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Inserm

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THÈSE

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Spécialité: Neurosciences

Molecular Characterization of NOS-synthesizing neurons and assessment of their function in the maturation of the hypothalamic – pituitary – gonadal axis

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L'apparition de la puberté et la régulation de la fertilité chez les mammifères sont contrôlées par un réseau neuronal complexe, situé principalement dans l'hypothalamus, et qui converge vers les neurones synthétisant l'hormone de libération des gonadotrophines (GnRH). Ces neurones régulent la sécrétion des gonadotrophines, la croissance et le fonctionnement des gonades. Le développement correct du système à GnRH, incluant des changements rapides dans l'expression et la signalisation de l'hormone GnRH au sein de cette population clairsemée de quelques centaines de neurones, est essentiel pour la maturation sexuelle et le fonctionnement normal de l'axe hypothalamo-hypophyso-gonadique.

Lors du développement embryonnaire, ces neurones migrent de la placode olfactive vers leur emplacement définitif, l'hypothalamus, pour y recevoir les connexions afférentes qui permettront une libération pulsatile de la GnRH et la libération subséquente des gonadotrophines (l'hormone de stimulation des follicules (FSH) et l'hormone lutéinisante (LH)). Dès les années 90, l'oxyde nitrique (NO) a été identifié comme molécule clé dans la décharge pré-ovulatoire de GnRH/LH. En effet, de nombreux travaux ont suggéré que des interactions entre les neurones exprimant la forme neuronale de l'enzyme de synthèse du NO (la nNOS) et le système GnRH étaient impliquées dans le contrôle central de la fonction de reproduction à l'âge adulte.

De plus, si le NO est reconnu depuis longtemps comme un acteur majeur du contrôle central de l'ovulation à l'âge adulte, la possibilité qu'il soit aussi impliqué dans la maturation sexuelle en régulant l'activité des neurones à GnRH à des stades précoces précédant la puberté n'a pas été explorée auparavant. Cependant, même si nous avons progressé dans la connaissance des interactions entre les neurones à nNOS et des différents acteurs importants de l'axe gonadotrope, l'identité moléculaire de ces neurones reste mal connue. Au cours de cette étude, nous avons recherché 1) l'identité moléculaire des neurones á nNOS dans

l'hypothalamus au cours de développement 2) si le NO régule la migration et l'intégration des neurones à GnRH dans l'hypothalamus et 3) si le NO régule la maturation sexuelle. Pendant ma thèse nous avons répertorié pour la première fois les différents neurotransmetteurs et les principaux récepteurs dans les neurones à nNOS au cours du développement post-natal.

De plus, les résultats de ma thèse montrent pour la première fois une implication de la signalisation du NO dans la migration des neurones à GnRH vers l'hypothalamus et font échos à l'identification d'une série de mutations de la NOS1 chez des patients atteints du syndrome de Kallmann, une maladie génétique congénitale rare qui associe une carence en GnRH, due à un défaut de migration neuronale, et une anosmie. Enfin, mes travaux montrent que le NO est un nouveau protagoniste dans la maturation post-natale du système à GnRH, la survenue de la puberté et l'acquisition de la capacité à se reproduire. Plus généralement, les résultats de ce travail de thèse permettent d'identifier de nouveaux mécanismes potentiellement responsables de troubles développementaux dans la mise en place des circuits neuronaux contrôlant l'axe gonadotrope chez les mammifères en général et l'homme en particulier. Nous espérons que ces résultats élargiront notre compréhension de la régulation de l'axe reproducteur, offrant ainsi des possibilités nouvelles de stratégies thérapeutiques contre les troubles de la fertilité.

Pendant les trois années de ma thèse, j'ai cherché à définir le rôle de la signalisation NO dans les événements qui se déroulent dans la région hypothalamique. Étant particulièrement intéressé par le NO libéré par les neurones synthétisants l'oxyde nitrique (nNOS) dans la région de l'hypothalamus, j'ai travaillé sur une caractérisation en profondeur des propriétés moléculaires et cellulaires, de la distribution anatomique, ainsi que de l'implication de l'expression de la protein nNos dans la physiologie du système reproducteur.

Les neurones á nNos sont connus pour être représentés par de nombreuses sous populations différentes de cellules en matière de spécification génétique, et de caractérisation moléculaire. L'hétérogénéité des neurones libérant du NO a été étudiée dans différentes régions du cerveau, alors que le phénotype des cellules nNOS résidant dans la région hypothalamique était largement inconnu. Puisque les neurones exprimant le nNOS ont été impliqués dans de nombreux événements survenant dans l'hypothalamus, comme la régulation de l'apport alimentaire, la dépense énergétique et la capacité de reproduction, l'identification moléculaire de ces neurones nous permettrait de décrire leur contribution aux processus métaboliques et reproductifs.

Dans la première étude, actuellement soumise dans le “Journal of comparative Neurology”, nous avons effectué une classification neuroanatomique des neurones nNOS dans l'hypothalamus pendant le développement postnatal. En examinant les stades de développement infantile, juvénile et adulte, nous avons décrit la distribution de protéine nNOS dans la plupart des régions hypothalamiques qui sont impliquées dans le contrôle de la fonction des neurones à GnRH. De plus, en utilisant les lignées de souris Vglut2, VGat et GAD-67 GFP (G42), nous avons déterminé la cohorte des principaux neurotransmetteurs et récepteurs exprimés par cette population neuronale dans l'hypothalamus adulte, et révélé de nouvelles sous populations nNOS recevant des signaux oestrogéniques et leptinergiques. Plus précisément, nous présentons des données confirmant que:

⇒ les neurones à nNOS se trouvent dans des noyaux hypothalamiques distincts avec la population la plus compacte identifiée dans la zone préoptique et plus spécifiquement dans la région de l’ organe vasculaire de la lame terminale (OVLT)

⇒ nNOS est exprimée de façon constitutive pendant le développement postnatal dans toutes les régions étudiées, la seule exception étant la région hypothalamique courbe

(ARH) où l'expression de nNOS était absente à P11. Les changements dans le niveau d'expression de la nNOS ont également été identifiés dans les régions du noyau noyau arqué (AHN), de l'hypothalamus latéral (LHA), du noyau suprachiasmatique hypothalamique (SCN), du noyau supraoptique (SON), de la zone périventriculaire préoptique (PVPo) et de l'hypothalamus postérieur (PH)

⇒ les neurones exprimant la nNOS sont divisés en sous-populations moléculaires distinctes en fonction du noyau hypothalamique dans lequel elles se trouvent; La grande majorité des cellules nNOS dans les régions de noyau ventro-médial (VMH), de l'hypothalamus dorsomédial (DMH), de la zone hypothalmique antéro-ventrale (AVPV), de la zone préoptique médiane (MePO) et de l'OVLT sont glutamatergiques. Au contraire, la population de nNOS résidant dans l'ARH est dans sa grande majorité gabaergique

⇒ dans l'ARH, des membres de la population de nNOS gabaergique sont identifiés comme des neurones GAD-67 positifs exprimants la parvalbumine

⇒ ER-α est co-exprimé par la plupart des neurones nNOS résidants dans les régions de ARH, VMH, AVPV et OVLT / MePO

Les résultats présentés dans cette étude mettent en evidence un rôle clé de la signalisation de NO dans les événements ayant lieu dans la région hypothalamique et révèlent de nouvelles populations de nNOS partageants éventuellement des rôles distincts dans le réseau hypothalamique neuronal contrôlant les processus reproducteurs et métaboliques.
Nous avons présenté des preuves démontrant que les cellules nNOS sont une population très diversifiée, représentée par de nombreuses sous populations neuronales différentes dans les différents noyaux hypothalamiques.

Dans la deuxième étude, j'ai cherché à décrire le rôle de la signalisation NO dans la physiologie de la reproduction. Des études précédentes de notre laboratoire ont souligné

l'importance de la signalisation NO dans les événements reproductifs au cours de l'âge adulte. Nous avons donc émis en hypothèse l'idée que ce neuromodulateur obscur pourrait réguler la maturation sexuelle de l'axe reproducteur en jouant un rôle catalyseur dans les événements menant à l'apparition pubertaire.

Les neurones à GnRH sont incontestablement les maîtres regulateurs du système reproducteur. Pour qu'ils puissent exercer leur rôle pivot dans l'établissement d'un phénotype fertile, leur activité et leur capacité neurosécrétoire doivent être régulées avec précision par des voies en amont. L'identification d'une série de mutations sur le gène humain Nos1 chez des patients présentant un hypogonadisme hypogonadotrope constitutif (CHH), ainsi que ceux prèsentant un retard constitutionnel de la croissance et de la puberté (CDGP), met non seulement en évidence un rôle-clé de nNOS dans l'établissement d'un phénotype fertile, mais surtout, ouvre la voie à une meilleure compréhension des conditions de l'infertilité idiopathique humaine. Dans ce but, nous présentons des données probantes soutenant un rôle nouveau de la signalisation hypothétique du NO au cours du développement embryonnaire, de la régulation de la migration neuronale de la GnRh, mais aussi du développement postnatal, de la régulation de la GnrH aux niveaux transcriptionnel et protéique. Plus précisément, nous présentons des données proposant que:

⇒ des mutations faux-sens dans le gène Nos1 sont liées à la présence de CHH dans des patients

⇒ NO signalisation est nécessaire pour la migration correcte des neurones à GnRH pendant le développement embryonnaire

⇒ l’ absence constitutive de NOS1 dans un modèle NOS1 ko entraîne un retard significatif dans le processus de maturation de l’ axe reproducteur

⇒ la suppression de la signalisation du NO spécifiquement pendant la période infantile aboutit à un phénotype de maturation retardée de l'axe reproducteur, semblable à celui décrit dans le modèle de NOS1 ko

⇒ l'arrivée de signaux oestrogéniques dans la région hypothalamique au cours de la période infantile est responsable de la maturation des neurones à nNOS résidants dans la zone préoptique

⇒ les cellules à nNOS sont nécessaires pour établir le dialogue entre les ovaires et l'hypothalamus

⇒ les neurones à nNOS jouent un rôle clé dans la régulation de la transcription du gène de la GnRH à P12

⇒ les neurones à nNOS régulent la libération de GnRH pendant la période infantile

Dans l'ensemble, nos données établissent un rôle nouveau pour le NO produit par les neurones hypothalamiques de nNOS dans le contrôle de la maturation de l'axe de reproduction. Le NO régule l'apparition de la puberté en agissant pendant une fenêtre de temps infantile restreinte pour réprimer la libération de GnRH, permettant ainsi l'activation synchronisée de la population neuronale de GnRH, conduisant à une poussée maximale de GnrH qui déclenchera l'apparition de la puberté. Les futures études se concentreront sur la façon dont les mutations nouvellement identifiées du gène Nos1 peuvent affecter la fonction des protéines nNOS et par la suite la libération de NO, ce qui nous permettrait de mieux comprendre les aspects importants de la pathologie CHH.

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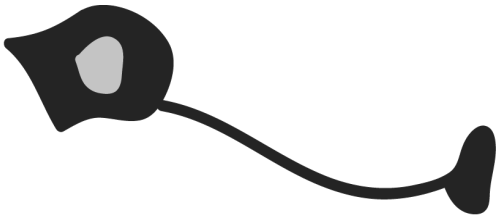
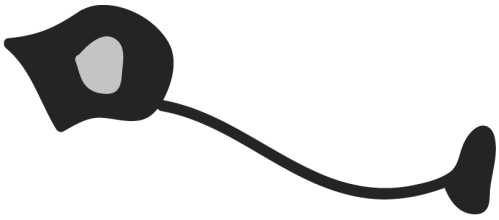
Abstract



The onset of puberty and the regulation of fertility in mammals are governed by a complex neural network, primarily in the hypothalamus, that converges onto gonadotropin-releasing hormone (GnRH)-producing neurons, the master regulators of gonadotropin secretion and postnatal gonadal growth and function. The proper development of the GnRH system, including timely changes in GnRH expression and signaling by this sparse population of a few hundred neurons, is essential for sexual maturation and the normal functioning of the hypothalamic-pituitary-gonadal axis. As the brain develops during embryogenesis, these neurons should move from the olfactory placode into the correct brain location in adequate numbers, and then establish the afferent connections that will allow the pulsatile release of GnRH peptide, and the subsequent release of the gonadotropins (follicle stimulating hormone, i.e FSH and luteinizing hormone, ie. LH). As early as in the 90's NO was presented as a key molecule in the preovulatory GnRH/LH surge, and results from different groups, have suggested the interaction of NOS-containing neurons with the GnRH system, and their involvement in the regulation of reproductive capacity. Even though nitric oxide has now been long recognized as a key player in the central hormonal regulation of ovulation during adulthood, no one has considered the possibility that it could act in an earlier stage as the master regulator of GnRH neurons before puberty, hence participating in the actual maturation of the neuroendocrine axis. The relationship of nNOS-expressing neurons with other important molecules of the hypothalamic axis has been well studied, whilst the molecular identity of this neuronal NOS-expressing population is poorly documented. . To this end, we address the hitherto unaddressed questions concerning 1) the molecular identity of nNOS-expressing neurons in the developing hypothalamus, 2) the putative involvement of the NO molecule in the migration of GnRH neurons and

the proper establishment of their afferent connections in the hypothalamic region and 3) the plausible determinant role of NO signaling in the maturation of the reproductive system. During this study we identified for the first time the cohort of the principal neurotransmitters and important receptors expressed by these cells in the hypothalamic region during development. Additionally, our results reveal for the first time an involvement of NO signaling in the migration of GnRH neurons in the hypothalamus and are in line with the identification of a series of NOS1 mutations in Kallmann syndrome (KS), a rare congenital genetic condition presenting a unique combination of GnRH deficiency, arising from a faulty migration of the neuronal population, and anosmia. Lastly, our study identifies NO as a novel protagonist during postnatal development, in the regulation of the onset of puberty and the acquisition of reproductive competence. Overall, the results of my PhD thesis identify putative new targets causing alterations of developmental programming under pathophysiological gestational environment in mammals in general, and in humans in particular. Here we thus provide new insights into the mechanisms by which the alteration of GnRH neuronal function leads to hypogonadotropic hypogonadism and infertility. We are hopeful that our results will expand our understanding of how the neuroendocrine axis is regulated and will possibly provide opportunities for therapeutic strategies against debilitating conditions.

Abbreviations



5'UTR	5'-untranslated region
A	Androstenedione
aco	Anterior commissure, olfactory limb
AgRP	Agouti-related peptide
Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aob	Accessory olfactory bulb
Arg	Arginine
ARH	Arcuate hypothalamic area
AVPV	Anteroventral periventricular nucleus
BH4	Tetrahydrobiopterin
C/EBPβ	CAAT/enhancer binding protein β
Ca^{+2}	Calcium
CA1	field CA1, Ammon's horn
CamK II	Ca2+/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CDGP	Constitutional delay of growth and puberty
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHH	Congenital hypogonadotropic hypogonadism
CNG	Cyclic-nucleotide gated channels
COM	Commissural nucleus
DG	Dentate gyrus
Di	Diestrus

DMH	Dorsomedial hypothalamic area
Dyn	Dynorphin
E	Estrous
EDRF	Endothelium relaxing factor
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERES	Estrogen response elements
FRET	Fluorescence resonance energy transfer
FSH	Follicle stimulating hormone
g	Gram
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GFP	Green fluoerescent protein
Glu	Glutamate
GnRH	Gonadotropin-releasing hormone
Gpr54	KiSS1-derived peptide receptor
GTP	Guanosine-5'-triphosphate
HCN channels	Hyperpolarisation-cyclic nucleotide activated channels
Hip	Hippocampus
HPG	Hypothalamic-pituitary-gonadal
Hz	Hertz

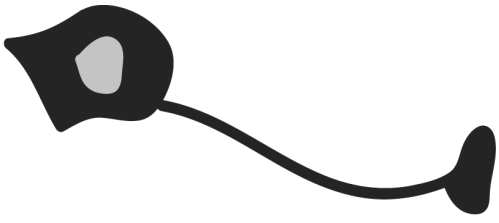
IHH	Idiopathic hypogonadotropic hypogonadism
iNOS	Inducible nitric oxide synthase
Kiss1, Kp	Kisspeptin
KNDy	Kisspeptin, Dynorphyn and Neurokinin B
KS	Kallmann syndrome
LacZ	β-galactosidase
LepR	Leptin receptor
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
lm	stratum lacunosum-moleculare
LPO	Lateral preoptic area
MBH	Mediobasal tuberal region
MePO	Median preoptic nucleus
MH	Medial habenula
miRNA	microRNA
mob	Main olfactory bulb
mol	mole
mRNA	Messenger RNA
MS	Medial septal nucleus
ms	Millisecond
n/fb j	Nasal/forebrain junction

NADPH	Nicotinamide adenine dinucleotide phosphate
nIHH	Normosmic IHH
NKb	Neurokinin B
NMDA	N-methyl-D-aspartate
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NR	Nuclear receptors
ns	Nanosecond
OB	Olfactory bulb
Ob/Ob	Obese (leptin deficient)
oe	Olfactory epithelium
ORF	Opening reading frame
OVLT	Organum vasculosum of the lamina terminalis
OVX	Ovariectomy
p-nNOS	Phosphorylated neuronal nitric oxide synthase
PAPA-NO	1-(3-aminopropyl)-2-hydroxy-3-oxo-1-propyltriazane
PDE	Phosphodiesterase
PKG	Protein kinase G
PMv	Ventral premammilary nucleus
PO	Posterior complex thalamus
POA	Preoptic area

POMC	Proopiomelanocortin
Pro	Proestrus
PSD-95	Postsynaptic density protein-95
PV	Parvalbumin
PVH	Paraventricular hypothalamic nucleus
Pvp	Posterior periventricular nucleus
SCN	Suprachiasmatic nucleus
sGC	Soluble guanylyl cyclase
SN	Substantia nigra
SNc	Substantia nigra, compact part
SNr	Substantia nigra, reticular part
T	Testosterone
TAG-1	Transiently expressed axonal glycoprotein
vfb	Ventral forebrain
vgat	Vesicular GABA transporter
vglut	Vesicular glutamate transporter
VMH	Ventromedial hypothalamic area
VMHvl	Ventrolateral part of the ventromedial hypothalamic nucleus
VMO	Vomeronasal organ
VNN	Vomeronasal nerve
VTA	Ventral tegmental area
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

α-MSH	Anorectic peptide α-melanocyte-stimulating hormone
μm	micrometer

Personal Statement



It was since senior school that I had decided I wanted to become a biology scientist, hence I decided to leave my hometown and follow my studies at the University of Crete (UOC), as it is widely known for its well organized, 4-year undergraduate program in Biomedical Sciences. Additionally, the close collaboration developed between the University of Crete and the pioneering Institute of Molecular Biology and Biotechnology (IMBB) as part of the Foundation for Research and Technology - Hellas (FORTH), provides numerous research opportunities and a unique environment for an undergraduate student to enter the world of science. Studying in the UOC has offered me a strong Biology background since I had to undertake courses from almost all biomedical fields, such as Zoology, Immunology, Developmental and Cellular Biology and Protein Biology. My preoccupation with several Neuroscience related courses, like the "Topics on Cell Cycle and Differentiation", "Neurobiology" and "Developmental Biology", triggered some initial curiosities of mine concerning how the brain works, that grew into a passion which later on convinced me to dedicate my studies in understudying the biological underpinnings of the human mind and cellular development. During the third year of my studies I applied for a Diploma thesis position to Dr. D. Karagogeos' research team; I was accepted as an undergraduate student to the laboratory of Developmental and Functional Biology (IMBB-FORTH), and from September '09 to June '10 I had the chance to be part of the high quality research conducted by Prof. D. Karagogeos' team, in the field of neuronal development and myelination.

The Dual masters in Brain and Mind sciences that University College London offered in collaboration with Université Pierre et Marie Curie in partnership with the Ecole Normale Superieure, was an amazingly well designed program which thrilled me, and

triggered my motivation and inspiration to study and work into research programs. During my master thesis projects I committed myself to the field of Cellular Neuroscience. During my work with Dr. M. Darmon (laboratory of Molecular and Cellular Biology of Serotoninergic Neurotransmission, Unit of Neuropharmacology, Hospital Pitié-Salpêtrière, UPMC) I studied the role of Yif1B protein in intracellular neuronal traffic and its implication in the targeting of 5HT1A receptor in dendrites. This work contributed to a publication where we presented data identifying for the first time, a role of Yif1B in the anterograde transport and the retainment of the tightly organized Golgi structure (Alterio et al., 2015).

Working under the supervision of Prof. J. Garthwaite (laboratory of Neural Signaling, Wolfson Institute for Biomedical Research, UCL), I studied the dynamics of Nitric oxide (NO) /cGMP signaling in NIE-115 neuroblastoma cell line using a novel cGMP-sensitive biosensor, with the aim to identify how the NO signaling pathway operates at physiological concentrations and in real time. The development of the δ-FlincG biosensor is a key advancement in the field; our study evidenced for the first time, the capability of a mitotic neuronal cell line to express the δ-FlincG novel biosensor, setting the ground for further studies into the NO signal transduction pathway under physiological conditions in real time. This study was eventually part of a publication presenting two improved FlincGs successfully expressed in neuroblastoma cells and in primary cultures of hippocampal and dorsal root ganglion cells, supporting their further use for real-time imaging of cGMP dynamics in both neural and non neuronal cells (Bhargava et al., 2013).

My collaboration with Prof J. Garthwaite, and of course my work in the field of NO signaling, inspired me and really triggered my curiosity concerning the implication of NO in several actions of the central and peripheral nervous system. Working with Dr V. Prevot during my PhD, allowed me to apply the knowledge I had acquired in the field of NO signaling to better designate the role of this obscure neurotransmitter in the physiology of reproduction. During the first year of my PhD I worked on the interaction between NO signaling and the leptinergic system, and contributed as a second author in a publication identifying nNOS neurons of the preoptic region as mediators of leptin signals responsible for regulating the hypothalamo-pituitary-gonadal axis (Bellefontaine et al., 2014). During the second year of my PhD I participated in a study identifying a novel role of NO signaling in the transcriptional regulation of the Gnrh promoter, embellishing our knowledge concerning the role of this gas neuromodulator in the central control of reproduction. In my thesis I will present you the main results of my project involving 1) the neuroanatomical and molecular characterization of the nNOS-expressing cells in the region of the brain, and 2) the identification of the role of hypothalamic NO signaling in the maturation of the reproductive axis.

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Konstantina Chachlaki, Samuel Malone, Paolo Giacobini, Erik Hrabovszky, Heike Münzberg, Fabrice Ango, Vincent Prevot (2016) Phenotyping of nNOS Neurons in the Postnatal and Adult Mouse Female Hypothalamus (J. Comp. Neurol. *submitted*).

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Training courses

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Scientific poster presentations

Chachlaki K., Bellefontaine N., Prevot V. (2014) Nitric oxide: the rising star in the neuroendocrine control of reproduction. COST meeting, Berlin 5-8 March, 2014

Chachlaki K., Bellefontaine N., Prevot V. (2014) Nitric oxide: the rising star in the neuroendocrine control of reproduction. Journée des doctorants JPARC, Lille

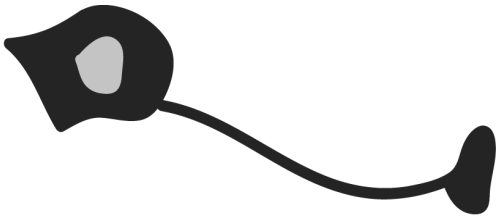
Chachlaki K., Bellefontaine N., Garthwaite J., Prevot V. (2014) Nitric oxide as a "volume transmitter" of neuroendocrine signals in the hypothalamic area. 8th International Congress of Neuroendocrinology, Sydney August 15-20, 2014

Chachlaki K., Bellefontaine N., Prevot V. (2014) Leptin requires NOS activity in the neuroendocrine response to fasting. 27th Conference of European Comparative Endocrinologists, Rennes August 25-29, 2014

Chachlaki K., Bellefontaine N., Garthwaite J., Prevot V. (2015) NO acts as a "volume transmitter" of neuroendocrine signals in the hypothalamic area and is implicated in the sexual maturation of the reproductive axis. 15ème Journée André VERBERT Faculté de Médecine de Lille, 14 septembre, 2015

Chachlaki K., Messina A., Garthwaite J., Prevot V., (2015) Nitric oxide says "NO" to promote and maintain reproductive capacity. BSN/SNE meeting, Lille September 23-25, 2015

Introduction



Chapter I

NO signaling pathway: components, regulation and function in the central nervous system

1.1 Identification of the NO signaling pathway in the region of the brain

Nitric oxide (NO) was discovered as a signaling molecule in the brain more than 20 years ago and still remains one of the most active areas of research. Following the identification of NO as the endothelium relaxing factor (EDRF) in 1987 (Ignarro et al., 1987; Palmer et al., 1987), many aspects of its role not only in the cardiovascular but also in the immune and nervous system, have been elucidated. The stimulation of soluble guanylate cyclase (sGC) activity by exogenous NO in the brain was described in 1977 (Arnold et al., 1977; Miki et al., 1977) however, it was not until 1988 when evidence was found that NO could be formed in the brain in response to activation of the NMDA subtype of glutamate receptor (Garthwaite et al., 1988). This explosive scientific interest concluded in NO being elected "the molecule of the year" in 1992, while only a few years after, in 1998, the significance of NO in life sciences was eventually accentuated, when the Nobel Prize for Physiology and Medicine was awarded to Robert Furchgott, Louis Ignarro and Ferid Murad for their substantial contribution to this field.

NO is produced intracellularly by NO synthase (NOS) through a reaction that converts L-arginine and oxygen into citrulline and NO. NOS are complex proteins found in three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), which differ in their structure, regulation and distribution, although they share 51-57% homology, suggesting a common ancestral NOS gene. Neuronal NOS was the first to be purified and cloned from the brain and is the one considered responsible for the NO production in neurons (Bredt et al., 1991). The spatial and temporal distribution of neural nitric oxide synthase mRNA and protein in

the rodent brain has been the subject of several studies: nNOS is present in most brain regions but staining is more dense in the molecular and granule cell layer of the cerebellar cortex, the olfactory bulb, the striatum, the hypothalamus and the hippocampus. Expression levels are also significant in the superior and inferior colliculi, the superficial layers of the cortex and in some circumventricular organs such as the organum vasculosum of the lamina terminalis and the subfornical organ (Fig. 1) (Bredt and Snyder, 1990; Schmidt et al., 1992; Giuili et al., 1994).

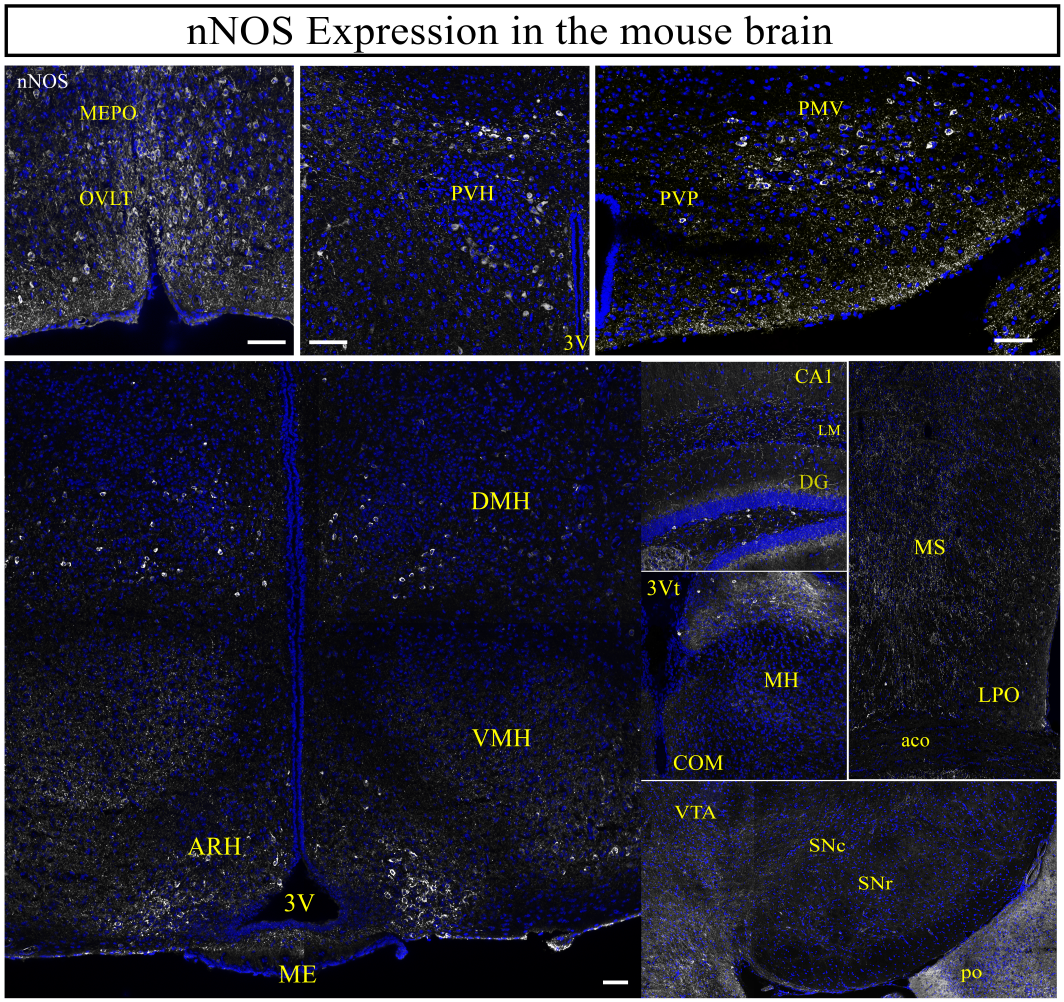


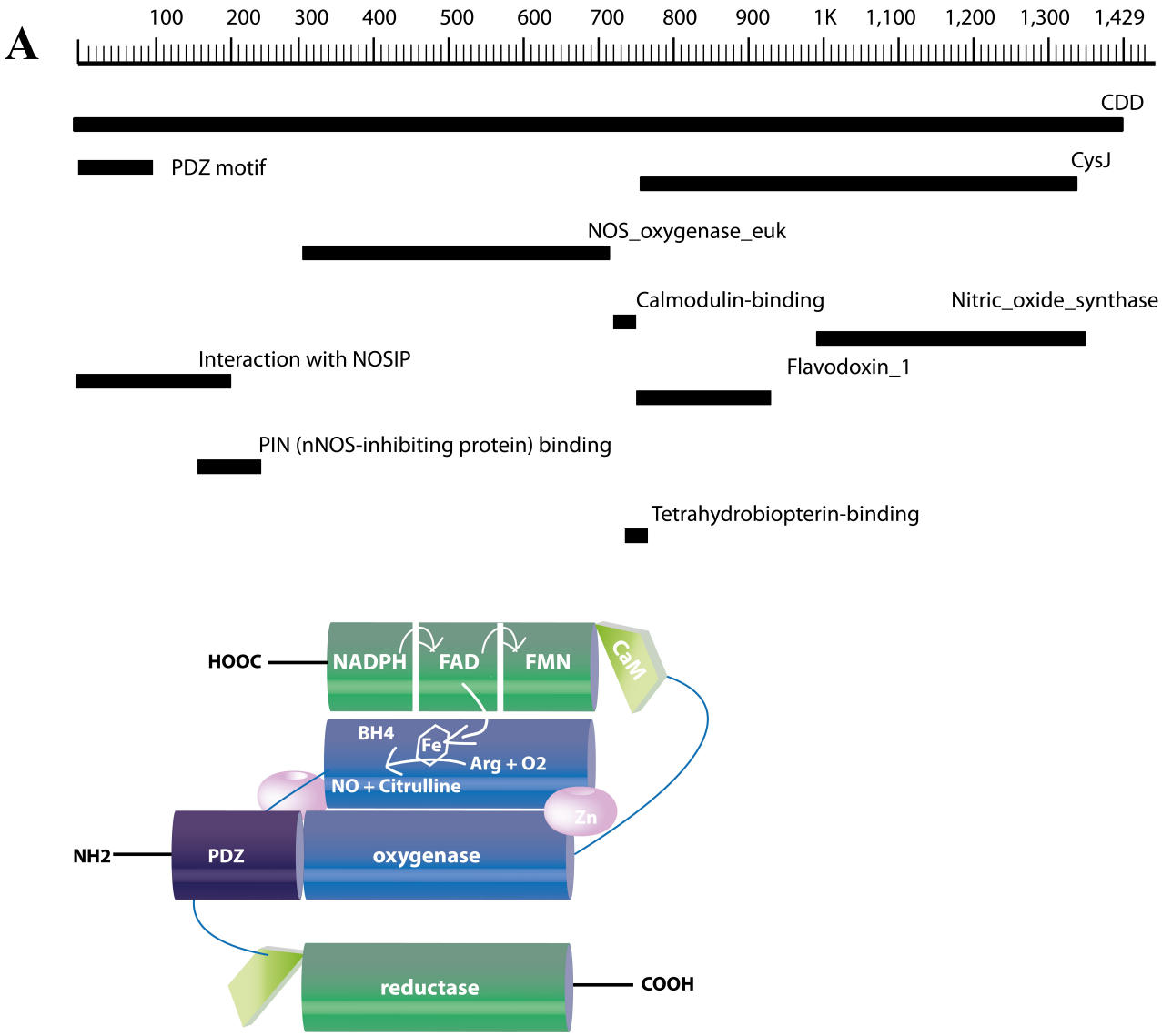
Figure 1 nNOS is expressed in distinct regions of the adult mouse brain. nNOS expression is demonstrated in white, nuclear staining is in blue. MEPO; medial preoptic area, OVLT; organum vasculosum laminae terminalis, PVH; paraventricular nucleus of the hypothalamus, PMV; ventral premammillary nucleus, PVP; posterior periventricular nucleus, ARH; arcuate nucleus, VMH; ventral hypothalamus, DMH; dorsomedial hypothalamus, ME; median eminence, 3V; 3rd ventricle, CA1; field

CA1, Ammon's horn, DG; dentate gyrus, LM; stratum lacunosum-moleculare (field CA1), MH; medial habenula, COM; commissural nucleus, MS; medial septal nucleus, LPO; lateral preoptic area, aco; anterior commissure, VTA; ventral tegmental area, PO; posterior complex thalamus, SNc; substantia nigra compact part, SNr; substantia nigra reticular part.

1.2 Neuronal NOS isoforms and posttranscriptional regulation

Genes playing key roles in biological processes undergo elaborate regulation of their mRNA expression. Mechanisms like alternative splicing create an allelic mRNA diversity essential for protein biodiversity, but also tissue or developmental-stage-specific protein function. Considering the importance of nNOS in various distinct biological functions, as well as its complex expression patterns, it comes as no surprise that mRNA diversity plays a role for the regulation of NO production throughout development and/or in specific cell types in physiological and pathological conditions. Multiple transcripts have been identified for the mouse nNOS gene, and most of the splicing events take place in the 5'-untranslated region (5'UTR), affecting the post-transcriptional regulation of the gene, or alter mRNA localization and stability. This diversity at the 5'terminus of nNOS mRNA transcripts does not affect the encoded protein sequence since these splicing patterns leave the opening reading frame (ORF) unaltered, instead affecting the efficiency of transcription (Wang et al., 1999; Newton et al., 2003). In humans, nNOS –or NOS1- is localized in chromosome 12, with its mouse ortholog being located in chromosome 5. The gene locus spans a region of 10,123 bps, and consists of 29 exons of which 28 are translated to generate the 150 kDa protein, nNOSα, the most commonly occuring isoform in the nervous system (Fig. 2). A unique characteristic of nNOSα is the existence of a 230-residue N-terminal region containing a PDZ domain, which allows nNOSα and the NMDA

receptor to associate with postsynaptic density protein-95 (PSD-95) (Brenman et al., 1996; Tochio et al., 1999). The influx of Ca^{+2} through open NMDA receptor channels and their subsequent binding to calmodulin leads to nNOS becoming catalytically active (Garthwaite et al., 1988). The serine-1412 phosphorylation of the NMDA-receptor-tethered nNOS by the protein kinase Akt (also known as protein kinase B) then leads to a rapid enhancement of NOS activity, with dephosphorylation being dependent on AMPA receptors and L-type Ca^{+2} channels (Adak et al., 2001; Rameau et al., 2007) (Fig. 2). Additionally, posttranslational regulation of nNOS activity can occur via the Ca^{+2}/calmodulin-dependent protein kinase (CamK) II, which co-resides with NMDA receptors and nNOS at synapses; CamKII phosphorylates nNOS on serine-847 and inhibits NO production by 50% by affecting Ca^{+2}/calmodulin binding (Hayashi et al., 1999; Komeima et al., 2000). However, this phosphorylation process appears to be slow in neurons (Rameau et al., 2004), suggesting that CaMKII is less likely to be a dynamic regulator of nNOS activity than a long-term gain controller (reviewed in Garthwaite, 2008).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NOS_oxygenase_euk
Calmodulin-binding
Nitric_oxide_synthase
Interaction with NOSIP
Flavodoxin_1
PIN (nNOS-inhibiting protein) binding
Tetrahydrobiopterin-binding
HOOC
NADPH
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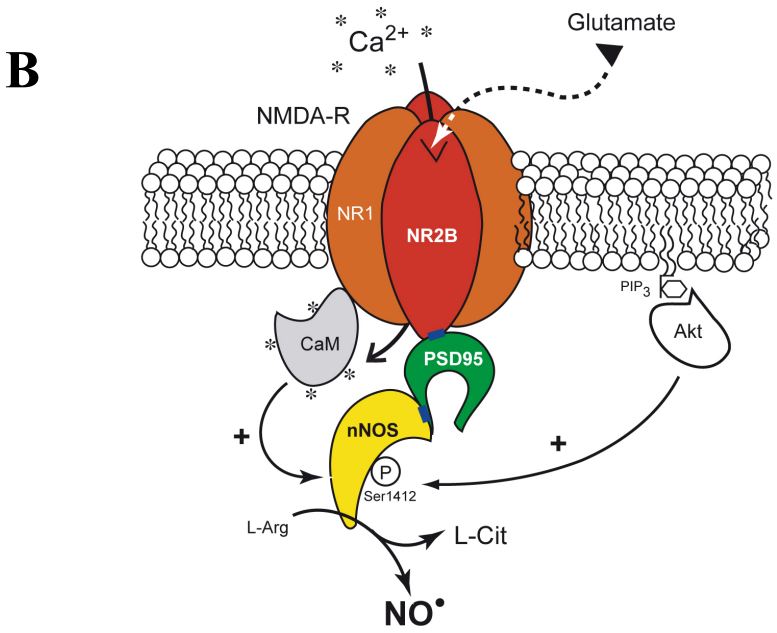
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Figure 2 Schematic figure showing (A) the dimeric conformation of mouse neuronal nitric oxide synthase alpha (nNOS α), and (B) the main activatory mechanism of the nNOS α variant. (A) The primary structure of nNOS α contains 1434 amino acids in its chain and has a predicted molecular weight of 160.8 kDa. Amino acids are numbered in the form of a scale, and regions encoding structural domains and cofactor-binding sites are shown in black bars according to their position on the chain of the nNOS α.. (B) nNOS α translocates from the cytosol to the membrane. Its physical interaction with the NR2B subunit of the NMDA receptors via PDZ domains (blue rectangles) involves the post-synaptic density-95 (PSD-95) scaffolding protein and the assembly of a ternary complex nNOS/PSD95/NMDAR. Binding of glutamate (glu) to the NMDA receptor allows Ca^{+2} entry into the neuron. Ca^{+2} influx activates nNOS α through calmodulin (CaM) binding leading to the production of NO, which is formed enzymatically from L-arginine (L-Arg) in equimolar amounts with L-citrulline (L-Cit). In parallel, membrane-tethered nNOS is also subjected to posttranscriptional modifications (such as phosphorylation via Akt) that modulates its catalytic activity. ARG ; L-arginine, BH4; tetrahydrobiopterin, CAM; calmodulin , PIN; nNOS-inhibiting protein , CysJ; sulfite reductase, alpha subunit (flavoprotein), CDD; conserved domain.

Apart from nNOS α, there are also other well-characterized splicing variants, namely the nNOS β and γ (Fig. 2, 3). nNOS β and γ are translated beginning with different first exons, generating 136 kDa and 125 kDa proteins, respectively. Since they skip exon 2, which contains the PDZ domain essential for anchoring nNOS to the neuronal membranes and for responding to the Ca2+ influx generated by activated NMDA receptors (Garthwaite et al., 1988), the variants are located in the cytosolic fraction. nNOSγ lacks significant catalytic activity (Eliasson et al., 1997; Putzke et al., 2000). However, while nNOSα is suggested to account for 95% of all nNOS catalytic activity under physiological conditions, nNOSβ is also expressed in various regions of the brain, representing ~5% of the total NOS mRNA, and accounts for the majority of nNOS catalytic activity in areas like the cochlear nucleus (Brenman et al., 1997; Eliasson et al., 1997). These variants are not only believed to play key roles in a tissue-specific manner, but also to undergo differential regulation of their expression according to developmental stage. For example, on embryonic day 15, a substantial amount of brain nNOS protein migrates as a 136 kDa band corresponding to nNOSβ

protein while, in the adult rat brain, nNOS migrates as a single band of 160 kDa (Brenman et al., 1997). These isoforms could therefore be responsible for producing basal levels of NO necessary for neuronal development at various stages. However, alternative splicing events also seem to be initiated under pathophysiological conditions. Interestingly, nNOSβ and γ have been reported to be upregulated in the reactive astrocytes of amyotrophic lateral sclerosis patients while nNOSα labeling is absent from these cells (Catania et al., 2001).

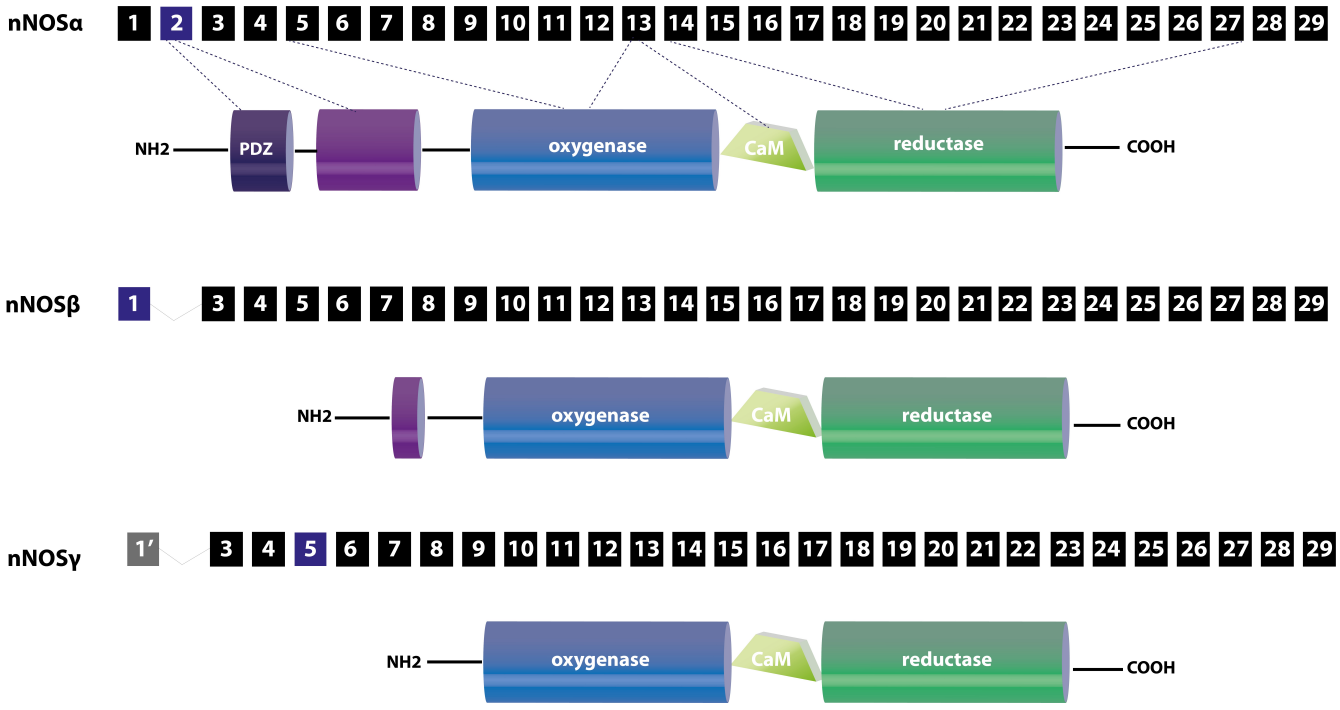


Figure 3 Schematic figures showing the nNOS α, β and γ splicing variants. nNOS α mRNA contains 29 exons and its translation results in a protein product of 160 kDa. The nNOS β and γ variants initiate their translation through different first exons skipping exon 2, which contains the PDZ domain. Note that all the three nNOS isoforms (α, β, γ) contain the oxygenase and the reductase domains (CysJ, flavodoxinJ, ferrodoxin), as well as the calmodulin and tetrahydrobiopterin domains. On the contrary, both nNOS β and γ isoforms, in addition to the lack of the PDZ domain, they are also missing the regions permitting interaction with NOSIP and PIN proteins. The alternative splicing events finally generate a 136 kDa and 125 kDa protein respectively.

1.3 NO activates the formation of cGMP upon stimulation of guanylate cyclase

As mentioned above, in neurons, Ca^{+2} influx through activated NMDA receptors is largely responsible for stimulation of NOS (Garthwaite et al., 1988), although other mechanisms for rising cytoplasmic [Ca^{+2}] such as voltage- gated Ca^{+2}-channels or release of Ca^{+2} from internal stores, can also be involved (Daniel et al., 2016). This rise in intracellular Ca^{+2} results in its binding to calmodulin creating a Ca^{+2}-calmodulin complex which can directly activate the constitutive isoforms of NOS (Toda and Okamura, 2003) and lead to NO production as long as Ca^{+2} levels are high. Once NO is released, it diffuses rapidly and stimulates the formation of cGMP by soluble guanylate cyclase (Garthwaite, 2016) (Fig. 4). NO-activated GC consists of a heme group acting as the ligand binding site and a transduction domain. Even though its heme group is of the type used in haemoglobulin for binding O2, NO- activated GC exhibits high preference for NO, initiating NO signaling in the presence of >10000 fold excess of O2 (Garthwaite, 2008). Upon binding of NO to the heme, a conformational change occurs due to the displacement of the histidine group, leading to the activation of the enzyme (Ignarro et al., 1991), which can now convert GTP to cGMP. In contrast to a more ubiquitous expression of NO, NO-dependent cGMP synthesis occurs mainly in neurons and astrocytes (Tanaka et al., 1997; Baltrons, María Antonia and García, 1999; Baltrons et al., 2008). cGMP can exert its functions through binding to three main targets: phosphodiesterases (PDEs), ion channels, i.e. cyclic-nucleotide gated channels (CNG) and hyperpolarisation-cyclic nucleotide activated channels (HCN), and cGMP dependent protein kinases (i.e PKGI and PKGII) which phosphorylate various substrates (Fig. 4).

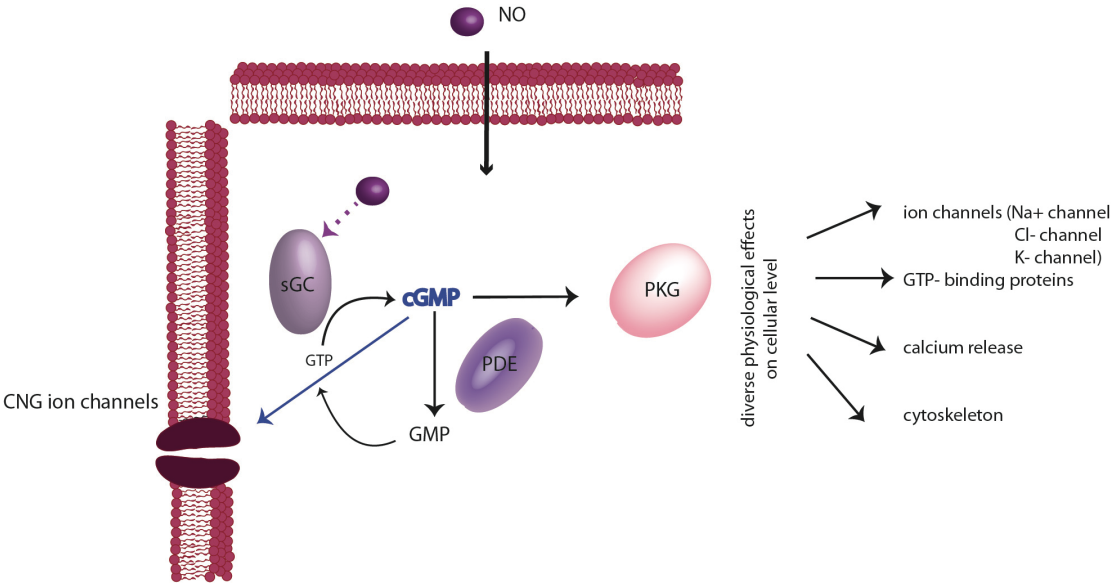


Figure 4 Schematic representation of the NO/cGMP signaling pathway. NO neurotransmitter is highly soluble and membrane permeable. Upon binding to NO-sensitive guanylyl cyclase, nitric oxide induces a conformational change resulting in the activation of the enzyme and the subsequent conversion of GTP to cGMP. The newly produced cGMP can interact with various intracellular proteins, including the cGMP- binding PDEs, cGMP-gated cation channel (CNG), and the protein kinase G (PKG), triggering thus the phosphorylation of many different substrates. The NO/cGMP pathway is therefore implicated in multiple distinct physiological processes such as cytoskeletal organization, Ca^{+2} release from intracellular stores, differentiation/proliferation of vascular smooth muscle etc.

Soluble guanylyl cyclase is a heterodimeric heme-protein containing two homologous subunits, α (82 kDa) and β (70 kDa), both of which need to be expressed in order for sGC protein to be active. Four different cDNA clones encoding sGCα (sGCα1 and sGCα2) and sGCβ (sGCβ1 and sGCβ2) have been identified, of which, sGCα1 and β1 are the most commonly found. More specifically, in situ hybridization studies have demonstrated that α1 and β1 are abundantly expressed in almost all tissues, and most of the regions of the brain (Matsuoka et al., 1992; Furuyama et al., 1993; Burgunder and Cheung, 1994). α2 is found in fewer tissues, but is highly expressed in the lung, colon, heart, spleen, uterus and placenta, with the highest amount of both protein and mRNA levels being found in the brain (Budworth et al., 1999; Gibb and Garthwaite,

2001; Mergia et al., 2003). β2 messenger on the other hand, is primarily found in the kidney, though low levels of β2 mRNA expression have been also identified in the brain region (i.e. cerebellum) (Yuen et al., 1990; Gibb and Garthwaite, 2001). There are contradicting studies concerning the activity of the β2- containing heterodimers; β2 has been claimed to be inactive when coexpressed with either α1 or α2, whilst showing cyclase activity as a homodimer (Koglin et al., 2001). In another study however, exposure of COS-7 cells expressing all the subunit combinations, to clamped NO concentrations, allowed the measurement of the activity of the NO-bound receptor and suggested that β2-containing heterodimers do possess NO-stimulated GC activity (with a maximal response comparable to the one of the homodimer). Although their maximal activities are relatively low, they still correspond to the physiological range that would be needed to activate cGMP-dependent protein kinases in normal cells (Gibbs, 2003). Bearing in mind that sGC is active only as an heterodimer, the distribution of the individual subunits does not necessarily correlate with the distribution of the active protein (Nedvetsky et al., 2002). The identification of a plasma membrane-localized GC, a protein that was believed to be merely soluble, only reinforced the proposed complexity of this molecule. As mentioned above, α1β1 heterodimer is the most commonly found isoform; being highly expressed within the cytoplasm it is presumably responsible for the production of a non-compartmentalized, source of cGMP (Bellingham and Evans, 2007). The α2β1 isoform is found in brain in comparable amounts to the α1β1 heterodimer and shares similar kinetics and enzymatic properties with the prevalent isoform, whilst only presents 30% identity in the N-terminal regulatory region (Mergia et al., 2003). This heterogeneity results in its association with PSD-95 and/or other synaptic scaffold proteins via a PDZ domain - a domain missing from the α1β1

isoform - and thus its localization at the plasma membrane. This unique characteristic of α2β1 GC protein was firstly demonstrated in 2001; Russwurm and colleagues showed that this heterodimer is not only expressed in the brain, but is also binding PSD-95 (and the related MAGUK proteins PSD 93, SAP 97, SAP 102) interacting preferentially with its third PDZ domain (Russwurm et al., 2001). As it is known, both NMDAR and NO synthase interact with the first two PDZ domains of PSD 95 (Cui et al., 2007). Thus, an interaction of the α2β1 GC heterodimer with PSD 95 could, potentially, result in the formation of a trimeric complex composed of NMDAR, NOS protein and α2β1 GC. Considering that nNOS is being specifically activated by Ca^{+2} influx through the NMDAR following the NOS/NMDAR complex formation, the addition of sGC to the above complex would create an increased proximity between the NO-producer (NOS) and the NO-sensor (sGC). The proximity of the above NO pathway key players would create a local NO signal transduction within a cellular compartment, avoiding an overall increase in the NO concentration (Russwurm et al., 2001) . Interestingly, Bellingham and Evans demonstrated that the physiological role of α2β1 isoform is to produce a localized membrane pool of cGMP which, firstly, is resistant to degradation by the phosphodiesterases (since they are merely found located in the cytoplasm) and secondly, due to its physical proximity to elements of the NO pathway, results to a more efficient transduction of the NO responses (Bellingham and Evans, 2007). Therefore, it is possible that these two isoforms have distinct roles in the brain region: the α1β1 isoform, being found in the cytoplasm, may play the role of the main NO transducer, being responsible for the production of a pool of cGMP that will freely diffuse to act on downstream targets, whereas the α2β1 isoform may have a more specific role in the local transduction of

the NO signal produced by the neuronal NOS to membrane targets, restraining the effects elsewhere within a cell.

1.4 Phosphodiesterases: a key member of the NO signaling pathway

The effects of cGMP occur through three main groups of cellular targets: PKGs, phosphodiesterases (PDEs) and cGMP-gated cation channels (Azevedo et al., 2013). From the above three targets however, PDE enzymes are of exquisite importance since they are responsible for catalyzing the hydrolysis of the 3'cyclic phosphate bond of cAMP and/or cGMP, thus modulating the duration and intensity of their intracellular response to the cGMP and cAMP downstream targets (for review see Azevedo et al., 2013). Among the members of the 11 known PDE families, 1,2,3,5,6,9,10 and 11 are those with the highest affinity for cGMP as the substrate, while PDE 5,6 and 9 are referred as cGMP-specific phosphodiesterases, with the rest of them (PDE 4,7 and 8) being considered as cAMP-specific (Garthwaite, 2008). The selectivity towards either cAMP or cGMP is suggested to be determined by what is known as "glutamine switch". According to the glutamine switch hypothesis a glutamine residue in the binding pocket of the PDEs is constrained by neighboring residues to a position favoring selectivity for either cAMP or cGMP; in PDEs with the ability to hydrolyze both cyclic nucleotides, this glutamine residue can rotate freely (Zhang et al., 2004). Studies thus far have described 21 different gene products coming from the above 11 PDE families (Azevedo et al., 2013). Nevertheless, theoretically, alternative splicing events taking place in these genes, would raise that number to more than 100 different mRNA products, the majority of which could be

translated into functional proteins (for review see Beavo, 1995; Omori and Kotera, 2007).

Apart from the above "genetic" variation found in the expression of the PDEs, post translational modifications and distinct biochemical mechanisms (i.e. phosphorylation, protein-protein interactions, allosteric binding of cAMP/cGMP etc.) come only to reinforce the complexity of these proteins. Now imagine that the above mechanisms of genetic and protein variations, can take place for several distinct PDE families in one single cell (Palmer and Maurice, 2000; Castro et al., 2006). In other words, many different modificatory interactions can be simultaneously expressed in a single cell type. Since each PDE protein has distinct hydrolysis rates and enzyme kinetics, their combinatory expression can further increase the complexity of their action, which is no other than the determination of the second messenger amounts available to act in downstream targets. The activity of cGMP-PDEs behaves in ways described by the Michaelis-Menten kinetics allowing to extensively study the changes of PDE activity according to GC activity and time. PDE5 is one of the most well reported members of the phosphodiesterase family; the general idea postulated is that the activity of PDE5 in its resting phase is very low, but becomes higher as the cellular cGMP concentration rises (Kass et al., 2007). cGMP is able to accumulate to significant concentrations before reversibly binding to the allosteric site of PDE5, resulting to the activation of the enzyme and its subsequent hydrolysis by the PDEs, whose activity may recover to its basal state in a quite slow mode (Garthwaite, 2010).

Though the complexity and importance of PDEs has lead to extensive research in the field of phosphodiesterase activity, very little is yet known about the subcellular localization of PDE families, or how they influence cyclic nucleotide signaling. PDE

families are distinctly expressed throughout the central and peripheral nervous system, in all tissues and various different cell types. In the brain region, several PDEs have been found expressed, though their specific localization and expression patterns remain relatively vague (Lakics et al., 2010). In the hypothalamic region recent evidence suggests a distinct role for PDE3B in transducing the action of leptin (Sahu and Sahu, 2015) without excluding the implication of more PDE families. This dual-specific PDE is shown to be expressed in proopiomelanocortin and neuropeptide Y neurons (Sahu et al., 2011), thus potentially implicating the hypothalamic PDE3B pathway in the regulation of food intake and energy homeostasis (Azevedo et al., 2013). This phosphodiesterase is of special interest because even though it has similar affinities for both cGMP and cAMP, the hydrolysis rate for cGMP is approximately 10 times lower than the one for cAMP (Azevedo et al., 2013). This could be an example of crosstalk between the cAMP and cGMP signaling pathways: by binding PDE3B, cGMP can block the hydrolysis of cAMP, increasing thus its concentration and prolonging its action.

1.5 cGMP- dependent protein kinases: PKG

PKG activation is probably the most widespread mechanism employed by cGMP to mediate its downstream signaling effects (for review see (Francis et al., 2010); PKG is a serine/threonine-specific protein kinase that phosphorylates a number of biologically important downstream molecules, like small G proteins (RhoA) or even members of the cGMP pathway, like PDEs and ion channels (for review see (Francis et al., 2010) having ultimately various downstream effects, such as alterations in activity, function, subcellular localization, cell differentiation and proliferation. PKG

is encoded by two genes in mammalian species, namely Prkg1 and Prkg2, resulting in the production of PKG type I (PKG-I) and type II (PKG-II) respectively (Hofmann, 2005). PKGI and II are homodimeric proteins, sharing the same structure, which involves a N-terminal domain, a regulatory domain that contains two non-identical cGMP-binding sites and a kinase domain, but having differing cellular and tissue distributions PKGI is predominantly localized in the cytoplasm, and is subjected to alternative splicing events resulting in the production of two variants, PKGIα and PKGIβ, which demonstrate different affinities for cGMP and different tissue distributions, with neurons expressing either the PKG-Iα or the PKG-Iβ isoform, platelets predominantly Iβ, and smooth muscle cells expressing both variants (Ruth et al., 1997; Pfeifer et al., 1999). PKGII on the other hand is myristoylated in its N-terminal resulting in its location at the plasma membrane of several brain cell nuclei, but not in cardiac and vascular myocytes (Fig. 4)

Chapter II

The role of the hypothalamic-pituitary-gonadal axis (HPG) in reproduction

2.1 The hypothalamus, a model system in which to study hormone- and activity-dependent brain plasticity

ὁρμῶν (hormôn),"to set in motion, to excite, to stimulate". This Greek word has been used for the past century to describe the chemical messengers used as means of communication between different organs in an animal. When Ernest Starling introduced the term hormone, back in 1905, practically nothing was known about the nature or the action of these messengers (Starling I-IV, 1905). We have come a long way since then; over the years it has become clear that hormones do play a catalytic role in the regulation of the organism's development, homeostasis, behavior, reproductive capacity and the overall modulation of physiological circuits. The hypothalamus is the single most important integrator of vegetative and endocrine regulation of the body. Accordingly, it controls diverse processes, such as cardiovascular function, sleep, metabolism, stress, thermoregulation, water and electrolyte balance, and reproduction. Within the hypothalamus specialized neuronal populations sense moment-to-moment changes in circulating levels of hormones and nutrients, regulating thus accordingly the neuroendocrine function (Elmquist et al., 2005). One of these hypothalamic neuronal populations are the GnRH neurons; GnRH cells, acting as integrators of metabolic and reproductive signals coming from both the central and the peripheral nervous system, are considered the main orchestrators of the physiological events initiated from the hypothalamic region. The proper development of GnRH neurons, GnRH expression, and GnRH signaling is essential for sexual maturation and the normal functioning of the HPG axis in mammals (Cattanach et al., 1977; Mason et al., 1986; Schwanzel-Fukuda et al., 1989).

2.2 GnRH neurons as master regulators of the reproductive function

In spite of their massive role on the orchestration of neuroendocrine signals, GnRH neurons are an extremely small population of cells, counting only 1,000−3,000 neurons across mammalian species. Morphologically, they have a simple bipolar or unipolar morphology, with long extended axons possessing spines like dendrites, that actively conduct action potentials (Herde et al., 2013). The number of spines increases at puberty (Campbell et al., 2005) enabling them to receive a larger number of synaptic inputs that will allow them a synchronous firing activity. The result of this synchronized GnRH firing is the secretion of the GnRH hormone; GnRH is a decapeptide produced by the GnRH nerve terminals located in the region of the median eminence. From there, GnRH will enter the portal vasculature and will travel to the pituitary where it will stimulate the synthesis and secretion of the gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropin hormones, as proposed by their name, will act on the gonads (i.e. the testes and ovaries) to control the production of sperm and eggs, and the secretion of sex steroids. Interestingly, GnRH neurons differentially regulate the secretion of LH and FSH; a high GnRH pulse frequency induces the LH secretion, while at low pulse frequency FSH release is promoted (reviewed by Constantin, 2011). The proper regulation of the GnRH secretory frequencies is indispensable for the correct release of LH and FSH, and subsequently the establishment of a functional operating system of the gonads. The fact that loss of GnRH signaling induces hypogonadism in a mouse model, while exogenous GnRH administration can rescue the phenotype provides definitive evidence for the role of GnRH in the regulation of FSH release (Charlton, 2004). Prior to puberty, GnRH neurons are receiving a plethora of inhibitory inputs that allow them to secrete very low concentrations of the GnRH

peptide in the pituitary. The increase in the GnRH pulsatile release from the hypothalamus later on during development, is induced by a combination of excitatory and inhibitory inputs, and is indispensable for the initiation of puberty (for review see Terasawa and Fernandez, 2001; Ojeda and Skinner, 2006; Plant and Witchel, 2006). Overall, the function of the GnRH system being of vital importance for the propagation of the species depends upon a finely balanced set of mechanisms. In fact, dysregulated GnRH pulse, GnRH deficiency or aberrant migration often associated with pathophysiological reproductive syndromes (discussed in Chapter IV).

2.2.1 GnRH neuronal migration during embryonic development

GnRH neurons are a unique population of neuroendocrine cells since, in contrast to other paraventricular neurosecretory neurons, they do not ascend from the neuroepithelium of the third ventricle. In fact, two independent studies more than twenty years ago discovered that GnRH neurons originate from stem cells in the olfactory placode, from where they migrate to enter the CNS during embryogenesis (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Around embryonic day (E) 11.5 GnRH cells initiate their journey towards the brain, leaving the epithelium of the medial olfactory pit. Their migratory process does not fall in the category of either the radial or the tangential movement; GnRH neurons migrate in close association with growing fibers of the vomeronasal nerve, following a series of guidance cues that involve among others, extracellular molecules, receptor tyrosine kinases, chemokines and a series of neurotransmitters (for review see Wray, 2010). GnRH neurons will eventually start penetrating the rostral forebrain around embryonic day (E) 12.5, continuing their migration ventrally, along a branch of the vomeronasal nerve, to

reach the basal forebrain (Tobet and Schwarting, 2006; Wierman et al., 2011). At postnatal day (P) 0, GnRH neurons have finally reached hypothalamus, their final destination, and have already established their afferent connections, extending their neuronal terminals towards the median eminence and the pituitary portal system (Fig. 5).

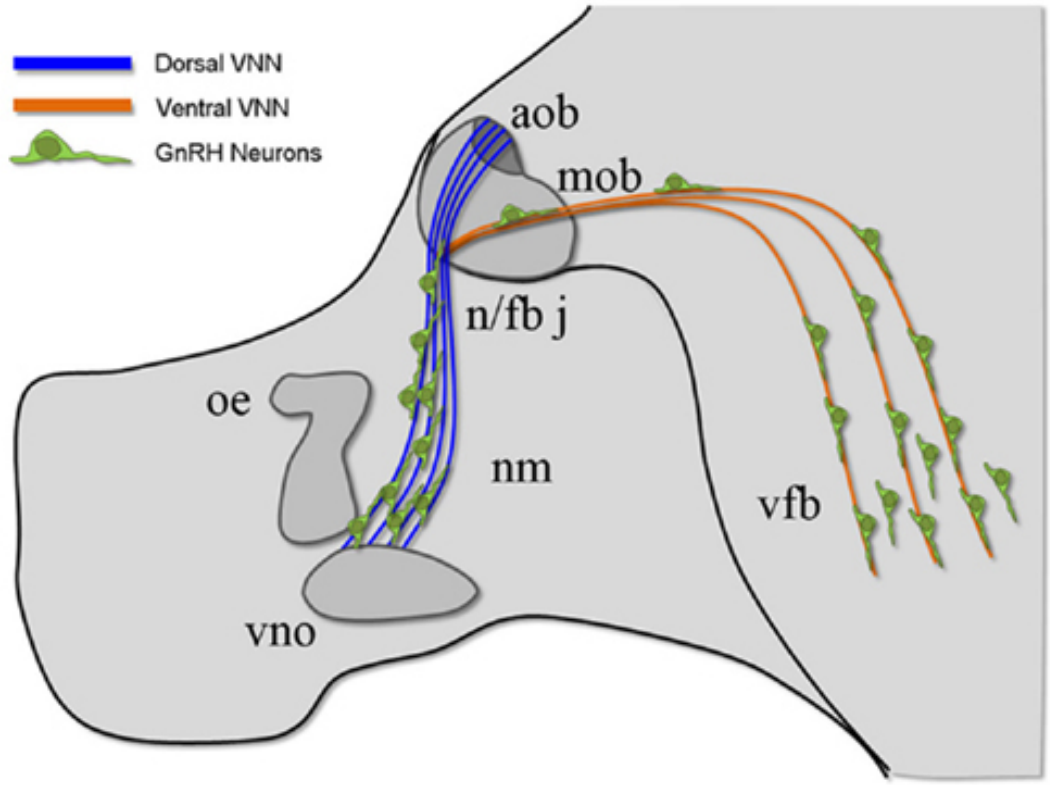


Figure 5 Schematic figure demonstrating the GnRH neuronal migratory route during embryonic developmental stage (E) 14.5. GnRH neurons are born outside of the brain, in the olfactory placode. They migrate in association with vomeronasal/ terminal nerve fibers, and with the help of distinct guidance cues, from the vomeronasal organ (vno) across the nasal/forebrain junction (n/fb j) into the forebrain. VNN, vomeronasal nerve; vno, vomeronasal organ; nm, nasal mesenchyme; oe, olfactory epithelium; n/fb j, nasal/forebrain junction; aob, accessory olfactory bulb; mob, main olfactory bulb; vfb, ventral forebrain. (from Messina and Giacobini, 2013)**.**

2.2.2 Postnatal maturation of the GnRH axis

At birth, GnRH neuronal somas are found abundantly distributed in the preoptic region, sending their axons into the pericapillary space of the median eminence. During postnatal development, the morphological characteristics, neurosecretory pattern, and overall neuronal activity of the GnRH neurons, are subjected to a series of complex maturational events that will affect the sexual maturation of the axis. In females, postnatal development of the reproductive axis consists of four stages: the neonatal period, extending from postnatal day (P) 0 to P7; the infantile period, P8-P21; the juvenile period, P22-P30 (approximately); and the peripubertal period which ends with the initiation of puberty (Ojeda et al., 1980).

During the neonatal period primordial follicles develop into secondary follicles that are present at postnatal day (P) 7. FSH and LH are improbable to exert direct actions on primordial follicles since functional gonadotropin receptors have not developed in them during this initial stage (O'Shaughnessy et al., 1997). Additionally, P7 GnRH-deficient hypogonadal (hpg) mice, demonstrate normal follicular development (Rajah et al., 1992). Hence, the process of folliculogenesis requires neither GnRH, nor LH/FSH to develop at this stage.

Later on, during the infantile period a series of neuroendocrine events stimulate the development of this pool of secondary follicles, into preantral and antral follicles (McGee et al., 1997), that are now responsive to FSH (O'Shaughnessy et al., 1997). In fact, the infantile FSH surge, occurring at P12, signifies the first activation of the GnRH system, known as mini-puberty, and is required for the proper maturation of the preantral follicles and their incoming into the antral stage (McGee et al., 1997; McGee and Hsueh, 2000). LH levels, though demonstrating small bursts of secretion,

still remain low while they do not participate in the ovarian development during this stage (Dohler and Wuttke, 1974; Zhang et al., 2001).

By the end of the infantile period, and the beginning of the juvenile stage, FSH levels decrease, with LH levels remaining low. However, towards the end of the juvenile period, sensitivity to the preovulatory levels of estradiol significantly increases to reach a peak around postnatal day (P) 30 (Andrews et al., 1981). The increase in estradiol levels exerts a stimulatory effect on LH secretion, which acquires a particularly accelerated pulsatile pattern (Kimura and Kawakami, 1982). This stimulatory effect of estradiol on LH release is believed to involve GnRH activation which is evident by the increased GnRH pulse frequency occurring during the juvenile period (Sarkar and Fink, 1979).

The arrival in the peripubertal stage is characterized by a diurnal pattern of LH release, which demonstrates increased amplitude (but unaltered frequency) in the afternoon. This activation of the gonadotropin secretion is a centrally driven gonad-independent phenomenon (Urbanski and Ojeda, 1990). At puberty, the ovary acquires the ability to secrete high level of estrogens for at least 24h, triggering the first GnRH/LH surge. This rising estrogen concentration enables the release of the GnRH peptide, subsequently resulting in the proestrus LH surge (Aiyer et al., 1974; Sarkar and Fink, 1979).

2.2.2.1 Establishment of GnRH afferent neuronal connections- the creation of a GnRH network

Even though GnRH neuronal projections towards the median eminence are already in place at birth they have not yet formed a mature neuronal network; GnRH neuronal projections are suggested to continue to develop during the first 3 weeks of postnatal development (Buchanan and Yellon, 1993; Heywood and Yellon, 1997). As a matter of fact, the axons of neurons residing in the ARH have been evidenced to reach GnRH neuronal soma during the second week of postnatal development, while they keep expanding throughout the infantile period (Bouret et al., 2004a; Caron et al., 2012a). The maturation of the ARH neuronal projections coincides with the development of the activation of the GnRH axis, as demonstrated by the concomitant increase of the FSH circulating levels at postnatal day (P) 12 (Stiff et al., 1974; Prevot et al., 2003). Thus, GnRH afferent connections received from the ARH axons are believed to contribute to the control of pulsatile GnRH release (Navarro et al., 2009; Ruka et al., 2013). Furthermore, recent studies have raised the intriguing possibility that these ARH fibers could mediate the maturation of the gonadal negative feedback action on the GnRH system, and thus further support the detrimental role of these afferent connections to the proper development of the GnRH neurosecretion (Caron et al., 2012a). ARH neuronal projections are not the only synaptic inputs established postanatally; regions like the ventromedial (VMH) and dorsomedial (DMH) hypothalamic areas also finalize their projectory pattern after birth (Bouret et al., 2004a). On the other side of the spectrum, neurons in the region of anteroventral periventricular nucleus (AVPV), known to mediate gonadal positive feedback, have already established their projections to the GnRH neuronal soma in the preoptic area

(POA) at birth (Polston and Simerly, 2006). Notably, synaptogenesis events are still taking place during the infantile period, formatting the synaptic density and the number of dendritic spines of the GnRH neuronal population (Campbell et al., 2005; Cottrell et al., 2006). All the above events certainly play an important role in the overall maturation of the GnRH neurons and their neuronal network, setting in motion the establishment of a mature and fully functional GnRH system that will orchestrate the initiation of puberty.

2.2.3 The main components of the GnRH neuronal circuit

GnRH neurons are undoubtedly governing the central control of reproduction. Their own activity however is controlled by a dynamic network of numerous internal and external signals, involving various different neurotransmitters and neuropeptides (Todman et al., 2005).

2.2.3.1 Glutamate and GABA neurotransmitter action

Glutamatergic inputs to the preoptic region are gradually increasing during the juvenile and peripubertal stages, to reach a maximum after the initiation of puberty (Goroll et al., 1994). The source of hypothalamic glutamate is not fully identified, whilst there are several neuronal populations located in the mediobasal hypothalamus (ARH, VMH and DMH), as well as in the preoptic region, shown to express glutamatergic transporters, and thus emerging as possible sources of glutamate (Eyigor and Jennes, 1996; Ottem et al., 2002; Lin et al., 2003; Donato et al., 2011). GnRH cells themselves have been also known to express NMDA or non-NMDA

glutamatergic receptors (Eyigor and Jennes, 1996; Gore et al., 1996), while several studies have attributed to these neurons a glutamatergic identity (Hrabovszky et al., 2004; Dumalska et al., 2008). In parallel to the gradual increase in the glutamatergic content during postnatal development described above, the ability of glutamate receptor agonists to stimulate GnRH release from hypothalamic explants also progressively increases during the infantile and juvenile stages (Bourguignon et al., 1990). Glutamatergic signaling is believed to be involved in the regulation of GnRH mRNA expression, neuronal activity and consequently the release of GnRH peptide (Urbanski and Ojeda, 1990; Petersen et al., 1991; Spergel et al., 1999), while studies have also highlighted the involvement of glutamate in the initiation of puberty and the acquisition of reproductive capacity, even though results about the actual pubertal regulation by glutamate are controversial (Hrabovszky and Liposits, 2008; Zuure et al., 2013; Cheong et al., 2015).

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the CNS and is synthesized via a reaction that involves the decarboxylation of glutamate to GABA and CO2. The enzyme responsible for the synthesis of GABA is the glutamic acid decarboxylase (GAD), and is present in two isoforms, GAD-67, and GAD-65, encoded by GAD1 and GAD2 genes respectively (Erlander et al., 2016). Both of these isoforms are found to be expressed in the brain, where GABA is a major neurotransmitter (Decavel and Van den Pol, 1990). Similarly to the increase in the glutamate content throughout the first stages of development, the levels of GAD mRNA are seen to increase in the preoptic region in parallel to the maturation of the axis (Davis et al., 1996; Roth et al., 1998). GnRH neurons express both GABAA and GABAB receptors (Sim et al., 2000; Pape et al., 2001; Temple and Wray, 2005;

Zhang et al., 2009), but also receive synaptic inputs from GABA neurons of the POA that co-express the estrogen receptor, and are thus considered as mediators of estrogen signals to GnRH neurons (Flügge et al., 1986; Curran‐Rauhut and Petersen, 2002). The functional role of GABA action on GnRH neurons has been the subject of a number of studies which attributed this inhibitory neurotransmitter many different roles, including, regulation of GnRH migration, mediation of puberty, control of estradiol positive and negative feedback and LH pulsatile release (Han et al., 2002; Christian and Moenter, 2007; Casoni et al., 2012). Overall, even if glutamategic and GABAergic systems have phenomenally an opposite function (since glutamate is considered an excitatory neurotransmitter, while GABA is mainly inhibitory), the two systems are tightly intertwined (van den Pol et al., 1998). The most evident example of such an interaction is the fact that glutamate itself is the natural precursor for GABA synthesis; hence any alterations in the availability of glutamate could directly result in altered GABA levels.

2.2.3.2 The action of Kisspeptin

Kisspeptin's significant role in the regulation of the onset of puberty and reproductive function has been broadly demonstrated (Clarkson and Herbison, 2009a; Kirilov et al., 2013; Tena-Sempere, 2013; Putteeraj et al., 2016). Hypothalamic Kiss mRNA is shown to increase at the time of sexual maturation, alongside with an increased responsiveness of GnRH to kisspeptin (Navarro et al., 2004; Han et al., 2005; Clarkson and Herbison, 2009b). Kisspeptin neuronal populations have been identified in the ARH and the AVPV. From the region of the AVPV, kisspeptin neurons send their axons to the MPOA to regulate the GnRH surge (Gu and Simerly, 1997). On the

other hand, the population of the ARH is proposed to modulate GnRH pulsatility by contacting GnRH axons at the level of the ME (Popolow et al., 1981). These two populations are not only sexually dimorphic, but are also differentially regulated by sex steroids via the action of ER- α (Gottsch et al., 2004; Smith et al., 2005; Clarkson et al., 2012). More specifically, it has been suggested that kisspeptin neurons residing in the AVPV participate in the gonadal positive-feedback mechanism (Smith et al., 2005; Wintermantel et al., 2006; Herbison, 2008), while the ARH kisspeptin population is responsible for mediating the estrogen- negative feedback (Cheng et al., 2010; d'Anglemont de Tassigny and Colledge, 2010; Alçin et al., 2013; Helena et al., 2015). Furthermore, in the ARH, kisspeptin neurons are shown to coexpress neurokinin B and dynorphin, which is not the case for the AVPV residing cells (Navarro et al., 2011; Hrabovszky et al., 2012). This unique characteristic of the ARH kisspeptin population further supports their involvement in the mediation of the ovarian estrogen negative feedback, since these KNDy (Kisspeptin, Dynorphyn and Neurokinin B) neurons, are believed to be the major targets for steroid hormones (for review see Lehman et al., 2010).

2.2.3.3 POMC-peptide and NPY/AgRP action

Besides kisspeptin neurons, other hypothalamic populations are also implicated in the regulation of reproductive capacity, and more specifically in the modulation of GnRH neuronal activity. One of these neuronal populations are the anorexigenic (appetite suppressing) proopiomelanocortin (POMC)-expressing neurons. POMC neurons are responsible for the release of two gene products: the anorectic peptide α-melanocyte-stimulating hormone (α-MSH) and the endogenous opioid β-endorphin. POMC

neurons of the ARH coexpress ER-α and have been shown to send their projections to the GnRH neurons of the POA (Simonian et al., 1999; de Souza et al., 2011). Interestingly, loss of ER-α expression from POMC neurons results to a failure of estrogens to mediate their negative feedback action (Xu et al., 2011). Additionally, GnRH neurons have been demonstrated to express receptors for β-endorphin (Lagrange et al., 1995), while α-MSH has been shown to promote the firing of GnRH in the POA (Roa and Herbison, 2012). Overall, hypothalamic POMC neurons, creating a link between central control of energy homeostasis and reproduction seem to have an important part in postnatal sexual maturation.

Similarly to the POMC population, NPY/AgRP neurons also morphologically and functionally interact with the GnRH neuronal network. NPY/ AgRP population is located in the ARH, where they secrete the agouti-related peptide (AgRP) in order to increase appetite and decrease metabolism and energy expenditure (Bäckberg et al., 2004). They as well, just like POMC cells, extend estrogen-responsive afferents to the POA, forming a neuronal network with the GnRH neurons (Simonian et al., 1999). Apart from mediating estrogenic signals, NPY/AgRP neurons can also modulate GnRH neuronal activity; NPY peptide has been shown to both excite and inhibit GnRH neurons depending on the NPY receptor activated (Roa and Herbison, 2012). The direct effect of NPY on the GnRH neuronal activity is also complemented by the ability of NPY signaling to affect pubertal onset and fertility (Minami et al., 1990). To sum up, both NPY/Agrp neurons as well as POMC neurons, by integrating a wide variety of metabolic signals that access the hypothalamus from the periphery (e.g. leptin) can coordinate both metabolic and reproductive functions in the hypothalamic region.

2.2.3.4 The action of Leptin

Leptin was identified in 1994 and has been since recognized as an important regulator of not only the metabolic, but also of the reproductive axis (Halaas et al., 1995). Leptin is an adipocyte-derived hormone secreted from the periphery in accordance to the size of body fat stores, acting thus to adjust food intake and energy homeostasis (Ahima et al., 1996; Casanueva and Dieguez, 1999). The action of leptin starts to get apparent after the first weeks of postnatal life; during the early juvenile period leptin levels serve as an indicator of the amounts of energy being stored, being positively correlated to adipocity (Frederich et al., 1995; Mistry et al., 1999). As mentioned above however, leptin is not only affecting the metabolic axis, but is also playing an active role in reproductive control. Several studies have identified this anorexigenic hormone as an important regulator of pubertal onset and adult fertility (Ahima et al., 1997; Cheung et al., 1997; Fernandez-Fernandez et al., 2006). Loss-of-function mutation of leptin or of its receptor, induces leptin deficiency, resulting in not only a metabolic phenotype of obesity, but also a failure to undergo puberty, and consequently, to infertility (Barash et al., 1996; Cohen et al., 2001). Reintroducing leptin receptor expression or applying exogenous leptin rescues the above phenotype (Chehab et al., 1996; de Luca et al., 2005), whereas transgenic overexpression of leptin has been shown to result in precocious puberty (Yura et al., 2000). The effects of leptin on the regulation of the GnRH neuronal system are indirect, through cells afferent to these neurons, since GnRH themselves are not expressing the leptin receptor (Quennell et al., 2009). Trying to identify the mediator of the leptin signals is not an easy task; in fact neither POMC and NPY/AgRP neurons of the ARH (which are known to transmit leptin signals in the hypothalamus) (van de Wall et al., 2008;

Williams and Elmquist, 2012; Balthasar et al., 2016), nor kisspeptin cells (Louis et al., 2011; True et al., 2011) seem to mediate the central action of leptin on reproduction, pointing towards a mechanism residing outside of the ARH (Ratra and Elias, 2014). One possible site of action seems to be the region of the ventral premammilary nucleus (PMv) (Leshan et al., 2009; Donato et al., 2011). Although the region of the ARH is possibly not mediating the central actions of leptin on reproduction, leptin receptor is expressed in the region implying a role for leptin in this hypothalamic area. Indeed, leptin is believed to act directly on LepR-expressing neurons of the ARH during the infantile period in order to promote the establishment of ARH projections to the POA (Bouret et al., 2004a; b).

2.3 Role of estrogens in the reproductive axis

Steroid hormones, or sex steroids, produced by the gonads (ovaries or testes) and the brain, play a catalytic role during mammalian brain development since they are not only required for the masculinization (and feminization) of neuronal circuits, but they are also responsible for promoting the normal realization of most neuroendocrine functions throughout an animals lifespan (Simerly, 1998; Brock et al., 2011). Sex steroids diffuse out of the cell and can modulate the protein expression of their neuronal targets either directly, by binding to steroid receptors located in the nucleus or the cytoplasm of their targets, or indirectly, by modulating the neurotransmitter release of afferent neurons, that will in turn, regulate protein expression of the target cells (Shivers et al., 1983). Steroid receptors belong to the family of nuclear receptors (NR) and are thus able to translocate to the nucleus and recruit general transcription factors, as well as the RNA polymerase II, upon binding of the hormone. As all of the

NRs, the transcriptional regulation of their target genes depends on the recruitment of the necessary coactivators/ corepressors, or more generally, coregulators. These "master regulators" enable the initialization of a highly elaborate sequence of events that exceeds beyond the actual transcription, orchestrating diverse signaling pathways to allow a long lasting implementation of coordinated programs pre- and post-developmentally (Jung et al., 2005). The significance of the role of both NRs and their coregulators is also demonstrated by their implication in different pathologies, like endocrine syndromes related to metabolism or reproduction (e.g. type 2 diabetes, obesity) and even some forms of cancer (Francis et al., 2003), rendering both excellent targets for therapeutic strategies.

In females, the primary sex hormone is estrogen secreted from the ovary, and more specifically the theca interna cells. Steroidal estrogen biosynthesis occurs using either androstenedione (A) or testosterone (T) as immediate precursors, and is principally stimulated by the action of FSH and LH on the ovary (Richards and Hedin, 1988). Like other steroid hormones, estrogen enters passively through the phospholipid membranes of the cell to bind and activate its two principal receptors, the estrogen receptor alpha (ER-α) and beta (ER-β). The formation of the estrogen/ estrogen receptor complex is known to activate the transcription of target genes by binding to specific estrogen response elements (EREs) (McDevitt et al., 2008). The fact that estrogen can physically enter all cells renders the presence of the ER a determinant of the downstream action of estrogen. In females, they promote the maturation of secondary sexual characteristic and the regulation of the estrous cycle, while in males they are important for the maturation of the sperm cells and the masculinization of the brain (for review see McCarthy, 2008).

2.3.1 Development of ovarian gonadal positive and negative feedback into the GnRH system

Estrogen can exert a dual effect on the hypothalamic GnRH secretion; the rising plasma estradiol levels during the follicular phase elicit a positive feedback action upon the GnRH network to stimulate the gonadotropin surge. During the later stages of the cycle however, estradiol is responsible for suppressing GnRH secretion by exerting its negative feedback effect on the hypothalamic axis (for review see Radovick et al., 2012). LH/GnRH surge is only induced when adequate, significantly high, estrogen levels arrive in the hypothalamus during the preovulatory period (Christian and Moenter, 2010). The sufficiently high estradiol signals during that stages are responsible for the activation of the GnRH neurons and thus the secretion of the GnRH peptide, initiating a chain reaction that will eventually trigger ovulation (Moenter et al., 1991; Pau et al., 1993). This stimulatory action of estrogen consists the "positive gonadal feedback", that occurs in the afternoon of proestrus in rodents and during the late follicular phase in humans (Herbison, 1998; Simerly, 2002). Anatomically, the positive feedback takes place in the preoptic area, in the regions of anteroventral AVPV and suprachiasmatic nucleus (SCN) (for review see Radovick et al., 2012). A series of studies suggest that ER-α-expressing neurons of the above region provide direct inputs onto GnRH neurons to mediate the estrogenic positive feedback action (for review see Herbison, 2008). One of these neuronal populations able to integrate positive estrogenic signals are believed to be the kisspeptin neurons (Adachi et al., 2007; Clarkson et al., 2008; Mayer et al., 2010).

During the estrous cycle estrogen plasma levels inhibit GnRH secretion and maintain GnRH neuronal pulse in low frequency levels (Levine, 1997; Herbison, 1998). The mechanisms through which estradiol promotes its "negative feedback" action to retain

GnRH pulse frequency and amplitude are rather unclear, but are proposed to involve ER-α- containing neurons of the arcuate nucleus (AHN), as well as the ER-β-expressing neurons (Wersinger et al., 1999; Kwakowsky et al., 2012).

Chapter III

The function of the NO signaling pathway in the region of the hypothalamus

Within the hypothalamus, neuroendocrine neurons have proven to be fascinating model systems for studying hormone- and activity-dependent plasticity both during development (Simerly, 2002; Tobet et al., 2009; Israel et al., 2014) and adulthood (Hatton, 1997; Bourque, 2008; Theodosis et al., 2008; Prevot et al., 2010; Giacobini et al., 2014; Bains et al., 2015; Grattan, 2015; Parkash et al., 2015). Neuronal NO has been shown to be produced in several hypothalamic areas and nuclei, including the preoptic region, the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, the ventromedial nucleus of the hypothalamus and the ventral premammillary nucleus (Fig. 1), where it regulates, among other functions, temperature, fluid balance, stress, glucose homeostasis and reproduction (Srisawat et al., 2000; Calabrese et al., 2007; Donato et al., 2010a; Bellefontaine et al., 2011; Branco et al., 2011; Rivier, 2014; Routh et al., 2014). Because hypogonadotropic hypogonadism is the most obvious phenotype of the mice lacking nNOS catalytic activity, hypothalamic GnRH neurons, which support sexual maturation and adult fertility, and the afferent neural network that modulates their activity (see for review (Herbison, 2015; Prevot, 2015) represent an intriguing model system in which to decipher the ways NO operates at the cellular and network level to coordinate the activity of a key physiological system controlled by the brain.

3.1 Neuronal NO neurons as an integral part of the hypothalamic GnRH neuronal network controlling ovarian cyclicity

In rodents, GnRH neurons are diffusely distributed and are particularly abundant in the preoptic region of the ventral forebrain. Their axons target the pericapillary space of the median eminence, where they release their neurohormone into the fenestrated

blood vessels of the pituitary portal system for delivery to the anterior pituitary. In the pituitary, GnRH elicits the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate gametogenesis and gonadal hormone secretion and thus support reproductive function (Fig. 6). As the final common target for the central control of reproduction, the activity of GnRH neurons is regulated by a complex array of excitatory and inhibitory transsynaptic and non-synaptic inputs (Christian and Moenter, 2010; Herbison, 2015; Prevot, 2015) and is subject to the direct and indirect influence of a plethora of internal and external signals (Evans and Anderson, 2012; Bellefontaine and Elias, 2014; Hazlerigg and Simonneaux, 2015; Tena-Sempere, 2015). While the neuronal network synaptically connected to GnRH neurons has been much explored using advanced genetic strategies in mice (Wintermantel et al., 2006; Boehm et al., 2016; Yoon et al., 2016), atypical neuronal and non-neuronal inputs such as the ones postulated to be involved in volume transmission are more difficult to comprehend (Agnati et al., 1995; Gundersen et al., 2015).

NO has long been recognized as a player in the central hormonal regulation of ovulation. In fact, back in the early 90's there was a burst of research activity, coming from different groups, focusing on the role of NO in the regulation of GnRH secretion both *in vitro* and *in vivo*, and suggesting NO as a key molecule in the preovulatory GnRH/LH surge (Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994). *In vitro*, the application of a NO donor was shown to stimulate GnRH release via the sGC-cGMP pathway in a dose-dependent manner, while *in vivo*, the intraventricular injection of a NOS inhibitor blunted pulsatile LH release, providing strong evidence that NO is essential for the generation of LH release (Moretto et al., 1993; Rettori et al., 1993). Specifically in the preoptic region, the chronic inhibition of NOS activity

was shown to disrupt rat estrous cyclicity (d'Anglemont de Tassigny et al., 2007a). All the above results, which present evidence for the marked disruption of LH pulsatile release upon NO inhibition, together with studies showing that nNOS mutations cause infertility in mice (Gyurko et al., 2002a) have laid the groundwork for the notion that NOS-containing neurons interact with the GnRH system and are thus involved in the central control of reproductive function.

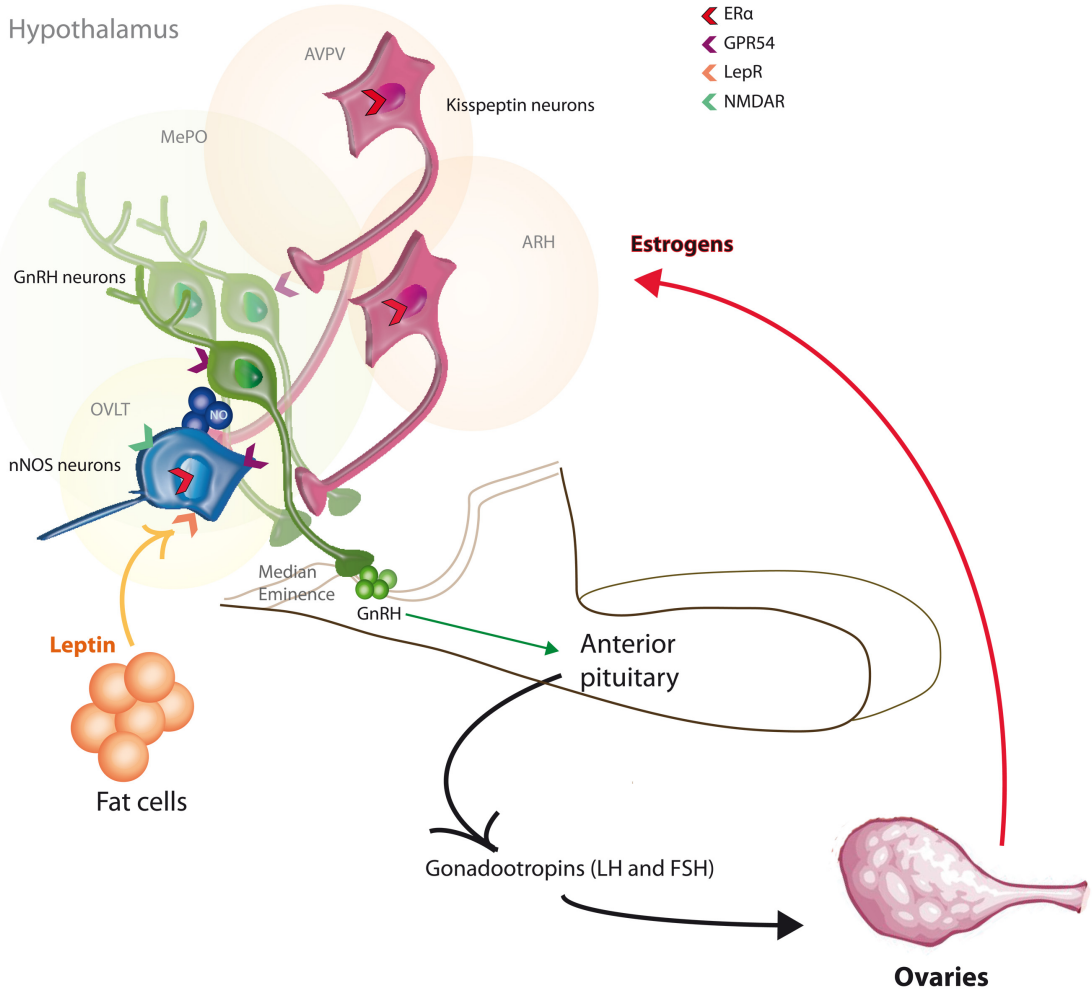


Figure 6 Schematic representation of the hypothalamo-pituitary-gonadal (HPG) axis. The cell bodies of the neuroendocrine neurons releasing gonadotropin releasing hormone (GnRH) are scattered throughout the preoptic region of the hypothalamus at the ventral surface of the brain, sending their neuronal processes to the median eminence where GnRH is released into the pituitary portal blood vessels for delivery to the anterior pituitary, where it stimulates the secretion of the gonadotropins: luteinizing hormone (LH) and folliculostimulating hormone (FSH). Blood-borne LH and FSH act on

target cells in the gonads (here the ovaries) to direct production of gametes, as well as the secretion of steroid hormones (e.g., estrogens in females). Within the brain, gonadal steroids influence GnRH secretion via neuroendocrine feedback loops. In contrast to the direct action of kisspeptin on GnRH cells, leptin is believed to indirectly regulate LH release since GnRH neurons do not express the LepR. The population of nNOS cells residing in the OVLT region, where the NOS synthesizing neurons surround GnRH cell bodies, tuning their neuronal excitability, probably plays this intermediary role. nNOS cells of the OVLT/ MePO that express the leptin receptor (LepR) act as mediators of leptin signals to control leptin-induced GnRH release, and thus tune the LH/ FSH surge. In parallel, this same population of nNOS cells also expresses, in addition to estrogen receptor alpha (ER-α) and NMDAR, the kisspeptin receptor (GPR54) and is surrounded by kisspeptin fibers, suggesting that nNOS cells can directly receive kisspeptin signals. ARH, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleus; MePO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis.

3.1.1 NO-synthesizing neurons anatomically and functionally interact with GnRH neuronal cell bodies in the preoptic region

The vast majority of hypothalamic NOS-expressing neuronal cells have been identified in the rostral part of the preoptic area, in the regions of median preoptic nucleus (MePO) and the organum vasculosum of the lamina terminalis (OVLT). nNOS-containing cells in the preoptic region are found in the vicinity of GnRH-containing perikarya, mainly surrounding GnRH cells which do not themselves express nNOS in the MePO (Herbison et al., 1996; Clasadonte et al., 2008), and around GnRH dendrites in the OVLT (Hanchate et al., 2012; Herde et al., 2013). The fact that nNOS neurons surround GnRH neuronal cell bodies and dendrites, creates an anatomical relationship between the two neuronal populations, which is complemented by the capacity of neuronal NO to modulate GnRH electrical activity (for review see Bellefontaine et al., 2011). Specifically, patch-clamp recordings from GnRH-GFP mouse brain slices reveal that NO can directly tune GnRH neuronal excitability (Clasadonte et al., 2008); the perfusion of hypothalamic slices with the

NO precursor L-arginine results in the nNOS-dependent postsynaptic inhibition of GnRH neuron firing. This reduction in the spontaneous discharge of GnRH cells was shown to require the sGC-cGMP signaling pathway and to involve potassium conductance (Clasadonte et al., 2008). Although this may vary across physiological conditions, the overall impact of NO on GnRH neurons is suggested to be beneficial, by facilitating synchronous activity among GnRH neurons that would otherwise act independently, and thus maximizing the release of this neurohormone into the blood as detailed further on.

3.1.2 Estrogen regulation of NO signaling in hypothalamic neurons

Estrogens have profound effects on brain structure and physiology (Maggi et al., 2004; Simerly, 2005; Israel et al., 2014; Hara et al., 2015; Nugent et al., 2015; Chachlaki and Prévot, 2016; Mahfouz et al., 2016). The manner in which sex steroids, and particularly estrogen, influence the reproductive circuitry has been the subject of many studies (Sarkar and Fink, 1980; Wintermantel et al., 2006; Christian and Moenter, 2010). It is widely known that natural fluctuations of estrogen levels across the estrous cycle lead to subsequent cyclic changes in protein levels and protein-protein interactions, that are important for the physiological processes taking place in the hypothalamic-pituitary axis (Woolley and McEwen, 1992; Weiner et al., 1994; Akama and McEwen, 2003). nNOS neurons located in the preoptic region of the hypothalamus express estrogen receptor α (ER-α) (Scordalakes et al., 2002; Sato et al., 2005), which is essential for the estrogen positive feedback that elicits the GnRH/LH surge triggering ovulation (Fig. 6) (Wintermantel et al., 2006).

Both amperometric measurements and microscopic visualization of NOS catalytic activity using immunofluorescence for L-citrulline (which is formed stoichiometrically with NO) reveal that, concomitantly with the preovulatory increase in plasma estrogen levels, NO levels in the preoptic region are significantly increased in proestrus when compared to diestrus (the stage of the estrous cycle when estrogen levels are low) (d'Anglemont de Tassigny et al., 2007a). Proteomic studies show that while nNOS expression does not vary across the estrous cycle, its activity is markedly modulated by its alternate coupling and uncoupling with NMDAR depending on the circulating levels of estrogens (d'Anglemont de Tassigny et al., 2007a, 2009; Parkash et al., 2010). As mentioned before, the physical interaction of nNOS with NMDAR at the plasma membrane promotes the coupling of Ca^{+2} influx to nNOS activity; the Ca^{+2} increase through NMDAR then results in its binding to calmodulin, creating a Ca^{+2}-calmodulin complex that can directly activate nNOS, inducing the production of NO (Garthwaite et al., 1988; Toda and Okamura, 2003). This differential association of nNOS with NMDA receptors during the estrous cycle involves the scaffolding protein PSD-95 (d'Anglemont de Tassigny et al., 2007a, 2009) and requires estrogen receptor activity (d'Anglemont de Tassigny et al., 2009) (Fig. 7). Importantly, the fact that selective NMDAR blockers have been shown to inhibit estradiol-induced NO release demonstrates that estrogen actually promotes the coupling of glutamatergic flux and NO production in preoptic neurons (d'Anglemont de Tassigny et al., 2009). The evidence that NO-producing neurons in the hypothalamus could be targets of glutamate, one of the main activators of the central reproductive axis (Urbanski and Ojeda, 1990; d'Anglemont de Tassigny et al., 2010), comes from neuroanatomical studies showing that virtually all-preoptic nNOS neurons express NMDA receptors (Bhat et al., 1995; d'Anglemont de Tassigny et al., 2007a). Besides, most NMDA

receptor-expressing neurons of the preoptic region have also been shown to contain ER-α (Chakraborty et al., 2003).

When nNOS is physically associated with NMDAR at the plasma membrane, the enzyme is susceptible to an activity-dependent Akt-mediated phosphorylation at Ser1412, which increases its sensitivity to Ca^{+2}-calmodulin and thus its activity (Rameau et al., 2007). Accordingly, the phosphorylation of nNOS at Ser1412 in the preoptic region is maximal on the afternoon of proestrus (when circulating estrogen levels are at their highest), and this phosphorylation-activated nNOS isoform has been shown to physically interact with the PSD-95/NMDAR complex at the plasma membrane (Parkash et al., 2010) (Fig. 7).

Altogether these findings strongly suggest that estrogens directly act on nNOS neurons of the preoptic region, coupling NO production to glutamate flux, which is well known for its positive feedback effect on GnRH secretion by the hypothalamus (Urbanski and Ojeda, 1990; Brann and Mahesh, 1991; Cheong et al., 2015). In addition, recent findings demonstrating that glutamatergic neurons also play a prominent role in mediating the negative estradiol feedback loop (Cheong et al., 2015), together with data showing that endogenous NO release imposes a tonic brake on LH secretion during the estrogen-mediated negative feedback phase (Hanchate et al., 2012), suggest that this glutamate/NO coupling could in fact be at work at all stages of the estrous cycle and be used by the neuroendocrine brain to convey both the inhibitory and the excitatory influences of estrogens on the reproductive axis. The resulting production of NO may then act on GnRH neuron cell bodies to synchronize their activity and adjust their firing behavior in a meaningful manner to enable the surge of GnRH during the estrogen positive feedback (Hanchate et al., 2012; Cheong et al., 2015) and to participate in restraining the activity of the GnRH system during

the negative feedback phase (Hanchate et al., 2012; Cheong et al., 2015). This "switch" between the activatory and the inhibitory influence of NO on the GnRH neuronal network could likely be linked to the level of activity of nNOS, as elaborated further down.

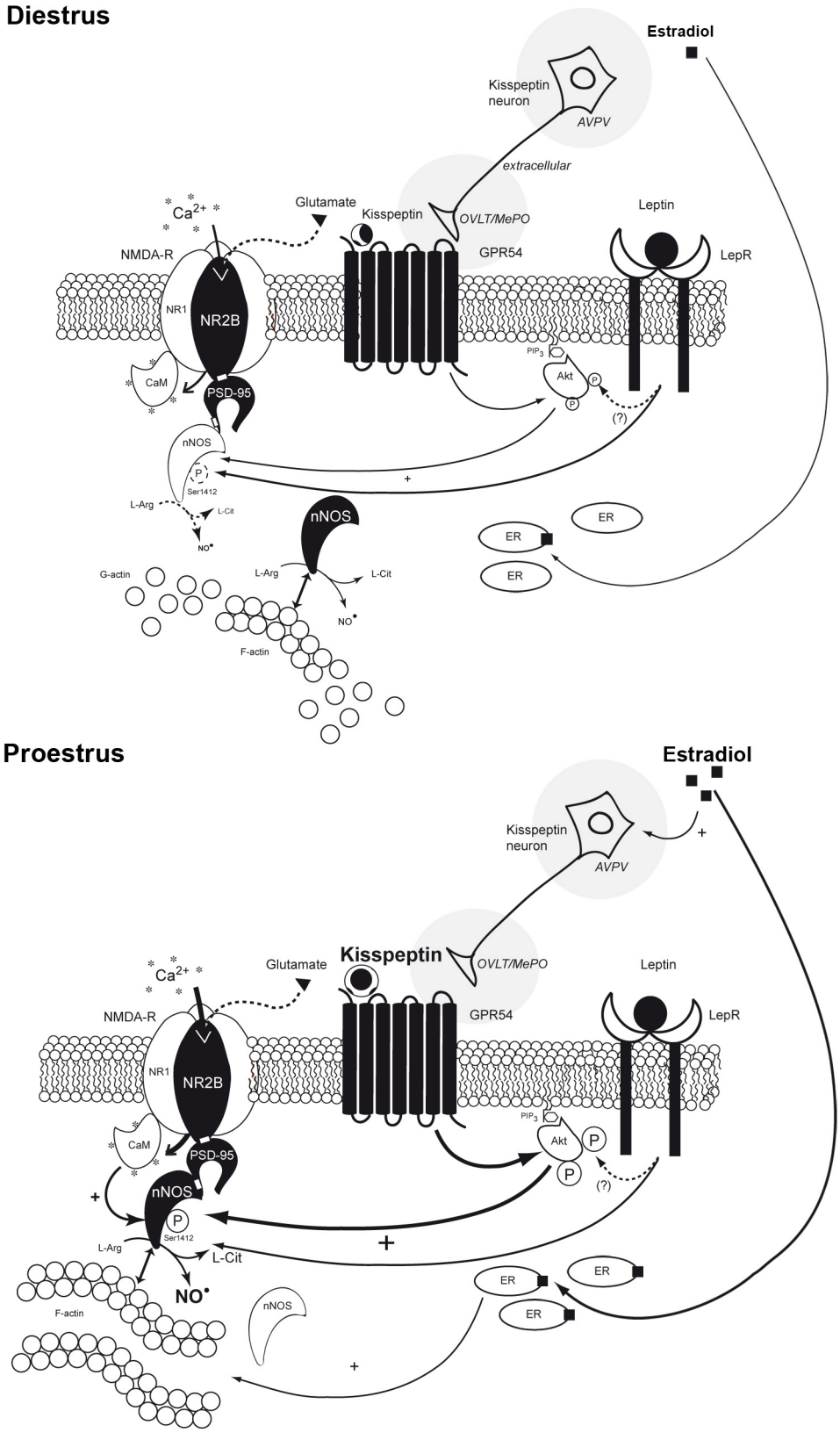


Figure 7 Schematic representations of the estradiol-, kisspeptin- and leptin- mediated changes in protein-protein interaction and phosphorylation during the ovarian cycle, proposed to be involved in the nNOS activity regulation in the hypothalamic preoptic area. Ca^{+2} influx through activated NMDA receptors is largely responsible for stimulation of nNOS via the Ca^{+2}/calmodulin

complex. The physical interaction of nNOS with NMDAR implicates the PSD-95 scaffolding protein, and the assembly of a ternary complex. Phosphorylation of nNOS is responsible for the modulation of its catalytic activity, with the addition of a phosphate group in its Ser1412 residue being responsible for the activation of the protein. Phosphorylation of the nNOS protein at Ser1412 has been shown to be regulated across the ovarian cycle, reaching its maximal levels on the day of proestrus. How is this phosphorylation being promoted in the hypothalamic area? There are three different pathways that are proposed to lead to the phosphorylation of the nNOS target: 1) Estradiol, requiring the estrogen receptor (ER) activity, is able to promote the association of nNOS with the NMDAR/PSD95 complex at the plasma membrane that is required for the activation of nNOS by phosphorylation. Natural fluctuations of estrogen levels across the ovarian cycle, i.e., low in diestrus and high in proestrus, regulate the amount of the nNOS/PSD95/NMDAR ternary complex that is formed. 2) Leptin, acting on the leptin receptor, sustains a basal level of phosphorylation of the nNOS protein physically associated with the NMDAR, possibly via the PI3K/AKT pathway. 3) On proestrus, the estradiol-activated kisspeptin/GPR54 signaling is suggested to promote the phosphorylation of nNOS protein on its Ser1412 activation site via the PI3K/AKT pathway, nNOS activity being required for the onset of the preovulatory GnRH/LH surge. L-Arg, L-arginine; L-Citr, L-citrullin; CaM, calcium calmodulin; GPR54, kispeptin receptor; LepR, leptin receptor. AVPV, anteroventral periventricular nucleus; MePO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis.

3.2 The emerging role of NO signaling in the action of kisspeptin neurons

GnRH neurons are undeniably an obligatory component of the central reproductive axis (Cattanach et al., 1977; Mason et al., 1986; Berghard et al., 2012). During the last decade, however, kisspeptin has been identified as playing an important role as an upstream regulator of the GnRH system (Oakley et al., 2008; Pinilla et al., 2012). In the rodent hypothalamus, kisspeptin neurons are distributed in the arcuate nucleus of the hypothalamus (ARH) in the tuberal region, and in the anteroventral periventricular nucleus (AVPV) in the preoptic region. ARH kisspeptin neurons that project towards the median eminence contact GnRH neuronal processes found there (Ciofi et al., 2006). Even though these ARH kisspeptin neurons also send projections to the preoptic region (Clarkson and Herbison, 2006; Caron et al., 2012a), GnRH cell bodies

in the POA appear to mainly receive afferents from AVPV kisspeptin neurons (Yip et al., 2015) which have been shown to be electrically active (Liu et al., 2011; Qiu et al., 2016). While AVPV kisspeptin neurons are considered to be key components of the surge mechanism, ARH kisspeptin neurons are the ones thought to be involved in the control of pulsatile GnRH release (Herbison, 2016). In fact, recent in vivo and in vitro optogenetic studies have shown that the selective activation of ARH kisspeptin neurons promotes GnRH neuronal activity (Han et al., 2015; Qiu et al., 2016) at stimulation frequencies similar to the ones required to elicit LH release (Campos and Herbison, 2014). Mutant studies have revealed the critical role of kisspeptin signaling in reproduction (Clarke and Dhillo, 2016). Humans and mice bearing a mutation in the kisspeptin gene or its receptor (Gpr54/Kiss1R), do not undergo puberty, are infertile and exhibit markedly reduced gonadal size due to low levels of LH and FSH hormones (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007b; Topaloglu et al., 2012). Moreover, kisspeptin and kisspeptin analogues have been shown to be potent post-synaptic activators of GnRH neuronal activity (Han et al., 2005; Decourt et al., 2016). Advanced genetic approaches have shown that the selective reintroduction of a functional Gpr54 in the GnRH neurons in an *Gpr54*-null background, is sufficient to rescue puberty onset and reproductive capacity in mice (Kirilov et al., 2013). Although unequivocally important, these direct effects of kisspeptin on GnRH neurons are, according to recent studies, insufficient to fully maintain the functionality of the gonadotropic axis throughout the reproductive life cycle (León et al., 2016). The compensation mechanism taking place during development in situations of early deficiency in kisspeptin neurons, giving rise to fertile individuals (Mayer and Boehm, 2011), reinforces the notion that kisspeptin signaling outside GnRH cells also plays a role in the central control of fertility.

Detailed maps of Gpr54-expressing cells, using modified mice that have *LacZ* knocked into the *Gpr54* locus, show Gpr54 promoter activity in the GnRH neuronal population alongside expression in some non-GnRH cells (Herbison et al., 2010). Interestingly, some of these GPR54 expressing cells were found in hypothalamic regions where GnRH and nNOS neurons are co-distributed, and where they are known to receive kisspeptin fibers likely arising from the AVPV (Yip et al., 2015), such as the MePO and the OVLT (Hanchate et al., 2012). X-gal histochemistry revealed that, in addition to GnRH neurons themselves, discrete populations of nNOS neurons in the MePO/OVLT, abundantly surrounded by kisspeptin fibers, also have an active Gpr54 promoter, which is not the case for the nNOS cell population of the medial septum, where the density of kisspeptin fibers is much lower (Hanchate et al., 2012). The notion that nNOS neurons express functional Gpr54 kisspeptin receptors is further supported by experiments showing that exogenous kisspeptin treatment promotes Gpr54-dependent Ser-1412 phosphorylation of nNOS in diestrus mice, at levels comparable to those seen in proestrus and in ovariectomized mice treaded with estradiol. Intriguingly, a null mutation of Gpr54 prevents estradiol effects on nNOS phosphorylation in ovariectomized mice, suggesting that estrogens require kisspeptin-Gpr54 signaling to influence nNOS activity during their positive feedback phase (Fig. 7). Thus, the increase in phosphorylation-activated nNOS during proestrus (Parkash et al., 2010; Hanchate et al., 2012) may be an integral part of the kisspeptin-dependent preovulatory activation of GnRH neurons (Hanchate et al., 2012). NO-producing, but also other non-GnRH kisspeptin-responsive cells (Pielecka-Fortuna et al., 2008), could potentially be used by kisspeptin neurons to facilitate synchronous activity among GnRH neurons and thus maximize the release of this hormone into the blood. . In line with this hypothesis, Gpr54-null mice in which Gpr54 expression has been

selectively restored in GnRH neurons exhibit blunted LH responses to key central activators of the gonadal axis, including both kisspeptin itself (León et al., 2016), and NMDA, which is known to elicit LH release in Gpr54- and Kiss1-null mice by acting, at least in part, on nNOS neurons when injected into the cerebral ventricles (d'Anglemont de Tassigny et al., 2010).

3.3 A role for preoptic NO in sexual maturation and the onset of fertility

Sexual maturation, puberty (the period when complete reproductive capacity is attained) and subsequent adult fertility are regulated by the hypothalamic-pituitary-gonadal axis (HPG). The activity of the HPG is itself orchestrated in the brain by neuroendocrine GnRH neurons, which are subjected to complex maturational events affecting their biosynthetic capacity, neurosecretory pattern and morphology (Prevot, 2015). This array of events, which is likely linked to the integration of postmigratory GnRH neurons into the neural network responsible for relaying bodily information to these core neurons, occurs at different phases of postnatal development (Prevot, 2015). The first activation of the axis occurs shortly after birth during the infantile period (a phenomenon referred to as mini-puberty) (Kuiri-Hänninen et al., 2014; Prevot, 2015), and results in the release of an FSH surge that stimulates the proliferation of immature Sertoli cells and spermatogonia in the testes of the male, and the growth of the first pool of ovarian follicles, destined to ovulate at puberty, in females. After weaning, the brain-driven maturation of the gonads leads to increasing production of gonadal steroids (e.g. estrogens and testosterone), which initiate a feedback loop that acts at different levels of the HPG axis, including the brain, to adapt its activity to both developmental needs and physiological demand, and hence

lead the way to the timely onset of puberty (Prevot, 2015). Puberty in males is characterized by the first occurrence of motile sperm in the epididymis, conferring on them the capacity for reproduction. Puberty in females is characterized by the first GnRH/LH surge leading to ovulation and the establishment of estrous cyclicity, the regularity of which conditions fertility during adulthood.

3.3.1 A role for NO in the control of GnRH expression during postnatal development?

Recent findings show that hypothalamic NO could be involved in the regulation of the expression of the gene encoding GnRH, which has been shown to increase during postnatal development (Prevot, 2015), and thus be part of the very mechanism regulating the clock-like precision of pubertal activation (Messina et al., 2016). During the second week of life, when nNOS activity increases in the preoptic region at minipuberty (Messina et al., 2016), coinciding with the arrival of kisspeptinergic and other fibers from the ARH at the preoptic region (Bouret et al., 2004a; Caron et al., 2012a), NO interacts with the transcription factor CAAT/enhancer binding protein β (C/EBPβ), encoded by the Cebpb gene, to repress the activity of the GnRH promoter (Belsham and Mellon, 2015). A selective switch in miRNA expression patterns in GnRH neurons appears to invert the balance between repressive and inductive signals controlling the expression of the GnRH promoter (Messina et al., 2016) by triggering decreased C/EBPβ expression while sustaining Gpr54 expression (Messina et al., 2016). In particular, the increased expression of miR155 (which represses *Cebpb*) at minipuberty, has been identified to act as one of the linchpins of this process, permitting the sustained increase of GnRH required for subsequent

sexual maturation (Messina et al., 2016). This miRNA-mediated flipping of the switch in the control of GnRH promoter activity at minipuberty intriguingly coincides with a dramatic change in the pattern of GnRH release (Glanowska et al., 2014). It is thus tempting to speculate that this kisspeptin-nNOS-GnRH neuronal microcircuit, along with the miRNA-gene network in GnRH neurons, could play an important role in the initiation of both rhythmic GnRH gene transcription, and pulsatile GnRH secretion (Choe et al., 2015) at puberty, in response to developmental and bodily cues received during the infantile period (Prevot, 2015).

3.3.2 nNOS neurons in the preoptic region are critically involved in the metabolic regulation of puberty onset

Nutritional status has long been recognized as a determining factor for the onset of puberty, as well as the maintenance of reproductive capacity. This notion is based on the fact that many aspects of reproductive physiology are energetically demanding (e.g. sexual maturation, pregnancy, lactation etc.), so a minimum store of energy is necessary to promote the normal growth and function of reproductive organs, and, of course, fertility (Kennedy, 1969; Biro et al., 2006; Bellefontaine and Elias, 2014). On the other hand, excess energy stores could have deleterious effects on reproductive physiology; precocious puberty, abnormal menstrual cycles, polycystic ovary syndrome and infertility are often associated with metabolic disorders, such as obesity, that can result from overnutrition (Friedman and Kim, 1985; Biro et al., 2006).

Leptin, a peptide hormone mainly produced by adipocytes, is a key link between metabolic homeostasis and reproductive competence (Chehab, 2014; Münzberg and

Morrison, 2016). Leptin deficiency, caused by the *Lep*$^{ob/ob}$ loss-of-function mutation in both humans and rodents, results in obesity, a failure to complete puberty, and consequently, infertility (Chehab et al., 1996; Montague et al., 1997). Chronic leptin administration to *Lep*$^{ob/ob}$ individuals induces the completion of sexual maturation and the restoration of fertility, indicating the pivotal role played by leptin (Chehab et al., 1996; Farooqi et al., 1999). There is compelling evidence to suggest that leptin regulates GnRH neurons via cells afferent to these neurons (Sullivan et al., 2003; Quennell et al., 2009) and/or those that interact morphologically with them (Louis et al., 2011). Seminal works have shown that states of negative energy balance, in which leptin levels are low (e.g., during fasting or when individuals subjected to strenuous exercise), result in decreased LH levels and the suppression of the estrous cycle, while leptin treatment restores fertility (Ahima et al., 1996; Nagatani et al., 1998; Welt et al., 2004). The recent experiments showing that the neuroendocrine reproductive axis was unable to sense the increase in leptin levels when NOS activity was pharmacologically blocked prior to leptin treatment in fasting mice (Bellefontaine et al., 2014), suggest that nNOS neurons could be one of the key neuronal populations relaying changes in leptin levels to GnRH neurons in the hypothalamus.

The mapping of leptin-responsive cells in the hypothalamus has revealed that several leptin-receptor-expressing (LepR) populations are NO-synthesizing neurons (Donato et al., 2010b; Leshan et al., 2012), and that the expression of the phosphorylation-activated nNOS can be regulated by circulating leptin in the preoptic region (Donato et al., 2010b; Bellefontaine et al., 2014), which contains neurons lying outside the blood-brain barrier at the level of the OVLT/MePO (Langlet et al., 2013; Prager-Khoutorsky and Bourque, 2015). While LepR expression in nNOS neurons has been shown to be crucial in the integration of metabolic signals (Leshan et al., 2012),

accumulating evidence shows that the preoptic region plays an active role in the control of energy homeostasis (Zhang et al., 2011; Yu et al., 2016). Preoptic nNOS neurons, in addition to regulating estrogen-dependent neural inputs, such as kisspeptin signals, are thus well poised to transmitting information regarding peripheral energy stores to GnRH neurons by sensing leptin (Fig. 7).

In line with the latter idea, leptin-promoted nNOS activation in the OVLT/MEPO is seen to be functionally associated with a rise in peripheral LH from nadir levels (Bellefontaine et al., 2014). The genetic deletion or pharmacological inhibition of nNOS prevents the leptin-induced LH release, which appears independent of kisspeptin/Gpr54 signaling (Bellefontaine et al., 2014). Obese leptin-deficient mice bearing a null mutation in the nNos gene or exposed to continuous nNOS inhibition in the preoptic region fail to undergo sexual maturation in response to chronic leptin administration even though they recover a lean phenotype (Bellefontaine et al., 2014). Finally, the selective deletion of LepR expression within the preoptic region in adult mice alters basal GnRH/LH secretion without affecting body weight (Bellefontaine et al., 2014). Together these results convincingly suggest that NO-signaling facilitates leptin action on reproduction and establishes the preoptic region as a novel site for the integration of leptin signaling on the GnRH neural network.

Although, GABA neurons have also been shown to play a role in relaying leptin signaling to the GnRH system (Zuure et al., 2013; Martin et al., 2014), most leptin-sensitive neurons of the preoptic region, likely including the nNOS neurons themselves, are glutamatergic (Yu et al., 2016). These data are reminiscent of what has been found in the ventral premammillary nucleus, another key site for leptin action in the onset of puberty and one which also contains numerous leptin-sensitive nNOS neurons (Leshan et al., 2009; Donato et al., 2010b), where LepR neurons are

likewise thought to be glutamatergic (Donato et al., 2011). Further work will be required to tease apart the relative importance of each of these different systems, which all appear to at least partially relay metabolic information to the neuroendocrine system controlling reproduction (Ratra and Elias, 2014).

3.4 Does neuronal NO set the rhythm?

The low molecular weight of NO (30 g mol^{-1}) confers on this molecule a large aqueous diffusion coefficient (3.3 μm^2 ms^{-1}) and a correspondingly rapid rate of aqueous diffusion (about 2.5 μm in 1 ms) (Garthwaite, 2016). In addition, its high lipid solubility (Lancaster, 1997) allows rapid permeation of biological membranes in which it concentrates about 4-fold and where it has a 10-fold lower diffusion rate than in aqueous buffer (Möller et al., 2005). NO would thus be capable of crossing the 3 nm hydrophobic membrane interior in about 3 ns. These physicochemical properties imply that NO will diffuse uniformly, in all directions, away from its site of synthesis, with the potential to influence the activity of cells located within an active area of brain tissue, irrespective of synaptic connectivity. NO is thus the prototypic candidate molecule for volume transmission (Agnati et al., 1995) with its biological effects being regulated by i) the catalytic properties of the nNOS enzyme, ii) its rate of inactivation and ii) the distance of NO targets from its source(s) (Garthwaite, 2016). Although it was initially thought that NO could be diffusing in active concentrations within a sphere of 200 μm diameter, thereby influencing 2 millions synapses (Wood and Garthwaite, 1994), the development of new advance techniques using cells expressing FRET-based biosensors to accurately measure NO concentrations in living brain slices, uncovered that NO produced at a synapse is only likely to function very

locally, specifically within submicrometer dimensions (Wood et al., 2011; Bhargava et al., 2013). Besides, calculations based on the pharmacokinetics of sGC upon its activation by NO, reveal that a single synaptic NO pulse results in a cGMP response within a concentration range that is probably too low to have any biological activity (Garthwaite, 2016). Indeed, it has been suggested that trains of NO pulses of about 10-20 Hz (a stimulation frequency similar to the one used to promote GnRH neuronal activity/secretion by kisspeptin neurons using an optogenetic approach (Han et al., 2015; Qiu et al., 2016) are needed to elicit an accumulation of cGMP to active concentration ranges at synapses (Garthwaite, 2016). Volume transmission could, however, occur in some parts of the central nervous system, where nNOS neuron cell bodies are densely packed, and thus potentially the combinatory activation of those neighboring nNOS cells- and the subsequent release of NO- could result in the accumulation of a biologically active concentration of cGMP. This could be the case for the OVLT/MePO area of the preoptic region, where a large population of nNOS expressing neurons resides among some GnRH cell bodies, and where GnRH neurons extend their dendrites and kisspeptin neurons their axons (Hanchate et al., 2012; Herde et al., 2013). The proposed mechanism here is that when nNOS activity is coordinated across the population of neurons by homeostatic blood-borne signals (e.g., estrogens and leptin) or transsynaptic inputs (e.g., kisspeptin and glutamate) (d'Anglemont de Tassigny et al., 2007a; Parkash et al., 2010; Hanchate et al., 2012; Bellefontaine et al., 2014), the NO concentration in between those neurons builds up to levels capable of stimulating neighboring GnRH neurons and coordinating their activity so as to promote the release of GnRH peptide into the pituitary portal blood circulation, and subsequently elicit LH release from the pituitary gland (Fig. 8). Realistic modeling of the size and distribution of the nNOS neurons in the

OVLT/MePO fully supports the proposed mechanism provided that two conditions are met: firstly, that the numbers of active nNOS neurons at the resting state are half or fewer than those active in the presence of the stimuli and, secondly, that the rate of inactivation of NO is high enough to permit GnRH neurons to discriminate between sparsely activated and a more fully activated population of nNOS neurons (Bellefontaine et al., 2014). The computed NO inactivation rate allowing this mode of signaling is equivalent to a half-life of 5 ms, a value neatly coinciding with the rate estimated for rat cerebellum using an experimental approach (Hall and Garthwaite, 2009).

Theoretical studies show that changes in nNOS activity and a diffuse type of NO neurotransmission can substantially influence the way in which networks process information. In the absence of structural changes, diffusive homeostasis maintains substantial heterogeneity in activity levels of individual neurons and confers networks with the capacity of representing input heterogeneity, and linearly responding over a broader range of inputs than those undergoing non-diffusive homeostasis (Sweeney et al., 2015). In contrast, if NO release influences the structure of synaptic connectivity and thus changes synaptic weight, neurons of the network would be more likely to coactivate with similar stimuli. This would provide possibilities for the interaction between functional organization driven by common sensory features, and functional organization driven by underlying spatial distribution of neurons within the network (Sweeney and Clopath, 2016). A future challenge would be to test experimentally these possibilities and investigate whether nNOS neurons of the periventricular preoptic region could be part of the central pattern generator network driving pulsatile release of GnRH (Tobet et al., 2009; Marder et al., 2014; Israel et al., 2016), or

whether they act as a filter to integrate information from the periphery with the input from the central pattern generator (Hanchate et al., 2012; Bellefontaine et al., 2014).

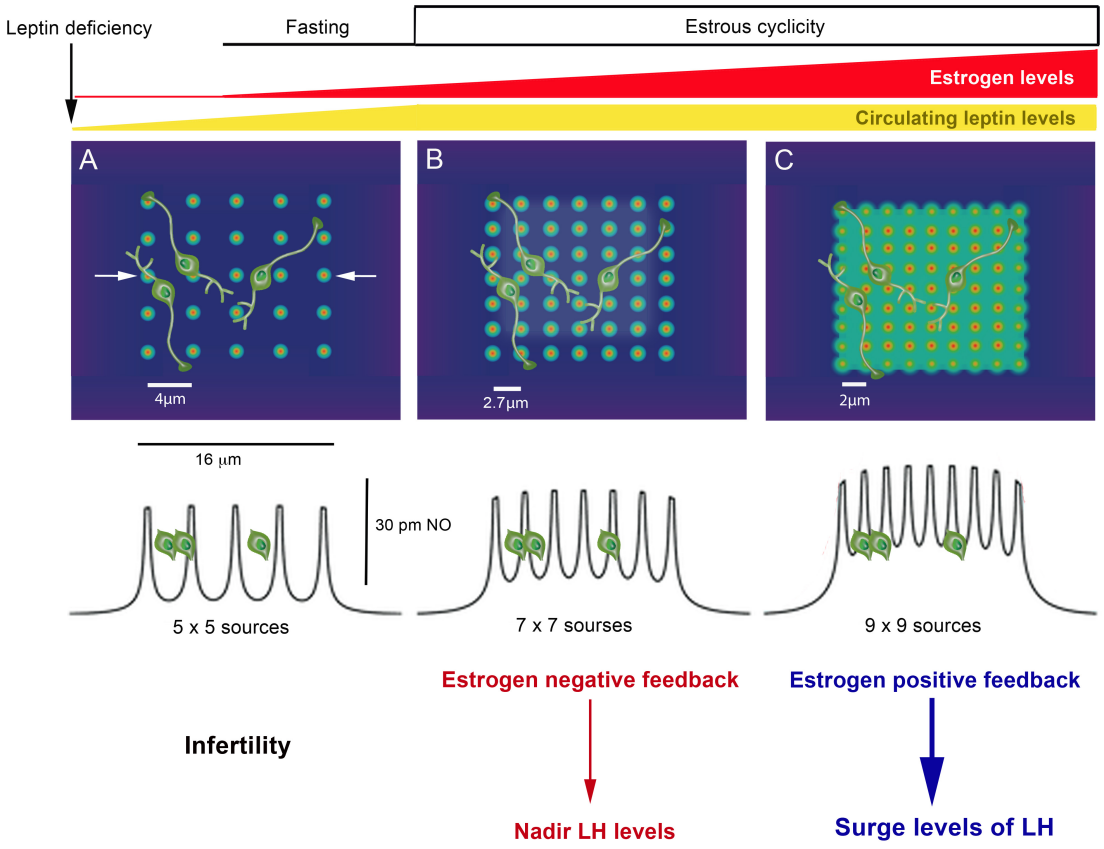


Figure 8 Model of NO signaling, resulting from the activation of nNOS cells surrounding GnRH neurons in the preoptic region of the hypothalamus (adapted from Garthwaite, 2016)

A, B and C show the computed profiles of steady state NO concentrations across the center of a cubic array of spherical surface sources. Spheres of radius = 0.2 µm and numbering 25 (A), 49 (B) and 81 (C) are illustrated in 2-dimensional arrays within a fixed area (16 µm × 16 µm). The spheres generate NO at their surfaces at the rate of 40 molecules s−1 and the resultant NO concentrations throughout the array at steady state are calculated as described in Bellefontaine and colleagues (Bellefontaine et al., 2014). According to the physiological (or pathological) stimuli, NO action can switch from being suboptimaly active (e.g.: in individuals with leptin deficiency or during fasting, A), to being active only locally (e.g.: during the negative estrogen feedback in diestrus, B) or being a "volume transmitter" capable of influencing GnRH cells located at a distance, irrespective of anatomical connectivity (e.g.: the positive estrogen feedback on proestrus, C). The upper panels (A, B and C) illustrate the distribution of NO within and outside the area of emitters and the traces bellow (black lines) are sample concentrations taken through the center of each array (marked by arrows in A, upper panel). In green are drawn hypothetical GnRH neuron cell bodies and dendrites enclosed in the cubic array under scrutiny to suggest how NO depending on its area of influence could switch from a restraining mode (when NO effluxes from MePO/OVLT nNOS neurons are low) to a synchronizing mode (when NO effluxes are high) on GnRH neuronal activity. NO could potentially be used by the neuroendocrine brain to facilitate synchronous activity among GnRH neurons that where previously operating independently and ultimately to maximize the release of its neurohormone into the blood.

Chapter IV

Human reproductive syndromes and clinical relevance of NO signaling

4.1 Hypogonadotropic Hypogonadism

Idiopathic congenital hypogonadotropic hypogonadism (CHH) is a rare reproductive condition resulting from GnRH deficiency. GnRH deficiency leads to extremely low levels of the LH and FSH hormones, and subsequently to undetectable concentrations of sex steroids. These aberrant hormonal levels, as expected, don't allow the initiation of puberty, leading to infertility in adults. The clinical phenotype of the disease varies according to the age of appearance (congenital vs. acquired) and the severity (complete vs. partial) (Boehm et al., 2015). Idiopathic hypogonadotropic hypogonadism (IHH) can be associated with the absent or reduced sense of smell, which defines the condition known as Kallmann syndrome. If HH individuals do not present perturbations of the olfactory system, they are referred to as normosmic IHH (nIHH) (for review see Seminara et al., 1998).

4.1.1 Kallmann Syndrome (KS)

KS was first recognized in 1856 (de San Juan, 1856) and a few years later, Kallmann et al. (Kallmann, 1944) identified the genetic nature of this condition, while several years afterwards, the absence of the olfactory bulb (OB) and its axons was identified in several cases of hypogonadotropic hypogonadism (de Morsier and Gauthier, 1963). The classic presentation of Kallmann syndrome is the association of hypogonadotropic hypogonadism with anosmia (i.e. lack of olfaction), arising from an atypical migration GnRH neurons during embryonic development (for review see Seminara et al., 1998). KS may be associated with a series of anomalies, including facial defects like the cleft lip and/or palate, short metacarpals, and renal agenesis

(Waldstreicher et al., 1996). All these variable phenotypes can even occur among individuals carrying the same mutation within a pedigree (Matsuo et al., 2000), and hence render KS a very complex disorder. KS has a prevalence of around 1 in 8,000 in male and 1/40,000 females, and is associated with an X chromosome deletion (Meitinger et al., 1990). There are more than 17 different putative loci for this disorder, while the list of mutations keeps getting updated as we gain more knowledge about this rare disorder (Bianco and Kaiser, 2009; Boehm et al., 2015; Kim, 2015).

4.1.2 Normosmic IHH (nIHH)

Normosmic IHH (nIHH) syndrome is associated with abnormalities of the activation or/and secretion of the GnRH peptide, whilst retaining a normal olfactory structure. nIHH is found in almost 50% of the cases of IHH (with KS accounting for the other 50%) (Boehm et al., 2015). Inactivating mutations in the GnRH receptor of the pituitary resulting to GnRH resistance, are the predominant cause of nIHH, accounting for 16-40 % of cases (de Roux et al., 1997; Bianco and Kaiser, 2009). Other genes usually involved in cases of nIHH are the GNRH1 (Chan et al., 2009), KISS1 (Silveira et al., 2010; Topaloglu et al., 2012) and KISS1R (de Roux et al., 2003), TAC3 and TAC3R (Topaloglu et al., 2009; Young et al., 2010), while there are rare examples of cases demonstrating mutations of LH or FSH β-subunits (Layman et al., 1997; Lofrano-Porto et al., 2007). The clinical manifestation of the disease is broadly similar to the one of KS; nIHH patients demonstrate absent or incomplete pubertal development and infertility, due to the impaired GnRH secretion, or insensitivity to GnRH (for review see Karges et al., 2012).

4.2 Clinical relevance of hypothalamic NO actions

Recognition that hypothalamic NO plays a role in the postnatal development of the neuroendocrine axis controlling sexual maturation and the onset of puberty (Messina et al., 2016) has important clinical implications, since age at puberty is associated with long-term health consequences (Witchel, 2016) and inhaled NO is a common treatment administered to infants to improve noncommunicable diseases, including those affecting the brain (Charriaut-Marlangue et al., 2013; Bhatraju et al., 2015). Noncommunicable diseases are chronic diseases of major public health importance such as cardiovascular diseases, obesity, cancer, type 2 diabetes, degenerative and mental disorders, and allergies, mainly attributable to environmental factors among which is the, timing of puberty (Patton and Viner, 2016; Witchel, 2016) .

In preterm infants, inhaled NO is routinely used to treat hypoxemic respiratory failures and pulmonary hypertension (Ambalavanan and Aschner, 2016). Preclinical studies show that inhaled NO significantly increases NO concentrations in the brain (Charriaut-Marlangue et al., 2012) and that, while it may exert both beneficial and detrimental effects depending of the dose administered and the time period of exposure (Charriaut-Marlangue et al., 2013), it could have neuroprotective effects in individuals with combined lung and brain injury (Pham et al., 2012). However, neurological surveys of preterm infants exposed to inhaled NO are rare and poorly reported. Equally unexplored are the effects of inhaled NO treatment on the awakening of the GnRH neuroendocrine axis at minipuberty - the onset of which occurs around 1 week of age and lasts for 3 months resulting in a transient gonadal maturation (Kuiri-Hänninen et al., 2014) - and its long-term consequences on pubertal development. Intriguingly, the amplitude of minipuperty varies tremendously depending on gestational age at birth, both in boys (Kuiri-Hänninen et al., 2011b) and

girls (Kuiri-Hänninen et al., 2011a): urinary FSH is seen to increase up to a 300-fold in preterm female infants (24-34 gestational weeks) when compared to full-term baby girls (37-42 weeks of gestation), with the former being at high risk to develop brain injury and neurodevelopmental disabilities (Back, 2016), together with reproductive defects (Swamy et al., 2008). This extremely high FSH surge in premature girls is associated with a marked delay of follicular development when compared to full-term born girls (Kuiri-Hänninen et al., 2011a). This phenomenon may be explained by an insufficient negative feedback inhibition of GnRH/FSH secretion by ovarian hormones, which are known to play key organizational roles in the infantile brain (Simerly, 2002; Bakker and Baum, 2008; Nugent et al., 2015). Inhaled NO could act to correct the aforementioned central neurohormone imbalance by inhibiting GnRH gene expression during this critical period of brain development (Messina et al., 2016); NO could thus potentially improve the pathological conditions related to preterm birth by enabling the proper unfolding of the sequence of events leading to normal postnatal brain maturation that requires a constant dialogue between the brain and the periphery.

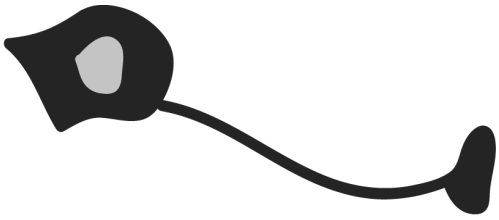
Interactions between hypothalamic neuroendocrine systems and peripheral hormones have been increasingly acknowledged to play a fundamental role in postnatal brain development, i.e. the hormonal programming of brain development (Steculorum et al.; Simerly, 2002; Bouret et al., 2004b; Bakker and Baum, 2008; Nugent et al., 2015), the impairment of which may lie at the origin of major neurological and psychiatric disorders. Early puberty timing is associated with heightened prevalence and intensity of depressive symptoms, adjustment problems, anxiety, and psychopathology (e.g., eating disorders) among adolescent females (Hoyt and Falconi, 2015; Patton and Viner, 2016). In addition, a possibility that puberty stimulates pathological brain

development in individuals with perinatally acquired brain abnormalities, underlies neurodevelopmental hypothesis of some psychotic disorders such as schizophrenia, the incidence of which markedly increases during the post pubertal years (Cohen et al., 1999), and for which association studies have identified nNOS as a genetic risk factor (O'Donovan et al., 2008; Freudenberg et al., 2015). Interestingly, a recent study treating 20 schizophrenic patients (age 19-40) with the NO donor sodium nitroprusside administered intravenously, showed a rapid (within 4 hours) improvement of symptoms that persisted 4 weeks after infusion (Hallak et al., 2013), reinforcing the view that the glutamate-NO-cGMP network plays a important role in the pathophysiology of this mental illness (Shim et al., 2016).

§

§ Parts of the above introduction have been incorporated in a Review, included in Annex, paragraph 10.1 (in preparation for submission)

Aim



During the three years of my PhD, I aimed to delineate the role of NO signaling in the events taking place in the hypothalamic region. Being particularly interested in the NO being released by the neuronal nitric oxide synthase (nNOS) population of the hypothalamus, I worked on an in depth characterization of the molecular and cellular properties, the anatomical distribution, as well as the implication of the nNOS-expressing neurons in the physiology of the reproductive system.

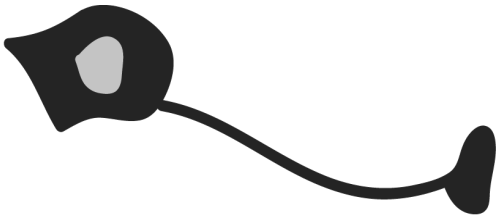
In the first study, in preparation for submission in the *Journal of Comparative Neurology,* we performed a neuroanatomical classification of the nNOS neurons in the developing hypothalamus. Examining the infantile, juvenile and adult developmental stages, we patterned the distribution and expression levels of the nNOS protein in most of the hypothalamic regions known to be involved in the control of GnRH neuronal function. We characterized the cohort of neurotransmitters and hormonal receptors expressed by this neuronal population during the three aforementioned stages of development. Up to now, the molecular identity of the nNOS hypothalamic population remained largely unknown. We presented evidence demonstrating that nNOS cells are a highly diverse population, represented by many different neuronal subpopulations in the different hypothalamic nuclei.

In the second study, I aimed to depict the role of NO signaling in the physiology of reproduction. Since previous studies from our lab highlighted the importance of NO signaling in the reproductive events during adulthood, we challenged the idea that this obscure neuromodulator could regulate the sexual maturation of the reproductive axis by playing a catalytic role in the events leading to pubertal onset. We presented highly significant genetic evidence identifying Nos1 mutations in probands of hypogonadic hypogonadism (CHH). We further assessed the role of NO in the migration of GnRH neurons since perturbations of this process are correlated with a form of CHH. Our

data support a role of NO signaling in the migration of GnRH neurons during embryonic development. Focusing on the infantile period, we identified a critical time window during which NO controls the initiation of puberty by regulating, not only the transcription of the Gnrh gene, but also the secretion of the GnRH peptide.

Overall, our data establish an eminent, novel role for the hypothalamic nNOS-derived NO in the control of the maturation of the reproductive axis, shading light into its key-role interactions with the components of the GnRH neuronal network. Future studies will focus on depicting how the newly identified mutations of the Nos1 gene can affect the function of the nNOS protein and subsequently the release of NO, enabling us to better understand important aspects of the CHH pathology.

Results



Chapter V

5.1 Phenotyping of nNOS Neurons in the Postnatal and Adult Mouse Female Hypothalamus

Aim

The aim of this first study was to characterize the anatomical distribution, as well as the molecular identity of the NOS-expressing hypothalamic neurons. Even though there are several studies focusing on the identification of molecularly divergent subpopulations of nNOS cells in distinct brain regions, the area of hypothalamus was overlooked; As a result our knowledge concerning the molecular properties of the nNOS neuronal population residing in the hypothalamus are extremely limited. During this study we 1) describe the neuroanatomical distribution of the nNOS-expressing cells in the hypothalamic region during development (infantile, juvenile and adult developmental stages), 2) present the molecular identity of the nNOS neurons, providing evidence for an either a gabaergic or glutamatergic phenotype, in distinct hypothalamic regions, and 3) portray the expression of estrogen receptor α from the distinct hypothalamic nNOS subpopulations.

Principal Results

⇒ nNOS neurons are found located in distinct hypothalamic nuclei with the most packed population identified in the preoptic area and most specifically in the region of the organum vasculosum laminae terminalis (OVLT)

⇒ nNOS is constitutively expressed during postnatal development in all the regions studied, the only exception being the arcuate hypothalamic region (ARH), where nNOS expression was absent at P11. Changes in the level of nNOS expression were also identified in the regions of anterior hypothalamic nucleus (AHN), lateral hypothalamus (LHA), suprachiasmatic hypothalamic

nucleus (SCN), supraoptic nucleus (SON), preoptic periventricular area (PVPo) and the posterior hypothalamus (PH)

⇒ nNOS-expressing neurons are divided into molecularly distinct subpopulations according to the hypothalamic nucleus they are located in ; the vast majority of nNOS cells in the regions of ventral hypothalamic area (VMH), dorsomedial hypothalamus (DMH), anteroventral hypothalmic area (AVPV), medial preoptic area (MePO), and OVLT are glutamatergic. On the contrary, the nNOS population residing in the ARH is in its vast majority gabaergic

⇒ in the ARH, members of the gabaergic nNOS population are identified as fast spiking parvalbumin positive GAD-67 neurons

⇒ ER-α is co-expressed by most of the nNOS neurons residing in the regions of ARH, VMH, AVPV and OVLT/MePO

Conclusion

Similarly to what has been described in extrahypothalamic structures, like for example in the region of the hippocampus, the nNOS population is highly divergent, being represented by many different neuronal subpopulations. Our results support a constitutive expression of the nNOS protein in most of the hypothalamic regions during development, whilst identifies a time-regulated expression of the protein in the region of ARH. In addition to this, nNOS neurons of the ARH are the only one identified in their vast majority as gabaergic cells, while the rest of the hypothalamic subpopulations studied were seen to be mainly glutamatergic. Estrogenic and NO signaling pathways are in closely collaborating in the hypothalamus, as proposed by the coespression of ER-α from the majority of nNOS neurons.

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Phenotyping of nNOS Neurons in the Postnatal and Adult Mouse Female Hypothalamus

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ABSTRACT

Nitric oxide synthesizing (nNOS) neurons play a role in major aspects of brain function. The heterogeneity of nNOS-expressing neurons has been studied in various brain regions, whilst the phenotype of nNOS cells residing in the hypothalamus remains largely unknown. Here we examined the distribution of cells that express nNOS in postnatal and adult female mouse brains using immunohistochemistry. In both adults and neonates, nNOS was largely restricted to regions of the hypothalamus involved in the control of bodily functions, such as energy balance and reproduction. Labeled cells were found in the paraventricular, ventromedial, dorsomedial nuclei of the hypothalamus, as well as in the lateral area of the hypothalamus. Intriguingly, the nNOS signal in the arcuate nucleus of the hypothalamus (ARH) was seen only after the second week of life. The most heavily and densely labeled population of cells was found in the organum vasculosum of the lamina terminalis (OV) and the median preoptic nucleus (MEPO), where lie most of the soma of the neuroendocrine neurons releasing GnRH and controlling reproduction. A great proportion of the nNOS-immunoreactive neurons of the OV/MEPO and ARH were seen to express ERα. Notably, almost all ERα-immunoreactive cells of the OV/MEPO were found to express nNOS. Moreover, the use of *Vglut2::Eyfp, Vgat::Eyfp* and *Gad67::Gfp* transgenic mouse lines revealed that, like GnRH neurons, most hypothalamic nNOS neurons have a glumatergic phenotype, but the nNOS neurons of the ARH that would be GABAergic. Altogether, these observations are consistent with the proposed role of nNOS neurons in bodily processes.

INTRODUCTION

Nitric oxide (NO) is a chemical transmitter with an extremely divergent role in the mammalian central and peripheral nervous system (Garthwaite, 2008; Garthwaite, 2016; Steinert et al., 2010). Therefore, it comes as no surprise that nitric oxide synthesizing (nNOS) neurons are represented by many different subpopulations of cells in terms of genetic specification and molecular characterization, whilst all of them having as a common ground their ability to produce NO. This heterogeneity in the origins of nNOS cells as well as in the nature of the neurotransmitters they may co-express has been the subject of many studies focusing on several regions of the brain, such as the hippocampus, the neocortex, the nucleus of the solitary tract and the olfactory bulb (Crespo et al., 2003; Fujimoto et al., 2016; Giuili et al., 1994; Lin, 2009; Tricoire and Vitalis, 2012; von Bartheld and Schober, 1997); however, our knowledge concerning the development and molecular identity of the cell populations expressing nNOS in the hypothalamus is extremely limited.

Results obtained from genetic mouse models harboring either mutations in the *Nos1* gene (Gyurko et al., 2002; Huang et al., 1993) or selective inactivation of specific genes in nNOS-expressing cells (Leshan et al., 2012), suggest that hypothalamic nNOS neurons play vital roles in the control of reproduction (Gyurko et al., 2002; Hanchate et al., 2012; Messina et al., 2016) and energy homeostasis (Leshan et al., 2012), as well as in the crosstalk between these two bodily functions (Bellefontaine et al., 2014). Intriguingly, this crosstalk is also thought to involve, at least in part, GABAergic neurons (Martin et al., 2014; Zuure et al., 2013). Whether GABA and NO are co-synthetized in discrete populations of hypothalamic neurons is just beginning to being explored (Marshall et al., 2016).

Regulation of reproduction mediated by hypothalamic nNOS neurons is principally thought to occur in the preoptic region at the median preoptic nucleus (MEPO) and the organum vasculosum of the lamina terminalis (OV) where nNOS and gonadotropin-releasing hormone (GnRH) neurons are co-distributed (d'Anglemont de Tassigny et al., 2007; Hanchate et al., 2012; Herbison et al., 1996) and functionally interact (Bellefontaine et al., 2014; Clasadonte et al., 2008). The activity of nNOS in the preoptic region has been shown to be tightly regulated by circulating estrogen levels (d'Anglemont de Tassigny et al., 2007; Parkash et al., 2010) and to be required for estrous cyclicity (d'Anglemont de Tassigny et al., 2007). In particular, nNOS

activity has been shown to restrain the activity of the hypothalamo-pituitary gonadal (HPG) axis when circulating levels of estrogens during the estrous cycle are low and to be required for the onset of the LH surge induced by the gonadal steroids in mice (Hanchate et al., 2012). Results from recent studies invalidating estrogen receptor alpha (ERα) expression selectively in glutamatergic neurons unexpectedly showed that glutamatergic neurons, which tightly regulate nNOS activity (Garthwaite et al., 1988) and are long-known to be potent activators of the HPG axis (Brann and Mahesh, 1991; Claypool et al., 2000; Urbanski and Ojeda, 1990), are not only playing a role in the estrogen-mediated positive feedback phase, which triggers the preovulatory GnRH surge, but also in the estradiol negative feedback loop (Cheong et al., 2015). These findings raise the hitherto unexplored possibility that discrete populations of glutamatergic neurons may express nNOS in the hypothalamus.

In addition to its regulatory role in the adult, data have also shown expression of nNOS in the hypothalamus during early life (Edelmann et al., 2007) and have suggested a role for NO in the maturation of GnRH neurons during postnatal development via the regulation of GnRH mRNA expression (Messina et al., 2016), a phenomenon that could be critical for sexual maturation (Prevot, 2015).

In the present study, we systematically analyzed the distribution of nNOS in the female mouse hypothalamus during postnatal development and in adults. In addition, to determine when nNOS neurons become competent in sensing estrogenic signal, we studied the distribution of cells expressing ERα in both developing and adult mice. Furthermore, we used *Vglut2::Eyf, Vgat::Eyfp* and *Gad67::Gfp* transgenic mouse models to further characterize the neurochemical phenotype of nNOS neurons in the hypothalamus.

MATERIALS AND METHODS

Animals and tissue preparation

All mice were group-housed in a temperature-controlled room (21-22°C) with a 12h light/dark cycle and *ad libitum* access to food and water. C57Bl/6J *Gad67::Gfp* (line G42) mice, in which GFP is expressed in parvalbumin-positive cells have been engineered as detailed elsewhere (Ango et al., 2004). C57Bl/6J *Vglut2::Eyfp* and *Vgat::Eyfp* mice were generously provided by Dr. Bradford B. Lowell, Beth Israel Deaconess Medical Center and Harvard Medical School (Vong et al., 2011). Postnatal

day 11 (P11, infantile period, n=4), P23 (juvenile period, n=4) and diestrous adult (P60-P80, n=8) female mice where were deeply anesthetized with Pentobarbital (50-90 mg/kg i.p.) and perfused transcardially with 0.9% saline, followed by an ice-cold 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB) pH 7.4 fixative solution. The brains were quickly removed from each perfused animals, post-fixed in the same fixative containing 20% sucrose for 4h (postnatal) or overnight (adult) at 4ºC, and immersed in 20% sucrose in 0.02M potassium phosphate buffer saline (KPBS) solution at 4ºC overnight. The brains were embedded in OCT (Optimal Cutting Temperature embedding medium, Tissue-Tek®, Sakura, France, Villeneuve d'Ascq), frozen in isopentane (-55°C) and stored at -80°C until sectioning. Using a cryostat, serial frozen sections (16-µm and 30-µm thick for P11-P23 and adult brains, respectively) were cut and collected on chrome-alum-gelatin-coated slides. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the Universities of Lille, Montpellier and Louisiana; 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).

Antibody characterization

All primary antibodies used are listed in table 1 and stained the appropriate pattern of cellular morphology and distribution as demonstrated in previous publications.

Immunohistochemistry

Sections were washed 3 times in PB 0.1M for 5 minutes each and then microwaved in 10 mM citrate buffer pH 6.0 (4 min at 800 W followed by 2 times 4 min at 400 W) for antigen unmasking. Sections were then washed in PB 0.1M, and incubated in blocking solution (5% donkey serum + 0.3% Triton X-100) in PB 0.1M for 60 min. Then, sections were incubated in rabbit anti-estrogen receptor α (Millipore) 1/500, or in guinea pig anti-Gnrh (home-made by Dr. Erik Hrabovszky, Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) and 1/10000, sheep anti-nNOS (generous gift from Dr. P. C. Emson, Medical ReseARHh Council, Laboratory for Molecular Biology, Cambridge, UK) and, when required, in 1/1000 and chicken anti-GFP antibody (Abcam) 1/500 in blocking solution for 48h at 4 ^{o}C. After the incubation in

the primary antibody, sections were rinsed with P.B. 0.1M for 3 times, 10 minutes each and then incubated in secondary antibody donkey anti-rabbit or anti-guinea pig Alexa-568 (Invitrogen), donkey anti-sheep Alexa-647 (Invitrogen) and donkey- anti-chicken 488 (Jackson ImmunoReseARHh) for 60 minutes at room temperature. Sections were then washed, counterstained with hoechst (1/10000) for 5 minutes, washed 2 times for 5 minutes and coverslipped with Mowiol coverslip mounting solution.

Image acquisition

Images were taken with a Zeiss Axio Imager Z2 microscope (Zeiss, Germany). Alexa 488 was imaged by using a 495 nm beam splitter with an excitation wavelength set at 450/490 nm and an emission wavelength set a 500/550 nm. Alexa 568 was imaged by using a 570 nm beam splitter with an excitation wavelength set a 538/562 nm and an emission wavelength set at 570/640 nm. Alexa 647 was imaged by using a 660 nm beam splitter with an excitation wavelength set a 625/655 nm and an emission wavelength set at 665/715 nm. Nuclear staining (Hoescst) was imaged by using a 395 nm beam splitter with an excitation wavelength set a 335/383 nm and an emission wavelength set at 420/470 nm. Images were taken with the objective Plan-Apochromat 20x/ 0.80 (numerical aperture) /M27 (thread type). Z-stack images were taken every 0.49 μm, for a total of i) 28.90 μm for adult sections, and iii) 6.86 μm for p11 and p23 sections. High magnification microphotographs represent maximal intensity projections derived from 20-25 triple-apoTome images collected using the z-stack module of the AxioVision 4.6 system and a Zeiss 63X oil immersion objective (N.A. 1.4). Images to be used for figures were adjusted for brightness and contrast by using Photoshop (Adobe Systems, San Jose, CA).

Cell counting and analysis

Analysis of ERα or GFP^{Gad67} expression in nNOS cells was undertaken by counting the numbers of single-labeled, dual-labeled nNOS neurons. Quantifications were performed in the MEPO and OV, represented by plate 17 of the Swanson's atlas (Swanson, 2004) and in the arcuate (ARH), ventromedial (VMH) and dorsomedial (DMH) nuclei of the hypothalamic represented by plates 28-30. Cell counts from OV/MEPO as well as ARH, VMH, DMH were determined by counting unilaterally the above regions, and as those were delimited by Hoechst nuclear staining, in a

surface area of 676 μm x 676 μm for the OVLT/MePO and of 1.28 mm x 1.87 mm for the ARH, VMH, DMH. All the above values for each mouse were averaged to determine mean counts for each age group and were then used to generate mean + SEM values for each group. All data were analyzed using the nonparametric multiple comparison Kurkal-Wallis test. Statistical significance was accepted if P was < 0.05.

RESULTS

The immunoreactive signal for nNOS in the CNS is highly abundant in the hypothalamus of female mice (Table 2), with a particularly dense distribution in the organum vasculosum of the lamina terminalis (OV) and the median preoptic nucleus (MEPO) (Fig. 1A). The nomenclature used in the paper corresponds to that described in the brain atlas of Swanson (Swanson, 2004). As referenced in Table 2 and bellow in the text, nNOS immunoreactivity was assessed as highest density, high density, moderate density, low density and undetectable based on both signal strength and the number of labeled cells. This label appeared to be specific because immunoreactive signal was not observed over cells when the nNOS antibody was used on sections obtained from nNOS-null mice (data not shown).

Expression of nNOS-immunoreactivity in adults

In the preoptic area of adult female mice, besides the compact expression of nNOS in the OV/MEPO (Fig. 1A), moderate levels of nNOS labeling were also found in the anteroventral periventricular nucleus (AVPV) (Fig. 1B) and the medial preoptic area (MPO) (Fig. 1B). A nNOS immunoreactive signal was clearly seen in cell bodies of the supraoptic nucleus (SO) (Fig. 1C), the paraventricular hypothalamic nucleus (PVH) (Fig. 1D) and the lateral hypothalamic area (LHA) (Fig.1C), but appeared to be absent from the suprachiasmatic hypothalamic nucleus (SCH) (Fig. 1C). In the tuberal region of the hypothalamus, nNOS immunoreactivity was also found in neuronal cell bodies of the dorsomedial hypothalamic nucleus (DMH) (Fig. 1E), the ventrolateral part of the ventromedial hypothalamic nucleus (VMHvl) (Fig. 1E), the arcuate nucleus of the hypothalamus (ARH) (Fig. 1E), the LHA (not shown), and the ventral premammillary nucleus (PMv) (Fig. 1F), whereas only rare nNOS-immunoreactive cell bodies where seen in the posterior periventricular nucleus (PVp) (Fig. 1F).

Expression of nNOS-immunoreactivity in postnatal brains

As referenced in Table 2 and summarized in Figure 2, the pattern of nNOS protein expression in the hypothalamus of P11 and P23 animals resembled that of adult mice, with two marked exceptions. Neuronal NOS immunoreactivity did not appear to vary across postnatal development (Table 2) in the OV/MEPO (Fig. 2A, 2B, Fig 1A), AVPV (Fig. 2C, 2D, Fig 1B) and MPO (Fig. 2C, 2D, Fig 1B), and the LHA (Fig. 2E, 2F, Fig 1C). In contrast, nNOS immunoreactivity was seen to decrease drastically in the SCH between P11 and P23 (Fig. 2E, 2F). In the tuberal region of the hypothalamus, nNOS expression was consistent between adult and postnatal brains in most nuclei, such as the VMH, DMH, Pvp and PMv (Table 2) (Fig. 2 I-L). However, striking differences in nNOS expression did exist between adult and P11 brains in the ARH. While nNOS-immunoreactive neurons could easily be seen in the ARH of P23 and adult female brains (Fig. 2J, Fig. 1E), nNOS immunoreactive signal was totally absent in the ARH at P11 (Fig. 2I, Table 2).

Subsets of nNOS neurons express ERα-immunoreactivity in the hypothalamus

To determine whether hypothalamic nNOS neurons can sense estrogens during postnatal development, we studied the pattern of estrogen receptor alpha (ERα) immunoreactivity. The analysis of double-labeled material clearly revealed that ERα immunoreactivity is detected in the majority of the nNOS-positive neurons in both the OV (P11: 74.65 % ± 12.15, P23: 67.84 % ± 2.77, adult: 82.71 % ± 3.01, n=3-4 per group) and the MEPO (P11: 56.74 % ± 10.47, P23: 53.65 % ± 3.46, adult: 50.15 % ± 5.97 , n=3-4 per group), with no significant difference in the percentage of double-labeled cells between ages (Fig. 3A and Fig. 4A). Interestingly, the vast majority of the ERα-expressing neurons of the OV (P11: 95.83 % ± 1.01, P23: 85.14 % ± 5.35, adult: 82.21 % ± 7.40, n=3-4 per group) and MEPO (P11: 67.66 % ± 6.89, P23: 82.04 % ± 7.36, adult: 87.59 % ± 4.17, n=3-4 per group) belonged in the population of the nNOS-expressing neurons, leaving only few single-labeled ERα-expressing neurons, which were not expressing the nNOS protein (Fig. 3A and Fig. 4A). In the AVPV, the hypothalamic structure known to play a key role in mediating the positive feedback of estradiol on the HPG axis (Herbison, 2016), nNOS that are much less abundant than in the OV/MEPO were also seen to express ERα at the different ages analyzed (Fig. 3B and Fig. 4B). In the ARH, another key hypothalamic structure involved in the feedback loop of gonadal estrogens (Herbison, 2016; Oakley et al., 2009; Pinilla et al.,

2012), ERα-immunoreactivity was detected as soon as P11, as previously reported by others (Brock et al., 2015), when nNOS immunoreactivity cannot yet be found. At P23, already more than 70% of the nNOS-immunoreactive neurons were seen to co-express ERα (72.65 % ± 5.60, n=4), a proportion that persisted into adulthood (72.29 % ± 7.29, n=3) (Fig. 3C and Fig. 4D). In the adult VMH, nNOS-immunoreactive cells located in the ventrolateral part of the nucleus were also seen to extensively colocalize with ERα (70.65 % ± 6.8, n=3) (Fig. 4C), in agreement with previous reports (Okamura et al., 1994). An age-dependent increase in the amount of nNOS and ERα double-positive cells was detected in the VMH (P11: 32.22 ± 1.92%, P23: 47.11 ± 1.62%, n=3-4 per group, Kurkal-Wallis test, p = 0.018; Dunn's Multiple Comparison Test, P11 *vs.* adult: $P < 0.05$) (Fig. 3C). In the DMH, ERα-containing cells were scattered and weakly stained, as opposed with the large population of nNOS expressing neurons residing in that nucleus; only occasional colocalization between nNOS and ERα immunoreactivities was detected at any stages of postnatal development (Fig. 3C and Fig. 4C). In the PMv, where an abundant population of nNOS neurons is found only sporadic ERα immunoreactivity was detected, however dual-labeled cells could be found (Fig. 3C and Fig. 4A).

Differential expression of *Vglut2* and *Vgat* promoters in hypothalamic nNOS neurons

Immunolabeling of nNOS in the hypothalamus of adult female *Vgat*::*Eyfp* and *Vglut2*::*Eyfp* mice revealed a strong segregation of the phenotype of nNOS-expressing neuronal cell populations depending on their anatomical localization. In the OV/MEPO, as well as in the DMH and VMH, more that 85% of the nNOS-immunoreactive neurons were seen to express *Vglut2* promoter activity (Fig. 5A-E), while less than 10% showed *Vgat* promoter expression, pointing towards a glutamatergic identity of the vast majority of the nNOS neurons in these hypothalamic nuclei (Fig. 5F-I). In contrast, *Vglut2* promoter activity was completely absent in the nNOS neurons of the ARH (Fig. 5E) and virtually all nNOS-immunoreactive cells of this hypothalamic nucleus were seen to exhibit *Vgat* promoter activity pointing to the GABAergic phenotype of arcuate nNOS neurons (Fig. 5J).

Distinct populations of nNOS neurons morphologically interact with hypothalamic GnRH neuronal cell bodies and nerve terminals.

Cellular analyses of dual-labeled material showed that most nNOS neurons morphologically associated with GnRH cell bodies and dendrites in the OV/MEPO were expressing *Vglut2* promoter activity (Fig. 6A-G). Interestingly, analysis of Eyfp labeling showed that *Vglut2* promoter activity was also expressed in the majority of the GnRH immunoreactive cells of the OV/MEPO (Fig 6A-G). These results are in agreement with previous studies attributing glutamatergic identity to GnRH neurons (Hrabovszky et al., 2004).

The use of the *Gad67::Gfp* G42 animal model, in which GFP is specifically expressed in paravalbumin-immunoreactive GABA neurons (Ango et al., 2004; Chattopadhyaya et al., 2004), allowed us to explore the specific relationship between this subpopulation of GABAergic neurons and nNOS- and GnRH-expressing cells in the hypothalamus. Although, a large population of GFP^{Gad67} neurons was seen to reside in the medial septal nucleus (MS) (data not shown), an extra-hypothalamic structure known to receive reciprocal connections from the hypothalamus (Swanson and Cowan, 1979), relatively few GFP^{Gad67} neurons were detected in the hypothalamus. In the preoptic region, while GFP^{Gad67} fibers were abundantly distributed in the OV/MEPO, the appearance of GFP neuronal soma only rarely occurred. OV/MEPO GFP^{Gad67} neurons were never seen to coexpress the nNOS protein (Fig. 6H, 6I). Remarkably, however, several of these GFP^{Gad67} neuronal soma were found to be immunoreactive for GnRH (Fig. 6I-N), leaving the possibility that at least portion of the non-glutamatergic GnRH neurons that were identified in *Vglut2::Eyfp* mice could be actually parvalbumin-expressing GABAergic neurons. In contrast, in the ARH, 30% of the GFP^{Gad67} neurons were seen to express nNOS-immunoreactivity in adult mice, however less that 5% on the ARH nNOS neurons were seen to express *Gad67* promoter activity (Fig. 6O, 6P, 6R). Noticeably, projections from GnRH neurons were observed on some of these nNOS-immunoreactive GFP^{Gad67} neurons in the ARH (inset Fig. 6I-T).

DISCUSSION

In the present study, we used immunohistochemistry to systematically examine the distribution of nNOS in the preoptic and the tuberal region of the hypothalamus in

female mice. Our findings are consistent with the proposed role of nNOS in regulating the neuroendocrine control of reproduction, and suggest that hypothalamic NO could also be involved in regulating body weight homeostasis. In addition, several sites in which nNOS expression changes during postnatal life were discovered. Finally, according to their distinct neurochemical repertoire, three main populations of hypothalamic nNOS neurons were identified.

It is well established that gonadal estrogens act via the brain to control the secretion of gonadotropins (follicle stimulating hormone, or FSH, and luteinizing hormone, or LH) by the pituitary gland, and thus control gonadal growth, folliculogenesis and ovulation in females. The onset of this feedback mechanism occurs after mini-puberty, which consists of an FSH surge occurring at P12 that triggers the growth of the first pool of ovarian follicles that will be ovulated at puberty (Prevot, 2015), concomitantly with the decrease in circulating levels of estrogen-binding α-fetoprotein between P12 and P16 (Bakker et al., 2006; Germain et al., 1978). The present study demonstrates that most nNOS neuronal cell populations expressing ERα in the adult hypothalamus also express ERα at P11. Intriguingly, however, our results show that nNOS expression in the ARH, in which ERα immunoreactivity is found in the infantile period at P11, only becomes apparent during the juvenile period at P23. Whether circulating estrogens drive nNOS expression in the ARH and whether nNOS expression in ERα immunoreactive cells is required for the onset of the estradiol negative feedback loop on the HPG axis requires further investigations.

Our dual-immunohistochemical data revealed that the most densely packed nNOS population of the hypothalamus was distributed in the OV/MEPO, a brain region in which NO-producing neurons are known to play a role in regulating GnRH neuronal activity and function (Bellefontaine et al., 2014; Clasadonte et al., 2008; d'Anglemont de Tassigny et al., 2007; Hanchate et al., 2012; Messina et al., 2016). Consistent with the critical involvement of estradiol in the control of nNOS activity in the preoptic region (d'Anglemont de Tassigny et al., 2007; d'Anglemont de Tassigny et al., 2009; Parkash et al., 2010), a majority of OV/MEPO nNOS neurons were seen to express ERα. We also found that ERα in the OV/MEPO was almost exclusively expressed in nNOS neurons, suggesting the importance of this discrete neuronal population in sensing gonadal information. OV neurons, by lying outside the blood-brain barrier, are likely to have greatly facilitated access to circulating hormones including gonadal

steroids (Langlet et al., 2013; Prager-Khoutorsky and Bourque, 2015), and are well poised to convey this information to GnRH neurons.

The present study also showed that in addition to being expressed in the OV/MEPO and the ARH, nNOS is also abundantly expressed in other hypothalamic nuclei such as the ventromedial and dorsomedial nuclei, as well as in the lateral hypothalamic area. Each of these nuclei and areas are known, like the ARH and more recently the MEPO, to be deeply involved in the regulation of energy homeostasis (Cone et al., 2001; Elmquist et al., 2005; Saper et al., 2002; Sawchenko, 1998; Schwartz et al., 2000; Yu et al., 2016; Zhang et al., 2011). Consistent with a potential role of nNOS in metabolic actions, recent data showed that mice lacking the receptor expression of the anorexigenic hormone leptin specifically in nNOS neurons, results in hyperphagic obesity (Leshan et al., 2012), and that introducing nNOS-null mutation in leptin-deficient mice significantly reduces the obese phenotype of these mice (Bellefontaine et al., 2014). Interestingly, our results showed that the great majority of nNOS cells in the ventromedial nucleus also express the receptor for estradiol, which in addition to its critical functions as reproductive hormone, also plays a vital role in the regulation of energy balance (Gao et al., 2007; Park et al., 2011). In contrast, nNOS/ERα dual-labelled cells were rarely seen in the dorsomedial nucleus and in the lateral hypothalamic area, as well as in the ventral prelimmamimary nucleus, which has been shown to relay metabolic information to GnRH neurons (Donato et al., 2011), despite abundant nNOS expression.

On the whole, our current findings on nNOS expression in adult female mouse agree well with those observed in the rat hypothalamus (Herbison et al., 1996; Yamada et al., 1996) and the mouse brain (Gotti et al., 2005; Sica et al., 2009), with some exceptions. Most differences observed between studies involved differences in signal intensity. For example although Gotti et al reported weak nNOS staining in the OV, we detected heavy staining in this area where nNOS neurons are found to be densely packed. These apparent discrepancies may likely be due to sex differences, since nNOS expression in the hypothalamus has been shown to be sexually dimorphic (Knoll et al., 2007).

The present study also demonstrated that most nNOS neurons of the hypothalamus shelter a glumatergic phenotype, but the nNOS neurons of the ARH that appear to be GABAergic. Notably, the ARH was the only hypothalamic nucleus in which a subpopulation of nNOS neurons was seen to express a *Gad67* promoter previously

shown to be active in the subset of GABAergic neurons expressing paravalbumin that usually reside in the medial septum, the cortical areas and the cerebellum (Ango et al., 2004; Chattopadhyaya et al., 2004). The prevalence of glutamatergic signaling in nNOS-expressing neurons of the OV/MEPO, suggests that the glutamate-mediated activation of nNOS via the NMDA receptor, which allows the Ca^{2+} influx activating the enzyme (Garthwaite et al., 1988), could occur locally and thus, incoming inputs from other brain regions may only play modulatory roles. While glutamatergic nNOS neurons in the OV/MEPO were seen to be intermingled with GnRH neuronal cells bodies, most of which are also glutamatergic phenotype (only few express *Gad67*), GABAergic nNOS neurons in the ARH were seen to be morphologically associated with GnRH axon terminals.

In conclusion, the results of this immunohistochemical study provide detailed and novel information about the spatial and temporal distribution of nNOS and ERα in the female mouse hypothalamus. These morphological data reveal sites in which nNOS expression changes during postnatal development and critically identifies distinct subsets of nNOS neurons in the hypothalamus according to their neurochemical phenotype (Fig. 7). These data support the role of hypothalamic nNOS in the regulation of reproductive and energy homeostasis functions both in developing and mature animals.

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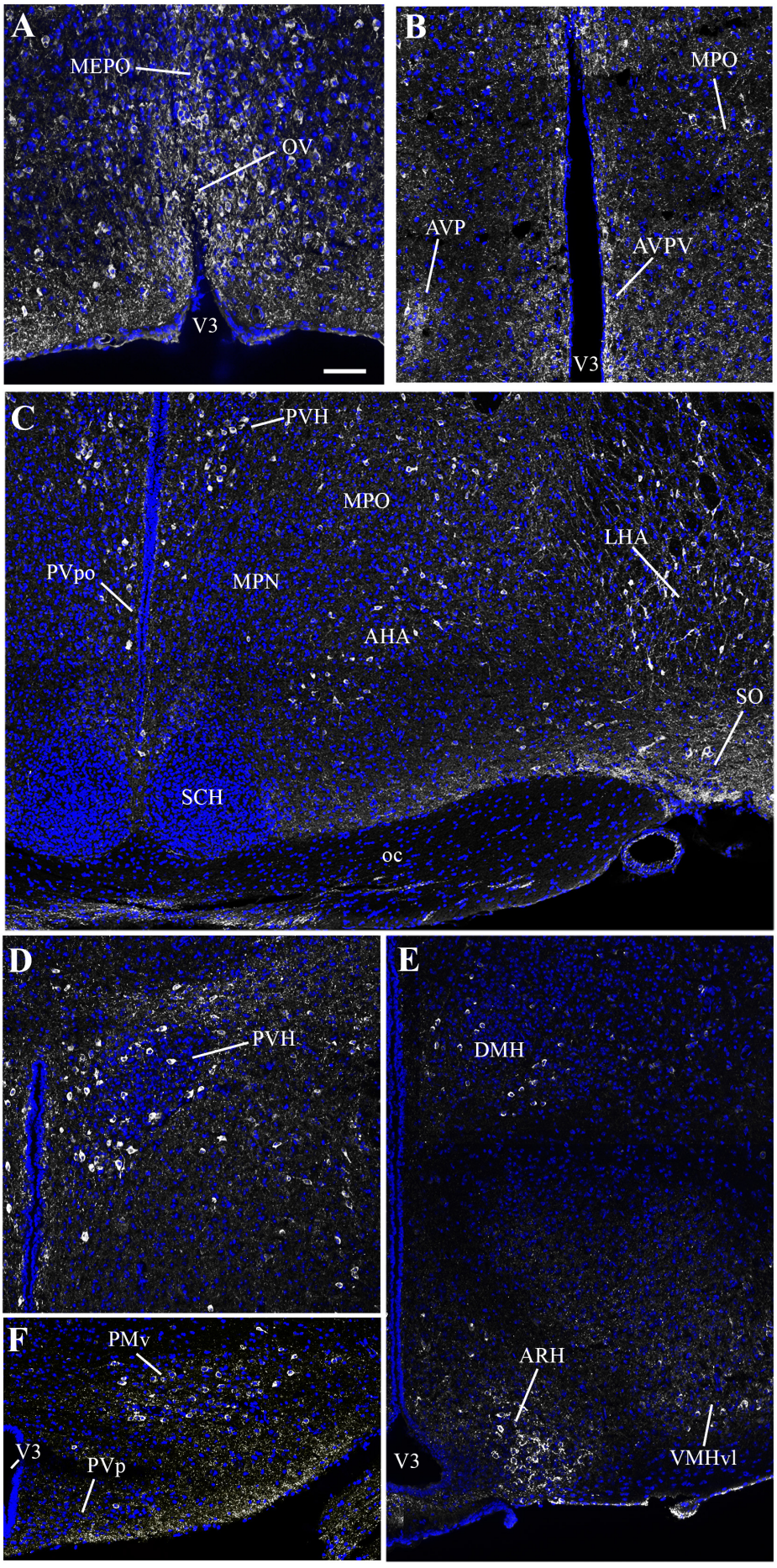
Table 1. Primary antibodies used in this study

Antibody	Animal	Antigen	Manufacturer and cat. no	Characterization
GFP	Chicken, polyclonal	Recombinant full length GFP	Abcam Ab 13970	(Jarvie and Hentges, 2012)
nNOS	Sheep, polyclonal	Full length Rat NOS1 protein	generous gift from Dr. P. C. Emson	(Herbison et al., 1996)
ER-alpha	Rabbit, polyclonal	TYYIPPEAEGFPNTI corresponding to the C-terminus of mouse/rat Estrogen receptor alpha	Millipore 06-935	(Friend et al., 1997)
GnRH	Guinea-pig, polyclonal	mammalian GnRH	generous gift from Dr. E. Hrabovszky	(Hrabovszky et al., 2011)

Table 2. Relative densities of nNOS immunoreactivity in the postnatal and adult brain

	p11	p23	Adult	
LPO: lateral preoptic area	++(+)	++(+)	++(+)	level 16-17
MPO: medial preoptic area	++	++	++	
OV: vascular organ of the lamina terminalis	++++	++++	++++	
MEPO: median preoptic nucleus	+++	+++	+++	
AVPV: anteroventral periventricular nucleus hypothalamus	+	+	+	level 18-19
AVP: anteroventral preoptic nucleus	++	++	++	
MPN: medial preoptic nucleus				
lateral part	+(+)	+(+)	+(+)	
medial part	(+)	(+)	(+)	level 21-23
AHN: anterior hypothalamic nucleus				
central part	++	(+)	(+)	
anterior part	++	++	+	
LHA: lateral hypothalamic area	++	++	+	
SCH: suprachiasmatic hypothalamic area	+	+/-	-	
SON:supraoptic nucleus	+(+)	++	++(+)	
PVHap: paraventricular nucleus, anterior parvicellular part	++	++	++	
Pva: periventricular hypothalamic	+	+	+	

nucelus, anterior part				
PVpo: preoptic periventricular nucleus	++	++	+(+)	
ARH: arcuate nucleus	-	+	+	
VMH: ventromedial hypothalamic nucleus				
dorsomedial part	+/-	+/-	+/-	
central part	-	-	-	level
ventromedial part	++	++	++	28-30
DMH: dorsomedial hypothalamic nucleus				
anterior part	++(+)	++(+)	++(+)	
posterior part	++	++	++	
PVp: periventricular hypothalamic nucleus, posterior part	(+)	(+)	(+)	
PMv: premammilary nucleus, ventral	++	++	++	level 32-33
PMd: premammilary nucleus, dorsal	+(+)	+(+)	++	
PH: posterior hypothalamic nucleus	++	++	+(+)	


A
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OV
V3
B
MPO
AVP
AVPV
V3
C
PVH
MPO
LHA
PVpo
MPN
AHA
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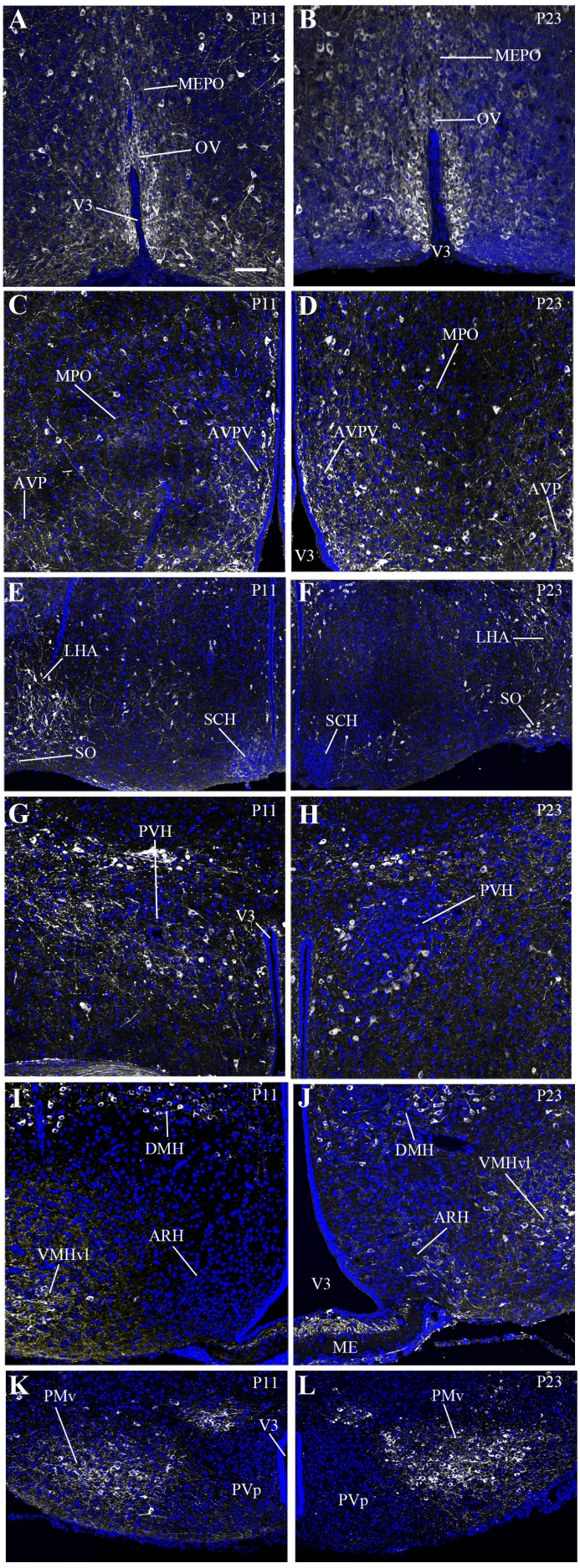
SCH
oc
D
PVH
E
DMH
ARH
V3
VMHvl
F
PMv
V3
PVp

Figure 1. Microphotographs showing the distribution of nNOS immunoreactivity in coronal sections of distinct hypothalamic nuclei in the adult female brain. nNOS immunoreactivity (white labeling) is readily visualized in neurons of the preoptic area; staining is evident in the regions of the organ vasculosum of the lamina terminalis (OV, A), median preoptic nucleus (MEPO, A), anteroventral preoptic nucleus (AVP, B), anteroventral periventricular nucleus (AVPV, B), and medial preoptic region (MPO, B). nNOS-immunoreactivity was also detected in the anterior hypothalamic nucleus (AHN, C), lateral hypothalamic area (LHA, C), supraoptic nucleus (SO, C), with rare neurons also present in the periventricular nucleus of the hypothalamus (PVp, F). nNOS-expressing neurons were also visualized in the paraventricular nucleus of the hypothalamus (PVH, D), the dorsomedial nucleus of the hypothalamus (DMH, E), the ventro-lateral part of the ventromedial nucleus of the hypothalamus (VMH, E), the arcuate nucleus of the hypothalamus (ARH, E) and the ventral perimammillary nucleus (PMv, F). Sections are counterstained using Hoechst (blue) to visualize cell nuclei and identify the morphological limits of each hypothalamic structure. 3V, third ventricle. Scale bar =100 μm in A, B, D and F, and 200 μm in C and E.


A
P11
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PMv
PVp

Figure 2. Representative photomicrographs demonstrating the expression of nNOS immunoreactivity in the infantile and juvenile hypothalamus. Note the absence of nNOS immunoreactivity (white labeling) in the arcuate nucleus of the hypothalamus (ARH) in infantile mice (P11; I) and its appearance in the ARH of juvenile mice (P23; J). On the contrary, nNOS immunoreactivity is present in the suprachiasmatic nucleus (SCH) in infantile mice (E), while it is mostly absent during the juvenile period (F). OV, organ vasculosum of the lamina terminalis (A, B); MEPO, median preoptic nucleus (A, B); AVP, anteroventral preoptic nucleus (C, D); AVPV, anteroventral periventricular nucleus of the hypothalamus (C, D); MPO, medial preoptic area (C, D); PVH, paraventricular nucleus of the hypothalamus (G, H); DMH, dorsomedial nucleus of the hypothalamus (I, J); VMH, ventro-lateral part of the ventromedial nucleus of the hypothalamus (I, J); PMv, ventral perimammillary nucleus (K, L); 3V, third ventricle. Sections are counterstained using Hoechst (blue) to visualize cell nuclei and identify the morphological limits of each hypothalamic structure. 3V, third ventricle. Scale bar =100 μm in A-D, G,H and 150 μm in K, L and 200 μm in E, F and I, J.

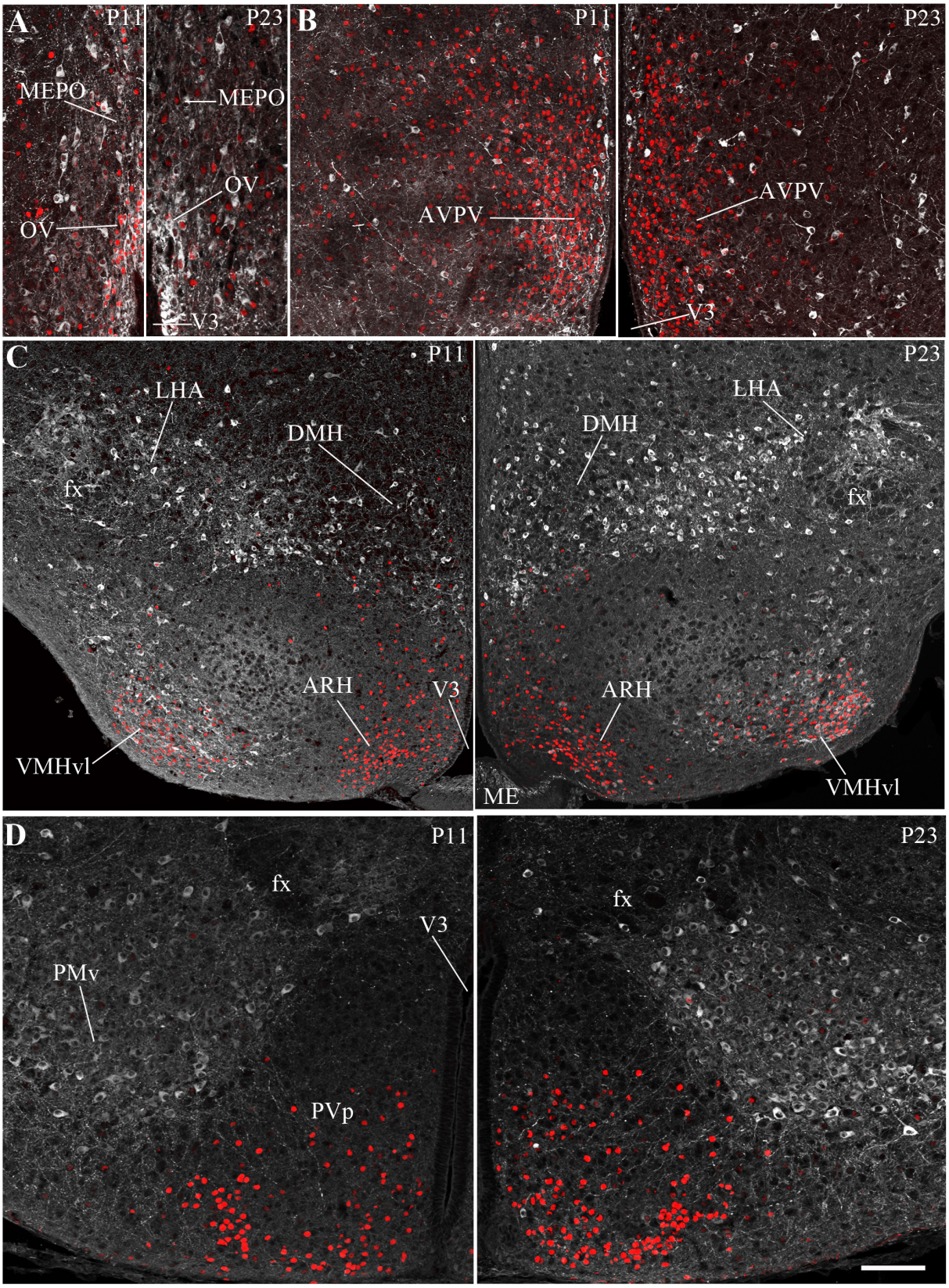


Figure 3. Representative images showing estrogen receptor alpha (ERα)- and nNOS-immunoreactivities in the hypothalamus of P-11 infantile and P-23 juvenile mice. nNOS (white labeling) and ERα (red nuclear staining)

immunoreactivities are highly colocalizing in the regions of the organ vasculosum of the lamina terminalis (OV), medial preoptic nucleus (MEPO) (A), anteroventral periventricular nucleus of the hypothalamus (AVPV, B), and the ventromedial hypothalamic nucleus (VMH) of the tuberal hypothalamus (C), in both infantile and juvenile mice, as well as in the arcuate nucleus of the hypothalamus (ARH) in juvenile mice (C). Scale bar =100 μm in A, D, 150 μm in B and 200 μm in C.

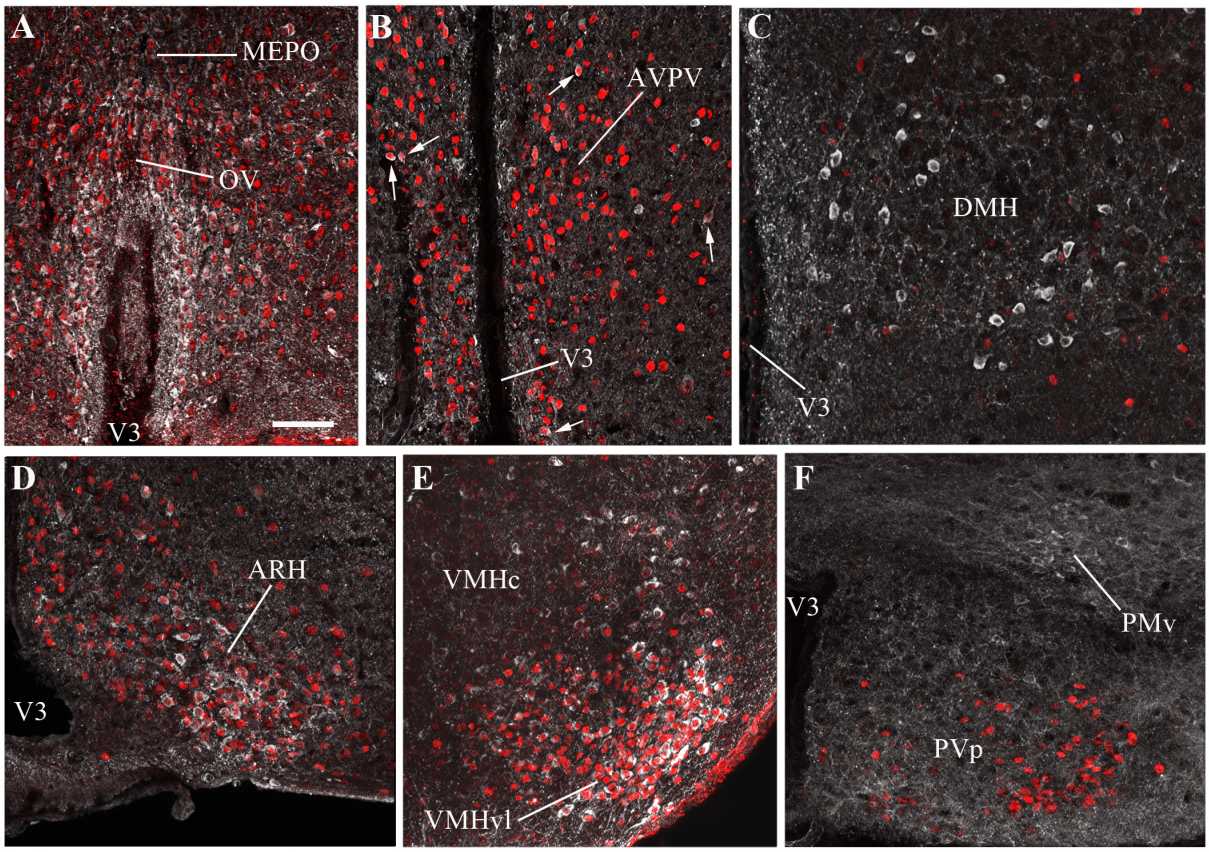


Figure 4. Representative images showing estrogen receptor alpha (ERα)- and nNOS-immunoreactivities in the hypothalamus of adult mice. Similarly to what was observed in juvenile mice (P23), nNOS (white labeling) and ERα (red nuclear staining) immunoreactivities are highly colocalizing in the regions of the organ vasculosum of the lamina terminalis (OV), medial preoptic nucleus (MEPO) (A), anteroventral periventricular nucleus of the hypothalamus (white arrows) (AVPV, B), arcuate nucleus of the hypothalamus (ARH; D) and ventromedial hypothalamic nucleus (VMH; E). On the contrary, colocalization is not evident in either the dorsomedial hypothalamic nucleus (DMH; C) or the periventricular hypothalamic nucleus (PVp; F) and the ventral premammillary nucleus (PMv; F). Scale bar =100 µm.

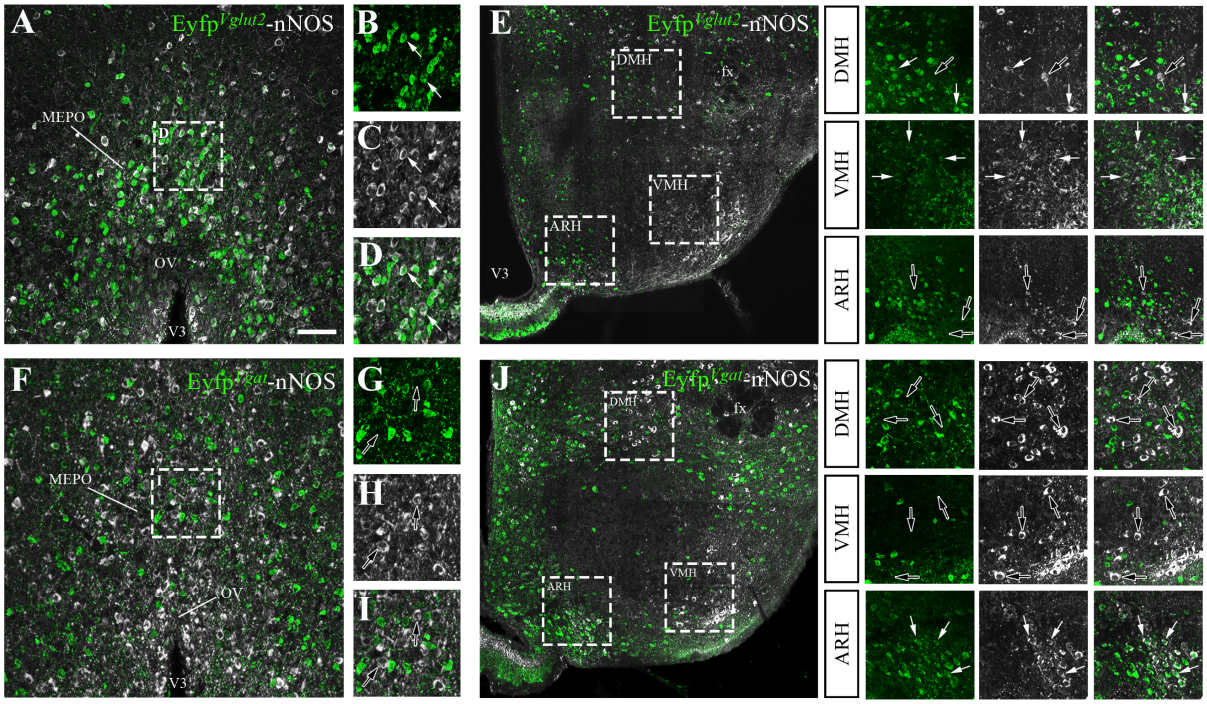


Figure 5. Representative images of nNOS immunoreactivity in *Vglut2::Eyfp*, and *Vgat::Eyfp* adult female mice. nNOS immunoreactive neurons (white labeling) of the preoptic region (A), the dorsomedial nucleus of the hypothalamus (DMH; E, upper inset) and the ventromedial nucleus of the hypothalamus (VMH; E, middle inset) express green-fluorescent-protein immunoreactivity (GFP-IR, green) in *Vglut2::Eyfp* mice, but not in *Vgat::Eyfp* mice (F, J). In contrast, nNOS neurons of the arcuate nucleus of the hypothalamus (ARH) express the GFP-IR in *Vgat::Eyfp* mice (J), but not in *Vglut2::Eyfp* mice (E, lower inset). White arrowheads show nNOS-IR and GFP-IR colocalization; empty arrowheads show nNOS single labeled cells. Scale bar =100 μm (50 μm in insets).

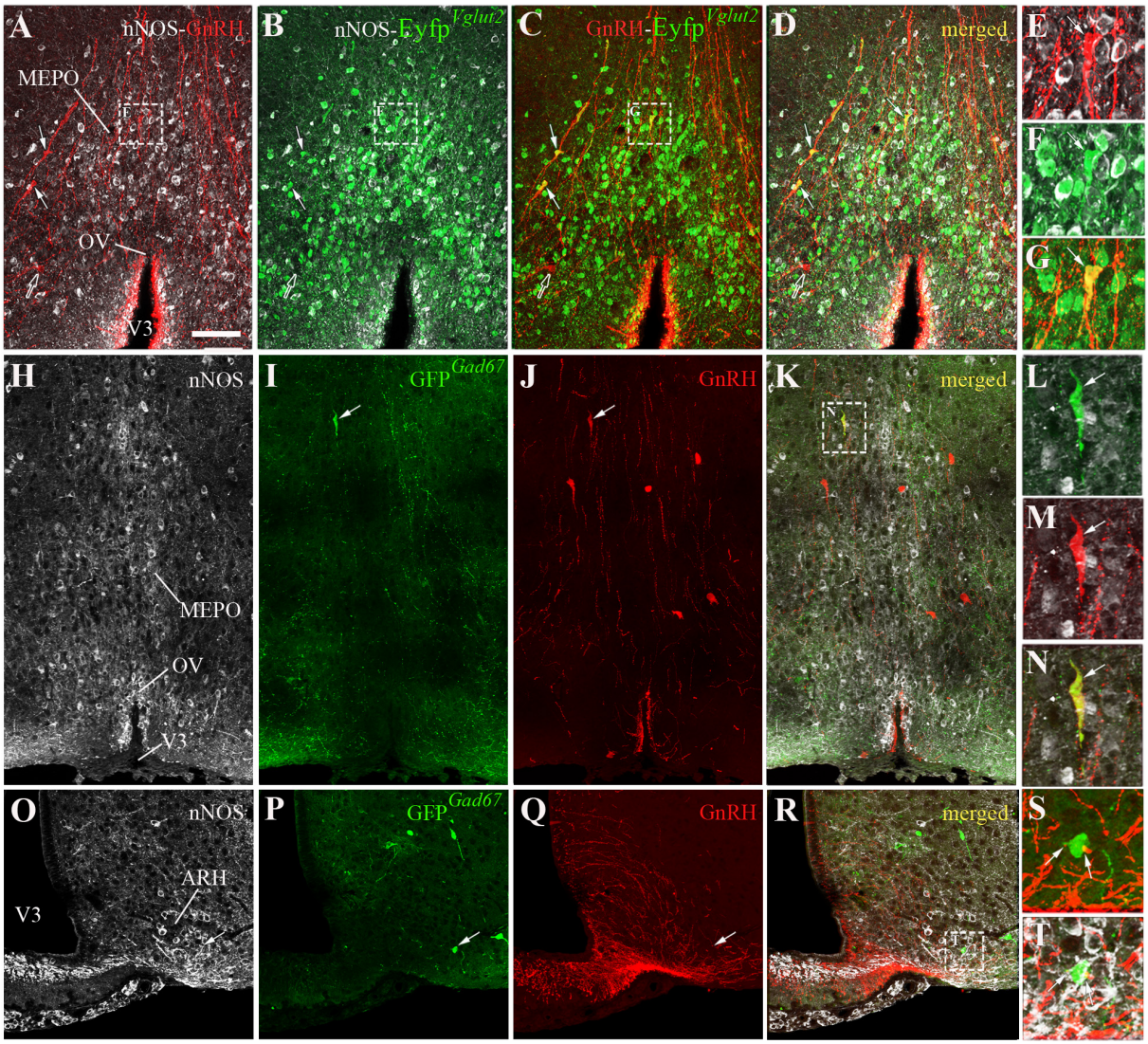


Figure 6. Representative images showing nNOS and GnRH immunoreactive neurons in *Vglut2::Eyfp* and *Gad67::Gfp* female mice. Colocalization of the green-fluorescent-protein immunoreactivity (GFP-IR, green) is evident in both nNOS immunoreactive cells (white; B, F) and GnRH-immunoreactive neurons (red; C, G) in the preoptic region of the adult *Vglut2::Eyfp* female mice (A-G). Expression of the green fluorescent protein (GFP) driven by the *Gad67* promoter is absent in nNOS immunoreactive cells (white) of the preoptic region (H-I), while it is occasionally expressed in GnRH immunoreactive neurons (red; I-N). In the arcuate nucleus of the adult female hypothalamus (ARH) nNOS immunoreactive neurons expressing the GFP^{Gad67} (O-P, T) are surrounded by GnRH immunoreactive fibers (red; Q, S-T). Scale bar =100 μm (25 μm in insets).

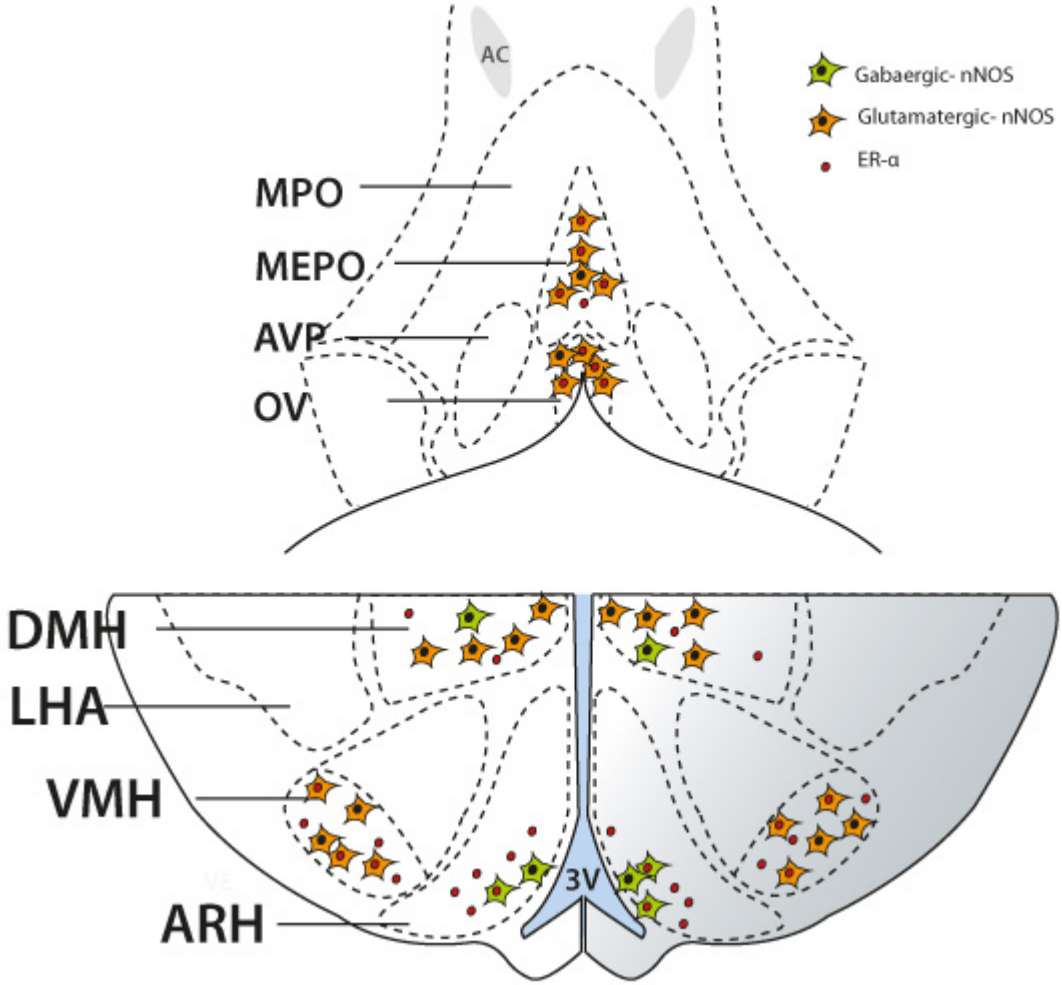
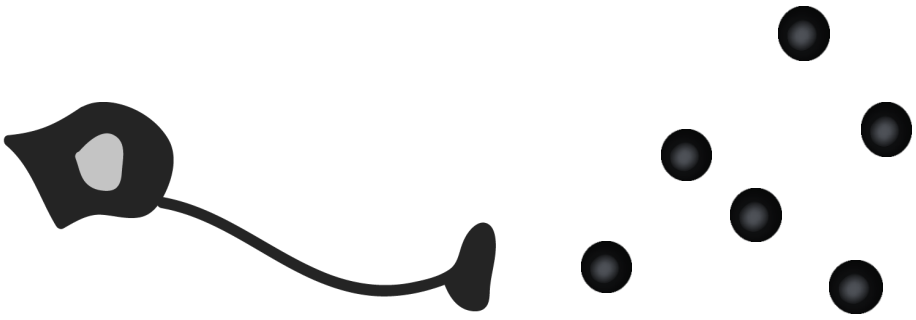


Figure 7. Summarizing scheme demonstrating the distribution and phenotype of nNOS immunoreactive neurons in the preoptic (upper panel) and the tuberal (bottom panel) regions of the hypothalamus of adult female mice. nNOS immunoreactive cells of the organ vasculosum of the lamina terminalis (OV) and the medial preoptic nucleus (MEPO) are in their majority of glutamatergic phenotype (orange) and a great proportion of them express the estrogen receptor α (ERα; red dot). In the tuberal region of the hypothalamus, nNOS immunoreactive neurons are divided in distinct subpopulations depending on the main neurotransmitter they express, and/or the expression of the estrogen receptor. More specifically, in the region of the arcuate nucleus (ARH), nNOS neurons are in their vast majority members of the GABAergic family of cells (green), and a high proportion of them express ERα. In the ventromedial nucleus of the hypothalamus (VMH), nNOS neurons are glutamatergic and most of them express ERα immunoreactivity. The dorsomedial nucleus of the hypothalamus (DMH) contains both GABAergic and

glutamatergic nNOS immunoreactive neurons, with glutamatergic nNOS neurons being the prevailing population residing in the region. Only rare nNOS neuron in the DMH were seen to express ERα.

Chapter VI



6.1 Role of hypothalamic nNOS-derived NO signaling in the control of the GnRH driven maturation of the reproductive axis

Aim

The aim of this study was to determine the functional role of NO signaling in the physiology of reproduction. More specifically, and considering the well-described role of nNOS neurons in the adult fertility, we sought to explore an unidentified action of NO in the events taking place during the minipuberty. More specifically, during this study we 1) identify the presence of NOS1 mutation in hypogonadotropic hypogonadism (CHH) probands, 2) describe a novel role of NO in the migration of GnRH neurons during embryonic development, 3) define the functional relationship between the estrogenic and NO pathways during postnatal development, and 4) characterize the involvement of nNOS neurons in the regulation of GnRH cells during minipuberty.

Principal Results

⇒ missense mutations in the Nos1 gene are linked with the presence of CHH in human probands

⇒ NO signaling is required for the proper migration of GnRH neurons during embryonic development

⇒ constitutive absence of NOS1 in a NOS1 ko model results in a significant delay in the process of sexual maturation

⇒ ablation of NO signaling specifically during the infantile period results in a phenotype of delayed maturation of the reproductive axis, similar to the one described in the NOS1 ko model

⇒ arrival of estrogenic signals in the hypothalamic region during the infantile period are responsible for the maturation of the nNOS neurons residing in the preoptic area

⇒ in turn, nNOS cells are required to set the dialogue between the ovaries and the hypothalamus

⇒ nNOS neurons play a key role in the regulation of GnRH transcription at P12

⇒ nNOS neurons regulate the GnRH release during the infantile period

Conclusion

Our results highlight a key, novel role of nNOS activity in the modulation of the GnRH system at the stages prior to pubertal onset. Our study suggests that NO signaling not only is required for the establishment of the GnRH neuronal network, during embryonic development but also for its regulation during the postnatal development of the axis. More specifically, NO signaling is necessary for the proper migration of the GnRH neurons into the hypothalamic region; Mutations in the Nos1 gene were identified in probands of CHH patients, supporting for a pivotal role of nNOS neurons in the coordination of the GnRH cells. During minipuberty nNOS could be of pivotal importance for the modulation of the FSH surge, playing a key role in filtering the negative and positive action of estrogens on the GnRH system during the infantile period. Overall, our results identify nNOS neurons as important regulators of the maturation of the hypothalamic-pituitary-gonadal axis.

Role of hypothalamic nNOS-derived NO signaling in the control of the GnRH driven maturation of the reproductive axis

Pilot study

ABSTRACT

GnRH neurons are undeniably the master regulators of the reproductive system. For them to exert their pivotal role in the establishment of a fertile phenotype, their activity and neurosecretory capacity needs to be precisely regulated by upstream pathways. Identification of a series of mutations on the Nos1 human gene in patients with Constitutive hypogonadotropic hypogonadism (CHH), as well as in probands related to Constitutional delay of growth and puberty (CDGP), not only highlights a key role of nNOS in the establishment of a fertile phenotype, but most importantly, paves the way for a better understanding of conditions of human idiopathic infertility. To this aim, we present evidence supporting a novel role of hypothalamic NO signaling in the regulation of the GnRH neuronal population: during embryonic development NO is required for the proper migration of GnRH cells, while postnatally, during the infantile period, it controls the transcriptional activation of Gnrh gene, and coordinates the events leading to the sexual maturation and the acquisition of a fertile phenotype.

INTRODUCTION

Sexual maturation, puberty and subsequent adult fertility, are all regulated by complex events taking place primarily in the hypothalamus, where several neuronal and hormonal pathways converge in order to regulate the biosynthetic capacity, neurosecretory pattern and morphology of GnRH neurons (Prevot, 2015). Maturation and function of the reproductive axis is a result of a long process, involving the timely regulation of events during the embryonic, infantile, pre-pubertal, and finally, the adult developmental stages. A prerequisite for the normal function of the axis is the proper migration of GnRH neurons from the olfactory placode, and their establishment into their principal site of residence, the hypothalamic preoptic region (Schwanzel-Fukuda et al., 1989). Defects in either the migration of GnRH neurons, or later on, their ability to secrete the GnRH peptide, result in severe infertility and delay in pubertal onset, and are related to a series of genetic disorders known as Congenital hypogonadotropic hypogonadism (CHH). Postnatally, the first activation of the GnRH axis is often referred to as mini-puberty and occurs during the infantile period, resulting in the surge of follicle stimulating hormone (FSH) that stimulates the growth of the first pool of ovarian follicles, destined to ovulate at puberty (Kuiri-Hänninen et al., 2014; Prevot, 2015). Pre-pubertally, during the early juvenile period, increased production of estrogens, is responsible for the initiation of a feedback loop eventually leading to the temporally-regulated activation of the GnRH system and thus pubertal onset (Andrews et al., 1981). Finally, in the adult brain, GnRH neurons act as master regulators of neuroendocrine signals receiving afferent neuronal connections, involving numerous neuropeptides and hormones, in order to govern communication between the ovaries, the pituitary and the brain, thus regulating the reproductive axis and ovarian cyclicity (Levine, 2015; Zeleznik and Plant, 2015). Even though several regulators of the GnRH neuronal fate have been identified, our

knowledge concerning the events leading to the timely acquisition of reproductive capacity is rather limited.

Nitric oxide (NO) is a gaseous neuromodulator produced by the nitric oxide synthesizing (NOS) proteins, and has long now been implicated in the central control of adult reproductive function (Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994). nNOS-expressing neurons of the hypothalamus have been demonstrated to not only interact with important players of the neuroendocrine brain, such as leptin, estrogen and kisspeptin (Parkash et al., 2010; Hanchate et al., 2012; Bellefontaine et al., 2014), but also to directly modulate GnRH neuronal activity and secretion (Clasadonte et al., 2008), while they were recently suggested to be part of the transcriptional regulation of the Gnrh promoter (Messina et al., 2016). In agreement with the above findings, mice deficient in nNOS protein are infertile (Gyurko et al., 2002b), reminiscent of the congenital hypogonadotropic hypogonadism condition in humans (Boehm et al., 2015). Overall, the role of NOS-containing neurons in the central control of the adult reproductive function has been well established. What remains to be determined; however, is whether NO could act in the immature reproductive axis, forming part of the mechanism regulating the clock-like precision of pubertal activation.

In this study we present a series of Nos1 gene mutations as these were identified in CHH and KS probands, as well as in patients with a Constitutional delay of growth and puberty (CDGP). We challenge the idea that NO is actively participating in sculpting the GnRH neuronal network, and is thus regulating sexual maturation and reproductive capacity. Furthermore, we use a novel cGMP-biosensor (FlincG3) (Wood et al., 2011; Bhargava et al., 2013) to accurately measure the amount of NO being released in the hypothalamus under physiological or pathophysiological conditions (i.e. when NOS1 protein is mutated). Our preliminary data demonstrate an important role of nitric oxide signaling during embryogenesis in the migration of GnRH, but also later on, during infantile period, in the

regulation of the events leading to the activation of the GnRH system and the subsequent initiation of puberty. We thus provide new insights into the mechanisms by which the alteration of GnRH neuronal function leads to hypogonadotropic hypogonadism that raise the intriguing possibility that NO could be a potential target for therapeutic strategies against debilitating conditions.

RESULTS

Mutations in the human Nos1 gene linked to human cases of hypogonadotropic hypogonadism

Kallmann syndrome (KS) and Congenital hypogonadotropic hypogonadism (CHH) are a group of rare disorders responsible for lack of pubertal maturation and infertility due to a defective GnRH secretion/action, subsequently resulting to insufficient production of the pituitary gonadotropins LH and FSH (Seminara et al., 1998; Bianco and Kaiser, 2009). KS patients, in contrast to the cases of CHH, present a unique combination of GnRH deficiency, arising from a faulty migration of the neuronal population, and anosmia (Kallmann, 1944; Schwanzel-Fukuda et al., 1989). Identification of genetic abnormalities related to KS/CHH has provided major insights into the pathways critical for the development, maturation and function of the gonadotrope axis, yet 50% of the cases are of unidentified origins (Boehm et al., 2015; Kim, 2015). Targeted genetic studies on KS and CHH patients revealed 7 heterozygous mutations in the Nos1 gene in 8 KS and 4 CHH probands, some of them being also present in patients with Constitutional delay of growth and puberty (CDGP): p.I1223M, p.R1001W, p.N725D, p.M619L, p.G595S, p.D394A, p.R260Q, p.D241N (Table 1). One mutation was identified in an unaffected individual (p.Y711C), still being significantly enriched in the patient cohort comparing to ExAC controls. All the above identified codon

alterations were located on different exons of the Nos1 gene, each leading to a distinct missense mutation on the protein sequence, affecting different domains of the NOS1 product (Fig. 1).

In vitro measurement of NO response elicited by the wildtype and mutated forms of NOS1

There have been many attempts to detect the NO concentration in cells and tissues (Hall and Garthwaite, 2009), but the huge variability in NO concentrations reported by different groups has been disappointing. Only a few years ago, Nausch et al., developed a new fluorescent cGMP biosensor named δ-FlincG, based on a previously described FRET- type cGMP sensor (Sato et al., 2006). δ-FlincG consists of the dual cGMP binding domain from cGMP dependent protein kinase fused to circularly permutated EGFP (Nausch et al., 2008). Its sensitivity to cGMP, which is in the submicromolar level, along with its selectivity for cGMP over cAMP and its rapid activation and deactivation kinetics, render this biosensor able to readout changes in the cellular cGMP concentration, and has been used in several cell lines and different experimental set ups, in a very efficacious way (Batchelor et al., 2010; Wood et al., 2011; Bhargava et al., 2013). More recently, the generation of a new FlincG variant (FlincG3), allows for an improved sensitivity and a superior dynamic range in the cGMP response (Bhargava et al., 2013). One of the cell lines already used as NO-detectors upon FlincG transfection is the HEKGC/PDE5 cells; even though HEK cells do not physiologically express NOS1, they express the sGC and PDE5, the two main components (apart from NOS) of the NO signaling pathway (Batchelor et al., 2010; Wood et al., 2011; Bhargava et al., 2013). In this first series of pilot experiments, we aimed to study the activity of the mutated forms of the NOS1 protein by measuring their ability to produce NO upon endogenous activation of the pathway, taking advantage of HEK-FlincG3 expressing cells. Endogenous activation of neuronal muscarinic acetylcholine receptors has been demonstrated to result in

the activation of NOS (Wang et al., 1994; Garthwaite, 1991) and HEK cells are known to express the muscarinic acetylcholine receptor M3 (Rümenapp et al., 2001; Luo et al., 2008).

Of the 9 mutations identified in CHH probands (table 1), p.R1001W, p.Y711D, p.G595S, and p.D394A, along with the plasmid expressing the wildtype sequence of the human Nos1 gene (referred to as wildtype), were chosen for the first series of pilot experiments. Co-transfection of HEK cells with FlincG3 and the wildtype or mutated sequence of the NOS1 yielded fluorescence levels similar to the ones seen when FlincG3 is singly transfected (Fig. 2a). Successful transfection of the NOS1 wildtype, or mutated forms was evaluated by immunofluorescence against the NOS1 protein and/or against the myc tag present in the mutated Nos1 plasmids (Fig.2b, c).

Stimulation of the endogenous NO production by superfusion of acetylcholine (100 uM) elicited a seemingly rapid fluorescence response that reached a peak within the first minute of the application in both the WT and mutated plasmids. Fluorescence response faded during the application (after about 1min), before recovering to baseline values on washout of acetylcholine. In the presence of either the NO synthase inhibitor L-nitroarginine (30μM), or the guanylate cyclase inhibitor ODQ (1 μM), the increase in fluorescence upon stimulation with Ach was abolished (data not shown), suggesting that the yielded fluorescence was indeed a result of cGMP produced via the activation of the NO pathway. Superfusion of a high concentration of the NO donor PAPA/NO (100 nM) at the end of the experiment allowed us to estimate the peak of the acetylcholine-evoked increase in fluorescence using the Hill equation and the published concentration-response curve parameters in FlincG3-transfected HEKGC/PDE5 cells (Bhargava et al., 2013) (Table 2).

Cells transfected with the wildtype form of the NOS1 responded in a constant way to application of ACh, yielding each time a response that corresponded to about 430 pM NO

(Fig. 3a; n= 2; assay no1: 400 pM, assay no2: 461 pM). The mutated cell cultures were responding to the application of ACh in a quite heterogeneous way (Fig. 3). In more detail:

Stimulation of mutant 117680472 (p.R1001W) yielded an increase in fluorescence that corresponded to about 356 pM NO (n= 1) (Fig 3b).

Stimulation of mutant 117703125 (p.Y711D) yielded an increase in fluorescence that corresponded to about 826, 95 pM NO (n= 2; assay no1: 191 pM , assay no2: 1,6 nM) (Fig 3c).

Stimulation of mutant 117710246 (p.G595S) yielded an increase in fluorescence that corresponded to about 125 pM NO (n= 1) (Fig 3d).

Finally, stimulation of mutant 117724018 (p.D394A) yielded an increase in fluorescence that corresponded to about 256 pM NO (n= 2; assay no1: 259 pM , assay no2: 253 pM) (Fig 3e).

Apart from the mutant p.Y711D that gave a highly divergent response (assay no1: 191 pM , assay no2: 1,6 nM), the rest of the duplicates of the assays were highly similar in matters of NO concentration released in each assay. Even though the mutations studied here seem to result in a weaker NO release, more replicates (independent transfection assays/ live cell imaging experiments) will be needed before to conclude on the capacity of mutated NOS1 to produce NO.

NO release can modulate GnRH neuronal migration during embryonic development

The finding that 8 probands presented KS is consistent with the existence of severe GnRH deficiency resulting from faulty migration of GnRH neurons from the olfactory placode in the forebrain. Considering the established role of NO in the regulation of GnRH neuronal activity and, as we demonstrated in the following study, its transcriptional regulation, we sought to investigate the possible involvement of NO in the migratory process of GnRH neurons during embryonic development. Immunohistochemical analyses of the distribution of nNOS-

immunoreactivity with respect to GnRH-immunoreactity strikingly revealed that nNOS and GnRH were co-expressed in migrating GnRH neurons both in the mouse embryo and in the human fetus (Fig. 5a). To begin to investigate the putative role of nNOS activity on GnRH neuronal migration, we blunted NO production in the nose of mouse embryos during the gestational day E12.5, when GnRH cells are just starting to penetrate the rostral forebrain, by injecting the NOS inhibitor L-NAME in the nose of living mouse embryos. 2 days after, as expected at E14.5, immunohistochemistry against GnRH in fixed sagittal sections of saline-injected embryos confirmed the presence of almost half of GnRH neurons in the region of the nose, while a significant population of GnRH cells had already successfully migrated in the region of the forebrain (Fig. 5d, e; 37,1 % ± 2.3 nose, 31.4 % ± 2.6 ob, and 31.7 % ± 2.3 vfb, n= 5). In contrast to the physiological migratory profile seen in the control group (Suppl. Fig 1a), L-NAME injection resulted in a major perturbation of the migratory process (Suppl. Fig. 1b). GnRH neurons were found in their vast majority as parts of aggregates constrained in the region of the nose (Fig. 5d, e; 56 % ± 5.6 nose, 28.4 % ± 3.4 ob, and 15.6 % ± 3.4 vfb, n= 4). Notably, only a minor percentage of GnRH cells was seen to have successfully migrated into the forebrain (Fig. 5d; LNAME nose vs vfb: one-way ANOVA, $p<0.001$, n=4). Intriguingly, this altered migratory pattern of GnRH neurons as exhibited upon inhibition of NO production, was accompanied by a significantly increased total number of GnRH cells, comparing to the amount of cells quantified in control embryonic tissue (Fig. 5c; total number of GnRH-ir cells in control vs LNAME-injected: $t_7= 3.730$, $p= 0.0074$, n=4-5 per group).

Role of nNOS in the maturation of the reproductive axis

Several of the newly identified mutations presented above were associated with the appearance of a Constitutional delay of growth and puberty (CDGP), pointing towards a delay in the activation of the HPG axis upon deregulation of the NO signaling pathway. Studies

coming from Nos-1 null mice have already demonstrated that nNOS expression is necessary for fertility in males and females, with hypogonadotropic hypogonadism being the most obvious phenotype of the mice lacking nNOS catalytic activity (Gyurko et al., 2002b). NOS activity specifically in the hypothalamic preoptic area, has been suggested to be crucial for the initiation of LH release in adult female mice (d'Anglemont de Tassigny et al., 2007a). To determine if the presence of nNOS protein is also required for the maturation of the reproductive axis we studied the vaginal opening and first estrous of Nos1 -/- mice, and their Nos1 +/- and Nos1 +/+ littermates. Nos1-null female mice underwent vaginal opening significantly later than Nos1 +/- and Nos1 +/+ mice (Fig.6a; one-way ANOVA, p= 0.0160, n=5-7 per group). Observation of vaginal smears revealed that loss of Nos1 protein results in a massive delay in the onset of puberty, with several Nos1 -/- females exhibiting pubertal onset only after reaching 3 months of age (Fig. 6b; one-way ANOVA, p= 0.0056, n=5-7 per group). Interestingly, this delay in the maturation of the reproductive axis was accompanied by a significantly liner phenotype of the nNOS -/- females comparing to both their wildtype and their Nos1 +/- littermates (Fig. 6c; one-way ANOVA, p= 0.0003, n=5-7 per group). Based on the above results, and considering the importance of nutritional status for the initiation of puberty and the proper regulation of sexual maturation, we could not exclude the possibility that NOS activity is indirectly affecting puberty onset via other metabolic pathways, such as via mediation of leptinergic signals, as demonstrated in the last manuscript presented in this thesis.

Postnatal activation of nNOS in the preoptic hypothalamic region

In a recent study we demonstrated that NOS activity significantly increases in the preoptic region during infantile period (Messina et al., 2016), a time known to be crucial for the establishment of the GnRH network (Bouret et al., 2004a; Caron et al., 2012a). To better

determine the time window during which the levels of NOS activity are being differentially regulated, and thus identify how NO release is being controlled during the stages prior to pubertal onset, we measured the levels of p-nNOS-ir in the OVLT during several different developmental stages (P7, P10, P12, P21 and P30). Immunoreactivity against nNOS protein remained unaltered throughout development, agreeing with the study presented in Chapter V (Fig. 7a). In contrast to the constitutive expression of nNOS protein in the OVLT, levels of p-nNOS-ir were almost undetectable till postnatal day 7, pointing towards a low nNOS activity prior to that age, and thus an awakening of the pathway during the later stages of the infantile period (Fig. 7a). Intriguingly, Intriguingly, the proportion of cells colocalizing p-nNOS and nNOS immunoreactivities increased along with the development of the axis (Fig.7 a,b; one-way ANOVA, p=0.018, n=3-4 per group), suggesting that the maturation of the nNOS neuronal network residing in the OVLT, takes place concomitantly to the maturation of the reproductive axis. Whilst, the difference in the ratio of nNOS/p-nNOS-ir reached significance only during the infantile period, exhibiting an important increase during P12 (P10 vs P12: t_4= 5.053, p= 0.0072, n= 3) and continuing to gradually increase during the juvenile period (P12 vs P21: t_5= 1.984, p= 0.1040, n= 3-4 per group; P12 vs P30: t_4= 1.641, p= 0.1761, n= 3 per group).

Role of infantile activation of nNOS on the maturation of reproductive axis and the regulation of reproductive capacity

As we demonstrated above, the ratio of nNOS/p-nNOS was significantly elevated during postnatal day 12, suggesting that the activity of the nNOS protein in the preoptic area prior to this developmental stage probably results only to a minor release of NO. To further depict the involvement of NOS activity in the process of reproductive maturation we blunted NO release in wildtype female mice from postnatal day 10 (when around 6% of the nNOS protein is

phosphorylated in the OVLT/MePO) till the end of the infantile period (P21). Daily injections of the NOS inhibitor LNAME markedly delayed the vaginal opening of mice when compared to their saline- injected littermates (Fig. 8a; t_{15}= 3.017, p= 0.0078, n= 9-10 per group). L-NAME injected mice exhibited a delayed pubertal onset (Fig. 8b; t_{17}= 2.343, p= 0.0213, n= 9-10 per group), while their body weight remained lower throughout life, with the difference becoming significant during the juvenile period, till, approximately, the time of pubertal onset (Fig. 8c; P26-40, 2way-ANOVA $p<0.05$, n= 9-10 per group). Interestingly, L-NAME injections during an earlier time window (P7-P12) did not result in a delay in either the vaginal opening, or the initiation of puberty ($p>0.2$, n= 5 per group), whilst lead to a significantly decreased body weight (P24-38, 2way-ANOVA $p<0.05$, n=5 per group), reminiscent to the one seen after P12-P21 LNAME injections (Suppl. Fig. 2). The diversity in the result of NO blockage according to the time window it was induced, points towards a distinct regulation of metabolic and reproductive axes by the NO signaling pathway. To determine whether the inactivation of NO signaling pathway during this critical time window could, apart from affecting pubertal onset, also induce changes to the overall process of sexual maturation thus eventually affecting the adult reproductive axis, we followed the estrous cyclicity of these P10-P21 LNAME-injected mice and their saline-injected littermates. While saline-injected mice did not exhibit any marked alteration of their estrous cycles, mice treated with LNAME showed a prolongation in the number of days spent in diestrus (t_7= 3.367, p= 0.0120, n= 7-8 per group), while they exhibited a fewer number of successful ovulatory events (Fig. 8d; t_7= 2.906, p= 0.0228, n= 7-8 per group). In line with this observation, proestrous LH showed a typical surge in adult (P75-90) controls but not in LNAME-treated mice (Fig. 8e; t_8= 3.944, p= 0.0043, n= 5 per group). Measurement of the fertility index of both groups in a period of 120 days revealed that blunting of NO release during the infantile

period resulted in a modest subfertility, agreeing with the events of unsuccessful ovulation observed in our LNAME-injected animals (Fig. 8f).

Effect of gonadal maturation on the activation of NO signaling during the infantile period

During the infantile period there is a marked rise in the production of estrogens by the ovary (Fortune and Eppig, 1979; Funkesnstein et al., 1980). This increase in the steroidogenic activity follows the peak of FSH levels at postnatal day 12 (Prevot et al., 2003b), and is thus responsible for the negative feedback of ovarian estradiol onto the hypothalamus, and hence the regulation of GnRH secretion (Prevot et al., 2005). Interestingly, the nNOS-expressing neurons of the POA have been demonstrated to express ERα throughout development (*Fig 3 of our previous chapter*); even during the neonatal period, when the nNOS population of the region is quiescent, these neurons can sense changes in the levels of estrogen (Suppl. Fig. 2). We asked the question of whether the activation of the gonads could be triggering the infantile activation of the estrogen- responsive, NO signaling pathway in the preoptic hypothalamic area. Ovariectomy of mice during postnatal day 12 allowed us to inhibit the action of estrogens on the hypothalamus during the time when p-nNOS levels were seen to significantly increase. Immunohistochemistry against p-nNOS at P21 OVX-mice and their control littermates, revealed a significant drop in the phosphorylation levels of nNOS in the OVLT (Fig.9; t_5= 3.783, p= 0.0129, n= 3-4 per group). The altered phosphorylation levels of the nNOS protein upon ovariectomy, around the time when estrogen negative feedback becomes operational in the hypothalamus, suggest a role of ovarian estrogen in the maturation of the NOS-expressing neurons of the preoptic area.

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nNOS activity is required to set the dialogue between the ovaries and the hypothalamus

To further dissect the role of NO in the negative feedback action of estradiol on the hypothalamic axis, we examined the effects of ovariectomy in saline-injected animals and mice treated with LNAME during the P10-P21 time window we identified above. Under physiological conditions, ovariectomy is known to induce an increase in the circulating levels of LH by relieving the hypothalamic-pituitary unit of the steroid-dependent inhibitory feedback control (Ojeda and Skinner, 2006). Mice were ovariectomized at P23 and sacrificed 96h later as previously described (Caron et al., 2012b). Ovariectomy raised the LH levels from their nadir values in both saline and LNAME-treated mice. Counterintuitively, inhibition of NOS markedly increased the plasma LH surge in LNAME-injected mice comparing to their saline-injected littermates (Fig.10; one-way ANOVA, p= 0.000, n=6,12 and 11 per group; t_{21}= 2.170, p= 0.0416 saline-injected OVX vs. LNAME-injected OVX, n= 11-12 per group). Overall, these results suggest an important role of nNOS activity for the action of ovarian estradiol on the hypothalamic axis during the infantile and early juvenile period.

Absence of nNOS protein results in increased FSH surge during postnatal day 12

According to our results, nNOS activity is in fact required for the action of ovarian estradiol on the hypothalamic axis during the stages prior to puberty. With the aim to further investigate the role of this nNOS/ estrogen interaction during the infantile and juvenile period we measured the levels of FSH in Nos1 +/+, Nos1 +/- and Nos1 -/- female mice during postnatal day 12, the time of the infantile FSH peak, as well as during the early juvenile period (P23), when the FSH levels are known to decrease to nadir values (Dohler and Wuttke, 1975). Constitutive absence of nNOS protein resulted in a massive increase in the FSH levels of P12 female mice comparing to their wildtype littermates, while the Nos1 +/- animals exhibited an intermediate phenotype (Fig. 11a; one-way ANOVA, p=0.0265, n=3-9 per

group). As expected, at the age of P23 the negative feedback action of ovarian estradiol on the hypothalamus resulted in a decrease of FSH levels in both Nos1 +/+ and Nos1 -/- (P12 vs P23: Nos1+/+, t_{10}= 3.696, p= 0.0041, n=5-7 per group; Nos1 -/-, t_6= 3.346, p= 0.0155, n=3-5 per group). However, FSH levels in P23 Nos1 -/- mice remained higher comparing to the levels measured in the Nos1 +/+ females, though the difference did not reach significance (Fig. 10b; one-way ANOVA, p= 0.0641, n= 5-9 per group).

Absence of nNOS protein results in an increase of GnRH expression levels

The higher FSH levels detected in our P12 Nos1 -/- mice indicate an increased secretion of GnRH peptide from the hypothalamus, and suggest a possible involvement of nNOS in the control of GnRH release. In fact, recent data have implicated infantile NO in the transcriptional regulation of the GnRH promoter (Messina et al., 2016), further supporting our hypothesis. To determine whether nNOS could directly regulate GnRH transcription, we verified the expression profile of the Gnrh transcript in GnRH neurons isolated by fluorescence-activated cell sorting (FACS) in P12 Nos1 +/+; Gnrh::Gfp, Nos +/-; Gnrh::Gfp and Nos1 -/-; Gnrh::Gfp mice. Real-time PCR analyses of FACS-isolated GFP-expressing GnRH neurons revealed a 4-fold increase in the Gnrh mRNA expression between Nos1 +/+ and Nos1 -/- mice (Fig. 11d; t_{14}= 3.971, p= 0.014, n=8), while Nos1 +/- exhibited an intermediate expression level (one-way ANOVA, p=0.0162, n=8). The increased GnRH mRNA expression in the absence of Nos1 activity (Nos1-null mice) agrees with our observation of increased GnRH content following blunting of NO release during embryonic development and further supports a role of nNOS in the inhibition of the GnRH system.

DISCUSSION

GnRH neurons are suggested to be the master regulators of the hypothalamic-pituitary-gonadal axis (HPG). Their role in orchestrating all the main reproductive aspects of the organism, i.e. sexual maturation, puberty and subsequent adult fertility, dictates a precise and dynamic regulation of their biosynthetic capacity and neurosecretory pattern. In other words, for GnRH neurons to fulfill their purpose in the regulation of the reproductive axis, a series of complex maturational events, initiated upstream of the GnRH neuronal network, need to occur. Our study identifies for the first time NO as an upstream regulator of the GnRH neuronal system, demonstrating a requirement of nNOS activity for the overall development of the reproductive axis and the maturation of the neuroendocrine populations implicated in the onset of puberty and the establishment of a fertile phenotype.

Genetic studies on KS and CHH patients revealed in total 9 missense mutations on the gene encoding for the NOS1 human protein, located mainly in the oxygenase and reductase domain of the NOS1 protein. More specifically Nos1 mutations were identified in 12 cases presenting hypogonadotropic hypogonadism, with the majority of them being prevalent in Kallmann syndrome (KS) probands. KS is a rare congenital genetic condition presenting a unique combination of GnRH deficiency, arising from a faulty migration of the GnRH neuronal population, and anosmia (Kallmann, 1944; Schwanzel-Fukuda et al., 1989). GnRH neurons, initially originating from the olfactory placode, are already located into their principal site of residence, the hypothalamic preoptic region, at postnatal day (P) 0. However, at embryonic day (E) 14.5 the GnRH neuronal population is still in the process of migration, with half of the migrating GnRH neurons being still in the nose and the other half having reached the ventral forebrain (Wray et al., 1989). Several mutations on proteins implicated in different

steps of this migratory process have been identified previously in KS probands (Matsuo et al., 2000; de Roux et al., 2003; Kim, 2015). The prevalence of NOS1 mutations in cases where GnRH deficiency is the main molecular phenotype, creates a link between nNOS impaired activity and GnRH defective release, and thus reinforces our findings of an nNOS-driven regulation of GnRH activity in the rodent postnatal brain. Focusing though on the migratory defect present in the KS we sought to investigate a possible involvement of NO prior to infantile period, in the migration of GnRH neurons during embryonic development. Our neuroanatomical studies reported the unprecedented findings that nNOS is expressed by GnRH neurons during their migration in the nasal compartment and that this nNOS expression gets extinguished once the GnRH neurons have reached the brain. Blunting NO release by in utero injection of the NOS inhibitor in the nose of embryonic day (E) 12.5 mouse embryos, resulted in a substantial perturbation in the migratory process of GnRH neurons. During the embryonic day (E) 14.5, in contrast with a almost evenly divided population of GnRH migrating cells seen in our saline-injected embryos, NO invalidation resulted in the formation of GnRH aggregates in the region of the nose, prohibiting thus the migration of these cells in the ventral forebrain region. Our pilot in vitro study results suggest that mutation of the NOS1 protein, at least when affecting the NOS1 protein domains mentioned in our results, leads to decreased enzymatic capacity of the nNOS, and as such to smaller amounts of NO being released by the protein. Hence, we could imagine that an abnormal release of NO because of the existence of a mutated NOS1 protein results in a failure of GnRH neurons to reach hypothalamus, and is the main cause of the migratory defect seen in KS patients bearing the newly-identified NOS1 mutations.

NOS-expressing neurons are an integral part of the hypothalamic GnRH neuronal network controlling ovarian cyclicity and ovulation, with NO being considered a key molecule in the preovulatory GnRH/LH surge. In vitro, application of a NO donor was shown to stimulate

GnRH release via the sGC-cGMP pathway, while in vivo, intraventricular inhibition of NOS activity diminished the pulsatile release of LH (Moretto et al., 1993; Rettori et al., 1993). More recently, chronic inhibition of NOS activity specifically in the preoptic hypothalamic area was shown to disrupt rat estrous cyclicity (d'Anglemont de Tassigny et al., 2007a). Out of the 9 heterozygous mutations in the Nos1 gene presented above, 7 were also correlated with the appearance of syndromes related to a delayed or completely absent pubertal onset. We presented evidence supporting a significant role of NO signaling prior to puberty, governing the events that will enable the acquisition of a fertile phenotype. Constitutive absence of Nos1 protein was shown to result in a severe delay in the sexual maturation of the female system, as suggested by the delayed vaginal opening and pubertal onset observed in our Nos1 -/- female mice. Interestingly though, NO exerts its role on the tuning of pubertal onset in a timely-regulated manner, acting during a restricted time window of the infantile period, known as mini-puberty. Transient ablation of NOS activity specifically during the infantile period (P10-P21) replicated the reproductive phenotype seen in the constitutive Nos1 knockout mouse model. Female mice demonstrated a delayed pubertal onset and a severely perturbed reproductive capacity even during adulthood, suggesting that NO signaling is required not only for the initiation of puberty, but generally for the establishment of a properly maturated reproductive axis that will ensure fertility is acquired and preserved during adulthood. The timely-precise action of NO signaling is in fact a result of the activation of the nNOS-expressing population residing in the OVLT. We show that this neuronal population has an altered phosphorylation profile depending on the developmental stage in question. Phosphorylation of the nNOS protein is an extremely important posttranslational modification since it leads to the activation of the enzyme, rendering it capable of producing NO (Rameau et al., 2007). Prior to P12, the extremely low expression of the p-nNOS protein is probably only resulting to a minor release of the neuromodulator, insufficient to control the

reproductive axis, as suggested by the absence of reproductive deficit when NOS activity was inhibited during an earlier time window. Hence, the significant increase in the phosphorylation levels of the nNOS at P12 signifies an increment in the levels of NO being released from this neuronal population, and therefore a possible action of this newly produced NO on the activity of the neighboring GnRH neurons.

Our results attribute this rise in the phosphorylation levels of the nNOS to the action of the ovarian steroids, known to significantly increase during the infantile period (Fortune and Eppig, 1979; Funkesnstein et al., 1980). Ablation of the ovarian source of estrogens during the infantile period diminished the phosphorylation levels of the hypothalamic nNOS protein during the later stages of the infantile period. The interaction of nNOS with estrogen is not surprising since in the adult brain natural fluctuations of estrogen levels across the estrous cycle have been already correlated to the activation of the nNOS protein (d'Anglemont de Tassigny et al., 2007a, 2009; Gingerich and Krukoff, 2008; Parkash et al., 2010). This estrogen-induced activation of the nNOS neuronal hypothalamic population during the infantile period enables a NO-driven maturation of the HPG axis by setting the dialogue between the ovaries and the hypothalamus. More specifically, and as expected, in the absence of ovarian estrogen, i.e. when the negative feedback on the hypothalamus is relieved, LH was seen to increase. When ovariectomy was coupled to the blunting of NO release during the critical, newly-identified, infantile period, LH surge levels presented a major increase, indicating that infantile hypothalamic NO action is necessary for the negative feedback of estradiol on the hypothalamic axis. Our data suggest that apart from inhibiting GnRH secretion by mediating the negative estrogenic feedback action, NO can directly negatively regulate the activity of GnRH. Constitutive absence of Nos1 activity in a Nos1-null mouse model, resulted in not only a vast increase of the P12 Gnrh mRNA content, but also of the

FSH surge levels. The observed increase in Gnrh transcription upon nNOS invalidation agrees with a recent study demonstrating a selective switch in miRNA expression patterns in GnRH neurons during mini-puberty, triggering the repression of NO-C/EBP signaling, thus permitting the sustained increase of GnRH required for subsequent sexual maturation (Messina et al., 2016). In other words, infantile nNOS-derived NO is acting as a break to retain GnRH transcription and subsequent neurosecretion, regulating thus the activation of the GnRH neuronal population, and driving the reproductive network to its maturation.

Overall our data unmask a previously unknown function of the hypothalamic infantile nNOS-derived NO in the regulation of GnRH neuronal activity and the subsequent maturation of the reproductive axis. We identify nNOS hypothalamic neurons as important regulators of the GnRH neuronal activity during both embryonic and postnatal development. More specifically, we determined infantile period, as a critical time window for the action of NO on the maturation of the GnRH neuronal network and subsequently the establishment of a fully functional reproductive axis. Infantile nNOS-derived NO appears to be required for the maturation of the HPG axis, acting not only in the level of the hypothalamus, but also being responsible for the establishment of an ovarian-hypothalamic dialogue, ensuring the proper maturation of the reproductive axis and promoting pubertal onset. More importantly, nNOS activation during P12 alters the balance of positive and negative signals, repressing Gnrh neuronal activity in the level of both the mRNA and the peptide during the infantile period, thus leading to maximal Gnrh production upon release of the NO break, enabling the onset of puberty. The identification of Nos1 mutations in human hypogonadotropic hypogonadic patients further supports the major role of hypothalamic NO neuronal signaling in the establishment of fertility. Hopefully, gaining further understanding on the prepubertal mechanisms leading to the acquisition of a functional reproductive system will improve our chances of treating idiopathic infertility conditions in humans.

Table 1 Identified mutations in the Nos1 gene. Targeted genetic studies on KS and CHH patients revealed 9 heterozygous mutations in the Nos1 gene, encoding for the nNOS protein in 8 KS and 4 CHH probands, some of them being also present in patients with Constitutional delay of growth and puberty (CDGP).

Position	Amino acid change	Nucleotide change	ExAC EUR (n=34,429)	Pedigree	Phenotype
117664523	p.Ile1223Met	c.3669A>G		P00300	CHH
117680472	p.Arg1001Trp	c.3001C>T	0,001%	P00464 (mother)	CDGP
117701743	p.Asn725Asp	c.2173A>G	0,004%	P00381	KS
117703125	p.Tyr711Cys	c.2132A>G		P00327 (father)	Unaff
117705934	p.Met619Leu	c.1855A>T	0,415%	P00311	CHH
				P00311 (father)	CDGP, cleft lip
				P00311 (sib)	cleft lip
				P00311 (sib)	CDGP, cleft lip
117710246	p.Gly595Ser	c.1783G>A	0,616%	P00043	KS
				P00435	KS
117724018	p.Asp394Ala	c.1181A>C	0,645%	P00459	KS
				P00459 (father)	CDGP
				P00459 (brother)	KS
				P00140	CHH
				P00489	CHH
				P00489 (mother)	Unaff
117749344	p.Arg260Gln	c.779G>A	0,002%	P00319	KS
117768154	p.Asp241Asn	c.721G>A	0,278%	P00022	KS
				P00022 (mother)	Unaff
				P00146	KS

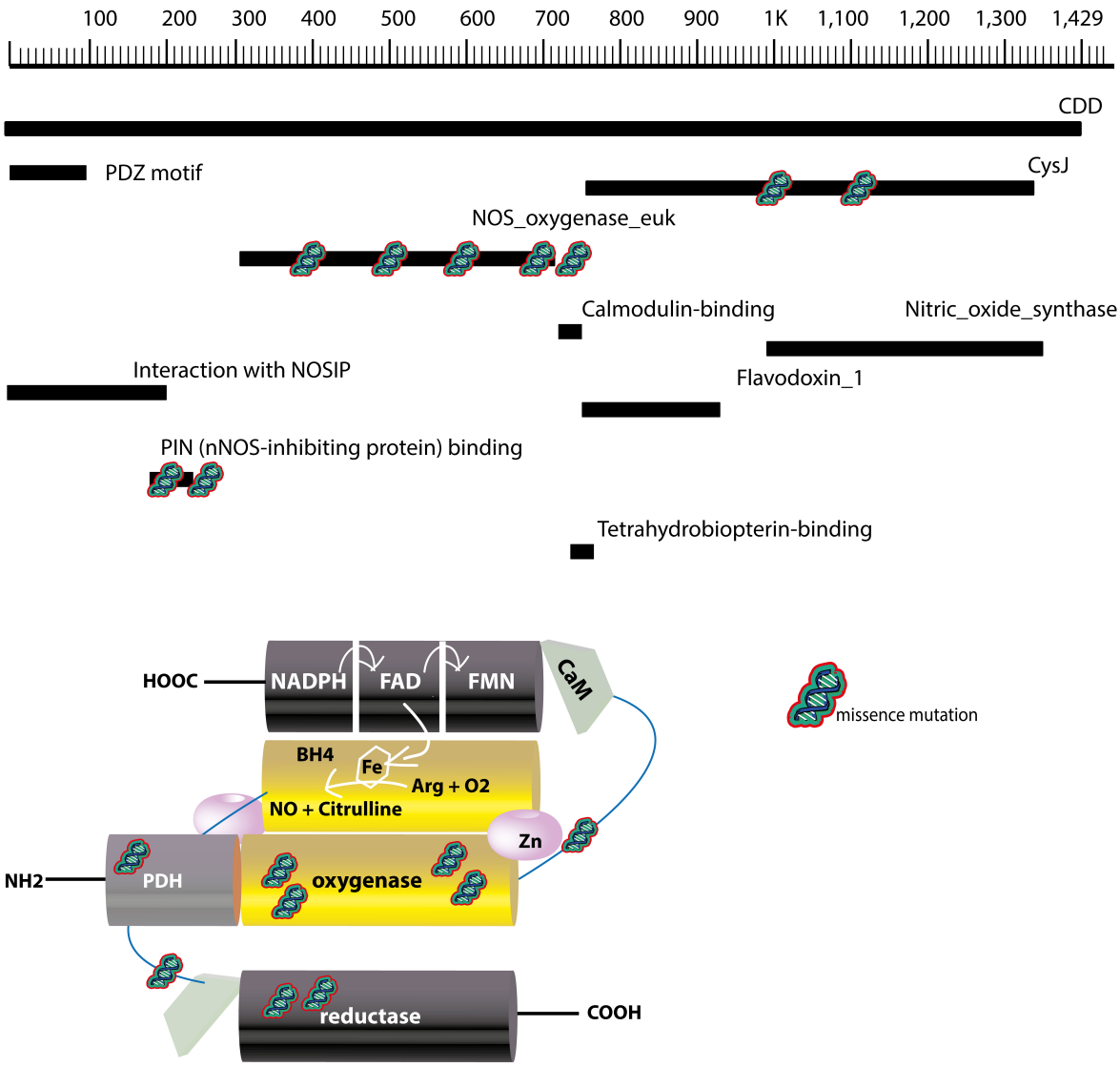

Figure 1 Schematic figure showing the position of the identified mutations on the human nitric oxide synthase (NOS). NOS1 mutations are mostly found localized on the oxygenase and the reductase domains (CysJ, flavodoxinJ, ferrodoxin) of the protein. Two of the mutations are located on the region permitting interaction with the PIN protein family. Binding sites for L-arginine (ARG), tetrahydrobiopterin (BH4), and calmodulin (CaM) are indicated.

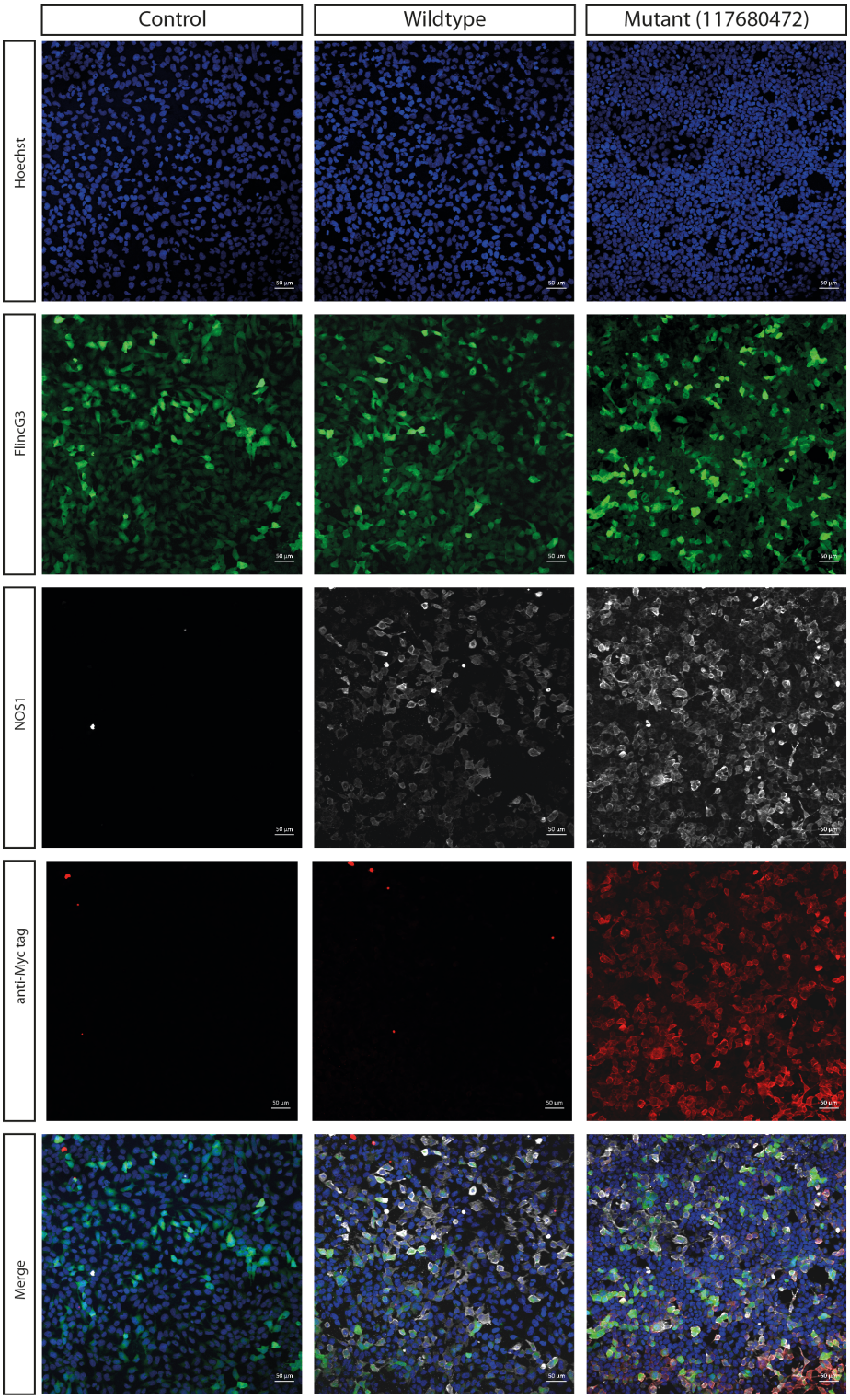

Figure 2 Immunolabeling demonstrating the transfection rate of HEK 293T cells. HEK cells were either transfected solely with the FlincG3 plasmid, serving as controls (first row), or co-transfected with either the NOS1 wildtype (middle row) or one of the NOS1 mutant plasmids (third row). Hoechst (nuclear staining) is shown in blue; FlincG3 transfection is shown in green. nNOS staining is shown in white, and myc tag staining is shown in red.

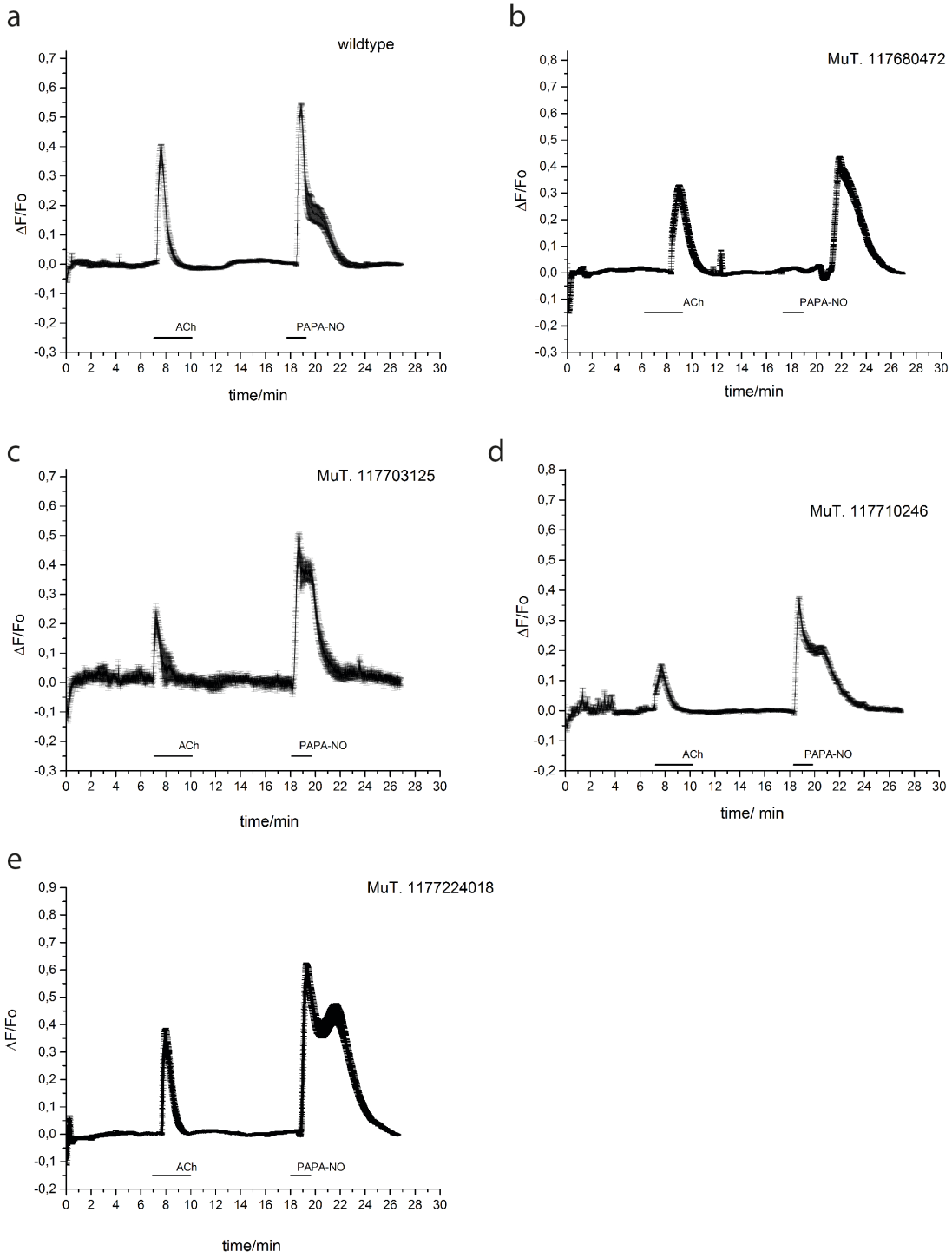

Figure 3 Behavior of NO-detector cells expressing the wildtype or mutated NOS1 protein upon endogenous stimulation of the NO signaling pathway. Response to acetylcholine (Ach; 100 µM) with application of a high concentration of PAPA/NO (1 µM) at the end in FlincG3 -transfected HEKGC/PDE5 cells expressing (a) the wildtype NOS1 protein, or (b-e) mutated forms of the protein as those identified in CHH probands. Black lines in a-e are means ± SEM.

Table 2 NO concentration-response curve parameters in FlincG3 -transfected HEKGC/PDE5 cells.

FlincG3	EC_{50} (pM)	n_H	R_{max}
	191 ± 37	1.10 ± 0.09	0.66 ± 0.03

[NO] = EC50/{[(Rmax/y) – 1]^1/n}

Rmax is the response to a saturating NO concentration (PAPA-NO application)
y is the endogenous response height (ACh)
n is the Hill slope (1.1)

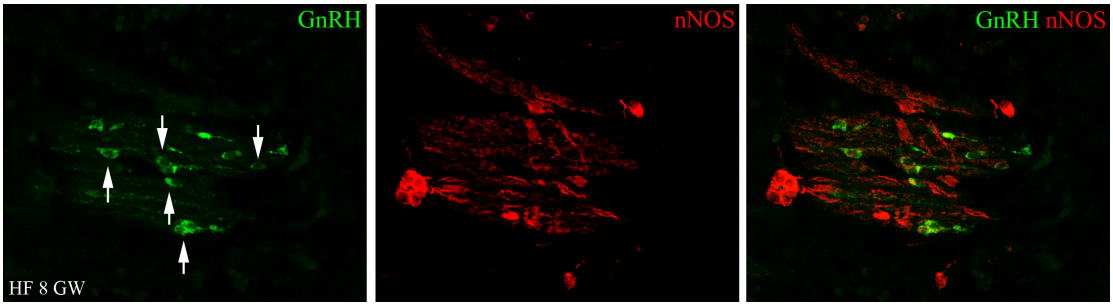

Figure 4 GnRH migrating neurons co-express the nNOS protein. 8-week old human fetus immunostaining showing GnRH neurons (in green) coexpressing the nNOS protein (in red) in the region of the nose during neuronal migration of the GnRH population.

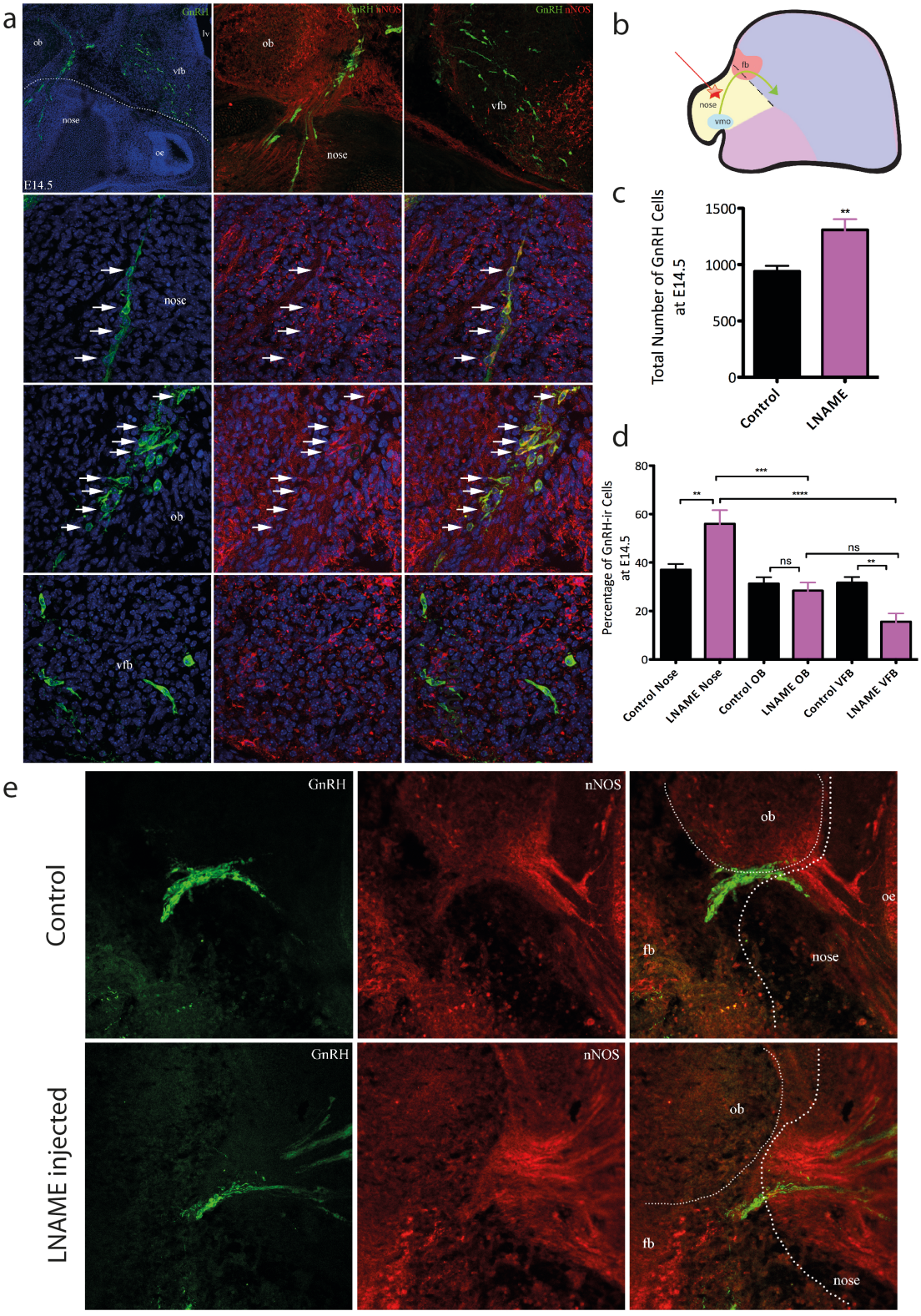

Figure 5 Inhibition of NO production in vivo results in a failure of GNRH neurons to properly migrate in the forebrain region. (a) Immunostaining showing migrating GnRH neurons (in green) coexpressing the nNOS protein (in red) in the region of the nose of a mouse embryo during embryonic day (E) 14.5 (b) In uterus injection of LNAME in the nose of Gnrh::Gfp embryos at E 12.5 (c) NOS inhibition results in an increased total number of GNRH neurons in sagittal sections of embryos (total

number of Gnrh-ir positive neurons Control vs LNAME: t7= 3.730, p= 0.074, n= 4-5 per group). (d) At E14.5, half of the migrating Gnrh::Gfp neurons are still in the nose, while the rest are found in the forebrain region in saline-injected embryos. (e) LNAME injection causes a major perturbation in the migratory process of GNRH cells, resulting in incomplete migration into the forebrain region and arrestment of the majority of the cells in the region of the nose (LNAME nose vs LNAME vfb: t6= 4.210, p= 0.056, n= 4-5 per group; Control vfb vs LNAME vfb: t7= 4.045, p= 0.0049, n=4-5 per group; Control nose vs LNAME nose: t7= 3.393, p= 0.016, n=4-5 per group).

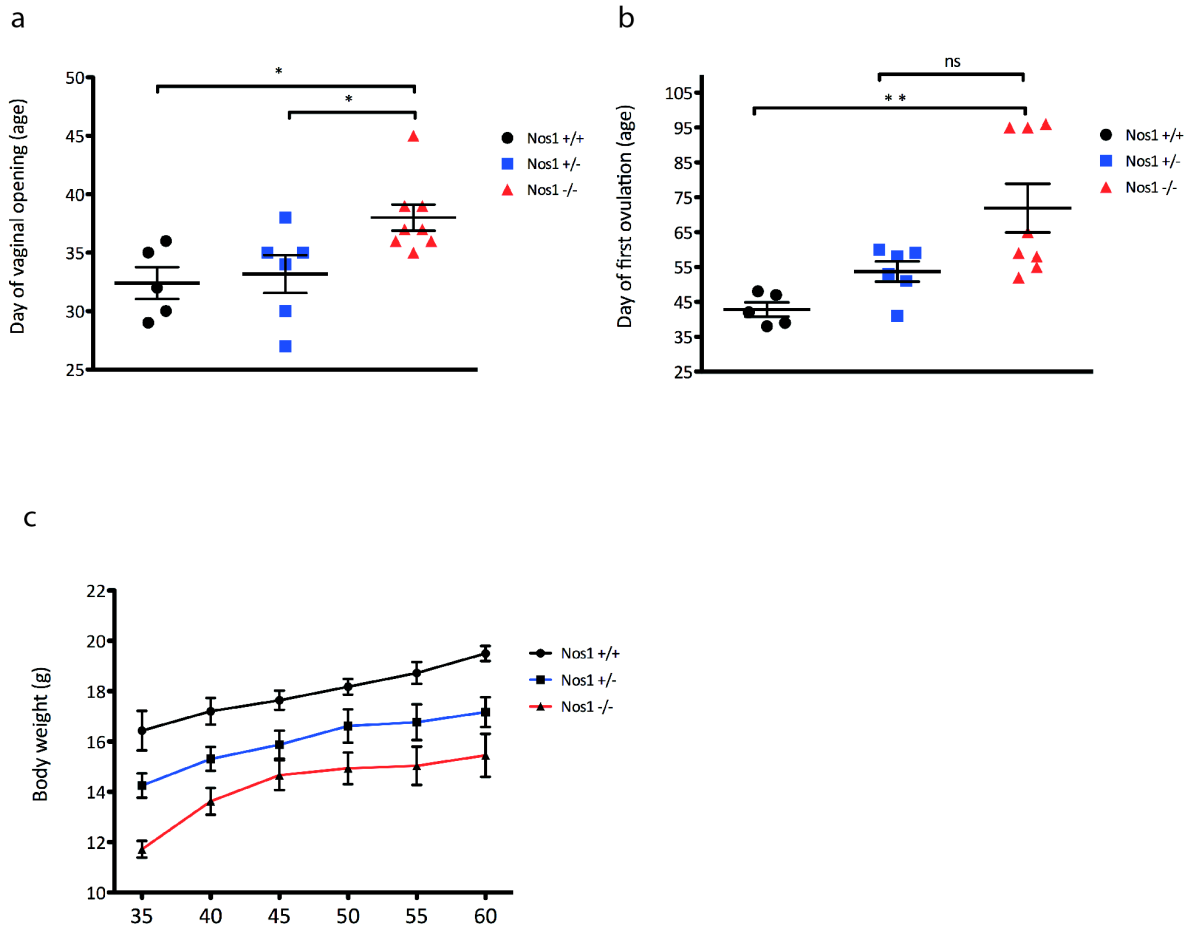

Figure 6 Constitutive absence of NOS1 protein results in delayed maturation of the reproductive axis. (a) Nos1 -/- female mice undergo vaginal opening with a delay of approximately 5 days comparing to their Nos1 +/+ and Nos1 +/- littermates (on-way ANOVA p= 0.0160, n= 5 Nos1 +/+, n= 6 Nos1 +/- and n=8 Nos1 -/-) and (b) reach puberty after 2 months of age, while the Nos1 +/- also exhibited significantly delayed sexual maturation comparing to their wildtype littermates (one-way ANOVA p= 0.0056, n= 5-8). (c) Nos1 -/- females have a linear phenotype as demonstrated by their decreased body weight during the first two months of age (one-way ANOVA, p= 0.0003, n=5-8 per group).

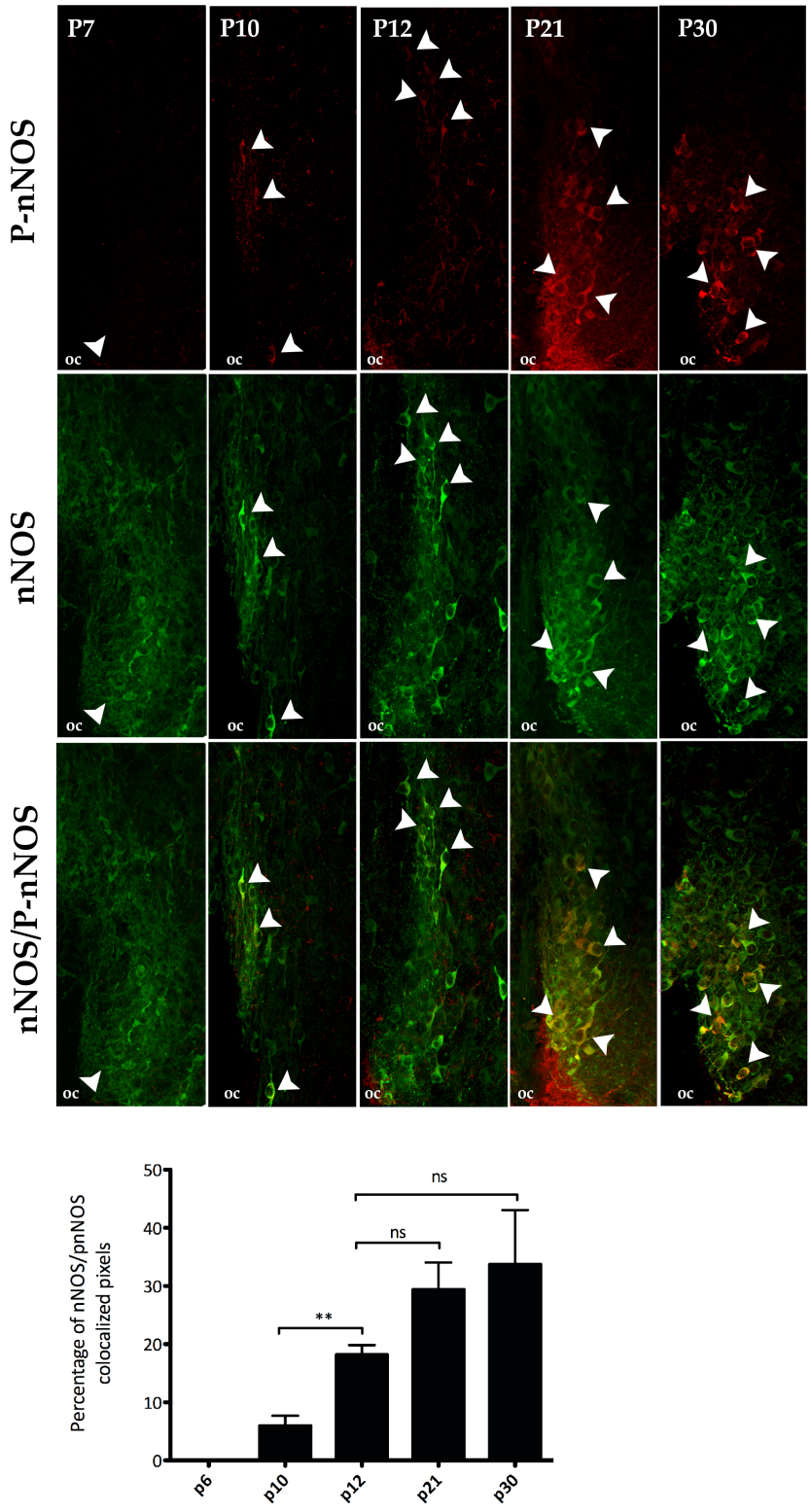

Figure 7 Progressive phosphorylation of the nNOS protein during postnatal development in the region of the OVLT leads to activation of the NO pathway at postnatal day 12. (a) nNOS (green) and P-nNOS (red) immunoreactivity in forebrain coronal sections of the OVLT in female mice during pre-pubertal postnatal day 7, 10, 12, 21, and 30. (P7 vs P10, p>0.05, P10 vs P12, p= 0.072, p= P12 vs P21, p>0.05, P12 vs P30, p>0.05, n=3-4 per group) Arrows show double-labeled neurons. oc, Optic

chiasm; 3V, third ventricle. Scale bar, 100 μm. (b) Bar graphs illustrate the mean ratio of the nNOS-ir positive pixels to that of p-nnos-ir positive pixels (one-way ANOVA, $p= 0.018$, $n = 3\text{-}4$ per age group). Error bars indicate SEM. $*p < 0.05$

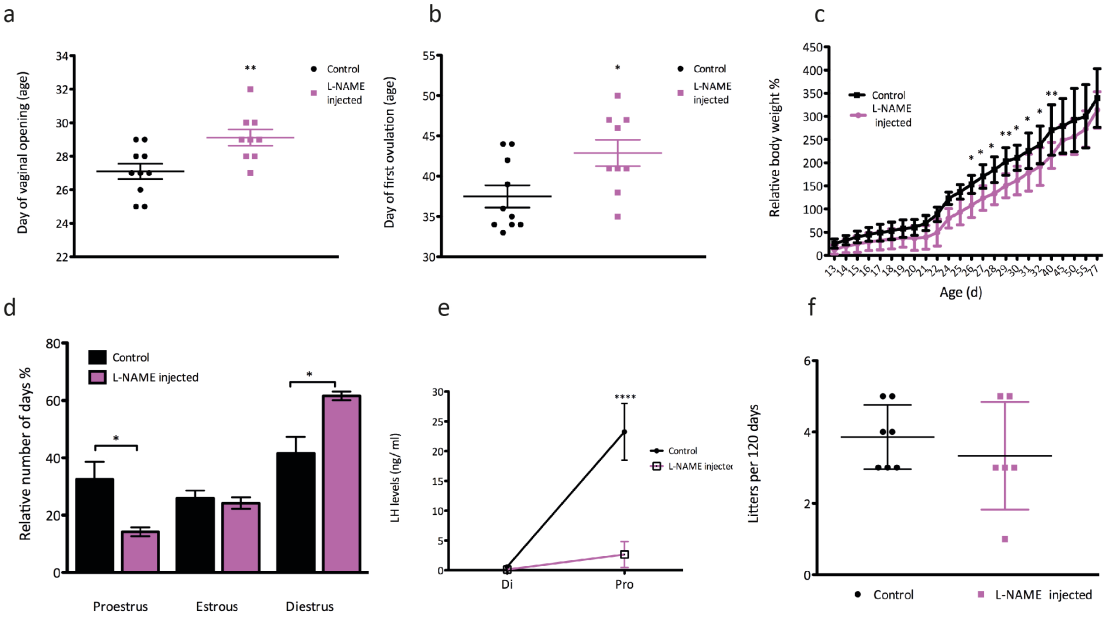

Figure 8 Ablation of NO release during the infantile period (p10-21) leads to a significant retardation of reproductive maturation and subfertility, reminiscent of the Nos1 -/- reproductive phenotype. (a) L-NAME injected mice exhibit delayed vaginal opening (t_{15}= 3.017, p= 0.0078, n= 9-10 per group) and (b) pubertal onset comparing to their saline-injected littermates (t_{17}= 2.343, p= 0.0213, n= 9-10 per group). (c) Inhibition of NO release during the infantile period leads to a decreased relative body weight during the juvenile period (2way-ANOVA, P26-40, $p<0.05$, n= 9-10 per group) and (d) perturbed estrous cyclicity in adulthood, accompanied by (e) lower circulating levels of LH (t_8= 3.944, p= 0.0043, n= 5 per group) and (f) minor subfertility (n= 5 per group).

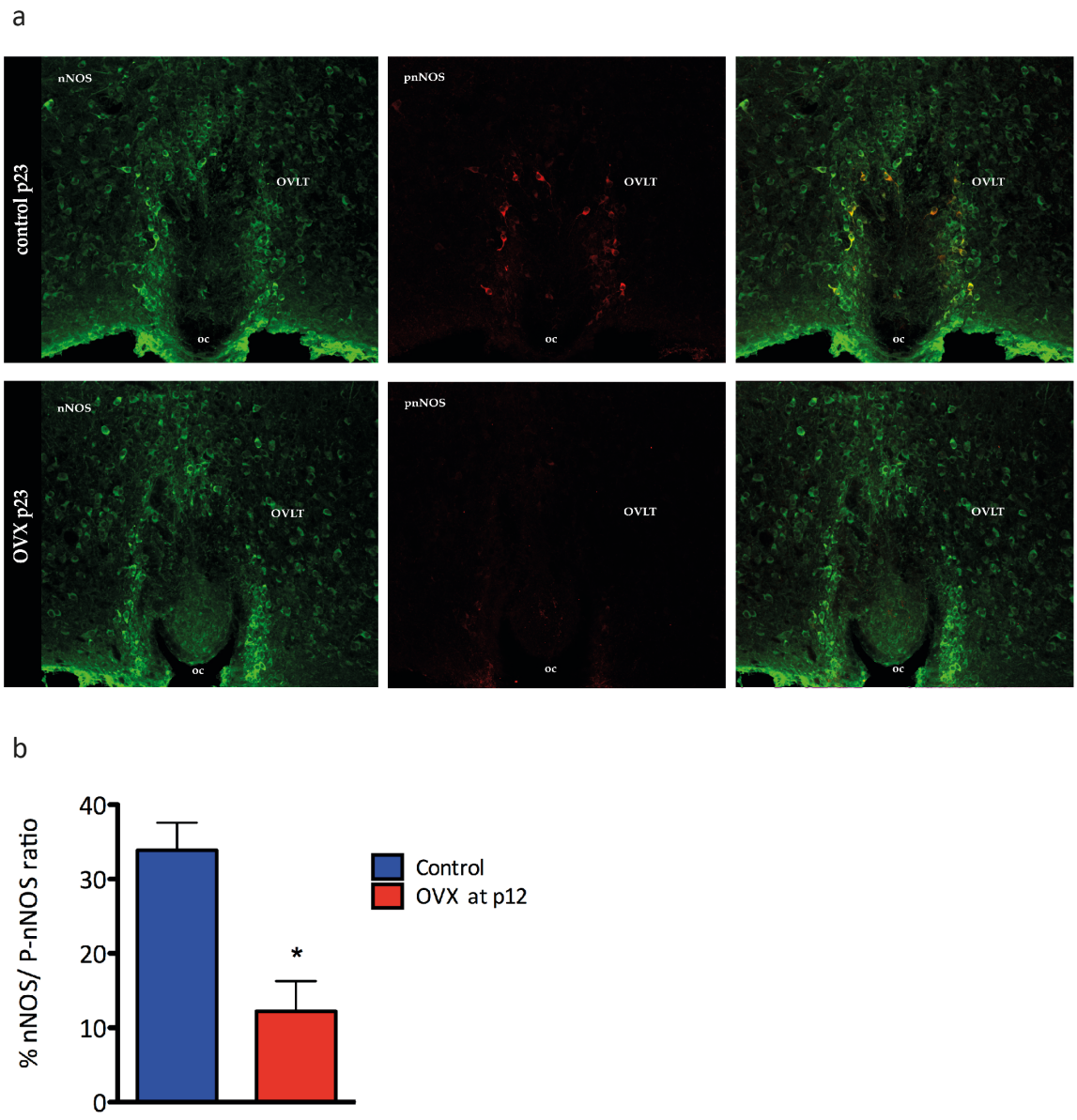

Figure 9 Arrival of ovarian estrogen into the hypothalamus during the infantile period triggers the activation of the nNOS-expressing neuronal population residing in the preoptic region. (a) nNOS (green) and P-nNOS (red) immunoreactivity in forebrain coronal sections of the OVLT in P23-OVX mice and their control littermates. (b) Ovariectomy of p12 wildtype mice resulted in decreased p-nNOS-ir levels during P23 (nNOS/p-nNOS-ir : Control, 33,88% ± 3.703, n=3 vs OVX, 12.23% ± 4.067, n=4; t5= 3.703, p= 0.0129, n=3-4 per group) Arrows show double-labeled neurons. oc, Optic chiasm; 3V, third ventricle. Scale bar, 100 µm.

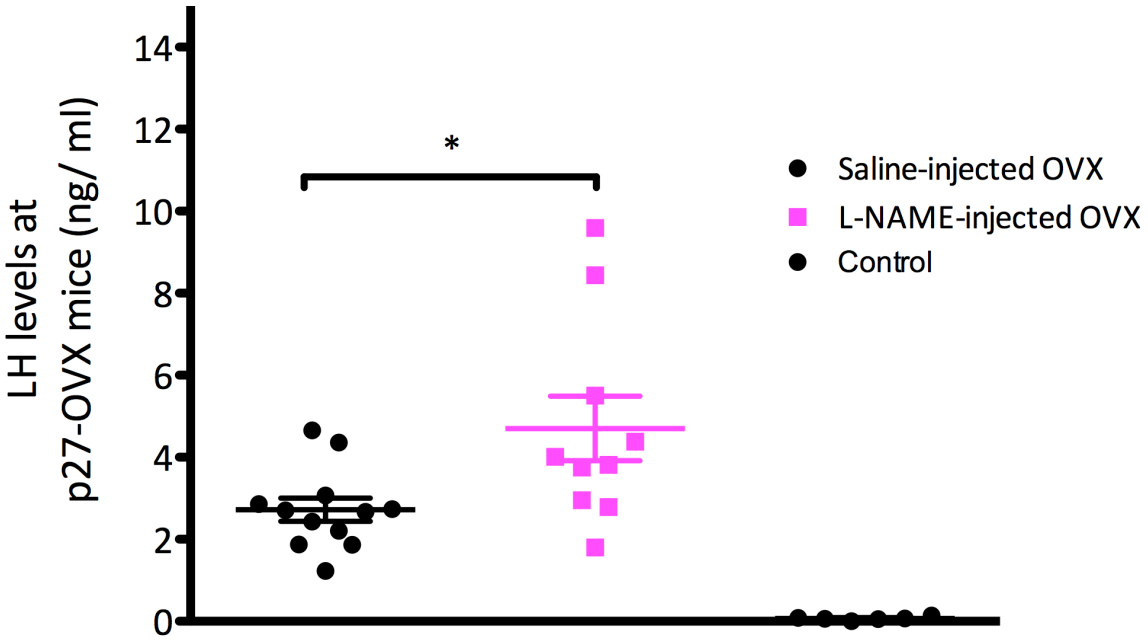

Figure 10 nNOS activity is required for the action of ovarian estrogen on the hypothalamus prior to puberty. Ablation of NO release during the infantile period (p10-21) and ovariectomy two days after the end of the injection period, lead to a significant incease in the LH hormonal levels, comparing to the values measured in OVX saline- injected mice (t_{21}= 2.170, p= 0.0416 saline-injected OVX vs. LNAME-injected OVX, n= 11-12 per group). As expected, non-OVX (control) mice exhibited very low levels of LH at P27.

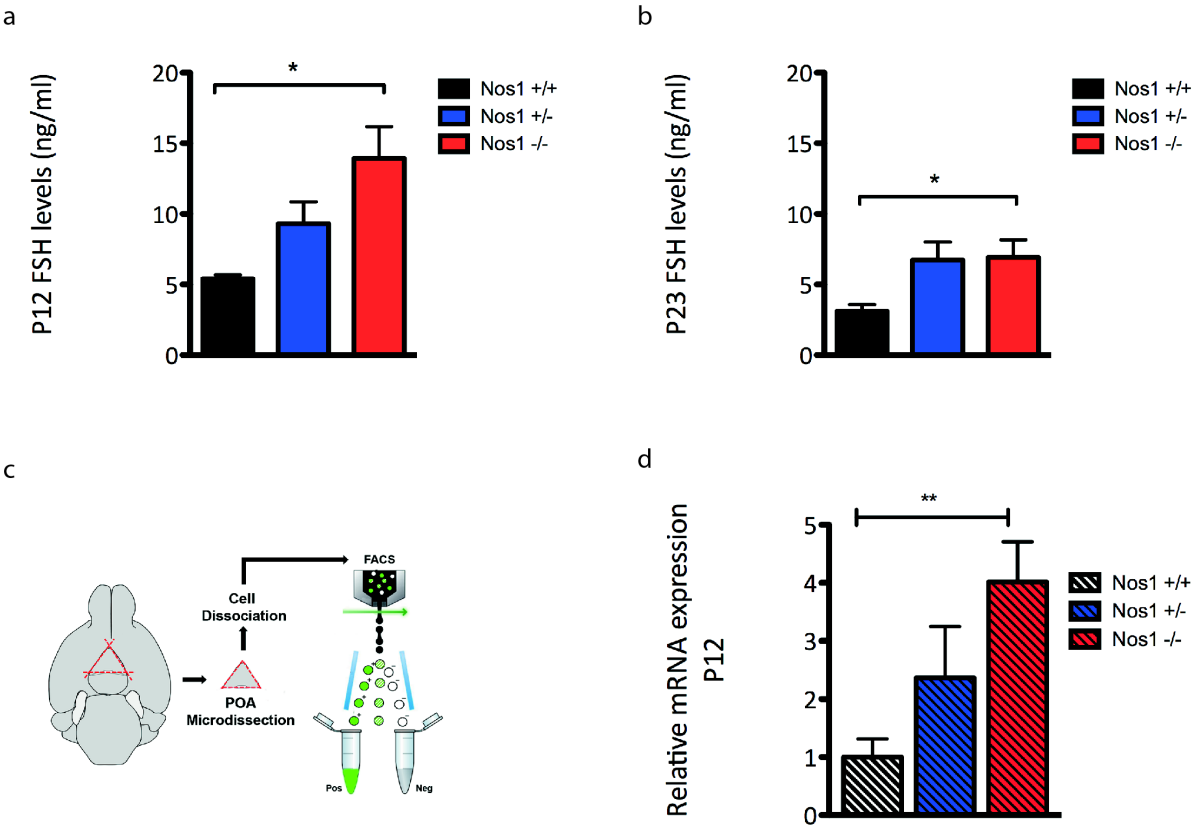

Figure 11 Nos1 is necessary for the regulation of the Gnrh mRNA levels as well as the GnRH release during the infantile period. (a) FSH levels at P12 (one-way ANOVA, P12: p= 0.0265, n=3-9 per group), and (b) P23 Nos1+/+, Nos1+/- and Nos1-/- mice (one-way ANOVA, p= 0.0641, n=5-9 per group). (c) GnRH-GFP neuron isolation by FACS from the preoptic region of Gnrh::Gfp mice (from Messina et al., 2016). (d) RT-PCR analysis of the expression of Gnrh in FACS-isolated GnRH-GFP neurons from Nos1+/+; Gnrh::Gfp, Nos1+/-; Gnrh::Gfp and Nos1-/-; Gnrh::Gfp mice at P12 (one-way ANOVA, p= 0.0162, n=7-8 per group; Nos1+/+ vs Nos1-/- : t14= 3.971, p=0.0014, n=7-8 per group).

MATERIALS AND METHODS

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 female C57BL/6J mice (Charles River Laboratories), Nos1-null (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).

Production of mutated-NOS1 plasmids

A cDNA containing the entire coding region of the human NOS1 (GenBank assembly accession; GRCh37.p13 / GCF_000001405.25), or the mutated coding regions identified in CHH patients (table) was inserted into pcDNA3.1+ using EcoRI-NotI plasmid expression vector. A his-tag was added to the 5'end of the WT sequence and a myc-tag to mutants sequences. All NOS1 plasmids were produced by *GeneCust*.

FlincG3 plasmid (pTriEx4-H6-FGAm) was kindly provided by Prof. Garthwaite (information can be found in the Addgene webiste).

Cell culture of NO detector cells (HEK 293T)

The HEK 293T cell line expressing NO-activated GC and phosphodiesterase-5 (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<40 times.

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

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 nitric oxide, cells were treated with the NO donor Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino] (PAPA/NO; 1μM, Enzo Life Sciences, Exeter, UK) for a duration of 90 sec. Endogenous NO release was stimulated upon application of acetylcholine (Ach; 100 μM, Sigma). 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). For in vivo application LNAME was applied in a dose of 50 mg/kg i.p. and 5 mM intranasally, diluted as previously described (Bellefontaine et al., 2014).

Puberty onset

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 the estrous cycle.

Fertility index

Female fertility index was calculated from the number of litters per females during a 120-day-long mating.

Ip injections of NOS-inhibitor (LNAME)

P10 female mice received bidaily injections of the NOS inhibitor 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 8H30 and 18H30, i.e. one hour after lights on and one hour before lights off according to a previously described protocol (Bellefontaine et al., 2014). 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 "Puberty onset" section above). When LNAME treated mice and their control littermates reached adulthood, blood samples were collected from the facial vein on Di1 and Pro for the measurement of LH hormone (described below). Mice were then either perfused for brain tissue collection and immunohistochemical studies, or used for fertility tests (see "Fertility index" section above).

In utero intranasal injection of LNAME in E12.5 embryos

Pregnant 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 LNAME (5mM) were injected into contralateral horns of each pregnant female. The needle was positioned vertically over the nose of the 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.

Hormone 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 (Martini et al., 2006). The accuracy of hormone measurements was confirmed by the assessment of rodent serum samples of known concentration (external controls).

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 P50) 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 10-100 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.

Ovariectomy

Female mice (P12) were bilaterally ovariectomized (OVX) under anaesthesia with isofluorane. OVX mice and their control littermates were sacrificed at P21.

Mice treated with LNAME or saline during the infantile period (P10-P21) were ovariectomized at P23. OVX/LNAME injected mice and their OVX/saline injected littermates were perfused (as described above) 96h afterwards (P27) as previously described (Caron et al., 2012a). Blood was collected, and LH levels were measured by ELISA (as described above).

Immunohistochemistry / Immunofluorescence

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

For nNOS/p-nNOS/GnRH immunostaining (Hanchate et al., 2012). 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 48-72 hours at 4°C in primary rabbit anti-p-nNOS (Thermoscientific) 1/500, guinea pig anti-Gnrh (a generous gift from Dr Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) 1/10000, and sheep anti-nNOS (generous gift from Dr. P. C. Emson (Medical ReseARHh Council, Laboratory for Molecular Biology, Cambridge, UK) 1/3000. Sections were washed 3 times for 5 minutes in PB 0.1M and then incubated in biotin donkey anti-rabbit (1:500; Jackson Laboratories) for 1 hour at room temperature. The sections were then washed and incubated in streptavidin Alexa 568 (1/500; Invitrogen), anti-guinea pig 488 (Jackson ImmunoResearch), and Alexa 647 donkey anti-sheep (1/500; Invitrogen). Sections

were then washed 5 times for 5 minutes each, and then counterstained with hoechst (1/10000) for 5 minutes. After washing 2 times for 5 minutes, sections were mounted on slides and coverslipped with Mowiol coverslip mounting solution.

For embryonic nNOS/GnRH/peripherin or TAG immunostaining. Sections were washed 3 times for 15 minutes each in TBS 1X and then incubated in blocking solution (10% NDS, 0.25% BSA, 0.03% Triton X-100 in TBS 1X) for 1 hour at room temperature. Sections were then incubated for 48 hours at 4°C in primary sheep anti-nNOS (1/1500; generous gift from Dr. P. C. Emson (Medical ReseARHh Council, Laboratory for Molecular Biology, Cambridge, UK), guinea pig anti-Gnrh (1/3000; a generous gift from Dr Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary), and either rabbit anti-peripherin (1/3000; Millipore), or goat anti-TAG1 (1/500; R&D systems). The sections were then washed and incubated in Alexa 488 donkey anti guinea pig (1/400; Invitrogen), Alexa 568 donkey anti rabbit or anti goat (1/400; Invitrogen), and Alexa 647 donkey anti-sheep (1/400; Invitrogen), in a sequential manner, followed by 3 washings of 5 minutes each between each incubation. Finally sections were counterstained with hoechst (1/10000) for 5 minutes. After washing 2 times for 5 minutes, sections were mounted on slides and coverslipped with Mowiol coverslip mounting solution.

Coverslips used for the live imaging experiments were fixed using 2% PFA for 20 min before being processed for immunolabeling. Cells were incubated in 0.1 % Triton X-100 for 10 min and then washed twice in PBS (5-10 min) before incubation with blocking solution (5% NDS, 2% BSA, 0.03% Triton X-100 in PBS 0.1 M) for 30 min at room temperature. Cells were washed and stained against NOS1 using a sheep anti-nNOS (generous gift from Dr. P. C. Emson (Medical ReseARHh Council, Laboratory for Molecular Biology, Cambridge, UK) 1/3000, or mouse anti-myc (1/1000; ThermoScientific), for 1h30 min at room temperature.

Cells were then washed and incubated in Alexa 568 donkey anti-mouse (1/400; Invitrogen), and Alexa 647 donkey anti-sheep (1/400; Invitrogen), for 1h in room temperature. After washing 2 times for 5 minutes, coverslips were mounted on slides using Mowiol coverslip mounting solution.

Imaging

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 the analysis of nNOS/p-nNOS ratio during development, Z-stack images (22 μm) were acquired with the 40X oil objective, using a numerical aperture of 0.50, and a zoom of 1.0. For the nNOS/p-nNOS ratio after ovariectomy Z-stack images (21 μm) were acquired with the 20X objective, using a numerical aperture of 1.30, and a zoom of 0.70. Illustrations were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA). Two-dimensional images presented here are maximal intensity projections of three-dimensional volumes along the optical axis.

FlincG3 fluorescence imaging: δ-FlincG 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 ^{o}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 ^{o}C.

Cell counting and analysis

Analysis was undertaken by counting the numbers of single-labeled, dual-labeled (nNOS staining colocalising with p-nNOS). The number of the above nNOS- expressing neuronal populations were counted in the region of organum vasculosum lamina terminalis (OVLT), represented by plate 16, of the L.W. Swanson brain map (Swanson, L.W. 2003). Cell counts in the OVLT were determined by placing a rectangle covering a surface of 1380x2701 pixels, over the midline of OVLT, and counting all immunoreactive nuclei unilaterally from each mouse. All the above values for each mouse were used to determine mean counts for each age/ treatment 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.

All analyses were performed using Prism 5 (GraphPad Software). Data were compared by a two-tailed unpaired Student's t-test or one-way analysis of variance for multiple comparisons followed by Tukey's post test. For comparisons between groups that included repeated measures two-way ANOVA test was used, followed by Bonferroni’s post test. The significance level was set at $P<0.05$ and data are represented as mean ± SEM values. The number of biologically independent experiments (n), p values, and age of the animals are indicated in the figure legends.

For in vitro experiments: 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 ($\Delta F/Fo$). Peak amplitudes for each cell giving a fluorescent signal were measured by taking the maximum $\Delta F/Fo$, 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.

Isolation of hypothalamic GnRH neurons using Fluorescence-Activated Cell Sorting

To obtain single-cell suspensions the preoptic region of $NOS^{-/-}$/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 wild-type 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).

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).

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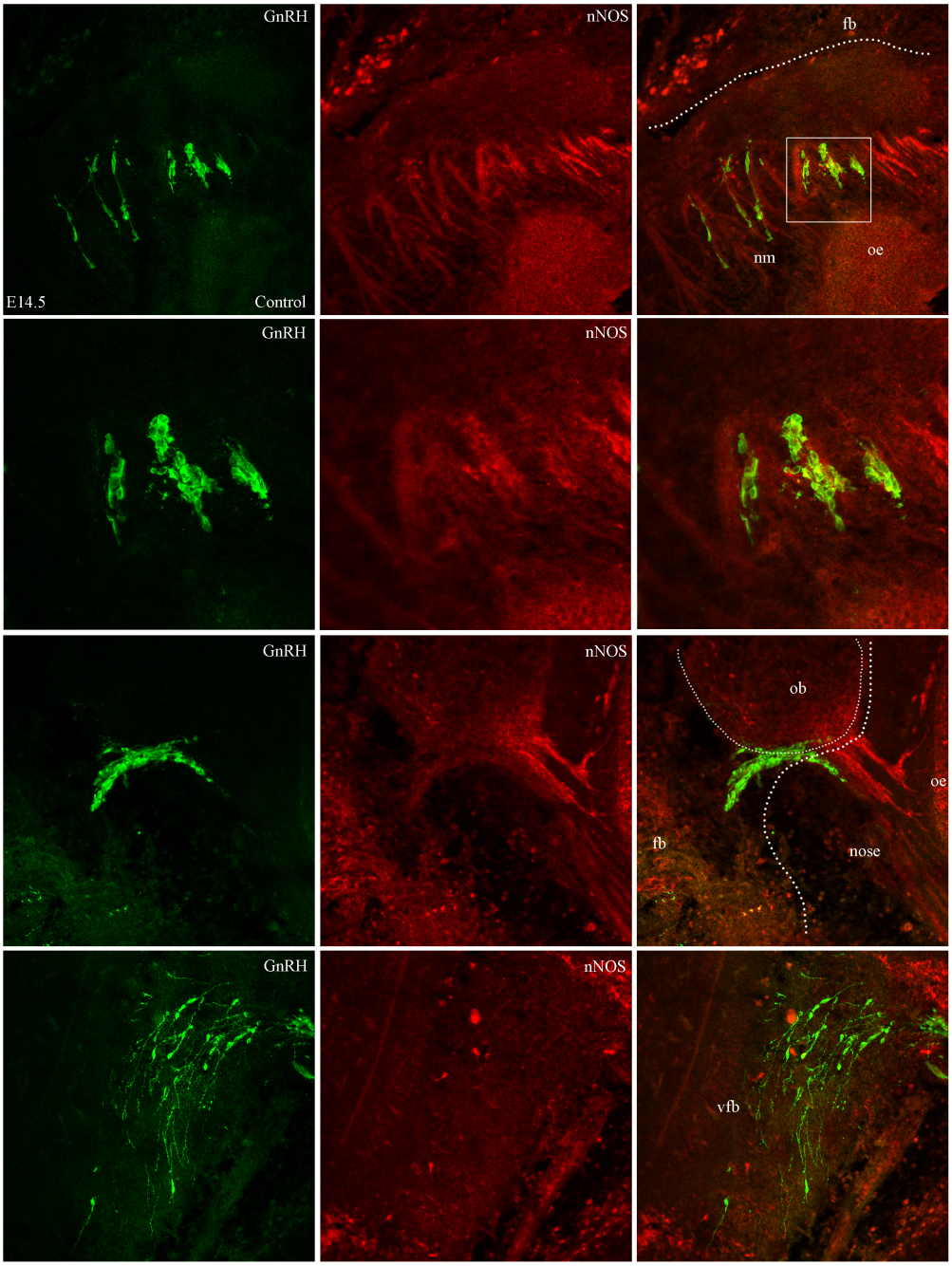
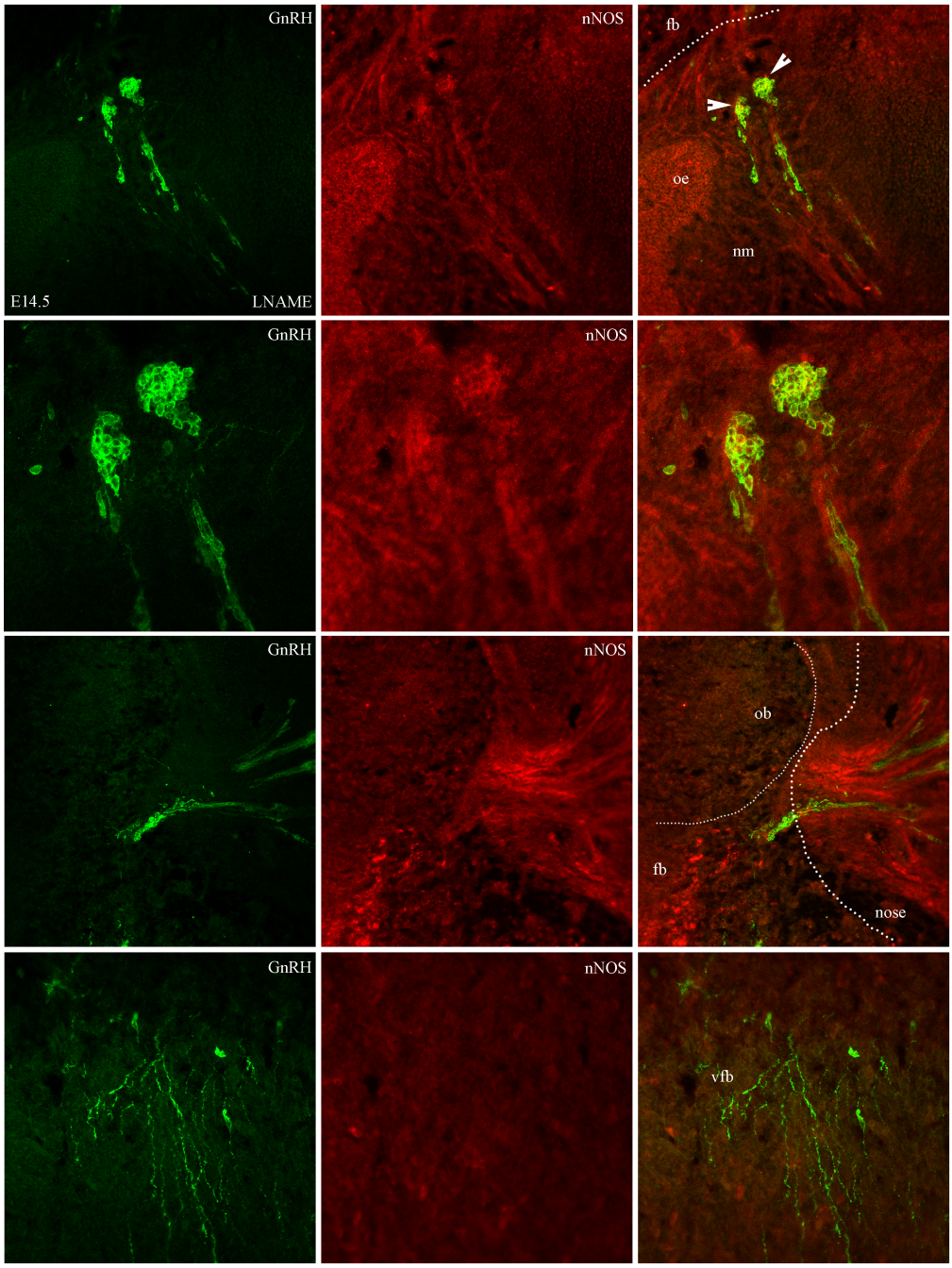
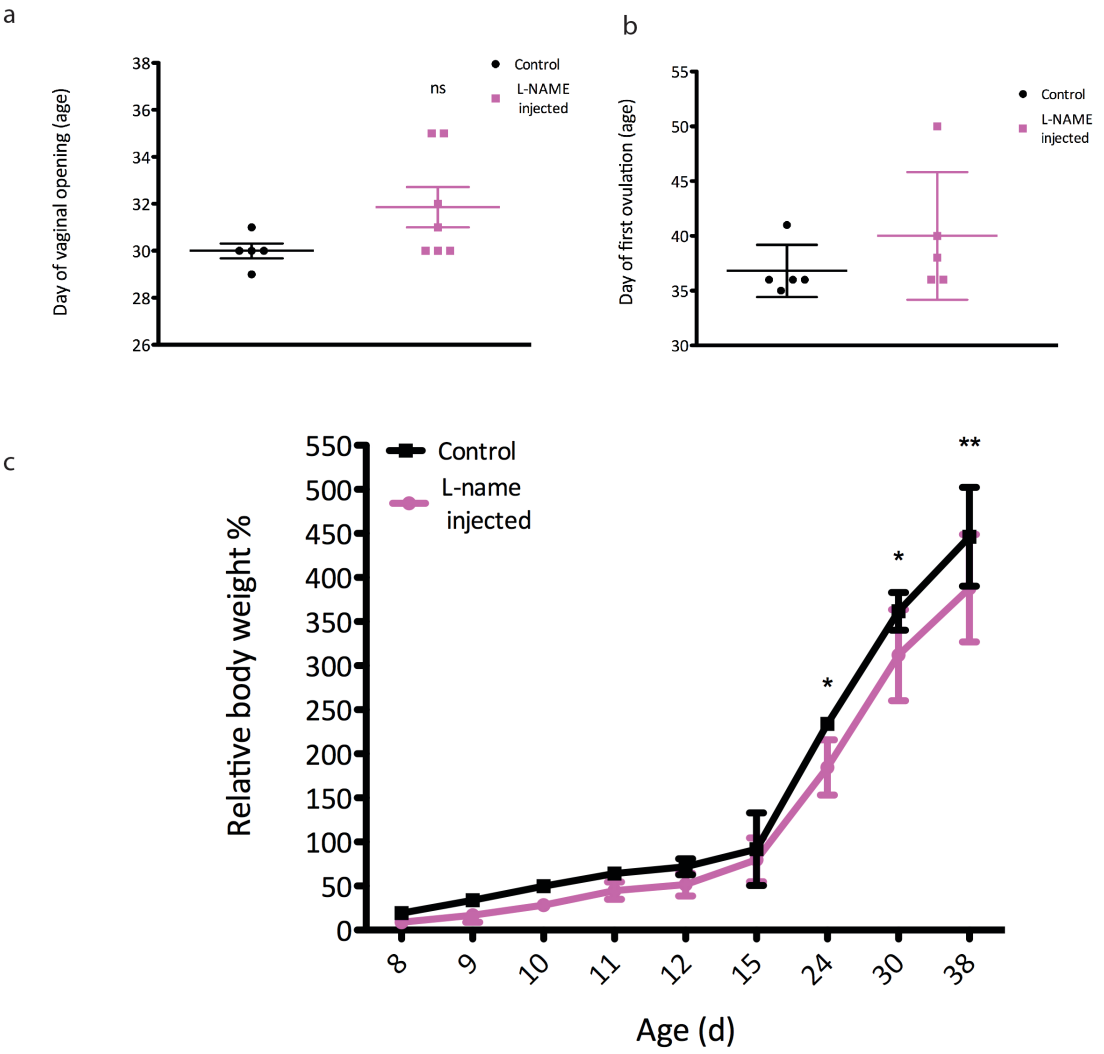
SUPPLEMENTARY



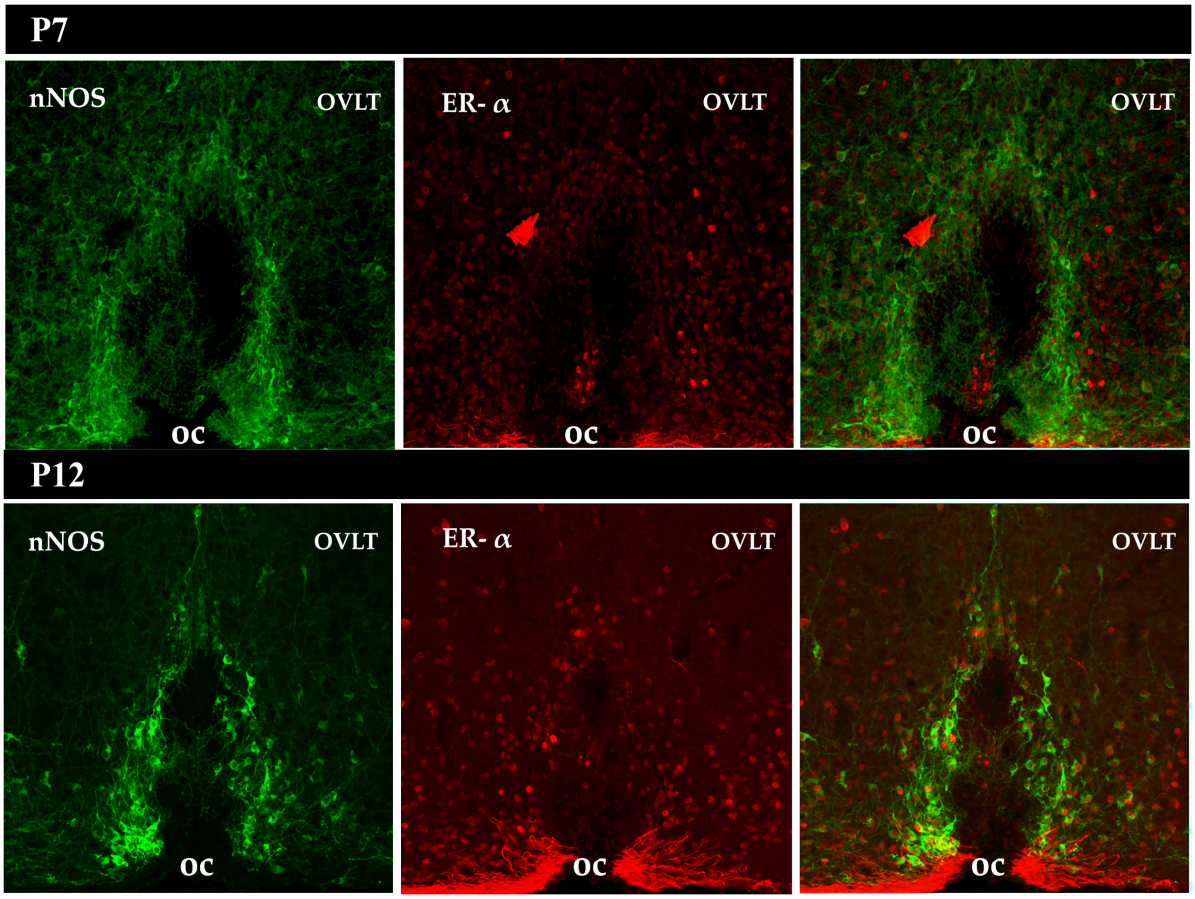
Supplementary Fig. 1a Migratory route of GnRH neurons in sagittal sections of mouse brain at embryonic day (E) 14.5. GnRH neurons (in green) migrate from the olfactory placode along a branch of the vomeronasal nerve, to reach the basal forebrain. Ob; olfactory bulb, fb; forebrain, vfb; ventral forebrain, oe; olfactory epithelium, nm; nasal mesenchyme.



Supplementary Fig. 1b Migratory route of GnRH neurons in sagittal sections of mouse brain at embryonic day (E) 14.5 after LNAME injection at E 12.5 GnRH neurons (in green) fail to properly migrate from the olfactory placode into the basal forebrain. GnRH cells are seen to form aggregates, staying constrained in the region of the nose. Ob; olfactory bulb, fb; forebrain, vfb; ventral forebrain, oe; olfactory epithelium, nm; nasal mesenchyme.



Supplementary Fig. 2 Ablation of NO release during the beginning of the infantile period (P7-12) leads to a significant decrease in body weight, whilst has no effect on the sexual maturation. (a) Inhibition of NO production during the P7- 12 time window does not affect vaginal opening ($p > 0.05$, n= 5 per group), or (b) pubertal onset ($p > 0.05$, n= 5 per group), however, (c) L-NAME injected mice exhibit decreased relative body weight (P24-38, 2way-ANOVA $p<0.05$, n=5 per group).



Supplementary Fig. 3 nNOS neurons residing in the preoptic area coexpress estrogen receptor α throughout development. (a) Coexpression of nNOS-ir (green) and ER-α (red) in the OVLT region during postnatal day (P) 7 and (b) postnatal day (P) 12. OVLT; organum vasculosum lamina terminalis, oc; optic chiasm

6.2 A microRNA switch regulates the rise in hypothalamic GnRH production before puberty

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The 'master molecule' gonadotropin-releasing hormone (GnRH) is being secreted by a few hundred hypothalamic neurons that form a complex neuroglial network controlling mammalian reproduction. Timely postnatal changes in GnRH expression are required for the orchestration of the events that govern puberty onset and adult fertility. During this study we identified that a selective switch in miRNA expression patterns in GnRH neurons inverts the balance between repressive and inductive signals controlling the expression of the GnRH promoter. More specifically, we present evidence suggesting that miR-155 acts both directly, by regulating Cebpb, a nitric oxide–mediated repressor of Gnrh, and indirectly, through Zeb1, to control pubertal onset. Knocking down the ability of miR-155 and/or miR-200 to repress Cebpb and Zeb-1 expression during the infantile period markedly disrupted the reproductive physiology of female mice, accelerating pubertal onset and increasing prepubertal LH values. Overall, these data highlight the involvement of NO pathway in the transcriptional regulation of Gnrh *in vivo*.

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A microRNA switch regulates the rise in hypothalamic GnRH production before puberty

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Brief summary

A switch operated by microRNAs regulates the control of puberty onset and adult fertility by the central nervous system by triggering increased hypothalamic GnRH mRNA expression at "mini-puberty", during the infantile period of postnatal development.

Abstract

A sparse population of a few hundred primarily hypothalamic neurons forms the hub of a complex neuroglial network that controls reproduction in mammals by secreting the "master molecule", gonadotropin-releasing hormone (GnRH). Timely postnatal changes in GnRH expression are essential for puberty and adult fertility. Here, we report that a multilayered microRNA-operated switch with inbuilt feedback controls governs increased GnRH expression during the infantile-juvenile transition, and that impairing microRNA synthesis in GnRH neurons leads to hypogonadotropic hypogonadism and infertility in mice. Two essential components of this switch, miR-200 and miR-155, respectively regulate *Zeb1*, a repressor of *Gnrh* transcriptional activators and *Gnrh* itself, and *Cebpb*, an NO-mediated repressor of *Gnrh* that acts both directly and through *Zeb1*, in GnRH neurons. This alteration in the delicate balance between inductive and repressive signals induces the normal GnRH-fuelled run-up to correct puberty initiation, and interfering with this process disrupts the neuroendocrine control of reproduction.

Introduction

The onset of puberty and the regulation of fertility in mammals are governed by a complex, primarily hypothalamic, neural network that converges onto gonadotropin-releasing hormone (GnRH)-producing neurons, the master regulators of gonadotropin secretion and postnatal gonadal growth and function [1]. Proper GnRH system development [2], including timely changes in GnRH expression and signaling by this sparse population of a few hundred neurons [3,4], is essential for sexual maturation and the normal hypothalamic-pituitary-gonadal (HPG) axis functioning. However, despite the identification of several factors influencing GnRH production and release [1], the mechanisms regulating the clocklike precision of the pubertal activation process remain largely unknown.

MicroRNAs (miRNAs) are short (~22-nucleotide) noncoding RNAs that silence gene expression posttranscriptionally, principally by binding to 3' untranslated regions (3'UTR) of target mRNAs (**Supplementary Fig. 1**). Mature miRNAs are known to be required for the normal differentiation and function of several cell types [5] and, in particular, to regulate somatic growth and fertility at the level of peripheral organs [6,7]. We therefore asked whether miRNAs could play a similarly critical role in the central neuroendocrine control of reproduction.

Here we show that a dramatic switch in miRNA expression patterns in infantile GnRH neurons inverts the balance between inductive and repressive signals, triggering increased hypothalamic GnRH expression and controlling the crucial transition from the early infantile phase, when its levels are low, to the GnRH-fuelled run-up to puberty. These data raise the intriguing possibility that miRNA-dependent epigenetic regulation of GnRH secretion could underlie the pathophysiology of human hypogonadotropic hypogonadism when a genetic cause is not evident, and hold therapeutic potential for puberty and infertility disorders of hypothalamic origin.

RESULTS

Dicer loss in GnRH neurons causes infertility

To determine whether miRNAs are involved in the crucial postnatal increase in GnRH expression, we generated mice in which Dicer, an RNAse-III endonuclease essential for miRNA biogenesis [8], was selectively inactivated in GnRH neurons. Animals harboring a floxed *Dicer* allele [5] were crossed with those expressing Cre recombinase under the control of the endogenous GnRH promoter [9] (**Fig. 1a**), which is expressed in hypothalamic GnRH neurons but not the gonads (**Supplementary Fig. 2a, b**). The resulting *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice were viable, born at Mendelian frequencies, and had body-weights at adulthood indistinguishable from *Dicer*$^{loxP/loxP}$ (wild-type) littermates ($t_{(8)}$=4.35, p=0.390, n=5 mice). All male and female mutant mice, however, exhibited severe hypogonadism (**Fig. 1b, c**; **Supplementary Fig. 2c, d**) and sterility (n=6 males; n= 6 females) (**Fig. 1d**). Serum levels of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were markedly reduced in both female and male adults (**Fig. 1e, f**). LH levels were readily increased by systemic GnRH injection, demonstrating intact pituitary function in *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice (**Supplementary Fig. 2e**), but were unaffected by the intracerebroventricular infusion of two potent stimulators of the GnRH system that trigger LH release in wild-type males: kisspeptin, which acts through its receptor Gpr54, and NMDA, which bypasses the kisspeptidergic system to stimulate GnRH neurons [10,11] (**Supplementary Fig. 2f, g**), suggesting that the hypogonadotropic hypogonadism observed was due primarily to hypothalamic GnRH deficiency. This possibility was further supported by the effective promotion of uterine growth (**Supplementary Fig. 2c**) and ovulation (**Supplementary Fig. 2h**) by treatment with gonadal steroids and pregnant mare gonadotropins, respectively, in *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice, indicating that gonadal function could be fully rescued by hormonal replacement downstream of GnRH neurons. Concerning postnatal sexual maturation, while some female *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice underwent vaginal opening (5 of 7) (**Fig. 1g**), this external sign of sexual maturation occurred significantly later than in *Dicer*$^{loxP/loxP}$ mice (Breslow-Wilcoxon test: $\chi^2_{(2)}$=9.363, p=0.0022, n=5

and 11 mice per group), and the absence of estrous cyclicity in vaginal smears showed that they never ovulated (n=7 mice) (**Fig. 1h**) despite normal somatic growth (two-way repeated-measures ANOVA, genotype: $F_{(1,\ 8)}$=0.0129, p=0.912; time: $F_{(11,\ 88)}$=178.9, p<0.0001; interaction: $F_{(11,\ 88)}$=5.19, p<0.0001; subject matching: $F_{(8,\ 88)}$=104, p=0.0001, n=5 mice per group) (**Fig. 1i**). Similarly, male *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice (n=5) did not exhibit balanopreputial separation, which occurred at 34.9±1.8 days of age in *Dicer*$^{loxP/loxP}$ littermates (n=11), or spermatozoa in seminiferous tubules (**Fig. 1c**).

Following these physiological data, neuroanatomical analyses revealed a complete absence of GnRH immunoreactivity in the hypothalamus of adult male and female mutants (**Fig. 2a**; **Supplementary Fig. 3**), reminiscent of Kallmann syndrome in humans [2], where GnRH neurons originating in the olfactory placode fail to migrate into the brain during development. To determine whether *Dicer* invalidation in mouse GnRH neurons similarly affected their migration, we examined them on embryonic day (E) 14.5, when half the migrating GnRH neurons are still in the nose, and at birth (postnatal day 0, P0), when GnRH neurons have completed their migration into the hypothalamic preoptic region, their principal site of residence, and sent projections to the median eminence, where they release their neurohormone into portal capillaries for delivery to the anterior pituitary [12,13]. Unlike the case in Kallmann patients, GnRH neurons displayed no migratory defects and GnRH immunoreactivity was comparable in *Gnrh*::Cre; *Dicer*$^{loxP/loxP}$ and *Dicer*$^{loxP/loxP}$ littermates at P0 ($t_{(4)}$=0.27, p=0.8, n=3 mice per group) (**Supplementary Fig. 4**). GnRH deficiency in mutant mice was thus not due to a developmental lack of GnRH neurons, but acquired postnatally.

Hypothalamic GnRH peptide content increases progressively between birth and puberty, quickening markedly during the infantile period (P7-P20) [14,15]. We thus examined hypothalamic GnRH immunoreactivity at different postnatal ages and found that its disappearance in *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice occurred gradually in both sexes, starting during the infantile period (**Fig. 2a**, **Supplementary Fig. 5**), and accelerating after weaning, during the juvenile period, i.e. P21 to ~P35 (two-way ANOVA: genotype, $F_{(1,45)}$=84.05, p<0.0001; time, $F_{(4,45)}$=19.56, p<0.0001; interaction, $F_{(4,45)}$=6.62, p=0.0003; Sikad's multiple

comparison test, *Dicer*$^{loxP/loxP}$ *vs. Gnrh*::Cre;*Dicer*$^{loxP/loxP}$: $t_{(45)}$=0.624, p=0.978, n=3 per group at P0; $t_{(45)}$=2.12, p=0.184, n=5 and 7, respectively, at P12; $t_{(45)}$=5.31, p<0.0001, n=6 and 7 at P21; $t_{(45)}$=7.98, p<0.0001, n=7 and 9 at P28; and $t_{(45)}$=6.32, p<0.0001, n=4 per group in adults) (**Fig. 2a**, **Supplementary Fig. 5**). Given the GnRH peptide extreme stability (for instance, GnRH immunoreactivity persists in axon terminals days after its disappearance from the cell body, **Supplementary Fig. 5**), we decided to construct a reporter system to more accurately track GnRH-expressing neurons postnatally. Unexpectedly, the generation of *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$;td*Tomato*$^{loxP/STOP}$ trigenic mice showed that in the absence of miRNAs, GnRH neurons did not die but simply lost GnRH immunoreactivity (**Fig. 2b**). In these mice, in which tdTomato, once triggered by GnRH transcriptional activation and thus Cre production during development, is expressed constitutively in GnRH neurons even without continued GnRH promoter activity, the number of tdTomato-expressing GnRH neurons did not significantly decrease, but around ~70% of them had lost GnRH peptide at P21 when compared to *Gnrh*::Cre;*Dicer*$^{+/+}$;td*Tomato*$^{loxP/STOP}$ littermates. To further pinpoint the stage at which GnRH expression is blocked in *Dicer*-deficient mice, we constructed *Gnrh::*Gfp;*Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ reporter mice, which express GFP under the control of an ectopic GnRH promoter only when the promoter is currently active, unlike tdTomato in the previous experiment. These mice showed a concomitant loss of GnRH peptide and GFP during postnatal development (**Supplementary Fig. 6**). Because the 3'UTR, and thus the putative miRNA target sequence, of the G*fp* transcript is completely distinct from the one coding *Gnrh*, this indicates that miRNAs probably did not regulate GnRH transcript stability and/or translation directly, but rather affected genes necessary for GnRH transcription, such as promoter modulators.

An infantile miRNA switch inverts GnRH promoter modulator expression

To tease apart the mechanism of miRNA action, we next verified the expression profile of the GnRH transcript as well as of GnRH promoter modulators in GnRH neurons isolated by fluorescence-activated cell sorting (FACS) at two stages: P7 and P12. As mentioned, P7 signals the beginning of the infantile period, while at P12 hypothalamic GnRH peptide

content undergoes a functionally relevant increase in normal mice [14,15] but begins to decrease in *Dicer*-deficient mice even though the vast majority of their GnRH neurons (~80%) still express detectable GFP (**Supplementary Fig. 6**). This increase leads to a phenomenon called "mini-puberty" in humans, characterized by the first centrally-driven gonad-independent activation of the HPG axis and a significant but transient surge in gonadotropin levels[15,16]. Real-time PCR analyses of FACS-isolated GFP-expressing GnRH neurons from *Gnrh::*Gfp;*Dicer*$^{loxP/loxP}$ mice (**Fig. 2c**; **Supplementary Fig. 7**), which are wild-type for *Dicer*, revealed a 200% increase in GnRH mRNA levels between P7 and P12 ($t_{(5)}$=-3.20, p=0.024, n=4 mice per group) (**Fig 2d**). This was accompanied by a significant upregulation of mRNA levels of several promoter activators known to affect GnRH expression [17,18] (**Fig. 2d**), while transcripts of three known GnRH repressors [17,18] remained unchanged (**Fig. 2d**). However, in GnRH neurons lacking *Dicer* (t-test, $t_{(5)}$=3.58, p=0.0159; n=4 mice per group) (**Fig. 2e**) isolated from *Gnrh::*Gfp;*Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ trigenic mice at P12, when GnRH mRNA should normally begin increasing, GnRH mRNA expression was significantly lower than in neurons from *Dicer* wild-type *Gnrh::*Gfp;*Dicer*$^{loxP/loxP}$ mice ($t_{(3.12)}$=5.88, p=0.0098, n=4 per group) (**Fig. 2e**). These altered expression levels were functionally relevant, corresponding to impaired FSH release during the infantile period (females: $t_{(19)}$=5.578, p<0.0001, n=10 and 11 per group; males: $t_{(20)}$=1.612, p=0.1226, n= 12 and 10 per group) (**Fig. 2f**). In addition to reduced GnRH transcript expression (**Fig.2e**), there was a marked decrease in the expression of most GnRH promoter activators (**Fig. 3a**) and a strong 15-fold increase in the transcript of the GnRH promoter repressor *Cebpb* (**Fig. 3b**) in *Dicer*-deficient GnRH neurons (**Fig. 3a,b**), supporting the hypothesis that miRNAs control GnRH transcript levels indirectly, by altering levels of GnRH promoter modulators.

miR-200 and *Zeb1* directly and indirectly control GnRH expression

The altered expression of GnRH promoter activators and repressors suggests that in the absence of miRNAs, repressors of these activators, in addition to repressors of GnRH itself, might be reciprocally increased. *In silico* analysis of the 3'UTR of these promoter modulators

(Aes, Dlx1, Dlx5, Meis1, Otx2, Pbx1, Pknox1, Pouf2f1, Cebpb, Msx1 and Tle4) revealed the presence of putative binding sites for several miRNAs ($p<0.01$; Table S1), confirming that miRNAs could regulate their expression. Concordantly, we uncovered a remarkable inversion of the expression profile of these and other putative regulatory miRNAs in FACS-sorted wild-type GnRH neurons between P7 and P12 (**Fig. 3c**), strongly suggesting that GnRH promoter activity during the infantile period answers to a miRNA-operated switch. To identify the molecular effectors and targets involved, we looked for miRNAs that were not only differentially expressed between P7 and P12 (**Fig. 3c**) but specifically enriched in GnRH neurons compared to non-GnRH cells at P12 (**Fig.3d**). Members of the miRNA-200 family, including miR-141 and miR-429 (**Supplementary Fig. 8a**), were among the most highly expressed miRNAs in infantile GnRH neurons when compared to non-GnRH-expressing cells (**Fig. 3d,e**). Within this family, the expression of miR-200a and miR-429 increased significantly between P7 and P12 (miR-200a, $t_{(4)}=9.178$, $p=0.0008$; miR-429, $t_{(5)}=3.931$, $p=0.0111$, n=3 and 4 mice per group) (**Fig. 3f**); these miRNAs are known to silence the transcriptional repressor Zeb1 in pituitary gonadotropes, where it regulates LH expression [6]. To verify whether the miR-200/*Zeb1* network [19] (**Supplementary Fig. 8a**) also controlled hypothalamic GnRH expression during postnatal development, we analyzed *Zeb1* expression in mice with or without miRNA production in GnRH neurons. Importantly, not only was *Zeb1* mRNA expressed in infantile GnRH neurons, its expression decreased significantly between P7 and P12 in *Gnrh::*Gfp;*Dicer*$^{+/+}$ mice ($t_{(6)}=4.86$, $p=0.0028$, n=4 per group) (**Fig. 4a**). RT-PCR in GnRH neurons isolated from *Gnrh::*Gfp;*Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ and *Gnrh::*Gfp;*Dicer*$^{loxP/loxP}$ littermates at P12 further showed that *Zeb1* mRNA was significantly upregulated in mice with *Dicer*-deficient GnRH neurons ($t_{(5)}=-3.38$, $p=0.0195$, n=3 and 4 mice per group) (**Fig. 4b**). *In silico* sequence analysis revealed varying numbers of putative Zeb1-binding sites in the genes of all GnRH transcriptional activators examined (**Supplementary Fig. 8b**), including Pou2f1, which, in addition to activating the GnRH gene directly, could increase miR-200 levels [20], thereby indirectly influencing GnRH activation by repressing Zeb1. Concordantly, we found that both human (**Supplementary Fig. 8c**) and

mouse GnRH promoters (**Fig. 4c**) contained putative Zeb1-binding sites. Chromatin immunoprecipitation assays in an immortalized mouse GnRH neuronal cell line expressing both promoters (**Fig. 4c**) (**Supplementary Fig. 8c**) revealed that several predicted as well as previously unknown sites were functionally active. Altogether these data support the idea that increased Zeb1 expression in the absence of miRNA biogenesis could mediate the downregulation of these activators and of GnRH in these neurons (**Fig. 3a**). To test this hypothesis, we infused a target-site blocker, TSB-200, into the brains of *Gnrh::*Gfp;*Dicer*$^{+/+}$ mice to selectively hamper miR-200b/200c/429 binding to *Zeb1* mRNA (**Supplementary Fig. 8d**). TSBs are small, freely diffusible locked-nucleic-acid (LNA) oligonucleotides with two notable characteristics: (i) they are highly stable (with a reported half-life *in vivo* on the order of weeks) and resistant to nuclease-mediated degradation *in vitro* and *in vivo*, thanks to their modified phosphorothioate backbone [21], and (ii) they are complementary to both the miRNA and the binding site being targeted, making them extremely specific (see for example **Supplementary Fig. 9b-e**). As shown by fluorescence-tagged sequences (**Supplementary Fig. 9a**), TSBs readily targeted the preoptic region and GnRH neurons 72h after infusion into the lateral ventricle of *Gnrh::*Gfp;*Dicer*$^{+/+}$ pups. TSB-200 significantly increased *Zeb1* mRNA levels in FACS-sorted GFP-positive GnRH neurons at P12 as compared to a scrambled sequence (t-test, $t_{(9)}$=-3.33, p=0.0087, n=5 and 6 mice per group) (**Fig. 4d**), but did not affect the transcripts for two other miR-200b/200c/429 target genes, *Lpin1* [22] (t-test, $t_{(10)}$=0.663, p=0.52, n=6 per group) and *Maf* [23] (t-test, $t_{(10)}$=-0.192, p=0.85, n=6 per group) (**Fig. 4d**). This increased *Zeb1* expression was specific to GnRH neurons (**Supplementary Fig. 10c**), in keeping with the enrichment of miR-200b/200c/429 in these cells (**Fig. 3d,e**), and was accompanied by a significant decrease in transcripts of two GnRH promoter activators that were also upregulated in GnRH neurons at P12 compared to P7 (**Fig. 2d**), *Meis1* (t-test, $t_{(8)}$=2.53, p=0.035, n=4 and 6 mice per group) and *Pou2f1* (t-test, $t_{(10)}$=4.15, p=0.002, n=6) (**Fig. 4e**); transcripts for the promoter activators *Aes*, *Otx2*, *Pbx1* and *Pknox1*, which did not significantly change between P7 and P12, were also unchanged by TSB-200 infusion. Interestingly, these genes also had fewer putative Zeb1-binding sites than those whose

expression was strongly modified by altered *Zeb1* expression (**Supplementary Fig. 8b**). As expected, *Gnrh* transcription was also downregulated by the blockade of miR-200/ *Zeb1* binding at P12 (t-test, $t_{(9)}$=5.37, p=0.0004, n=4 and 7 mice per group) (**Fig. 4f**). This repression of *Gnrh* was apparently mediated by the inhibition of GnRH promoter activity, as shown by the strong decrease in *Gfp* reporter mRNA expression (t-test, $t_{(10)}$=14.1, p<0.0001, n=6 mice per group) (**Fig. 4f**), suggesting that the miR-200/*Zeb1* network is actively involved in the switch in GnRH transcriptional control in the infantile mouse hypothalamus (**Supplementary Fig. 8e**).

Hampering miR-155/*Cebpb* binding lowers *Gnrh* mRNA by activating *Zeb-1*

Contrary to the downregulation of GnRH promoter activators in mice with *Dicer*-deficient GnRH neurons, the mRNA for a GnRH repressor, *Cebpb*, which codes for CAAT/enhancer binding protein β (C/EBPβ), was highly upregulated (see **Fig. 3b** above). We thus reasoned that C/EBPβ repression by miRNAs would increase radically in infantile wild-type mice. The miRWalk database, which provides validated information on miRNA-target interactions [24], showed that among miRNAs upregulated in GnRH neurons at P12 (**Fig. 3c,d**) was miR-155, whose seed target region is present in the *Cebpb* transcript 3'UTR (p=0.0077, Table S2, **Supplementary Fig. 9b, c**) [25]. To determine whether the ten-fold increase in miR-155 expression between P7 and P12 ($t_{(4)}$=-4.85, p=0.0083, n=3 mice per group) (**Fig. 5a**) played an actual role in regulating C/EBPβ expression in GnRH neurons, we used TSB-155 to selectively hamper miR-155/*Cebpb* binding in the hypothalamus of infantile mice (**Supplementary Fig. 9d, e**). TSB-155 infusion into *Gnrh::*Gfp;*Dicer*$^{+/+}$ mice markedly increased *Cebpb* mRNA levels in GnRH neurons when compared to a scrambled sequence ($t_{(9)}$=-3.41, p=0.0038, n=5 and 6 mice per group) (**Fig. 5b**), but did not change mRNA levels of *Lpin1* ($t_{(10)}$=0.601, p=0.56, n=6) or *Maf* ($t_{(10)}$=0.406, p=0.69, n=6) (**Fig. 5b**), which are also validated miR-155 targets [26,27]. Importantly, this TSB-155-mediated upregulation of the *Cebpb* transcript was associated with a strong inhibition of both GnRH promoter activity, as

indicated by *Gfp* mRNA levels ($t_{(10)}$=14, p<0,0001, n=6 mice per group) (**Fig. 5c**), and *Gnrh* mRNA levels ($t_{(8)}$=3.57, p=0.0073, n=4 and 6 mice per group) (**Fig. 5c**). However, we found using ChIPBase (an integrated resource and platform for decoding transcription factor binding maps based on ChipSeq data [28]) that the *Zeb1* gene contained binding sites for C/EBPβ in six different regulatory regions [29,30] (**Supplementary Fig. 11a**), but no binding site for miR-155, suggesting that C/EBPβ could regulate GnRH mRNA levels not only directly but indirectly, by influencing *Zeb1* expression. In agreement with this hypothesis, TSB-155, which selectively upregulates *Cebpb* expression, also increased the *Zeb1* transcript in *Dicer*$^{+/+}$ GnRH neurons ($t_{(9)}$=-2.89, p=0.0177, n=5-6 per group) (**Fig. 5c**). Furthermore, *in silico* and ChipSeq analyses revealed several binding sites for both C/EBPβ- and Zeb1 on the *Gpr54* gene (**Supplementary Fig. 8b** and **Supplementary Fig. 11b**), suggesting that in addition to their direct and promoter-activator-mediated effects, C/EBPβ and Zeb1 could repress GnRH gene transcription indirectly by blocking kisspeptidergic signaling through Gpr54, known to increase GnRH transcription by promoting the nuclear translocation of the GnRH promoter activator Otx2 [31] (**Supplementary Fig. 11c**). Supporting this hypothesis, RT-PCR analysis revealed that in animals with Dicer-deficient GnRH neurons, *Gpr54* mRNA levels were also strongly reduced ($t_{(6)}$=4.22, p=0.0056, n=4 mice per group) (**Fig. 5d**), to a similar extent as that of other GnRH activators (**Fig. 3a**).

Blocking infantile NO release partially rescues GnRH expression

C/EBPβ is also known to mediate the nitric oxide (NO)-induced repression of *Gnrh* expression [17]. However, we have recently shown that activation of neuronal NO synthase (nNOS) in preoptic neurons, itself regulated by Kisspeptin-Gpr54 binding [32], is required for sexual maturation [33]. In wild-type mice in the present study, nNOS activation by its phosphorylation at Ser1412[36] increased significantly in the preoptic region between P7 and P12, when GnRH neuronal network synaptic connectivity is being established [34,35] (p-nNOS/NOS ratio, Mann-Whitney test: U=0, p=0.0286, n=4 animals per group) (**Fig. 5e**). To

determine whether the marked decrease in GnRH expression in *Dicer*-deficient mice could be related to this infantile increase in NO-C/EBPβ signaling, we next treated P12 *Gnrh::*Gfp*;Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice with the NOS inhibitor N-G-Nitro-L-arginine methyl ester (L-NAME; 50 mg/kg, i.p.) and assessed GnRH mRNA expression in FACS-isolated neurons 12h later. Reduced NO production following L-NAME treatment partially but significantly rescued *Gnrh* transcript levels ($t(8)=-2.40$, $p=0.043$, n=6 and 7 mice per group) (**Fig. 5f**). In addition to the alleviation of the previously demonstrated repressive effect of C/EBPβ on the GnRH promoter [17], the increased GnRH expression could have resulted from the inhibition of the transcriptional activation of Zeb1 by C/EBPβ (**Supplementary Fig. 11a**), as indicated by the 2.4-fold increase in transcripts for Pou2f1 (in L-NAME-injected vs. saline-injected mice; t-test: $t(9)=2.92$, $p=0.017$, n=5 and 6 per group; **Supplementary Fig. 9f**). Increased NO levels during this period crucial for subsequent GnRH neuronal function might therefore actually participate in the multilayered transcriptional repression of the *Gnrh* gene in the absence of posttranscriptional silencing of C/EBPβ by miRNAs (**Supplementary Fig. 9g**). In other words, not only do miRNAs control reproductive capacity by controlling the switch in GnRH promoter activity in infantile GnRH neurons, they do so through an intricate mechanism involving crosstalk between inductive and repressive pathways.

miR-200 family/*Zeb-1* binding controls GnRH function

To assess the functional involvement of miR-200 family members and miR-155 in GnRH neurons in puberty onset, we performed a single bolus injection of the same TSB-200 and/or TSB-155 into the brains of female wild-type mice at P9, and monitored sexual maturation after weaning. Fluorescently-labeled TSBs were detectable in the brain up to 2 weeks post-injection (**Supplementary Fig. 9a**), in keeping with previous reports showing that they remained active for at least a week *in vivo* [37]. Surprisingly, while growth (**Supplementary Fig. 12a**) was similar in TSB- and scrambled-sequence-injected mice, TSB-injected mice exhibited a precocious onset of puberty when compared to control mice (**Fig. 6a**). This effect was most pronounced in TSB-200-infused mice, with 50% of animals displaying their first

estrus by P32, i.e. more than 10 days before control mice. In line with these results, TSB-200-infused mice showed significantly elevated peripubertal afternoon surges of plasma LH at P38, while changes in LH levels induced by TSB-155 treatment did not reach statistical significance when compared to control mice, in which plasma LH levels were at the limit of detection (**Fig.6b**). The combination of TSB-200 and TSB-155, however, only minimally increased LH levels (**Fig. 6b**; one-way ANOVA: $F(3, 32)=3.475$, $p=0.0272$, n=8,9,9,10 mice per group; Tukey's multiple comparisons test: control *vs.* treated groups: TSB-200, $q_{(32)}=4.03$, $p=0.0361$; TSB-155, $q_{(32)}=1.4$, $p=0.754$; TSB-200+TSB-155, $q_{(32)}=0.151$, $p=0.9996$), and induced earlier puberty onset by about 5 days when compared to control mice, similar to the effect of TSB-155 treatment alone (**Fig. 6a**; Breslow-Wilcoxon test: TSB vs. control treatment: TSB-200, $\chi^2_{(1)}=10.86$, $p=0.01$; TSB-155, $\chi^2_{(1)}=5.181$, $p=0.0228$; TSB-200+155, $\chi^2_{(1)}=5.797$, $p=0.0161$, n=8,9,9,10 mice per group). To verify whether this discrepancy in the impact of blocking miR-200/429/*Zeb1* binding vs. miR-155/*Cebpb* binding on GnRH promoter activity at P12 (**Fig. 4f** and **Fig. 5c**, respectively) and on subsequent sexual maturation (**Fig 6a**) was linked to differences in the specific cell types targeted by TSB-155 and TSB-200 in the hypothalamus, we analyzed the effects of TSB injection on *Cebpb* and *Zeb1* expression in non-GnRH cells at P12. In agreement with the level of enrichment of miRNA expression in GnRH neurons compared to non-GnRH hypothalamic cells, which is about 50 fold higher for miR200/429 (**Fig. 3e**) than for miR-155 (**Supplementary Fig. 10a**), *Cebpb* mRNA levels were significantly increased in non-GnRH cells of the preoptic region after TSB-155 infusion (**Supplementary Fig. 10b**), while TSB-200 treatment altered neither *Cebpb* nor *Zeb1* transcripts in these cells (**Supplementary Fig. 10b, c**). In contrast to TSB-155, therefore, TSB-200 selectively targets GnRH neurons. Altogether, these data suggest that, in keeping with the complex feedback mechanisms at work (**Supplementary Fig. 13**), selectively applying the brakes to GnRH production in an excessive manner (**Fig. 4f**) and then releasing them prematurely can trigger precocious puberty, possibly due to a rebound effect (**Supplementary Fig. S12b**). In line with this hypothesis, in females treated with TSB-200 at P9, there was a normalization of the levels of

Zeb1 mRNA ($t_{(7)}$=0.0797, p=0.939, n=4 and 5 mice per group) (**Fig. 4d**) and *Gnrh* mRNA levels ($t_{(7)}$=0.3079, p=0.767, n=4 and 5 per group) (**Fig. 4f**) in FACS-sorted GnRH neurons at P28 (i.e. shortly before the observed date of puberty onset), accompanied by a significant elevation in afternoon circulating levels of LH, when compared with control female mice ($t_{(8)}$=2.344, p=0.0471, n=4 and 6 per group) (**Fig. 6c**).

To determine whether the miR-200/*Zeb1* network could also control GnRH function outside of this critical infantile time-window leading up to puberty, we next infused TSB-200 into the hypothalamic preoptic region of cycling adult female mice and followed their estrous cycles (**Fig. 6d**). While scrambled-sequence-infused control mice did not exhibit any marked alteration of their estrous cycles, most mice treated with TSB-200 (5 out of 6) showed a slight prolongation in the number of days spent in diestrus (a basal stage when LH is released at nadir levels) and eventually reached proestrus (when the preovulatory surge of LH occurs), but displayed an incomplete cycle at least once during the first two weeks after injection (**Fig. 6d**). In line with this observation, proestrous LH levels one week after injection showed a characteristic surge in controls but not in TSB-200-treated mice (**Fig. 6e**; one-way ANOVA: F(2, 14)=4.371, p=0.0335, n=5 and 6 mice per group; control *vs.* TSB-200 >1 week, $q_{(14)}$=3.956, p=0.0357; control *vs.* TSB-200 >2 weeks, $q_{(14)}$=1.02, p=0.754). In 4 of the 6 animals treated with TSB-200, LH levels recovered and displayed normal preovulatory surges by the third week after TSB infusion (**Fig. 6d**).

DISCUSSION

The balance between inductive and repressive signals is a process essential for successful reproduction in mammals [38,39]. Our data provide the first physiological and neuroanatomical evidence that miRNAs, by modulating this balance in the GnRH gene network during the infantile period, play an essential role in the postnatal development of the hypothalamic neuroendocrine neurons controlling the onset of puberty and fertility in mammals, in addition to their known involvement in somatic growth, including the development of peripheral sexual organs [6,7]. This infantile "critical period", lasting just a few days, corresponds to the first

centrally-driven gonad-independent activation of the HPG axis and resulting surge in gonadotropin levels. This phenomenon, known as "mini-puberty" in humans, is the first of three activational periods that primes the HPG axis for puberty and adult fertility, setting in motion, for example, the growth of the first wave of ovarian follicles that will ovulate at puberty in females and the development of the testes in males (see for review [15]). We show that during this critical period, a switch in miRNA expression in turn flips a switch in a multilayered array of GnRH gene activators and repressors, permitting the sustained increase of the neurohormone required for subsequent sexual maturation. Two miRNA species act as the linchpins of this process: the miR-200/429 family, which is not only upregulated during this critical period but selectively enhanced in GnRH neurons, and miR-155, which appears to act on other hypothalamic cell types as well, and mediates, for example, the effects of a concomitant release of NO upstream of GnRH neurons. Interfering with the binding and function of these two key species blunts the infantile increase in *GnRH* expression, and the *in vivo* alteration of the miR-200/429-transcription factor micronetwork leads to the disruption of normal puberty onset as well as normal estrous cyclicity in adulthood.

The fact that GnRH neurons form an extremely small population (~800 neurons in a mouse brain) and their scattered distribution in a continuum from the olfactory bulb to the hypothalamus, albeit with a strong concentration in the hypothalamic preoptic area, have made genetic and epigenetic studies in these neurons a veritable technical challenge until now. Our successful separation of GnRH from non-GnRH cells in the preoptic region of *GnRH*::Gfp mice has allowed us to determine miRNA expression profiles specific to this limited population of neurons in the postnatal brain, i.e. once differentiation and the establishment of connectivity, including their projection over several millimeters to the hypothalamic median eminence, are complete. The difficulty of this approach is evident from the fact that other such studies, even those focusing on more abundant neuronal types, have not always succeeded [40]. Using this approach, we were not only able to study changes in miRNA expression in GnRH neurons at different stages of development, but to analyze the

effects of the selective invalidation of miRNA biogenesis and acute manipulation of specific miRNA networks on the expression of transcription factors, GnRH promoter activity and GnRH expression in the postnatal brain *in vivo*.

We demonstrated the specific role of miRNAs in sustaining postnatal GnRH expression by generating *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice, in which Cre-mediated *Dicer* excision should have occurred as soon as the GnRH promoter was first activated during embryonic development, i.e. at E14.5 [41], early enough for any miRNA effects on GnRH neuronal migration or integration into hypothalamic circuits to be apparent. Our findings show that miRNA processing in GnRH neurons is required for sexual development, but not GnRH neuronal migration *in vivo*; the latter observation is in contrast with the reported involvement of miRNAs in the migration of other brain neuronal populations [42]. Indeed, GnRH expression appears normal during the perinatal and early infantile periods, and the process of sexual maturation commences normally and leads to vaginal opening in 80% of our mice. However, these mice never achieve puberty because of the progressive decline in GnRH promoter activity from the second week of postnatal life (P7 to P12) and its complete extinction during the juvenile period. Our data indicate that miRNA species whose expression is upregulated in GnRH neurons during this critical period include miR-155 and members of the miR-200 family. Notably, the significant 100-fold enrichment of miR-200 family members is restricted to GnRH neurons, indicating a highly specific function for these miRNA species in the regulation of infantile GnRH expression. We have identified several genes targeted directly or indirectly by miR-155 and miR-200/429, whose repression (e.g. Cebpb and Zeb1) or activation (e.g. Pou2f1 and Meis1, which are both targets for Zeb1) is required for normal GnRH expression at P12, corresponding to the first centrally-driven activation of the HPG axis and resulting gonadotropin surge in mice (**Supplementary Fig. 13**). In particular the miR-200/429 family and its target Zeb1, which can directly repress the GnRH promoter, also seem to form a double-negative loop with the key GnRH promoter activator Pou2f1, while miR-155 counteracts NO/Cebpb-dependent GnRH repression. Nor can we rule out the possibility that some promoter modulators act as both activators and repressors depending

upon physiological conditions and cellular context, or exert a reciprocal regulatory effect on the miRNAs that control their expression. For instance, Cebpb, a GnRH promoter repressor, nevertheless increases Zeb1 levels in GnRH neurons but not in non-GnRH hypothalamic cells (which do not express miR-200/429), while Zeb1 is known to provide negative feedback to miR-200/429 [19].

The expression patterns of various known GnRH promoter modulators at P12 are not the same. While activators that are upregulated specifically in GnRH neurons in wild-type mice at P12 show reduced expression with the blockade of miR-200/429 binding (with the exception of Dlx5, which has no Zeb1 binding site and is thus probably regulated by other miRNA-effector pathways), the expression of one known activator, Dlx1[43], is increased. Apart from a possible indirect effect, the fact that unlike Pou2f1 or Meis1, Dlx1 expression shows a downward trend in wild-type GnRH neurons at P12 could explain this seeming paradox. The lack of effect of a miRNA-200/429 blockade on the expression of GnRH activator genes that contain no or few Zeb1-binding sites and that are not differentially expressed during the infantile-juvenile transition in GnRH neurons (but that could nevertheless play a role in baseline GnRH production or its regulation in other physiological contexts) further highlights the importance of the miR-200/Zeb1/Cebpb switch in the prepubertal increase in GnRH expression.

Both miR-155 (by interfering with NO/Cebpb-mediated signaling by nNOS neurons, themselves activated by Kisspeptin [32]), and miR-200/429 (through Zeb1) could influence GnRH expression through another route: by modulating the intricate relationship between different cells of the hypothalamic GnRH network through their effect on the expression of the Kisspeptin receptor Gpr54, and thus on Kisspeptin-Gpr54-mediated activation of GnRH expression (**Supplementary Fig. 13**). Notably, kisspeptidergic fibers arising from the arcuate nucleus of the hypothalamus (ARH), like the axons of all ARH neurons, first reach the preoptic region around P12 and only achieve their final distribution by the end of the infantile period in both males and females [34,35]. The subsequent formation of new synaptic contacts with GnRH cell bodies and dendritic pruning [44] could be among the triggers of the miRNA-

mediated switch in the control of GnRH promoter activity during this period (**Supplementary Fig. 13**). NO itself, through its involvement in synapse formation, elimination and efficacy[45,46], and later, through its essential function in puberty initiation [33] and its participation in the repression of GnRH expression [17], could play a more complex role than suspected heretofore. The kisspeptin/nNOS/GnRH neuronal microcircuit and miRNA-gene network in GnRH neurons (**Supplementary Fig. 13**) would thus be ideally positioned to ignite rhythmic GnRH gene transcription and pulsatile GnRH secretion [47] at puberty in response to developmental and bodily cues during the infantile period [15].

Other microcircuits and feedback loops, possibly involving the expression of miR-155 in other cell types, could also exist, as suggested by the fact that simultaneously neutralizing both miR-200/429/Zeb1 binding and miR-155/Cebpb binding results in a considerably weaker effect on peripubertal LH levels than miR-200/429 blockade alone. Intriguingly, while blocking miR-200/429/Zeb1 binding during this infantile-juvenile critical period suppresses GnRH promoter activity at the cellular level, these animals go on to display precocious, not delayed, puberty. Our data suggest that this counterintuitive finding could in fact stem from a rebound effect, where the prolonged inhibition of GnRH expression (TSBs, once injected, remain at the injection site for around 2 weeks) results in a compensatory spike once the inhibition is relieved (**Supplementary Fig. 12b**). Such a phenomenon has been demonstrated previously in human populations, where children whose GnRH system is blunted by exposure to endocrine disruptors of agricultural origin undergo precocious puberty when they migrate to countries that do not use them [48].

While our study was primarily performed in mice, miRNA expression profiles available from RNAseq repositories for human tissues (e.g. http://www.gtexportal.org/home) indicate that miR155 is also widely distributed in the hypothalamus and other brain regions in humans. In contrast, the expression of miR-200 family members is, as described in mice [6], below the limit of detection in human brain tissues including the hypothalamus, an observation consistent with the restriction of these miRNA species to the sparse GnRH neuronal population. The Allen Human Brain Atlas (http://human.brain-map.org) also shows

the coexpression of *ZEB1* and *CEBPB* with *GNRH1* in regions of the diencephalon overlapping the hypothalamus (**Supplementary Fig. 15**). Altogether, these *in silico* data, combined with our ChIP assays identifying ZEB1 binding sites in the human *GNRH1* promoter (Fig S7C), suggest that the miRNA-GnRH promoter modulator network identified in this study (**Supplementary Fig. 13**) may also be physiologically relevant for HPG axis activation during "mini-puberty" and continued sexual maturation in humans [49].

To conclude, our data are the first to indicate not only that miRNAs are active in the hypothalamic GnRH network, but that miRNA-200/429 and miRNA-155 are key components of a complex developmental switch that controls GnRH promoter activity, illustrated in the models presented in **Supplementary Fig. 13**, whose correct functioning is essential for the normal initiation of puberty and adult fertility. Moreover, this dramatic switch in gene expression appears to involve a multilayered and interconnected array of miRNAs and their target genes in GnRH neurons, with its own inbuilt feedback control mechanism, that alters the balance between inductive and repressive signals during the infantile critical period and thus leads to increased GnRH production. Deciphering the mechanisms through which miRNAs contribute to precocious or delayed puberty through their actions on GnRH neural network function may thus not only provide novel insights into the epigenetic regulation of maturational processes, fascinating in itself, but pave the way to a better understanding of idiopathic infertility [50] and the elaboration of new diagnostic and therapeutic options in humans.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M., F.L., K.C., J.R., S.R., F.G., M.T.-S., P.G. and V.P. designed the experiments. A.M., F.L., K.C., J.R., N.J., S.G., F.G., J.P., P.G. and V.P. performed experiments. A.M. and V.P. analyzed the data. All authors discussed the results and A.M., J.R., S.R., M. T.-S., P.G. and V.P. wrote the manuscript.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interest

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Figure 1. Lack of miRNA biogenesis in GnRH neurons leads to hypogonadotropic hypogonadism and infertility. (**a**) Genetic strategy to invalidate *Dicer* expression specifically in GnRH-expressing cells in mice. (**b**) Smaller testes and ovaries and thread-like uteri in mutant mice. (**c**) Hematoxylin-eosin-stained ovarian and testicular sections of mutant *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice and their wild-type *Dicer*$^{loxP/loxP}$ littermates. Asterisks show corpora lutea and arrows, spermatozoa. Scale bars: 500 μm (left and middle panels) and 30 μm (right panels). Pictures shown in **b** and **c** are representative of what has been observed in more than 10 animals per sex and per genotype. (**d**) Both male and female *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice exhibit are infertile. N.D.: not detected. (**e**, **f**) Circulating levels of LH (top panels) and FSH (bottom panels) in GnRH cells of control (blue) and *Dicer* mutants (red): diestrous females (LH, t-test, $t_{(12)}=2.82$, p=0.0155, n=6 and 8 mice per group; FSH, t-test, $t_{(13)}=2.46$, p=0.0287, n=7 and 8 mice per group) and males (LH, t-test, $t_{(15)}=2.16$, p=0.047, n=6 and 11 mice per group; FSH, t-test, $t_{(15)}=16.2$, p<0.0001, n=6 and 11 mice per group). (**g** to **i**) While some *Gnrh*::Cre; *Dicer*$^{loxP/loxP}$ female mice undergo vaginal opening during postnatal development (**g**), they never reach puberty (**h**) despite normal somatic growth (**i**). Values shown are means ± SEM.

Figure 2. A switch in the control of GnRH gene expression is operated by miRNAs during postnatal developmental. (**a**) Gradual disappearance of GnRH-immunoreactive (GnRH-IR) neurons in *Gnrh*::Cre;*Dicer*$^{loxP/loxP}$ mice (red bars) during postnatal development. Data from mice wild-type for *Dicer* appear in blue. P0, postnatal day 0; N.D., not detected (***: p<0.001). (**b**) Neuronal lineage tracing shows normal distribution of GnRH neurons in P21 juvenile mice with GnRH deficiency (*Gnrh*::Cre;*Dicer*$^{loxP/loxP}$;td*Tomato*$^{loxP/STOP}$), when compared to control littermates (*Dicer*$^{loxP/loxP}$;td*Tomato*$^{loxP/STOP}$). Arrowheads show GnRH-immunonegative Tomato-positive neurons. OVLT, organum vasculosum of the lamina terminalis. Scale bars, 200 μm; 10 μm (inset). These images are representative of what has been observed in three distinct animals per genotype. (**c**) GnRH-GFP neuron isolation by FACS from the preoptic region of *Gnrh::*Gfp mice. Data are obtained from mice wild-type for *Dicer* at P7 (light blue) and P12 (dark blue). (**d-e**) Real-time PCR analysis of expression levels of GnRH mRNA (**d,e**), *Dicer* (**e**), GnRH promoter activators (t-test, P7 *vs.* P12, *Aes,* $t_{(5)}$=1.08, p=0.329; *Dlx1,* $t_{(5)}$=1.22, p=0.277; *Dlx5*, $t_{(5)}$=-3.62, p=0.015; *Meis1*, $t_{(5)}$=-3.17, p=0.024; *Otx2*, $t_{(5)}$=0.725, p=0.500; *Pbx1*, $t_{(5)}$=1.83, p=0.126; *Pknox1*, $t_{(5)}$= 1.42, p=0.216; *Pou2f1*, $t_{(5)}$=-6.14, p=0.0017; *Cebpb*, $t_{(5)}$=0.83, p=0.444; *Msx1*, $t_{(5)}$=0.702, p=0.514; *Tle4*, $t_{(5)}$= 0.61, p=0.566; n=3 and 4 mice per group) (light grey shaded area in **d**) and repressors (dark grey shaded area in **d**) in FACS-sorted GnRH-GFP neurons. (*: p<0.05; **: p<0.01). Values are expressed relative to P7 or wild-type values, as appropriate, set at 1. (**f**) FSH levels in females (t-test, $t_{(19)}$=5.58, p<0.0001, n=10 and 11 animal per group) and males (t-test, $t_{(20)}$=1.61, p=0.123, n=10 and12 mice per group) at P15. In panels **a,e and f**, data from mice wild-type for *Dicer* appear in blue and data from mice in which *Dicer* was selectively deleted in GnRH neurons are shown in red. Values shown are means ± SEM.

Figure 3. MiRNAs regulate the expression of GnRH promoter modulators in infantile GnRH neurons. (**a** and **b**) RT-PCR analysis of the expression of GnRH promoter activators (**a**) and repressors (**b**) in FACS-sorted GnRH-GFP neurons from mice selectively lacking *Dicer* in GnRH neurons (red bars) or wild-type for *Dicer* (blue bars) (all values are expressed relative to wild-type values, set at 1) (t-test, $Dicer^{loxP/loxP}$ *vs. Gnrh*::Cre; $Dicer^{loxP/loxP}$: **a;** *Aes*, $t_{(5)}$=9.58, p=0.0002; *Dlx1*, $t_{(5)}$=3.78, p=0.032; *Dlx5*, $t_{(5)}$=2.41, p=0.061; *Meis1*, $t_{(5)}$=4, p=0.018; *Otx2*, $t_{(5)}$=4.34, p=0.0074; *Pbx1,* $t_{(6)}$=1.21, p=0.271; *Pknox1*, $t_{(6)}$=2.63, p=0.039; *Pou2f1*, $t_{(5)}$=10, p=0.0002; n=3 and 4 mice per group; **b;** *Cebpb,* $t_{(6)}$= 4.79, p=0.003; *Msx1*, $t_{(6)}$=0.537, p=0.61 ; *Tle4*, $t_{(6)}$=1.22, p=0.27, n=4 animal per group) (*: p<0.05; **: p<0.01; ***: p<0.001). **(c,d**) All miRNAs whose expression levels in GnRH neurons change between P7 and P12, organized according to the direction of the change (**c**), and miRNAs (including family members of those identified in **c**) that are specifically enriched in GnRH neurons as compared to surrounding non-GnRH cells at P12 (**d**). See also Table S5. Pos: GFP-expressing neurons; Neg: GFP-negative cells. ΔΔCT analyses were performed using GnRH neurons (GFP positive cells, Pos) at P7 as the control group in **c** and using non-GnRH cells (GFP negative cells, Neg) at P12 as the control group in **d**. As shown in the index, shades of blue indicate a decrease in expression compared to control, while shades of red indicate an increase. (**e** and **f**) RT-PCR analysis of the expression of miR-200 family members in FACS-sorted cells from the preoptic region microdissected from *Gnrh::*Gfp mice wild-type for *Dicer* either at P12 (**e**; t-test, Pos *vs.* Neg: miR-141, $t_{(5)}$=3.37, p=0.0199; miR-200a, $t_{(4)}$=13.57, p=0.0002; miR-200b, $t_{(5)}$=2.775, p=0.0391; miR-200c, $t_{(5)}$=4.016, p=0.0102; miR-429, $t_{(5)}$=4.778, p=0.005; n=3 and 4 mice per group) or at P7 and P12 (**f**; t-test, P7 *vs.* P12: miR-141, $t_{(5)}$=0.6652, p=0.5354; miR-200a, $t_{(4)}$=9.178, p=0.0008; miR-200b, $t_{(5)}$=0.9572, p=0.3824; miR-200c, $t_{(5)}$=0.2604, p=0.805 ; miR-429, $t_{(5)}$=3.931, p=0.011; n= 3 and 4 animal per group). Values shown are means ± SEM.

Figure 4. Knocking down the ability of miR-200 to repress *Zeb1* expression in the brain alters infantile GnRH promoter activity and *Gnrh* mRNA expression. (**a** and **b**) RT-PCR analysis of the expression of *Zeb1* in GnRH-GFP neurons isolated from *Gnrh::*Gfp; *Dicer*$^{+/+}$ mice at P7 and P12 (**a**) or from mice in which *Dicer* expression is invalidated or not in GnRH neurons (**b**). (**c**) Diagram showing the distribution of putative *Zeb1* binding sites on the mouse *Gnrh* gene (upper panel) and their validation using a Zeb1 chromatin immunoprecipitation assay in an immortalized mouse cell line secreting GnRH and cultured in the presence or absence of fetal bovine serum (fbs). Values are expressed relative to the immunoprecipitation of chromatin containing the GnRH promoter region with irrelevant IgG species, arbitrarily set at 1 (dotted red line). Enh, enhancer; Pro, promoter. T-test, serum-free medium (sfm) *vs.* fbs: *Enh1*, $t_{(6)}$=4.756, p=0.0031; *Enh2*, $t_{(6)}$=2.320, p=0.0595; *Enh-Pro1*, $t_{(6)}$=0.9063, p=0.3997; *Enh-Pro2*, $t_{(6)}$=5.462, p=0.0016; *Pro1*, $t_{(6)}$=0.2104, p=0.8403; *Pro2*, $t_{(6)}$=2.467, p=0.0486, n=4 mouse per group. (**d-f**) Effect of the i.c.v. injection of TSB-200 (green bar) or a scrambled sequence (CTRL, blue bar) on the expression of *Zeb1 , Lpin1* and *Maf* (**d**), mRNA for GnRH promoter activators (t-test, control *vs.* TSB-200: *Aes*, $t_{(10)}$=0.22, p=0.823; *Dlx1*, $t_{(8)}$=2.63, p=0.03; *Dlx5*, $t_{(10)}$=0.43, p=0.67; *Otx2*, $t_{(8)}$= 0.183, p=0.0859; *Pbx1*, $t_{(10)}$=1.69, p=0.12; *Pknox1*, $t_{(9)}$=-2.024, p=0.074, n=4 and 6 animal per group) (**e**), GnRH transcript (**f**) and GnRH promoter activity (**f**) in GnRH-GFP neurons of infantile mice of 12 days of age. Pink zones in **d** and **f** show data that were obtained from P28 mice**.** Values are expressed relative to P7, wild-type or control values, as appropriate, set at 1. (*: p<0.05; **: p<0.01). Values shown are means ± SEM.

Figure 5. miR-155 modulates *Cebpb, Gnrh* and *Zeb1* mRNA expression in infantile GnRH neurons. (**a**) RT-PCR analysis of the expression of miR-155 in FACS-isolated GnRH-GFP neurons between P7 and P12 (**: p<0.01). (**b,c**) Effect of the i.c.v. injection of TSB-155 (yellow bar) or a scrambled sequence (Ctrl, blue bar) on the expression of *Cebpb, Lpin1* and *Maf* (**b**), GnRH promoter activity (**c**), and GnRH (**c**) and *Zeb1* (**c**) transcripts in GnRH-GFP neurons of infantile mice. (**d**) RT-PCR analysis of expression levels of *Gpr54* mRNA in FACS-sorted GnRH-GFP neurons (**p<0.01). (**e**) Western blot (*left panel*) and quantitative (*right panels*) comparison of phosphorylated and total nNOS protein expression in the preoptic region of wild-type mice between P7 and P12 (n=3 per age). Actin was used as a loading control to ensure that equal amounts of proteins were loaded for P7 and P12. Values are expressed relative to P7, set at 1. Full-length blots are presented in **Supplementary Fig. 15**. (**f**) L-NAME treatment partially rescues *Gnrh* mRNA expression in P12 mice harboring a *Dicer* deficiency in GnRH neurons. *Gnrh* mRNA levels were arbitrarily set at 1 in control P12 $Dicer^{loxP/loxP}$ mice (blue line). (*: p<0.05; ****: p<0.0001). Values are expressed relative to untreated wild-type values, set at 1. Values shown are means ± SEM.

Figure 6. miR-200 binding to *Zeb1* modulates the function of GnRH neurons both in infantile and adult mice. (**a**) Knocking down the ability of miR-200 and/or miR-155 to repress *Zeb1* and *Cebpb* expression, respectively, during the infantile period markedly accelerates puberty. (**b**) Circulating levels of LH in P38 peripubertal mice (arrow in A), in which TSB-200, TSB-155, TSB-200+155 or scrambled control sequences were infused into the brain during the infantile period (*: $p<0.05$). (**c**) LH levels in P28 juvenile mice, in which TSB-200 or scrambled control sequences were infused into the brain during the infantile period (*: $p<0.05$). (**d**) Estrous cyclicity before and after the infusion of TSB-200 into the preoptic region of adult mice (red arrows). Upward arrows show the time of blood collection in control- (blue) and TSB-200-treated (green) mice. (**e**) LH levels in blood samples collected during proestrus as indicated by the colored arrows in **d**. Values shown are means ± SEM.

ONLINE METHODS

Animals

All mice were group-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. C57Bl/6J *Gnrh::*Cre (Tg(*Gnrh1::*Cre)1Dlc), C57Bl/6J *Dicer*$^{LoxP/LoxP}$, and C57Bl/6J *Gnrh::*Gfp mice were a generous gift of Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA) [9], Dr. Brian Harfe (University of Florida, FL) [5] and Dr. Daniel J. Spergel (Section of Endocrinology, Department of Medicine, University of Chicago, IL) [51], respectively. *tdTomato*$^{loxP/STOP}$ mice (B6.Cg-*Gt(ROSA)26Sortm9(CAG-tdTomato)Hze*/J) were purchased from the Jackson laboratory (Maine, USA). Mice were genotyped by PCR using primers listed in table S3. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the Universities of Lille and Cordoba; 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 in specified in the text and/or figure legends, except for experiments assessing GnRH neuronal migration, postnatal GnRH expression and real time-PCR analyses on FACS-sorted GnRH neurons, in which animals of both sexes were used. Investigators were to the genotype or treatment group of animals except when morphological/physiological differences were too obvious to be ignored. Heterozygous *Gnrh::*Cre;*Dicer*$^{LoxP/+}$ littermates were excluded from the analyses.

Physiological measurements

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

Puberty onset. 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 the estrous cycle. Weaned males were checked daily for balanopreputial separation.

Hormone level measurements. Protocols and doses for in vivo testing of the LH response to GnRH, kisspeptin-10 and NMDA were as described in detail elsewhere [52]. Serum LH and FSH levels were measured using radioimmunoassay kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodo-gen tubes, following the instructions of the manufacturer (Pierce, Rockford, IL). Hormone concentrations were determined using reference preparations of LH-RP-3 and FSH-RP-2 as standards. Intra- and inter-assay coefficients of variation were <8 and 10% for LH and <6 and 9% for FSH, respectively. The sensitivity of the assay was 3.75 pg/tube for LH and 20 pg/tube for FSH. The accuracy of hormone measurements was confirmed by the assessment of rodent serum samples of known concentration (external controls).

Pharmacological inhibition of NOS activity

N-G-nitro-L-arginine methyl ester, HCl (L-NAME; N5751, Sigma) was used to inhibit the activity of nitric oxide synthase in infantile mice. *Gnrh::*Gfp; *Gnrh::*Cre; *Dicer*$^{LoxP/LoxP}$ mice received a single intraperitoneal injection of L-NAME (50 mg/kg, i.p.) or vehicle (saline) on P11 and were killed 12hr later for GnRH neuron isolation by FACS.

Gonadal histology and quantitative analysis

Ovaries and testes were collected from 3-month-old *Dicer*$^{LoxP/LoxP}$ and *Gnrh::*Cre; *Dicer*$^{LoxP/LoxP}$ mice, immersion-fixed in 4% PFA solution and stored at 4°C. Paraffin-embedded ovaries and testes were sectioned at a thickness of 5 µm (histology facility, University of Lille 2, France) and stained with hematoxylin-eosin.

Tissue preparation

For immunohistochemical analysis, embryos were obtained after cervical dislocation from timed-pregnant *Gnrh::*Cre; *Dicer*$^{LoxP/+}$ crossed with *Gnrh::*Cre; *Dicer*$^{LoxP/LoxP}$ male mice. Embryos were washed thoroughly in cold 0.1 m PBS, fixed in fixative solution [4% paraformaldehyde (PFA), 0.2% picric acid 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 (P0 to P28) and adult mice (3–5 months old) were anesthetized with 50-100 mg/kg of Ketamine-HCl and 5-10mg/kg Xylazine-HCl and perfused transcardially with 2-10 ml of saline, followed by 10-100 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.

Immunohistochemistry and GnRH neuron quantification

Tissues were cryosectioned (Leica cryostat) at 16µm for embryos and pre-weaning postnatal mice, and at 35µm (free-floating sections) for post-weaning and adult brains. Immunohistochemistry was performed as previously reported [53,54], using Alexa-Fluor 488- (1:400) and Cy3-conjugated (1:800) secondary antibodies (Invitrogen, A11008). Fluorescent specimens were mounted using 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich). The primary antisera used were as follows: 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) [55]. The GnRH neuronal population being very limited in the mouse brain (about 800), all neurons were counted by eye under the microscope in two out of three series of brain (adult) or head (embryos and P0) sections encompassing the entire nasal and/or forebrain regions, as reported previously [53].

Digital image acquisition

Sections were analyzed using an Axio Imager.Z1 ApoTome microscope (Zeiss, Germany), equipped with a motorized stage and an AxioCam MRm camera (Zeiss, Germany). Specific filter cubes were used for the visualization of green (EX: 475/40 nm, DM: 500 nm, BA: 530/50 nm), red (EX: 550/25 nm, DM: 570 nm, BA: 605/70 nm), and UV (bisbenzimide) or AMCA (amino-methyl-coumarin-acetate) fluorescence (EX: 365 nm, DM: 395 nm, BA: 445/50 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 (N.A. 0.8). High magnification photomicrographs represent maximal intensity projections derived from a series of triple-ApoTome adjacent images collected at 0.310 μm intervals using the z-stack module of the AxioVision 4.6 system and a Zeiss 40X oil immersion objective (N.A. 1.3). Some images were also acquired using a Zeiss 63X oil-immersion objective (N.A. 1.3). 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 (λ=500nm). Adobe Photoshop (Adobe Systems, San Jose, CA) was then used to process the images, adjust brightness and contrast, and merge them.

Isolation of hypothalamic GnRH neurons using Fluorescence-Activated Cell Sorting

The preoptic regions of *Gnrh::*Gfp; *Dicer*$^{LoxP/LoxP}$ and *Gnrh::*Gfp; *Gnrh::*Cre; *Dicer*$^{LoxP/LoxP}$ mice were microdissected and enzymatically dissociated using a Papain Dissociation System (Worthington, Lakewood, NJ) to obtain single-cell suspensions. 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 wild-type animals, as indicated in figure S5. For each animal, 400 to 800

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).

Quantitative RT-PCR analyses

For gene expression 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): *Aes* (Aes-Mm00507847_m1), *Cebpb* (Cebpb-Mm00843434_s1), *Dicer* (Mm00521722_m1), *Dlx1*(Dlx1-Mm00438424_m1), *Dlx5* (Dlx5-Mm00438430_m1), *Gfp* (Gfp-Mr03989638_mr), *Gnrh1* (Gnrh1-Mm01315605_m1), *Gpr54* (*Gpr54*-Mm00475046_m1), *Lpin1* ((Lpin1-Mm00550511_m1), *Maf* (Maf-Mm02581355_s1), *Meis1* (Meis1-Mm00487664_m1), *Msx1* (Msx1-Mm00440330_m1), *Otx2* (Otx2-Mm00446859_m1), *Pbx1* (Pbx1-Mm04207617_m1), *Pknox1* (Pknox1-Mm00479320_m1), *Pou2f1* (Pou2f1-Mm00448332_m1), *Tle4* (Tle4-Mm01195172_m1), *Zeb1* (Zeb1-Mm00495564_m1). Control housekeeping genes: *r18S* (18S-Hs99999901_s1); ACTB (Actb-Mm00607939_s1).

MicroRNA expression analyses were performed using stem-loop RT-PCR based TaqMan Rodent MicroRNA Arrays (Applied Biosystems). Briefly, miRNAs obtained from FACS-sorted GnRH neurons were reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) in combination with the stem-loop Megaplex primer pool sets A and B according to the manufacturer's instructions. A linear preamplification step was performed using the TaqMan® PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems) and quantitative real-time PCR were performed using TaqMan Low-Density Arrays (Applied Biosystems) 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), with R18S and Actin as control housekeeping mRNAs and U6sRNA as control housekeeping miRNA following a standardized procedure [56]. Assay-centric heat-maps were generated with Data Assist 3.0.1 by unsupervised hierarchical clustering (complete linkage) using Pearson's correlation as a distance measure.

Zeb1 chromatin immunoprecipitation (ChIP) assays

Immortalized GnRH neurons (GN11 cells) were cultured in DMEM + 10% FBS (Life Technology). Prior to experimental assays, cells were washed twice in PBS and incubated overnight in serum-free medium. The next morning, 10% FBS was added for 2h. Chromatin isolation was performed using the ChIP-IT Express kit (Active Motif) following the manufacturer's instructions. After DNA shearing by sonication (9 min total, 20s pulses at 70% power followed by a 40s pause; on ice), protein-bound DNA was immunoprecipitated at 4°C overnight using 5 µg of a polyclonal goat anti-rabbit Zeb1 antibody (E20; Santa Cruz Biotechnology; SC-10572) or control goat IgGs (Santa Cruz Biotechnology; sc-2028). ChIP-DNA fragments were purified using a QIAquick PCR purification kit (Qiagen), and analyzed by qPCR on a CFX96 thermal cycler (Biorad) using GoTaq® qPCR Master Mix (Promega) and the primers indicated in Table S4.

Western blot analyses

The preoptic area of the hypothalamus was dissected from each animal using Wecker scissors (Moria, France) under a binocular magnifying glass, and protein extracted in 200 µl of lysis buffer (25 mM Tris, pH 7.4, β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin A, 10 µg/ml apoprotinin, 100 µg/ml PMSF, and 1% Triton-X100) by trituration of the fragments through 22 and 26 gauge needles in succession. Tissue lysates were cleared by centrifugation at 14000 rpm for 15 min at 4°C. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA) and equal amounts of protein were mixed with 4X

sample buffer (Invitrogen). Samples were boiled for 5 min and stored at -80°C until use. Samples were reboiled for 5 min after thawing and electrophoresed for 75 min at 150 V in 7% Tris-acetate, or for 50 min at 200 V in precast 4–12% MES SDS-polyacrylamide gels according to the protocol supplied with the NuPAGE system (Invitrogen). After size fractionation, the proteins were transferred onto 0.2 μm pore-size polyvinylidene difluoride membranes (LC2002; Invitrogen) in the blot module of the NuPAGE system (Invitrogen) for 75 min at room temperature. Membranes were blocked for 1 h in blocking buffer [TBS with 0.05% Tween 20 (TBST) and 5% nonfat milk] at room temperature, and incubated overnight at 4°C with the appropriate primary antibody diluted in blocking buffer (rabbit polyclonal anti-RFP, 600-401-379, 1:1000, Rockland antibodies & assays; rabbit polyclonal anti-nNOS, sc-8309, 1:500, Santa Cruz technologies; rabbit polyclonal anti-Ser^{1412} phospho-nNOS, PA1-032; 1:1000, Affinity BioReagents; goat polyclonal anti-actin, sc-1616, 1:1000). Membranes were washed four times with TBST the following day before being exposed to HRP-conjugated secondary antibodies (Vector, peroxidase-labeled anti rabbit and anti goat IgGs PI-1000 and PI9500, respectively) diluted in blocking buffer for 1 h at room temperature. Immunoreactions were visualized using the ECL detection kit (NEL101; PerkinElmer, Boston, MA). Immunoblots were scanned using a desktop scanner (Epson Expression 1680 PRO) and Adobe Photoshop, and band intensities were determined using ImageJ software (NIH, Bethesda).

Target site blockers (TSBs)

To selectively hamper the silencing activity of miR-155 and miR-200b/200c/429 on *Cebpb* and *Zeb1* respectively in vivo, we used custom-designed target sites blockers with phosphorothioate backbone modifications from Exiqon (miRCURY LNA^{TM} microRNA Target Site Blockers, in vivo ready). TSB sequences are designed with a large arm that covers the miRNA binding site and a short arm outside the miRNA seed to ensure target specificity. In detail, one sequence was generated to protect the unique miR-155-binding site in the *Cebpb* 3'UTR (TSB-155, Fig. S7). Five sequences were generated to protect the five different miR-

200b/200c/429-binding sites in the *Zeb1* 3'UTR (Fig. S7) and mixed at equimolar ratios (TSB-200).

Stereotactic brain infusions of TSBs

*Gnrh::*Gfp *mice* were placed in a stereotaxic frame (Kopf® Instruments, California) under anesthesia (isoflurane), and a burr hole was drilled 1 mm lateral to the bregma, according to a mouse brain atlas (Paxinos and Franklin, 2004). A 10 µl Hamilton syringe was slowly inserted into the bottom of the left lateral ventricle (3.5 mm deep relative to the dura), and 1µl of the different treatment solutions (TSB-200, 50µM, n = 6; TSB-155, 50µM, n = 6) or vehicle (PBS, pH7.4, n = 6) was injected using an infusion pump (KD Scientific, Holliston, MA) over 4 min. Mice were randomly assigned to TSB injection. Animals were subjected to intracranial surgery at P9 and killed at P12.

Identification of putative Zeb1-binding sites

To identify putative Zeb1- and C/EBPβ-binding sites in the promoter regions of target genes, 1.5 Kb of the genomic sequence upstream of the transcription initiation site of GnRH, Gpr54 and the GnRH promoter activators under study, were analyzed using ALGEN PROMO 3.0 software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and jaspar database (http://jaspar.genereg.net/).

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. Typically, $n \geq 5$ mice from at least three different litters for each group were used to study sexual maturation and fertility; $n \geq 3$ mice from three different litters were collected to perform quantitative RT-PCR analyses in cells isolated by FACS; and $n \geq 3$ mice from three different litters each group were collected for anatomy and immunostaining. No

randomization method was used to assign subjects in the experimental groups or to collect and process data.

Statistics

All analyses were performed using Prism 5 (GraphPad Software) and assessed for normality (Shapiro-Wilk test) and variance, when appropriate. Sample sizes were chosen according to standard practice in the field. Data were compared using an unpaired two-tailed Student's t test, a one-way ANOVA for multiple comparisons or a two-way repeated-measures ANOVA. A Mann-Whitney U test (non-parametric) was performed when appropriate. A non-parametric unpaired test (Wilcoxon–Mann–Whitney) was used to compare Western blot data. The significance level was set at $p < 0.05$. Data are indicated as means ± SEM. The number of biologically independent experiments, p values and degrees of freedom are indicated either in the main text or in the figure legends.

A Supplementary Methods Checklist is available.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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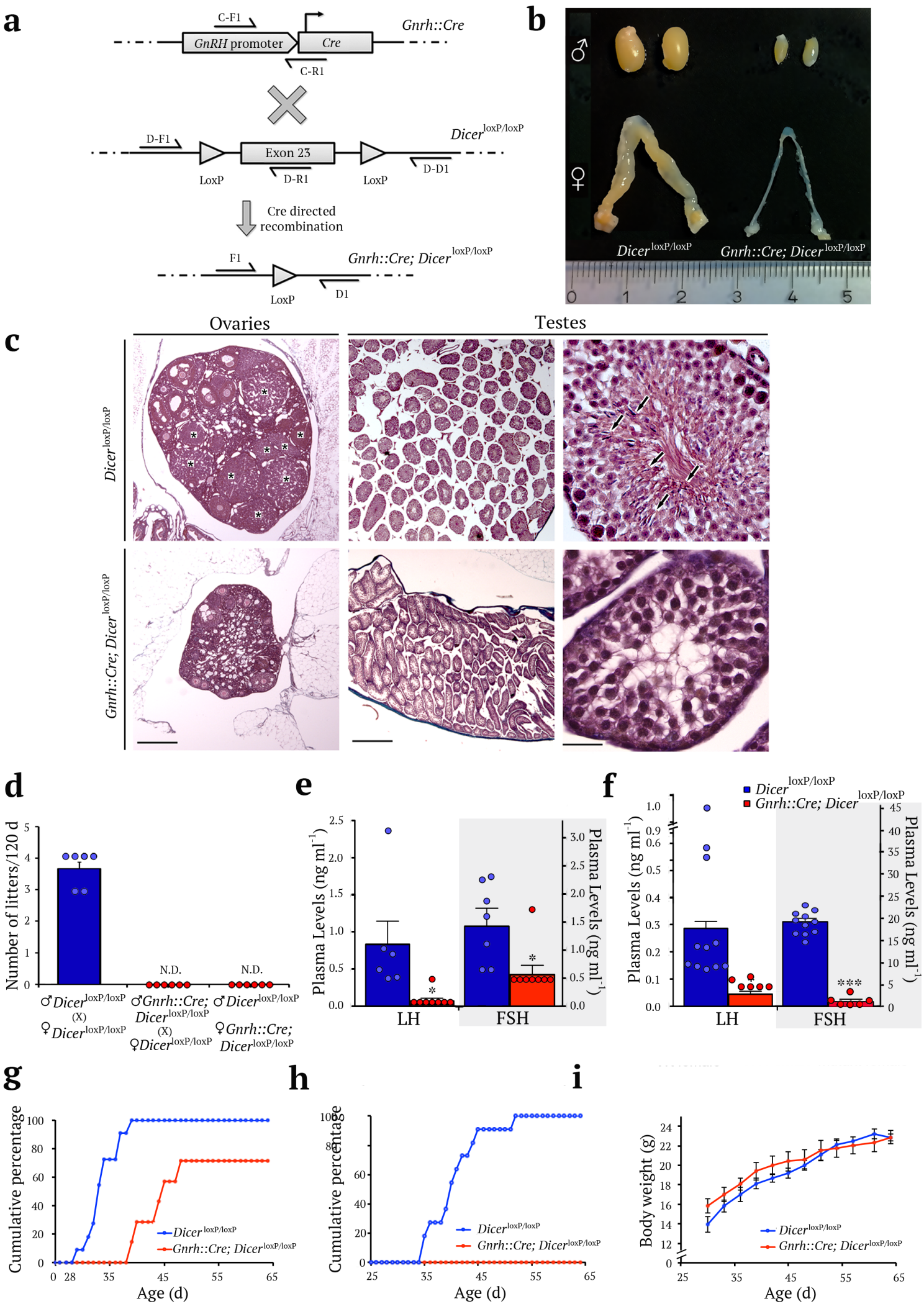
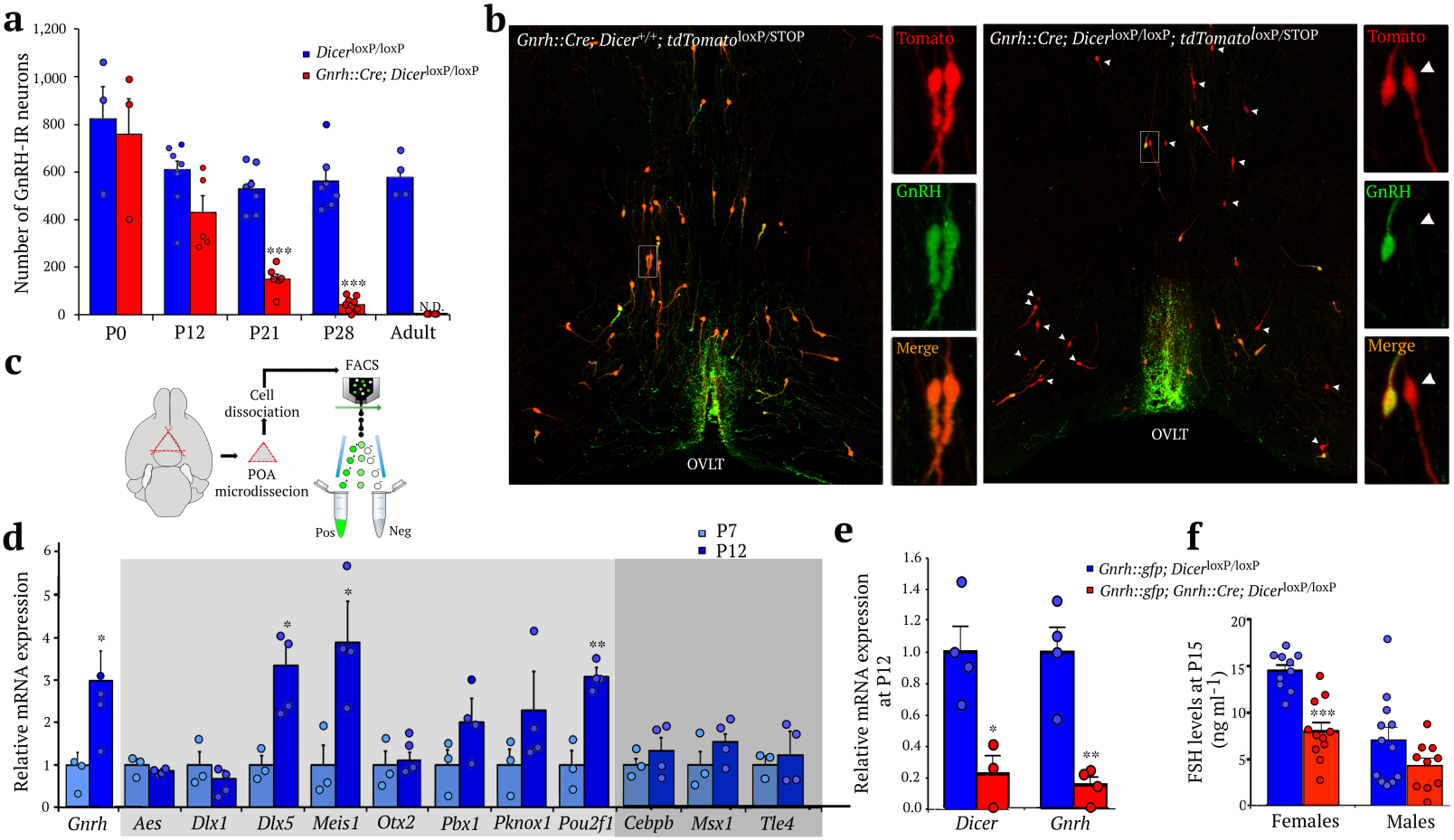
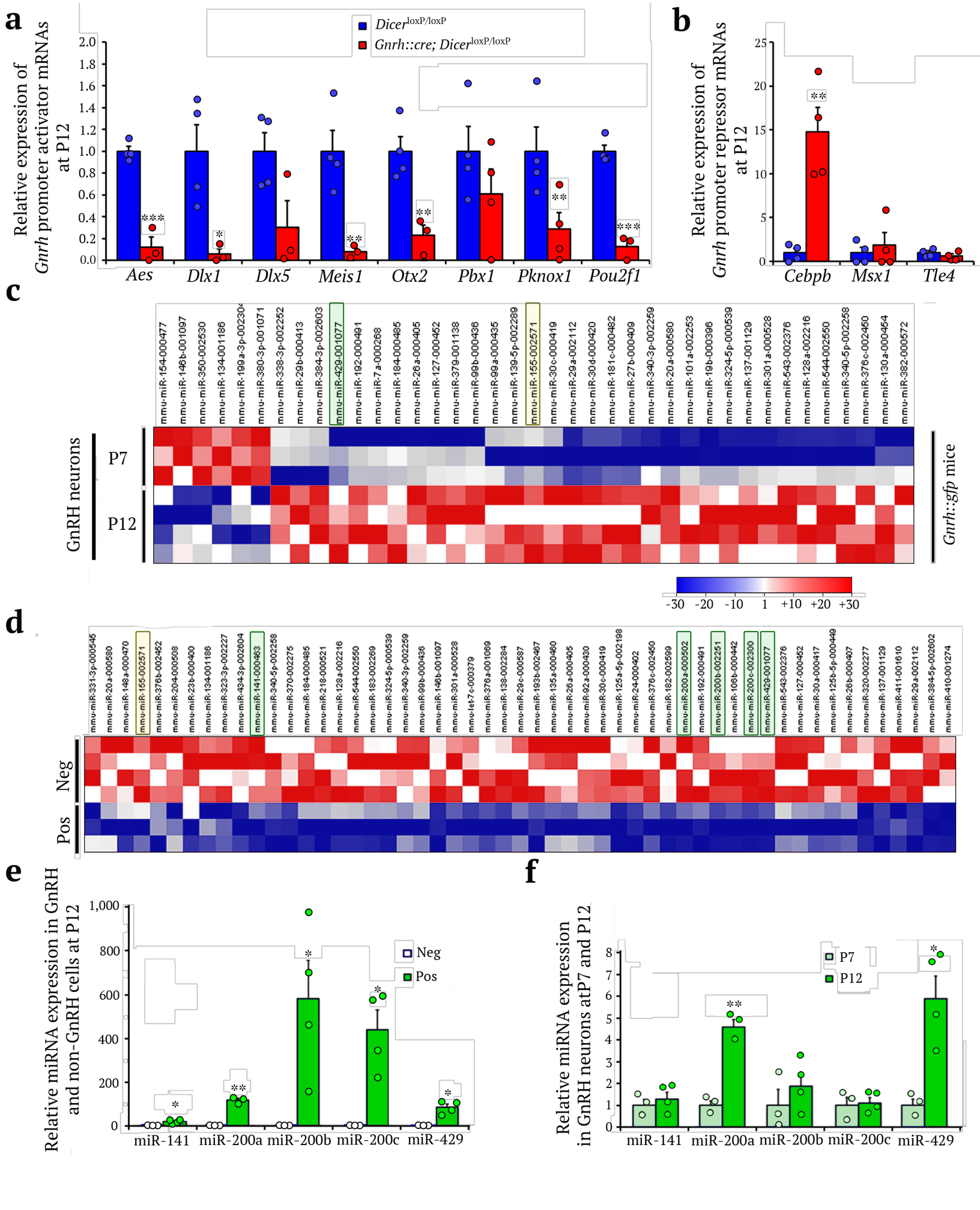
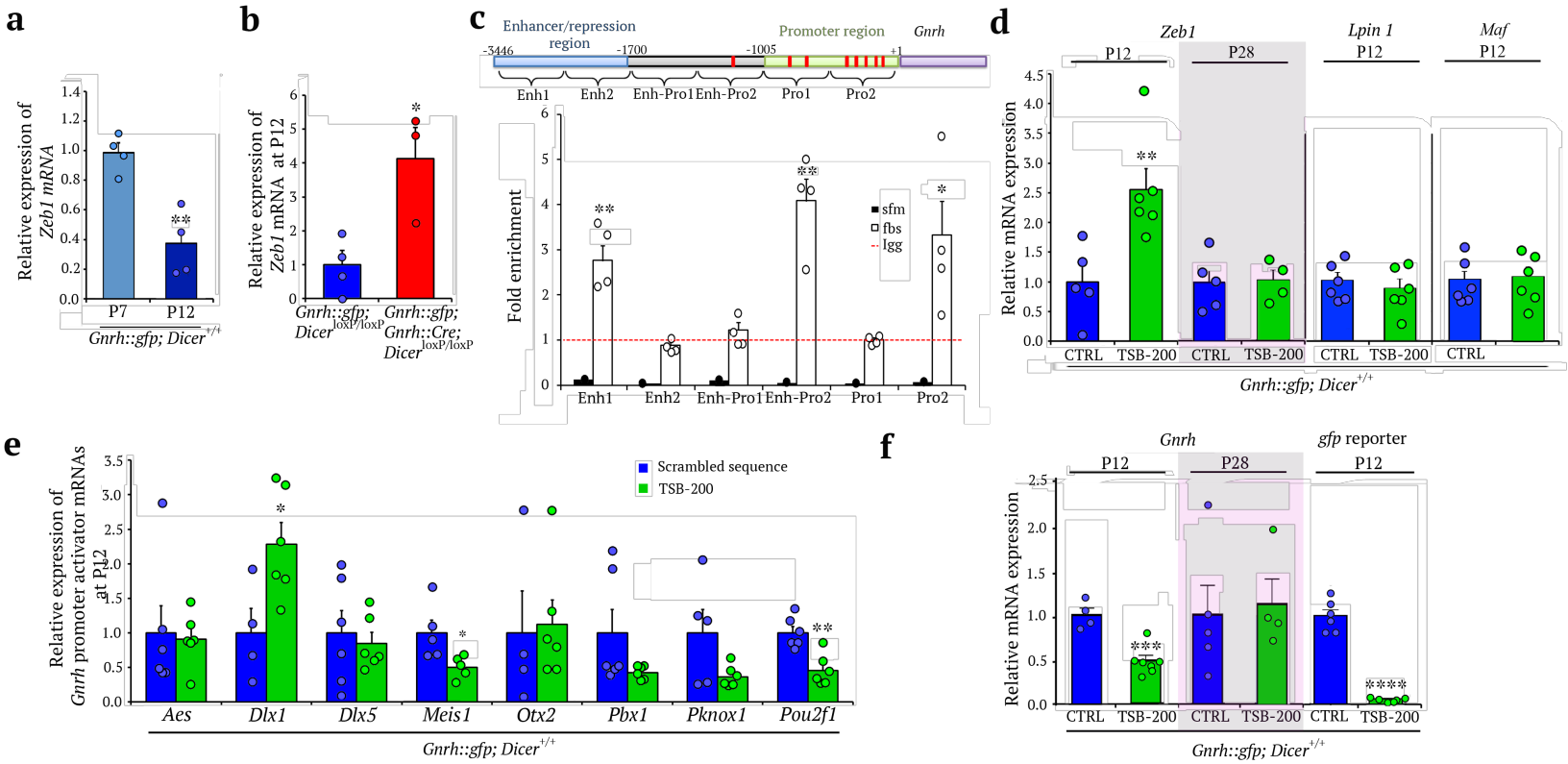
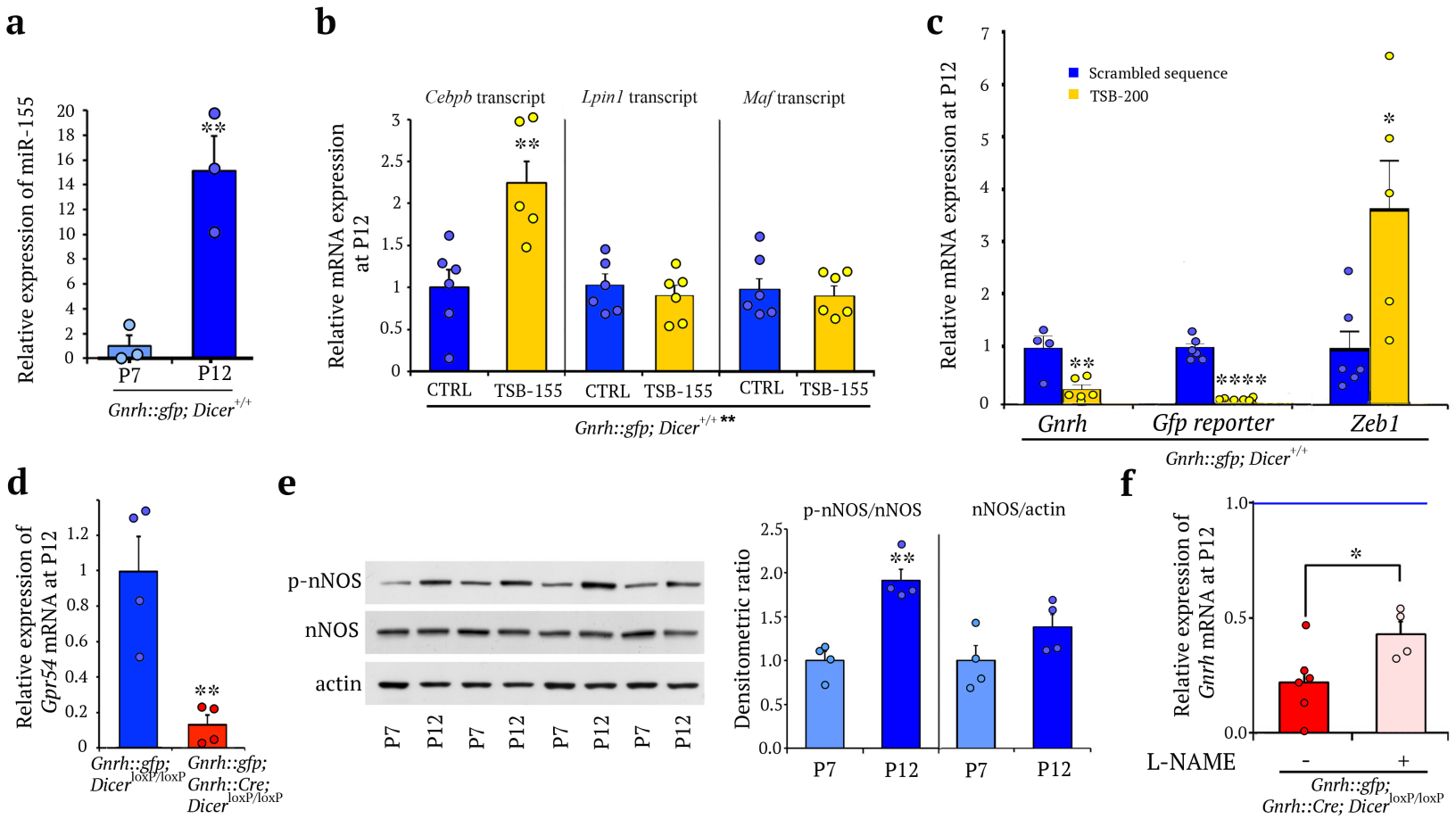
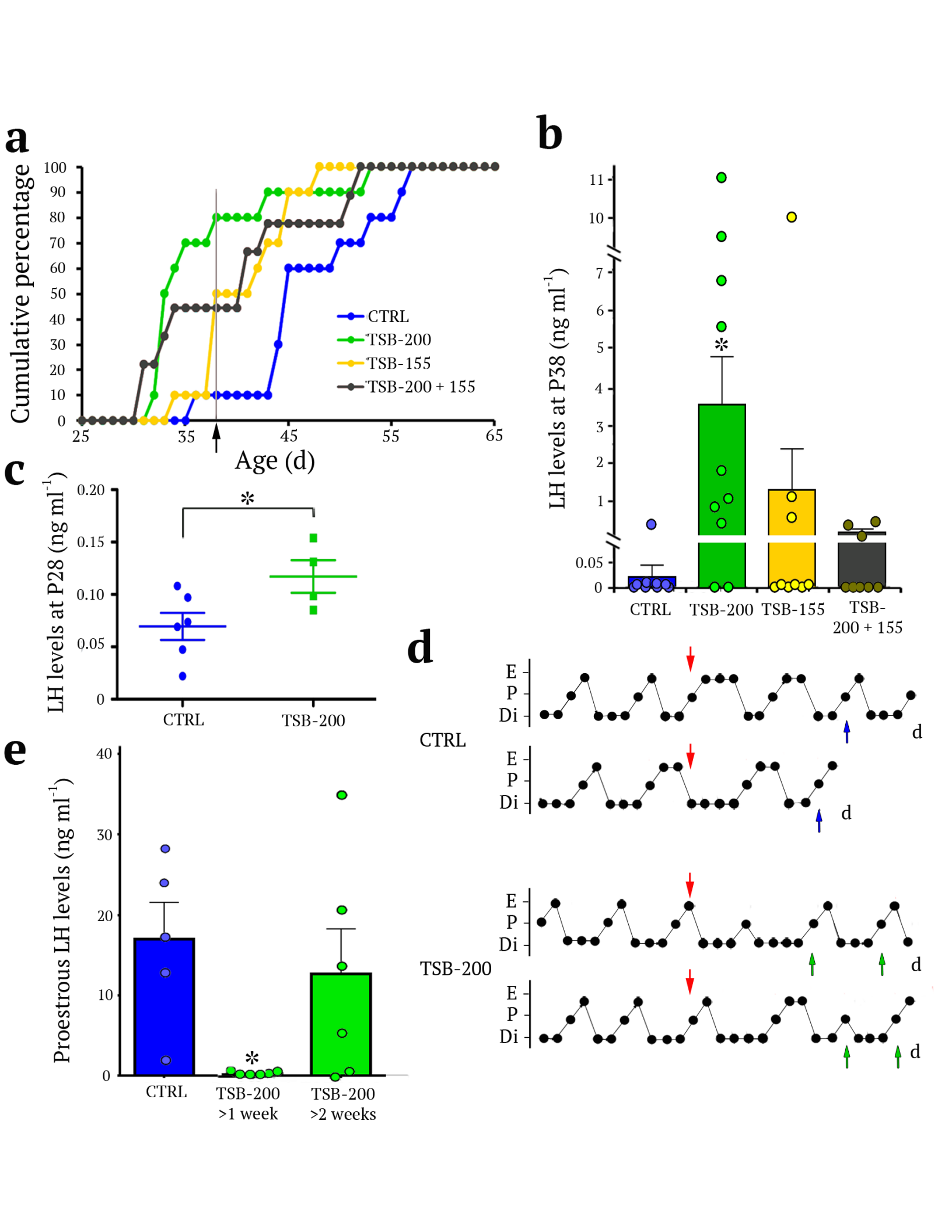

a
C-F1
GnRH promoter
Cre
Gnrh::Cre
C-R1
D-F1
Exon 23
Dicer loxP/loxP
LoxP
D-R1
LoxP
D-D1
Cre directed recombination
F1
Gnrh::Cre; Dicer loxP/loxP
LoxP
D1
b
♂
♀
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
0 1 2 3 4 5
c
Ovaries
Testes
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
d
Number of litters/120 d
N.D.
N.D.
♂Dicer loxP/loxP (X) ♀Dicer loxP/loxP
♂Gnrh::Cre; Dicer loxP/loxP (X) ♀Dicer loxP/loxP
♂Dicer loxP/loxP ♀Gnrh::Cre; Dicer loxP/loxP
e
Plasma Levels (ng ml-1)
LH
FSH
f
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
Plasma Levels (ng ml-1)
LH
FSH
g
Cumulative percentage
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
Age (d)
h
Cumulative percentage
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
Age (d)
i
Body weight (g)
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
Age (d)


a
Number of GnRH-IR neurons
1,200
1,000
800
600
400
200
0
Dicer loxP/loxP
Gnrh::Cre; Dicer loxP/loxP
***
***
N.D.
P0
P12
P21
P28
Adult
b
Gnrh::Cre; Dicer +/+; tdTomato loxP/STOP
Gnrh::Cre; Dicer loxP/loxP; tdTomato loxP/STOP
Tomato
GnRH
Merge
OVLT
c
FACS
Cell dissociation
POA microdissecion
Pos
Neg
d
P7
P12
Relative mRNA expression
Gnrh
Aes
Dlx1
Dlx5
Meis1
Otx2
Pbx1
Pknox1
Pou2f1
Cebpb
Msx1
Tle4
e
Relative mRNA expression at P12
Dicer
Gnrh
f
Gnrh::gfp; Dicer loxP/loxP
Gnrh::gfp; Gnrh::Cre; Dicer loxP/loxP
FSH levels at P15 (ng ml-1)
Females
Males


a
Relative expression of Gnrh promoter activator mRNAs at P12
Dicer loxP/loxP
Gnrh::cre; Dicer loxP/loxP
Aes
Dlx1
Dlx5
Meis1
Otx2
Pbx1
Pknox1
Pou2f1
b
Relative expression of Gnrh promoter repressor mRNAs at P12
Cebpb
Msx1
Tle4
c
GnRH neurons
P7
P12
Gnrh::gfp mice
-30 -20 -10 1 +10 +20 +30
d
Neg
Pos
e
Relative miRNA expression in GnRH and non-GnRH cells at P12
Neg
Pos
miR-141
miR-200a
miR-200b
miR-200c
miR-429
f
Relative miRNA expression in GnRH neurons atP7 and P12
P7
P12
miR-141
miR-200a
miR-200b
miR-200c
miR-429


a
Relative expression of Zeb1 mRNA
P7
P12
Gnrh::gfp; Dicer+/+
b
Relative expression of Zeb1 mRNA at P12
Gnrh::gfp; Dicer loxP/loxP
Gnrh::gfp; Gnrh::Cre; Dicer loxP/loxP
c
Enhancer/repression region
Promoter region
Gnrh
-3446
-1700
-1005
+1
Enh1
Enh2
Enh-Pro1
Enh-Pro2
Pro1
Pro2
Fold enrichment
sfm
fbs
Igg
d
Zeb1
Lpin 1
Maf
P12
P28
Relative mRNA expression
CTRL
TSB-200
Gnrh::gfp; Dicer+/+
e
Relative expression of Gnrh promoter activator mRNAs at P12
Scrambled sequence
TSB-200
Aes
Dlx1
Dlx5
Meis1
Otx2
Pbx1
Pknox1
Pou2f1
Gnrh::gfp; Dicer+/+
f
Gnrh
gfp reporter
Relative mRNA expression
CTRL
TSB-200
Gnrh::gfp; Dicer+/+


a
Relative expression of miR-155
P7
P12
Gnrh::gfp; Dicer+/+
b
Cebpb transcript
Lpin1 transcript
Maf transcript
Relative mRNA expression at P12
CTRL
TSB-155
Gnrh::gfp; Dicer+/+ **
c
Scrambled sequence
TSB-200
Relative mRNA expression at P12
Gnrh
Gfp reporter
Zeb1
Gnrh::gfp; Dicer+/+
d
Relative expression of Gpr54 mRNA at P12
Gnrh::gfp; Dicer loxP/loxP
Gnrh::gfp; Gnrh::Cre; Dicer loxP/loxP
e
p-nNOS
nNOS
actin
P7
P12
p-nNOS/nNOS
nNOS/actin
Densitometric ratio
f
Relative expression of Gnrh mRNA at P12
L-NAME
Gnrh::gfp; Gnrh::Cre; Dicer loxP/loxP


a
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
25
35
45
55
65
Age (d)
CTRL
TSB-200
TSB-155
TSB-200 + 155
b
LH levels at P38 (ng ml-1)
11
10
7
6
5
4
3
2
1
0.05
0
*
CTRL
TSB-200
TSB-155
TSB-
200 + 155
c
LH levels at P28 (ng ml-1)
0.20
0.15
0.10
0.05
0
*
CTRL
TSB-200
d
CTRL
TSB-200
E
P
Di
d
e
Proestrous LH levels (ng ml-1)
40
30
20
10
0
*
CTRL
TSB-200
>1 week
TSB-200
>2 weeks

6.3 Leptin-dependent neuronal NO signaling in the preoptic hypothalamus facilitates reproduction

Nicole Bellefontaine, Konstantina Chachlaki, Jyoti Parkash, Charlotte Vanacker, William Colledge, Xavier d'Anglemont de Tassigny, John Garthwaite, Sebastien G. Bouret, and Vincent Prevot

Leptin, a peptide hormone mainly produced by adipocytes, is a key link between metabolic homeostasis and reproductive competence. There is compelling evidence to suggest that leptin regulates GnRH neurons via cells afferent to these neurons and/or those that interact morphologically with them. The mapping of leptin-responsive cells in the hypothalamus has revealed that several leptin-receptor expressing populations are NO-synthesizing neurons and that leptin can induce the activation of nNOS by its phosphorylation. In this study we demonstrate that leptin-promoted nNOS activation in the OVLT/MePO is associated with a rise in peripheral LH from nadir levels. Leptin concentration has been shown to fall during starvation and leptin-deficient ob/ob mice present neuroendocrine abnormalities similar to those seen during conditions of food deprivation; here we considered the possibility that NOS-expressing neurons are actively implicated in the consequences of fasting in the neuroendocrine axis. Our results propose nitric oxide signaling pathway as a significant player in the control of ovarian cyclicity and the initiation of the preovulatory LH surge. Most importantly we present evidence suggesting that NO acts as a volume transmitter to mediate the leptin signal to the GnRH neurons of the preoptic area, controlling thus the synchronization of the GnRH cells, and subsequently the release of LH.

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LEPTIN FACILITATES REPRODUCTION THROUGH NEURONAL NITRIC OXIDE SIGNALING IN THE HYPOTHALAMIC PREOPTIC REGION

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Short title: Leptin requires nitric oxide to promote fertility

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Conflict of interest: The authors have declared that no conflict of interest exists

Abstract

Puberty and adult fertility require a minimum level of energy availability. Leptin, an adipocyte-derived hormone that signals the long-term status of peripheral energy stores, is a key metabolic messenger to the neuroendocrine reproductive axis. Humans and mice lacking leptin (*Lep*$^{ob/ob}$) or the leptin receptor (*LepR*$^{db/db}$) fail to complete puberty and are infertile. The restoration of leptin levels in these individuals results in sexual maturation, which requires the pulsatile, coordinated delivery of GnRH to the pituitary and the resulting surge of luteinizing hormone (LH). However, the neural circuits controlling the leptin-mediated awakening of the reproductive axis are not fully understood. Here we show that leptin requires the mediation of neurons that synthesize the freely-diffusible, volume based transmitter nitric oxide (NO) in the preoptic region of the hypothalamus, to coordinate fertility, and promotes the phosphorylation of neuronal NO synthase (nNOS) in these neurons. The genetic deletion of nNOS or its pharmacological inhibition in the preoptic region blunts the stimulatory action of leptin on LH secretion and prevents the restoration of fertility in *Lep*$^{ob/ob}$ female mice treated with leptin. Leptin could thus play a central role in regulating the hypothalamo-pituitary-gonadal axis *in vivo* by acting on nNOS neurons of the preoptic region.

Introduction

Reproduction in mammals is favored when there is sufficient energy available to permit the survival of offspring. Minimum energy stores are thus necessary for the initiation of puberty and the maintenance of reproductive capacity. Consequently, undernourishment is associated with delays in sexual maturation and the suppression of fertility during adulthood. At the other end of the spectrum, the rising prevalence of metabolic disorders involving excess energy stores, including obesity, has been linked with a rise in the incidence of precocious puberty, abnormal menstrual cycles, and infertility (1, 2). Given this alarming increase in metabolic disorders and their association with conditions such as infertility, a great deal of research has been devoted to elucidating the mechanisms underlying these disorders and their relationship to the neuroendocrine reproductive axis (3). Leptin, an adipocyte-derived hormone secreted in proportion to body fat mass, has been linked to the regulation of both metabolic and reproductive function. Chronically elevated leptin levels, both a characteristic of obesity and a condition known to accelerate puberty (4, 5) and prolong the menstrual cycle (5), may indeed account for the increased prevalence of reproductive impairment in obese patients.

Leptin deficiency, caused by the *Lep*$^{ob/ob}$ loss-of-function mutation in both humans and rodents, results in obesity, a failure to complete puberty, and consequently, infertility (6, 7). Chronic leptin administration to *Lep*$^{ob/ob}$ individuals induces the completion of sexual maturation and the restoration of fertility, indicating the key role played by leptin (7, 8). There is compelling evidence to suggest that leptin regulates neurons producing gonadotropin-releasing hormone (GnRH neurons) via cells afferent to these neurons (9, 10) and/or those that interact morphologically with them (11). However, despite its crucial implications for mammalian reproduction, the identification of the sites of action and specific cell populations involved in the transmission of leptin signaling to the neuroendocrine reproductive axis remains elusive. In this report, we show that neurons synthesizing nitric oxide (NO) could act

as mediators of leptin signaling to the central reproductive axis, and identify the preoptic region as a novel site for the integration of this signal into GnRH neuronal function.

Results

Leptin activates a subset of NO-synthesizing neurons in the preoptic region of the hypothalamus

NO is known to play an important role in the neuroendocrine control reproduction (12, 13), and has been directly implicated in the regulation of GnRH neuronal activity (14). NO is produced in the vicinity of GnRH-containing perikarya in the preoptic region by neuronal NO synthase (nNOS)-expressing neurons (14), which we have recently shown to be an integral part of the neuronal network controlling ovarian cyclicity and ovulation (15). Whether, in addition to being a trigger for the preovulatory activation of GnRH neurons, these nNOS neurons are also able to sense signals required for the maturation and function of the reproductive axis, such as leptin, is not known. The mapping of leptin-responsive cells in the hypothalamus has revealed that several LepR populations are NO-synthesizing neurons (16, 17), and that the enzymatic activity of nNOS can be regulated by leptin in rostral areas of the hypothalamus (16). To examine whether nNOS neurons within the preoptic region are leptin-sensitive, we performed double immunofluorescence analyses for nNOS and the leptin-induced phosphorylation of STAT3 (P-STAT3), a widely used technique for identifying leptin-responsive cells (18). In cycling diestrous mice, 30-40% of NO-synthesizing neurons in the dorsal part of the medial preoptic area (MEPO) and the organum vasculosum of the lamina terminalis (OVLT) were shown to express P-STAT3 (Supplemental Figure 1). We next investigated the putative effect of leptin treatment on nNOS activity by studying its state of phosphorylation (19). Interestingly, western blot analyses showed an acute increase in P-nNOS expression in the preoptic region of diestrous mice 15 min after leptin injection (Figure 1A). The phosphorylation of nNOS was found to be restricted to the OVLT and the MEPO, in which 80% of nNOS expressing cells displayed P-nNOS immunoreactivity following leptin injection (Figure 1B,C) ($n=4$, $p<0.05$, Student's t-test for vehicle *vs.* leptin treatment). Leptin-induced nNOS phosphorylation in these neurons coincided with a rise in luteinizing hormone (LH) levels from their nadir (Figure 1A,D) ($n=5$, $p<0.05$, one-way ANOVA). Together, these

results suggest that nNOS neurons within and in direct proximity to the OVLT, a site devoid of the blood brain barrier (20) and to which GnRH neurons extend dendrites (21), may acutely sense changes in leptin levels and rapidly relay this information to GnRH neurons, which in turn stimulate LH release.

NO signaling originating from the preoptic region is important for leptin-induced LH release and is independent of kisspeptin/GPR54 signaling

Hypothalamic neurons that release the neuropeptide kisspeptin, a fundamental player in the reproductive axis (22-24), functionally interact with nNOS neurons (which express the kisspeptin receptor GPR54) within the preoptic region to regulate GnRH/LH release (15). Kisspeptin neurons in the arcuate nucleus (ARH) putatively contain the leptin receptor, suggesting that leptin-induced LH release involves kisspeptin-GPR54 signaling (3). However, this hypothesis is somewhat controversial, since only 5% of kisspeptin neurons in the ARH have been confirmed to express the leptin receptor (11), and the selective deletion of this receptor in kisspeptin neurons results in normal sexual maturation and continued fertility (25). To ascertain whether kisspeptin-GPR54 signaling is required for leptin-induced LH release in the current model, leptin was administered to *Gpr54*$^{-/-}$ mice and their wild-type littermates. Leptin stimulated the release of LH in both wild-type and *Gpr54*$^{-/-}$ mice at diestrus within 15 minutes of its administration (Figure 2A) ($n=4-5$, $p<0.05$), suggesting that kisspeptin-GPR54 signaling is not required for the effects of leptin on LH release. This result is in stark contrast to that found in mice lacking *nNos*, in which leptin did not trigger an increase in LH levels (Figure 2A) ($n=4-5$, $p=0.93$). To confirm that NO signaling is involved in the leptin-induced LH release, wild-type mice were injected intraperitoneally (i.p.) with a NOS inhibitor, L-NAME, 3 hours prior to leptin administration. Consistent with the results obtained in *nNos*$^{-/-}$ mice, leptin did not induce any increase in LH levels when co-administered with L-NAME (Figure 2B). Since we have previously shown that the chronic inhibition of nNOS activity in preoptic neurons disrupts estrous cyclicity (26), we sought to examine whether NO signaling was involved in the leptin-induced release of LH in the preoptic region by injecting L-NAME

intracranially (i.c.) into the preoptic region prior to peripheral leptin administration. L-NAME completely suppressed the release of LH, whereas in vehicle-injected animals, LH levels rose in response to leptin (Figure 2B) (n= 4-6, $p<0.05$).

Together, these data indicate that NO signaling, particularly within the preoptic region, is involved in the acute stimulatory effect of leptin on LH release.

Mathematical modelling of hypothalamic NO concentrations before and after leptin treatment supports a synchronizing effect

The aforementioned results could be explained if, prior to leptin treatment, NO exists as a discrete cloud restricted to the vicinity of the active nNOS neurons whereas, after leptin, greater numbers of active nNOS neurons, as indicated by P-nNOS expression, leads to a build-up of NO in the intervening tissue to concentrations sufficient to engage GnRH neurons located therein, leading to GnRH (and subsequent LH) release.

To test the plausibility of this hypothesis, active nNOS neurons in the preoptic region were modeled as a 3-dimensional array of NO-emitting spheres (Supplementary Results & Supplementary Figure 2). From the analysis, it is concluded that the above hypothesis is fully supported, subject to two main conditions: first, that the numbers of active nNOS neurons under low-leptin conditions are half or fewer of those present in the fully active state achieved after leptin treatment (Figure 1C) and second, that NO is subject to a similar rate of inactivation to that found in the cerebellum (see Supplementary Results). Accordingly, the model supports the idea that leptin treatment can switch the mode of operation of NO from being active only locally, to being a "volume transmitter" capable of influencing cells located at a distance, irrespective of anatomical connectivity (Figure 2C).

Leptin requires NOS activity in the neuroendocrine response to fasting

The seminal work by Ahima et al. (28) demonstrated that suppression of fertility during an acute severe fast can be rescued through bi-daily administration of leptin (28). Because leptin acts, at least in part, through NO signaling to stimulate LH release we sought to assess

whether nNOS activity plays an active role in leptin-sensing during conditions of severe food deprivation. We employed a modified experimental design (28), in which wildtype mice were deprived of food for 24 hours on the day of diestrus I and treated with either vehicle or leptin twice daily for the duration of the fast. To determine the function of nNOS activity, mice were treated with either L-NAME (50mg/kg, i.p) or saline prior to each leptin or vehicle injection. Predictably, food deprivation for 24 hours during diestrus 1 impaired the ability of mice to enter proestrus two days afterwards (Figure 3A,B) and thus suppressed the preovulatory-LH surge (Figure 3C), while leptin administration completely restored both estrous cyclicity and LH levels in fasted mice when compared to ad libitum fed mice (n=6-7, p=0.02) (Figure 3). Remarkably, the administration of L-NAME concurrently with leptin completely abolished the ability of exogenous leptin to rescue fertility in fasting conditions (Figure 3 A-C), suggesting that NO-signaling plays a role in leptin-sensing during periods of food deprivation.

Altogether, these data indicate that NO signaling is not only involved in the acute, but also in the long-term stimulatory effect of leptin on the neuroendocrine reproductive axis.

nNOS signaling in the preoptic region is necessary for the recovery of sexual maturation in *Lep*$^{ob/ob}$ mice by leptin

Chronic leptin administration to *Lep*$^{ob/ob}$ mice is sufficient to correct the sterile phenotype (7). Here we modified the experimental design such that each mouse received leptin (3mg/kg, i.p.) daily for 28 days to restore puberty in *Lep*$^{ob/ob}$ mice. In the first step of the experiment, *Lep*^{ob} mice were crossed with *nNos* null mice to create a double transgenic mouse lacking both leptin and nNOS (*nNos*$^{-/-}$; *Lep*$^{ob/ob}$) in order to establish the necessity of NO signaling. In the second step, local inhibition of nNOS in the preoptic region was used to examine the role of NO signaling in this discrete brain area. To do this, the NOS-inhibitor L-NAME was chronically delivered into the preoptic region for the duration of the leptin treatment regime.

nNos$^{-/-}$; *Lep*$^{ob/ob}$ mice were infertile despite a much lower initial body weight than their *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ littermates (Fig. 4A,E) (n=4, p<0.01, two-way ANOVA). Interestingly, the targeted mutation of the leptin receptor in nNOS cells results in an obese phenotype (29),

while our *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice displayed a less dramatic obese phenotype, thus raising the possibility that nNOS neurons, which are not leptin sensitive, are also involved in energy homeostasis. Predictably, the mice incurred substantial weight loss during the leptin regime (Figure 4A). Pubertal activation was never detected in *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice, while the first estrous cycle occurred 13 days from the start of the leptin regime in *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ littermates and remained variable thereafter, typically lasting 4-5 days (Figure 4B,E). The *nNos* null single mutant mice (*nNos*$^{-/-}$; *Lep*$^{+/+)}$ demonstrated an ovulatory pattern, as reported in other studies (15, 30), suggesting that the lack of nNOS alone is not responsible for the continued infertility of *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice.

Three to five days prior to the end of the leptin regime, on the day of diestrus I, females were placed for 62 hours in a cage that had previously held a sexually experienced male. This experimental protocol has previously been shown to induce a natural peak of LH release, necessary for ovulation in cycling females (31). Strikingly, while LH levels in *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ female mice remained below the limit of detection, *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ females displayed a quintessential surge in LH levels (Figure 4F). Importantly, *nNos*$^{-/-}$; *Lep*$^{+/+}$ littermates demonstrated detectable basal LH levels, as well as an increase in plasma LH following exposure to male odors, as described above (n=4, 0.39±0.29 ng/ml after exposure to male odor *vs.* 0.13 ±0.014 ng/ml under basal conditions, p=0.05), providing evidence that the lack of LH release in *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ females after the leptin regime was not due to the *nNos* null mutation but rather to the impairment of leptin signal transmission to GnRH neurons via nNOS cells. At the gonadal level, the presence of corpora lutea was noted in *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ females following the leptin regime, demonstrating that *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ females had mature ovaries and had undergone ovulation, while the ovaries of *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ females remained immature (Figure 4G). Importantly, *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice did not show any developmental alteration of the GnRH system, either with respect to the number of GnRH neurons in the preoptic region or with respect to the innervation of the median eminence, when compared with the other genotypes (Figure 5). *Lep*$^{ob/ob}$ female mice without leptin treatment had dense GnRH-ir within the median eminence, as previously described

(25), while leptin restored GnRH-ir to wildtype levels, suggesting a problem in transport and/or release of the GnRH peptide in *Lep*$^{ob/ob}$ animals, likely contributing to the infertile phenotype. Yet the *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice treated with leptin maintained normal GnRH-ir at the median eminence demonstrating that their continuing infertile phenotype is likely not attributed to a blockade of the GnRH peptide within the nerve terminals (Figure 5B,C).

To further assess the role of NO signaling in the preoptic region, *Lep*$^{ob/ob}$ mice were given a chronic infusion of L-NAME directly into the preoptic region in conjunction with peripheral leptin administration. Remarkably, *Lep*$^{ob/ob}$ mice treated with L-NAME phenocopied *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice, in which leptin did not induce sexual maturation but did decrease body weight, while vehicle-infused animals showed their first estrus 16 days from the start of leptin treatment and recovered estrous cyclicity (Figure 4C, D), providing further evidence of the dissociation between leptin's influence on reproduction and metabolism (25). Furthermore, at the end of the treatment regime, all vehicle-infused *Lep*$^{ob/ob}$ mice had detectable levels of plasma LH, similar to basal LH levels in control wild-type littermates (n=3-6, 0.10 ± 0.01 ng/ml in *Lep*$^{+/+}$ and *Lep*$^{ob/+}$ vs. 0.15 ± 0.07 ng/ml in vehicle-infused *Lep*$^{ob/ob}$ mice, p =0.29), while LH levels remained below the limit of detection in all animals in the L-NAME-infused group (n = 4).

Together, these data suggest that leptin, acting through NO signaling particularly within the preoptic region, is critical for the progression of sexual maturation in leptin-deficient mice.

Leptin receptor signaling within the preoptic region controls basal LH secretion

To further study the physiological relevance of LepRb signaling in the preoptic region, we used intracranial injections of the TAT-Cre fusion protein, whose cellular uptake is enhanced compared to Cre recombinase (32), into the preoptic region of female mice (Supplementary Figure 3) to target neurons in *LepR*$^{loxP/loxP}$ mice. Control experiments with *tdTomato*$^{loxP/+}$ reporter mice showed that a single injection of TAT-Cre into the preoptic region caused gene recombination in neurons of this region, including those expressing nNOS, but not in other LepRb expressing brain areas such as the ARH and the ventral premammillary nucleus

(PMv) (Supplementary Figure 3). Control experiments showed that phosphorylation of STAT3 induced by peripheral leptin administration is selectively blunted in the prepotic region of *LepR*$^{loxP/loxP}$ mice, while retained in other LepR expressing regions of the hypothalamus (Figure 6A). This selective inhibition of LepR expression in the preoptic region that did not alter body weight (Figure 6B) led to a significant increase in uterine weight (Figure 6C) and basal LH serum levels in diestrous mice (Figure 6D; n=9-10, Two-way ANOVA, $p < 0.05$). In contrast, 15-minute exogenous leptin treatment failed to promote further increases in LH levels in these mice (Figure 6D, n=9-10, Two-way ANOVA, $p = 0.35$). Together these data demonstrate that LepR receptor expression in the preoptic region plays an active role in the control of basal GnRH/LH secretion.

Discussion

The dialogue between metabolic homeostasis and reproductive competence has been extensively documented over many decades yet with the rapid increase in the rates of obesity and diabetes and the subsequent deleterious effects on reproduction, the need to investigate the crosstalk between metabolism and reproduction has become more urgent. In this study, we report that nNOS neurons, in particular those residing within the preoptic region, are an integral component of leptin action on the neuroendocrine reproductive axis. Leptin promotes the rapid phosphorylation of the catalytic subunit of the nNOS protein within the preoptic region, an event associated with a rise in peripheral LH from nadir levels. Genetic deletion, as well as pharmacological inhibition, of nNOS prevents leptin induced LH release. Remarkably, this effect appears independent of kisspeptin/GPR54 signaling. Leptin - deficient mice bearing a null mutation in the *nNos* gene or exposed to continuous nNOS inhibition fail to undergo sexual maturation in response to chronic leptin administration. Finally, we have provided evidence that LepRb expression within the preoptic region may play an active role in the control of basal GnRH/LH secretion. Together these results demonstrate that NO-signaling facilitates leptin action on reproduction and establishes the

preoptic region as a novel site for the integration of leptin signaling on the GnRH neural network.

NO is a freely diffusible neurotransmitter, its biological effects being regulated by the catalytic properties of the nNOS enzyme, its rate of inactivation, and distance from its source (33-35). The preoptic region of the hypothalamus is one of the major sites for the expression of nNOS within the basal forebrain (36, 37), where the release of NO has been linked to the secretion of GnRH and stimulation of the preovulatory surge of GnRH/LH (15, 38-40). The importance for nNOS activity in reproductive functioning is highlighted by the detrimental effects on fertility in nNOS-deficient female mice, ranging from sporadic ovulation and blunted preovulatory LH secretion to complete hypothalamic hypogonadism (15, 30, 41). Intriguingly, nNOS neurons within the preoptic region have been shown to be a key integrator of neural signals involved in reproduction (15, 39, 42, 43), in which the local activation of nNOS and subsequent production of NO exerts a tonic inhibitory tone on surrounding GnRH neurons (14, 15). Recently, the peripheral metabolic hormone leptin has also been found to directly activate nNOS neurons (16) and induce the phosphorylation of nNOS within the preoptic region (present study). Moreover, the targeted deletion of LepR in nNOS neurons has a profound effect on energy balance providing evidence that nNOS neurons are crucial in the integration of metabolic signals (29). Since mice deficient in nNOS are insensitive to the stimulatory effects of leptin on GnRH/LH secretion and the directed inhibition of nNOS in wildtype mice blunts leptin action, we extend the findings that nNOS neurons are involved in the coordination of metabolic signals and provide the first evidence that NO signaling within the preoptic region is critical to leptin's effect on the reproductive axis.

To evaluate the specific role of leptin receptor signaling in the preoptic region, we employed stereotaxic techniques to deliver a TAT-Cre recombinant protein to LepR floxed mice. The directed deletion of LepR within the OVLT/MEPO, the site where leptin activates nNOS (16), increased basal LH release suggesting that a minimal amount of leptin signaling in the preoptic region is required for regular basal GnRH secretion. Indeed, it appears that leptin exerts a tonic inhibitory effect on GnRH/LH secretion, an effect strikingly similar to action of

NO transmission on GnRH neurons during the estrogen-mediated negative feedback phase (15). Thus it is tempting to speculate that LepR signaling through NO transmission is mediating this effect. Furthermore, exogenous administration of leptin fails to further promote LH secretion indicating that POA leptin receptor signaling is involved in the coordination of leptin's stimulatory effect. One caveat to note is that the use of intracranial injections inherently provides variability and heterogeneity in injection sizes, injection sites, and leakage of the TAT-Cre protein into surrounding nuclei. However, leptin receptor functioning appears completely undisturbed in caudal parts of the hypothalamus and LH hormonal profile remains consistent within groups and thus we are confident that the effects we observe are primarily mediated through knockdown of LepRbs within the preoptic region.

Previous studies have demonstrated that states of negative energy balance, where leptin levels are low, results in decreased LH levels and the suppression of the estrous cycle, while leptin treatment restores fertility (28, 44-46). Although leptin is considered stimulatory in nature, it is rather the fall in leptin levels that is the critical signal required for the organism to sense acute and long term changes in energy balance and initiate a neuroendocrine response (28). Interestingly, in our fasting paradigm, leptin actively restores LH levels, while the inhibition of nNOS activity prior to leptin treatment renders the neuroendocrine reproductive axis unable to sense the increase in leptin levels. Thus, the presence of nNOS activity and subsequent volume transmission of NO appears to be necessary for sensing the changes in leptin levels to relay to GnRH neurons, a scenario that our computer modeling showed to be plausible.

In addition to modulating leptin signaling, nNOS neurons also act as an interface for kisspeptin signaling (15). However, targeted deletion of the leptin receptor in kisspeptin neurons does not disrupt fertility (25, 47), nor is the selective reactivation of the leptin receptor in kisspeptin neurons in mice otherwise null for the leptin receptor (LepR null) sufficient to promote sexual maturation (47). In agreement, in our *gpr54*$^{-/-}$ mouse model, leptin evokes a rise in LH levels which is not mitigated by the complete absence of

kisspeptin-GPR54 signaling, suggesting that leptin does not require GRP54 signaling to stimulate the neuroendocrine reproductive axis.

The lack of pubertal activation in *Lep*$^{ob/ob}$ mice in which nNOS was site-specifically inhibited argues for the importance of leptin signaling within the preoptic region in the onset of the neuroendocrine control of fertility. However, nNOS is also expressed in various other leptin-receptor-containing regions of the hypothalamus, making it impossible to exclude the involvement of NO signaling originating from regions other than the preoptic. Among these leptin-sensitive regions is the ventral portion of the PMv (16, 17, 29, 48), another hypothalamic area involved in the neuroendocrine regulation of fertility (25, 29), and in which the majority of the neurons that express nNOS are also capable of sensing leptin (16, 29). Intriguingly, leptin-sensitive PMv neurons are also glutamatergic and project to the preoptic region (17, 25). Since most of the nNOS neurons in the preoptic region express the NMDA receptor (26), which is involved in the onset of puberty (49) and regulates nNOS activity (19, 26, 50, 51), it is tempting to speculate that glutamatergic neurons of the PMv morphologically and functionally interact with nNOS neurons of the preoptic region to regulate the activity of GnRH neurons, thus synchronizing the effects of leptin in the two regions. Curiously, cre-mediate excision of the leptin receptor in glutamatergic neurons results in no striking metabolic or reproductive phenotype (52, 53), suggesting that leptin signaling through glutamate is not required for fertility. Yet, the reactivation of the leptin receptor in mice otherwise null for the leptin receptor within the PMv, which houses predominantly glutamatergic neurons, is sufficient to rescue fertility (25). Thus, the coordination between leptin sensing glutamatergic PMv neurons and preoptic nNOS neurons may nonetheless occur in normal conditions and potentially regulate GnRH release.

Consistent with our model, female mice with a selective deletion of the leptin receptor in nNOS neurons demonstrate a substantial delay in the onset of puberty (29). The fact that reproductive capacity remained uncompromised in these mice during adulthood presumably points to the redundancy in the reproductive axis (29). As the deletion of the leptin receptor in nNOS neurons occurred during early development likely allowed for the generation of

compensatory changes. Consistent with this hypothesis, functional redundancies in the neuroendocrine reproductive axis have been demonstrated in several cases, including GnRH (54, 55) and kisspeptin neurons (23). As reproduction is such a critical and energy-consuming process, redundancies in the metabolic pathway controlling reproduction would ensure the survival of the species. In the present study, experiments were designed to inhibit nNOS activity and LepRb expression exclusively during adulthood, precluding the development of compensatory mechanisms, as seen in both the metabolic and reproductive neural circuitry (23, 56). In the future, a nNOS inducible-cre mouse would be needed to clarify the role for leptin signaling in these neurons during development and adulthood.

The directed inhibition of nNOS in obese leptin-deficient females did not prevent leptin induced weight loss during the four week leptin regime. Yet the progression through puberty was not observed despite the substantial decrease in body weigh in these mice. Our study, in conjunction with data from Elias and colleagues demonstrating that the selective reactivation of the leptin receptor in LepR null mice restores fertility but does not rescue body weight (25), provides compelling evidence that leptin can act through multiple independent pathways to influence metabolism and reproductive fecundity. It should be noted, however, that the termination of our experiment was four weeks from the start of the leptin regime and the possibility remains that the leptin-deficient nNOS null mice and the $Lep^{ob/ob}$ mice treated with the nNOS inhibitor may have regained reproductive functioning with further time on leptin treatment.

In the present study we show that, in addition to regulating estrogen-dependent neural inputs, such as kisspeptidergic signals, the same population of NO-synthesizing neurons also sense leptin, thereby transmitting information regarding peripheral energy stores to GnRH neurons. Together, these data give rise to the provocative idea that nNOS-neurons of the OVLT/MEPO are a vital cell population for the rapid integration and transmission of both gonadal and metabolic signals in the neuroendocrine brain.

Material and Methods

Animals

All experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and French bylaws regarding mammalian research, and approved by the Institutional Animal Care and Use Committee of the Saban Research Institute of the Children's Hospital Los Angeles and the University of Lille. Experiments were performed on adult (3-6 months old) female C57Bl/6J mice (Charles River Laboratories), *nNos*-null mice (*nNos*$^{-/-}$, B6.129S4-*Nos1tm1Plh*/J) (30), *Lep*-null mice (*Lep*$^{ob/ob}$, B6.V-*Lepob*/J) (Jax mice; Jackson Laboratory), *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ double mutants, 129S6/SvEv *Gpr54*-null mice (*Gpr54*$^{-/-}$) (22); *LepR*$^{loxP/loxP}$ mice (Jax mice; Jackson Laboratories), and their respective wild-type littermates. The *nNOS*-null, *Lep*-null, and *LepR*$^{loxp/loxp}$ mice were maintained on a C57B6/J background, while the *Gpr54*-null mice were maintained on a mixed 129/SV background.

Drugs

Recombinant murine leptin (3mg/kg, i.p.) and the NOS inhibitor, NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 50mg/kg, i.p.; 5mM i.c.) were purchased from Peprotech and Calbiochem, respectively.

Immunohistochemistry

The detection of nNOS, P-nNOS and P-STAT3 was carried out using protocols that we have previously described in refs. (15) and (18). Detailed methods are provided in *SI Materials and Methods*.

Western blot analyses

The detection of P-nNOS and nNOS by western blotting was carried out as described previously (15).

Stereotaxic surgery

L-NAME (5mM) or sterile saline was infused into the preoptic region (coordinates from bregma: +0.5mm anteroposterior, +/-0.2mm lateral, -5.3mm in depth) of mice *in vivo* using a Hamilton syringe or infusion cannula connected to a subcutaneously implanted osmotic minipump (Alzet, CA) for acute and chronic infusion, respectively. The tat-cre protein was injected bilaterally into floxed animals at the same coordinates. Detailed methods are provided in *SI Materials and Methods*.

Mathematical model

Detailed methods are provided in SI Materials and Methods.

Tat-cre delivery

A tat-Cre fusion protein produced as detailed previously (32, 57) was stereotaxically infused into the preoptic region (300 nl over 5 min at 2.1 mg/ml; AP: +0.5 mm, ML: 0.2 mm DV: - 5.3 mm) of isoflurane-anesthetized $LepR^{loxP/loxP}$ and $LepR^{+/+}$ 2-month old littermates 6 days before experiments.

Plasma LH assay

Plasma LH was measured using a Rodent LH ELISA kit (Endocrine technologies, Newark, CA) with a sensitivity of 0.01 ng/ml and 7% intra-assay and 10% inter-assay coefficients of variance.

Statistics

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Differences between two groups were analyzed with an unpaired Student's t-test. One-way ANOVA or multivariate ANOVA followed by a Tukey post hoc test was used to draw

comparisons between 3 or more groups. P-values of less than 0.05 were considered to be statistically significant.

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Figures

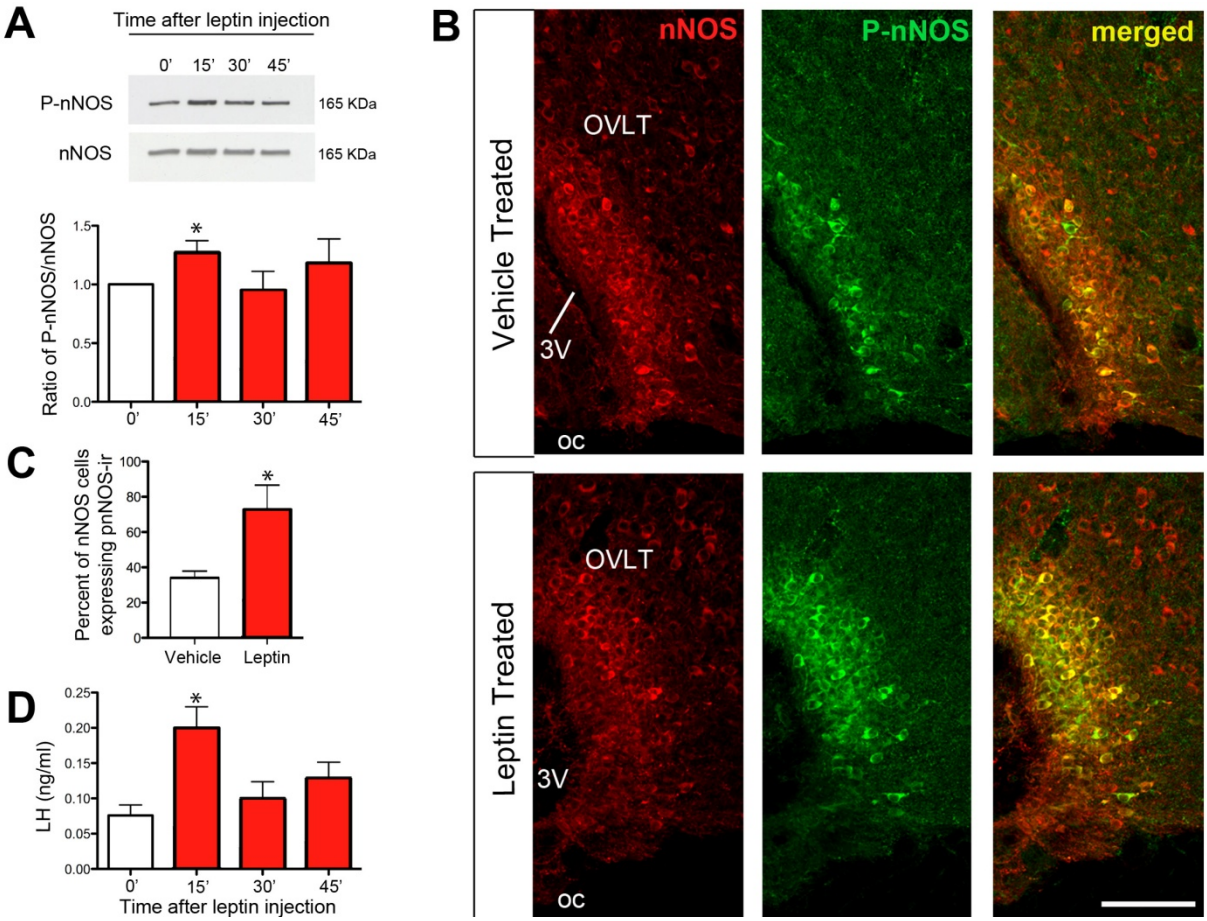


Figure 1. Leptin activates neuronal nitric oxide synthase in the preoptic region and increases circulating LH levels. (A) Upper panel: Representative western blots for phosphorylated and total nNOS at the times indicated (in minutes) following leptin treatment. Leptin promotes the phosphorylation of nNOS acutely at 15 minutes. (B) Coronal sections of the organum vasculosum of the lamina terminalis (OVLT) showing an increase in the percentage of nNOS cells expressing P-nNOS immunoreactivity (ir) 15 minutes after leptin stimulation. 3V: third ventricle; oc: optic chiasm. Scale bar: 100 μm. (C) Quantification of immunolabeling shown in B. (D) Circulating LH levels surge 15 minutes after leptin administration. *: $p<0.05$.

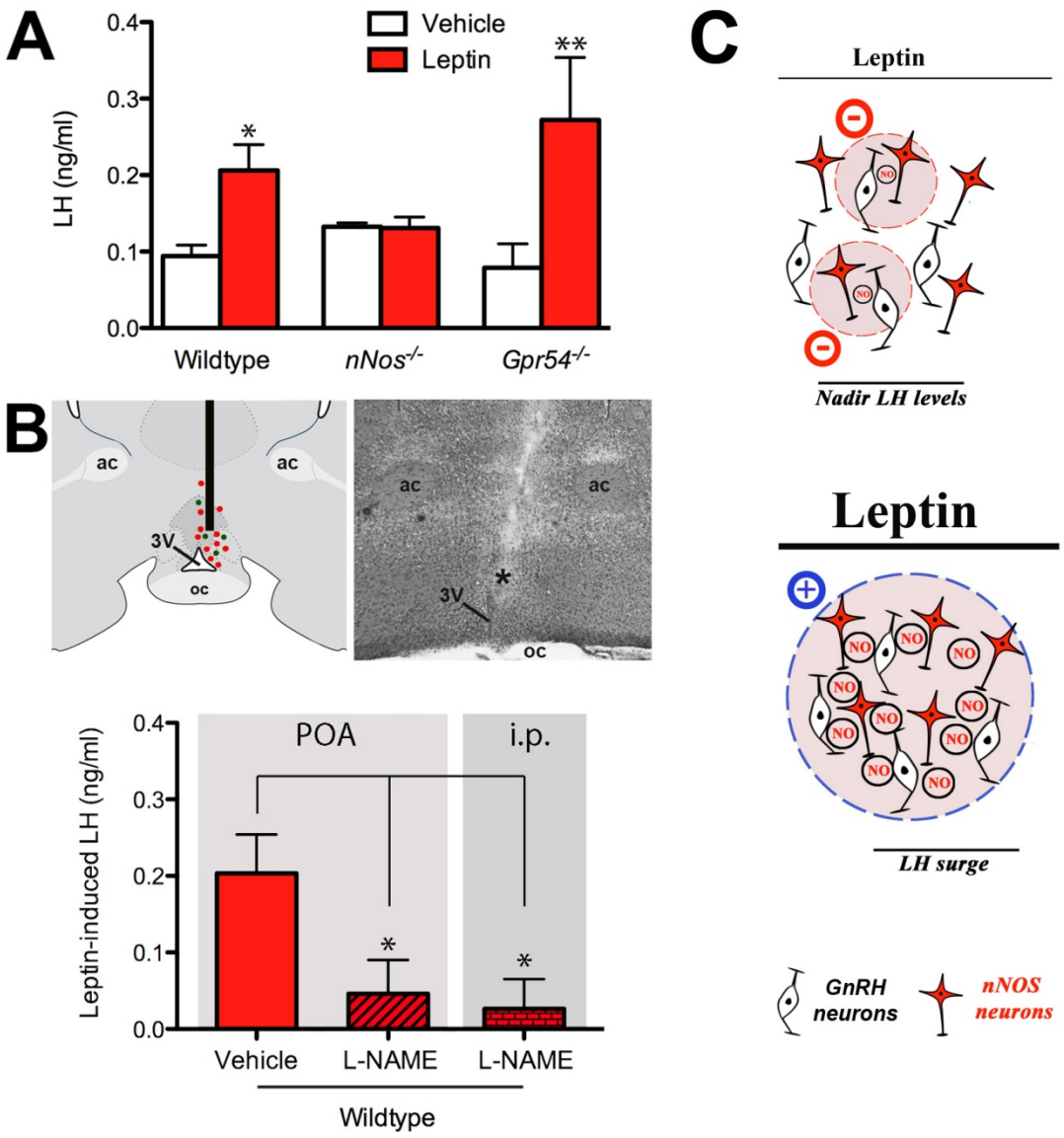


Figure 2. The blockade of nNOS blunts leptin's ability to induce LH release. (A) nNOS activity is required for leptin-induced LH release, independently of kisspeptin-GPR54 signaling. (B) The pharmacological inhibition of nNOS specifically within the preoptic region (POA) is sufficient to block leptin-induced LH release. The diagram and the corresponding photomicrograph show the target site into which L-NAME was stereotaxically infused in the POA, where GnRH (green dots) and nNOS (red dots) neuronal cell bodies are intermingled. Different letters represent statistical significance between groups. 3V: third ventricle; oc: optic chiasm; ac: anterior commissure; *: trajectory of the implanted needle. In bar graphs, *: $p<0.05$, **: $p<0.01$. (C) Schematic representation of the hypothetical mechanisms of regulation of LH secretion in the absence (upper panel) or presence (bottom panel) of

exogenous leptin treatment in diestrus mice (see Supplementary Figure 2 for mathematical modeling).

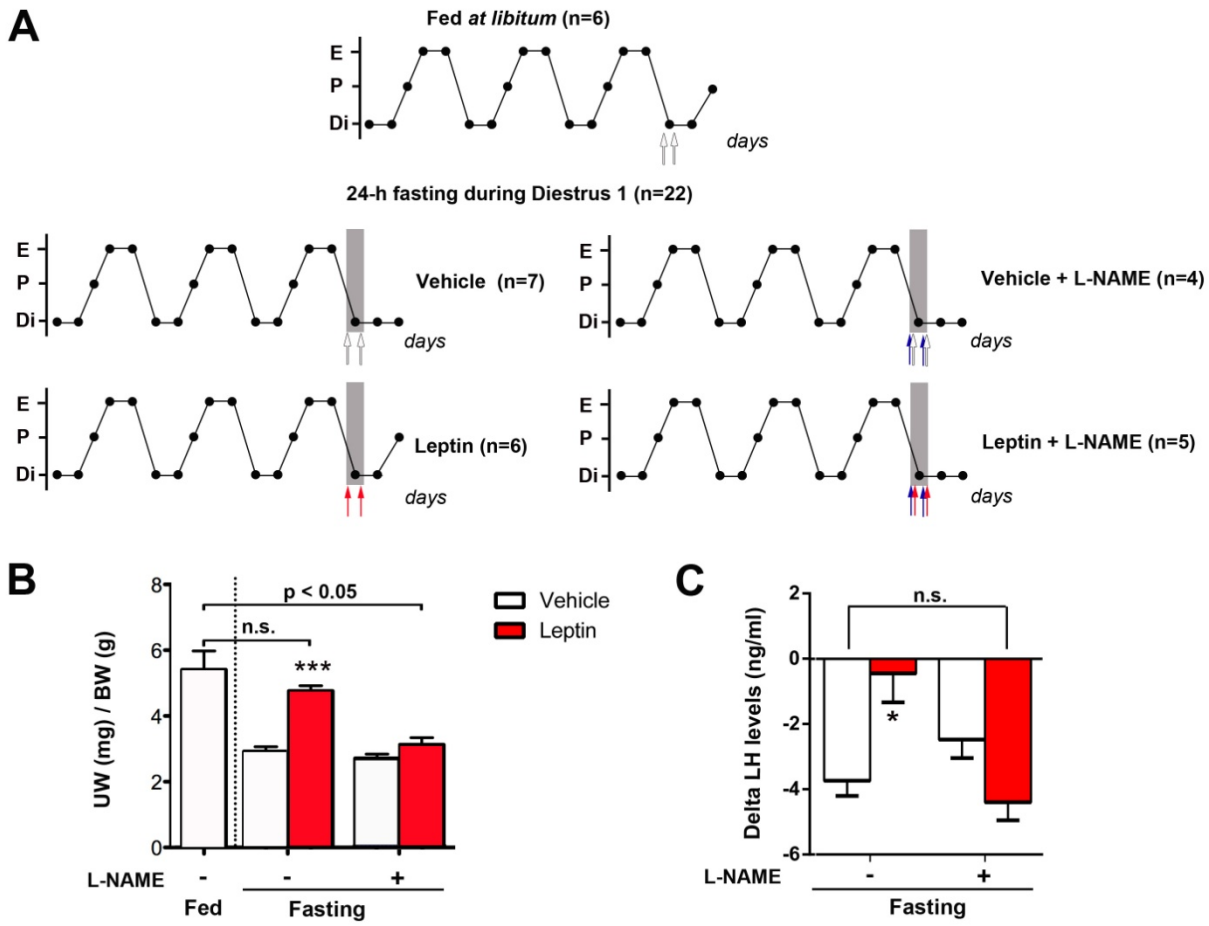


Figure 3. The pharmacological inhibition of nNOS with L-NAME prevents the rescue of the estrous cycle and LH levels by leptin in fasted mice. (A) Representative 15-day estrous cycles of animals subjected or not to 24h-fasting in diestrus 1 three days before death (gray shadow). Mice were subjected to leptin (red arrows), L-NAME (blue arrows) or vehicle (white arrows) injection twice daily on diestrus 1 three days before death. Di, diestrus; P, proestrus; E, estrus. (B) Leptin treatment in fasting mice rescued proestrous-like uterine weight (UW), whereas concomitant L-NAME injection blunted this effect. BW, body weight. (C) Leptin treatment restores surge levels of LH in mice subjected to a fasting of 24h in Diestrus 1 and killed on the expected day of proestrus, i.e., two days after. L-NAME treatment impedes this leptin rescuing effect in fasting mice. *: p<0.05 and ***: p<0.001 leptin *vs.* vehicle.

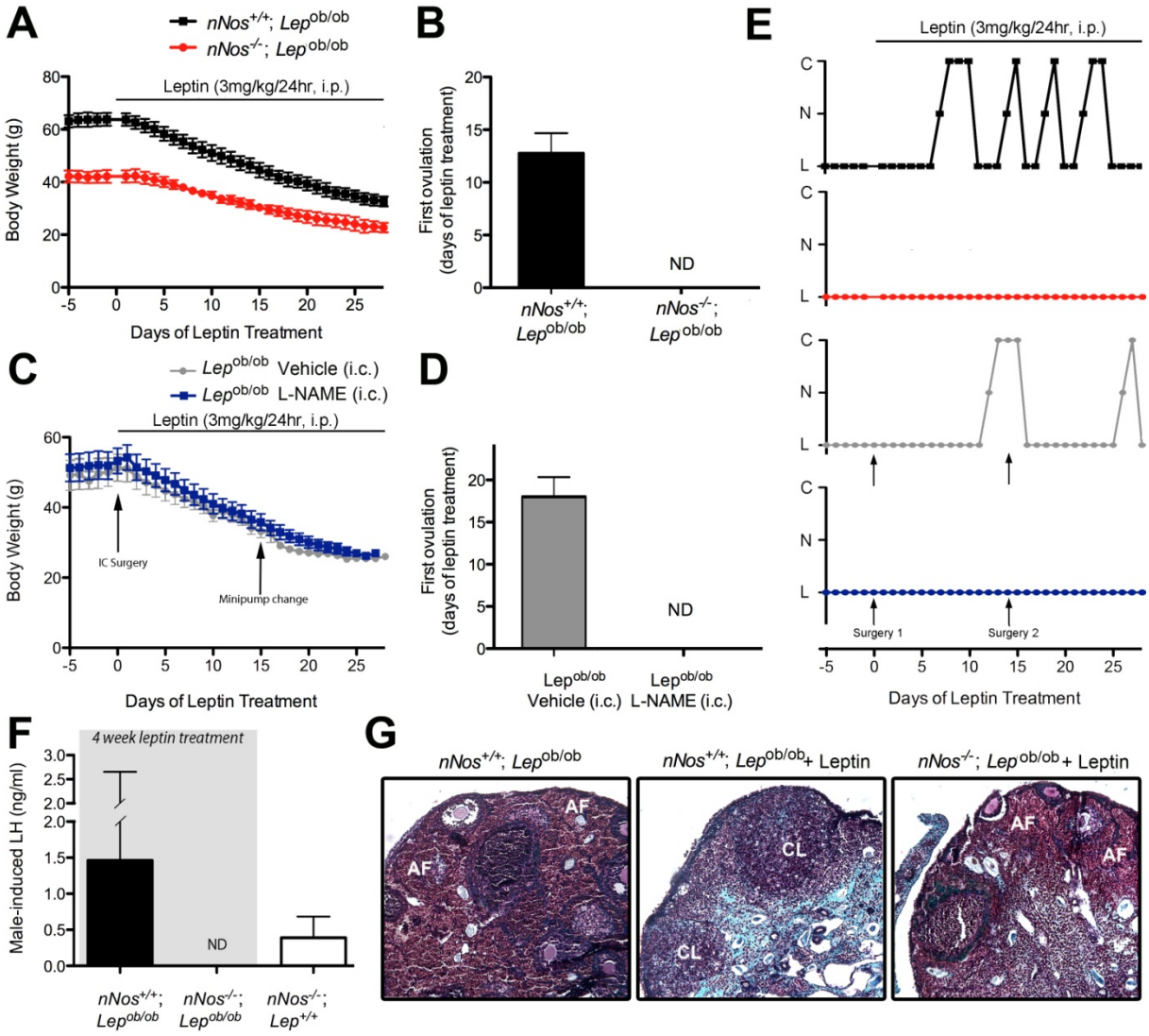


Figure 4. A deficiency in NO signaling renders leptin unable to induce puberty in *Lep*$^{ob/ob}$ mice. (A) *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice displayed a lower body weight than *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ littermates prior to the leptin regime, although both groups lost weight with leptin treatment. (B) *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice never underwent first vaginal estrus. N.D.: not detected. (C) No difference in body weight was observed between *Lep*$^{ob/ob}$ mice infused with either vehicle or L-NAME, although both groups responded to leptin with a decrease in body weight. (D) The *Lep*$^{ob/ob}$ mice treated chronically with L-NAME never demonstrated first vaginal estrus. (E) Blockade of nNOS in *Lep*$^{ob/ob}$ mice either by genetic or pharmacological means resulted in a lack of estrous cyclicity, while this was corrected in control mice. C, Cornified (estrus); N, nucleated (proestrus); L, lymphocytic (diestrus). (F) Blockade of nNOS in *Lep*$^{ob/ob}$ mice leads to non-detectable levels of LH. *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ and *nNos*$^{-/-}$; *Lep*$^{+/+}$ mice showed partial to full surge-like LH levels following exposure to male odor. (G) Ovarian sections from *nNos*$^{+/+}$;

Lep$^{ob/ob}$ mice with no leptin treatment, *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ following 28 days of leptin treatment, and *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice following 28 days of leptin treatment. Note the presence of corpora lutea (CL) in *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ mice treated with leptin. AF: atretic follicles; ND: not detected; i.c.: intracranial.

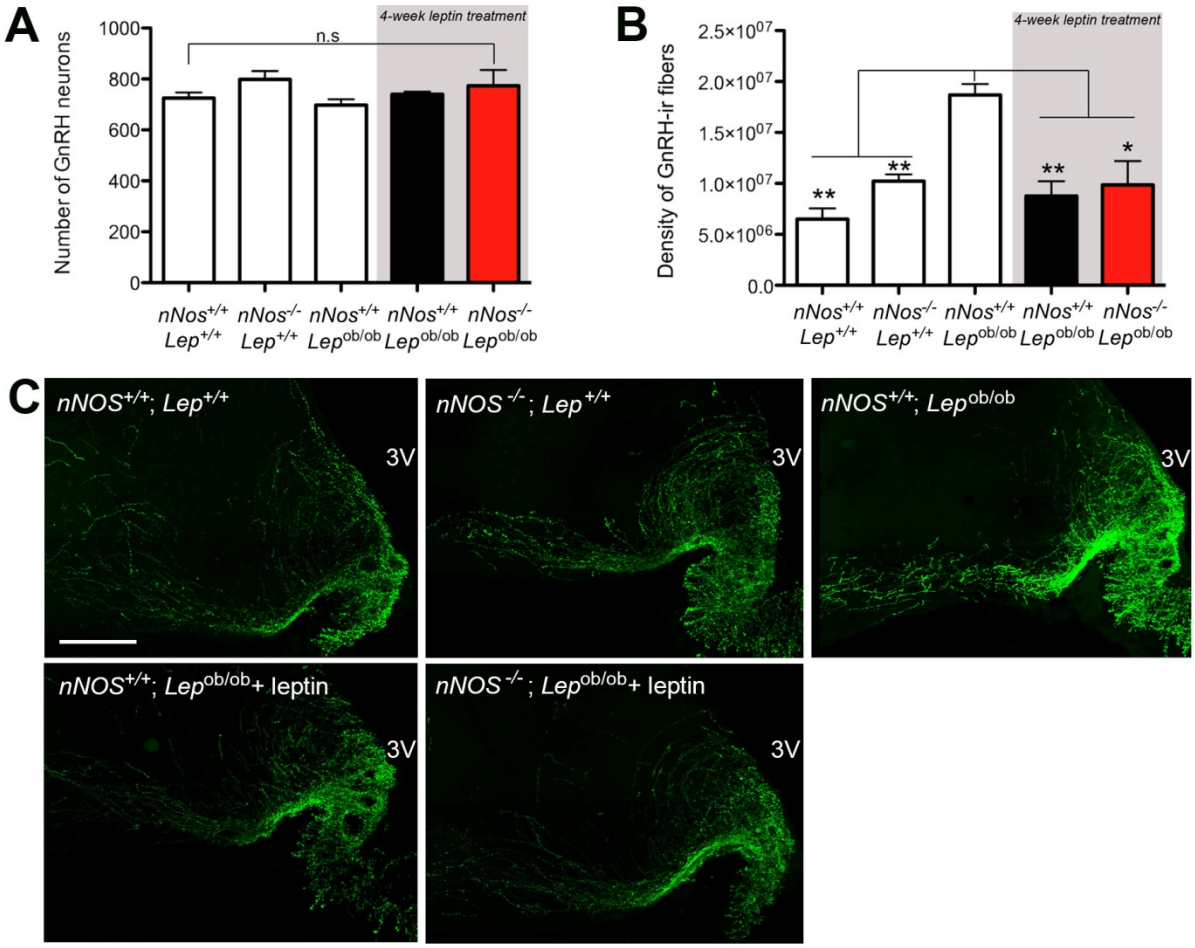


Figure 5. The number of GnRH neurons in the preoptic region (A) and the density of GnRH-immunoreactive (ir, green) fibers in the median eminence (B,C). Interestingly, the *nNos*$^{+/+;}$ *Lep*$^{ob/ob}$ mice show an increased GnRH immunoreactivity, while *nNOS*$^{-/-}$; *Lep*$^{ob/ob}$ and *nNOS*$^{+/+}$; *Lep*$^{ob/ob}$ female mice treated with leptin are equivalent to those of wild-type and *nNOS*$^{-/-}$ *Lep*$^{+/+}$ mutant mice.. 3V: third ventricle; n.s.: not statistically significant. Scale bar: 200 μm.

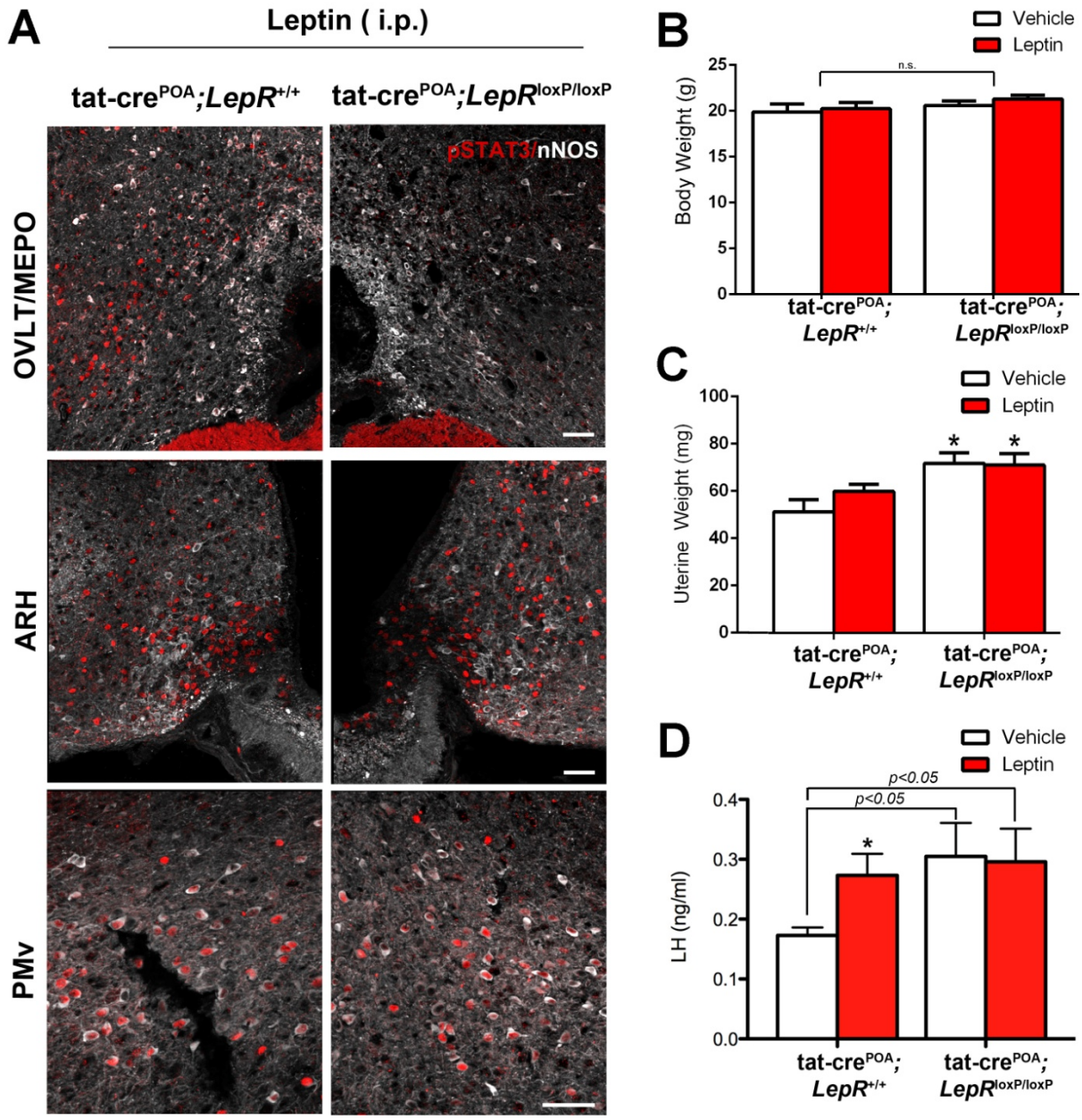
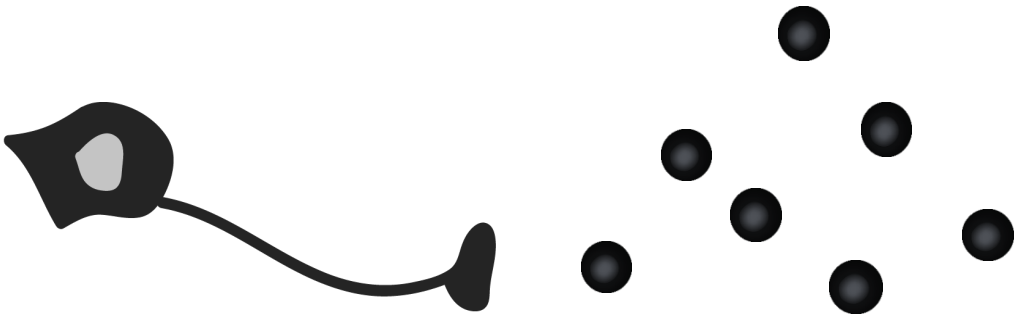


Figure 6. Site-specific deletion of LepR disrupts basal LH levels in female mice. (A) Bi-lateral injections of tat-cre protein into the OVLT/MEPO prevented P-STAT3 45 minutes following peripheral leptin injection in the preoptic region (POA) of *LepR*$^{loxP/loxP}$, but not in *LepR*$^{+/+}$ littermates. However, leptin was still able to induce P-STAT3 in caudal areas of the hypothalamus such as the ARH and PMv in tat-cre$^{POA;}$; *LepR*$^{loxP/loxP}$ mice. (B) Body weight did not differ between tat-cre$^{POA;}$; *LepR*$^{+/l+}$ and tat-cre$^{POA;}$; *LepR*$^{loxP/loxP}$ mice. (C) Lack of leptin signaling in the OVLT/MEPO resulted in higher uterine weight in tat-cre$^{POA;}$; *LepR*$^{loxP/loxP}$ mice when compared to tat-cre injected wildtype littermates. (D) Strikingly, basal levels of LH is increased in females lacking leptin receptor signaling in the preoptic region when compared

to wildtype mice injected with tat-cre. When injected with leptin, tat-cre$^{POA;}$; *LepR*$^{loxP/loxP}$ mice were unable to respond further with a rise in LH levels. OVLT: organum vasculosum of the lamina terminalus; ARH: arcuate nucleus; PMv: ventral premammillary nucleus. Scale bars: 50μm.

Chapter VII

Discussion



7.1 The nNOS- expressing population is characterized by a great heterogeneity in terms of molecular and genetic identity

Since the original discovery of the endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki (Furchgott and Zawadzki, 1980) and its identification as nitric oxide (NO) by two independent groups (Ignarro et al., 1987; Palmer et al., 1987), a great number of scientific studies contributed to our knowledge concerning the functions of this molecule in biological systems. The first demonstration of a neuronal NO production in the region of the brain by Garthwaite (Garthwaite et al., 1988) opened many research possibilities, that lead to our understanding that this very simple molecule is in fact participating in some of the most elaborate physiological processes of the brain (for review see Calabrese et al., 2007). To accomplish its undeniably distinct functions, NO production is governed by an extremely heterogeneous neuronal network of nitric oxide synthases (NOS) (Giuili et al., 1994; Burette et al., 2002; Lin, 2009; Tricoire and Tania, 2012).

In the region of the hypothalamus, nitric oxide has long been recognized as an important part of the regulatory mechanisms behind central hormonal control of reproductive and metabolic functions. More than 20 years ago, different groups reported a role of NO in the regulation of GnRH secretion both in vitro and in vivo (Bonavera et al., 1993, 1994; Moretto et al., 1993; Rettori et al., 1993; Bhat et al., 1995; Vanhatalo and Soinila, 1995; Prevot et al., 1998). Unfortunately, despite the initial burst of scientific reports implicating NO in the GnRH/LH surge, NO was quite overlooked in the years to come. It was not till the following decade that the significance of NO in the central hormonal regulation of reproduction was once more underlined; several research groups contributed in our better understanding of this obscure neuronal population by implicating nNOS in principal hypothalamic

functions, such as energy homeostasis (Leshan et al., 2012; Pfeifer et al., 2013; Sutton et al., 2014; Borgquist et al., 2015), food intake (Otukonyong et al., 2000; Riediger et al., 2006; Sutton et al., 2014; Wellhauser et al., 2016), lordosis behavior (García-Juárez et al., 2012), and reproduction (Dufourny and Skinner, 2002a; Sato et al., 2005; d'Anglemont de Tassigny et al., 2007a; Clasadonte et al., 2008; Sica et al., 2009; Parkash et al., 2010; Hanchate et al., 2012; Bellefontaine et al., 2014; Messina et al., 2016).

All the above reports of the distinct functions of nNOS cells, in combination with our newly identified actions of NO are once more highlighting the divergent role of NO signaling, raising the intriguing possibility that, as in other major brain regions, nNOS- expressing neurons of the hypothalamus could be represented by distinct subpopulations according to their anatomical distribution and/or function.

7.1.1 nNOS cells residing in the tuberal region of the hypothalamus are differentially regulated according to their neuroanatomical distribution and the developmental stage in question

Interestingly, only subtle differences were seen in the expression levels of hypothalamic nNOS protein levels across the distinct developmental stages studied, with the only striking exception being the nNOS-expression in the region of the ARH. The nNOS population residing there was not seen in place during the infantile period studied (P11), but appeared to be present later on, in the beginning of the juvenile stage (P23). Hence from all the major hypothalamic regions where nNOS cells were seen located, the ARH population was the only one demonstrating a developmentally regulated expression of the nNOS protein. Further investigation of this nNOS population revealed another unique characteristic of these NO-releasing neurons; in

contrast to the nNOS cells located in the DMH and VMH regions that were seen to largerly coexpress the vglut2 promoter, suggesting a glutamatergic identity, the nNOS population residing in the ARH was the only nNOS subpopulation of the MBH observed to be in its vast majority GABAergic, with a subpopulation of cells identified as PV+/GAD-67+ fast-spiking GABAergic nNOS neurons. So the question arose: what is the reason of this diversity within the nNOS residing in the ARH and what could be the mechanism triggering this timely- regulated nNOS expression in these GABAergic neurons?

During the juvenile period preovulatory levels of estradiol are known to markedly increase (Andrews et al., 1981). As the hypothalamic-pituitary axis is becoming susceptible to this increasing ovarian-estrogen production, the development of the negative feedback by estrogen takes place, regulating eventually the initiation of puberty (Meijs-Roelofs and Kramer, 1979; Prevot, 2015; Mayer et al., 2010; Dubois et al., 2016; Greenwald-Yarnell et al., 2016). Here we presented data demonstrating that the vast majority of nNOS neurons of the ARH region are actually coexpressing estrogen receptor α, agreeing with previous reports demonstrating the colocalization of NADPH-diaphorase and estrogen receptor in the rat and the sheep hypothalamic nuclei (Okamura et al., 1994; Dufourny and Skinner, 2002b). The expression of ER-α from the NOS-producing neurons of the region suggests that this population of nNOS cells is indeed sensitive to alterations in the estrogens levels, raising the intriguing possibility that by acting as recipients of estrogen they could be promoting its negative feedback action on the hypothalamus (Meijs-Roelofs and Kramer, 1979; Prevot, 2015). Of note, in the adult female all of the nNOS+/ PV+/GAD-67+ neurons present in the ARH were also seen to co-express ER-α. This newly described nNOS+/ PV+/GAD-67+/ ER-α+ neuronal population could potentially play a unique role in

mediating the negative feedback action of estrogen but further studies are necessary to depict its function.

7.1.2 The nNOS neurons playing a vital role in puberty onset and estrous cyclicity are glutamatergic

According to our results, and in contrast to what was observed in the ARH of the tuberal region of the hypothalamus, nNOS neurons were evidenced to be glutamatergic in the AVPV, OVLT and MePO. nNOS cells of the preoptic region have been in fact evidenced as integral parts of the network regulating GnRH neuronal activity and secretion (d'Anglemont de Tassigny et al., 2007a; Clasadonte et al., 2008; Parkash et al., 2010; Bellefontaine et al., 2014; Messina et al., 2016). Thus the newly-identified glutamatergic phenotype of these nNOS-expressing neurons is in agreement with studies demonstrating an involvement of glutamatergic signaling in the regulation of GnRH mRNA expression, neuronal activity and consequently the release of GnRH peptide (Urbanski and Ojeda, 1990; Petersen et al., 1991; Spergel et al., 1999). Most importantly, the results we presented in both of our studies agree on a constitutive expression of nNOS protein, from the beginning of the infantile period till the later stages of adulthood, in the neuronal populations residing in the preoptic area. Considering the fact that preovulatory LH release and initiation of puberty are governed by GnRH neurons residing in the POA, an implication of NO in the control of pubertal onset seemed rather plausible. In our second study we tackled the above question, i.e. whether NO signaling, and more specifically nNOS-derived NO, could indeed regulate sexual maturation. Constitutive absence of nNOS protein (in our Nos1- null mouse model) was seen to result in a severe delay of the sexual

maturation, as suggested by the delayed vaginal opening and pubertal onset observed. More specifically, the use of L-NAME, a wellknown inhibitor of NOS, showed that NOS activity mainly plays a critical role for the maturartion of the HPG axis during the infantile period between minipuberty and weaning.

7.2 The maturation of the nNOS population of the OVLT is tightly linked to the concomitant maturation of the gonads

Even though we demonstrated that there is no difference in the levels of nNOS expression in the neuronal population of the OVLT during postnatal development, we did identify a developmentally regulated maturation of the NOS neuronal network residing in the region. Phosphorylation of the nNOS protein is an extremely important posttranslational modification since it leads to the activation of the enzyme, rendering it capable of producing NO (Rameau et al., 2007). Prior to P12, we observed an extremely low expression of the p-nNOS protein in the region of the OVLT, which however robustly increased between P12 and weaning. Interestingly, the activation of the nNOS neurons of the preoptic area was seen to happen almost concomitantly with the appearance of the nNOS population in the region of the ARH. For the latter population we hypothesized that the timely-regulated nNOS expression was linked to the ovarian steroids, known to significantly increase during the infantile period (Fortune and Eppig, 1979; Funkesnstein et al., 1980). What if this was also the case for the nNOS neurons of the OVLT, who are in fact largerly coexpressing ER- α throughout development? Our second study actually validated this hypothesis; ablation of the ovarian source of estrogens during the infantile period diminished the phosphorylation levels of the hypothalamic nNOS protein during the later stages of

the infantile period. The interaction of nNOS with estrogen is not surprising since in the adult brain natural fluctuations of estrogen levels across the estrous cycle have been already correlated to the activation of the nNOS protein (d'Anglemont de Tassigny et al., 2007b, 2009; Gingerich and Krukoff, 2008; Parkash et al., 2010). This estrogen-induced activation of the nNOS neuronal hypothalamic population during the infantile period enables a NO-driven maturation of the HPG axis by setting the dialogue between the ovaries and the hypothalamus. When ovariectomy after weaning was coupled to the selective blunting of NO release during the infantile period, LH levels presented a major increase, indicating that infantile hypothalamic NO action is necessary for the implementation of an appropriate negative feedback loop of gonadal estrogens on the hypothalamus.

7.3 Infantile NO by modulating Gnrh transcription and secretion is required for the initiation of puberty and the establishment of a mature and functional reproductive axis

The significant increase in the phosphorylation levels of the nNOS during postnatal day (P) 12 signifies an increment in the amount of NO being released from this neuronal population, and therefore a possible direct action of this newly produced NO on the neighboring GnRH neurons. Transient ablation of NOS activity specifically during the infantile period (P10-P21) replicated the reproductive phenotype seen in the constitutive NOS1 knockout mouse model. Female mice demonstrated a delayed pubertal onset and a severely perturbed reproductive capacity during adulthood, suggesting that infantile NO signaling is required not only for the initiation of puberty, but generally for the establishment of a fully matured and functional reproductive axis. We reckon that NO exerts this role by directly tempering with the expression levels of the Gnrh mRNA, thus subsequently modulating GnRH secretion.

As a matter of fact, we demonstrated that constitutive absence of NOS1 activity resulted in not only a vast increase in the P12 Gnrh mRNA content, but also in the FSH surge levels at minipuberty. This observed increase of Gnrh transcription upon nNOS invalidation agrees with our recent study, where Messina and collegues demonstrated a selective switch in miRNA expression patterns in GnRH neurons during mini-puberty that triggered the repression of NO-C/EBP signaling, thus permitting the sustained increase of GnRH required for subsequent sexual maturation (Messina et al., 2016). In other words, both of our studies support a role of infantile nNOS-derived NO as a break to retain GnRH transcription and subsequent neurosecretion, regulating the activation of the GnRH neuronal population, and driving the reproductive network to its maturation.

7.4 Identification of NOS1 mutations in humans with hypogonadotropic hypogonadism

Even though our study focused on the postnatal action of NO, specifically during the infantile period, primarily using a mouse model we also presented important novel data demonstrating the discovery of a series of mutations on the Nos1 gene. Genetic studies on KS and CHH patients revealed in total 9 missense mutations on the gene encoding for the NOS1 human protein, located mainly in the oxygenase and reductase domain of the nNOS. Out of these 9 heterozygous mutations in the Nos1 gene, 7 were correlated with the appearance of syndromes related to a delayed or completely absent pubertal onset. More specifically NOS1 mutations were identified in 12 cases presenting hypogonadotropic hypogonadism, with the majority of them being prevalent in Kallmann syndrome (KS) probands. Kallmann syndrome is a rare

congenital genetic condition presenting a unique combination of GnRH deficiency, arising from a faulty migration of the neuronal population, and anosmia. GnRH neurons, initially originating from the olfactory placode, are already located into their principal site of residence, the hypothalamus, at birth. Several mutations on proteins implicated in different steps of this migratory process have been identified previously in KS probands. The prevalence of NOS1 mutations in cases where GnRH deficiency is the main molecular phenotype, creates a link between nNOS impaired activity and GnRH defective release, and thus reinforces our findings of an nNOS driven regulation of GnRH activity in the rodent postnatal brain.

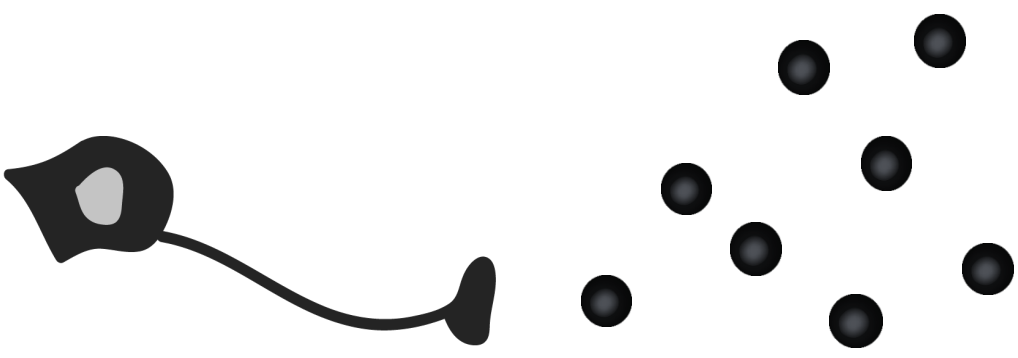
7.4.1 Novel role of NO signaling in the migration of GnRH neurons during embryonic development

During this study we identified for the first time that nNOS was coexpressed by migrating GnRH neurons located in the region of the nose in both a human fetus at 8 weeks of gestation, as well as in a mouse embryo during embryonic day (E) 14.5. nNOS expression was also evident in he scaffold of vomeronasal/terminal nerve fibers along which GnRH cells migrate. Interestingly, once GnRH neurons had entered into the ventral forebrain they were no longer seen to coexpress the nNOS protein. Considering this newly identified coexpression of GnRH and the nNOS, but also the migratory defect present in the cases of KS, we sought to investigate a possible involvement of NO prior to the infantile period, in the migration of GnRH neurons during embryonic development. Blunting NO release by in utero injection of the NOS inhibitor in the nose of embryonic day (E) 12.5 mouse embryos resulted in a substantial perturbation in the migratory process of GnRH neurons. During the

embryonic day (E) 14.5, in contrast with an almost evenly divided population of GnRH migrating cells seen in our saline-injected embryos, NO invalidation resulted in the formation of GnRH aggregates in the region of the nose, prohibiting thus the migration of these cells in the ventral forebrain region. Hence, we could imagine that an abnormal release of NO because of the existence of a mutated nNOS protein results in a failure of GnRH neurons to reach the hypothalamus, and is the main cause of the migratory defect seen in KS patients bearing the newly identified Nos1 mutations.

Chapter VIII

Concluding Remarks



During my PhD we characterized the neuroanatomical distribution of nNOS neurons of the hypothalamic area during development. We provided new information about their molecular identity by identifying the cohort of neurotransmitters and hormone receptors expressed by the distinct subpopulations of nNOS cells during development. Additionally, focusing on the functional aspects of the nNOS cells residing in the POA we unmasked previously unknown functions of the hypothalamic infantile nNOS-derived NO in the regulation of GnRH neuronal activity and the subsequent maturation of the reproductive axis. We identified that NO exerts its role on the tuning of pubertal onset in a timely-regulated manner, acting during a restricted time window of the infantile period, known as mini-puberty.

Furthermore, we generated data supporting a novel role of NO in the regulation of GnRH migration during embryogenesis. These results are in agreement with the identification of a series of NOS1 mutations in cases of CHH, presented in this study, that further highlight the major role of hypothalamic NO neuronal signaling in the establishment of fertility.

Overall, my PhD identifies nNOS hypothalamic neurons as important regulators of the GnRH neuronal activity during both embryonic and postnatal development. The in depth analysis of the newly reported mutations in the Nos1 gene will certainly improve our understanding on the mechanisms through which nNOS can modulate the fate of the reproductive axis, improving our chances of treating human infertility.

Chapter IX

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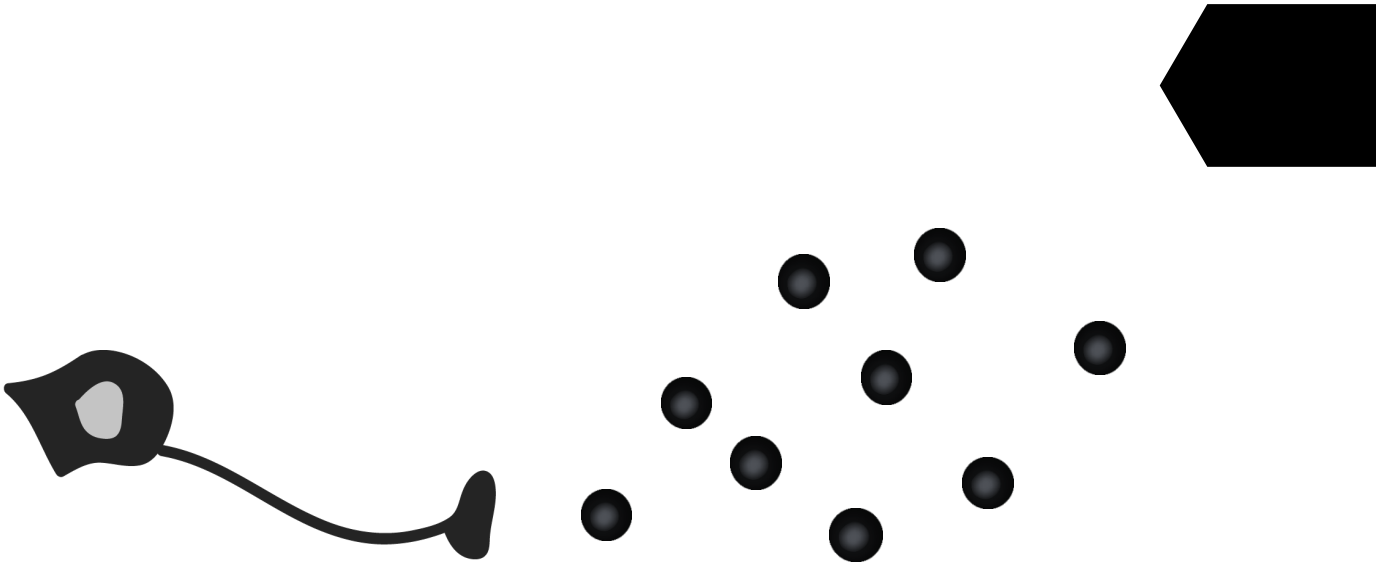

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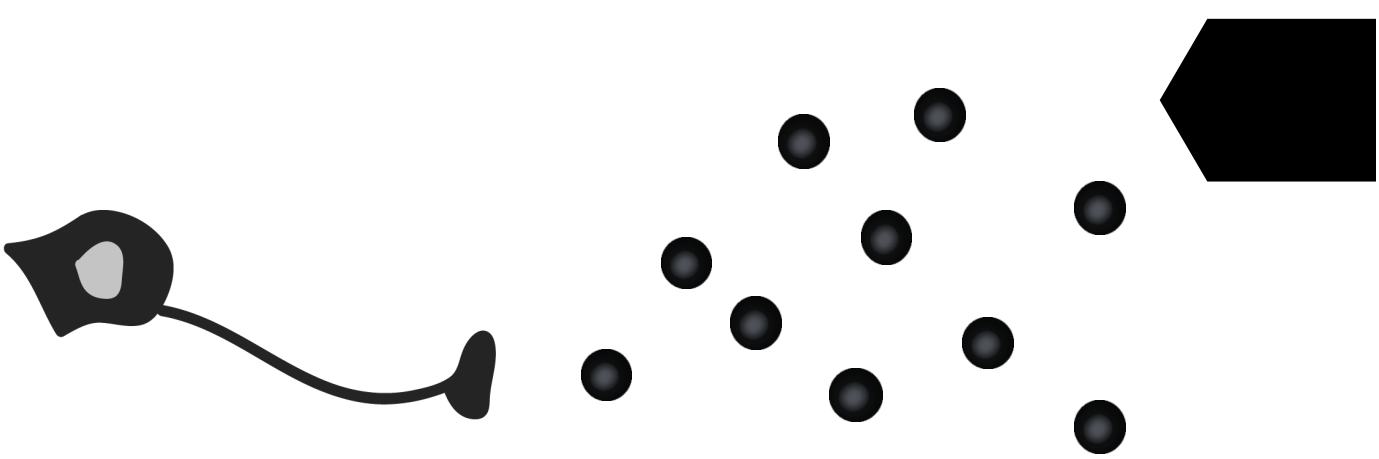
Annex



10.1 Review

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The gentle art of saying NO: how nitric oxide gets things done in the preoptic region of the brain

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Abstract

The chemical signaling molecule nitric oxide, which freely diffuses through aqueous and lipid environments, subserves an array of functions in the mammalian central nervous system, such as the regulation of synaptic plasticity, blood flow and neurohormone secretion. In this review, we consider the cellular and molecular mechanisms by which nitric oxide evokes short-term and long-term changes in neuronal activity, and highlight recent studies revealing that discrete populations of neurons synthesizing nitric oxide in the hypothalamus constitute integrative systems that support life by relaying metabolic and gonadal signals to the neuroendocrine brain, and thus gate the onset of puberty and adult fertility. We also discuss the putative involvement and therapeutic potential of nitric oxide in the pathophysiology of brain diseases for which hormonal imbalances during postnatal development could be risk factors.

Introduction

The role of nitric oxide (NO), originally known as endothelium relaxing factor (EDRF) as a signaling molecule in the brain was uncovered more than 30 years ago. Nevertheless, it still remains the subject of active research. Although the stimulation of soluble guanylate cyclase (sGC) activity by exogenous NO in the brain was described in 1977 (1, 2), it was not until 1988 that evidence was found that NO could be formed in the brain in response to the activation of the NMDA subtype of glutamate receptors (3). NO is produced intracellularly by NO synthase (NOS) through a reaction that converts L-arginine and oxygen into citrulline and NO. NOS is a complex protein with three isoforms that are homologous, although they differ in their structure, regulation and distribution: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Neuronal NOS was the first to be purified and cloned from the brain and is considered responsible for NO production in neurons (4). The spatial and temporal distribution of nNOS mRNA and protein in the rodent brain has been the subject of several studies. While it's presence is evident in most brain regions, labelling is more dense in the molecular and granule cell layer of the cerebellar cortex, the olfactory bulb, the striatum, the hypothalamus and the hippocampus. Expression levels are also significant in the superior and inferior colliculi, the superficial layers of the cortex and in some circumventricular organs such as the organum vasculosum of the lamina terminalis and the subfornical organ (5-10).

This review will concentrate on the physiological implications of nNOS activity and NO signaling in the regulation of brain function. In particular, we will focus our attention on the role of neuronal NO in the control of key physiological functions controlled by the hypothalamus, including survival of the species. We will also discuss how the deregulation of NO production might be involved in the pathophysiology of brain diseases for which hormonal imbalances during postnatal development could be a risk factor.

Neuronal NOS isoforms and posttranscriptional regulation

Genes playing key roles in biological processes undergo elaborate regulation of their mRNA expression. Mechanisms like alternative splicing create the allelic mRNA diversity essential for protein biodiversity, but also for tissue- or developmental-stage-specific protein function. Considering the importance of nNOS in various distinct biological functions as well as its

complex expression patterns, it comes as no surprise that mRNA diversity plays a role in the regulation of NO production throughout development and/or in specific cell types under physiological and pathological conditions. Multiple transcripts have been identified for the mouse nNOS gene, and most splicing events take place in the 5'-untranslated region (5'UTR), affecting the post-transcriptional regulation of the gene, or alter mRNA localization and stability. This diversity at the 5' terminus of nNOS mRNA transcripts does not affect the encoded protein sequence since these splicing patterns leave the open reading frame (ORF) unaltered, instead affecting the efficiency of transcription (11, 12). In humans, nNOS or NOS1 is located on chromosome 12, with its mouse ortholog being located on chromosome 5. The gene locus spans a region of 10,123 bps and consists of 29 exons, of which 28 are translated to generate the 150 kDa protein, nNOSα, the most commonly occuring isoform in the nervous system (Figure 1A). A unique characteristic of nNOSα is the existence of a 230-residue N-terminal region containing a PDZ domain, which allows nNOSα and the NMDA receptor to associate with postsynaptic density protein-95 (PSD-95) (13, 14) (Figure 1B). The influx of Ca^{2+} through open NMDA receptor channels and their subsequent binding to calmodulin leads to nNOS becoming catalytically active (3) (Figure 1B). The serine-1412 phosphorylation of the NMDA-receptor-tethered nNOS by the protein kinase Akt (also known as protein kinase B) (Figure 1B) then leads to a rapid enhancement of NOS activity, with dephosphorylation being dependent on AMPA receptors and L-type Ca^{2+} channels (15, 16). Additional, posttranslational regulation of nNOS activity can occur via Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), which co-resides with NMDA receptors and nNOS at synapses and phosphorylates nNOS at serine-847, inhibiting NO production by 50% by affecting Ca^{2+}/calmodulin binding (17, 18). However, this phosphorylation process appears to be slow in neurons (19), suggesting that CaMKII is less likely to be a dynamic regulator of nNOS activity than a long-term gain controller (reviewed in (20)).

Apart from nNOSα, there are other well-characterized splice variants, namely nNOSβ and γ (Figure 1A). nNOSβ and γ are translated beginning with different first exons, generating 136 kDa and 125 kDa proteins, respectively. Since they skip exon 2, which contains the PDZ domain essential for anchoring nNOS to the neuronal membranes and for responding to the Ca^{2+} influx generated by activated NMDA receptors (3), the variants are located in the

cytosolic fraction. nNOSγ lacks significant catalytic activity (21, 22). However, while nNOSα is suggested to account for 95% of all nNOS catalytic activity under physiological conditions, nNOSβ is also expressed in various regions of the brain, representing ~5% of the total NOS mRNA, and accounts for the majority of nNOS catalytic activity in areas like the cochlear nucleus (21, 23). These variants are not only believed to play key roles in a tissue-specific manner, but also to undergo differential regulation of their expression according to developmental stage. For example, on embryonic day 15, a substantial amount of brain nNOS protein migrates as a 136 kDa band corresponding to nNOSβ protein while, in the adult rat brain, nNOS migrates as a single band of 160 kDa (23). These isoforms could therefore be responsible for producing basal levels of NO necessary for neuronal development at various stages. However, alternative splicing events also seem to be initiated under pathophysiological conditions. Interestingly, nNOSβ and γ have been reported to be upregulated in the reactive astrocytes of amyotrophic lateral sclerosis patients while nNOSα labeling is absent from these cells (24).

NO activates the formation of cGMP upon stimulation of guanylate cyclase

As mentioned above, in neurons, Ca2+ influx through activated NMDA receptors is largely responsible for the stimulation of NOS (3), although other mechanisms for increasing cytoplasmic [Ca2+], such as voltage- gated Ca2+-channels or the release of Ca2+ from internal stores, can also be involved (25). This rise in intracellular Ca2+ results in its binding to calmodulin, creating a Ca2+-calmodulin complex that can directly activate the constitutive isoforms of NOS (26) and lead to NO production as long as Ca2+ levels are high. Once NO is released, it diffuses rapidly and stimulates the formation of cGMP by sGC (27). NO-activated GC consists of a heme group, which acts as the ligand binding site, and a transduction domain. Even though this heme group is of the type used in hemoglobin to bind O2, NO-activated GC exhibits a marked preference for NO, initiating NO signaling even in the presence of a >10000-fold excess of O2 (20)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008)(Garthwaite, 2008) . Upon the binding of NO to the heme group, a conformational change occurs due to the displacement of the histidine group, leading to the activation of the

enzyme (28), which can now convert GTP to cGMP. NO-dependent cGMP synthesis occurs mainly in neurons and astrocytes (29-31). cGMP can exert its functions through binding to three main targets: phosphodiesterases (PDEs), ion channels, i.e. cyclic-nucleotide gated channels (CNGs) and hyperpolarization-activated cyclic nucleotide-gated channels (HCN), and cGMP-dependent protein kinases (i.e PKGI and PKGII), which phosphorylate various substrates (Figure 2).

Lessons from nNOS-mutant mouse models

The first line of mice deficient in nNOS protein was generated in 1993; these mice lacked exon 2 of the nNOS gene, leading to non-obvious neuroanatomical and behavioral deficits (32). The targeted disruption of the gene leads, however, to a significant enlargement of the stomach (32), consistent with the role of NO in infantile pyloric stenosis (33). Interestingly, in homozygous mutant mice, a low amount of residual NOS is still present, probably accounting for the absence of severe deficits in this mouse model (32)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993)(Huang et al., 1993). Only a couple of years after the characterization of the above nNOS exon 2 KO model, another study identified the existence of alternatively spliced RNA isoforms (nNOSβ and nNOSγ) in mice with targeted deletions of exon 2. The nNOSβ variant was demonstrated to possess enough residual activity to account for the modest level of impairment seen in the above model (21). A more recent mouse model, this time bearing a deletion of exon 6, which encodes the catalytic heme-binding domain of all NOS variants, resulted in viable animals, but, in contrast to the exon 2 KO model, these displayed a strong reproductive phenotype (34) reminiscent of congenital hypogonadotropic hypogonadism in humans (35). Indeed, KN2 mice show pyloric stenosis, like the exon 2 KO model, but also display small gonads and infertility, with females showing altered hypothalamic content of gonadotropin releasing hormone (GnRH), the neuropeptide controlling the onset of puberty and fertility (36), and abnormal circulating levels of gonadotropins, and males presenting mating problems (34). Overall, the exon 6 KO model clearly demonstrated the necessity of neuronal NO signaling for the neuroendocrine and behavioral control of reproductive function.

The hypothalamus, a model system in which to study hormone- and activity-dependent brain plasticity

The hypothalamus is the single most important integrator of vegetative and endocrine regulation of the body. It controls several vital bodily processes including cardiovascular regulation, sleep, metabolism, stress, thermoregulation, water and electrolyte balance and reproduction. Within the hypothalamus, neuroendocrine neurons have proven to be fascinating model systems for studying hormone- and activity-dependent plasticity both during development (37-39) and in adulthood (40-47). Neuronal NO has been shown to be produced in several hypothalamic areas and nuclei, including the preoptic region, the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, the ventromedial nucleus of the hypothalamus and the ventral premammillary nucleus, where it regulates, among other functions, temperature, fluid balance, stress, glucose homeostasis and reproduction (48-54). Because hypogonadotropic hypogonadism is the most obvious phenotype of mice lacking nNOS catalytic activity, hypothalamic GnRH neurons, which are essential for sexual maturation and adult fertility, and the afferent neural network that modulates their activity (see for review (36, 55)) appear to be an intriguing model system in which to decipher the ways in which NO operates at the cellular and network level to coordinate a key physiological function of the brain.

Neuronal NO neurons as an integral part of the hypothalamic GnRH neuronal network controlling ovarian cyclicity

In rodents, GnRH neurons are diffusely distributed and are particularly abundant in the preoptic region of the ventral forebrain. Their axons target the pericapillary space of the median eminence, where they release their neurohormone into the fenestrated blood vessels of the pituitary portal system for delivery to the anterior pituitary. In the pituitary, GnRH elicits the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate gametogenesis and gonadal hormone secretion and thus support reproductive function (Figure 3). As the final common target in the central control of reproduction, the activity of GnRH neurons is regulated by a complex array of excitatory and inhibitory transsynaptic and non-synaptic inputs (36, 55, 56) and is subject to the direct and

indirect influence of a plethora of internal and external signals (57-60). While the neuronal network synaptically connected to GnRH neurons has been much explored using advanced genetic strategies in mice (61-63), atypical neuronal and non-neuronal inputs such as those postulated to be involved in volume transmission are more difficult to comprehend (64, 65).

NO has long been recognized as a player in the central hormonal regulation of ovulation. In fact, back in the early '90s, there was a burst of research activity from different groups focusing on the role of NO in the regulation of GnRH secretion both *in vitro* and *in vivo*, and suggesting NO as a key molecule in the preovulatory GnRH/LH surge (66-68)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994)(Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994). *In vitro*, the application of an NO donor was shown to stimulate GnRH release via the sGC-cGMP pathway in a dose-dependent manner, while *in vivo*, the intraventricular injection of a NOS inhibitor blunted pulsatile LH release, providing strong evidence that NO was essential for the generation of LH release (67, 68). Specifically, in the preoptic region, the chronic inhibition of NOS activity was shown to disrupt rat estrous cyclicity (69). All the above results, which present evidence for the marked disruption of LH pulsatile release upon NO inhibition, together with studies showing that nNOS mutations cause infertility in mice (34), have laid the groundwork for the notion that NOS-containing neurons interact with the GnRH system and are thus involved in the central control of reproductive function.

NO-synthesizing neurons anatomically and functionally interact with GnRH neuronal cell bodies in the preoptic region

The vast majority of hypothalamic NOS-expressing neuronal cells have been identified in the rostral part of the preoptic area, in the median preoptic nucleus (MePO) and in the organum vasculosum of the lamina terminalis (OVLT). nNOS-containing cells in the preoptic region are found in the vicinity of GnRH-containing perikarya, mainly surrounding GnRH cells which do

not themselves express nNOS in the MePO (70, 71), and around GnRH dendrites in the OVLT (72, 73). The fact that nNOS neurons surround GnRH neuronal cell bodies and dendrites creates an anatomical relationship between the two neuronal populations, which is complemented by the capacity of neuronal NO to modulate GnRH electrical activity (for review see (52)). Specifically, patch-clamp recordings from GnRH-GFP mouse brain slices reveal that NO can directly tune GnRH neuronal excitability (70); the perfusion of hypothalamic slices with the NO precursor L-arginine results in the nNOS-dependent postsynaptic inhibition of GnRH neuron firing. This reduction in the spontaneous discharge of GnRH cells has been shown to require the sGC-cGMP signaling pathway and to involve potassium conductance (70). Although this may vary across physiological conditions, the overall impact of NO on GnRH neurons is suggested to be beneficial, by facilitating synchronous activity among GnRH neurons that would otherwise act independently, and thus maximizing the release of this neurohormone into the blood as detailed further on.

Estrogen regulation of NO signaling in hypothalamic neurons

Estrogens have profound effects on brain structure and physiology (39, 74-79). The manner in which sex steroids, and particularly estrogen, influence the reproductive circuitry has been the subject of many studies (56, 63, 80). It is widely known that natural fluctuations of estrogen levels across the estrous cycle lead to subsequent cyclic changes in protein levels and protein-protein interactions that are important for the physiological processes taking place in the hypothalamic-pituitary axis (81-83). nNOS neurons located in the preoptic region of the hypothalamus express estrogen receptor α (ERα) (84, 85), which is essential for the positive feedback by estrogen that elicits the GnRH/LH surge triggering ovulation (63) (Figure 3).

Both amperometric measurements and microscopic visualization of NOS catalytic activity using immunofluorescence for L-citrulline (which is formed stoichiometrically with NO) reveal that, concomitantly with the preovulatory increase in plasma estrogen levels, NO levels in the preoptic region are significantly increased in proestrus when compared to diestrus (the stage of the estrous cycle when estrogen levels are low) (69). Proteomic studies show that while nNOS expression does not vary across the estrous cycle, its activity is markedly modulated by its alternate coupling and uncoupling with NMDAR depending on the circulating levels of

estrogens (69, 86, 87). As mentioned before, the physical interaction of nNOS with NMDAR at the plasma membrane promotes the coupling of Ca2+ influx to nNOS activity; the Ca2+ increase through NMDAR then results in its binding to calmodulin, creating a Ca2+-calmodulin complex that can directly activate nNOS, inducing the production of NO (3, 26). This differential association of nNOS with NMDAR during the estrous cycle involves the scaffolding protein PSD-95 (86, 88) and requires estrogen receptor activity (86) (Figure 4). Importantly, the fact that selective NMDAR blockers have been shown to inhibit estradiol-induced NO release demonstrates that estrogen actually promotes the coupling of glutamatergic flux and NO production in preoptic neurons (86). The evidence that NO-producing neurons in the hypothalamus could be targets of glutamate, one of the main activators of the central reproductive axis (89, 90), comes from neuroanatomical studies showing that virtually all-preoptic nNOS neurons express NMDA receptors (88, 91). Besides, most NMDA receptor-expressing neurons of the preoptic region have also been shown to contain ERα (92).

When nNOS is physically associated with NMDAR at the plasma membrane, the enzyme is susceptible to an activity-dependent Akt-mediated phosphorylation at Ser1412, which increases its sensitivity to Ca^{2+}-calmodulin and thus its activity (16). Accordingly, the phosphorylation of nNOS at Ser1412 in the preoptic region is maximal on the afternoon of proestrus (when circulating estrogen levels are at their highest), and this phosphorylation-activated nNOS isoform has been shown to physically interact with the PSD-95/NMDAR complex at the plasma membrane (87) (Figure 4).

Altogether these findings strongly suggest that estrogens directly act on nNOS neurons of the preoptic region, coupling NO production to glutamate flux, which is well known for its positive feedback effect on GnRH secretion by the hypothalamus (89, 93, 94). In addition, recent findings demonstrating that glutamatergic neurons also play a prominent role in mediating the estradiol negative feedback loop (94) together with data showing that endogenous NO release imposes a tonic brake on LH secretion during the estrogen-mediated negative feedback phase (72) suggest that this glutamate/NO coupling could in fact be at work at all stages of the estrous cycle and be used by the neuroendocrine brain to convey both inhibitory and excitatory signaling by estrogens to the reproductive axis. The resulting production of NO may then act on GnRH neuronal cell bodies to synchronize their activity and adjust their firing behavior in a

meaningful manner to enable the surge of GnRH during the estrogen positive feedback phase (72, 94) and to participate in restraining the activity of the GnRH system during the negative feedback phase (72, 94). The switch between the activatory and the inhibitory influence of NO on the GnRH neuronal network could likely be linked to the level of activity of nNOS, as elaborated further down (Figure 5).

The emerging role of NO signaling in the action of kisspeptin neurons

GnRH neurons are undeniably an obligatory component of the central reproductive axis (95-97). During the last decade, however, kisspeptin has been identified as playing an important role as an upstream regulator of the GnRH system (98, 99). In the rodent hypothalamus, kisspeptin neurons are distributed in the arcuate nucleus of the hypothalamus (ARH) in the tuberal region, and in the anteroventral periventricular nucleus (AVPV) in the preoptic region. ARH kisspeptin neurons that project towards the median eminence contact GnRH neuronal processes found there (100). Even though these ARH kisspeptin neurons also send projections to the preoptic region (101, 102), GnRH cell bodies in the POA appear to mainly receive afferents from AVPV kisspeptin neurons (103), which have been shown to be electrically active (104, 105). While AVPV kisspeptin neurons are considered to be key components of the surge mechanism, ARH kisspeptin neurons are the ones thought to be involved in the control of pulsatile GnRH release (106). In fact, recent *in vivo* and *in vitro* optogenetic studies have shown that the selective activation of ARH kisspeptin neurons promotes GnRH neuronal activity (105, 107) at stimulation frequencies similar to those required to elicit LH release (108). Mutant studies have revealed the critical role of kisspeptin signaling in reproduction (109). Humans and mice bearing a mutation in the kisspeptin gene or its receptor (Gpr54/Kiss1R), do not undergo puberty, are infertile and exhibit markedly reduced gonadal size due to low levels of LH and FSH (110-113). Moreover, kisspeptin and kisspeptin analogues have been shown to be potent post-synaptic activators of GnRH neuronal activity (114, 115). Advanced genetic approaches have shown that the selective reintroduction of a functional Gpr54 into GnRH neurons in a *Gpr54*-null background is sufficient to rescue puberty onset and reproductive capacity in mice (116). Although unequivocally important, these direct effects of kisspeptin on GnRH neurons are, according to recent studies, insufficient to fully maintain the functionality of

the gonadotropic axis throughout the reproductive life cycle (117). Detailed maps of Gpr54-expressing cells, using genetically modified mice that have *LacZ* knocked into the *Gpr54* locus, show Gpr54 promoter activity in the GnRH neuronal population alongside expression in some non-GnRH cells (118). Interestingly, some of these Gpr54-expressing cells are found in hypothalamic regions such as the median preoptic nucleus (MePO) and the organum vasculosum of the lamina terminalis (OVLT) (72) in which GnRH and nNOS neurons are co-distributed, and where GnRH neurons are known to receive kisspeptin fibers likely arising from the AVPV (103). X-gal histochemistry reveals that, in addition to GnRH neurons themselves, discrete populations of nNOS neurons in the MePO/OVLT, abundantly surrounded by kisspeptin fibers, also have an active Gpr54 promoter ,which is not the case for the nNOS cell population of the medial septum, where the density of kisspeptin fibers is much lower (72). The notion that nNOS neurons express functional Gpr54 is further supported by experiments showing that exogenous kisspeptin treatment promotes Gpr54-dependent Ser-1412 phosphorylation of nNOS in diestrous mice at levels comparable to those seen in proestrus and in ovariectomized mice treated with estradiol. Intriguingly, a null mutation of *Gpr54* prevents estradiol effects on nNOS phosphorylation in ovariectomized mice, suggesting that estrogens require kisspeptin-Gpr54 signaling to influence nNOS activity during their positive feedback phase (Figure 4). Thus, the increase in phosphorylation-activated nNOS during proestrous (72, 87) may be an integral part of the kisspeptin-dependent preovulatory activation of GnRH neurons (72). NO-producing as well as other non-GnRH kisspeptin-responsive cells (119) could potentially be used by kisspeptin neurons to facilitate synchronous activity among GnRH neurons and thus maximize the release of this hormone into the blood. In line with this hypothesis, *Gpr54*-null mice in which Gpr54 expression has been selectively restored in GnRH neurons exhibit blunted LH responses to key central activators of the gonadal axis, including both kisspeptin itself (117) and NMDA, which is known to elicit LH release in *Gpr54*- and *Kiss1*-null mice by acting, at least in part, on nNOS neurons when injected into the cerebral ventricles (90).

A role for preoptic NO in sexual maturation and the onset of fertility

Sexual maturation, puberty (the period when complete reproductive capacity is attained) and subsequent adult fertility are regulated by the hypothalamic-pituitary-gonadal axis (HPG). The activity of the HPG is itself orchestrated in the brain by neuroendocrine GnRH neurons, which are subjected to complex maturational events affecting their biosynthetic capacity, neurosecretory pattern and morphology (36). This array of events, which is likely linked to the integration of postmigratory GnRH neurons into the neural network responsible for relaying bodily information to these core neurons, occurs at different phases of postnatal development (36). The first activation of the axis occurs shortly after birth during the infantile period (a phenomenon referred to as mini-puberty) (36, 120), and results in the release of an FSH surge that stimulates the proliferation of immature Sertoli cells and spermatogonia in the testes of the male, and the growth of the first pool of ovarian follicles, destined to ovulate at puberty, in females. After weaning, the brain-driven maturation of the gonads leads to increasing production of gonadal steroids (e.g. estrogens and testosterone), which initiate a feedback loop that acts at different levels of the HPG axis, including the brain, to adapt its activity to both developmental needs and physiological demand, and hence lead the way to the timely onset of puberty (36). Puberty in males is characterized by the first occurrence of motile sperm in the epididymis, conferring on them the capacity for reproduction. Puberty in females is characterized by the first GnRH/LH surge leading to ovulation and the establishment of estrous cyclicity, the regularity of which conditions fertility during adulthood.

A role for NO in the control of GnRH expression during postnatal development?

Recent findings show that hypothalamic NO could be involved in regulating the expression of the GnRH gene, which has been shown to increase during postnatal development (36), and thus be part of the very mechanism regulating the clock-like precision of pubertal activation (121). During the second week of life, when nNOS activity increases in the preoptic region at minipuberty (121), coinciding with the arrival of kisspeptinergic and other fibers from the ARH at the preoptic region (101, 122), NO interacts with the transcription factor CAAT/enhancer binding protein β (C/EBPβ), encoded by the Cebpb gene, to repress the activity of the GnRH promoter (123). A selective switch in miRNA expression patterns in GnRH neurons appears to invert the balance between repressive and inductive signals controlling the expression of the

GnRH promoter (121) by triggering decreased C/EBPβ expression while sustaining Gpr54 expression (121). In particular, the increased expression of miR155 (which represses *Cebpb*) at minipuberty has been identified as one of the linchpins of this process, permitting the sustained increase of GnRH required for subsequent sexual maturation (121). This miRNA-mediated flipping of the switch in the control of GnRH promoter activity at minipuberty intriguingly coincides with a dramatic change in the pattern of GnRH release (124). It is thus tempting to speculate that this kisspeptin-nNOS-GnRH neuronal microcircuit, along with the miRNA-gene network in GnRH neurons, could play an important role in the initiation of both rhythmic GnRH gene transcription, and pulsatile GnRH secretion (125) at puberty, in response to developmental and bodily cues received during the infantile period (36).

nNOS neurons in the preoptic region are critically involved in the metabolic regulation of puberty onset

Nutritional status has long been recognized as a determining factor for the onset of puberty, as well as the maintenance of reproductive capacity. This notion is based on the fact that many aspects of reproductive physiology are energetically demanding (e.g. sexual maturation, pregnancy, lactation etc.), so a minimum store of energy is necessary to promote the normal growth and function of reproductive organs, and, of course, fertility (59, 126, 127). On the other hand, excess energy stores could have deleterious effects on reproductive physiology; precocious puberty, abnormal menstrual cycles, polycystic ovary syndrome and infertility are often associated with metabolic disorders, such as obesity, that can result from overnutrition (127, 128).

Leptin, a peptide hormone mainly produced by adipocytes, is a key link between metabolic homeostasis and reproductive competence (129, 130). Leptin deficiency, caused by the *Lep*$^{ob/ob}$ loss-of-function mutation in both humans and rodents, results in obesity, a failure to complete puberty, and consequently, infertility (131, 132). Chronic leptin administration to *Lep*$^{ob/ob}$ individuals induces the completion of sexual maturation and the restoration of fertility, indicating the pivotal role played by leptin (132, 133). There is compelling evidence to suggest that leptin regulates GnRH neurons via cells afferent to these neurons (134, 135) and/or those that interact morphologically with them (136). Seminal works have shown that states of

negative energy balance, in which leptin levels are low (e.g., during fasting or when individuals subjected to strenuous exercise), result in decreased LH levels and the suppression of the estrous cycle, while leptin treatment restores fertility (137-139). The recent experiments showing that the neuroendocrine reproductive axis was unable to sense the increase in leptin levels when NOS activity was pharmacologically blocked prior to leptin treatment in fasting mice (140), suggest that nNOS neurons could be one of the key neuronal populations relaying changes in leptin levels to GnRH neurons in the hypothalamus.

The mapping of leptin-responsive cells in the hypothalamus has revealed that several leptin-receptor (LepR)-expressing populations are NO-synthesizing neurons (54, 141), and that the activation of nNOS by its phosphorylation can be regulated by circulating leptin in the preoptic region (54, 140), which contains neurons lying outside the blood-brain barrier at the level of the OVLT/MePO (142, 143). LepR expression in nNOS neurons has been shown to be crucial for the integration of metabolic signals (144), accumulating evidence shows that the preoptic region plays an active role in the control of energy homeostasis (145, 146). Preoptic nNOS neurons, in addition to regulating estrogen-dependent neural inputs such as kisspeptin signals, are thus well poised to transmit information regarding peripheral energy stores to GnRH neurons by sensing leptin (Figure 4).

In line with the latter idea, leptin-promoted nNOS activation in the OVLT/MePO is seen to be functionally associated with a rise in peripheral LH from nadir levels (140). The genetic deletion or pharmacological inhibition of nNOS prevents the leptin-induced LH release, which appears independent of kisspeptin/Gpr54 signaling (140). Obese leptin-deficient mice bearing a null mutation in the *nNos* gene or exposed to continuous nNOS inhibition in the preoptic region fail to undergo sexual maturation in response to chronic leptin administration even though they recover a lean phenotype (140). Similarly, Finally, the selective deletion of LepR expression within the preoptic region in adult mice alters basal GnRH/LH secretion without affecting body weight (140). Together these results convincingly suggest that NO-signaling facilitates leptin action on reproduction and establishes the preoptic region as a novel site for the integration of leptin signaling into the GnRH neural network.

Although GABA neurons have also been shown to play a role in relaying leptin signaling to the GnRH system (147, 148), most leptin-sensitive neurons of the preoptic region, likely

including nNOS neurons themselves, are glutamatergic (146). These data are reminiscent of what has been found in the ventral premammillary nucleus, another key site for leptin action in the onset of puberty and one which also contains numerous leptin-sensitive nNOS neurons (54, 141), where LepR neurons are likewise thought to be glutamatergic (149). Further work will be required to tease apart the relative importance of each of these different systems, which all appear to at least partially relay metabolic information to the neuroendocrine system controlling reproduction (150).

Does neuronal NO set the rhythm?

The low molecular weight of NO (30 g mol^{-1}) confers on this molecule a high aqueous diffusion coefficient (3.3 μm^2 ms^{-1}) and a correspondingly rapid rate of aqueous diffusion (about 2.5 µm in 1 ms) (27). In addition, its high lipid solubility (151) allows its rapid permeation of biological membranes in which it concentrates about 4-fold and in which its diffusion rate is 10-fold lower than in an aqueous milieu (152). NO would thus be capable of crossing the 3 nm hydrophobic membrane in about 3 ns. These physicochemical properties imply that NO diffuses uniformly in all directions, away from its site of synthesis, with the potential to influence the activity of cells located within a given area of brain tissue, irrespective of synaptic connectivity. NO is thus the prototypical candidate molecule for volume transmission (153), with its biological effects being regulated by i) the catalytic properties of the nNOS enzyme, ii) its rate of inactivation and iii) the distance of NO targets from its source(s) (27). Although it was initially thought that NO could be diffusing at active concentrations within a sphere with a diameter of 200 µm, thereby influencing 2 millions synapses (154), the development of advanced techniques using cells expressing FRET-based biosensors to accurately measure NO concentrations in living brain slices has shown that NO produced at a given synapse is only likely to function very locally, specifically within submicrometer distances (155, 156). Besides, calculations based on the pharmacokinetics of sGC upon its activation by NO reveal that a single synaptic NO pulse results in a cGMP response within a concentration range that is probably too low to have any biological activity (27). Indeed, it has been suggested that trains of NO pulses of about 10-20 Hz (a stimulation frequency similar to that used to promote GnRH neuronal activity/secretion by kisspeptin neurons using an optogenetic approach (105, 107)) are needed to elicit the

accumulation of cGMP to an active concentration range at synapses (27). Volume transmission could, however, occur in some parts of the central nervous system where nNOS neuron cell bodies are densely packed, and the potential cumulative activation of these neighboring nNOS cells and subsequent release of NO could result in a biologically active concentration of cGMP. This could be the case in the OVLT/MePO area of the preoptic region, where a large population of nNOS-expressing neurons resides among some GnRH cell bodies, and where GnRH neurons extend their dendrites and kisspeptin neurons their axons (72, 73). The proposed mechanism here is that when nNOS activity is coordinated across the population of neurons by homeostatic blood-borne signals (e.g. estrogens or leptin) or transsynaptic inputs (e.g. kisspeptin or glutamate) (69, 72, 87, 140), the NO concentration between these neurons builds up to levels capable of stimulating neighboring GnRH neurons and coordinating their activity so as to promote the release of GnRH peptide into the pituitary portal blood circulation, and thus elicit LH release from the pituitary gland (Figure 5). Realistic modeling of the size and distribution of nNOS neurons in the OVLT/MePO fully supports the proposed mechanism, provided that two conditions are met: firstly, that the number of nNOS neurons that are active in the resting state is half or fewer than those that are active in the presence of the stimulus, and, secondly, that the rate of inactivation of NO is high enough to permit GnRH neurons to discriminate between sparsely activated and more fully activated populations of nNOS neurons (140). The computed NO inactivation rate allowing this mode of signaling is equivalent to a half-life of 5 ms, a value that corresponds well with the rate estimated for the rat brain using an experimental approach (157).

Theoretical studies show that changes in nNOS activity and a diffuse type of NO neurotransmission can substantially influence the way in which networks process information. In the absence of structural changes, diffusive homeostasis maintains substantial heterogeneity in the activity levels of individual neurons and confers networks with the capacity to represent input heterogeneity, and to respond linearly over a broader range of inputs than networks undergoing non-diffusive homeostasis (158). In contrast, if NO release influences the structure of synaptic connectivity and thus changes synaptic weight, neurons of the network would be more likely to be coactivated by similar stimuli. This would provide an opening for the interaction between functional organization driven by common sensory features, and functional

organization driven by the underlying spatial distribution of neurons within the network (159). A future challenge would be to experimentally test these possibilities and investigate whether nNOS neurons of the periventricular preoptic region could be part of the central pattern generator network that drives pulsatile GnRH release (39, 160, 161), or whether they act as a filter to integrate information from the periphery with input from the central pattern generator (72, 140).

Clinical relevance

The recognition that hypothalamic NO plays a role in the postnatal development of the neuroendocrine axis controlling sexual maturation and the onset of puberty (121) has important clinical implications. Interactions between hypothalamic neuroendocrine systems and peripheral hormones have been increasingly acknowledged to play a fundamental role in postnatal brain development, i.e. the hormonal programming of brain development (37, 77, 162-164), the impairment of which may lie at the origin of major neurological and psychiatric disorders. Age at puberty falls among the known risk factors for several noncommunicable diseases of major importance to public health, such as cardiovascular disease, obesity, type 2 diabetes, cancer and neurodegenerative and mental disorders (165-167). In particular, early puberty is associated with the heightened prevalence and intensity of depressive symptoms, adjustment problems, anxiety, and psychopathological disorders (e.g. eating disorders) among adolescent females (167, 168). In addition, the possibility that puberty stimulates or unmasks pathological brain development in individuals with perinatally acquired brain abnormalities underlies the neurodevelopmental hypothesis of some psychotic disorders such as schizophrenia, the incidence of which markedly increases during the post-pubertal years (169). Indeed, association studies have identified nNOS as a genetic risk factor for some of these disorders (170, 171). Interestingly, a recent study treating 20 schizophrenic patients (age 19-40) with the NO donor sodium nitroprusside administered intravenously revealed a rapid (within 4 hours) improvement of symptoms that persisted for 4 weeks after infusion (172), reinforcing the view that the glutamate-NO-cGMP network plays a important role in the pathophysiology of this mental illness (173). Considering the key position of the GnRH neuronal network at the crossroads between reproductive and metabolic function, it would not

be surprising if the abnormal timing or magnitude of NO signaling were to play an important role in a large number of the disorders mentioned above.

In addition to putative endogenous fluctuations in NO production, whether physiological or pathological, the use of exogenous NO in the clinical setting to treat several noncommunicable diseases, including those affecting the brain (174, 175), is worth noting. In preterm infants, inhaled NO is routinely used to treat hypoxemic respiratory failure and pulmonary hypertension (176). Preclinical studies show that inhaled NO significantly increases NO concentrations in the brain (177) and that, while it may exert both beneficial and detrimental effects depending of the dose administered and the time period of exposure (174), it could have neuroprotective effects in individuals with combined lung and brain injury (178). However, the neurological follow-up, especially in the medium-to-long term, of neurodevelopmentally vulnerable preterm infants exposed to inhaled NO is rare and poorly reported. Equally unexplored are the effects of inhaled NO treatment on the awakening of the GnRH neuroendocrine axis at minipuberty - which occurs around 1 week of age and lasts for around 3 months, resulting in transient gonadal maturation (120) - and its eventual consequences on pubertal development. Intriguingly, the amplitude of minipuberty varies tremendously depending on gestational age at birth in both boys (179) and girls (180): urinary FSH (the reflection of the GnRH-mediated activation of the pituitary) is seen to increase up to 300-fold in preterm female infants (24-34 gestational weeks) and to peak for an extended period of time in preterm male infants (even if urinary FSH levels in premature girls are remarkably higher than in premature boys) (179, 180), who are also at high risk of developing brain injury and neurodevelopmental disorders (181) as well as reproductive perturbations (182), when compared to full-term infants (37-42 weeks of gestation). This extremely high FSH surge in premature girls is associated with a marked delay in follicular development when compared to full-term girls at birth (180). This phenomenon may be explained by insufficient negative feedback inhibition of GnRH/FSH secretion by ovarian hormones, which are known to play key organizational roles in the infantile brain (37, 77, 164). In boys, urinary FSH levels are positively correlated to testicular growth and preterm boys show significantly higher androgen levels than full-term boys (179). Through programming, hyperandrogenism in infancy could influence the characteristics of growth, body composition, fat distribution,

blood pressure, and lipid and glucose metabolism, thereby contributing to risk factors of many chronic diseases. Inhaled NO, by inhibiting GnRH gene expression during this critical period of brain development (121), could potentially correct the aforementioned central neurohormone imbalance and its pathological consequences in preterm infants by enabling the proper unfolding of the sequence of events leading to normal postnatal brain maturation.

Concluding remarks

NO is a versatile signaling molecule that plays key roles in the development and survival of mammalian species by endowing brain neural networks with the ability to make continual adjustments to function in response to moment-to-moment changes in physiological inputs throughout life. NO can modulate neuronal activity through various modes of action: it can act as an intracellular messenger (183), operate at the level of the individual synapse (184, 185), or subserve a more diffuse type of signal transmission in which NO from multiple sources in a given tissue volume accumulates, generating a local cloud of NO capable of acting on target cells, irrespective of their anatomical connectivity (140). This diffuse type of signaling has actually been highlighted as a plausible mechanism for establishing synaptic connectivity during development (186, 187) and controlling the synchronized unfolding of gene expression in select populations of neurons (121). These modes of action could play an essential role not only in puberty initiation (140), a critical time period for brain development during which many mental disorders become visible (188), but also later on in the establishment and maintenance of fertility (34, 72, 140). NO has in fact been hailed as a putative treatment for schizophrenia in adults (172), and is commonly used in preterm infants to prevent neurological and other damage to their immature and highly vulnerable organs (174, 175). Puberty could also be an interesting therapeutic window for the use of inhaled NO to treat mental health problems with an onset during adolescence, and prevent them from persisting through adulthood (188).

With regard to the specific question of puberty and fertility, determining whether hypothalamic NO could contribute to the molecular basis of congenital hypogonadotropic hypogonadism in humans (35), and whether nNOS could be a novel biomarker of this complex genetic disease in which gene-environment interactions are increasingly recognized to play an important role

(189, 190), are just two of the challenges to be faced in the future. To advance our mechanistic understanding of how preoptic nNOS neurons, which promptly, effectively, and reproducibly sense fluctuations in homeostatic signals throughout life (69, 72, 87, 140), coordinate GnRH neuronal network activity during the reproductive cycle, technological advances are necessary in addition to theoretical insights. For example, a combination of advanced fluorescent probes that act as biosensors of cGMP at physiological levels to study NO propagation in living hypothalamic brain slices (156), and electrophysiological recordings upon metabolic and reproductive challenges, will allow us to seek experimental corroboration of the mathematical model that is currently being built (Figure 5) (27, 140).

To summarize, we have certainly come a long way in deciphering the role of this obscure molecule in the neuroendocrine brain. However, the many questions that still remain unanswered merely provide new and exciting avenues for future research.

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Figures

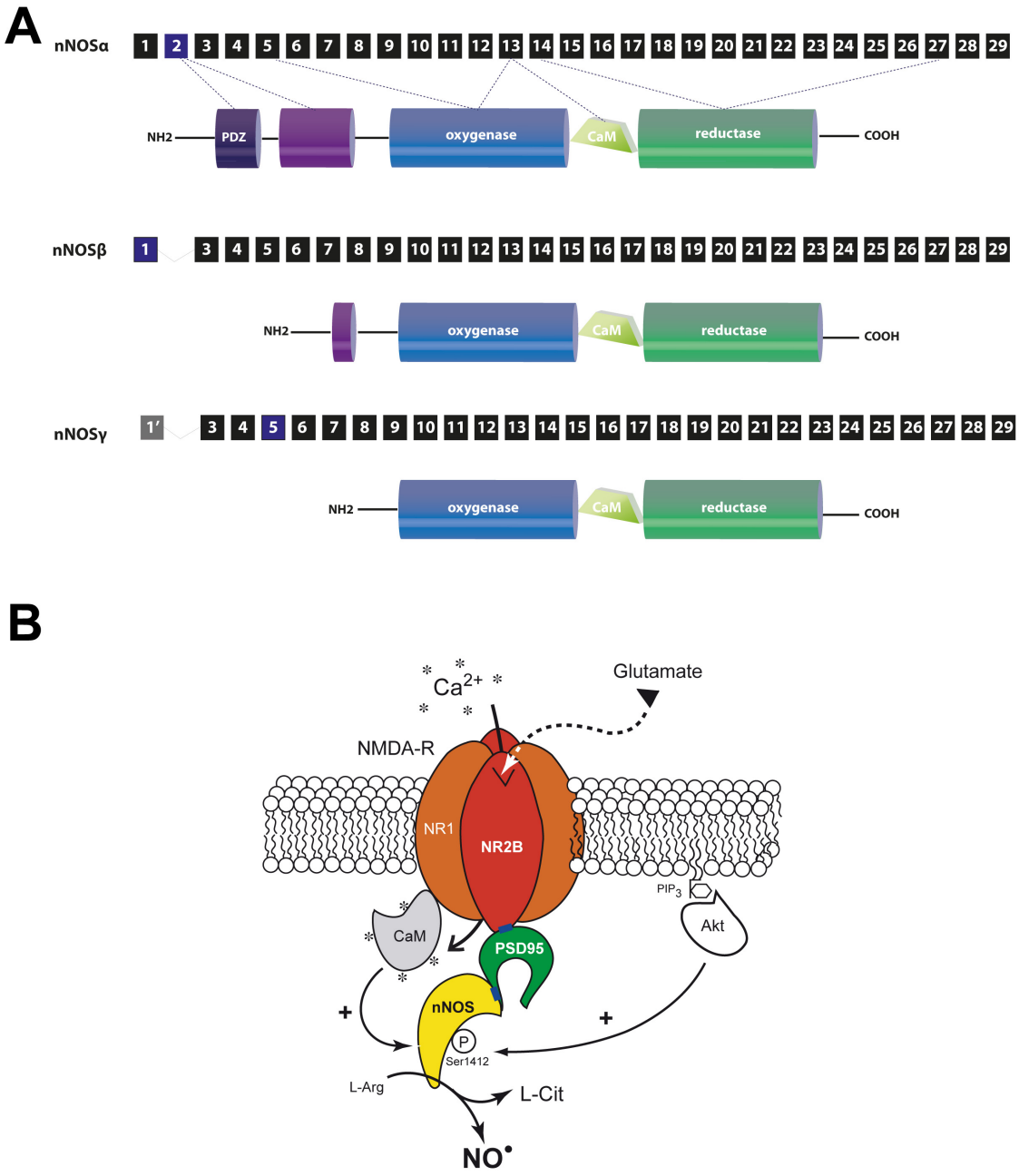

Figure 1. Schematic figures showing (A) the nNOS α, β and γ splicing variants, and (B) the main activatory mechanism of the nNOS α variant. nNOS α mRNA contains 29 exons and its translation results in a protein product of 160 kDa. The nNOS β and γ variants initiate their translation through different first exons skipping exon 2, which contains the PDZ domain. All the three nNOS isoforms (alpha, beta, gamma) contain the oxygenase and the reductase domains (CysJ, flavodoxinJ, ferrodoxin), as well as the calmodulin and tetrahydrobiopterin domains. On the contrary, both nNOS beta and gamma isoforms in addition to the lack of the PDZ domain, they are also missing the regions permitting interaction with NOSIP and PIN

proteins. The alternative splicing events finally generate a 136 kDa and 125 kDa protein respectively. (B) The translocation of nNOS α from the cytosol to the membrane, its physical interaction with the NR2B subunit of the NMDA receptors via PDZ domains (blue rectangles) involves the post-synaptic density-95 (PSD-95) scaffolding protein and the assembly of a ternary complex nNOS/PSD95/NMDAR. Binding of glutamate (glu) to the NMDA receptor allows Ca^{+2} entry into the neuron. Ca^{+2} influx activates nNOS α through calmodulin (CaM) binding leading to the production of NO, which is formed enzymatically from L-arginine (L-Arg) in equimolar amounts with L-citrulline (L-Cit). In parallel, membrane-tethered nNOS is also subjected to posttranscriptional modifications (such as phosphorylation via Akt) that modulates its catalytic activity.

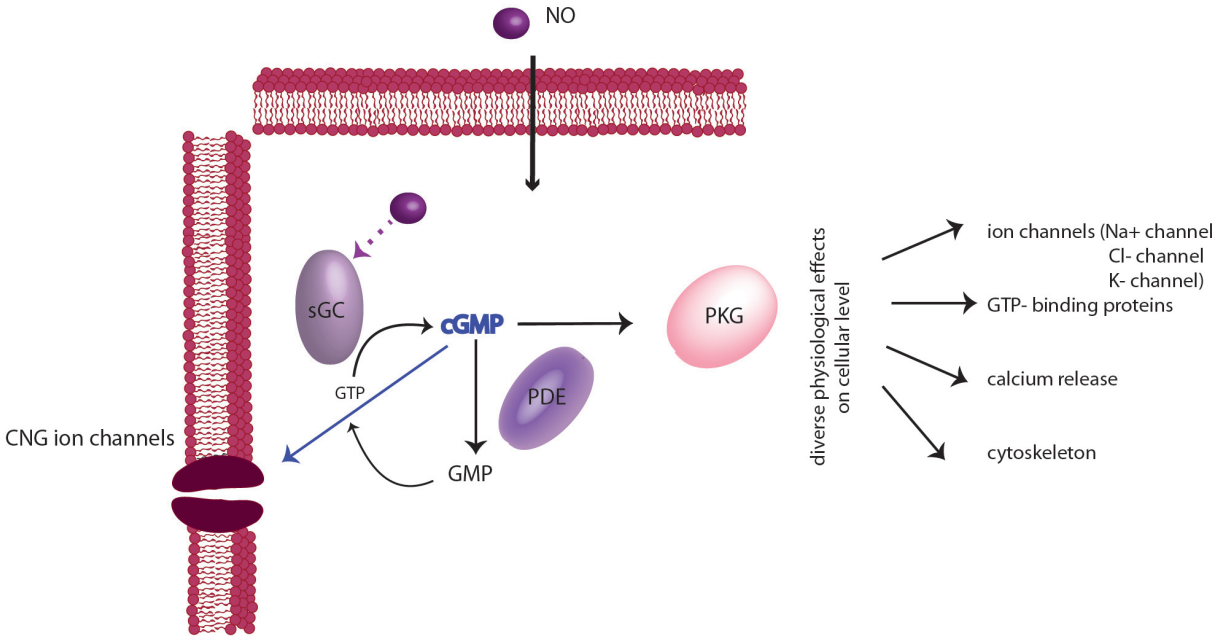

Figure 2. Schematic representation of the NO/cGMP signaling pathway. NO neurotransmitter is highly soluble and membrane permeable. Upon binding to NO-sensitive guanylyl cyclase, nitric oxide induces a conformational change resulting in the activation of the enzyme and the subsequent conversion of GTP to cGMP. The newly produced cGMP can interact with various intracellular proteins, including the cGMP- binding PDEs, cGMP-gated cation channel (CNG), and the protein kinase G (PKG), triggering thus the phosphorylation of many different substrates. The NO/cGMP pathway is therefore implicated in multiple distinct physiological processes such as cytoskeletal organization, Ca^{+2} release from intracellular stores, differentiation/proliferation of vascular smooth muscle etc.

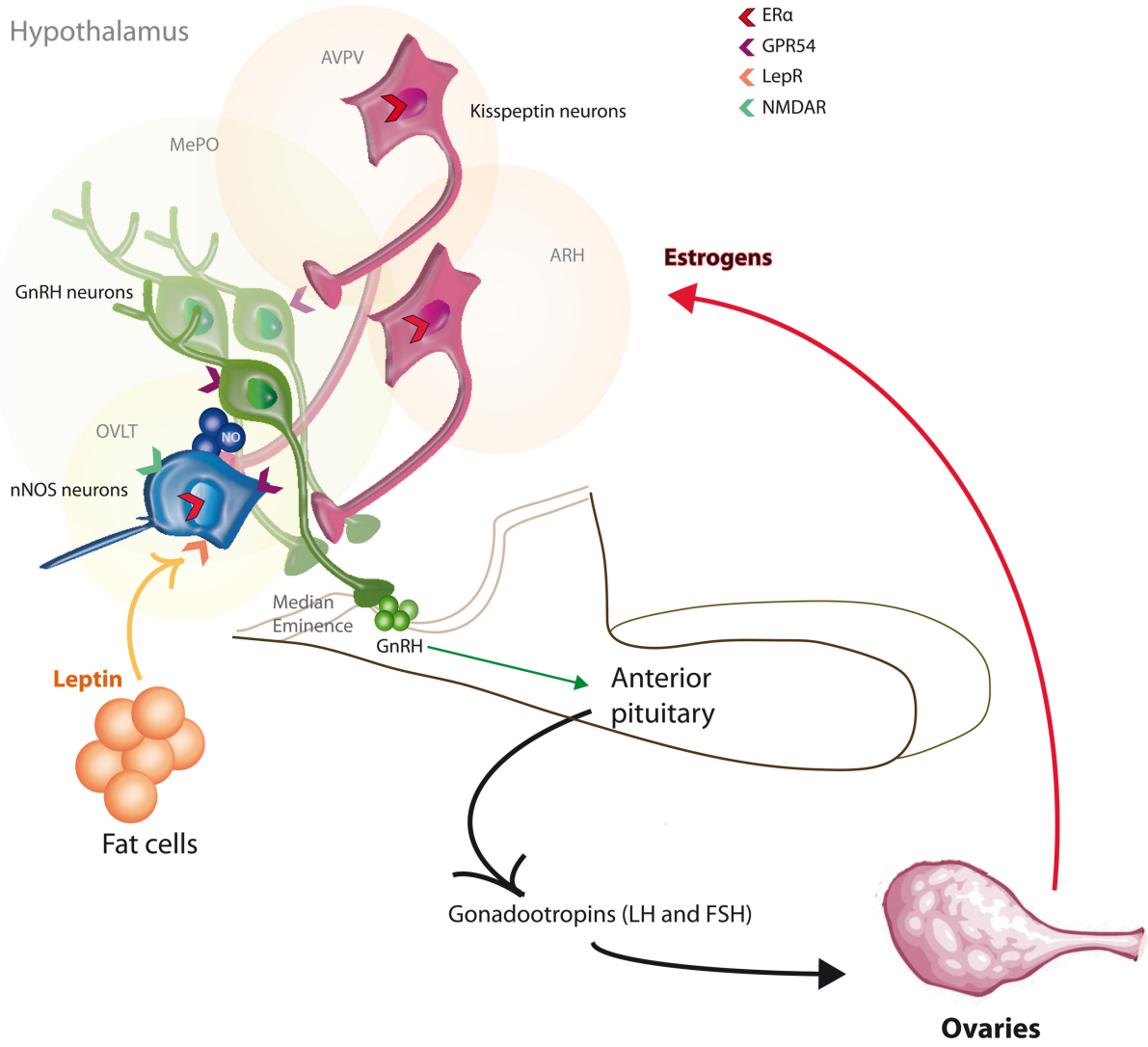

Figure 3. Schematic representation of the hypothalamo-pituitary-gonadal (HPG) axis. The cell bodies of the neuroendocrine neurons releasing gonadotropin releasing hormone (GnRH) are scattered throughout the preoptic region of the hypothalamus at the ventral surface of the brain, sending their neuronal processes to the median eminence where GnRH is released into the pituitary portal blood vessels for delivery to the anterior pituitary, where it stimulates the secretion of the gonadotropins: luteinizing hormone (LH) and folliculostimulating hormone (FSH). Blood-borne LH and FSH act on target cells in the gonads (here the ovaries) to direct production of gametes, as well as the secretion of steroid hormones (e.g., estrogens in females). Within the brain, gonadal steroids influence GnRH secretion via neuroendocrine feedback loops. In contrast to the direct action of kisspeptin on GnRH cells, leptin is believed to indirectly regulate LH release since GnRH neurons do not express the LepR. The population of nNOS cells residing in the OVLT region, where the NOS synthesizing neurons surround GnRH cell bodies, tuning their neuronal excitability, probably plays this intermediary role. nNOS cells of the OVLT/ MePO that express the leptin receptor (LepR) act as mediators of leptin signals to control leptin-induced GnRH release, and thus

tune the LH/ FSH surge. In parallel, this same population of nNOS cells also expresses, in addition to estrogen receptor alpha (ERα) and NMDAR, the kisspeptin receptor (GPR54) and is surrounded by kisspeptin fibers, suggesting that nNOS cells can directly receive kisspeptin signals. ARH, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleus; MePO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis.

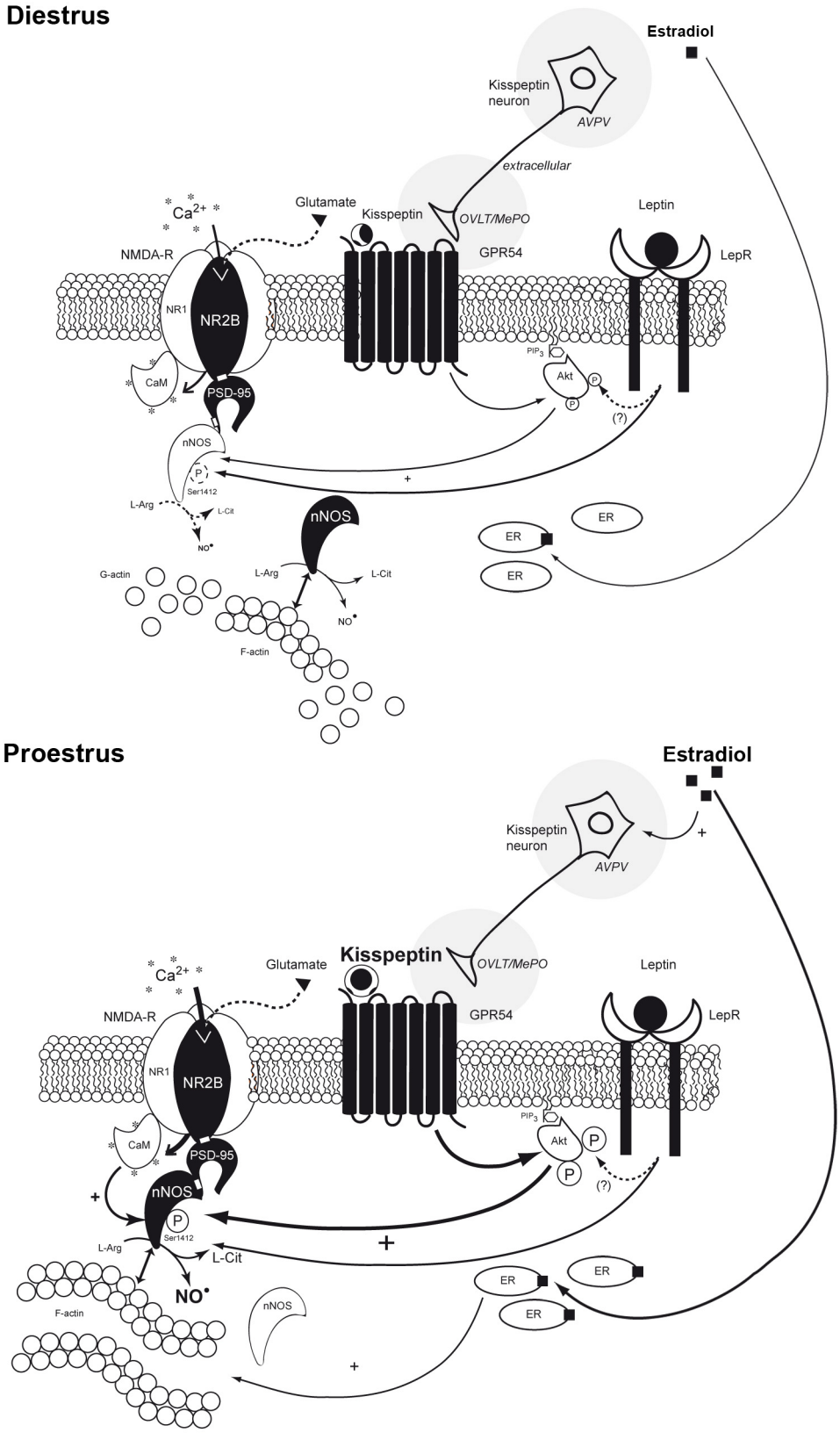

Figure 4. Schematic representations of the estradiol-, kisspeptin- and leptin- mediated changes in protein-protein interaction and phosphorylation during the ovarian cycle, proposed to be involved in the nNOS activity regulation in the hypothalamic preoptic area. Ca2+ influx through activated NMDA receptors is largely responsible for stimulation of

nNOS via the Ca2+/calmodulin complex. The physical interaction of nNOS with NMDAR implicates the PSD-95 scaffolding protein, and the assembly of a ternary complex. Phosphorylation of nNOS is responsible for the modulation of its catalytic activity, with the addition of a phosphate group in its Ser1412 residue being responsible for the activation of the protein. Phosphorylation of the nNOS protein at Ser1412 has been shown to be regulated across the ovarian cycle, reaching its maximal levels on the day of proestrus. How is this phosphorylation being promoted in the hypothalamic area? There are three different pathways that are proposed to lead to the phosphorylation of the nNOS target: 1) Estradiol, requiring the estrogen receptor (ER) activity, is able to promote the association of nNOS with the NMDAR/PSD95 complex at the plasma membrane that is required for the activation of nNOS by phosphorylation. Natural fluctuations of estrogen levels across the ovarian cycle, i.e., low in diestrus and high in proestrus, regulate the amount of the nNOS/PSD95/NMDAR ternary complex that is formed. 2) Leptin, acting on the leptin receptor, sustains a basal level of phosphorylation of the nNOS protein physically associated with the NMDAR, possibly via the PI3K/AKT pathway. 3) On proestrus, the estradiol-activated kisspeptin/GPR54 signaling is suggested to promote the phosphorylation of nNOS protein on its Ser1412 activation site via the PI3K/AKT pathway, nNOS activity being required for the onset of the preovulatory GnRH/LH surge. L-Arg, L-arginine; L-Citr, L-citrullin; CaM, calcium calmodulin; GPR54, kispeptin receptor; LepR, leptin receptor. AVPV, anteroventral periventricular nucleus; MePO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis.

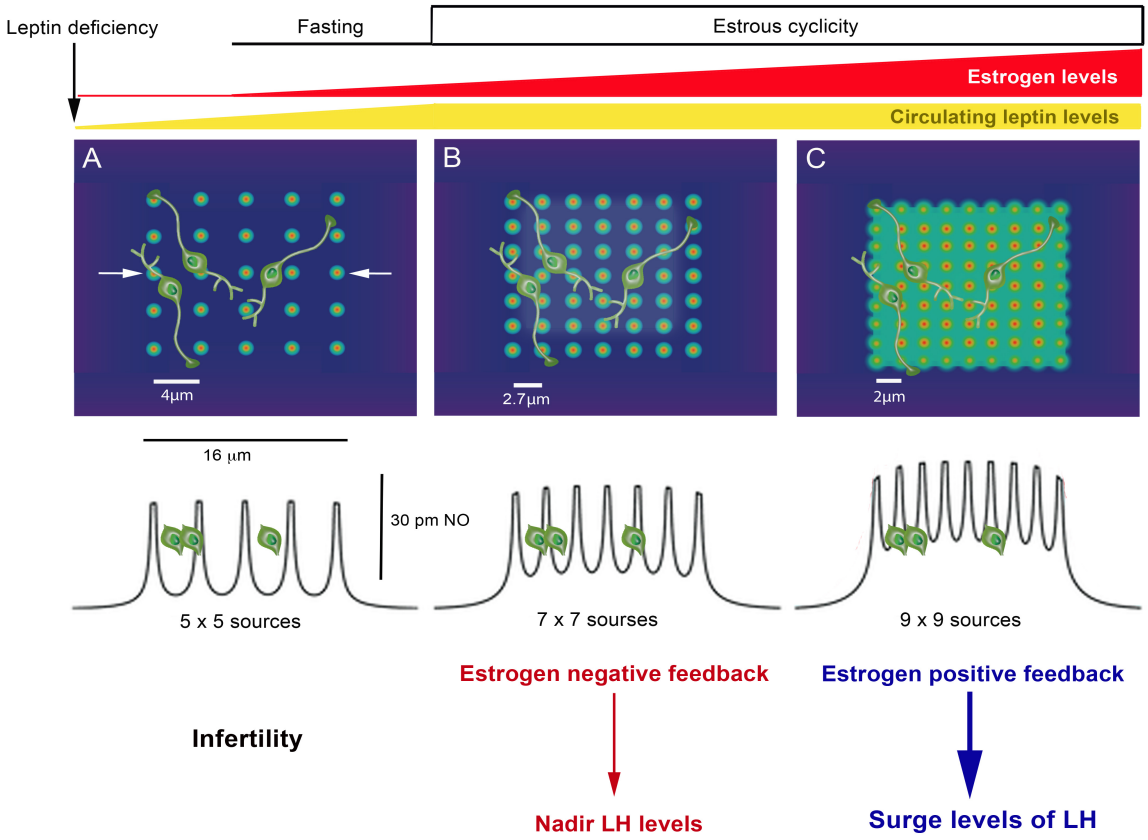

Figure 5. Model of NO signaling, resulting from the activation of nNOS cells surrounding GnRH neurons in the preoptic region of the hypothalamus. A, B and C show the computed profiles of steady-state NO concentrations across the center of a cubic array of spherical surface sources. Spheres of radius = 0.2 µm and numbering 25 (A), 49 (B) and 81 (C) are illustrated in 2-dimensional arrays within a fixed area (16 µm × 16 µm). The spheres generate NO at their surfaces at the rate of 40 molecules s−1 and the resultant NO concentrations throughout the array at steady state are calculated as described in Bellefontaine and colleagues (140). According to the physiological (or pathological) stimuli, NO action can switch from being suboptimaly active (e.g.: in individuals with leptin deficiency or during fasting, A), to being active only locally (e.g.: during the negative estrogen feedback in diestrus, B) or being a “volume transmitter” capable of influencing GnRH cells located at a distance, irrespective of anatomical connectivity (e.g.: the positive estrogen feedback on proestrus, C). The upper panels (A, B and C) illustrate the distribution of NO within and outside the area of emitters and the traces bellow (black lines) are sample concentrations taken through the center of each array (marked by arrows in A, upper panel). In green are drawn hypothetical GnRH neuron cell bodies and dendrites enclosed in the cubic array under scrutiny to suggest how NO depending on its area of influence could switch from a restraining mode (when NO effluxes from MePO/OVLT nNOS neurons are low) to a synchronizing mode (when NO effluxes are high) on GnRH neuronal activity. NO could potentially be used by the neuroendocrine brain to facilitate synchronous activity among GnRH neurons that where

previously operating independently and ultimately to maximize the release of its neurohormone into the blood.

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Commentary

Coexpression profiles; could they be a stepping-stone in our pursuit of steroid receptor pathways?

More than just a coincidence: when coexpression profiles reveal hidden gene networks

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The large-scale automation of neuroscience has enabled the construction of genome-wide atlases, of which the Allen Brain Atlas (ABA), which allows the 3-dimensional visualization of the expression profile of 21,500 genes in the male mouse brain down to single-cell-level resolution, is the most comprehensive (1). In this issue of PNAS, Mahfouz et *al.* (2) used the ability of the ABA to pinpoint the anatomical locations of expressed genes to uncover transcripts whose expression profiles correlate with those of steroid receptors, to begin to understand their function and specificity of action in different brain regions. This study shows that mapping combinatorial interactions among specific sets of genes represents a significant leap forward in our understanding of how tissue specificity for a given signaling pathway is determined, and in identifying the potential relationship between otherwise-unrelated brain areas in terms of the adaptive response to specific biological and environmental challenges.

Steroid receptors are pleiotropic transcription factors belonging to the superfamily of nuclear receptors, whose activity is induced by steroid hormones - lipophilic signaling molecules derived from cholesterol and primarily produced by the gonads and the adrenal cortex. In the mammalian brain, steroid hormones mediate the feedback from these steroid-generating organs on the neuroendocrine hypothalamus to control bodily functions (reproduction, metabolism, stress, inflammation, osmoregulation), but also play a fundamental organizational role during brain development, trigger adult brain plasticity and are involved in cognitive and emotional regulation (3-10).

The idea of having an anatomical map combined with a quantitative expression map of nuclear receptor genes dates back to 2007, when Gofflot and colleagues created an interactive database of 49 nuclear receptor genes spanning more than 100 different regions of the mouse brain (11). These researchers used two complementary approaches to meet the challenge of obtaining both cell-level resolution and an unbiased expression profile of large anatomical regions: real-time PCR provided a broad estimate of nuclear receptor expression levels in selected brain regions, while spatial expression patterns were more closely studied using high-resolution in situ hybridization (ISH). The study by Mahfouz and colleagues (2) significantly extends this approach to identify novel aspects of steroid hormone action via the spatial correlation of the expression

patterns of steroid receptors with those of genes that could potentially be steroid hormone targets or even downstream receptor coregulators.

Six well-studied steroid receptors were chosen by the authors to co-star in the “Guilt by association” play; their expression profiles, though already reported in the literature, were validated using 3D spatial gene expression data from the ABA (1). Based on the “Guilt by Association” principle, genes with similar spatial expression profiles are assumed to share similar biological functions, forming a neighborhood network of potential partners (12). Hence, for each receptor, the authors ranked potential partner genes based on their spatial coexpression in various brain structures in different parts of the brain, and tested the assumption that genes strongly coexpressed within a given brain region are related to a localized functional role of the steroid receptor. Genome-wide coexpression analyses indeed showed the strong coexpression of known glucocorticoid receptor (GR) transcriptional targets in the hippocampus and known estrogen receptor alpha (ESR1) transcriptional targets in the hypothalamus. Interestingly, these analyses revealed an unexpected coexpression of *Esr1*-related genes with *Esr1* outside known sites of action of estrogens, calling into question our understanding of the coordinated response of the brain to this gonadal steroid. Equally intriguing was the finding that among the top 10 genes coexpressed with *Gr* as well as known glucocorticoid-responsive genes across the whole brain, none were strongly coexpressed in the hypothalamus, indicating that GR signaling in the hypothalamus is distinct from GR signaling in the rest of the brain. These results show that spatial coexpression analysis has great potential for the identification of novel steroid receptor targets and putative region-specific pathways or gene networks. One should, however, be very careful while interpreting these unanticipated coexpression patterns; these representations do not necessarily reflect a causal relationship, but rather a likelihood of the association of certain genes that could indeed identify an actual functional relationship after further validation via expression measurement techniques.

Alternatively, the coexpression patterns described by Mahfouz et *al*. (2) could pinpoint potential steroid receptor coregulators rather than target genes, since each correlation merely indicates a possible associative, rather than causative, link between expressed genes. To explore

this possibility, the authors analyzed the coexpression patterns of each and every steroid receptor as well as a set of published nuclear receptor coregulators. The results revealed a great heterogeneity of coexpression patterns across distinct brain regions, pointing towards selective, region-specific coregulation. For example, *Pias2* and *Ncoa4*, two coactivators of GR and of the androgen receptor (AR), were found to be highly coexpressed with *Gr* in midbrain and hypothalamic regions, respectively, but not with *Ar*, although the relative abundance of its transcript is higher than that of *Gr* in these regions. Conversely, the authors uncovered the high coexpression of the mineralocorticoid receptor (*Mr*) with several nuclear receptor coregulators not thought to regulate MR function in the hippocampus, thus identifying putative novel coregulators of the MR pathway in this brain region. These results highlight the region-specific actions of coregulators and support the authors' notion that a brain-wide qualitative approach measuring mRNA levels using ISH could indeed be used to identify the spatially restricted regulation of steroid receptor function.

An important limitation of this approach, however, lies in the fact that it relies on the quality of the ISH, which is insufficient for some genes and/or datasets. Consequently, there is a risk of false negative results that goes hand in hand with the use of a genome-wide approach to identify region-specific targets and coregulators. This is something that the authors do not neglect to underline. However, their findings constitute a rich resource for the further prediction and validation of upstream or downstream genes using quantitative approaches like qPCR or ChIP analysis.

The strength and utility of the approach proposed in the paper of Mahfouz et *al.* (2) is further demonstrated by the prediction of *Magel2* as a transcriptional target for ESR1. Brain estrogen receptors alpha were selectively activated using the synthetic estrogen diethylstilbestrol (DES) in castrated male mice; this resulted in an increase in mRNA expression for estrogen-responsive genes. Among the 10 genes most significantly coexpressed with *Esr1,* they identified *Magel2*, a previously unidentified ESR1 target that shows strong coexpression with *Ers1* in the hypothalamus according to the ABA database. The measurement of *Magel2* expression using qPCR and quantitative double ISH in the hypothalamus after DES-induced activation revealed a

significant increase in *Magel2* mRNA levels after DES treatment, predicting that *Magel2* could be a target of ESR1. The loss of *Magel2* expression has been shown to contribute to several aspects of Prader-Willi syndrome (13), including hypogonadotropic hypogonadism (13, 14), providing support for a link with estrogen regulation. As noted above, though, coexpression analyses remain a rather indirect measurement of interaction, and chromatin immunoprecipitation assays followed by next-generation sequencing (ChIP-Seq) to identify ESR1 binding sites in the *Magel2* promoter region will be required to fully validate this hypothesis.

The findings convincingly show that the spatial correlation of steroid receptors with genome-wide mRNA expression across different regions of the mouse brain using web-based repositories provides a novel *in silico* assay with which to explore novel aspects of steroid hormone action and obtain a glimpse of how the brain, by integrating ever-fluctuating combinatorial levels of circulating steroid hormones, orchestrates the adaptive response of the organism. Could this study be the stepping-stone to deciphering even more unknown steroid receptor pathways and networks not only in the mouse brain but also in the human brain (Fig. 1)? Certainly one could envision that this method will act as a catalyst for the elucidation of the molecular mechanisms underlying sex steroid actions, but also for more efficient drug production against neuroendocrine disorders. Clearly, as exciting as this possibility is, there is a great deal of work yet to be done in order to overcome its limitations in terms of the sensitivity of the high-resolution ISH, and improve its predictive power. Still, as neuroscientists we cannot but acknowledge the unparalleled opportunity for prediction that this technique represents, and that too using data already available in the literature and online databases, to explore uncharted territories in brain function.

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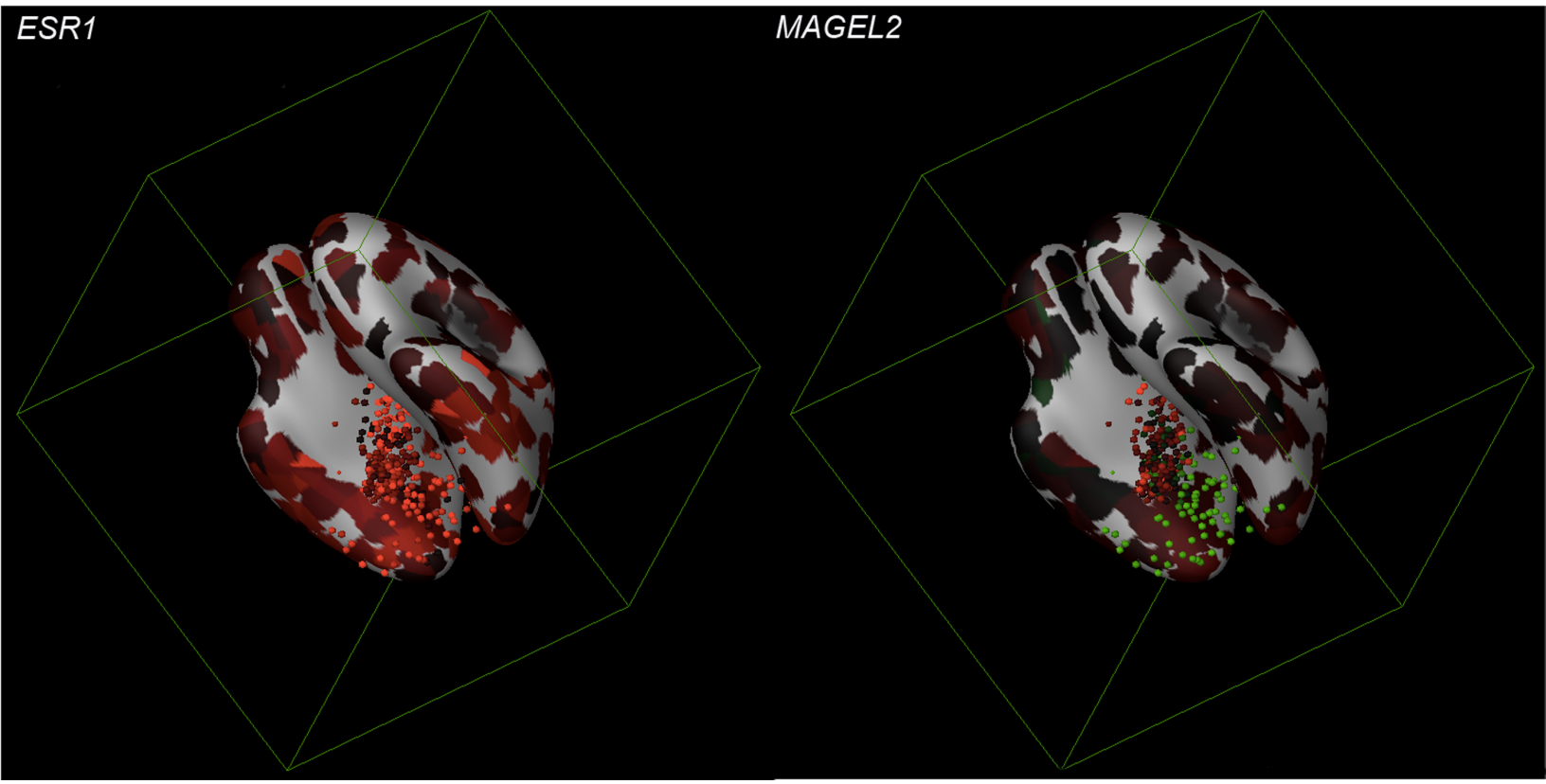
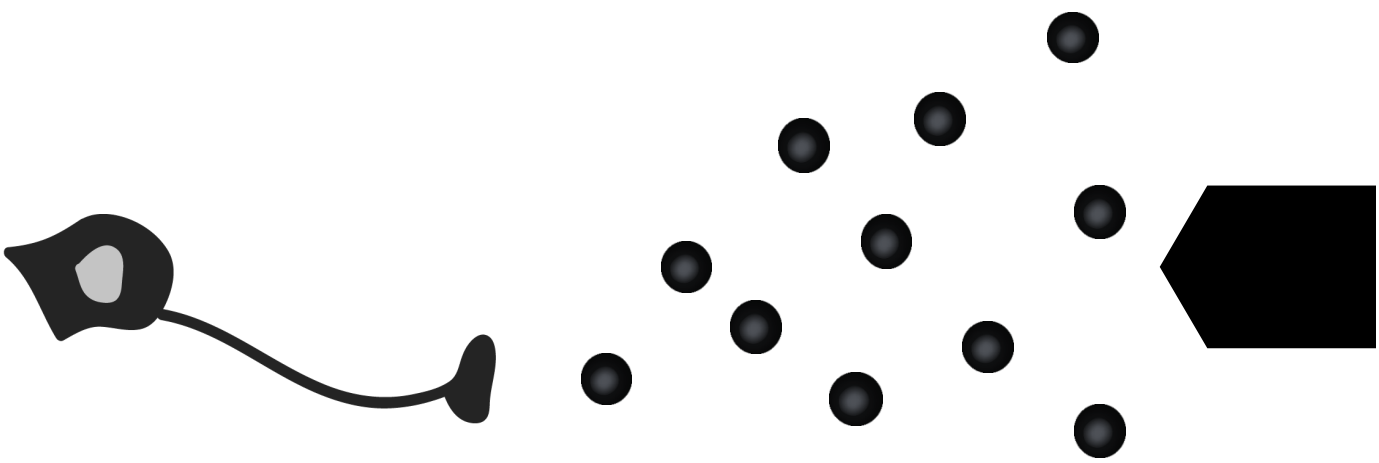
Fig. 1. Three-dimensional gene expression in the human brain. The model shows the expression of *ESR1* and *MAGEL2* mapped onto a reference atlas. The expression of these genes is indicated by the dots. The color of the dots indicates the expression level – green (low expression) through to red (high expression). Images were produced by the Brain explorer 2 software application, an interactive version of the Allen Human Brain Atlas.


ESR1
MAGEL2

Acknowledgements



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