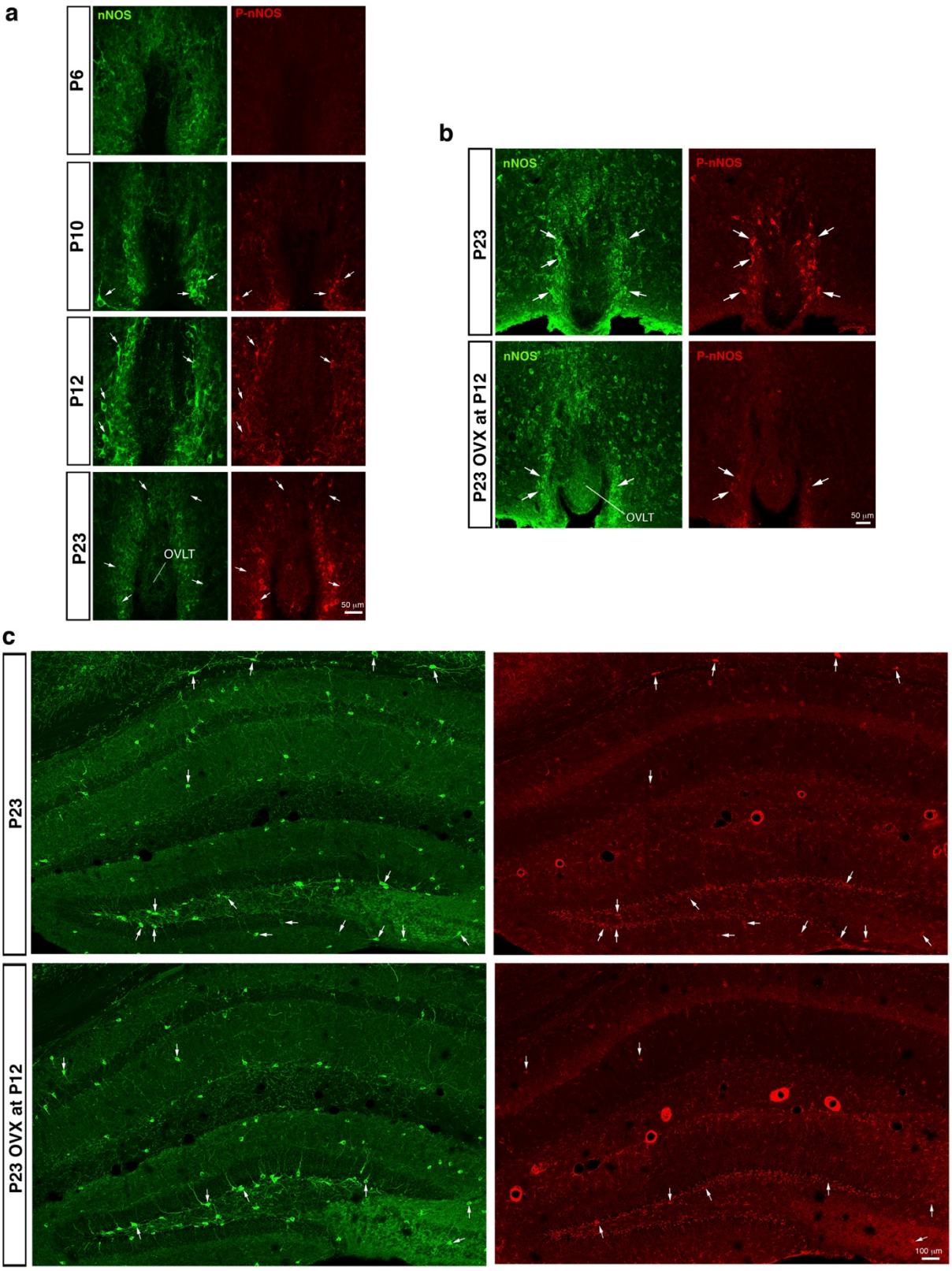


Figure S5. Immunofluorescent images of the Nos1 (green) / P-Nos1 (red) labeling in the preoptic region and hippocampus of female mice. Progressive phosphorylation of the Nos1 protein during postnatal development in the preoptic region at the level of the organosum of the lamina terminalis (OVLT) leads to activation of the NO pathway at P12. Nos1 (green) and P-Nos1 (red) immunoreactivity in forebrain coronal

sections of the OVLT in female mice during pre-pubertal P7, 10, 12, and 23. Ovariectomy at p12 blunted the increase in the phosphorylation of Nos1 at P23 in the preoptic region. Ovariectomy at p12 blunted the increase in the phosphorylation of Nos1 at P23 in the hippocampus.

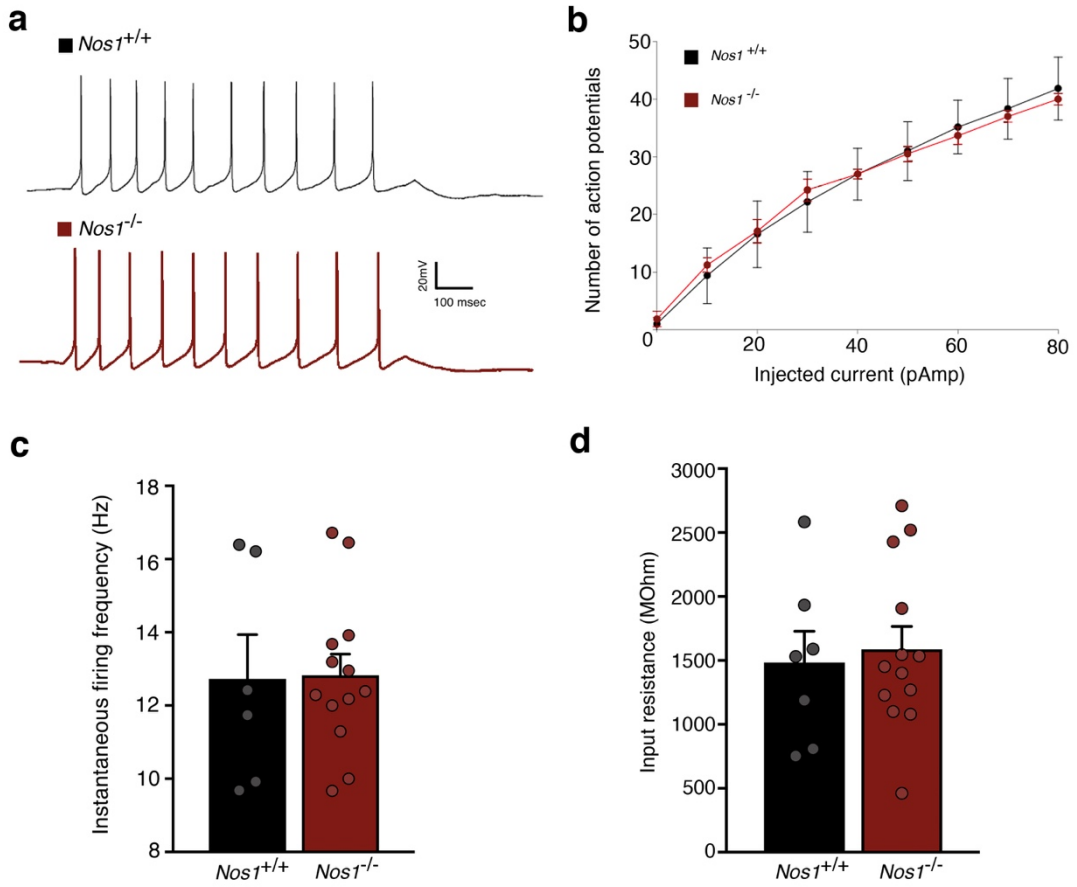


Figure S6. The increased GnRH excitability in *Nos*$^{-/-}$ mice (Figure 4c,4d) is not due to cell autonomous changes since the response of GnRH neurons to evoked firing does not differ between *Nos1*$^{+/+}$ and *Nos1*$^{-/-}$ animals. (**a**) Representative traces of firing evoked by a 10pA current injection in GnRH neurons from *Nos1*$^{+/+}$ (top trace in black) and *Nos*$^{-/-}$ (bottom trace in brown) mice. (**b**) Frequency-Current curve of evoked firing response in GnRH neurons from *Nos1*$^{+/+}$ (black) and *Nos1*$^{-/-}$ (red) mice over a range of current injections. (**c**) Instantaneous firing frequency after 10pA current injection in GnRH neurons from *Nos1*$^{+/+}$ and *Nos1*$^{-/-}$ animals. (**d**) Input resistance in GnRH neurons from *Nos1*$^{+/+}$ and *Nos1*$^{-/-}$ animals.

Nucleotide Change	Amino acid change	dbSNP number	Number of probands	MAF % gnomAD controls	In vitro studies	
					NO release in cells transfected with mutant protein	Enzymatic activity of NOS1 mutant protein
c.721G>A	p.Asp241Asn	rs76090928	2	0.193	↓↓↓	↓↓↓
c.779G>A	p.Arg260Gln	rs547371716	1	0.003	↓↓	↓↓
c.784T>G	p.Phe262Val	rs200602438	1	0.003	↓↓↓	↓↓↓
c.1783G>A	p.Gly595Ser	rs41356652	3	0.392	↓↓	↓↓
c.1855A>T	p.Met619Leu	rs79487279	1	0.226	↓↓↓	↓↓↓
c.2591G>A	p.Gly864Asp	rs9658445	1	0.436	↓↓↓	↓↓↓
c.3370G>A	p.Glu1124Lys	rs372660293	1	0.005	↓↓↓	↓↓↓
c.3669A>G	p.Ile1223Met	-	1	Pr	↓↓	↓

Supplemental Table 1. Genetic and functional characterization of *NOS1* rare sequence variants in CHH patients. Nucleotide and protein changes are based on reference on reference cDNA sequence NM_000620.4. CHH cohort included 180 patients. MAF, minor allele frequency; Pr, private; For in vitro studies ↓ = decreased, $p< 0.05$ compared to wt; ↓↓ = decreased, $p<0.01$ compared to wt; ↓↓↓ = severely decreased, $p<0.001$ compared to wt.

5.4.2 Supplemental Results

5.4.2.1 Pharmacologically-induced infantile NO deficiency causes infertility

To further explore the physiological role of infantile NO in the maturation of the reproductive axis, we specifically blunted the production of NO in wt mice between P10 and P21 by the daily intraperitoneal injection of NOS inhibitor L-NAME. Strikingly, this pharmacologically-induced infantile NO deficiency (Figure 4a-d) recapitulated the reproductive phenotype of the genetic *Nos1*-deficient mice (Figure 4e-f) with a significant delay in vaginal opening (Figure 4a) and pubertal onset (Figure 4b). Further, infantile NO deficiency led to a defective adult reproductive capacity, as indicated by a prolongation in the percentage of days spent in diestrus and fewer successful ovulatory events (Figure 4c). In line with this observation, the typical LH preovulatory surge was blunted in most infantile-NO-deficient mice (Figure 4d) when compared to vehicle-treated animals (P75-90). Surprisingly, when NO production was abolished at P7-P12, an early infantile period when FSH levels are rising (Prevot, 2015), there was no effect on sexual maturation (Figure S6). This clearly defines a critical time-window between P10 and P21 for the action of infantile NO on reproductive fitness.

5.4.3 Supplemental material and methods

5.4.3.1 Human tissues

Human hypothalamic tissues were obtained at autopsies from the Forensic Medicine Department of the University of Debrecen, Hungary, with the permission of the Regional

Committee of Science and Research Ethics (DEOEC RKEB/IKEB: 3183-2010). Permission to use 9 gestational-week-old human Fetuses was obtained from the French Agence de Biomédecine (PFS16-002). Processing of human tissues and immunochemistry protocols are detailed in the **Supplementary Appendix**.

5.4.3.2 Genetic analyses

We evaluated coding exons and intronic splice regions (≤6 bp from the exons) of the known CHH genes for pathogenic and likely pathogenic variants according to ACMG guidelines (Richards et al., 2015). The included CHH genes are: *ANOS1* (NM_000216.2)*, SEMA3A* (NM_006080)*, FGF8* (NM_033163.3)*, FGF17* (NM_003867.2)*, SOX10* (NM_006941)*, IL17RD* (NM_017563.3)*, AXL* (NM_021913)*, FGFR1* (NM_023110.2)*, HS6ST1* (NM_004807.2)*, PCSK1* (NM_000439)*, LEP (*NM_000230), *LEPR* (NM_002303)*, FEZF1 (*NM_001024613)*, NSMF* (NM_001130969.1)*, PROKR2* (NM_144773.2)*, WDR11* (NM_018117)*, PROK2* (NM_001126128.13)*, GNRH1* (NM_000825.3)*, GNRHR* (NM_000406.2)*, KISS1* (NM_002256.3)*, KISS1R* (NM_032551.4)*, TAC3* (NM_013251.3)*, and TACR3* (NM_001059.2).

Position-specific evolutionary preservation tool (PANTHER-PSEP)(Tang et al., 2016) was used to determine whether the identified *NOS1* missense variants were at sites conserved among species, including *pig, rabbit, rat, mouse and ferret (GenBank accession numbers F1RKF2, O19132, D3ZEW7, Q9Z0J4 and M3XUN6 respectively*) and to predict their putative damaging effect.

5.4.3.3 In silico analysis

ConSurf web surver (http://consurf.tau.ac.il) was used for the identification of evolutionary conservation of amino acid positions in human NOS1 (Ashkenazy et al., 2016). The degree of amino acid evolutionary conservation reflects its natural tendency to be mutated. Aim of the method was to investigate whether any of the identified mutations (p.Gly595Ser and p.Met619Leu) are important for structure and/or function based on the evolutionary pattern of Nos1. Homology sequence search was conducted based on amino acid sequence from the human crystal structure of Nos1 (PDB ID code: 5VUV). PSI-BLAST homolog search algorithm and UniProt database were used for the generation of a Multiple Sequence Alignment (MSA) with ClustalW algorithm and homologs were selected automatically. Maximum of 50 sequences, closest to the reference sequence of Nos1, were used for the analysis out of the

homolog search algorithm. Maximal and minimal % ID between sequences were set at 95 and 35 respectively.

5.4.3.4 Analyses of NOS1 mutants

A cDNA containing the entire coding region of the human *NOS1* transcript isoform 1 (RefSeq. NM_000620.4; GenBank assembly accession; GRCh37.p13 / GCF_000001405.25), was inserted into a modified pcDNA3.1+ expression vector containing a his-tag at the 5'end (GeneCust). Similarly, plasmid encoding *NOS1* mutants (Asp241Ans, Arg260Gln, Gly595Ser, Met619Leu and Ile1223Met) were obtained using modified pcDNA3.1+ expression vector containing a myc-tag at the 5'end of the coding region (GeneCust). The plasmid encoding remaining *NOS1* mutants (p.Phe262Val, p.Gly864Asp and p.Glu1124Lys) were generated by site directed mutagenesis using QuickChange XLII Kit (Stratagene) and confirmed by Sanger sequencing. FlincG3 NO-detector plasmid (pTriEx4-H6-FGAm) has been produced as described previously (Bhargava et al., 2013).

5.4.3.5 Cell culture of NO detector cells

The HEK 293T cell line expressing NO-activated GC and PDE5, previously referred to as GChighPDE5low cells (Batchelor et al., 2010), were provided by Professor Doris Koesling (Ruhr-Universitat Bochum, Bochum). HEK 293T were cultured under standard conditions in a DMEM-based medium containing 5% fetal bovine serum and appropriate selection antibiotics; they were replated before reaching 80% confluency and were passaged<35 times.

Transfection was performed on cells growing on poly-D-lysine-coated coverslips using Fugene6 (Roche Applied Science) according to the manufacturer's protocol, in a transfection rate of 3:1 (Fugene6/DNA). Flincg3 plasmid was co-transfected, in an one step process, with the NOS1 plasmid used in each experiment.

5.4.3.6 Live imaging

FlincG3 fluorescence imaging: FlincG3 has a broad excitation spectrum with peaks at 491 and 410 nM and an emission maximum at 507 nm. Time series were recorded using an Axio Observer Z1, with a camera (Orca LT) and a 20X air objective (numerical aperture 0.8, Zeiss), under software control (Zen Imaging Software, Zeiss). Fluorescent HEK 293T cells were excited at a wavelength set at 495, with an emission set at 519. Exposure levels were set at 300 ms and the intensity level at 8%. The chamber was superfused at 1.5 ml/min and temperature set

at 37°C with imaging solution containing: KCl 2 mM, KH2PO4 1.18 mM, glucose 5.5 mM, HEPES 10 mM, NaCl 140 mM, CaCl2 1.5 mM. The solution was adjusted to a pH of 7.4 and osmolality to 285-290 mOsmol/kg at a temperature of 37°C.

FlincG3 fluorescence data analyses: Epifluorescent signals were captured by camera, corrected for the background levels, and displayed as the change in intensity relative to baseline divided by the baseline intensity (ΔF/Fo). Peak amplitudes for each cell giving a fluorescent signal were measured by taking the maximum ΔF/Fo, subtracting the mean baseline and then subtracting the difference between the peak ΔF/Fo of the baseline and the mean baseline for that cell. These calculations were made with 14 OriginPro 8.5 software.

5.4.3.7 Compounds used for in vitro and in vivo experiments

All of the compounds used were delivered to the HEK 293T cells through superfusion. To explore the ability of the transfected cell line respond to NO, cells were treated with the NO donor (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino] diazen-1-ium-1,2-diolate (PAPA/NO; 1µM, Enzo Life Sciences, Exeter, UK) for a duration of 90 sec. Endogenous NO release was stimulated upon application of calcimycin (A23187; 50 nM diluted in DMSO, Abcam) for a duration of 1 min. The responses to NO could be inhibited by both the NOS inhibitor (L-NAME; 30 µM, Calbiochem) and the NO receptor blocker 1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin -1-one (ODQ; 1 µM, Sigma, Dorset, UK). ODQ is shown to selectively and potently inhibit guanylyl cyclase and thus it can block the accumulation of cGMP in response to NO donors (Garthwaite et al., 1995).

The Abcam NOS activity assay kit (ab211084) was used in HEK cells transfected with the wt and/or the mutated plasmids according to the manufacturer's instructions.

For *in vivo* application NO synthesis was blocked using the NOS blocker L-NAME LNAME (Merck, Ref. 483125; 50 mg/kg i.p. and 5 mM intranasally), diluted as previously described (Bellefontaine et al., 2014). The activity of PDE5 was inhibited by the use of sildenafil (SIGMA, Ref. PZ0003; 15 mg/kg, i.p.) diluted in DMSO. KINOX 450 ppm mole/mole inhaled NO gaz was generously supplied by Prof. Laurent Storme, Lille University Hospital.

5.4.3.8 Animals

All C57Bl/6J mice were housed under specific pathogen-free conditions in a temperature-controlled room (21-22°C) with a 12h light/dark cycle and ad libitum access to food and water. Experiments were performed on male and female C57BL/6J mice (Charles River Laboratories), *Nos1*-deficient (*Nos1*$^{-/-}$, B6.129S4-Nos1tm1Plh/J) mice(Huang et al., 1993) and *Gnrh*::*Gfp* mice (a generous gift of Dr. Daniel J. Spergel, Section of Endocrinology, Department of Medicine, University of Chicago, IL) (Spergel et al., 1999). *Nos1*$^{-/-}$; *Gnrh*::*Gfp* mice were generated in our animal facility by crossing *Nos1*$^{-/+}$ mice with *Gnrh*::*Gfp* mice. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the Universities of Lille; all experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU) and were approved by the French Department of Research (APAFIS#2617-2015110517317420v5).

5.4.3.9 Examination of physiology

Weaned female mice were checked daily for vaginal opening. After vaginal opening, vaginal smears were performed daily and analyzed under the microscope to identify the puberty onset (i.e. first appearance of two consecutive days where vaginal smears contained cornified cells) and eventually the specific day of the estrous cycle. Male mice were checked daily for balano-preputial separation, as an external sign of puberty onset.

5.4.3.10 In utero intranasal injections of L-NAME

Pregnant wt females were anesthetized with isoflurane, placed ventral side up and covered with a sterile surgical cloth. Abdominal hair was removed from a small surface around the incision. Skin and connective tissue were carefully cut and a small incision in the abdominal wall allowed the exposure of the uterine horn. Each horn was carefully pulled out of the abdomen and placed on top of it, while it was kept moist with fresh DPBS throughout the surgical process. Saline and the NOS inhibitor L-NAME (5mM) were injected into contralateral horns of each pregnant female. The needle was positioned vertically over the nose of the E12.5 embryo, and introduced until it was estimated to reach the nasal septum. Following administration of the substance, the needle was held steady for a few seconds before being gently withdrawn. The uterus was then returned to the abdomen and rehydrated with a small

amount of DPBS. The incisions were closed with surgical sutures and the female was singly housed until embryo harvesting (E14.5).

5.4.3.11 Intraperitoneal injections of L-NAME in infantile mice

P10 wt female mice received bi-daily injections of L-NAME (50 mg/kg, i.p.) or a control (saline), during infantile period, till the day of weaning (P21). L-NAME or the control where administered at 8H00 and 18H00, i.e. one hour after lights on and one hour before lights off according to a previously described protocol [23]. In the end of the treatment with the NOS inhibitor, mice were monitored for the assessment of pubertal onset and the study of estrous cyclicity (see "Examination of physiology" section above). When L-NAME treated mice and their control littermates reached adulthood, blood samples were collected from the facial vein on diestrus I (DiI) and proestrus (Pro) for the measurement of LH hormone (described below). Mice were then perfused for brain tissue collection and immunohistochemical studies.

5.4.3.12 Hormonal level measurements

Plasma LH was measured using a highly sensitive Enzyme-linked Immunosorbent Assay (ELISA) as described elsewhere (Steyn et al., 2013). Serum FSH levels were measured using radioimmunoassay as previously described (Messina et al., 2016). The accuracy of hormone measurements was confirmed by the assessment of rodent serum samples of known concentration (external controls).

5.4.3.13 Inhaled NO administration

Protocol was adapted from previous publications by others (Pansiot et al., 2010, Hua-Huy et al., 2016). *Nos1*$^{-/-}$ mother with her pups (*Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$) was placed inside a cage ("inhaled NO chamber") constantly perfused with 20 ppm NO, a dose commonly administered to premature infants at birth (Ambalavanan et al., 2016) that induces the expression of cGMP (Figure S7). Treatment started when pups reached P10 (lights on) and ended at P23, when mice were weaned and removed from the inhaled NO chamber.

5.4.3.14 Sildenafil administration

P10 *Nos1*$^{+/+}$, *Nos1*$^{+/-}$ and *Nos1*$^{-/-}$ mice received daily injections of the PDE5 inhibitor sildenafil (15 mg/kg, i.p.) during infantile period, till the day of weaning (P23). Sildenafil was administered at 8H00, i.e. one hour after lights are on. In the end of the treatment with the

NOS inhibitor, mice were monitored for the assessment of vaginal opening, pubertal onset and balano-preputial separation (see “Examination of physiology” section above).

5.4.3.15 Behavioral tests

For all behavioral tests, the animals were coded so the investigator was blind to the genotype/phenotype of each animal.

5.4.3.15.1 Novel object recognition test

Recognition memory was assessed using the novel object recognition (NOR) test. During the habituation phase on day 1, each mouse was allowed to explore the open-field arena for 30 minutes. On day 2, two identical objects (A+A) were placed within the open-field arena on opposite sides of the cage, equidistant from the cage walls. Each mouse was placed within the two objects and allowed to explore them for 15 minutes. Day 3 consisted of two phases, a familiarization and a test phase. During the familiarization phase (trial 1) that lasted 15 minutes, mice explored two other identical objects (B+B). After this phase, the mouse was placed back in its home cage for 1 hour before starting the test phase. During the test phase, one object of trial 1 and a completely new object (B+C) were placed within the open-field area and mice were allowed to explore them for 5 minutes (trial 2). The object recognition score was calculated as the time spent exploring the new object (trial 2) over the total exploration time, and is used to represent recognition memory function.

5.4.3.15.2 Olfactory habituation/dishabituation test

The habituation/dishabituation test was used to assess the ability to differentiate between different odors. This olfactory test included a presentation of acetophenone (00790, Sigma) for habituation and octantal (05608, Sigma) for dishabituation, or vice versa. Before the test, mice were allowed to explore the open-field area and an empty odor box for 30 minutes. After this habituation period, mice were sequentially presented with one odor for four consecutive trials for a duration of 1 minute, and an inter-trial interval of 10 minutes was maintained to ensure the replacement of the odor. After four consecutive trials, a second odor was presented during a 1-minute trial. Odors (20µl of 1:1000 dilution) were administered on a filter paper and placed in a perforated plastic box, to avoid direct contact with the odor stimulus. Measures consisted in recording the total amount of time the mouse spent sniffing the object during the different trials.

5.4.3.15.3 Social olfactory preference test

Social olfactory preference test consisted of two phases, a familiarization and a test phase. During the familiarization phase, all mice were allowed to freely explore the open-field arena and were exposed to urine samples from an adult C57BL6/J WT stud male and estrous female for 30 min. After 30 min in clean bedding, mice were allowed to explore the same urine samples for 10 min, during which the behavior towards the urine samples was recorded. For each test, 50 µl of either male or female urine was administered on a filter paper and placed in a perforated plastic box, to avoid direct contact with the odor stimulus. The distribution of the time sniffing the urine samples was used as an indication of the interest to gain further information from the scent source.

5.4.3.15.4 Hearing tests

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine and levomepromazin (100 mg/kg and 5 mg/kg respectively) and placed on a servo-controlled heating pad that maintained their core temperature at 37°C. Audiological tests were performed in a sound-proof booth. Distortion-product otoacoustic emissions (DPOAES) probes cochlear mechanics and ABR thresholds and suprathreshold waveforms, both of which are sensitive to detect auditory-pathway disorders. The predominant DPOAE at frequency $2f_1$-f_2 was recorded in response to two primary tones f_1 and f_2, with f_2/f_1 = 1.20, at equal sound levels (Cub^{e}Dis system, Mimosa Acoustics; ER10B microphone, Etymotic Res.). Frequency f_2 was swept at 1/10th octave steps from 4 to 20 kHz, and DPOAE level was plotted against frequency f_2 at increasing primary tone levels, from 20 to 70 dB SPL in 10 dB steps, then to 75 dB SPL. The ABRs elicited by calibrated tone bursts in the 5-40 kHz range (repetition rate 17/s) were derived by synchronously averaging electroencephalograms recorded between subcutaneous stainless-steel electrodes at the vertex and ipsilateral mastoid, with the help of a standard digital averaging system (CED1401+). One hundred 10-ms long epochs were averaged, except within 10 dB of the ABR threshold (defined as the smallest tone-burst level giving rise to at least one repeatable wave above background noise level, 100 nV in an anesthetized mouse), for which 300 epochs were collected. Next, ABRs in response to 10-kHz tone bursts at increasing levels, stepwise from 15 to 105 dB were collected and their waves were labelled from I to IV in chronological order, for the latency of wave II to be extracted at every stimulus level.

5.4.3.16 Tissue preparation

Embryos were washed thoroughly in cold 0.1 m PBS, fixed in fixative solution [4% paraformaldehyde (PFA), in 0.1 m PBS, pH 7.4] for 6–8 h at 4°C and cryoprotected in 20% sucrose overnight at 4°C. The following day, embryos were embedded in OCT embedding medium (Tissue-Tek), frozen on dry ice, and stored at −80°C until sectioning.

Postnatal (P10 to P30) and adult mice (2–6 months old) were anesthetized with 50-100 mg/kg of Ketamine-HCl and perfused transcardially with 2-10 ml of saline, followed by 10-50 ml of 4% PFA, pH7.4. Brains were collected, postfixed in the same fixative for 2 h at 4°C, embedded in OCT embedding medium (Tissue-Tek), frozen on dry ice, and stored at −80°C until cryosectioning.

5.4.3.17 Immunohistochemistry/immunofluorescence

Tissues were cryosectioned (Leica cryostat) sagitally at 16µm for embryos and coronally at 35µm (free-floating sections) for postnatal mice (pre-weaning, post-weaning and adult) and 16µm for iNO box post-weaning mice.

General procedure. As described previously (Hanchate et al., 2012, Bellefontaine et al., 2014), sections were washed 3 times for 15 minutes each in PB 0.1M and then incubated in blocking solution (5% NDS, 0.3% Triton X-100 in PB 0.1M) for 1 hour at room temperature. Sections were then incubated for 12-72 hours at 4°C with primary antibodies followed after rinses by secondary antibodies (1h at room temperature), all diluted in PB 0.1M containing 0.3% Triton X-100. Sections were then rinsed and counterstained with Hoechst (0.0001% in PB 0.1M; 5 min), rinsed again and coverslipped under Mowiol.

Primary antibodies. For p-Nos1 and Nos1 detection, rabbit anti-Ser1412 phospho-Nos1 (1:500; Thermoscientific, PA1-032) and sheep anti-Nos1 (1:3000; generous gift from Dr. P. C. Emson, Medical Research Council, Laboratory for Molecular Biology, Cambridge, UK). For Nos1 and NK3R detection, sheep anti-Nos1 (1:3000; P.C. Emson) and rabbit anti-NK3R (coded IS-7/7; 1:4000; allows the selective visualization in the ARH nucleus of kisspeptin neurons by labeling their somatodendritic domain (Mittelman-Smith et al., 2012)). For cGMP detection, sheep antiserum to formaldehyde-fixed cGMP (1:1000; H.W.M. Steinbusch, Maastricht University). For Nos1 and GnRH detection, sheep anti-Nos1 (1:1500; P.C. Emson) and guinea pig anti-GnRH (1:3000) (Hrabovszky et al., 2011).

Secondary antibodies. Included where appropriate (all from Jackson Laboratories) biotinylated donkey anti-rabbit (followed by strepavidin-Alexa 568 diluted 1:500; Invitrogen), TRITC donkey anti-rabbit, Alexa 488 donkey anti-sheep, Alexa 647 donkey anti-sheep, Alexa 488 donkey anti-guinea pig, Alexa 568 donkey anti-rabbit or anti-goat.

5.4.3.18 Triple-immunofluorescent detection of kisspeptin, NOS1 and GnRH in adult human brains

Human hypothalamic tissues from four postmenopausal female individuals (aged 58, 70, 88 and 90ys; *post mortem* interval<24 h) were obtained at autopsies from the Forensic Medicine Department of the University of Debrecen, with the permission of the Regional Committee of Science and Research Ethics (DEOEC RKEB/IKEB: 3183-2010). The postmenopausal model was chosen for its very high level of Kp expression in the infundibular region (Hrabovszky et al., 2011). The included subjects were not known to suffer from neurological or endocrine disorders prior to death. Dissection and immersion-fixation of hypothalamic tissue blocks, section preparation and immunohistochemical pretreatments of serial coronal sections covering also the infundibular region were carried out as in previous immunofluorescent experiments(Skrapits et al., 2014). For simultaneous triple-immunofluorescent labeling of NOS1, GnRH and Kp, previously characterized primary antibodies were applied to the sections in a cocktail consisting of rabbit KP (AAS26420C; Antibody Verify; 1:1000)(Borsay et al., 2014), sheep NOS1 (1:1000, gift from Dr. P. C. Emson) and guinea pig GnRH (#1018; 1:1000) (Hrabovszky et al., 2011) (4C; 24h). Then, the sections were transferred into a cocktail of anti-rabbit-Cy3 (1:1000)+anti-sheep-FITC (1:250)+anti-guinea pig-Cy5 (1:500) secondary antibodies (Jackson ImmunoResearch) for 12 h at 4C. The triple-labeled specimens were mounted, coverslipped with Mowiol and analyzed with confocal microscopy (Zeiss LSM780 microscope). High resolution images were captured using a 20×/0.8 NA objective, a 1–3× optical zoom and the Zen software (CarlZeiss). Different fluorochromes were used and detected with the following laser lines: 488 nm for FITC, 561 nm for Cy3, 633 nm for Cy5. Emission filters were as follows: 493–556 nm for FITC, 570–624 nm for Cy3 and 638–759 nm for Cy5. To avoid the emission crosstalk between the fluorophores, the red channel (Cy3) was recorded separately from the green (FITC)/far-red (Cy5) channels ('smart setup' function). To illustrate the results, confocal Z-stacks (Z-steps: 0.941-1.000 µm, pixel dwell time: 1.27-1.58 µs, resolution: 1024×1024 pixels, pinhole size: set at 1 Airy unit) were used.

5.4.3.19 Digital image acquisition

Immunofluorescent preparations were analyzed on the LSM 710 Zeiss confocal microscope. Excitation wavelengths of 493/562 nm, 568/643 and 640/740 were selected to image Alexa 488, Alexa 568 and Alexa 647 secondary antibodies. All images were taken with the objective EC Plan-Neofluar M27 (thread type). For investigating GnRH neuronal migration in embryonic tissue, sagittal sections of the head were acquired with the 20X objective, using a numerical aperture 0.50, and a zoom of 1.0. For the analysis of hypothalamic Nos1/p-Nos1 ratio during development, Z-stack images were acquired with the 40X oil objective, using a numerical aperture of 0.50, and a zoom of 1.0. For the analysis of hippocampal Nos1/p-Nos1 ratio after OVX, Z-stack images with tiles were acquired with the 20X objective, using a numerical aperture of 0.80, and a zoom of 1.0 All images were captured in a stepwise fashion over a defined z-focus range corresponding to all visible staining within the section and consistent with the optimum step size for the corresponding objective and the wavelength. Two-dimensional images presented here are maximal intensity projections of three-dimensional volumes along the optical axis. Illustrations were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).

5.4.3.20 Cell counting

Analysis was undertaken by counting the numbers of single-labeled, dual-labeled (Nos1 staining colocalising with p-Nos1). The number of the above Nos1- expressing neuronal populations were counted in the region of the OVLT, represented by plate 16, of the L.W. Swanson brain map(Swanson, 2004) as described previously(Chachlaki et al., 2017). All the above values for each mouse were used to determine mean counts for each age group which were then used to generate mean + SEM values for each group. For the embryonic tissue sagittal sections of the brain were examined in a Zeiss Axio Imager Z2 microscope. Alexa 488 was imaged by using a 495 beam splitter with an excitation wavelength set at 450/490 and an emission wavelength set a 500/550, allowing the identification of immunocytochemically labeled GnRH neurons. All GnRH neuronal nuclei throughout each tissue section were visualized and counted.

5.4.3.21 Isolation of hypothalamic GnRH neurons using fluorescent-activated cell sorting

To obtain single-cell suspensions the preoptic region of *Nos1*$^{-/-}$; *Gnrh*::*Gfp*; mice was microdissected and enzymatically dissociated using a Papain Dissociation System (Worthington, Lakewood, NJ). FACS was performed using an EPICS ALTRA Cell Sorter Cytometer device (BD Bioscience). The sort decision was based on measurements of GFP fluorescence (excitation: 488nm, 50 mW; detection: GFP bandpass 530/30 nm, autofluorescence bandpass 695/40nm) by comparing cell suspensions from *Gnrh*::*Gfp* and wt animals. For each animal, approximately 200 GFP-positive cells were sorted directly into 10µl extraction buffer: 0.1% Triton® X-100 (Sigma-Aldrich) and 0.4 U/µl RNaseOUT™ (Life Technologies).

5.4.3.22 Quantitative RT-PCR analyses

mRNAs obtained from FACS-sorted GnRH neurons were reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies) and a linear preamplification step was performed using the TaqMan® PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems). Real-time PCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System using exon-boundary-specific TaqMan® Gene Expression Assays (Applied Biosystems): *Gnrh1* (Gnrh1-Mm01315605_m1). Control housekeeping genes: *r18S* (18S-Hs99999901_s1), *ACTB* (Actb-Mm00607939_s1).

5.4.3.23 Brain slice electrophysiology preparation and recordings

Infantile *Nos1*$^{+/+}$;*Gnrh::Gfp* and *Nos1*$^{-/-}$; *Gnrh*::*Gfp* littermates (P13-P20) were anaesthetized with isoflurane, and, after decapitation, the brain was rapidly removed and put in ice-cold oxygenated (O_2 95% / CO_2 5%) artificial cerebrospinal fluid (ACSF) containing the following (in mM): 120 NaCl, 3.2 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, pH 7.4 (with O_2 95% / CO_2 5%). After removal of the cerebellum, the brain was glued and coronal slices (150 µm thickness) were cut throughout the septum and POA using a vibratome (VT1200S; Leica). Before recording, slices were incubated at 34ºC to recover for 1 h. After recovery, slices were placed in a submerged recording chamber (32.8ºC; Warner Instruments) and continuously perfused (2 ml/min) with oxygenated ACSF. *GFP*-positive GnRH neurons in the hypothalamic POA were visually identified with a 40 X objective magnification in an upright Leica DM LFSA

microscope under a FITC filter and their cell body observed by using IR-differential interference contrast. Whole-cell patch-clamp recordings were performed in current-clamp with bridge mode by using a Multiclamp 700B amplifier (Molecular Devices). Data were filtered at 1 kHz and sampled at 5 kHz with Digidata 1440A interface and pClamp 10 software (Molecular Devices). Pipettes (from borosilicate capillaries; World Precision Instruments) had resistance of 6-8 MΩ when filled with an internal solution containing the following (in mM): 140 K-gluconate, 10 KCl, 1 EGTA, 2 Mg-ATP and 10 HEPES, pH 7.3 with KOH. Bridge balance was adjusted to compensate for pipette resistance. All recordings were analyzed with Clampfit 10 (Molecular Devices). Junction potentials were determined to allow correction of membrane potential values. Electrical membrane properties were measured in current-clamp mode by applying a series of current pulses from – 60 to + 80 pA (1 s, 10 pA increments). Input resistance (R_{in}) was determined by measuring the slope of the linear portion of the current-voltage (I-V) curve. All data are presented as mean ± standard deviation.

5.4.3.24 Statistical analyses

All analyses were performed using Prism 7 (Graphpad Software, San Diego, CA) and assessed for normality (Shapiro–Wilk test) and variance, when appropriate. Sample sizes were chosen according to standard practice in the field. The investigators were blinded to the group allocation during the experiments. For each experiment, replicates are described in the figure legends. For animal studies, data were compared using an unpaired two-tailed Student's *t*-test or a one-way ANOVA for multiple comparisons against the control condition followed by Dunnett multiple comparison *post-hoc* test. Data not following normal distribution were analyzed using either a Mann-Whitney *U* test (comparison between two experimental groups) or Wilcoxon/Kruskal-Wallis test (comparison between three or more experimental groups) followed by a Dunn's *post hoc* analysis. The number of biologically independent experiments, sample size, *P* values, age and sex of the animals are all indicated in the main text or figure legends. All experimental data are indicated as mean ± s.e.m. The significance level was set at $P < 0.05$. Symbols in figures correspond to the following significance levels: ns. $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, ***$P < 0.0001$.

5.4.4 Detailed description of clinical characteristics of all probands

Family A, Patient II-3

***NOS1* p.Met619Leu (heterozygous)**

The male proband originating from a rural area in Switzerland came to medical attention because of absent puberty (testicular volume < 1 ml) at age 17. He was previously known for a cleft lip treated by surgery at birth as well as for unilateral cryptorchidism with a left inguinal testis, which subsequently spontaneously descended to the scrotum. Laboratory assessment revealed frank T deficiency associated with undetectable LH and low FSH. The patient reported normal sense of smell, which was confirmed some years later by formal testing. Other important clinical features were a slight intellectual disability, Oligodontia, sensorineural hearing loss requiring use of cochlear implants as well as hypertelorism and short fourth metacarpal in physical examination. The patient was started on T replacement, which was discontinued after 1-2 years because of intolerance of injections. After a loss of contact for several years, we reassessed the patient at age 23 with a new laboratory work up which confirmed HH. Family history was complex with presence of delayed puberty and cleft lip in the patient's father and delayed puberty in the patient's mother (menarche 17 years). The proband was the third of 5 brothers. The eldest reports history of mild delayed puberty (growth spurt and first shaving at 17 years) with no cleft lip or other dysmorphic features. Normal T level was verified at age 29. The second brother, born in 1991, had history of surgically corrected cleft lip and delayed puberty, which spontaneously progressed to eugonadism at adult life. The fourth brother exhibited cleft lip, delayed puberty and renal dysplasia (renal duplication). The youngest brother was still 12 years old during the last assessment rendering impossible the definitive assessment of his pubertal status. He had also presented with cleft lip at birth and physical status was notable for brachydactyly. No variants in known CHH genes were detected in the proband. Nevertheless, he harbored a rare RSV in *NOS1*, which was inherited by his father and was present in the second and fourth brother. Interestingly, the patient's mother harbored a heterozygous *GNRHR* variant (p.Q106R), which was also present in the youngest brother.

Family B, Patient II-2

***NOS1* p.Gly868Asp (heterozygous)**

The anosmic Caucasian female, as well as her monozygotic twin sister, presented with primary amenorrhea and absent breast development. The combination of HH and anosmia led to diagnosis of KS. Both sisters had syndromic features with sensorineural deafness, intellectual disability and incomplete dentition. Congenital heart disease was also present. Family history was remarkable for delayed puberty in the patients' father based on reported late voice deepening (16-17 years). The patients' mother as well as their elder brother and younger sister presented with normal puberty. All family members had normal sense of smell, verified by formal testing. The proband does not harbor any variant in known CHH genes. A rare variant in NOS1 was detected in both affected sisters and their father who exhibits a partial phenotype (delayed puberty).

Family C, Patient II-1

***NOS1* p.Asp241Asn (heterozygous)**

This Caucasian male was first brought to medical attention in his country of origin (Serbia) at age 1 year for micropenis and bilateral cryptorchidism. Biochemical data on HPG-axis were unavailable. No hormonal treatment was offered for micropenis. A pituitary MRI was performed at age 6 years showing normal aspect and size of pituitary gland but hypoplastic olfactive nerves. Hyposmia was subsequently confirmed by formal smell testing. The patient received a first orchidopexy in Serbia at age 7 years, followed by a surgical revision after his arrival in Switzerland at age 14 years. At this moment complete endocrine assessment revealed absent puberty with prepubertal testes and frank HH. Physical examination was notable for intellectual disability, obesity (BMI 33.7 kg/m^2), hypertelorism and bilateral ureteral stenosis accompanied by mild hydronephrosis with otherwise normal kidneys. An audiogram was normal. Family history was negative for delayed puberty, infertility and reduced sense of smell. Family DNA was not available. Given the above data, KS was suspected and the patient began systemic T since 14 years old. At 18 years old, the testicles remained small (0.5-1 ml). Due to change of physician, no interruption of the androgen replacement was performed at adult life. During the last contact with his current physician at 22 years old, the patient continued to be on T. He had developed an epilepsy and was not regularly working due to the associated mental retardation.

Family D, Patient II-1

***NOS1* p.Asp241Asn (heterozygous)**

The Caucasian male proband had presented with intrauterine growth retardation attributed to placental dysfunction according to medical records. At birth, micropenis, bilateral cryptorchidism and grade 1 hypospadias were reported. Multiples clinical features (dilated aortic root, inferior lens dislocation, high-arched palate and history of fracture-dislocations of left hip and left knee) led to diagnosis of Marfan's syndrome, confirmed by the detection of a *FBN1* mutation. The patient consulted our clinic at age 18 for absent puberty. Testicular volumes at diagnosis were 1 ml (right) and 2 ml (left). Several associated phenotypes (anosmia, synkinesia, clinodactyly) argued in favor of KS. T was < 1.0nmol/l) in the setting of undetectable gonadotropins (LH <0.5 U/l, FSH <0.5 U/l). Family history was negative for delayed puberty and defective sense of smell in the two parents. The latter was confirmed by formal testing. Anosmia and clinodactyly in the deceased maternal grandmother was reported by the patient's mother. The patient's younger brother was also known for Marfan's syndrome and craniosynostosis of the jaw, treated surgically. Interestingly, his puberty and sense of smell were normal, while physical examination was negative for synkinesia and clinodactyly. The proband harbors a heterozygous NOS1 mutation, inherited by his mother. Both his father and younger brother were wt.

Family E, Patient II-1

***NOS1* p.Arg260Gln (heterozygous)**

The anosmic proband was first evaluated for absent puberty at age 15.5 years. Testicular volume was estimated at 1.5 ml bilaterally. Physical examination was unremarkable except for the presence of a surnumerary tooth. T was frankly reduced (0.2 nmol/l), accompanied by low gonadotropins (LH 0.1 U/l, FSH 0.6 U/l). A cranial MRI was performed and confirmed pituitary hypoplasia and absent OBs. Based on the above data, KS was diagnosed and T was subsequently started. The patient's mother had normal menarche at age 12 years and was subjectively normosmic, later confirmed by formal testing (UPSIT). The patient's father possibly had delayed puberty but this information could not be verified due to advanced AD. The patient harbors a heterozygous NOS1 mutation, while her mother was wt. A genetic sample of the father was not available due to the impossibility to guarantee informed consent.

Family F, Patient II-1

***NOS1* p.Phe262Val (heterozygous)**

This CHH proband was first noted to have absent puberty when admitted aged 56 years with life-changing injuries following major self-harm episode, precipitated by a change in financial circumstances, but exacerbated by social isolation and poor self-esteem arising from lack of virilisation. He was referred to us when a surgical nurse performing urethral catheterisation noted his poor genital development. He received the standard psychological counselling for self-harm, but particularly appreciated a bedside visit by a local "expert patient"; he consistently declined any formal psychotherapy. There was a history of childhood surgery for grade 1 hypospadias. Although clearly distressed by his lack of physical development, he declined to explore the reasons why neither he (not, in his adolescence, his parents) has ever sought medical attention for this problem. Genital status revealed presence of micropenis without cryptorchidism but with testes of prepubertal size (3 ml). Initial hormonal status indicated HH (LH & FSH <0.5 IU/l) with low serum T (<1.0 nmol/l). T was subsequently begun at age 56 years as previously reported(Pazderska et al., 2018). Physical examination did not reveal any CHH-associated phenotypes, although the digits could not be objectively assessed for clinodactyly due to post-traumatic deformity, contractions and amputation. Olfactory function did appear to be absent, but was most likely from smoke-inhalation damage to the OE, as he clearly recalled being previously able to sense simple odorants such as the smell of flowers, coffee brewing and food being cooked. Additionally, cranial MRI showed normal OBs and sulci. Family history was unremarkable for pubertal timing and sense of smell. The patient, who harbors a NOS1 variant, has categorically refused to involve his parents in the genetic investigations. Apart from his endocrine management, he is undergoing a prolonged and intensive process of rehabilitation and reconstructive surgery.

Family G, Patient II-1

***NOS1* p.Gly595Ser (heterozygous)**

This male anosmic proband first consulted an urologist at age 17 years for absence of puberty (testicular volume 2 ml) and was diagnosed with simple pubertal delay for which he received monthly injections of Sustanon® 50 mg on 4 to 5 occasions, but was not offered further follow-up thereafter. At age 23.5 years, he asked to be referred to our clinic and we confirmed frank HH (T <0.5 ml, LH <1.0 U/l, FSH <1.05 U/l). Frequent sampling every 20 minutes confirmed an apulsatile LH profile, compatible with severe GnRH deficiency. Testes were just under 4 ml

bilaterally. Given the concomitant presence of anosmia, verified by formal testing (UPSIT: 8/40), we concluded to the diagnosis of KS. T replacement was restarted at age 24 years. Interestingly, this proband also had congenital deafness of the left ear of sensorineural origin, as confirmed by an audiogram. Clavicles were significantly shortened bilaterally. Family history was negative for pubertal delay with, in particular, menarche of the patient's mother at 12.5 years old. A formal test (UPSIT) showed normal sense of smell in the patient's mother, however, it was impossible to obtain in the patient's father due to his death during the follow-up. The proband does not carry any variants in known CHH genes, but he harbors a *NOS1* mutation, inherited by his mother.

Family H, Patient II-1
***NOS1* p.Gly595Ser (heterozygous)**
The male proband of European Ascent sought medical advice at age 16 years due to pubertal delay. There was no history of cryptorchidism nor micropenis. Personal history included closure of a brachial cyst of at age 5 years. T was frankly low (1.8 nmol/l) associated with low-normal gonadotropins. The initial testicular volume was estimated at 5 ml. Olfaction was subjectively normal. After receiving T replacement for 14 years, the patient presented a spontaneous increase of testicular volume at age 30 years, consistent with CHH reversal. The retrieved function of the HPG-axis was confirmed after withdrawal of T replacement. The patient fathered a son at age 32 years following spontaneous conception. The patient's son was at good health with unremarkable genital status. At age 33 years, the patient was still eugonadal (T 13.9 nmol/l, LH 5.0 U/l, FSH 8.9 U/l). Family history was notable for delayed puberty in the patient's sister (menarche at 16 years). No variants were detected in known CHH genes. The patient harbors, though, a *NOS1* variant. Genetic samples from the patient's parents and sister have been requested but their reception and analysis was not possible before the publication of this article.

Family I, Patient II-1
***NOS1* p.Gly595Ser (heterozygous)**
The anosmic male proband presented at age 21 years with complete absence of puberty and initial testicular volume of 2 ml bilaterally. The patient reported concomitant lack of smell leading to diagnosis of KS (LH & FSH <0.5 IU/l & T <1.0 nmol/l). There were not any other associated phenotypes. Family history was negative for pubertal and olfactory abnormalities in both parents (verified by formal smell testing) and the two patient's brothers. After

receiving T replacement since age of 21 years, he was subsequently switched to combined gonadotropins at age 27 years and achieved fertility via IVF of a son without micropenis/cryptorchidism at age 32 years and a normal daughter via frozen embryo transfer two years later. This proband harbors a heterozygous *NOS1* variant, inherited from his father. He is also carrier of a *FGFR1* variant, p.Pro776Ser, which is considered as of uncertain significance according to the ACMG guidelines. In particular, this variant has never been functionally assessed. Sequencing of the parents confirmed that the FGFR1 variant is not *de novo* (it is inherited by his mother) and its pathogenicity based on this pedigree cannot be established.

Family J, Patient II-1
***NOS1*, p.Glu1158Lys (heterozygous)**

This female patient presented with complete absence of puberty, manifested as primary amenorrhea and absent breast development at age 17. The diagnosis remained unclear at that moment and the patient was put on estrogen-progestin pills. She was later treated for infertility by ovarian stimulation (gonadotropins) in her country of origin (Kosovo) but suffered a miscarriage. At age 31 years, she consulted our clinic for a second opinion. After review of previous medical records and a new laboratory assessment which confirmed HH (E_2 0.07 nmol/l, LH 0.1 U/l, FSH 0.5 U/l) with otherwise intact pituitary function, CHH was suspected. Sense of smell was subjectively conserved but formal testing revealed anosmia (Sniffin' Sticks, 10/16, < 5th percentile). The patient also reported scoliosis. Cranial MRI showed reduced size of pituitary gland without midline defects. We concluded to KS diagnosis. The patient was subsequently put on fertility treatment by pulsatile GnRH, which restored ovulatory cycles and led to pregnancy after 4 cycles. Both parents and her four siblings (two brothers and two sisters) have normal pubertal timing. However, her mother was found to be anosmic (UPSIT 14/40). The patient does not carry any variants in known CHH genes but harbors a *NOS1* mutation, inherited by her father.

Family K, Patient II-1
***NOS1*, p.Ile1257Met (heterozygous)**

This female patient of Portuguese origin presented at age of 17 years with primary amenorrhea. Initial hormonal assessment showed low serum E_2 associated with low gonadotropines (LH < 0.1 U/l, FSH 0.23 U/l). The testing of other pituitary axis was normal. Cranial MRI showed normal pituitary gland with intact OBs and olfactory strips. The patient

has a slight mental deficiency. She has an apparent narrowing of the bony internal acoustic pores bilaterally with moderate hearing impairment of the left side of conducting origin. An ultrasound revealed normal kidneys. Sense of smell was normal based on patient's subjective assessment. A formal olfactory assessment was not performed due to subsequent hospitalization of the patient for psychiatric reasons and loss of contact with the local endocrinologist. Family history was negative for pubertal delay and decreased sense of smell. The patient does not harbor variants in known CHH genes. We detected, however, the presence of a heterozygous *NOS1* variant, inherited by her mother.

DISCUSSION AND PERSPECTIVE

In this Ph.D. thesis, we provide evidence that GnRH – widely known for its key regulatory role on the regulation of puberty onset and fertility – plays an unexpected yet critical role in non-reproductive functions, more specifically cognitive function.

Recently, the GnRH neuroendocrine system has been proposed to regulate systemic ageing and age-related cognitive impairments (Zhang et al., 2013, Zhang et al., 2017). The idea that prohibiting the hypothalamic release of modulators including GnRH may accelerate ageing is accordant with the finding that hypogonadal mice, which hold a spontaneous mutation that inactivates the GnRH gene (Mason et al., 1986), display Alzheimer dementia-like changes during ageing (Drummond et al., 2012). As the concentrations of GnRH in the CSF are proportional to the levels found in portal blood vessels delivering the neurohormone to the anterior pituitary gland to control fertility (Van Vugt et al., 1985, Caraty et al., 1989, Caraty et al., 2008), a deficiency in hypothalamic GnRH could additionally alter homeostasis in neuronal populations expressing GnHR-Rs in brain regions involved cognition, such as the hippocampus, in rodents (Granger et al., 2004) and humans (Wilson et al., 2006). The CSF could thus represent a source of GnRH potentially signalling to brain areas involved in cognition. Additionally, Casoni and colleagues demonstrated the presence of GnRH cell bodies and fibers in the hippocampus of human embryos (Casoni et al., 2016), which could constitute a direct route of action. The results presented in this manuscript are in accordance with the idea that GnRH could target extrahypothalamic brain areas such as the hippocampus, and that therefore, dysregulations in the GnRH system could underlie cognitive impairments. Dysregulations in the network regulating GnRH expression are associated with an impaired cognitive function in our murine models, while GnRH replacement therapies and/or targeting the underlying miR-network dysregulation can reverse the observed phenotype.

Our results revealed *NOS1* as a frequently mutated gene underlying CHH, a genetic disorder rooted in a GnRH deficiency. Several of these patients exhibited non-reproductive comorbidities, such as olfactory impairments and intellectual disability. Indeed, independent studies showed that the NO system is implicated in the regulation of various behavioral manifestations, including hyperactivity, sexual and aggressive behaviors (Vincent et al., 1992, Demas et al., 1997, Prast et al., 2001). Indeed, Nelson and colleagues reported a disruption of the *Nos1* gene in mice to be associated with inappropriate aggressiveness and altered sexual behavior (Nelson et al., 1995). Here, we demonstrate that *Nos1* KO mice exhibit olfactory and

cognitive impairments, as well as a disruption of the GnRH regulation and reproductive axis. *Nos1* KO mice showed an exacerbated and prolonged minipuberty, which is thought to be absent in CHH, followed by delayed puberty and altered fertility. Interestingly, both the reproductive and non-reproductive phenotypes could be reversed by NO replacement, administering exogenous NO or a PDE5 inhibitor during the critical time window of minipuberty.

CHAPTER 4. Progressive GnRH insufficiency underlies olfactory and cognitive dysfunction in Down syndrome

In this study, we show that GnRH plays an unexpected role in the pathophysiology of olfactory and cognitive deficits in a mouse model of DS. Our data raise the intriguing possibility that the onset of intellectual and olfactory impairment in DS patients (Grieco et al., 2015) may be due to the progressive alteration of GnRH expression during postnatal development, which primarily alters the production of the GnRH peptide in extrahypothalamic regions, as well as affecting pituitary LH secretion reflecting the pulsatile release of GnRH in the median eminence of the hypothalamus.

In individuals with DS, a mild deviation from neurotypically developing trajectories in early childhood culminates in decreasing cognitive abilities during adolescence that continue into adulthood (Grieco et al., 2015), a phenomenon paralleled by the progressive impairment of olfactory function (Nijjar et al., 2002). Although neurodegenerative changes occur even in the absence of clinical signs of dementia, cognitive decline in middle to late adulthood is thought to be associated with dementia representative of AD (Ballard et al., 2016, Lott et al., 2019). Our data suggest that the impairment of the sense of smell precedes the onset of cognitive alterations. While these defects occur before the development of any AD-like proteinopathy in the brain (i.e., APP overexpression), they are concomitant with gradual changes in GnRH expression starting with a mild alteration in transcriptional levels during the infantile period (at minipuberty) and culminating in a marked reduction in GnRH protein expression in adulthood. The trisomy in the mouse DS model appears to induce a disequilibrium in the miRNA-gene network that modulates the *Gnrh* promoter during the infantile-to-juvenile switch in GnRH neuronal function (Messina et al., 2016). While altered GnRH expression in adult trisomic mice is associated with cognitive deficits, it does not perturb the pattern of

GnRH-triggered release of LH pulses by pituitary gonadotropes, instead altering the level of LH secretion, with a decrease in pulse amplitude in addition to increased basal levels. While, to our knowledge, no study has been designed to determine the pattern of GnRH/LH secretion in individuals with DS, who also show elevated basal gonadotropin levels (Hsiang et al., 1987), the alteration of this pattern in trisomic mice is reminiscent of the changes in the LH secretion profile documented in elderly women (Hall et al., 2000) and men (Deslypere et al., 1987) prone to developing cognitive decline and dementia (Kivipelto et al., 2018). Targeting either the disequilibrium in miR-200 hypothalamic expression or the alteration in the pattern of GnRH secretion, resulted in the successful restoration of both olfactory and cognitive performance. In particular, using subcutaneous osmotic pumps to deliver pulsatile GnRH, a treatment also currently used to manage infertility in patients with HH (Boehm et al., 2015), to restore wt-like LH secretion patterns and behavior in Ts65Dn mice, raises the possibility of conducting similar interventional studies in individuals with DS to improve their intellectual performance, well-being and integration into the society they live in (Engler et al., 2017).

From a broader perspective, the vital role of GnRH in the regulation of cognition in mice is in line with previous transcriptomic studies showing that genes involved in GnRH signaling are among the most downregulated in discrete cortical areas of post-mortem brains from AD patients (Wang et al., 2016), as well as in the hippocampus of a mouse AD mouse model (Chatterjee et al., 2018). Splicing alterations in GnRH-related genes during normal aging have also been demonstrated in the murine hippocampus (Benito et al., 2015). Pulsatile GnRH delivery may thus also hold therapeutic potential in aging-related cognitive decline, including AD.

CHAPTER 5. Defects in NOS1 activity cause GnRH deficiency in human and mice: evidence for reversal after NO treatment in infantile mice

Our study reveals that 8% of probands with CHH harbor heterozygous mutations in *NOS1*. This implicates *NOS1* as one of the most frequently mutated genes in congenital GnRH deficiency, together with *FGFR1* and *CHD7* (Cassatella et al., 2018). All eight *NOS1* mutations are in highly conserved aa and are confirmed loss-of-function *in vitro*. Mutations were inherited in an autosomal dominant fashion with reduced penetrance. It is known that NOS1 requires homodimerization to enzymatically convert L-arginine and oxygen into L-citrulline and NO

(Stuehr, 1997) and that NOS1 mutants cause dominant negative effects by forming heterodimers with wt NOS1, thus reducing cellular levels of active NOS1 dimers and NO production *in vitro* (Phung et al., 1999). Herein, we used *Nos1* KO mice lacking exon 2 (with some residual Nos1 activity (Huang et al., 1993, Eliasson et al., 1997)) and demonstrated that NOS1 deficiency impairs the onset of puberty and fertility in a dose-dependent fashion. Taken together, these data are consistent with the heterozygous loss-of-function *NOS1* mutations found in CHH patients.

Notably, *NOS1* is the first gene involved in CHH that encodes an enzyme synthesizing a neurotransmitter, suggesting a contribution of neuronal network homeostasis to the disease. Importantly, several probands exhibited other neuronal defects, including anosmia (KS), intellectual disability, and sensorineural hearing loss — phenotypes also seen in *Nos1*-deficient mice.

Although absent or partial puberty is the hallmark of CHH, it is currently thought that the transient activation of the HPG-axis during minipuberty is also absent in most cases (Quinton et al., 2017). Minipuberty occurs during the first postnatal months in humans (Kuiri-Hanninen et al., 2011, Kuiri-Hanninen et al., 2011) and the second week of life in rodents (Prevot, 2015), and is characterized by a transient surge in GnRH production leading to gonadal activation. *Nos1* deficient mice unexpectedly exhibited exacerbated and prolonged minipuberty followed by delayed puberty and altered fertility. In particular, we observed increased GnRH neuronal activity, a higher expression of the *Gnrh* transcript in the hypothalamus, and abnormally elevated and sustained levels of FSH. Through pharmacological inhibition of NO production, we recapitulated the reproductive defects observed in *Nos1*-deficient mice, and defined a critical window of NO activity in shaping minipuberty and subsequent sexual maturation. These data point toward a major role of infantile NO in postnatal maturation of the central neuroendocrine circuits driving pulsatile GnRH release. In addition, the effect of OVX in infantile mice indicates that NO signaling is dependent on feed-back secretion from maturing gonads that is essential for the completion of minipuberty, which appears to play a key role in the postnatal programing of puberty onset and adult fertility. This novel insight gives rise to the intriguing hypothesis that the dynamics of the GnRH release during minipuberty in humans might also shape puberty onset and adult fertility. Future studies evaluating intensity and length of minipuberty could confirm such findings in CHH or CDGP patients.

The existence of the non-reproductive phenotypes in CHH and *Nos1*-deficient mice suggest a NO-dependent impairment of other brain neuronal circuits (Weitzdoerfer et al., 2004, Steinert et al., 2011, Gao et al., 2015). Anosmia in CHH is thought to result from a defect in axonal targeting of olfactory neurons, the migratory scaffold for GnRH neuron migration (Teixeira et al., 2010, Hanchate et al., 2012, Boehm et al., 2015). Through pharmacological inhibition of Nos1 activity during embryonic development, we identified a transient defect in GnRH neuron migration that normalized in adulthood. This is consistent with a temporally restricted action of NO on GnRH neuron migration rather than an effect on the migratory scaffold (i.e. olfactory axons) as shown for the migration of other neuronal populations (Mandal et al., 2013, Marin, 2013). Further, the olfactory deficits of *Nos1*$^{-/-}$ mice (i.e. altered olfactory processing) are in line with the modulation of synaptic plasticity and neuronal circuit synchronization by NO in the OB and other brain regions (Roskams et al., 1994, Kendrick et al., 1997, Kwan et al., 2012). Taken together, these studies raise the possibility that anosmia could be due to a functional defect caused by an impairment of the maturational processes involved in the processing of olfactory information, at least in some KS patients.

Estrogen positively impacts the maturation of neuronal circuits in several brain areas (McEwen, 2002, McCarthy, 2008, Denley et al., 2018) and contributes to the postnatal increase of Nos1-dependent NO production in infantile mice both in the hypothalamus and the hippocampus. Thus, it is tempting to speculate that the rise in FSH-induced estrogen production during minipuberty could act as a synchronous trigger on Nos1 neurons to promote maturation of neuronal circuits involved in both reproductive and non-reproductive functions. In this respect, it will be crucial to carefully study the minipuberty and its link to neurodevelopmental disorders. Notably, preterm infants have been shown to display abnormally higher serum FSH levels during the minipuberty and have an increased risk to develop decreased reproductive capacity (Swamy et al., 2008), intellectual disability and hearing loss (Moster et al., 2008, D'Onofrio et al., 2013).

Our results clearly demonstrate that NO plays a key role in shaping minipuberty in mice. Further, the exacerbated and prolonged HPG-axis activity during infancy resulting from NO deficiency is associated with both pubertal defects and infertility. Remarkably, we demonstrate a reversal of reproductive and non-reproductive phenotypes following the administration of exogenous NO or a PDE5 inhibitor (Sildenafil) during the critical postnatal

time window of minipuberty. These results confirm a role for NO in the maturation of neuronal circuits. It is tempting to speculate that similar treatments may be used in patients with NOS1 deficiency to induce a reversal of the defects of the HPG-axis. It is known that 10-20% of CHH patients exhibit a reversal after normalization of sex steroid milieu following hormone therapy (Raivio et al., 2007). Estrogen, a powerful trigger of the expression of several genes including *NOS1* (Garcia-Duran et al., 1999), induces NOS1 activity in hypothalamic neurons (d'Anglemont de Tassigny et al., 2007, d'Anglemont de Tassigny et al., 2009), and could thus contribute to the reversal observed in a CHH patient harboring *NOS1* mutations (Family H). Further, PDE5-inhibitor treatment is used in men with sexual dysfunction and leads to increased serum T (Carosa et al., 2004). However, a central action of PDE5 inhibitors has not been investigated. This raises an intriguing possibility to treat disorders of pubertal development or infertility using NO or PDE5 inhibitors.

The broad spectrum of actions for NO on the development and homeostasis of cardiovascular, immune, and central nervous systems is well-known. Our current study expands the reach of this critical molecule to include sexual maturation and reproduction. Finally, our data points to NO as a potential target for therapeutic intervention in a large spectrum of reproductive disorders and neurological disorders.

Perspective : the role of NO in the Ts65Dn mouse model

The *Nos1* KO mouse model, as demonstrated in the second study of this Ph.D. manuscript, presents a strong reproductive phenotype. Similar to what is thought in CHH (Quinton et al., 2017), minipuberty has been found to be exacerbated and prolonged in *Nos1* KO mice, followed by a delay in puberty onset and infertility. Corresponding to the alteration of the HPG-axis in the *Nos1* KO model, Ts65Dn male mice present with a delayed puberty onset and infertility. It has been shown that several miRNAs, such as miR-155, are overexpressed in the DS brain (Elton et al., 2010). miR-155 has been suggested to shape minipuberty, as its levels are significantly higher during this period (Messina et al., 2016) and is known to interact with Cebpb, which exerts a NO induced repression on GnRH expression.

It is well described that the NO system is implicated in the regulation of various behaviors, including hyperactivity, sexual and aggressive behaviors (Vincent et al., 1992, Demas et al., 1997, Prast et al., 2001). Alterations in the above mentioned behaviors (Escorihuela et al., 1995, Coussons-Read et al., 1996, Klein et al., 1996), as well as deficits in learning and memory (Reeves et al., 1995, Demas et al., 1996), have been described in the Ts65Dn mouse model, suggesting the implication of Nos1-derived NO signaling in DS. Moreover, similarities in cognitive impairments have been observed between the *Nos1* KO and Ts65Dn mouse model.

Consistently with the aforementioned findings, Gotti and colleagues demonstrated that Ts65Dn mice exhibit a reduced number of Nos1 neurons and reduced Nos1 activity in the hypothalamic PVN compared to their wt littermates.

Interestingly, NO replacement therapies, either the administration of exogenous NO or Sildenafil (a PDE-5- inhibitor), during the critical minipuberty window were able to restore the reproductive and non-reproductive phenotypes in the *Nos1* KO mice. In Ts65Dn mice, showing similar aberrancies, the observed non-reproductive phenotype was rescued by different GnRH replacement therapies.

Given the observed deficiency of NO production in the hypothalamus of Ts65Dn mice, as well as the similarities between the reproductive phenotype and observed comorbidities between the Ts65Dn mouse model of DS and *Nos1* KO mouse model, the question arises whether defects in NO signaling could be implicated in the etiology of the observed phenotype in the Ts65Dn mouse model. For this reason, first, the nNOS populations in key regions for sexual maturation, cognition and olfaction should be phenotyped at different stages of development to understand if the observed phenotype could result from an alteration in NOS activation during minipuberty. Second, it would be important to examine whether the observed phenotype can be described to some actors of the NO signalling and/or NO-cGMP pathway. Furthermore, it would be very interesting to examine whether NO replacement therapies, more specifically the administration of sildenafil or inhaled NO, during the infantile period would be able to rescue the cognitive and olfactory deficits observed in Ts65Dn mice, as it has been demonstrated to be effective in *Nos1* KO mice.

CONCLUSIONS

In this present Ph.D. thesis, we sought to examine (i) whether GnRH neurons and the regulation of GnRH expression - widely known to control fertility - are involved in non-reproductive functions, more specifically progressive cognitive and olfactory decline, and (ii) whether these deficits can be restored targeting the GnRH-system.

The main conclusions of our first study (chapter 4) are as follows:

- Male Ts65Dn mice, a mouse model of DS, present with an impaired reproductive phenotype; e.g. impaired sexual maturation, hypogonadism and infertility.
- A progressive loss of GnRH expression is observed in Ts65Dn mice, and can be explained by a dysregulated miRNA-transcription factor network including a downregulation of most miR-200 family members.
- The progressive loss of GnRH expression is concomitant with a decline in both cognitive and olfactory performance, independent of Alzheimer-like pathology, in this mouse model of DS.
- Different GnRH replacement therapies, as listed below, are able to reverse the olfactory and cognitive impairments observed in Ts65Dn mice.
    - Grafting neonatal POA transplants into the 3v.
    - Viral-vector mediated overexpression of miR-200b, a member of the miR-200 family that is significantly downregulated in this mouse model.
    - Chemogenetic activation of GnRH neuronal activity using DREADD technology.
    - Pulsatile administration of GnRH using subcutaneous programmable mini-pumps, a treatment currently used to manage infertility in patients with HH.

The main conclusions of our second study (chapter 5) are as follows:

- *NOS1* mutations lead to CHH and KS, characterized by a congenital GnRH deficiency.
- There is a critical time window for Nos1 action in shaping minipuberty and sexual maturation.
- *Nos1* deficient mice present with an impaired puberty onset and fertility, as well as other comorbidities such as cognitive and olfactory impairments.
- Restoration of NO activity by the administration of inhaled NO or PDE5 inhibitors is able to rescue the reproductive, as well as the non-reproductive phenotype in *Nos1* deficient mice.

REFERENCES

AA, A. L.-H., Z. M. Lei and C. V. Rao (1997). "Neurons from fetal rat brains contain functional luteinizing hormone/chorionic gonadotropin receptors." Biol Reprod **56**(5): 1071-1076.

AA, A. L.-H., Z. M. Lei and C. V. Rao (1997). "Novel expression of functional luteinizing hormone/chorionic gonadotropin receptors in cultured glial cells from neonatal rat brains." Biol Reprod **56**(2): 501-507.

Adelman, J. P., A. J. Mason, J. S. Hayflick and P. H. Seeburg (1986). "Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat." Proc Natl Acad Sci U S A **83**(1): 179-183.

Aguiar-Oliveira, M. H., A. H. O. Souza, C. R. P. Oliveira, V. C. Campos, L. A. Oliveira-Neto and R. Salvatori (2017). "MECHANISMS IN ENDOCRINOLOGY: The multiple facets of GHRH/GH/IGF-I axis: lessons from lifetime, untreated, isolated GH deficiency due to a GHRH receptor gene mutation." Eur J Endocrinol **177**(2): R85-R97.

Ahima, R. S., C. B. Saper, J. S. Flier and J. K. Elmquist (2000). "Leptin regulation of neuroendocrine systems." Front Neuroendocrinol **21**(3): 263-307.

Ahmed, I., T. Ghafoor, N. A. Samore and M. N. Chattha (2005). "Down syndrome: clinical and cytogenetic analysis." J Coll Physicians Surg Pak **15**(7): 426-429.

Ahmed, M. M., X. Sturgeon, M. Ellison, M. T. Davisson and K. J. Gardiner (2012). "Loss of correlations among proteins in brains of the Ts65Dn mouse model of down syndrome." J Proteome Res **11**(2): 1251-1263.

Albertson, A. J., A. Navratil, M. Mignot, L. Dufourny, B. Cherrington and D. C. Skinner (2008). "Immunoreactive GnRH type I receptors in the mouse and sheep brain." J Chem Neuroanat **35**(4): 326-333.

Albright, D. L., A. M. Voda, M. H. Smolensky, B. P. Hsi and M. Decker (1990). "Seasonal characteristics of and age at menarche." Chronobiol Int **7**(3): 251-258.

Alexander, S. E., S. Aksel, J. M. Hazelton, R. R. Yeoman and S. M. Gilmore (1990). "The effect of aging on hypothalamic function in oophorectomized women." Am J Obstet Gynecol **162**(2): 446-449.

Ambalavanan, N. and J. L. Aschner (2016). "Management of hypoxemic respiratory failure and pulmonary hypertension in preterm infants." J Perinatol **36 Suppl 2**: S20-27.

Andersen, K., L. J. Launer, M. E. Dewey, L. Letenneur, A. Ott, J. R. Copeland, J. F. Dartigues, P. Kragh-Sorensen, M. Baldereschi, C. Brayne, A. Lobo, J. M. Martinez-Lage, T. Stijnen and A. Hofman (1999). "Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group." Neurology **53**(9): 1992-1997.

Antonarakis, S. E. (1998). "10 years of Genomics, chromosome 21, and Down syndrome." Genomics **51**(1): 1-16.

Antonarakis, S. E. (2017). "Down syndrome and the complexity of genome dosage imbalance." Nat Rev Genet **18**(3): 147-163.

Antonarakis, S. E. and C. J. Epstein (2006). "The challenge of Down syndrome." Trends in Molecular Medicine **12**(10): 473-479.

Antonarakis, S. E., R. Lyle, R. Chrast and H. S. Scott (2001). "Differential gene expression studies to explore the molecular pathophysiology of Down syndrome." Brain Res Brain Res Rev **36**(2-3): 265-274.

Antonarakis, S. E., R. Lyle, E. T. Dermitzakis, A. Reymond and S. Deutsch (2004). "Chromosome 21 and down syndrome: from genomics to pathophysiology." Nat Rev Genet **5**(10): 725-738.

Apaja, P. M., K. T. Harju, J. T. Aatsinki, U. E. Petaja-Repo and H. J. Rajaniemi (2004). "Identification and structural characterization of the neuronal luteinizing hormone receptor associated with sensory systems." J Biol Chem **279**(3): 1899-1906.

Arai, A. C. (2009). "The role of kisspeptin and GPR54 in the hippocampus." Peptides **30**(1): 16-25.

Ascoli, M., F. Fanelli and D. L. Segaloff (2002). "The lutropin/choriogonadotropin receptor, a 2002 perspective." Endocr Rev **23**(2): 141-174.

Ashkenazy, H., S. Abadi, E. Martz, O. Chay, I. Mayrose, T. Pupko and N. Ben-Tal (2016). "ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules." Nucleic Acids Res **44**(W1): W344-350.

Atkins, C. M., J. C. Selcher, J. J. Petraitis, J. M. Trzaskos and J. D. Sweatt (1998). "The MAPK cascade is required for mammalian associative learning." Nat Neurosci **1**(7): 602-609.

Aydogan, U., A. Aydogdu, H. Akbulut, A. Sonmez, S. Yuksel, Y. Basaran, O. Uzun, E. Bolu and K. Saglam (2012). "Increased frequency of anxiety, depression, quality of life and sexual life in young hypogonadotropic hypogonadal males and impacts of testosterone replacement therapy on these conditions." Endocr J **59**(12): 1099-1105.

Azman, B. Z., R. Ankathil, I. Siti Mariam, M. A. Suhaida, M. Norhashimah, A. B. Tarmizi, M. A. Nor Atifah, T. P. Kannan and B. A. Zilfalil (2007). "Cytogenetic and clinical profile of Down syndrome in Northeast Malaysia." Singapore Med J **48**(6): 550-554.

Baba, Y., H. Matsuo and A. V. Schally (1971). "Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses." Biochem Biophys Res Commun **44**(2): 459-463.

Balaban, R. S., S. Nemoto and T. Finkel (2005). "Mitochondria, oxidants, and aging." Cell **120**(4): 483-495.

Ballard, C., W. Mobley, J. Hardy, G. Williams and A. Corbett (2016). "Dementia in Down's syndrome." Lancet Neurol **15**(6): 622-636.

Barja, G. (2013). "Updating the mitochondrial free radical theory of aging: an integrated view, key aspects, and confounding concepts." Antioxid Redox Signal **19**(12): 1420-1445.

Barker, D. J., P. D. Winter, C. Osmond, B. Margetts and S. J. Simmonds (1989). "Weight in infancy and death from ischaemic heart disease." Lancet **2**(8663): 577-580.

Barnes, L. L., R. S. Wilson, J. L. Bienias, J. A. Schneider, D. A. Evans and D. A. Bennett (2005). "Sex differences in the clinical manifestations of Alzheimer disease pathology." Arch Gen Psychiatry **62**(6): 685-691.

Barria, A., D. Muller, V. Derkach, L. C. Griffith and T. R. Soderling (1997). "Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation." Science **276**(5321): 2042-2045.

Barron, A. M., G. Verdile, K. Taddei, K. A. Bates and R. N. Martins (2010). "Effect of chronic hCG administration on Alzheimer's-related cognition and A beta accumulation in PS1KI mice." Endocrinology **151**(11): 5380-5388.

Batchelor, A. M., K. Bartus, C. Reynell, S. Constantinou, E. J. Halvey, K. F. Held, W. R. Dostmann, J. Vernon and J. Garthwaite (2010). "Exquisite sensitivity to subsecond, picomolar nitric

oxide transients conferred on cells by guanylyl cyclase-coupled receptors." Proc Natl Acad Sci U S A **107**(51): 22060-22065.

Bayen, E., K. L. Possin, Y. Chen, L. Cleret de Langavant and K. Yaffe (2018). "Prevalence of Aging, Dementia, and Multimorbidity in Older Adults With Down Syndrome." JAMA Neurol **75**(11): 1399-1406.

Beauvillain, J. C. and G. Tramu (1980). "Immunocytochemical demonstration of LH-RH, somatostatin, and ACTH-like peptide in osmium-postfixed, resin-embedded median eminence." J.Histochem.Cytochem. **28**(9): 1014-1017.

Belchetz, P. E., T. M. Plant, Y. Nakai, E. J. Keogh and E. Knobil (1978). "Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone." Science **202**(4368): 631-633.

Belle, M., D. Godefroy, G. Couly, S. A. Malone, F. Collier, P. Giacobini and A. Chedotal (2017). "Tridimensional Visualization and Analysis of Early Human Development." Cell **169**(1): 161-173 e112.

Belle, M., D. Godefroy, C. Dominici, C. Heitz-Marchaland, P. Zelina, F. Hellal, F. Bradke and A. Chedotal (2014). "A simple method for 3D analysis of immunolabeled axonal tracts in a transparent nervous system." Cell Rep **9**(4): 1191-1201.

Bellefontaine, N., K. Chachlaki, J. Parkash, C. Vanacker, W. Colledge, X. d'Anglemont de Tassigny, J. Garthwaite, S. G. Bouret and V. Prevot (2014). "Leptin-dependent neuronal NO signaling in the preoptic hypothalamus facilitates reproduction." J Clin Invest **124**(6): 2550-2559.

Bendayan, R., A. M. Piccinin, S. M. Hofer, D. Cadar, B. Johansson and G. Muniz-Terrera (2017). "Decline in Memory, Visuospatial Ability, and Crystalized Cognitive Abilities in Older Adults: Normative Aging or Terminal Decline?" Journal of aging research **2017**: 6210105-6210105.

Benito, E., H. Urbanke, B. Ramachandran, J. Barth, R. Halder, A. Awasthi, G. Jain, V. Capece, S. Burkhardt, M. Navarro-Sala, S. Nagarajan, A. L. Schutz, S. A. Johnsen, S. Bonn, R. Luhrmann, C. Dean and A. Fischer (2015). "HDAC inhibitor-dependent transcriptome and memory reinstatement in cognitive decline models." J Clin Invest **125**(9): 3572-3584.

Bernabeu, R., M. L. de Stein, C. Fin, I. Izquierdo and J. H. Medina (1995). "Role of hippocampal NO in the acquisition and consolidation of inhibitory avoidance learning." Neuroreport **6**(11): 1498-1500.

Bernert, G., M. Sustrova, E. Sovcikova, R. Seidl and G. Lubec (2001). "Effects of a single transdermal nicotine dose on cognitive performance in adults with Down syndrome." J Neural Transm Suppl(61): 237-245.

Berry, A., Y. Tomidokoro, J. Ghiso and J. Thornton (2008). "Human chorionic gonadotropin (a luteinizing hormone homologue) decreases spatial memory and increases brain amyloid-beta levels in female rats." Horm Behav **54**(1): 143-152.

Bertrand, I. and D. Koffas (1946). "Cas d'idioti mongolienne adulte avec nombreuses plaques senile et concretions calcaires pallidales." Rev Neurol (Paris) **78**(338).

Bhargava, Y., K. Hampden-Smith, K. Chachlaki, K. C. Wood, J. Vernon, C. K. Allerston, A. M. Batchelor and J. Garthwaite (2013). "Improved genetically-encoded, FlincG-type fluorescent biosensors for neural cGMP imaging." Front Mol Neurosci **6**: 26.

Bianchi, P., S. Bettini, S. Guidi, E. Ciani, S. Trazzi, F. Stagni, E. Ragazzi, V. Franceschini and R. Bartesaghi (2014). "Age-related impairment of olfactory bulb neurogenesis in the Ts65Dn mouse model of Down syndrome." Exp Neurol **251**: 1-11.

Blagosklonny, M. V. (2008). "Aging: ROS or TOR." Cell Cycle **7**(21): 3344-3354.
Blair, J. A., R. Palm, J. Chang, H. McGee, X. Zhu, X. Wang and G. Casadesus (2016). "Luteinizing hormone downregulation but not estrogen replacement improves ovariectomy-associated cognition and spine density loss independently of treatment onset timing." Horm Behav **78**: 60-66.
Blake, C. A. and C. H. Sawyer (1974). "Effects of hypothalamic deafferentation on the pulsatile rhythm in plasma concentrations of luteinizing hormone in ovariectomized rats." Endocrinology **94**(3): 730-736.
Bliss, T. V. and G. L. Collingridge (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." Nature **361**(6407): 31-39.
Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner and W. E. Wright (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-352.
Boehm, U., P. M. Bouloux, M. T. Dattani, N. de Roux, C. Dode, L. Dunkel, A. A. Dwyer, P. Giacobini, J. P. Hardelin, A. Juul, M. Maghnie, N. Pitteloud, V. Prevot, T. Raivio, M. Tena-Sempere, R. Quinton and J. Young (2015). "Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism--pathogenesis, diagnosis and treatment." Nat Rev Endocrinol **11**(9): 547-564.
Bohr, V. A. and R. M. Anson (1995). "DNA damage, mutation and fine structure DNA repair in aging." Mutat Res **338**(1-6): 25-34.
Bonavera, J. J., P. S. Kalra and S. P. Kalra (1996). "L-arginine/nitric oxide amplifies the magnitude and duration of the luteinizing hormone surge induced by estrogen: involvement of neuropeptide Y." Endocrinology **137**(5): 1956-1962.
Borsay, B. A., K. Skrapits, L. Herczeg, P. Ciofi, S. R. Bloom, M. A. Ghatei, W. S. Dhillo, Z. Liposits and E. Hrabovszky (2014). "Hypophysiotropic gonadotropin-releasing hormone projections are exposed to dense plexuses of kisspeptin, neurokinin B and substance p immunoreactive fibers in the human: a study on tissues from postmenopausal women." Neuroendocrinology **100**(2-3): 141-152.
Bouillanne, O., M. Rainfray, O. Tissandier, A. Nasr, A. Lahlou, X. Cnockaert and F. Piette (1996). "Growth hormone therapy in elderly people: an age-delaying drug?" Fundam Clin Pharmacol **10**(5): 416-430.
Bouligand, J., C. Ghervan, J. A. Tello, S. Brailly-Tabard, S. Salenave, P. Chanson, M. Lombes, R. P. Millar, A. Guiochon-Mantel and J. Young (2009). "Isolated familial hypogonadotropic hypogonadism and a GNRH1 mutation." N Engl J Med **360**(26): 2742-2748.
Bourguignon, J. P., A. Gerard, G. Purnelle, V. Czajkowski, C. Yamanaka, M. Lemaitre, J. M. Rigo, G. Moonen and P. Franchimont (1997). "Duality of glutamatergic and GABAergic control of pulsatile GnRH secretion by rat hypothalamic explants: II. Reduced NR2C- and GABAA-receptor-mediated inhibition at initiation of sexual maturation." J Neuroendocrinol **9**(3): 193-199.
Bovicelli, L., L. F. Orsini, N. Rizzo, V. Montacuti and M. Bacchetta (1982). "Reproduction in Down syndrome." Obstet Gynecol **59**(6 Suppl): 13S-17S.
Braden, T. D. and P. M. Conn (1992). "Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors." Endocrinology **130**(4): 2101-2105.
Breton-Provencher, V., M. Lemasson, M. R. Peralta, 3rd and A. Saghatelyan (2009). "Interneurons produced in adulthood are required for the normal functioning of the olfactory bulb network and for the execution of selected olfactory behaviors." J Neurosci **29**(48): 15245-15257.

Breving, K. and A. Esquela-Kerscher (2010). "The complexities of microRNA regulation: mirandering around the rules." Int J Biochem Cell Biol **42**(8): 1316-1329.

Bridoux, A., C. Laloux, P. Derambure, R. Bordet and C. Monaca Charley (2013). "The acute inhibition of rapid eye movement sleep by citalopram may impair spatial learning and passive avoidance in mice." J Neural Transm (Vienna) **120**(3): 383-389.

Brookmeyer, R., D. A. Evans, L. Hebert, K. M. Langa, S. G. Heeringa, B. L. Plassman and W. A. Kukull (2011). "National estimates of the prevalence of Alzheimer's disease in the United States." Alzheimers Dement **7**(1): 61-73.

Brunjes, P. C. and L. L. Frazier (1986). "Maturation and plasticity in the olfactory system of vertebrates." Brain Res **396**(1): 1-45.

Bryan, K. J., J. C. Mudd, S. L. Richardson, J. Chang, H. G. Lee, X. Zhu, M. A. Smith and G. Casadesus (2010). "Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits." J Neurochem **112**(4): 870-881.

Buée-Scherrer, Condamines, Mourton-Gilles, Jakes, Goedert, Pau and Delacourte (1996). "AD2, a phosphorylation-dependent monoclonal antibody directed against tau proteins found in Alzheimer's disease." Brain Res Mol Brain Res.

Jul;39(1-2):79-88.

Burke, S. N. and C. A. Barnes (2006). "Neural plasticity in the ageing brain." Nat Rev Neurosci **7**(1): 30-40.

Burnham, V., C. Sundby, A. Laman-Maharg and J. Thornton (2017). "Luteinizing hormone acts at the hippocampus to dampen spatial memory." Horm Behav **89**: 55-63.

Campbell, R. E., G. Gaidamaka, S. K. Han and A. E. Herbison (2009). "Dendro-dendritic bundling and shared synapses between gonadotropin-releasing hormone neurons." Proc Natl Acad Sci U S A **106**(26): 10835-10840.

Campbell, R. E. and T. M. Plant (2018). Morphology of the Adult GnRH Neuron. The GnRH Neuron and its Control. A. E. Herbison and T. M. Plant**:** 121-148.

Campbell, W. A., J. Lowther, I. McKenzie and W. H. Price (1982). "Serum gonadotrophins in Down's syndrome." J Med Genet **19**(2): 98-99.

Caraty, A., A. Locatelli and G. B. Martin (1989). "Biphasic response in the secretion of gonadotrophin-releasing hormone in ovariectomized ewes injected with oestradiol." J Endocrinol **123**(3): 375-382.

Caraty, A. and D. C. Skinner (2008). "Gonadotropin-Releasing Hormone in Third Ventricular Cerebrospinal Fluid: Endogenous Distribution and Exogenous Uptake." Endocrinology **149**(10): 5227-5234.

Carlson, M. C., L. Hasher, R. T. Zacks and S. L. Connelly (1995). "Aging, distraction, and the benefits of predictable location." Psychol Aging **10**(3): 427-436.

Carosa, E., P. Martini, F. Brandetti, S. M. Di Stasi, F. Lombardo, A. Lenzi and E. A. Jannini (2004). "Type V phosphodiesterase inhibitor treatments for erectile dysfunction increase testosterone levels." Clin Endocrinol (Oxf) **61**(3): 382-386.

Carr, J. (1994). "Long-term-outcome for people with Down's syndrome." J Child Psychol Psychiatry **35**(3): 425-439.

Carr, J. (2005). "Stability and change in cognitive ability over the life span: a comparison of populations with and without Down's syndrome." J Intellect Disabil Res **49**(Pt 12): 915-928.

Casadesus, G., E. L. Milliken, K. M. Webber, R. L. Bowen, Z. Lei, C. V. Rao, G. Perry, R. A. Keri and M. A. Smith (2007). "Increases in luteinizing hormone are associated with declines in cognitive performance." Molecular and Cellular Endocrinology **269**(1): 107-111.

Casoni, F., B. I. Hutchins, D. Donohue, M. Fornaro, B. G. Condie and S. Wray (2012). "SDF and GABA interact to regulate axophilic migration of GnRH neurons." J Cell Sci **125**(Pt 21): 5015-5025.

Casoni, F., S. A. Malone, M. Belle, F. Luzzati, F. Collier, C. Allet, E. Hrabovszky, S. Rasika, V. Prevot, A. Chedotal and P. Giacobini (2016). "Development of the neurons controlling fertility in humans: new insights from 3D imaging and transparent fetal brains." Development **143**(21): 3969-3981.

Cassatella, D., S. R. Howard, J. S. Acierno, C. Xu, G. E. Papadakis, F. A. Santoni, A. A. Dwyer, S. Santini, G. P. Sykiotis, C. Chambion, J. Meylan, L. Marino, L. Favre, J. Li, X. Liu, J. Zhang, P. M. Bouloux, C. Geyter, A. Paepe, W. S. Dhillo, J. M. Ferrara, M. Hauschild, M. Lang-Muritano, J. R. Lemke, C. Fluck, A. Nemeth, F. Phan-Hug, D. Pignatelli, V. Popovic, S. Pekic, R. Quinton, G. Szinnai, D. l'Allemand, D. Konrad, S. Sharif, O. T. Iyidir, B. J. Stevenson, H. Yang, L. Dunkel and N. Pitteloud (2018). "Congenital hypogonadotropic hypogonadism and constitutional delay of growth and puberty have distinct genetic architectures." Eur J Endocrinol **178**(4): 377-388.

Cecchini, M. P., D. Viviani, M. Sandri, A. Hähner, T. Hummel and C. Zancanaro (2016). "Olfaction in People with Down Syndrome: A Comprehensive Assessment across Four Decades of Age." PloS one **11**(1): e0146486-e0146486.

Chachlaki, K., J. Garthwaite and V. Prevot (2017). "The gentle art of saying NO: how nitric oxide gets things done in the hypothalamus." Nat Rev Endocrinol **13**(9): 521-535.

Chachlaki, K., S. A. Malone, E. Qualls-Creekmore, E. Hrabovszky, H. Munzberg, P. Giacobini, F. Ango and V. Prevot (2017). "Phenotyping of nNOS neurons in the postnatal and adult female mouse hypothalamus." J Comp Neurol **525**(15): 3177-3189.

Chakravarti, S., W. P. Collins, J. D. Forecast, J. R. Newton, D. H. Oram and J. W. Studd (1976). "Hormonal profiles after the menopause." British medical journal **2**(6039): 784-787.

Chapman, R. M., M. Mapstone, M. N. Gardner, T. C. Sandoval, J. W. McCrary, M. D. Guillily, L. A. Reilly and E. DeGrush (2011). "Women have farther to fall: gender differences between normal elderly and Alzheimer's disease in verbal memory engender better detection of Alzheimer's disease in women." J Int Neuropsychol Soc **17**(4): 654-662.

Charlton, H. M., A. J. Jones, D. Whitworth, M. J. Gibson, G. Kokoris, E. A. Zimmerman and A. J. Silverman (1987). "The effects of the age of intracerebroventricular grafts of normal preoptic area tissue upon pituitary and gonadal function in hypogonadal (HPG) mice." Neuroscience **21**(1): 175-181.

Charriaut-Marlangue, C., P. Bonnin, H. Pham, G. Loron, P. L. Leger, P. Gressens, S. Renolleau and O. Baud (2013). "Nitric oxide signaling in the brain: a new target for inhaled nitric oxide?" Ann Neurol **73**(4): 442-448.

Chatterjee, S., R. Cassel, A. Schneider-Anthony, K. Merienne, B. Cosquer, L. Tzeplaeff, S. Halder Sinha, M. Kumar, P. Chaturbedy, M. Eswaramoorthy, S. Le Gras, C. Keime, O. Bousiges, P. Dutar, P. Petsophonsakul, C. Rampon, J. C. Cassel, L. Buee, D. Blum, T. K. Kundu and A. L. Boutillier (2018). "Reinstating plasticity and memory in a tauopathy mouse model with an acetyltransferase activator." EMBO Mol Med **10**(11).

Chen, J. J., H. D. Rosas and D. H. Salat (2011). "Age-associated reductions in cerebral blood flow are independent from regional atrophy." Neuroimage **55**(2): 468-478.

Chertow, B. S. (1981). "The role of lysosomes and proteases in hormone secretion and degradation." Endocr Rev **2**(2): 137-173.

Chiba, A., Y. C. Sohn and Y. Honma (1996). "Immunohistochemical and Ultrastructural characterization of the terminal nerve ganglion cells of the ayu, Plecoglossus altivelis (Salmoniformes, Teleostei)." Anat Rec **246**(4): 549-556.

Childs, G. V. and G. Unabia (1997). "Cytochemical studies of the effects of activin on gonadotropin-releasing hormone (GnRH) binding by pituitary gonadotropes and growth hormone cells." J Histochem Cytochem **45**(12): 1603-1610.

Chowdhury, R., M. Guitart-Masip, C. Lambert, P. Dayan, Q. Huys, E. Duzel and R. J. Dolan (2013). "Dopamine restores reward prediction errors in old age." Nat Neurosci **16**(5): 648-653.

Christian, C. A. and S. M. Moenter (2007). "Estradiol induces diurnal shifts in GABA transmission to gonadotropin-releasing hormone neurons to provide a neural signal for ovulation." J Neurosci **27**(8): 1913-1921.

Cingolani, P., A. Platts, L. Wang le, M. Coon, T. Nguyen, L. Wang, S. J. Land, X. Lu and D. M. Ruden (2012). "A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3." Fly (Austin) **6**(2): 80-92.

Clarke, I. J. (2015). "Hypothalamus as an endocrine organ." Compr Physiol **5**(1): 217-253.

Clarke, I. J., J. T. Cummins, M. E. Crowder and T. M. Nett (1987). "Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma luteinizing hormone in ovariectomized-hypothalamo pituitary disconnected ewes. I. Effect of changing frequency of gonadotropin-releasing hormone pulses." Biol Reprod **37**(4): 749-754.

Clarkson, J., W. C. Boon, E. R. Simpson and A. E. Herbison (2009). "Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset." Endocrinology **150**(7): 3214-3220.

Clarkson, J., S. Y. Han, R. Piet, T. McLennan, G. M. Kane, J. Ng, R. W. Porteous, J. S. Kim, W. H. Colledge, K. J. Iremonger and A. E. Herbison (2017). "Definition of the hypothalamic GnRH pulse generator in mice." Proc Natl Acad Sci U S A **114**(47): E10216-E10223.

Clarkson, J. and A. E. Herbison (2009). "Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinising hormone surge." J Neuroendocrinol **21**(4): 305-311.

Clasadonte, J., P. Poulain, J. C. Beauvillain and V. Prevot (2008). "Activation of neuronal nitric oxide release inhibits spontaneous firing in adult gonadotropin-releasing hormone neurons: a possible local synchronizing signal." Endocrinology **149**(2): 587-596.

Claxton, A., L. D. Baker, C. W. Wilkinson, E. H. Trittschuh, D. Chapman, G. S. Watson, B. Cholerton, S. R. Plymate, M. Arbuckle and S. Craft (2013). "Sex and ApoE genotype differences in treatment response to two doses of intranasal insulin in adults with mild cognitive impairment or Alzheimer's disease." J Alzheimers Dis **35**(4): 789-797.

Conn, P. M., C. A. McArdle, W. V. Andrews and W. R. Huckle (1987). "The molecular basis of gonadotropin-releasing hormone (GnRH) action in the pituitary gonadotrope." Biol Reprod **36**(1): 17-35.

Considine, R. V., M. K. Sinha, M. L. Heiman, A. Kriauciunas, T. W. Stephens, M. R. Nyce, J. P. Ohannesian, C. C. Marco, L. J. McKee, T. L. Bauer and et al. (1996). "Serum immunoreactive-leptin concentrations in normal-weight and obese humans." N Engl J Med **334**(5): 292-295.

Constantin, S. (2011). "Physiology of the gonadotrophin-releasing hormone (GnRH) neurone: studies from embryonic GnRH neurones." Journal of neuroendocrinology **23**(6): 542-553.

Constantin, S., C. Jasoni, N. Romano, K. Lee and A. E. Herbison (2012). "Understanding calcium homeostasis in postnatal gonadotropin-releasing hormone neurons using cell-specific Pericam transgenics." Cell Calcium **51**(3-4): 267-276.

Contestabile, A., M. Parrini, S. Naskar, M. Alberti, A. Rocchi, M. Nanni, G. Ronzitti, F. Mingozzi and L. Cancedda (2016). "53. A Neuro-Specific Gene Therapy Approach to Treat Cognitive Impairment in Down Syndrome by RNA Interference." Molecular Therapy **24**: S23-S24.

Coppus, A. M., H. M. Evenhuis, G. J. Verberne, F. E. Visser, P. Eikelenboom, W. A. van Gool, A. C. Janssens and C. M. van Duijn (2010). "Early age at menopause is associated with increased risk of dementia and mortality in women with Down syndrome." J Alzheimers Dis **19**(2): 545-550.

Corpas, E., S. M. Harman and M. R. Blackman (1993). "Human growth hormone and human aging." Endocr Rev **14**(1): 20-39.

Coussons-Read, M. E. and L. S. Crnic (1996). "Behavioral assessment of the Ts65Dn mouse, a model for Down syndrome: altered behavior in the elevated plus maze and open field." Behav Genet **26**(1): 7-13.

Couzens, D., M. Cuskelly and M. Haynes (2011). "Cognitive development and Down syndrome: age-related change on the Stanford-Binet test (fourth edition)." Am J Intellect Dev Disabil **116**(3): 181-204.

Couzens, D., M. Haynes and M. Cuskelly (2012). "Individual and environmental characteristics associated with cognitive development in Down syndrome: a longitudinal study." J Appl Res Intellect Disabil **25**(5): 396-413.

Cristofalo, V. J., R. G. Allen, R. J. Pignolo, B. G. Martin and J. C. Beck (1998). "Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation." Proc Natl Acad Sci U S A **95**(18): 10614-10619.

Curran-Rauhut, M. A. and S. L. Petersen (2002). "Regulation of glutamic acid decarboxylase 65 and 67 gene expression by ovarian steroids: identification of two functionally distinct populations of GABA neurones in the preoptic area." J Neuroendocrinol **14**(4): 310-317.

Czieselsky, K., M. Prescott, R. Porteous, P. Campos, J. Clarkson, F. J. Steyn, R. E. Campbell and A. E. Herbison (2016). "Pulse and Surge Profiles of Luteinizing Hormone Secretion in the Mouse." Endocrinology **157**(12): 4794-4802.

d'Anglemont de Tassigny, X., C. Campagne, B. Dehouck, D. Leroy, G. R. Holstein, J. C. Beauvillain, V. Buee-Scherrer and V. Prevot (2007). "Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction." J Neurosci **27**(23): 6103-6114.

d'Anglemont de Tassigny, X., C. Campagne, S. Steculorum and V. Prevot (2009). "Estradiol induces physical association of neuronal nitric oxide synthase with NMDA receptor and promotes nitric oxide formation via estrogen receptor activation in primary neuronal cultures." J Neurochem **109**(1): 214-224.

D'Onofrio, B. M., Q. A. Class, M. E. Rickert, H. Larsson, N. Langstrom and P. Lichtenstein (2013). "Preterm birth and mortality and morbidity: a population-based quasi-experimental study." JAMA Psychiatry **70**(11): 1231-1240.

Dai, D.-F., Y. A. Chiao, D. J. Marcinek, H. H. Szeto and P. S. Rabinovitch (2014). "Mitochondrial oxidative stress in aging and healthspan." Longevity & Healthspan **3**(1): 6.

Daikoku, S., I. Koide, M. Chikamori-Aoyama and Y. Shimomura (1993). "Migration of LHRH neurons derived from the olfactory placode in rats." Arch Histol Cytol **56**(4): 353-370.

David, M. A., F. Fraschini and L. Martini (1966). "Control of LH secretion: role of a "short" feedback mechanism." Endocrinology **78**(1): 55-60.

Davis, A. M., D. R. Grattan, M. Selmanoff and M. M. McCarthy (1996). "Sex differences in glutamic acid decarboxylase mRNA in neonatal rat brain: implications for sexual differentiation." Horm Behav **30**(4): 538-552.

Davisson, M., E. Akeson, C. Schmidt, B. Harris, J. Farley and M. A. Handel (2007). "Impact of trisomy on fertility and meiosis in male mice." Hum Reprod **22**(2): 468-476.

Davisson, M. T., L. J. Bechtel, E. C. Akeson, A. Fortna, D. Slavov and K. Gardiner (2001). "Evolutionary breakpoints on human chromosome 21." Genomics **78**(1-2): 99-106.

Davisson, M. T., C. Schmidt and E. C. Akeson (1990). "Segmental trisomy of murine chromosome 16: a new model system for studying Down syndrome." Prog Clin Biol Res **360**: 263-280.

Davisson, M. T., C. Schmidt, R. H. Reeves, N. G. Irving, E. C. Akeson, B. S. Harris and R. T. Bronson (1993). "Segmental trisomy as a mouse model for Down syndrome." Prog Clin Biol Res **384**: 117-133.

De Falco, F. A., E. D'Angelo, G. Grimaldi, F. Scafuro, F. Sachez and G. Caruso (1994). "[Effect of the chronic treatment with L-acetylcarnitine in Down's syndrome]." Clin Ter **144**(2): 123-127.

de la Torre, R. and M. Dierssen (2012). "Therapeutic approaches in the improvement of cognitive performance in Down syndrome: past, present, and future." Prog Brain Res **197**: 1-14.

De Morsier, G. and G. Gauthier (1963). "[OLFACTO-GENITAL DYSPLASIA]." Pathol Biol **11**: 1267-1272.

de Roux, N., E. Genin, J. C. Carel, F. Matsuda, J. L. Chaussain and E. Milgrom (2003). "Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54." Proc Natl Acad Sci U S A **100**(19): 10972-10976.

de Roux, N., E. Genin, J. C. Carel, F. Matsuda, J. L. Chaussain and E. Milgrom (2003). "Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54." Proc.Natl.Acad.Sci.U.S.A **100**(19): 10972-10976.

de Roux, N., J. Young, M. Misrahi, R. Genet, P. Chanson, G. Schaison and E. Milgrom (1997). "A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor." N Engl J Med **337**(22): 1597-1602.

de San Juan, A. (1856). "Falta total de los nerviosolfactorios con anosmia en un individuo en quien existia una atrofia congenita de los testiculos y miembro viril." Siglo Med **131**(211).

de Souza, F. M. S., N. Busquet, M. Blatner, K. N. Maclean and D. Restrepo (2011). "Galantamine improves olfactory learning in the Ts65Dn mouse model of Down syndrome." Scientific reports **1**: 137-137.

Delabar, J. M., D. Theophile, Z. Rahmani, Z. Chettouh, J. L. Blouin, M. Prieur, B. Noel and P. M. Sinet (1993). "Molecular mapping of twenty-four features of Down syndrome on chromosome 21." Eur J Hum Genet **1**(2): 114-124.

Dellu, F., A. Contarino, H. Simon, G. F. Koob and L. H. Gold (2000). "Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice." Neurobiol Learn Mem **73**(1): 31-48.

Delmas, S., R. Porteous, D. H. Bergin and A. E. Herbison (2018). "Altered aspects of anxiety-related behavior in kisspeptin receptor-deleted male mice." Scientific Reports **8**(1): 2794.

Demas, G. E., M. J. Eliasson, T. M. Dawson, V. L. Dawson, L. J. Kriegsfeld, R. J. Nelson and S. H. Snyder (1997). "Inhibition of neuronal nitric oxide synthase increases aggressive behavior in mice." Mol Med **3**(9): 610-616.

Demas, G. E., R. J. Nelson, B. K. Krueger and P. J. Yarowsky (1996). "Spatial memory deficits in segmental trisomic Ts65Dn mice." Behav Brain Res **82**(1): 85-92.

den Dunnen, J. T. and S. E. Antonarakis (2000). "Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion." Hum Mutat **15**(1): 7-12.

Denley, M. C. S., N. J. F. Gatford, K. J. Sellers and D. P. Srivastava (2018). "Estradiol and the Development of the Cerebral Cortex: An Unexpected Role?" Front Neurosci **12**: 245.

DePristo, M. A., E. Banks, R. Poplin, K. V. Garimella, J. R. Maguire, C. Hartl, A. A. Philippakis, G. del Angel, M. A. Rivas, M. Hanna, A. McKenna, T. J. Fennell, A. M. Kernytsky, A. Y. Sivachenko, K. Cibulskis, S. B. Gabriel, D. Altshuler and M. J. Daly (2011). "A framework for variation discovery and genotyping using next-generation DNA sequencing data." Nat Genet **43**(5): 491-498.

Deslypere, J. P., J. M. Kaufman, T. Vermeulen, D. Vogelaers, J. L. Vandalem and A. Vermeulen (1987). "Influence of age on pulsatile luteinizing hormone release and responsiveness of the gonadotrophs to sex hormone feedback in men." J Clin Endocrinol Metab **64**(1): 68-73.

Dhillo, W. S., O. B. Chaudhri, M. Patterson, E. L. Thompson, K. G. Murphy, M. K. Badman, B. M. McGowan, V. Amber, S. Patel, M. A. Ghatei and S. R. Bloom (2005). "Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males." J Clin Endocrinol Metab **90**(12): 6609-6615.

Di Bona, D., A. Plaia, S. Vasto, L. Cavallone, F. Lescai, C. Franceschi, F. Licastro, G. Colonna-Romano, D. Lio, G. Candore and C. Caruso (2008). "Association between the interleukin-1beta polymorphisms and Alzheimer's disease: a systematic review and meta-analysis." Brain Res Rev **59**(1): 155-163.

Dickson, D. W. (1997). "The pathogenesis of senile plaques." J Neuropathol Exp Neurol **56**(4): 321-339.

Dierssen, M. (2012). "Down syndrome: the brain in trisomic mode." Nat Rev Neurosci **13**(12): 844-858.

Dierssen, M., Y. Herault and X. Estivill (2009). "Aneuploidy: from a physiological mechanism of variance to Down syndrome." Physiol Rev **89**(3): 887-920.

Dierssen, M., E. Marti, C. Pucharcos, V. Fotaki, X. Altafaj, K. Casas, A. Solans, M. L. Arbones, C. Fillat and X. Estivill (2001). "Functional genomics of Down syndrome: a multidisciplinary approach." J Neural Transm Suppl(61): 131-148.

Dierssen, M., J. Ortiz-Abalia, G. Arque, M. M. de Lagran and C. Fillat (2006). "Pitfalls and hopes in Down syndrome therapeutic approaches: in the search for evidence-based treatments." Behav Genet **36**(3): 454-468.

Doty, R. L. (2012). "Olfactory dysfunction in Parkinson disease." Nat Rev Neurol **8**(6): 329-339.

Drummond, E. S., R. N. Martins, D. J. Handelsman and A. R. Harvey (2012). "Altered expression of Alzheimer's disease-related proteins in male hypogonadal mice." Endocrinology **153**(6): 2789-2799.

Duchon, A., M. Raveau, C. Chevalier, V. Nalesso, A. J. Sharp and Y. Herault (2011). "Identification of the translocation breakpoints in the Ts65Dn and Ts1Cje mouse lines: relevance for modeling Down syndrome." Mamm Genome **22**(11-12): 674-684.

Dungan, H. M., D. K. Clifton and R. A. Steiner (2006). "Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion." Endocrinology **147**(3): 1154-1158.

Ebling, F. J. P. (2005). "The neuroendocrine timing of puberty." Reproduction **129**(6): 675-683.

Editorial, T. L. (2013). "Strengthening connections between Down syndrome and AD." **12**(10): 931.

Elias, C. F. and D. Purohit (2013). "Leptin signaling and circuits in puberty and fertility." Cell Mol Life Sci **70**(5): 841-862.

Eliasson, M. J., S. Blackshaw, M. J. Schell and S. H. Snyder (1997). "Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain." Proc.Natl.Acad.Sci.U.S.A **94**(7): 3396-3401.

Elton, T. S., S. E. Sansom and M. M. Martin (2010). "Trisomy-21 gene dosage over-expression of miRNAs results in the haploinsufficiency of specific target proteins." RNA Biol **7**(5): 540-547.

Emanuele, N., R. Oslapas, E. Connick, L. Kirsteins and A. M. Lawrence (1981). "Hypothalamic LH may play a role in control of pituitary LH release." Neuroendocrinology **33**(1): 12-17.

Emanuele, N. V., J. Anderson, E. Andersen, E. Connick, G. Baker, L. Kirsteins and A. M. Lawrence (1983). "Extrahypothalamic brain luteinizing hormone: characterization by radioimmunoassay, chromatography, radioligand assay and bioassay." Neuroendocrinology **36**(4): 254-260.

Encinas, J. M., T. V. Michurina, N. Peunova, J. H. Park, J. Tordo, D. A. Peterson, G. Fishell, A. Koulakov and G. Enikolopov (2011). "Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus." Cell Stem Cell **8**(5): 566-579.

Engler, A. and E. Schulze (2017). "POSEIDON - Bringing Assistive Technology to People with Down Syndrome: Results of a Three Year European Project." Stud Health Technol Inform **236**: 169-175.

English, J. D. and J. D. Sweatt (1997). "A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation." J Biol Chem **272**(31): 19103-19106.

Epstein, C. J. (1986). "Developmental genetics." Experientia **42**(10): 1117-1128.

Epstein, C. J. (1990). "The consequences of chromosome imbalance." Am J Med Genet Suppl **7**: 31-37.

Epstein, C. J., J. R. Korenberg, G. Anneren, S. E. Antonarakis, S. Ayme, E. Courchesne, L. B. Epstein, A. Fowler, Y. Groner, J. L. Huret and et al. (1991). "Protocols to establish genotype-phenotype correlations in Down syndrome." Am J Hum Genet **49**(1): 207-235.

Erturk, A., K. Becker, N. Jahrling, C. P. Mauch, C. D. Hojer, J. G. Egen, F. Hellal, F. Bradke, M. Sheng and H. U. Dodt (2012). "Three-dimensional imaging of solvent-cleared organs using 3DISCO." Nat Protoc **7**(11): 1983-1995.

Erturk, A. and F. Bradke (2013). "High-resolution imaging of entire organs by 3-dimensional imaging of solvent cleared organs (3DISCO)." Exp Neurol **242**: 57-64.

Escorihuela, R. M., A. Fernandez-Teruel, I. F. Vallina, C. Baamonde, M. A. Lumbreras, M. Dierssen, A. Tobena and J. Florez (1995). "A behavioral assessment of Ts65Dn mice: a putative Down syndrome model." Neurosci Lett **199**(2): 143-146.

Esiri, M. M. (2007). "Ageing and the brain." J Pathol **211**(2): 181-187.

Esiri, M. M. and G. K. Wilcock (1984). "The olfactory bulbs in Alzheimer's disease." J Neurol Neurosurg Psychiatry **47**(1): 56-60.

Espeland, M. A., S. R. Rapp, S. A. Shumaker, R. Brunner, J. E. Manson, B. B. Sherwin, J. Hsia, K. L. Margolis, P. E. Hogan, R. Wallace, M. Dailey, R. Freeman, J. Hays and S. Women's Health Initiative Memory (2004). "Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study." JAMA **291**(24): 2959-2968.

Everett, J. W. (1964). "Central Neural Control of Reproductive Functions of the Adenohypophysis." Physiol Rev **44**: 373-431.

Everett, J. W., C. H. Sawyer and J. E. Markee (1949). "A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat." Endocrinology **44**(3): 234-250.

Everitt, B. J. and T. Hokfelt (1990). "Neuroendocrine anatomy of the hypothalamus." Acta Neurochir Suppl (Wien) **47**: 1-15.

Farbman, A. I. (1992). "Cell Biology of Olfaction." Cambridge University Press.

Farooqi, I. S. and S. O'Rahilly (2014). "20 years of leptin: human disorders of leptin action." J Endocrinol **223**(1): T63-70.

Ferguson, S. S. (2001). "Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling." Pharmacol Rev **53**(1): 1-24.

Fernandez, F., W. Morishita, E. Zuniga, J. Nguyen, M. Blank, R. C. Malenka and C. C. Garner (2007). "Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome." Nat Neurosci **10**(4): 411-413.

Finch, C. E. (1976). "The regulation of physiological changes during mammalian aging." Q Rev Biol **51**(1): 49-83.

Fjell, A. M., L. McEvoy, D. Holland, A. M. Dale, K. B. Walhovd and I. Alzheimer's Disease Neuroimaging (2014). "What is normal in normal aging? Effects of aging, amyloid and Alzheimer's disease on the cerebral cortex and the hippocampus." Prog Neurobiol **117**: 20-40.

Flatt, T. and P. S. Schmidt (2009). "Integrating evolutionary and molecular genetics of aging." Biochim Biophys Acta **1790**(10): 951-962.

Fleming, J. E., I. Reveillaud and A. Niedzwiecki (1992). "Role of oxidative stress in Drosophila aging." Mutat Res **275**(3-6): 267-279.

Freeman, S. B., L. F. Taft, K. J. Dooley, K. Allran, S. L. Sherman, T. J. Hassold, M. J. Khoury and D. M. Saker (1998). "Population-based study of congenital heart defects in Down syndrome." Am J Med Genet **80**(3): 213-217.

Frisch, R. E. and J. W. McArthur (1974). "Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset." Science **185**(4155): 949-951.

Frisch, R. E. and R. Revelle (1970). "Height and weight at menarche and a hypothesis of critical body weights and adolescent events." Science **169**(3943): 397-399.

Fuentealba, P., R. Begum, M. Capogna, S. Jinno, L. F. Marton, J. Csicsvari, A. Thomson, P. Somogyi and T. Klausberger (2008). "Ivy cells: a population of nitric-oxide-producing, slow-spiking GABAergic neurons and their involvement in hippocampal network activity." Neuron **57**(6): 917-929.

Fukusumi, S., R. Fujii and S. Hinuma (2006). "Recent advances in mammalian RFamide peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP." Peptides **27**(5): 1073-1086.

Gaillard, A. L., B. H. Tay, D. I. Perez Sirkin, A. G. Lafont, C. De Flori, P. G. Vissio, S. Mazan, S. Dufour, B. Venkatesh and H. Tostivint (2018). "Characterization of Gonadotropin-Releasing Hormone (GnRH) Genes From Cartilaginous Fish: Evolutionary Perspectives." Front Neurosci **12**: 607.

Gallo, R. V., J. H. Johnson, S. P. Kalra, D. I. Whitmoyer and C. H. Sawyer (1972). "Effects of luteinizing hormone on multiple-unit activity in the rat hippocampus." Neuroendocrinology **9**(3): 149-157.

Gao, Y. and S. A. Heldt (2015). "Lack of neuronal nitric oxide synthase results in attention deficit hyperactivity disorder-like behaviors in mice." Behav Neurosci **129**(1): 50-61.

Garcia-Duran, M., T. de Frutos, J. Diaz-Recasens, G. Garcia-Galvez, A. Jimenez, M. Monton, J. Farre, L. Sanchez de Miguel, F. Gonzalez-Fernandez, M. D. Arriero, L. Rico, R. Garcia, S. Casado and A. Lopez-Farre (1999). "Estrogen stimulates neuronal nitric oxide synthase protein expression in human neutrophils." Circ Res **85**(11): 1020-1026.

Garthwaite, J., E. Southam, C. L. Boulton, E. B. Nielsen, K. Schmidt and B. Mayer (1995). "Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one." Mol.Pharmacol. **48**(2): 184-188.

Ghezzo, A., S. Salvioli, M. C. Solimando, A. Palmieri, C. Chiostergi, M. Scurti, L. Lomartire, F. Bedetti, G. Cocchi, D. Follo, E. Pipitone, P. Rovatti, J. Zamberletti, T. Gomiero, G. Castellani and C. Franceschi (2014). "Age-related changes of adaptive and neuropsychological features in persons with Down Syndrome." PloS one **9**(11): e113111-e113111.

Gianetti, E., C. Tusset, S. D. Noel, M. G. Au, A. A. Dwyer, V. A. Hughes, A. P. Abreu, J. Carroll, E. Trarbach, L. F. Silveira, E. M. Costa, B. B. de Mendonca, M. de Castro, A. Lofrano, J. E. Hall, E. Bolu, M. Ozata, R. Quinton, J. K. Amory, S. E. Stewart, W. Arlt, T. R. Cole, W. F. Crowley, U. B. Kaiser, A. C. Latronico and S. B. Seminara (2010). "TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood." J Clin Endocrinol Metab **95**(6): 2857-2867.

Gibbs, R. B. (2000). "Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats." Neurobiol Aging **21**(1): 107-116.

Gonzalez-Martinez, D., N. Zmora, E. Mananos, D. Saligaut, S. Zanuy, Y. Zohar, A. Elizur, O. Kah and J. A. Munoz-Cueto (2002). "Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (Dicentrarchus labrax) using antibodies to the corresponding GnRH-associated peptides." J Comp Neurol **446**(2): 95-113.

Goodman, R. and F. Karsch (1981). "Biological Clocks in Seasonal Reproductive Cycles." Wright, Bristol: 223.

Gordon, C. M., K. E. Ackerman, S. L. Berga, J. R. Kaplan, G. Mastorakos, M. Misra, M. H. Murad, N. F. Santoro and M. P. Warren (2017). "Functional Hypothalamic Amenorrhea: An

Endocrine Society Clinical Practice Guideline." J Clin Endocrinol Metab **102**(5): 1413-1439.

Gore, A. C., T. Oung and M. J. Woller (2002). "Age-related changes in hypothalamic gonadotropin-releasing hormone and N-methyl-D-aspartate receptor gene expression, and their regulation by oestrogen, in the female rat." J Neuroendocrinol **14**(4): 300-309.

Gore, A. C. and J. L. Roberts (1997). "Regulation of gonadotropin-releasing hormone gene expression in vivo and in vitro." Front Neuroendocrinol **18**(2): 209-245.

Goroll, D., P. Arias and W. Wuttke (1994). "Ontogenic changes in the hypothalamic levels of amino acid neurotransmitters in the female rat." Brain Res Dev Brain Res **77**(2): 183-188.

Granger, A., V. Ngo-Muller, C. Bleux, C. Guigon, H. Pincas, S. Magre, D. Daegelen, A. Tixier-Vidal, R. Counis and J. N. Laverriere (2004). "The promoter of the rat gonadotropin-releasing hormone receptor gene directs the expression of the human placental alkaline phosphatase reporter gene in gonadotrope cells in the anterior pituitary gland as well as in multiple extrapituitary tissues." Endocrinology **145**(2): 983-993.

Greenberg, D. A. and K. Jin (2006). "Turning neurogenesis up a Notch." Nat Med **12**(8): 884-885.

Gregg, D. W. and T. M. Nett (1989). "Direct effects of estradiol-17 beta on the number of gonadotropin-releasing hormone receptors in the ovine pituitary." Biol Reprod **40**(2): 288-293.

Grieco, J., M. Pulsifer, K. Seligsohn, B. Skotko and A. Schwartz (2015). "Down syndrome: Cognitive and behavioral functioning across the lifespan." Am J Med Genet C Semin Med Genet **169**(2): 135-149.

Griffiths, S., H. Scott, C. Glover, A. Bienemann, M. T. Ghorbel, J. Uney, M. W. Brown, E. C. Warburton and Z. I. Bashir (2008). "Expression of long-term depression underlies visual recognition memory." Neuron **58**(2): 186-194.

Griswold, M. D. and D. McLean (2006). CHAPTER 19 - The Sertoli Cell. Knobil and Neill's Physiology of Reproduction (Third Edition). J. D. Neill. St Louis, Academic Press**:** 949-975.

Gu, G. B. and R. B. Simerly (1997). "Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat." J Comp Neurol **384**(1): 142-164.

Guedj, F., J. L. Pennings, M. A. Ferres, L. C. Graham, H. C. Wick, K. A. Miczek, D. K. Slonim and D. W. Bianchi (2015). "The fetal brain transcriptome and neonatal behavioral phenotype in the Ts1Cje mouse model of Down syndrome." Am J Med Genet A **167A**(9): 1993-2008.

Guevara-Aguirre, J., P. Balasubramanian, M. Guevara-Aguirre, M. Wei, F. Madia, C. W. Cheng, D. Hwang, A. Martin-Montalvo, J. Saavedra, S. Ingles, R. de Cabo, P. Cohen and V. D. Longo (2011). "Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans." Sci Transl Med **3**(70): 70ra13.

Gupta, D. and J. E. Morley (2014). "Hypothalamic-pituitary-adrenal (HPA) axis and aging." Compr Physiol **4**(4): 1495-1510.

Gyurko, R., S. Leupen and P. L. Huang (2002). "Deletion of exon 6 of the neuronal nitric oxide synthase gene in mice results in hypogonadism and infertility." Endocrinology **143**(7): 2767-2774.

Hall, J. (2012). "Boston Naming Test: Gender Differences in Older Adults with and without Alzheimer's Dementia." Psychology **03**: 485-488.

Hall, J. E., H. B. Lavoie, E. E. Marsh and K. A. Martin (2000). "Decrease in gonadotropin-releasing hormone (GnRH) pulse frequency with aging in postmenopausal women." J Clin Endocrinol Metab **85**(5): 1794-1800.

Hamalainen, T., M. Poutanen and I. Huhtaniemi (1999). "Age- and sex-specific promoter function of a 2-kilobase 5'-flanking sequence of the murine luteinizing hormone receptor gene in transgenic mice." Endocrinology **140**(11): 5322-5329.

Hamernik, D. L. and T. M. Nett (1988). "Gonadotropin-releasing hormone increases the amount of messenger ribonucleic acid for gonadotropins in ovariectomized ewes after hypothalamic-pituitary disconnection." Endocrinology **122**(3): 959-966.

Han, S. K., M. L. Gottsch, K. J. Lee, S. M. Popa, J. T. Smith, S. K. Jakawich, D. K. Clifton, R. A. Steiner and A. E. Herbison (2005). "Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty." J Neurosci **25**(49): 11349-11356.

Hanchate, N. K., P. Giacobini, P. Lhuillier, J. Parkash, C. Espy, C. Fouveaut, C. Leroy, S. Baron, C. Campagne, C. Vanacker, F. Collier, C. Cruaud, V. Meyer, A. Garcia-Pinero, D. Dewailly, C. Cortet-Rudelli, K. Gersak, C. Metz, G. Chabrier, M. Pugeat, J. Young, J. P. Hardelin, V. Prevot and C. Dode (2012). "SEMA3A, a gene involved in axonal pathfinding, is mutated in patients with Kallmann syndrome." PLoS Genet **8**(8): e1002896.

Hanchate, N. K., J. Parkash, N. Bellefontaine, D. Mazur, W. H. Colledge, X. d'Anglemont de Tassigny and V. Prevot (2012). "Kisspeptin-GPR54 signaling in mouse NO-synthesizing neurons participates in the hypothalamic control of ovulation." J Neurosci **32**(3): 932-945.

Hanney, M., V. Prasher, N. Williams, E. L. Jones, D. Aarsland, A. Corbett, D. Lawrence, L. M. Yu, S. Tyrer, P. T. Francis, T. Johnson, R. Bullock, C. Ballard and M. t. researchers (2012). "Memantine for dementia in adults older than 40 years with Down's syndrome (MEADOWS): a randomised, double-blind, placebo-controlled trial." Lancet **379**(9815): 528-536.

Harada, C. N., M. C. Natelson Love and K. L. Triebel (2013). "Normal cognitive aging." Clin Geriatr Med **29**(4): 737-752.

Hardy, J. A. and G. A. Higgins (1992). "Alzheimer's disease: the amyloid cascade hypothesis." Science **256**(5054): 184-185.

Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol **11**(3): 298-300.

Harman, D. (1972). "The biologic clock: the mitochondria?" J Am Geriatr Soc **20**(4): 145-147.

Hart, R. W. and R. B. Setlow (1974). "Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species." Proc Natl Acad Sci U S A **71**(6): 2169-2173.

Hasunuma, I. and K. Terakado (2013). "Two novel gonadotropin-releasing hormones (GnRHs) from the urochordate ascidian, Halocynthia roretzi: implications for the origin of vertebrate GnRH isoforms." Zoolog Sci **30**(4): 311-318.

Hauser-Cram, P., M. E. Warfield, J. P. Shonkoff, M. W. Krauss, C. C. Upshur and A. Sayer (1999). "Family influences on adaptive development in young children with Down syndrome." Child Dev **70**(4): 979-989.

Haxby, J. V. (1989). "Neuropsychological evaluation of adults with Down's syndrome: patterns of selective impairment in non-demented old adults." J Ment Defic Res **33 (Pt 3)**: 193-210.

Hayflick, L. (1991). "Aging under glass." Mutat Res **256**(2-6): 69-80.

He, C., P. Kraft, C. Chen, J. E. Buring, G. Pare, S. E. Hankinson, S. J. Chanock, P. M. Ridker, D. J. Hunter and D. I. Chasman (2009). "Genome-wide association studies identify loci associated with age at menarche and age at natural menopause." Nat Genet **41**(6): 724-728.

Henderson, V. W. (2004). "Hormone therapy and Alzheimer's disease: benefit or harm?" Expert Opin Pharmacother **5**(2): 389-406.

Herbison, A. (2006). Physiology of the Adult Gonadotropin-Releasing Hormone Neuronal Network. **1:** 1415-1482.

Herbison, A. E. (2016). "Control of puberty onset and fertility by gonadotropin-releasing hormone neurons." Nat Rev Endocrinol **12**(8): 452-466.

Herbison, A. E. (2018). "The Gonadotropin-Releasing Hormone Pulse Generator." Endocrinology **159**(11): 3723-3736.

Herbison, A. E., J. I. Hubbard and N. E. Sirett (1984). "LH-RH in picomole concentrations evokes excitation and inhibition of rat arcuate neurones in vitro." Neurosci Lett **46**(3): 311-315.

Herbison, A. E. and S. M. Moenter (2011). "Depolarising and hyperpolarising actions of GABA(A) receptor activation on gonadotrophin-releasing hormone neurones: towards an emerging consensus." Journal of neuroendocrinology **23**(7): 557-569.

Herbison, A. E. and J. R. Pape (2001). "New evidence for estrogen receptors in gonadotropin-releasing hormone neurons." Front Neuroendocrinol **22**(4): 292-308.

Herbison, A. E., S. X. Simonian, P. J. Norris and P. C. Emson (1996). "Relationship of Neuronal Nitric Oxide Synthase Immunoreactivity to GnRH Neurons in the Ovariectomized and Intact Female Rat." Journal of Neuroendocrinology **8**(1): 73-82.

Herde, M. K., K. J. Iremonger, S. Constantin and A. E. Herbison (2013). "GnRH neurons elaborate a long-range projection with shared axonal and dendritic functions." J Neurosci **33**(31): 12689-12697.

Herrlich, A., B. Kuhn, R. Grosse, A. Schmid, G. Schultz and T. Gudermann (1996). "Involvement of Gs and Gi proteins in dual coupling of the luteinizing hormone receptor to adenylyl cyclase and phospholipase C." J Biol Chem **271**(28): 16764-16772.

Hirono, M., M. Igarashi and S. Matsumoto (1972). "The direct effect of HCG upon pituitary gonadotrophin secretion." Endocrinology **90**(5): 1214-1219.

Hogervorst, E., K. Yaffe, M. Richards and F. A. Huppert (2009). "Hormone replacement therapy to maintain cognitive function in women with dementia." Cochrane Database Syst Rev(1): CD003799.

Hojager, B., H. Peters, A. G. Byskov and M. Faber (1978). "Follicular development in ovaries of children with Down's syndrome." Acta Paediatr Scand **67**(5): 637-643.

Holmes, D. (2014). "Metabolism: kisspeptin signalling linked to obesity." Nat Rev Endocrinol **10**(9): 511.

Howieson, D. B., L. A. Holm, J. A. Kaye, B. S. Oken and J. Howieson (1993). "Neurologic function in the optimally healthy oldest old. Neuropsychological evaluation." Neurology **43**(10): 1882-1886.

Hrabovszky, E., C. S. Molnar, M. T. Sipos, B. Vida, P. Ciofi, B. A. Borsay, L. Sarkadi, L. Herczeg, S. R. Bloom, M. A. Ghatei, W. S. Dhillo, I. Kallo and Z. Liposits (2011). "Sexual

dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women." Front Endocrinol (Lausanne) **2**: 80.

Hsiang, Y. H., G. D. Berkovitz, G. L. Bland, C. J. Migeon and A. C. Warren (1987). "Gonadal function in patients with Down syndrome." Am J Med Genet **27**(2): 449-458.

Hua-Huy, T., S. Duong-Quy, H. Pham, J. Pansiot, J. C. Mercier, O. Baud and A. T. Dinh-Xuan (2016). "Inhaled nitric oxide decreases pulmonary endothelial nitric oxide synthase expression and activity in normal newborn rat lungs." ERJ Open Res **2**(1).

Huang, P. L., T. M. Dawson, D. S. Bredt, S. H. Snyder and M. C. Fishman (1993). "Targeted disruption of the neuronal nitric oxide synthase gene." Cell **75**(7): 1273-1286.

Huynh, T. V., A. A. Davis, J. D. Ulrich and D. M. Holtzman (2017). "Apolipoprotein E and Alzheimer's disease: the influence of apolipoprotein E on amyloid-beta and other amyloidogenic proteins." J Lipid Res **58**(5): 824-836.

Ionescu, A., F. Dufrasne, M. Gelbcke, I. Jabin, R. Kiss and D. Lamoral-Theys (2012). "DYRK1A kinase inhibitors with emphasis on cancer." Mini Rev Med Chem **12**(13): 1315-1329.

Irvine, K., K. R. Laws, T. M. Gale and T. K. Kondel (2012). "Greater cognitive deterioration in women than men with Alzheimer's disease: a meta analysis." J Clin Exp Neuropsychol **34**(9): 989-998.

Jayasena, C. N., A. Abbara, S. Narayanaswamy, A. N. Comninos, R. Ratnasabapathy, P. Bassett, J. T. Mogford, Z. Malik, J. Calley, M. A. Ghatei, S. R. Bloom and W. S. Dhillo (2015). "Direct comparison of the effects of intravenous kisspeptin-10, kisspeptin-54 and GnRH on gonadotrophin secretion in healthy men." Hum Reprod **30**(8): 1934-1941.

Jayasena, C. N. and S. Franks (2014). "The management of patients with polycystic ovary syndrome." Nat Rev Endocrinol **10**(10): 624-636.

Jennes, L., B. Dalati and P. M. Conn (1988). "Distribution of gonadrotropin releasing hormone agonist binding sites in the rat central nervous system." Brain Res **452**(1-2): 156-164.

Jennes, L., O. Eyigor, J. A. Janovick and P. M. Conn (1997). "Brain gonadotropin releasing hormone receptors: localization and regulation." Recent Prog Horm Res **52**: 475-490; discussion 490-471.

Jervis, G. A. (1948). "Early senile dementia in mongoloid idiocy." Am J Psychiatry **105**(2): 102-106.

Jiang, J. H., Z. He, Y. L. Peng, W. D. Jin, Z. Wang, R. W. Han, M. Chang and R. Wang (2015). "Kisspeptin-13 enhances memory and mitigates memory impairment induced by Abeta1-42 in mice novel object and object location recognition tasks." Neurobiol Learn Mem **123**: 187-195.

Kahlem, P., M. Sultan, R. Herwig, M. Steinfath, D. Balzereit, B. Eppens, N. G. Saran, M. T. Pletcher, S. T. South, G. Stetten, H. Lehrach, R. H. Reeves and M. L. Yaspo (2004). "Transcript level alterations reflect gene dosage effects across multiple tissues in a mouse model of down syndrome." Genome Res **14**(7): 1258-1267.

Kaplan, P. S. and J. S. Werner (1986). "Habituation, response to novelty, and dishabituation in human infants: tests of a dual-process theory of visual attention." J Exp Child Psychol **42**(2): 199-217.

Karch, C. M. and A. M. Goate (2015). "Alzheimer's disease risk genes and mechanisms of disease pathogenesis." Biol Psychiatry **77**(1): 43-51.

Kasai, H., N. Takahashi and H. Tokumaru (2012). "Distinct initial SNARE configurations underlying the diversity of exocytosis." Physiol Rev **92**(4): 1915-1964.

Kauffman, A. S., A. Wills, R. P. Millar and E. F. Rissman (2005). "Evidence that the Type-2 Gonadotrophin-Releasing Hormone (GnRH) Receptor Mediates the Behavioural

Effects of GnRH-II on Feeding and Reproduction in Musk Shrews." Journal of Neuroendocrinology **17**(8): 489-497.

Kavsek, M. and M. H. Bornstein (2010). "Visual habituation and dishabituation in preterm infants: a review and meta-analysis." Res Dev Disabil **31**(5): 951-975.

Keenan, D. M., P. Y. Takahashi, P. Y. Liu, P. D. Roebuck, A. X. Nehra, A. Iranmanesh and J. D. Veldhuis (2006). "An ensemble model of the male gonadal axis: illustrative application in aging men." Endocrinology **147**(6): 2817-2828.

Keenan, D. M. and J. D. Veldhuis (2004). "Divergent gonadotropin-gonadal dose-responsive coupling in healthy young and aging men." Am J Physiol Regul Integr Comp Physiol **286**(2): R381-389.

Kendrick, K. M., R. Guevara-Guzman, J. Zorrilla, M. R. Hinton, K. D. Broad, M. Mimmack and S. Ohkura (1997). "Formation of olfactory memories mediated by nitric oxide." Nature **388**(6643): 670-674.

Kerr, J. B. (1992). "Functional cytology of the human testis." Baillieres Clin Endocrinol Metab **6**(2): 235-250.

Kerr, J. B., K. L. Loveland, M. K. O'Bryan and D. M. de Kretser (2006). CHAPTER 18 - Cytology of the Testis and Intrinsic Control Mechanisms. Knobil and Neill's Physiology of Reproduction (Third Edition). J. D. Neill. St Louis, Academic Press**:** 827-947.

Khoshnood, B., R. Greenlees, M. Loane, H. Dolk, E. P. M. Committee and E. W. Group (2011). "Paper 2: EUROCAT public health indicators for congenital anomalies in Europe." Birth Defects Res A Clin Mol Teratol **91 Suppl 1**: S16-22.

King, J. C. and E. L. Anthony (1983). "Biosynthesis of LHRH: inferences from immunocytochemical studies." Peptides **4**(6): 963-970.

Kirkwood, T. B. and A. Kowald (2012). "The free-radical theory of ageing--older, wiser and still alive: modelling positional effects of the primary targets of ROS reveals new support." Bioessays **34**(8): 692-700.

Kishi, M., X. Liu, T. Hirakawa, D. Reczek, A. Bretscher and M. Ascoli (2001). "Identification of two distinct structural motifs that, when added to the C-terminal tail of the rat LH receptor, redirect the internalized hormone-receptor complex from a degradation to a recycling pathway." Mol Endocrinol **15**(9): 1624-1635.

Kishnani, P. S., J. H. Heller, G. A. Spiridigliozzi, I. Lott, L. Escobar, S. Richardson, R. Zhang and T. McRae (2010). "Donepezil for treatment of cognitive dysfunction in children with Down syndrome aged 10-17." Am J Med Genet A **152A**(12): 3028-3035.

Kivipelto, M., F. Mangialasche and T. Ngandu (2018). "Lifestyle interventions to prevent cognitive impairment, dementia and Alzheimer disease." Nat Rev Neurol **14**(11): 653-666.

Klein, S. L., L. J. Kriegsfeld, J. E. Hairston, V. Rau, R. J. Nelson and P. J. Yarowsky (1996). "Characterization of sensorimotor performance, reproductive and aggressive behaviors in segmental trisomic 16 (Ts65Dn) mice." Physiol Behav **60**(4): 1159-1164.

Kobayashi, M., M. Amano, M. H. Kim, K. Furukawa, Y. Hasegawa and K. Aida (1994). "Gonadotropin-releasing hormones of terminal nerve origin are not essential to ovarian development and ovulation in goldfish." Gen Comp Endocrinol **95**(2): 192-200.

Koc, A., A. P. Gasch, J. C. Rutherford, H. Y. Kim and V. N. Gladyshev (2004). "Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging." Proc Natl Acad Sci U S A **101**(21): 7999-8004.

Kokoris, G. J., N. Y. Lam, M. Ferin, A. J. Silverman and M. J. Gibson (1988). "Transplanted gonadotropin-releasing hormone neurons promote pulsatile luteinizing hormone secretion in congenitally hypogonadal (hpg) male mice." Neuroendocrinology **48**(1): 45-52.

Kondo, Y., S. Mizuno, K. Ohara, T. Nakamura, K. Yamada, S. Yamamori, C. Hayakawa, T. Ishii, Y. Yamada and N. Wakamatsu (2006). "Two cases of partial trisomy 21 (pter-q22.1) without the major features of Down syndrome." Am J Med Genet A **140**(3): 227-232.

Koopman, J. J., D. van Heemst, D. van Bodegom, M. S. Bonkowski, L. Y. Sun and A. Bartke (2016). "Measuring aging rates of mice subjected to caloric restriction and genetic disruption of growth hormone signaling." Aging (Albany NY) **8**(3): 539-546.

Korbel, J. O., T. Tirosh-Wagner, A. E. Urban, X. N. Chen, M. Kasowski, L. Dai, F. Grubert, C. Erdman, M. C. Gao, K. Lange, E. M. Sobel, G. M. Barlow, A. S. Aylsworth, N. J. Carpenter, R. D. Clark, M. Y. Cohen, E. Doran, T. Falik-Zaccai, S. O. Lewin, I. T. Lott, B. C. McGillivray, J. B. Moeschler, M. J. Pettenati, S. M. Pueschel, K. W. Rao, L. G. Shaffer, M. Shohat, A. J. Van Riper, D. Warburton, S. Weissman, M. B. Gerstein, M. Snyder and J. R. Korenberg (2009). "The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies." Proc Natl Acad Sci U S A **106**(29): 12031-12036.

Kramer, P. R. and S. Wray (2000). "Midline Nasal Tissue Influences Nestin Expression in Nasal-Placode-Derived Luteinizing Hormone-Releasing Hormone Neurons during Development." Developmental Biology **227**(2): 343-357.

Kuhn, D. E., G. J. Nuovo, A. V. Terry, Jr., M. M. Martin, G. E. Malana, S. E. Sansom, A. P. Pleister, W. D. Beck, E. Head, D. S. Feldman and T. S. Elton (2010). "Chromosome 21-derived microRNAs provide an etiological basis for aberrant protein expression in human Down syndrome brains." J Biol Chem **285**(2): 1529-1543.

Kuiri-Hanninen, T., S. Kallio, R. Seuri, E. Tyrvainen, A. Liakka, J. Tapanainen, U. Sankilampi and L. Dunkel (2011). "Postnatal developmental changes in the pituitary-ovarian axis in preterm and term infant girls." J Clin Endocrinol Metab **96**(11): 3432-3439.

Kuiri-Hanninen, T., U. Sankilampi and L. Dunkel (2014). "Activation of the hypothalamic-pituitary-gonadal axis in infancy: minipuberty." Horm Res Paediatr **82**(2): 73-80.

Kuiri-Hanninen, T., R. Seuri, E. Tyrvainen, U. Turpeinen, E. Hamalainen, U. H. Stenman, L. Dunkel and U. Sankilampi (2011). "Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys." J Clin Endocrinol Metab **96**(1): 98-105.

Kumar, T. R., Y. Wang, N. Lu and M. M. Matzuk (1997). "Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility." Nat Genet **15**(2): 201-204.

Kwan, K. Y., M. M. Lam, M. B. Johnson, U. Dube, S. Shim, M. R. Rasin, A. M. Sousa, S. Fertuzinhos, J. G. Chen, J. I. Arellano, D. W. Chan, M. Pletikos, L. Vasung, D. H. Rowitch, E. J. Huang, M. L. Schwartz, R. Willemsen, B. A. Oostra, P. Rakic, M. Heffer, I. Kostovic, M. Judas and N. Sestan (2012). "Species-dependent posttranscriptional regulation of NOS1 by FMRP in the developing cerebral cortex." Cell **149**(4): 899-911.

Kwekkeboom, D. J., F. H. de Jong, A. M. van Hemert, J. P. Vandenbroucke, H. A. Valkenburg and S. W. Lamberts (1990). "Serum gonadotropins and alpha-subunit decline in aging normal postmenopausal women." J Clin Endocrinol Metab **70**(4): 944-950.

Lacreuse, A., M. E. Wilson and J. G. Herndon (2002). "Estradiol, but not raloxifene, improves aspects of spatial working memory in aged ovariectomized rhesus monkeys." Neurobiol Aging **23**(4): 589-600.

Lagrange, A. H., O. K. Ronnekleiv and M. J. Kelly (1995). "Estradiol-17 beta and mu-opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback?" Endocrinology **136**(5): 2341-2344.

Lakshminrusimha, S., B. Mathew and C. L. Leach (2016). "Pharmacologic strategies in neonatal pulmonary hypertension other than nitric oxide." Semin Perinatol **40**(3): 160-173.

Lane, R. K., T. Hilsabeck and S. L. Rea (2015). "The role of mitochondrial dysfunction in age-related diseases." Biochim Biophys Acta **1847**(11): 1387-1400.

Langlet, F., A. Mullier, S. G. Bouret, V. Prevot and B. Dehouck (2013). "Tanycyte-like cells form a blood-cerebrospinal fluid barrier in the circumventricular organs of the mouse brain." J Comp Neurol **521**(15): 3389-3405.

Laron, Z. (2008). "The GH-IGF1 axis and longevity. The paradigm of IGF1 deficiency." Hormones (Athens) **7**(1): 24-27.

Lasaite, L., J. Ceponis, R. T. Preiksa and B. Zilaitiene (2014). "Impaired emotional state, quality of life and cognitive functions in young hypogonadal men." Andrologia **46**(10): 1107-1112.

Launer, L. J., K. Andersen, M. E. Dewey, L. Letenneur, A. Ott, L. A. Amaducci, C. Brayne, J. R. Copeland, J. F. Dartigues, P. Kragh-Sorensen, A. Lobo, J. M. Martinez-Lage, T. Stijnen and A. Hofman (1999). "Rates and risk factors for dementia and Alzheimer's disease: results from EURODEM pooled analyses. EURODEM Incidence Research Group and Work Groups. European Studies of Dementia." Neurology **52**(1): 78-84.

Laws, S. C., M. J. Beggs, J. C. Webster and W. L. Miller (1990). "Inhibin increases and progesterone decreases receptors for gonadotropin-releasing hormone in ovine pituitary culture." Endocrinology **127**(1): 373-380.

LeBlanc, E. S., J. Janowsky, B. K. Chan and H. D. Nelson (2001). "Hormone replacement therapy and cognition: systematic review and meta-analysis." JAMA **285**(11): 1489-1499.

Lebrethon, M. C., A. Aganina, M. Fournier, A. Gerard, A. S. Parent and J. P. Bourguignon (2007). "Effects of in vivo and in vitro administration of ghrelin, leptin and neuropeptide mediators on pulsatile gonadotrophin-releasing hormone secretion from male rat hypothalamus before and after puberty." J Neuroendocrinol **19**(3): 181-188.

Lee, D. A., J. L. Bedont, T. Pak, H. Wang, J. Song, A. Miranda-Angulo, V. Takiar, V. Charubhumi, F. Balordi, H. Takebayashi, S. Aja, E. Ford, G. Fishell and S. Blackshaw (2012). "Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche." Nat Neurosci **15**(5): 700-702.

Leger, M., A. Quiedeville, V. Bouet, B. Haelewyn, M. Boulouard, P. Schumann-Bard and T. Freret (2013). "Object recognition test in mice." Nat Protoc **8**(12): 2531-2537.

Lehman, M. N., L. M. Coolen and R. L. Goodman (2010). "Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion." Endocrinology **151**(8): 3479-3489.

Lehman, M. N., F. J. Karsch, J. E. Robinson and A. J. Silverman (1988). "Ultrastructure and synaptic organization of luteinizing hormone-releasing hormone (LHRH) neurons in the anestrous ewe." J Comp Neurol **273**(4): 447-458.

Lei, Z. M., C. V. Rao, J. L. Kornyei, P. Licht and E. S. Hiatt (1993). "Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain." Endocrinology **132**(5): 2262-2270.

Lejeune, J., M. Gautier and R. Turpin (1959). "[Study of somatic chromosomes from 9 mongoloid children]." C R Hebd Seances Acad Sci **248**(11): 1721-1722.

Lettre, G., A. U. Jackson, C. Gieger, F. R. Schumacher, S. I. Berndt, S. Sanna, S. Eyheramendy, B. F. Voight, J. L. Butler, C. Guiducci, T. Illig, R. Hackett, I. M. Heid, K. B. Jacobs, V. Lyssenko, M. Uda, I. Diabetes Genetics, Fusion, Kora, L. C. Prostate, T. Ovarian Cancer Screening, S. Nurses' Health, SardiNia, M. Boehnke, S. J. Chanock, L. C. Groop, F. B. Hu, B. Isomaa, P. Kraft, L. Peltonen, V. Salomaa, D. Schlessinger, D. J. Hunter, R. B. Hayes, G. R. Abecasis, H. E. Wichmann, K. L. Mohlke and J. N. Hirschhorn (2008). "Identification of ten loci associated with height highlights new biological pathways in human growth." Nat Genet **40**(5): 584-591.

Lewkowitz-Shpuntoff, H. M., V. A. Hughes, L. Plummer, M. G. Au, R. L. Doty, S. B. Seminara, Y. M. Chan, N. Pitteloud, W. F. Crowley, Jr. and R. Balasubramanian (2012). "Olfactory phenotypic spectrum in idiopathic hypogonadotropic hypogonadism: pathophysiological and genetic implications." J Clin Endocrinol Metab **97**(1): E136-144.

Lezak, M., D. Howieson and D. Loring (2012). "Neuropsychological assessment. 5th edn Oxford University Press." Oxford, New York, ISBN **10**: 9780195395525.

Li, H. and R. Durbin (2009). "Fast and accurate short read alignment with Burrows-Wheeler transform." Bioinformatics **25**(14): 1754-1760.

Li, J., Y. Tang and D. Cai (2012). "IKKbeta/NF-kappaB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes." Nat Cell Biol **14**(10): 999-1012.

Liu, X., X. Jian and E. Boerwinkle (2013). "dbNSFP v2.0: a database of human non-synonymous SNVs and their functional predictions and annotations." Hum Mutat **34**(9): E2393-2402.

Liu, Y., Z. Lin, M. Liu, H. Wang and H. Sun (2017). "Overexpression of DYRK1A, a Down Syndrome Candidate gene, Impairs Primordial Germ Cells Maintenance and Migration in zebrafish." Sci Rep **7**(1): 15313.

Lopez Rodriguez, D., V. Delli, C. Aylwin, A. Gerard, S. Blacher, E. Tirelli, A. Noel, J.-P. Bourguignon, A. Lomniczi and A. S. Parent (2019). "OR23-1 Transgenerational Effects of Endocrine Disrupting Chemicals on Pubertal Timing through Epigenetic Reprogramming of the Hypothalamus." Journal of the Endocrine Society **3**(Suppl 1): OR23-21.

Lott, I. T. and E. Head (2019). "Dementia in Down syndrome: unique insights for Alzheimer disease research." Nat Rev Neurol **15**(3): 135-147.

Lukacs, H., E. S. Hiatt, Z. M. Lei and C. V. Rao (1995). "Peripheral and intracerebroventricular administration of human chorionic gonadotropin alters several hippocampus-associated behaviors in cycling female rats." Horm Behav **29**(1): 42-58.

Lyle, R., F. Bena, S. Gagos, C. Gehrig, G. Lopez, A. Schinzel, J. Lespinasse, A. Bottani, S. Dahoun, L. Taine, M. Doco-Fenzy, P. Cornillet-Lefebvre, A. Pelet, S. Lyonnet, A. Toutain, L. Colleaux, J. Horst, I. Kennerknecht, N. Wakamatsu, M. Descartes, J. C. Franklin, L. Florentin-Arar, S. Kitsiou, E. Ait Yahya-Graison, M. Costantine, P. M. Sinet, J. M. Delabar and S. E. Antonarakis (2009). "Genotype-phenotype correlations in Down syndrome identified by array CGH in 30 cases of partial trisomy and partial monosomy chromosome 21." Eur J Hum Genet **17**(4): 454-466.

Makimura, H., T. M. Mizuno, J. W. Mastaitis, R. Agami and C. V. Mobbs (2002). "Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake." BMC Neurosci **3**: 18.

Malone, S. A. (2017). Morphological, molecular and genetic aspects of the GnRH neuronal migratory process in mice and humans, University of Lille 2, Centre de Recherche Jean-Pierre Aubert Neurosciences et Cancer.

Mammen, A. L., K. Kameyama, K. W. Roche and R. L. Huganir (1997). "Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II." J Biol Chem **272**(51): 32528-32533.

Mandal, S., A. Stanco, E. S. Buys, G. Enikolopov and J. L. Rubenstein (2013). "Soluble guanylate cyclase generation of cGMP regulates migration of MGE neurons." J Neurosci **33**(43): 16897-16914.

Manfredi-Lozano, M., J. Roa, F. Ruiz-Pino, R. Piet, D. Garcia-Galiano, R. Pineda, A. Zamora, S. Leon, M. A. Sanchez-Garrido, A. Romero-Ruiz, C. Dieguez, M. J. Vazquez, A. E. Herbison, L. Pinilla and M. Tena-Sempere (2016). "Defining a novel leptin-melanocortin-kisspeptin pathway involved in the metabolic control of puberty." Mol Metab **5**(10): 844-857.

Manini, T. M., S. L. Hong and B. C. Clark (2013). "Aging and muscle: a neuron's perspective." Curr Opin Clin Nutr Metab Care **16**(1): 21-26.

Mann, D. M. and M. M. Esiri (1989). "The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's syndrome." J Neurol Sci **89**(2-3): 169-179.

Marin, O. (2013). "Cellular and molecular mechanisms controlling the migration of neocortical interneurons." Eur J Neurosci **38**(1): 2019-2029.

Markakis, E. A. (2002). "Development of the neuroendocrine hypothalamus." Front Neuroendocrinol **23**(3): 257-291.

Martin, K., N. Santoro, J. Hall, M. Filicori, M. Wierman and W. F. Crowley, Jr. (1990). "Clinical review 15: Management of ovulatory disorders with pulsatile gonadotropin-releasing hormone." J Clin Endocrinol Metab **71**(5): 1081A-1081G.

Martin, T. F. J. (2012). "Role of PI(4,5)P(2) in vesicle exocytosis and membrane fusion." Sub-cellular biochemistry **59**: 111-130.

Mason, A. J., J. S. Hayflick, R. T. Zoeller, W. S. Young, 3rd, H. S. Phillips, K. Nikolics and P. H. Seeburg (1986). "A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse." Science **234**(4782): 1366-1371.

Matsuo, H., Y. Baba, R. M. Nair, A. Arimura and A. V. Schally (1971). "Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence." Biochem Biophys Res Commun **43**(6): 1334-1339.

McCarthy, M. M. (2008). "Estradiol and the developing brain." Physiol Rev **88**(1): 91-124.

McEwen, B. (2002). "Estrogen actions throughout the brain." Recent Prog Horm Res **57**: 357-384.

McKeown, D. A., R. L. Doty, D. P. Perl, R. E. Frye, I. Simms and A. Mester (1996). "Olfactory function in young adolescents with Down's syndrome." Journal of neurology, neurosurgery, and psychiatry **61**(4): 412-414.

McNay, D. E., N. Briancon, M. V. Kokoeva, E. Maratos-Flier and J. S. Flier (2012). "Remodeling of the arcuate nucleus energy-balance circuit is inhibited in obese mice." J Clin Invest **122**(1): 142-152.

Mehta, P. D., A. J. Dalton, S. P. Mehta, K. S. Kim, E. A. Sersen and H. M. Wisniewski (1998). "Increased plasma amyloid beta protein 1-42 levels in Down syndrome." Neurosci Lett **241**(1): 13-16.

Mehta, P. D., B. A. Patrick, A. J. Dalton, P. S. Aisen, M. E. Emmerling, E. A. Sersen and H. M. Wisniewski (1999). "Increased levels of tau-like protein in patients with Down syndrome." Neurosci Lett **275**(3): 159-162.

Messina, A., N. Ferraris, S. Wray, G. Cagnoni, D. E. Donohue, F. Casoni, P. R. Kramer, A. A. Derijck, Y. Adolfs, A. Fasolo, R. J. Pasterkamp and P. Giacobini (2011). "Dysregulation of Semaphorin7A/beta1-integrin signaling leads to defective GnRH-1 cell migration, abnormal gonadal development and altered fertility." Hum Mol Genet **20**(24): 4759-4774.

Messina, A. and P. Giacobini (2013). "Semaphorin signaling in the development and function of the gonadotropin hormone-releasing hormone system." Front Endocrinol (Lausanne) **4**: 133.

Messina, A., F. Langlet, K. Chachlaki, J. Roa, S. Rasika, N. Jouy, S. Gallet, F. Gaytan, J. Parkash, M. Tena-Sempere, P. Giacobini and V. Prevot (2016). "A microRNA switch regulates the rise in hypothalamic GnRH production before puberty." Nat Neurosci **19**(6): 835-844.

Mielke, M. M., J. M. Leoutsakos, C. D. Corcoran, R. C. Green, M. C. Norton, K. A. Welsh-Bohmer, J. T. Tschanz and C. G. Lyketsos (2012). "Effects of Food and Drug Administration-approved medications for Alzheimer's disease on clinical progression." Alzheimers Dement **8**(3): 180-187.

Millar, R. P. (2005). "GnRHs and GnRH receptors." Anim Reprod Sci **88**(1-2): 5-28.

Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-275.

Min, K. S., X. Liu, J. Fabritz, J. Jaquette, A. N. Abell and M. Ascoli (1998). "Mutations that induce constitutive activation and mutations that impair signal transduction modulate the basal and/or agonist-stimulated internalization of the Lutropin/Choriogonadotropin receptor." J Biol Chem **273**(52): 34911-34919.

Minami, S., S. A. Frautschy, P. M. Plotsky, S. W. Sutton and D. K. Sarkar (1990). "Facilitatory role of neuropeptide Y on the onset of puberty: effect of immunoneutralization of neuropeptide Y on the release of luteinizing hormone and luteinizing-hormone-releasing hormone." Neuroendocrinology **52**(1): 112-115.

Minegishi, T., M. Tano, M. Igarashi, S. Rokukawa, Y. Abe, Y. Ibuki and K. Miyamoto (1997). "Expression of follicle-stimulating hormone receptor in human ovary." Eur J Clin Invest **27**(6): 469-474.

Mittelman-Smith, M. A., H. Williams, S. J. Krajewski-Hall, J. Lai, P. Ciofi, N. T. McMullen and N. E. Rance (2012). "Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight." Endocrinology **153**(6): 2800-2812.

Miyamoto, K., Y. Hasegawa, M. Nomura, M. Igarashi, K. Kangawa and H. Matsuo (1984). "Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species." Proc Natl Acad Sci U S A **81**(12): 3874-3878.

Mockett, R. J., B. H. Sohal and R. S. Sohal (2010). "Expression of multiple copies of mitochondrially targeted catalase or genomic Mn superoxide dismutase transgenes does not extend the life span of Drosophila melanogaster." Free Radic Biol Med **49**(12): 2028-2031.

Moenter, S. M., M. A. Silveira, L. Wang and C. Adams (2019). "Central aspects of systemic oestradiol negative- and positive-feedback on the reproductive neuroendocrine system." J Neuroendocrinol: e12724.

Moffat, S. D., A. B. Zonderman, E. J. Metter, M. R. Blackman, S. M. Harman and S. M. Resnick (2002). "Longitudinal assessment of serum free testosterone concentration predicts memory performance and cognitive status in elderly men." J Clin Endocrinol Metab **87**(11): 5001-5007.

Moffat, S. D., A. B. Zonderman, E. J. Metter, C. Kawas, M. R. Blackman, S. M. Harman and S. M. Resnick (2004). "Free testosterone and risk for Alzheimer disease in older men." Neurology **62**(2): 188-193.

Moguilevsky, J. A., S. Carbone, B. Szwarcfarb and D. Rondina (1991). "Sexual maturation modifies the GABAergic control of gonadotrophin secretion in female rats." Brain Res **563**(1-2): 12-16.

Molitch, M., M. Edmonds, E. E. Jones and W. D. Odell (1976). "Short-loop feedback control of luteinizing hormone in the rabbit." Am J Physiol **230**(4): 907-910.

Molofsky, A. V., S. G. Slutsky, N. M. Joseph, S. He, R. Pardal, J. Krishnamurthy, N. E. Sharpless and S. J. Morrison (2006). "Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing." Nature **443**(7110): 448-452.

Mongiat, L. A., M. O. Fernandez, V. A. Lux-Lantos, L. G. Guilgur, G. M. Somoza and C. Libertun (2006). "Experimental data supporting the expression of the highly conserved GnRH-II in the brain and pituitary gland of rats." Regul Pept **136**(1-3): 50-57.

Monroe, S. E., R. B. Jaffe and A. R. Midgley, Jr. (1972). "Regulation of human gondotropins. 13. Changes in serum gonadotropins in menstruating women in response to oophorectomy." J Clin Endocrinol Metab **34**(2): 420-422.

Montagne, A., S. R. Barnes, M. D. Sweeney, M. R. Halliday, A. P. Sagare, Z. Zhao, A. W. Toga, R. E. Jacobs, C. Y. Liu, L. Amezcua, M. G. Harrington, H. C. Chui, M. Law and B. V. Zlokovic (2015). "Blood-brain barrier breakdown in the aging human hippocampus." Neuron **85**(2): 296-302.

Montaner, A. D., L. Mongiat, V. A. Lux-Lantos, M. K. Park, W. H. Fischer, A. G. Craig, J. E. Rivier, D. Lescheid, D. Lovejoy, C. Libertun, N. M. Sherwood and G. M. Somoza (2001). "Structure and biological activity of gonadotropin-releasing hormone isoforms isolated from rat and hamster brains." Neuroendocrinology **74**(3): 202-212.

Montecucco, C. and J. Molgo (2005). "Botulinal neurotoxins: revival of an old killer." Curr Opin Pharmacol **5**(3): 274-279.

Montecucco, C. and G. Schiavo (1994). "Mechanism of action of tetanus and botulinum neurotoxins." Mol Microbiol **13**(1): 1-8.

Moore, C. S., C. Hawkins, A. Franca, A. Lawler, B. Devenney, I. Das and R. H. Reeves (2010). "Increased male reproductive success in Ts65Dn "Down syndrome" mice." Mamm Genome **21**(11-12): 543-549.

Morales-Delgado, N., B. Castro-Robles, J. L. Ferran, M. Martinez-de-la-Torre, L. Puelles and C. Diaz (2014). "Regionalized differentiation of CRH, TRH, and GHRH peptidergic neurons in the mouse hypothalamus." Brain Struct Funct **219**(3): 1083-1111.

Moretto, M., F. J. Lopez and A. Negro-Vilar (1993). "Nitric oxide regulates luteinizing hormone-releasing hormone secretion." Endocrinology **133**(5): 2399-2402.

Moster, D., R. T. Lie and T. Markestad (2008). "Long-term medical and social consequences of preterm birth." N Engl J Med **359**(3): 262-273.

Murman, D. L. (2015). "The Impact of Age on Cognition." Semin Hear **36**(3): 111-121.

Murphy, C. and S. Jinich (1996). "Olfactory dysfunction in Down's Syndrome." Neurobiol Aging **17**(4): 631-637.

Navarro, V. M., J. M. Castellano, S. M. McConkey, R. Pineda, F. Ruiz-Pino, L. Pinilla, D. K. Clifton, M. Tena-Sempere and R. A. Steiner (2011). "Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat." Am J Physiol Endocrinol Metab **300**(1): E202-210.

Navarro, V. M., M. L. Gottsch, C. Chavkin, H. Okamura, D. K. Clifton and R. A. Steiner (2009). "Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse." J Neurosci **29**(38): 11859-11866.

Nelson, L., J. K. Johnson, M. Freedman, I. Lott, J. Groot, M. Chang, N. W. Milgram and E. Head (2005). "Learning and memory as a function of age in Down syndrome: a study using animal-based tasks." Prog Neuropsychopharmacol Biol Psychiatry **29**(3): 443-453.

Nelson, R. J., G. E. Demas, P. L. Huang, M. C. Fishman, V. L. Dawson, T. M. Dawson and S. H. Snyder (1995). "Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase." Nature **378**(6555): 383-386.

Nett, T. M., M. E. Crowder, G. E. Moss and T. M. Duello (1981). "GnRH-receptor interaction. V. Down-regulation of pituitary receptors for GnRH in ovariectomized ewes by infusion of homologous hormone." Biol Reprod **24**(5): 1145-1155.

Nijjar, R. K. and C. Murphy (2002). "Olfactory impairment increases as a function of age in persons with Down syndrome." Neurobiol Aging **23**(1): 65-73.

Nussey, S. and S. Whitehead (2001). Endocrinology: An Integrated Approach. Oxford.

Oakley, A. E., D. K. Clifton and R. A. Steiner (2009). "Kisspeptin signaling in the brain." Endocr Rev **30**(6): 713-743.

Ohm, T. G. and H. Braak (1987). "Olfactory bulb changes in Alzheimer's disease." Acta Neuropathol **73**(4): 365-369.

Ojeda, S. R., W. W. Andrews, J. P. Advis and S. S. White (1980). "Recent advances in the endocrinology of puberty." Endocr Rev **1**(3): 228-257.

Ojeda, S. R., C. Roth, A. Mungenast, S. Heger, C. Mastronardi, A. S. Parent, A. Lomniczi and H. Jung (2006). "Neuroendocrine mechanisms controlling female puberty: new approaches, new concepts." Int J Androl **29**(1): 256-263; discussion 286-290.

Okere, C. O. and H. Kaba (2000). "Increased expression of neuronal nitric oxide synthase mRNA in the accessory olfactory bulb during the formation of olfactory recognition memory in mice." Eur J Neurosci **12**(12): 4552-4556.

Olson, L. E., R. J. Roper, L. L. Baxter, E. J. Carlson, C. J. Epstein and R. H. Reeves (2004). "Down syndrome mouse models Ts65Dn, Ts1Cje, and Ms1Cje/Ts65Dn exhibit variable severity of cerebellar phenotypes." Dev Dyn **230**(3): 581-589.

Ottis, P., K. Koppe, B. Onisko, I. Dynin, T. Arzberger, H. Kretzschmar, J. R. Requena, C. J. Silva, J. P. Huston and C. Korth (2012). "Human and rat brain lipofuscin proteome." Proteomics **12**(15-16): 2445-2454.

Palm, R., J. Chang, J. Blair, Y. Garcia-Mesa, H. G. Lee, R. J. Castellani, M. A. Smith, X. Zhu and G. Casadesus (2014). "Down-regulation of serum gonadotropins but not estrogen replacement improves cognition in aged-ovariectomized 3xTg AD female mice." J Neurochem **130**(1): 115-125.

Pangalos, C., D. Avramopoulos, J. L. Blouin, O. Raoul, M. C. deBlois, M. Prieur, A. A. Schinzel, M. Gika, D. Abazis and S. E. Antonarakis (1994). "Understanding the mechanism(s) of

mosaic trisomy 21 by using DNA polymorphism analysis." Am J Hum Genet **54**(3): 473-481.

Pansiot, J., G. Loron, P. Olivier, R. Fontaine, C. Charriaut-Marlangue, J. C. Mercier, P. Gressens and O. Baud (2010). "Neuroprotective effect of inhaled nitric oxide on excitotoxic-induced brain damage in neonatal rat." PLoS One **5**(6): e10916.

Pantic, V. R. (1995). "Biology of hypothalamic neurons and pituitary cells." Int Rev Cytol **159**: 1-112.

Parent, A. S., G. Teilmann, A. Juul, N. E. Skakkebaek, J. Toppari and J. P. Bourguignon (2003). "The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration." Endocr Rev **24**(5): 668-693.

Park, D. C. and S. G. Yeo (2013). "Aging." Korean J Audiol **17**(2): 39-44.

Parker, S. E., C. T. Mai, M. A. Canfield, R. Rickard, Y. Wang, R. E. Meyer, P. Anderson, C. A. Mason, J. S. Collins, R. S. Kirby, A. Correa and N. National Birth Defects Prevention (2010). "Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006." Birth Defects Res A Clin Mol Teratol **88**(12): 1008-1016.

Patritti-Laborde, N., A. R. Wolfsen, D. Heber and W. D. Odell (1979). "Pituitary gland: site of short-loop feedback for luteinizing hormone in the rabbit." J Clin Invest **64**(4): 1066-1069.

Paul, V. and P. Ekambaram (2011). "Involvement of nitric oxide in learning & memory processes." The Indian journal of medical research **133**(5): 471-478.

Paxinos, G. and K. B. J. Franklin (2004). The mouse brain in stereotaxic coordinates. London, Academic Press.

Pazderska, A., Y. Mamoojee, S. Artham, M. Miller, S. G. Ball, T. Cheetham and R. Quinton (2018). "Safety and tolerability of one-year intramuscular testosterone regime to induce puberty in older men with CHH." Endocr Connect **7**(1): 133-138.

Peegel, H., J. Randolph, Jr., A. R. Midgley and K. M. Menon (1994). "In situ hybridization of luteinizing hormone/human chorionic gonadotropin receptor messenger ribonucleic acid during hormone-induced down-regulation and the subsequent recovery in rat corpus luteum." Endocrinology **135**(3): 1044-1051.

Phung, Y. T. and S. M. Black (1999). "Use of chimeric forms of neuronal nitric-oxide synthase as dominant negative mutants." IUBMB Life **48**(3): 333-338.

Pingault, V., V. Bodereau, V. Baral, S. Marcos, Y. Watanabe, A. Chaoui, C. Fouveaut, C. Leroy, O. Verier-Mine, C. Francannet, D. Dupin-Deguine, F. Archambeaud, F. J. Kurtz, J. Young, J. Bertherat, S. Marlin, M. Goossens, J. P. Hardelin, C. Dode and N. Bondurand (2013). "Loss-of-function mutations in SOX10 cause Kallmann syndrome with deafness." Am J Hum Genet **92**(5): 707-724.

Pinson, A., D. Franssen, A. Gerard, A. S. Parent and J. P. Bourguignon (2017). "Neuroendocrine disruption without direct endocrine mode of action: Polychloro-biphenyls (PCBs) and bisphenol A (BPA) as case studies." C R Biol **340**(9-10): 432-438.

Pohl, C. R. and E. Knobil (1982). "The role of the central nervous system in the control of ovarian function in higher primates." Annu Rev Physiol **44**: 583-593.

Popolow, H. B., J. C. King and A. A. Gerall (1981). "Rostral medial preoptic area lesions' influence on female estrous processes and LHRH distribution." Physiol Behav **27**(5): 855-861.

Pradhan, M., A. Dalal, F. Khan and S. Agrawal (2006). "Fertility in men with Down syndrome: a case report." Fertil Steril **86**(6): 1765 e1761-1763.

Prast, H. and A. Philippu (2001). "Nitric oxide as modulator of neuronal function." Prog Neurobiol **64**(1): 51-68.

Prevot, V. (2015). Puberty in mice and rats. Knobil and Neill's Physiology of Reproduction. T. M. Plant and A. J. Zeleznik. New York, Elsevier**:** 1395-1439.

Prevot, V. (2015). Puberty in mice and rats. Knobil and Neill's Physiology of Reproduction. T. M. Plant and J. Zeleznik. New York, Elsevier**:** pp 1395-1439.

Prevot, V., D. Croix, C. M. Rialas, P. Poulain, G. L. Fricchione, G. B. Stefano and J. C. Beauvillain (1999). "Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor." Endocrinology **140**(2): 652-659.

Prevot, V., C. M. Rialas, D. Croix, M. Salzet, J. P. Dupouy, P. Poulain, J. C. Beauvillain and G. B. Stefano (1998). "Morphine and anandamide coupling to nitric oxide stimulates GnRH and CRF release from rat median eminence: neurovascular regulation." Brain Res **790**(1-2): 236-244.

Pritchard, M. A. and I. Kola (1999). "The "gene dosage effect" hypothesis versus the "amplified developmental instability" hypothesis in Down syndrome." J Neural Transm Suppl **57**: 293-303.

Pueschel, S. M. (2006). "The effect of acetyl-L-carnitine administration on persons with Down syndrome." Res Dev Disabil **27**(6): 599-604.

Purnelle, G., A. Gerard, V. Czajkowski and J. P. Bourguignon (1997). "Pulsatile secretion of gonadotropin-releasing hormone by rat hypothalamic explants without cell bodies of GnRH neurons [corrected]." Neuroendocrinology **66**(5): 305-312.

Quinton, R., Y. Mamoojee, C. N. Jayasena, J. Young, S. Howard, L. Dunkel, T. Cheetham, N. Smith and A. A. Dwyer (2017). "Society for Endocrinology UK guidance on the evaluation of suspected disorders of sexual development: emphasizing the opportunity to predict adolescent pubertal failure through a neonatal diagnosis of absent minipuberty." Clin Endocrinol (Oxf) **86**(2): 305-306.

Rabbitt, P. (2005). "Frontal brain changes and cognitive performance in old age." Cortex **41**(2): 238-240.

Radovick, S., J. E. Levine and A. Wolfe (2012). "Estrogenic regulation of the GnRH neuron." Front Endocrinol (Lausanne) **3**: 52.

Raivio, T., J. Falardeau, A. Dwyer, R. Quinton, F. J. Hayes, V. A. Hughes, L. W. Cole, S. H. Pearce, H. Lee, P. Boepple, W. F. Crowley, Jr. and N. Pitteloud (2007). "Reversal of idiopathic hypogonadotropic hypogonadism." N Engl J Med **357**(9): 863-873.

Rasier, G., J. Toppari, A. S. Parent and J. P. Bourguignon (2006). "Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: a review of rodent and human data." Mol Cell Endocrinol **254-255**: 187-201.

Rasmussen, D. D. (1993). "Episodic gonadotropin-releasing hormone release from the rat isolated median eminence in vitro." Neuroendocrinology **58**(5): 511-518.

Raz, N. and K. M. Rodrigue (2006). "Differential aging of the brain: patterns, cognitive correlates and modifiers." Neurosci Biobehav Rev **30**(6): 730-748.

Reeves, J. J., A. Arimura and A. V. Schally (1971). "Changes in pituitary responsiveness to luteinizing hormone-releasing hormone (LH-RH) in anestrous ewes pretreated with estradiol benzoate." Biol Reprod **4**(1): 88-92.

Reeves, R. H., L. L. Baxter and J. T. Richtsmeier (2001). "Too much of a good thing: mechanisms of gene action in Down syndrome." Trends Genet **17**(2): 83-88.

Reeves, R. H., N. G. Irving, T. H. Moran, A. Wohn, C. Kitt, S. S. Sisodia, C. Schmidt, R. T. Bronson and M. T. Davisson (1995). "A mouse model for Down syndrome exhibits learning and behaviour deficits." Nat Genet **11**(2): 177-184.

Rehman, S., Z. Usman, S. Rehman, M. AlDraihem, N. Rehman, I. Rehman and G. Ahmad (2018). "Endocrine disrupting chemicals and impact on male reproductive health." Translational andrology and urology **7**(3): 490-503.

Reinholdt, L. G., Y. Ding, G. J. Gilbert, A. Czechanski, J. P. Solzak, R. J. Roper, M. T. Johnson, L. R. Donahue, C. Lutz and M. T. Davisson (2011). "Molecular characterization of the translocation breakpoints in the Down syndrome mouse model Ts65Dn." Mamm Genome **22**(11-12): 685-691.

Renaud, L. P., J. B. Martin and P. Brazeau (1975). "Depressant action of TRH, LH-RH and somatostatin on activity of central neurones." Nature **255**(5505): 233-235.

Renaud, L. P., J. B. Martin and P. Brazeau (1976). "Hypothalamic releasing factors: physiological evidence for a regulatory action on central neurons and pathways for their distribution in brain." Pharmacol Biochem Behav **5**(Suppl 1): 171-178.

Resnick, S. M. and V. W. Henderson (2002). "Hormone therapy and risk of Alzheimer disease: a critical time." JAMA **288**(17): 2170-2172.

Richards, S., N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, W. W. Grody, M. Hegde, E. Lyon, E. Spector, K. Voelkerding, H. L. Rehm and A. L. Q. A. Committee (2015). "Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology." Genet Med **17**(5): 405-424.

Richman, S., V. Edusa, A. Fadiel and F. Naftolin (2006). "Low-dose estrogen therapy for prevention of osteoporosis: working our way back to monotherapy." Menopause **13**(1): 148-155.

Rivero-Muller, A., K. C. Jonas, A. C. Hanyaloglu and I. Huhtaniemi (2013). "Di/oligomerization of GPCRs-mechanisms and functional significance." Prog Mol Biol Transl Sci **117**: 163-185.

Roa, J. and A. E. Herbison (2012). "Direct regulation of GnRH neuron excitability by arcuate nucleus POMC and NPY neuron neuropeptides in female mice." Endocrinology **153**(11): 5587-5599.

Robbins, T. W., M. James, A. M. Owen, B. J. Sahakian, A. D. Lawrence, L. McInnes and P. M. Rabbitt (1998). "A study of performance on tests from the CANTAB battery sensitive to frontal lobe dysfunction in a large sample of normal volunteers: implications for theories of executive functioning and cognitive aging. Cambridge Neuropsychological Test Automated Battery." J Int Neuropsychol Soc **4**(5): 474-490.

Rocca, W. A., B. R. Grossardt and L. T. Shuster (2014). "Oophorectomy, estrogen, and dementia: a 2014 update." Mol Cell Endocrinol **389**(1-2): 7-12.

Roche, K. W., R. J. O'Brien, A. L. Mammen, J. Bernhardt and R. L. Huganir (1996). "Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit." Neuron **16**(6): 1179-1188.

Rose, M. R. (1991). Evolutionary Biology of Aging. New York, Oxford University Press.

Roskams, A. J., D. S. Bredt, T. M. Dawson and G. V. Ronnett (1994). "Nitric oxide mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons." Neuron **13**(2): 289-299.

Rudolph, K. L., S. Chang, H. W. Lee, M. Blasco, G. J. Gottlieb, C. Greider and R. A. DePinho (1999). "Longevity, stress response, and cancer in aging telomerase-deficient mice." Cell **96**(5): 701-712.

Rueda, N., J. Florez and C. Martinez-Cue (2012). "Mouse models of Down syndrome as a tool to unravel the causes of mental disabilities." Neural Plast **2012**: 584071.

Ryan, B., G. Joilin and J. M. Williams (2015). "Plasticity-related microRNA and their potential contribution to the maintenance of long-term potentiation." Frontiers in molecular neuroscience **8**: 4-4.

Sago, H., E. J. Carlson, D. J. Smith, J. Kilbridge, E. M. Rubin, W. C. Mobley, C. J. Epstein and T. T. Huang (1998). "Ts1Cje, a partial trisomy 16 mouse model for Down syndrome, exhibits learning and behavioral abnormalities." Proc Natl Acad Sci U S A **95**(11): 6256-6261.

Sago, H., E. J. Carlson, D. J. Smith, E. M. Rubin, L. S. Crnic, T. T. Huang and C. J. Epstein (2000). "Genetic dissection of region associated with behavioral abnormalities in mouse models for Down syndrome." Pediatr Res **48**(5): 606-613.

Salthouse, T. (2012). "Consequences of age-related cognitive declines." Annu Rev Psychol **63**: 201-226.

Salthouse, T. A. (2010). "Selective review of cognitive aging." J Int Neuropsychol Soc **16**(5): 754-760.

Salthouse, T. A., N. M. Fristoe, T. T. Lineweaver and V. E. Coon (1995). "Aging of attention: does the ability to divide decline?" Mem Cognit **23**(1): 59-71.

Sangiao-Alvarellos, S., M. Manfredi-Lozano, F. Ruiz-Pino, V. M. Navarro, M. A. Sanchez-Garrido, S. Leon, C. Dieguez, F. Cordido, V. Matagne, G. A. Dissen, S. R. Ojeda, L. Pinilla and M. Tena-Sempere (2013). "Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty." Endocrinology **154**(2): 942-955.

Scaglia, H., M. Medina, A. L. Pinto-Ferreira, G. Vazques, C. Gual and G. Perez-Palacios (1976). "Pituitary LH and FSH secretion and responsiveness in women of old age." Acta Endocrinol (Copenh) **81**(4): 673-679.

Schally, A. V., A. Arimura, Y. Baba, R. M. Nair, H. Matsuo, T. W. Redding and L. Debeljuk (1971). "Isolation and properties of the FSH and LH-releasing hormone." Biochem Biophys Res Commun **43**(2): 393-399.

Schapiro, M. B., J. V. Haxby, C. L. Grady, R. Duara, N. L. Schlageter, B. White, A. Moore, M. Sundaram, S. M. Larson and S. I. Rapoport (1987). "Decline in cerebral glucose utilisation and cognitive function with aging in Down's syndrome." J Neurol Neurosurg Psychiatry **50**(6): 766-774.

Schapiro, M. B., J. S. Luxenberg, J. A. Kaye, J. V. Haxby, R. P. Friedland and S. I. Rapoport (1989). "Serial quantitative CT analysis of brain morphometrics in adult Down's syndrome at different ages." Neurology **39**(10): 1349-1353.

Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. DasGupta and C. Montecucco (1992). "Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin." Nature **359**(6398): 832-835.

Schmidt, R., E. Kienbacher, T. Benke, P. Dal-Bianco, M. Delazer, G. Ladurner, K. Jellinger, J. Marksteiner, G. Ransmayr, H. Schmidt, E. Stogmann, J. Friedrich and C. Wehringer (2008). "[Sex differences in Alzheimer's disease]." Neuropsychiatr **22**(1): 1-15.

Schwanzel-Fukuda, M. and D. W. Pfaff (1989). "Origin of luteinizing hormone-releasing hormone neurons." Nature **338**(6211): 161-164.

Segaloff, D. L., R. Sprengel, K. Nikolics and M. Ascoli (1990). "Structure of the lutropin/choriogonadotropin receptor." Recent Prog Horm Res **46**: 261-301; discussion 301-263.

Seidl, R., M. Tiefenthaler, E. Hauser and G. Lubec (2000). "Effects of transdermal nicotine on cognitive performance in Down's syndrome." Lancet **356**(9239): 1409-1410.

Selkoe, D. J. (1999). "Translating cell biology into therapeutic advances in Alzheimer's disease." Nature **399**(6738 Suppl): A23-31.

Selmanoff, M., C. Shu, S. L. Petersen, C. A. Barraclough and R. T. Zoeller (1991). "Single cell levels of hypothalamic messenger ribonucleic acid encoding luteinizing hormone-releasing hormone in intact, castrated, and hyperprolactinemic male rats." Endocrinology **128**(1): 459-466.

Seminara, S. B., F. J. Hayes and W. F. Crowley, Jr. (1998). "Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations." Endocr Rev **19**(5): 521-539.

Seminara, S. B., S. Messager, E. E. Chatzidaki, R. R. Thresher, J. S. Acierno, Jr., J. K. Shagoury, Y. Bo-Abbas, W. Kuohung, K. M. Schwinof, A. G. Hendrick, D. Zahn, J. Dixon, U. B. Kaiser, S. A. Slaugenhaupt, J. F. Gusella, S. O'Rahilly, M. B. Carlton, W. F. Crowley, Jr., S. A. Aparicio and W. H. Colledge (2003). "The GPR54 gene as a regulator of puberty." N.Engl.J.Med. **349**(17): 1614-1627.

Seminara, S. B., S. Messager, E. E. Chatzidaki, R. R. Thresher, J. S. Acierno, Jr., J. K. Shagoury, Y. Bo-Abbas, W. Kuohung, K. M. Schwinof, A. G. Hendrick, D. Zahn, J. Dixon, U. B. Kaiser, S. A. Slaugenhaupt, J. F. Gusella, S. O'Rahilly, M. B. Carlton, W. F. Crowley, Jr., S. A. Aparicio and W. H. Colledge (2003). "The GPR54 gene as a regulator of puberty." N Engl J Med **349**(17): 1614-1627.

Senti, K. A. and J. Brennecke (2010). "The piRNA pathway: a fly's perspective on the guardian of the genome." Trends Genet **26**(12): 499-509.

Sergeant, N., J. P. David, D. Champain, A. Ghestem, A. Wattez and A. Delacourte (2002). "Progressive decrease of amyloid precursor protein carboxy terminal fragments (APP-CTFs), associated with tau pathology stages, in Alzheimer's disease." J Neurochem **81**(4): 663-672.

Shao, H., J. C. Breitner, R. A. Whitmer, J. Wang, K. Hayden, H. Wengreen, C. Corcoran, J. Tschanz, M. Norton, R. Munger, K. Welsh-Bohmer, P. P. Zandi and I. Cache County (2012). "Hormone therapy and Alzheimer disease dementia: new findings from the Cache County Study." Neurology **79**(18): 1846-1852.

Shapiro, B. L. (2001). "Developmental instability of the cerebellum and its relevance to Down syndrome." J Neural Transm Suppl(61): 11-34.

Sheridan, R., J. Llerena, Jr., S. Matkins, P. Debenham, A. Cawood and M. Bobrow (1989). "Fertility in a male with trisomy 21." J Med Genet **26**(5): 294-298.

Shishkina, G. T., T. S. Kalinina and N. N. Dygalo (2004). "Attenuation of alpha2A-adrenergic receptor expression in neonatal rat brain by RNA interference or antisense oligonucleotide reduced anxiety in adulthood." Neuroscience **129**(3): 521-528.

Shumaker, S. A., C. Legault, S. R. Rapp, L. Thal, R. B. Wallace, J. K. Ockene, S. L. Hendrix, B. N. Jones, 3rd, A. R. Assaf, R. D. Jackson, J. M. Kotchen, S. Wassertheil-Smoller, J. Wactawski-Wende and W. Investigators (2003). "Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the

Women's Health Initiative Memory Study: a randomized controlled trial." JAMA **289**(20): 2651-2662.

Simonian, S. X., D. P. Spratt and A. E. Herbison (1999). "Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat." J Comp Neurol **411**(2): 346-358.

Singh, M., J. W. Simpkins, J. W. Simpkins, H. A. Bimonte-Nelson, H. A. Bimonte-Nelson, R. D. Brinton and R. D. Brinton (2013). "Window of opportunity for estrogen and progestin intervention in brain aging and Alzheimer's disease." Brain Res **1514**: 1-2.

Sisk, C. L. and D. L. Foster (2004). "The neural basis of puberty and adolescence." Nature Neuroscience **7**(10): 1040-1047.

Skinner, D. C. and A. Caraty (2002). "Measurement and possible function of GnRH in cerebrospinal fluid in ewes." Reprod Suppl **59**: 25-39.

Skrapits, K., B. A. Borsay, L. Herczeg, P. Ciofi, S. R. Bloom, M. A. Ghatei, W. S. Dhillo, Z. Liposits and E. Hrabovszky (2014). "Colocalization of cocaine- and amphetamine-regulated transcript with kisspeptin and neurokinin B in the human infundibular region." PLoS One **9**(8): e103977.

Slezak, M., A. Grosche, A. Niemiec, N. Tanimoto, T. Pannicke, T. A. Munch, B. Crocker, P. Isope, W. Hartig, S. C. Beck, G. Huber, G. Ferracci, M. Perraut, M. Reber, M. Miehe, V. Demais, C. Leveque, D. Metzger, K. Szklarczyk, R. Przewlocki, M. W. Seeliger, D. Sage-Ciocca, J. Hirrlinger, A. Reichenbach, S. Reibel and F. W. Pfrieger (2012). "Relevance of exocytotic glutamate release from retinal glia." Neuron **74**(3): 504-516.

Sliger, M., T. Lander and C. Murphy (2004). "Effects of the ApoE epsilon4 allele on olfactory function in Down syndrome." J Alzheimers Dis **6**(4): 397-402; discussion 443-399.

Soper, B. D. and R. F. Weick (1980). "Hypothalamic and extrahypothalamic mediation of pulsatile discharges of luteinizing hormone in the ovariectomized rat." Endocrinology **106**(1): 348-355.

Spergel, D. J., U. Kruth, D. F. Hanley, R. Sprengel and P. H. Seeburg (1999). "GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice." J.Neurosci. **19**(6): 2037-2050.

Stadtman, E. R. (1992). "Protein oxidation and aging." Science **257**(5074): 1220-1224.

Starling, E. (1905). "The Croonian Lectures ON THE CHEMICAL CORRELATION OF THE FUNCTIONS OF THE BODY." The Lancet **166**(4275): 339-341.

Stearns, P. E., K. E. Droulard and F. H. Sahhar (1960). "Studies bearing on fertility of male and female mongoloids." Am J Ment Defic **65**: 37-41.

Stefanidis, K., P. Belitsos, A. Fotinos, N. Makris, D. Loutradis and A. Antsaklis (2011). "Causes of infertility in men with Down syndrome." Andrologia **43**(5): 353-357.

Steinert, J. R., S. W. Robinson, H. Tong, M. D. Haustein, C. Kopp-Scheinpflug and I. D. Forsythe (2011). "Nitric oxide is an activity-dependent regulator of target neuron intrinsic excitability." Neuron **71**(2): 291-305.

Steyn, F. J., Y. Wan, J. Clarkson, J. D. Veldhuis, A. E. Herbison and C. Chen (2013). "Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice." Endocrinology **154**(12): 4939-4945.

Stojilkovic, S. S., J. Reinhart and K. J. Catt (1994). "Gonadotropin-releasing hormone receptors: structure and signal transduction pathways." Endocr Rev **15**(4): 462-499.

Struwe, F. (1929). "Histopathologische Untersuchungen uber Entstehung und Wesen der senilen Plaques." Z Ges Neurol Psychiat **122**: 291-307.

Studd, J. W., S. Chakravarti and W. P. Collins (1978). "Plasma hormone profiles after the menopause and bilateral oophorectomy." Postgrad Med J **54 Suppl 2**: 25-30.

Stuehr, D. J. (1997). "Structure-function aspects in the nitric oxide synthases." Annu Rev Pharmacol Toxicol **37**: 339-359.

Sturgeon, X. and K. J. Gardiner (2011). "Transcript catalogs of human chromosome 21 and orthologous chimpanzee and mouse regions." Mamm Genome **22**(5-6): 261-271.

Sun, H., J. K. Karow, I. D. Hickson and N. Maizels (1998). "The Bloom's syndrome helicase unwinds G4 DNA." J Biol Chem **273**(42): 27587-27592.

Swamy, G. K., T. Ostbye and R. Skjaerven (2008). "Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth." JAMA **299**(12): 1429-1436.

Swanson, L. W. (2004). Structure of the rat brain. Amsterdam, Elsevier Science Publishers.

Szczypka, M. S., K. Kwok, M. D. Brot, B. T. Marck, A. M. Matsumoto, B. A. Donahue and R. D. Palmiter (2001). "Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice." Neuron **30**(3): 819-828.

Szwarcfarb, B., S. Carbone, M. L. Stein, J. Medina and J. A. Moguilevsky (1994). "Sexual differences in the effect of the GABAergic system on LH secretion and in the hypothalamic ontogenesis of GABAA receptors in prepubertal rats." Brain Res **646**(2): 351-355.

Tang, H. and P. D. Thomas (2016). "PANTHER-PSEP: predicting disease-causing genetic variants using position-specific evolutionary preservation." Bioinformatics **32**(14): 2230-2232.

Teixeira, L., F. Guimiot, C. Dode, C. Fallet-Bianco, R. P. Millar, A. L. Delezoide and J. P. Hardelin (2010). "Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions." J Clin Invest **120**(10): 3668-3672.

Tejedor, F. J. and B. Hammerle (2011). "MNB/DYRK1A as a multiple regulator of neuronal development." FEBS J **278**(2): 223-235.

Telegdy, G., A. Adamik, M. Tanaka and A. V. Schally (2010). "Effects of the LHRH antagonist Cetrorelix on affective and cognitive functions in rats." Regul Pept **159**(1-3): 142-147.

Telegdy, G., M. Tanaka and A. V. Schally (2009). "Effects of the LHRH antagonist Cetrorelix on the brain function in mice." Neuropeptides **43**(3): 229-234.

Teles, M. G., L. F. Silveira, C. Tusset and A. C. Latronico (2011). "New genetic factors implicated in human GnRH-dependent precocious puberty: the role of kisspeptin system." Mol Cell Endocrinol **346**(1-2): 84-90.

Temple, J. L., R. P. Millar and E. F. Rissman (2003). "An Evolutionarily Conserved Form of Gonadotropin-Releasing Hormone Coordinates Energy and Reproductive Behavior." Endocrinology **144**(1): 13-19.

Tena-Sempere, M. (2013). "Keeping puberty on time: novel signals and mechanisms involved." Curr Top Dev Biol **105**: 299-329.

Tenenbaum-Rakover, Y., M. Commenges-Ducos, A. Iovane, C. Aumas, O. Admoni and N. de Roux (2007). "Neuroendocrine phenotype analysis in five patients with isolated hypogonadotropic hypogonadism due to a L102P inactivating mutation of GPR54." J Clin Endocrinol Metab **92**(3): 1137-1144.

Themmen, A. P. N. and I. T. Huhtaniemi (2000). "Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function." Endocr Rev **21**(5): 551-583.

Thompson, I. R. and U. B. Kaiser (2014). "GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression." Mol Cell Endocrinol **385**(1-2): 28-35.

Timiras, P. S. (1978). "Biological perspectives on aging." Am Sci **66**(5): 605-613.

Tobet, S. A., E. P. Bless and G. A. Schwarting (2001). "Developmental aspect of the gonadotropin-releasing hormone system." Mol Cell Endocrinol **185**(1-2): 173-184.

Tobet, S. A., I. K. Hanna and G. A. Schwarting (1996). "Migration of neurons containing gonadotropin releasing hormone (GnRH) in slices from embryonic nasal compartment and forebrain." Brain Res Dev Brain Res **97**(2): 287-292.

Tobet, S. A. and G. A. Schwarting (2006). "Minireview: recent progress in gonadotropin-releasing hormone neuronal migration." Endocrinology **147**(3): 1159-1165.

Topaloglu, A. K., J. A. Tello, L. D. Kotan, M. N. Ozbek, M. B. Yilmaz, S. Erdogan, F. Gurbuz, F. Temiz, R. P. Millar and B. Yuksel (2012). "Inactivating KISS1 mutation and hypogonadotropic hypogonadism." N Engl J Med **366**(7): 629-635.

Tosato, M., V. Zamboni, A. Ferrini and M. Cesari (2007). "The aging process and potential interventions to extend life expectancy." Clin Interv Aging **2**(3): 401-412.

Trifunovic, A., A. Wredenberg, M. Falkenberg, J. N. Spelbrink, A. T. Rovio, C. E. Bruder, Y. M. Bohlooly, S. Gidlof, A. Oldfors, R. Wibom, J. Tornell, H. T. Jacobs and N. G. Larsson (2004). "Premature ageing in mice expressing defective mitochondrial DNA polymerase." Nature **429**(6990): 417-423.

Trimble, W. S., T. S. Gray, L. A. Elferink, M. C. Wilson and R. H. Scheller (1990). "Distinct patterns of expression of two VAMP genes within the rat brain." J Neurosci **10**(4): 1380-1387.

Turzillo, A. M., G. B. DiGregorio and T. M. Nett (1995). "Messenger ribonucleic acid for gonadotropin-releasing hormone receptor and numbers of gonadotropin-releasing hormone receptors in ovariectomized ewes after hypothalamic-pituitary disconnection and treatment with estradiol." J Anim Sci **73**(6): 1784-1788.

Turzillo, A. M., J. L. Juengel and T. M. Nett (1995). "Pulsatile gonadotropin-releasing hormone (GnRH) increases concentrations of GnRH receptor messenger ribonucleic acid and numbers of GnRH receptors during luteolysis in the ewe." Biol Reprod **53**(2): 418-423.

Turzillo, A. M. and T. M. Nett (1995). "Effects of estradiol on concentrations of gonadotropin-releasing hormone receptor messenger ribonucleic acid following removal of progesterone." Endocrine **3**(10): 765-768.

Tuscher, J. J., L. R. Taxier, A. M. Fortress and K. M. Frick (2018). "Chemogenetic inactivation of the dorsal hippocampus and medial prefrontal cortex, individually and concurrently, impairs object recognition and spatial memory consolidation in female mice." Neurobiol Learn Mem **156**: 103-116.

Urbanski, H. F. and S. R. Ojeda (1990). "A role for N-methyl-D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty." Endocrinology **126**(3): 1774-1776.

Van Dyke, D. C. (2003). "Down Syndrome: Visions for the 21st Century." American Journal of Human Genetics **72**(5): 1354-1354.

Van Raamsdonk, J. M. and S. Hekimi (2009). "Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans." PLoS Genet **5**(2): e1000361.

Van Raamsdonk, J. M. and S. Hekimi (2012). "Superoxide dismutase is dispensable for normal animal lifespan." Proc Natl Acad Sci U S A **109**(15): 5785-5790.

Van Vugt, D. A., W. D. Diefenbach, E. Alston and M. Ferin (1985). "Gonadotropin-releasing hormone pulses in third ventricular cerebrospinal fluid of ovariectomized rhesus monkeys: correlation with luteinizing hormone pulses." Endocrinology **117**(4): 1550-1558.

Vaziri, H. and S. Benchimol (1998). "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span." Curr Biol **8**(5): 279-282.

Veldhuis, J. D. (2008). "Aging and hormones of the hypothalamo-pituitary axis: gonadotropic axis in men and somatotropic axes in men and women." Ageing Res Rev **7**(3): 189-208.

Vidal, A., Q. Zhang, C. Medigue, S. Fabre and F. Clement (2012). "DynPeak: an algorithm for pulse detection and frequency analysis in hormonal time series." PLoS One **7**(7): e39001.

Vijg, J. and Y. Suh (2013). "Genome instability and aging." Annu Rev Physiol **75**: 645-668.

Villeda, S. A., J. Luo, K. I. Mosher, B. Zou, M. Britschgi, G. Bieri, T. M. Stan, N. Fainberg, Z. Ding, A. Eggel, K. M. Lucin, E. Czirr, J. S. Park, S. Couillard-Despres, L. Aigner, G. Li, E. R. Peskind, J. A. Kaye, J. F. Quinn, D. R. Galasko, X. S. Xie, T. A. Rando and T. Wyss-Coray (2011). "The ageing systemic milieu negatively regulates neurogenesis and cognitive function." Nature **477**(7362): 90-94.

Vina, J., C. Borras, K. M. Abdelaziz, R. Garcia-Valles and M. C. Gomez-Cabrera (2013). "The free radical theory of aging revisited: the cell signaling disruption theory of aging." Antioxid Redox Signal **19**(8): 779-787.

Vincent, S. R. and H. Kimura (1992). "Histochemical mapping of nitric oxide synthase in the rat brain." Neuroscience **46**(4): 755-784.

von Zglinicki, T. (2002). "Oxidative stress shortens telomeres." Trends Biochem Sci **27**(7): 339-344.

Vranekovic, J., I. B. Bozovic, Z. Grubic, J. Wagner, D. Pavlinic, S. Dahoun, F. Bena, V. Culic and B. Brajenovic-Milic (2012). "Down syndrome: parental origin, recombination, and maternal age." Genet Test Mol Biomarkers **16**(1): 70-73.

Wahjoepramono, E. J., L. K. Wijaya, K. Taddei, K. A. Bates, M. Howard, G. Martins, K. deRuyck, P. M. Matthews, G. Verdile and R. N. Martins (2011). "Direct exposure of guinea pig CNS to human luteinizing hormone increases cerebrospinal fluid and cerebral beta amyloid levels." Neuroendocrinology **94**(4): 313-322.

Waldstreicher, J., S. B. Seminara, J. L. Jameson, A. Geyer, L. B. Nachtigall, P. A. Boepple, L. B. Holmes and W. F. Crowley, Jr. (1996). "The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human." J Clin Endocrinol Metab **81**(12): 4388-4395.

Wang, H. J., G. E. Hoffman and M. S. Smith (1995). "Increased GnRH mRNA in the GnRH neurons expressing cFos during the proestrous LH surge." Endocrinology **136**(8): 3673-3676.

Wang, L. and S. M. Moenter (2019). "Differential roles of hypothalamic AVPV and arcuate kisspeptin neurons in estradiol feedback regulation of female reproduction." Neuroendocrinology.

Wang, M., P. Roussos, A. McKenzie, X. Zhou, Y. Kajiwara, K. J. Brennand, G. C. De Luca, J. F. Crary, P. Casaccia, J. D. Buxbaum, M. Ehrlich, S. Gandy, A. Goate, P. Katsel, E. Schadt, V. Haroutunian and B. Zhang (2016). "Integrative network analysis of nineteen brain regions identifies molecular signatures and networks underlying selective regional vulnerability to Alzheimer's disease." Genome Med **8**(1): 104.

Wang, Q. F., P. G. Farnworth, J. K. Findlay and H. G. Burger (1988). "Effect of purified 31K bovine inhibin on the specific binding of gonadotropin-releasing hormone to rat anterior pituitary cells in culture." Endocrinology **123**(5): 2161-2166.

Weimbs, T., K. Mostov, S. H. Low and K. Hofmann (1998). "A model for structural similarity between different SNARE complexes based on sequence relationships." Trends Cell Biol **8**(7): 260-262.

Weiner, R. I., W. Wetsel, P. Goldsmith, G. Martinez de la Escalera, J. Windle, C. Padula, A. Choi, A. Negro-Vilar and P. Mellon (1992). "Gonadotropin-releasing hormone neuronal cell lines." Front Neuroendocrinol **13**(2): 95-119.

Weinert, B. T. and P. S. Timiras (2003). "Invited review: Theories of aging." J Appl Physiol (1985) **95**(4): 1706-1716.

Weitzdoerfer, R., H. Hoeger, E. Engidawork, M. Engelmann, N. Singewald, G. Lubec and B. Lubec (2004). "Neuronal nitric oxide synthase knock-out mice show impaired cognitive performance." Nitric Oxide **10**(3): 130-140.

Wen, S., W. Ai, Z. Alim and U. Boehm (2010). "Embryonic gonadotropin-releasing hormone signaling is necessary for maturation of the male reproductive axis." Proc Natl Acad Sci U S A **107**(37): 16372-16377.

Wetsel, W. C. and S. Srinivasan (2002). "Pro-GnRH processing." Prog Brain Res **141**: 221-241.

Whittemore, K., E. Vera, E. Martinez-Nevado, C. Sanpera and M. A. Blasco (2019). "Telomere shortening rate predicts species life span." Proc Natl Acad Sci U S A **116**(30): 15122-15127.

Wiklund, I., J. Karlberg and L.-Å. Mattsson (1993). "Quality of life of postmenopausal women on a regimen of transdermal estradiol therapy: A double-blind placebo-controlled study." American Journal of Obstetrics and Gynecology **168**(3, Part 1): 824-830.

Wilson, A. C., M. S. Salamat, R. J. Haasl, K. M. Roche, A. Karande, S. V. Meethal, E. Terasawa, R. L. Bowen and C. S. Atwood (2006). "Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression." J Endocrinol **191**(3): 651-663.

Wilson, R. C., J. S. Kesner, J. M. Kaufman, T. Uemura, T. Akema and E. Knobil (1984). "Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey." Neuroendocrinology **39**(3): 256-260.

Wintermantel, T. M., R. E. Campbell, R. Porteous, D. Bock, H. J. Grone, M. G. Todman, K. S. Korach, E. Greiner, C. A. Perez, G. Schutz and A. E. Herbison (2006). "Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility." Neuron **52**(2): 271-280.

Wirsig-Wiechmann, C. R., L. D. Houck, P. W. Feldhoff and R. C. Feldhoff (2002). "Pheromonal activation of vomeronasal neurons in plethodontid salamanders." Brain Res **952**(2): 335-344.

Wise, M. E., D. Nieman, J. Stewart and T. M. Nett (1984). "Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone." Biol Reprod **31**(5): 1007-1013.

Wise, P. M., M. L. Kashon, K. M. Krajnak, K. L. Rosewell, A. Cai, K. Scarbrough, J. P. Harney, T. McShane, J. M. Lloyd and N. G. Weiland (1997). "Aging of the female reproductive system: a window into brain aging." Recent Prog Horm Res **52**: 279-303; discussion 303-275.

Wiseman, F. K., K. A. Alford, V. L. Tybulewicz and E. M. Fisher (2009). "Down syndrome--recent progress and future prospects." Hum Mol Genet **18**(R1): R75-83.

Wisniewski, K. E., A. J. Dalton, C. McLachlan, G. Y. Wen and H. M. Wisniewski (1985). "Alzheimer's disease in Down's syndrome: clinicopathologic studies." Neurology **35**(7): 957-961.

Wray, S., P. Grant and H. Gainer (1989). "Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode." Proc Natl Acad Sci U S A **86**(20): 8132-8136.

Wray, S. and G. Hoffman (1986). "A developmental study of the quantitative distribution of LHRH neurons within the central nervous system of postnatal male and female rats." J Comp Neurol **252**(4): 522-531.

Wray, S., A. Nieburgs and S. Elkabes (1989). "Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode." Brain Res Dev Brain Res **46**(2): 309-318.

Wyss-Coray, T. (2016). "Ageing, neurodegeneration and brain rejuvenation." Nature **539**(7628): 180-186.

Xu, Y., L. D. Faulkner and J. W. Hill (2011). "Cross-Talk between Metabolism and Reproduction: The Role of POMC and SF1 Neurons." Front Endocrinol (Lausanne) **2**: 98.

Yaffe, K., L. Y. Lui, J. Zmuda and J. Cauley (2002). "Sex hormones and cognitive function in older men." J Am Geriatr Soc **50**(4): 707-712.

Yang, S. N., F. Lu, J. N. Wu, D. D. Liu and W. Y. Hsieh (1999). "Activation of gonadotropin-releasing hormone receptors induces a long-term enhancement of excitatory postsynaptic currents mediated by ionotropic glutamate receptors in the rat hippocampus." Neurosci Lett **260**(1): 33-36.

Yasin, M., A. C. Dalkin, D. J. Haisenleder, J. R. Kerrigan and J. C. Marshall (1995). "Gonadotropin-releasing hormone (GnRH) pulse pattern regulates GnRH receptor gene expression: augmentation by estradiol." Endocrinology **136**(4): 1559-1564.

Yen, S. S. and C. C. Tsai (1971). "The effect of ovariectomy on gonadotropin release." J Clin Invest **50**(5): 1149-1153.

Yoon, H., L. W. Enquist and C. Dulac (2005). "Olfactory inputs to hypothalamic neurons controlling reproduction and fertility." Cell **123**(4): 669-682.

Young, W. C., R. W. Goy and C. H. Phoenix (1964). "Hormones and Sexual Behavior." Science **143**(3603): 212-218.

Yu, C. E., J. Oshima, Y. H. Fu, E. M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G. M. Martin, J. Mulligan and G. D. Schellenberg (1996). "Positional cloning of the Werner's syndrome gene." Science **272**(5259): 258-262.

Zhang, G., J. Li, S. Purkayastha, Y. Tang, H. Zhang, Y. Yin, B. Li, G. Liu and D. Cai (2013). "Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH." Nature **497**(7448): 211-216.

Zhang, Y., M. S. Kim, B. Jia, J. Yan, J. P. Zuniga-Hertz, C. Han and D. Cai (2017). "Hypothalamic stem cells control ageing speed partly through exosomal miRNAs." Nature **548**(7665): 52-57.

Zhao, L., Z. Mao and R. D. Brinton (2009). "A select combination of clinically relevant phytoestrogens enhances estrogen receptor beta-binding selectivity and neuroprotective activities in vitro and in vivo." Endocrinology **150**(2): 770-783.

Zhao, L., S. K. Woody and A. Chhibber (2015). "Estrogen receptor beta in Alzheimer's disease: From mechanisms to therapeutics." Ageing Res Rev **24**(Pt B): 178-190.

Zhuo, M. and R. D. Hawkins (1995). "Long-term depression: a learning-related type of synaptic plasticity in the mammalian central nervous system." Rev Neurosci **6**(3): 259-277.

Ziegler, S. G. and J. E. Thornton (2010). "Low luteinizing hormone enhances spatial memory and has protective effects on memory loss in rats." Horm Behav **58**(5): 705-713.

Zou, Y.-M., D. Lu, L.-P. Liu, H.-H. Zhang and Y.-Y. Zhou (2016). "Olfactory dysfunction in Alzheimer's disease." Neuropsychiatric disease and treatment **12**: 869-875.

Zuhlke, C., U. Thies, I. Braulke, A. Reis and C. Schirren (1994). "Down syndrome and male fertility: PCR-derived fingerprinting, serological and andrological investigations." Clin Genet **46**(4): 324-326.

The role of GnRH in the age-related cognitive decline in some disorders including Down syndrome

Résumé

Il est maintenant établi que les neurones à gonadotropin-releasing hormone (GnRH) jouent un rôle majeur dans la régulation d'un réseau neuronal complexe qui contrôle le début de la puberté et la fertilité, principalement au sein de l'hypothalamus. De plus, il est de plus en plus décrit que la GnRH serait impliquée dans une multitude de fonctions, autre que celle de la reproduction. Ici, nous montrons que les neurones à GnRH sont essentiels au maintien de la fonction cognitive et à l'olfaction. Une première étude montre la présence d'une dérégulation dans la transcription de micro-ARN (miR) dans un modèle murin de Trisomie 21 ou Syndrome de Down (souris Ts65Dn) associée à une perte progressive de l'expression de la GnRH. Cette perte est concomitante à un déclin cognitif et olfactif. De manière intéressante, ces déficits sont réversibles à la suite d'une surexpression du miR-200, induite par un vecteur viral. La fonction de la GnRH est, quant à elle, restaurée grâce à une greffe de neurones à GnRH ou à l'administration pulsatile de GnRH. Une deuxième étude démontre que des mutations dans le gène codant pour la synthase d'oxyde nitrique neuronale (*NOS1*) peuvent être un facteur à l'origine de deux troubles génétiques associés à un déficit en GnRH : l'hypogonadisme hypogonadotropique congénital et le syndrome de Kallmann. De façon similaire à l'Homme, les souris déficientes en *Nos1*, ne présentent pas uniquement un retard de puberté et une infertilité mais également des déficits cognitifs et olfactifs. En outre, l'inhalation de d'oxide nitrique (NO) au cours de la période infantile est restaure, dans ce modèle, la maturation sexuelle, la cognition et l'olfaction. Dans leur ensemble, les résultats recueillis dans cette thèse de doctorat, montrent que la GnRH joue un rôle critique et inattendu dans le maintien de la fonction cognitive et l'olfaction, offrant ainsi de nouvelles opportunités de stratégies thérapeutiques contre de nombreux troubles neuro-développementaux et neuro-dégénératifs.