UNIVERSITE DROIT ET SANTE DE LILLE II
Ecole doctorale Biologie – Santé

Thèse

Pour l'obtention du grade de

DOCTEUR DE L'UNIVERSITE DE LILLE II

Spécialité : Neuroscience

Présentée par

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Etude du rôle de l'expression du récepteur Neuropilin-1 et de l'exocytose Calcium-dépendante dans le neurone à GnRH sur le développement et la maturation du système à GnRH et la physiologie de la reproduction

Qui sera soutenue le 28 septembre 2015 devant le jury composé de :

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J'exprime tous mes remerciements à

Madame le Professeur Anne-Simone Parent et Monsieur le Docteur Nicolas de Roux, qui m'ont fait l'honneur d'accepter d'être les rapporteurs de ce travail. Ils ont consacré leur temps précieux à son évaluation et sa critique. Qu'ils trouvent ici le témoignage de toute ma considération.

Monsieur le Docteur Didier Vieau pour sa présence en temps qu'examinateur. Je lui suis très reconnaissante pour l'intérêt qu'il a déjà pu porter à mon travail au cours des comités de suivi de thèse. Merci pour le soutien qu'il m'a accordé depuis qu'il a été mon professeur à l'Université de Lille 1.

Monsieur le Docteur Philippe Ciofi pour sa présence en temps qu'examinateur. Merci pour ses précieux conseils et ses encouragements. Qu'il soit remercié de siéger parmi ce jury.

Monsieur le Docteur Vincent Prévot pour avoir encadré mes travaux de Master 2 et de thèse. Merci pour votre confiance, votre soutien et votre grand enthousiasme. Merci surtout pour m'avoir accordé votre confiance et avoir cru en moi il y a quatre ans quand je suis arrivée au laboratoire. Grâce à vous j'ai accompli un long chemin qui me permet de soutenir ma thèse aujourd'hui.

Je voudrais remercier vivement

Jean Claude Beauvillain pour ses grandes qualités scientifiques et humaines, pour ses précieux conseils et son soutien depuis mon arrivée au laboratoire.

Ariane Sharif, pour le temps qu'elle m'a consacré et pour son grand soutien. Mais aussi merci à ma collègue de TP d'histo pour son aide et sa joie de vivre.

Bénédicte Dehouck pour sa gentillesse, son écoute et sa bonne humeur.

Odile Viltart pour nos conversations scientifiques et personnelles, pour sa grande sincérité et son soutien.

Paolo Giacobini pour son aide précieuse au cours des manips mais aussi pour ses conseils d'expert. Merci aussi pour ta bonne humeur et tes anecdotes croustillantes.

Un grand merci à

Danièle pour le temps précieux qu'elle a consacré dans la réalisation de la microscopie électronique pour mes études, et pour tous ses bons conseils. Merci pour tous nos bons moments et pour nos folies au laboratoire. Au final je n'aurais pas fait tant de bêtises que ça…

Anne pour toutes nos discussions, pour son grand soutien, pour son aide et pour tout le temps qu'elle consacre pour nous tous au laboratoire. Merci pour ta bonne humeur et ta gentillesse.

Qu'est ce qu'on ferait sans vous …

Marie Claire, ma super responsable des TP d'histo, pour sa confiance et sa grande joie de vivre. Cette expérience m'a donné beaucoup de confiance et je l'en remercie.

Un énorme merci à

Sarah, ma collègue des premiers jours, pour sa complicité, son amitié, sa bonne humeur à toute épreuve et sa grande aide (liste non-exaustive). J'ai du mal à me dire que le but pour moi est de partir… Qu'est ce que je vais faire sans toi ?

Nadia, pour son assurance, sa zénitude, sa confiance, son grand soutien, pour nos aventures et pour sa folie bien cachée. ευχαριστώ πολύ Νάντια μου.

Special thanks to

Irene, my office mate for years, for her joy and her madness, and for the craziness she brought to the lab (and outside).

Giuliana, for her kindness, her care and her grande sorriso which always makes me happy.

My Dear Sam, for its fun, generosity, originality, and because he makes me feel I'm the best… hahaha.

Nicole, for her care, her trust and her help. You're the one who taught me so many things.

Naresh, for its trust and encouragement, even if we didn't collaborate a lot in the lab.

Je tiens à remercier

Emilie pour son aide précieuse, sa gentillesse, mais aussi et surtout pour son grand soutien dans les moments difficiles.

Sophie Croizier pour le temps qu'elle m'a consacré dans les manips, pour son aide et son soutien. Merci pour ton peps et ta bonne humeur.

Andrea, JP, Filippo, pour leurs bons conseils et leur grande aide dans la réalisation de mon projet, mais aussi pour tous les bons moments passés au labo ou ailleurs. Ils ont laissé un grand vide dans le labo.

Mathieu et Gustav, mes compagnons de bureau, pour leur aide et leur soutien, et pour tous nos bons moments.

Fanny, Eglantine, Sophie Stec, les anciennes qui m'ont montré l'exemple et qui ont mis la barre très haute.

Manon, pour sa confiance, sa gentillesse et sa grande aide

Je remercie sincèrement

Thomas et Karim pour tout le travail qu'ils font pour nous, pour leur grande gentillesse et leur bonne humeur

Delphine Cappe, Julien Devassine, Yann Lepage, Delphine Tailleu, et plus généralement toute l'équipe de l'animalerie pour leur aide précieuse.

Sophie Lesage, Nathalie Perrais, Michèle Ramirez pour leur joie de vivre et leur gentillesse.

Je tiens finalement à remercier

Mes parents, grâce à qui j'ai pu accomplir tout ce chemin, qui m'ont donné les moyens d'arriver où je suis, qui m'ont toujours fait confiance et qui m'ont encouragée et soutenue en toutes circonstances.

A mes amis et ma famille qui ont cru en moi, pour leur compréhension, leur soutien et leurs encouragements.

A Charles pour sa présence, son amour, sa franchise. Il y a peu de choses que tu ne saches déjà. Tu as su me supporter, me réconforter et me booster pendant ces trois années de thèse… quel challenge ! Et dire que ce n'est que le début… Merci pour ta compréhension et ton soutien, et pour tous nos beaux projets futurs.

Le travail que j'ai réalisé au cours de ces trois années de thèse a donné lieu aux communications scientifiques suivantes :

ARTICLES SCIENTIFIQUES

1. Vanacker C., Duquenne M., Messina A., Mazur D., Hrabovszky E., Pfrieger FW., Giacobini P., Prevot V. **Calcium-dependent exocytosis in GnRH neurons is required for sexual maturation and body weight homeostasis but not hypothalamic targeting in female mice.** *Endocrinology*. Révision favorable.

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

3. Giacobini P., Parkash J., Campagne C., Messina A., Casoni F., Vanacker C., Langlet F., Hobo B., Cagnoni G., Hanchate NK., Mazur D., Taniguchi M., Mazzone M., Verhaagen J., Ciofi P., Bouret SG., Tamagnone L., Prevot V. (2014) **Semaphorin 3A is an Endothelial-cell-derived factor that controls GnRH axon growth in the adult brain**. *PLoS Biol*. 2014 Mar 11;12(3):e1001808.

4. Osterstock G., El Yandouzi T., Romano N., Carmignac D., Langlet F., Coutry N., Guillou A., Schaeffer M., Chauvet N., Vanacker C., Galibert E., Dehouck B., Robinson IC., Prévot V., Mollard P., Plesnila N., Méry PF. (2014) **Sustained Alterations of hypothalamic tanycytes during post traumatic hypopituitarism in male mice**. *Endocrinology.* 155(5):1887-1898.

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

REVUE SCIENTIFIQUE

1. Vanacker C., Sharif A., Prevot V. **Dynamic Control of Neural Reproductive Centers by Endothelial cells**. *In: Endothelial Cell Plasticity in the Normal*

and Injured Central Nervous System (Herrera EM, Phillips-Farfán BV, Ospina GG, eds). CRC Press, January 1, 2015.

ARTICLE SCIENTIFIQUE EN PREPARATION

1. Vanacker C., Casoni F., Messina A., Hanchate NK., Croizier S., Bouret SG., Giacobini P., Prevot V. **GnRH neuronal migration and central precocious puberty : a role for Neuropilin-1 expression in GnRH neurons**. *En préparation.*

COMMUNICATIONS DANS LE CADRE DE CONGRES NATIONAUX ET INTERNATIONAUX

Communications orales

Vanacker C., Casoni F., Messina A., Hanchate NK., Giacobini P., Prévot V. Neuropilin-1 expression in GnRH neurons is required for a normal onset of puberty and GnRH neuron distribution, *18th meeting of LARC Neuroscience Network, Caen France*

Vanacker C., Casoni F., Messina A., Hanchate NK., Giacobini P., Prevot V. (September 2014) Neuropilin-1 expression in GnRH neurons is implicated in GnRH cell development, *Société de Neuroendocrinologie, Paris France*

Vanacker C., Hanchate NK., Giacobini P., Prevot V. (Juin 2013) Neuropilin-1 expression in GnRH neurons is necessary for a normal puberty onset, *FENS-IBRO Training school in Neuroendocrinology, Prato Italie*

Communications affichées

Vanacker C., Casoni F., Messina A., Hanchate NK., Giacobini P., Prévot V. (Mai 2015) Neuropilin-1 expression in GnRH neurons is required for a normal onset of puberty and GnRH neuron distribution. *Société des Neurosciences, Montpellier France*

Vanacker C., Duquenne M., Messina A., Mazur D., Hrabovszky E., Pfrieger FW., Giacobini P., Prevot V. (Mai 2015) Calcium-dependent exocytosis in GnRH neurons is required for sexual maturation and body weight homeostasis but not hypothalamic targeting in female mice. *DN2M meeting, Lille France*

Vanacker C., Casoni F., Messina A., Hanchate NK., Giacobini P., Prevot V. (August 2014) Neuropilin-1 expression in GnRH neurons is necessary for the normal onset of puberty and GnRH cells development, *Conference of European comparative endocrinologists (CECE2014), Rennes France*

Vanacker C., Casoni F., Messina A., Hanchate NK., Giacobini P., Prevot V. (Août 2014) Neuropilin-1 expression in GnRH neurons is necessary for the

normal onset of puberty and GnRH cells development, *8th International congress of Neuroendocrinology (ICN 2014), Sydney Australie*

Vanacker C., Casoni F., Hanchate NK., Giacobini P., Prevot V. (Juillet 2014) Neuropilin-1 expression in GnRH neurons is required for the normal onset of puberty and GnRH cell distribution in the adult brain*, FENS forum of Neurosciences, Milan Italie*

Vanacker C., Casoni F., Hanchate NK., Giacobini P., Prevot V. (Mai 2014) Neuropilin-1 expression in GnRH neurons is implicated in GnRH cell development, *Journées thématiques de la Société des Neurosciences, Lille France*

Vanacker C., Casoni F., Hanchate NK., Giacobini P., Prevot V. (Octobre 2013) Neuropilin-1 expression in GnRH neurons is necessary for the normal onset of puberty, *17th meeting of LARC Neuroscience Network, Rouen France, Poster*

Vanacker C., Hanchate NK., Giacobini P., Prevot V. (Juin 2013) Neuropilin-1 expression in GnRH neurons is necessary for a normal puberty onset, *FENS-IBRO Training school in Neuroendocrinology, Prato Italie*

Vanacker C., Hanchate NK., Giacobini P., Prevot V. (october 2012) Neuropilin-1 expression in GnRH neurons is required for the normal onset of puberty, *16th meeting of LARC Neuroscience Network, Porthmouth England*

Vanacker C., Hanchate NK., Giacobini P., Prevot V. (september 2012) Neuropilin-1 expression in GnRH neurons is required for the normal onset of puberty, *Société de Neuroendocrinologie, Banyuls France*

Résumé

L'acquisition de la fertilité chez les mammifères est le résultat d'un long processus de développement et de maturation de l'axe gonadotrope. Cette fonction cruciale à la survie des espèces est orchestrée par une poignée de cellules loca lisées au niveau de l'aire préoptique hypothalamique chez le rongeur, sécrétant la gonadotropin-releasing hormone (GnRH). La GnRH stimule la sécrétion de LH et de FSH par l'adénohypophyse, qui stimulent à leur tour les gonades. Les neurones à GnRH naissent dans l'épithélium voméronasal pendant le développement embryonnaire et migrent le long des axones voméronasaux pour atteindre l'hypothalamus. A la naissance le système est entièrement en place, toutefois il subira une phase de maturation avant d'atteindre la puberté, signant le début de la fertilité. Chez l'homme, un défaut de sécrétion de GnRH peut conduire à un hypogonadisme hypogonadotrope idiopathique (IHH) caractérisé par une subfertilité et une puberté retardée voire absente, ou même à un syndrome de Kallmann. Dans une grande partie des cas ce défaut de sécrétion est lié à un défaut de développement prénatal et à une diminution du nombre de neurones à GnRH dans dans l'hypothalamus. Depuis peu, la grande famille des semaphorines, déjà connues pour leurs effets chimiotactiques dans certains types cellulaires, et en particulier la semaphorine3A (Sema3A) via son récepteur la Neuropilin-1 (Nrp1), a été décrite comme un facteur indispensable au développement du système à GnRH et décrit comme un « gène Kallmann ». Toutefois son rôle spécifique dans les neurones à GnRH reste à élucider. Le **premier objectif** de ma thèse a donc été de déterminer le rôle de l'expression du récepteur Nrp1 dans les neurones à GnRH. Le suivi de la maturation sexuelle des animaux *Gnrh::cre, Nrp1*$^{loxp/loxp}$ (qui n'expriment pas la Nrp1 exclusivement dans les neurones à GnRH) a révélé l'apparition d'une puberté précoce et d'un phénotype de surpoids en comparaison aux animaux contrôles, corrélé à une accumulation des cellules à GnRH dans l'aire préoptique. L'étude de l'embryogenèse du système à GnRH chez ces animaux a démontré une augmentation du nombre de cellules à GnRH pendant leur migration. Nos résultats obtenus *in vivo* et *in vitro* suggèrent que la signalisation Nrp1 a un impact sur la survie des neurones à GnRH, et qu'elle module la motilité des cellules en migration et influe leur positionnement dans le cerveau. Le **deuxième objectif** de ma thèse a été d'étudier le rôle de l'exocytose dépendante du calcium et donc de la neurosécrétion dans les neurones à GnRH sur leur développement. Le suivi de la physiologie d'animaux *Gnrh::cre*; iBot, dont l'exocytose dépendante du calcium est abolit par clivage de protéine VAMP2/synaptobrevin 2 dans le neurone à GnRH, a révélé l'apparition de deux phénotypes distincts selon la pénétrance du transgène : un groupe ayant une puberté normale et un poids comparable aux animaux contrôles, et un groupe ayant une puberté retardée voire inexistante associé à un surpoids. Ces derniers présentent un IHH, une augmentation du tissu adipeux périgonadique et une hyperleptinémie, alors que la distribution des neurones à GnRH dans le cerveau n'est pas altérée. Ces données mettent en évidence le fait que l'activité de neurosécrétion dans les neurones à GnRH ne serait pas nécessaire pour leur développement embryonnaire, mais qu'elle pourrait jouer un rôle dans le maintien de l'homéostasie énergétique.

Ces deux études mettent en avant un lien étroit entre axe gonadotrope et métabolisme énergétique chez les mammifères et ont dévoilés de nouveaux mécanismes qui pourraient être impliqués dans la physiopathologie de la reproduction chez l'homme.

AA : Acide Aminé
ac : commissures antérieures
ADNc : Acide DésoxyriboNucléique complémentaire
AHA : Aire Hypothalamique Antérieure
AMPA : α-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid receptor
AMPc : Adénosine MonoPhosphate cyclique
α-MSH : α-melanocyte-stimulating hormone
ARC : noyau arqué
ARNm : Acide RiboNucléique messager
AVPV : Antero Ventral Peri-ventricular nucleus
BDNF : Brain-Derived Neurotrophic Factor
DBB : Bandes Diagonales de Broca
Ca^{2+} : Calcium
CAM : calmoduline
CCK : Cholescystokinine
CGRP : calcitonine gene-related peptide
CHD7 : Chromodomaine Helicase-DNA binding protein 7
Cre : protéine cre recombinase
CUB : domaine amino-terminal Complement-Binding
DCC : Deleted in Colorectal Cancer
Dyn : Dynorphine
E : âge Embryonnaire
EAA : acides aminés excitateurs
ECM : Matrice Extra-Cellulaire
EGF : Epidermal Growth Factor
EphA : Récepteur aux Ephrines
ER : Récepteur aux Estrogènes
ERK : extracellular signal-regulated kinase
FAK : Focal Adhesion Kinase
FGF : Fibroblast Growth Factor
FGFR : Récepteur au Fibroblast Growth Factor
FLK1 : Récepteur au VEGF de type 2
FSH : Hormone Folliculo-Stimulante
FV/VIII : Fatceur de coagulation V/VIII
GABA : Acide-Γ-Amino-Butyrique
GABAR : Récepteur à l' Acide-Γ-Amino-Butyrique
GAD-67 : Glutamic Acid Decarboxylase
GAP : GnRH Associated Protein
GAP : GTP-ase Activating Protein
GFP : Green Fluorescent Protein
GH : Hormone de croissance
GMP-c : Guanosine MonoPhospahte cyclique
GnRH : Gonadotrophine-releasing hormone, Gonadolibérine
GnRHR : Récepteur à la Gonadolibérine
GPCR : récepteur couplé à une protéine G
CPP/iCPP : Puberté Centrale Précoce (idiopathique)
GPR(54) : récpeteur couplé à une protéine G (54)

CRAM : CRMP-associated molecule
CRMP : collapse response mediator protein
HGF : Hepatocyte Growth Factor
HH/iHH/niHH : Hypogonadisme Hypogonadotrope (normosmique, idiopathique)
hpg : hypogonadique
HPG : Hypothalamic-Pituitary-Gonadal axis, axe Gonadotrope
HRP : Horseradish Peroxidase Protein
ICV : Intra-Cérébro-Ventriculaire
Ig : Immunoglobuline
IGF-1 : Insuline-like Growth Factor 1
IP : Intra-Péritonéal
IR : Récepteur à l'Insuline
Itg : Intégrine
IPT : domaine Immunoglobuline-like, Plexine, et facteur de Transmission
KA : Récepteur au Kainate
Kal-1 : Gène Kallmann 1/ Anosmin-1
kDa : KiloDalton
KDR : Récepteur au VEGF de type 2 (voir aussi FLK1)
KiSS : Kisspeptine
KNDy : Kisspeptine/Neurokinine B/Dynorphine
KS : Syndrome de Kallmann
KO : knock out
LDCV : large dense core vesicle, vésicule à cœur dense
Lep : Leptine
LepR : Récepteur à la Leptine
LH : Hormone Lutéinisante
LHRH : Luteinizing Hormone-Releasing Hormone
L-NAME : NG-nitro-L-arginine Methyl Ester
LRF : Luteinizing hormone Releasing factor
MAM : Meprin, A5, Mu domain
MAPK : mitogen-activated protein kinase
MBH : Hypothalamus Médio-Basal
ME : Eminence Médiane
MS : Septum Médian
NELF : Nasal Embryonic LHRH Factor
NCAM : Neural Cell Adhesion Molecule
NHLH2 : human nescient helix-loop-helix 2
NKB : Neurokinine B
NMDA : N-Methyl-D-Aspartate
NMDA R : Récepteur au N-Methyl-D-Aspartate
NO : monoxyde d'azode
NOS : monoxyde d'azote synthase
NPV : Noyaux Para-Ventriculaires
NPY : Neuropeptide Y
NRG : NeuRéGuline
NSO : Noyaux Supra-Optiques
Nrp : Neuropiline

NT : NeuroTensine
OB : bulbe olfactif
oc : chiasma optique
OE : Epithélium Olfactif
OEC : Olfactory Ensheating Cells, cellules gliales enveloppantes
OTX2 : Orthodenticle Homeobox 2
OVLT : organe vasculaire de la lame terminale
OVX : ovariectomisé
P : âge Postnatal
p : groupement phosphate
PG : prostaglandine
PIP2 : PhophatidylInositol-4,5-DiPhosphate
PKC : Protéine Kinase C
PLC : Phospholipase C
Plxn : plexine
PMN/PMV : Noyaux PréMammilaires
POA/mPOA/rPOA
POMC : ProOpioMélanoCortine
PR : Récepteur à la Progestérone
PROK : Prokinéticine
PROKR : Récepteur à la Prokinéticine
PSI : domaine plexines, sémaphorines, intégrines
PSA (NCAM) : Acide Polysialique (forme polysialilée de la Neural Cell Adhesion Molecule)
PVpo : noyau PériVentriculaire de l'aire PréOptique
RFRP : RFamide (arginine-phenylalanine-amide) related peptide
RP3V : Aire Rostrale Périventriculaire du troisième Ventricule
Sema : Sémaphorine
SF-1 : Steroidogenic Factor
SNAP : Synaptosomal-Associated Molecule/SNARE-Associated Molecule
SNARE (V-SNARE/t-SNARE) : N-Ethylmaleimide Sensitive factor Attachment protein Receptor (v : vesiculaire, t : membrane plasmique)
SSV : Small Synaptic Vesicle, petite vésicule synaptique
TACR : Thachykinin Receptor
TGF : Transforming Growth Factor
Vamp/Synaptobrévine : Vesicle Associated Membrane Protein
VEGF : Vascular Endothelial Growth Factor
VEGFR : Récepteur au Vascular Endothelial Growth Factor
VGLUT : Vesicular Glutamate Transporter
VL/LV : Ventricule Latéral
VNN : Nerf VoméroNasal
VNO : Organe VoméroNasal
VMH : Hypothalamus Ventro-Médian
3V : troisième Ventricule

Chapitre 1

Chapitre 2

Chapitre 3

La fonction de reproduction est l'un des trois piliers régissant la survie des espèces avec la nutrition et la croissance. La transmission du patrimoine génétique est, en effet, une préoccupation première pour les êtres vivants. La fonction de reproduction est orchestrée par tout un réseau neuronal au sein de l'hypothalamus, ayant pour finalité la sécrétion de la neurohormone Gonadotrophine-releasing hormone (GnRH). La GnRH stimule la sécrétion des gonadotrophines par l'adénohypophyse, qui agissent en périphérie pour contrôler la maturation des gonades, la puberté, la gamétogénèse, et le maintien de la fertilité. Un défaut en GnRH peut être responsable d'une subfertilité et d'une puberté retardée, voire absente, appelée hypogonadisme hypogonadotrope. La sécrétion pulsatile et cyclique de cette neurohormone est elle même régulée par un large éventail de facteurs afférents, ayant un rôle modulateur sur le système à GnRH. Ce dernier est également à l'écoute des signaux périphériques, tels que les stéroïdes gonadiques, mais encore les signaux métaboliques, qui ont un rôle régulateur sur la sécrétion de GnRH. Le système à GnRH est donc un système complexe et hautement régulé.

L'intégrité du système à GnRH est également liée à son origine embryonnaire. En effet, les cellules à GnRH constituent une population atypique de neurones, puisqu'elles naissent, en partie, à l'extérieur du cerveau, plus précisément au sein de la placode olfactive, et migrent pendent le développement embryonnaire pour atteindre l'hypothalamus. A la naissance, le système est en place mais n'est, toutefois, pas encore prêt à fonctionner, puisqu'il nécessite une longue étape de maturation post-natale avant que l'individu acquiert la capacité à se reproduire. Ainsi, un défaut dans la migration des neurones à GnRH, ou dans la maturation du système à GnRH, peut également conduire à un hypogonadisme hypogonadotrope. De nombreuses molécules interviennent dans la prolifération, l'apoptose, la migration, et le guidage des cellules pendant l'ontogénèse du système nerveux. Récemment, plusieurs membres de la famille des molécules de guidance appelées sémaphorines, ainsi que leurs récepteurs, ont montré un rôle dans la mise en place du système à GnRH et dans son fonctionnement à l'âge adulte. La sémaphorines 3A a notamment montré un rôle dans l'ontogénèse du système à GnRH. Le gène codant pour cette protéine a, en effet, été caractérisé comme un gène responsable du

syndrome de Kallmann, chez l'Homme et chez la souris, qui est diagnostiqué par un hypogonadisme hypogonadotrope associé à une anosmie. Le rôle direct de la sémaphorine 3A et de la signalisation via son récepteur, la Neuropiline-1, sur la migration des cellules à GnRH, reste toutefois à élucider.

Outre le rôle de facteurs afférents sur le système à GnRH, le rôle de la sécrétion autocrine/paracrine des neurones à GnRH dans leur propre fonctionnement, aussi bien dans leur migration, dans leur maturation, et leur sécrétion, et donc son rôle dans la physiologie de la reproduction, n'a pas été clairement déterminé. En effet, des facteurs autres que la GnRH sont sécrétés par les neurones à GnRH, mais les connaissances quant aux rôles de ces facteurs sur la physiologie du neurone à GnRH sont très réduites.

Au cours de mon travail de thèse, nous nous sommes demandés dans quelle mesure des signaux intrinsèques au neurones à GnRH, notamment leur sécrétion autocrine/paracrine, et des signaux extrinsèques aux neurones à GnRH, et en particulier de rôle de la signalisation sémaphorine 3A/Neuropiline-1, pouvaient affecter non seulement leur migration, mais encore la fonction de reproduction et la physiologie de l'organisme.

Avant de présenter les résultats issus de ce travail, je rappellerai, dans un premier temps, le fonctionnement de l'axe gonadotrope et la fertilité chez l'adulte, en me basant particulièrement sur les connaissances apportées par l'utilisation du rat et de la souris comme modèles animaux. Au sein d'un deuxième chapitre je décrirai le développement prénatal (la migration) et postnatal (la maturation) du système à GnRH et introduirai le rôle des facteurs les plus importants dans ce développement, ainsi que les pathologies de la reproduction qui en découlent, connus chez l'Homme et chez les rongeurs. Le troisième chapitre sera consacré aux rôles connus de la sémaphorine 3A et de la Neuropiline-1 dans le système nerveux central et dans la fonction de reproduction.

Chapitre 1 : Le système à GnRH et la physiologie de la reproduction

Chez les mammifères, la reproduction est contrôlée par une petite population de neurones hypothalamiques qui synthétisent la Gonadotropin-Releasing Hormone (GnRH) ou Gonadolibérine. Les terminaisons axonales de ces neurones sont localisées au niveau de la zone externe de l'éminence médiane hypothalamique où la GnRH est libérée et transportée via le sang porte hypothalamo-hypophysaire jusqu'à l'adénohypophyse. Elle peut ainsi se fixer à son récepteur exprimé par les cellules gonadotropes qui sécrèteront en réponse l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH). Ces gonadotrophines sont nécessaires à la maturation des gonades, à la gamétogenèse, à la synthèse des hormones stéroïdes gonadiques (estrogènes, progestérone, testostérone), ainsi qu'au maintien du cycle ovarien chez la femelle.

1. Le système à GnRH

1.1 La découverte du décapeptide

L'existence d'une hormone gonadotrope sécrétée par le cerveau avait été suspectée dès les années 1950 (Harris 1950), notamment grâce à l'isolement d'extraits hypothalamiques capable de stimuler la libération de LH par l'hypophyse antérieure (chez le rat, McCann & Ramirez 1964). Toutefois la grande difficulté rencontrée pour doser les taux extrêmement faibles de GnRH n'a permis sa découverte officielle qu'une vingtaine d'années plus tard : il s'agit d'une neurohormone constituée de 10 acides aminés (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, Schally et al. 1971). D'abord appelé « Luteinizing hormone releasing factor » ou LRF, puis « Luteinizing hormone-Releasing Hormone » ou LHRH, ce décapeptide est actuellement nommé GnRH puisqu'il est capable de stimuler la sécrétion de LH mais également de FSH. Ce n'est que dans les années 1980 qu'est décrit le gène codant pour la GnRH, ainsi que son ADNc chez l'homme (Seeburg & Adelman 1984) et chez le rat (Adelman et al. 1986). C'est à cette même période qu'est isolée une deuxième isoforme de la GnRH chez le poulet (chicken GnRH-II, Miyamoto & Hasegawa 1984) et une troisième chez le poisson (Salmon GnRH-III, Sherwood et al. 1983). Dès lors de nombreuses formes de GnRH très conservées au cours de l'évolution sont découvertes, toutes portant le nom de l'animal chez qui elles ont été caractérisées, et sont classées en trois groupes évolutifs distincts. On trouvera alors la GnRH-I qui est la forme hypothalamique, la GnRH-II mesencéphalique (ces deux formes sont retrouvées des poissons jusqu'aux primates humains et non-humains, mais pas chez la souris) et la GnRH-III télencéphalique

exclusivement décrite chez le poisson. Les cellules exprimant la GnRH-II et la GnRH-III auraient un rôle dans le comportement sexuel (Eisthen et al. 2000; Robert P. Millar et al. 2004), alors que les cellules exprimant la GnRH-I hypothalamiques ont des capacités neuroendocrines. La GnRH-I est en effet indispensable à la fonction de reproduction, comme le montre la souris « hpg » découverte en 1977 (Cattanach et al. 1977), dont la mutation autosomale récessive du gène codant pour la GnRH-I est liée à un hypogonadisme et à une infertilité (Mason et al. 1986). Le clonage du gène GnRH-I, puis l'injection du transgène GnRH à des embryons de souris hpg, a permis la récupération d'un phénotype normal chez ces souris. Cette technique novatrice, en présage de la thérapie génique, a permis de comprendre son rôle crucial dans la fonction de reproduction (Seeburg et al. 1987).

1.2 La synthèse de la GnRH

Le gène codant la GnRH est composé de 4 exons. Il a été localisé sur le chromosome 8 chez l'homme (Yang-Feng et al. 1986) et sur le chromosome 14 chez la souris (Williamson et al. 1991). La caractérisation de l'ADNc du précurseur de la GnRH par Seeburg & Adelman, en 1984, permet alors de comprendre la biosynthèse du décapeptide. La GnRH est synthétisée à partir de la préproGnRH de 92 acides aminés, constituée d'un peptide signal de 23 acides aminés, du décapeptide GnRH, d'un site de protéolyse et d'amidation (Gly-Lys-Arg), et du peptide GAP « GnRH associated peptide » de 56 acides aminés. La préprohormone subit une maturation post-traductionnelle consistant en l'élimination du peptide signal et à la cyclisation de la glutamine en pyroglutamine, au niveau du groupement NH_2 en position terminale, grâce à l'action de protéases au niveau d'acides aminés basiques. Ceci aboutit à la libération d'un peptide de 13 acides aminés et du peptide GAP. Une autre coupure enzymatique, au niveau de la glycine 11 du peptide de 13 acides aminés, permet la libération du décapeptide GnRH amidé en COOH terminal. Le peptide GAP a, d'une part, un effet inhibiteur sur la sécrétion de prolactine et, d'autre part, une action stimulatrice sur la sécrétion des gonadotrophines chez le rat (Nikolics et al. 1985; Yu et al. 1989). Majoritairement trouvée dans le cerveau, la GnRH est également présente dans d'autres tissus comme le placenta, l'utérus, les ovaires, les testicules et les tissus mammaires (Khodr & Siler-Khodr 1980; Chou et al. 2004).

2. Neuroanatomie du système GnRH

2.1 Corps cellulaires

Les neurones à GnRH constituent une petite population de 800 à 2000 cellules dispersées dans le cerveau antérieur chez les mammifères (pour revue : Barry et al. 1985). La localisation des neurones à GnRH a été précisément décrite dans l'hypothalamus de rongeur dans les années 70 (Leonardelli et al. 1973; Baker et al. 1974; Barry et al. 1973; Silverman et al. 1979). Ces petites cellules bipolaires et fusiformes, irrégulières et à l'aspect épineux (King et al. 1982) sont localisées dans la « région préoptique », constituée par les régions septale, préoptique, et hypothalamique chez le rongeur adulte. Les neurones à GnRH sont distribués bilatéralement en formant un continuum en forme de V sur un plan horizontal, dont l'apex est dirigé rostralement et localisé dans les bandes diagonales de Broca (DBB), et dont les pieds atteignent l'hypothalamus médio-basal (MBH), de part et d'autre du 3^{e} ventricule, en passant par les noyaux septaux médian et triangulaire, l'aire préoptique (POA) périventriculaire médiane et latérale (King et al. 1982; Merchenthaler et al. 1984). Sur un axe dorso-ventral, la distribution des neurones à GnRH est souvent représentée comme ayant la forme d'un « Y inversé ». On constate cette distribution sur une coupe coronale (Figure 1, 2^{e} vue coronale), le pied du Y étant constitué par les neurones du septum médian (MS), et les deux bras du Y, qui projettent caudalement, sont séparés par le début du 3^{e} ventricule, autour de l'organe vasculaire de la lame terminale (OVLT), où les cellules à GnRH sont particulièrement concentrées (King et al. 1982). Chez l'homme, les neurones à GnRH sont plus abondants dans l'aire infundibulaire de l'hypothalamus (MBH) que dans l'aire préoptique en comparaison aux rongeurs (King et al. 1985).

Il existe toutefois une population de neurones à GnRH extra-hypothalamique décrite chez la souris, qui a été beaucoup moins étudiée, et qui n'est pas directement en lien avec le rôle de la GnRH dans la stimulation de la sécrétion des gonadotrophines par l'adénohypophyse. Cette population est localisée au sein des bulbes olfactifs, où la plupart des cellules ainsi que des fibres immunoréactives pour la GnRH sont associées au nervus teminalis (nerf terminal, ou nerf cranial 0). Bien que très proches et souvent confondus avec les nerfs olfactifs, les deux nerfs terminaux ne sont pas connectés aux bulbes olfactifs mais projettent vers les aires préoptiques médiale et septale, et seraient un vestige embryonnaire, ou encore liés à la détection des phéromones (Fields & Douglas 2007). Ainsi les neurones à GnRH du système olfactif sont localisés au niveau de la racine septale du nerf

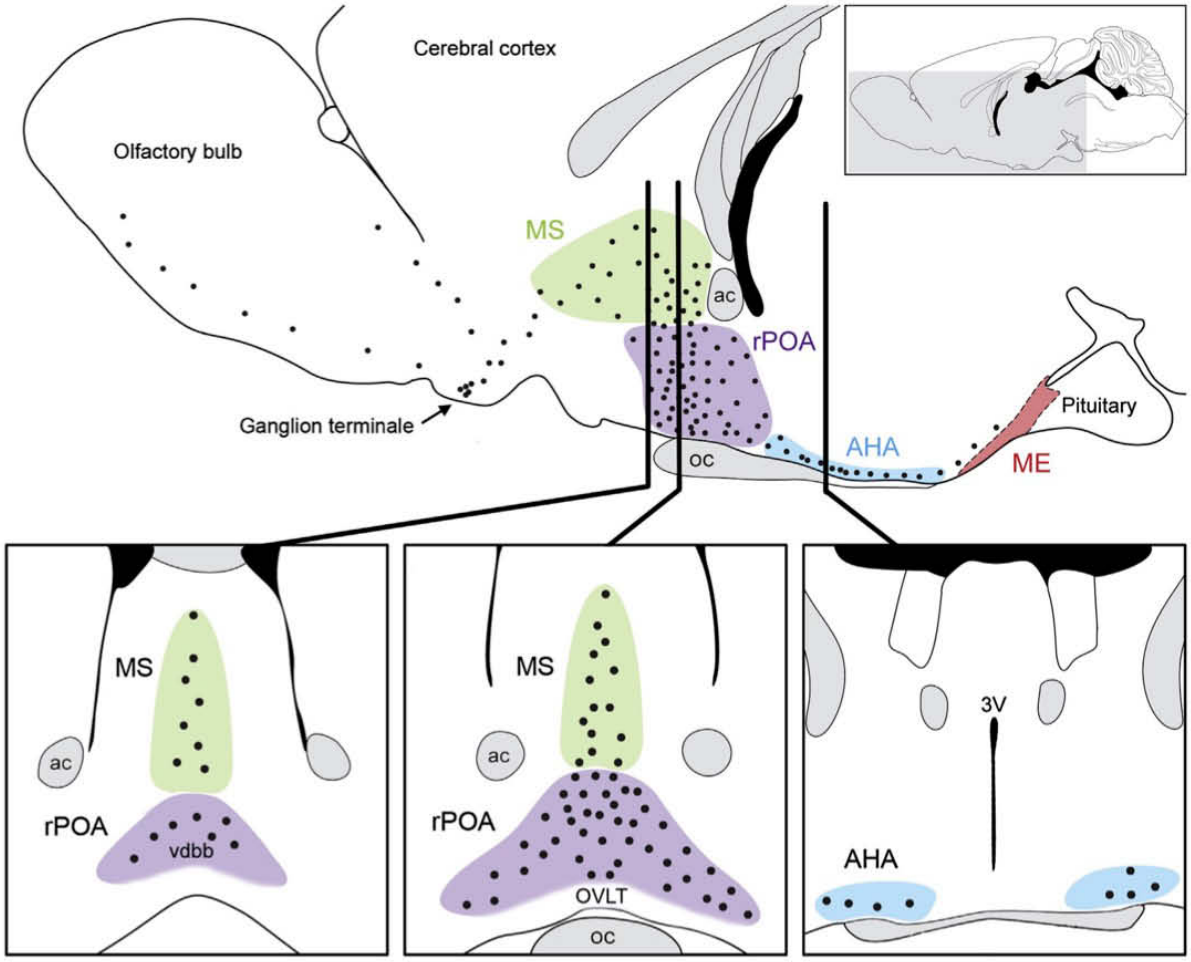


Figure 1. Distribution des neurones à GnRH dans le cerveau chez la souris. Schéma montrant la distribution des neurones à GnRH (points noirs) dans le cerveau de souris sur une vue saggitale (haut) et trois vues coronales (bas). La première vue coronale représente la région du septum médian (MS), la deuxième représente la région de l'organe vasculaire de la lame terminale (OVLT), région qui contient le plus de neurones à GnRH. Ces deux régions font parti de l'aire préoptique rostrale (rPOA). La 3^{e} vue coronale représente la région la plus caudale de l'hypothalamus antérieur (AHA). Des cellules à GnRH sont également présentes dans les bulbes olfactifs. *Abréviations : vdbb, branche verticale des bandes diagonales de Broca ; ac, commissures antérieures ; oc, chiasma optique ; ME, éminence médiane ; 3V, troisième ventricule.* Adapté de Plant et Zeleznik, Knobil and Neill's, Physiology of reproduction, 4^{e} édition, Diagram courtesy of Dr Michel Herde.

terminal, dans le ganglion terminal, dans le réseau même de nerfs amyélinisés fasciculés du nerf terminal, ainsi que dans différentes couches des bulbes olfactifs principaux et accessoires et la partie basale de l'épithélium nasal (Jennes & Stumpf 1980; Jennes 1987; Schwanzel-Fukuda et al. 1987). Cette distribution caractéristique n'est pas sans rappeler l'origine extra-cérébrale des cellules à GnRH, ainsi que leur longue étape de migration pendant la formation embryonnaire du cerveau. La description du développement prénatal du système à GnRH fera l'objet de la première partie du chapitre 2.

2.2 Projections

Les projections axonales et dendritiques issues des cellules à GnRH ont d'abord été caractérisées par immunohistochimie (King et al. 1982). Elles projettent majoritairement vers des organes circumventriculaires. Les axones des neurones à GnRH ciblent en particulier l'éminence médiane, organe circumventriculaire bordant le plancher du troisième ventricule, et qui constitue physiologiquement et anatomiquement l'interface entre hypothalamus et hypophyse. On parle alors d'un tractus préoptico-infundibulaire ayant pour origine l'aire préoptique médiale/septale et projetant au niveau de l'éminence médiane et de la tige pituitaire (Merchenthaler et al. 1980). Beaucoup de cellules à GnRH de l'aire préoptique projettent également leur dendrite vers l'OVLT, à proximité de capillaires fenestrés (Herde et al. 2011). 85% des neurones à GnRH projetant leur dendrite dans l'OVLT seraient impliqués dans le pic préovulatoire de GnRH/LH (Herde et al. 2011). Il est très probable que les neurones à GnRH aient, via leur contact avec l'OVLT, un accès direct aux signaux périphériques (Prevot 2011), bien que le rôle exact de cette innervation très dense et ramifiée n'ait pas encore été précisément déterminé (Caraty & Skinner 2008). Toutefois, les notions de dendrite et d'axone issus du neurone à GnRH ont récemment été remises en question par une étude de Herbison, qui montre que l'axone possède en vérité des propriétés de dendrite, morphologiquement par la présence d' « épines dendritiques », et physiologiquement par l'initiation de potentiels d'actions dans cette région (Herde et al. 2013b). C'est grâce à l'utilisation de traceurs rétrogrades, injectés soit dans l'éminence médiane (Silverman et al. 1987), soit dans la circulation générale (Witkin 1990), que la localisation précise des neurones à GnRH impliqués dans la neurosécrétion est établie. Ainsi, 90% des neurones à GnRH de la région préoptique se projetteraient vers des organes circumventriculaires (ME, OVLT, et organe subfornical) d'après Witkin (1990). D'après des études de Silverman et al. (1987), ce sont près de 50% des neurones à GnRH qui se projettent vers la zone externe de l'éminence médiane, sans regroupement dans une région

particulière. Une étude de Jennes et al. (1987) a démontré qu'une sous-population de neurones à GnRH localisés dans les bulbes olfactifs de souris était en contact avec des capillaires fenestrés, puisque ces derniers ont pu être marqués par une protéine HRP rétrograde (ne passant pas la barrière hémato-encéphalique) par injection intravasculaire, et pourraient donc être impliqués dans une régulation d'événements de type endocrine. Les auteurs émettent l'hypothèse que ces cellules seraient probablement connectées au tissu conjonctif sous-épithélial et/ou périglandulaire du nez. Cette même étude montre que d'autres cellules à GnRH de cette même région sont capables de capter la protéine HRP lorsqu'elle est injectée dans le liquide céphalo-rachidien mais aussi quand elle est administrée par infusion intra-nasale. Ces résultats surprenants démontrent que les neurones à GnRH localisés dans le système olfactif de la souris peuvent influencer une grande variété de sites cibles, via le sang, le liquide cérébro-spinal, ou encore par des contacts synaptiques/asynaptiques, possiblement avec la muqueuse nasale. Par ailleurs, la GnRH pourrait jouer un rôle de neuromodulateur ou de neurotransmetteur au sein du cerveau puisqu'une portion de la population de cellule à GnRH, de la région préoptique comme du bulbe olfactif, se projette dans diverses régions du système nerveux central telle que l'amygdale moyenne (Witkin et al. 1982; Merchenthaler et al. 1984; Jennes 1987).

3. Mode de sécrétion de la GnRH

La GnRH est une neurohormone d'origine hypothalamique. Elle est en effet synthétisée dans le cytoplasme somatique et est acheminée jusqu'à la terminaison axonale de la cellule, à proximité des capillaires fenestrés du système porte hypothalamo-hypophysaire, où elle est sécrétée. Ces capillaires irriguent l'hypophyse, et permettent ainsi la détection de la GnRH, localement très concentrée, par les cellules gonadotropes de la partie antérieure. En réponse à une augmentation de la concentration de GnRH, les cellules gonadotropes synthétisent et sécrètent la LH et la FSH.

3.1 Exocytose

Les vésicules contenant la GnRH

Les événements menant à l'exocytose des vésicules de neurosécrétion des neurones à GnRH n'ont pas encore été complètement caractérisés, et très peu de choses sont connues sur les mécanismes intracellulaires qui la régissent. Des études en microscopie électronique

ont permis de localiser le peptide dans de larges vésicules à cœur dense (ou large dense core vesicles, LDCV) dans le soma, les dendrites, les axones, et les terminaisons axonales des neurones (Lehman et al. 1988; Prevot et al. 1998; Prevot et al. 1999). Ces granules de sécrétion sont préférentiellement acteurs de la voie régulée de sécrétion, et sont donc mobilisés en réponse à un signal externe (Gumbiner & Kelly 1982), par opposition à la voie constitutive de sécrétion qui fait plutôt intervenir des petites vésicules synaptiques (ou small synaptic vesicles, SSV). Les signaux extracellulaires sont convertis en signaux intracellulaires tels qu'une augmentation des taux de calcium (Ca^{2+}) intracellulaire, d'AMPc ou de protéines kinases. L'exocytose dépendante du calcium joue un rôle majeur dans la sécrétion des neurotransmetteurs par les neurones, ainsi que dans la sécrétion des hormones par les cellules endocrines (pour revue, voir Kasai et al. 2012).

Les protéines SNAREs

Les vésicules de sécrétion sont d'abord stockées au niveau de la terminaison axonale, et arrimées à la membrane plasmique au niveau du site d'exocytose. Ce phénomène, encore appelé arrimage, a été décrit pour les LCDV dans les neurones pré-synaptiques (Deák et al. 2009). L'amorçage, qui peut être dépendant des taux de Ca^{2+}, constitue une étape de facilitation, où des protéines cytoplasmiques viennent réguler l'assemblage des protéines liées à la membrane plasmique appelées soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). Il existe une protéine SNARE vésiculaire (v-SNARE) appelée synaptobrévine ou vesicle-associated membrane protein (VAMP), et deux protéines SNAREs cibles de la membrane plasmique (t-SNARE) que sont la syntaxine et SNAP25. Ces trois protéines partagent un motif « SNARE » d'environ 60 acides aminés, qui participe à la formation du complexe SNARE, par interaction des trois protéines (Weimbs et al. 1998; Fasshauer et al. 1998). Quand ces trois protéines sont assemblées, elles forment un complexe tertiaire très stable, constitué par l'association de quatre hélices α (VAMP et syntaxine sont constituées d'une hélice α, SNAP25 de deux), qui va apporter l'énergie nécessaire pour la fusion des deux membranes (Fasshauer et al. 1998). On observe alors un réarrangement des phospholipides membranaires qui laisse place à un pore de fusion, et qui permet une continuité entre le milieu intérieur de la vésicule et le milieu extérieur de la cellule. La cinétique de l'étape de fusion peut être accélérée par l'augmentation de la concentration en Ca^{2+} cytosolique, notamment grâce à la détection du calcium par la synaptotagmine qui est un senseur calcique (Augustine et al. 1999). La protéine VAMP2 est l'isoforme de VAMP préférentielle des neurones. Elle intervient, en

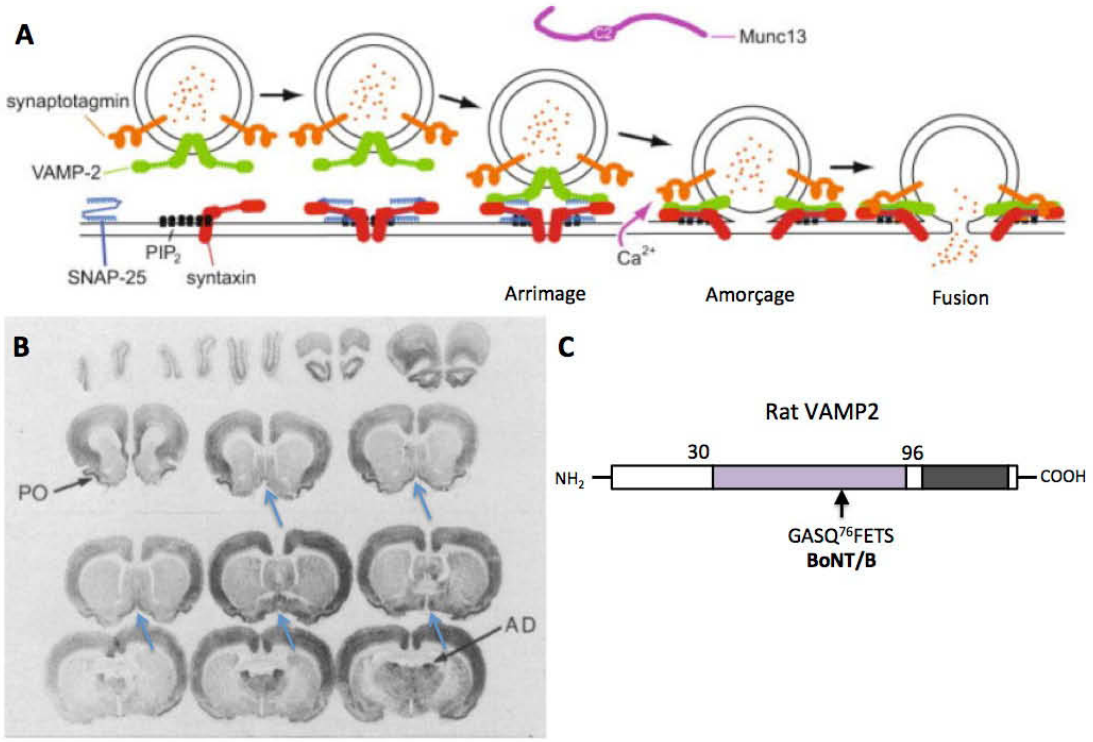


Figure 2. La protéine VAMP2 et son rôle dans l'exocytose. (A) Schéma montrant les étapes d'exocytose calcium-dépendante d'une vésicule. La SNARE vésiculaire représentée par VAMP-2 interagit avec les SNAREs de la membrane plasmique (SNAP-25 et syntaxine). Sous l'effet d'un signal calcique, et notamment la fixation de calcium (Ca2+)/calmoduline au niveau de VAMP2, ces protéines changent de conformation et la synaptotagmine est recrutée au sein du complexe. Cette conformation permet la fusion de la membrane vésiculaire et de la membrane plasmique pour permettre le relargage du neurotransmetteur dans la synapse. D'autres molécules interviennent dans le processus d'exocytose, comme la protéine Munc13 et son domaine C2 calcium-dépendant, ou le phosphatidylinositol diphosphate (PIP2). Adapté de Martin 2012. (B) Photographie aux rayons-X de tranches de cerveau de rat révélant l'expression de *Vamp2* par hybridation *in situ*, dans le cerveau antérieur, des bulbes olfactifs jusqu'à l'hypothalamus médio-basal. La Vamp2 est exprimée dans la région préoptique, incluant le septum médial, l'OVLT et l'AVPV (flèches bleues), région où sont localisés les neurones à GnRH. *Abréviations : PO, cortex olfactif primaire ; AD, noyau thalamic antéro-dorsal.* Adapté de Trimble et al. 1990. (C) Schéma représentant la protéine VAMP2/synaptobrévine 2. Elle possède un court domaine terminal COOH intracellulaire, suivi d'un segment transmembranaire (gris). La partie centrale extracellulaire de la VAMP2 est très conservée en fonction des espèces et des isoformes (violet), et participe à la formation du complexe SNARE avec ses partenaires sur la membrane plasmique. Ce domaine contient plusieurs sites de clivage, dont celui de la toxine botulique de sérotype B (**BoNT/B**). Le domaine NH2 (blanc) ne fait pas parti du complexe SNARE, et est très peu conservé entre les isoformes.

effet, dans l'exocytose de type ultra-rapide de petites vésicules, ainsi que dans l'exocytose lente de granules constituant la voie régulée de sécrétion (Kasai et al. 2012) et est nécessaire à la neurotransmission (Schoch et al. 2001). Cette isoforme est particulièrement exprimée par le cerveau et par l'aire préoptique (Figure 2) (Trimble et al. 1990). Elle est aussi exprimée par les cellules endocrines (Kasai et al. 2012). En plus de son rôle clé dans la neurotransmission, la VAMP2 est impliquée dans la neuritogénèse (Gupton & Gertler 2010), l'orientation chimiotactique de la pousse des projections neuronales (Tojima et al. 2007) et le branchement des axones (C.-L. Wang et al. 2007). Elle intervient aussi dans la sécrétion de protéines à destination de la matrice extracellulaire, et est ainsi impliquée dans la migration neuronale (Vohra et al. 2006). Vamp2 a un rôle modulateur dans ces différents processus, via l'interaction avec d'autres protéines telles que les intégrines, ou la sémaphorine 3A (Gupton & Gertler 2010; Vohra et al. 2006). Les neurotoxines botuliques (BoNT) sont très utilisées dans l'étude des protéines SNAREs. Ce sont des toxines naturelles produites par les bactéries anaérobies de type clostridium et sont responsables d'une pathologie mortelle appelée le botulisme, qui se manifeste par une paralysie musculaire (Erbguth & Naumann 1999; Montecucco & Molgó 2005). Ces toxines sont des enzymes protéases reconnaissant des sites hautement spécifiques des protéines SNARE, et capables de les cliver (Montecucco & Schiavo 1994; Montal 2010). Le sérotype B de la toxine botulique (BoNT/B) est inhibe spécifiquement la sécrétion de neurotransmetteurs par la reconnaissance et le clivage de la protéine VAMP2 (Figure 2C) (Schiavo et al. 1992; Schiavo et al. 2000).

L'exocytose de la GnRH

Une étude de Waters et al. (1998) montre que l'exocytose de la GnRH dans un explant d'éminence médiane est dépendante d'une augmentation de Ca^{2+} intracellulaire, détectée par des protéines liant le Ca^{2+} nommées calmodulines (CAM), qui activent à leur tour des enzymes protéinases K II, dépendantes du complexe Ca^{2+}/CAM. Ce mécanisme est généralement amorcé par l'arrivée d'un potentiel d'action engendrant l'ouverture des canaux calciques voltage-dépendants, permettant une entrée massive de Ca^{2+} dans la cellule (Greengard et al. 1993). En effet, en 2015, l'équipe de Moenter montre qu'un potentiel d'action suivi d'une augmentation de Ca^{2+} intracellulaire est nécessaire à la sécrétion de GnRH par l'éminence médiane, pour assurer son rôle neuroendocrine (Glanowska & Moenter 2015). Il existe toutefois une sécrétion de GnRH indépendante de la présence d'un potentiel d'action, c'est la sécrétion via les dendrites, à proximité du corps cellulaire, au sein de l'aire préoptique. En effet, un rôle central et moins connu de la GnRH a été décrit en tant

que neuromodulateur, notamment d'autorégulation négative de la sécrétion de GnRH, qui agirait en fin de pic préovulatoire (DePaolo et al. 1987). Si on fait la parallèle avec d'autres modèles neuroendocrines (ocytocine, vasopressine), ce mécanisme pourrait être indépendant d'un signal extracellulaire apporté par un ligand (Castel et al. 1996).

La GnRH est ainsi détectée dans la tige hypophysaire (chez le rat, de Greef et al. 1987), dans le sang porte hypothalamo-hypophysaire, mais aussi dans le liquide cérébrospinal du troisième ventricule (chez la brebis, Skinner et al. 1997) notamment à proximité de l'éminence médiane.

3.2 Une sécrétion cyclique et pulsatile

L'une des caractéristiques fondamentales des neurones à GnRH pourrait être leur capacité à générer des potentiels d'action, de façon unitaire ou en bouffées. En effet, sur des coupes de cerveaux ou même en culture, les neurones à GnRH sont spontanément actifs de manière épisodique (Terasawa 1998; Moenter et al. 2003) avec des périodes d'augmentation de décharges, ponctuées par des périodes de quiescence. Ces mécanismes intrinsèques aux neurones à GnRH sont encore peu connus, même s'ils sont décrits comme étant une activité pacemaker intrinsèque, ou encore le « GnRH pulse generator » (Moenter et al. 2003). Chez le mâle comme chez la femelle, la sécrétion pulsatile de GnRH est nécessaire à la fertilité, puisque l'administration continue de GnRH chez le singe rhésus inhibe l'activité de l'axe gonadotrope jusqu'à la suppression de la sécrétion de gonadotrophines et, par conséquent, conduit à une infertilité (Belchetz et al. 1978). Chez la femelle mammifère, les changements de fréquence et d'intensité de sécrétion de la GnRH stimulent la libération de LH ou de FSH à des moments spécifiques du cycle ovarien, créant un environnement favorable au développement folliculaire (Clarke et al. 1987; Marshall & Griffin 1993). Ces paramètres sont sous l'influence du rétrocontrôle des stéroïdes gonadiques. Les œstrogènes ont ainsi été décrits comme ayant un rôle biphasique sur la sécrétion de GnRH, pouvant à la fois inhiber l'activité de décharge des neurones à GnRH à faible concentration, alors que de fortes concentrations physiologiques la stimulent (chez la souris (Chu, Andrade, M. a Shupnik, et al. 2009)). La même équipe montre en 2005 (Christian et al. 2005) que la fréquence et l'intensité de décharge des neurones à GnRH chez des souris ovariectomisées ne changent pas au cours de la journée, alors que les cellules à GnRH des souris ovariectomisées supplémentées en estradiol, montrent de bas niveaux d'activité le matin (rétrocontrôle négatif) et de hauts niveaux d'activité en fin d'après-midi (rétrocontrôle positif). Il a été démontré chez la rate que la survenue du pic préovulatoire de

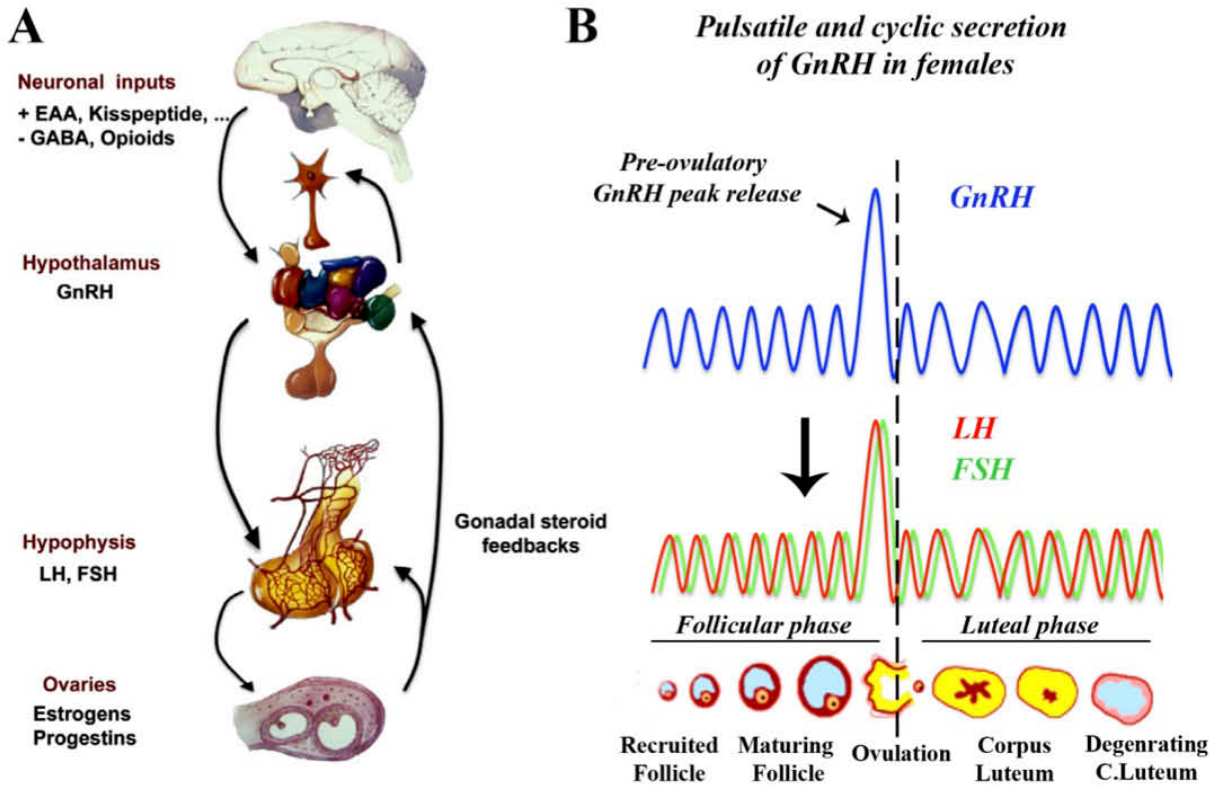


Figure 3. L'axe gonadotrope chez la femelle. (A) Les neurones à GnRH, situés dans l'hypothalamus, constituent la voie finale de régulation de l'axe gonadotrope. Ils sont sujets à la modulation par des facteurs afférents tels que des acides aminés excitateurs (EAA), les kisspeptines, la GABA, les opioïdes, … Les neurones à GnRH envoient leur terminaison axonale au niveau de l'éminence médiane, en apposition avec les capillaires du système porte hypothalamo-hypophysaire, où ils sécrètent la neurohormone. Ainsi, la GnRH stimule la sécrétion de LH et de FSH par les cellules gonadotropes de l'adénohypophyse dans la circulation périphérique. Ces gonadotrophines stimulent la maturation des ovaires, la folliculogénèse, l'ovulation et la synthèse d'estrogènes et de progestérone. Ces hormones stéroïdes agissent par un rétrocontrôle, positif ou négatif, sur la sécrétion de GnRH. Adapté de Prévot et al. (2010). (B) La sécrétion de GnRH est pulsatile et induit la sécrétion de LH et de FSH. Elle est aussi cyclique. Au cours de la phase folliculaire, le profil de sécrétion de FSH permet le recrutement et la maturation de follicules ovariens, qui sécrètent des estrogènes au fur et à mesure de leur croissance. A faible concentration, ces estrogènes exercent un rétrocontrôle négatif sur la sécrétion de GnRH, et de FSH/LH. Les follicules arrivés à maturité sécrètent un maximum d'estrogènes, qui déclenchent alors, par rétrocontrôle positif, une décharge maximale de GnRH dans le système porte hypothalamo-hypophysaire. Ce pic préovulatoire de GnRH engendre la sécrétion d'un pic de LH, responsable de l'ovulation. Pendant la phase lutéale, on observe la lutéinisation du corps jaune (ou corps lutéal), qui dégénèrera, ou deviendra corps progestatif en cas de fécondation.

GnRH est due à une augmentation de l'amplitude des potentiels d'action (Levine & Ramirez 1982). Ce pic de sécrétion massif de GnRH dans le sang porte hypothalamo-hypophysaire précède et induit le pic préovulatoire de LH (Sarkar et al. 1976).

Chez la rate et chez la souris, le cycle ovarien se décompose en 4 phases : le metestrus et le diestrus (ou diestrus 1 et 2) qui sont deux phases durant lesquelles la sécrétion de GnRH est basale, le proestrus caractérisé par un pic préovulatoire de GnRH, qui induit un pic de LH responsable de l'ovulation, qui se produit pendant la dernière phase d'estrus (Figure 3B). D'après Fink & Henderson (1977), le principal facteur responsable de la survenue du pic préovulatoire de GnRH pendant l'après midi du proestrus est directement en lien avec une augmentation des taux d'oestradiol plasmatique entre le soir du diestus 2 et le jour du proestrus, agissant ainsi comme un rétrocontrôle positif sur la sécrétion de GnRH.

4. Les récepteurs à GnRH

La GnRH agit sur les cellules gonadotropes de l'adénohypophyse via des récepteurs à GnRH (GnRHRs) pour stimuler la sécrétion de LH et de FSH. Des mutations inactivatrices du gène *GnRHR* conduisent à une hypogonadisme hypogonadotrope chez l'homme (de Roux et al. 1997). Le clonage de ces récepteurs a permis de mettre en évidence qu'ils appartiennent à la famille des récepteurs à 7 domaines transmembranaires couplés à une protéine G (GPCR) rhodopsine-like (Stojilkovic et al. 1994). Après liaison de la GnRH au niveau des boucles extracellulaires et changement de conformation, ces récepteurs activent en effet la protéine $G_{q/11}$ qui permet l'activation de la phospholipase C (PLC) (Millar et al. 2004). Cette dernière permet l'augmentation des taux de Ca^{2+} intracellulaires et active la protéine kinase C (PKC), essentiels à la synthèse des gonadotrophines ainsi qu'à leur sécrétion (Conn et al. 1987). Les GnRHRs clonés chez les vertébrés peuvent être divisés en 3 groupes. Le plus représenté chez les mammifères est le GnRHR de type I, liant principalement la GnRH-I mais aussi de manière moins spécifique la GnRH-II (pour revue voir Millar et al. 2004). En plus d'être largement exprimés dans les cellules gonadotropes de l'hypophyse, les GnRHRs ont été décrits (souvent assiciés avec la GnRH) dans beaucoup d'autres tissus incluant le cerveau et le système reproducteur (pour revue voir Cheng & Leung 2005).

5. Les gonadotrophines

La LH et la FSH sont synthétisées et sécrétées en réponse à la fixation de la GnRH sur son récepteur, et initient chez la femelle des événements au niveau des cellules cibles des ovaires.

La FSH est nécessaire à la maturation des follicules ovariens. La première phase de développement du follicule ovarien se fait indépendamment des gonadotrophines, puis le follicule commence à exprimer les récepteurs à la FSH au niveau des cellules folliculaires de la granulosa, qui lui permettront de poursuivre sa maturation et de préparer l'expulsion de l'ovocyte (bloqué en prophase de 1^{e} division méiotique, Borum 1961). On observe donc une augmentation progressive de la sécrétion de FSH jusqu'à maturation complète d'un (chez la femme) ou plusieurs (chez les mammifères à portées) follicules. Seul un petit nombre de follicules atteindra la maturité, la majeure partie d'entre eux subissant une mort programmée, encore appelée atrésie folliculaire.

La LH est quant à elle impliquée dans l'ovulation du follicule mature (ou de De Graaf), dans la reprise de division méiotique de l'ovocyte et son ovulation en métaphase 2, et dans la formation du corps lutéal qui prépare l'utérus à la nidation via la sécrétion de progestérone.

Les follicules sont d'abord exposés à des taux de LH peu élevés pendant le processus de maturation. L'augmentation de la production d'androgènes, précurseurs d'autres hormones stéroïdes, permet la production des estrogènes grâce à l'activité aromatase des cellules de la granulosa. En plus de faciliter les actions de la FSH, l'augmentation des taux d'estrogènes agit comme un rétrocontrôle positif sur l'axe hypothalamo-hypophysaire pour induire le pic de LH préovulatoire (pour revue voir Gougeon 2010). Ainsi les gonadotrophines subissent un cycle qui assure la maturation des follicules et leur ovulation. Dans des conditions pathologiques comme le syndrome des ovaires polykystiques, les cellules de la thèque produisent des taux très élevés d'androgènes, ce qui induit une perturbation du cycle des gonadotrophines et interrompt la maturation correcte des follicules (Aghadavod et al. 2015).

6. Les neuropeptides coexprimés par les neurones à GnRH

Au fil des années, plusieurs neuropeptides ont été détectés dans les neurones à GnRH, comme la proopiomélanocortine (POMC) chez le cochon d'Inde (Beauvillain et al. 1981), la galanine (Coen et al. 1990; Merchenthaler et al. 1990), la cholécystokinine (CCK) ou la neurotensine (Ciofi 2000) chez différentes espèces de mammifères. Les neurotransmetteurs glutamate et GABA sont également coexprimés par ces cellules. Nous verrons ici plus en détails les neuropeptides les plus décrits dans les neurones à GnRH du rat et de la souris.

6.1. La Galanine

La galanine est retrouvée en majorité dans les neurones à GnRH de l'aire préoptique. Chez le rat et la souris, il existe un dimorphisme sexuel puisque près de 70% des neurones à GnRH des femelles expriment la galanine contre 20% des neurones des mâles (Merchenthaler et al. 1990; Rajendren & Gibson 1999). La synthèse de la galanine par les neurones à GnRH est dépendante de l'activité et/ou des stéroïdes gonadiques, avec une expression maximale le jour du proestrus, au moment du pic de GnRH/LH (Marks et al. 1994; Marks et al. 1993) mais aussi une augmentation de l'expression de la galanine au moment de la puberté (Rossmanith et al. 1994). La galanine est une molécule utilisée dans la signalisation du métabolisme énergétique et de la reproduction et est sécrétée par beaucoup d'autres neurones. Les neurones à GnRH eux-mêmes perçoivent la galanine comme un signal excitateur (Todman et al. 2005). Elle pourrait avoir un rôle autocrine, éventuellement modulateur, dans la facilitation de la sécrétion de la GnRH au moment du pic préovulatoire.

6.2 Le Glutamate

Le glutamate est le principal neurotransmetteur excitateur du système nerveux central (Storm-Mathisen et al. 1983), capable d'influencer tous les types de neurones endocrines hypothalamiques (van den Pol et al. 1990). Le marquage immunohistochimique des protéines VGLUT (1, 2, 3), qui sont les protéines de transport vésiculaire du L-glutamate, met en évidence l'existence d'axones glutamatergiques innervant les neurones à GnRH au niveau de l'aire préoptique (Kocsis 2003). Les neurones à GnRH sont en effet directement et indirectement sensibles au glutamate (voir chapitre 2, 2.2.2). La protéine VGLUT 2 a également été détectée par immunohistochimie et microscopie confocale directement dans

les neurones à GnRH (Hrabovszky et al. 2004). Plusieurs récepteurs ionotropiques au glutamate, notamment le récepteur au NMDA de type 1 et le récepteur au kainate de type 2, sont également détectés au niveau des terminaisons axonales des neurones à GnRH (Yin et al. 2007), et même sur les mêmes vésicules à cœur dense qui contiennent la GnRH. De plus, l'infusion d'explants d'éminence médiane avec du glutamate induit la sécrétion de GnRH dans le milieu (Kawakami et al. 1998). Ces données mettent en évidence un rôle complexe du glutamate sur le contrôle de la sécrétion de la GnRH par différents mécanismes stimulateurs, mais aussi modulateurs, probablement par une action autocrine/paracrine, localement, au niveau de l'éminence médiane (pour revue voir Hrabovszky & Liposits 2008).

6.3 Le GABA

L'acide gamma-amino butyrique (GABA) est le neurotransmetteur inhibiteur principal du cerveau. Les taux de GABA sont étroitement liés à ceux du glutamate, puisque le GABA est synthétisé à partir du glutamate via l'action de l'enzyme glutamic acid decarboxylase (GAD). Le rôle fonctionnel de l'expression de GABA par les neurones à GnRH est peu connu. L'étude d'un modèle murin dans lequel la protéine GAD-67, est sélectivement surexprimée sous le contrôle du promoteur GnRH montre une importance cruciale du GABA exprimé dans les cellules à GnRH dans leur migration mais aussi dans le contrôle de la sécrétion de la GnRH post-natale. On observe en effet chez ces souris une migration aberrante des cellules dans le nez, sans toutefois affecter le nombre de cellules dans l'hypothalamus. Ce phénotype est lié à une augmentation de la fréquence des pulses de GnRH ainsi qu'à des taux de gonadotrophines élevés pendant les premières semaines de vie post-natale, et une fonction de reproduction altérée chez les femelles adultes (Heger et al. 2003a).

6.4 La Cholecystokinine et la neurotensine

L'expression de la cholecystokinine (CCK) et de la neurotensine (NT) est décrite dans des aires neuroendocrines de l'hypothalamus (Meister 1993; Micevych et al. 1997; Simerly 1998). Ces protéines ont aussi été détectées par immunohistochimie dans les neurones à GnRH uniquement chez la rate (Ciofi 2000). En effet, environ 55% des neurones à GnRH de l'OVLT, de la région préoptique, et de la région suprachiasmatique, expriment également la CCK, et 30% expriment à la fois la CCK et la NT chez la rate. Ce dimorphisme est probablement lié à la nature des stéroïdes synthétisés, qui permettrait le maintien, ou non, de l'expression de ces deux peptides (Ciofi 2000). Ils seraient sécrétés par les terminaisons à

GnRH et pourraient agir localement au niveau de l'éminence médiane, ou au niveau de l'hypophyse. Néanmoins, la CCK et la NT n'ont pas été retrouvées dans les neurones à GnRH chez la souris.

7. Les signaux nerveux et hormonaux contrôlant le système à GnRH

7.1 L'action des estrogènes sur les neurones à GnRH

Il est maintenant bien établi que les neurones à GnRH n'expriment que l'isoforme béta du récepteur aux estrogènes (ERβ) (chez la souris (Skynner et al. 1999), chez le rat (Hrabovszky et al. 2000), ainsi que chez l'Homme (Hrabovszky et al. 2007)). C'est pourtant l'isoforme alpha (ERα) qui a été démontrée comme nécessaire et suffisante pour le mécanisme de rétrocontrôle positif des estrogènes sur l'axe gonadotrope chez la souris. En effet, la lignée de souris ERαKO globale est totalement infertile avec des taux de gonadotrophines sévèrement affectés, voire absents, alors que les mutants ERβKO ont un phénotype plus discret, avec différents degrés de subfertilité (Krege et al. 1998; Dupont et al. 2000), et des pics de LH observables (Wintermantel et al. 2006). ERβ n'est en effet pas essentiel à la régulation de la libération de GnRH via les estrogènes (Dorling et al. 2003). Il apparaît toutefois que ERβ pourrait médier des effets rapides dans les neurones à GnRH (Moenter & Chu 2012), en modulant des canaux potassiques (K+)/adénosine tri-phosphate (ATP) (Zhang et al. 2010) ou en altérant leur vitesse de décharge (Chu, Andrade, M. A. Shupnik, et al. 2009; Romanò & Herbison 2012). ERβ semble aussi être impliqué dans la régulation stéroïde-dépendante de la synthèse de galanine par les neurones à GnRH (Merchenthaler et al. 2005).

L'absence de ERα dans les cellules à GnRH implique donc que les estrogènes agissent sur les neurones à GnRH par des voies indirectes. Le marquage de ERα a montré que les neurones susceptibles d'être impliqués dans le rétrocontrôle positif estrogeno-dépendant étaient concentrés principalement dans 3 noyaux hypothalamiques : le noyaux antéroventral périventriculaire (AVPV), le noyaux périventriculaire de l'aire préoptique (PVpo), et l'aire préoptique médiale (mPOA/RP3V) (Herbison 2008). Un plus petit nombre de neurones a aussi été trouvé au sein du noyaux arqué (ARC) (Wintermantel et al. 2006). Ces neurones comprennent des neurones exprimant le glutamate (Eyigor et al. 2004), le GABA (Herbison 1997), la dynorphine, l'enképhaline (Simerly 1991), la galanine (Bloch et al. 1992), la substance P (Okamura et al. 1994), le « calcitonine gene-related peptide » (CGRP) (Allan E.

Herbison & Theodosis 1992), la kisspeptine (Clarkson et al. 2008; Smith et al. 2006) et la neurotensine (Herbison & Theodosis 1992). Toutefois l'avancée la plus considérable a été réalisée grâce aux travaux de Campbell et al. en 2007, qui ont permis de mettre en évidence les neurones exprimant ERα et faisant directement synapse avec les neurones à GnRH. La population de cellules ayant un rôle critique dans le rétrocontrôle estrogéno-dépendant n'est pas encore bien déterminée. Cependant les neurones à kisspeptine, les neurones glutamatergiques et les neurones GABAergiques sont les principaux candidats pour ce rôle.

La kisspeptine et le modèle des « neurones KNDy »

Il devient de plus en plus évident que les neurones à kisspeptine constituent une afférence très importante pour les neurones à GnRH. Ils sont essentiels à leur fonctionnement normal et à leur activité en réponse à une variété de stimuli incluant le rétrocontrôle estrogénique. La kisspeptine est une protéine de 53 acides aminés, codée par le gène KiSS1 (Roa et al. 2008; Oakley et al. 2009), qui par clivage enzymatique donne différents produits dont la kisspeptine-10 (de 10 acides aminés) qui semble être très impliquée dans la fonction de reproduction (Gottsch et al. 2004). Les kisspeptines sont de puissants stimulateurs de la sécrétion de GnRH. L'administration centrale ou périphérique de kisspeptine entraine la sécrétion de LH et de FSH à des doses maximales (Gottsch et al. 2004; Thompson et al. 2004). De fait, la majorité des neurones à GnRH expriment le récepteur de la kisspeptine, la protéine GPR54 (Irwig et al. 2004). Le rôle crucial de la signalisation KiSS1/GPR54 est d'abord mis en évidence par la découverte d'une déletion et d'une mutation inactivatrice du gène GPR54 chez des patients souffrant d'un hypogonadisme hypogonadotrope (HH) (de Roux et al. 1997; Seminara et al. 2003; de Roux et al. 2003). Parallèlement, l'inactivation du récepteur GPR54 chez une lignée de souris conduit au même phénotype que celui observé chez l'homme (Funes et al. 2003). Ce phénotype étant lié à un défaut de sécrétion de GnRH, il semblerait alors que le récepteur GPR54 et son ligand, la kisspeptine, soient impliqués dans une régulation située en amont de la sécrétion de GnRH, comme par exemple dans le « pulse generator », et pour le moins essentiel à son fonctionnement. D'autres expériences ont mis en évidence un rôle crucial de la kisspeptine dans le contrôle de la sécrétion de GnRH et de gonadotrophines, par des études de gonadectomie. L'élimination des facteurs gonadiques chez le rat, mâle et femelle, conduit non seulement à une augmentation des taux de gonadotrophines, mais encore à une augmentation de l'expression du gène KiSS1 dans l'hypothalamus (V. M. Navarro et al. 2004; Irwig et al. 2004). Les neurones à kisspeptine sont subdivisés en deux populations chez

le rongeur, au vu de leur localisation au sein de deux noyaux distincts : l'AVPV et l'ARC (Gottsch et al. 2004; Irwig et al. 2004), alors que chez la femme et chez le singe on les trouve uniquement dans l'ARC (Rometo et al. 2007).

La population de neurones à Kisspeptine de l'AVPV projette directement ses axones sur les neurones à GnRH (Wintermantel et al. 2006) et sont activés en même temps que les neurones à GnRH, au moment du pic préovulatoire, comme il a été démontré grâce à des marquages immunohistochimiques ciblant cFos (Smith et al. 2006; Clarkson et al. 2008). Ces données sont consistantes avec des données plus récentes montrant une forte activité des neurones à Kisspeptine avant le proestrus, et une diminution de cette activité après le pic de GnRH, dans cette région (de Croft et al. 2012). Cette population aurait donc un rôle préférentiel dans l'activation des neurones à GnRH pour générer le pic préovulatoire de GnRH/LH, et est donc impliquée dans le rétrocontrôle positif médié par les estrogènes.

Les neurones à kisspeptine de l'ARC projettent leurs axones vers l'aire préoptique (Caron et al. 2012), mais ils ne semblent pas être en contact direct avec les neurones à GnRH (Kalló et al. 2012; True et al. 2011). La voie par laquelle ces neurones sont impliqués dans l'apparition de la puberté, et dans le « pulse generator » n'est pas encore clairement établie. La biosynthèse de la kisspeptine par les neurones de l'ARC est régulée par les estrogènes, de manière opposée à celle des neurones de l'AVPV. Une gonadectomie augmente l'expression du gène Kiss1, ce qui pourrait suggérer que la population de neurones à kisspeptine de l'ARC serait impliquée dans le rétrocontrôle négatif médié par les estrogènes. Néanmoins, une étude plus récente montre que ces neurones sont électriquement totalement silencieux pendant le diestrus, comme chez des femelles ovariectomisées (de Croft et al. 2012). Ces données infirmeraient l'hypothèse du rôle de cette population de neurones à kisspeptine dans le rétrocontrôle estrogénique négatif.

Les neurones à Kisspeptine de l'ARC ont un haut degré de colocalisation avec la neurokinine B (NKB) e la Dynorphine (Dyn) chez plusieurs espèces de mammifères, comme le mouton (Goodman et al. 2007), la souris (Navarro et al. 2009), la chèvre (Wakabayashi et al. 2010) et le rat (Mittelman-Smith et al. 2012; Ciofi et al. 2006). La NKB est en effet aussi impliquée dans le contrôle de la fonction de reproduction (Topaloglu et al. 2009) et en particulier au niveau de la modulation de la sécrétion de la GnRH au niveau de l'éminence médiane (Krajewski et al. 2005). Une étude chez le rat montre le rôle essentiel des « neurones KNDy » dans la sécrétion de la LH et de la FSH, et en particulier son implication

dans le rétrocontrôle négatif des estrogènes dans le système à GnRH, puisque la suppression spécifique de cette population empêche l'augmentation des taux de LH plasmatiques suite à une ovariectomie (Mittelman-Smith et al. 2012). Cette population de neurones semble également impliquée dans la modulation du poids corporel par les estrogènes, puisque sans elle, les rates ovariectomisées ne présentent pas d'augmentation de poids, comparé à leurs homologues uniquement ovariectomisées (Mittelman-Smith et al. 2012). La présence des neurones KNDy chez l'Homme semble toutefois être très variable en fonction de l'âge et du sexe (Hrabovszky et al. 2012; Hrabovszky et al. 2010).

Le Glutamate

Comme précisé précédemment (Chapite 1, 6.2), les neurones glutamatergiques sont présents dans la région préoptique (Clements et al. 1990; Dumalska et al. 2008), et projettent leurs axones vers les corps cellulaires des neurones à GnRH (Kiss et al. 2003). De plus, les neurones à GnRH expriment les récepteurs au glutamate AMPA, NMDA et kainate, et sont sensibles au glutamate (Iremonger et al. 2010). Des études montrent qu'il existerait une augmentation du nombre de synapses glutamatergiques au moment du pic préovulatoire de GnRH/LH (Ottem et al. 2004), ainsi qu'une augmentation du taux de glutamate à proximité des corps cellulaires à GnRH chez la rate au même moment (Jarry et al. 1995). La majorité des neurones glutamatergiques chez le rat expriment en effet ERα, mais aussi ERβ (Moore & Evans 1999; Kiss et al. 2013). Les estrogènes activeraient, en effet, les neurones glutamatergiques pour stimuler les neurones à GnRH au cours du rétrocontrôle positif (Tada et al. 2013).

Le GABA

Le GABA joue un rôle majeur dans le système à GnRH post-natal (ainsi que dans le déclenchement de la puberté, voire chapitre 2, 2.2.3). Il agit très probablement via la présence de terminaisons GABAergiques, contactant directement les neurones à GnRH dans l'aire préoptique (Leranth et al. 1985; Witkin 1992) ainsi que leurs terminaisons chez la souris (Cottrell et al. 2006), et via l'expression des récepteurs au GABA de type A et B par les neurones à GnRH (Jung et al. 1998; Herbison & Moenter 2011; Liu & Herbison 2011). D'après des études récentes, les neurones GABAergiques inervant les neurones à GnRH proviendraient de l'AVPV (Liu et al. 2011), l'aire préoptique latérale (Penatti et al. 2010), ou encore les noyaux supraoptiques (Christian & Moenter 2007). Différentes études montrent que les niveaux de GABA à proximité des corps cellulaires des neurones à GnRH sont élevés

le matin du proestrus chez le rat, puis diminuent rapidement dans l'après midi, juste avant le pic préovulatoire de GnRH/LH (Jarry et al. 1995; Mitsushima et al. 2002; Christian & Moenter 2007). L'effet exact du GABA sur la sécrétion de GnRH est pour le moins controversé (Herbison & Moenter 2011; Constantin et al. 2012). Le GABA exercerait un effet inhibiteur puissant et probablement direct sur les neurones à GnRH (Jarry et al. 1991; Scott & Clarke 1993; Mitsushima et al. 1994) et une diminution de son action est nécessaire à la survenue du pic de GnRH/LH (Herbison & Dyer 1991). Toutefois des études *in vitro* montrent que le GABA est capable de stimuler la sécrétion de GnRH (Moenter & DeFazio 2005), via la stimulation du récepteur au GABA de type A (DeFazio et al. 2002). Ces effets opposés pourraient être médiés par la signalisation via des récepteurs différents, notamment l'effet inhibiteur du GABA sur les neurones à GnRH ferait suite à l'activation du récepteur au GABA de type B (Liu et al. 2011). Les cellules GABAergiques de la région préoptique sont également sensibles aux estrogènes (Flügge et al. 1986; Herbison 1997). Il semblerait d'après une étude de Ottem et al. (2004) qu'une sous-population de neurones glutamatergiques et GABAergiques de l'aire préoptique sensibles aux estrogènes sont en fait une seule et même population de neurones exprimant les deux neurotransmetteurs.

D'après les données précédentes, il apparaît donc évident que les estrogènes jouent un rôle essentiel dans le contrôle du fonctionnement des neurones à GnRH. Ces estrogènes sont aussi connus pour avoir un impact important dans le contrôle du métabolisme et de l'homéostasie énergétique (Clegg 2012; Xu et al. 2011; Gao et al. 2007). L'ovariectomie chez le rongeur provoque une importante augmentation du poids corporel (Mauvais-Jarvis et al. 2013), alors que la perte des estrogènes, notamment chez la femme ménopausée, peut aussi conduire à des problèmes de prise de poids (Mauvais-Jarvis et al. 2013). Ces effets sont depuis longtemps reconnus mais toutefois mal caractérisés. Des études récentes qui tentent de trouver la région du cerveau faisant le lien entre balance énergétique et fonction de reproduction cibleraient l'ARC, pour la suppression de la prise alimentaire, et le VMH, pour l'action des estrogènes sur la thermogenèse (Xu et al. 2011).

7.2 L'action de la progestérone sur les neurones à GnRH

La progestérone est l'hormone stéroïde ovarienne dominante dans la circulation périphérique au cours du cycle de reproduction des mammifères. Les taux de progestérone augmentent pendant la phase lutéale qui suit l'ovulation. Cette augmentation inhibe la sécrétion pulsatile de GnRH et de LH en diminuant la fréquence de sécrétion (Goodman &

Karsch 1980; Goodman et al. 1981; Karsch et al. 1987), et prévient l'apparition d'un autre pic de sécrétion en réponse aux fluctuations d'estrogènes qui accompagnent les vagues de croissance folliculaire (Kasa-Vubu et al. 1992; Scaramuzzi et al. 1971; Dierschke et al. 1973). Il existe peu d'études focalisées sur les rongeurs concernant l'effet de la progestérone sur le contrôle de la sécrétion de GnRH, et la plupart des études ont été faites chez le mouton. Les quelques études sur les rongeurs montrent que l'inhibition de la synthèse des stéroïdes gonadiques bloque l'ovulation, et que la supplémentation en progestérone la restaure (Lipner et al, 1988). De plus, un modèle murin transgénique chez qui le récepteur à la progestérone (PR) est supprimé ne montre aucune ovulation (Lydon et al. 1995). Pendant la période de diestrus chez le rongeur, quand les taux de progestérone sont élevés, la progestérone stimule la sécrétion de Sema7A par les tanycytes au niveau de l'éminence médiane, et participe ainsi à l'expansion des pieds tanycytaires, qui recouvrent les terminaisons à GnRH. La progestérone aurait ainsi un rôle dans le contrôle de la sécrétion de GnRH, en limitant l'accès de la neurohormone à l'espace péricapillaire (Parkash et al. 2015). Chez la femme, la progestérone représente le régulateur majeur de la fréquence des pics de GnRH, avec la même action suppressive que vu précédemment (Gill et al. 2002; Soules et al. 1984).

7.3 L'action des facteurs orexigéniques et anorexigéniques circulants sur l'activité des neurones à GnRH

Il n'est pas surprenant que les peptides orexigènes et anorexigènes aient un rôle dans la fonction de reproduction, puisque la fonction de reproduction ainsi que son développement sont fortement liés à l'état énergétique de l'individu. Ainsi des perturbations métaboliques chez l'homme mais aussi chez les modèles animaux (surpoids, obésité et anorexie) peuvent avoir de lourdes répercussions sur la fonction de reproduction (subfertilité voire infertilité).

Le rôle crucial de la leptine

La leptine est une hormone périphérique synthétisée par les adipocytes, faisant état des stocks énergétiques disponibles dans le corps. Cette hormone a un effet satiétogène sur le cerveau (voir Meister 2000 pour revue). Via la forme longue de son récepteur, situé notamment dans l'ARC, la leptine contrôle la prise alimentaire et la balance énergétique. Elle active la production du peptide a-melanocyte-stimulating hormon (αMSH) par les neurones à POMC (Korner et al. 1999), ayant un effet anorexigéne (satiétogène), et inhibe, d'autre

part, la production de neuropeptide Y (NPY) (Schwartz et al. 1996), qui a quant à lui un effet orexigéne.

La leptine a un rôle crucial dans la fonction de reproduction. Toutefois, les récepteurs à la leptine (LepRs) ne sont pas exprimés par les neurones à GnRH. Ils sont exprimés dans l'hypothalamus et par l'hypophyse antérieure (Zamorano et al. 1997). Les LepR sont aussi coexprimés par les cellules présentant les ERs dans la région préoptique, l'ARC et l'AVPV, ce qui suggère que la leptine pourrait agir via la même circuiterie que les estrogènes pour moduler les processus neuroendocrines concernant la fonction de reproduction (Diano et al. 1998). La leptine agit comme un signal permissif autorisant le système à accomplir la maturation pubertaire lorsque les ressources métaboliques sont jugées adéquates à un tel changement (voir chapitre 2, partie 2). Chez l'adulte, elle stimule, en effet, la sécrétion de LH et de FSH *in vitro* (Yu et al. 1997) et *in vivo* (Watanobe 2002), et stimule même directement la sécrétion de GnRH (Watanobe 2002; Woller et al. 2001). L'évidence du rôle crucial de la leptine dans la fonction de reproduction est aussi illustrée par les modèles murins présentant une mutation spontanée du gène codant pour la leptine (gène *Lep*, souris *ob/ob*) ou pour son récepteur (gène *Lepr*, souris *db/db*) qui développent une hyperphagie, une obésité morbide et une infertilité (Halaas et al. 1995; Farooqi 2002; Begriche et al. 2008). L'infertilité n'est toutefois pas due à la prise de poids, car la perte de poids n'est pas suffisante à restaurer la fonction de reproduction chez ces animaux. Chez des modèles animaux obèses, ou même encore chez l'homme, les taux de leptine sont paradoxalement élevés, mais sont en fait liés à une leptinorésistance centrale (Frederich et al. 1995) et il a été montré, plus récemment, un défaut d'accès de la leptine au cerveau (Balland et al. 2014). Des souris soumises à 48H de jeûne voient leurs taux de leptine chuter, et on constate parallèlement une diminution des taux de LH et une augmentation de la durée du cycle ovarien. Par contre, une administration chronique de leptine au cours du jeûne augmente les taux de LH et prévient l'effet sur le cycle ovarien (Ahima et al. 1996).

L'existence d'une connexion entre leptine et kisspeptine dans la modulation du système à GnRH a longtemps été suspectée et discutée par plusieurs études (Smith et al. 2006). Cette hypothèse s'essouffle avec la preuve que très peu des neurones à kisspeptine expriment en fait le LepR (Cravo et al. 2011). Elle est finalement totalement infirmée par l'équipe d'Elias, qui montre que la suppression du gène *Lepr* dans les neurones à kisspeptine n'a aucun effet sur la puberté ni la fertilité (Donato et al. 2011a).

Les neurones exprimant la neuronal Nitric Oxide Synthase (nNOS), enzyme impliquée dans la synthèse de monoxyde d'azote (NO), qui est un gaz ayant une fonction de neurotransmetteur, semblent être particulièrement impliqués dans le mécanisme de signalisation de la leptine. En effet d'après l'étude de Bellefontaine et al. (2014), la leptine est capable d'activer les neurones à nNOS de la région préoptique, concomitamment à une augmentation des taux de LH. Par ailleurs, le blocage de la nNOS par un inhibiteur spécifique infusé centralement, empêche la leptine d'induire un pic de LH. Ces données mettent en évidence un rôle central des neurones à nNOS (pour plus de détails se référer au chapitre 1, 1.7.4) dans la transmission du signal engendré par la leptine dans le contrôle de la fertilité.

Les noyaux prémammilaires (PMV) sont des noyaux hypothalamiques contenant une grande proportion de neurones exprimant le récepteur à la leptine, sensibles à la leptine, et ont été depuis peu décrits comme cible préférentielle de la leptine dans la modulation de puberté et de la fertilité (Elias et al. 2000; Donato et al. 2011; Donato & Elias 2011). La lésion des PMV interrompt la cycle ovarien chez la rate (Donato et al. 2009), et la réexpression sélective des LepR dans les PMV chez des souris *db/db* est capable d'induire la puberté (Donato et al. 2011). Ces neurones coexpriment d'autres neurotransmetteurs, notamment le glutamate et le NO, et contactent directement les neurones à GnRH et les neurones à kisspeptine (Leshan et al. 2009; Donato et al. 2010). Ces données indiquent que les PMV pourraient être impliqués dans la stimulation des neurones à GnRH et des neurones à kisspeptine par la leptine, et ont très probablement un rôle clé dans le contrôle de la fonction de reproduction par la leptine (Elias & Purohit 2013).

Des études récentes montrent l'importance des neurones GABAergiques dans le contrôle de la fonction de reproduction par la leptine. En effet, la suppression du LepR dans les neurones exprimant le GABA chez la souris conduit à un retard pubertaire, à une acyclicité et à une subfertilité (Zuure et al. 2013; Martin et al. 2014). Le LepR dans les neurones GABAergiques serait ainsi nécessaire à l'induction du pic préovulatoire de GnRH. Ces mêmes études démontrent, à l'inverse, que la suppression du LepR dans les neurones glutamatergiques n'affecte pas la fonction de reproduction. L'effet du senseur GABAergique, et non glutamatergique, du signal adipeux dans le contrôle de l'axe gonadotrope pourrait passer par l'activation des neurones à kisspeptine (Martin et al. 2014). Toutes ces populations de neurones suspectées dans la signalisation de la leptine ne se suffisent

probablement pas à elles-mêmes et sont en étroite relation dans le contrôle de la fonction de reproduction.

L'insuline

L'insuline est l'hormone périphérique pancréatique principale impliquée dans le contrôle de la glycémie (Hirsch 1999), et a un rôle majeur dans la régulation de la fonction de reproduction (Pralong 2010). Elle est, tout d'abord, une hormone régulatrice majeure de la synthèse de leptine, elle est donc très liée à son action biologique (Cantley 2014). L'insuline joue aussi un rôle stimulateur de l'axe gonadotrope (Gamba & Pralong 2006 pour revue), comme le montrent les cas pathologiques présentant des taux d'insuline bas, comme par exemple les cas de diabètes non-contrôlés, qui sont associés à des taux de gonadotrophines bas, voire nuls, et à une subfertilité (Pralong 2010). Un modèle murin transgénique qui n'exprime pas le récepteur à l'insuline (IR), sélectivement dans les neurones, développe une obésité hyperphagique, mais aussi un hypogonadisme lié à une déficience en GnRH (Bruning 2000). La suppression de IR dans les neurones à GnRH spécifiquement n'est, toutefois, pas suffisante pour empêcher le déclenchement de la puberté ou affecter la fonction de reproduction chez les souris, mâles comme femelles (Divall et al. 2010). L'insuline a une structure voisine du facteur de croissance insuline-like growth factor-1 (IGF-1) qui semble avoir un rôle direct sur les cellules à GnRH. L'élimination du récepteur IGF-1 dans les neurones à GnRH conduit à une puberté retardée chez le mâle et chez la femelle (Divall et al. 2010). Le rôle précis de l'insuline dans le contrôle de la fonction de reproduction n'est toutefois pas encore bien décrit.

La ghréline

La ghréline a, tout d'abord, un rôle dans la régulation de la balance énergétique et dans la prise alimentaire. Elle est sécrétée par l'estomac et est le seul facteur circulant connu à ce jour comme ayant une action orexigène (Cummings & Overduin 2007). Les taux de ghréline sont d'ailleurs négativement corrélés à l'index de masse corporel (BMI pour body mass index) (van der Lely et al. 2004), et opèrent comme un signal à long-terme d'insuffisance énergétique. Elle a donc un rôle opposé à celui de la leptine, qui est un signal d'abondance énergétique, avec qui elle interagit (Zigman & Elmquist 2003). La ghréline a donc un rôle inhibiteur sur l'axe gonadotrope, par opposition au rôle permissif de la leptine. Elle a un rôle important dans l'apparition de la puberté, puisqu'une augmentation des taux de ghréline, pendant cette période critique, supprime les taux de gonadotrophines

circulantes, et provoque un retard pubertaire chez la souris (Tena-Sempere 2008b; Tena-Sempere 2008a). Inversement les taux de ghréline chez l'homme chutent au moment de la puberté (Soriano-Guillén et al. 2004). Chez la femelle rongeur, l'injection centrale ou périphérique de ghréline induit l'inhibition de LH, de FSH (Fernández-Fernández et al. 2005; Furuta et al. 2001; Fernández-Fernández et al. 2004) ainsi que des taux d'estrogènes et de progestérone (Fang et al. 2012).

Les orexines

Les neuropeptides orexigéniques centraux peuvent aussi affecter la fonction de reproduction. C'est le cas des orexines A et B, clivées à partir d'un précurseur commun : la prépro-orexine (de Lecea et al. 1998). Les axones exprimant l'orexine A ont été trouvés à proximité des corps cellulaires des neurones à GnRH, alors que son récepteur, l'orexine-récepteur 1, est exprimé dans les cellules à GnRH (Campbell et al. 2003). Il apparaît que la quantité d'orexine A dans l'hypothalamus est augmentée spécifiquement pendant la phase de proestrus chez le rat (Porkka-Heiskanen et al. 2004). L'orexine A induit la sécrétion de GnRH à partir d'explants hypothalamiques de rates prélevés en proestrus (Russell et al. 2001). Ces peptides ont donc probablement un rôle dans la stimulation du système à GnRH au cours de certaines périodes du cycle.

7.4 Les autres facteurs neuronaux impliqués dans le contrôle de la fonction de reproduction

Les monoamines

Les monoamines sont des neurotransmetteurs dérivés d'acides aminés. On distingue notamment les catécholamines (qui comprennent l'adrénaline, la noradrénaline et la dopamine) et les tryptamines (dont la sérotonine) respectivement dérivés de la tyrosine et du tryptophane.

Le rôle des catécholamines, et principalement de la noradrénaline dans la régulation du cycle ovarien, a été décrit depuis très longtemps (Sawyer & Hollinshead 1947). Il a été notamment montré que différents antagonistes du récepteur α-adrénergique bloquaient l'ovulation (Everett et al. 1949; Sawyer et al. 1949). Les neurones à catécholamines du tronc cérébral seraient des cibles du rétrocontrôle par les stéroïdes, puisque la plupart d'entre eux expriment le gène d'activation précoce cfos en même temps que l'initiation du pic de LH

(Lee et al. 2000). Une partie de ces neurones expriment ERα et envoient leurs projections vers l'aire préoptique hypothalamique. Il existe énormément de publications quant à l'effet potentiel des catécholamines sur la sécrétion de GnRH, qui diffèrent toutefois en fonction des techniques utilisées et du modèle animal. De manière générale, il apparaît que la noradrénaline aurait plutôt un effet stimulateur sur le système à GnRH (Anselmo-Franci et al. 1999), qui pourrait être direct (Lee et al. 1995), ou indirect en facilitant la transmission d'autres neurotransmetteurs (Herbison 1998). Bien que le rôle de l'adrénaline reste controversé et surtout difficile à dissocier de celui de la noradrénaline, il semblerait que l'adrénaline stimule la pulsatilité et la sécrétion de la GnRH (Smith & Jennes 2001). Les effets de la dopamine, quant à elle, restent difficiles à interpréter. Elle stimule la sécrétion de GnRH issue de fragments hypothalamiques chez le rat (Rotsztejn et al. 1976) mais semble avoir des effets différents en fonction des espèces étudiées. Chez la brebis, la dopamine est toutefois connue pour être le principal médiateur du rétrocontrôle négatif médié par les estrogènes sur la sécrétion de GnRH pendant la période d'anoestrus (Herbison 1998).

La sérotonine montre aussi des effets différents sur le système à GnRH en fonction des études. La majorité des résultats sont cependant en faveur d'un effet stimulateur de la sérotonine sur le pic de LH préovulatoire (Rasmussen et al. 1981). Parce que la sécrétion de sérotonine est diurne et cyclique, elle pourrait intervenir dans le contrôle du rythme circadien dans le déclenchement du pic préovulatoire de GnRH/LH (Héry et al. 1982; Héry et al. 1978).

Les peptides opioïdes endogènes

Les dynorphines, les enképhalines et la β-endorphine sont des dérivés opioïdes endogènes et agissent via les récepteurs de type μ, δ et κ, qui sont couplés aux protéines G. Les dérivés opioïdes endogènes ont été démontrés comme impliqués dans l'inhibition de la sécrétion de GnRH, et par conséquent de LH, au cours du cycle ovarien (Ching 1983). Il semblerait que ces peptides jouent un rôle dans le rétrocontrôle médié par les stéroïdes gonadiques. Une partie des neurones à β-endorphine possède en effet ERα (Simonian et al. 1999), et leur action inhibitrice serait alors levée au moment du pic préovulatoire de GnRH/LH sous l'action des estrogènes (Petersen et al. 1993). Bien que moins étudiés, les autres peptides opioïdes semblent avoir des effets similaires. L'expression de prodynorphine et de proenképhaline diminue en effet en phase de proestrus chez la rate (Simerly et al. 1996).

Le Neuropeptide Y (NPY)/Agouti-related peptide (AgRP)

Le NPY est un peptide orexigène sécrété par une population de neurones de l'ARC, coexprimé avec le peptide Agrp (Hahn et al. 1998), qui favorise l'appétit et la prise alimentaire. Le NPY stimule, par ailleurs, la sécrétion de LH et de FSH *in vivo* (Bauer-Dantoin et al. 1993; Freeman 1993; Sutton et al. 1988) et de GnRH à partir d'hypothalamus médiobasal *in vitro* (Crowley & Kalra 1987). De plus les taux de LH sont atténués chez des souris déficientes en NPY (Xu et al. 2000). Les neurones à NPY établissent en effet des connexions synaptiques directes avec les corps cellulaires de neurones à GnRH (Li et al. 1999). Le rôle du NPY dans la stimulation des neurones à GnRH apparaît toutefois complexe, et dépend du récepteur mobilisé (de Y1 à Y5) (Raposinho et al. 1999; Roa & Herbison 2012). Les différentes études existantes sont plutôt en faveur d'un rôle neuromodulateur du NPY sur la sécrétion de GnRH. Ainsi le NPY pourrait jouer un rôle dans un premier temps dans le déclenchement du pic préovulatoire de GnRH/LH, puis dans son atténuation, ce qui serait consistant avec le fait qu'il participerait au rétrocontrôle médié par les estrogènes (Simonian et al. 1999). Le peptide AgRP a, lui aussi, des rôles variables, tantôt excitateurs, tantôt inhibiteurs, sur différentes populations de neurones à GnRH (Roa & Herbison 2012).

La proopiomélanocortine (POMC)/a-melanocyte-stimulating hormone (aMSH)

Les neurones à POMC, population de neurones anorexigéniques (qui diminue l'appétit) situés dans l'ARC, présentent des contacts synaptiques avec les neurones à GnRH (Leranth et al. 1988). Leur implication dans le contrôle de la fonction de reproduction à été mise en lumière par la suppression de ERα dans les neurones à POMC chez la souris, ce qui conduit à une infertilité, en bloquant le rétrocontrôle négatif médié par les estrogènes (Xu et al. 2011). Les neurones à GnRH n'expriment pourtant pas le récepteur à la POMC (Mitchell et al. 1997). Toutefois, l'αMSH, peptide anorexigènique issu de la POMC, est capable d'activer les neurones à GnRH de l'aire préoptique via l'activation post-synaptique directe de son récepteur à la melanocortine de type 3 (MC3R) et 4 (MC4R) chez la souris adulte (Roa & Herbison 2012).

L'ocytocine

L'ocytocine jouerait également un rôle neuromodulateur sur la sécrétion de GnRH. L'administration centrale d'anticorps dirigés contre l'ocytocine, au cours de la phase de proestrus, bloque le pic de LH (Johnston et al. 1990). L'effet excitateur de ce peptide

interviendrait ainsi au niveau des terminaisons à GnRH (Selvage & Johnston 2001) en stimulant l'activité de la NOS, provoquant successivement l'augmentation des taux de NO puis de prostaglandine E2 (PGE2) par les astrocytes, qui induirait alors la sécrétion de GnRH (Rettori, Canteros & McCann 1997a; Rettori et al. 1997b; Parent et al. 2008).

Le RFamide-related peptide 3 (RFRP-3)

Comme la kisspeptine, le RFRP-3 est un membre de la famille des neuropeptides « arginine-phenylalanine-amide » (RFamide), mais ses effets sur la sécrétion des hormones de la reproduction lui sont diamétralement opposés. La RFRP-3 est l'orthologue de la gonadotropin-inhibitory hormone (GnIH) d'abord découverte chez l'oiseau, et ensuite découverte chez les mammifères et notamment chez le rongeur. Le RFRP-3 est produit par les neurones du noyau dorso-médian hypothalamique (DMN) (Kriegsfeld et al. 2006). Il a la capacité d'inhiber la sécrétion des gonadotrophines par l'hypophyse *in vitro* (Tsutsui et al. 2000), et *in vivo*, notamment chez le hamster (Kriegsfeld et al. 2006) et chez le rat (Murakami et al. 2008). Ces neurones, qui expriment ERα, ont été observés à proximité des neurones à GnRH (Kriegsfeld et al. 2006), alors que les neurones à GnRH expriment leur récepteur de haute affinité, le GPR74 (Ducret et al. 2009; Wu et al. 2009). Même s'il n'existe pas à l'heure actuelle d'étude qui le prouve, la population de neurones exprimant le RFRP-3 pourrait ainsi être impliquée dans le rétrocontrôle stéroïde sur la sécrétion de GnRH/LH, d'autant qu'elle a également été impliquée dans la modulation des neurones à kisspeptine chez la souris (Rizwan et al. 2009).

Le monoxyde d'azote (NO)

Le NO est un gaz qui joue le rôle de neurotransmetteur, et qui est également impliqué dans le contrôle neuroendocrine. Contrairement aux neurotransmetteurs peptidiques classiques ou aux amines, le NO n'est pas transporté par des vésicules synaptiques, n'interagit pas avec des récepteurs protéiques, et n'est pas sécrété exclusivement au niveau d'une synapse. Après sa synthèse, il diffuse à travers les membranes biologiques pour exercer son action, uniquement limité par sa demi-vie d'environ 1 seconde (Garthwaite & Boulton 1995), pouvant ainsi parcourir environ 200mm (voir Bellefontaine et al. 2011 pour revue). Le NO est un dérivé de la production de L-citrulline, à partir de L-arginine, par l'enzyme NOS (Förstermann et al. 1991). Il existe trois isoformes différentes de NOS : la NOS neuronale (nNOS ou type 1), la NOS exprimée dans les macrophages (mNOS ou type 2) et la NOS endothéliale (eNOS ou type 3) (Arzumanian et al.

2003). Les nNOS et eNOS sont constitutives alors que la mNOS est induite. La nNOS est exprimée dans les régions PVN, OVLT, mPOA, ARC, ME (Bhat et al. 1995; Prevot, Bouret, Stefano, et al. 2000; Knauf et al. 2001), mais ne colocalise pas avec les neurones à GnRH (Mahachoklertwattana et al. 1994). Elle est retrouvée notamment au niveau des neurones coexprimant le récepteur NMDA (Bhat et al. 1995), et par des neurones qui entourent le corps cellulaire des neurones à GnRH (Herbison et al. 1996; Clasadonte et al. 2008). Le NO est en effet un modulateur clé de la sécrétion de GnRH (Moretto et al. 1993; Rettori et al. 1993), et contribue à l'induction de la décharge préovulatoire de GnRH/LH (Bonavera et al. 1994). En effet l'infusion intracérébrale d'un inhibiteur de la NOS, le NG-nitro-L-arginine methyl ester (L-NAME), dans l'aire préoptique ou dans l'éminence médiane, provoque une importante dérégulation du cycle ovarien chez la rate (d'Anglemont de Tassigny et al. 2007; De Seranno et al. 2004). Comme le montre l'étude de Clasadonte et al., le NO affecte directement l'excitabilité des neurones à GnRH (Clasadonte et al. 2008) en les inhibant. Parallèlement, la production de NO varie en fonction du cycle ovarien dans la région préoptique et est significativement augmentée le jour du proestrus. L'hypothèse proposée est que la production de NO dans l'aire préoptique inhiberait les neurones à GnRH, dans le but de potentialiser la décharge préovulatoire de GnRH le jour du proestrus. Il a donc été suspecté comme molécule permettant la synchronisation des neurones à GnRH. L'augmentation des taux de NO pendant cette période du cycle serait dépendante des taux d'estrogènes (d'Anglemont de Tassigny et al. 2007; d'Anglemont de Tassigny et al. 2009), qui sont capables de moduler l'expression et la synthèse protéique de la nNOS (Pu et al. 1998; d'Anglemont de Tassigny et al. 2007). De manière intéressante, le NO pourrait aussi avoir une action indirecte sur la sécrétion de GnRH via l'interaction morphologique qu'il existe entre les neurones à nNOS et les neurones à Kisspeptine dans la région préoptique (Hanchate, Parkash, et al. 2012).

Les terminaisons à GnRH localisées au niveau de l'éminence médiane, à proximité des capillaires fenestrés du système porte, constituent un site clé de la modulation de la sécrétion de la GnRH (V Prevot et al. 2010; Vincent Prevot et al. 2010a). Les deux types de NOS sont présentes au niveau de l'éminence médiane. La nNOS est exprimée par les terminaisons des neurones magnocellulaires qui projettent sur la neurohypophyse, et la plus représentée, la eNOS, est abondamment exprimée par les cellules endothéliales (Yamada et al. 1996; Herbison et al. 1996). Le développement de méthodes ampérométriques ont permis la mesure en temps réel de la sécrétion de NO (Knauf et al. 2001), et ont permis de

démontrer que le NO est spontanément sécrétés de manière pulsatile par l'éminence médiane, et ce de manière similaire à la sécrétion de GnRH. De même, le traitement d'explants d'éminences médianes de rates prélevés en proestrus avec un inhibiteur de eNOS inhibe la sécrétion de NO, mais également de GnRH (Knauf et al. 2001), ce qui démontre que ces deux évènements sont liés.

Il existe bien d'autres neuropeptides ayant montré un impact sur la sécrétion de LH sous une variété de conditions expérimentales ou de modèles animaux différents, pour lesquels il est donc plus difficile d'interpréter le rôle exact. Parmi eux l'arginine-vasotocine (Cheesman et al. 1977), la cholecystokinine (Vijayan, Samson & McCann 1979), la neurotensine et la substance P (Vijayan & McCann 1979), la galanine, (Todd et al. 1998), la gastrine (Vijayan et al. 1978), l'angiotensine II (Steele et al. 1985), le Vasoactive Intestinal Peptide (VIP) (Vijayan, Samson, Said, et al. 1979; Weick & Stobie 1995).

7.5 Les facteurs gliaux impliqués dans le contrôle de la fonction de reproduction

Il est désormais établi que les cellules non-neuronales du système nerveux central participent de manière active au fonctionnement du cerveau, et ceci est le cas pour le contrôle de la fonction de reproduction. Les cellules gliales (astrocytes, oligodendrocytes et microglie) mais aussi les cellules endothéliales, sont à l'origine d'émission de molécules bioactives qui ont un effet modulateur sur la sécrétion de GnRH. La majorité de ces signaux proviennent cependant des astrocytes.

Le « Transforming growth factor α » (TGFα), les neurégulines (NRG) et la prostaglandine E2 (PGE2)

Le TGFα et les neurégulines sont des membres de la famille des Epidermal Growth Factor (EGF) (Massagué et al. 1990), qui sont synthétisés par les différentes population de cellules de l'hypothalamus, comme les astrocytes (Jun Ma et al. 1992; Ojeda et al. 2000), et stimulent la sécrétion de GnRH par l'éminence médiane *in vitro* (Ojeda et al. 1990). L'expression de TGFα augmente dans l'hypothalamus au moment du pic préovulatoire de GnRH/LH (Jun Ma et al. 1992). Le TGFα et les NRG se lient aux récepteurs membranaires erbB1, erbB4 et erbB2 présents sur les astrocytes hypothalamiques (Jun Ma et al. 1992; Ma et al. 1999) et induisent l'hétérodimérisation erbB1/erbB2 et erbB4/erbB2. Elles se lient aussi aux récepteurs erbB1 et erbB2 exprimés par les tanycytes, qui sont les cellules épendymogliales de l'éminence médiane (Prevot, Cornea, et al. 2003) et induisent

l'hétérodimérisation erbB1/erbB2. En effet, les souris transgéniques dont la signalisation erbB4 et erbB1 est perturbée sont subfertiles et voient leur sécrétion de GnRH altérée (Prevot, Rio, et al. 2003). L'initiation de ces voies de signalisation aboutie ensuite à la libération d'autres facteurs, tels que la PGE2 (Ojeda, Roth, et al. 2006; Prevot et al. 2007), par les cellules gliales (astrocytes (Ma & Ojeda 1997) et tanycytes (Prevot, Cornea, et al. 2003)). La signalisation issue de la liaison de la PGE2 à son récepteur EP2, exprimé par les corps cellulaires à GnRH, induit l'activité des neurones à GnRH (Clasadonte et al. 2011). La PGE2 est également capable de stimuler la sécrétion de GnRH par la liaison à son récepteur E-prostanoïde de type 1 (EP1), exprimé au niveau des terminaisons nerveuses à GnRH (Rage et al. 1997). Parallèlement, la PGE2 induit un phénomène de plasticité au niveau de l'éminence médiane en agissant sur le changement de morphologie des tanycytes (voir chapitre 1, 7.5). Cette signalisation est mise en jeu au niveau des astrocytes par le glutamate (Dziedzic et al. 2003). Cette action pourrait être potentialisée par les estrogènes, qui modifient l'expression du TGFα dans les astrocytes hypothalamiques (Galbiati et al. 2002).

Le « Transforming Growth Factor b1 » (TGFb1)

Le TGFβ1, sécrété par les astrocytes, aurait une action directe au niveau des corps cellulaires des cellules à GnRH, dans le but de stimuler la synthèse et la sécrétion de GnRH (Melcangi et al. 1995; Galbiati et al. 1996). Les neurones à GnRH sont, en effet, entourés d'astrocytes exprimant l'ARNm TGFβ1 au niveau de l'aire préoptique *in vivo* (S. Bouret et al. 2004), et expriment les récepteurs de type 1 et 2 au TGFβ (Bouret et al. 2004; Prevot et al. 2000). La famille du TGFβ serait également impliquée dans le rétrocontrôle médié par les estrogènes, puisque l'estradiol stimule la production de TGFβ1 par les astrocytes hypothalamiques (Buchanan 2000). De plus l'expression du TGFβ varie au cours du cycle ovarien, avec une augmentation le jour du pic préovulatoire de GnRH/LH (Melcangi et al. 2001).

Le «basic Fibroblast Growth Factor» (bFGF ou FGF2)

Le bFGF est un facteur de croissance exprimé par les astrocytes hypothalamiques. Il est capable d'induire la sécrétion de GnRH à partir d'explants d'éminence médiane (Ojeda et al. 1990). Il serait particulièrement impliqué dans la biosynthèse de la neurohormone et dans sa maturation (Tsai et al. 1995; Voigt et al. 1996). Les souris déficientes en FGF

récepteur 1 (FGFR1) présentent une puberté retardée mais un cycle ovarien normal (Tata et al. 2012).

L' « Insuline-like Growth Factor » (IGF-1)

Une étude à montré que l'IGF-1 est un facteur capable d'agir directement au niveau de l'éminence médiane pour induire la sécrétion de GnRH chez des rates prépubères (Hiney et al. 1991). Toutefois, une autre étude montre que l'IGF-1 a un effet inhibiteur sur la sécrétion de GnRH à partir d'explants hypothalamiques (Bourguignon et al. 1993). Les tanycytes pourraient être à l'origine de cette modulation de la sécrétion de GnRH via l'IGF-1 (Garcia-Segura et al. 1999), non pas en le synthétisant, mais en captant et en accumulant l'IGF-1 périphérique (Garcia-Segura et al. 1999; Danilovich et al. 1999).

7.6 La plasticité morphologique du système à GnRH

Les études neuroanatomiques mettent en lumière l'existence d'une interaction physique très importante entre les cellules gliales et les neurones à GnRH, au niveau de leur corps cellulaire et de leurs terminaisons dans l'éminence médiane. Cette interaction est dynamique et participe au contrôle de la fonction de reproduction.

La plasticité structurale au niveau de la région préoptique

Chez la plupart des espèces de mammifères étudiées, il a été noté qu'environ 90% du soma des cellules à GnRH est en directe apposition avec des processus astrocytaires (chez le mouton (Lehman et al. 1988); le rat (Jennes et al. 1985); le singe (Witkin et al. 1991); et l'homme (Baroncini et al. 2007)). Ce recouvrement glial est sensible à la présence de stéroïdes gonadiques dans l'environnement. En effet, chez le singe ovariectomisé, le nombre de prolongements astrocytaires recouvrant le soma des cellules à GnRH augmente, tandis que le nombre de contacts synaptiques sur les neurones à GnRH diminue, et qu'une supplémentation en estrogènes rétablit la morphologie initiale (Witkin et al. 1991). Chez le rat le recouvrement astrocytaire ainsi que le nombre de prolongements par astrocyte présente une plasticité cyclique et diminue en proestrus (Cashion et al. 2003). Il est très probable que cette plasticité gliale puisse moduler les afférences des neurones à GnRH. Toutefois, aucune corrélation n'existe à ce jour entre le pourcentage de recouvrement des neurones à GnRH et un niveau d'activité de ces neurones (Jansen et al. 2003; Perera & Plant 1997). Il n'y a pas non plus d'information concernant « l'enrobage » des dendrites des neurones à GnRH.

L'interaction cellule endothéliale-tanycyte-terminaison à GnRH

Le troisième ventricule est bordé par des cellules épendymaires, et au niveau de l'éminence médiane par les cellules épendymaires spécialisées appelées tanycytes. Les corps cellulaires des tanycytes constituent la zone épendymaire de l'éminence médiane, alors que leurs prolongements s'étendent jusqu'à la zone externe. Sous la couche épendymaire, la zone interne de l'éminence médiane contient des astrocytes et les axones provenant des noyaux supra optiques (NSO) et des noyaux paraventriculaires (NPV), en direction de la neurohypophyse. La zone externe de l'éminence médiane contient majoritairement les pieds tanycytaires et les terminaisons neuroendocrines adénohypophysiotropes. On y trouve de nombreux facteurs contenus dans les terminaisons qui peuvent être libérés dans le sang porte pour agir comme neurohormones, ou agir localement de façon autocrine/paracrine en temps que neuromodulateurs. La zone externe est bordée par un réseau de capillaires fenestrés qui font partie du système porte hypothalamo-hypophysaire, et qui émettent des anses infundibulaires dans le parenchyme hypothalamique. Ces capillaires ne possèdent pas de propriétés de barrière hématoencéphalique, contrairement aux capillaires retrouvés dans le parenchyme cérébral. Le tissu nerveux est délimité par une lame basale parenchymateuse, faisant face à une lame basale sous-endothéliale, bordant l'endothélium des capillaires, séparées l'une de l'autre par un espace péricapillaire.

Au niveau de l'éminence médiane, les terminaisons nerveuses des neurones endocrines adénohypophysiotropes contactent la lame basale parenchymateuse, et sont donc en étroite apposition avec l'espace péricapillaire, pour déverser leurs substances dans le sang porte (Ibata et al. 1986; Ohtsuka et al. 1983; Shioda & Nakai 1983; Liposits et al. 1987). Les terminaisons à GnRH, comme le montrent des études de microscopie électronique, sont clairement enveloppées par des pieds tanycytaires et sont ainsi éloignées de l'espace péricapillaire (Prevot et al. 1998; King & Rubin 1995; Kozlowski & Coates 1985; Ugrumov et al. 1989). Ce phénomène n'aurait été observé que pour les terminaisons à GnRH, ce qui suggèrerait qu'il est propre au système à GnRH. L'accès direct des terminaisons à GnRH à l'espace péricapillaire, et la formation d'une jonction neurovasculaire, ou neurohémale, met en jeu des remaniements structuraux au niveau de l'éminence médiane. En particulier, au moment du pic préovulatoire de GnRH, on observe la rétraction des pieds tanycytaires ainsi que le rapprochement des terminaisons des neurones à GnRH de l'espace péricapillaire, concomitamment avec une augmentation de perméabilité des vaisseaux eux-

mêmes (Prevot et al. 1999; King & Letourneau 1994). Ce mécanisme aurait un rôle dans la facilitation de la sécrétion de la GnRH dans le système porte au moment du pic (Prevot et al. 2010b).

Les mécanismes de plasticité au cours du cycle sont de mieux en mieux décrits, et ont notamment été largement étudiés au laboratoire. La voie de signalisation via la famille des récepteurs erbB et la PGE2, précédemment décrite, est impliquée dans le changement dynamique de morphologie des pieds tanycytaires. Ainsi le TGFα , sécrété par les cellules gliales en réponse à l'augmentation des taux d'estrogènes (Lomniczi et al. 2006), agit sur son récepteur erbB pour induire la sécrétion de PGE2 par les tanycytes et les astrocytes. Cette signalisation induit à son tour la sécrétion de TGFβ1, qui induit à son tour l'activation de métalloprotéinases, qui agissent sur la matrice extracellulaire, pour permettre la rétraction tanycytaire (Lomniczi et al. 2006). D'autre part l'endothélium des capillaires portes est aussi profondément impliqué dans le mécanisme de rétraction tanycytaire. Les cellules endothéliales expriment la eNOS, et sont donc capables de produire le NO en réponse à une augmentation des taux d'estrogènes au moment du proestrus. Ceci a deux conséquences majeures : premièrement le NO agit directement sur la sécrétion de GnRH par les terminaisons, et deuxièmement le NO active la guanylyl cyclase (pour augmenter les taux de guanosine monophosphate cyclique (GMPc)) et la cyclooxygenase (pour induire la sécrétion de PGE2) dans le but de faciliter la rétraction tanycytaire (De Seranno et al. 2004; de Seranno et al. 2010; Knauf et al. 2001).

Les cellules endothéliales sont aussi impliquées dans le rapprochement des terminaisons à GnRH de l'espace péricapillaire, via la sécrétion de facteurs de guidance axonale : les sémaphorines. D'après l'étude de Giacobini et al. (2014), à laquelle j'ai participé au cours de ma thèse (voir annexes), la sémaphorine 3A (Sema3A), qui est une forme sécrétée de sémaphorine, est exprimée par les cellules endothéliales au niveau de l'éminence médiane. De plus le taux de Sema3A varie au cours du cycle ovarien au sein de l'éminence médiane, avec une augmentation maximale au moment du proestrus chez la rate. D'autre part son récepteur spécifique, la Neuropiline-1 (Nrp1), est exprimé par les cellules à GnRH, aussi bien au niveau du corps cellulaire que des terminaisons. Enfin l'infusion d'un anticorps neutralisant anti-Nrp1 au sein de l'éminence médiane pendant sept jours interrompt le cycle ovarien chez la rate. La diminution de l'expression du gène *sema3a,* par recombinaison induite par l'injection de protéine TATcre (une protéine cre recombinase

capable d'être internalisée par des cellules) chez la souris *sema3a*$^{loxp/loxp}$, provoque la diminution des taux de LH après induction par le mâle (Giacobini et al. 2014).

La sémaphorine 7A (Sema7A) est une sémaphorine membranaire exprimée par les tanycytes de l'éminence médiane. Elle voit également son expression varier au cours du cycle ovarien sous l'influence des taux d'estrogènes, ainsi que des taux de progestérone et de TGFβ1 mais, contrairement à la Sema3A, la Sema7A est majoritairement exprimée en phase de Diestrus. Elle est, en fait, impliquée dans le processus d'enveloppement des terminaisons à GnRH quand la sécrétion de GnRH est minimale. De manière intéressante, la diminution de liaison de la Sema7A à son récepteur empêche la repousse tanycytaire et l'enveloppement des terminaisons neuroendocrines à GnRH pendant la phase de diestrus, mais interrompt aussi le cycle ovarien et altère les taux de LH (Parkash et al. 2015).

Ces études très complémentaires mettent en évidence l'importance de la plasticité au sein de l'éminence médiane dans le contrôle du cycle ovarien chez la femelle.

D'autres molécules sont impliquées dans cette plasticité, c'est le cas de la 2 ,8 linked polysialic acid (PSA) on neural cell adhesion molecule (PSA-CAM), qui est la forme polysialylée de la NCAM, marqueur de plasticité structurale. Cette protéine réduit l'adhésion cellulaire, permet le remaniement cellulaire, et est exprimée dans l'éminence médiane (Kaur et al. 2002), et plus généralement dans l'hypothalamus (Parkash & Kaur 2007). Elle a été directement impliquée dans la couverture gliale des terminaisons à GnRH, puisque sa suppression *in vivo* interrompt le cycle ovarien chez la rate (Kumar et al. 2012). L'augmentation de son expression dans l'éminence médiane au moment du proestrus est probablement influencée par les estrogènes (Garcia-Segura et al. 1995).

7.7 Le rôle des phéromones dans la sécrétion de GnRH

Les animaux ont développé des stratégies sensorielles et comportementales très évoluées pour détecter et reconnaître des dangers ou des signaux intéressants pour leur survie. C'est le cas également pour la reconnaissance de partenaires sexuels. Chez les mammifères, incluant le rat et la souris, les cavités nasales contiennent deux systèmes sensoriels majeurs impliqués dans la détection de messagers chimiques : le système olfactif principal, et le système olfactif secondaire ou voméronasal (Tirindelli et al. 2009; Boehm 2006). Des études de lésions des structures olfactives ont permis de lier le système olfactif primaire à l'odorat, et donc à la détection d'une grande variété de molécules odorantes

volatiles, alors que le système voméronasal médie la détection de signaux espèce-spécifique, impliqués dans le contrôle du comportement sexuel, de l'accouplement et du comportement agressif (Dulac & Torello 2003). Ce dernier est aussi très important dans la modulation du comportement maternel (Brouette-Lahlou et al. 1999). Plusieurs équipes dont celles de Bronson, Bruce et Vandenbergh font partie des premières à mettre en évidence que les phéromones, présents notamment dans les urines ou dans les glandes lacrymales, sont de puissants outils capables de moduler la physiologie de la reproduction (Bruce 1959). Les phéromones issues d'un mêle sexuellement mature sont capables d'induire un pic naturel de LH chez la femelle après 62h (Bronson 1973a; Giacobini, et al. 2014). De la même manière, l'exposition d'une femelle prépubère, entre 26 et 30 jours, à des phéromones de mâles sexuellement actifs, peut induire la puberté. Ainsi la cohabitation d'un mâle mature et de femelles prépubères conduit rapidement à une augmentation des taux de LH et de FSH chez les femelles, suivit d'une ovulation (Bronson & Desjardins 1974; Bronson & Stetson 1973b). Notons toutefois que, pour atteindre ainsi précocement la puberté, les femelles juvéniles doivent atteindre un poids critique de 17g minimum. De manière intéressante, des mâles hébergés ensembles vont avoir tendance à avoir des taux d'androgènes bas et une spermiogénèse ralentie (Mckinney & Desjardins 1973), et des femelles vivant en groupe peuvent voir leur cycle ovarien modifié, voire même supprimé (effet de Lee-Boot, Van Der Lee & Boot 1955). Les phéromones peuvent donc avoir des effets négatifs ou positifs sur les sécrétions de gonadotrophines et sur la fonction de reproduction. Une voie neuronale multisynaptique ayant pour origine les organes voméronasaux, et passant via les bulbes olfactifs accessoires et l'amygdale moyenne, projette en effet vers l'hypothalamus pour envoyer des informations environnementales aux neurones à GnRH. C'est en effet ce qui a été démontré grâce à des études de traçage, mais également grâce à l'étude de l'activation de cfos (Boehm et al. 2005; Yoon et al. 2005). Chez l'homme, l'organe voméronasal existe pendant l'embryogenèse, mais semble ne plus être fonctionnel après la naissance. De plus les bulbes olfactifs accessoires sont absents chez l'homme (Monti-Bloch et al. 1998). Pourtant, l'hypothèse que des phéromones puissent être détectées par les hommes et les femmes est très controversée. En effet des phéromones existent et sont sécrétées chez l'humain, notamment par les glandes apocrines axillaires, qui s'activent au moment de la puberté, et il semblerait que l'homme, mais surtout la femme, y soient sensibles (pour revue, voir Verhaeghe et al. 2013). En effet une partie des phéromones seraient prise en charge par le système olfactif principal chez l'humain.

En conclusion, les neurones à GnRH constituent une population étendue dans l'hypothalamus et très hétérogène. Ils représentent la voie finale commune dans la régulation centrale de la reproduction, et font donc partie d'un véritable système à GnRH, car leur sécrétion est hautement et finement régulée par tout un éventail de facteurs (neurotransmetteurs, hormones stéroïdes, hormones métaboliques, facteurs neuronaux, facteurs gliaux,…). La liste de modulateurs qui vient d'être exposée est non-exhaustive puisqu'il existe encore d'autres systèmes connus pour influencer le système à GnRH, comme par exemple le rythme circadien.

Chapitre 2 : Le Développement du système à GnRH

De développement du système à GnRH dépend l'intégrité de l'axe gonadotrope. Les neurones à GnRH subissent, dans un premier temps, un long processus de migration pendant le développement embryonnaire. A la naissance, ils ont pris place au sein de l'hypothalamus et envoyé leurs projections au niveau de l'éminence médiane. Toutefois, ils subissent encore un processus de maturation, qui permettra l'activation du système gonadotrope au moment de la puberté. Toutes ces étapes sont nécessaires à l'acquisition de la fertilité.

1. Le développement prénatal du système à GnRH

Les neurones à GnRH constituent une population particulière, puisqu'ils naissent à l'extérieur du cerveau, alors que la majorité des neurones hypothalamiques proviennent du neuroépithélium bordant les ventricules. Les neurones à GnRH ont pour origine l'épithélium présomptif de l'organe voméronasal, et migrent à travers le parenchyme nasal, la lame criblée, les bulbes olfactifs, en rencontrant des environnement différents, et en utilisant les axones des nerfs voméronasaux pour se guider. Enfin, ils pourront rejoindre leur localisation finale dans l'hypothalamus en suivant la branche caudale du nerf voméronasal, et projeter leurs terminaisons en direction de l'éminence médiane.

1.1 L'ontogenèse du système à GnRH

1.1.1 La naissance des neurones à GnRH

En 1989, deux équipes différentes mettent en évidence l'apparition de cellules à GnRH au sein de la placode olfactive d'embryon de souris, au dixième jour de vie embryonnaire (E10) et E11, par immunohistochimie et par hybridation *in situ* (Wray, Nieburgs, et al. 1989; Wray, Grant, et al. 1989). D'après leurs observations à différents stades embryonnaires entre E10 et E18, les neurones à GnRH semblent apparaître au sein de l'épithélium voméronasal et se déplacer le long de « rails » à travers le septum nasal. Ces rails sont constitués des nerfs terminaux et des nerfs voméronasaux, qui dégénèrent après la naissance (Tobet & Chickering 1996). Il fut alors proposé que la placode olfactive ne génère pas seulement des neurones sensoriels olfactifs et des cellules de soutien, mais qu'elle est aussi à l'origine des neurones à GnRH. L'analyse de l'origine des neurones à GnRH a pour limite le fait qu'il n'existe aucun marqueur permettant d'identifier l'intégralité des

neurones à GnRH excepté la GnRH elle-même. Il est donc impossible de marquer ces cellules avant qu'elles ne commencent à exprimer la GnRH. Une seconde origine des neurones à GnRH a été suspectée chez le poisson zèbre en 2003 (Whitlock et al. 2003) et a été confirmée récemment grâce à l'utilisation d'animaux transgéniques (Forni et al. 2011). En effet, une sous population de neurones à GnRH provient des crêtes neurales, qui sont à l'origine des nerfs crâniens. Cette sous-population représenterait environ 30% de la population totale de neurones à GnRH.

1.1.2 Les modalités de migration des neurones à GnRH

L'étroite relation entre les neurones à GnRH et le système olfactif

Après leur naissance à E10,5, les neurones à GnRH commencent immédiatement à migrer dans le parenchyme nasal en apposition avec les nerfs voméronasaux, qu'ils utilisent comme rails pour être guidés jusqu'au cerveau. Il utilisent un mode de migration axophilique (le long d'un axone) (Wray 2010; Schwanzel-Fukuda & Pfaff 1989). L'étroite relation entre les neurones à GnRH et le système olfactif est illustrée par le syndrome de Kallmann (KS) qui a été très décrit chez l'humain. Cette pathologie est caractérisée par une anosmie (perte de l'odorat) et un hypogonadisme hypogonadotrope (hypogonadisme lié à un défaut de maturation central de l'axe gonadotrope). L'analyse de fœtus atteints du syndrome de Kallmann a révélé une absence de croissance des axones olfactifs notamment dans le cerveau. Les cellules à GnRH restent alors bloquées au niveau de la lame criblée, à proximité des bulbes olfactifs, ou même dans la région nasale (Schwanzel-Fukuda & Pfaff 1989; Quinton et al. 1997). Ainsi, les molécules impliquées dans la croissance des axones voméronasaux affectent aussi la migration des neurones à GnRH, et peuvent même les empêcher de rejoindre leur destination. Ce phénomène résulte en une distribution et un nombre anormaux de cellules à GnRH dans l'hypothalamus, et peut être lié à un retard pubertaire et un hypogonadisme. Chez les mammifères, la majorité des axones olfactifs immunoréactifs pour la périphérine (protéine de filament intermédiaire exprimée spécifiquement par les neurones du système périphérique) projettent vers les bulbes olfactifs principaux et accessoires. Chez l'embryon de souris, il a été mis en évidence une branche qui pénètre le système nerveux central, pivote et projette ventralement, vers l'hypothalamus en développement. Cette branche est appelée nerf terminal (ou nervus terminalis) ou branche ventrale du nerf voméronasal (Wray 2010).

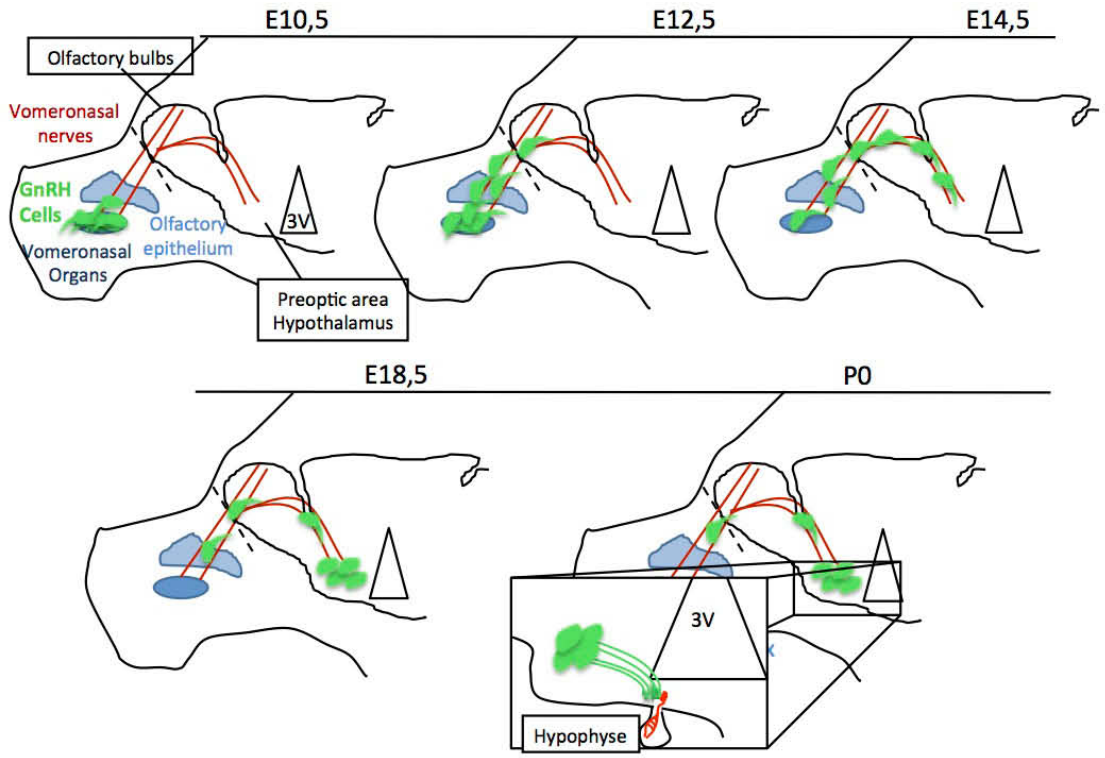


Figure 4. Distribution des neurones à GnRH au cours de leur migration embryonnaire chez la souris. Schéma montrant la migration des neurones à GnRH pendant l'embryogenèse. A E10,5, les cellules à GnRH naissent dans l'épithélium présomptif de l'organe voméronasal, puis elles commencent à migrer le long des axones des nerfs voméronasaux. A E12,5, les cellules se trouvent en majorité dans le nez, atteignent la lame criblée en formation (représentée par la ligne pointillée) et entrent dans le cerveau par les bulbes olfactifs. A E14,5, on retrouve environ 50% des cellules dans le nez et 50% dans le cerveau. Les neurones à GnRH empruntent la branche ventrale du nerf terminal pour migrer dans le cerveau. A E18,5, la majorité des neurones à GnRH a rejoint le cerveau antérieur et finit son positionnement dans l'aire préoptique de l'hypothalamus. En migrant, les neurones à GnRH commencent à envoyer leurs terminaisons vers les organes circumventriculaires.

Un large éventail de facteurs sont impliqués dans la mise en place des nerfs voméronasaux, et impactent par conséquent la migration des neurones à GnRH. Ainsi ces deux systèmes rencontrent, au cours de leur développement, différents environnementsJe détaillerai les facteurs les mieux décris dans les modèles animaux au cours du développement.

Le développment des nerfs voméronasaux et terminaux

Les nerfs voméronasaux expriment des récepteurs impliqués dans leur mise en place. C'est le cas de Deleted in Colorectal Cancer (DCC), qui est un récepteur des molécules de guidance, notamment de la netrine-1 (Deiner & Sretavan 1999; Schwarting et al. 2001). Chez des souris déficientes en DCC, la branche ventrale du nerf voméronasal projette de manière aberrante au niveau du cortex au lieu de l'hypothalamus. Les cellules à GnRH qui suivent ces rails migrent vers le cortex plutôt que vers l'hypothalamus (Schwarting et al. 2004). Les facteurs de guidance axonal, ayant un rôle de répulsion ou d'attraction des prolongements axonaux en croissance, s'avèrent particulièrement impliqués dans la projection des axones voméronasaux.

De même, des protéines sécrétées telles que la prokinéticine 2 (PROK2), et son récepteur de type 2 (PROKR2), qui ont un effet trophique sur des populations neuronales, ont un rôle certain dans la projection des axones voméronasaux. Des souris déficientes en PROK2 montrent des bulbes olfactifs atrophiés associés à une diminution de cellules à GnRH dans l'hypothalamus (Matsumoto et al. 2006).

La CCK est un facteur qui réduit la poussée des axones olfactifs, et indirectement, la migration des neurones à GnRH, via le récepteur CCK-1R. Ainsi, la migration des neurones à GnRH est accélérée chez des souris déficientes en CCK-1R (Giacobini et al. 2004).

Les récepteurs Neuropiline (Nrp) sont des récepteurs aux facteurs de guidance, notamment aux Semaphorines (Sema), molécules très exprimées pendant l'ontogenèse du système nerveux. Ces récepteurs sont exprimés par les nerfs olfactifs et terminaux et sont nécessaire à leur développement correct (Cariboni, et al. 2011)). Le rôle des Nrp et de leurs ligands dans la mise en place du système à GnRH a été récemment étudiés, puisque les souris KO pour les Nrp présentent une subfertilité associée à une altération des nerfs olfactifs (Cariboni et al. 2007). Ces souris présentent en effet une accumulation des cellules à

GnRH dans le nez, suggérant qu'elles ne migrent pas dans le cerveau. Cette étude met en évidence l'expression des Sema3 (notamment Sema3A et Sema3F) et leurs récepteurs Nrp-1, Nrp-2 et PlexinA1 dans la placode olfactive embryonnaire. Les auteurs montrent *in-vitro* que les Sema3A et 3F exercent un effet inhibiteur sur la migration de cellules à GnRH immortalisées (GN11).

Plus récemment, une étude réalisée au laboratoire, à laquelle j'ai participé au cours de mon Master 2, a mis en lumière le rôle de la Sema3A dans l'ontogenèse du système à GnRH. La suppression du domaine de liaison de la Sema3A sur son récepteur spécifique Nrp-1 provoque chez la souris un phénotype similaire au syndrome de Kallmann rencontré chez l'homme, puisqu'on observe chez ces animaux un hypogonadisme hypogonadotrope lié à un défaut de projection des nerfs olfactifs et voméronasaux et une anosmie (Hanchate, et al. 2012). Le rôle de cette signalisation dans la migration des cellules à GnRH reste encore à être approfondi.

Une migration autonome des cellules à GnRH : focus sur la région nasale

Dès leur naissance, les neurones à GnRH commencent leur migration de manière autonome. Cette étape nécessite l'augmentation de la motilité des neurones à GnRH ainsi que l'action de facteurs qui vont promouvoir l'adhérence ou non des cellules avec les axones voméronasaux, leur changement de morphologie et leur motilité. Comme les cellules du système nerveux central, les neurones à GnRH en migration adoptent une forme bipolaire, étendent un prolongement principal vers la zone de migration, effectuent la translocation du noyau vers le prolongement principal (nucléokinésie), puis éliminent le prolongement resté en retrait (Marín et al. 2010). Ils auraient une vitesse de 13 à 20 mm par heure (Marín et al. 2010; Casoni & Wray 2008). Plusieurs facteurs sont connus pour affecter la migration des neurones à GnRH dans la région nasale.

La Neural Cell Adhesion Molecule (NCAM) est une molécule d'adhésion extra-cellulaire permettant l'ancrage physique de la cellule à son environnement. La présence d'acides polysialiques (PSA) sur la NCAM atténue ses capacités d'adhésion. La forme PSA-NCAM est ainsi associée à des mécanismes tels que la migration, la synaptogenèse ou la croissance axonale (Gascon et al. 2007). La molécule NCAM et sa forme polysialilée, sont impliquées dans le mouvement des cellules à GnRH au sein de la région nasale et sont exprimées par les axones voméronasaux. L'injection d'un anticorps anti-NCAM dans le nez

d'embryons à E10 altère la migration des neurones à GnRH, avant d'atteindre le cerveau (Schwanzel-Fukuda et al. 1994; Yoshida et al. 1999).

Le Nasal Embryonic LHRH Factor (NELF) a été identifié dans les neurones à GnRH en migration. Sa neutralisation sur des explants de placodes olfactives a montré qu'il était impliqué dans la projection des axones olfactifs et dans la migration des cellules à GnRH (Kramer & Wray 2000) (Tableau 1).

La famille du FGF est également impliquée dans la migration des cellules à GnRH. Le traitement d'explants de placodes olfactives à E10,5 par un antagoniste des récepteurs au FGF abolit la différenciation des neurones à GnRH. Ce facteur est donc essentiel dans la mise en place du système à GnRH, ce qui a pu être confirmé dans les pathologies d'hypogonadisme hypogonadotrope (HH) chez l'humain, dans le cas de la mutation du gène *anosmin*, impliqué dans le fonctionnement du récepteur FGF (Dodé & Hardelin 2004). La mutation des gènes codant pour le FGF8 et sont récepteur le FGFR1, ont également été mis en évidence chez des individus atteints d'HH (Falardeau et al. 2008; Raivio et al. 2009) (Tableau 1).

L'Hepatocyte Growth Factor (HGF) est détecté dans la région nasale pendant l'embryogenèse, et son récepteur Met est exprimé par les neurones à GnRH en migration. L'étude de Giacobini et al. en 2007 montre que le traitement d'explants de placodes olfactives par un anticorps neutralisant anti-HGF réduit la migration des neurones à GnRH ainsi que la poussée des fibres olfactives, alors que l'ajout d'HGF accroit la distance parcourue par les neurones à GnRH. L'HGF exerce donc un effet motogène sur la migration des neurones à GnRH.

Des facteurs de guidage axonal impliqués dans la répulsion et l'attraction ont aussi des effets directs sur les cellules à GnRH en migration. C'est le cas de la signalisation par les récepteurs EphA et leurs ligands, les ephrins. La suppression du récepteur EphA5 dans les neurones à GnRH empêche leur migration hors de la région nasale (Gamble et al. 2005).

La sémaphorine 7A (Sema7A), ainsi que son récepteur, l'intégrine-β1, régulent la motilité des neurones à GnRH pendant leur migration. L'invalidation de l'intégrine-β1 résulte

en une diminution d'environ 30% de cellules à GnRH dans le cerveau adulte et est liée à un retard pubertaire (Messina et al. 2011; Jyoti Parkash et al. 2012).

La migration des neurones à GnRH : entrée dans le cerveau

Une fois l'arrivé des neurones à GnRH au niveau de la lame criblée plusieurs signaux sont nécessaires à la poursuite de leur migraton. Les nerfs voméronasaux se divisent et envoient une branche ventrale dans le cerveau antérieur, qui permet de guider les neurones à GnRH jusqu'à l'hypothalamus. Plus tard la branche ventrale voméronasale se rétracte et dégénère, et les cellules à GnRH arrêtent progressivement leur migration.

Au niveau de la lame criblée, une population de neurones olfactifs libèrent du GABA, qui a un effet inhibiteur sur la migration des cellules à GnRH dans d'explants de placodes olfactives (Fueshko et al. 1998; Casoni et al. 2012). Parallèlement, des observations chez l'embryon de poulet ont révélé l'existence d'une « pause » des cellules à GnRH à l'entrée du cerveau (Mulrenin et al. 1999). Il a ainsi été suggéré que le GABA puisse être responsable de cet arrêt de migration d'environ trois jours. La raison de cette pause reste à déterminer, mais il se pourrait que des remaniements au sein des cellules à GnRH soient nécessaires à un changement drastique d'environnement, entre le nez et le cerveau.

Dans le cerveau, le GABA a également montré un rôle dans le maintien de l'interaction entre les neurones à GnRH et le nerf terminal, et interviendrait dans l'orientation des neurones dans le cerveau antérieur (Bless et al. 2000). A l'inverse, l'activation du récepteur au glutamate NMDA-R1 inhibe le mouvement des cellules à GnRH dans le cerveau (Simonian & a E. Herbison 2001). La sérotonine et la noradrénaline auraient aussi un rôle stimulateur sur la migration des neurones à GnRH dans le cerveau (Pronina et al. 2003(a); Pronina et al. 2003(b)).

La possible intervention d'une régulation autocrine et/ou paracrine des neurones à GnRH sur leur propre migration a été suggérée par l'étude de Heger et al. (2003b). En effet une sous-population de neurones à GnRH synthétise du GABA au cours de sa migration dans le nez (Tobet & Chickering 1996). La surexpression de GAD-67, enzyme clé de la synthèse de GABA, dans les neurones à GnRH pendant leur migration, conduit à une migration aberrante et une diminution du nombre de cellules dans l'hypothalamus. Toutefois la production et la sécrétion de facteurs coexprimés par les neurones à GnRH pendant leur migration sont peu

Nature de la protéine codée	*Gène*	*Rôle indirecte migration*	*Rôle directe migration*	*Référence*
Molécules d'adhésion	*N-CAM/PSA-N-CAM*	+	+	(Yoshida et al. 1999)
	β3Gnt1	NON	+	(Bless et al. 2006)
Facteurs de guidance	*Netrin/DCC*	+	?	(Schwarting et al. 2001; Schwarting et al. 2004)
	EphA5	NON	+	(Gamble et al. 2005)
	Reelin	NON	+	(Cariboni et al. 2005)
	NELF	+	?	(Kramer & Wray 2000)
	Sema3A/Nrp1	+	?	(Hanchate et al. 2012)
	Sema4D/plexin-b1	NON	+	(Giacobini et al. 2008)
	Sema7A/β1-integrin	NON	+	(Messina et al. 2011)
Facteurs de croissance	*FGF8/FGFR1*	+	+/ ? (niHH)	(Villanueva & de Roux 2010)
	HGF/Met	+	+	(Giacobini et al. 2007)
	Axl Tyro3	?	+	(Pierce et al. 2008)
	VEGF164	NON	+	(Cariboni et al. 2011)
GPCR	*PROK2/PROKR2*	+	?	(Matsumoto et al. 2006)
	CXCR4/CXCR7	+	?	(Schwarting et al. 2006)
Neurotrans-metteurs	*GAD67*	+	-	(Vastagh et al. 2015; Heger et al. 2003)
	CCK/CCK1R	+	+	(Giacobini et al. 2004)
Facteurs de transcription	*CHD7*	+	?	(Layman et al. 2011)
	EBF2	+	?	(Corradi et al. 2003)
	Necdin	?	+	(Miller et al. 2009)
	NHLH2	+/ ?	+/ ?	(Cogliati et al. 2007)
	OTX2	?	+	(Layman et al. 2011; Diaczok et al. 2011)
	LHX2	+	?	(Berghard et al. 2012)
	SOX10	+	+/ ?	(Pingault et al. 2013)

Tableau 1 : Gènes mutés étudiés chez des patients HH et KS et leur rôle dans le développement du système olfactif et la migration des neurones à GnRH. Ici a été reporté, lorsqu'il est connu, le rôle direct ou indirect de chaque molécule codée par ces gènes sur la migration des neurones à GnRH. L'atteinte des nerfs voméronasaux, par la mutation d'un gène, impacte indirectement la migration des neurones à GnRH, puisqu'il leur sert de guide. Ainsi, la plupart du temps, le rôle intrinsèque du gène dans la migration des neurones à GnRH n'a pu être déterminé. Le rôle direct est justifié par une atteinte de la motilité, de la guidance, ou de la survie des neurones à GnRH sans atteinte du VNN. Le symbole + correspond à un rôle stimulateur dans le développement du système, conclut quand une suppression du gène concerné résulte en un défaut de développement. Le symbole – correspond à un effet inhibiteur du gène dans le développement du système. Le symbole ? s'applique quand le rôle précis du gène dans la mise en place du système n'est pas décrit dans la littérature. *Abréviations : GPCR, récepteur couplé à une protéine G, niHH, hypogonadisme hypogonadotrope idiopathique et normosmique.* Pour les abréviations des gènes voir la liste des abréviations.

décrites, et l'intervention d'une régulation autocrine/paracrine des cellules est méconnue. La seule certitude à ce sujet est que, la GnRH elle-même, bien qu'exprimée pendant l'embryogenèse, n'est pas nécessaire à la migration des cellules, puisqu'on constate une migration normale des neurones à GnRH chez les souris hpg (John C. Gill et al. 2008a).

1.2 La mise en place des fibres à GnRH

L'éminence médiane est le principal lieu de projection des axones neuroendocrines à GnRH. Les premières projections sont détectées chez le rongeur à E15-E16 (Wierman et al. 2011). Des greffes de région préoptique d'embryon dans le troisième ventricule d'animaux adultes montrent en effet que l'éminence médiane est un lieu préférentiel de projection pour les neurones à GnRH nouvellement installés (Gibson et al. 1984). De même, *ex vivo*, des cocultures organotypiques de région préoptique et d'hypothalamus médio-basal ont montré que les projections issues des neurones à GnRH s'étendent préférentiellement vers les extraits médio-basaux (Rogers et al. 1997; Gibson et al. 2000). Il semblerait donc que cette région soit particulièrement impliquée dans la sécrétion de facteurs chimiotropes, sécrétant probablement des facteurs attractifs diffusibles, pendant le développement, mais aussi visiblement encore à l'âge adulte (voir Chapitre 1 partie sécrétion de la GnRH). Des facteurs comme le Brain-Derived Neurotrophic Factor (BDNF) et le FGF ont été impliqués dans l'élongation et le ciblage des neurones à GnRH vers l'éminence médiane, grâce à leurs effets neurotrophiques (Cronin et al. 2004; Gill et al. 2004; Gill & Tsai 2006). La Sema7A et son récepteur, l'intégrine-β1, sont également nécessaires à l'extension des terminaisons endocrines à GnRH vers l'éminence médiane puisque ce processus est impacté de près de 70% chez des souris déficientes en intégrine-β1 (Jyoti Parkash et al. 2012).

2. Le développement postnatal du système à GnRH : de la naissance à l'acquisition de la fertilité

A la naissance, les neurones à GnRH ont atteint leur destination dans l'hypothalamus et projettent leur axone vers l'espace péricapillaire des vaisseaux du système porte en commençant à sécréter la GnRH dans le sang. En clair, le système à GnRH semble prêt à fonctionner. Ce n'est toutefois qu'à partir de la puberté que l'individu devient fertile. Le système à GnRH va en effet subir, depuis la naissance jusqu'à l'âge adulte, une maturation complexe qui va affecter la biosynthèse de la GnRH, le profil de sécrétion des neurones, leur morphologie, leurs afférences, ce qui aboutira enfin à un système à GnRH

intégré et fonctionnel tel qu'il a été décrit précédemment, et donc, à l'acquisition de la capacité à se reproduire.

2.1 Les changements de sécrétion de GnRH, de gonadotrophines et d'hormones stéroïdes régissent la succession de quatre périodes de maturation

La maturation postnatale de l'axe gonadotrope peut être subdivisée en 4 périodes chez les rongeurs, mâles comme femelles, en fonction de paramètres physiologiques et morphologiques, comme proposé par Ojeda et al. (1980). D'abord, la période néonatale comprend la première semaine de vie à partir de la naissance, ou jour post-natal 0 (P0), puis la période infantile s'étend de P7-8 à P21 (jour du sevrage), ensuite la période juvénile s'étend de P25 à P40, et enfin la période péripubertaire s'étend jusqu'à la première ovulation chez la femelle (environ P50 en fonction des souches), et est considérée jusqu'à P60 chez le mâle. La puberté chez la femelle rongeur est ainsi marquée premièrement par l'ouverture vaginale, signe externe de maturation qui est provoqué par l'augmentation des taux d'estrogènes, et deuxièmement par la première ovulation, due à l'activation centrale de l'axe gonadotrope.

2.1.1 La sécrétion de GnRH dans la maturation de l'axe gonadotrope

L'expression du gène de la GnRH n'est requis ni pour la migration des cellules à GnRH pendant l'embryogenèse, ni pour leur maturation, puisque l'absence d'expression de GnRH chez les souris hpg (voir Chapitre 1, 1.1) ne perturbe ni le positionnement des cellules à GnRH (Mason et al. 1986) ni leurs propriétés intrinsèques d'activité spontanée (John C Gill et al. 2008). L'augmentation de l'expression du gène *Gnrh1* dans l'hypothalamus est toutefois nécessaire à l'apparition de la puberté et à la mise en place d'un cycle ovarien fonctionnel (Navratil et al. 2007; Grosse et al. 2000). L'expression d'activateurs et de répresseurs du promoteur GnRH va influencer le taux d'expression de GnRH, et est important dans l'apparition de la puberté. C'est le cas par exemple de l'activateur « orthodenticle homeobox 2 » (OTX2), dont l'invalidation spécifiquement dans les neurones à GnRH provoque une diminution importante de l'expression de la GnRH chez l'adulte et une puberté retardée (Kelley et al. 2000).

Chez les rongeurs, les taux de GnRH augmentent de manière importante pendant la période néonatale (Aubert et al. 1985). Ensuite, ces taux restent constants jusqu'à l'âge de P12, puis augmentent pendant le reste de la période infantile, continuent d'augmenter pendant la période juvénile, pour atteindre leur maximum au cours de la période

péripubertaire, et notamment au moment de la première ovulation chez la femelle (Cattanach et al. 1977; Hompes et al. 1982; Prevot, Cornea, et al. 2003). Chez le mâle, on n'observe pas de pic, mais les taux de GnRH augmentent progressivement jusqu'à l'âge adulte. La GnRH est sécrétée de manière pulsatile dès l'âge de P5 (Matagne et al. 2004), et des changements dans la fréquence et l'intensité de ces décharges vont réguler la sécrétion des gonadotrophines.

2.1.2 La Période néonatale

Pendant la période néonatale, la GnRH commence à induire la sécrétion de LH et de FSH. Chez la femelle, à la naissance, les ovaires sont constitués de cordes d'ovogonies (McGee & Hsueh 2000), dont le stock est déterminé et ne prolifèrera plus. Les follicules primordiaux apparaissent vers P3, et les premiers follicules secondaires (ovocytes de type I entourés de plusieurs couches de cellules folliculaires) apparaissent vers P7. Toutefois, à ces âges, les gonadotrophines n'ont probablement pas d'action sur les follicules en croissance, qui ne possèdent pas encore de récepteurs à FSH ou LH (O'Shaughnessy et al. 1997).

2.1.3 Période infantile

Pendant la période infantile, le profil de sécrétion de GnRH, notamment de basse fréquence (Kaiser et al. 1997; Dalkin et al. 1989), provoque chez la femelle une augmentation des taux de FSH, qui atteignent un pic très important à P12 (chez le rat comme chez la souris (Dahl et al. 1988; Döhler & Wuttke 1975)). C'est à ce moment que les premiers follicules secondaires deviennent sensibles à la FSH en exprimant son récepteur (O'Shaughnessy et al. 1997). L'augmentation des taux de FSH provoque alors l'entrée en maturation d'un pool de follicules qui deviendront préantraux, puis antraux, puis mûrs, et ovuleront environ 3 semaines plus tard au moment de la puberté (Gelety & Magoffin 1997; McGee et al. 1997). Ainsi, des altérations de sécrétion de FSH pendant cette période critique chez des modèles murins transgéniques induisent une puberté retardée ainsi qu'une réduction de la fertilité (Prevot, Rio, et al. 2003). La sécrétion de LH est également élevée et pulsatile pendant la période infantile (Döhler & Wuttke 1975; Döhler & Wuttke 1974) mais ne semble malgré tout pas jouer un rôle prépondérant dans le développement ovarien. A l'inverse, chez le mâle, les cellules de Leydig sécrètent la testostérone en réponse à la LH, et des modèles transgéniques présentant des taux de FSH bas peuvent atteindre la puberté, développer une spermatogenèse plus ou moins normale et être fertiles (Kumar et al. 1997). A partir de P7, les ovaires commencent à sécréter des taux croissants d'estrogènes

(Funkenstein et al. 1980). Toutefois, le rétrocontrôle négatif des estrogènes sur la sécrétion de GnRH n'est pas encore fonctionnel. On parlera d'ailleurs de maturation gonado-indépendante de l'axe hypothalamo-hypophysaire dans les périodes infantiles et juvéniles.

2.1.4 Période juvénile

A la fin de la période infantile, qui se situe au moment du sevrage (P21), les taux de gonadotrophines circulantes sont très réduits (Döhler & Wuttke 1975; Ojeda & Ramírez 1972). Cependant, la fréquence des pulses de GnRH à l'entrée de la période juvénile augmente et avoisine les valeurs retrouvées chez l'adulte, qui correspondent à un pulse toutes les 30min environ (Matagne et al. 2004). Cette fréquence de sécrétion de GnRH est connue pour stimuler massivement la sécrétion de LH (Kaiser et al. 1997). La sécrétion pulsatile de LH accélère en effet à la fin de la période juvénile (Urbanski & Ojeda 1987; Andrews & Ojeda 1981) pour atteindre également une fréquence de 30min. Parallèlement, les taux de stéroïdes gonadiques, issus de la maturation des follicules chez la femelle, continuent d'augmenter, et la sensibilité du système à GnRH au rétrocontrôle gonadique augmente considérablement pour atteindre son maximum à la fin de la période juvénile (Andrews et al. 1981a). Les taux d'estrogènes ne sont alors pas suffisants pour induire un rétrocontrôle positif sur le système, et le rétrocontrôle négatif, qui se met en place progressivement au cours de la période juvénile, ne semble pas affecter la maturation des neurones à GnRH (White & Ojeda 1981).

2.1.5 Période péripubertaire

Le passage à la période péripubertaire est marqué par l'apparition d'un rythme diurne dans la concentration de LH circulante, avec des différences entre le matin et l'après-midi. Chez la rate entre P30 et P38, le taux de LH plasmatique l'après midi est environ 2 fois supérieur à celui du matin, sans modification visible de la fréquence des décharges (Urbanski & Ojeda 1987), ce qui est consistant avec l'amplitude des pics de sécrétion de GnRH pendant cette même période (Sisk et al. 2001). Ces différences diurnes ne sont probablement pas en lien avec le rétrocontrôle médié par les estrogènes, puisque des augmentations de LH plasmatique sont également observées chez des rates ovariectomisées avant la période péripubertaire (Urbanski & Ojeda 1987). L'existence d'un minipic de GnRH/LH en milieu d'après midi, qui consiste en un épisode d'augmentation plus soutenu de LH, est décrit chez une partie des rates étudiées, et cette proportion semble augmenter avec l'âge, au fur et à mesure que l'on se rapproche de la puberté (Urbanski & Ojeda 1987; Sisk et al. 2001). Ce

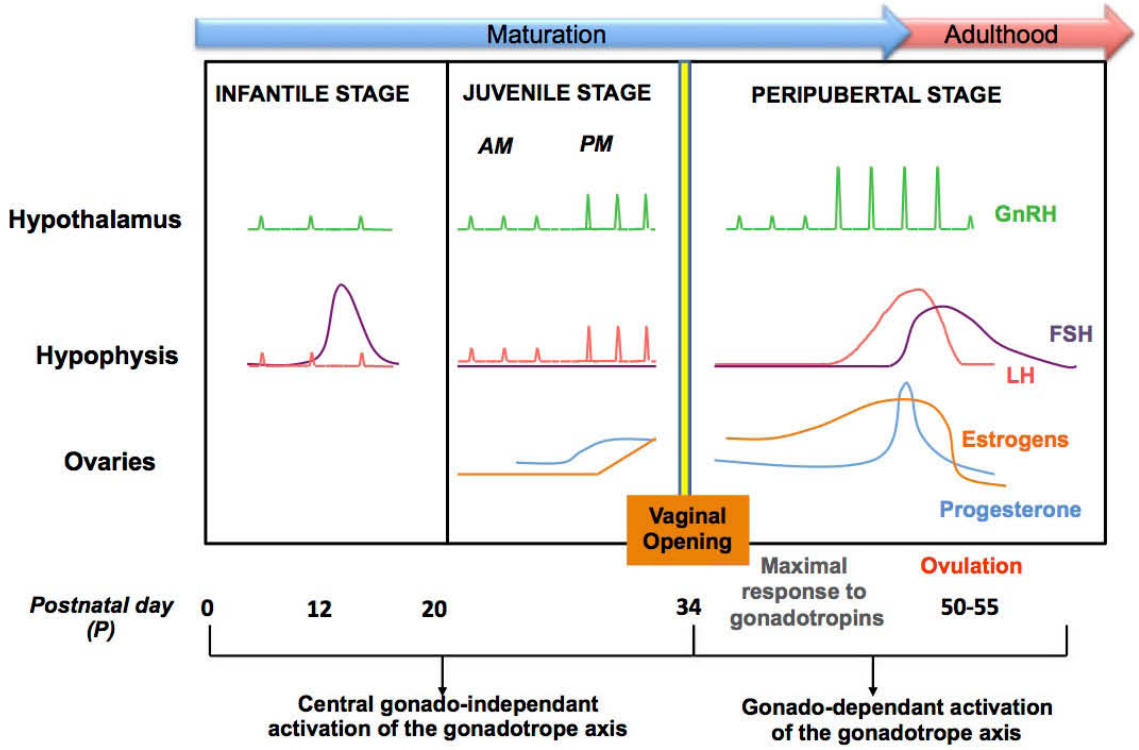


Figure 5. Maturation postnatale de l'axe gonadotrope chez le rongeur. Schéma montrant le profil de sécrétion de GnRH, des gonadotrophines (LH et FSH), et des stéroïdes gonadiques (estrogènes et progestérone) au cours de la maturation postnatale de l'axe gonadotrope chez la femelle. Au cours de la période infantile, la sécrétion de GnRH est caractérisée par une fréquence et une amplitude faibles. Ce profil induit une sécrétion de LH de faible fréquence et de faible amplitude, et provoque un pic de sécrétion de FSH à l'âge de P12. Ce pic de FSH induit l'entrée en maturation d'un groupe de follicules qui ovulera à la puberté. Le sevrage signe l'entrée en phase juvénile, et avec elle l'augmentation de la fréquence de sécrétion de GnRH, et un changement diurne de l'amplitude de sécrétion, avec notamment une augmentation de l'amplitude des décharges l'après-midi. La sécrétion de LH suit le même profil que la sécrétion de GnRH. L'augmentation de production de stéroïdes conduit à l'apparition du signe d'entrée en phase péripubertaire : l'ouverture vaginale. Pendant les phases infantile et juvénile, le rétrocontrôle ovarien se met progressivement en place. Toutefois la maturation de l'axe gonadotrope est indépendante de la maturation des gonades. Pendant la période péripubertaire, le rétrocontrôle hormonal ovarien affecte la sécrétion de GnRH. Les taux d'estrogènes atteignent progressivement une amplitude maximale induisant un rétrocontrôle positif sur la sécrétion de GnRH, et déclenchant alors le premier pic préovulatoire de GnRH, et par conséquent le pic préovulatoire de LH et de FSH, responsable de la première ovulation. *Abréviations : AM, matin ; PM, après-midi.*

minipic est quant à lui directement lié à l'augmentation du taux d'estrogènes circulants (Urbanski & Ojeda 1986).

2.1.6 Puberté

Contrairement aux périodes infantile et juvénile, où les changements de taux de gonadotrophines sont gonado-indépendants, le déclenchement de la puberté se fait par une activation gonado-dépendante de l'axe gonadotrope. Les ovaires ont alors acquit la capacité à sécréter des taux élevés d'estrogènes et de progestérone, qui agissent par rétrocontrôle positif sur le système à GnRH (Parker & Mahesh 1976; Smith et al. 1975). C'est un événement clé qui induit la première décharge préovulatoire de GnRH/LH/FSH, et par conséquent, la première ovulation chez la femelle (Ojeda et al. 1976; Sarkar & Fink 1979). Des traitements à l'œstradiol, à la progestérone, ou aux deux, chez la souris, sont en effet capables d'induire une ovulation chez des femelles sexuellement immatures (Bronson 1975). Chez le rongeur mâle, l'arrivée de la puberté se traduit par la présence de spermatozoïdes mobiles dans l'épididyme, et par la capacité à se reproduire (McKinney & Desjardins 1973). Ces événements surviennent à l'issue d'une augmentation des taux de testostérone circulants (McGee & Narayan 2013), liée à l'augmentation des taux de LH (Ellis & Desjardins 1982). La spermatogenèse complète chez le rongeur requiert 40 à 54 jours, ce qui correspond approximativement à l'âge de la puberté chez ces espèces (Hess & Renato de Franca 2008). Même s'il est possible d'observer des signes externes de puberté précoce chez certains modèles transgéniques (Themmen APN & Huhtaniemi 2000), comme par exemple une avancée de la séparation préputiale, la spermatogenèse ne peut pas être avancée chez le mâle. Il est donc impossible chez le rongeur mâle d'observer une vraie puberté précoce (McGee & Narayan 2013), ce qui n'est pas le cas chez l'homme.

2.2 Le remodelage morphologique du système à GnRH au cours du développement postnatal

2.2.1 Le remodelage structural des neurones à GnRH au cours de la maturation post-natale

En 1986, l'équipe de Wray (Wray & Hoffman 1986) décrit deux types de cellules à GnRH, selon leur apparence morphologique : des cellules à GnRH « lisses » et des cellules à GnRH irrégulières à l'aspect épineux. Le nombre de cellules lisses diminue, et le nombre de cellules épineuses augmente avec l'âge, et se stabilise au moment de la puberté. Il

semblerait que les cellules lisses se transforment en cellules épineuses au cours de la maturation de la fonction de reproduction. L'étude de Cottrell et al. (2006) met en évidence une profonde modification de la morphologie des neurones à GnRH entre la période infantile (P10-15) et l'âge adulte (P60). Pendant la période infantile, une proportion (environ 40%) de cellules à GnRH apparaît complexe et très ramifiée. Cette population ne représente plus que 10% à P35 et à l'âge adulte, alors que la population majeure est constituée de cellules bipolaires. Parallèlement la densité d'épines dendritiques augmente entre la période infantile et l'âge adulte. Il existe donc une phase de remodelage, qui consiste en un élagage des dendrones pour laisser place à des cellules bipolaires, coïncidant avec une augmentation de la quantité d'épines dendritiques sur ces dendrones, qui reçoivent des afférences synaptiques excitatrices (Gray 1959).

2.2.2 Le développement des connexions afférentes aux neurones à GnRH participe à la maturation du système à GnRH

Comme vu au sein du chapitre 1, les neurones à GnRH reçoivent une multitude d'informations par l'intermédiaire de synapses afférentes excitatrices ou inhibitrices. Le remodelage structural des neurones à GnRH qui a lieu au cours de leur maturation est hautement corrélé à la maturation concomitante des systèmes afférents.

Les neurones de l'AVPV, qui sont impliqués dans le rétrocontrôle positif médié par les estrogènes chez la femelle mature (Herbison 2008), ont déjà contacté le l'aire préoptique médiale, et font synapse avec les neurones à GnRH à la naissance (Polston & Simerly 2006). La population de neurones de l'ARC, dont l'implication est suspectée dans le rétrocontrôle négatif médié par les estrogènes, n'est quant à elle pas présente à la naissance. Les premières connexions entre le noyau arqué et l'aire préoptique s'opèrent à P12, mais n'atteignent leur distribution finale qu'à la fin de la période infantile (S. G. Bouret et al. 2004a; Caron et al. 2012), au moment où le rétrocontrôle négatif des estrogènes commence à opérer chez la femelle (Andrews et al. 1981b; Prevot, Cornea, et al. 2003).

Le glutamate

Le glutamate est, comme vu précédemment, un puissant stimulateur du système à GnRH. Des données montrent que la quantité de glutamate déshydrogenase, enzyme qui catalyse la synthèse du glutamate, augmente dans l'hypothalamus de rate ayant atteint la puberté. A l'inverse, on observe au même moment une diminution de la glutamine

synthase, qui catalyse le métabolisme du glutamate en glutamine (Erecińska & Silver 1990). Le taux de glutamate dans l'hypothalamus augmente, en effet, pendant le développement postnatal, et atteint un maximum après le déclenchement de la puberté (Goroll et al. 1994). Parallèlement, la capacité du glutamate (ou d'agonistes glutamatergiques) à stimuler la sécrétion de GnRH augmente progressivement pendant les périodes infantile et juvénile (Bourguignon et al. 1990). Ce phénomène est très probablement corrélé à l'augmentation de l'expression de la sous-unité NR1 du récepteur NMDA, nécessaire à son activité, pendant les périodes infantile et péripubertaire chez la souris (Simonian & A. E. Herbison 2001). Ces données sont consistantes avec le fait que l'activation du récepteur NMDA est nécessaire à l'apparition de la puberté (Bourguignon et al. 1990). Le traitement avec un agoniste du récepteur NMDA est en effet capable d'avancer la puberté chez la rate, alors qu'un traitement avec un antagoniste du même récepteur la retarde (MacDonald & Wilkinson 1990). Pendant la maturation postnatale du système à GnRH, il existe en effet une augmentation des afférences glutamatergiques vers les neurones à GnRH, qui est nécessaire à l'apparition de la puberté. L'augmentation des afférences excitatrices a un rôle crucial dans la modulation de la pulsatilité des neurones à GnRH et très probablement un rôle facilitateur dans le déclenchement de la décharge de GnRH préovulatoire.

Le GABA

L'effet global du GABA sur le système à GnRH et sur l'apparition de la puberté est inhibiteur (Ojeda, Lomniczi, et al. 2006). En effet, des expériences de push-pull perfusion ont démontré que les taux de GABA dans l'aire préoptique diminuent pendant les périodes juvénile et péripubertaire (Goroll et al. 1993), ce qui serait consistant avec l'hypothèse d'une levée d'inhibition GABAergique, concomitante avec une augmentation d'afférences excitatrices glutamatergiques, dans l'apparition de la puberté. Parce que le glutamate est le précurseur du GABA, qu'ils ont des effets opposés, et parce qu'ils sont coexprimés dans les mêmes neurones de l'AVPV (coexprimant également ERa), il se pourrait que l'interaction entre ces deux neurotransmetteurs ait un rôle majeur dans l'apparition de la puberté. Même s'il a été montré que l'action du GABA via le récepteur au GABA de type A peut avoir un effet excitateur sur la sécrétion de GnRH (DeFazio et al. 2002), il semblerait que l'expression de ce récepteur diminue dans les neurones à GnRH au cours de la période infantile (Sim et al. 2000).

La Kisspeptine

La mutation inactivatrice du récepteur GPR54 chez l'homme et chez la souris (de Roux et al. 2003; Seminara et al. 2003), est lié à un défaut de maturité sexuelle et même à un impubérisme. Ces observations ont conduit à l'hypothèse que la kisspeptine et son récepteur ont un rôle essentiel dans l'apparition de la puberté. En effet, l'expression des gènes *KiSS1* et *GPR54* chez le rongeur, mâle et femelle, est basse pendant la période juvénile, et subit une augmentation importante, dont le pic coïncide avec l'apparition de la puberté (V. M. Navarro et al. 2004), ce qui suggère que l'activation de la signalisation KiSS1/GPR54 prend place pendant la période péripubertaire. L'administration de Kisspeptine-10 à des femelles immatures est capable d'induire la maturation précoce de l'axe gonadotrope (Navarro et al. 2004), ce qui suggère que la signalisation KiSS1/GPR54 semble aussi être suffisante au déclenchement des événements neuroendocrine conduisant à la puberté. Parallèlement, on observe une augmentation de la sensibilité des neurones à GnRH à la kisspeptine, chez le rat et la souris, pendant la période péripubertaire (Castellano et al. 2006; Han et al. 2005). Alors que, chez la souris juvénile, 27% des neurones à GnRH sont activés par l'administration de kisspeptine, 90% d'entre eux sont dépolarisés par la kisspeptine chez l'adulte. Des études en immunohistochimie dans l'hypothalamus de souris montrent que la population de neurones à kisspeptine de l'AVPV est sujet à un développement postnatal important, avec une apparition de l'immunoréactivité pour la protéine pendant la période infantile, et une augmentation pendant la période péripubertaire. De plus, on observe parallèlement une augmentation de l'apposition des terminaisons à kisspeptine et des corps cellulaire à GnRH au moment de la transition pubertaire (Clarkson & Herbison 2006). Il existe donc une maturation du système à kisspeptine concomitante à celle du système à GnRH au moment de l'apparition de la puberté.

La PGE2 et les cellules gliales

L'activation des neurones à GnRH lors de la puberté n'est pas uniquement médiée par des afférences neuronales, mais aussi par des facteurs gliaux qui ont un rôle critique dans la modulation de l'âge de la puberté (Lomniczi & Ojeda 2009). La PGE2, facteur astrocytaire capable d'induire la sécrétion de GnRH, comme vu précédemment (voir Chapitre 1, 7.5), est également impliquée dans la maturation postnatale de l'axe gonadotrope. Il a été montré que les taux de PGE2 augmentent dans l'hypothalamus au moment de la première ovulation (Ojeda & Campbell 1982) et que ce phénomène est

dépendant de l'augmentation des taux d'estrogènes (Amateau & McCarthy 2002). L'inhibition de la voie de signalisation des récepteurs erbB1, erbB2, ou erbB4, impliqués dans la stimulation de la sécrétion de PGE2 par les cellules gliales, induit un retard pubertaire chez le rongeur. (Prevot, Rio, et al. 2003; Prevot et al. 2005). L'activation des récepteurs erbB a aussi été impliquée dans le réarrangement morphologique des astrocytes hypothalamiques (Sharif et al. 2009), ce qui pourrait moduler le recouvrement astrocytaire des neurones à GnRH *in vivo* (Vincent Prevot et al. 2010a).

2.3 Les facteurs métaboliques intervenant dans l'apparition de la puberté

2.3.1 L'hypothèse de Frisch et la preuve d'un rôle du poids dans le déclenchement de la puberté chez la femme

Le fait que la puberté soit corrélée au poids corporel est connu depuis plusieurs milliers d'années, en particulier chez la femme, quand les symboles de bonne santé et de fertilité sont représentés par des personnages gras et bien portants (Référence à la Vénus de Willendorf qui serait une idole de la fécondité, Beaune 1993). Il est bien connu qu'une femme doit avoir des stocks énergétiques, notamment de masse grasse, suffisants pour pouvoir mener à bien une grossesse suivie d'une lactation, le poids ayant même été un critère attractif majeur dans le choix de sa partenaire (c'est encore le cas dans de nombreux pays peu ou non-industrialisés). La raison de cette tendance naturelle est qu'il faut entre 50 000 et 80 000 Kcal pour produire un enfant viable, et après cela environ 1000 Kcal par jour pour le nourrir (Frisch & Revelle 1970). De même, une femme aurait besoin d'un stock suffisant d'énergie pour pouvoir développer le ménarche (soit l'arrivée des premières règles), processus énergivore déterminé comme étant la puberté chez la femme, et donc l'acquisition de la capacité à se reproduire. Ces observations constituent la base de l'hypothèse de Frisch, selon laquelle l'âge du ménarche serait en lien avec l'atteinte d'un poids corporel suffisant plutôt qu'avec une période chronologique (Frisch & Revelle 1970; Frisch 1984). Des études épidémiologiques montrent en effet que les femmes des pays riches atteignent la puberté plus tôt que dans les pays pauvres. C'est aussi le cas des femmes les plus grandes et ayant un poids corporel plus important au sein d'une même population (St George et al. 1994). D'autre part, une balance énergétique négative a aussi un impact sur l'arrivée de la puberté puisque des femmes subissant des restrictions alimentaires drastiques, comme c'est par exemple le cas dans l'anorexie, ou qui pratiquent une activité sportive intense, peuvent avoir un retard pubertaire (Hoggard et al. 1998).

2.3.2 Le rôle de la leptine dans le déclenchement de la puberté

Les recherches des mécanismes moléculaires sous-jacents à l'hypothèse d'un poids critique permissif, dans déclenchement de la puberté, ont rapidement impliqué la leptine, dont les taux circulants sont le reflet de la proportion de masse grasse stockée (Meister 2000). Comme vu précédemment, la perte de fonction de la leptine ou de son récepteur résulte en une obésité morbide, et une infertilité, soit une incapacité à atteindre la puberté et à générer une cycle ovarien. Ce phénotype peut néanmoins être contré par une administration de leptine (Chehab et al. 1996). A l'inverse, la surexpression du gène de la leptine, artificiellement causée par une stratégie transgénique chez le rongeur, ou encore la supplémentation en leptine par administration, provoque le déclenchement d'une puberté précoce (Yura et al. 2000; Chehab et al. 1997). Toutefois de manière naturelle, la leptine n'avance pas la puberté chez un animal nourri *ad libitum* (Cheung et al. 1997), ce qui suggère qu'elle a principalement un rôle permissif dans le déclenchement de la puberté.

La leptine a une deuxième fonction au cours de la maturation du système à GnRH, puisqu'elle joue un rôle majeur dans l'établissement des projections des neurones du noyau arqué vers l'aire préoptique pendant la période infantile (Bouret & Simerly 2004). Chez le rat et la souris, la concentration de leptine plasmatique augmente pendant les deux premières semaines de vie pour atteindre un pic entre P12 et P16 (Ahima et al. 1998). Ce pic de leptine postnatal provoque la croissance des neurones de l'ARC en agissant directement sur son récepteur (S. G. Bouret et al. 2004b; Bouret et al. 2012). L'origine de ce pic de leptine pendant la période infantile n'est pas précisément caractérisée, toutefois plusieurs pistes indiquent que la modification de la nutrition néonatale aurait un impact certain sur ses taux (Bouret et al., 2007), et peut entraîner des effets délétères à long terme comme l'altération de l'apparition de la puberté (Caron et al. 2012; Castellano et al. 2011). La leptine pourrait même provenir de la mère puisqu'elle est détectée dans le lait maternel (Houseknecht et al. 1997).

2.4 La puberté chez l'Homme

La puberté est une période qui dure de 3 à 5 ans en moyenne chez l'humain. Elle commence avec l'apparition des caractères sexuels secondaires et se termine avec l'acquisition de gamètes fécondants et fécondables, et notamment avec le ménarche chez la femme. Elle est suivie d'une période « pré-adulte » de quelques années, le point culminant

de la fécondité étant atteint aux environs de l'âge de 20 ans chez la femme, et de l'âge de 24 ans chez l'homme (Tanner 1962). Le stade de développement pubertaire peut être évalué grâce à une échelle descriptive élaborée par Tanner (1962).

Le développement des caractères sexuels secondaires précède la puberté et est régi par des événements endocrines. Il est défini par l'apparition des poils axillaires et pubiens, qui est dépendante de l'augmentation de la sécrétion des androgènes par les testicules chez l'homme, et par les glandes cortico-surrénales chez la femme (Sklar et al. 1980; Palmert et al. 2001). L'augmentation de la production d'hormones androgènes par la glande surrénale est appelée adrénarche et n'est pas directement liée à l'activation de l'axe gonadotrope. Chez la femme, l'apparition de la pilosité sexuelle ne donne donc pas d'information directe sur la maturation des gonades ou de l'axe gonadotrope. L'augmentation de la sécrétion de stéroïdes gonadiques pendant la période péripubertaire, mais aussi et surtout d'hormone de croissance (GH), est à l'origine d'une accélération de la croissance osseuse et pondérale.

La difficulté pour définir l'âge de la puberté chez l'humain est essentiellement dûe à la difficulté de mesurer des critères objectifs. Chez l'homme, le premier signe de développement pubertaire est l'augmentation du volume testiculaire au delà de 3 mm^3 (Tanner 1962; Grumbach 2002), sous l'influence de la sécrétion de la LH et de la FSH. Chez la femme, les marqueurs utilisés pour définir l'âge de la puberté sont dans un premier temps le thélarche (ou le développement des glandes mammaires), suivi par le ménarche (ou l'apparition des premières règles). Ces deux événements sont significativement corrélés (Tanner 1962), mais donnent toutefois des informations différentes, puisque le ménarche correspond à la finalité d'un processus complexe de maturation, alors que le thélarche est plus simplement sous l'influence de l'action des estrogènes, dont le taux augmentent pendant la période péripubertaire. Ainsi, l'âge du thélarche peut être influencé par l'exposition à des estrogéno-mimétiques, et être indépendant de l'activation de l'axe hypothalamo-hypophyso-ovarien (voire 2.6 : facteurs environnementaux). Parallèlement, les taux de FSH induisent la croissance et la maturation folliculaire conduisant à l'augmentation de la taille des ovaires. (Tanner 1962; Grumbach 2002).

L'âge du ménarche chez la femme est distribué selon une courbe Gaussienne, avec une médiane située entre 12 et 13,3 ans en fonction du pays étudié au alentours des années 2000 (Parent et al. 2003). En effet, on constate une différence de l'âge moyen et de l'âge

médian du ménarche en fonction de l'origine ethnique. Cet âge était de 12,6 ans en France dans les années 2000, alors qu'il était de 13 ans au Danemark. De manière intéressante, les études de cohortes ont montré des différences d'âge de la puberté entre différentes populations ethniques aux USA : les américaines de type caucasien ont le ménarche à 12,9 ans, alors que les afro-américaines ont le ménarche à 12,2 ans, selon un étude de 1997 de USA PROS (Herman-Giddens et al. 1997). Ces données mettent en évidence une variabilité génétique dans l'apparition de la puberté. L'âge du ménarche change en fonction des régions du monde, mais semble aussi très corrélé au niveau de vie. En effet, dans les pays les plus défavorisés, l'âge du ménarche est élevé. Par exemple il est compris entre 13,4 et 13,8 ans au Guatemala et en Colombie (Khan et al. 1995; Ireton et al. 2002), alors qu'il est de 12,3 ans en moyenne dans une population plus aisée au Venezuela (Macías-Tomei et al. 2000). De même au Venezuela, les femmes les plus défavorisées ont un ménarche à 12,9 ans dans cette même étude. L'âge de la puberté est visiblement impacté par les facteurs environnementaux, tel que la qualité de la nutrition. De manière globale, il existe une tendance à la diminution de l'âge de la puberté au fil du temps. Entre le milieu du 19^{e} et le milieu du 20^{e} siècle, l'âge du ménarche est passé d'environ 17 à 14 ans aux Etats Unis et en Europe de l'Ouest (Zacharias & Wurtman 1969; Wyshak & Frisch 1982; Parent et al. 2003). Ce phénomène est hautement lié à l'amélioration du niveau de vie ainsi qu'à l'industrialisation, puisqu'il varie en fonction des pays. L'âge critique des pubertés particulièrement avancées ou précoces, et des pubertés retardées, a évolué avec l'évolution de l'âge de la puberté. Aujourd'hui la puberté est considérée comme précoce chez la femme avant l'âge de 8 ans, et chez l'homme avant l'âge de 9 ans (Bridges et al. 1994; Klein 1999). On qualifie de puberté précoce centrale (CPP), les pubertés précoces liées à une activation centrale précoce de l'axe gonadotrope. La puberté précoce centrale peut être décrite chez des patients atteints de pathologies du système nerveux central (tumeurs ou malformations) ou est dite « idiopathique » quand elle est d'origine inconnue (ICPP). Elle est beaucoup plus fréquente chez la fille que chez le garçon. La puberté retardée est définie chez le garçon par l'absence d'augmentation de volume testiculaire au delà de l'âge de 14 ans, et chez la fille par l'absence de thélarche à 13 ans et de ménarche au delà de 15 ans (Hasegawa 2006). Ce phénotype est plus souvent décrit chez le garçon, et se traduit par un HH, c'est à dire un hypogonadisme hypogonadotrope lié à un défaut d'activation de l'axe gonadotrope, idiopathique s'il est d'origine inconnue (iHH). Quand ce phénotype est associé à une anosmie ou une hyposmie, qui correspond à un défaut d'olfaction, on parle alors de syndrome de Kallmann (KS).

2.5 Les facteurs génétiques impliqués dans le déclenchement de la puberté

Beaucoup de facteurs interviennent dans le contrôle du déclenchement de la puberté. L'un des principaux facteurs déterminant l'âge de la puberté est le facteur génétique. Des études chez des jumeaux ont en effet révélées que les gènes constituent le principal facteur de variabilité dans la maturation de l'axe gonadotrope et l'accès à la fertilité (Eaves et al. 2004; Mustanski et al. 2004). Ces gènes clés qui jouent un rôle dans le déclenchement de la puberté ont été mis en évidence grâce à l'étude de cas où la puberté est anormale, comme l'iHH, le KS, ou la puberté précoce.

2.5.1 Hypogonadisme hypogonadotrope normosmique (HH)

L'apparition de la puberté peut-elle être attribuée à l'activation d'un seul et unique gène ? C'est visiblement le cas puisque de nombreux gènes ont été directement impliqués, par leur unique mutation, dans les cas d'HH, et jouent donc un rôle critique dans l'activation de l'axe gonadotrope au moment de la puberté (Tableau 1). L'HH, caractérisé par des taux d'hormone gonadique bas et à un retard pubertaire, mais avec un sens de olfaction intacte, a d'abord été associé à des mutations dans le gène du *GnRHR* (de Roux et al. 1997). Plus récemment, des mutations du *GnRH-1* ont été identifiées (Chan et al. 2009; Bouligand et al. 2009). Plus généralement, les polymorphismes des gènes codant pour le neuropeptide GnRH-1 et pour son récepteur sont la cause de retards pubertaires (Sedlmeyer et al. 2005). La mutation du gène *GPR54*, récepteur des kisspeptines, conduit également à un HH (de Roux et al. 2003; Seminara et al. 2003). Le rôle de la signalisation KiSS1/GPR54 a déjà été abordé précédemment. La mutation des gènes TAC3 et TAC3R, qui codent respectivement pour la NKB et son récepteur, a aussi été identifiée chez les patients HH (Topaloglu et al. 2009). Ces dernières données mettent en évidence le rôle prépondérant des neurones KNDy dans le déclenchement de la puberté. L'HH qui est fortement corrélé à une obésité peut aussi résulter d'un défaut des gènes codant pour la leptine (*LEP*) ou pour son récepteur (*LEPR*). D'autres gènes ont été impliqués dans le développement de l'axe gonadotrope et liés à un HH, comme le récepteur nucléaire orphelin *DAX1* (dosage-sensitive sex reversal adrenal hypoplasia critical region on the X-chromosome gene), le steroidogenic factor-1 (*SF-1*), ou la prohormone convertase 1 (*PC-1*). C'est le cas également des mutations des gènes de facteurs de transcription hypophysaires *HESX-1*, *LHX3* ou *PROP1*, qui conduisent à des défauts de sécrétion de gonadotrophines (pour revue Gajdos et al. 2010; Herbison 2007)

2.5.2 Syndrome de Kallmann (KS)

De nombreux gènes nécessaires au fonctionnement de l'axe gonadotrope, mais aussi nécessaires au développement du système olfactif, ont été identifiés grâce à l'étude du KS chez l'homme, et en mimant ce syndrome chez l'animal. Le KS est une pathologie génétique rare, avec une incidence accrue chez l'homme (4 hommes pour une femme), lié à des taux d'hormone gonadique bas, à un retard pubertaire et à un sens de l'olfaction défaillant. La mutation du gène *KAL-1* (Franco et al. 1991; Hardelin et al. 1993) lié au chromosome X, et du gène du *FGFR1* (Dodé et al. 2003), impliqué dans la forme autosomale dominante de la maladie, représentent approximativement 20% des patients atteints par le KS. Des mutations du gène *PROKR2*, un récepteur couplé à une protéine G, et de son ligand *PROK2*, ont été identifiées dans des cohortes de patients présentant un KS (Dodé et al. 2006). La facteur NELF, qui joue un rôle dans la migration des neurones à GnRH et dans le développement des axones olfactifs (Kramer & Wray 2000) a également été impliqué dans le KS, bien que des polymorphismes du gène soient aussi impliqués dans l'iHH (Miura et al. 2004). Enfin, plus récemment, des mutations du gène *SEMA3A* ont été détectées chez des patients atteints du KS, et parallèlement modélisé chez la souris au laboratoire (Hanchate et al. 2012). Cette étude, ainsi qu'une autre étude publiée la même année (Young et al. 2012) mettent en évidence le rôle de la SEMA3A dans le développement du système à GnRH et de l'axe gonadotrope.

Les mutations des gènes impliqués dans le KS peuvent avoir différents degrés d'impact sur la maladie. C'est le cas des mutations du gène *FGF8*, ligand du FGFR1, qui ont été observées chez des patients HH avec une variabilité de phénotypes olfactifs chez l'homme et chez la souris (Falardeau et al. 2008). Récemment des mutations du gène *CHD7*, un gène responsable du syndrome de CHARGE, qui partage des caractéristiques développementales avec le KS, ont été identifiées à la fois chez des patients HH et des patients KS (Kim et al. 2008).

Toutes ces mutations qui sont plus ou moins bien décrites ne constituent toutefois que 30% des cas de HH et de KS (Herbison 2007). Il existe en effet une grande variabilité de phénotypes cliniques, avec des degrés différents allant du retard pubertaire avec une fertilité plus ou moins normale, jusqu'à la complète infertilité. Il est important de considérer que le retard pubertaire peut résulter de polymorphismes des gènes vus précédemment, ou d'autres gènes, mais surtout d'une accumulation de plusieurs mutations. Les perturbations

de l'apparition de la puberté auraient une étiologie majoritairement oligogénique, affectant des gènes faisant partie d'un réseau complexe (Ojeda et al. 2010; Herbison 2007; Caronia et al. 2011).

2.5.3 Puberté précoce

La puberté précoce est caractérisée par une avancée de la maturation de l'axe gonadotrope, notamment visible chez l'Homme, par l'apparition des caractères sexuels secondaires avant l'âge de 8 ans chez la fille, et avant l'âge de 9 ans chez le garçon. Le taux d'incidence de pubertés précoces idiopathiques chez la fille est actuellement de 2,68/10000 par an (2011 à 2013, d'après les données de l'institut de veille sanitaire. Chez le garçon il est de 0,28/10000 par an. Peu de mutations génétiques sont associées à la puberté précoce. La plupart d'entre elles sont en effet des mutations activatrices qui sont plus rares que les mutations inhibitrices. La plus connue est une mutation autosomale dominante du récepteur GPR54 qui cause une activation prolongée de la voie de signalisation liée au GPR54 en réponse à la liaison de la kisspeptine (Teles et al. 2008). Une étude très récente mais qui nécessite encore des précisions a détecté des polymorphismes directement au sein du gène KiSS1 et de son promoteur chez des enfants atteints d'ICPP (Mazaheri et al. 2015). Le polymorphisme des gènes TAC3 et TACR3 a également été détecté en 2014 et en 2015 chez des individus présentant une ICPP (Krstevska-Konstantinova et al. 2014; Xin et al. 2015). De manière intéressante, il semblerait que les gènes qui avaient été précédemment impliqués dans des iHH ou des KS puissent être également impliqués dans des ICPP. Les mutations concernant ces gènes auraient probablement un impact différent sur leur fonction, pouvant être, par exemple, responsables d'une perte d'inhibition ou encore d'une activation. D'autres polymorphismes ont été détectés chez des patientes ayant un ménarche avancé (Perry et al. 2009; Sulem et al. 2009).

La mutation du récepteur à la LH entraine une puberté précoce chez l'Homme et dans un modèle animal. Toutefois, cette puberté précoce n'est pas considérée comme une ICPP puisque la LH a majoritairement un impact en périphérie, sur les gonades, et qu'elle n'a pas pour origine une activation précoce centrale. La même mutation chez les femelles induit une puberté précoce qui est corrélée à de sévères irrégularités du cycle, à une anovulation, une augmentation du poids, un hyperandrogénisme, des ovaires polykystiques, et une infertilité (Hai et al. 2015).

Chez la femme, la puberté précoce n'est pas systématiquement corrélée à une diminution de la fertilité, bien qu'elle soit un critère de diagnostic du syndrome des ovaires polykystiques (PCOS), avec d'autres critères comme l'hyperandrogénisme, la virilisation congénitale, ou les taux élevés de LH plasmatique (Nicandri & Hoeger 2012). Elle représente toutefois une véritable pathologie pour l'homme comme pour la femme, avec une croissance accélérée, une maturation précoce du squelette et donc une soudure des épiphyses avancée, conduisant à une petite taille (Carel et al. 2009). Elle est aussi corrélée à une prolongation de l'exposition aux hormones stéroïdes au cours de la vie, et est liée à une augmentation de l'incidence de cancers du sein chez la femme (Stoll et al. 1994). Dans certaines études de cohortes, elle a aussi pu être corrélée à une augmentation de la prévalence de l'obésité et du surpoids (Colmenares et al. 2014) ainsi que de pathologies comme le diabète (Stöckl et al. 2012).

2.6 Les facteurs environnementaux impliqués dans le déclenchement de la puberté

Les facteurs environnementaux qui peuvent impacter le développement du système à GnRH au cours de la puberté sont très variés. L'apport alimentaire, en particulier les aspects qualitatifs et quantitatifs de la nutrition, ont un impact certain sur l'arrivée de la puberté, comme vu précédemment (Chapitre 2, partie 2.3.1). Les jeunes filles présentant un ménarche avancé sont plus susceptibles à l'obésité (Stark et al. 1989). Toutefois, le surpoids à l'âge du ménarche pourrait être la cause, ou la conséquence de ce ménarche avancé (Parent et al. 2003). Les études de cohortes ayant montré l'importance de l'origine ethnique dans l'apparition de la puberté ont aussi montré que ces différences ethniques était corrélé à des différences d'index de masse corporel, de taux d'insuline en réponse au glucose, de dépenses énergétiques, et possiblement d'activité physique (Anderson et al. 2003; Gower & Higgins 2003). Les cas les plus significatifs montrant un impact de l'environnement dans la maturation de l'axe gonadotrope sont illustrés par la migration d'enfants depuis des pays défavorisés vers des pays développés. Il existe, en effet, une forte incidence d'apparition précoce de la puberté, associé à une augmentation du poids et de la taille (Adolfsson & Westphal 1981) chez les enfants adoptés issus de pays défavorisés. La raison de cette corrélation est mal connue, même si la nutrition dans le pays d'accueil est suspectée. La plupart des enfants suivis avaient en effet une courbe de croissance altérée, et ont subi un phénomène de rattrapage de la courbe de croissance à l'arrivée dans le pays d'accueil. Ces données pourraient suggérer l'existence d'une période critique du développement pondéral pendant l'enfance qui pourrait alors inluencer l'arrivée de la puberté (Cooper et al. 1996). La

qualité de la nutrition est également suspectée dans ce phénomène. En effet, l'exposition à des substances comme les phytoestrogènes, qui sont présents majoritairement dans les aliments végétaux (maïs, blé, soja, graines), influencent la régulation de l'axe gonadotrope (Soriguer et al. 1995), et auraient des effets agonistes ou antagonistes sur la sécrétion de gonadotrophines en fonction de l'état hormonal (Kuiper et al. 1998). Plus dramatiquement, la présence d'estrogéno-mimétiques dans l'environnement peut être à l'origine de dommages sur le système endocrinien (Marshall 1993; Toppari et al. 1996). C'est le cas de pesticides à la demi-vie très longue, interdits dans les années 1960 (Partsch & Sippell; Key & Reeves 1994), et dont on retrouve les métabolites dans le sang encore aujourd'hui (Krstevska-Konstantinova et al. 2001). Une exposition fétale ou périnatale à de telles substances est un paramètre critique dans la maturation précoce de l'axe gonadotrope (McLachlan 2001). Chez l'homme, ces substances affectent la fertilité en diminuant la concentration en spermatozoïdes dans le sperme (Sharpe & Skakkebaek 1993). Des estrogéno-mimétiques, encore appelés perturbateurs endocriniens, sont encore utilisés de nos jours. C'est le cas du bisphénol A, de phtalates, de médicaments (stéroïdes contraceptifs rejetés dans la nature), de détergeant et de pesticides organochlorés ou azotés (Rhomberg et al. 2012).

Le développement embryonnaire et le développement postnatal sont deux évènements étroitement liés dans l'acquisition de la fertilité. De nombreux facteurs sont essentiels à ce développement et ont été mis en évidence grâce à des mutations qui résultent en des pathologies de la puberté et de la fertilité chez l'Homme, et qui ont pu être modélisé chez le rongeur pour être approfondi.

Chapitre 3 : Le rôle de la semaphorine 3A et de la Neuropiline-1 dans le système nerveux central et dans le contrôle du système à GnRH

Le développement du système nerveux central est régulé par un large éventail de facteurs qui agissent sur la prolifération, l'apoptose, la différenciation et la migration depuis le site de production des cellules jusqu'à leur destination, ainsi que dans le développement des projections neuronales. Le guidage des projections axonales et dendritiques jusqu'à leur cible, et l'intégration correcte des cellules dans leur réseau, nécessite des signaux externes attractifs et répulsifs (Huber et al. 2003). Parmi les nombreuses classes de molécules participant à l'ontogenèse du système nerveux, la grande famille des sémaphorines, aujourd'hui largement étudiée, a initialement été décrite dans le cerveau en formation (Luo et al. 1995). Leurs effets sont maintenant bien décrits dans le cerveau adulte ainsi que dans la formation du système nerveux périphérique (Giacobini et al. 2014; J. Parkash et al. 2012; Masuda et al. 2014). Les sémaphorines sont aussi impliquées dans de nombreux processus développementaux et pathologiques, comme le développement du système cardiovasculaire, la réponse immunitaire, et la progression tumorale (Neufeld & Kessler 2008; Perälä et al. 2012; Zhou et al. 2008).

1. Les sémaphorines

Les sémaphorines (Sema) constituent une famille de glycoprotéines sécrétées ou transmembranaires (Pasterkamp & Kolodkin 2003). Elles sont subdivisées en 8 classes, sur la base de leur structure et les similarités de leur séquence peptidique (Figure 6) (Goodman et al. 1999). Les Sema de classes 1 et 2 sont retrouvées chez les invertébrés, celles faisant parti des classes 3 à 7 sont retrouvées chez les vertébrés, et enfin les Sema de classe 8 sont exprimées exclusivement par les virus (Figure 6). Individuellement, les Sema sont désignées par le chiffre de leur classe suivi d'une lettre terminale. Il existe ainsi une vingtaine de Sema différentes (Goodman et al. 1999). Elles sont connues pour leurs effets chimiotactiques sur le guidage correct d'axones (Liu et al. 2004; Xiao et al. 2003) et participent ainsi à la mise en place d'aires cérébrales (hippocampe, cortex, bulbes olfactifs) (Sahay et al. 2003; Homman-Ludiye & Bourne 2014; Taniguchi et al. 2003).

1.1 Structure

Les différentes Sema sont des protéines phylogénétiquement très conservées au cours de l'évolution, en particulier grâce à leur « domaine SEMA » d'environ 400 acides aminés (Love et al. 2003; Antipenko et al. 2003). Elles possèdent également un domaine PSI (plexins, semaphorins and integrin), ainsi qu'un domaine immunoglobuline-like (Ig)

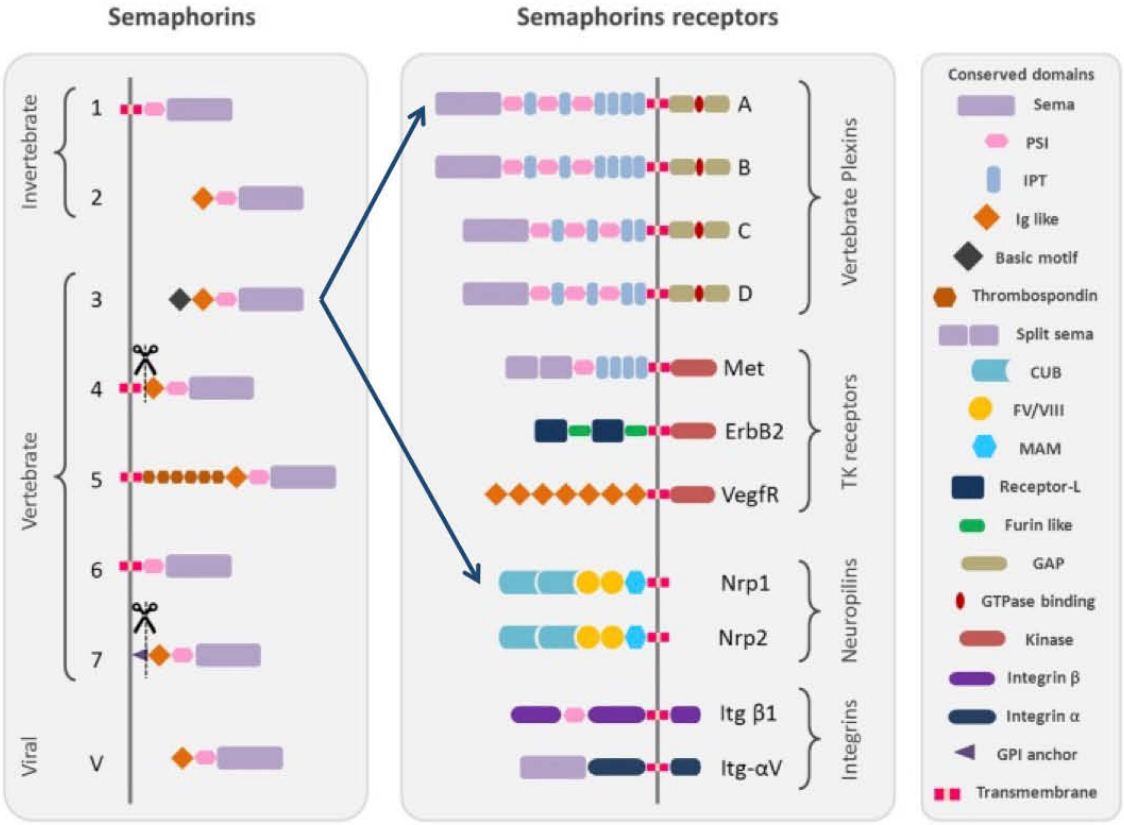


Figure 6. La famille des sémaphorines et leurs récepteurs. Représentation schématique de la structure protéique des sémaphorines et de leurs récepteurs. Les sémaphorines 1 et 2 des invertébrés se lient aux plexines A et B retrouvées chez les invertébrés. La classe de sémaphorines 3 se lie principalement aux plexines de type A, mais nécessitent la dimérisation de la plexine à une corécepteur obligatoire : la neuropiline. Elles peuvent se lier aussi à la plexine D1. La classe de semaphorines 4 a pour récepteurs les plexines B. Les sémaphorines de type 5 se lient aux plexines B, et en particulier à la plexine B3. Les sémaphorines de la classe 6 se fixent aux plexines de type A, préférentiellement à la plexine A1. Les semaphorines 7 se lient aux plexines C ainsi qu'aux intégrines. Les sémaphorines de type viral, ou sémaphorines 8, ont pour récepteur principal la plexine C1. Les récepteurs tyrosine kinase sont des récepteurs qui peuvent se dimériser avec les récepteurs aux sémaphorines. La plexine B1 peut se coupler au récepteurs Met ou erbB2 pour lier la Sema4D. La Nrp1 se dimérise avec VegfR dans la signaliation du VEGF. *Abréviations : PSI, domaine plexins, semaphorins, integrins ; IPT, domaine Ig-like, plexins and transcription factor ; CUB, amino-terminal complement-binding ; FV/VIII, facteur de coagulation V/VIII ; MAM, domaine Meprin, A5, Mu ; GPI, glycosyl-phosphatidylinositol ; VegfR, vascular endothelial growth factor receptor.* Adapté de Messina & Giacobini 2013.

pour la plupart. Elles possèdent ensuite des particularités, par exemple la classe des Sema3 possède deux sites de clivage par des enzymes convertases (voir Kruger et al. 2005 pour revue).

1.2 Les récepteurs aux sémaphorines

Les plexines constituent les principaux récepteurs aux Sema. Cette famille est subdivisée en quatre sous-groupes de A à D sur la base de leur homologie. Comme les Sema, les plexines possèdent un domaine SEMA. Elles possèdent également trois domaines PSI, et trois domaines IPT (Ig-like, plexins and transcription factors) dans leur domaine extracellulaire (Antipenko et al. 2003). Sur leur domaine intracellulaire, les plexines possèdent deux domaines « GTPase-activating protein » (GAP), ayant une forte homologie avec les petites protéines GTPases RasGAP, ainsi qu'un domaine de liaison aux GTPases qui peut accueillir la liaison des protéines GTPases Ras, Rac, RhoD, ou encore RND1 (Perälä et al. 2012). Les Sema étant des protéines transmembranaires ou sécrétées, les plexines interagissent avec leur ligand sur les cellules adjacentes, lors d'une sécrétion juxtacrine, ou dans l'environnement extracellulaire, pour des molécules paracrines. Après la liaison à leur ligand, les plexines déclenchent la signalisation intracellulaire, excepté pour la liaison à la famille des Sema3, qui nécessite obligatoirement la présence d'un corécepteur, appelé neuropiline (Kolodkin et al. 1997; He & Tessier-Lavigne 1997; Gu et al. 2005). Les neuropilines sont des protéines transmembranaires de 900 acides aminés, avec un domaine intracellulaire très réduit, sans activité enzymatique intrinsèque (Takagi et al. 1991). Elles fonctionnent en tant que partenaires de liaison aux Sema3 dans le complexe corécepteur avec la plexine. Les neuropilines se dimérisent aussi avec les récepteurs au Vascular Endothelial Growth Factor (VEGFR) après liaison avec le VEGF (Barr et al. 2008).

1.3 La signalisation induite par les sémaphorines

La fixation des Sema à leurs récepteurs induit la phosphorylation de résidus tyrosine au niveau du domaine intracellulaire des plexines (Tamagnone et al. 1999). Le changement de conformation du domaine intracellulaire permet alors le recrutement de petites protéines GTPases. La machinerie intracellulaire diffère en fonction des plexines activées. Par exemple, l'activation des plexines A et B induit l'inhibition de l'adhérence des intégrines à la matrice extracellulaire. En général, toutes les sémaphorines induisent la réorganisation des microtubules et du cytosquelette d'actine, via l'activation de la voie des Mitogen-Activated Protein Kinases (MAPK) (Gu & Ihara 2000; Aurandt et al. 2006; Bechara et al. 2008).

2. La Neuropiline-1

2.1 Structure et interactions

La neuropiline-1 (Nrp1) est un récepteur transmembranaire de haute affinité pour la Sema3A. La Nrp1 est, en effet, nécessaire à la signalisation Sema3A puisque les neurones sensoriels issus de souris déficientes en Nrp1, ou exposés à un anticorps neutralisant anti-Nrp1, ne répondent pas à la Sema3A *in vitro* (He et al. 1997; Kolodkin et al. 1997). La Nrp1 peut aussi lier d'autres Sema de classe 3 avec moins d'affinité (Bender & Mac Gabhann 2013). La partie extracellulaire de la protéine Nrp1 est complexe, contenant deux motifs « amino-terminal complement-binding » (CUB), suivis de deux domaines ayant une forte homologie avec des facteurs de coagulation V/VIII (FV/VIII), impliqués dans la liaison au domaine SEMA des sémaphorines, et au domaine C-terminal de la Sema3A (He et al. 1997; Antipenko et al. 2003; Nakamura et al. 1998). La Nrp1 possède aussi un domaine « Meprin, A5, Mu » (MAM), impliqué dans la formation d'homodimères (Nakamura et al. 1998). La Nrp1 peut en effet interagir avec son ligand sous forme de monomère, par exemple avec la forme de 65kDa de la Sema3A, ou sous forme d'homodimère, quand elle lie la forme dimérique covalente de 95kDa (Antipenko et al. 2003). La queue cytoplasmique de 40 acides aminés de la Nrp1 n'est pas impliquée dans la propagation du signal induite par le ligand. Elle ne comporte, en effet, aucun motif peptidique le permettant. Ainsi, après fixation de la Sema3A à la Nrp1, la transduction du signal se fait par l'interaction de la Nrp1 avec un corécepteur de type plexine A, mettant en jeu les domaines CUB et MAM juxtamembranaires (Nakamura et al. 1998). La plexine principalement recrutée par la liaison de la Sema3A à la Nrp1 est la plexine A1, même s'il existe aussi une interaction avec les plexines A2 et A4, alors que la plexine A3 serait plutôt impliquée dans la signalisation par la Nrp2 (Wu et al. 2014a; Schwarz et al. 2008).

Le récepteur Nrp1 est aussi impliqué dans la signalisation du VEGF (Gu et al. 2003). La Nrp1 est en effet capable de lier le VEGF164 grâce à son domaine FV/VIII (Gu et al. 2002), et d'agir comme corécepteur, en formant un hétérodimère avec le récepteur VEGFR2 (encore appelés KDR ou FLK1) (Gu et al. 2003; voir Vieira et al. 2007 pour revue). Cette signalisation a d'abord été principalement étudiée dans la mise en place du système cardiovasculaire, au vu du rôle angiogénique du VEGF. Néanmoins, le VEGF est maintenant reconnu pour son effet neurotrophique (Rosenstein et al. 2003; Gu et al. 2003). La Sema3A et le VEGF164 peuvent entrer en compétition dans la liaison au

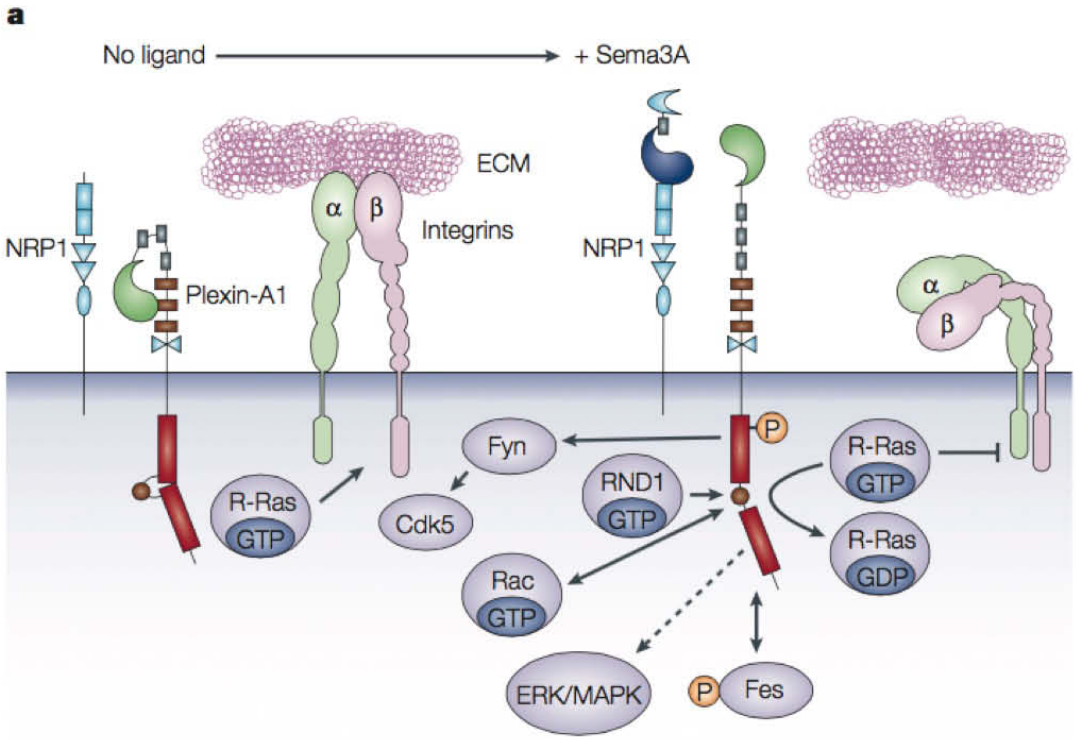


Figure 7. Voie de signalisation de la sémaphorine 3A suite à la fixation à son récepteur Neuropiline-1. La fixation de la Sema3A à son récepteur Nrp1 induit le changement de conformation de la plexine A1 et par conséquent le déclenchement de plusieurs événements intracellulaires. Le domaine de liaison aux GTPases permet le recrutement de petites protéines GTPases (R-Ras, RND1, Rac). Le recrutement de R-Ras provoquel'inhibition de l'adhésion des intégrines à la matrice extracellulaire. L'activation des protéines kinases Fes et Fyn induit le recrutement des « collapse response mediator » (CRMP) et des « CRMP-associated molecule » (CRAM) (ou protéines cdk5), impliqués dans la dynamique des microtubules. Enfin les protéines « mitogen-activated protéines kinases » (MAPK), en en particulier « extracellular signal-regulated kinase » (ERK) est activée et induit la réorganisation des microtubules et du cytosquelette d'actine. L'ensemble de cette signalisation induite le « collapse » du cône de croissance. *Abréviations : ECM, matrice extracellulaire ; P, groupement phosphate.* Adapté de Kruger et al. 2005.

récepteur Nrp1. Il est notamment décrit que la Sema3A antagonise l'effet stimulateur du VEGF164 sur la prolifération endothéliale (Miao et al. 1999).

On pensait initialement que la Sema3A ne se liait pas au récepteur Nrp2 (Chen et al. 1997). Ceci a été récemment infirmé par d'autres études. A l'heure actuelle on considère que la Sema3A peut se fixer à la Nrp2 (Nasarre et al. 2009; Cariboni et al. 2011).

2.2 La signalisation intracellulaire issue de l'interaction Sema3a/Nrp1

Suite à la liaison de la Sema3A à son récepteur Nrp1, la plexine A1 met en jeu la voie signalisation intracellulaire (Figure 7). L'activité GAP (qui permet de recruter des GTPases) de la plexine A1 nécessite la présence de la protéine RND1 et permet l'activation de la GTPase R-Ras, qui induit alors l'inhibition de l'adhérence des intégrines à la matrice extracellulaire. La liaison de la Sema3A induit aussi l'activation des protéines kinases Fes et Fyn qui recrutent les protéines « collapse response mediator » (CRMP) et la « CRMP-associated molecule » (CRAM) (Mitsui et al. 2002) qui sont impliquées dans la dynamique des microtubules (Gu & Ihara 2000). La plexine A1 mobilise également l'activation de la MAPK « extracellular signal-regulated kinase » (ERK), qui induit la réorganisation des microtubules et du cytosquelette d'actine (Campbell & Holt 2003). L'ensemble de cette signalisation est nécessaire à l'activité de « collapse » du cône de croissance neuronal (effondrement associé à la désorganisation du cytosquelette), qui caractérise la Sema3A depuis sa découverte (Luo et al. 1993; Kolodkin et al. 1993).

2.3 Les effets de la signalisation Sema3A/Nrp1

Développement des projections neuronales

La signalisation issue de la liaison de la Sema3A à son récepteur Nrp1 est connue pour son rôle inhibiteur sur le cône de croissance (Bechara et al. 2008). La Sema3A étant une molécule sécrétée, elle diffuse localement et agit selon un gradient de concentration dans l'environnement extracellulaire. Ainsi, elle a un rôle chimiorépulsif, en orientant la pousse axonale dans le sens opposé au gradient (Shelly et al. 2011). La Sema3A peut aussi avoir un rôle attractif, car elle stimule la croissance dendritique, et est impliquée dans le branchement des dendrites (Bagnard et al. 1998; Fenstermaker et al. 2004). Ces effets opposés seraient médiés par des taux différents de guanosine monophosphate cyclique (GMPc). Ainsi, cette signalisation est largement impliquée dans

le développement, la morphogenèse et la distribution des projections neuronales (Wu et al. 2014b; Tran et al. 2007) et guide donc l'intégration des neurones dans leur environnement (Cioni et al. 2013; Schwarz et al. 2008). Par conséquent, la signalisation par la Sema3A et la Nrp1 est impliquée dans la formation d'aires cérébrales, par exemple, dans la formation des aires corticales (Homman-Ludiye & Bourne 2014).

Migration

En plus d'un rôle dans le guidage et le branchement des projections neuronales, la signalisation Sema3A/Nrp1 guide aussi la migration des cellules. La Sema3A est en effet largement exprimée sur le trajet de migration radiale des neuroblastes originaires de la zone sous-ventriculaire pendant l'embryogenèse, en particulier par des cellules endothéliales (Meléndez-Herrera et al. 2008) et guide ainsi la migration correcte des cellules (Chen et al. 2008). La perturbation de la signalisation Sema3A/Nrp1 pendant l'embryogenèse conduit à une migration inappropriée des interneurones corticaux (Zimmer et al. 2010). Enfin, cette signalisation intervient dans le guidage de la migration des cellules issues des crêtes neurales (Eickholt et al. 1999).

3. La signalisation par le récepteur Nrp1 dans le développement du système à GnRH

Différentes Sema et leur récepteur ont montré leur importance dans le développement du système à GnRH (voir pour revue Giacobini 2015). Néanmoins, nous nous focaliserons ici uniquement sur les données connues à ce jour concernant la signalisation Sema3A/Nrp1.

3.1 La signalisation Sema3A/Nrp1 dans le développement du système olfactif

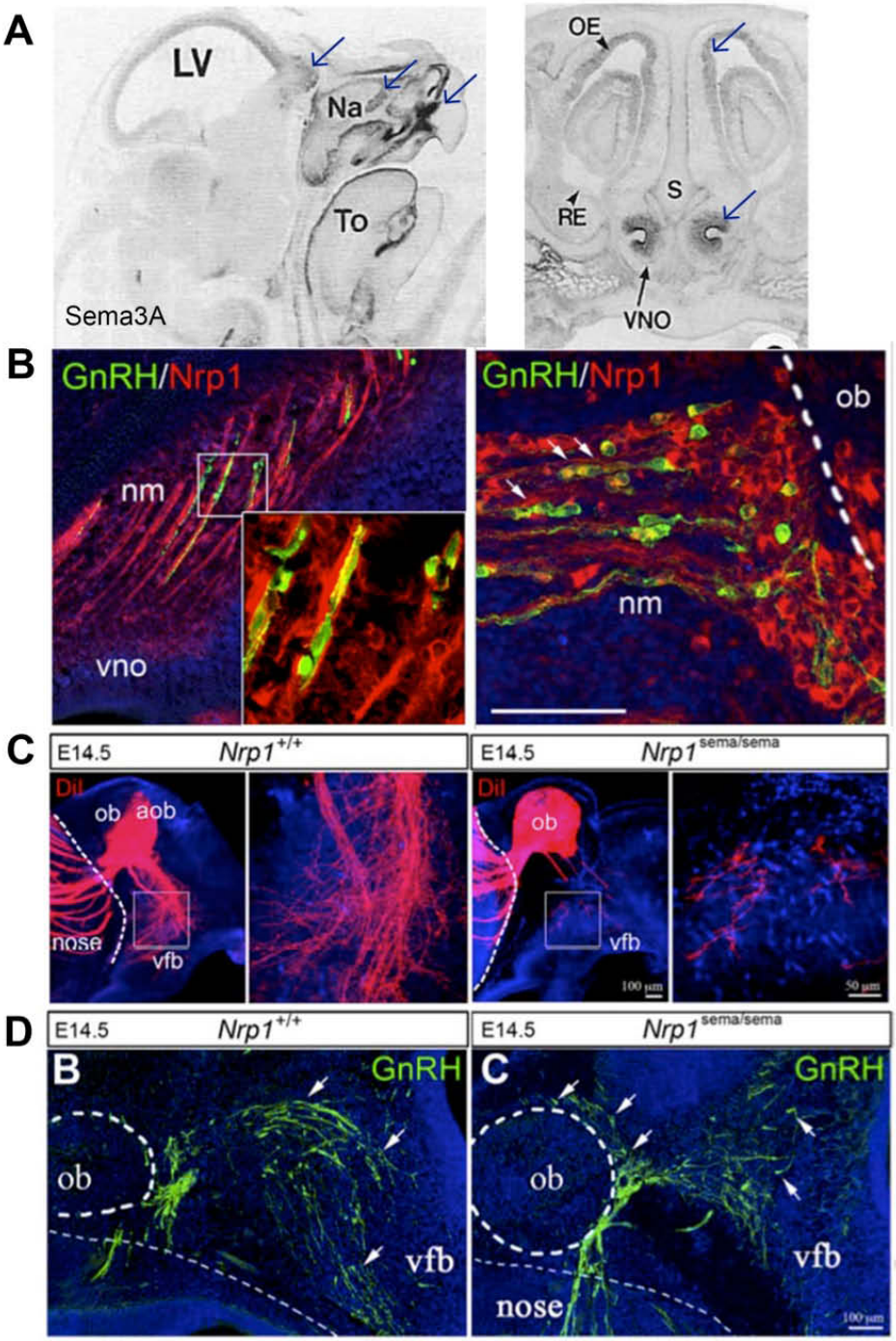
La Sema3A est exprimée par l'épithélium olfactif et voméronasal de souris pendant l'embryogenèse (Figure 8A), et est exprimée dans le parenchyme nasal, notamment par une sous-population d'axones olfactifs et voméronasaux, ainsi que par la couche externe de cellules bordant les bulbes olfactifs. Elle est aussi exprimée par une population de cellules faisant partie de la masse migratoire, qui migrent du nez au cerveau pendant l'embryogenèse, et qui deviennent des cellules gliales spécialisées dans le guidage des neurones olfactifs en les enveloppant, appelées cellules gainantes olfactives, ou « olfactory ensheating cells » (OEC) (Pasterkamp et al. 1998; Schwarting et al. 2000; Cummings & Brunjes 1995). Le récepteur Nrp1 est exprimé par une sous-population d'axones voméronasaux, différente et complémentaire de celle exprimant la

Sema3A. Les axones exprimant la Nrp1 s'étendent jusqu'aux bulbes olfactifs, en évitant soigneusement les régions exprimant la Sema3A (Schwarting et al. 2000). Ces deux molécules sont intimement liées au développement du système olfactif et voméronasal. Des souris déficientes en Sema3A montrent en effet des glomérules olfactifs atypiques et sous-développés, ainsi qu'une projection aberrante des axones voméronasaux et olfactifs (Schwarting et al. 2000; Taniguchi et al. 2003). La Sema3A est ainsi impliquée dans la fasciculation des nerfs voméronasaux et terminaux, qui servent de guide aux neurones à GnRH, ainsi que dans le développement du système olfactif. Par ailleurs, les neurones à GnRH eux-mêmes expriment le récepteur Nrp1, aussi bien chez la souris que chez l'Homme (Figure 8B) (Hanchate, Giacobini, et al. 2012).

3.2 L'impact de la signalisation Sema3A/Nrp1 sur la migration des neurones à GnRH

Plusieurs études, dont une menée au laboratoire, ont en effet démontré que la signalisation engendrée par la liaison de la Sema3A à la Nrp1 était impliquée dans le développement du système à GnRH pendant l'embryogenèse (Cariboni et al. 2011a; Cariboni et al. 2011b). Les souris déficientes en Sema3A présentent une migration des cellules à GnRH anormale associé à un hypogonadisme hypogonadotrope (Cariboni et al. 2010). Plus récemment, il a été montré que des souris dont le domaine de liaison de la Sema3A au niveau du récepteur Nrp1 était invalidé, présentaient un hypogonadisme hypogonadotrope associé à une anosmie, et donc un phénotype similaire au syndrome de Kallmann retrouvé chez l'Homme (Hanchate, Giacobini, et al. 2012). Dans ce modèle, les axones voméronasaux, et par conséquent les neurones à GnRH, s'accumulent au niveau de la lame criblée, à l'entrée des bulbes olfactifs, et ne parviennent pas à entrer dans le cerveau (Figure 8C et 8D). La projection anormale des nerfs voméronasaux en l'absence de signalisation par la Sema3A conduit à des défauts considérables du système à GnRH et du système olfactif chez ce mutant. Parallèlement, la mutation du gène *Sema3a* a été retrouvé chez près de 6% des patients atteints du syndrome de Kallmann, associé à d'autres gènes Kallmann connus (Tableau 1) (Hanchate, Giacobini, et al. 2012). Le défaut de migration des neurones à GnRH dans ces modèles animaux est néanmoins indirectement causé par le défaut de projection des nerfs voméronasaux. Le rôle intrinsèque de la signalisation par la Sema3A et la Nrp1 dans la migration des neurones à GnRH n'est pas encore connu.

Dans une étude de Cariboni et al. (2011), la suppression conditionnelle du récepteur Nrp1 spécifiquement dans les précurseurs neuronaux conduit à une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Nrp1sema/sema
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GnRH
ob
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nose
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Figure 8. **L'expression de la semaphorine 3A et de la Neuropiline-1 dans le système à GnRH chez l'embryon**. (A) Vue saggitale (gauche) et coronale (droite) d'une coupe d'embryon à E15 montrant l'expression de la Sema3A, révélée par hybridation *in situ*. On constate que la Sema3A est exprimée le long du trajet migratoire des neurones à GnRH : dans l'épithélium olfactif, l'organe voméronasal, le parenchyme nasal et au niveau des bulbes olfactifs. Adapté de Giger et al. 1996. (B) La protéine Nrp1 (rouge) est exprimée par les neurones à GnRH (vert) chez la souris (gauche) et chez le fœtus humain (droite). On constate, en effet, un marquage jaune résultant de la colocalisation des deux marquages (échelle 100mm). (C) Un colorant fluorescent antérograde et lipophile a été injecté dans la lumière de l'organe voméronasal chez des embryons à E14,5 pour marquer les axones voméronasaux (rouge). L'image de gauche montre le résultat du marquage chez un embryon sauvage ($Nrp1^{+/+}$), tandis que l'image de droite montre un embryon dont le domaine de liaison de la Sema3A sur le récepteur Nrp1 est muté ($Nrp1^{sema/sema}$), au niveau de la transition entre le nez et le cerveau, sur une coupe saggitale. Chez le sauvage, le nerf voméronasal s'étend dans la partie médiale du bulbe olfactif (ob) et du bulbe accessoire (aob) et envoie une branche ventrale dans le cerveau antérieur (vfb). On constate en revanche, chez l'embryon mutant, que la branche ventrale du nerf voméronasal est déstructurée voire totalement absente, en comparaison à l'embryon contrôle. (D) L'image de gauche montre le trajet de migration des neurones à GnRH chez un embryon sauvage à E14,5 dans la région de la jonction entre nez et cerveau. Les cellules à GnRH pénètrent dans le cerveau antérieur. Chez l'animal mutant (image de droite), la distribution des neurones à GnRH est aberrante. Les cellules restent bloquées au niveau du bulbe olfactif, ou se perdent dorsalement. Ce défaut est lié à un défaut de développement du système olfactif. Ce phénotype est comparable au syndrome de Kallmann qu'on retrouve chez l'Homme. *Autres abréviations : LV, ventricule latéral ; Na, cavité nasale ; To, langue ; RE, épithélium respiratoire, ou olfactif ; S, septum nasal ; VNO/vno, organe voméronasal ; nm, mésenchyme frontonasal ; nl, couche nerveuse*. Adapté de Hanchate et al. 2012 et Giacobini 2015

diminution du nombre total de neurones à GnRH. En association avec des expériences *in vitro*, cette étude montrerait que la signalisation induite par la fixation du VEGF au récepteur Nrp1, et non au VEGFR2, dans les neurones à GnRH, intervient dans la survie des cellules à GnRH au cours de leur migration. Les auteurs proposent donc un modèle dans lequel la Sema3A et le VEGF agiraient en coopération pour assurer d'une part le développement et le maintien des nerfs voméronasaux, et d'autre part la survie des neurones à GnRH, pour parvenir à une migration complète du système à GnRH pendant l'embryogenèse.

3.3 La signalisation Sema3A/Nrp1 dans la modulation des projections à GnRH

L'étude de Giacobini et al. (2014) est la seule étude rapportant le rôle de la Sema3A dans la neuritogénèse de neurones à GnRH immatures, et a été réalisée au laboratoire. Une culture primaire de neurones à GnRH issus d'embryons à E12 est naturellement entourée de cellules exprimant la Sema3A qui ont été isolées à partir du compartiment nasal. Après 24h, les neurones à GnRH étendent des neurites dans leur milieu. L'ajout d'un anticorps neutralisant anti-Sema3A au milieu de ces cultures conduit à une élongation réduite des projections issues des neurones à GnRH. Cet effet positif de la Sema3A sur la neuritogénèse est retrouvé chez une lignée de cellules matures immortalisées sécrétant la GnRH (GnV-3). Les neurites se développent en direction d'un gradient croissant de Sema3A, établit grâce à la transfection de cellules COS avec le gène *Sema3a*. L'ajout d'un anticorps neutralisant anti-Nrp1 dans le milieu réduit significativement la taille des neurites issues des cellules GnV-3. Ces données montrent que la Sema3A a une rôle, non seulement dans la formation des projections des neurones à GnRH immatures, mais encore dans la neuritogénèse des neurones à GnRH matures. Cette même étude montre le rôle de la Sema3A, via sa liaison au récepteur Nrp1, dans la plasticité des projections à GnRH *in vivo*, chez l'adulte (Giacobini et al. 2014) (voir Chapitre 1, 7.6). Brièvement la Sema3A sécrétée par les cellules endothéliales des capillaires de l'éminence médiane induit la pousse des axones à GnRH à proximité de l'espace péricapillaire des vaisseaux en proestrus chez la souris, et facilite ainsi la sécrétion de GnRH pendant le pic préovulatoire de GnRH.

Les données de la littérature rapportées dans cette introduction mettent en évidence l'existence de relations complexes au sein du système à GnRH impliquant une grande variété de facteurs et de types cellulaires permettant de réguler finement l'axe gonadotrope. Ces processus de régulation interviennent pendant l'embryogénèse, pour assurer le bon développement du système à GnRH, et pendant la vie postnatale pour permettre la mise en place de la sécrétion de GnRH. Ces éléments participent grandement au contrôle de la sécrétion cyclique et pulsatile de la GnRH, dans le but d'assurer un environnement hormonal propice au maintien de la fertilité. Les facteurs moléculaires ayant un rôle dans le contrôle de la fonction de reproduction sont variés. Ils émanent de neurones mais aussi de cellules gliales et endothéliales qui constituent l'environnement des corps cellulaires à GnRH ou de leurs terminaisons, sont parfois sécrétés par les neurones à GnRH eux-mêmes, ou encore proviennent de la périphérie. En effet, l'homéostasie gonadique, mais aussi la balance énergétique, constituent des acteurs clés du contrôle de la fonction de reproduction.

Lors de ce travail de thèse, nous avons cherché à comprendre le rôle de facteurs intrinsèques et extrinsèques aux neurones à GnRH, non seulement au cours de la migration des neurones à GnRH pendant l'embryogenèse, mais également dans la physiologie de la reproduction. Nous nous sommes pour cela intéressés, d'une part, aux facteurs exprimés par les neurones à GnRH et, en particulier, au rôle de leur sécrétion dans la mise en place du système et dans la fonction de l'axe gonadotrope, en tentant d'élucider en partie le mécanisme d'exocytose utilisé. D'autre part, nous nous sommes intéressés au rôle de molécules, en particulier la sémaphorine 3A et son récepteur exprimé par les neurones à GnRH, la Neuropiline-1, ayant précédemment été montré pour avoir un rôle dans le développement du système à GnRH, mais dont le rôle direct dans les neurones à GnRH restait à déterminer. En parallèle, nous nous sommes demandés si, en plus de ce qui est connu sur la communication entre la balance énergétique et la fonction de reproduction, l'axe gonadotrope pouvait affecter le métabolisme énergétique, en particulier dans certains cas pathologiques. Pour cela nous avons choisi d'articuler ce

travail de thèse autour de deux différentes études, basées sur des modèles animaux originaux.

Les différents objectifs de ma thèse ont donc été les suivants :

- Déterminer le rôle de la sécrétion des neurones à GnRH, et notamment de l'exocytose dépendante de l'expression des protéines VAMP, dans la mise en place et l'intégrité du système à GnRH et ses conséquences sur l'organisme.

- Déterminer le rôle de la signalisation Neuropiline-1 dans les neurones à GnRH sur leur migration et la mise en place de la fonction de reproduction.

ARTICLE 1 :

Calcium-dependent exocytosis in GnRH neurons is required for sexual maturation and body weight homeostasis but not hypothalamic trageting in female mice

Le premier projet de mon travail de thèse s'est porté sur des paramètres fondamentaux intrinsèques aux neurones à GnRH, qui étaient encore inconnus. Il a été de déterminer le rôle de l'exocytose dépendante de la protéine Vamp2 dans la sécrétion par les neurones à GnRH, et dans leur développement.

Lors de cette étude, nous avons mis en évidence le rôle fondamental de la protéine Vamp2 dans l'exocytose par les neurones à GnRH. Nous avons ainsi démontré que l'ablation de la protéine Vamp2 conduit à un hypogonadisme hypogonadotrope lié à un trouble de la balance énergétique. Nous avons enfin démontré que l'exocytose par les neurones à GnRH n'étaient pas nécessaire à leur migration correcte et à l'intégration à leur réseau.

L'ensemble de ces données a fait l'objet d'un article qui est en révision favorable dans *Endocrinology*.

Submission: January 11, 2016
Endocrinology

Calcium-dependent exocytosis in GnRH neurons is required for sexual maturation and body weight homeostasis but not hypothalamic targeting in female mice

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Running head: No alteration of GnRH neuron hypothalamic targeting by block of exocytosis

Number of text pages: 30
Number of figures: 6
Number of tables: 0
Number of words (abstract): 221

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Abstract

Puberty is initiated by activation of the hypothalamic-pituitary-gonadal axis. The initial steps involve GnRH release by hypothalamic neurons into the pituitary portal circulation triggering of gonadotropin release by the pituitary. Intriguingly, GnRH signaling has been shown to be dispensable in the proper development and maintenance of GnRH neurons. However, whether calcium-dependent transmitter release plays a role in this process remains unclear. To address this question, we generated mice in which activity-dependent exocytosis is blocked by the Cre recombinase-dependent expression of the *Clostridial botulinum* neurotoxin serotype B light chain, which cleaves vesicle-associated membrane protein 2. Here we show that toxin expression in GnRH neurons promotes GnRH deficiency leading to hypogonadotropic hypogonadism in a subpopulation of female mice that also develop overweight and hyperleptinemia. This effect depends on the actual proportion of GnRH neurons expressing the transgene, which does not alter the anatomic placement and projections of GnRH neurons in the hypothalamus. These data establish the existence of a threshold effect for congenital GnRH deficiency in which small environmental changes in individuals harboring an identical pool of genes may have major consequences on their reproductive and metabolic status throughout life.

Introduction

Hypothalamic GnRH-producing neurons are the master regulators of gonadotropin secretion and postnatal gonadal growth. The proper development of GnRH neurons, GnRH expression and GnRH signaling are essential for sexual maturation and the normal functioning of the hypothalamo-pityitary-gonadal (HPG) axis in mammals (1-4). Unlike other paraventricular neurosecretory neurons that arise from the neuroepithelium of the third ventricle (5, 6), GnRH neurons originate from both the neural crest and ectodermal progenitors in the olfactory placode (7), and subsequently migrate from the nose to the brain during embryogenesis (8, 9). At birth, GnRH neurons have reached their final destination within the hypothalamus where they are diffusely distributed and contact the median eminence where their neuroendocrine axon terminals open into the pituitary portal blood for delivery of GnRH to the pituitary. Some factors controlling development of GnRH neurons have been identified during these last 25 years (10).

The expression of the GnRH is dispensable for the migration and maturation of GnRH neurons as shown by *hpg* mice (11), which harbor a naturally-occurring deletion of the gene (2). Moreover, lack of the GnRH peptide does not preclude the spontaneous generation of bursts of action potentials in GnRH neurons (11) nor the projection of GnRH neurosecretory axons to the median eminence (11). However, it is possible that the autocrine-paracrine release of neuropeptides other than GnRH such as galanin (12-15) and neurotransmitters including glutamate (16) and GABA (17, 18) control the development of the GnRH system. GABA is known to inhibit GnRH neuronal migration (19-21) and to disrupt GnRH neuronal migratory fate and female reproductive function in mice where GAD-67, a key enzyme in GABA synthesis, is selectively overexpressed under the control of the GnRH promoter (17). Galanin is co-released with GnRH from large dense core vesicles (LDVs) in neuroendocrine neurons (13) and inhibits cell migration and increase neuronal

differentiation of SVZ-derived neuroblats *in vitro* (22). Moreover, there is evidence that synaptic release is required for the development of the GnRH neural network. A recent study has shown that patients harboring mutations in the gene coding for Rab3a, a protein associated with synaptic vesicles (23) exhibit gonadotropic axis deficiency (24). The same study shows that Rab3a haploinsufficiency in mice causes infertility associated with a reduction in GnRH number (24).

To test whether calcium-dependent exocytotosis influences the development of the gonadtropic axis we generated transgenic mice that express *Clostridial botulinum* neurotoxin serotype B light chain (BoNT/B) selectively in GnRH neurons using iBot mice (25) and a GnRH-Cre line (26). BoNT/B inhibits transmitter release by selectively cleaving vesicle associated membrane protein 2/synaptobrevin 2 (VAMP2) (27, 28), a component of the soluble Nethylmaleimide-sensitive factor-attachment protein receptor (SNARE) complex required both for ultrafast (synaptic vesicles) and slow (LDCVs) synaptic exocytosis in neurons (29). We report that expression of BoNT/B in GnRH neurons blunts the function of the hypothalamic-pituitary gonadal (HPG) axis in female animals without perturbing the anatomical placement and projections of the neurons. Interestingly, HPG dysfunction in these mice is associated with a marked metabolic phenotype.

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. *Gnrh::cre* (Tg(*Gnrh1::*Cre)1Dlc), Tg(CAG-BoNT/B,EGFP)U75-56wp/J (iBot mice) and *Gnrh::gfp* mice were a generous gift of Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA) (26), Dr. Frank Pfrieger (University of Strasbourg) (25) and Dr. Daniel J. Spergel (Section of

Endocrinology, Department of Medicine, University of Chicago, IL) (30), respectively. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the University of Lille; all experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU).

Isolation of hypothalamic GnRH neurons using Fluorescence-Activated Cell Sorting

The preoptic regions of *Gnrh::gfp* 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, about 400 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): *Gnrh1* (Gnrh1-Mm01315605_m1), *Vamp1* (Vamp1-Mm00772307_m1), *Vamp2* (Vamp2-Mm01325243_m1), *Vamp3* (Vamp3-Mm01268442_g1), *Vamp4* (Vamp4-Mm00450315_m1), *Vamp5* (Vamp5-Mm00444144_m1), *Vamp7* (Vamp7-

Mm00807071_m1), *Vamp 8* (Vamp8-Mm00450314_m1). Control housekeeping genes: *r18S* (18S-Hs99999901_s1); ACTB (Actb-Mm00607939_s1).

Physiological measurements

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.

Male-induced LH surge. The male-pheromone-induced preovulatory GnRH/LH surge assay was adapted from Bronson and Stetson (31) as described previously by us (32). LH was assayed using a protocol previously described by others (33). A 96-well high-affinity binding microplate (9018; Corning) was coated with 50 µL of capture antibody (monoclonal antibody, anti-bovine LH beta subunit, 518B7; University of California) at a final dilution of 1:1 000 (in $NaHCO_3/NaH_2CO_3$ solution, pH=9.6) and incubated overnight at 4°C. Then wells were incubated with 200 µL of blocking buffer [5% (w/v) skim milk powder in 1x PBS-T (1x PBS with 0.05% Tween 20)] for 2 hours at room temperature (RT). A standard curve was generated using a 2-fold serial dilution of mLH (reference preparation, AFP-5306A; National Institute of DIABETES and Digestive and Kidney Diseases−National Hormone and Pituitary Program [NIDDK-NHPP]) in 1% (w/v) BSA−1x PBS-T. The wells were then incubated with 50 µL of detection antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb; NIDDK-NHPP) at a final dilution of 1:10 000 for 1.5 hours (at RT). Each well containing bound substrate was incubated with 50 µL of horseradish peroxidase–conjugated antibody (polyclonal goat anti-rabbit, PI-1000 Vector Laboratories) at a final dilution of 1:2000. After a 1.5-hour incubation, 100 µL of o-phenylenediamine (002003; Invitrogen), substrate containing 0.1% H_2O_2 was added to each well and left at RT for 10 to 15 minutes. The reaction was stopped by addition of 50 µL of 3 M HCl to each well, and absorbance of each well was read at a wavelength of 490 nm

(Multiskan Ascent Thermo Labsystems, Ascent Software). The concentration of LH in whole blood samples was determined by interpolating the OD values of unknowns against a nonlinear regression of the LH standard curve.

Measure of body composition by MRI

MRI analyses have been performed using a published protocol (34). Briefly, a 7 T Bruker Biospec (Ettlingen, Germany) imaging system equipped with a 40 cm horizontal bore magnet was used to acquire images using T1-weighted and axial and coronal Rapid Acquisition with Relaxation Enhancement (RARE) sequences. Multi-slice coronal (FOV = 3.6 Å~ 3.6 cm, 14 contiguous slices of 1.5mm thick) and axial (FOV = 3 Å~ 3 cm, 18 contiguous slices of 2mm thick) RARE images were collected with a TR of 400ms, a TE of 9ms, a 256 Å~ 256 data matrix, and a number of repetitions (NEX) of 2.

Ovarian histology and quantitative analysis

Ovaries were collected from 3-month-old mice, immersion-fixed in 4% PFA solution and stored at 4°C. Paraffin-embedded ovaries were sectioned at a thickness of 5 µm and stained with hematoxylin-eosin.

Tissue preparation

For immunohistochemical analysis, adult female 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

Tissues were cryosectioned (Leica cryostat) at 35µm (free-floating sections). Immunohistochemistry was performed as previously reported (35, 36) using Alexa-Fluor 488- (1:400) and Cy3-conjugated (1:800) secondary antibodies (Invitrogen). Fluorescent specimens were mounted using 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich). The primary antisera used were the guinea pig anti-GnRH (1:10000) (37) and rabbit anti EGFP (1:1000; Abcam) (25). Analyses of the total GnRH cell number and GnRH fiber density have been performed as described previously (32).

Electron microscopy. Immunogold electron microscopic analyses of GnRH nerve terminals in the median eminence have been performed using a published protocol (38). The rabbit anti-GnRH (1:10000) we used was a generous gift from Prof. G. Tramu (Centre Nationale de la Recherche Scientifique, URA 339, Université Bordeaux I, Talence, France) (39).

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 One-way ANOVA (Bonferroni's multiple comparison test) and an unpaired two-tailed Student's t test. For non-normally distributed values Kruskal-Wallis (one way analysis) and Mann-Whitney tests were used. The significance level was set at $p < 0.05$. Data are indicated as means ± SEM. The number of animals, p values and degrees of freedom are indicated either in the main text or in the figure legends.

Results

GnRH neurons abundantly express VAMP2 transcript *in vivo*

We first investigated the expression profile of VAMPs in GnRH neurons isolated by fluorescent-activated cell sorting from the preoptic region of *Gnrh::gfp* mice at birth (Fig. 1A). Real-time PCR analyses revealed that the most abundant VAMP expressed in the preoptic region both in GnRH and non-GnRH cells was VAMP2 (Figure 1B). Transcripts for VAMP1, VAMP5 and VAMP8 were also expressed in the preoptic region (Figure 1B), whereas VAMP3, VAMP4 and VAMP7 mRNAs were barely detectable.

Expression of BoNT/B in GnRH neurons *in vivo* leads to overweight and hyperleptinemia in a subpopulation of female mice

To reveal the role of calcium-dependent transmitter release from GnRH neurons in mouse development, we induced BoNT/B expression in GnRH neurons by crossing iBot mice (25) with a GnRH-Cre line (26) (Fig. 2A), which expresses the Cre recombinase in the hypothalamus but not in the gonads (40). The resulting *Gnrh::cre*; iBot bigenic mice were viable, born at mendelian frequencies and had body weights at weaning indistinguishable from *Gnrh::cre* monogenic littermates ($t_{(16)}$=1.67, p=0.12, n=8 to 10 per group). Intriguingly, at peripubertal ages, i.e., between postnatal day 40 (P40) and P50, a subgroup of bigenic female mice started to put on significantly more weight than their littermates and stayed overweight for all their life (Fig. 2B). MRI analysis of adult female P80 female mice revealed a dramatic 300% increase in intra-abdominal fat of overweight bigenic mice when compared to lean bigenic and monogenic littermates as measured by MRI (Fig. 2C). In agreement with increased in fat mass, overweight bigenic mice exhibited a marked increase in circulating leptin levels when compared to monogenic littermates ($H_{(2)}$ = 7.1, P<0.05, n=5 to 11 per group) (Figure 2D).

Puberty onset is dramatically altered in overweight female mice expressing BoNT/B- mice

Vaginal opening in female *Gnrh::cre*; iBot mice was significantly delayed by 3-4 days compared to monogenic mice ($t_{(30)}$=2.81, p=0.009, n=13 to 19 per group), where vaginal introitus occurred between P28 and P37 (** p<0.01) (Fig. 3A). Daily collection and analysis of vaginal secretions from the day of vaginal opening revealed a dramatic delay in puberty onset measured by age at first ovulation in overweight bigenic mice (Fig. 3B). Notably, 3 out of 4 mice never reached puberty, whereas bigenic mice with normal weight curve reached puberty (i.e. age at first ovulation) at an age comparable to controls ($t_{(13)}$=1.39, p=0.19, n=4 to 10 per group) (Fig. 3B).

Overweight BoNT/B-expressing mice exhibit hypogonadotropic hypogonadism

Daily inspection of vaginal cytology in adult P60-P80 mice revealed an absence of normal estrous cyclicity in overweight bigenic mice, while lean bigenic littermates exhibited a cycle similar to monogenic controls (U = 21, P=0,89, n=5 to 10 per group) (Fig. 4A). To further probe the HPG axis, mice were subjected to a male-pheromone-induced preovulatory GnRH/LH surge protocol. While monogenic and lean bigenic mice exposed to male pheromones showed uteri full of fluid weighing more than 100 mg, overweight *Gnrh::cre*; iBot mice presented thread-like uteri weighing less than 7 mg (Fig. 4B and 4C). Strikingly, while the LH plasma levels in overweight bigenic female mice remained bellow the limit of detection, they were strongly increased in monogenic females (Fig. 4D). Lean bigenic littermates demonstrated proestrous-like levels of LH (Fig. 4D). Even though these latter appeared to be lower than the one seen in controls, the difference was not statistically significant due to the high variability of the LH values between individuals in the lean bigenic group ($H_{(2)}$=8,334, p>0.05, n= 3 to 5 per group). Morphometric analysis of ovaries revealed that there was a 1.7 fold reduction in the size of ovaries between overweight Gnrh*::cre*; iBot and monogenic littermates (length: $F_{(2.19)}$=9.85,

p=0.001, width: $F_{(2.19)}$=9.9, p=0.001, n= 5 to 10 per group) (Fig. 4E and 4F), whereas lean bigenic mice and monogenic female mice had ovaries of similar sizes (length: $F_{(2.19)}$=9.85, p=0.172, width: $F_{(2.19)}$=9.9, p=1, n= 5 to 10 per group) (Fig. 4E and 4F).

Expression of BoNT/B in GnRH neurons does not alter GnRH neuron development

We next investigated whether hypogonadotropic hypogonadism in overweight Gnrh*::cre*; iBot female mice was due to altered migration and neurite extention in GnRH neurons. Comparison of the size and distribution of the GnRH neuronal population in the brains of monogenic mice with overweight Gnrh*::cre*; iBot littermates unexpectedly showed that the total number of GnRH neurons and their rostral to caudal distribution was not different between the two groups ($t_{(4)=}$1.973, p=0,12, n=5 per group) (Figure 5A, 5B and 5C). These data thus suggest that similar GnRH neuronal migration patterns likely occurred developmentally between neurons in which exocytosis is hampered by the expression of BoNT/B and intact neurons. Likewise, GnRH immunostaining patterns were similar in the OVLT and the median eminence of both control and BoNT/B-expressing mice (($t_{(9)=}$0.21, p=0,84, n=5 per group) (Fig. 5A) suggesting that BoNT/B expression does not alter GnRH axon and/or dendrite extension. Electron microscopic inspection of the external zone of the median eminence showed no obvious changes in the ultrastructure of GnRH neuron axon terminals at the proximity of the pericapillary space of the pituitary portal blood vessels in overweight bigenic female mice compared to control monogenic littermates (n=5 to 10 per group; Fig. 5D). Together, these data suggest that the hypogonadotropic hypogonadism was not caused by developmental changes in GnRH neurons.

Penetrance of Cre-mediated recombination in iBot mice varies depending on individuals

The variable impact of BoNT/B expression in GnRH neurons on body weight and fertility was possibly caused by interindividual differences in transgene expression. To test this, we used immunohistochemical staining for EGFP to detect the transgene in GnRH neurons. The average fraction of EGFP+ neurons among all GnRH neurons was higher in overweight Gnrh*::cre*; iBot female mice than in lean Gnrh*::cre*; iBot littermates ($t_{(8)}$=2.6, p=0,032, n=4 per group) (Fig. 6) indicating variable targeting efficacy in the transgenic line.

Discussion

We report here that the blockade of calcium-dependent exocytosis in GnRH neurons by the selective expression of BoNT/B in these neurons leads to the progressive onset of hypogonadotropic hypogonadism in a subpopulation of mice that develop overweight at the age of puberty, while lean littermates exhibit normal puberty onset and regular estrous cycle. Retrospective analyses show that phenotypic differences between lean and overweight BoNT/B-expressing female mice are undistinguishable at vaginal opening, which occurs at an age where no divergence in body weight is detected yet. Body weight in bigenic mice is indeed seen to diverge only after P40. These results demonstrate that activation of the GnRH system during the juvenile period exerts a strong weight-reducing effect. This view is consistent with the fact that overweight BoNT/B-expressing female mice have disrupted puberty leading to hypogonadism and hence to low circulating levels of estrogens, which are well known for their anti-obesity effects (41-43). Our results are also consistent with the recent study showing that hypogonadal kisspeptin receptor knockout and *hpg* female mice exhibit metabolic dysfunctions (44) and that patients with either congenital or functional forms of hypogonadotropic hypogonadism have an increased prevalence of obesity (45, 46).

Exocytosis is mediated by a fundamental mechanism requiring the assembly of SNARE proteins (29). Our results show that VAMP2 is the vesicle SNARE protein preferentially expressed in GnRH neurons. This results is consistent with the known expression in the brain of VAMP2, which has been shown to be particularly enriched in the preoptic region (47) and the expression of VAMP2, but not VAMP1, in GnRH-releasing GT cells isolated from mice (48). In addition to its key role in activity-dependent neurotransmission (49), VAMP2 has been reported to being involved in neuritogenesis (50), growth cone chemoattraction (51) and terminal axon branching (52), as well as to likely play a role in neuronal migration (53). Even though, VAMP2 has been shown to modulate some of these processes by interfering with molecular pathways, such as the ones of integrins (50) and semaphorin 3A (54) that are known to control GnRH neuronal migration (35, 36, 55), axonal extention (55) and terminal axon plasticity (38, 56), our results show that the inhibition of its activity via the selective expression of BoNT/B in GnRH neurons affects neither GnRH neuronal distribution in the hypothalamus nor GnRH neuroendocrine projections to the median eminence. Because the *GnRH::Cre* line used to drive expression of the toxin has been shown to be active early during development (57), these results suggest that activity-dependent autocrine-paracrine release of neurotransmitters including peptides and putative trophic factors, but also calcium-triggered receptor delivery to the membrane (54) do not seem to play a major role in GnRH neuron development.

Neuronal haploinsufficiency of Rab3a, which is a highly abundant protein in synaptic vesicles used for docking and priming of exocytosis vesicles (58), has been shown to cause gonadotropic deficiency associated with the presence of a smaller total number of GnRH-immunoreactive neurons in mice (24). One hypothesis was that the latter alteration was either due to a defect in GnRH neuron differentiation in the olfactory placode or to an alteration of GnRH neuronal migration (24). Our results suggesting that development of GnRH neurons is normal in mice in which exocytosis has been selectively blunted in GnRH-expressing cells, would suggest that defective

number of GnRH neurons in Rab3a-deficient mice might not be a cell-autonomous trait. One possibility would be that this defect may rather involve deficiency in other neuronal cell populations, including abnormal development of the peripheral olfactory system forming the axonal scaffold for the intracerebral migration of GnRH cells (59), as demonstrated previously for other genetic animal models (35, 60).

The divergent impact of BoNT/B expression in GnRH neurons appears to be linked to the actual proportion of GnRH neurons expressing the transgene. These results strongly suggest that in our animal model Cre-mediated recombination is variable. This variability reveals a previously unknown threshold effect: when release is impaired in less than 50% of the GnRH neurons puberty onset and estrous cyclicity are normal. When more than 50% of the GnRH neurons are affected, mice show a marked delay in puberty onset and developed hypogonadotropic hypogonadism. Because the EGFP transgene is located downstream to an IRES sequence (Fig. 2A), we probably underestimate the proportion of GnRH neurons expressing BoNT/B (61). The threshold hypothesis is in good agreement with previous studies, which examined the relationship between embryonic GnRH neuronal migration and sexual maturation during postnatal development (62) or the threshold effect of killing Kisspeptin-receptor-expressing GnRH neurons on puberty onset and estrous cyclicity (63) Taken together, these studies may contribute to explain why some “disease genes” known to cause congenital hypogonadotropic hypogonadism in some patients do not lead to such defect in other individual, in which their expression is either asymptomatic or only causes reversible forms of GnRH-deficiency during adult life (64); it might depend on the proportion of GnRH neurons targeted by these mutations and several mutations may even be required to fully affect the neuroendocrine control of reproduction (35, 65, 66). However, our results also indicate that small differences in the proportion of GnRH neurons with altered development and/or function can have a big impact on the HPG axis once the threshold of disease-affected GnRH neurons is crossed. These data also show that

genetic and/or environmental changes as small as the ones that can occur in a litter of individuals harboring homogeneous genetic background may dramatically impact the expression of reproductive and metabolic phenotypes promoted by transgene expression in GnRH neurons.

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale, Inserm, and the University of Lille School of Medicine, France (grant number U1172 to V.P.), the Fondation pour la Recherche Medicale (Equipe FRM, DEQ20130326524, France to V.P), the Agence Nationale de la Recherche (ANR, France: RoSes and GnRH to P.G; Astrex to F.W.P.) and EU COST action (BM1105 to V.P., P.G. and E.H.). We thank Nathalie Jouy (cell sorting core facility), Florent Auger (*in vivo* imaging core facility), Marie-Hélène Gevaert and Rose-Marie Siminski (histology core facility), Delphine Taillieu, Julien Devassine and Delphine Cappe (animal core facility) of the University of Lille for their expert technical assistance and Dr. S. Rasika for the editing of our manuscript.

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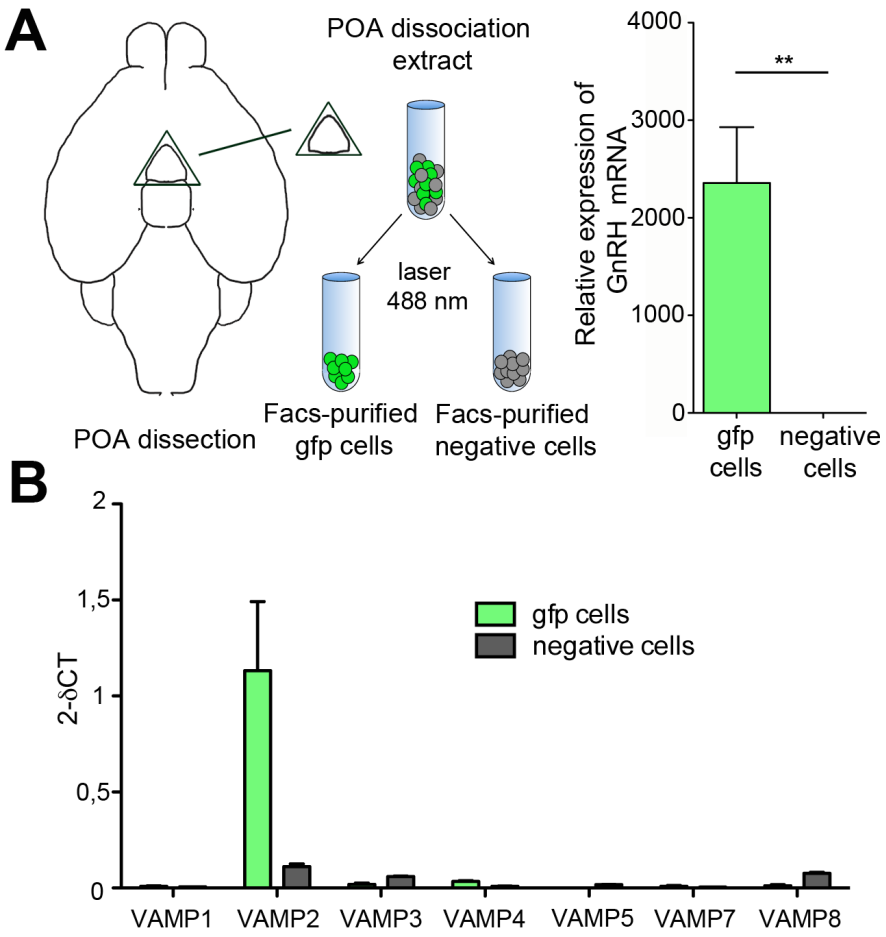

Figure 1. VAMP mRNA expression in GnRH neurons at birth. (A) Schematic diagram illustrating the steps of GFP-positive GnRH cell isolation from the preoptic region (POA) (ventral view of mouse brain) using FACS (laser 488 nm), and relative mRNA expression from real time PCR analysis of GnRH transcript in comparison with GFP-negative cells (GFP(-) cells). The GnRH transcript appears to be selectively expressed in GFP-positive cells (GFP (+) cells) ($t_{(6)}$ =4.12, p=0.0062, n=4). (**B**) Real-time PCR analysis ($2^{-deltaCT}$) of VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, VAMP7 and VAMP8 transcripts in FACS isolated GFP-positive and GFP-negative cells. Bar graphs show means +/- SEM.

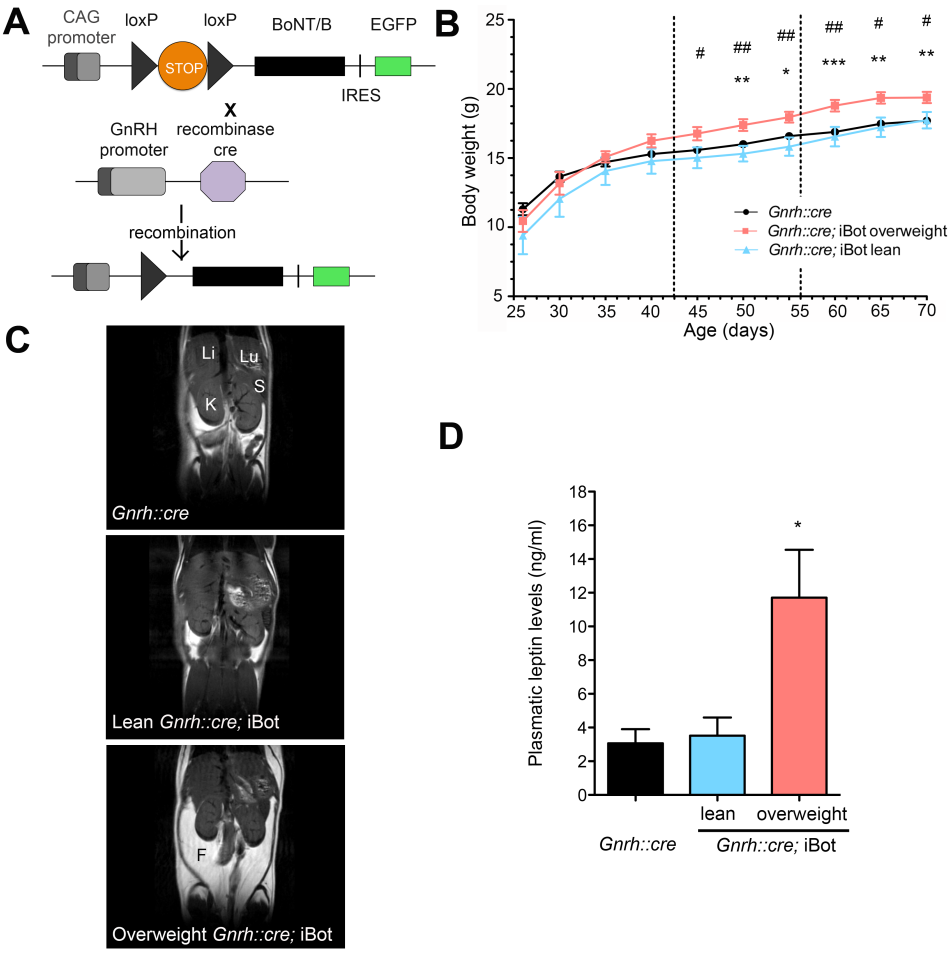

Figure 2

Figure 2. Overweight and hyperleptinemia in a subpopulation of female mice expressing BoNT/B in GnRH neurons. (**A**) Genetic strategy to express BoNT/B selectively in GnRH-expressing cells in mice using iBot mice and *Gnrh::Cre* line. (**B**) Body weight curves from postnatal day 25 (P25) to P70 in female transgenic mice. Note that a subgroup of bigenic *Gnrh::cre*; iBot mice exhibited an increase in body weight during the peripubertal period (dashed lines frame the period o puberty onset in *Gnrh::cre* mice) (repeated measures ANOVA, $F_{(2,18)} = 71.04$, $p<0.0001$). *Gnrh::cre*; iBot animals have been split into two distinct groups according to their body weight: overweight and lean. *Gnrh ::cre vs*. overweight *Gnrh ::cre*; iBot, * $P<0.05$, ** $P<0.01$; lean *Gnrh ::cre;* iBot *vs.* overweight *Gnrh ::cre*; iBot, # $P<0.05$. (**C**) Representative MRI pictures of the abdominal region in female mice. Fat mass (F) appears in white.

Lu : lung, Li : liver, S : stomac, Ki : kidney. (**D**) Plasmatic leptin levels in female littermates. Values shown are means ± SEM.

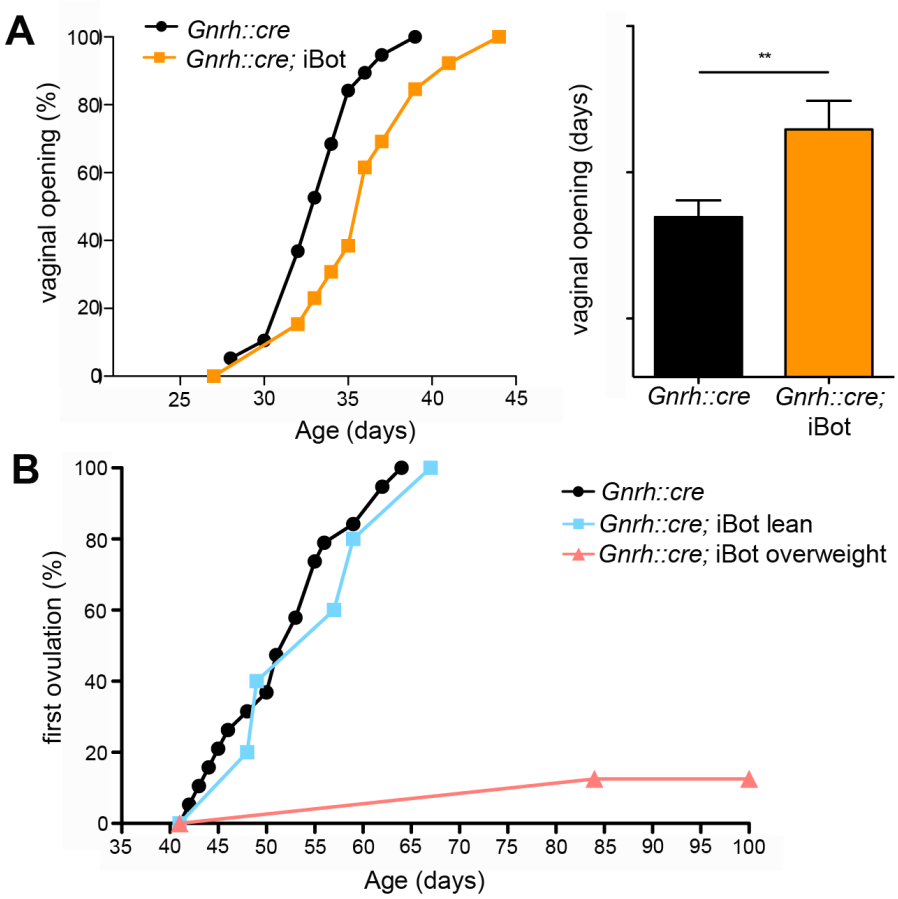

Figure 3. Puberty onset in overweight and lean transgenic mice. (**A**) Cumulative percentage data for vaginal opening (left panel) and corresponding bar graphs (right panel). **p<0.01. (**B**) Cumulative percentage data for first estrus. Note that 87.5% of the overweight Gnrh::cre; iBot female mice never reached puberty. Bar graphs show means ± SEM.

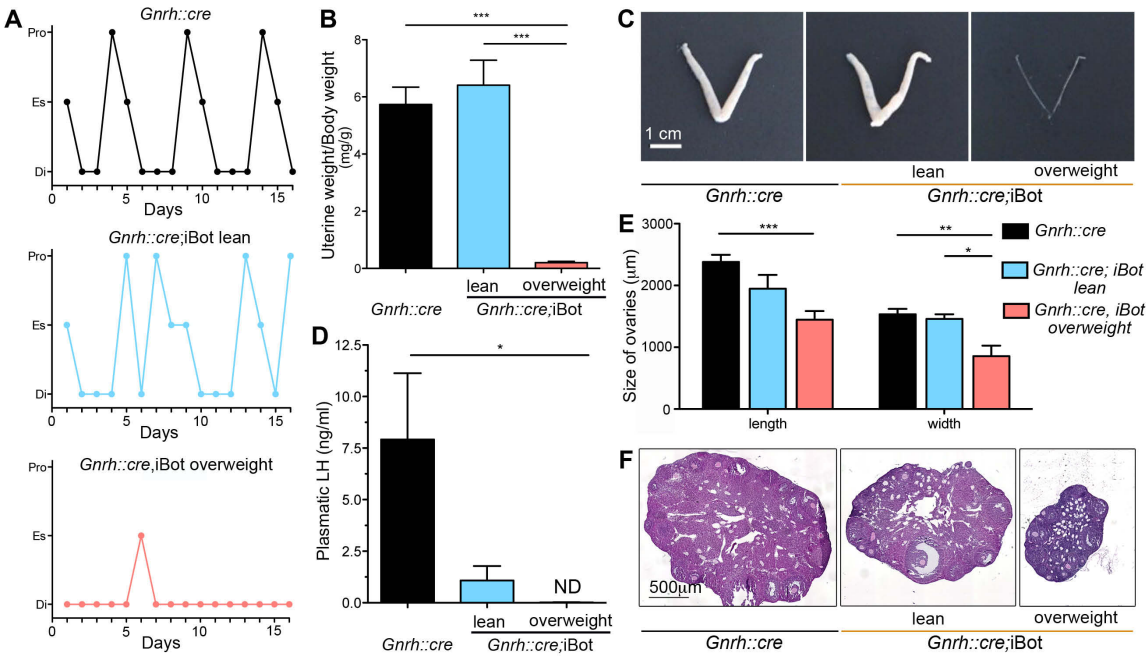

Figure 4

Figure 4. Adult fertility in overweight and lean transgenic mice. (**A**) Representative estrous cycle profiles in *Gnrh::cre* and *Gnrh::cre*; iBot littermates. Di : diestrus, Es : estrus, Pro : proestrus. (**B**) Mean uterine weight (mg/g BW) in females subjected to a male-induced LH-surge protocol. One-way ANOVA $F_{(2,20)}$ =23.36, p<0.0001, overweight *Gnrh::cre vs.* other groups p<0.0001 (***). (**C**) Representative picture illustrating uterine size in each group of mice. (**D**) Plasmatic LH levels following exposure to male odor (* p<0.05). ND, not detected. (**E**) Mean ovarian size in adult *Gnrh::cre* and *Gnrh::cre*; iBot mice (** p<0.01). (**F**) Representative micrographs of ovarian section after histochemical staining with hematoxylin-eosin illustrating ovarian size in each group of mice. Bar graphs show means ± SEM.

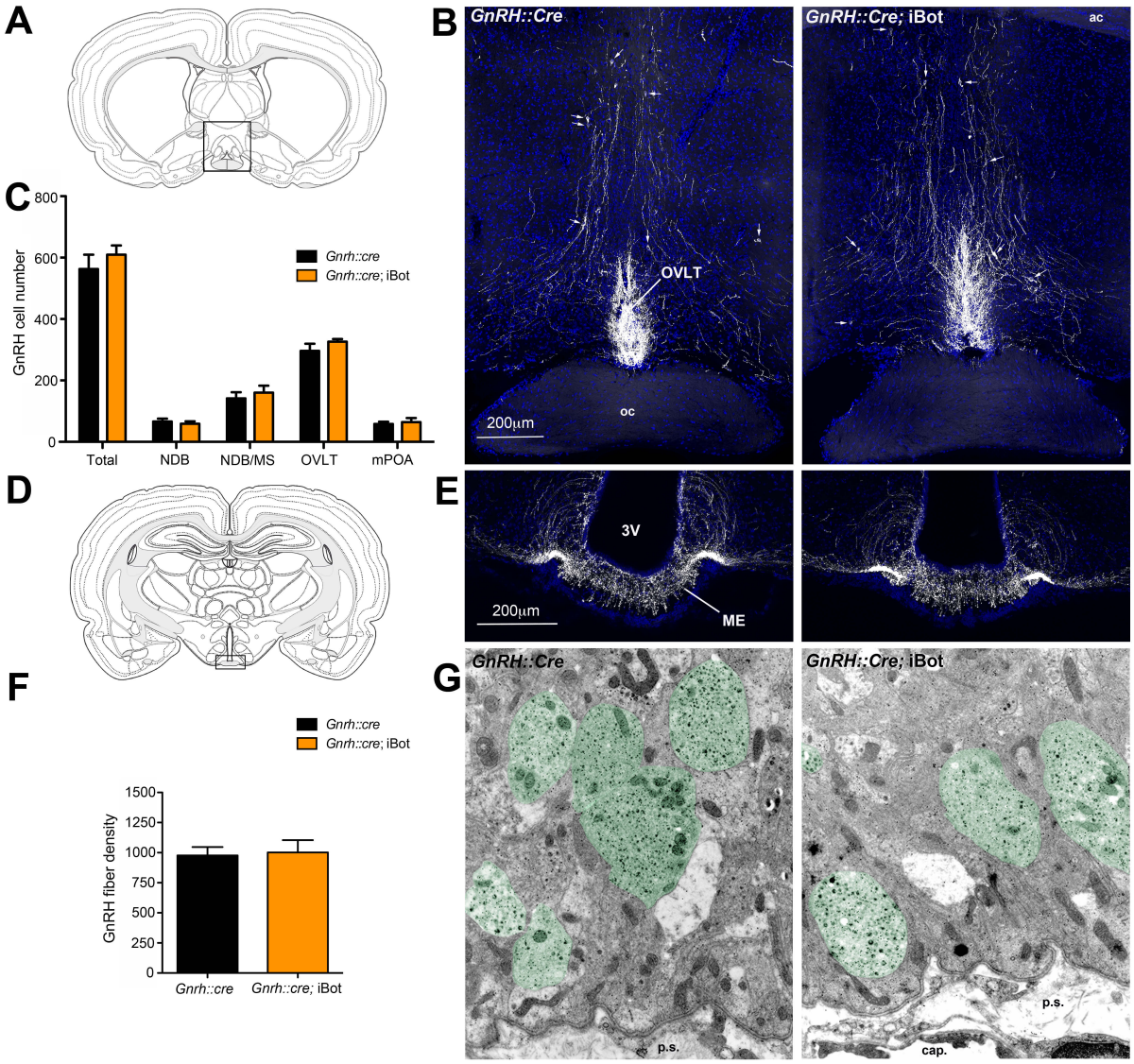

Figure 5

Figure 5. Expression of BoNT/B in GnRH neurons altered neither distribution of GnRH neuronal cell bodies in the preoptic region nor GnRH-immunoreactivity in the median eminence. (A) Drawing showing the level of the coronal brain section at the organum vasculosum of the lamina terminalis (OVLT). (**B**) Representative immunofluorescence images showing GnRH immunoreactivity (white) and nuclear staining (blue) in coronal sections of the preoptic region at the level of the OVLT in adult female mice. oc, optic chiasma; ca, anterior commissure. Arrows show GnRH cell bodies. (**C**) Bar graphs showing the distribution of GnRH neuronal cell bodies (white arrows) in the hypothalamus of adult transgenic mice with indicated genotype.

NDB, diagonal band of Broca; MS, medial septum; mPOA, median preoptic area. (**D**) Drawing showing the level of the coronal brain section at the ME. (**E**) Representative immunofluorescence images showing GnRH immunoreactivity (white) and nuclear staining (blue) in coronal sections at the level of the ME in adult female mice. 3V, third ventricle, (**F**) Bar graphs showing GnRH fiber density in the hypothalamus of adult transgenic mice with indicated genotype. (**G**) Representative electron microphotograph of the external zone of the ME showing 15-nm gold particle-labeled GnRH nerve terminals (green) at the proximity of the pericapillary space (p.s.). cap, fenestrated pituitary portal blood capillary. Bar graphs show means ± SE. bar graphs showing GnRH fiber density in adult female mice of different genotypes.

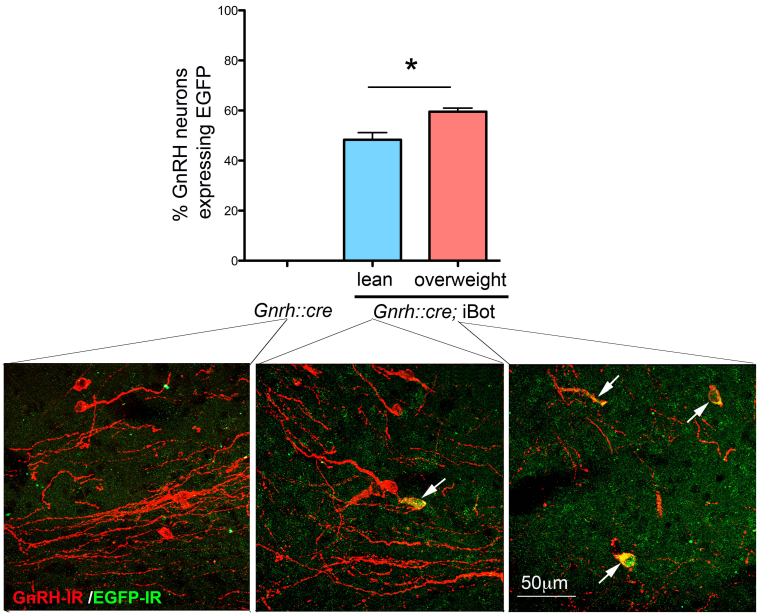

Figure 6

Figure 6. Efficiency of the Cre-mediated recombination in *Gnrh::cre*; iBot mice. *Upper panel*, percentage of hypothalamic GnRH neurons expressing EGFP in *Gnrh::cre* and *Gnrh::cre*; iBot mice. *Lower panels* representative images showing EGFP-immunoreactivity (green) in GnRH-immunoreactivive neurons (red) of *Gnrh::cre*; iBot mice (yellow cells shown by white arrows, right panels), but not in *Gnrh::cre* littermates (left panel). * $p < 0.05$. Values shown are means ± SEM.

Des résultats complémentaires à cette étude ont été obtenus plus récemment. A la suite de notre première étude, nous nous sommes demandé, d'une part, si la fonction hypophysaire était intacte dans notre modèle animal exprimant la toxine botulique dans les neurones à GnRH. D'autre part, nous avons voulu savoir si le phénotype observé chez les femelles était le même chez les mâles.

Les données supplémentaires présentées ci-après montrent que les animaux mutants déclenchent une sécrétion de LH en réponse à une injection périphérique de GnRH. Le suivi de la puberté chez le mâle montre que les mâles mutants présentent une puberté retardée associée à un poids inférieur comparé aux animaux contrôles.

Ces résultats apportent de nouvelles informations à notre étude. La fonction hypophysaire est, en effet, intacte dans notre modèle animal, ce qui suggère que le phénotype d'hypogonadisme hypogonadotrope est uniquement lié à un défaut de sécrétion de GnRH. Deuxièmement, ces résultats mettent en évidence un dimorphisme sexuel entre les mâles et les femelles dans ce modèle animal quand à l'impact de la balance énergétique.

Matériel et Méthodes supplémentaires

Injection intrapéritonéale de GnRH

Le sang a d'abord été prélevé chez tous les animaux par la veine mandibulaire, pendant la phase de diestrus (pour les animaux ayant un cycle). De la GnRH-1 synthétique (GeneCust) a été injectée par voie intrapéritonéale à la concentration de 1,25 mg/mL (200 ml par animal). Le sang a été récupéré 15 min après injection par la veine mandibulaire. Le plasma est centifugé à 6600 RPM pendant 15 minutes et le surnagent est gardé sur glace jusqu'à stockage à -80°C. Le dosage de la LH est décrit dans le matériel et méthode de l'étude précédente.

Suivi des animaux et détermination de la puberté par la séparation préputiale

Les animaux ont été pesés chaque jours depuis le sevrage jusqu'à l'âge de P50. La séparation préputiale a été vérifiée chaque jour depuis le sevrage jusqu'au décollement complet du prépuce. L'urine de chaque mâle a été récupérée chaque jour entre le sevrage et l'âge de P50. L'urine est centrifugée à 13000 RPM pendant 5 min et conservée à -80°C.

Western blot et révélation de la protéine MUP (major urinary protein)

Les échantillons d'urine sont chauffés à 100°C pendant 5 min. Les échantillons sont déposés sur un gel de polyacrylamide MES SDS à 4-12% (Invitrogen) pour une électrophorèse de 1H à 200V. Les protéines sont ensuite transférées sur une membrane de polyvinylidene difluoride (PVDF) à pores de 0,2 mm (Invitrogen) pendant une heure sur glace. Les membranes sont incubées une heure dans un tampon Tris salin (TBS ; 0,05 M Tris, pH 7,4, 0,15 M NaCl) avec 0,05% de Tween 20 (TBST) et 5% de lait non-gras à température ambiante. Les membranes sont exposées à l'anticorps primaire Rabbit polyclonal anti-MUP 1 :1000 (R&D Systems) sur une nuit à 4°C. Les membranes sont ensuite détectées par un anticorps secondaire horseradish peroxidase-conjugated anti-rabbit 1 :500 (Sigma) dans du

TBST 5% lait pendant une heure à température ambiante. Les membranes sont révélées grâce à un substrat chimioluminescent (NEL101 ; PerkinElmer, Boston, MA).

Résultats

Les animaux *Gnrh ::cre* ; iBot, déficients en protéine Vamp2 dans les neurones à GnRH, ont une fonction hypophysaire intacte.

Afin de déterminer si la fonction hypophysaire gonadotrope est affectée dans notre modèle animal, et par conséquent connaître l'origine exacte de défaut de sécrétion de LH chez les femelles mutantes, nous avons effectué une injection de GnRH par voie intrapéritonéale et récupéré le sang périphérique, par la veine mandibulaire, 15 min après injection pour dosage de la LH. Les taux de LH avant le dosage sont similaires entre animaux contrôles et mutants, le sang ayant été prélevé en phase de diestrus, quand la LH est basale. 15 min après injection de GnRH, les taux de LH sont significativement augmentés par rapport aux taux basaux dans les deux groupes ($t_{(20)}$=9,28, p<0,0001, n=11 groupe contrôle; $t_{(14)}$=9,46, p<0,0001, n=8 groupe mutant). Les taux de LH après injection sont similaires entre animaux contrôles et mutants ($t_{(17)}$=0,09, p=0.92, n=8 à 11 par groupe). La capacité à sécréter la LH n'est donc pas affectée chez les animaux mutants. Ceci suggère que l'hypogonadisme observe est probablement et uniquement en lien avec un défaut de secretion de la GnRH.

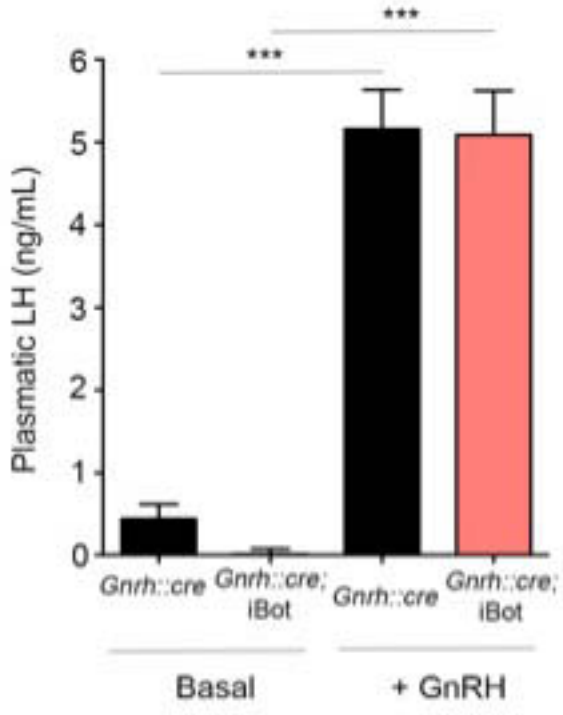

Figure 1

Figure 1. La fonction hypophysaire est intacte chez les animaux déficients en protéine Vamp2 dans les neurones à GnRH. Taux de LH avant et 15 min après injection de GnRH. Moyenne +/- SEM. *** p<0,001

Les mâles *Gnrh ::cre* ; iBot présentent une puberté retardée, et un poids inférieur aux contrôle.

Le suivi révèle, dans un premier temps, une séparation préputiale retardée de plus de 10 jours en moyenne chez les animaux déficients en protéine Vamp2 dans les neurones à GnRH en comparaison aux contrôles ($t_{(10)}$=5,27, p=0.0004, n=5 à 7 par groupe). Ce paramètre a été associé à l'apparition de la protéine MUP, dépendante des taux de testostérone, dans les urines. Le jour d'apparition de la protéine MUP est significativement retardée d'environ 5 jours, chez les animaux mutants ($t_{(8)}$=2,39, p=0,043, n=4 à 7). De manière intéressante, le poids est significativement inférieur chez les animaux mutants compare aux animaux contrôles dès l'âge de 35 jours ($t_{(10)}$=2,85, p=0,02, n=5 à 7 par groupe) jusqu'à P50 ($t_{(10)}$=3,40, p=0,007, n=5 à 7 par groupe). Ces données mettent en lumière un retard de la maturation de l'axe gonadotrope chez le male, similaire à ce qui a été observé chez les femelles.

Inversement aux femelles, les mâles présentent, quant à eux, un poids inférieur aux animaux contrôles. Il existe donc un dimorphisme sexuel dans notre modèle animal déficient en protéine Vamp2 dans les neurones à GnRH.

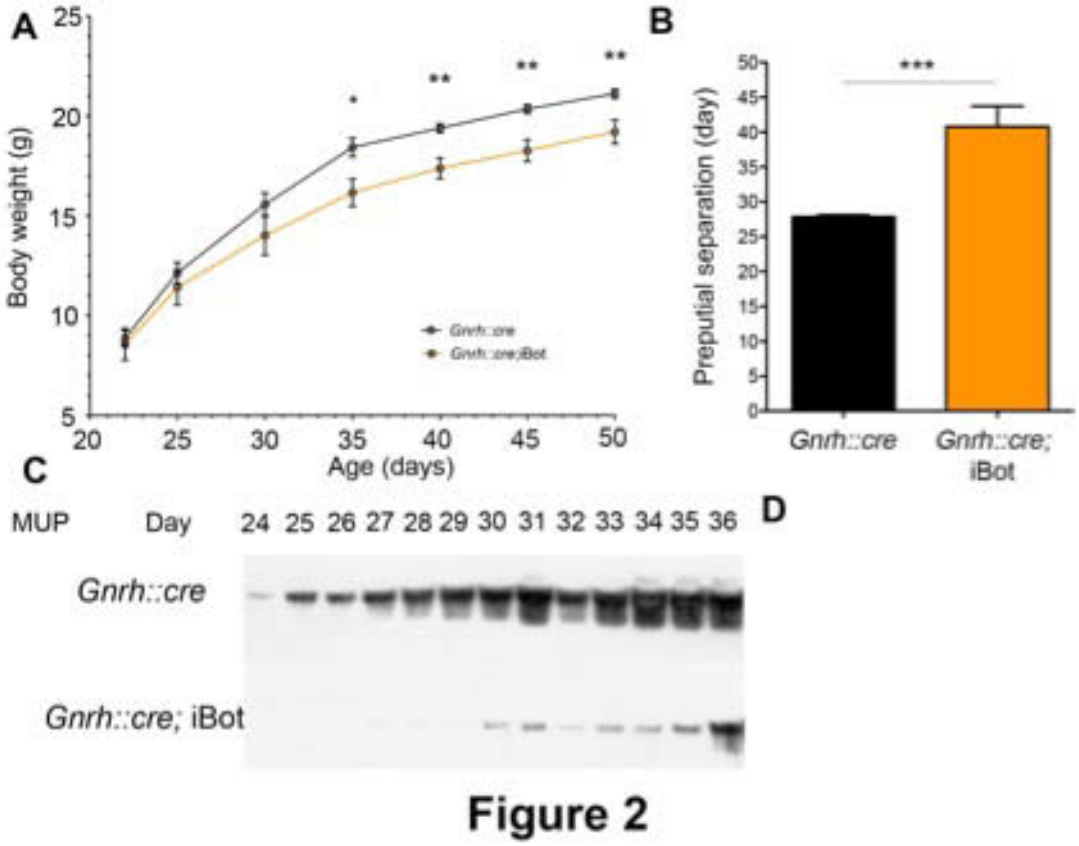

Figure 2

Figure 2. Les mâles déficients en protéine Vamp2 dans les neurones à GnRH présentent un retard pubertaire et un poids inférieur aux contrôles. (A) Evolution du poids corporel entre P22 et P50 (ANOVA, $F_{(2,18)}$ = 71.04, p<0.0001) (* p<0,05; ** p<0,001)/ (B) Jour moyen de la séparation préputiale (*** p<0,001). (C) Cinétiques représentatives de l'apparition de la protéine MUP dans les urines des animaux contrôles (en haut) et mutants (en bas) détecté par western blot. (D) Jour moyen de l'apparition de la MUP dans les urines (* p<0,05). Moyenne +/- SEM.

ARTICLE 2 :

GnRH neuronal migration and central precocious puberty : a role for Neuropiline-1 expression in GnRH neurons

Le second projet de mon travail de thèse a été de déterminer le rôle intrinsèque de l'expression du récepteur Neuropiline-1 dans les neurones à GnRH, et de comprendre son rôle dans la fonction de reproduction.

Lors de cette étude, nous avons mis en évidence le rôle de la Neuropiline-1 dans la survie des neurones à GnRH pendant l'embryogenèse et dans leur accumulation dans le cerveau. Chez l'adulte, nous avons montré que la distribution des neurones à GnRH est altérée, et que les cellules se retrouvent accumulées dans l'hypothalamus. Ces observations neuroanatomiques sont liées à un phénotype de puberté précoce centrale, ainsi qu'à un trouble de la balance énergétique chez l'adulte.

L'ensemble de ces données a fait l'objet d'un article qui est en cours de préparation.

GnRH neuronal migration and central precocious puberty: a role for Neuropilin-1 expression in GnRH neurons

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Running head: GnRH neuronal migration controls the timing of puberty onset

Number of text pages:
Number of figures: 5

Number of tables: 0

Number of words (abstract): 153

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Abstract

Semaphorin3A (Sema3A), a new Kallmann gene, and its receptor, neuropilin 1 (Nrp1) are required for GnRH neuronal migration during embryogenesis. Disruption of Sema3A signaling indeed alters the projections of vomeronasal axons towards the olfactory bulb that are used as a scaffold for neuronal migration between the nose and the brain by GnRH neurons in mice and causes hypogonadotropic hypogonadism in humans. However, the specific role of *Nrp1* expression in GnRH neurons themselves remains to be elucidated. Here, we show that the selective invalidation of *Nrp1* expression in GnRH neurons leads to juvenile overweight and precocious puberty, a phenomenon accompanied by an increased migration of GnRH neurons into the hypothalamus. These data raise the intriguing possibility that the timing of sexual maturation during postnatal development could be modulated by the number of GnRH neurons that reach the hypothalamus during embryogenesis and that GnRH neurons may play an active role in the control peripubertal metabolism.

Introduction

Reproduction and transmission of the genetic material is one of the major preoccupations of the sexually produced individuals after nutrition. In mammals, fertility, which is under the control of the hypothalamic-pituitary-gonadal (HPG) axis, is directly orchestrated by a small population of neuroendocrine neurons releasing the GnRH neurohormone that dives gonadotropins secretion in the pituitary (1–3). The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), in turn act on peripheral reproductive organs to regulate the onset of puberty, gametogenesis, and estrous cyclicity (4). GnRH-secreting neurons originate from both the nasal placode and the neural crest during embryonic development and migrate to the hypothalamus along vomeronasal/terminal nerves (5–9).

The complex developmental events leading to correct GnRH neuronal migration and secretion are tightly regulated by the specific spatiotemporal expression patterns of growth factors, adhesion molecules, diffusible attractants and repellents (8,10). The semaphorins constitute one of the largest families of phylogenetically conserved proteins, serving as guidance cues. Although originally identified as embryonic axon guidance cues, semaphorins are now known to regulate multiple processes crucial for neuronal network formation (11,12).

Of these, Sema3A, which exert both repulsive and attractive effects on migrating cells during embryogenesis (13–16), is widely expressed on the migratory path of the GnRH cells during embryonic development (17). The action of Sema3A through its receptor Neuropilin-1 (Nrp1) recently appeared to be essential for the normal development of the gonadotrope axis (18). A disruption of this signalling pathway during embryogenesis leads to a Kallmann-like phenotype, caracterized by hypogonadotrope hypogonadism (HH) and anosmia, in mice and *SEMA3A* has recently been described as a new Kallmann gene in humans (19,20). Alteration of Sema3A/Nrp1 signaling in mice appears to affect GnRH neuronal migration by

impairing the targeting of the vomeronasal axons to the brain that are used as scaffolds for their migration. However, the putative role of Nrp1 expression in GnRH neurons in GnRH system development remains to be elucidated.

In this study we examined the role of Nrp1 expression in GnRH neurons using a transgenic mouse model in which Nrp1 expression was selectively knocked out in GnRH neurons. We report that these mice exhibit central precocious puberty. This phenomenon is associated with an accumulation of GnRH cells in the medial preoptic region likely caused by cell-autonomous migratory defects during embryogenesis.

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. *Gnrh::cre* (Tg(*Gnrh1::*Cre)1Dlc), and *Gnrh::gfp* mice were a generous gift of Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA) (21), and Dr. Daniel J. Spergel (Section of Endocrinology, Department of Medicine, University of Chicago, IL) (22), respectively. *Nrp1*$^{loxp/loxp}$ (B6.129(SJL)-Nrp$^{tm2Ddg/J}$) mice were purchase from Jackson Laboratory, Maine, USA (23). Genotyping was released by polymerase chain reaction (PCR) and the following primers: *Nrp1*-sens 5'-AGGTTAGGCTTCAGGCCAAT-3' *Nrp1*-antisens 5'-GGTACCCTGGGTTTTCGATT-3' ; *Gnrh ::cre-s*ens 5'-CTGGTGTAGCTGATGATCCG-3' *Gnrh ::cre*-antisens 5'-ATGGCTAATCGCCATCTTCC-3' ; *Gnrh ::gfp*-sens 5'-GAAGTACTCAACCTACAACGGAAG-3' *Gnrh ::gfp*-antisens 5'GCCATCCAGTTCCACAGAATTGG -3'. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the University of Lille; all experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU).

Isolation of hypothalamic GnRH neurons using Fluorescence-Activated Cell Sorting

The preoptic regions of *Gnrh::cre; Nrp1*$^{loxp/loxp}$; *Gnrh::gfp* and *Nrp1*$^{loxp/loxp}$; *Gnrh::gfp* 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, about 400 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): *Gnrh1* (Gnrh1-Mm01315605_m1), *Nrp1* (Nrp1-Mm01253208_m1), *Nrp2* (Nrp2-Mm00803099_m1), *PlexinA1* (PlexinA1- Mm00501110_m1), *PlexinA2* (PlexinA2-Mm00801930_m1), *PlexinA3* (PlexinA3- Mm00501170_m1), *PlexinA4* (PlexinA4-Mm00558881_m1). Control housekeeping genes: *r18S* (18S-Hs99999901_s1); ACTB (Actb-Mm00607939_s1).

Physiological measurements

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.

Male-induced LH surge. The male-pheromone-induced preovulatory GnRH/LH surge assay was adapted from Bronson and Stetson (24) as described previously by us (25). LH was assayed using a protocol previously described by others (26). A 96-well high-affinity binding microplate (9018; Corning) was coated with 50 µL of capture antibody (monoclonal antibody, anti-bovine LH beta subunit, 518B7; University of

California) at a final dilution of 1:1 000 (in NaHCO3/NaH2CO3 solution, pH=9.6) and incubated overnight at 4°C. Then wells were incubated with 200 µL of blocking buffer [5% (w/v) skim milk powder in 1x PBS-T (1x PBS with 0.05% Tween 20)] for 2 hours at room temperature (RT). A standard curve was generated using a 2-fold serial dilution of mLH (reference preparation, AFP-5306A; National Institute of DIABETES and Digestive and Kidney Diseases−National Hormone and Pituitary Program [NIDDK-NHPP]) in 1% (w/v) BSA−1x PBS-T. The wells were then incubated with 50 µL of detection antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb; NIDDK-NHPP) at a final dilution of 1:10 000 for 1.5 hours (at RT). Each well containing bound substrate was incubated with 50 µL of horseradish peroxidase–conjugated antibody (polyclonal goat anti-rabbit, PI-1000 Vector Laboratories) at a final dilution of 1:2000. After a 1.5-hour incubation, 100 µL of o-phenylenediamine (002003; Invitrogen), substrate containing 0.1% H2O2 was added to each well and left at RT for 10 to 15 minutes. The reaction was stopped by addition of 50 µL of 3 M HCl to each well, and absorbance of each well was read at a wavelength of 490 nm (Multiskan Ascent Thermo Labsystems, Ascent Software). The concentration of LH in whole blood samples was determined by interpolating the OD values of unknowns against a nonlinear regression of the LH standard curve.

Measure of body composition by CT Scan

Body composition analysis of fat/lean mass was perfom using a CTScan LaTheta 100 X-ray Computed Tomography scanner (LCT-100A; Zinsser Analytic) (Collden et al.).

BrdU injections

5-Bromo 2-deoxyuridine (Sigma) was injected intraperitonaly at the concentration of 3mg/Kg to pregnant mice at E9,5 and 10,5 or at 11,5.

Tissue preparation

For immunohistochemical analysis, adult female 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 50-100 ml of 4% PFA, pH7.4. Brains were collected, postfixed in the same fixative for 2 h at 4°C, cryoprotected with PBS 20% sucrose, and embedded in OCT embedding medium (Tissue-Tek), frozen in isopenthane cooled with nitrogen, and stored at −80°C until cryosectioning (coronal slices). Embryos were removed from uterus of pregnant females, after sacrifice, at embryonic age 12,5 (E12,5), E14,5, E18,5, or were sacrified at postnatal day 0 (P0). The entire head was dissected and post-fixed from 4 h to one night according to the age at 4°C, then cryoprotected with PBS 20% sucrose, and embedded in OCT, frozen in isopenthane cooled with nitrogen, and stored at 80°C until saggital cryosectioning.

Nasal explants

Embryos were obtained from timed-pregnant animals. Nasal pits of E11,5 were isolated under aseptic conditions in Gey's Balanced Salt Solution (Invitrogen) enriched with glucose (Sigma-Aldrich) and plated. Explants were placed onto glad coverslips coated with 10 μl of chicken plasma (Cocalico Biologicals). 10 μl of thrombin (Sigma-Aldrich) was then added to adhere (thrombin/plasma clot) the explant to the coverslip. Explant were maintained in defined in serum-free medium (SFM) (27) containing 2.5 ml/ml Fungizone (Sigma/Aldrich) at 37°C with 5% CO_2 for 7 days *in vitro*. From culture day 3, fresh medium containing fluorodeoxyuridine (8 x 10^{-5} M; Sigma-Aldrich) was provided to inhibit the proliferation of dividing olfactory neurons and fibroblasts. The medium was replaced with fresh SFM every 3 days.

Immunohistochemistry

Tissues were cryosectioned (Leica cryostat) at 35µm (free-floating sections). Immunohistochemistry was performed as previously reported (19,28) using Alexa-Fluor 488- (1:500), Alexa-Fluor 568- (1:500) and Alexa-Fluor 650- (1:500) secondary antibodies (Invitrogen), and Hoechst 33258 (pentahydrate bis-benzimide, Invitrogen). Fluorescent specimens were mounted using 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich). The primary antisera used were the rabbit anti-GnRH 1:3000 (gift from G. Tramu, CNRS URA 339, Université de Bordeaux) (29) or guinea pig anti-GnRH (1:10000) (30), Goat anti-Nrp1 (1:400; R&D), Rabbit anti-Peripherin (1:1000; Millipore) and Rat anti-BrdU (1:300; AbD serotec). Analyses of the total GnRH cell number and GnRH fiber density have been performed as described previously (25).

Image Analysis

For confocal observations and analyses, an inverted laser scanning Axio observer microscope (LSM 710, Zeiss) with EC Plan NeoFluor 10×/0.3 NA, 20×/0.5 NA, and 40×/1.3 NA (Zeiss) objectives was used (Imaging Core Facility of IFR114 of the University of Lille 2, France). ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop CS5 (Adobe Systems, San Jose, CA) were used to process, adjust, and merge the photomontages.

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 One-way ANOVA (Bonferroni's multiple comparison test) and an unpaired two-tailed Student's t test. For non-normally distributed values Kruskal-Wallis (one way analysis) and Mann-Whitney tests were used. The significance level was set at $p <$

0.05. Data are indicated as means ± SEM. The number of animals, *p* values and degrees of freedom are indicated either in the main text or in the figure legends.

Results

Nrp1 expression is abolished in GnRH cells of Gnrh::cre; Nrp1$^{loxp/loxp}$ mice.

To investigate the role of Nrp1 receptor signaling in GnRH neurons in mouse, we specifically invalidated *Nrp1* expression in GnRH neurons, by crossing *Nrp1*$^{loxP/loxP}$ mice (23) with a *Gnrh::cre* line (21). Triple transgenic mice were also produced by crossing them with *Gnrh::gfp* (31) (Fig. 1B). The resulting *Gnrh::gfp; Gnrh::cre*; *Nrp1*$^{loxP/loxP}$ and *Gnrh::cre*; *Nrp1*$^{loxP/loxP}$ mice were viable and born at mendelian frequencies. We first investigated the expression of the Nrp1 Sema3A-binding receptor, and its coreceptors from the Plexin family, which are the signal transducing subunit in Sema3A signaling (32), in GnRH neurons isolated by fluorescent-activated cell sorting (FACS) from the preoptic region of *Gnrh::cre; Nrp1*$^{loxp/loxp}$;*Gnrh::gfp* and *Nrp1*$^{loxp/loxp}$;*Gnrh::gfp* mice at P0 (Fig. 1C). Real time PCR analyses showed a dramatic decrease in *Nrp1* transcript in GnRH neurons from *Gnrh::cre; Nrp1*$^{loxp/loxp}$;*Gnrh::gfp* compared to *Nrp1*$^{loxp/loxp}$; *Gnrh::gfp* ($t_{(9)}$=3.85, p=0.004, n=5 to 6 per group), without changing *Nrp2* expression ($t_{(9)}$=1.65, p=0.13, n=5 to 6 per group) (Fig. 1D). This is accompanied by a decrease in plexinA1 expression ($t_{(9)}$=3.09, p=0.01, n=5 to 6 per group), which is one of the preferential coreceptors of Nrp1 upon Sema3A binding (32). The expression of the Nrp1 protein was also investigated during embryonic development. *Nrp1*$^{loxp/loxp}$ (control) embryos expressed Nrp1 protein in GnRH neurons at E14.5, as shown by the colocalisation of Nrp1 (red) and GnRH (green) immunostainings (yellow) (Fig. 1E). *Gnrh::cre; Nrp1*$^{loxp/loxp}$ (mutant) littermates did not show any detectable Nrp1 protein expression in GnRH neurons, but still

expressed Nrp1-immunoreactivity in other structures such as the vomeronasal nerve (Fig. 1E). These data validated our animal model.

The lack of Nrp1 expression in GnRH neurons impacts the pattern of GnRH cell migration and survival during embryogenesis

To determine whether Nrp1 expression has a role during GnRH ontogenesis, we analyzed GnRH cell distribution at different key embryonic stages. Peripherin immunostaining, a marker of peripheral axons, showed no alteration in the vomeronasal tract in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ embryos (data not shown). GnRH neurons were quantified along the migratory path from nose to brain at E12.5 (when the majority of the cells are born but still in the nose), at E14.5 (when half of the cells normally reached the brain whereas the other half is still in the nose), at E18.5 (at the end of the migration, when most of the cells have reached their final destination in ventral forebrain (VFB)) and at P0 (when the GnRH cell migration is supposedly over) (8) (Fig. 2A). Surprisingly, the total number of GnRH neurons was significantly increased in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ embryos at E14,5 ($t_{(6)}$=2.82, p=0.03, n=3 to 5 per group) and E18,5 ($t_{(7)}$=2.42, p=0.04, n=4 to 5 per group), compared to controls. By analyzing more precisely the distribution between nose, olfactory bulbs, considered as nasal/brain junction, and VFB at E14,5, we noticed that these extranumerous cells are located in the brain ($t_{(6)}$=2.98, p=0.02, n=3 to 5 per group), meaning that they already reached the brain at this embryonic stage (Fig. 2C and 2D). To further investigate the migration profile of GnRH neurons lacking Nrp1 during development, we took advantage of organotypic explant of nasal placode from E11.5 embryos. GnRH neurons maintained in nasal explants exhibit many characteristics displayed by GnRH neurons in vivo. In this model system, GnRH neurons migrate from the nasal epithelium (NE) to the periphery, along peripherin-positive olfactory axons. After 8 days of culture, the total number of GnRH neurons was significantly higher in nasal explants from *Gnrh::cre; Nrp1*$^{loxp/loxp}$ embryos ($t_{(6)}$=3.51, p=0.01, n=3 to 5 per

group). The distribution of GnRH cells was similar between nasal placode from mutant and control embryos, but significantly more cells were found beyond a radius of 8 millimeter in mutant nasal placode ($t_{(5)}$=2.85, p=0.04, n=3 to 5 per group). The increase of total number of cells is associated with an increase in the number of cells that migrated further. To further understand whether this increase of quantity of GnRH cells was due to an increase in proliferation or a decrease in apoptosis, we used incorporation of BrdU in dividing cells after sequential injection of pregnant females at E9.5 and E10.5, during the proliferation pic of the GnRH progenitors (33,34). At this time point, GnRH is not yet expressed, and the recombination didn't occur. Quantification of BrdU-positive GnRH cells, by double immunostaining in the embryos at E14.5 (Fig. 2I), showed a total increase in double labeled cells ($t_{(6)}$=5.06, p=0.002, n=4 per group), specifically at the level of the olfactory bulbs ($t_{(6)}$=3.51, p=0.01, n=4 per group), and in the VFB ($t_{(6)}$=4.06, p=0.007, n=4 per group) (Fig. 2J). These data suggest that more GnRH neurons arising from progenitors born at E9-E10 survived until E14.5. When BrdU is administrated at E11.5, when GnRH starts to be expressed in the nasal placode, the presence of BrDU-positive GnRH cells 24 hours later is undetectable. These data suggest, consistently with the literature, that GnRH cells don't proliferate anymore after differentiation and that the suppression of Nrp1 expression in GnRH neurons does not alter this neuronal differentiation (data not shown). Together these data indicate a putative role for Nrp1 receptor in the survival of GnRH cells to destination, during embryogenesis, which is linked to a decrease of GnRH cell death. Interestingly, the total number of cells doesn't appeared to be different between *Gnrh::cre; Nrp1*$^{loxp/loxp}$ and *Nrp1*$^{loxp/loxp}$ animals at P0 ($t_{(6)}$=0.98, p=0.37, n=3 to 5 per group). This result could suggest a catch up or a compensatory effect later in the development.

The lack of Nrp1 expression in GnRH neurons results in a differential distribution of GnRH cell bodies in the adult brain

We next investigated the distribution of GnRH neurons in adult mutant mice. We quantified GnRH cell bodies in the hypothalamus, and in all the anterior parts of the brain including olfactory bulbs. The total number of GnRH neurons in the brain was the same between control and mutant mice ($t_{(16)}$=0.97, p=0.34, n=9 per group) (Fig. 3A). While a significant amount of cells still reside at the level of the olfactory bulbs in control mice (30%), this amount was significantly decreased in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ mice (21%) ($t_{(15)}$=2.51, p=0.02, n=9 per group) (Fig. 3B, 3C and 3D). Paralleling these changes at the level of the olfactory bulb, the proportion of GnRH cells in the hypothalamus was significantly increased in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ mice compared to their control littermate ($t_{(15)}$=2.09, p=0.05, n=9 per group) (Fig. 3B). Interestingly, we noticed a specific accumulation on GnRH cells in the region of OVLT (organum vasculosum of lamina terminalis) in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ ($t_{(14)}$=2.13, p=0.05, n=9 per group) (Fig. 3E, 3F and 3G), which is the area implicated in the LH surge (35). The differential distribution of GnRH neurons observed in adult brains suggests the intriguing possibility that Nrp1 expression in GnRH neurons regulates the critical time window during which GnRH neurons migrate and/or modulates the survival of GnRH neurons in specific areas of the brain.

Female mice lacking Nrp1 expression in GnRH neurons exhibit central precocious puberty

In order to evaluate the integrity of the hypothalamo-pituitary gonadal axis in our animal model, we investigated the peripubertal period of maturation by assessing vaginal opening and first ovulation. Even if no differences were observed in the timing of vaginal opening ($t_{(22)}$=1.136, p=0.27, n=12 per group) (data not shown), first ovulation was significantly advanced in mutant mice compared to their control littermates ($t_{(22)}$=3.47, p=0.002, n=12 per group) (Fig. 4A and 4B). While *Nrp1*$^{loxp/loxp}$

females first ovulated at P50, *Gnrh::cre; Nrp1*$^{loxp/loxp}$ ovulated at P45 (Fig. 4A). The analysis of plasmatic LH at P45 showed significantly higher LH levels in mutant females than in control littermates ($t_{(9)}$=2.59, p=0.03, n=5 to 6 per group) (Fig. 4C). In line with these results, the uterine weight in mutant females was also significantly increased at P45 ($t_{(14)}$=2.49, p=0.03, n=6 to 10 per group), suggesting an increase in estrogen impregnation earlier in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ when compared to controls (Fig. 4D). Together these data showed a precocious puberty, linked to central advanced maturation of the gonadotrope axis, in *Gnrh::cre; Nrp1*$^{loxp/loxp}$. Intriguingly, the body weight at P45 was significantly higher (about 1g) in mutant animals than in controls ($t_{(22)}$=2.38, p=0.026, n=12 per group) (Fig. 4E), but the weight at puberty was identical between the two groups ($t_{(19)}$=0.94, p=0.36, n=11 per group) (Fig. 4F) showing that premature activation of the HPG axis may accelerate growth.

Adult mice lacking Nrp1 expression in GnRH neurons exhibit impaired energy balance

Body weight of *Gnrh::cre; Nrp1*$^{loxp/loxp}$ females was seen to be significantly increased from peripubertal period on (Fig. 5A) when compared to *Nrp1*$^{loxp/loxp}$ littermates. In adults, overweight was associated to an increased fat volume at 4 months ($t_{(14)}$=2.2, p=0.04, n=8 per group) (Fig. 5B), with no change in the lean mass volume ($t_{(14)}$=0.04, p=0.97, n=8 per group) (data not shown) in mutant mice when compared to control. This fat was predominantly consistuted of visceral/perigonadal fat ($t_{(14)}$=2.17, p=0.05, n=8 per group) (Fig. 5B).

Discussion

In the current report, we showed that the lack of Nrp1 receptor in GnRH neurons may impact GnRH neurons survival and/or migration. Analysis of embryonic developmental showed that more of GnRH neurons reach the ventral forebrain at E14.5 in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ emrbyos when compared to control littermates. Adult

mutant animals showed an abnormal distribution of GnRH neurons in the brain, with an increased number of GnRH neurons in the medial preoptic region in mutant mice. Female mice selectively lacking Nrp1 expression in GnRH neurons underwent central precocious puberty. This premature activation of the HPG axis was associated with overweight from weaning on, and increased fat mass in adults.

We showed a decrease of *Nrp1*, but not *Nrp2* transcript in GnRH cells from *Gnrh::cre; Nrp1*$^{loxp/loxp}$ animals which suggests that the loss of Nrp1 in GnRH neurons was not compensated with an increase in Nrp2 expression in those neurons. Interestingly, PlexinA1 expression was seen to be significantly decreased in mutant when compared to control GnRH cells, which suggests that Neuropilin-1 expression could indirectly impact the expression of its obligate coreceptors. Indeed, Nrp1 receptor, which is responsible of the ligand-binding step, doesn't have any intracellular domain, and needs the formation of heterodimeric complexes to initiate intracellular sinaling. For example, upon Sema3A binding, Nrp1 heterodimerises either with PlexinA1 or Plexin A4 (36,37). Nrp1 is also involved in VEGF signaling and can form heterodimers with the VEGF receptors (23).

We showed an increased survival of GnRH cells lacking Nrp1 receptor in E14.5 embryos, with a bigger population localized in ventral forebrain. We took advantage of BrdU integration in dividing cells to show that this population is born before the differentiation of the progenitors into GnRH expressing cells, and so, before the recombination in our transgenic mouse model. This is in favor of an anti apoptotic effect of the removal of Nrp1 receptor in GnRH neurons. These data could be, *a priori*, contradictory with some data from the literature showing an increased survival effect of GnRH cells through Nrp1 receptor, via VEGF164 pro-survival signaling factor (38). Indeed Nrp1 receptor is widely described as taking part of an anti-apoptotic processus, because signalization through Nrp1 is able to increase survival, in particular in tumor and endothelial cells, via the action of VEGF (39–41). However, some other studies identify Sema3A via its action through Nrp1 as an apoptotic

factor in non-neuronal cells (42,43). Although further investigation are required to determine which specific pathway is implicated in the phenotype we describe, our data suggest that Nrp1 may regulate GnRH cell population across development by modulating GnRH neuron survival. Cariboni and colleagues proposed a model in which the cooperation between VEGF-mediated neuronal survival and Sema3A-mediated axon guidance is required for normal GnRH neuronal migration (38). We propose that Nrp1-mediated apoptosis could also be involved in the regulation of the size of the GnRH neuronal population throughout development.

Furthermore, the differential distribution of GnRH cell bodies in *Gnrh::cre; Nrp1*$^{loxp/loxp}$ versus *Nrp1*$^{loxp/loxp}$ adult brains, with no difference in the total number of cells, may also suggest a role for Nrp1 in the modulation of the migration of GnRH neurons in the brain. This is consistent with previous reports showing a role of Sema3A in the migration and orientation of neuronal cells during ontogenesis of the brain (13,14,44). This is the first report linking increased number of GnRH neurons in the hypothalamus to central precocious puberty. Our hypothesis is that more GnRH neurons located in the area involved in the control of the LH surge (35) may trigger earlier maturation of the system, due to increased GnRH release.

Finally, our transgenic animal model may be a good model for studying effects of central precocious puberty, as it mimics some features encounters in women with precocious puberty such as overweight. Precocious puberty, which is more often found in women, is a pathology widely correlated to increased prevalence of metabolic diseases including overweight, obesity and diabetes (45,46), but also breast cancer (47), and is a criteria for polycystic ovary syndrome diagnosis (48).

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale, Inserm, and the University of Lille School of Medicine, France (grant number U1172 to V.P.), the Fondation pour la Recherche Medicale (Equipe FRM, DEQ20130326524, France to V.P), the Agence Nationale de la Recherche (ANR, France: RoSes and GnRH to P.G; Astrex to F.W.P.) and EU COST action (BM1105 to V.P., P.G. and E.H.). We thank Nathalie Jouy (cell sorting core facility), Meryem Tardivel (Confocal microscopy core facility), Delphine Taillieu, Julien Devassine and Delphine Cappe (animal core facility) of the University of Lille for their expert technical assistance and Dr. S. Rasika for the editing of our manuscript.

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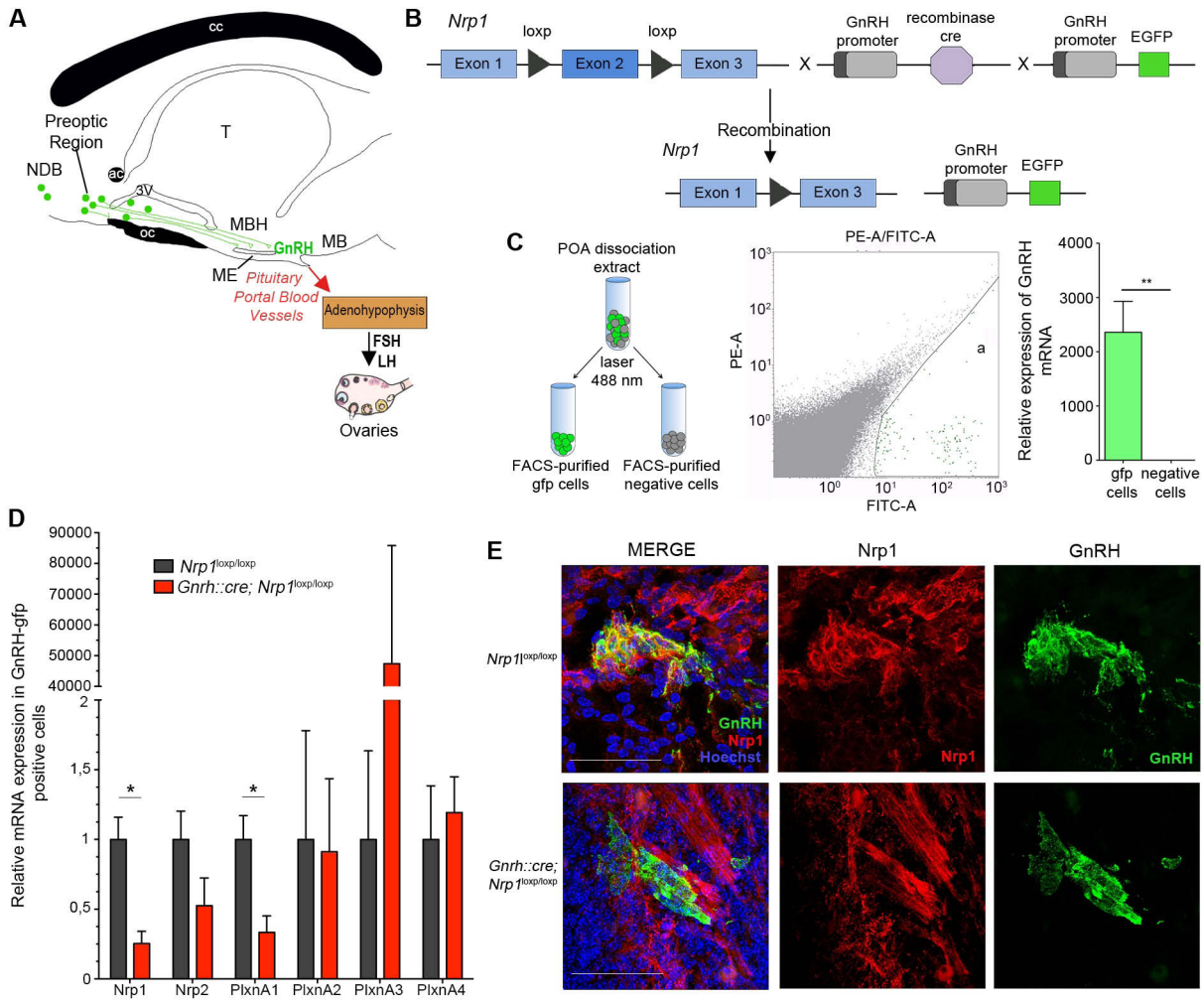

Figure 1

Figure 1. Nrp1 expression is selectively suppressed in GnRH neurons of *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ animals *in vivo.* (**A**) Schematic diagram illustrating the anatomy of the hypothalamic pituitary-gonadal axis in a sagittal view. In rodents, GnRH cell bodies (green circles) are diffusely distributed in the anterior part of the brain, particularly in the preoptic region, and send neuroendocrine axons (green fibers) toward the ME of hypothalamus. The neurohormone is secreted into pituitary portal blood vessels for delivery to the anterior pituitary, where GnRH elicits secretion of the gonadotrophins LH and FSH. In females, these hormones secreted into peripheral blood stimulate ovary function, as secretion of gonadal steroïds estrogen and progesterone, folliculogenesis, and ovarian cycle. Cc, corpus calosum ;

ac, anterior comissure ; 3V, third ventricle ; T, thalamus ; NDB, diagonal band of Broca nucleus ; MBH, medio-basal hypothalamus ; ME, median eminence ; MB, mamillary bodies ; FSH, follicle stimulating hormone ; LH, luteinizing hormone. (**B**) Genetic strategy to selectively suppress Nrp1 in GnRH-expressing cells, using *Nrp1*$^{loxp/loxp}$ mice crossed with *Gnrh::cre* mice. We also crossed these mice with *Gnrh::gfp* mice in order to generate triple transgenic mice. (**C**) Gfp-positive cells isolation by FACS : schematic diagram (left) and dot plot (middle) showing the selection of gfp-positve population (a). Relative mRNA expression from real time PCR analysis of GnRH transcript in GFP-positive cells, in comparison with GFP-negative cells. The GnRH transcript appears to be selectively expressed in GFP-positive cells ($t_{(6)}$ =4.12, p=0.0062, n=4). (**D**) Relative mRNA expression from real-time PCR analysis of Neuropilin-1 (Nrp1), Neuropilin 2 (Nrp2), plexin A1 (PlxnA1), PlxnA2, PlxnA3 and PlxnA4 transcripts in FACS isolated GFP-positive GnRH cells from control *Nrp1*$^{loxp/loxp}$ (grey) and mutant *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ (red) P0 mice. * $P<0.05$, ** $P<0.01$ (**E**) Representative immunofluorescence images showing migrating GnRH cells on their migratory path in sagittal slice from E14.5 control and mutant embryos. GnRH cells (green) express Nrp1 protein (red) in control *Nrp1*$^{loxp/loxp}$ animals whereas Nrp1 is undetectable in GnRH cells from mutant Gnrh*::cre*; *Nrp1*$^{loxp/loxp}$ animals. Scale bar, 50 μm. Bar graphs show means ± SE.

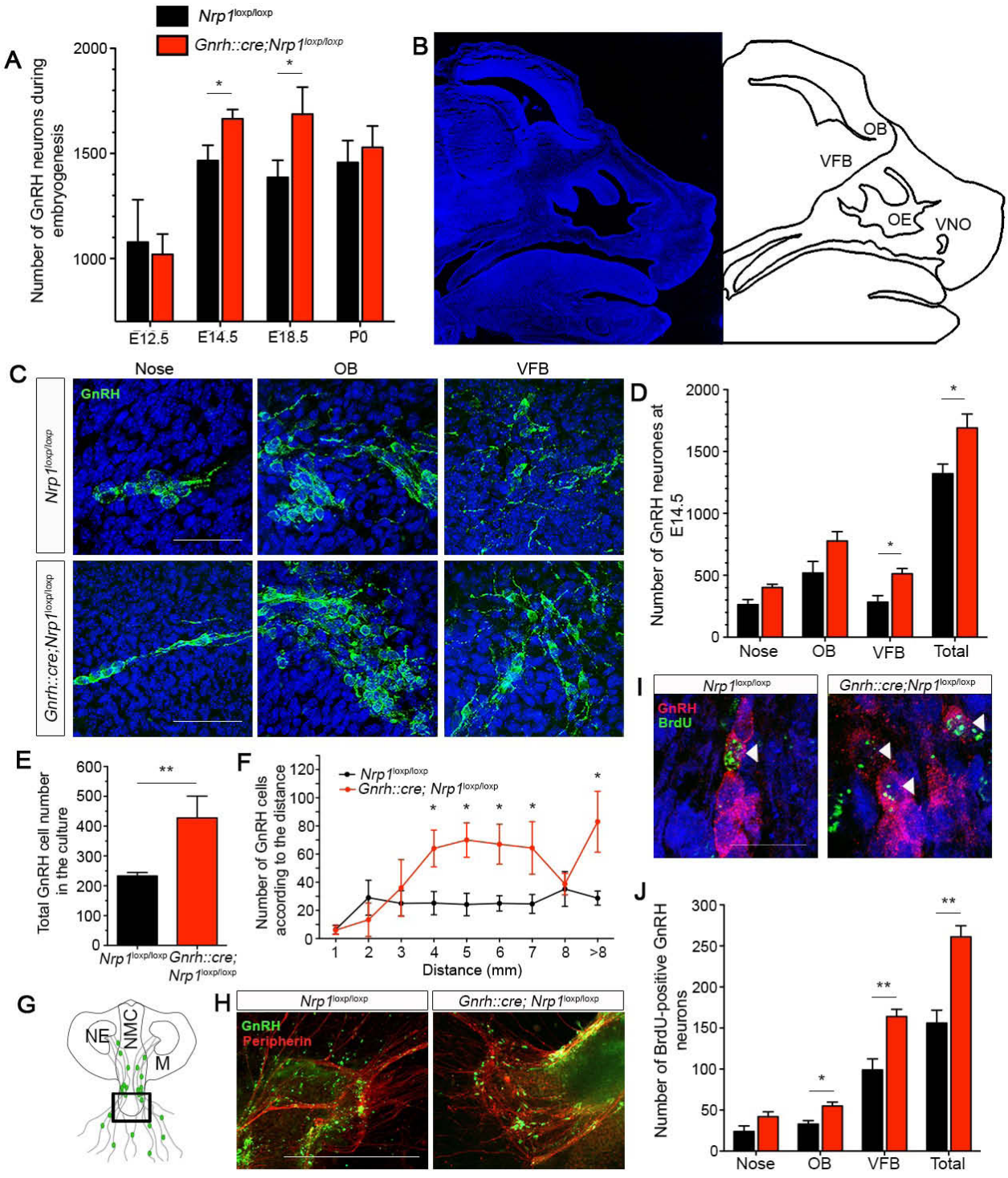
A
Nrp1loxp/loxp
Gnrh::cre;Nrp1loxp/loxp
Number of GnRH neurons during embryogenesis
2000
1500
1000
E12.5
E14.5
E18.5
P0
B
OB
VFB
OE
VNO
C
Nose
OB
VFB
GnRH
Nrp1loxp/loxp
Gnrh::cre;Nrp1loxp/loxp
D
Number of GnRH neurones at E14.5
2000
1500
1000
500
0
Nose
OB
VFB
Total
I
Nrp1loxp/loxp
Gnrh::cre;Nrp1loxp/loxp
GnRH
BrdU
E
Total GnRH cell number in the culture
600
500
400
300
200
100
0
Nrp1loxp/loxp
Gnrh::cre; Nrp1loxp/loxp
F
Nrp1loxp/loxp
Gnrh::cre; Nrp1loxp/loxp
Number of GnRH cells according to the distance
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
>8
Distance (mm)
J
Number of BrdU-positive GnRH neurons
300
250
200
150
100
50
0
Nose
OB
VFB
Total
G
NE
NMC
M
H
Nrp1loxp/loxp
Gnrh::cre; Nrp1loxp/loxp
GnRH
Peripherin

Figure 2

Figure 2. Suppression of Nrp1 expression in GnRH neurons leads to increased survival of GnRH cells during embryogenesis and accumulation of cells in the brain. (**A**) Quantification of total number of GnRH neurons during embryogenesis, at E12.5, E14.5, E18.5 and P0 (*p<0.05). (**B**) Representative sagittal slice from E14.5 stained with hoechst and schematic representation of the different areas where GnRH cells are located during migration. VNO, vomeronasal organ ; OE, olfactory epithelium ; OB, olfactory bulb ; VFB, ventral forebrain. (**C**) Representative immunohistofluorescence showing migrating GnRH neurons at the level of the nose, the olfactory bulbs (OB) and the ventral forebrain (VFB). Scale bar, 50 μm. More cells were detected in the brain of *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ embryos. (**D**) Distribution of GnRH neurons from nose to brain in E14.5 control and mutant embryos, and total number of cells (*p<0.05). (**E**) Total number of GnRH neurons quantified after 8 days of culture in nasal explants isolated from *Nrp1*$^{loxp/loxp}$ and *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ embryos (** p<0.01). (**F**) Distance of GnRH neurons from starting point, which was considered between the two olfactory eptihelia. Cells were quantified within a radius of 1 to 8 millimiters, and more than 8 millimiters (>8). (**G**) Schematic of a nasal explant. GnRH neurons (green circles) emerge from a nasal epithelium (NE), and follow olfactory axons to the midline and then into the periphery of the explant. NMC, nasal midline cartilage ; M, surrounding mesenchyme. (**H**) Representative immunofluorescence of nasal explants at the level of the end of the nasal cartilage. GnRH cells (green) migrate on peripherin-positive olfactory axons (red). More GnRH cells was observed in *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ explants. Scale bar, 500 μm. (**I**) Representative immunofluorescence of GnRH BrdU-positive cells in control and mutant E14.5 embryos. More double positive cells were quantified in *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ embryos (white arrow). Scale bar, 20 μm (**J**) Distribution of GnRH Brdu-postive cells from nose to brain in E14.5 control and mutant embryos and total number of cells (*p<0.05; ** p<0.01)

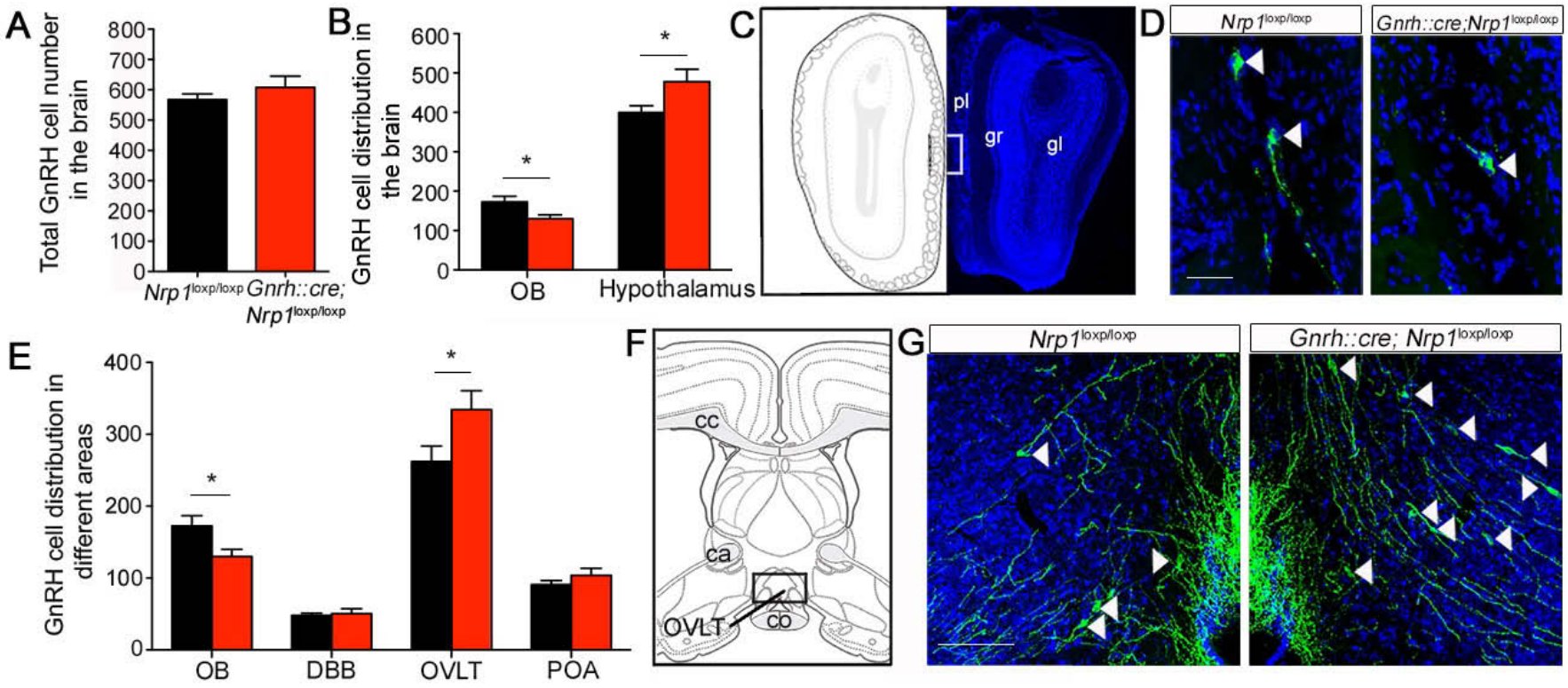
A
Total GnRH cell number in the brain
800
700
600
500
400
300
200
100
0
Nrp1loxp/loxp
Gnrh::cre; Nrp1loxp/loxp
B
GnRH cell distribution in the brain
600
500
400
300
200
100
0
*
*
OB
Hypothalamus
C
pl
gr
gl
D
Nrp1loxp/loxp
Gnrh::cre;Nrp1loxp/loxp
E
GnRH cell distribution in different areas
400
300
200
100
0
*
*
OB
DBB
OVLT
POA
F
cc
ca
OVLT
co
G
Nrp1loxp/loxp
Gnrh::cre; Nrp1loxp/loxp

Figure 3

Figure 3. Suppression of Nrp1 expression in GnRH neurons leads to differential distribution of GnRH neurons in adult brain. (**A**) Total number of GnRH in the brain of control and mutant adult mice. (**B**) Distribution of GnRH neurons between olfactory bulbs (OB) and hypothalamus in the brain of control and mutant adult mice (*p<0.05). (**C**) Schematic of an olfactory bulb and representative coronale slice stained with hoechst. Pl, plexiforme layer; gr, granular layer; gl, glomerular layer. (**D**) Representative immunohistofluorescence showing GnRH cells in the olfactory bulbs of control and mutant adult mice. Less GnRH cells were observed in *Gnrh::cre*; *Nrp1*$^{loxp/loxp}$ bulbs (white arrow). Scale bar, 50 μm (**E**) Distribution of GnRH neurons in olfactory bulbs (OB) and in the differents regions of hypothalamus (*p<0.05). DBB, diagonal band of Broca ; OVLT, organum vasculosum of lamina terminalis ; POA, preoptic area. (F) Schematic representation of a coronal slice at the level of the OVLT. Cc, corpus calosum ; ac, anterior comissure ; oc, optic chiasma. (G) Representative immunohistofluorescence of coronal slice at the level of the OVLT from control (left) and mutant (right) adult mice. More GnRh cells were observed

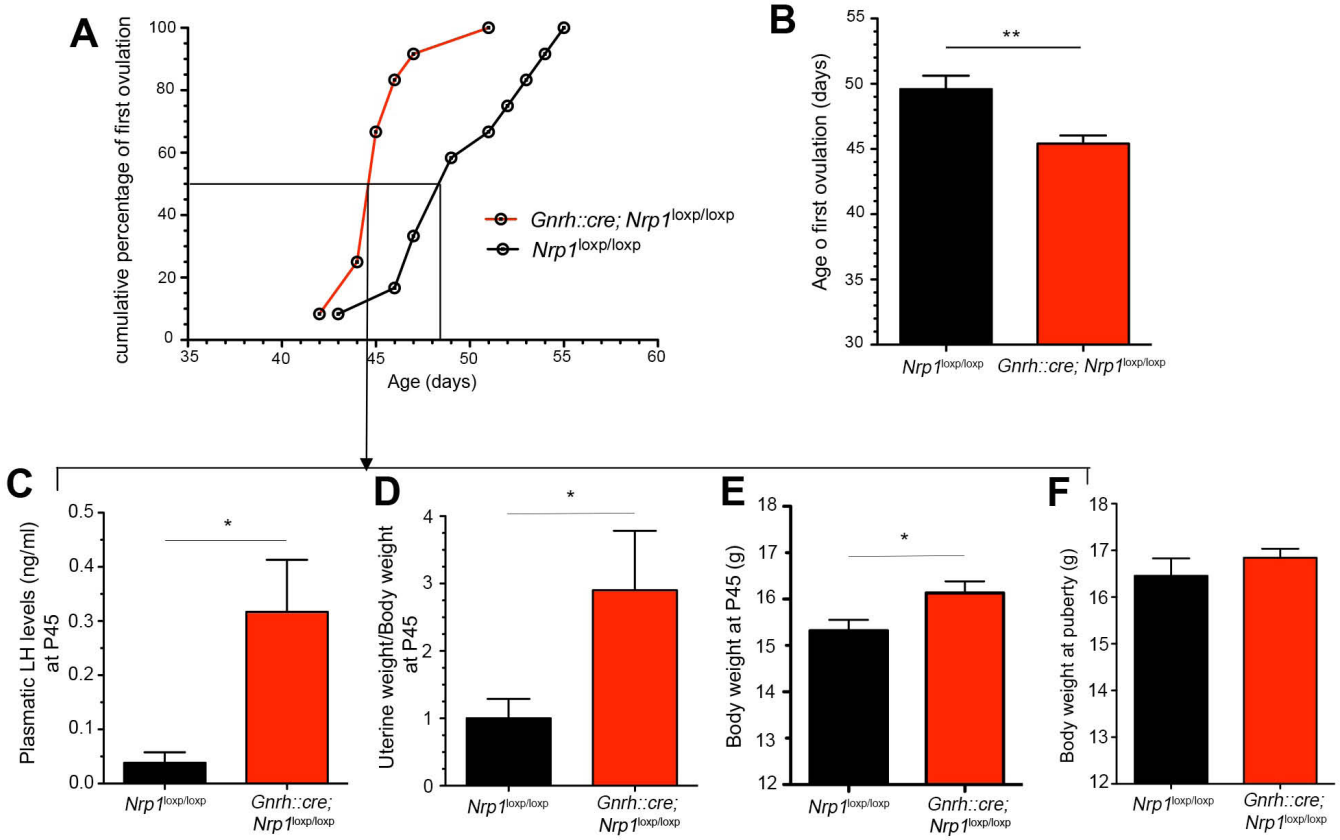

Figure 4

Figure 4. Supression of Nrp1 expression in GnRH neurons leads to central precocious maturation of the gonadotrope axis. (**A**) Cumulative percentage data for first ovulation, caracterized by the first estrus. (**B**) Corresponding bar graph (** p<0.01) (**C**) Plasmatic LH levels at P45 in control and mutant mice (* p<0.05). (**D**) Mean uterine weight normalized on body weight (mg/g BW) (* p<0.05). (**E**) Body weight at P45 (* p<0.05). (**F**) Body weight at puberty.

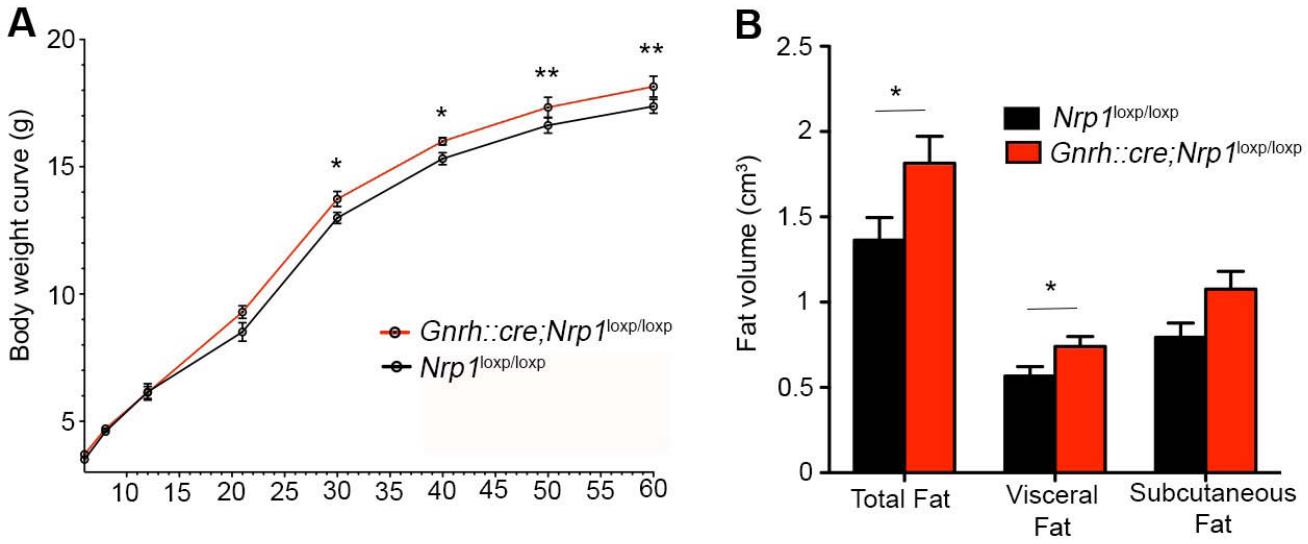

Figure 5

Figure 5. The lack of Nrp1 un GnRH neurons leads to perturbation in energy metabolism in female adult mice. (**A**) Body weight curve from infantile period to adulthood (repeated measures ANOVA, $F_{(2,18)}$ = 81.12, p<0.01) (* P<0.05, **P<0.01). (**B**) Total fat volume and distribution of visceral and subcutaneous fat (cm^3) (* P<0.05).

REVUE SCIENTIFIQUE :

Dynamic Control of Neural Reproductive Centers by Endothelial Cells

Au cours de ma thèse, j'ai eu l'occasion de produire un chapitre de livre avec l'aide d'Ariane Sharif et de Vincent Prévot. Cette revue met en lumière le rôle étroit qu'il existe entre le système vasculaire, et en particulier les cellules endothéliales, et le système nerveux.

Cette revue scientifique fait l'objet d'un chapitre dans *Endothelial Cell Plasticity in the Normal and Injured Central Nervous System (Esperanza Meléndez Herrera, Bryan V. Philips-Farfàn, Gabriel Gutiérrez Ospina Eds)*

Dynamic control of neural reproductive centers by endothelial cells

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It is generally accepted that neurons, glia and brain capillaries are organized into well-structured neuro-glio-vascular units, in which individual astroglial cells support the function of specific neuronal populations and territories, and communicate with associated segments of the microvasculature (Iadecola and Nedergaard, 2007; Barres, 2008; Halassa and Haydon, 2010; Prevot et al., 2010b). These microfunctional domains are likely to play an important role in maintaining a precisely regulated microenvironment for reliable neuronal signaling in an ever-changing physiological context. Gaining new insights into how cellular events that involve neurons, astroglia and vascular cells are orchestrated is therefore fundamental to an improved understanding of brain function.

Blood vessels and axons employ similar mechanisms and follow common guidance cues for growth and navigation during embryonic development (Carmeliet and Tessier-Lavigne, 2005; Larrivee et al., 2009). Blood vessels influence axonal trajectories to reach the appropriate destinations (Makita et al., 2008). In the adult brain, blood vessels communicate with neurons and glia to meet physiological demands (Iadecola, 2004). Endothelial cells are positioned to sense peripheral inputs and ideally suited to convey signals that could influence neuronal structure and synaptic plasticity. However, whether these cells are capable of influencing the function of the mature central nervous system remains largely understudied.

The median eminence of the hypothalamus, which constitutes the ventral border of the third ventricle, provides an excellent model in which to investigate the complex relationship between neurosecretion, function-related morphological plasticity involving neuronal-glial-endothelial interactions, and the expression of key physiological functions. The median eminence is one of the seven so-called circumventricular organs (CVO) (Duvernoy and Risold, 2007; Langlet et al., 2013a) and thus constitutes a window of exchanges between the hypothalamus and the periphery that is facilitated by the presence

of permeable brain capillaries featuring fenestrated endothelium (Ciofi et al., 2009; Mullier et al., 2010; Ciofi, 2011; Schaeffer et al., 2013). In contrast to the other CVOs, it appears that the most important function associated with the lack of blood-brain barrier in the median eminence is that it permits the release of neurohormones produced by neuroendocrine cells from terminals into the pituitary portal circulation. It is also important to acknowledge that the cellular processes through which neuroendocrine terminals release their neuropeptides into the circulation could be subjected to the direct modulatory influence of blood-borne factors acting on this region. The peculiar cytoarchitecture of the median eminence is mainly conferred by tanycytes, which are specialized unciliated ependymoglial cells that form a belt lining the floor of the third ventricle (Page, 1994). One dominant feature of tanycytes is their marked polarization; although tanycyte cell bodies line the border of the third ventricle, they also send processes to the vascular walls, where they make contacts through “end-feet” specializations (Sharif et al., 2013). In addition, tanycytes were recently shown to express efficient tight junction complexes at their apex that bestow them with properties of the blood-brain barrier (Mullier et al., 2010), which are highly plastic and depend on the metabolic status of the individual and are tightly regulated by communication processes set in motion between tanycytes and vascular endothelial cells (Langlet et al., 2013b; Myers, 2013; Prevot et al., 2013). Although tanycytes are the dominant cell type, astrocytes also reside within the internal zone of the median eminence (Figure 1).

Reproductive function requires the coordinated and timely activation of gonadotropin-releasing hormone (GnRH) neurons (Herbison and Neill, 2006). These neurons, which in rodents are located in the preoptic region of the hypothalamus, extend their neurosecretory axons to the median eminence, where they make contact with basal lamina and open into the pericapillary space of the primary hypophyseal portal plexus. Upon reaching the pituitary portal system, GnRH travels to the pituitary to stimulate the synthesis and secretion of pituitary gonadotropins: luteinizing hormone (LH) and follicle stimulating 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. Within the brain, gonadal steroids influence GnRH secretion via neuroendocrine feedback loops (Figure 2).

The present chapter will review the findings that have unveiled some of the cell-cell communication processes involving non-neuronal cells such as vascular endothelial cells, astrocytes and tanycytes,

which locally regulate both GnRH neurohaemal junction formation and GnRH release within the median eminence. These modulatory mechanisms use signaling molecules that may represent some of the synchronizing cues to coordinate GnRH release from the scattered GnRH neuroendocrine terminals that may allow for the occurrence of functionally meaningful episodes of GnRH secretion and thus contribute to fertility. More specifically, endothelial-cell-derived signals appear to regulate GnRH neurosecretion via two alternative but not mutually exclusive pathways: i)- promoting retraction of the tanycytic distal processes that ensheath GnRH nerve terminals and ii)- regulating outgrowth of GnRH neuroendocrine axons. Here, we will first detail the structural changes regulating the establishment of the GnRH neurovascular junction during the ovarian cycle. We will then discuss the molecular mechanisms underlying the morphological alteration of the tanycitic sheath controling the direct access of GnRH neurons to the pericapillary space. Finally, we will review the evidences suggesting that vascular endothelial cells play a dynamic role in the control of these morphofunctional changes.

Neuronal-glial-vascular interactions in the median eminence modulate neurohaemal junction formation and neurosecretion at the GnRH nerve terminals

At the time of the preovulatory GnRH/LH surge, morphological remodeling in the external zone of the median eminence results in the formation of direct neurovascular contacts for the GnRH neurons (Prevot et al., 1998; Prevot et al., 1999a). Tanycytes release the engulfed terminals, retract from their initial position along the basal lamina and bring the pericapillary space to the GnRH neuroendocrine terminals (Figure 3). Some GnRH nerve terminals sprout toward the basal lamina of the endothelial wall, with which they eventually make direct contact. Both of these phenomena seem to occur concurrently on the day of proestrus, and they allow the GnRH nerve terminals to directly contact the pericapillary space so that GnRH can reach the fenestrated capillaries of the pituitary portal vasculature more efficiently and thus trigger the preovulatory GnRH/LH surge. During the last decade, significant efforts have been directed toward deciphering the cell-cell mechanisms and the underlying signaling pathways responsible for these structural changes that allow neuroendocrine GnRH neurons to establish detectable neurovascular junctions every four days during the rat estrous cycle. It is becoming increasingly clear that these plastic changes require the cooperative actions of multiple signals whose cellular sources and

targets are alternatively tanycytes, astrocytes, vascular endothelial cells and the GnRH neurons themselves.

Molecular mechanisms involved in the retraction of tanyctic processes during the ovarian cycle

Tanycytes together with cerebellar Bergman glia and retinal Müller cells are the persistent radial glial cells of the mature central nervous system (Reichenbach and Wolburg, 2005); they are bipolar ependymoglial cells that extend long processes throughout the tissue. Remarkably, tanycytes share some properties with embryonic radial glia and neural stem cells (Kriegstein and Alvarez-Buylla, 2009); they express immature glial cell markers such as vimentin (Chauvet et al., 1995; Chauvet et al., 1998; Prieto et al., 2000; Mullier et al., 2010) and nestin (Xu et al., 2005; Baroncini et al., 2007) *in vivo*, they give rise to neurons when they divide (Xu et al., 2005; Lee et al., 2012; Haan et al., 2013; Robins et al., 2013) and they are capable of supporting the migration of neuronal elements (Chauvet et al., 1995; Chauvet et al., 1996; Chauvet et al., 1998; Prieto et al., 2000). These highly plastic features of tanycytes may be conferred by the peculiar microvascular bed of the median eminence that could provide a permissive environment for neural stem cell expansion, neuronal differentiation and parenchymal migration (Louissaint et al., 2002; Goldman and Chen, 2011) by releasing regulatory signals such as nitric oxide (NO) (Prevot et al., 2000a) and BDNF (Givalois et al., 2004).

Insights into cell-cell signaling mechanisms underlying tanycyte plasticity in the reproductive neuroendocrine brain were gained recently from studies demonstrating that, in addition to being involved in dynamic communication processes between astrocytes and GnRH neurons (Clasadonte et al., 2011b), glial erbB signaling may also account for part of the plastic remodeling taking place in the external zone of the median eminence during the estrous cycle (Ojeda et al., 2008; Clasadonte et al., 2011a). The use of primary cultures of tanycytes from the median eminence as a model system showed that these cells express functional erbB-1 (epidermal growth factor receptor or EGFR) and erbB-2 receptors but lack the erbB-4 receptors (Prevot et al., 2003a), an expression repertoire identical to the one seen in tanycytes *in vivo* (Ma et al., 1992; Ma et al., 1999). Within the median eminence, tanycytes also express transforming growth factor alpha (TGFα) (Ma et al., 1992), which like all other EGF-related peptides, is synthesized as a membrane-bound precursor protein that has to be cleaved by zinc-dependent metalloproteinases to release a mature form that can activate erbB-1 receptors in an auto- or

paracrine way (Junier, 2000). Experiments using phorbol myristate treatment, which promotes the processing of erbB ligand precursors via a cellular signaling pathway involving protein kinase C (Pandiella and Massague, 1991; Prenzel et al., 1999), demonstrated that cultured tanycytes also expressed EGF-like peptides capable of activating endogenous erbB-1 receptors (Prevot et al., 2003a). Like hypothalamic astrocytes, tanycytes respond to TGFα by releasing PGE_2; however, in contrast to hypothalamic astrocytes, tanycytes also release transforming growth factor beta 1 (TGFβ_1). TGFβ_1 is a glial growth factor known to stimulate GnRH mRNA expression in both native and immortalized GnRH cells (Galbiati et al., 2001; Bouret et al., 2004). Although TGFβ_1 is known to stimulate GnRH release from GnRH-secreting cell lines (Melcangi et al., 1995; Buchanan et al., 2000), it does not stimulate GnRH release from median eminence GnRH nerve terminals (Ojeda et al., 1990), suggesting that this growth factor facilitates GnRH secretion via direct action exerted on GnRH neuronal cell bodies. The intracellular mechanisms underlying the stimulatory effect of TGFα on TGFβ_1 release in tanycytes involve the formation of PGE_2, as increases in the release of both PGE_2 and TGFβ_1 after initial exposure of the cells to TGFα require cyclooxygenase (COX) activity (Prevot et al., 2003a). Unexpectedly, morphometric studies revealed that TGFα and TGFβ_1 had dramatically opposite effects on tanycyte plasticity *in vitro* (Prevot et al., 2003a). After a 12-h treatment TGFβ_1 induced the retraction of tanycytic processes, whereas TGFα promoted tanycytic outgrowth. Noticeably, longer exposure of tanycytes to TGFα caused tanycytic retraction in 60% of the cultures, an effect that was abolished by immunoneutralization of TGFβ_1, indicating that the retraction is attributable to TGFα-induced TGFβ_1 formation (Prevot et al., 2003a). These studies also showed that TGFβ_1-promoted retraction of tanycytes requires the activity of matrix metalloproteinases (Prevot et al., 2003a), which were shown to be expressed and differentially active during the estrous cycle in the median eminence (Estrella et al., 2004). In summary, stimulation of erbB-1 receptors in tanycytes results in biphasic plastic changes characterized by an initial phase of outgrowth and a secondary phase of retraction (Figure 4). Although the initial outgrowth is independent of the TGFβ system, the subsequent retraction requires PGE_2 synthesis and a PGE_2-dependent increase in the production of TGFβ_1. Importantly, tanycytes were shown to express TGFβ serine/threonin receptors *in vivo* (Prevot et al., 2000b; Bouret et al., 2004).

Taken together, these data provide strong support for the concept that activation of erbB-1 signaling may be a key component of the cell-to-cell communication pathways used by tanycytes to facilitate GnRH release *in vivo*.

Because circulating levels of estrogens rise dramatically at the onset of the preovulatory GnRH/LH surge (Smith et al., 1975), and because median eminence tanycytes were shown to express estrogen receptors (Langub and Watson, 1992), estrogens are likely to be key humoral factors involved in the orchestration of the glia-to-neuron communication processes that allow GnRH neurons to directly contact the pituitary portal blood vessels at proestrus. *In vivo*, estrogens have been shown to activate erbB-mediated signaling events by stimulating the expression of both TGFα (Ma et al., 1992; Ma et al., 1994a) and erbB-1 (Ma et al., 1994b) and to enhance the effect of PGE_2 on GnRH release by increasing the synthesis of PGE_2 receptors in hypothalamic GnRH neurons (Rage et al., 1997). Recent data demonstrate that the activity of tumor necrosis factor α converting enzyme (TACE), the major proteolytic enzyme responsible for shedding of TGFα from its transmembrane precursor (Peschon et al., 1998; Sahin et al., 2004), increases selectively in the median eminence at the onset of the preovulatory GnRH/LH surge (Lomniczi et al., 2006), suggesting that the bioavailability of the mature form of TGFα is maximal at this stage of the estrous cycle. Along the same lines, estrogens were shown to up-regulate TGFβ_1 mRNA levels in the hypothalamus (Galbiati et al., 2001), to stimulate TGFβ_1 release in cultured hypothalamic astrocytes (Buchanan et al., 2000) and to modulate the expression of hypothalamic TGFβ receptors (Bouret et al., 2002).

In addition, tanycytes are also a target for other factors, including peptidergic hormones that may influence structural plasticity at the GnRH nerve terminals during the estrous cycle (Garcia-Segura et al., 1996). For example, insulin-like growth factor 1 (IGF-I), which is taken up by tanycytes in a gonadal steroid-controlled manner from the portal blood (Duenas et al., 1994; Garcia-Segura et al., 2008) and which has a well-established stimulatory effect on GnRH release (Hiney et al., 1996) may contribute to the modulation of these plastic changes.

Intriguingly, in seasonal birds, subtractive hybridization analysis performed in the tuberal region of the quail recently identified a gene encoding TGFα as being differentially regulated during the photoperiodic

control of reproduction (Takagi et al., 2007). In situ hybridization experiments showed that TGFα mRNA expression was markedly and rapidly induced in tanycytes of the median eminence during long days (Takagi et al., 2007), which is when GnRH secretion is induced and GnRH nerve terminals are observed in close proximity to the basal lamina in the quail median eminence (Yamamura et al., 2004), as described in a previous paragraph. Notably, intracerebroventricular infusion of physiological doses of TGFα induced LH secretion and gonadal growth under short-day conditions, when birds are sexually inactive (Takagi et al., 2007). These results raise the exciting possibility that the activation of TGFα-erbB signaling may be a key component of the cell-to-cell communication pathways used by the neuroendocrine brain to regulate structural changes between tanycytes and GnRH nerve terminals during the photoperiodic control of reproduction in seasonal breeders.

Endothelial-cell-promoted neuronal-glial structural plasticity

Using immunopanning techniques to purify the vascular endothelial cells of the median eminence and co-culture systems with isolated tanycytes, studies performed a decade ago have shown that median eminence endothelial cells can convey signals to tanycytes to promote acute remodeling of their actin cytoskeleton (De Seranno et al., 2004). These changes in tanycyte cytoarchitecture prompted by endothelial cells appear to involve the highly labile and diffusible factor NO (Garthwaite, 2008). Experiments showed that pre-treatment of endothelial cells with L-NAME, an inhibitor of NO synthase (NOS) activity (De Seranno et al., 2004), or infection of endothelial cells with and adenovirus expressing a dominant negative form of endothelial NOS (eNOS) (de Seranno et al., 2010) blunted endothelial cell-promoted actin cytoskeleton remodeling in tanycytes, whereas tanycyte treatment with physiological doses of NO donors mimicked the co-culture effects (De Seranno et al., 2004). NO, which travels readily across cellular membranes, mediates most of its effects by binding to the prosthetic heme group of the enzyme NO-sensitive guanylyl cyclase (sGC), resulting in increased production of cGMP (Koesling and Friebe, 1999; Bredt, 2003; Garthwaite, 2008). NO can also regulate the activity of COX 1 and 2 (other heme containing enzymes), and thus elicits prostaglandin release (Salvemini et al., 1993). The findings that the inhibition of either sGC or COX abrogates NO-mediated actin cytoskeleton remodeling in

tanycytes (De Seranno et al., 2004) indicated that the intracellular mechanisms underlying the stimulatory effect of NO on tanycyte plasticity involve the participation of both cGMP and prostaglandins/thromboxanes. Additional co-culture experiments showed that increased NO production by endothelial cells elicited by the NO precursor L-arginine can trigger tanycyte retraction (Fig. 8a) (de Seranno et al., 2010). These *in vitro* results suggest that endothelial NO may be capable of inducing neuronal-glial remodeling in the median eminence. Using electron microscopy of isolated median eminence explants, it was found that the activation of endogenous NO production via L-arginine treatment induces rapid structural changes, allowing a direct access of GnRH neurosecretory axons to the portal vasculature, likely by promoting tanycyte end-foot retraction (De Seranno et al., 2004).

Recent studies revealed that estradiol acts on both purified endothelial cells and isolated tanycytes that express estrogen receptors *in vitro* (de Seranno et al., 2010) to trigger endothelial-to-glia communication that leads to a sudden and massive retraction of tanycyte processes in an NO-dependent manner (de Seranno et al., 2010). Several lines of evidence support the conclusion that the estradiol-induced acute tanycyte retraction mediated by NO depends mainly on the action of COX enzyme byproducts. First, inhibition of COX activity abrogates NO-mediated actin cytoskeleton remodeling in tanycytes (De Seranno et al., 2004). Second, isolated tanycytes secrete PGE_2 both under control and under stimulated conditions (Prevot et al., 2003a). Thirdly, estradiol upregulates COX-1 and COX-2 expression in tanycytes while leaving the expression of sGC unchanged (de Seranno et al., 2010). Finally, in simple tanycyte cultures, PGE_2 mimics the estrogen-induced acute cellular retraction of tanycytes seen in co-cultures with endothelial cells, whereas membrane-permeable cGMP analogues do not (de Seranno et al., 2010). These results provide evidence for a major role for a COX product such as PGE_2 in the estradiol-induced tanycyte retraction mediated by endothelial cells, although additional factors released by tanycytes and/or endothelial cells may also contribute to this process (Figure 4).

Direct evidence for the ability of PGE_2 to control neuronal-glial plasticity at the neurovascular junction was obtained from experiments in which PGE_2 was applied directly to median eminence explants at concentrations known to stimulate GnRH release (Ojeda and Negro-Vilar, 1985; Ojeda et al., 1990; Prevot et al., 2003b), and structural remodeling was observed in a matter of minutes. PGE_2 treatment caused the advancement of GnRH neurosecretory terminals toward the pericapillary space, a

phenomenon that probably results from the retraction of tanycyte end-feet (de Seranno et al., 2010). Intriguingly, PGE_2 failed to promote direct neurovascular contacts between GnRH axons and the vascular wall. This is in contrast to the effects of L-arginine treatment mentioned earlier, and it suggests that additional downstream signaling pathways, such as those involving cGMP, are required for NO to fully exert its effects on neuronal-glial plasticity. The recent demonstration of an involvement of NO-cGMP signaling in axonal elongation and/or growth cone orientation (Song et al., 1998; Seidel and Bicker, 2000; Nishiyama et al., 2003; De Seranno et al., 2004) supports this interpretation.

Results obtained in adult rats *in vivo* corroborate the role of PGE_2 in the mechanism that controls neuronal-glial plasticity at the neurovascular junction. Intracerebral infusion of the same COX antagonist that blocks NO-mediated actin cytoskeleton remodeling in primary cultures (indomethacin) (De Seranno et al., 2004) into the median eminence resulted in a marked impairment of the ovarian cycle (de Seranno et al., 2010), which requires the coordinated delivery of GnRH into the hypothalamo-hypophyseal portal system. Local inhibition of PG synthesis arrested the ovarian cycle in either the diestrus or the estrus phase (de Seranno et al., 2010), when GnRH release is low (Levine and Ramirez, 1982) and GnRH neuroendocrine terminals are enclosed by tanycyte end-feet (Prevot et al., 1998; Prevot et al., 1999a). These results highlight the physiological importance of eicosanoids in the cell-cell communication processes regulating GnRH release and they are in full agreement with pioneering data obtained more than 30 years ago demonstrating that PGE_2 synthesis increases within the hypothalamic region containing the median eminence during the onset of the first preovulatory surge at puberty (Ojeda and Campbell, 1982), as well as with the findings that intracerebral infusion of PGE_2 promotes LH release (Harms et al., 1973) and, in contrast, inhibitors of PG synthesis alter the onset of the steroid-induced LH surge in ovariectomized female rats (Ojeda et al., 1975).

Altogether, these results raise the possibility that endothelial NO is a molecule involved in the process by which estrogen stimulates GnRH secretion at proestrus (Figure 4). However, is NO produced in the median eminence? Several studies using either L-arginine, NO donors or NOS inhibitors have demonstrated that this gaseous transmitter can stimulate GnRH release from GnRH neuroendocrine terminals in the median eminence (Rettori et al., 1992; Bonavera et al., 1993; Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1994; Seilicovich et al., 1995; Bonavera et al., 1996; Canteros et al.,

1996; Kohsaka et al., 1999). The use of amperometric methods to measure NO production in isolated median eminence explants revealed that estradiol promotes acute increases in the release of both NO and GnRH (Prevot et al., 1999b). Amperometric measurements also showed that NO is released spontaneously in isolated median eminence explants throughout the estrous cycle, with levels increasing dramatically at the onset of the preovulatory surge (Knauf et al., 2001). Critically, blockade of NO production with L-NIO, a selective endothelial nitric oxide synthase (eNOS) inhibitor, prevented estradiol- and proestrus-stimulated release of both GnRH and NO (Prevot et al., 1999b; Knauf et al., 2001) (Figure 5). In addition, it was found that increases in median eminence NO release can be induced in ovariectomized rats by injection of estrogens (Knauf et al., 2001), that intracerebroventricular injection of antisense oligodeoxynucleotides to eNOS inhibits the gonadal-steroid-induced GnRH/LH surge in ovariectomized rats (Aguan et al., 1996), and that inhibition of NO synthesis in the median eminence with a local infusion of NOS inhibitors blunts estrous cyclicity (De Seranno et al., 2004).

Taken together, these findings provide strong evidence that vascular endothelial cells are crucial targets for the action of estrogen on the structural remodeling that takes place at the projection site of GnRH neurons and on GnRH neurosecretion during the estrous cycle. Estrogen may induce endothelial cells and tanycytes in the median eminence to produce NO and upregulate cyclooxygenase expression, respectively. Endothelial NO diffuses into neural tissue and acts on tanycytes to stimulate PGE_2 production, which in turn acts with additional factors that remain unidentified to promote the acute retraction of tanycyte end-feet that allows GnRH nerve terminals to form direct neurovascular junctions.

Do endothelial cells talk directly to GnRH neurons themselves? Electron microscopic data suggest that on the day of proestrus some GnRH axons sprout new terminals that appear to be attracted by the vascular wall (Prevot et al., 1999a). Interestingly, mature GnRH neurons were shown to express growth-associated-43 protein (GAP-43), an intrinsic determinant of axonal growth (Aigner et al., 1995; Benowitz and Routtenberg, 1997), with the proportion of GnRH perikarya expressing GAP-43 mRNA reaching a maximum on the day of proestrus (Prevot et al., 2000c). Conceivably, an accumulation of GAP-43 in GnRH nerve terminals at proestrus could enhance the responsiveness of individual GnRH nerve endings to growth-promoting factors (Benowitz and Routtenberg, 1997). Screening for molecular cues known to play a role in the control of axon guidance indicated Semaphorin3A (Sema3A) as a candidate

(Campagne et al., 2008; Giacobini and Prevot, 2013). Semaphorins are chemotropic factors recently shown to intervene in the control of GnRH neuronal migration during embryogenesis (Cariboni et al., 2007; Giacobini et al., 2008; Messina et al., 2011). Among them, Sema3A, by binding to the transmembrane protein neuropilin-1 (Nrp1), has been shown to be essential for the patterning of the axonal scaffold used by GnRH neurons in their migration from nose to brain during embryonic life (Cariboni et al., 2011; Hanchate et al., 2012). Within the adult brain, *in situ* hybridization revealed robust Sema3A mRNA expression in the capillary zone of the median eminence, whereas only scant hybridization signal was found in the nervous tissue (Campagne et al., 2008). Immunohistochemical stainings showed that Sema3A immunoreactivity is restricted to portal blood capillaries of the median eminence (Campagne et al., 2008). Western blot analyses investigating whether Sema3A protein expression changes during the reproductive cycle, showed that the expression of the 65-kDa Sema3A isoform increased significantly on the afternoon of proestrus (Campagne et al., 2008). Further experiments showed that GnRH nerve terminals might be able to sense Sema3A: expression of Neuropilin-1 receptor was observed in GnRH neurons at both the mRNA and the protein level and the proportion of GnRH perikarya expressing Neuropilin-1 mRNA appears to be maximal at proestrus, a phase of the estrous cycle when GnRH nerve terminals have direct access to the portal vasculature (Campagne et al., 2008). The use of electron microscopy further demonstrated that activation of Sema3A/Neuropilin-1 signaling in the median eminence promotes sprouting of GnRH axons towards the pericapillary space of the pituitary portal blood vessels (Campagne et al., 2008). Finally, local *in vivo* neutralization of Neuropilin-1 activity was shown to disrupt estrous cyclicity (Campagne et al., 2008). Taken together these results raise the exciting possibility that endothelial cells of the median eminence may use chemotropic factors of the semaphorin family to regulate GnRH axonal plasticity.

Conclusion

The evidence reviewed in this chapter demonstrates the vital role of vascular endothelial cells, astrocytes and tanycytes in cellular mechanisms that regulate function-related structural plasticity of the GnRH system. As such, their roles need to be integrated into current models of the central control of fertility, in which neurons are not the sole contributors to the neuroendocrine release of GnRH. Rather,

neurons, astrocytes and vascular endothelial cells are components of a basic unit of functional organization, which we can conceptualize as a neuro-glio-vascular unit (Nedergaard et al., 2003) that must coordinate their actions to maintain homeostasis of the reproductive neuroendocrine brain. In a broader perspective, the demonstration that non-neural cells (vascular endothelial cells) participate in morphological and functional plasticity of neurons and glial cells in the adult central nervous system opens new avenues of investigation in neuroscience.

Acknowledgments

This research was supported by the Agence National pour la Recherche (ANR, France) grants ANR-07-NEURO-026-03 and ANR-09-BLAN-0267, the Fondation pour la Recherche Médicale (Equipe FRM 2005 & DEQ20130326524, France) and the European INTERREG program TC2N.

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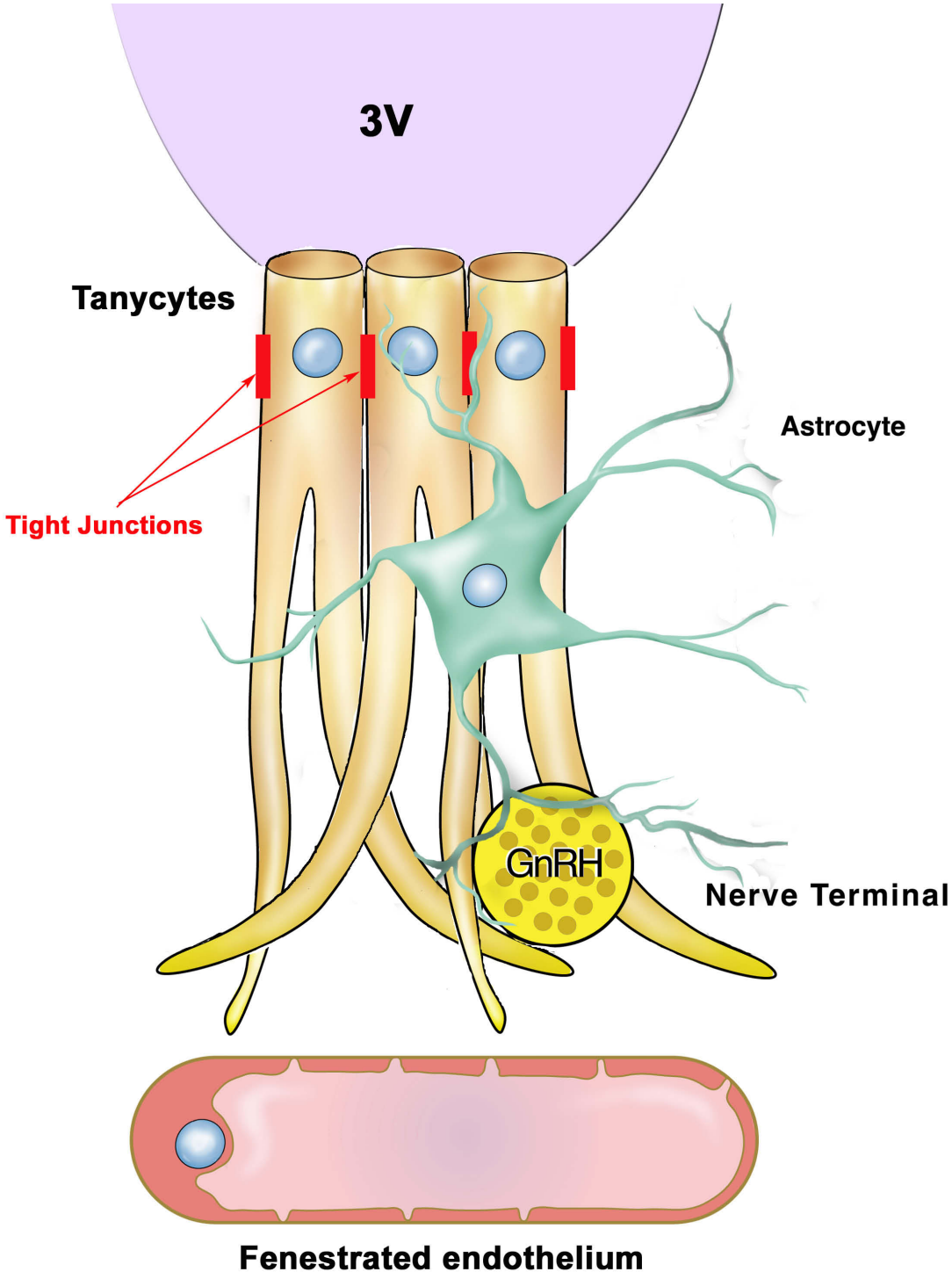

Figure 1.

Schematic representation of the cell types (tanycytes, astrocytes and endothelial cells) and neuronal elements (neuroendocrine terminals) that reside within the median eminence of the hypothalamus. The median eminence of the hypothalamus is the brain structure forming the floor of the third ventricle (3V). The median eminence, which is one of the circumventricular organs of the brain is capable of conveying information from the brain to the periphery via the release of neurohormones into the circulation and,

conversely sensing information reaching the brain via the bloodstream. From (Prevot et al., 2010a) with permission.

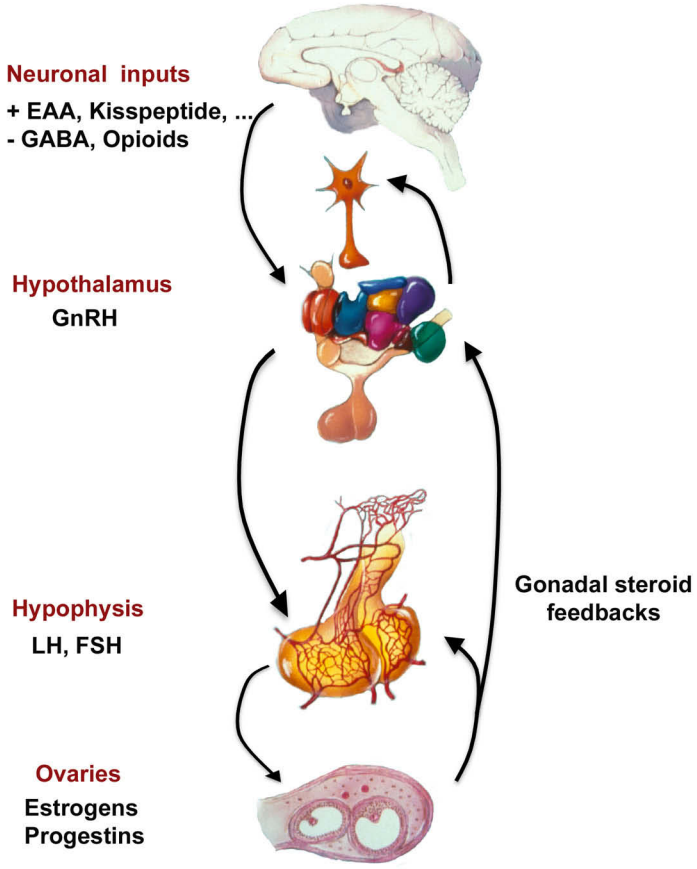

Figure 2. Neuronal control of the pituitary gonadal axis. As hypothalamic GnRH neurons are the final common pathway for central control of gonatropin secretion, they are subjected to a complex array of excitatory and inhibitory transynaptic inputs that modulate their activity. GnRH neuroendocrine neurons project to the median eminence where they make contact with basal lamina and open into the pericapillary space of the primary hypophyseal portal plexus. Upon reaching the pituitary portal system, GnRH travels to the pituitary to stimulate the synthesis and secretion of pituitary gonadotropins: luteinizing hormone (LH) and follicle stimulating homone (FSH). Blood-borne LH and FSH act on target cells in the gonads (here the ovaries) to direct production of gametes, as well as the secretion of steroid hormones. Within the brain, gonadal steroids influence GnRH secretion via neuroendocrine feedback loops. EAA, excitatory amino acids; GABA, gamma- aminobutyric acid. From (Prevot, 2010; Prevot et al., 2010b) with permission.

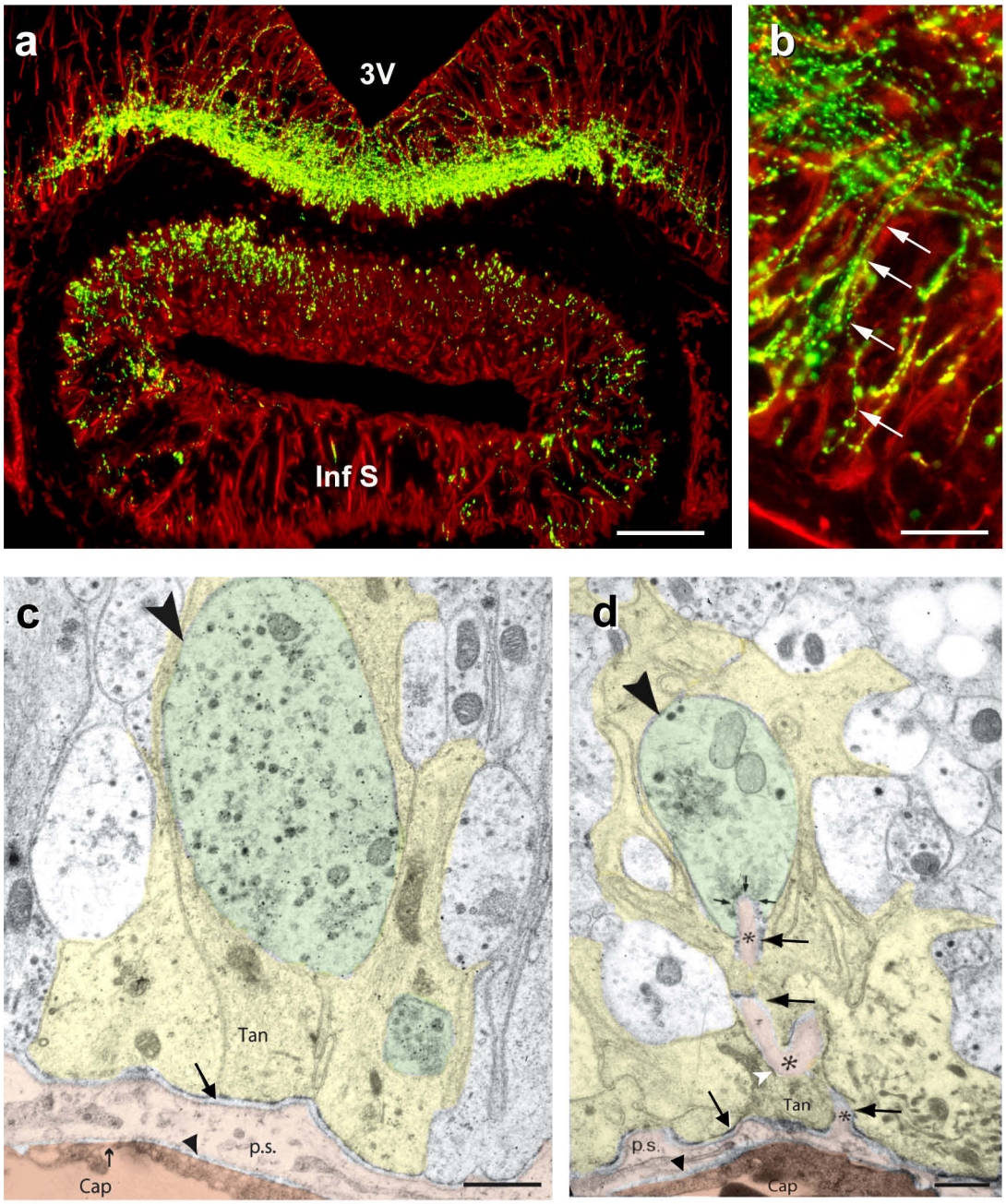

Figure 3. Close association of GnRH nerve terminals with tanycytic processes in the median eminence of the hypothalamus. a: Fluorescent micrograph of a frontal section showing dense GnRH-immunoreactivity (green) and tanycytic processes visualized by their immunoreactivity for vimentin (red) in the external zone of the median eminence. 3V, third ventricle; Inf S, infundibular stem. b: High magnification image showing GnRH axon fibres (green) alongside tanycytic processes (red) projecting to the capillary zone of the median eminence (arrows). c-d: Electron micrographs illustrating the dynamic changes occurring in the external zone of the median eminence that control the direct access of GnRH nerve terminals to the pericapillary space during the estrous cycle in the rat. c: Electron micrograph of

GnRH-immunoreactive terminals (large arrowhead, green) in the external zone of the median eminence in close proximity to fenestrated capillaries (Cap, red) of the portal vasculature. At most of the stages of the reproductive cycle, GnRH nerve terminals (labeled with 15-nm gold particles) are entirely embedded in tanycytic endfeets (Tan, yellow), which prevent them from contacting the pericapillary space (p.s., pink) delineated by the parenchymatous basal lamina (arrow). Arrowhead, endothelial basal lamina; short arrows, fenestration of the endothelium. Scale bar: 0.5μm. d: On the day of proestrus, the time of the occurrence of preovulatory GnRH/LH surge, a significant fraction of GnRH nerve endings (large arrowhead, green) directly contact the pericapillary space (p.s.,pink). Scale bar: 0.5 μm. From (Prevot et al., 1998; Prevot et al., 1999a) with permission.

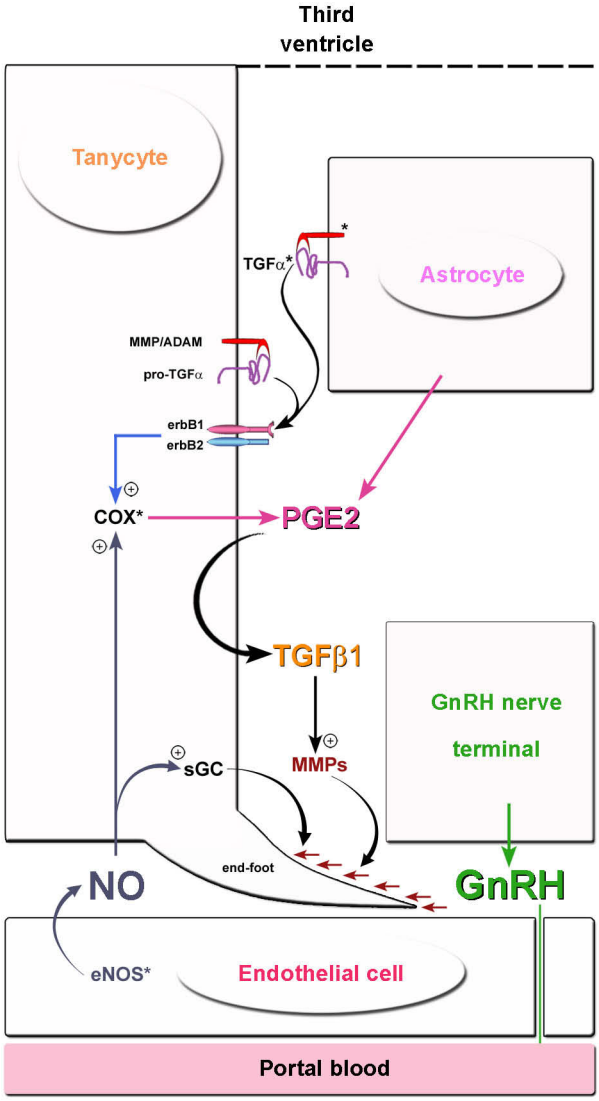

Figure 4. Morphological plasticity of the glial coverage of GnRH neurons in the median eminence. Under conditions of low gonadotropin output such as in diestrus, tanycytes envelop neurosecretory GnRH nerve terminals and, through their end-feet, prevent them from directly contacting the perivascular space and releasing GnRH into the vascular compartment. TGFα, which is produced by tanycytes and astrocytes, activates erbB1/erbB2 heterodimers to stimulate the production of PGE2 and the subsequent PGE2-dependent release of TGFβ1 from tanycytes. Astrocytes also provide a source of PGE2 in response to EGF peptides. Tanycyte-derived TGFβ1 acts in an autocrine manner to induce the retraction of tanycytic processes through the metalloproteinase (MMP)-mediated degradation of extracellular matrix. Endothelial cells also regulate the morphological plasticity of tanycytes via the release of NO, which diffuses to tanycytes to stimulate the enzymatic activity of COX and of the NO-sensitive soluble guanylyl cyclase (sGC). Notably, the mechanism by which the activation of sGC promotes actin

cytoskeleton remodelling in tanycytes remains unknown. The retraction of tanycytic end-feet enables GnRH nerve endings to make direct contact with the pericapillary space (i.e., the space delineated by the parenchymatous basal lamina on one side and by the endothelial basal lamina on the other side; not shown). The released GnRH then enters the portal blood via the fenestrations of endothelial cells. Asterisks indicate the molecular components shown to be positively regulated by gonadal steroids in primary cultures of astrocytes, tanycytes and endothelial cells (see main text for details). From (Sharif et al., 2013) with permission.

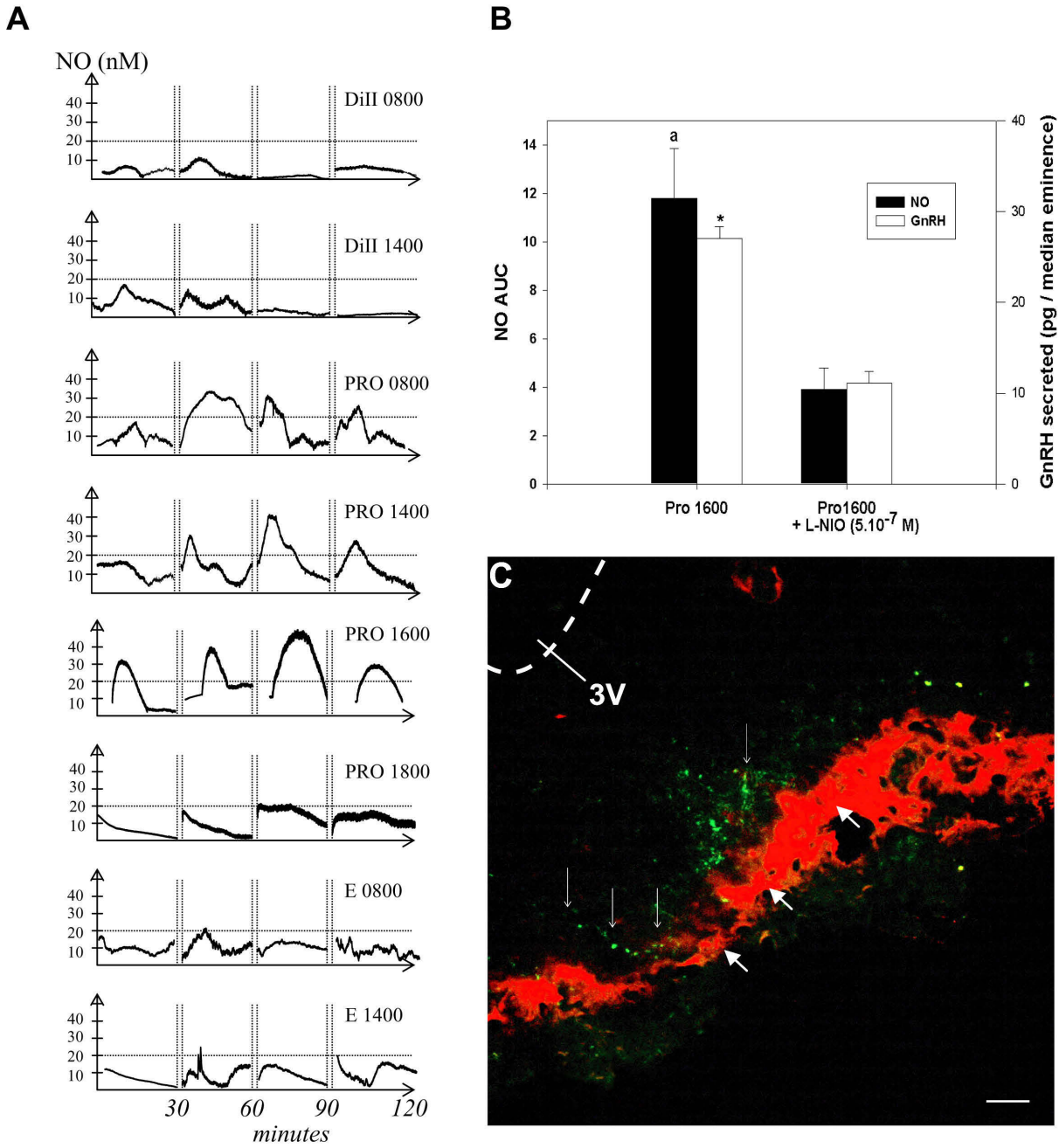

Figure 5. In the median eminence of the hypothalamus, endothelial nitric oxide (NO) secretion may represent one of the synchronizing cues that by coordinating GnRH release from GnRH neuroendocrine terminals that are distributed over 2 mm within the median eminence allows the occurrence of functionally meaningful episodes of GnRH secretion. (A) Real time amperometric measurement of spontaneous NO release from median eminence explants at different stages of the rat estrous cycle. DiII, diestrus II; PRO, proestrus; E, estrus. (B) On the afternoon of proestrus, the preovulatory GnRH/NO release is blocked with L-NIO, an NOS inhibitor selective for eNOS at 0.5 μM. * and a, significantly different from treated samples, p<0:05: AUC: area under the curve during a 30 min period. (C)

Photomicrograph showing GnRH axonal fibers in the external zone of the median eminence (green fluorescence, arrows) in close apposition to eNOS-immunoreactive portal vasculature (red fluorescence, arrowheads). 3V, third ventricle. The dotted lines outline the third ventricle. Scale bar: 75 μm. From (Prevot et al., 2000a; Knauf et al., 2001) with permission.

La sécrétion de la GnRH implique l'exocytose dépendante du calcium en faisant intervenir la protéine d'exocytose VAMP2

Les résultats présentés au cours de notre étude sont les premiers à montrer que le gène *Vamp2* est exprimé par les neurones à GnRH, et que l'expression de la toxine botulique dans les neurones à GnRH conduit à un hypogonadisme hypogonadotrope. La toxine botulique est une enzyme clivant les protéines VAMP, et en particulier la protéine VAMP2 (Schiavo et al. 2000; Montecucco et al. 2005). Les résultats obtenus suggèrent donc que la protéine VAMP2 est impliquée dans l'exocytose de la GnRH. Les neurones à GnRH sont des neurones pour le moins atypiques. De part leur origine embryonnaire extracérébrale (Wray, Nieburgs, et al. 1989; Schwanzel-Fukuda & Pfaff 1989), la plasticité de leurs terminaisons (Prevot et al. 1999), ou encore leur dendrone (Herde et al. 2013b), qui surplombe aujourd'hui la notion d'axone, ces cellules ne répondent pas aux caractéristiques classiques des neurones. Cependant, nous montrons que les cellules à GnRH utilisent visiblement un mode d'exocytose typique des neurones, à savoir une exocytose dépendante de la protéine VAMP2.

Les taux de LH, qui sont directement et étroitement liés aux taux de GnRH, sont indétectables chez les femelles exprimant la toxine botulique dans les neurones à GnRH du groupe « overweight ». Elles présentent donc un hypogonadisme lié à un défaut de sécrétion de GnRH, en raison de l'expression de la toxine. La galanine, comme la GnRH, est retrouvée dans les vésicules à cœur dense des neurones endocrines (Liposits et al. 1995). Il est donc très probable que la sécrétion de galanine par les neurones à GnRH soit également touchée. Enfin, La protéine VAMP2 est exprimée aussi bien par les vésicules à cœur dense que par les petites vésicules synaptiques des neurones (H. Kasai et al. 2012). Il est possible que la toxine botulique empêche également la sécrétion d'autres facteurs par les neurones à GnRH, tels que le GABA et le glutamate, qui interviennent dans la modulation de la GnRH. Des études complémentaires sont nécessaires pour répondre à cette question.

Un développement normal du système à GnRH chez les animaux déficients en protéines VAMP2 dans les neurones à GnRH

Chez les animaux exprimant la toxine botulique dans les neurones à GnRH, et donc déficients en protéine VAMP2, on trouve un nombre de cellules à GnRH normal, ainsi qu'une densité de fibres à GnRH normale dans le cerveau. Ceci suggère une complète mise en place du système pendant le développement embryonnaire. Ces données mettent en évidence le fait que la sécrétion de facteurs par les neurones à GnRH pendant l'embryogenèse n'est pas nécessaire à leur migration correcte, ni à la projection normale de leurs fibres. Les images de microscopie électronique (étude n°1, figure 5) montrent des terminaisons à proximité de l'espace péricapillaire, comparables aux contrôles. Anatomiquement les terminaisons à GnRH sont normales. Ces données sont consistantes avec ce qui a été décrit précédemment chez les souris hpg, déficientes en GnRH, et chez qui la migration et la maturation des neurones à GnRH est normale (John C. Gill et al. 2008b).
Nous montrons également dans les résultats supplémentaires à cette étude qu'une injection de GnRH périphérique est capable d'induire en 15 minutes une sécrétion de LH de même amplitude dans tous les groupes. Ces données sous-entendent que la fonction hypophysaire chez les animaux mutants n'est pas affectée. Le phénotype d'hypogonadisme hypogonadotrope n'est donc pas lié à un défaut de sécrétion de gonadotrophines par l'hypophyse, mais uniquement à un défaut de sécrétion de GnRH par l'hypothalamus.

Une notion de seuil de sécrétion de la GnRH dans l'acquisition de la fertilité

Les animaux exprimant la toxine botulique dans les neurones à GnRH ont été subdivisés en deux groupes distincts en fonction de leur phénotype, qui est corrélé au pourcentage de neurones à GnRH exprimant le transgène, et subissant par conséquent le phénomène de recombinaison. Ces données mettent en évidence la notion d'un seuil minimal de sécrétion de GnRH requis pour mener à bien la fonction de reproduction. Dans notre cas, les femelles présentant environ 40% de recombinaison affectant les neurones à GnRH présentaient un phénotype comparable aux animaux contrôles (lean), alors que ceux qui présentent environ 60% de cellules ayant subi la recombinaison présentent une puberté retardée, voire absente, un surpoids, et son acycliques (overweight). D'après une étude de Herbison et al. (2008), seuls 12% des neurones à GnRH dans le cerveau de femelles sont nécessaires au déclenchement d'un pic de LH, même si ces animaux sont subfertiles, et 34%

seulement sont requis pour présenter un cycle ovarien et des ovulations. Il est possible que nos quantifications aient été sous-estimées, étant donné que le gène rapporteur EGFP utilisé dans notre stratégie transgénique, soit présent en aval d'une séquence IRES. La séquence IRES est, en effet, associée à une diminution de l'expression du gène en aval jusqu'à 20% par rapport au gène en amont (Mizuguchi et al. 2000).

Nous avons subdivisé les animaux mutants en deux groupes. Toutefois, il se pourrait que d'autres phénotypes soient observables. En effet, à l'analyse des ovaires des femelles du groupe « overweight », l'une d'elle a montré les signes d'une ovulation, alors que les autres n'en ont présenté aucun. Le pourcentage de recombinaison des neurones à GnRH pourrait varier entre 45 et 60%, et être lié à des phénotypes différents, allant du phénotype semblable au contrôle, jusqu'à la totale infertilité.

Balance énergétique et axe gonadotrope : un dialogue dans les deux sens ?

La GnRH, ou les facteurs exprimés par les neurones à GnRH, pourraient avoir directement ou indirectement une incidence sur le métabolisme énergétique. S'il existe un grand nombre d'études montrant un rôle direct de la balance énergétique sur la fonction de reproduction, il n'existe aucune étude montrant un rôle direct de la sécrétion de GnRH sur le métabolisme énergétique.

Dans notre étude, les femelles « overweight » ont un défaut de sécrétion de GnRH, sont hypogonadiques et présentent une augmentation du poids par rapport à leurs sœurs « lean » et contrôles. Il est connu que l'hypogonadisme est lié à un surpoids. Ceci est notamment décrit chez des femelles ovariectomisées (Kamei et al. 2005). Ce phénomène serait probablement en lien avec les hormones périphériques. En effet, les estrogènes constituent un signal anorexigène, la suppression de ce signal pouvant conduire à une obésité (Gao et al. 2007; Rogers et al. 2009; Xu et al. 2011). Ces observations sont consistantes avec d'autres modèles de souris présentant un hypogonadisme hypogonadotrope (Good et al. 1997).

L'hypogonadisme hypogonadotrope et le syndrome de Kallmann chez l'Homme sont fortement corrélés à un surpoids et à des troubles métaboliques, tels que l'obésité et le diabète (Fichna et al. 2011). Ces deux pathologies sont plus souvent retrouvées chez les hommes que chez les femmes, qui possèdent des taux d'estrogènes très bas (Stamou et al. 2015). La testostérone a aussi montré un rôle anti-obésité, mais elle a surtout un rôle dans le développement de la masse maigre musculaire (Kelly & Jones 2015), et la supplémentation

en testostérone n'empêche pas le développement de l'obésité chez des hommes atteints du syndrome de Kallmann (Van Dop et al. 1987). Il pourrait donc exister un rôle direct, qui ne passerait pas par l'action des stéroïdes gonadiques, de l'axe gonadotrope sur la balance énergétique. Cette hypothèse n'est pas décrite et nécessite, pour y répondre, des études complémentaires, qui pourraient être apportées à l'aide de notre modèle déficient en sécrétion de GnRH.

L'étude du mâle et la mise en évidence d'un dimorphisme sexuel chez les animaux déficients en protéine VAMP2

Dans les résultats supplémentaires à cette étude, nos résultats préliminaires démontrent que les mâles exprimant la toxine botulique dans les neurones à GnRH présentent également une puberté retardée, caractérisée à l'aide d'un signe externe de maturation de la gonadotrope : la séparation préputiale. Ce phénomène est corrélé au retard de détection de phéromones, et notamment de la protéine MUP, dans les urines. Les différentes protéines MUP sont sécrétées en réponse à la testostérone chez le mâle. Ces données, ainsi que des études précédentes, confirment que la technique de détection de la MUP par western blot, qui est une technique non-invasive, est un bon indicateur de maturation sexuelle chez le mâle (McGee & Narayan 2013; Vesper et al. 2006).

A l'inverse du phénotype décrit chez les femelles, les résultats préliminaires obtenus montrent que le poids est significativement inférieur chez le mâle déficient exprimant la toxine botulique dans les neurones à GnRH. Ces différences pourraient s'expliquer par l'existence d'un dimorphisme sexuel concernant le système à GnRH. Il existe en effet un dimorphisme sexuel au niveau des neurones afférents aux neurones à GnRH (Hrabovszky et al. 2011), mais le dimorphisme majeur intrinsèque aux neurones à GnRH constitue l'expression de galanine, quasiment inexistante chez le mâle, et qui concerne environ 45% des neurones à GnRH chez la femelle (Liposits et al. 1995). Toutefois, le phénotype observé chez le mâle, dans ce modèle animal, va à l'encontre de ce qui est observé chez les individus atteints d'hypogonadisme ou de syndrome de Kallmann, comme vu dans le paragraphe précédent. Ces observations suggèrent que les stéroïdes gonadiques ne sont pas seuls acteurs d'un dialogue avec la balance énergétique dans notre modèle. L'explication de ces phénotypes opposés, entre mâle et femelle, nécessite toutefois de plus amples analyses, notamment de prise alimentaire et de dépense énergétique.

La suppression de l'expression du récepteur Nrp1 dans les neurones à GnRH chez notre modèle animal transgénique est spécifique

Grâce à notre stratégique transgénique, basée sur le croisement de souris *Nrp1*$^{loxp/loxp}$ (présentant l'exon 2 du gène *Nrp1* flanqué de séquence loxp) et de souris *Gnrh ::cre* (exprimant la cre recombinase sous le contrôle du promoteur GnRH), nous avons sélectivement diminué l'expression du récepteur Nrp1 dans les neurones à GnRH. Cette modification n'induit pas de compensation par l'expression de la Nrp2, qui est, elle aussi, capable de lier la Sema3A, avec moins d'affinité (Nasarre et al. 2009; Cariboni, Davidson, Rakic, et al. 2011), puisque son expression reste inchangée entre les animaux mutants et contrôles. Néanmoins l'expression de la plexine A3, qui se dimérise notamment avec la Nrp2 dans la signalisation par les Sema3 (Schwarz et al. 2008), apparaît particulièrement augmentée dans les cellules à GnRH issues de mutants, même si les grandes variations obtenues n'ont pas permis de mettre en évidence une différence significative. D'autres analyses sont nécessaires pour savoir si la voie de la signalisation impliquant la plexine A3 est augmentée dans les neurones à GnRH déficients en Nrp1. De manière intéressante, on constate que l'expression de la plexine A1 est diminuée dans les neurones à GnRH défiscients en Nrp1. La plexine A1 est la plexine la plus décrite dans la dimérisation à la Nrp1 la signalisation induite pas la Sema3A (Wu et al. 2014a). La modulation de l'expression de la plexine A1 par la Nrp1, ou l'inverse, n'est pas décrite dans la littérature. Ces données suggèrent qu'en plus d'être des partenaires importants et nécessaires dans la liaison et la signalisation de la Sema3A, la Nrp1 et la plexine A1 interagissent aussi par leurs taux d'expression.

Nous avons démontré, par immunohistofluorescence, que la suppression de la protéine Nrp1 ne se faisait que dans les neurones à GnRH, et que les autres structures, telles que les axones voméronasaux, exprimaient toujours ce récepteur.

L'expression de la Neuropiline-1 dans les neurones à GnRH est impliquée dans la survie des cellules et dans leur migration pendant l'embyogenèse

Nous avons montré que les souris déficientes en Nrp1 dans les neurones à GnRH, présentaient un plus grand nombre de cellules à GnRH pendant l'embryogenèse. Ce phénomène passe visiblement par une diminution de l'apoptose des neurones à GnRH au cours de la migration embryonnaire. Le récepteur Nrp1 aurait donc un rôle dans la

régulation de la survie des neurones à GnRH, en favorisant l'apoptose. Le rôle de la Nrp1 dans la survie des cellules n'est pas nouveau. En effet, à l'inverse de ce qu'on observe dans notre étude, la Nrp1 est connue pour son effet anti-apoptotique notamment dans des populations de cellules endothéliales ou tumorales (Karjalainen et al. 2011; Barr et al. 2008; L. Wang et al. 2007). Cet effet est toutefois lié à la signalisation par le VEGF164, qui est un facteur induisant la survie (Cariboni, Davidson, Dozio, et al. 2011). Nos résultats semblent aussi, de premier abord, aller à l'encontre de l'étude de Cariboni et al. (2011, décrit précédemment : Chapitre 3, 3.2), qui conclut à un rôle anti-apoptotique de la Nrp1 dans les neurones à GnRH, induit par la liaison du VEGF164, et qui aurait donc un rôle dans leur survie au cours de la migration embryonnaire. Toutefois, il existe des études, réalisées dans des populations non-neuronales, qui ont montré que la Nrp1 pouvait avoir un effet apoptotique, via la fixation de la Sema3A (Fu et al. 2012; Guttmann-Raviv et al. 2007). Il semblerait donc que la Nrp1 puisse avoir à la fois un rôle apoptotique, mieux décrit, et un rôle anti-apoptotique, en fonction du ligand rencontré. Même si de plus amples analyses sont nécessaires pour déterminer le ou les ligand(s) en cause dans notre modèle animal, la Nrp1 est impliquée dans la régulation de la taille de la population de cellules à GnRH pendant le développement. Cet effet pourrait passer par la fixation du ligand Sema3A.
A P0, on n'observe plus de différence du nombre total de cellules à GnRH entre les animaux mutants et leurs contrôles. Ceci pourrait être lié à une compensation de l'absence de la Nrp1, qui se produirait entre E18,5 et P0. Ces données sont consistantes avec ce qui est observé à l'âge adulte, puisque le nombre total de neurones à GnRH dans le cerveau à P60 est le même entre les animaux contrôles et mutants.

L'expression de la Neuropiline-1 dans les neurones à GnRH pourrait également être impliquée dans la migration autonome des cellules à GnRH

Le surplus de cellules retrouvé chez les animaux mutants a déjà atteint le cerveau antérieur à E14,5. L'absence de Nrp1 dans les cellules à GnRH pourrait donc également affecter la migration de ces cellules. Ce phénomène est fortement suggéré par les résultats obtenus à l'âge adulte. En effet, le nombre total de neurones à GnRH dans le cerveau adulte est inchangé entre les animaux contrôles et les mutants, alors que leur distribution est altérée. On observe une accumulation de cellules dans l'hypothalamus, et en particulier dans la région de l'OVLT chez les animaux mutants. Ceci pourrait être causé par une modification de modalités de migration des cellules, telles que la vitesse ou la durée.

D'autres analyses sont toutefois nécessaires pour déterminer le rôle précis de la Nrp1 dans la migration autonome des cellules à GnRH.

L'expression de la Neuropiline-1 dans les neurones à GnRH est impliquée dans le déclenchement de la puberté

Notre étude constitue le premier rapport décrivant une maturation sexuelle précoce centrale associée à une accumulation de neurones à GnRH dans l'hypothalamus chez la souris. Même si de plus amples analyses sont nécessaires à la compréhension du lien entre ces deux phénomènes, on pourrait supposer que l'augmentation du nombre de cellules à GnRH dans l'OVLT, région impliquée dans le déclenchement du pic préovulatoire de LH (Herde et al. 2011), est à l'origine d'une plus grande sécrétion de GnRH. Les taux de GnRH nécessaires au développement central de l'axe gonadotrope pourraient donc être atteints plus tôt chez les animaux mutants. Ceci est consistant avec les données concernant les taux de LH à P45, directement corrélés aux taux de GnRH, et qui sont plus élevés chez les femelles mutantes. Les données concernant la fonction de reproduction de ces femelles à l'âge adulte sont comparables aux femelles contrôles. Elles présentent en effet des taux de LH comparables en phase de proestrus et en phase de diestrus, ainsi qu'un cycle ovarien normal et une fertilité normale. On pourrait, toutefois, s'attendre à ce que ces femelles développent d'autres troubles en lien avec la fonction de reproduction, tels que des ovaires polykystiques, ou encore une sénescence précoce.

Au vu du rôle de la Sema3A dans la neuritogénèse des neurones à GnRH chez l'embryon, comme chez l'adulte (Giacobini, Parkash, Campagne, Messina, Casoni, Vanacker, Langlet, Hobo, Cagnoni, Gallet, Hanchate, Mazur, Taniguchi, Mazzone, Verhaagen, Ciofi, Sébastien G Bouret, et al. 2014), il est probable que l'intégration des neurones à GnRH à leur réseau soit impactée par la déficience en Nrp1. Les observations de marquages de la GnRH sur des coupes de région préoptique et d'éminence médiane ne semblent pas montrer de différences de quantité ou de taille des fibres issues des cellules à GnRH. Toutefois, de plus amples analyses sont nécessaires à la discussion de ce point. Par ailleurs, l'étude de Giacobini et al. (2013) rapporte également que, sans la signalisation Nrp1/Sema3A dans les terminaisons à GnRH chez la rate et la souris, le cycle ovarien est affecté. Ce phénomène est lié à l'incapacité des terminaisons à GnRH à se rapprocher de l'espace péricapillaire pendant la période de proestrus. Il pourrait exister un phénomène de compensation dans la plasticité de l'éminence médiane au cours du cycle ovarien dans notre modèle animal, par exemple

par l'intervention d'autres sémaphorines, puisque les femelles mutantes ont un cycle ovarien normal.

L'absence de Neuropiline-1 dans les neurones à GnRH conduit à un surpoids chez la femelle

Les femelles déficientes en Nrp1 dans les neurones à GnRH développent un surpoids, en comparaison à leurs sœurs contrôles, à partir de la période péripubertaire. Toutefois, le poids à la puberté est le même entre les animaux mutants et contrôles. Ces résultats suggèrent, dans notre cas où la mutation est présente exclusivement dans les neurones à GnRH, que la maturation précoce de l'axe gonadotrope précède la croissance pondérale. A l'âge adulte, ces mêmes animaux présentent un surpoids, lié à une augmentation des taux de masse grasse. La balance énergétique est donc dérégulée chez les femelles mutantes. L'ensemble de ces données est un argument en faveur d'un dialogue de l'axe gonadotrope vers la balance énergétique, indépendamment des taux de stéroïdes gonadiques périphériques. Le phénotype observé chez les souris déficientes en Nrp1 dans les neurones à GnRH est consistant avec les troubles observés chez les individus atteints de puberté précoce chez l'Homme. La puberté précoce centrale idiopathique est, en effet, une véritable pathologie, plus souvent retrouvée chez la femme, corrélée à une augmentation de l'incidence de troubles métaboliques, incluant le surpoids, l'obésité, ou encore le diabète (Colmenares et al. 2014; Stöckl et al. 2012). C'est pourquoi, notre modèle animal pourrait être un bon modèle dans l'étude de la puberté précoce centrale.

De manière générale, ce travail de thèse apporte des connaissances fondamentales sur le fonctionnement des neurones à GnRH, leur sécrétion, leur migration, et le dialogue entre l'axe gonadotrope et le métabolisme énergétique dans le modèle animal murin. Dans un premier temps, ce travail apporte de nouvelles connaissances concernant le mécanisme d'exocytose régissant la sécrétion des neurones à GnRH. Il met en évidence le rôle de la sécrétion par les neurones à GnRH dans la fonction de reproduction et dans le métabolisme énergétique, et montre que cette sécrétion n'est pas nécessaire au développement et à la mise en place correcte du système à GnRH. Dans un second temps, ce travail montre que le récepteur Nrp1 a un rôle direct dans la survie et la migration des neurones à GnRH au cours du développement embryonnaire. Ce phénotype est lié à une maturation précoce de l'axe gonadotrope, à l'apparition d'une puberté précoce, ainsi qu'à des troubles métaboliques chez l'adulte.

Néanmoins, beaucoup d'interrogations ressortent, de ce travail. Il nous faut, d'une part, comprendre si le phénotype métabolique observé chez les femelles exprimant la toxine botulique dans les neurones à GnRH est exclusivement la conséquence de l'hypogonadisme, et dû défaut de stéroïdes gonadiques qui en découle, ou s'il peut être du, directement, au défaut de sécrétion de GnRH. Pour cela, nous envisageons, dans un premier temps, d'inclure à notre étude un troisième groupe d'animaux, qui présentera une sécrétion de GnRH normale, mais qui sera gonadectomisé et qui ne sera pas supplémenté en stéroïdes gonadiques. Nous analyserons ainsi, grâce à l'utilisation d'une plateforme métabolique au sein de notre centre de recherche, l'apport et la dépense énergétique, entre les groupes « contrôle », « mutant », et « gonadectomisé ». Nous pourrons ainsi déterminer si la sécrétion de GnRH a un rôle direct sur la balance énergétique. D'autre part, nous réaliserons une tentative de récupération du phénotype « overweight », par injection chronique centrale de GnRH ou de galanine, et nous pourrons ainsi déterminer lequel de ces peptides a un impact sur le métabolisme énergétique.

Nous sommes, par ailleurs, en cours de développement de nouveaux outils d'immunohistochimie, de western blot, et de PCR en temps réel, pour détecter directement la VAMP2 ou la VAMP2 clivée, ainsi que la toxine botulique. Ces outils nous permettront ainsi de quantifier plus précisément la quantité de cellules à GnRH affectées par la mutation,

et d'établir une corrélation entre le pourcentage de recombinaison et la sévérité du phénotype métabolique.

D'autre part, il nous faut comprendre si le phénotype observé pendant l'embryogenèse, dans notre modèle animal déficient en Nrp1 dans les neurones à GnRH, est lié aux effets du ligand Sema3A, ou du ligand VEGF164. Pour cela, nous étudierons l'effet d'injections intranasales d'inhibiteur de la sémaphorine 3A chez des embryons. Parallèlement, l'utilisation de cultures organotypiques de placodes olfactives, issues d'embryons, nous permettra de suivre, par technique de « time-lapse », la migration des cellules à GnRH exprimant la GFP. Le traitement de ces cultures avec de la sémaphorine 3A, du VEGF164, ou l'inhibiteur de sémaphorine 3A, nous permettra ainsi de déterminer le rôle direct de ces molécules sur la motilité des cellules à GnRH en migration.

De manière intéressante, nos deux études ont mis en évidence de différentes façons le dialogue qui est suspecté entre l'axe gonadotrope et la balance énergétique. Il est nécessaire de déterminer s'il existe un lien direct entre la sécrétion de la GnRH et le contrôle de la prise alimentaire. Anatomiquement, les fibres à GnRH qui se projettent vers l'éminence médiane couvrent en grande partie le noyau arqué. S'il devait y avoir un lien direct entre les neurones à GnRH et ceux du noyau arqué qui contrôlent la balance énergétique, il pourrait être localisé à cet endroit. Ainsi, grâce à l'injection de virus cre-dépendants, qui expriment des protéines présynaptiques fluorescentes, chez des animaux *Gnrh::cre*, nous envisageons d'analyser l'existence potentielle de synapses entre les neurones à GnRH et d'autres populations neuronales, en particulier au sein du noyau arqué.

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Submission: May 30, 2012
PLoS Genetics

SEMA3A, a gene involved in axonal pathfinding, is mutated in patients with Kallmann syndrome

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Short title: *SEMA3A* mutations and Kallmann syndrome

Funding. This research was supported by Equipe FRM (to VP), ANR-07-NEUR-NeoVasc (to VP), ANR-2010-JCJC-1404-01 (to PG), ANR-09-GENO-017-01 (to PL, JY, JPH, CD), GIS maladies rares (project A09051KS), Compagnia di San Paolo-2008.1224, Torino, Italy (to PG), and the European Research Council COST action BM1105. NKH was a Ph.D. student supported by Inserm U837.

Competing interests. The authors have declared that no competing interests exist.

Abstract

Kallmann syndrome (KS) associates congenital hypogonadism due to gonadotropin-releasing hormone (GnRH) deficiency, and anosmia. The genetics of KS involves various modes of transmission, including oligogenic inheritance. Here, we report that *Nrp1*$^{sema/sema}$ mutant mice that lack a functional semaphorin-binding domain in neuropilin-1, the obligatory coreceptor of semaphorin-3A, have a KS-like phenotype. Pathohistological analysis of these mice indeed showed abnormal development of the peripheral olfactory system and defective embryonic migration of the neuroendocrine GnRH cells to the basal forebrain, which results in increased mortality of newborn mice and reduced fertility in adults. We thus screened 386 KS patients for the presence of mutations in *SEMA3A* (by Sanger sequencing of all 17 coding exons and flanking splice sites), and identified nonsynonymous mutations in 24 patients, specifically, a frameshifting small deletion (D538fsX31) and seven different missense mutations (R66W, N153S, I400V, V435I, T688A, R730Q, R733H). All the mutations were found in heterozygous state. Seven mutations resulted in impaired secretion of semaphorin-3A by transfected COS-7 cells (D538fsX31, R66W, V435I) or reduced signaling activity of the secreted protein in the GN11 cell line derived from embryonic GnRH cells (N153S, I400V, T688A, R733H), which strongly suggests that these mutations have a pathogenic effect. Notably, mutations in other KS genes had already been identified, in heterozygous state, in five of these patients. Our findings indicate that semaphorin-3A signaling insufficiency contributes to the pathogenesis of KS, and further substantiate the oligogenic pattern of inheritance in this developmental disorder.

Author Summary

Kallmann syndrome is a hereditary developmental disease that affects both the hormonal reproductive axis and the sense of smell. There is a developmental link between the reproductive and olfactory disorders: neuroendocrine cells producing the gonadotropin-releasing hormone that is deficient in the patients normally migrate from the nose to the forebrain along olfactory nerve fibers during embryonic life, and they fail to do so in the patients. Affected individuals usually do not undergo spontaneous puberty. Hormone replacement therapy is the treatment to initiate virilization in males or breast development in females, and later, to develop fertility in both sexes. This is a genetically heterogeneous disease. Mutations in any of eight causative genes identified so far have been found in approximately 30% of the affected individuals, thus indicating that other genes remain to be discovered. We report on the identification, in 6% of the KS patients, of various loss-of-function mutations in the gene coding for semaphorin-3A, a secreted protein involved in the navigation of olfactory nerve fibers during embryogenesis. The fact that many of these mutations were also detected in clinically unaffected individuals indicates that they must combine with other genetic defects to produce the disease phenotype.

Introduction

Kallmann syndrome (KS, MIM 147950, 244200, 308700, 610628, 612370, 612702) is an inherited neurodevelopmental disorder defined as the association of hypogonadotropic hypogonadism, due to gonadotropin-releasing hormone (GnRH) deficiency, and the inability to smell (anosmia or hyposmia), related to abnormal development of the peripheral olfactory system (olfactory nerves and olfactory bulbs). The genetics of KS involves various modes of transmission, specifically, autosomal recessive, autosomal dominant with incomplete penetrance, X-chromosome linked, and also oligogenic inheritance [1,2]. Pathohistological studies of fetuses with olfactory bulb agenesis have shown that the reproductive phenotype of KS results from a pathological sequence in embryonic life, whereby premature interruption of the olfactory, vomeronasal and terminal nerve fibers in the frontonasal region disrupts the migration of neuroendocrine GnRH cells, which normally migrate from the nose to the brain along these nerve fibers [3,4]. What causes the primary failure of these fibers to establish proper contact with the forebrain is, however, still unknown. Since KS is genetically heterogeneous, identification of the various genes involved and the study of appropriate animal models are expected to provide valuable clues. Barely 30% of the KS patients have mutations in any of the eight genes known so far, specifically, *KAL1* (ID 3730) [5-7], *FGFR1* (ID 2260) [8], *FGF8* (ID 2253) [9], *PROKR2* (ID 128674), *PROK2* (ID 60675) [10], *WDR11* (ID 55717) [11], *HS6ST1* (ID 9394) [12], *CHD7* (ID 55636) [13,14], and current efforts thus concentrate on the identification of other genes that contribute to this disorder. One strategy is based on close pathohistological examination of targeted mutant mice that may reproduce the human KS phenotype. Here, we show that *Nrp1*$^{sema/sema}$ mutant mice, which are defective for the semaphorin-binding domain of the membrane coreceptor neuropilin-1, have a KS-like phenotype, and we provide genetic evidence that insufficient semaphorin-3A signaling can contribute to the KS phenotype in man.

Results/Discussion

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

In the mouse, GnRH cells begin to leave the epithelium of the medial olfactory pit around embryonic day 11.5 (E11.5). They migrate in the frontonasal region in close association with growing fibers of the vomeronasal and terminal nerves, then penetrate into the rostral forebrain together with the central processes of these nerves, and continue their migration towards the hypothalamic region along a branch of the vomeronasal nerve that projects to the basal forebrain or along fibers of the terminal nerve itself [15-17] (Figure 1A). Proper navigation of growing axons depends on guidance cues, which include semaphorins, a large and diverse family of secreted and membrane-associated proteins [18]. Among these, there is semaphorin-3A (Sema3A), a secreted protein with repulsive effects on primary olfactory axons expressing the coreceptor neuropilin-1 (Nrp1) [19-21]. The role of semaphorins in the navigation of vomeronasal/terminal axons and embryonic GnRH cells is still unclear, but previous studies in rodents have shown that migrating GnRH cells are morphologically associated with Nrp1-immunoreactive axons and are themselves immunoreactive [22,23]. Indeed, we were able to confirm these findings in E14.5 mouse embryos, and extend them to a 9-week old human fetus (Figure 1B-D), using specific antibodies to Nrp1 (Figure S1) in immunohistofluorescence experiments. Notably, the caudal branch of the vomeronasal nerve that accompanies GnRH cells in their intracerebral path was also Nrp1-immunoreactive in the mouse embryos (Figure 1C). These observations suggested that semaphorin signaling through Nrp1 imparts guidance information to axons of the vomeronasal neurons and migrating GnRH cells.

Migration of GnRH cells to the basal forebrain is defective in *Nrp1*$^{sema/sema}$ mutant mice

We thus analyzed *Nrp1*$^{sema/sema}$ mutant mice that harbor inactivating aminoacid substitutions in the semaphorin-binding domain of Nrp1. Unlike *Nrp1*$^{-/-}$ knockout mice, which die around E12.5 [24], these mice survive until birth [25]. In *Nrp1*$^{sema/sema}$ newborn mice (n=4), many axons of olfactory receptor neurons were stuck at the dorsal aspect of the cribriform plate and did not project to the olfactory bulb glomeruli (Figure 2A). Olfactory cues are thought to play an important role in suckling behavior [26]. Analysis of six litters at postnatal day 1 (P1) indeed showed that 7 out of 8 *Nrp1*$^{sema/sema}$ pups had little or no milk in their stomachs, whereas most *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/+}$ littermates (18 out of 21) had full stomachs. These findings account for the decreased survival rate of homozygous, but not

heterozygous, mutant pups [25], and strongly suggest that the sense of smell is affected in *Nrp1*$^{sema/sema}$ mice. Most importantly, DiI axonal labeling at E14.5 showed abnormal projection of the vomeronasal nerve to the ventral forebrain in the mutant embryos (n=4) (Figure 2B). Since this projection forms the axonal scaffold for the intracerebral migration of GnRH cells [17,27], we analyzed the distribution of these cells in E14.5 and newborn mice. At E14.5, a significant accumulation of GnRH cells in the nasal compartment and concomitant decreased cell number within the brain already indicated abnormal cell migration in the mutants (n=4) (Figure 2E). In addition, while GnRH cells normally turn ventrally towards the basal forebrain, in *Nrp1*$^{sema/sema}$ embryos, many GnRH cells were found to migrate dorsally and medially towards the cortex and the thalamus, respectively, along aberrantly projecting axonal fibers (Figures 2C, S2). Incidentally, conditional mutant mice that lack Nrp1 only in GnRH cells (*GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice) displayed a normal distribution of these cells between the nose and the brain at E14.5 as well as a normal number of these cells in the adult brain (Figure S3 and data not shown), thus confirming that the defective migration we found in *Nrp1*$^{sema/sema}$ embryos is not a cell-autonomous trait. The migration defect was still conspicuous at birth (Figure 2D), a time when neuroendocrine GnRH cells have completed their migration in normal mice [3]. Brains of *Nrp1*$^{sema/sema}$ newborn mice (n=4) indeed contained 38% fewer GnRH cells, which were dispersed in the ventral forebrain, while there was a 36% increase in the number of GnRH cells detected in the rostral forebrain compared with *Nrp1*$^{+/+}$ littermates (n=5, $p<0.01$ for both comparisons) (Figure 2E). This GnRH-cell migration defect in *Nrp1*$^{sema/sema}$ animals resulted in decreased GnRH immunoreactivity in the median eminence of the hypothalamus (Figure 2D), which is the projection field of neuroendocrine GnRH cells.

Of the *Nrp1*$^{sema/sema}$ newborn mice, only four males and two females survived into adulthood. Both females had delayed pubertal activation, specifically, the first ovulation occurred more than 10 days later than in *Nrp1*$^{sema/+}$ heterozygous littermates, and monitoring of the ovarian cycle from P60 showed that one female stayed in the diestrous stage (a stage with low gonadotropin outputs) throughout the 3-week study period, while the other female had disrupted ovarian cyclicity (data not shown). Male reproductive capacity was assessed by breeding the young adult (P90) *Nrp1*$^{sema/sema}$ males with confirmed wild-type dams, and monitoring the occurrence of litters over 10-13 months. While *Nrp1*$^{sema/+}$ males (n=4) produced about one litter per month, as did *Nrp1*$^{+/+}$ males, the fertility index (number of

litters per month) was markedly reduced in the *Nrp1*$^{sema/sema}$ males, which only gave birth to 2 to 4 litters (fertility index: 0.29±0.04 *vs.* 1.08±0.12 in *Nrp1*$^{sema/+}$; Student's t-test, $p<0.001$). Moreover, neuroanatomical analysis of *Nrp1*$^{sema/sema}$ adult brains showed significantly reduced GnRH cell populations in the preoptic and hypothalamic regions (384±67 GnRH cells, n=4) compared to *Nrp1*$^{sema/+}$ littermates (767±49 GnRH cells, n=4; Student's t-test, $p<0.001$), whereas *Nrp1*$^{sema/+}$ mice did not differ from *Nrp1*$^{+/+}$ mice (701±11 GnRH cells, n=4; Student's t-test, $p>0.05$). Therefore, the GnRH cell migration defect found in *Nrp1*$^{sema/sema}$ mouse embryos was not corrected during later development, and caused subfertility in adult homozygous mutants.

SEMA3A loss-of-function mutations in Kallmann syndrome patients

The KS-like phenotype of *Nrp1*$^{sema/sema}$ mice, and that, even more pronounced, of *Sema3a*$^{-/-}$ mice [22], prompted us to ask whether insufficient Sema3A signaling through Nrp1 might also be involved in the human disorder. We sought mutations, by Sanger sequencing, in the 17 coding exons of *SEMA3A* (ID 10371) and flanking splice sites, in 386 unrelated KS patients (297 males and 89 females). All of them had confirmed hypogonadotropic hypogonadism and anosmia or hyposmia, and some already harbored a mutation in one of the five KS genes we had previously analyzed, specifically, *KAL1* (13 patients), *FGFR1* (30 patients), *FGF8* (3 patients), *PROKR2* (30 patients), or *PROK2* (12 patients). Nonsynonymous mutations in *SEMA3A* were found in 24 patients (20 males and 4 females), all in heterozygous state (Table 1). They consist of a frameshifting deletion of 14 nucleotides (c.del1613_1626; p.D538fsX31), and seven different missense mutations (p.R66W, p.N153S, p.I400V, p.V435I, p.T688A, p.R730Q, p.R733H) that affect evolutionarily conserved aminoacid residues located in different domains of the protein (Figure 3). In addition, the p.R730Q and p.R733H mutations, which both remove basic residues in the C-terminal basic motif of Sema3A, are predicted to affect in vivo proteolytic processing by furin-like endoproteases at residue R734 [28]. Notably, all the missense mutations, but not the frameshifting mutation, have been reported in the Exome Variant Server database, with allele frequencies in the European American population below 0.03% except for p.N153S (0.4%) and p.V435I (1.3%). Three of these mutations (p.R66W, p.V435I, p.R730Q) were also

detected in our sample of 386 unrelated Caucasian controls (see Table 1). We thus studied the effects of the eight mutations on the signaling activity of Sema3A using the GN11 cell line, derived from murine embryonic GnRH cells [29-31], and conditioned media from transfected COS-7 cells producing Sema3A either from the wild-type *SEMA3A* cDNA or from cDNAs harboring the mutations. We found that the conditioned medium from COS-7 cells transfected with the wild-type *SEMA3A* cDNA was as potent at inducing phosphorylation of FAK (focal adhesion kinase) and ERK1/2 (extracellular signal-regulated kinases 1 and 2) in GN11 cells as the purified recombinant human Sema3A (100 µg/L). By contrast, Sema3As harboring the N153S, I400V, T688A, or R733H missense mutations were ineffective, despite normal production and secretion of the proteins by COS-7 cells, shown by western blot analysis of the conditioned media. The R66W and V435I mutant proteins were not detected in the conditioned medium, which indicates defective secretion. Likewise, the c.del1613_1626 (p.D538fsX31) frameshifting mutation resulted in the absence of protein secretion, as expected (Figure 4). From these results, we were able to conclude that all the mutations, except p.R730Q, are loss-of-function mutations that affect the secretion or signaling activity of Sema3A, which strongly argues in favor of their pathogenic effect in the KS patients. In addition, the p.R730Q mutation may still have a pathogenic effect not detected in our experimental system, especially since this mutation is expected to impair proteolytic processing of Sema3A in vivo, as mentioned previously. Notably, the patients carrying the p.T688A and p.I400V mutations, and three patients carrying the p.V435I mutation also carry, in heterozygous state, p.Y217D, p.R268C (two patients), p.H70fsX5, and p.G687N pathogenic mutations in *KAL1*, *PROKR2*, *PROK2*, and *FGFR1*, respectively (Table 1), which further substantiates the digenic/oligogenic mode of inheritance of KS [1,2]. Based on the seemingly normal reproductive phenotype of *Sema3a*$^{+/-}$ heterozygous mice [21,22], we suggest that the monoallelic mutations in *SEMA3A* are not sufficient to induce the abnormal phenotype in the patients, but contribute to the pathogenesis of KS through synergistic effects with mutant alleles of other disease-associated genes. Accordingly, the other KS patients who carry monoallelic mutations in *SEMA3A* are also expected to carry at least one pathogenic mutation in another gene (see footnote). Although *NRP1* (ID 8829) might be viewed as one of the best candidates, we did not find a mutation within its 17 coding exons and flanking splice sites in any of these patients, nor did we in a group of 100 KS patients without *SEMA3A*

mutations, which indicates that mutations in *NRP1*, if any, are infrequent. It is also possible that some of the additional mutations affect other proteins involved in Sema3A-signaling, such as members of the plexin family of transmembrane receptors [18]. A whole-exome sequencing strategy should prove useful to explore the spectrum of genes which, when mutated, can lead to a KS phenotype in conjunction with *SEMA3A* mutations.

Note: While this article was under review, Young et al. reported the coexistence of KS and a large deletion in *SEMA3A*, in heterozygous state, in two siblings and their clinically affected father (*Hum. Reprod.*, 2012; 27:1460-1465). Our findings do not support mere autosomal dominant Mendelian inheritance in this family, and suggest that another, as yet unidentified genetic hit combines with *SEMA3A* haploinsufficiency to produce the disease phenotype.

Materials and methods

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

Animals and human fetus. All experiments on mice were carried out in accordance with Directive 86/609/EEC of the Council of the European Communities regarding the mammalian research and French bylaw. *Nrp1*$^{sema/+}$ mice (B6.129(C)-Nrp1tm1Ddg/J) [25] were purchased from the Jackson laboratory (Maine, USA), maintained on a controlled 12h:12h light cycle, provided with food and water ad libitum, and genotyped as described previously [25]. E14.5 (plug day, E0.5), P0, and adult *Nrp1*$^{+/+}$, *Nrp1*$^{sema/+}$ and *Nrp1*$^{sema/sema}$ mice were obtained and processed for immunohistofluorescence analyses as previously described [30]. In addition, homozygous *Nrp1*$^{loxP/loxP}$ mice (B6.129(SJL)-Nrp1tm2Ddg/J) [25] from the Jackson laboratory were crossed with a transgenic mouse line expressing the cre recombinase under the control of the GnRH gene promoter (*GnRH::cre* mice) [32], a gift from C. Dulac (Harvard university, Cambridge, USA) to obtain *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice that lack Nrp1 in GnRH cells only. *Nrp1*$^{loxP/loxP}$ and *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice were used for immunofluorescence analyses at E14.5 and adult stages.

The human fetus was obtained from a voluntary terminated pregnancy, with parent's written informed consent. Gestational age was established by crown-rump length measurement. The fetus was fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, for three weeks at 4°C, and then immersed in 0.1 M PBS containing 30% sucrose for two days at 4°C. The head was embedded in OCT embedding medium (Tissue-Tek®), frozen, and sagittal cryosections (20 µm thick) were taken and processed for immunohistofluorescence.

Immunohistofluorescence. Immunohistofluorescence experiments were carried out as described previously [30]. Primary antibodies were: rabbit anti-GnRH (dilution 1:3000), a gift from G. Tramu (University of Bordeaux, France); rabbit anti-peripherin (dilution 1:1000), AB1530 (Millipore); goat anti-neuropilin1 (dilution 1:400), AF566 (R & D systems); goat anti-olfactory marker protein (dilution 1:6000), a gift from F. L. Margolis (University of Maryland, Baltimore, USA).

DiI labeling of nerve fibers. Vomeronasal nerve fibers were traced anterogradely with the lipophilic fluorescent dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) as previously described [17]. After diffusion of the tracer, serial sagittal sections (100 µm thick) were taken through the forebrain, and analyzed using a LSM 710 confocal microscope (Zeiss) and the ImageJ analysis software (NIH, Bethesda, USA).

Cell cultures. COS-7 cells and GN11 cells were grown in monolayers in 5% CO_2 at 37 °C, in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 1 mM sodium pyruvate, 2 mM glutamine, 50 mM glucose and supplemented with 10% fetal bovine serum (Invitrogen), 100 µ g/ml streptomycin and 100 U/ml penicillin.

Signaling activity of wild-type and mutant Sema3A in GN11 cells. A cDNA containing the entire coding region of the human *SEMA3A* (GenBank NM_006080) was inserted into a pRK5 plasmid expression vector. Recombinant plasmids containing *SEMA3A* cDNAs harboring each of the eight mutations identified in the KS patients were then engineered using the QuickChange mutagenesis protocol (Stratagene). COS-7 cells were transiently transfected using a fast-forward protocol (Lipofectamine 2000, Invitrogen) [30]. Conditioned medium was collected 48 h after transfection, tested for the presence of Sema3A by western blot analysis using an anti-Sema3A antibody (Santa Cruz, sc-

10720, dilution 1:100), and then processed for signaling activity experiments in the GN11 cell line. Briefly, subconfluent GN11 cells were grown overnight in serum-free medium, and then stimulated for 20 min with human recombinant Sema3A (R&D systems) at 100 µg/L, or with the concentrated conditioned media from transfected COS-7 cells. Western blot experiments [30] were carried out on cell lysates using antibodies to P-ERK (#9101L) and ERK (#9102L) from Cell Signaling (dilution 1:1000), or P-FAK (sc56901) and FAK (sc81493) from Santa Cruz (dilution 1:500).

DNA sequencing. Informed consent was obtained from all individuals analyzed. Genomic DNAs were prepared from white blood cells using a standard procedure. Each of the *SEMA3A* and *NRP1* coding exons and flanking splice sites was PCR-amplified from genomic DNA using a specific primer pair (see Tables S1 and S2 for primer sequences), and sequenced using either PCR oligonucleotide as sequencing primer. The mutations were confirmed by sequencing two independent PCR products on both DNA strands. Exons 2, 5, 11, 14, and 17 of *SEMA3A*, which harbor the mutations identified in some patients, were analyzed by denaturing high performance liquid chromatography (DHPLC) scanning on an automated HPLC instrument (Wave technology) in 386 unrelated Caucasian controls, followed by Sanger sequencing of the exon in case of abnormal DHPLC profile.

Acknowledgments

We thank the patients and family members for their contribution to the study. We thank Julien Devassine (Animal facility, IFR114) for the maintenance of transgenic mouse lines, Meryem Tardivel (Imaging Core Facility, IFR114) and Julie Sarfati (Inserm U1016) for technical assistance, and the Centre National de Séquençage (Evry, France) for sequencing facilities.

Author contributions

PG, JPH, VP, CD conceived and designed the experiments. NKH, PG, PL, JP, CE, CF, CL, SB, C. Campagne, CV, FC, C. Cruaud, VM, CD carried out the experiments and analyzed the data. AGP, DD, CCR, KG, CM, GC, MP, JY contributed materials/analysis tools. PG, JPH, VP, CD wrote the article.

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Legends to figures

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

(A) Schematic representation of the head of a mouse embryo at E14.5, showing the scaffold of vomeronasal/terminal nerve fibers (in red) along which GnRH cells (in blue) migrate from the nose to the brain. Several areas along this migratory path have been shown to produce Sema3A, including the frontonasal mesenchyme and the olfactory bulb region [21,33]**.** Boxes indicate the locations of the sagittal sections shown in (B) and (C). Abbreviations: oe, olfactory epithelium; vno, vomeronasal organ; nm, frontonasal mesenchyme; mob, main olfactory bulb; aob, accessory olfactory bulb; vfb, ventral forebrain; 3V, third ventricle. (B) Sagittal section of the frontonasal region in an E14.5 mouse embryo. In the frontonasal mesenchyme (nm), migrating GnRH-immunoreactive cells (green) are morphologically associated with Nrp1-immunoreactive nerve fibers (red) originating in the vomeronasal organ (vno). Single plane confocal images at higher magnification (insets) show that GnRH cells are Nrp1-immunoreactive (green+red = yellow staining). (C) Sagittal section of the ventral forebrain (vfb) in an E14.5 mouse embryo. The peripherin-immunoreactive (green) fibers of the caudal branch of the vomeronasal nerve (arrows) are also Nrp1-immunoreactive (red), as shown by their yellow staining (green+red). (D) Sagittal section of the olfactory epithelium (oe) and olfactory bulb (ob) regions (left panel) and detail of the frontonasal region (right panel) in a 9 week-old human fetus. Clusters of GnRH-immunoreactive cells (green, arrowheads) are visible in the frontonasal mesenchyme (nm) and the rostral forebrain (fb). In the frontonasal region, these cells migrate in close contact with Nrp1-immunoreactive axons (red). Note that migrating GnRH cells are also Nrp1-immunoreactive, as shown by their yellow staining (green+red) in the right panel (arrows). Scale bars: 100 µm (25 µm in insets).

Figure 2. **Defects in olfactory and vomeronasal axons, and GnRH cell migration in *Nrp1*$^{sema/sema}$ mutant mice**

(A) Coronal sections of the right olfactory epithelium (oe) and olfactory bulb (ob) regions (left panels), and detail of the olfactory bulb showing the olfactory nerve layer (nl) and glomerular layer (gl) (right panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ newborn (P0) mice. Axons of the olfactory receptor neurons were immunostained (red) using an antibody directed against the olfactory marker protein (OMP). In the *Nrp1*$^{sema/sema}$ mouse, the immunostaining is both enlarged below the olfactory bulb ventro-medial aspect (asterisks) and markedly reduced in the glomerular layer (arrowheads) compared to wild-type. (B) Sagittal sections of the rostral and ventral forebrain regions (left panels), and detail of the caudal branch of the vomeronasal nerve (right panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ E14.5 mouse embryos. A crystal of the DiI lipophilic fluorescent dye has been placed in the vomeronasal organ lumen to anterogradely label vomeronasal axons. The vomeronasal nerve extends across the medial aspect of the olfactory bulb and projects both dorsally, to the accessory olfactory bulb, and caudally, to the

ventral forebrain (vfb). In the mutant mouse, fibers in the caudal branch are scarce compared to wild-type. (C) Sagittal sections of the rostral and ventral forebrain regions at E14.5, immunostained for GnRH (green). Note the abnormal distribution of GnRH-immunoreactive cells in the *Nrp1*$^{sema/sema}$ mouse (arrows). (D) Coronal sections of the preoptic region (upper panels) showing GnRH neuroendocrine cells (green) and their projections in the median eminence (me, arrows) (lower panels) in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ newborn (P0) mice. The immunostaining is reduced in the *Nrp1*$^{sema/sema}$ mouse. (E) Quantitative analysis (mean ± s.d.) of GnRH cell distributions in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ mice at E14.5 and P0. * and ** denote statistically significant differences between genotypes in the indicated head regions (two-way ANOVA followed by Tukey's range test) with $p<0.05$ and $p<0.01$, respectively. Note that the total numbers of GnRH cells are not statistically different between *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ mice at E14.5 or P0 (Student's t-test, $p>0.05$). Other abbreviations: cx, cerebral cortex; ovlt, organum vasculosum of lamina terminalis; 3v, third ventricle. Scale bars: 100 µm (50 µm in inset).

Figure 3. Diagram of Sema3A with the mutations found in Kallmann syndrome patients

Sequence chromatograms of the mutations are shown together with the positions of the corresponding aminoacid residues in the protein domains. Abbreviations: sema, semaphorin; PSI, plexin/semaphorin/integrin; Ig, immunoglobulin-like; C, cysteine residue involved in Sema3A dimerization (interchain disulfide bond) [18].

Figure 4. Defective secretion or signaling activity of Sema3A proteins harboring the mutations identified in Kallmann syndrome patients

(A) Western blot analysis of conditioned media from transfected COS-7 cells producing wild-type (WT) or mutated Sema3A proteins. The p.D538fsX31 frameshifting mutation, and the p.V435I and p.R66W missense mutations result in the absence of a secreted protein. (B) Upper panels: Representative western blots for the phosphorylated and total forms of FAK (left panel) and ERK1/2 (right panel) in GN11 cells following a 20 min incubation with serum-free medium (negative control), 100 ng/ml of purified recombinant human Sema3A, or the conditioned media from transfected COS-7 cells producing wild-type or mutated Sema3A proteins. Lower panels: Bar graphs illustrate the mean ratio (± s.d.) of the western blot signal intensity obtained for phosphorylated FAK (P-FAK) or ERK1/2 (P-ERK1/2) to that of total FAK or ERK1/2, respectively. Each experiment was carried out three times independently. * denotes statistically significant difference with wild-type Sema3A (one-way ANOVA followed by Fisher's LSD test, $p<0.05$). a.u.: arbitrary units (pixel density).

Supporting information

Figure S1. The anti-neuropilin1 (Nrp1) polyclonal antibody AF566 (R & D systems) selectively recognizes the semaphorin-binding domain of the protein. Top panel: western blot analysis of Nrp1 in protein extracts from the hypothalamus of *Nrp1*$^{+/+}$, *Nrp1*$^{sema/+}$ and *Nrp1*$^{sema/sema}$ mice (antibody used at 1:1000 dilution). Bottom panel: immunohistofluorescence analysis of Nrp1 in the median eminence of *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ newborn mice (antibody used at 1:400 dilution). Scale bar: 200 μm.

Figure S2. Many GnRH cells migrate along ectopic nerve fibers in the brain of *Nrp1*$^{sema/sema}$ mutant mice. Immunohistofluorescence analysis of sagittal sections of the rostral and ventral forebrain regions in *Nrp1*$^{+/+}$ and *Nrp1*$^{sema/sema}$ mice at E14.5, with anti-GnRH (green) and anti-peripherin (red) antibodies. Insets show detailed views of the normal and the aberrant GnRH cell migratory pathway in the wild-type and the mutant mouse, respectively. In both cases, migrating GnRH cells appear to follow peripherin-immunoreactive axonal fibers (arrows). Abbreviations: cx, cortex; nm, frontonasal mesenchyme; ob, olfactory bulb; vfb**,** ventral forebrain. Scale bar: 50 μm (20 μm in insets).

Figure S3. GnRH cell migration is not affected in *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ conditional knockout mice that lack Nrp1 only in GnRH cells. (A) Immunohistofluorescence analysis of the frontonasal region (sagittal sections, single plane confocal microscopy images) in *Nrp1*$^{loxP/loxP}$ and *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice at E14.5, with anti-GnRH (green) and anti-Nrp1 (red) antibodies. As expected, Nrp1 immunoreactivity of the GnRH cells (yellow) is detected in the *Nrp1*$^{loxP/loxP}$ mouse, but not in the *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mouse. Abbreviations: ob, olfactory bulb; fb, forebrain. Scale bar: 50 μm (20 μm in insets). (B) *Nrp1*$^{loxP/loxP}$ and *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice display similar distributions of GnRH cells between the nose and the brain at E14.5 (Kruskal-Wallis test, $p>0.05$).

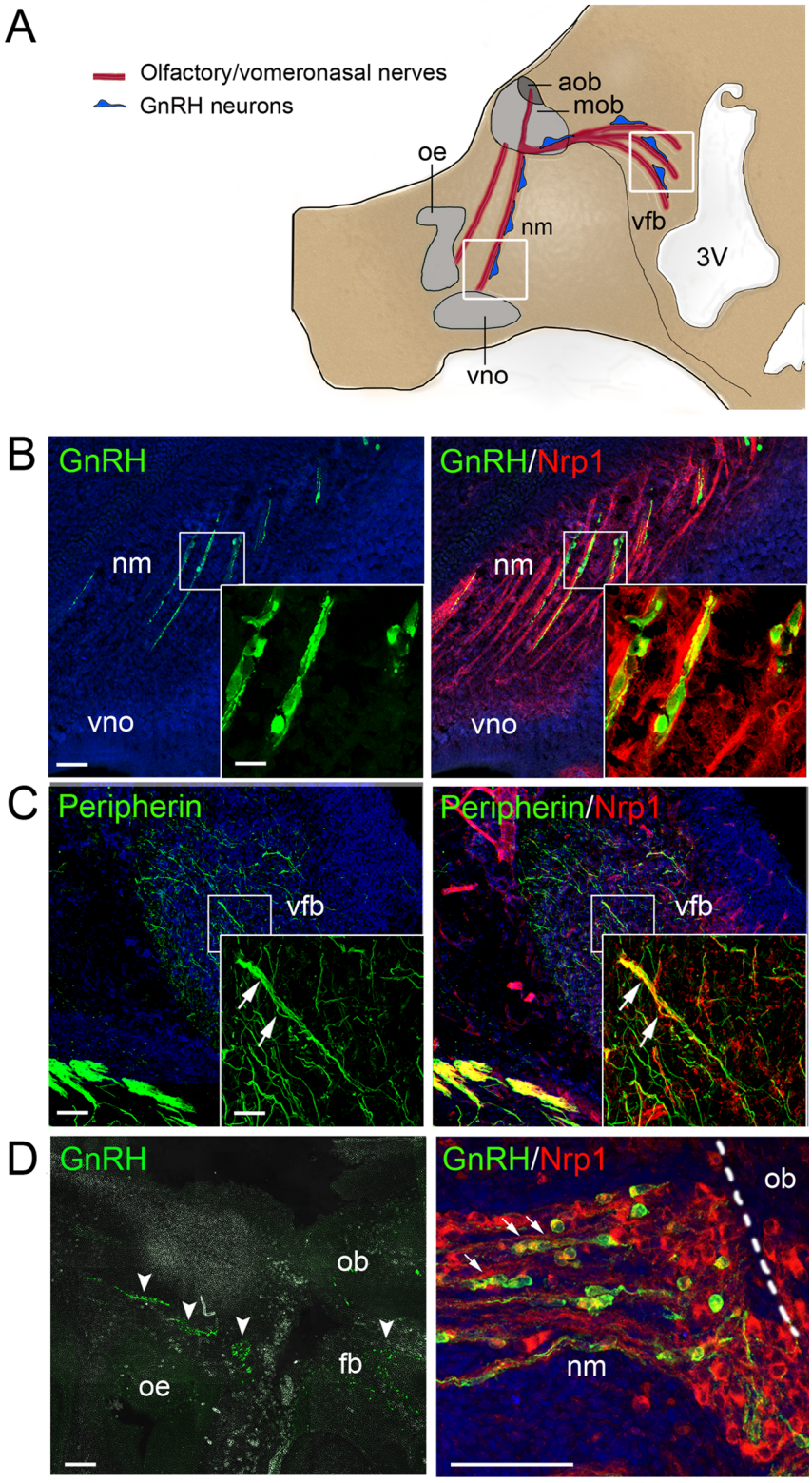
Table S1. *SEMA3A* sequencing primers

Table S2. *NRP1* sequencing primers

Nucleotide change	Exon	Aminoacid change	Protein domain	Allele frequency in control subjects	gender (M/F) of patients	Additional mutation in the patient	
c.197C>T	2	p.R66W	sema	1/772	M		
c.458A>G	5	p.N153S	"	0/772	2M		
c.1198A>G	11	p.I400V	"	0/772	M	*PROKR2*	p.R268C
c.1303G>A	"	p.V435I	"	13/772	11M+2F		
"	"	"	"	"	M	*PROKR2*	p.R268C
"	"	"	"	"	F	*PROK2*	p.H70fsX5
"	"	"	"	"	M	*FGFR1*	p.G687R
c.del1613_1626	14	p.D538fsX31	PSI	0/772	M		
c.2062A>G	17	p.T688A	interdomain	0/772	M	*KAL1*	p.Y217D
c.2189G>A	"	p.R730Q	basic motif	1/772	F		
c.2198G>A	"	p.R733H	"	0/772	M		

Table 1. *SEMA3A* mutations identified in Kallmann syndrome patients

Figure 1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GnRH neurons
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3V
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B
GnRH
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GnRH/Nrp1
nm
vno
C
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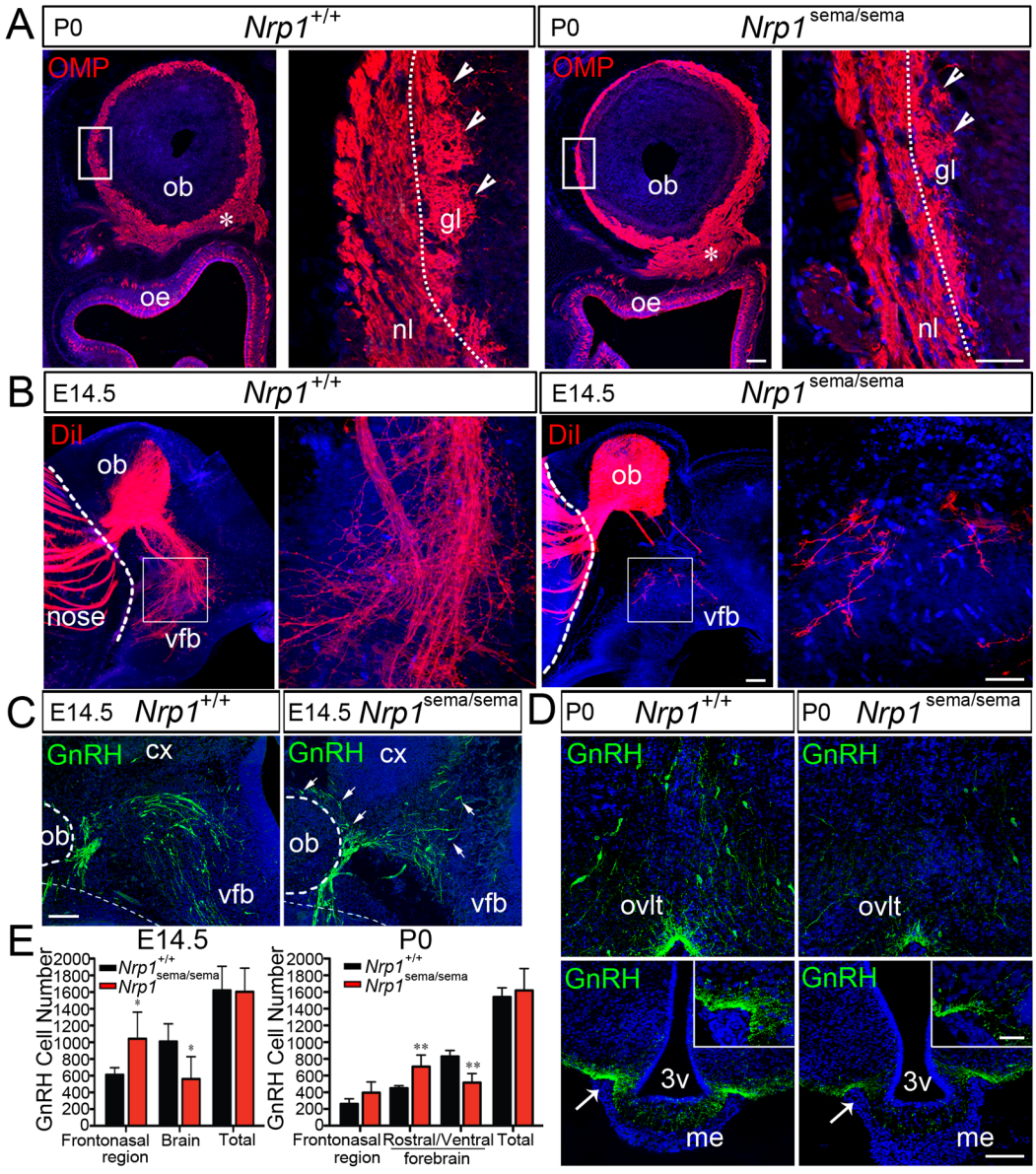
GnRH/Nrp1
ob
nm

Figure 2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DiI
ob
nose
vfb
C
E14.5 Nrp1+/+
E14.5 Nrp1sema/sema
GnRH
cx
ob
vfb
D
P0 Nrp1+/+
P0 Nrp1sema/sema
GnRH
ovlt
3v
me
E
E14.5
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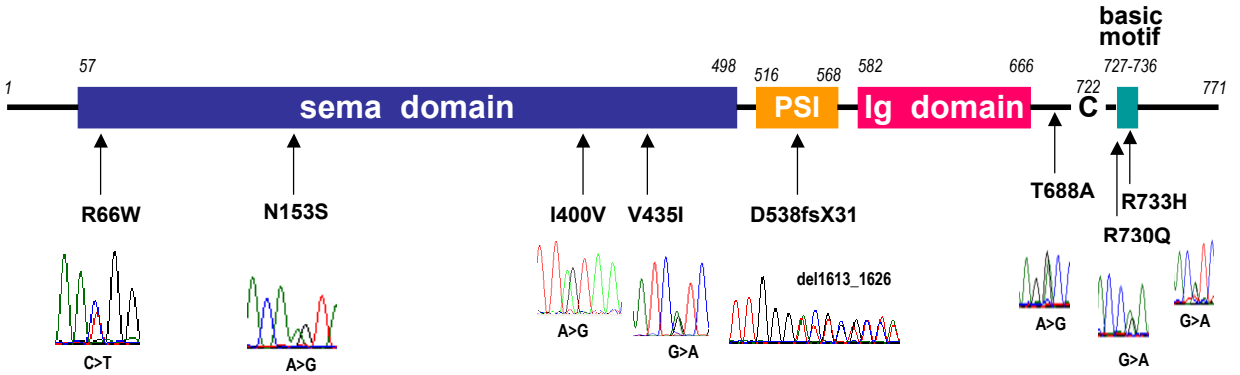
Nrp1sema/sema
GnRH Cell Number
Frontonasal region
Brain
Total
Rostral/Ventral forebrain

Figure 3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V435I
D538fsX31
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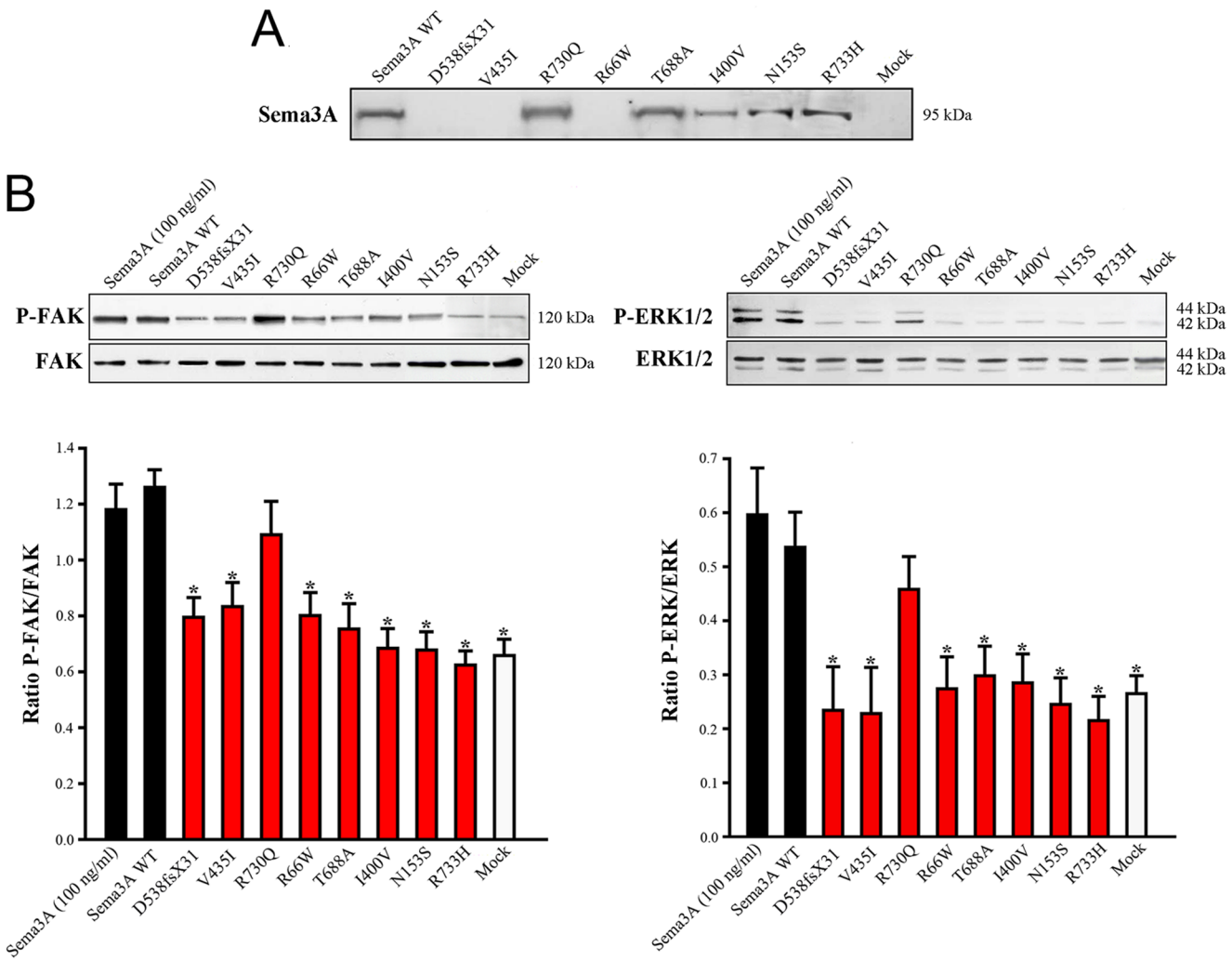
Figure 4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95 kDa
B
Sema3A (100 ng/ml)
Sema3A WT
D538fsX31
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R66W
T688A
I400V
N153S
R733H
Mock
P-FAK
FAK
120 kDa
120 kDa
P-ERK1/2
ERK1/2
44 kDa
42 kDa
44 kDa
42 kDa
Ratio P-FAK/FAK
Ratio P-ERK/ERK

Sustained Alterations of hypothalamic tanycytes during post traumatic hypopituitarism in male mice

Abbreviated title. Hypothalamic glial cells remodeling after CCI

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Section: Neuroendocrinology

Word count: 4697

Disclosure information: G.O., T.E., N.R., D.C., F.L., N.Co., A.G., M.S., N.Ch., C.V, E.G., B.D., I.R., V.P., P.M., N.P., PF.M. have nothing to declare.

ABSTRACT (256 words)

Traumatic brain injury is a leading cause of hypopituitarism, which compromises patients' recovery, quality of life and life span. To date there are no means other than standardized animal studies to provide insights into the mechanisms of post-traumatic hypopituitarism. We have found that growth hormone (GH) levels were impaired after inducing a controlled cortical impact (CCI) in mice. Furthermore, GH releasing hormone (GHRH) stimulation enhanced GH to lower level in injured than in control or sham mice. Since many characteristics were unchanged in the pituitary glands of CCI mice, we looked for changes at the hypothalamic level. Hypertrophied astrocytes were seen both within the arcuate nucleus and the median eminence, two pivotal structures of the GH axis, spatially remote to the injury site. In the arcuate nucleus, GHRH neurons were unaltered. In the median eminence, injured mice exhibited unexpected alterations. First, the distributions of claudin-1 and ZO-1 between tanycytes were disorganized, suggesting tight junction disruptions. Second, endogenous IgG were increased in the vicinity of the third ventricle, suggesting abnormal barrier properties after CCI. Third, icv. injection of a fluorescent-dextran derivative, highly stained the hypothalamic parenchyma only after CCI, demonstrating an increased permeability of the third ventricle edges. This alteration of the third ventricle might jeopardize the communication between the hypothalamus and the pituitary gland. In conclusion, the phenotype of CCI mice had similarities to the post traumatic hypopituitarism seen in humans with intact pituitary gland and pituitary stalk. It is the first report of a pathological status where tanycytes dysfunctions appear as a major acquired syndrome.

Keywords: growth hormone, hypothalamus, pituitary gland, neuropeptides, action potential, astrocyte, tanycyte, neuroinflammation, aging, neuroendocrinology

INTRODUCTION (445 words)

Traumatic brain injury (TBI) triggers deficits with varying degrees in onset, severity and outcome [1]. There is a relationship between the severity of the trauma, survival rates and release times from the emergency unit [2,3]. In rodents also, the severity of the trauma dictates the extent of the brain damages and of the behavioral deficits [4]. The underlying processes are studied at the molecular level in these models [1,5-7].

Efforts were made in understanding the chronic phase of TBI. Long lasting alterations in white and grey matters, changes in astroglial phenotypes, down-regulation of synaptic markers [8-10] were targeted by therapies, but these efforts were unsuccessful. Non-neural mechanisms also participate in post-traumatic deficits, and some brain injuries induce cognitive impairments in rodents lacking anatomical lesions [11]. Accordingly, endocrine dysfunctions are known consequences of TBI in patients, and endocrine axis remain impaired when assayed up to 5 years after the injury [12,13]. The risk of chronic hormonal shortage correlates with the severity of the trauma. Importantly, while pituitary functions generally improve with time, new hormonal deficiencies are often found in patients [12]. GH deficiency is the most common chronic deficiency at 1 and 3 years after TBI (mean of 23% patients [12]). This is striking given the neuroprotective role of GH and/or peripheral GH-induced IGF-I [14-16], and the negative relationship between GH shortage and outcome from TBI [17]. It would be valuable to cure post-traumatic GH shortage, a cause of frailty and poor quality of life in patients [12,18]. At the anatomical level, some, but not all patients show pituitary stalk and/or gland lesions, and hypothalamic lesions can be found in the absence of pituitary sequelae [12,19-21]. Previous experimental data show that the hypothalamic levels of inflammatory markers increase during post traumatic GH shortage induced by controlled cortical impacts (CCI) in rats [22]. Yet, the description of these central changes, and their relationship with the activity of the GH axis, awaits investigations. What are the structures involved: the hypothalamus containing neuroendocrine cell bodies; the median eminence containing neuroendocrine nerve endings; or the pituitary gland? How might these structures, remote from injury sites, be involved in post-traumatic alterations? What are

1 the cells involved: the neuroendocrine neurons, the neighboring neural cells (astrocytes, microglial
2 cells, tanycytes), or the endocrine cells?

3 Standardized animal models are essential in understanding the links between the detrimental clinical
4 outcomes of TBI and some cellular dysfunctions, including hormonal secretions. Therefore, we have
5 examined the post-traumatic consequences of CCI in mice. We are reporting that the barrier properties
6 of the tanycytes undergo delayed alterations in mice. This was associated with impaired GH serum
7 levels. These findings seemed relevant to human clinical cases where post traumatic hypopituitarism is
8 not associated with alterations in pituitary gland and pituitary stalk.

MATERIALS & METHODS (600 words)

Male mice, housed under constant temperature (22-24°C) and humidity (60%), had free access to food and water. Studies, complying with the animal welfare guidelines of the EC, were approved by the Direction of Veterinary departments of Herault and the Languedoc Roussillon Institutional Animal Care and Use Committee (#CEEA-LR-11026).

Traumatic brain injury protocol

Anaesthesia and surgery. Controlled cortical impact (CCI) was induced as previously described [23] on adult mice (10-12 weeks) anaesthetized with isoflurane (see Supplemental Methods).

Hormonal assays.

In vivo blood sampling. As previously described [25], mice were anaesthetized by inhalation of isoflurane (1.5% in O_2). A catheter was inserted into the jugular vein and 50 µL blood was sampled before and after 5 and 15 min of an injection, via the same catheter, of 1µg hGHRH 1–29NH_2 (Bachem AG, Switzerland) diluted in vehicle buffer (NaCl 0.9%, heparin 20 unit/mL, and BSA 0.05%). In some experiments, 50 µL blood was collected for prolactin, LH assays before the first GH sample. Blood samples were spun down at 1600g (4°C, 10min) and plasma was stored at −20°C until assay.

Hormonal detection. Plasmatic mouse GH and mouse prolactin were assayed by RIA using reagents kindly provided by Dr. Parlow and the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD, USA) as previously described [26-28]. ELISA methods for pituitary GH assay [29] and plasmatic mouse LH detection [30] were described previously.

Real-time RT qPCR.

Pituitary glands and hypothalami were dissected from terminally anaesthetized mice, and processed as previously described [31-32] (Supplemental Methods).

Histology and immunocytochemistry

The basic procedures for immunofluorescence, morphometry, fenestrated capillary loops counts, and tight junction organization have been described previously [33-36] (Supplemental Methods).

In vivo tissue stainings

Intracerebroventricular (icv.) delivery. Mice were deeply anaesthezised with a mixture of ketamine (80µg/g) and xylazine (10µg/g), given ip in saline, and were placed in a stereotaxic frame. The injection into the left lateral ventricle was processed as described [37]. One µl of sterile physiological saline containing 12.5mg.ml^{-1} tetramethylrhodamine-conjugated dextran (3000MW, lysine fixable, Molecular Probes) was injected during 2min, and the injecting needle removed within 1min. Sacrifice of the mice by decapitation occurred 5min after the start of the icv. injection, and the brain was immersed into PFA 4% within 2 additional min. Brain slices (175 µm-thick) were then prepared for two-photon imaging, performed with a Carl-Zeiss 7MP 2-photon microscope with a Carl-Zeiss W Plan Apo x20, 1.0 NA objective. Multiphoton excitation was achieved using a Ti:Sapphire Chameleon Laser (Coherent) tuned to 880nm. Emitted fluorescence was recorded from 610 to 700nm.

In vivo blood vessels staining. Blood vessels within the lateral lobe of the pituitary gland and within the median eminence were visualized using a modification of the method of Budry et al. [38]. In brief, terminally anesthetized mice were perfused via the heart with 3ml of PBS at 37°C followed by 6ml of a solution of fluorecein isothiocyanate conjugated gelatin (6%) in PBS at a velocity of 1.5ml/min. Tissues were then prepared for two-photon imaging, performed as described above, except that the emitted fluorescence was recorded from 500 to 550nm for the FITC signal.

Electrophysiology

Slice preparation for electrophysiological recordings, patch-clamp recordings of adult male GHRH-GFP mice and data analysis were performed as described previously [24,34] (Supplemental Methods).

Statistics. Data were expressed as mean ± standard-error-of-the-mean (sem). For electrophysiological experiments, n is the number of neurons. Statistical tests were performed with Prism (GraphPad software, La Jolla, CA). Non parametric statistical tests and ANOVA were used according to data sets, as indicated. Multiple comparisons tests were selected when series of data were >2. Statistical difference between groups was assumed when $p<0.05$.

RESULTS (2085 words)

Seven days after a unilateral parietal impact, the brains of CCI mice exhibited cortical lesions (1.7 ± 0.8 mm^3, n=6) and contusions (14.2 ± 3.0 mm^3, n=6) which expanded outside the cortex and included subcortical areas (see Supplemental Figure S1; [23]). The impact site was characterized by hypertrophied astrocytes, activation of microglial cells, elevated immunostaining for endogenous IgG, infiltration of peripheral bone marrow derived cells and increased NG-2 immunostaining (data not shown). After 30 days, cortical lesions and contusions were still evident, (1.0 ± 0.4 and 11.3 ± 1.4 mm^3 respectively, n=6) with strong alterations in blood supply and the apposition of an astrocytic scar (Supplemental Figure S1). Thus, this protocol induced a severe traumatic injury [23].

In vivo GH secretion.

GH secretion is impaired in a long lasting manner in humans after TBI [12], and in rats after CCI [22]. The basal circulating GH levels were similar in control (4.8 ± 0.7 ng/ml, n=21, Figure 1A) and sham mice 7 days (6.9 ± 0.6 ng/ml, n=11, Figure 1A) and 30 days after surgery (data not shown). GH levels were also in the same ranges of values in mice, early, 7 days after CCI (3.7 ± 0.7 ng/ml, n=39). In contrast, basal GH levels were strongly lowered 30 days after CCI (0.33 ± 0.03 ng/ml, n=17; $p<0.001$ vs control). Since GH deficiency is diagnosed by stimulation testing in humans [12], GH levels were investigated after injection of growth hormone releasing hormone (GHRH) in anesthetized mice [18,25]. GHRH stimulated GH levels were similar in control (64.6 ± 8.8 ng/ml; $p<0.001$ vs basal) and sham mice (68.5 ± 17 ng/ml; $p<0.001$ vs basal), while they were lower in CCI mice, early (32.1 ± 6.1 ng/ml; $p<0.001$ vs basal; $p<0.005$ vs. control) as well as late (24.5 ± 4.2 ng/ml, $p<0.005$ vs basal; $p<0.01$ vs control) after injury.

Since GH deficiency is defined according to a cut-off value in humans [12,39], plasma GH levels were classified according to three ranges of concentrations in mice (Figure 1B). In control mice, the spontaneous GH levels almost equally distributed between a lower [0.3-3ng/ml] and an intermediate [3-30ng/ml] ranges. This distribution was very similar 7 days after CCI, but was totally shifted in the lower category of values 30 days after CCI. This shift was not seen in sham mice. Upon stimulation

with GHRH, GH levels were distributed in intermediate (about 30%) and high (about 70%) concentrations ranges in control, as well as in sham mice. Distributions of GH levels were somewhat opposite in CCI mice were ~60% of the samples were in the low ranges of concentrations, and ~15% in the lowest [0.3-3ng/ml] range. The status of other pituitary hormones was then examined 30 days after surgery. Strikingly, the prolactin and the LH levels (Figure 1C,D) were slightly enhanced in CCI mice, at variance with the changes in GH. The hypopituitarism seemed selective of the GH axis. In the following experiments, we focused on the long lasting changes of the GH axis, 30 days after the CCI.

The pituitary gland level of the GH axis.

Pituitary dysfunction often underlies GH deficiency [18]. However, GH contents of pituitary glands were similar in control (51.5±7.1µg, n=8), sham (65.3±2.8µg, n=9) and CCI mice (79.7±14.7µg, n=8; Figure 2A). Furthermore, pituitary contents of GH mRNA were identical in control, sham and CCI mice (Figure 2B). Accordingly, the expression of the GHRH receptor, which drives GH secretion and expression of the main hypothalamic GH regulatory factors [18], was not changed after trauma in mice (Figure 2C). Next, the involvement of SST, the main hypothalamic inhibitory factor [18], was investigated. The expressions of sst1, stt2A, sst2B and sst5, the pituitary SST receptors of the GH axis, were not modified by CCI in mice (Figure 2C,D). Since GH secretion is not only shaped by GH cells but also by pituitary blood supply [25], the vascularization of pituitary gland was visualized in mice after intracardiac perfusion with the fluorescent gelatin-FITC derivative (Figure 2E). The compound filled a network of large and small diameter blood vessels which was similar in control (n=6), sham (n=3) and CCI mice (n=3). In the present study, pituitary glands, as well as pituitary stalks, never showed signs of anatomical alteration, lesion or infarction (data not shown). Altogether, these results suggested that the pituitary gland was not the primary injury site of the GH axis after CCI.

The hypothalamus is durably modified after CCI.

It is well-known that TBI can induce hypothalamic lesions in patients [12,13,21], and that CCI in rats triggered an increase in hypothalamic GFAP [22]. Thus, the distribution of hypothalamic GFAP was examined after CCI in mice. As shown in Figure 3A, GFAP immunolabeling was homogeneous in hypothalami from control and sham mice. The distribution of GFAP was much higher 30 days after CCI in the vicinity of the third ventricle and within the arcuate nucleus. On average (Figure 3B), the GFAP immunoreactive volume of the median eminences amounted 0.038 ± 0.001 mm^3 in control mice (n=8), and this was very similar in sham mice (0.048 ± 0.001 mm^3, n=8). However, it was 0.082 ± 0.004 mm^3 (n=9) 30 days after CCI, almost twice as large as in control and sham ($p<0.05$). Thirty days after CCI, there was also a ~50% increase in the number of GFAP-positive cell bodies (control: 270 ± 10, n=8; sham: 221 ± 9, n=8; CCI: 384 ± 12, n=9, $p<0.05$ vs control, $p<0.05$ vs sham). In an upper periventricular region, long cellular processes exhibited both vimentin and GFAP immunolabelings (Supplemental Figure S2). In contrast, GFAP only marginally colocalized with vimentin within the median eminence and within the bulk of the arcuate nucleus (Supplemental Figure S2). Altogether, GFAP seemed to originate from astrocytes [5,35] and not from tanycytes [35,40] at the ventral side of the third ventricle. The lateral subtypes of tanycytes, however, seemed to strongly induce the expression of GFAP. Thus, CCI triggered an activation of glial cells in the vicinity of GHRH cell bodies and nerve terminals.

Properties of GHRH neurons at the arcuate nucleus.

Glial cells participate in neuronal activity [41], and the activation of astrocytes impairs neuronal activity [5,42,43]. The electrical activity of GHRH neurons, which trigger GH release, was then studied with the patch-clamp technique. First, their action potential properties were not different in sham and CCI mice (Supplemental Figure S3). Second, their spontaneous electrical activity was recorded in brain slices from sham and CCI mice, 30 days after surgical procedures (Figure 4A&B). Both neurons exhibited irregular firing rates in the 1Hz range (like in GHRH neurons from control mice [44]). The instantaneous frequencies of the action potentials of each GHRH neuron were plotted as cumulated distributions [33], and were averaged in either sham or CCI groups (Figure 4C). The

mean distributions of action potentials frequencies were not altered by CCI, 30 days after surgery, their mean mid-points being 1.6±0.4 Hz (sham, n=23) and 1.1±0.3 Hz (CCI, n=14). The activity of each GHRH neuron was also characterized by the skewness of the action potential frequencies [44]. On average (Figure 4D), this index of clustering was not different in GHRH neurons from sham and CCI mice, 30 days after surgery (respectively, 1.3 ± 0.4 and 0.9 ± 0.3). In conclusion, the presence of reactive astrocytes did not impair the activity of GHRH neurons. Furthermore, the number of GHRH neurons was similar in sham and CCI mice (respectively, 1743±83, n=4 and 1501±171, n=4) ruling out a neurotoxic effect of reactive glial cells. Since the expression of GHRH was also unchanged by CCI in mice (Supplemental Figure S4), it seemed that CCI did not impair GHRH neurons at the cell body level. Note that the expression of the other major hypothalamic GH regulating neuropeptide, SST, was also unchanged by CCI in mice (Supplemental Figure S4).

Morphological properties at the median eminence level of the GH axis.

The presence of reactive astrocytes in the the median eminence of CCI mice suggested a possible alteration of the barrier properties at this level. Indeed, astrocytes can build up a scar, between an intact parenchyma and the injury site, which restricts the detrimental consequences of blood vessels disruptions [5]. This issue was first examined by immunofluorescence. In the Figure 5A, an anti-claudin-1 antibody labeled a network of tight junctions between tanycytes and an anti-MECA 32 antibody decorated the fenestrated capillaries [35]. While there was no difference in MECA-32 labeling between the sham and CCI mouse there was a clear loss of claudin-1 immunostaining in the CCI mouse as compared to sham (see also Mullier et al. [35] for control). On average, the number of MECA 32-postive fenestrated capillaries were similar in control (n=4), sham (n=4) and CCI (n=4) mice, 30 days after surgery (Figure 5B). Furthermore, this capillary network labeled by intracardiac perfusion with gelatin-conjugated fluorescein-isothiocyanate [25,45], was preserved in the median eminence after CCI, as compared to control and sham mice (Supplemental Figure S5). In contrast, the alteration in Claudin-1 staining suggested that CCI selectively modified tanycytes at the median eminence. The distribution of Zonula Occludens-1 (ZO-1), a more general marker of tight junctions

[35], was also strongly modified in CCI mice both at the ventral side of the third ventricle (Figure 5C) and at its lateral side lining the arcuate nucleus (Figure 5D). The mean density of ZO-1 in tanycyte cell bodies lining the median eminence in CCI mice (n=4) was lower than those of control (n=4, difference of means=20.2, $p<0.05$) and sham (n=4, difference of means=18.7, $p<0.05$), 30 days after surgery (Figure 5E). Similarly in tanycytes lining the arcuate nucleus (Figure 5F), the mean density of ZO-1 was significantly altered in CCI mice (n=4) as compared with control difference of means=13, $p<0.05$) and sham (n=4, difference of means=12.6, $p<0.05$). The density of occludin, another protein of the tight junctions of the tanycytes [35] was also lowered in CCI mice (data not shown). Collectively, these data established that the morphology of the epithelial layer of tanycytes was severely modified in a chronic manner after CCI.

Barrier properties at the median eminence level.

The functional consequences of the above morphological changes have never been studied. Thus, the parenchymal distribution of IgG, a major protein of the cerebrospinal fluid, was examined (Figure 6A). IgG were trapped within the hypothalamic parenchyma of a control mouse in a narrow band consisting in part of the median eminence and the borders of the third ventricle [46]. While the thickness of this IgG immunopositive area was not different in a sham mouse, it was much wider in late CCI mouse. On average (Figure 6C), the IgG immunopositive volume in control (0.015 ± 0.008 mm^3/hypothalamus, n=5) and sham mice (0.017 ± 0.001 mm^3/hypothalamus, n=5) were significantly more compact than in the CCI mice (0.031 ± 0.002 mm^3/hypothalamus, n=5; $p<0.05$ vs control or sham). This phenomenon was also observed in early CCI mice when compared to controls and shams ($p<0.05$ for both comparisons, Figure 6B). This enrichment in parenchymal IgG in CCI mice was primarily due to its presence in more lateral areas (as shown in Figure 6), rather than to its appearance in the rostro-caudal direction. Nevertheless, in late CCI mice, IgG was present in up to two additional rostral sections (which were included in the analysis). This alteration of the parenchymal distributions of a large molecule, IgG, further suggested that the morphological changes at the tanycytic barrier influenced the permeability of the ventral side of the third ventricle. The dynamic properties of the

tanycytic layer were then investigated using icv injections of a lysine-fixable tetramethylrhodamine-conjugated dextran. In the coronal sections of the control and the sham mice shown in Figure 6D, the injection of the dye into the lateral ventricle did not increase the fluorescence signal within the hypothalamic parenchyma. In contrast, the dye accumulated in cell bodies within the arcuate nucleus and diffused within the median eminence in CCI mice. On average (Figure 6E), the maximal lateral diffusion of the dextran was much larger in the arcuate nucleus from late CCI mice (285.9 ± 61.1 µm, n=6) than in those of control and sham mice (respectively, 23.1 ± 23.1 µm, n=4 and 90.3 ± 46.6 µm, n=7, both $p<0.05$ vs late CCI). In addition (Figure 6F), the mean surface labeled by the dye in late CCI mice (9819.8 ± 2187.6 μm^2, n=6) was increased as compared to control and sham mice (respectively, 1598.5 ± 1598.5 μm^2, n=4 and 2213.7 ± 1208.1 μm^2, n=7, both $p<0.05$ vs late CCI). Thus, there was a profound alteration of the permeability at the ventral side of the third ventricle.

DISCUSSION (1369 words)

Our data established that the barrier properties of tanycytes were compromised in mice subjected to cortical injury, an unexpected and new mechanism of post traumatic alteration. The interface between the parvocellular hypothalamus and the pituitary gland, including the median eminence, was clearly disorganized when circulating GH levels were severely diminished. The hypopituitarism found in this animal model of traumatic brain injury had similarities to human cases of post traumatic hypopituitarism with intact pituitary gland and stalk.

Plasma levels of GH in control and sham mice showed a large range of concentrations, known to mirror the pulsatile pattern of GH secretion [18,47]. This distribution was completely flattened in CCI mice, where GH levels were barely detectable. Since these alterations were significant 30 days but not 7 days after CCI, the dampening of the spontaneous pulsatile release of GH appeared as a delayed consequence of CCI. Similarly, the number of patients with hypopituitarism increases with time after TBI [12]. Yet, GH levels are evaluated after provocative tests in the clinical setting. In addition, the effect of GHRH on GH secretion is dampened in an inflammatory context [48]. Thus, GH levels were assayed after GHRH stimulations in CCI mice. While GHRH-induced GH secretion raised GH levels in all groups of mice, the mean GH levels were low in CCI mice. In addition, 18 % of the samples from late CCI mice (3 out of 17) had less than 3 ng/ml GH, and this level is a cutoff range characterizing GH deficiency in humans [12]. Importantly, while the relative effect of GHRH was weak in early CCI mice (29.5 ± 4.8 fold increase, n=39), it was not different in control (50.8 ± 16 fold increase, n=21) and late CCI mice (75.3 ± 13.2 fold increase, n=18, $p<0.05$ vs early CCI). This robust responsiveness of pituitary glands of late CCI mice suggested that they did not primarily account for the CCI-induced GH shortage. Indeed, the GH contents of pituitary glands in CCI mice were not changed. This was corroborated by the expression levels of other markers of the GH axis which contribute to GH expression and release [18]. In addition, while GH contents are lower in the anterior pituitaries of CCI rats, this decrease is by no means proportional to the 75% decrease in circulating

GH [22]. Nevertheless, GHRH receptors and SST receptors will deserve a study at the protein level, since subtle alterations, either at the receptor level or at the underlying transduction pathways, might participate in the CCI-induced GH shortage.

GH insufficiencies increase the numbers of GHRH neurons in Dwarf rats and Ames mice [49,50], because GH has a negative feedback on *ghrh* transcription [47,51,52]. In contrast, GHRH neuronal population and GHRH expression were normal in CCI mice, like in other GH deficient mice (Gq11-deficient mice and CREB mutant mice) where GHRH levels do not seem to correlate with GH levels [53,54]. Of note, SST plays a critical role in this central GH feedback [52], and hypothalamic SST expression was not changed after CCI in mice. At the functional level, the electrical properties of GHRH neurons were also similar in sham and CCI mice. For a comparison, hippocampal neurons undergo alterations in resting properties, intrinsic ionic currents and synaptic currents after brain injury [55,56], and show no or incomplete recovery in rats [57,58]. In the vicinity of the GHRH neurons, however, both astrocytes and tanycytes were modified in CCI mice. Such a long distance propagation of the cortical injury to the hypothalamus is not surprising, as cognitive impairments correlate with thalamic microglial activations remote from the original site of focal injury in patients after TBI [59]. In CCI mice, the primary impact likely induces a top-down compression of the ventricles. The pressure exerted onto the third ventricle might generate a second injury site, at the borders of the third ventricle. Indeed, tanycytes showed post-traumatic leakage. This mechanism might also explain the presence of an early astrocytic scar-like image within the median eminence where the tanycytes normally build-up a tight barrier [35]. It remains to determine if/how secondary mechanisms are involved in the changes observed in the hypothalamus of CCI mice. Diffuse axonal injury might not be relevant since GHRH neurons, as well as GHRH and SST expression levels were not changed in CCI mice. Alterations of the hypothalamic vasculature seemed unlikely since IgG leakage was not increased around blood-brain capillaries and fenestrated capillaries (which exhibited a normal morphology).

In CCI mice, tanycytes did no longer show the classical pattern of claudin-1 and ZO-1 [35]. Since these proteins are engaged in tight junctions, such changes likely account for the post traumatic increase of the permeability of the third ventricle. The layer of tanycytes seemed compromised, exhibiting some necrotic cell bodies or some microglial-like phenotypes (unpublished transmission electronic microscopy data). Accordingly, microglial activation triggers tight junction disruptions [60]. Despite widespread alterations of tanycytes, the diffusion of the dextran through the third ventricle was not homogenous. Indeed, the honeycomb pattern of junction proteins of tanycytes at the median eminence, but not at the arcuate nucleus, correlates with the lowest permeability [35]. Thus, the CCI-induced alteration of junction proteins (ZO-1, occludin) might differentially alter the permeability properties of these closely related compartments. In addition, the astrocytic scar–like structure [5] seen below the ventral edge of the third ventricle might restrict fluid movements within the median eminence as compared to the arcuate nucleus. So far, a rupture of the tanycytic barrier has only been reported in the context of an experimental alteration [40,61], but not under pathological conditions [5,7].

There are two mechanisms whereby tanycytes might play a role in post traumatic hypopituitarism. First, the permeability of the luminal side of tanycytes is contributing to the pressure balance between the cerebrospinal fluid and the interstitial fluid [62]. Depending on this balance, 1) gradients of soluble cerebral factors are measured between the cerebrospinal fluid and the parenchyma, and 2) blood borne factors of the portal vessels diffuse within the brain, or are excluded from the parenchyma of the median eminence. An increase in the permeability of the tanycytes would induce an improper distribution of incoming and outgoing messengers, and an alteration of the environment of neuroendocrine, including GHRH, nerve terminals. This might compromise the hypothalamic control of pituitary functions. Accordingly, the parenchymal distribution of IgG was enlarged in CCI mice, reflecting such altered pressure balance. Second, the improper pattern of tight junctions alters cell polarity of epithelial cells [63]. This might modify the distal endfeet of tanycytes which regulates the secretion of hypothalamic neuropeptides, such as GnRH [64]. Indeed, endfeet dynamics are dependent

upon multiple erbB receptors signaling [64], and erbB receptors pathways are modified by changes in cell polarity, including changes in tight junction density [63]. However, despite the fact that GHRH was found in tanycytes [65], the relationships between tanycytes and GHRH nerve terminals are still hypothetical. Similarly, the role of tanycytes in the activity of tubero-infundibular dopaminergic nerve terminals is not known, Since plasmatic GH was lowered while plasmatic LH was slightly increased in CCI mice, tanycytes might have specific roles on the diverse neuroendocrine axis as reviewed [40,64]. Nevertheless, tanycytes will have to be included in our future understanding of the chronic changes after CCI (see Fleiss & Gressens [7]). So far, they were better known for beneficial effects under pathophysiological conditions, supporting axonal regeneration [66] and behaving as neuronal precursors [67].

Brain injury initiates long term cellular changes of unknown neurological relevance [59,68-70], and animal models provide invaluable information as to the cellular mechanisms underlying TBI. Here, we found that CCI in mice induced a long lasting GH shortage, with similarities to the clinical situation of hypopituitarism with preserved pituitary structures. Progress into the cellular and molecular mechanisms involved in this pathology will require a better understanding of the function of tanycytes on the different hypothalamic neuronal populations controlling the anterior pituitary. In the light of their specialized morphology and function, these cells might be viewed as interesting therapeutical targets. In addition, it will be interesting to investigate if new hypothalamic messengers, possibly inflammatory factors, are modifying the intercellular signaling of hypothalamic neurohormones action at the pituitary gland level in CCI mice. This might provide pharmacological opportunities in the treatment of post-traumatic hypopituitarism.

Acknowledgments. We thank the staff of the animal room facilities (BioCampus, Montpellier) for assistance with mice survey; Ségolène Debiesse (IGF, Montpellier) for genotyping; Pierre Fontanaud (IGF, Montpellier) for computer programming; the staff from the Plate-forme Régionale de Recherche en Imagerie Cellulaire (Inserm U-413, IFR MP23, Université de Rouen, Mont Saint Aignan). We thank Dr. François Rassendren, Lauriane Ullman for participating in early pilot experiments; Professor Britta Engelhardt (University of Bern, Switzerland) for the generous gift of the anti-MECA-32 antibody ; Emmet O'Reilly for careful reading of the manuscript. This work was supported by grants from The Royal College of Surgeons in Ireland, the Institut National de la Santé et de la Recherche Médicale; Centre National de la Recherche Scientifique; the Universities of Montpellier 1 & 2; the National Biophotonics and Imaging Platform of Ireland; the Institut Fédératif de Recherches n°3; the Région Languedoc Roussillon; the Agence Nationale de la Recherche (Gliodiabesity project); the Ministère délégué à la Recherche et aux Nouvelles Technologies and by a core funding from the Medical Research Council of United Kingdom.

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Figure legends.

Figure 1. Cortical impact selectively decreased circulating GH in mice. ***A,*** GH levels in blood samples from control (*squares*), sham (*circles*) and CCI mice (7 days: *diamonds;* 30 days: *triangles*). Spontaneous GH levels were impaired late (30 days) after CCI, but not earlier (7 days). GH levels in early and late CCI mice were lower than normal after a GHRH challenge. Symbols are individual data; bars and lines are the means and sem of the numbers of experiments indicated within brackets. Jugular blood sampling of sedated mice was performed 5 min before and after the jugular injection of 1µg hGHRH, as indicated in Methods. ***B,*** (same data as in ***A***) proportions of GH levels classified according to three ranges of concentrations showing an abnormal distribution in late CCI mice under basal conditions (i.e., <3 ng/ml); as well as altered proportions of high GHRH-elevated GH levels (i.e. >30 ng/ml) in early and late CCI mice. For basal values, differences from control (*), sham ($) and early CCI (&) are shown as *,$ $p<0.05$; ***, $$$, &&&, $$$ $p<0.005$, using a Kruskal-Wallis test with Dunn's multiple comparison test. This statistical procedure was also performed amongst GHRH-induced GH levels. Other comparisons of the GHRH-induced GH levels with the corresponding basal levels are shown as ££ $p<0.01$, using a two-tailed unpaired t-test with Welch's correction. ***C&D,*** prolactin (***C***) and LH (***D***) levels in blood samples from control, sham and late CCI mice. $ indicates $p<0.05$, using one-way analysis of variance with a post-hoc Bonferroni's multiple comparison test. The bars and lines are the means and sem of the numbers of experiments indicated.

Figure 2. Cortical injury did not modify pituitary markers. ***A&B,*** pituitary contents of GH (***A***) and expression of GH mRNA (***B***) in control, sham and late CCI mice (30 days after surgery). ***C&D,*** pituitary contents of GHRH receptor mRNA (***C***) and SST receptors mRNA (***D***) in control, sham and late CCI mice (30 days after surgery). mRNA contents were quantified by RT-real time qPCR. The bars and lines are the means and sem of the numbers of experiments indicated. ***E,*** vascular networks in the lateral areas of pituitary glands from control (n=3), sham (n=3) and late CCI mice (30 days after

surgery, n=3). The blood vessels are visualized after iv. injection of gelatin-FITC in anaesthetized mice. Each picture is a 3-D stack of 102 µm depth with a step of 2 µm. Scale bar: 150 µm.

Figure 3. Cortical injury triggered astrocytic activation in mouse hypothalamus. ***A,*** representative sections of brains from control, sham and CCI mice, 30 days after surgery, immunostained for glial fibrillary acidic protein (GFAP). Scale bars: 120 µm. ***B & C***, morphological analysis of GFAP stainings in control, sham and CCI mice (early, 7 days; late, 30 days) expressed as the total volume of the anti-GFAP immunostained area within the median eminence (***B,*** see Methods) and as the number of GFAP-positive cell bodies within the arcuate nucleus (***C***). Note that the number of GFAP-positive cell bodies only increased 30 days after CCI. The bars and lines are the means and sem of the numbers of experiments indicated. Statistical difference from control and sham values are shown respectively as *,$ $p<0.05$, using one-way analysis of variance with a post-hoc Bonferroni's multiple comparison test.

Figure 4. Cortical injury did not modify the spontaneous activity of GHRH neurones. ***A & B*** *top panels*, raw traces of action potentials recorded with the extracellular loose patch technique in the voltage clamp mode, in GHRH neurons of a sham GHRH-GFP mouse (***A***) and of a late CCI GHRH-GFP mouse (***B***). This spontaneous electrical activity was analyzed as, either the instantaneous frequency of action potentials (*middle panel*), or the discharge density (*lower panel*) where the number of action potentials is divided by a bin width (chosen as the mean action potential interval, respectively 0.7 s and 1.1 s in ***A & B***). ***C,*** Cumulated histograms of the mean instantaneous frequency of action potentials recorded in sham (*squares*) and late CCI (*triangles*) GHRH-GFP neurons. Symbols and bars represent means and SEM of the numbers of experiments, respectively. ***D,*** mean skewness of the discharge density histograms in the recordings of GHRH neurons from sham and late CCI mice. The bars and lines are the means and sem of the numbers of experiments indicated.

Figure 5. Cortical injury altered the morphology of the barriers at basal hypothalamus. ***A,*** Claudin-1 (green) and MECA 32 (red) immunoreactivities in coronal brain sections from sham and late (30 days) CCI mice. Sections were counterstained with the cell nucleus marker Hoechst (blue). Scale bar: 100 µm. ***B***, quantification of MECA-32 positive loops within median eminences of controls, shams and CCI mice. ***C & D,*** ZO-1 immunoreactivity of tanycyte cell bodies facing the median eminence (***C***) and the arcuate nucleus (***D***) in coronal brain sections from control, sham and CCI mice, 30 days after surgery. Scale bar: 20 µm. ***E & F***, quantification of the ZO-1 staining of the tanycyte cell bodies lining the median eminence (***E***) and the arcuate nucleus (***F***) in controls, shams and CCI mice (see Methods). In ***B, E & F***, the bars and lines are the means and sem of the numbers of experiments indicated. Statistical differences with control and sham are shown as * $p<0.05$, using a one-way analysis of variance test with a post hoc Bonferronis multiple-comparison test.

Figure 6. Cortical injury altered the physiological barrier properties at the median eminence. ***A,*** Endogenous IgG immunoreactivity (*pink*) of control, sham and late CCI GHRH-GFP mice, 30 days after surgery. Sections were also stained with an anti eGFP antibody (*green*) to visualize GHRH neurons and counterstained with the nuclear marker DAPI (*blue*). Scale bar: 120 µm. ***B&C,*** analysis of mean IgG stainings in control, sham and early (***B***) or late (***C***) CCI mice expressed as the IgG immunopositive volume within the ventral tuberal hypothalamus (see Methods). ***D,*** Enhanced dye spreading within the arcuate nucleus and the median eminence of the late CCI mouse, as compared to control and sham mice. Mice were injected icv with a Lysine-fixable 3000 MW tetramethylrhodamine-conjugated dextran, which was visualized in 175 µm-thick stacks of images of coronal slices encompassing the median eminence. Scale bar: 200 µm. ***E&F,*** averages of the maximal diffusion distances within the arcuate nucleus (***E***) and of the maximal labeled surface of the median eminence (***F***) of the fluorescent dextran. In ***B,C,E&F***, the bars and lines are the means and sem of the numbers of experiments indicated. Statistical differences with control and sham are shown respectively as *,$ $p<0.05$ using a one-way analysis of variance with a post-hoc Bonferroni's multiple comparison test.

Submission: January 4, 2016
Paper in press in The Journal of Clinical Investigation

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

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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

The transition to puberty and adult fertility both require a minimum level of energy availability. The adipocyte-derived hormone leptin signals the long-term status of peripheral energy stores and serves as a key metabolic messenger to the neuroendocrine reproductive axis. Humans and mice lacking leptin or its receptor fail to complete puberty and are infertile. Restoration of leptin levels in these individuals promotes sexual maturation, which requires the pulsatile, coordinated delivery of gonadotropin-releasing hormone (GnRH) to the pituitary and the resulting surge of luteinizing hormone (LH); however, the neural circuits that control the leptin-mediated induction of the reproductive axis are not fully understood. Here we found that leptin coordinated fertility by acting on neurons in the preoptic region of the hypothalamus and inducing the synthesis of the freely diffusible volume-based transmitter nitric oxide (NO), through the activation of neuronal NO synthase (nNOS) in these neurons. The deletion of the gene encoding nNOS or its pharmacological inhibition in the preoptic region blunted the stimulatory action of exogenous leptin on LH secretion, and prevented the restoration of fertility in leptin-deficient female mice by leptin treatment. Together, these data indicate that leptin plays a central role in regulating the hypothalamo-pituitarygonadal axis in vivo through the activation of nNOS in 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.

Acknowledgments

This research was supported by the NEUROBESE International Associated Laboratory (Inserm, SABAN, University of Lille 2; to V.P. and S.G.B.), the Fondation pour la Recherche Médicale (Equipe FRM 2005 & DEQ20130326524, France to V.P.; Régulation Métabolique to S.G.B.), the Agence National pour la Recherche (ANR-09-BLAN-0267, France, to V.P.), the EUFP7 Integrated Project (Grant agreement 266408, Full4Health, to S.G.B.), the BBSRC (grant BB/F01936X/1 to WHC) and the National Institutes of Health (Grant DK84142 to S.G.B.). N.B. was supported by a doctoral fellowship from the University of Lille 2. We would like to thank Dr. Carol F. Elias for her insight discussions on the manuscript. We would also like to thank Dr. S. Rasika for the editing of our manuscript, and Delphine Taillieu, Julien Devassine, Delphine Cappe and Melanie Besegher (animal facility, IFR 114) for expert technical assistance.

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Figures

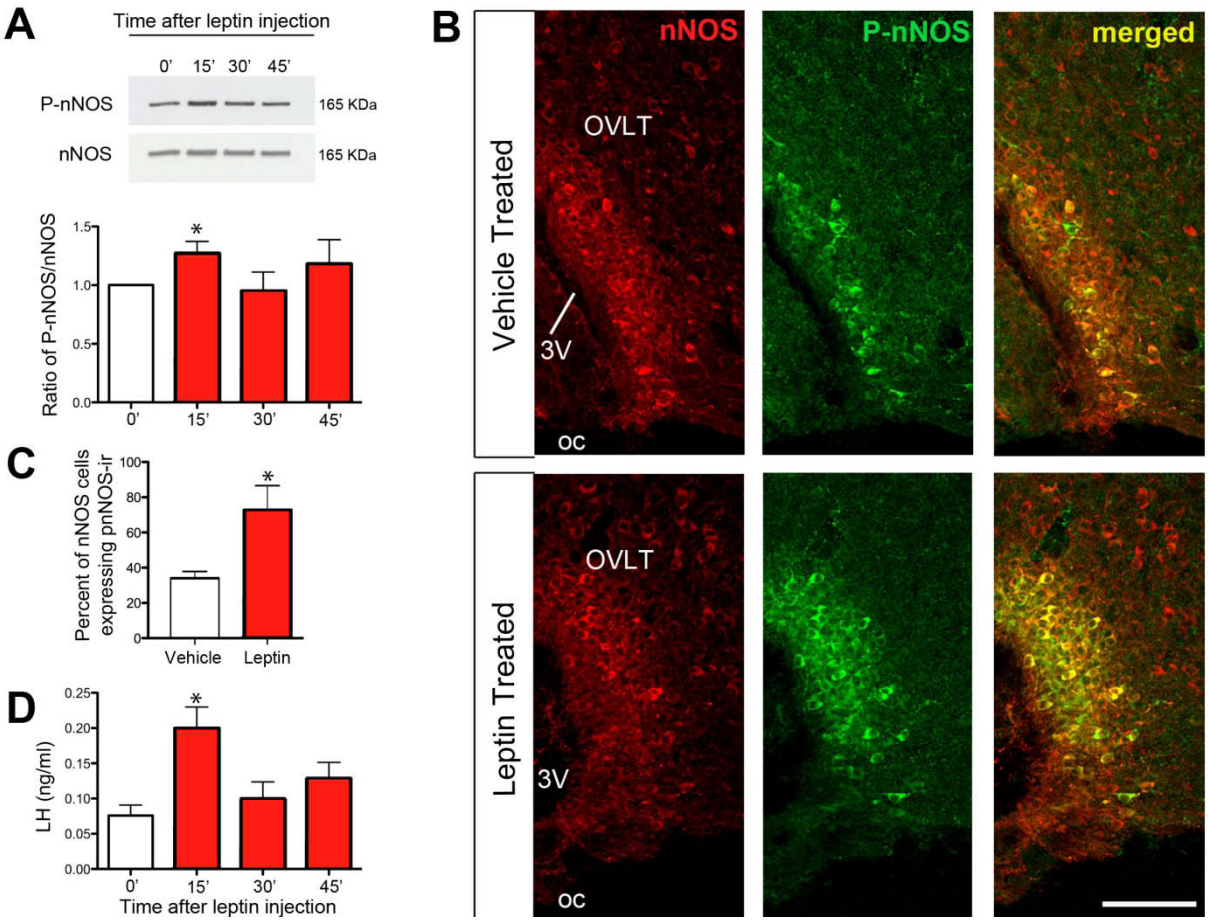


Figure 1. Leptin activates neuronal nitric oxide synthase in the preoptic region and increases circulating LH levels. (A) Upper panel: Representative western blots for phosphorylated and total nNOS at the times indicated (in minutes) following leptin treatment. Leptin promotes the phosphorylation of 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. *: $p<0.05$.

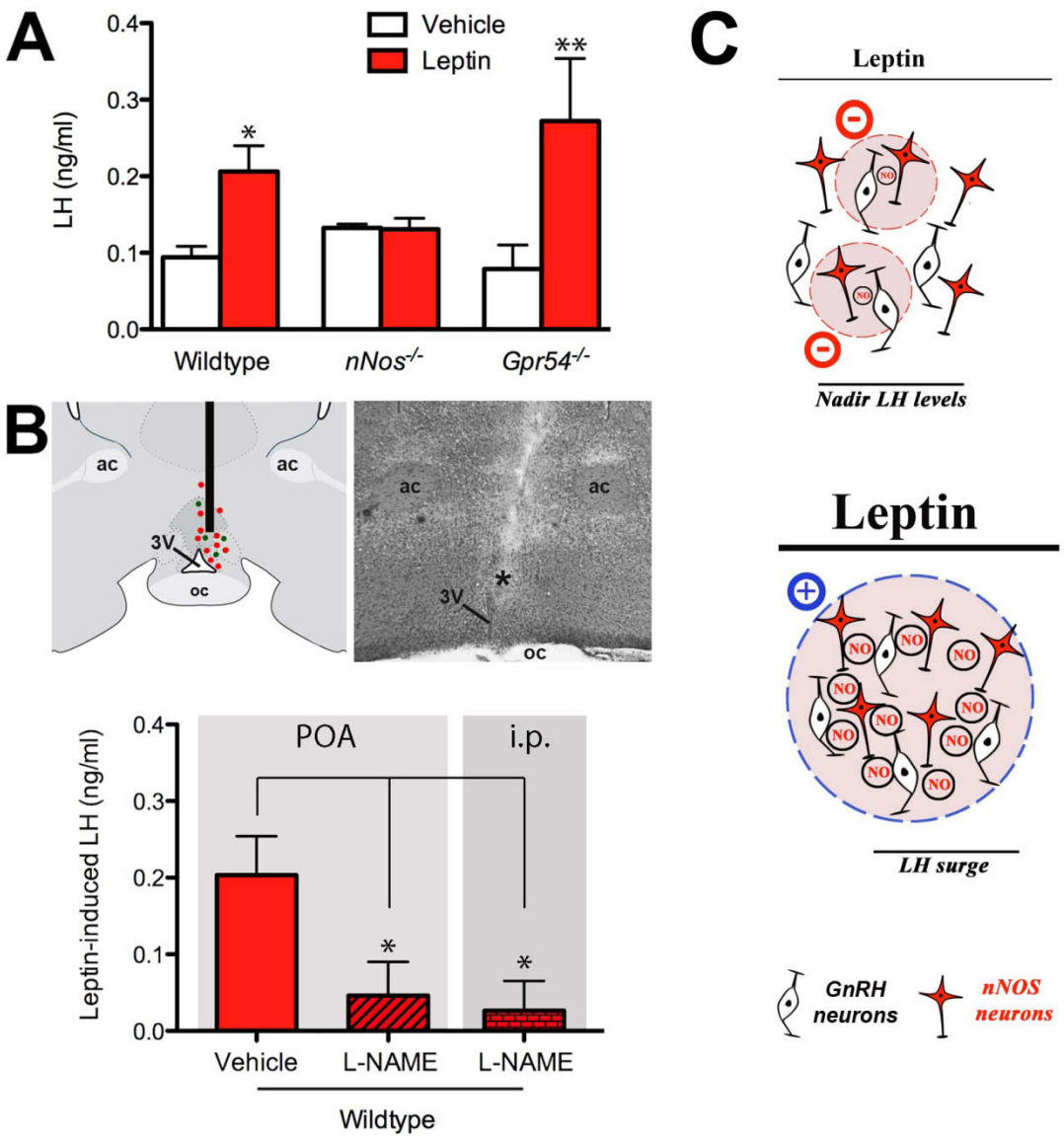


Figure 2. The blockade of nNOS blunts leptin's ability to induce LH release. (A) nNOS activity is required for leptin-induced LH release, independently of kisspeptin-GPR54 signaling. (B) The pharmacological inhibition of nNOS specifically within the preoptic region (POA) is sufficient to block leptin-induced 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).

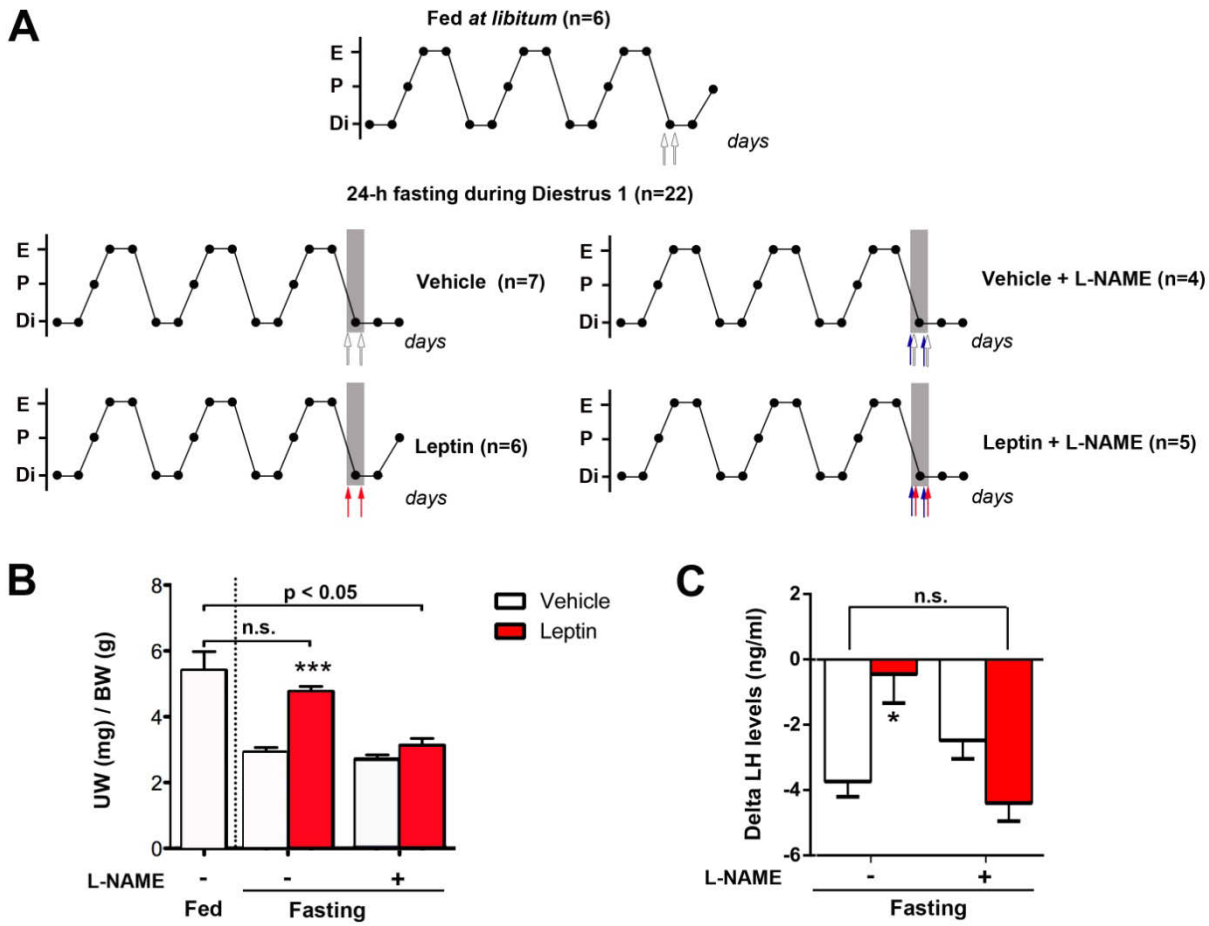


Figure 3. The pharmacological inhibition of nNOS with L-NAME prevents the rescue of the estrous cycle and LH levels by leptin in fasted mice. (A) Representative 15-day estrous cycles of animals subjected or not to 24h-fasting in diestrus 1 three days before death (gray shadow). Mice were subjected to 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<0.01 and ****: p<0.0001 leptin *vs.* vehicle.

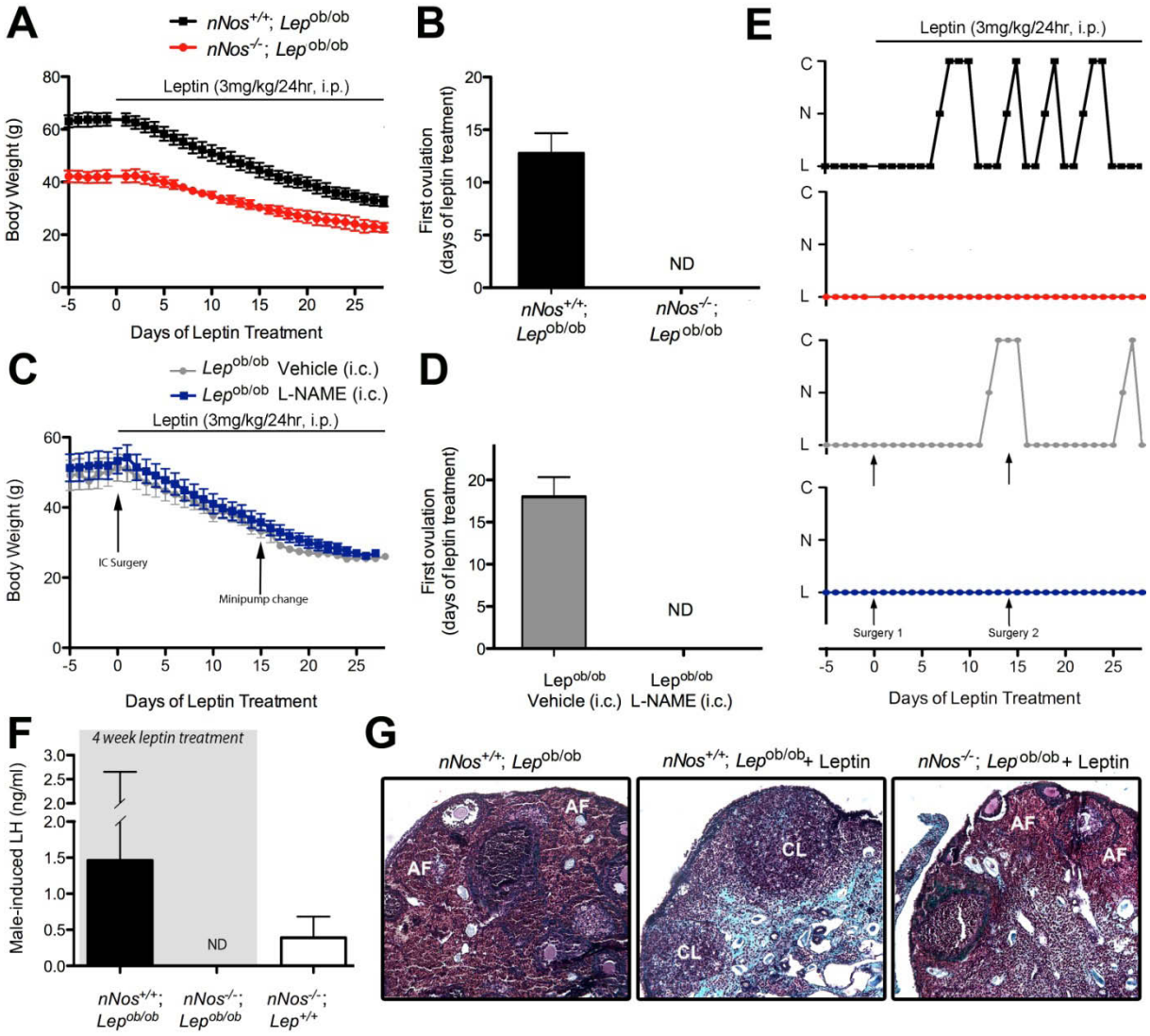


Figure 4. A deficiency in NO signaling renders leptin unable to induce puberty in *Lep*$^{ob/ob}$ mice. (A) *nNos*$^{-/-}$; *Lep*$^{ob/ob}$ mice displayed a lower body weight than *nNos*$^{+/+}$; *Lep*$^{ob/ob}$ littermates prior to the leptin regime, although both groups lost weight with leptin treatment. (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.

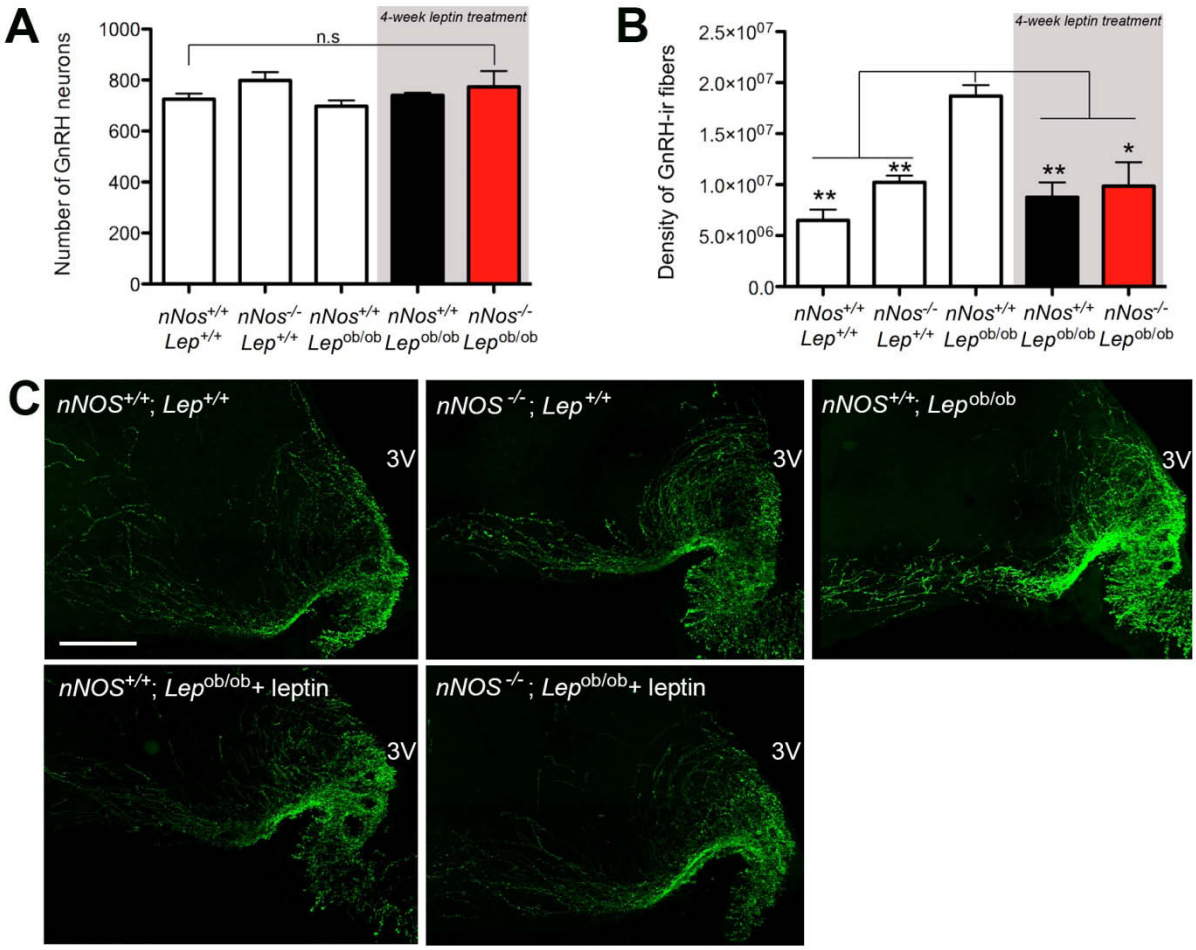


Figure 5. The number of GnRH neurons in the preoptic region (A) and the density of GnRH-immunoreactive (ir, green) fibers in the median eminence (B,C). Interestingly, the *nNos*$^{+/+;}$ *Lep*$^{ob/ob}$ mice show an increased GnRH immunoreactivity, while *nNOS*$^{-/-}$; *Lep*$^{ob/ob}$ and *nNOS*$^{+/+}$; *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.

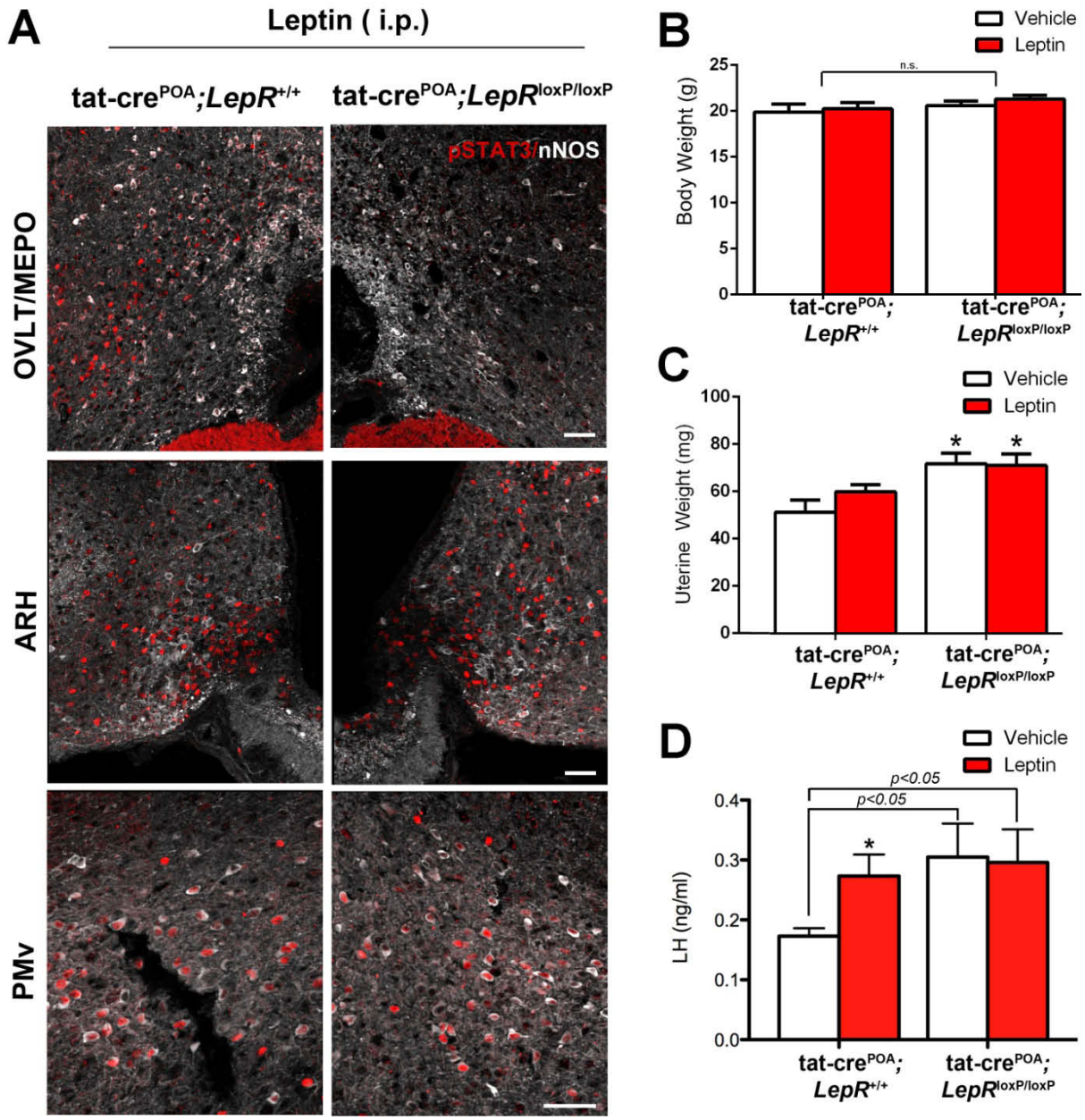


Figure 6. Site-specific deletion of LepR disrupts basal LH levels in female mice. (A) Bi-lateral injections of tat-cre protein into the OVLT/MEPO prevented P-STAT3 45 minutes following peripheral leptin injection in the preoptic region (POA) of *LepR*$^{loxP/loxP}$, but not in *LepR*$^{+/+}$ littermates. 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*$^{+/+}$ 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.

Supplemental Results

Computer modeling of NO signaling in the median preoptic nucleus before and after leptin treatment

nNOS neurons in the preoptic region in the presence of leptin were modeled on the basis of a stereological analysis of nNOS neurons in sections of the median preoptic nucleus. From measurements made in 55 □m-thick tissue sections, the mean diameter of nNOS neurons was 15 ± 0.3 □m and there were 80 ± 3 of these cells per 24000 □m^2. After applying Abercrombie's correction (1), the packing density comes to 4.76 x 10^4 nNOS neurons/mm^3. To simulate this tissue, nNOS neurons were modeled as a 3-dimensional cubic array of spheres (9x9x9), each 15 □m in diameter, and having the same nearest-neighbour spacing (28 □m) that would exist if the nNOS neurons were similarly packed. [With this geometry, the distance to the nearest neighbour is given by (packing density)$^{-1/3}$; (2)]. Because active nNOS is presumed to be associated with the cell membrane, NO was emitted from the surfaces of the spheres. Diffusion from a continuous spherical surface source is given by Equation 10.4.10 of Carslaw and Jaeger (3). The equation was modified to incorporate the x-, y- and z-coordinates and to sum the contributions of all the spheres located within these coordinates (*n* in each plane). The resulting equation was:

$$[NO](x,y,z,t) = \frac{1}{10^3 N_{av}} \sum_{a=0}^{n-1} \sum_{b=0}^{n-1} \sum_{c=0}^{n-1} \left| \frac{Q}{8\pi R r' \sqrt{\pi D}} \int_0^t \frac{1}{\sqrt{t-t'}} \exp\left[-\frac{(R-r')^2}{4D(t-t')} - \lambda(t-t') \right] - exp\left[-\frac{(R+r')^2}{4D(t-t')} - \lambda(t-t') \right] \right| dt'$$

where $R = \sqrt{(x-x_a')^2 + (y-y_b')^2 + (z-z_c')^2}$

and, with the units given in parentheses, *[NO]* = NO concentration (M), N_{av} = Avagadro's number (molecules/mole), *Q* = rate of NO release (molecules/s), *r′* is the

radius of the spherical source surface (m), D = NO diffusion coefficient (m^2/s), t = time (s), λ = NO inactivation rate constant (s^{-1}), x,y,z are distances in the 3 dimensions (m), x'_a,y'_b and z'_c are the coordinates of the n sources in each dimension, indexed (a,b and c) to reside at regularly-spaced intervals.

A range of values for the rate of NO inactivation was considered (λ = 1-150 s^{-1}) and, to ensure steady-state conditions even at the slowest rate (λ = 1 s^{-1}), the sources were set to run for 10 s. The resultant NO concentration profile is proportional to the rate of NO release (Q), the value of which was selected on the basis of the measured NO synthase activity in the hypothalamus, namely15 nmol/min/g tissue (4). Assuming 1 ml/g tissue, this activity corresponds to 3.46 x 10^{-15} mol/s for the array volume, or 2.84 x 10^6 NO molecules/s for each constituent sphere. Given that brain tissue has a relatively small extracellular space [20 % of the volume, (5)], the NO diffusion coefficient (D) was taken to be its intracellular value [8.48 x 10^{-10} m^2/s; (6)]. The equation was solved using the "Adaptive" integrator in Mathcad 14 (Parametric Technology Corp., Needham, MA, USA).

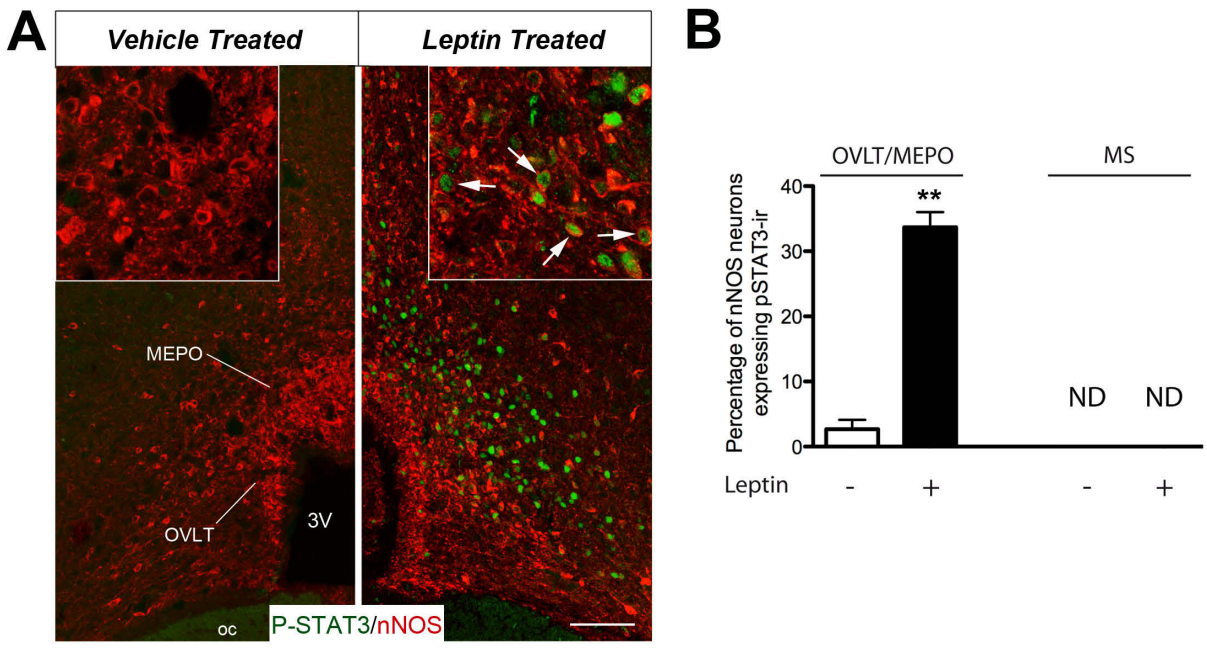
A key determinant of the amplitude and spread of NO signals is the rate of NO inactivation and we have considered a range of NO inactivation rate constants (λ), from 1 s^{-1}, which is approximately the value expected if loss of NO was solely through interaction with hemoglobin in the microcirculation (7), up to 150 s^{-1}, which is the value deduced for slices of cerebellum at subnanomolar NO concentrations (8). When λ = 1 s^{-1} the resulting NO concentration is high (approximately 200 nM) and roughly uniform over the lattice (Supplemental Figure 2A), whereas with higher rates of inactivation, the profiles of NO over each sphere become progressively smaller and more distinct (see Supplemental Figure 2B top panel for λ = 100 s^{-1}). Even at high rates of inactivation, however, appreciable intercellular NO concentrations are

predicted to exist. For example, with $\lambda = 100\ s^{-1}$, the steady-state intercellular concentrations at their lowest points (troughs) reach 1.3 nM, which is about 15 % of the concentration found at the NO-emitting spherical surfaces.

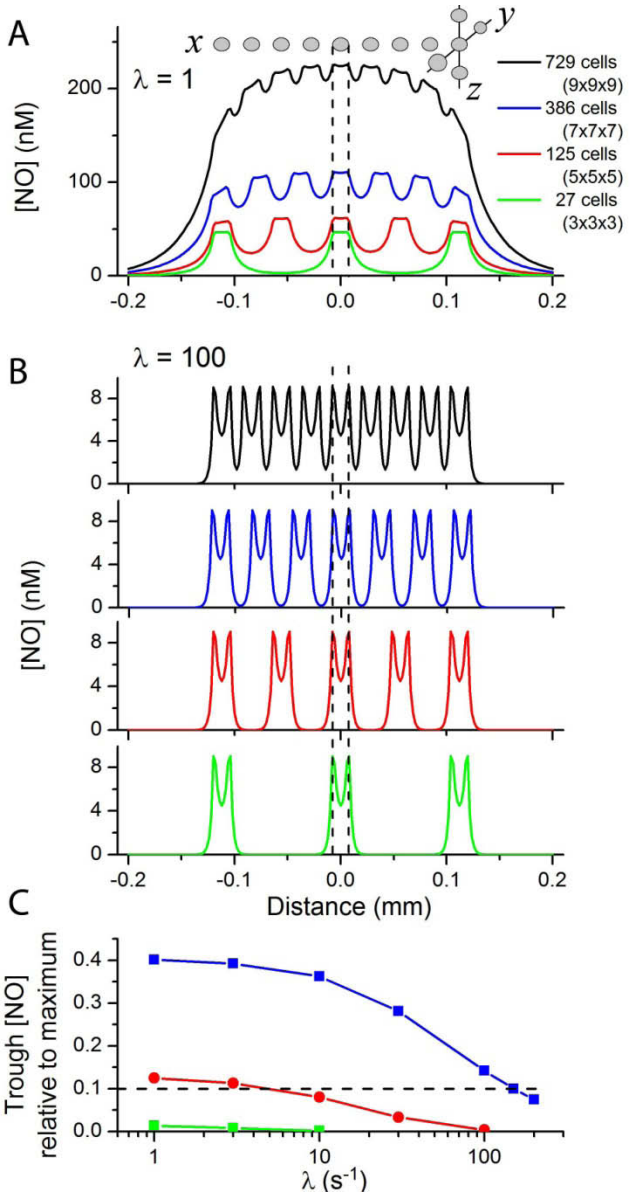
In order to simulate the pre-leptin situation, the number of sources was reduced, while keeping the overall dimensions of the lattice the same. With a 7x7x7 lattice (containing about half the number of NO-emitting spheres), trough NO concentrations with $\lambda = 100\ s^{-1}$ are predicted to be reduced to about 15 % of those found in the fully active lattice, although the concentrations at the sources are unaltered (Supplemental Figure 2B, second panel). With further reductions in the density of spheres, the trough NO concentrations become increasingly negligible (Supplemental Figure 2B, lower panels) provided that the rate of NO inactivation is reasonably high (Supplemental Figure 1A).

From the relationship between the trough NO concentrations, the density of spheres, and the NO inactivation rate (Supplemental Figure 2C), it can be concluded that, providing the numbers of active nNOS neurons in the tissue in the pre-leptin situation are half or less of the numbers in the fully active population (post-leptin), and provided that the rate of NO inactivation is relatively high, leptin would augment the NO concentration in the vicinity of the intermingled GnRH neurons by at least 10-fold. Parenthetically, we also note that the values of the computed NO concentrations arrived at by assuming a rate of NO synthesis based on the hypothalamic NOS activity, together with a high rate of NO inactivation, are very similar to the concentrations (low nM) deduced from measurements of NO release to exist within the nNOS-rich regions of cerebellar slices following stimulation with NMDA, concentrations that also cohere with the measured cerebellar nNOS activity (9).

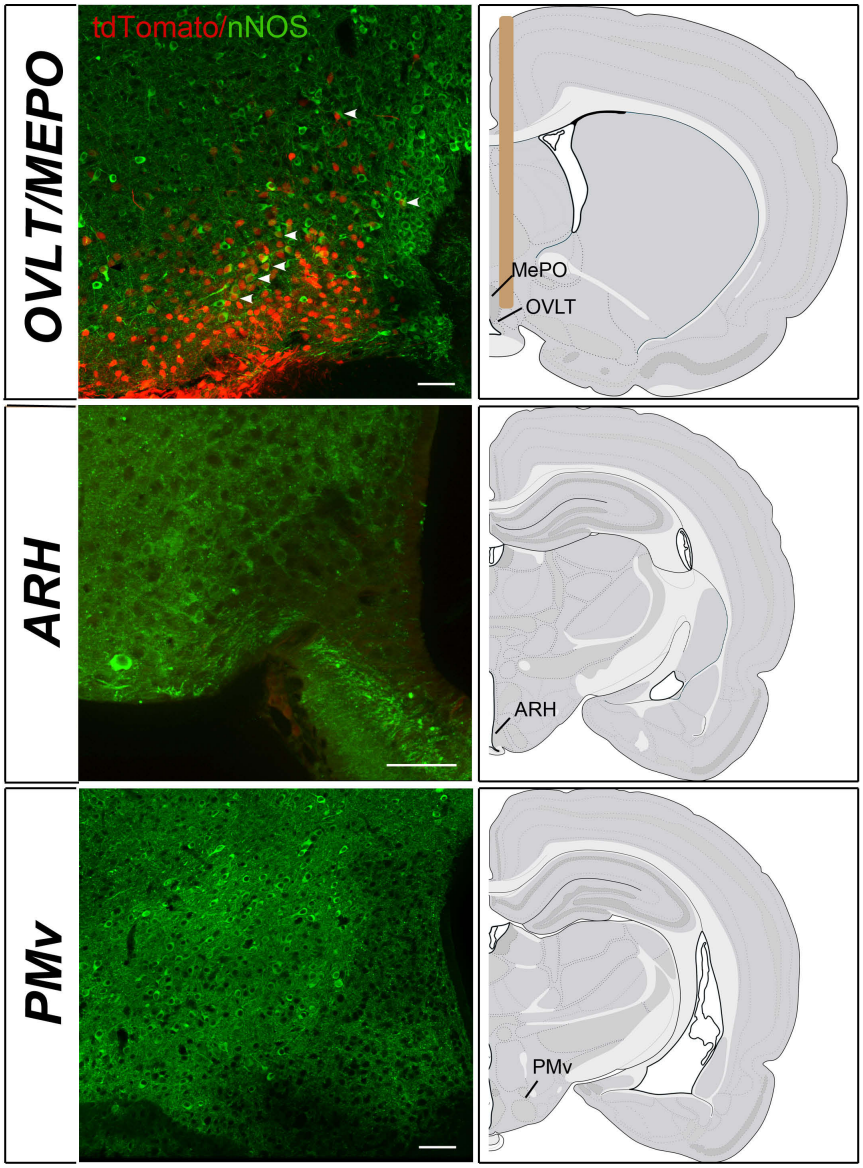
Supplemental Figures



Supplemental Figure 1. Intraperitoneal injection of leptin promotes STAT3 phosphorylation in nNOS neurons in the preoptic region, but not in the medial septum (MS). (A) Coronal section of the preoptic region showing a marked increase in P-STAT3 expression (green) in leptin *vs.* vehicle treated diestrous mice at the level of the organum vasculosum of the lamina terminalis (OVLT) and the medial preoptic area (MePO) where numerous nNOS-immunoreactive neurons reside (red). Arrows in inset show double-labelled neurons. 3V, third ventricle; oc, optic chiasm. Scale bar: 100 μm (30 μm in insets). (B) Graphs represent the proportion of nNOS neurons expressing P-STAT3 immunoreactivity in the OVLT/MePO and MS in leptin (+) or vehicle (-) treated animals. N.D.: not detected. ** $P < 0.01$ *vs.* vehicle.

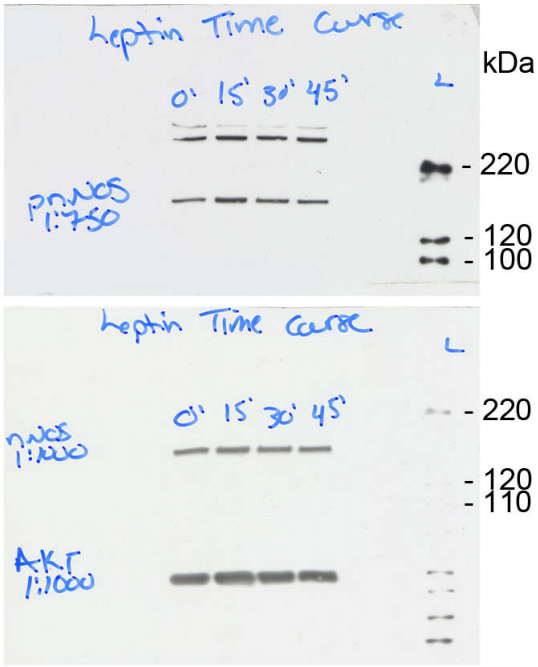


Supplemental Figure 2. Model of NO signaling in the preoptic region of the hypothalamus. A and B show the computed profiles of steady-state NO concentrations across the center of a cubic array of spherical surface sources. The 9x9x9 array is depicted diagrammatically at the top of panel A and the vertical broken lines indicate the boundaries of the central sphere. Different numbers of active spheres are considered (color-coded as in the legend to panel A) with NO inactivation rate constants (λ) of 1 s^{-1} (A) and 100 s^{-1} (B). In C, the lowest NO concentrations found in the inter-sphere region (the troughs) are expressed relative to the values found when all spheres in a 9x9x9 array are active, at different values of λ. The horizontal broken line indicates trough concentrations that are 10 % of those found in the 9x9x9 array. The color-coding is as in A and B.



Supplemental Figure 3. Representative images showing Tomato expression (red) in nNOS (green)-immunoeactive neuons (arrowheads) in the OVLT/MEPO, but not the ARH and PMv of *dtTomato*$^{loxP/+}$ mice in which the tat-cre recombinant protein has been infused into the preoptic region. Scale bars: 50 μm.

Figure 1 A



Supplementary Figure 4. Full-length photographs of the western blots presented in Figure 1A.

Supplemental Experimental Procedures

Immunofluorescent stainings

Immunohistochemistry of P-STAT3 and nNOS. Mice were given a lethal overdose of chloral hydrate (400mg/kg) then perfused transcardially with cold saline (0.9%) followed by cold 2% paraformaldehyde in 0.1M sodium phosphate buffer (PB) pH 7.4. Brains were collected immediately after perfusions and post-fixed for 2 hours in a 20% sucrose- 2% paraformaldehyde solution. Following postfixation brains were cryoprotected in 20% sucrose-KPBS 0.02M solution overnight, then imbedded in OCT, flash frozen, and stored at -80°C until further processing. Coronal sections were taken at 30um through the preoptic area directly onto Superfrost slides and stored in antifreeze at -20°C. Sections were washed 4 times in KPBS 0.02M for 15minutes each. Sections were then treated with a 0.5% H_2O_2 +0.5% NaOH solution in KPBS for 20minutes at room temperature. The sections were washed 5 times for 5 minutes each in KPBS. Sections were then treated with 0.3% glycine in KPBS for 10 minutes at room temperature and then washed 5 times for 5 minutes each in KPBS. Following the washes, the slides were treated with a 0.03% SDS solution in KPBS for 10 minutes at room temperature and then washed 5 times 5 minutes in KPBS. Sections were incubated in blocking solution (4% NDS + 0.4% Triton X-100 +1% BSA in KPBS 0.02M) for 90 minutes. Then sections were then incubated in rabbit anti-P-STAT3 (Tyr705) (Cell Signaling) 1/1000 and sheep anti-nNOS (generous gift from Dr. P. C. Emson (Medical Research Council, Laboratory for Molecular Biology, Cambridge, UK) in a KPBS 0.02M solution containing 1% NGS + 0.4% Triton X-100 + 1% BSA for 48 hours at 4°C. After the incubation in the primary antibody, sections were rinsed with KPBS 0.02M 8 times for 5 minutes each. The sections were then incubated in secondary antibody donkey anti-rabbit Alexa-568 (Invitrogen) and

donkey anti-sheep Alexa-488 (Invitrogen) for 2 hours at room temperature. Sections were washed in KPBS 5 times for 5 minutes each then counterstained with bis-benzamide for 3 minutes and washed 2 times for 5 minutes and coverslipped with 65% glycerol in KPBS 0.02M.

Immunofluorescence for pnNOS and nNOS. Mice were given a lethal overdose of chloral hydrate (400mg/kg) then perfused transcardially with cold saline (0.9%) followed by cold 4% paraformaldehyde in 0.1M PB pH 7.4. Brains were collected after perfusions and post-fixed for 4 hours in 4% paraformaldehyde followed by cold PB 0.1M. The following day brains were sliced coronally to collect free-floating sections using a Vibratome V1200ST at 50um and were immediately processed for immunohistochemistry. 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 72hours at 4°C in primary rabbit anti-pnNOS (Thermoscientific) and sheep anti-nNOS. Sections were washed 3 times for 5 minutes in PB 0.1M and then incubated in donkey anti-rabbit biotin (1:500; Jackson Laboratories) for 1 hour at room temperature. The sections were then washed and incubated in streptavidin Alexa 488 (1/400; Invitrogen) and donkey anti-sheep Alexa 568 (1/400; Invitrogen). Sections were washed, mounted, and coverslipped with 65% glycerol in PB 0.1M.

Western blot analyses

Protein extraction. The preoptic region was dissected from each animal and protein extracted for western blotting as described previously. Briefly, mice were killed by decapitation following treatment. After the rapid removal of the brain, the meninges and optic chiasm were removed and the preoptic region was dissected under a

binocular magnifying glass with the Wecker scissors (Moria, France). The external limits for this dissection were: laterally, the external border of the medial preoptic area and dorsally, the internal border of the anterior commissures. Anteroposteriorly, the dissected region was comprised between the atlas levels 16 and 20 of the Swanson Atlas (10). After dissection, each fragment was placed in a microcentrifuge tube, snap frozen in liquid nitrogen and stored at -80°C.

Protein extracts were prepared from each preoptic region sample in 200 µl of lysis buffer (25 mM Tris, pH 7.4, β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin A, 10 µg/ml apoprotinin, 100 µg/ml PMSF, and 1% Triton-X100) by homogenization of the fragments through 22 and 26 gauge needles in succession. Tissue lysates were cleared by centrifugation at 14000 rpm for 15 min at 4°C. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA) and equal amount of protein were mixed with 4X sample buffer (Invitrogen). Samples were boiled for 5 min and stored at -80°C until use.

Immunoblot. Samples were reboiled for 5 min after thawing and electrophoresed for 75 min at 150 V in 8-12% Tris-acetate polyacrylamide gels according to the protocol supplied with the NuPAGE system (Invitrogen). After size fractionation, the proteins were transferred onto 0.2 µm pore-size polyvinylidene difluoride membranes (LC2002; Invitrogen) in the blot module of the NuPAGE sytem (Invitrogen) for 75 min at RT. Membranes were blocked for 1 h in blocking buffer [TBS with 0.05% Tween 20 (TBST) and 5% nonfat milk] at RT, and incubated overnight at 4°C with primary rabbit anti-pnNOS (Thermoscientific) diluted in blocking buffer. Membranes were washed four times with TBST the following day before being exposed to HRP-conjugated secondary antibodies diluted in blocking buffer for 1 h at RT. Immunoreactions were

visualized using the ECL detection kit (NEL101; PerkinElmer, Boston, MA). Immunoblots were scanned using a desktop scanner (Epson Expression 1680 PRO) and Adobe Photoshop, and band intensities were determined using ImageJ software (NIH, Bethesda).

Fasting-induced suppression of LH levels

Vaginal smears were taken from adult female mice for two complete estrous cycles prior to the fast. On the day of diestours I, female mice were placed under food deprivation for 24 hours to disrupt fertility. At the beginning of the light cycle (7H30) food was removed and subsequently treated with bidaily injections of the NOS inhibitor L-NAME (50mg/kg, i.p.) or a control (saline) one hour prior to leptin (3mg/kg, i.p.) or vehicle injections. The timing of the bidaily injections are as follows: L-NAME or the control where administered at 8H30 and 18H30, while leptin or vehicle were administered at 9H30 and 19H30. Food was returned at 7H30 exactly 24 hours following the initial food deprivation. Mice were sacrificed on the evening of the predicted proestrous precisely at 19H30 (lights-off) and blood was collected for later analyses.

Stereotaxic surgery

Acute stereotaxic surgery and peripheral leptin treatment. On the day of diestrous I, mice were deeply anesthetized using 4% isoflurane to O_2 then transferred to the stereotaxic apparatus and anesthetized with 1.5-2.5% isoflurane to O_2 for the duration of the surgery with constant monitoring of temperature and breathing rate. Mice were administered L-NAME (5mM) or sterile saline into the POA (coordinates: +0.5mm bregma, -0.5mm lateral, -5.3mm dorsal-ventral) via a Hamilton syringe at a

rate of 50nl/minute. The syringe was left untouched for 3 minutes following injection. Generally, mice were fully mobile 5 minutes following the removal of the anesthesia and were allowed to recover under a heat lamp 15 minutes prior to leptin treatment. Leptin was administered intraperitoneally 15 minutes following surgery and sacrificed by live decapitation 15 minutes following the injection. Blood was collected for LH level analysis and the brain removed for correct placement of the syringe.

Chronic L-NAME Infusion and Daily Peripheral Leptin Treatment for 28 Days. Cannulae (Plastics One) were attached via a catheter to a 14-day osmotic minipump (Alzet; model 2002). The minipumps contained either vehicle (sterile saline 0.9%) or L-NAME (5mM) and were primed overnight in sterile saline at 37°C. $Lep^{ob/ob}$ mice were anesthetized using a 2% Avertin (Sigma) solution. The cannula was implanted into the POA as described above and the minipump placed into the subcutaneous space in the back. Mice were allowed to recover for 24 hours prior to the start of peripheral leptin administration. $Lep^{ob/ob}$ mice were weighed and administered leptin (3mg/kg) once daily at the end of the light cycle for 28 days. As the duration of the minipump was 14 days a second surgery was required to change the minipump to continue the treatment. On the morning of the 14^{th} day of the leptin regime, the minipump was changed by applying a local anesthetic directly on the back surrounding the area of the minipump before an incision was made. Mice were allowed to recover under a heat lamp for the afternoon prior to the daily leptin injection.

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Submission: January 4, 2016
PLOS Biology

Brain Endothelial Cells Control Fertility through Ovarian-Steroid-Dependent Release of Semaphorin 3A

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Running Title: **Brain endothelial cells control fertility**

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Number of text pages: 48
Number of figures: 7
Number of Supplemental Figures: 5
Number of tables: 0
Number of words (abstract): 187

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Abstract

Neuropilin-1 (Nrp1) guides the development of the nervous and vascular systems, but its role in the mature brain remains to be explored. Here we report that the expression of the 65kDa isoform of Sema3A, the ligand of Nrp1, by adult vascular endothelial cells, is regulated during the ovarian cycle and promotes axonal sprouting in hypothalamic neurons secreting gonadotropin-releasing hormone (GnRH), the neuropeptide controlling reproduction. Both the inhibition of Sema3A/Nrp1 signaling and the conditional deletion of Nrp1 in GnRH neurons counteract Sema3A-induced axonal sprouting. Furthermore, the localized intracerebral infusion of Nrp1- or Sema3A-neutralizing antibodies *in vivo* disrupts the ovarian cycle. Finally, the selective neutralization of endothelial-cell Sema3A signaling in adult *Sema3a*$^{loxP/loxP}$ mice by the intravenous injection of the recombinant TAT-Cre protein alters the amplitude of the preovulatory luteinizing hormone surge, likely by perturbing GnRH release into the hypothalamo-hypophyseal portal system. Our results identify a previously unknown function for 65 kDa Sema3A-Nrp1 signaling in the induction of axonal growth, and raise the possibility that endothelial cells actively participate in synaptic plasticity in specific functional domains of the adult central nervous system thus controlling key physiological functions such as reproduction.

Author summary

Within the developing embryo, endothelial cells release chemotropic signals such as Semaphorin 3A (Sema3A) that, by binding to the transmembrane protein neuropilin-1 (Nrp1), actively regulate not only vascular development and branching, but also neuronal migration and axon guidance. Whether or not subsets of endothelial cells in the adult brain retain the ability to secrete molecules capable of such long-range diffusion in order to influence neuronal function is unknown. Here we show that endothelial-cell-derived Sema3A promotes the cyclic outgrowth of axons of the fraction of hypothalamic neurons releasing the neuropeptide controlling reproduction, gonadotropin-releasing hormone (GnRH), into blood vessels of the pituitary portal blood system, which deliver neurohormones to the anterior pituitary gland. Notably, this endothelial-cell-mediated sprouting of GnRH axons appears to regulate neuropeptide release at key stages of the estrous cycle, such as at proestrus, when the surge of GnRH that triggers ovulation occurs, and to be under the control of ovarian steroids. These findings establish a previously unknown mechanism of axonal plasticity in the adult brain involving vascular Sema3A, and have broad implications for endothelial-cell-to-neuron communication as a general mediator of axonal plasticity in the mature nervous system. Moreover, they suggest a model in which vascular endothelial cells are dynamic signaling components that relay peripheral information to the brain to control key physiological functions, including species survival.

Blurb

- Endothelial-cell-derived Sema3A is an outgrowth-promoting factor that regulates axonal plasticity and neurohormone release in the adult brain in response to the ovarian cycle.

Abbreviasions list

Sema3A, semaphorin 3A; Nrp1, neuropilin-1; GnRH, gonadotropin-releasing hormone; PV1, plasmalemmal vesicle-associated protein 1; DARPP-32, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000; TSH, thyroid-stimulating hormone; ER, estrogen receptor, E_2, 17β-estradiol 3-benzoate; P, progesterone; OVX, ovariectomized; GFP, green fluorescent protein; GAP-43, Growth associated protein-43.

Introduction

Blood vessels and axons employ similar mechanisms and follow common guidance cues to grow and navigate tissues during embryonic development [1,2]. Blood vessels influence the trajectories taken by axons to reach their appropriate end organs [3]. In the adult brain, they communicate with neurons and glia in order to meet physiological demands [4,5]. Endothelial cells are well positioned to sense peripheral inputs and ideally suited to convey signals that could influence neuronal structure and synaptic plasticity. However, whether they are capable of influencing axonal plasticity in the mature central nervous system remains to be elucidated. Recent evidence suggests that the semaphorins, members of a family of secreted guidance molecules, continue to be expressed in the postnatal brain and may have important implications for neuronal plasticity and nervous system physiology [6]. Of these, Sema3A, which exerts both repulsive and attractive effects on growing axons [7,8,9], is also expressed in endothelial cells during vascular development [10,11]. Interestingly, Sema3A acts as a guidance factor during the migration of a particular population of neuroendocrine neurons that secrete the fertility-regulating neuropeptide gonadotropin-releasing hormone (GnRH) [12,13], and that moreover retain a high degree of plasticity in the mature brain [14]. In particular, GnRH neurons, which project to the hypothalamic median eminence and release their neurohormone into a specialized capillary network for delivery to the anterior pituitary (Figure 1A), are known to undergo extensive axonal growth towards the vascular wall during critical time windows in adulthood, such as at the onset of the preovulatory surge, when massive GnRH release has to occur to trigger ovulation [14], and are thus an ideal system in which to analyze endothelial-axonal interactions during adult nervous system homeostasis.

In this study, we examined whether this periodic sprouting of GnRH axon terminals in the median eminence of the adult hypothalamus was regulated by endothelial cells, through the release of Sema3A and the activation of its cognate receptor, neuropilin 1 (Nrp1) [15,16,17]. We report that endothelial cells of the median eminence do indeed release the 65 kDa

isoform of Sema3A (p65-Sema3A) at key stages of the ovarian cycle, that Nrp1 is expressed in GnRH axons, and that Sema3A-Nrp1 signaling is required for the extension of GnRH axon terminals towards the vascular plexus on the day of the preovulatory surge. We also demonstrate that the selective inhibition of Sema3A expression in endothelial cells of the median eminence and the transient local manipulation of Sema3A signaling *in vivo* alter the preovulatory release of GnRH, suggesting that the endothelium-to-neuron communication mediated by 65kDa Sema3A-Nrp1 signaling is of functional relevance in the adult brain. Our results thus indicate a hitherto unidentified role for brain vascular endothelial cells in mediating the cyclic plasticity of GnRH axons in the adult hypothalamus, and consequently, in reproductive physiology.

Results

Sema3A is expressed by the endothelial cells of portal blood vessels in the median eminence of the adult hypothalamus

Sema3A is mainly known as a developmental signal regulating axon guidance. In order to assess the potential role of Sema3A as a guidance cue for hypothalamic GnRH neurons controlling the ovarian cycle, we first investigated its expression in the median eminence of adult animals. In situ hybridization of adult female rat brain sections revealed that the mRNA for Sema3A was selectively expressed in endothelial cells of the vascular compartment of the median eminence (Figure 1B). Only a weak hybridization signal was seen in the ependymal layer and in the internal and external axon layers. Brain sections hybridized with the sense probe (negative control) did not exhibit any detectable labeling in the median eminence (data not shown). Further analysis by cell sorting, using an affinity-purified antibody to plasmalemmal vesicle-associated protein 1 (PV1) [18] a component of the fenestral diaphragms [19] selectively expressed by endothelial cells of the median eminence (Figure 1B; Figure 1C, PV1 mRNA expression in fluorescent vs. non-fluorescent cells $t_{(6)}$=4.080, p=0.007, n=4), revealed that Sema3A expression was restricted to PV1-positive cells (Figure S1; Figure 1C, $t_{(6)}$=2.636, p=0.039, n=4), unlike β3-tubulin, DARPP-32 and thyroid-stimulating hormone (TSH), markers for neurons, tanycytes and endocrine cells, respectively, which were expressed only by non-PV1-positive cells and not found in the same fraction as Sema3A (Figure S1). Immunofluorescence analysis in adult female mice using a Sema3A-specific antibody [13], revealed bright Sema3A immunoreactivity in the capillary zone of the median eminence that extended into the nervous tissue where it progressively vanished (Figure 1D). Together, these findings indicate that Sema3A is expressed *in vivo* in the median eminence of the mature brain, and is localized in vascular endothelial cells of the pituitary portal system, onto which GnRH neurons abut.

Vascular endothelial cells isolated from the median eminence release p65-Sema3A

To further investigate the site of origin of Sema3A in portal blood vessels and to determine whether fenestrated endothelial cells from the rat median eminence can release Sema3A, we used a sequential panning method for their purification, as described previously [20,21]. Consistent with our findings *in vivo* [18,21], purified median eminence endothelial cells in culture expressed PV-1 and were labeled by *Bandeiraea simplicifolia* lectin (Figure S2). We confirmed Sema3A mRNA expression in purified median eminence endothelial cells by RT-PCR expression analysis (Figure 1E). Next, we used immunoblotting to analyze the conditioned medium of purified median eminence endothelial cells and compared it with total protein extracts from median eminence explants, revealing several bands corresponding to the different known isoforms (the secreted 65 kDa and 95 kDa forms, and the 125 kDa precursor; see Figure 1F) of Sema3A [22]. Notably, the conditioned medium of purified median eminence endothelial cells only contained a smear at 125 kDa and a discrete band for p65-Sema3A *(*Figure 1F), which appears to be the furin cleavage product of the 95 kDa isoform (Figure S2B). This confirms that fenestrated endothelial cells of the median eminence express Sema3A and release its 65 kDa isoform into the extracellular space.

p65-Sema3A release in the median eminence is regulated by the ovarian cycle

To determine whether Sema3A expression in the median eminence varies during the ovarian cycle, we performed western blotting experiments during the onset of the preovulatory surge at proestrus (when GnRH nerve terminals are close to portal plexus vessels) and during diestrus (when GnRH nerve terminals are distant from the endothelial wall) [23]. Remarkably, we found that p65-Sema3A expression was significantly increased on the day of proestrus as compared to diestrus (Figure 1G; p65-Sema3A, Di16h *vs*. Pro16h; n=5 independent experiments, $p < 0.01$, *t* test). This regulation appeared to be selective for p65-Sema3A, as the expression of other Sema3A isoforms did not change significantly during the ovarian cycle (Figure 1G; p95-Sema3A: 0.226 ± 0.0449 arbitrary units at Di16h *vs*. 0.133 ± 0.0411 arbitrary units at Pro16h, n=5, $t_{(8)}=1.522$, p=0.167; p125-Sema3A: 0.427 ± 0.0455 arbitrary units at Di16h *vs.* 0.379 ± 0.0519 arbitrary units at Pro16h, n=5, $t_{(8)}=0.698$, p=0.505). These

data indicate that the levels of p65-Sema3A in the median eminence are maximal on the day of proestrus, when circulating levels of estradiol are also high and are known to exert their positive feedback effect on the hypothalamo-pituitary-gonadal axis [24,25]. To determine whether these changes are sex-steroid-dependent, we ovariectomized (OVX) adult cycling female rats and subsequently treated them with subcutaneous injections of sesame oil, alone or containing 17β-estradiol 3-benzoate (E_2), progesterone (P) or E_2 + P. As shown in Figure 1H, estradiol induced a significant increase in p65-Sema3A expression in the median eminence of OVX rats when compared with the other treatment groups, whereas progesterone inhibited this increase (n = 5 rats per treatment, $p < 0.05$, one-way ANOVA). Interestingly, additional RT-PCR analyses revealed that PV1-positive endothelial cells expressed mRNA for the estrogen receptor ERα (Figure 1C) and that this expression was particularly enriched in the median eminence of adult female rats (PV1-positive *vs.* PV1-negative cells $t_{(6)}=2.793$, p=0.031, n=4).

Altogether, these results provide direct evidence that the release of p65-Sema3A by fenestrated endothelial cells of the median eminence is strictly regulated during the ovarian cycle, being maximal during proestrus, under the action of circulating estradiol.

GnRH neurons express the Sema3A receptor neuropilin-1

We next investigated whether adult GnRH neurons express Nrp1, the obligate receptor of Sema3A, by performing double *in situ* hybridization experiments using ^{33}P-labeled Nrp1 and Dig-UTP-labeled GnRH antisense cRNA probes (Figure 2A). High levels of Nrp1 mRNA were observed in cells of the diagonal band of Broca (DBB) and in several nuclei of the rostral and medial preoptic regions, e.g. the anteroventral periventricular nucleus, the median preoptic nucleus and the medial preoptic nucleus (data not shown). The distribution of neurons expressing GnRH mRNA was similar to that described in previous *in situ* hybridization studies [26,27,28], i.e. the highest density was seen in the rostral preoptic region, followed in decreasing order by the medial preoptic area and the DBB. Nrp1 mRNA was expressed at detectable levels in 38.2 ± 2.5% of GnRH neurons in diestrus (n = 4 animals, more than 200

GnRH neurons were considered per animal) and in 50.0 ± 1.5% of GnRH neurons in proestrus (n=4 animals, $t_{(6)}$=4.039, p=0.007) with no preferential anatomical distribution. Nrp1 mRNA was also expressed in the median eminence, the projection field of GnRH neurons (Figure 2B). However the hybridization signal was not seen in the neural layers, which contain various types of glial cells associated with neuroendocrine axons [14], but instead was detected in the capillary zone (Figure 2B, inset).

To determine whether Nrp1 protein was present in GnRH axon terminals abutting onto the vascular plexus, we performed double immunolabeling studies with antibodies to Nrp1 and GnRH in the median eminence of the adult brain. Strong Nrp1 immunoreactivity was seen in the external zone of the median eminence (Figure 2C) at every anteroposterior level examined. Nrp1 labeling was distributed in the same regions as the majority of GnRH axon terminals, and, consistent with Nrp1 mRNA expression by GnRH neuronal cell bodies, GnRH-containing fibers were also found to contain Nrp1 protein (Figure 2C, arrows). However, many Nrp1-positive axon-like fibers did not contain GnRH (Figure 2C), suggesting that additional neuroendocrine systems express this receptor. In agreement with *in situ* hybridization data, endothelial cells of the pituitary portal blood vessels were also found to express Nrp1 immunoreactivity (Figure S3).

Thus, the spatial and temporal pattern of expression of Sema3A in the median eminence together with that of its receptor, Nrp1, in GnRH neurons is consistent with a functional role for Sema3A signaling in promoting GnRH axonal growth towards the vascular plexus at proestrous, when a massive release of the neurohormone into the pituitary portal circulation is required to trigger the preovulatory surge of gonadotropins.

Sema3A promotes GnRH axonal growth towards the endothelial wall of portal blood vessels

In order to assess whether Sema3A can promote the outgrowth of GnRH axons *in situ*, we analyzed hypothalamic explants containing the median eminence, maintained *ex vivo* in artificial cerebrospinal fluid. Explants obtained from either diestrous or preovulatory

proestrous rats were exposed to 1µg/ml Sema3A for 30 min, then fixed and processed for electron microscopy. Using 15 nm gold-particle labeling, we revealed a striking transformation of GnRH nerve terminals as a function of the presence or absence of Sema3A in diestrous rats. Indeed, the distance between GnRH nerve terminals (green) and the pericapillary space of pituitary portal blood vessels (p.s., pink) appeared to be significantly shorter in Sema3A-treated explants *vs.* controls (Figure 3A). Quantitative morphometric analysis showed that while the total number of GnRH nerve terminals at a distance of 10 µm or less from the parenchymatous basal lamina (which delineates the pericapillary space) did not vary significantly among treatments (n = 4 animals per condition; more than 100 GnRH-immunoreactive axon terminals were considered per explant, one-way ANOVA, $F_{(2,11)}$=0.224, p=0.803), their distribution was markedly changed (Figure 3B). In fact, the fraction of GnRH nerve terminals found at a distance of less than 1 µm from the pericapillary space increased by 400% in diestrous median eminence explants exposed to 1µg/ml Sema3A for 30 min when compared to controls (Figure 3B, left panel; n = 4 hypothalamic explants per condition; p < 0.001, one-way ANOVA). Importantly, Sema3A-mediated effects on GnRH axonal growth in diestrous explants were abolished upon pretreatment with an Nrp1-neutralizing antibody (Figure 3B, left panel; n = 4 hypothalamic explants per condition; p < 0.01, one-way ANOVA). In contrast, in median eminence explants obtained from animals in proestrus, when Sema3A is heavily released, GnRH axons naturally extend up to the pericapillary space. In this context, exogenous Sema3A treatment did not further affect the elongation of GnRH nerve terminals towards the pericapillary space (Figure 3B, right panel; n = 4 hypothalamic explants per condition; p > 0.05, one-way ANOVA). However, exposing proestrous median eminence explants to neutralizing antibodies to either the Nrp1 or Sema3A caused GnRH nerve endings to retract from the pericapillary space (Figure 3B, right panel; n = 4 hypothalamic explants per condition; p < 0.01, one-way ANOVA), suggesting that GnRH axon extension towards the endothelial wall at the transition between diestrus and proestrus is attributable to Nrp1 activation by Sema3A.

Sema3A-regulated structural changes at the neurovascular junction depend on Nrp1 expression in GnRH neurons

To assess whether the structural changes promoted by Sema3A at the GnRH neurovascular junction require neuronal expression of Nrp1, we generated mice in which *Nrp1* expression was selectively knocked out in GnRH neurons. Animals harboring the conditional *Nrp1* allele [29] were crossed with a mouse line expressing Cre recombinase under the control of the endogenous GnRH gene promoter [30] (Figure 3C). To verify the efficacy of our genetic targeting strategy, we analyzed Nrp1 expression in GnRH neurons of wild-type (*Nrp1*$^{loxP/loxP}$) and mutant (*GnRH::*Cre*; Nrp1*$^{loxP/loxP}$) littermates by immunofluorescence. In wild-type mice, the expression patterns of GnRH and Nrp1 partially overlapped within the median eminence (Figure 3D, arrow), as seen in rats (Figure 2C). In contrast, upon Cre-mediated deletion of *Nrp1* in GnRH-positive neurons, Nrp1 expression was abolished in the external zone of the median eminence where GnRH axons are found (Figure 3D, asterisk), while it was maintained in other neuroendocrine axonal populations (Figure 3D). Electron microscopic analyses of hypothalamic explants from diestrous mice treated with Sema3A (as in Figure 3A,B) confirmed the extension of GnRH nerve terminals towards the pericapillary space in the median eminence of *Nrp1*$^{loxP/loxP}$ mice (Figure 3E; $p < 0.05$, two-way ANOVA, control *vs.* Sema3A; n = 3-4 hypothalamic explants per condition; 150 GnRH-immunoreactive nerve terminals were considered per explant), while this was not observed in *GnRH::*Cre*; Nrp1*$^{loxP/loxP}$ littermates (Figure 3E; $p = 0.23$, control *vs.* Sema3A), indicating that Nrp1 expression in GnRH neurons is required to mediate this functional regulation by Sema3A *in vivo* .

Sema3A promotes neurite outgrowth from GnRH neurons *in vitro*

In order to evaluate the role of Sema3A on neurite elongation in GnRH-expressing neurons, we took advantage of our ability to obtain primary cultures of GnRH neurons from the nose of 12.5-day-old *GnRH-GFP* embryos (E12.5) (Figure 4A). As expected, primary GFP-positive neurons were seen to be surrounded by numerous Sema3A positive cells (Figure 4B), which

have been co-isolated with GnRH neurons from the nasal compartment [31]. While 24-h treatment with Sema3A had no effect on GnRH neurite elongation (data not shown), the addition of a Sema3A-neutralizing antibody to the culture medium for the same time period caused significant shortening of GnRH neuronal processes (Figure 4C). These data strongly suggest that the production of Sema3A by the surrounding cells was responsible for neurite elongation in these GnRH neurons.

To further explore the role of Sema3A on neurite outgrowth in mature GnRH-expressing neurons, we took advantage of the GnV-3 cell line, one of eleven clones of GnRH-expressing cells obtained by the conditional immortalization of cultured adult rat hypothalamic cells. GnV-3 cells grow in culture in the presence of doxycycline, but stop proliferating and undergo differentiation upon drug removal, exhibiting many of the features of mature adult GnRH neurons, including neurite growth [32]. Rat median eminence explants were cultured in proximity to aggregates of GnV-3 neuronal cells. After 72 hours of co-culture, neurites grew to the same extent on both the proximal and distal sides of GnV-3 cell aggregates (Figure 5A). To test whether Nrp1 was involved in GnRH neurite growth in response to factors released by the median eminence, Nrp1-neutralizing antibodies were added to the medium. These antibodies significantly attenuated the growth-promoting effect of the median eminence on GnV-3 neurites (Figure 5B).

Data shown in Figure 1 indicate that endothelial cells of the median eminence are a major source of p65-Sema3A, the expression of which is induced by estradiol during proestrus. To date, no biological function has been attributed to this 65 kDa isoform of Sema3A. In order to determine whether it is involved in the GnRH axonal-growth-promoting effect described above, we performed a second set of experiments using three-dimensional matrix co-cultures. Briefly, aggregates of GnV-3 cells were cultured for 72 hours along with aggregates of mock-transfected COS-7 cells or COS-7 cells secreting the 95 kDa full-length (Sema3A-FL) or recombinant 65 kDa Sema3A, in the presence or absence of the Nrp1-neutralizing antibody (Figure 5C). Remarkably, Sema3A-FL and p65-Sema3A were equally effective at promoting neurite elongation in GnV-3 cells, whereas the Nrp1 antibody stunted this

Sema3A-dependent outgrowth (Figure 5D). Altogether these findings suggest that p65-Sema3A, which is highly expressed in the median eminence during proestrus, unlike the relatively scarce 95 kDa or 125 kDa forms, acts on GnRH neuroendocrine axons through Nrp1 to promote their elongation.

The targeted infusion of Nrp1- or Sema3A-neutralizing antibodies into the median eminence is sufficient to disrupt the reproductive cycle

Consistent with the fact that Nrp1 is also expressed in GnRH neurons during embryogenesis [12,13,33], we have observed that *GnRH::*Cre*; Nrp1*$^{loxP/loxP}$ mice exhibit some alterations in the development of the GnRH system, although they display a comparable number of GnRH terminals in the median eminence as Nrp-expressing mice (Figure 3D, E). To study the physiological relevance of Sema3A-Nrp1 signaling in the mature brain independent of any potential developmental effects, however, we treated adult female rats with a regular 4-day estrous cycle with the Nrp1- or Sema3A-neutralizing antibodies found to inhibit the Sema3A-induced outgrowth of GnRH axon terminals *in situ* (see Figure 3). The antibodies were locally infused into the median eminence (Figure 6A) at a rate of 0.1µg/h for 7 days, via a cannula connected to a subcutaneously-implanted osmotic minipump. Estrous cycle monitoring by daily inspection of vaginal smears for 1 week following the initiation of treatment revealed a clear disruption of the cyclic pattern (Figure 6A). In fact, both Nrp1- and Sema3A-antibody-infused animals showed a preponderance of days in the diestrous phase, which is associated with reduced release of GnRH [34] and increased distance of GnRH axon terminals from the pericapillary space [23], and a concomitant reduction of days in proestrus (Figure 6B; n=5-6 per group, $p < 0.05$, one-way repeated measures ANOVA, during *vs*. before infusion). In contrast, animals infused with the vehicle alone (PBS) displayed normal 4-day estrous cycles (n=6) (Figure 6A,B). One week after the initiation of treatment, the animals were sacrificed and subjected to control immunoprecipitation or immunofluorescent experiments to verify that the infused Nrp1- and Sema3A- antibodies had successfully targeted receptors and ligands in the median eminence, respectively (Figure S4).

Immunoprecipitation and immunoblot analyses indicated that the infused Nrp1 antibodies did bind to, and could therefore effectively block, about 50% of the endogenous pool of Nrp1 contained in the median eminence (Figure S3A). Immunofluorescence analysis of the binding of the Sema3A-neutralizing antibody showed that it selectively targeted the external zone of the median eminence, where pituitary portal blood vessels and neuroendocrine terminals are localized (Figure S4B). Together with our *ex vivo* results, these data suggest that Sema3A-Nrp1 signaling is required for the neuroendocrine control of the ovarian cycle in the adult rat brain.

Endothelial-cell-derived Sema3A modulates the amplitude of the preovulatory LH surge

To further study the physiological relevance of Sema3A-Nrp1 endothelial-cell-to-neuron signaling in the mature brain, we used an intravenous injection of the TAT-Cre fusion protein, whose cellular uptake is enhanced compared to Cre recombinase [35] particularly in the median eminence of living animals [36], to target endothelial cells in *Sema3a*$^{loxP/loxP}$ mice. Control experiments with *tdTomato*$^{loxP/+}$ reporter mice showed that a single injection of TAT-Cre into the tail vein caused gene recombination in tanycytes, which do not express Sema3A (see Figure 1C), and in the capillary zone harboring Sema3A mRNA-expressing endothelial cells in adult females (Figure 1B, Figure 7A). Quantitative RT-PCR analyses showed that Sema3A mRNA expression was decreased by 50% in the median eminence of virgin female *Sema3a*$^{loxP/loxP}$ mice treated with TAT-Cre and subjected to a male-pheromone-induced preovulatory GnRH/LH surge protocol [37], when compared to vehicle-treated mice (Figure 7C; n=7-8 per group, t-test, $p<0.01$), while it remained unchanged in the adjacent mediobasal hypothalamus (Figure 7C; n=5-7 per group, t-test, $p>0.05$), where Sema3A mRNA is abundantly expressed (Figure 1B) and is known to play a key role in the control of GnRH release [38]. This selective attenuation of Sema3A expression in endothelial cells of the median eminence led to a significant decrease in preovulatory luteinizing hormone (LH) serum levels (Figure 7B; n=7-8 per group, t-test, $p<0.05$), used as an index of GnRH release

[39]. Finally, real-time PCR analyses of Sema3A expression in the median eminence of wild-type mice across the estrous cycle revealed that Sema3A mRNA levels were significantly higher in proestrus than in diestrus (1±0.11 arbitrary units at proestrus *vs*. 0.54±0.09 arbitrary units at diestrus, n=7 and 3, respectively, $t_{(8)}$=2.602, p=0.032). Together, these data suggest that the ovarian cycle modulates Sema3A expression in endothelial cells of the median eminence, which in turn promotes the elongation of GnRH neuroendocrine axons on the day of proestrus to control the amplitude of the preovulatory GnRH/LH surge.

Discussion

The reproductive cycle of mammals is critically regulated by hypothalamic GnRH neurons [25], which periodically extend their axons in the median eminence towards the pericapillary space, into which they release the GnRH neuroendocrine signal during a specific time window [23,40]. The potential role of vascular endothelial cells in controlling this cyclic growth of axon terminals has not been investigated. Our *in vivo* and *in vitro* findings collectively indicate that Sema3A is a vascular factor promoting GnRH axonal growth in the adult brain and playing a pivotal role in orchestrating the central control of reproduction. We propose that Sema3A released by fenestrated endothelial cells of the hypothalamo-hypophyseal portal blood vessels cyclically induces GnRH neurons to extend their terminals towards the pericapillary space, this directionality being controlled by the glial scaffold along which GnRH axonal fibers travel within the median eminence (see for review [14]). In turn, this mechanism regulates neuropeptide release at key stages of the ovarian cycle, such as at proestrus, when the preovulatory surge of GnRH occurs.

Our ultrastructural analyses in *GnRH::cre*; *Nrp1*$^{loxP/loxP}$ mice, which do not exhibit any defect in GnRH axonal targeting when compared to *Nrp1*$^{loxP/loxP}$ mice (Figure 3D,E), as well as the effect of locally restricted Sema3A infusion on GnRH axonal growth (Figure 3B), strongly indicate that the effects of Sema3A on axonal plasticity within the median eminence depend on direct Sema3A-Nrp1 signaling in post-developmental GnRH terminals. The functional consequence of endothelial Sema3A secretion on GnRH axonal plasticity has, in addition, been demonstrated by the selective invalidation of Sema3A expression in the median eminence of adult *Sema3a*$^{loxP/loxP}$ mice by the intravenous injection of the recombinant TAT-Cre protein. Indeed, this approach, which further circumvents any putative developmental effect that might occur with the use of classic promoter-driven Cre expression technology, confirms that the endothelial-Sema3A-promoted elongation of GnRH axons modulates the amplitude of the preovulatory GnRH/LH surge on the day of proestrus.

The molecular pathways that underlie this cyclic Sema3A-Nrp1-mediated GnRH axonal sprouting are unknown, although they appear to be intrinsic to GnRH neurons since Sema3A

promotes GnRH neurite outgrowth both in tissue explants and in isolated cell cultures. A recent study has intriguingly suggested that Sema3A could promote axonal growth by inducing protein kinase G activity [41]. Notably, Sema3A receptors are broadly expressed in the axon terminals of other neuroendocrine systems and this signal has been proposed to serve as a coordinator of structural and functional synaptic plasticity in various neuronal circuits [6]. In our study, only about 50% of the Nrp1 expressed in the median emincence was neutralized by antibody infusion. It would be of interest to investigate the effects of more complete Nrp1 invalidation in adult animals, as well as the potential role of endothelial Sema3A in the growth of hypothalamic neuronal projections controlling other anterior pituitary functions, such as the growth-, stress- and thyroid-hormone axes.

An intriguing finding of this study is that endothelial cells of the median eminence appear to selectively release the 65 kDa isoform of Sema3A. Interestingly, we show that estradiol mimics ovarian-cycle effects on p65-Sema3A production in ovariectomized rats and that, in agreement with a previous *in vitro* study [42], endothelial cells of the median eminence express the estrogen receptor ERα. The mechanisms underlying these changes in protein levels within the median eminence are unknown but likely involve changes in *Sema3a* transcription, rather than its translation or post-translational processing such as furin cleavage. In line with this idea, analysis of the *Sema3a* gene using the ALGEN PROMO 3.0 software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) predicts the presence of a putative estrogen-receptor-binding element at 100 bp upstream of the transcription initiation site; further experiments will be required to determine whether this presumptive binding site is actually functional. Even though the biological activity of p65-Sema3A has been validated in heterologous systems mimicking growth-cone collapse [43] and in co-culture systems using sympathetic ganglion explants [22], this isoform was originally described as a proteolytic by-product of p95-Sema3A, with reduced functional activity [22]. Similarly, it has been reported recently that the anti-angiogenic activity of the 61 kDa proteolytic fragment of Sema3B is dramatically reduced compared to the full-length 83 kDa isoform [44]. In contrast, here we demonstrate that 65 kDa and 95 kDa Sema3A

isoforms are equally effective at promoting GnRH neurite elongation ex vivo (Figure 5C, D), indicating that the proteolytic cleavage of Sema3A does not interfere with its axonal-growth-promoting activity. In conjunction with the fact that the expression of the 65 kDa isoform of Sema3A, unlike the 125 kDa precursor and the best-known 95 kDa secreted isoform, is subject to cyclic changes, being maximal on the day of proestrus, these results uncover for the first time a physiological role for p65-Sema3A in the adult brain.

In conclusion, we show that in the median eminence of the hypothalamus, p65-Sema3A is an endothelial-cell-derived protein that acts on Nrp1 receptors in GnRH neuroendocrine processes, which have previously been seen to express axonal markers such as GAP-43 [27], to promote their growth towards the target vascular wall during a time window of the reproductive cycle that is critical to ovulation. Because ovarian-cycle-regulated GnRH axonal elongation in the adult brain is likely to depend on the coordinated action of many extracellular factors, endothelial p65-Sema3A may work in concert or in competition with other secreted molecules including VEGF, nitric oxide, TGF-β1 and BDNF, which are particularly enriched in the capillary zone of the median eminence [21,36,45,46] and may influence axonal plasticity by modulating the endothelial expression of or responsiveness to semaphorins [8,47,48,49]. These findings have implications for the possible roles of p65-Sema3A in adult brain function. Finally, our results raise the intriguing possibility that vascular semaphorins may play important and unexpected roles in the adult neural plasticity underlying several other key physiological processes such as learning, stress and the control of energy homeostasis [50,51,52,53].

Methods

Ethics Statement

All experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding mammalian research, and were approved by the Institutional Animal Care and Use Committee of Lille and the animal experimentation committee of the Royal Netherlands Academy of Arts and Sciences in Amsterdam.

Animals

Rats. Female Sprague Dawley rats (Janvier, Saint-Berthevin, France) weighing 250-300 g were used for *in situ* hybridization, immunohistofluorescence, immunoprecipitation, western blotting of tissue explants, electron microscopy and intracerebral infusion experiments. Vaginal smears were examined daily, and only rats that exhibited at least two consecutive 4-day estrous cycles were used for experiments. Diestrus 1 and 2 were defined by the predominance of leukocytes in the vaginal lavage, the day of proestrus was characterized by the predominance of round nucleated epithelial cells, and estrus was distinguished by a large number of clustered cornified squamous epithelial cells.

C57BL/6 Mice. *Nrp1*$^{loxP/loxP}$ [29] and *tdTomato*$^{loxP/+}$ mice were purchased from JAX® mice (Jackson laboratory, Maine, USA), while *Sema3a*$^{loxP/loxP}$ mice were generated as described previously [54]. *GnRH::Cre*$^{+/-}$ mice were a generous gift from Dr. Catherine Dulac (Howard Hughes Medical Institute, MA, USA) [30]. *GnRH-GFP* mice were kindly provided by Dr. Daniel J. Spergel (Section of Endocrinology, Department of Medicine, University of Chicago, Illinois)[55]. Animals were genotyped by PCR using tail DNA samples. Genomic DNA was extracted using the NucleoSpin®Tissue kit (Cat. No. 740.952.250, Macherey-Nagel, Hoerdt, France). PCR was carried out using GoTaq DNA Polymerase (Promega, USA) under the following cycling conditions: 95 °C, 2 min; 95 °C, 1 min, 57.3°C, 1 min, 72°C, 1 min, 35 cycles; 72°C, 5 min; 4°C until analysis. The primer sequences used were: Cre-sense 5'-ATGGCTAATCGCCATCTTCC-3', Cre-antisense 5'-CTGGTGTAGCTGATGATCCG-3'; Nrp1-

sense 5'-AGGTTAGGCTTCAGGCCAAT-3', Nrp1-antisense 5'-GGTACCCTGGGTTTTCGATT-3'; Tomato-sense 5'-CTGTTCCTGTACGGCATGG-3'; Tomato-antisense 5'-GGCATTAAAGCAGCGTATCC-3'.

Adult (2-4-month-old) female rats and mice were housed in a room with controlled photoperiod (12h/12h light cycle) and temperature (21-23°C). Animals were allowed access to tap water and pelleted food *ad libitum*.

Primary culture of median eminence endothelial cells

The purification of endothelial cells of the median eminence was realized by sequential immunopanning. Endothelial cells of the median eminence were isolated from 10-day-old rats using a procedure adapted from a protocol kindly provided by Dr. Ben Barres (Stanford, CA) [20], as described previously [21]. In brief, median eminence explants were enzymatically dissociated at 37°C for 90 min using a solution of papain (33 U/ml) (Worthington/Cooper, Lakewood, NJ) in MEM/HEPES (Invitrogen) containing L-cysteine (0.4 mg/ml) (Sigma) and DNase (125 U/ml) (Sigma). Tissues were then triturated in a solution containing ovomucoid trypsin inhibitor solution (2 mg/ml) (Boehringer Mannheim, Mannheim, Germany), DNase (125 U/ml), and BSA (1 mg/ml) (Sigma), to obtain a suspension of single cells. The suspension was filtered through a 20µm nylon mesh. After centrifugation at 550 x g, single cells were successively panned on a Petri dish coated with an anti-CD90 mouse monoclonal antibody, which recognizes the rat Thy1.1 antigenic determinant (MRC-OX7; Serotec, Oxford, UK), to deplete macrophages and fibroblasts, and on a second Petri dish coated with rat neural antigen (RAN)-2 ascites (LGC Promochem, Molsheim, France) to deplete meningeal cells and type-1 astrocytes; the remaining cells were incubated in a Petri dish coated with an affinity-purified rabbit antibody raised against PV1, which selectively recognizes fenestrated vascular endothelial cells of the median eminence [18]. Purified endothelial cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 2% penicillin/streptomycin until they reached confluency. They were then recovered by trypsin digestion and plated in 10cm dishes or on poly-D-lysine (Sigma) coated

coverslips. Three primary cultures from three independent litters were used in the present study.

To produce endothelial cell-conditioned medium (EC-CM), cell monolayers were cultured in 10-cm dishes for 48h in DMEM (devoid of phenol red; Invitrogen) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 5µg/ml insulin (Sigma), and 100µM putrescine (Sigma). For western blot analysis, 10ml of EC-CM were concentrated using a Centriplus® centrifugal filter device (size cutoff of 10 kDa, Cat. 4411, YM10; Millipore, Bedford, MA) to obtain a final volume of 30-40µl. The concentrated medium was mixed with NuPAGE® LDS sample buffer 4X (Invitrogen) to obtain a final concentration of 1X, boiled for 5 min, and stored at -80°C until loading.

Inhibition of furin proteolytic activity in mouse endothelial cells

Confluent cultures of mouse endothelial cells SVEC4-10 were incubated in serum-free DMEM medium for 48 hours, either in presence or absence of the furin protease selective inhibitor Dec-RVKR-CMK (100µM; Bachem). Cell-conditioned media were concentrated with a size cutoff of 50 kDa (Vivaspin, Sartorius) and a sample size equivalent to the medium collected from a 2 cm^2 cell monolayer was separated by SDS-PAGE and eventually analyzed by Western blotting.

Reverse transcription-PCR amplification

Complementary DNA fragments derived from mRNAs encoding Sema3A were generated by reverse transcription (RT)-PCR of total RNA extracted from the neonatal rat brain, adult female rat median eminence or primary cultures of median eminence endothelial cells. One µg of Trizol (Life Technologies, Grand Island, NY)-extracted RNA was reverse transcribed to cDNA in a final volume of 10µl containing 200 U of SuperScript II reverse transcriptase (Invitrogen), 20 U of RNase inhibitor (Promega, Madison, WI), and 0.5µg of oligo-dT primer. After a 1h incubation at 42°C, the reaction was stopped by heating at 94°C for 5 min. PCR was performed by using 1µl of each reverse transcription reaction and Hotstart Taq DNA

polymerase (Qiagen, France) in a volume of 50µl. The thermocycling conditions were 15 min at 95°C for enzyme activation, followed by 35 cycles at 94°C for 1 min, annealing at 53°C for 1 min, 72°C for 1 min, followed by a final extension period of 10 min at 72°C. A 364 bp DNA fragment (sense 5'-TCATCCTGAGGACAACAT-3', antisense 5'-GCATATCTGACCTATTCT-3') corresponding to nucleotides 444-807 (NM017310) was amplified. PCR with the substitution of cDNA with RNA served as a control. All cDNAs generated by RT-PCR were verified by sequencing. β-actin cDNA was amplified with primers 5'-AACTGACAGACTACCTCA-3' and 5'-GCTCATAGCTCTTCTCCA-3' to verify the quality of samples (not shown).

In situ hybridization

Tissue preparation. The brains of adult female rats (n=4 per experiment) were fixed by transcardiac perfusion with ice-cold 4% paraformaldehyde in 0.1M borate buffer at pH 9.5, postfixed in the same fixative containing 10% sucrose for 2h at 4°C, and immersed in 20% sucrose in 0.02M potassium phosphate buffered saline prepared with DEPC-treated water at 4°C overnight, embedded in Tissue-Tek (Miles, Elkhart, IN), and frozen in liquid nitrogen. Coronal sections (30µm) cut on a cryostat were mounted onto gelatin-subbed and poly-L-lysine coated slides, dried under vacuum for 4h, and stored in boxes with dessicants at -80°C until use.

^{33}P-labeled cRNA probes. Plasmids were provided by Dr. Marc Tessier-Lavigne (University of California, San Francisco, USA). The lyophilized plasmid vector pBluescript II SK containing a PstI/BamHI fragment of 1181 bp corresponding to nucleotides 429-1610 (Genbank X85993) of the mouse Sema3A complementary DNA (cDNA) was used. To generate antisense ^{33}P-labeled cRNA, the plasmids were linearized by digestion with NotI and subjected to *in vitro* transcription with T7 RNA polymerase. For generation of sense ^{33}P-labeled cRNA, the plasmids were linearized by digestion with XhoI and subjected to *in vitro* transcription with T3 RNA polymerase, according to previously described protocols (Bouret et

al., 2004). The lyophilized plasmid vector pBluescript II SK containing a 1285 bp PstI fragment of rat Nrp1 corresponding to 490bp of the 5'UTR and 795bp of the coding region was used. SacI and T3 RNA polymerase were used to synthesize the antisense probe, and HindIII and T7 RNA polymerase were used to synthesize the sense probe.

Digoxigenin-labeled GnRH cRNA probe. the plasmid vector GST7 containing a 330bp BamHI/ HindIII insert of GnRH cDNA was linearized with HindIII for antisense and with BamHIII for sense probes. The riboprobes were synthesized *in vitro* in a 10µl transcription reaction volume containing 1µg of linearized GnRH cDNA, 1µl of a 2mM solution of Digoxigenin-11-dUTP (Roche Diagnostics, Meylan, France), 2µl of a mixture of GTP, CTP and ATP (2.5mM each) diluted from 10mM stocks, 1µl of DTT 100 mM, 1µl of RNasin, RNA polymerase (T7 for antisense and SP6 for sense), and 10X transcription buffer. This mixture was incubated at 37°C (T7) or at 40°C (SP6) for 1h. Residual DNA was digested with DNAse.

In situ hybridization. Single-label *in situ* hybridization for Sema3A or for Nrp1 was performed as we have previously described [45]. Briefly, after proteinase K digestion (10µg/ml at 37°C; Boehringer Mannheim, Indianapolis, IN) and acetylation (0.0025% acetic acid at room temperature) for 30 min, the sections were dehydrated through an ascending ethanol series and dried under vacuum for 4h. The ^{33}P-labeled cRNA probes were heated at 65°C for 5 min with 500µg/ml yeast tRNA (Sigma, Saint Quentin Fallavier, France) and 50µM dithiothreitol (DTT) (Euromedex) in DEPC (Sigma)-treated water and then diluted to an activity of $5x10^6$ cpm/µl with hybridization buffer containing 50% formamide (Boehringer Mannheim), 0.25 M sodium chloride, 1x Denhardt's solution (Sigma), and 10% dextran sulfate (Pharmacia). Eighty µl of this hybridization solution were pipetted onto the sections, which were covered with a glass coverslip and sealed with DPX (Electron Microscopy Sciences) before incubation for 20h at 58°C. Then, the slides were washed four times (5 min each) in 4X Saline Sodium Citrate (SSC) before digestion with RNase (20µg/ml for 30 min at 37°C; Sigma), and rinsed at room temperature in decreasing concentrations of SSC (2X, 1X, 0.5X

for 10 min each) containing 1 mM DTT, to final stringency in 0.1X SSC at 65°C for 30 min. After dehydration in an ascending ethanol series, the sections were vacuum-dried, dipped in NTB-2 liquid emulsion (Kodak), dried and stored in the dark. Emulsion-coated slides were developed after 1 month for Sema3A or after 14 days for Nrp1 with Kodak D-19 developer. The sections were then counterstained with thionine, dehydrated, cleared in xylenes, and mounted with DPX.

For double-label *in situ* hybridization, prehybridization, hybridization and posthybridization procedures were similar to those described above, with the exception that the sections were not dehydrated after the last 0.1X SSC rinse, but were further processed for the localization of digoxigenin-labeled hybrids. Briefly, after rinsing in 2X SSC, sections were blocked overnight with 2X SSC buffer containing 0.3% Triton X-100 and 2% normal goat serum, then washed in buffer 1 (100mM Tris-HCl and 150mM NaCl, pH 7.4), and incubated for 5 h with alkaline-phosphatase-conjugated anti-digoxigenin (Roche) diluted 1:1000 with buffer 1 containing 1% normal goat serum. After rinsing in buffer 1 and in buffer 2 (100mM Tris-HCl, 50mM MgCl2, and 100mM NaCl, pH9.5), sections were incubated in nitro blue tetrazolium (NBT) + 5-bromo-4-chloro-3-indolyl phosphate (BCIP) chromogen solution (Sigma) for 12h, and the reaction stopped with buffer 3 (10mM Tris-HCl, 1mM EDTA, pH 8). Sections were then quickly dehydrated in ethanol, dried, and dipped in K5 emulsion (Ilford, Saint-Priest, France).

Quantitative analysis. Nrp1 mRNA was quantified in GnRH neurons as follows. GnRH mRNA-expressing cells were observed under brightfield illumination, and Nrp1 mRNA-expressing cells were observed under darkfield illumination. Thus, each digoxigenin-labeled GnRH neuron was examined for the presence of silver grains, indicating Nrp1, using alternate bright- and darkfield observations. About 200 GnRH cells were studied per animal. The total number of GnRH mRNA-expressing cells and the number of GnRH cells coexpressing Nrp1 mRNA was counted per section. From these data, the proportion of GnRH neurons expressing Nrp1 mRNA per animal was calculated and averaged.

Fluorescence-activated cell-sorting analysis

Median eminences from female P90 rats (n = 3) were microdissected and enzymatically dissociated using a Papain Dissociation System (Worthington, Lakewood, NJ) to obtain single-cell suspensions. Subsequently, dissociated cells were resuspended in Hanks' balanced salt solution (HBSS; Invitrogen) containing 1% BSA. Endothelial cells were labeled for 30 min at 4° C using an affinity-purified rabbit antibody raised against PV1 (1:100), which selectively recognizes fenestrated vascular endothelial cells of the median eminence [18], followed by a 15-min incubation at 37 C with an Alexa-Fluor 488 anti-rabbit secondary antibody (1:100, Invitrogen).

FACS was performed using an EPICS ALTRA Cytometer device (Beckman Coulter, Inc.). Sorted GFP-positive cells and GFP-negative cells (yield: 30000 cells isolated from each animal) were collected into two separate tubes containing 500 µl of sterile HBSS (Invitrogen) and subsequently centrifuged for 1 min at 7500 *g* (maximum) to pellet the cells. HBSS was then aspired and 8 µl of a solution containing 1 µl of 0.1% Triton X-100 and 7 µl of Prime RNase inhibitor (diluted 1:100 in diethylpyrocarbonate-treated water; Invitrogen) was added. Captured cells were used to synthesize first-strand cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. Controls without reverse transcriptase were performed to demonstrate the absence of contaminating genomic DNA. RNA isolated from the adult rat brain was also reverse transcribed and used as a positive control. PCR was performed at 35 cycles on a thermocycler (30 s denaturation at 94°C, 30 s annealing at 55–65°C, and 2 min elongation at 72°C). PCR primer pairs were as follows: Sema3A forward primer, 5′-ATGAATGCAAGTGGGCTGGA-3′; Sema3A reverse primer, 5′-CGGTCCTGATGGGATGATGG-3′; PV1 forward primer, 5′- TGAAGGAGGGCAACAAGACC-3′ PV1 reverse primer, 5′-AACGGTAGACCAGCGAATCC-3′; β3-tubulin forward primer, 5′-CGTCTCTAGCCGAGTGAAGTC-3′; β3-tubulin reverse primer, 5′-TCCGAGTCCCCCACATAGTT-3′; DARP32 forward primer, 5′-

CCTCATAGAGCGCGGGATTT-3′; DARP32 reverse primer, 5′-CGGATCATCTCCACCTGTCG-3′; TSH forward primer, 5′- GAGAGTGTGCCTACTGCCTG-3′; TSH reverse primer, 5′- CATCCCGGTATTTCCACCGT-3′; GAPDH forward primer, 5′-GGACCAGGTTGTCTCCTGTG-3′; GAPDH reverse primer, 5′-ATTCGAGAGAAGGGAGGGCT-3′. Qualitative RT-PCR experiments were run three times on sorted cells from 3 different animals. 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): PV1 (PV1_Rn00571706_m1); Sema3A (Sema3A_Rn00436469_m1); ERα (Esr1_Rn01640372_m1); ERβ (Esr2_Rn00562610_m1); housekeeping ribosomal RNA (Rn45_Rn03928990_g1).

Immunohistochemistry

Two-month-old female rats and monogenic and bigenic mouse littermates were perfused transcardially with 4% paraformaldehyde in 0.1M PBS, pH7.4. Rat and mouse brains were postfixed in the same fixative containing 20% sucrose for 2h at 4°C, immersed in 20% sucrose in 0.1M phosphate buffered saline overnight at 4°C, embedded in Tissue-Tek and frozen in liquid nitrogen. Coronal sections (14µm) were cut on a cryostat and mounted onto chrome-alum-gelatin coated slides, and subjected to fluorescent labeling. Briefly, the sections were washed in 0.1M PBS, then incubated for 10 min at room temperature in blocking solution containing 2% normal donkey serum (D9663; Sigma) and 0.3% Triton X-100 in 0.1M PBS. Sections were then incubated overnight at 4°C with the primary antibodies diluted in the same solution. Primary antibodies used were rabbit polyclonal antibodies diluted 1:3000 for GnRH [56] and PV-1 [18] and a goat polyclonal antibody to the extracellular domain of rat Nrp1 (AF 566; R&D Systems) diluted 1:400. Sections were washed in 0.1M PBS, and labeling revealed by incubation for 1h at room temperature with Alexa Fluor 568-conjugated anti-rabbit or Alexa Fluor 488-conjugated anti-rabbit antibodies (1:400; Molecular Probes) or biotin-conjugated donkey anti-goat IgGs (1:400; Jackson

Immunoresearch, West Grove, PA) followed by Alexa Fluor 488- or Alexa Fluor 568-conjugated streptavidin (1:500) for 1h. Vascular endothelial cells were visualized with tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Bandeiraea simplicifolia* lectin (1:600; Sigma). After washes, slices were coverslipped with Permafluor medium (434990; Immunon, Pittsburgh, PA).

For the detection of Sema3A, female mouse brains were quickly harvested, embedded in ice-cold Tissue Tek, frozen in isopentane (-55°C) and stored at -80°C until use. Brains were cut into 20 µm-thick coronal sections and processed for immunohistochemistry as follows. Slide-mounted sections were (1) fixed by immersion for 1 minute in methanol/acetone (vol/vol) at -20°C; (2) blocked for 30 minutes using a solution containing 4% normal goat serum and 0.3% Triton X-100; (3) incubated overnight at 4°C with a rabbit polyclonal anti-Sema3A (1:50, sc-10720; Santa Cruz Biotechnology, Santa Cruz, CA) which selectively recognizes Sema3A in western blots (Figure 1E,F,G and Figure S4), and a rat anti-mouse PV1 (MECA32 clone, 1:200, gift from Professor Britta Engelhardt, Switzerland) followed by 1h at room temperature with a cocktail of secondary Alexa Fluor-conjugated antibodies (1:500, Molecular Probes, Invitrogen, San Diego, CA); (4) counterstained with Hoechst (1:10000, Molecular Probes, Invitrogen), and coverslipped using Mowiol (Calbiochem, USA).

Protein extraction and immunoprecipitation from tissue explants

Median eminences were obtained from cycling diestrous and proestrous rats killed at 16h. After dissection, each fragment was placed in a microcentrifuge tube, snap frozen in dry ice, and stored at -80°C. Protein extracts of a set of two median eminences were prepared by trituration of the fragments through 22 and 26 gauge needles in succession in 200µl of lysis buffer (25mM Tris, pH 7.4, β-glycerophosphate, 1.5mM EGTA, 0.5mM EDTA, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, 10µg/ml leupeptin and pepstatin A, 10µg/ml apoprotinin, 100µg/ml PMSF, and 1% Triton X-100) for straight analysis, or in 750µl for immunoprecipitation. After 30 min of gentle rocking at 4°C, the tissue lysates were cleared by centrifugation at 14000 rpm for 15 min. For straight analysis, the protein content of

supernatants was determined using BCA protein assays (Pierce Chemical, Rockford, IL), and equal amounts of proteins were mixed with SB4X to obtain a final volume of 50 microliters in 1X NuPAGE LDS sample buffer (Invitrogen). For immunoprecipitation, 60µl of protein A-sepharose (1:1 slurry in lysis buffer, P3391; Sigma) were added to the supernatants in order to remove endogenous IgGs (preclearing). The samples were then rocked for 30 min at 4°C, the beads were centrifuged for 15 s at 14,000 rpm, and the supernatants collected. Equal amounts of protein (350 µg) in 750µl of lysis buffer were incubated with 2µg of anti-Nrp1 (AF566, R&D systems) with gentle rocking overnight at 4°C.Thereafter, 60µl of protein A-sepharose beads were added to the antibody-antigen complex and incubated for 3h at 4°C. The sepharose beads were collected by centrifugation. Beads were then washed twice with ice-cold lysis buffer, and boiled for 5min in 50µl of 2X NuPAGE® LDS sample buffer (Invitrogen). Samples were stored at -80°C until use.

Western blotting

Samples were boiled again for 5 min after thawing and electrophoresed for 1h at 150 V in precast 3-8% Tris-acetate gels or for 35 min at 200 V in precast 4-12% MES polyacrylamide-SDS gels (Invitrogen). Then, the proteins were transferred onto 0.2µm pore-size polyvinylidene difluoride (PVDF) membranes (Invitrogen) for 1h at room temperature (RT). Blots were incubated for 1h in Tris-buffered saline (TBS; 0.05M Tris, pH 7.4, 0.15M NaCl) with 0.05% Tween 20 (TBS-T) and 5% nonfat milk at RT, or in TBS with 1% Tween 20 for 1h at RT. The membranes were exposed to the primary antibody (goat polyclonal anti-Nrp1,1:100, AF566, R&D Systems, or rabbit polyclonal anti-Sema3A, 1:100, sc-10720, Santa Cruz Biotechnology) diluted in TBS-T with 5% nonfat milk overnight at 4°C with gentle rocking. Immunoreactions were detected with horseradish peroxidase-conjugated secondary antibodies (Sigma) in TBS-T with 5% nonfat milk for 1h at room temperature, and developed using enhanced chemiluminescence (NEL101; PerkinElmer, Boston, MA). When necessary, the membranes were stripped (PBS; 5min at 100°C), and incubated with a goat polyclonal

antibody against actin (1:1000; Santa Cruz Biotechnology). Protein expression was densitometrically analyzed using Scion Image software (Scion Corporation, MA, USA)

Assessment of ultrastructural changes in GnRH nerve terminals induced by Sema3A

To determine whether Sema3A promotes GnRH nerve terminal plasticity, *ex vivo* experiments were carried out according to previously described protocols [21]. Female rats weighting 250-300g were killed on diestrus 2 (n=12) or proestrus (n=12) by decapitation. Four animals were used per condition. After rapid removal of the brain, hypothalamic explants were microdissected without damaging the median eminences. Explants were placed in 12–well plates and preincubated for 30 min at 37°C in 1mL of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4.5 mg/ml D-dextrose and 5μM tetrodotoxin, with or without Nrp1- or Sema3A-neutralizing antibodies (15μg/ml, AF566 and MAB-1250, respectively, R&D Systems), under an atmosphere of air containing 5% CO_2. The Nrp1-neutralizing antibody has been shown to selectively target the semaphorin-binding domain of Nrp1 [13]. In addition, we confirmed the specificity of the Sema3A-neutralizing antibody, which specifically detects Sema3A in the conditioned media from transfected COS-7 cells (Figure S4C), using immunohistochemistry (Figure S4B). After this preincubation, tissues were placed in fresh medium with or without a recombinant human Semaphorin-3A/Fc chimera (1000ng/ml; 1250-S3, R&D Systems) for an additional 30 min incubation period. Explants were then processed for electron microscopy as described previously [23]. Briefly, tissues were fixed by immersion in a solution of 2% paraformaldehyde, 0.2% picric acid and 0.1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 2h at 4°C. Tissues were postfixed with 1% OsO_4 in phosphate buffer for 1h at room temperature. After dehydration, tissues were embedded in Araldite. Semithin sections (1-2 μm -thick) were used to progressively approach and identify the portion of the median eminence targeted for ultrastructural studies, i.e. the area where the pituitary stalk becomes distinct from the base of the hypothalamus but still remains attached to it by the hypophyseal portal vasculature [23]. This area, which does not extend beyond 20μm, contains high numbers of GnRH fibers. To detect GnRH

immunoreactivity, ultrathin sections (80-90 nm thick) collected on Parlodion 0.8%/isoamyl acetate-coated 100 mesh grids (EMS, Fort Washington, PA) were treated using an immunogold procedure described previously [23]. Briefly, after a preliminary treatment with H_2O_2 (10%; 8 min) and a blocking step in TBS (0.1 M Tris, pH 7.4, 0.15 M NaCl) containing 1% normal goat serum and 1% bovine albumin serum (TBSB) (10 min at room temperature), the grids were floated on a drop of the following reagents and washing solutions: (1) rabbit anti-GnRH (1: 5000) in TBSB for 60h at 4°C, (2) TBS to remove excess antibodies (three times for 10 min), (3) colloidal gold (18 nm)-labeled goat anti-rabbit immunoglobulins (Jackson ImmunoResearch) 1:20 in TBS for 90 min at room temperature, (4) TBS (three times for 10 min), and (5) distilled water (three times for 10 min). The sections were then counterstained with uranyl acetate and lead citrate before observation. The specificity of the GnRH antisera used has been discussed previously [56]. Ultrathin immunolabeled sections were examined with a Zeiss transmission electron microscope 902 (Leo, Rueil-Malmaison, France) and images were acquired using a Gatan Orius SC1000 CCD camera (Gatan France, Grandchamp, France). Morphometric analysis was performed by an investigator blind to hypothalamic explant treatment on digitalized images taken at an original magnification of 12,000 X from 10-15 ultrathin sections per animal, with a space of 25 sections between them, to avoid taking the same GnRH nerve terminal into consideration twice (the diameter of a GnRH nerve terminal rarely exceeds 2 µm). All GnRH-immunoreactive nerve terminals located at less than 10 µm from the parenchymatous basal lamina (i.e., the pial surface of the brain) were taken into consideration, i.e. more than 100 distinct axon terminals per animals (i.e., almost all GnRH nerve terminals abutting onto the pituitary portal blood vessels in the aforementioned 20-µm-thick region of the median eminence). Immunolabeled terminals confined to a distance of 10 µm or less from the basal lamina were imaged and the distance from the nerve terminal to the pericapillary space recorded.

Similar electron microscopic analyses were performed in 60-day-old diestrous *Nrp1*$^{loxP/loxP}$ (n = 8) and *GnRH*::Cre; *Nrp1*$^{loxP/loxP}$ (n = 8) mice. Three to four animals were used per condition.

Functional assay in GnRH primary cultures

Timed-pregnant *GnRH-GFP* mice were anesthetized with an intraperitoneal injection of 200 mg/kg ketamine and killed by cervical dislocation. E12.5 embryos were harvested and nasal regions were dissected from each embryo and dissociated using the Papain Dissociation System (Worthington, Lakewood, NJ) to obtain single-cell suspensions.

Dissociated nasal tissue containing *GnRH-GFP* cells, mesenchymal cells and olfactory/vomeronasal cells were cultured in DMEM/F12 (Invitrogen) supplemented 1% L-glutamine (Invitrogen) and D-(+)-glucose (final concentration 1%) at 37°C with 5% CO_2 for 24 hrs in the presence or absence of mouse monoclonal anti-Sema3A (15 μg/ml) [57] neutralizing antibody (R&D system, MAB-1250) before processing for immunocytochemistry (cntr: N = 3 independent experiments, n = 146 cells; treated: N = 3 independent experiments, n = 143 cells). Anti-Sema3A binding in living cells was visualized using an Alexa Fluor 568-conjugated anti-mouse antibody (1:400; Invitrogen), and GFP using an anti-GFP chicken primary antibody (1:1000, ab13970, Abcam) and an Alexa Fluor 488-conjugated anti-chicken secondary antibody (1:400; Jackson Immunoresearch, West Grove, PA) in 4%-paraformaldehyde-fixed cultures.

Quantification of GnRH fiber length was performed on digitized photomicrographs using the NeuronJ plugin of ImageJ software (National Institute of Health); 10-20 pictures were taken for each culture well and a total of 200 cells for each treatment conditions was analyzed. Twelve embryos were used for the control group and 13 for the treatment group. All experiments used primary cultures generated from different individuals on multiple culture dates. Data are presented as means ± SEM. For comparison between the two groups, a two-tailed unpaired Student's t test was used. Normality of the data was tested with the Shapiro-Wilk test.

Cell lines

COS-7 and SVEC4-10 cells (ATCC) cells were grown in a monolayer at 37 C in a 5% CO2 atmosphere, in DMEM (Life Technologies, Inc.) containing 1 mM sodium pyruvate, 2 mM glutamine (Life Technologies, Inc.), 100 µg/ml streptomycin, 100 U/ml penicillin and 4500 mg glucose (ICN Biomedicals, Inc.), supplemented with 10% FBS (Invitrogen). The medium was replaced at 2-day intervals. Subconfluent cells were routinely harvested by trypsinization and seeded onto 58 cm^2 dishes (100,000 cells). For all experiments, only cells within six passages were used.

GnV-3 cells are one of eleven clones of GnRH-expressing cells obtained by the conditional immortalization of cultured adult rat hypothalamic cells [58]. GnV-3 cells express markers of well-differentiated neurons and do not express markers of glial cells [59]. Cells were grown in Proliferation medium consisting of Neurobasal A medium with B27 supplement (20 µl/ml, Invitrogen-Gibco), PSN (1X, Invitrogen), Glutamax I (Invitrogen), doxycycline hydrochloride (0.5µg/ml, Sigma), FBS (10µl/ml, Biological Industries) and βFGF (5ng/ml, Invitrogen). Doxycycline promotes the proliferation of these conditionally immortalized cells. To induce the differentiation of GnV-3 cells, the culture medium was replaced by differentiation medium (containing Neurobasal A, B27 supplement, PSN, Glutamax I and βFGF).

In vitro cell transfection

COS-7 cells were transiently transfected using the Fast-forward protocol. Briefly, a 58 cm^2 subconfluent dish was split into four dishes in OptiMEM medium (Invitrogen) about 1h before high-efficiency liposome transfection (Lipofectamine 2000, Invitrogen). Each dish was transfected with 2-4 µg of DNA construct (full-length human Semaphorin 3A-myc cDNA plasmid, 65 kDa truncated human Semaphorin 3A-myc cDNA plasmid and empty vector for control). The latter construct was generated by site-directed mutagenesis using a QuickChange II XL site-directed mutagenesis kit (Agilent Technologies) to introduce a Stop codon after the conserved Arginine residue 555, corresponding to the furin cleavage site.

Cell aggregates

Three-dimensional matrix assays were performed by co-culturing GnV-3 cell aggregates with median eminence explants dissected from adult female rats as described above. In another set of experiments, GnV-3 cell aggregates were co-cultured with COS-7 cells transfected either with full-length Sema3A (Sema3A-FL) or 65 kDa Sema3A, or with the control vector, as previously described [60].

For the aggregates, cells were collected by trypsinization, resuspended in 5 µl of growth-factor-free Matrigel (BD Biosciences, San Jose, CA) (10^6 cells/ml for both GnV-3 and COS-7) and placed on the lid of a culture dish. As cell aggregates were formed in the droplets, they were plated onto Millicell inserts coated with growth-factor free Matrigel (Millipore) and maintained in culture for 72h. Cultures were grown for 2–3 days in Neurobasal medium containing B27 and gentamycin before staining. To test whether Sema3A acts on GnRH-1 processes in an Nrp1-dependent fashion, a rat-Nrp1-neutralizing antibody (1 µg/ml, R&D Systems, AF566) was added to the growth medium of the co-cultures. The following day, the cultures were fixed with 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4 and permeabilized with 0.3% Triton X-100 (Sigma) for 1h at room temperature. Finally, they were stained with Alexa 568-X phalloidin (Molecular Probes, Eugene, OR) for 45 min at 37°C before image analysis. Quantification of GnV3 fiber growth was performed on digitized photomicrographs using the NeuronJ plugin of ImageJ software (National Institute of Health).

Image analysis

For confocal observations and analyses, an inverted laser scanning Axio observer microscope (LSM 710, Zeiss) with EC Plan NeoFluor 10x/0.3 NA, 20x/0.5 NA and 40x/1.3 NA (Zeiss) objectives was used (Imaging Core Facility of IFR114 of the University of Lille 2, France). ImageJ (National Institute of Health, Bethesda, USA) and Photoshop CS5 (Adobe Systems, San Jose, CA) were used to process, adjust and merge the photomontages.

Intracerebral infusion of Neuropilin-1- or Sema3A- neutralizing antibodies

To determine the importance of Nrp1 in the central control of reproductive function, *in vivo* experiments were performed to neutralize Nrp1 receptor-ligand interactions within the median eminence. Anti- Nrp1 or -Sema3A IgGs (R&D Systems) were chronically infused into the median eminence (bregma -3.6 mm, 9.5 mm depth from the skull surface) [61] through a stereotaxically-implanted infusion cannula (Plastics One, Roanoke, VA) connected to a subcutaneously-implanted osmotic minipumps (model 1007D; Alzet, Palo Alto, CA). The pump had a flow rate of 0.5µl /h and a capacity of 100µl, resulting in a delivery period of 7 days. Each pump was loaded with sterile DPBS (invitrogen) containing the Nrp1-neutralizing antibody (0.25µg/µl final) or no antibody. After connection to the infusion device and overnight priming in 0.9% NaCl at 37°C, the assembly was implanted into cycling 190-200g female rats with regular estrous cycles. Estrous cycles were monitored before and after surgery.

Following infusion for 7 days, animals were killed to assess the implantation site of the cannula and check for exhaustion of the infused solution. Median eminences from Nrp1 and PBS-infused animals were collected, snap frozen in dry ice and store at -80°C. To determine whether the infused Nrp1 antibodies actually targeted the median eminence and bound endogenous Nrp1, protein extracts from median eminences were prepared and subjected to immunoprecipitation using Nrp1 antibodies as described above. The sepharose beads from the preclearing step allowed IgGs to be collected from the median eminences. The beads were then separated by centrifugation, washed twice with ice-cold lysis buffer, and boiled for 5min in 50µl of 2X NuPAGE® LDS sample buffer (Invitrogen). After centrifugation, the supernatants were analyzed by western blotting for Nrp1. Anti-Sema3A-treated animals and their PBS-treated controls were perfused transcardially with fixative and processed for immunohistochemistry as described above. Alexa Fluor 488-conjugated anti-mouse antibodies (1:400) were used to detect the binding of the intracranially infused Sema3A

antibodies *in situ*; vascular endothelial cells were visualized using (TRITC)-conjugated *Bandeiraea simplicifolia* lectin.

TAT-Cre delivery

A TAT-Cre fusion protein produced as detailed previously [35] was injected into the tail vein (40 μl at 2.1 mg/ml) of mice one week before they were placed for 62 hours in a cage that had previously held a sexually experienced male to induce, a protocol used to induce a preovulatory surge in adult virgin mice [37].

Quantitative RT-PCR analyses from mouse hypothalamic explants

For *Sema3a* gene expression analysis, mRNAs obtained from microdissected median eminence and mediobasal hypothalamus explants were reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies). Real-time PCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System using the SEMA3A (Sema3a_Mm00436469_m1) exon-boundary-specific TaqMan® Gene Expression Assays (Applied Biosystems).

Plasma LH assay

Plasma LH was measured using a Rodent LH ELISA kit (Endocrine Technologies, Newark, CA) with a sensitivity of 0.03 ng/ml and 7% intra-assay and 10% inter-assay coefficients of variance.

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 the standard practice in the field. Before statistical analysis, percentages were subjected to arc-sine transformation to convert them from a binomial to a normal distribution [62]. Data were compared by a two-tailed unpaired Student's t test, one-way ANOVA for multiple

comparisons or by two-way repeated-measures ANOVA. A Tukey's *post hoc* test was performed when appropriate. The significance level was set at $p < 0.05$. Data groups are indicated as mean ± SEM. The number of biologically independent experiments, *P* values and degrees of freedom are indicated in the figure legends.

Acknowledgments

This research was supported by the Agence National pour la Recherche (ANR, France) grants ANR-07-NEURO-026-03 (VP), ANR-09-BLAN-0267 (VP) and ANR-2010-JCJC-1404-01 (PG), ANR 11 BSV1 02102 (to S.G.B. and P.C.) the Fondation pour la Recherche Médicale (Equipe FRM 2005 & DEQ20130326524, France, VP), the European INTERREG program TC2N (to VP and SGB), the Institut Fédératif de Recherche 114 (IFR114, France; electron microscopy core facility), the University of Lille 2, Lille, France (grant: Appel à Projets du Conseil Scientifique de l'Université Lille 2 to P.G.), the Compagnia di San Paolo-2008.1224 (Turin, Italy, PG and LT) and the EU FP7 program Plastisice (to JV). CC was supported by a doctoral fellowship from the Inserm and the Région Nord Pas de Calais. We thank Meryem Tardivel (imaging core facility, IFR114), Nathalie Jouy (cell sorting core facility, IFR114), Delphine Taillieu and Julien Devassine (animal core facility, IFR 114) for their expert technical assistance. We thank Dr. Catherine Dulac (Howard Hughes Medical Institute, MA, USA) and Dr. Daniel Spergel (Section of Endocrinology, Department of Medicine, University of Chicago, Illinois) for their generous gift of the *GnRH::Cre*$^{+/-}$ and *GnRH-GFP* mouse lines, respectively, Dr. François Pralong (CHUV, Lausanne) for his generous gift of the GnV3 cell line and Dr. S. Rasika for the editing of our manuscript.

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Figure legends

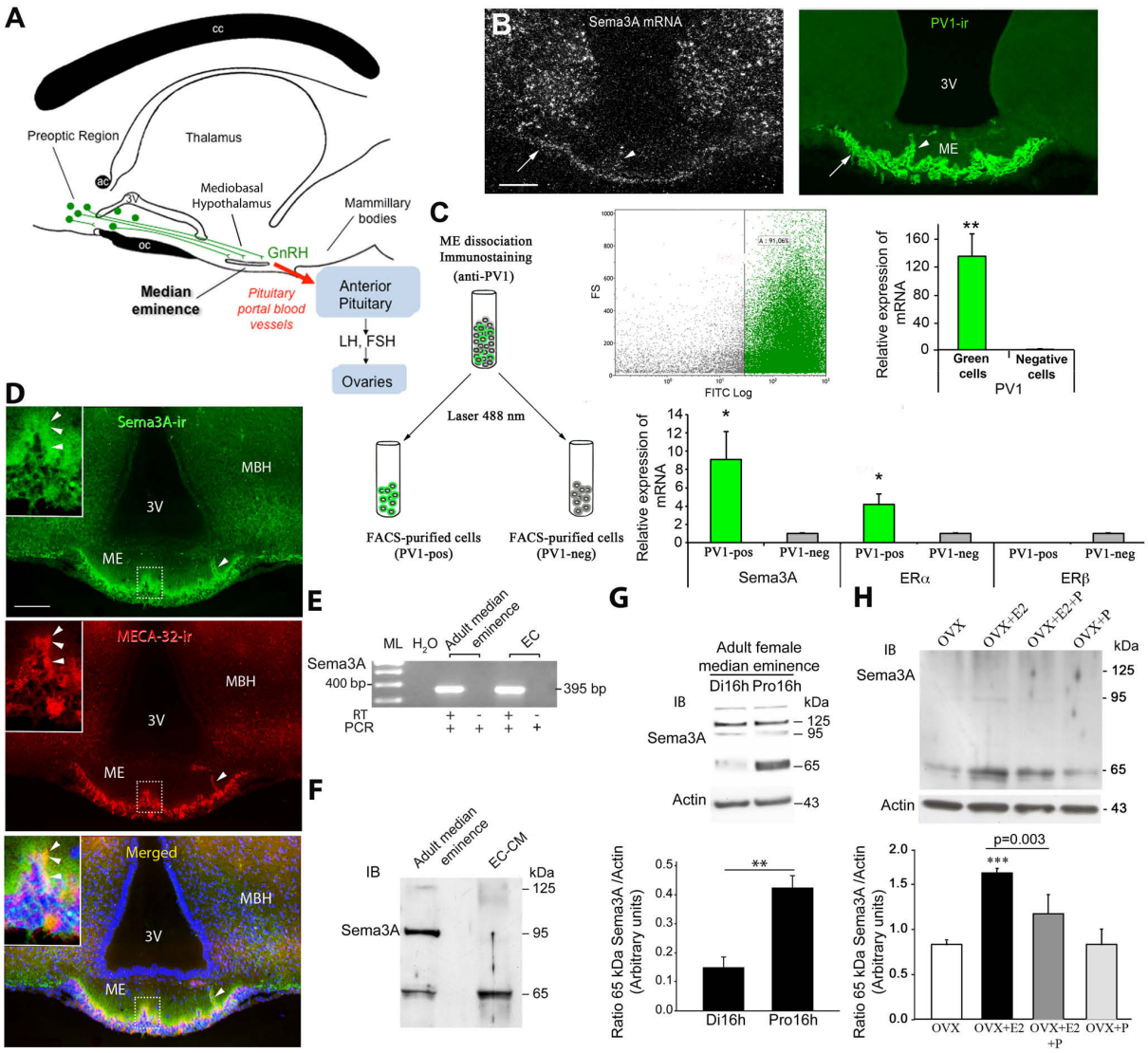

Figure 1. **Sema3A expression in median eminence vascular endothelial cells during the ovarian cycle.** (A**)** Schematic diagram illustrating the anatomy of the hypothalamic-pituitary-gonadal axis in a sagittal view. In rodents, GnRH cell bodies (green circles) are diffusely distributed in the preoptic region and send neuroendocrine axons (green fibers) towards the median eminence of the hypothalamus, where they release the neurohormone into pituitary portal blood vessels (red arrow) for delivery to the anterior pituitary. At the adenohypophysis, GnRH elicits the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate gametogenesis and gonadal-steroid secretion and thus support reproductive function. cc: corpus calosum, ac: anterior comissure, oc: optic chiasma, 3V: third ventricle. (B) Representative dark-field photomicrographs of a

coronal section of an adult female rat median eminence (ME), showing Sema3A mRNA localized using a radioactive probe (bright dots indicating silver grains, *top panel*). Note the presence of Sema3A mRNA in the capillary zone of the ME (white arrow) and in intrainfundibular capillary loops (arrowhead) containing PV1-immunoreactive fenestrated endothelial cells (*bottom panel*, green immunofluorescence), and its relative paucity in the parenchyma. Sema3A mRNA expression is also seen in various nuclei of the mediobasal hypothalamus (MBH) that lie adjacent to the ME but do not contain PV1-immunoreactive bood vessels. V3: third ventricle. Scale bar: 100 µm. (C) PV1-positive cell (PV1-pos) isolation by FACS (schematic diagram and dot plot, top) and real time PCR analysis of PV1, Sema3A, estrogen receptor alpha (ERα) and ERβ transcripts. (D) Representative immunofluorescence images showing the localization of Sema3A immunoreactivity (green) in coronal sections of the ME of adult female mice. Fenestrated vascular endothelial cells are labeled by the monoclonal antibody MECA32, which binds to mouse PV1 (red). Note that Sema3A immunoreactivity is localized in portal blood capillaries of the external zone of the ME (inset) as well as some intrainfundibular capillary loops present in the nervous parenchyma (arrowhead); Sema3A immunolabelling is of very high intensity at the level of the capillary zone, but is also seen in the adjacent nervous parenchyma, progressively vanishing at deeper levels of the tissue. Nuclei are counterstained in blue using Hoechst. Insets: High-magnification images of the areas indicated by dashed lines. Scale bar: 100 µm (30 µm in inset). (E) Detection by RT-PCR of Sema3A mRNA in total RNA extracts from ME explants microdissected from adult female rats and. immunopurified ME endothelial cells (EC) ML, 100 bp molecular ladder; H_2O, PCR negative control without cDNA; +/-, Sema3A amplicon (395 bp) with (+) or without (-) RT. (F) Western blot analysis of Sema3A protein levels in the adult ME and 48h median eminence EC-conditioned medium (EC-CM). Each lane was loaded with 35 µg of protein. While all Sema3A isoforms (65, 95 and 125 kDa) are detected in protein extracts from the adult female ME, only the 65kDa Sema3A isoform is present in the EC-CM. (G) Western blot (top) and quantitative analysis (bottom, relative to actin) of Sema3A protein levels showing a difference in 65kDa Sema3A expression between the

afternoon of diestrus (Di16h) and proestrus (Pro16h), whereas the protein levels of 125 and 95 kDa Sema3A remain unchanged. Band intensity was quantified using Scion software. **: $t_{(8)}$=4.709, p=0.0015 (n = 5 independent experiments). (H) Western blot analysis for Sema3A (top; upper image) and actin (top; lower image) in the ME of control ovariectomized (OVX) female rats and those treated with 17β-estradiol 3-benzoate (E2), progesterone (P) or E2 + P (n = 5 independent experiments per treatment). E2 induces a significant increase in 65 kDa Sema3A expression in the ME of OVX rats when compared with the other treatment groups one-way ANOVA, $F_{(8,11)}$=27.779, p<0.001; Tukey's test, ***p<0.001), whereas progesterone inhibits this increase (Tukey's test, p=0.003). Bar graph: mean ratio (± SEM) of Sema3A expression to that of actin (p<0.05, one-way ANOVA). Data are represented as means ± SEM.

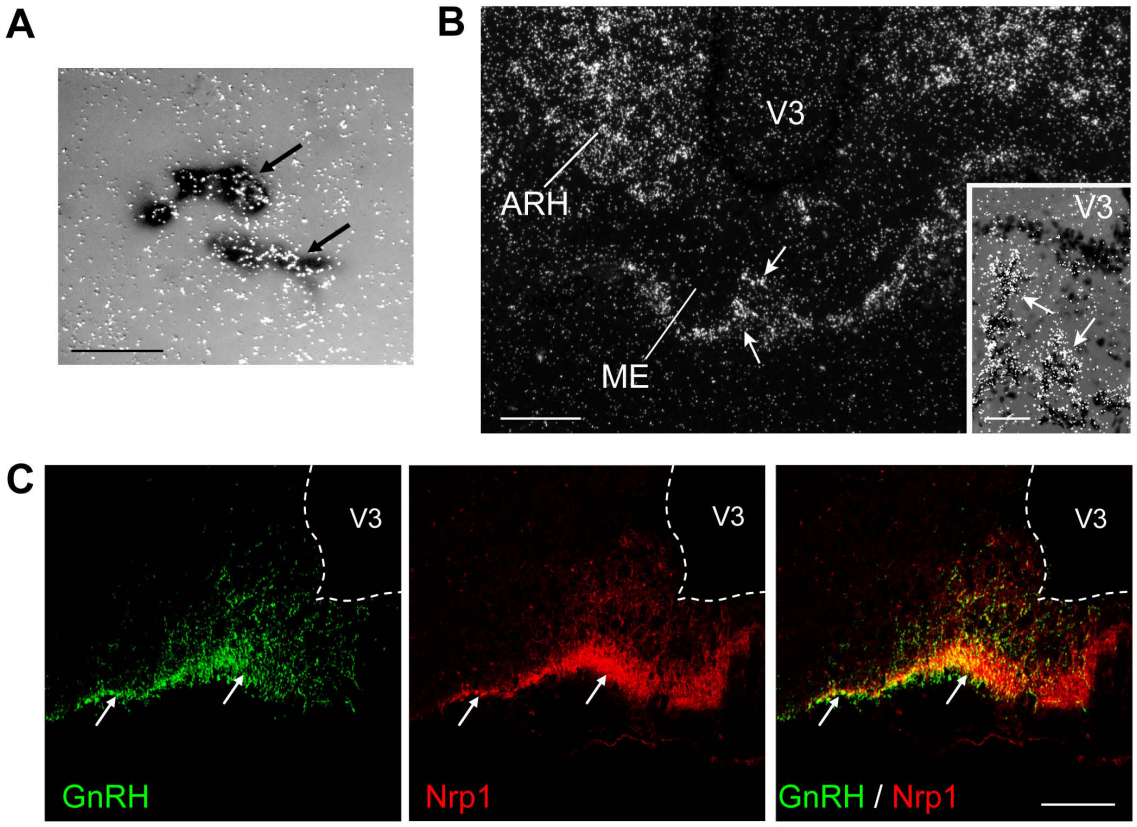

Figure 2

Figure 2. **Neuropilin-1 (Nrp1), the obligate Sema3A receptor, is expressed in adult GnRH neurons.** (A) Simultaneous bright-field and epi-illumination photomicrograph showing cells (arrows) labeled with a digoxigenin-conjugated probe for GnRH mRNA (dark staining) and a radioactive probe for Nrp1 mRNA (bright silver grains) in the preoptic region of an adult female rat. Scale bar: 20μm. (B) Representative dark-field photomicrograph of Nrp1 mRNA (bright dots) in an adult female rat median eminence (ME) localized using a radioactive probe. Note the absence of signal in the parenchyma of the ME but intense expression of Nrp1 mRNA in the capillary zone of the ME (white arrows). V3: third ventricle; ARH: arcuate nucleus of the hypothalamus. Scale bar: 150 μm. Inset: High-magnification image of a different field in the same area under simultaneous bright-field and epi-illumination to visualize cell nuclei counterstained for Nissl. Scale bar: 50 μ m. (C) Photomicrographs showing the distribution of GnRH (green) and Nrp1 (red) immunoreactivity in the ME of an

adult female rats. Note that Nrp1 and GnRH are colocalized in the external layer of the ME (arrows). Scale bars: 100 μm.

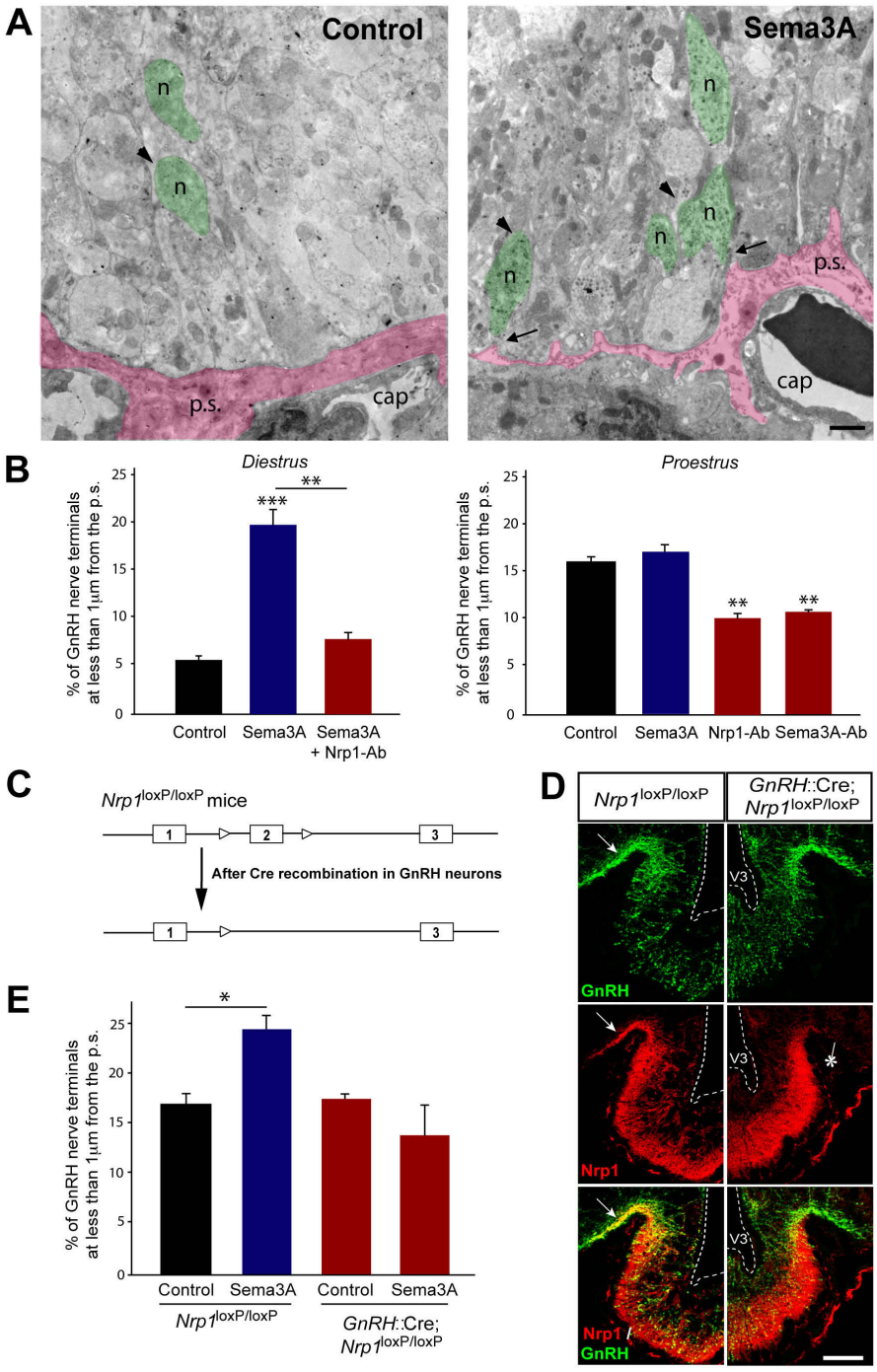

Figure 3

Figure 3. Sema3A-Nrp1 signaling promotes GnRH axonal growth in the median eminence (ME) of the adult female rodent brain. (A) Representative electron micrographs of GnRH-immunoreactive axon terminals (green) from diestrous female rat hypothalamic explants containing the ME, incubated for 30 min in the presence (right panel) or absence (left panel) of Sema3A. Under basal unstimulated conditions (left panel), GnRH nerve

endings (n, arrowhead, green) are distant from the pericapillary space (p.s., pink). Sema3A treatment (right panel) causes GnRH axon terminals to advance towards the pericapillary space (p.s., pink), from which they remain separated by only a few nanometers (arrows). Cap: pituitary portal blood capillaries. Scale bar: 1 µm. (B) Quantitative analysis of the percentage of GnRH nerve terminals located less than 1 µm from the pericapillary space in the external zone of the ME, in explants from diestrous (*left panel*) and proestrous (*right panel*) rats treated with Sema3A, a Nrp1-neutralizing antibody (Nrp1-Ab), a Sema3A-neutralizing antibody (Sema3A-Ab), both Nrp1-Ab and Sema3A, and in controls. *Left panel*, one-way ANOVA, $F_{(2,11)}$=54.875, p<0.001; *right panel*, one-way ANOVA, $F_{(3,12)}$=37.093, p<0.001. Tukey's test, ***p<0.001, **: p<0.01 for pairs of groups as indicated; n = 3-4 animals per group. (C) Genetic strategy to invalidate Nrp1 expression specifically in GnRH-expressing cells in mice. (D) Immunofluorescence analysis of coronal brain sections from adult female *Nrp1*$^{loxP/loxP}$ (left) and *GnRH::*Cre; *Nrp1*$^{loxP/loxP}$ (right) littermates using antibodies to GnRH (green) and Nrp1 (red). Note the markedly reduced Nrp1 immunoreactivity in the dorsolateral part of the median eminence, where most GnRH axon fibers occur, in *GnRH::*Cre; *Nrp1*$^{loxP/loxP}$ mice (asterisk) when compared to Nrp1$^{loxP/loxP}$ animals (arrow), confirming the efficient ablation of *Nrp1* in GnRH neurons of the former. Scale bar: 50 µm. (E) Quantitative analysis of the percentage of GnRH nerve terminals located less than 1 µm from the pericapillary space in the external zone of the median eminence in explants from control *Nrp1*$^{loxP/loxP}$ and *GnRH::*Cre; *Nrp1*$^{loxP/loxP}$ mice and those treated with Sema3A. One-way ANOVA, $F_{(3,15)}$=9.894, p=0.0015. Tukey's test, *: p<0.05; n = 3-4 animals per group. Data are represented as means ± SEM.

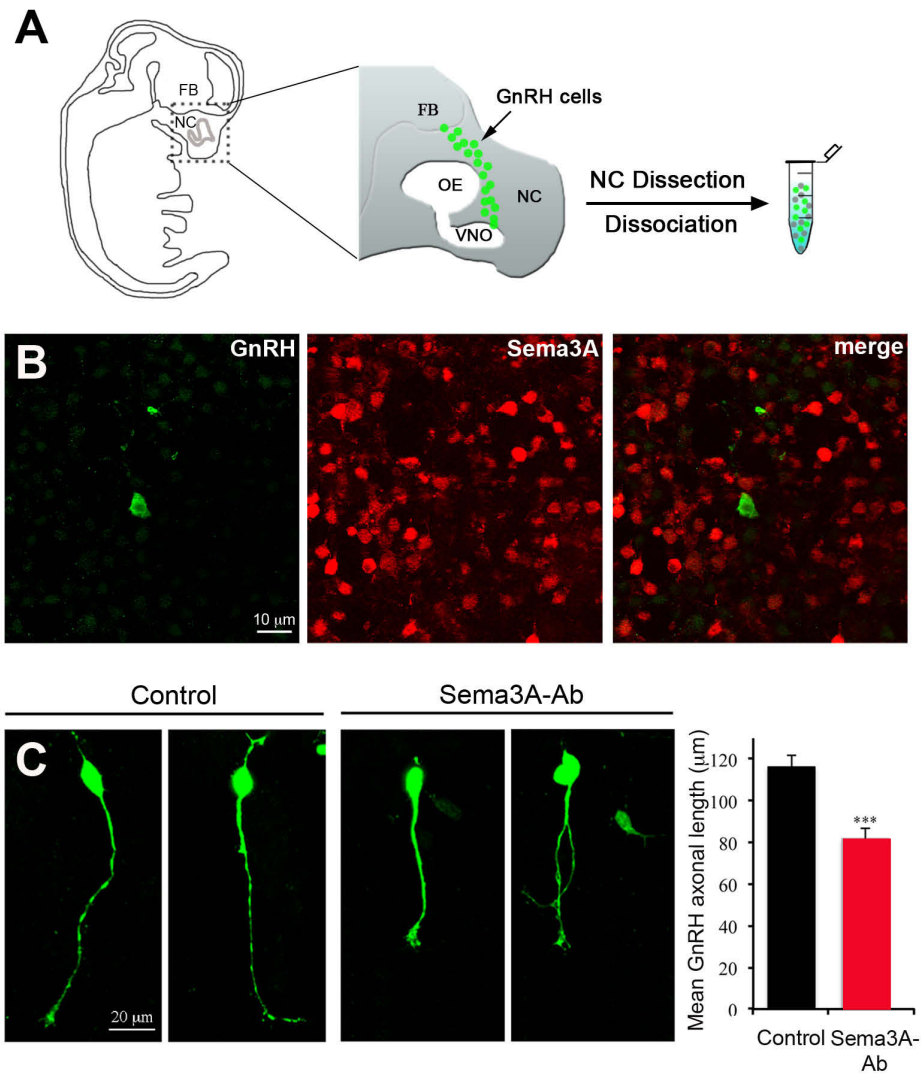

Figure 4

Figure 4. Sema3A immunoneutralization causes the retraction of axon-like processes in primary GnRH neurons *in vitro*. (A) Schematic representation of a sagittal view of a mouse embryo at E12.5, showing the distribution of GnRH neurons (green dots) within the head. Primary cultures were performed from microdissected nasal compartment (NC) explants, which contain most GnRH neurons at this embryonic stage. FN: forebrain; OE: olfactory epithelium; VNO: vomeronasal organ. (B) Representative images showing the binding of the Sema3A-neutralizing antibody (red) to cultured cells surrounding GFP-expressing GnRH neurons (green). (C) Representative images showing the morphology of cultured GnRH neurons under control conditions and after treatment with the Sema3A-

neutralizing antibody and bar graph quantifying the mean length of their axon-like processes (control: N=3 independent experiments, n=146 cells; Sema3A-Ab: N=3 independent experiments, n=143 cells). Total number of cultures = 24 from 4 litters. Data are represented as means ± SEM. Unpaired Student's t test, $t_{(285)}$=4.823, p<0.0001.

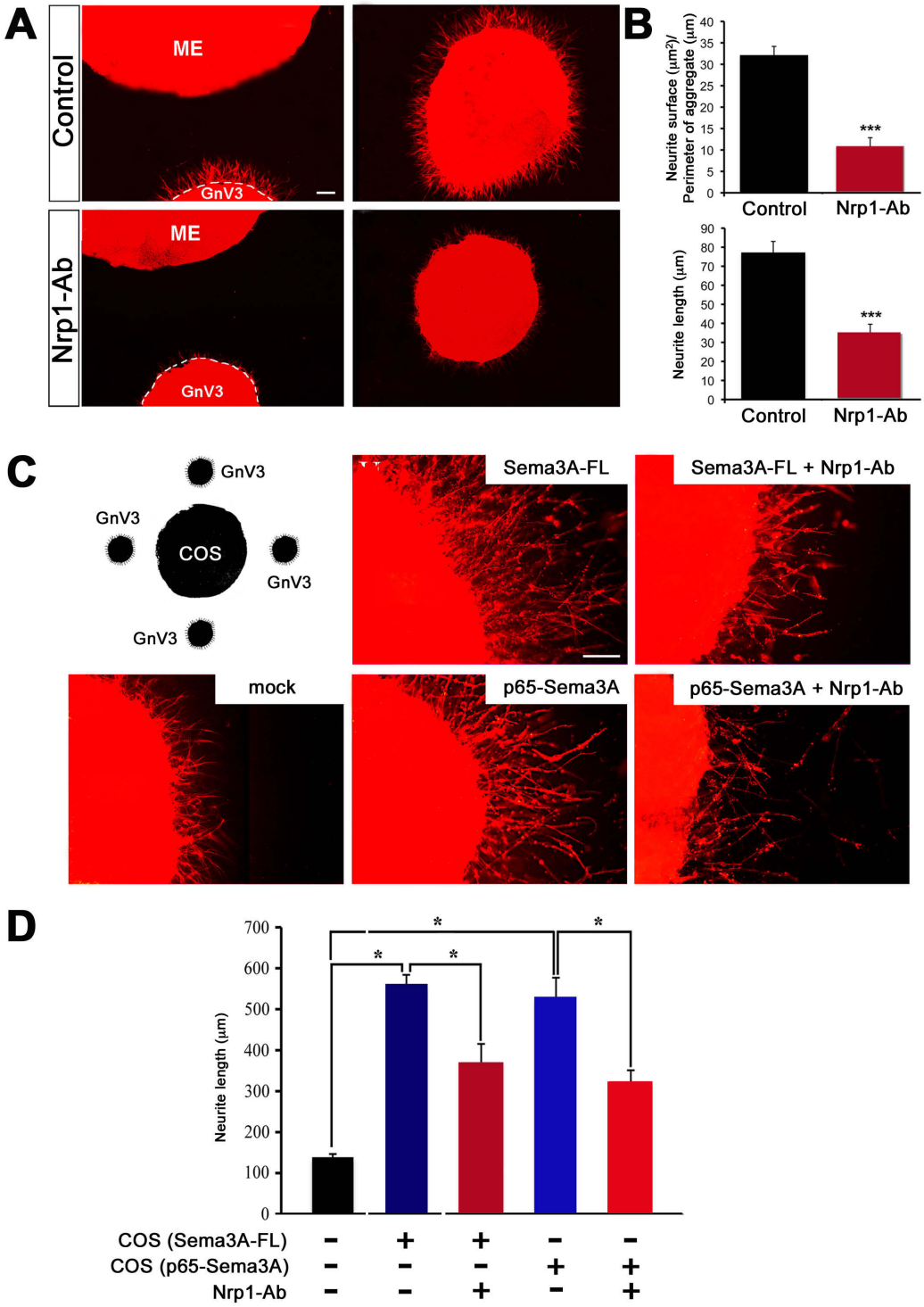

Figure 5

Figure 5. 65 kDa Sema3A-Nrp1 signaling is responsible for GnRH neurite sprouting. (A) Three-dimensional matrix assays using co-cultures of median eminence (ME) explants dissected from adult female rats (control n=3; Nrp1-Ab n=4) and cell aggregates of

immortalized GnV-3 cells, in the absence (top panels) or presence (bottom panels) of an Nrp1-neutralizing antibody (Nrp1-Ab). Co-cultures were fixed and stained with Alexa 588-X phalloidin. GnV-3 cell aggregates show neurite extension under control conditions, whereas neurite sprouting is strongly inhibited by Nrp1-Ab. (B) Quantitative analysis of the area covered by phalloidin staining surrounding the aggregates (top panel; n = 3 in Controls, n = 4 in Nrp1-Ab-treated aggregates; unpaired Student's t test, $t_{(5)}$=7.424, p<0.001) and GnV-3 neurite length (bottom panel; n=3 in Controls, n=4 in Nrp1-Ab-treated aggregates; unpaired Student's t test, $t_{(5)}$=5.610, p<0.005), respectively. (C) Co-cultures of GnV-3 cell aggregates placed around aggregates of COS-7 cells transfected with full-length (95kDa) Sema3A (Sema3A-FL), 65 kDa Sema3A (p65-Sema3A) or the control vector (n=7), in the presence (Sema3A-FL + Nrp1-Ab, n=4; p65-Sema3A + Nrp1-Ab, n=6) or absence of Nrp1-Ab (Sema3A-FL, n=4; p65-Sema3A, n=4), as shown in he schematic drawing. Sema3A-FL and p65-Sema3A are equally effective at inducing GnV-3 neurite growth when compared to control conditions (middle panels), while neurite growth is prevented by the Nrp1-neutralizing antibody (right panels). (D) Quantitative analysis of GnV-3 neurite length (one-way ANOVA with Tukey's *post hoc* test, $F_{(4,24)}$=38.058, p<0.0001). Data are represented as means ± SEM. Scale bars: 100 μm in A, 50 μm in C.

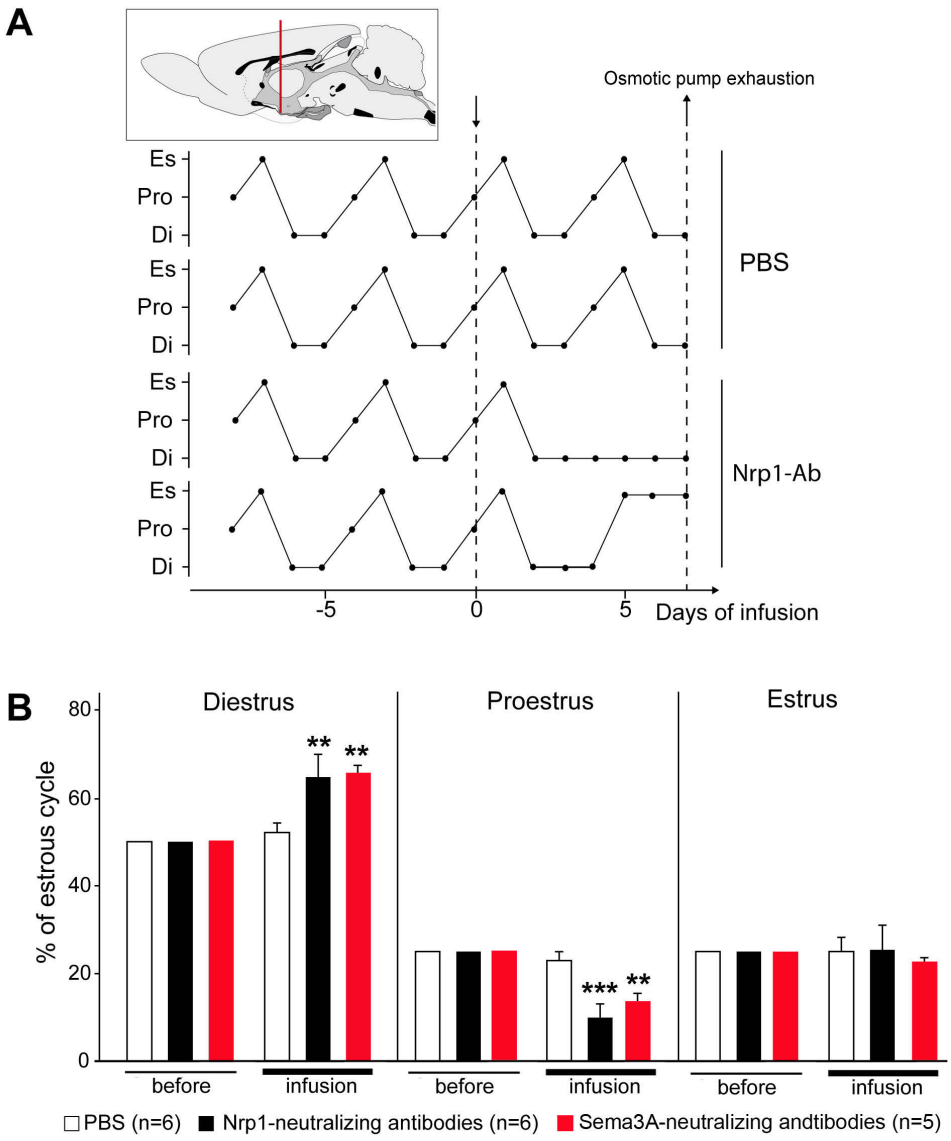

Figure 6

Figure 6. Neutralization of Nrp1 and Sema3A activity in the median eminence (ME) *in vivo* impairs adult reproductive function in rats. (A) *Upper panel*, schematic diagram representing the stereotaxic implantation of a 28 gauge infusion cannula connected to a subcutaneously-implanted mini-osmotic pump in the ME of cycling female rats, for the delivery of Nrp1- or Sema3A-neutralizing antibodies (0.2 μg/μl, 0.5 μl/h). *Lower panel*, representative estrous cycle profiles showing the disruption of estrous cyclicity by the infusion of Nrp1-Ab but not of PBS into the ME. Infusion was started on day 0 (downward arrow) and ended 7 days later (upward arrow), when the pump contents were exhausted. Di,

diestrus; Pro, proestrus; Es, estrus. (B) Quantitative analysis of alterations in ovarian cyclicity (number of days in each phase) caused by Nrp1-Ab or Sema3A-Ab infusion (n=6 animals in the PBS and Nrp1 groups; n=5 animals in the Sema3A group). Diestrus, two-way repeated-measures ANOVA, $F_{(14,33)}$=19.073, p<0.001, Tukey's test, **: p=0.003 and p=0.005 between before and after infusion within Nrp-1-and Sema3A treated groups, respectively. Proestrus, two-way repeated measures ANOVA, $F_{(14,33)}$=31.119, p<0.001, Tukey's test, ***: p<0.001 and **: p=0.002 between before and after infusion within the Nrp-1-and Sema3A-treated groups, respectively. Estrus, two-way repeated measures ANOVA, $F_{(14,33)}$=0.084, p=0.776. Data represented as means ± SEM.

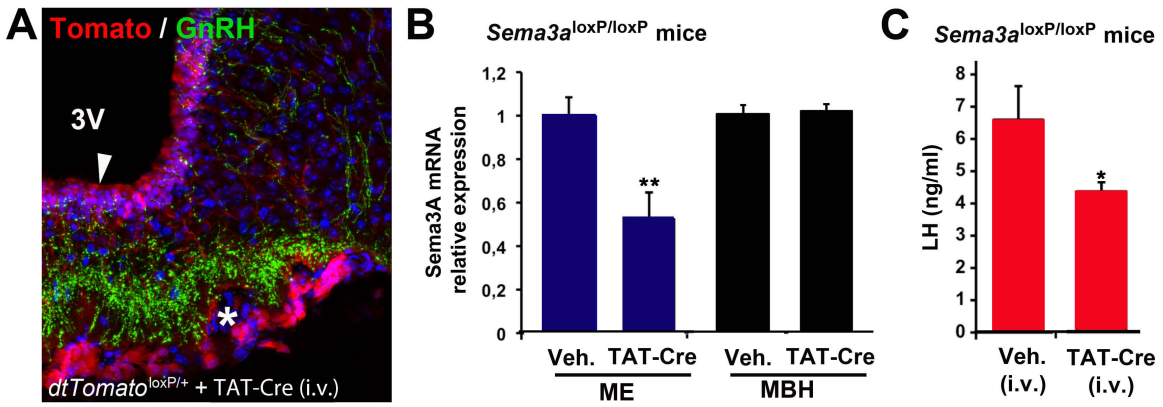

Figure 7

Figure 7. Targeted *Sema3a* gene deletion in endothelial cells of the median eminence (ME) alters the amplitude of the preovulatory GnRH/LH surge in mice. (A) Representative image showing Tomato expression (red) in the capillary zone (asterisk) onto which GnRH axon terminals abut (green) in the ME of *tdTomato*$^{loxP/+}$ mice into which the TAT-Cre recombinant protein was injected intravenously (i.v.). Note that Tomato is also expressed in tanycytes (arrowhead), whose cell bodies line the floor of the third ventricle (3V) but which do not express Sema3A mRNA (see Figure 1B). ARH, arcuate nucleus of the hypothalamus. Scale bar: 100 µ m. (B) Quantitative RT-PCR analysis of Sema3A mRNA expression in the ME, $t_{(13)}$=3.372, **: p=0.005, and in the adjacent mediobasal hypothalamus (MBH), $t_{(10)}$=-0.287, p=0.780, in *Sema3a*$^{loxP/loxP}$ mice treated i.v. with vehicle (n=7) or TAT-Cre (n=8 and 5, respectively). (C) Preovulatory LH levels in TAT-Cre (n=8) or vehicle-injected (n=7) *Sema3a*$^{loxP/loxP}$ mice, $t_{(13)}$=2.188, *p=0.048. Data are represented as means ± SEM.

Supporting information

Figure S1. RT-PCR analysis of Sema3A, PV1, β3-Tubulin (β3Tub), DARPP-32, thyroid-stimulating hormone (TSH) and GAPDH transcripts (gel image) in PV1-positive (PV1-pos) and PV1-negative cells isolated by FACS from the median eminence of adult female rats.

Figure S2. Cultured mouse endothelial cells. **(**A) Immunopurified endothelial cells of the median eminence cultured *in vitro* are labeled with TRITC-conjugated Bandeiraea simplicifolia lectin (BsII, red) and exhibit PV1 immunoreactivity (green). Scale bar: 20 μm. (B) SVEC4-10 mouse endothelial cells mainly release p65 Sema3A, the proteolytic product of a 95 kDa precursor, released by furin cleavage.

Figure S3. Nrp1 is expressed by vascular endothelial cells of the median eminence (ME). Confocal images showing the localization of Nrp1 immunoreactivity (green) in coronal sections of the ME of adult female rats. Vascular endothelial cells are labeled with TRITC-conjugated Bandeiraea simplicifolia lectin (BsII, red). Note that in addition to its abundance in the parenchyma of the ME (top panels), Nrp1 immunoreactivity is also found in endothelial cells of portal blood capillaries (bottom panels, arrows). Bottom panels are high-magnification images of the framed areas shown in A. Scale bars: 100 μm in top and 50 μm in bottom panels.

Figure S4. Nrp1- and Sema3A- neutralizing antibodies were efficiently delivered into the median eminence (ME) of adult female rats. (A) Immunoprecipitation (IPP) and immunoblot (IB) analyses showing Nrp1 targeting by Nrp1-neutralizing antibodies (Nrp1-Ab) infused into the ME. At the end of the infusion period, MEs were microdissected, proteins extracted, and equal amounts of proteins incubated with protein A-sepharose beads to precipitate free IgGs (preclearing). The precipitated proteins were subjected to western blotting and the supernatant used for immunoprecipitation. Note that in protein extracts from PBS-infused animals, no Nrp1 immunoreactivity was seen in the precleared fraction of the samples, while a strong Nrp1 immunoreactive signal was obtained after immunoprecipitation. In contrast, in

protein extracts from the ME of Nrp1-Ab-treated rats, Nrp1 immunoreactivity was found in both the precleared and immunoprecipitated fractions of samples, showing the proportion of endogenous Nrp1 receptors bound and unbound by the infused antibody, respectively. SB: well loaded with sample buffer only. (B) Representative images showing the binding of intracranially infused Sema3A-neutralizing antibodies (green fluorescence, Alexa 488) in the ME. Arrows show the injection site. Note that the Sema3A- neutralizing antibodies selectively targets the capillary zone of the ME, in which vascular endothelial cells are labeled with TRITC-conjugated Bandeiraea simplicifolia lectin (BSLI), and the surrounding nervous parenchyma. ARH, arcuate nucleus of the hypothalamus (ARH). (C) Representative Western blot image of conditioned media from transfected COS-7 cells producing the 65kDa or the 95 kDa full length Sema3A proteins.

Figure S5. Full-length photographs of each of the western blots presented in Figure 1F, Figure 1G, Figure 1H and Supplementary Figure 1 and Supplementary Figure 3 (IB, immunoblot).

Etude du rôle de l'expression du récepteur Neuropilin-1 et de l'exocytose Calcium-dépendante dans le neurone à GnRH sur le développement et la maturation du système à GnRH et la physiologie de la reproduction

L'acquisition de la fertilité chez les mammifères est le résultat d'un long processus de développement et de maturation de l'axe gonadotrope. Cette fonction cruciale à la survie des espèces est orchestrée par une poignée de cellules localisées au niveau de l'aire préoptique hypothalamique chez le rongeur, sécrétant la gonadotropin-releasing hormone (GnRH). La GnRH stimule la sécrétion de LH et de FSH par l'adénohypophyse, qui stimulent à leur tour les gonades. Les neurones à GnRH naissent dans l'épithélium voméronasal pendant le développement embryonnaire et migrent le long des axones voméronasaux pour atteindre l'hypothalamus. A la naissance le système est entièrement en place, toutefois il subira une phase de maturation avant d'atteindre la puberté, signant le début de la fertilité. Chez l'homme, un défaut de sécrétion de GnRH peut conduire à un hypogonadisme hypogonadotrope idiopathique (IHH) caractérisé par une subfertilité et une puberté retardée voire absente, ou même à un syndrome de Kallmann. Dans une grande partie des cas ce défaut de sécrétion est lié à un défaut de développement prénatal et à une diminution du nombre de neurones à GnRH dans dans l'hypothalamus. Depuis peu, la grande famille des semaphorines, déjà connues pour leurs effets chimiotactiques dans certains types cellulaires, et en particulier la semaphorine3A (Sema3A) via son récepteur la Neuropilin-1 (Nrp1), a été décrite comme un facteur indispensable au développement du système à GnRH et décrit comme un « gène Kallmann ». Toutefois son rôle spécifique dans les neurones à GnRH reste à élucider. Le **premier objectif** de ma thèse a donc été de déterminer le rôle de l'expression du récepteur Nrp1 dans les neurones à GnRH. Le suivi de la maturation sexuelle des animaux *Gnrh::cre, Nrp1*$^{loxp/loxp}$ (qui n'expriment pas la Nrp1 exclusivement dans les neurones à GnRH) a révélé l'apparition d'une puberté précoce et d'un phénotype de surpoids en comparaison aux animaux contrôles, corrélé à une accumulation des cellules à GnRH dans l'aire préoptique. L'étude de l'embryogenèse du système à GnRH chez ces animaux a démontré une augmentation du nombre de cellules à GnRH pendant leur migration. Nos résultats obtenus *in vivo* et *in vitro* suggèrent que la signalisation Nrp1 a un impact sur la survie des neurones à GnRH, et qu'elle module la motilité des cellules en migration et influe leur positionnement dans le cerveau. Le **deuxième objectif** de ma thèse a été d'étudier le rôle de l'exocytose dépendante du calcium et donc de la neurosécrétion dans les neurones à GnRH sur leur développement. Le suivi de la physiologie d'animaux *Gnrh::cre*; iBot, dont l'exocytose dépendante du calcium est abolit par clivage de protéine VAMP2/synaptobrevin 2 dans le neurone à GnRH, a révélé l'apparition de deux phénotypes distincts selon la pénétrance du transgène : un groupe ayant une puberté normale et un poids comparable aux animaux contrôles, et un groupe ayant une puberté retardée voire inexistante associé à un surpoids. Ces derniers présentent un IHH, une augmentation du tissu adipeux périgonadique et une hyperleptinémie, alors que la distribution des neurones à GnRH dans le cerveau n'est pas altérée. Ces données mettent en évidence le fait que l'activité de neurosécrétion dans les neurones à GnRH ne serait pas nécessaire pour leur développement embryonnaire, mais qu'elle pourrait jouer un rôle dans le maintien de l'homéostasie énergétique.

Ces deux études mettent en avant un lien étroit entre axe gonadotrope et métabolisme énergétique chez les mammifères et ont dévoilés de nouveaux mécanismes qui pourraient être impliqués dans la physiopathologie de la reproduction chez l'homme.