Cell neogenesis in the postnatal hypothalamus as a new mechanism of control of the reproductive function

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Cell neogenesis in the postnatal hypothalamus as a new mechanism of control of the reproductive function

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The woman has been locked for centuries. When she has access to culture she is like a starving person.
And the food is much more useful to those who are hungry than those who are already full.

-Rita Levi-Montalcini

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Resumé

Malgré sa complexité, le cerveau intègre en permanence de nouvelles cellules – à la fois neuronales et gliales – au-delà du développement embryonnaire et ce, tout le long de la vie. La période postnatale est caractérisée par une gliogenèse intense. A l'âge adulte, de nouveaux neurones et cellules gliales sont produits dans des régions restreintes à partir de cellules souches/progénitrices (CSP) localisées dans des niches. Les deux niches de CSP adultes les mieux décrites sont la zone sous-ventriculaire des ventricules latéraux, qui produit de nouveaux interneurones olfactifs, et la zone sous-granulaire du gyrus denté de l'hippocampe, où de nouveaux neurones en grain sont produits localement. Des travaux menés ces dernières années ont montré qu'une neuro- et une gliogenèse avaient aussi lieu dans l'hypothalamus postnatal, une petite région du diencéphale ventral qui régule des processus physiologiques vitaux tels que le métabolisme, la reproduction, le sommeil et la thermorégulation. Si l'identité des CSP hypothalamiques reste débattue, de nombreux travaux s'accordent sur l'importance de la neurogenèse hypothalamique postnatale dans le contrôle du métabolisme. Cependant, la possibilité que la genèse postnatale de cellules contribue aussi au contrôle de la fonction de reproduction, une autre fonction clé de l'hypothalamus, restait à explorer.

L'objectif premier de mon travail de thèse était de rechercher si la genèse de cellules dans l'hypothalamus postnatal est impliquée dans le contrôle de la reproduction, une fonction physiologique qui requière un haut degré de plasticité. La fonction de reproduction est orchestrée par une petite population de neurones produisant la neurohormone Gonadotrophin-Releasing Hormone (GnRH). Ces neurones, qui naissent en dehors du cerveau, sont en place dans la région préoptique (RPO) de l'hypothalamus à la naissance. Cependant, ils doivent subir une maturation postnatale pour acquérir le profil de sécrétion qui leur permettra d'initier la puberté et d'assurer la fertilité de l'individu.

Dans une **première étude**, grâce à une combinaison d'approches *in vitro* et *in vivo*, nous avons mis en évidence une vague d'astrogenèse dans l'environnement des neurones à GnRH au sein de la RPO au cours des deux premières semaines de vie postnatale chez la ratte. Nos résultats suggèrent que les neurones à GnRH utilisent la prostaglandine D2 pour attirer les progéniteurs environnants et que ce recrutement est important pour la maturation sexuelle.

Dans une **deuxième étude**, nous avons recherché si de nouvelles cellules naissent à l'âge adulte dans des régions hypothalamiques qui contrôlent la fonction de reproduction. Nous montrons que des cellules sont produites dans la RPO chez la ratte adulte et que leur taux

varie au cours du cycle oestral, suggérant une régulation par les stéroïdes sexuels. De plus, nous montrons que la survenue d'une gestation stimule la néogenèse cellulaire dans une zone de la RPO qui contrôle le comportement maternel.

Si la néogenèse hypothalamique adulte a surtout été étudiée chez les rongeurs de laboratoire, il reste à déterminer si ce phénomène existe aussi chez l'homme. Pour aborder cette question, nous avons évalué dans une **troisième étude** l'expression de marqueurs de CSP dans l'hypothalamus humain adulte, comparativement au rongeur (souris, rat) et à un primate lémurien, le microcèbe. Nous montrons que l'hypothalamus humain adulte contient des populations de cellules au profil antigénique de CSP, dont certaines semblent propres à l'homme.

Au total, ces travaux montrent que de nouvelles cellules naissent dans des régions hypothalamiques qui contrôlent la fonction de reproduction au cours de la vie postnatale et à l'âge adulte chez la ratte, et que ce phénomène est important pour la maturation sexuelle. L'observation de CSP putatives dans l'hypothalamus humain adulte suggère que la capacité de l'hypothalamus à produire de nouvelles cellules à l'âge adulte existe aussi dans notre espèce.

Abstract

Despite its complexity, the brain keeps adding new cells – both neuronal and glial – beyond embryonic development and throughout life. The postnatal period is characterized by intense and widespread gliogenesis. During adulthood, both glio- and neurogenesis occur in restricted locations from stem/progenitor cells (NPC) residing in niches. The two best-described niches of adult NPC are the subventricular zone of the lateral ventricles, which provides new interneurons to the olfactory bulb, and the subgranular zone of the hippocampal dentate gyrus that locally produces new granule cells. The last decade has seen an accumulation of studies showing that neuro- and gliogenesis also occur in the postnatal hypothalamus, a small portion of the ventral forebrain surrounding the third ventricle that regulates essential physiological processes such as metabolism, reproduction, sleep and thermoregulation. Even though the identity of hypothalamic NPC remains a matter of debate, a growing body of evidence points to postnatal hypothalamic neurogenesis relevance for the control of metabolism. However, a possible contribution of postnatal hypothalamic cell generation to the central control of reproduction, another key function of the hypothalamus, remained to be explored.

The main aim of my doctoral researches was to evaluate whether the generation of new cells in the postnatal hypothalamus contributes to the central control of reproduction, a physiological function known to require a high degree of plasticity. The reproductive function is controlled by a small population of neurons producing the neurohormone Gonadotrophin-Releasing Hormone (GnRH). These neurons, which are born in the nasal placodes, are in place at birth in the preoptic area (POA) of the hypothalamus. However, they need a postnatal maturation to reach a mature secretory pattern that will trigger puberty and subsequent fertility.

In a **first study**, using a combination of *in vitro* and *in vivo* experiments, we showed that a wave of astrogenesis occurs in the POA from local progenitors in the environment of GnRH neurons during the first weeks of postnatal life in the female rat. We identified prostaglandin D2 as a factor used by GnRH neurons to attract progenitors in their vicinity and showed that impaired progenitor recruitment alters sexual maturation.

In a **second study**, we evaluated whether cell neogenesis still occurs during adulthood in hypothalamic regions relevant for the reproductive function. Our results showed that new cells are born in the POA of adult female rats. The rate of cell neogenesis varies across the estrus cycle, suggesting a regulatory influence of gonadal steroids. Moreover, we showed

that gestation impacts the rate of cell neogenesis in a POA region implicated in the control of maternal behavior.

While cell neogenesis in the adult hypothalamus has been mainly studied in laboratory rodents, it remains to be known whether this phenomenon also occurs in humans. To start addressing this question, we evaluated in a **third study** the expression of a panel of NPC markers in the adult human hypothalamus and compared it to that found in rodents (mouse, rat) and a lemur primate, the grey mouse lemur. Our results showed that the adult human hypothalamus contains populations of cells with an antigenic profile of NPC, some of which appear specific to humans.

Altogether, this work shows that new cells are born in hypothalamic regions controlling reproduction throughout postnatal and adult life in female rats, and that this process is required for sexual maturation. The identification of NPC marker-expressing cells in the adult human hypothalamus suggests that the capacity for cell neogenesis also exists in the hypothalamus of our species.

Résumé substantiel

Tous les jours, nous interagissons avec un environnement en constante évolution à partir duquel nous recevons une multitude de stimuli différents qui, avec un certain nombre de signaux internes, convergent vers notre cerveau. La délicate coordination entre ces deux acteurs est fondamentale pour toutes les réponses physiologiques.

Nous sommes confrontés à un système où la stabilité, doit coexister avec la plasticité, car le cerveau est un tissu complexe possédant des millions de connexions. La plasticité est la capacité à modifier et adapter les structures et les fonctions pendant la maturation, les pathologies ou les défis environnementaux, au niveau neuronal ou non neuronal, améliorant ainsi les résultats fonctionnels pour mieux s'adapter aux conditions environnementales.

Aujourd'hui nous savons que la plasticité neurale est effectivement maintenue à l'âge adulte, cependant une telle déclaration n'aurait pas été possible il y a 50 ans, quand les premières expériences montrant la capacité du cerveau adulte à générer de nouveaux neurones dans des conditions physiologiques ont commencé à secouer le dogme central de la neuroscience: « dans le système nerveux tout est immuable ».

La région du cerveau principalement impliquée dans la discussion entre l'environnement et l'organisme est l'hypothalamus. Cette petite portion du diencéphale, présente dans le système nerveux de tous les vertébrés, est un régulateur homéostatique des processus comportementaux et physiologiques comme le métabolisme, la reproduction, la thermorégulation, les rythmes circadiens, la peur, les comportements sexuels, toutes les fonctions complexes dépendantes du statut interne de l'animal (par exemple les niveaux de nutriments) mais aussi sur les informations reçues de l'environnement (par exemple les heures de lumière et d'obscurité, le voisinage d'un danger ou d'un partenaire).

Compte tenu de son rôle essentiel dans le maintien de l'homéostasie physiologique, il ne faut pas s'étonner du niveau élevé de plasticité observé dans la population neuronale et gliale hypothalamique. Néanmoins, l'existence d'une population de cellules souches neurales hypothalamiques n'a été abordée que superficiellement jusqu'à présent. Les deux niches de cellules souches neurales généralement acceptées chez les mammifères adultes se trouvent au niveau de la zone subventriculaire (SVZ) des ventricules latéraux et de la zone subgranulaire (SGZ) de l'hippocampe. En outre, quelques études pionnières ont été consacrées à explorer la possibilité que la production de nouveaux neurones et/ou cellules gliales dans l'hypothalamus adulte puisse contribuer à la flexibilité nécessaire pour intégrer continuellement des signaux provenant d'autres noyaux cérébraux ainsi que de la périphérie et de l'environnement afin de maintenir l'homéostasie énergétique dans le corps.

L'hypothalamus est le centre de contrôle de toutes les fonctions végétatives du cerveau des vertébrés. En fonction des signaux concernant l'état physiologique de l'organisme, ainsi que des informations sur l'environnement, cette région doit adapter sa réponse pour coordonner l'action des différents organes par interaction avec l'hypophyse. Les changements dans les entrées synaptiques, ainsi que les interactions neurones-glie ont déjà été décrits comme des caractéristiques de plasticité importantes pour que l'hypothalamus exerce son rôle. De plus, des études initiales chez des rongeurs sur le rôle de la néogenèse cellulaire dans le contrôle du métabolisme, permettent de penser que l'hypothalamus puisse représenter une niche de cellules souches neurales adultes. Cela suggère que l'hypothalamus conserve un niveau de plasticité encore plus élevé que supposé précédemment et ouvre de nombreuses questions sur le fait de savoir si la prolifération cellulaire intervient seulement à l'âge adulte ou

pendant le développement postnatal, et de savoir quelles fonctions hypothalamiques peuvent être touchées par ce processus.

La reproduction est la fonction physiologique la plus importante pour une espèce, même si un seul animal peut survivre s'il n'est pas fertile, la perpétuation de la vie dépend de la reproduction. Cette fonction complexe, qui se développe sur une période post-natale, est fortement influencée par l'environnement et l'état métabolique de l'animal, en particulier chez les femmes qui doivent adapter fortement leur corps et leur cerveau pour être en mesure de s'assurer un processus aussi exigeant que la grossesse et les soins à la progéniture, les éléments essentiels pour assurer la survie de l'espèce.

Les régulateurs principaux du système reproducteur sont les neurones GnRH, une petite population de neurones parvocellulaires localisés dans l'aire preoptique de l'hypothalamus, qui ne sont fonctionnels qu'au stade de la puberté, et qui nécessitent donc une période de développement postnatal, dépendant de l'établissement des signaux neuronaux et non neuronaux ainsi que des signaux hormonaux qui agissent pour moduler la synthèse et la libération de la GnRH.

Les objectifs de mon travail ont été:

(i) étudier le rôle de la néogenèse cellulaire dans l'aire preoptique dans la régulation du processus de maturation des neurones GnRH au cours du développement postnatal, un moment où la plupart des cellules gliales sont produites dans le cerveau;

(ii) caractériser la prolifération cellulaire au cours du cycle œstral dans l'aire preoptique et l'éminence médiane à l'âge adulte, les régions où les corps et les

fibres des neurones GnRH sont respectivement trouvés, et d'évaluer son implication possible avec la fonction de reproduction;

(iii) évaluer le phénotype progéniteurs/cellules souches des cellules trouvées le long du 3ème ventricule dans l'hypothalamus humain adulte en comparant l'expression d'un panel de progéniteurs neuronaux et de marqueurs de cellules souches entre le cerveau humain et celui d'autres espèces.

Néogenèse cellulaire dans l'hypothalamus postnatal chez la rate: implication dans la maturation sexuelle

Les neurones GnRH sont une petite population de neurones retrouvés au niveau de la région préoptique. Ils contrôlent la reproduction grâce à leur libération de l'hormone gonadotrophine qui induit la libération des gonadotrophines, LH et FSH, qui agiront au niveau des gonades pour induire la gonadogenèse et la libération des hormones gonadiques. Après la migration embryonnaire des neurones GnRH de la placode olfactive, où ils sont nés, ils s'installent dans l'hypothalamus où ils se trouvent à la naissance. Cependant, une maturation postnatale est nécessaire pour permettre le début de la puberté et la cyclicité œstrale qui en découle.

Le développement postnatal des neurones à GnRH chez les rats femelles peut être divisé en 4 stades, une période néonatale, de la naissance à P7; une période infantile, de P8 au sevrage, une période débutant par la première activation centrée de l'axe HPG, marqué par un pic de FSH à P12; une période juvénile, de P21 à P30, et une période péripubertaire. Au cours de cette maturation postnatale, la libération de GnRH augmente progressivement et

culmine au moment de la première poussée de LH, provoquant la première ovulation, qui chez le rat correspond au jour de l'ouverture vaginale.

Pendant ce temps, les neurones GnRH reçoivent des inputs neuronaux et non neuronaux provenant de la même région ainsi que d'autres noyaux de l'hypothalamus, car d'autres fonctions hypothalamiques, telles que l'état métabolique ou l'horloge circadienne, interviennent dans le contrôle de la reproduction et contribue donc à régler le moment où le système acquiert sa maturation complète.

On sait maintenant qu'un rôle important dans la régulation de l'activité de neurones à GnRH est exercé également par les astrocytes de la zone préoptique qui interviennent dans la signalisation des facteurs de croissance de la famille EGF puisque les neurones à GnRH n'expriment pas leurs récepteurs. En réponse à cette voie de signalisation, les astrocytes agissent sur les neurones à GnRH par l'action de la Prostaglandine (PG) E2 qui peut induire la libération de GnRH par la stimulation du déclenchement de ces neurones. Il a également été montré que cette diaphonie est nécessaire au début de la puberté et à l'établissement d'une cyclicité œstrale.

Cependant, alors que les neurones sont nés au cours du développement embryonnaire du cerveau, on sait que les cellules gliales apparaissent au cours du développement postnatal, dans un processus qui n'est pas encore entièrement compris.

Le premier objectif de ma thèse était donc d'étudier si la néogenèse cellulaire dans la zone préoptique pouvait être impliquée dans le processus de maturation des neurones GnRH.

Nous avons utilisé la bromodésoxyuridine (BrdU) pour analyser la prolifération dans la région préoptique de rats femelles à différents âges postnataux. Une intense activité prolifératives caractérise la période infantile et ces cellules se différencient principalement en

astrocytes. Les astrocytes nouveau-nés sont en relation morphologique avec les neurones à GnRH, par rapport à l'ensemble de la population neuronale de la zone préoptique et leur nombre augmente au fil du temps. Nous avons montré que les neurones à GnRH sont capables de recruter des cellules néonatales à proximité de leur corps cellulaires par la libération de prostaglandine D2 et la signalisation sur son récepteur (DP1). De plus, cette activité proliférative est nécessaire à la maturation des neurones GnRH et au début de la puberté, et le recrutement spécifique des cellules néonatales est impliqué dans la maturation du système GnRH.

Néogenèse cellulaire dans l'hypothalamus adulte des rates

La reproduction dépend de l'ovulation féminine, qui se produit de manière cyclique. Les rats (ainsi que les souris) ont des cycles très courts qui peuvent être divisés en 4 étapes: 2 jours de diestrus, un jour de proestrus quand la montée de GnRH et de LH survient, induisant l'ovulation, et un jour d'estrus. Les hormones gonadiques augmentent graduellement alors que les follicules mûrissent au niveau des ovaires, et exercent à la fois une rétroaction négative et une rétroaction positive, ces dernières se produisant lorsque leurs niveaux sont suffisamment élevés pour causer la poussée de la GnRH et de la LH.

La neurogenèse et la gliogenèse ont été décrites dans les régions hypothalamiques contrôlant la fonction de GnRH, telles que le noyau antéro-ventral périventriculaire (AVPV) de l'hypothalamus, où se trouvent les neurones kisspeptin responsables de la rétroaction positive des œstrogènes sur les neurones GnRH; au niveau de l'organum vasculeux de la lamina terminalis (OVLT), où se trouvent les corps cellulaires des neurones GnRH, et au

niveau de l'éminence médiane, où les neurones GnRH projettent leurs terminaisons; ce qui soulève la possibilité que ce processus puisse également participer aux mécanismes de plasticité impliqués dans la régulation neuroendocrinienne de la reproduction.

Le second objectif de mon doctorat était d'explorer la possibilité que la génération de nouvelles cellules dans l'hypothalamus adulte représente un mécanisme de plasticité impliqué dans le contrôle du système à GnRH.

Nous avons observé que la génération de nouvelles cellules se poursuit au-delà de la puberté dans les régions hypothalamiques critiques pour le contrôle des neurones à GnRH: la région préoptique et l'éminence médiane, en outre et que les niveaux de prolifération cellulaire varient selon le cycle œstral. De plus, les cellules nées dans la région préoptique des rats femelles, juste avant l'ovulation, survivent préférentiellement si la gestation survient. Compte tenu du rôle critique de la zone préoptique dans l'apparition du comportement maternel, nous proposons l'hypothèse que l'ajout de nouvelles cellules dans cette région pendant la grossesse participe aux changements adaptatifs qui permettent à ce comportement de se développer.

La niche des cellules souches/progénitrices dans l'hypothalamus humain adulte

Le cerveau adulte contient des niches de cellules souches neurales qui ajoutent continuellement de nouveaux neurones à des circuits sélectionnés tout au long de la vie. Chez l'homme également, les deux principales niches neurogéniques, la SVZ et la SGZ, ont

fait l'objet d'études approfondies, montrant un certain niveau de variabilité par rapport aux autres espèces. Cependant, malgré les données récentes identifiant l'hypothalamus adulte comme une troisième niche neurogénique chez les rongeurs, cette région n'a pas encore été étudiée chez les humains.

Afin d'évaluer si une niche de cellules souches neurales existe également dans l'hypothalamus adulte humain, nous avons effectué plusieurs marqueurs d'immunofluorescence pour évaluer l'expression d'un panel de marqueurs de cellules souches/progénitrices neurales (Sox2, nestine, vimentine, GLAST, GFAP) dans l'hypothalamus humain et nous les avons comparés à la souris, au rat et à une espèce de primate non humain, le lémur gris (Microcebus murinus).

Nos résultats montrent que l'hypothalamus humain adulte contient trois populations distinctes de cellules qui expriment les cinq marqueurs des cellules progénitrices neurales:

i) un ruban de petites cellules étoilées qui tapisse la paroi du troisième ventricule derrière un espace hypocellulaire, similaire à celui précédemment décrit le long des ventricules latéraux humains;
ii) des tanycytes qui tapissent le plancher du troisième ventricule dans la région tubéreuse;
iii) une population de petites cellules étoilées dans le noyau suprachiasmatique.

Dans l'hypothalamus de la souris, du rat et du Microcebus murinus, les marqueurs des cellules progénitrices neurales sont co-exprimés dans les tanycytes mais pas dans le noyau suprachiasmatique, et ces espèces n'ont pas de ruban ventriculaire. Notre travail identifie ainsi trois populations cellulaires avec le profil antigénique des cellules progénitrices neurales dans l'hypothalamus humain adulte, dont deux semblent spécifiques aux humains.

Ce travail montre que de nouvelles cellules naissent dans les régions hypothalamiques contrôlant la reproduction tout au long de la vie postnatale et adulte chez les rats femelles, et que ce processus est nécessaire pour la maturation sexuelle. L'identification des cellules exprimant les marqueurs des cellules progénitrices neurales dans l'hypothalamus humain adulte suggère que la capacité de néogenèse cellulaire existe également dans l'hypothalamus de notre espèce.

Poster and oral communications

Oral communications:

- 2017: Pellegrino G, Allet C, Leroy D, Siepmann J, Baroncini M, Prevot V, Sharif A. GnRH neurons shape their glial environment during postnatal maturation through the action of PGD2. The 21st Annual meeting of the LARC-Neuroscience. Lille, France. 13 November 2017. Selected on abstract.

Posters:

- 2017 : Pellegrino G, Allet C, Leroy D, Siepmann J, Baroncini M, Prevot V, Sharif A. Postnatal astrogenesis is critical for sexual maturation. 13e EUROGLIA meeting, Edinburgh, 8-11 July 2017.
- 2017 : Pellegrino G, Allet C, Leroy D, Siepmann J, Baroncini M, Prevot V, Sharif A. GnRH neurons use PGD2 to shape their glial environment during postnatal maturation. Endo 2017, Orlando, Florida, USA, 1-4 April 2017.
- 2017 : Romero-Ruiz A, Torres-Jiménez E, Pellegrino G, Sharif A, Argente J, Chowen J, Roa J, Pinilla L, Colledge W.H, Tena-Sempere M. Identification of a novel kisspeptin signalling pathway in glial cells. Endo 2017, Orlando, Florida, USA, 1-4 April 2017.
- 2017 : Sharif A, Pellegrino G, Trubert C, Wyart E, Leroy D, Baroncini M, Maurage CA, Fontaine C, Prévot V. Une niche de cellules souches dans l'hypothalamus humain adulte? 22es Journées du Collège des Histologistes, Embryologistes et Cytogénéticiens, Reims, France.
- 2015 : Sharif A, Allet C, Pellegrino G, Leroy D, Caillet A, Loyens A, Siepmann J, Corfas G, Ojeda SR & Prevot V. The birth of astrocytes in the environment of GnRH neurons during early post-natal development is required for the timely onset of puberty in rodents. Colloque de la Fondation Ipsen, Paris, France.
- 2015 : Pellegrino G, Prevot V, Baroncini M, Sharif A. Cell neogenesis in the hypothalamus: a new mechanism of control of the reproductive function? 40ème colloque de la Société de Neuroendocrinologie (SNE-BSN joint meeting), Lille, France.

- 2015 : Sharif A, Allet C, Pellegrino G, Leroy D, Caillet A, Loyens A, Siepmann J, Corfas G, Ojeda SR & Prevot V. « The timely onset of puberty in rodents requires postnatal astrogenesis in the environment of GnRH neurons ». 12ème congrès de la Société des Neurosciences Françaises, Montpellier, France.
- 2015 : Pellegrino G, Prevot V, Baroncini M, Sharif A. « Cell neogenesis in the hypothalamus: a new mechanism of control of the reproductive function? » COST Third Annual Training School in Neuroendocrinology, Prato, Italy.
- 2015 : Sharif A, Allet C, Pellegrino G, Leroy D, Caillet A, Loyens A, Siepmann J, Corfas G, Ojeda SR & Prevot V. Postnatal astrogenesis and female sexual maturation in rodents. Reprosciences 2015, Rennes, France.

Abbreviations

α-MSH	α-melanocyte stimulating hormone
AgRP	agouti-related protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
aNSC	adult neural stem cells
APC	adenomatosis polyposis coli
AR	androgen receptor
ARC	arcuate nucleus
AVP	anteroventral nucleus
AVPV	anteroventral periventricular nucleus
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
BLBP	brain lipid binding protein
BMP	bone morphogenetic proteins
BrdU	bromodeoxyuridine
CCK	cholecystokinin
cGMP	cyclic GMP
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CNTFR	CNTF receptor
COX	cyclooxygenases
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DA	dopamine
DCX	doublecortin
DG	dentate gyrus
DMH	dorsomedial hypothalamus
eNSC	embryonic neural stem cells

EGF	epidermal growth factor
EGFP	enhanced GFP
EGFR	EGF receptor
eNOS	endothelial NOS
EP	E-prostanoid receptor
ER	oestrogen receptor
ESC	embryonic stem cells
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GFAP	glial fibrillary acidic protein
GFP	green fluorescence protein
GHRH	growth hormone releasing hormone
GLAST	GLutamate ASpartate Transporter
GnRH	gonadotropin releasing hormone
Gpr54	kisspeptin receptor
GW	gestational week
HFD	high fat diet
HH	hypothalamic hamartomas
HPA	hypothalamic–pituitary–adrenal
HPG	hypothalamic–pituitary–gonadal
HPT	hypothalamic–pituitary–thyroid
ICM	inner cell mass
i.c.v.	intracerebroventricular
IGF-I	insulin-like growth factor I
IKKβ	IκB kinase-β
iNOS	inducible NOS

i.p.	intraperitoneal
IPC	intermediate progenitor cells
Kiss1r	kisspeptin receptor
LH	luteinizing hormone
LepR	leptin receptor
LHA	lateral hypothalamus
MAP2	microtubule-associated protein 2
MAPK	mitogen activated protein kinase
ME	median eminence
MEPO	median preoptic nucleus
MPO	medial preoptic area
MRI	magnetic resonance imaging
NF-κB	nuclear factor-κB
NG2	nerve/glial antigen 2
nIPC	neuronal IPC
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	NO synthase
nNOS	neuronal NOS
NPY	neuropeptide Y
NRG	neuregulins
NT	neurotensin
OB	olfactory bulb
OEC	olfactory ensheathing cell
oIPC	oligodendrocyte IPC
OVLT	organum vasculosum of the lamina terminalis
PB	phosphate buffer

PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
PGE2	prostaglandin E2
PGD2	prostaglandin D2
PHDA	periventricular hypophyseal DA neuron
PKA	protein kinase A
POA	preoptic area
POMC	proopiomelanocortin
PR	progesterone receptor
PSA-NCAM	polysialylated-neural cell adhesion molecule
PVN	paraventricular nucleus
RG	radial glia
RMS	rostral migratory stream
SCN	suprachiasmatic nucleus
SDN	sexually dimorphic nucleus
Sema7A	semaphorin7A
SGZ	subgranular zone
SHH	sonic hedgehog
SON	supraoptic nucleus
Sox	sex-determining region Y-related Homolog box
SS	somatostatin
SVZ	subventricular zone
SynCAM1	synaptic cell adhesion molecule 1
TACE	TGF-α-converting-enzyme
TGF	transforming growth factor
TF	transcription factors
THDA	tuberohypophyseal DA neurons

TIDA	tuberoinfundibular DA neurons
TRH	thyrotropin releasing hormone
VEGF	vascular endothelial growth factor
VMH	ventromedial hypothalamus
VZ	ventricular zone

INTRODUCTION

Every day we interact with a constantly changing environment from which we receive a multitude of different stimuli that, together with a number of internal signals, converge into our brain. Coordination between these two players is fundamental to any physiological response as well as extremely delicate: the number, position, subtype and connectivity web of neural cells need to be tightly controlled and can be easily perturbed. Therefore we are in front of a system where stability, being the brain a complex tissue with millions of intricate connections, has to coexist with plasticity. This is the ability to modify and adapt structures and functions during maturation, pathologies or environmental challenges, at many levels of organization of both neural and non-neural elements, improving functional outcome to better suit the prevailing environmental conditions.

Although early life experiences are those which mainly shape our brain circuits, as they occur at a moment when the brain itself is still developing, neural plasticity is indeed maintained also during adulthood.

Such a statement would have been considered impossible until no more than 50 years ago, when the first experiments showing the ability of the adult brain to generate new neurons in physiological conditions started shaking one of the central dogma of neuroscience: “In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.” (Degeneration and Regeneration of the Nervous System, Ramon y Cajal, 1928).

The discovery of neural stem cells in mammalian brains which could generate new neurons and glial cells in adult animals was a real paradigm shift; however, realising that this process could be influenced by external stimuli highlighted the role of brain plasticity in integrating external information with internal cues to elaborate adapted behavioral and physiological responses; it gave this process a purpose.

The brain region mainly involved in the cross talk between the environment and the organism itself is the hypothalamus. This small portion of the diencephalon, found in all vertebrate nervous systems, is a homeostatic regulator of behavioral and physiological processes as metabolism, reproduction, thermoregulation, circadian rhythms, fear, sexual behaviors, all complex functions dependent on the internal status of the animal (e.g. the nutrient levels) but also on information received from the environment (e.g. the hours of light and dark, the vicinity of a danger or of a mate).

Given its critical role in maintaining physiological homeostasis, it should come with no surprise that high levels of plasticity are observed in the hypothalamic neuronal and glial populations. Nonetheless the existence of a population of hypothalamic neural stem cells has only started to be addressed until now. A few pioneering studies explored the possibility that the production of new neurons and/or glial cells in the adult hypothalamus may contribute to the flexibility necessary to continuously integrate signals coming from other brain nuclei as well as from the periphery and the environment in order to maintain the correct energy homeostasis in the body.

Nevertheless, little is known about the implication of this process in any of the other hypothalamic functions. Reproduction is the most important physiological function for a species. Even though a single animal can survive if not fertile, perpetuation of life depends upon reproduction. This complex function, which matures over the postnatal period, is strongly influenced by the environment and the metabolic state of the animal, especially in females that have to strongly adapt their body and brain to be able to sustain a process as demanding as pregnancy and care of the offspring, all essential elements to ensure survival of the species.

After a general introduction on how neurons and glial cells are produced in the brain, I will first introduce our current knowledge on adult neurogenesis in the two canonical niches, the SVZ and the SGZ. I will then present the current data on the emerging field of cell neogenesis in the post-natal hypothalamus and finally give an overview of how the hypothalamus controls the reproductive function.

Chapter 1

Cell neogenesis in the brain throughout life

1.1 STEM CELLS

In the 1960s the work from James Till and Ernest McCulloch for the first time introduced the concept of developmental hierarchies of cells, where a single stem cell can give rise to multiple differentiated offspring (Daley, 2015; W. Xu, Lakshman, & Morshead, 2017). Indeed, despite the great level of diversity found among the different cells of an organism, they all derive from the unicellular zygote, result of fertilisation between two gametes (Ohnuki & Takahashi, 2015). During embryogenesis, the zygote transforms into a morula and then a blastocyst through mitotic cell division before implantation. The blastocyst is composed by an inner cell mass (ICM), that will form the embryo, and an outer layer called trophoblast, which will give rise to the placenta and yolk sac (Ohnuki & Takahashi, 2015; W. Xu et al., 2017).

Stem cells have not been easy to characterise due to their lack of specific markers (Reynolds & Rietze, 2005). However, they have to fulfil two main characteristics: ability to self-renew and to differentiate. According to their differentiation potency, cells can be distinguished as:

Totipotent: only the zygote and early blastomeres, which can form all the tissues of the embryo itself as well as the supportive extraembryonic tissues.

Pluripotent: cells with robust self-renewal properties and unbiased potential to form all somatic cell lineages and germ cells; these are the embryonic stem cells (ESCs) isolated from the ICM of the blastocyst.

Multipotent: cells that can give rise to all cell types within the same lineage; these are progenitor cells, for example the hematopoietic cells, which can differentiate into the different types of blood cells.

Bi/Unipotent: these cells can also be referred to as progenitor cells as they still retain the ability to proliferate but are committed to differentiate into one or two specific cell types (Daley, 2015; Dulak et al., 2015; Martello & Smith, 2014).

For many years, embryonic development has been depicted as a one-way process and compared to a ball rolling downhill, with the range of a cell's fate becoming progressively restricted as if cells would roll towards a stable differentiated state from which it was impossible to "escape" (Daley, 2015; Ohnuki & Takahashi, 2015). However, in the late 1980's, ground breaking experiments led to the discovery that pluripotency is a transcription

factor–determined state and can therefore be experimentally induced even in fully differentiated somatic cells, opening the era of the induced pluripotent stem cells (Martello & Smith, 2014; Takahashi & Yamanaka, 2006).

The classical function of somatic stem cells, found in adults within specific germinal niches, is that to sustain homeostatic tissue turnover and enable repair throughout life (Martello & Smith, 2014). Multipotent stem cells are able to divide symmetrically, to expand the stem cell pool or asymmetrically, to maintain the stem cell pool while giving rise to the cell types of the tissue in which they reside (Xu et al., 2017). The direct descendants of stem cells are progenitor cells: cells with a more restricted lineage potential but higher proliferative capacity (Xu et al., 2017).

Adult stem cells can be found in either a quiescent or an active state. The latter are responsible for replenishment of the tissue, while the former are a backup to replace active stem cells. The reasons behind this strategy may be to prevent exhaustion of the stem cell pool and the accumulation of potentially tumorigenic mutations. The quiescent pool can be activated by stochastic mechanisms, feedback signals derived from the loss of the active stem cells, or by extensive tissue damage. The coexistence of these two different pools in the same regions highlights the importance of the presence of a niche where specific factors are produced to maintain the specific proliferative state of the cells (Li & Clevers, 2010).

1.2 EMBRYONIC AND POSTNATAL DEVELOPMENT

1.2.1 Neurogenesis occurs during embryonic life

ESCs can be isolated from the developing mouse blastocyst around embryonic day (E) 3.5; by E6.5 the ICM comprises three germ layers: the endoderm, the mesoderm and the ectoderm (Ohnuki & Takahashi, 2015; Xu et al., 2017). The endoderm will form the digestive tube with the glands that open into it, the urinary bladder, the epithelial parts of trachea, the lungs, the thyroid, the parathyroids (Kiecker et al., 2016; Solnica-Krezel & Sepich, 2012); the mesoderm will form muscles, bones, cartilage, connective tissue, adipose tissue, the circulatory system, the lymphatic system, dermis, the genitourinary system, serous membranes, and the notochord (Kiecker et al., 2016; Solnica-Krezel & Sepich, 2012; Takemoto, 2013); finally the ectoderm will develop in the surface ectoderm, from which epidermis, hair, nails will derive; the neural crests that will give peripheral nervous system,

melanocytes (Nitzan & Kalcheim, 2013); and the neural tube that gives rise to the brain, spinal cord, posterior pituitary and the retina (Kiecker et al., 2016; Solnica-Krezel & Sepich, 2012; Takemoto, 2013).

The process of invagination and closure of the ectoderm, through which the neural tube is formed, is called neurulation; this is a finely regulated process divided into different steps, each of them dependent on the establishment of precise morphogen gradients (Takemoto, 2013). The neural tube will give rise to the central nervous system (CNS) therefore being subjected to a rostro-caudal as well as dorso-ventral differentiation process. In its rostral portion, from which the brain will derive, the neural tube balloons into three primary vesicles: the forebrain (cerebral hemispheres, thalamus, hypothalamus, and retina), the midbrain (tectum and motor pathways of the basal ganglia), and the hindbrain (cerebellum and medulla oblongata) (Darnell & Gilbert, 2017). The inner cavity of these vesicles will later be filled with cerebrospinal fluid (CSF) and will give the four ventricles. Meanwhile the dorsoventral axis of the more caudal portion, which will give rise to the spinal cord, is modified by signals coming from the immediate environment: the dorsal side receives sensory inputs, whereas motor signals emanate from the ventral side (Darnell & Gilbert, 2017).

Neurons and macroglia of the brain derive from neuroepithelial cells that can be called embryonic neural stem cells (eNSCs), which line the cerebral ventricles, and undergo different amplification steps giving rise to intermediate progenitor cells (IPCs) (Fig. 1). Cortical neurogenesis begins around E9-10 in mice. By this time, neuroepithelial cells begin to acquire features of glial cells, such as expression of astroglial markers like GLAST (glutamate aspartate transporter), BLBP (brain lipid binding protein), nestin, vimentin and are referred to as radial glia (RG) (Kriegstein & Alvarez-Buylla, 2009; Rowitch & Kriegstein, 2010). These cells also express GFAP (glial fibrillary acidic protein) in some species, such as in primates, and in mice only from E16 (Choi, 1988). RGs stay in contact with the surface of the ventricle and the pia, with their cell bodies found at the level of the ventricular zone (VZ) and connected by adherens junctions (Kriegstein & Alvarez-Buylla, 2009).

RG cells proliferate and divide asymmetrically to maintain the eNSC pool and to generate either a neuron or an IPC. These IPCs can be committed to the neuronal lineage (nIPC) or to the oligodendrocyte one (oIPC) (Fig. 1).

During cell division, the pial endfeet of the RGs do not retract and the daughter neurons often migrate along these processes. IPCs in turn divide symmetrically to generate either two IPCs or two neurons. RGs but not IPCs are characterised by interkinetic nuclear migration during their cell cycle progression, with nuclei in M-phase being closer to the ventricular surface, nuclei in S-phase migrating towards the apical side and nuclei in G1 or G2-phase found in an intermediary position between the two (Kriegstein & Alvarez-Buylla, 2009). RG cells are able to sense the gradients of the different morphogens that characterise each region of the developing CNS and in turn respond by producing different subtypes of neurons according to their position and stage of development, completing the process of neurogenesis before birth (Kriegstein & Alvarez-Buylla, 2009; Rowitch & Kriegstein, 2010) (Fig.1).

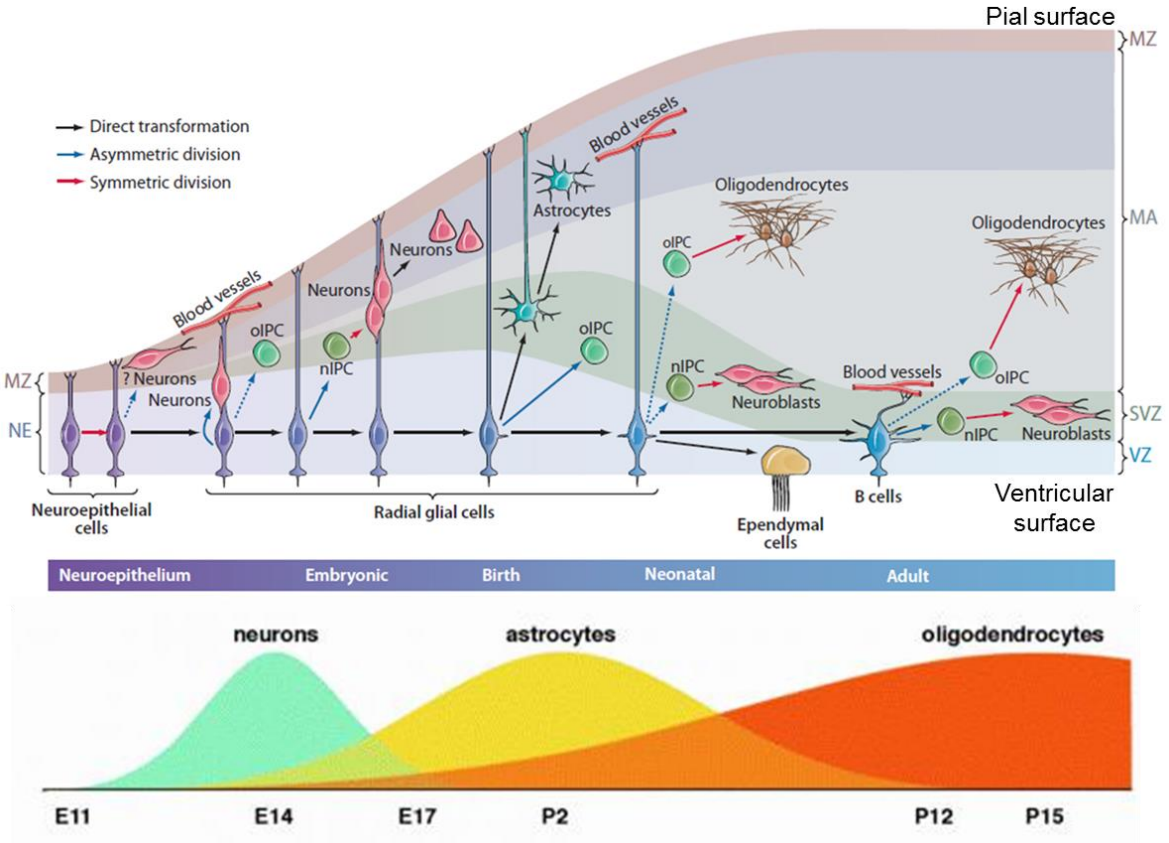


Figure 1: Neural stem cells (NSCs) from embryo to adult. (Upper panel) During brain development neuroepithelial cells elongate and convert into radial glial (RG) cells that contact the ventricle apically and the meninges, basal lamina, and blood vessels basally. RG can give rise to different type of intermediate progenitors, committed towards a neuronal (nIPC) or oligodendrocytic (oIPC) fate. At the end of embryonic development, some RGs convert into ependymal cells, others detach and convert into astrocytes while a subpopulation retains apical contact and continue functioning as NSCs in the neonate and adult animal. Dashed arrows are hypothetical. **(Lower panel)** Waves of neuro-, astro- and oligodendro-genesis from embryological to postnatal development. MA: mantle; MZ: marginal zone; NE: neuroepithelium; nIPC: neurogenic progenitor cell; oIPC: oligodendrocytic

progenitor cell; RG: radial glia; SVZ: subventricular zone; VZ: ventricular zone (From Kriegstein and Alvarez-Buylla 2009, Annual Review Neuroscience and Sauvageot & Stiles 2002, Current Opinion in Neurology).

1.2.2 Gliogenesis occurs during postnatal life

1.2.2.1 Astrogenesis

At the end of the embryological development, RG cells disappear in most brain regions (Kriegstein & Alvarez-Buylla, 2009). They will either self-consume by symmetric neurogenic divisions or generate glial and ependymal cells; however, in many mammalian species, RGs can still be found in the adult brain acting as NSCs (Götz et al., 2015; Kriegstein & Alvarez-Buylla, 2009) (see chapter 1.3). The translocation of RG and transformation to astrocytes, mostly studied in the cortex, has been directly visualised by using retroviral labelling and time-lapse imaging (Noctor et al., 2008).

Since neurons and astrocytes derive from the same progenitor cells, a tight regulation of the switch from a neuronal to an astrocytic potential, which relies upon exogenously secreted cues and intrinsic chromatin changes, is critical for normal brain formation. The main mechanism by which astrogenesis is repressed is by inactivation of the JAK/STAT pathway which plays an important role in astrogenesis. Once JAKs are activated, by autophosphorylation, they phosphorylate STAT3 which forms homodimers that are translocated into the nucleus to induce the expression of astrocytic genes, such as GFAP, therefore promoting astrocytic differentiation (Mallamaci, 2013; Qian et al., 2000). Moreover, early in forebrain development, the promoters of GFAP and S100B are methylated, therefore silenced, and neuroepithelial cells are insensitive to cytokines. At the onset of astrogenesis, RG cells change their differentiation competence through demethylation of the promoters of astrocytic genes, possibly through epigenetic regulation as for instance the chromatin state of the GFAP promoter changes significantly to allow active gene transcription (Mallamaci, 2013). Even though the precise timing of this process remains unclear, due to a lack of definitive markers to distinguish astrocyte precursors from radial glia, it is clear that astrocyte number increases abruptly after neurogenesis completion. Accordingly, $GFAP^+$ cells, still rare at birth (< 2.0%), double in number every 3-4 days in the first postnatal week, peaking around P3 (Qian et al., 2000) (Fig. 1) and reaching their absolute plateau around P21 (Bandeira et al., 2009). The injection of enhanced green

fluorescence protein (GFP)-encoding retroviruses into the cortex of P0-P2 mice showed that almost 50% of astrocytes were labelled 10 days after the injection, suggesting that most astrocytes are generated by local proliferating progenitors (Mallamaci, 2013). Moreover at this stage, neurons secrete gliogenic cytokines promoting gliogenesis themselves. Once new astrocytes are generated glial identity needs to be retained and in order to ensure that, neuronal genes must be repressed. Disruption in any of these mechanisms can potentially affect the timing or efficiency of neurogenesis and astrogenesis, altering the relative ratio between these two cell types, which may contribute to the progression of a variety of neurodevelopmental disorders (Sloan & Barres, 2014).

1.2.2.2 Oligodendrogenesis

Oligodendrocytes originate in multiple locations and at different moments during development, starting during embryogenesis and continuing during postnatal development. Oligodendrocyte progenitor cells are nerve/glial antigen 2 (NG2)-expressing proliferating cells distributed throughout the brain; the origin of these cells remains unclear, but they are likely to be derived from RG cells (Nishiyama et al., 2016).

NG2 cells can be considered as a type of oligodendrocyte precursors that have not yet become terminally differentiated into oligodendrocytes, and which remain capable of symmetrically divide to replenish their pool, throughout all stages of development. A property of NG2 cells that distinguishes them from astrocytes is their robust migratory ability. NG2 cells reach their peak density during the first postnatal week, after which it declines even though they will occupy defined domains through the entire parenchyma. Genetic fate mapping studies have shown that NG2 cells undergo proliferation in response to a stab wound and some differentiate into oligodendrocytes but not astrocytes. Nevertheless, it has been shown that NG2 cells could be manipulated to differentiate into neurons in the injured CNS (Nishiyama et al., 2016).

1.3 NSCs IN THE ADULT BRAIN: THE TWO CANONICAL REGIONS

NCSs can still be found in the adult brain within restricted regions where they can give rise to glial as well as neuronal cells (Alvarez-Buylla & Lim, 2004; Gage, 2000; Ming & Song, 2005; Nottebohm, 2004). The two most studied and characterised regions, where the presence of

adult NSCs (aNSCs) has been widely accepted, are the subventricular zone of the lateral ventricles (SVZ), adjacent to the ependymal ciliated single cell layer that lines the wall of the lateral ventricles, and the subgranular zone of the dentate gyrus of the hippocampus (SGZ) (Alvarez-Buylla & Lim, 2004; Gage, 2000; Ming & Song, 2005). However, increasing evidences are pointing towards the hypothesis that more regions retain the ability to produce new neural cells, such as the striatum (Bédard et al., 2006; Luzzati et al., 2007; Nato et al., 2015) and the hypothalamus (Djogo et al., 2016; Kokoeva et al., 2005; Mcnay et al., 2012; Migaud et al., 2010; Pencea et al., 2001; Sharif et al., 2014; Xu et al., 2005) (see chapter 1.7.3).

The origin of aNSCs in the SVZ has been identified only recently in a subpopulation of quiescent eNSCs (Fuentealba et al., 2015; Furutachi et al., 2015). It appears that around E13.5 and 15.5, a subset of eNSCs strongly slow down their cell cycle, maintaining their undifferentiated state and in this way persisting until postnatal stages when they become reactivated. The rest of them continue dividing to ensure the development of the CNS and are lost during the process (Furutachi et al., 2015). Moreover, it has been shown that as early as E11.5, these cells have acquired a regional specification, due probably to the expression of distinct combinations of transcription factors (TFs), which they will retain throughout postnatal life and that will determine their progeny (Fuentealba et al., 2015).

NSCs of the DG derive from stem cells located in the subpallium region during embryonic development. However, their exact embryonic origin has not been fully elucidated since the DG is generated by a mosaic of stem cells with different embryonic origins. Embryonic formation of the DG begins around E13.5 in mice. Hippocampal RG cells, which express typical markers such as nestin and GLAST at E15.5, proliferate in a domain of the ventricular zone called the dentate neuroepithelium, which is adjacent to the cortical hem (Li et al., 2013; Rolando & Taylor, 2014). From here, these cells will migrate through the dentate migratory stream and spread, covering the DG. At the end of the first postnatal week, following a process of reorganisation, NSCs are found in the SGZ where they will persist throughout life (Rolando & Taylor, 2014).

1.3.1 Identity of aNSCs – to be or not to be

Adult NSCs are not a homogeneous population. It is true that the classical definition of stem cells implies multipotency and self-renewal. However, these characteristics often emerge

only at a population level. This means that most aNSCs are committed to a specific cell type or even to a neuronal subtype; besides, most of them remain quiescent most of their life making them difficult to identify and isolate as there is no single specific marker for aNSCs or progenitors (Kriegstein & Alvarez-Buylla, 2009; Magnusson & Frisen, 2016) (Fig. 2). These cells retain some features typical of the RG cells and were phenotypically identified as classical astrocytes. Like RGs, they present a primary cilium, localised on their apical surface, which expresses the glycoprotein prominin 1 (CD133 in human), and a long basal process which contacts surrounding blood vessels in mice (Mirzadeh et al., 2008) (Fig. 2). At the same time, they express astrocytic genes such as GFAP or GLAST and they share their ultrastructural and electrophysiological characteristics (Götz et al., 2015; Kriegstein & Alvarez-Buylla, 2009). This brought up the question of whether all astrocytes retain a latent capacity to behave as stem cells. Only astrocytes of the SVZ and SGZ are clearly specialised and behave as NSCs, but the intrinsic potential of parenchymal astrocytes is not well known yet.

For instance, debates are still ongoing on whether new neurons are produced in the striatum and what their physiological function might be. Low levels of striatal neurogenesis have been reported under physiological conditions in rabbits, rats and squirrel monkeys (Bédard et al., 2002; Dayer et al., 2005; Magnusson et al., 2014; F. Luzzati, 2006), although these data have not always been replicated (Magnusson & Frisen, 2016). Moreover, strokes triggeres bursts of proliferation in striatal astrocytes in rodents (Magnusson et al., 2014), increasing neurogenesis in this region; this capacity to remain quiescent and being activated following a stimulus is reminiscent of aNSCs behaviour (Magnusson & Frisen, 2016).

The situation changes in other areas. For instance, cortical astrocytes, can reactivate their proliferation within a week after a stab wound injury (Buffo et al., 2008; Sirko et al., 2013); these subsets of proliferating astrocytes are enriched in the vicinity of the vasculature (Bardehle et al., 2013) and, even though they express some more immature markers such as nestin, they remain within their lineage, not showing neurogenic capabilities *in vivo* (Bardehle et al., 2013; Buffo et al., 2008). These differences might be explained in part by the changing microenvironment. For example, experiments where adult progenitor cells of the SVZ or SGZ, were transplanted into the cortex showed that they would lose the ability to generate neurons and would only give rise to glial cells, proving that the cortex is a non-permissive environment for neuronal differentiation (Herrera et al., 1999). Nevertheless,

astrocytes are extremely heterogeneous in their form and function throughout the brain, as well as in their mRNA expression profile patterns to an extent that has only started to be unfolded.

Cell differentiation is a complex process involving many different transcription factors which interact to build a fixed network that will determine a cell's properties and identity; such a network will also be self-stabilised by interactions among its different components (Magnusson & Frisen, 2016). However, these transcriptional networks can present variable degrees of stability, giving in turn to the cell different grades of plasticity; in such a case two or more transcriptional networks are expressed and compete against each other keeping the cell on the edge between them two, a situation where even small perturbations would trigger a switch in the cell's identity (Graf & Enver, 2009). The hypothesis is that astrocytes may represent an example of such a state, with no distinct line between "classical" astrocytes and aNSCs but more like a continuum from parenchymal astrocytes that would be able to dedifferentiate to a multipotent intermediate state (Llorens-Bobadilla et al., 2015; Magnusson & Frisen, 2016). In this case, astrocytes from non-neurogenic niches, such as the cortex, may not lack neurogenic capacity but rather possess a less plastic astrocytic identity (Magnusson & Frisen, 2016). For instance, it has been shown that disruption of Notch signalling or overexpression of Sex-determining region Y (SRY)-related HMGbox 2 (SOX2) activates a neurogenic state in mouse striatal astrocytes (Magnusson et al., 2014; Niu et al., 2013). Thus, it appears that Notch promotes astrocyte differentiation while Sox2 stabilises a neurogenic network including activation of Ascl1, which regulates many neurogenic genes (Magnusson & Frisen, 2016).

The same approach applies to the switch between a quiescent and an active state within aNSCs; quiescence is a reversible state where cell cycle is arrested but can be rapidly reinitiated in response to environmental cues. The use of single-cell analysis techniques has allowed the characterisation of what are now considered as two different populations within the aNSC pool. In different studies, aNSCs have been purified from both the SVZ and the SGZ, taking advantage of their coexpression of GFAP, or GLAST, and prominin 1 (Fig. 2). In the case of the SVZ, this purified population has proved to divide, to give rise to olfactory bulb (OB) interneurons with no preference towards a specific subtype and these cells were not found in the parenchyma (Beckervordersandforth et al., 2010; Llorens-Bobadilla et al., 2015; Shin et al., 2015). The first feature that allowed the authors to distinguish between active

and quiescent NSCs was the lack of EGF receptor (EGFR) expression in the latter (Codega et al., 2014; Llorens-Bobadilla et al., 2015) (Fig. 2). Single-cell transcriptomic analysis revealed a decrease in the expression of glial genes going through the intermediate states that bring from a quiescent to an active state. Quiescent NSCs are characterised by a glycolytic metabolism, low expression levels of genes involved in protein synthesis and high levels of membrane receptors, cell-cell adhesion molecules and ion channels, probably to promptly sense changes in the microenvironment and respond to them (Llorens-Bobadilla et al., 2015); they are also characterised by strong expression of Notch receptors and Notch's target genes and surprisingly, at the level of the SVZ, they did not show expression of Nestin (Codega et al., 2014; Shin et al., 2015) (Fig. 2).

On the contrary, active NSCs prefer a lipidic metabolism, they express high levels of genes involved in protein synthesis and cell cycle, as they enter mitosis, they also highly express Notch ligands, suggesting the possibility they may act on the quiescent pool to maintain them in a dormant state through Notch signalling (Llorens-Bobadilla et al., 2015). At the same time however, it appears that quiescent NSCs may regulate their dormancy by an autocrine control since they express high levels of the bone morphogenetic proteins (BMP) signalling pathway, known to promote quiescence (Mira et al., 2010), i.e. BMP receptor, BMP ligands and targets. Finally, quiescent NSCs are able to enter a primed-quiescent state upon brain injury, switching towards the more active state (Llorens-Bobadilla et al., 2015; Shin et al., 2015).

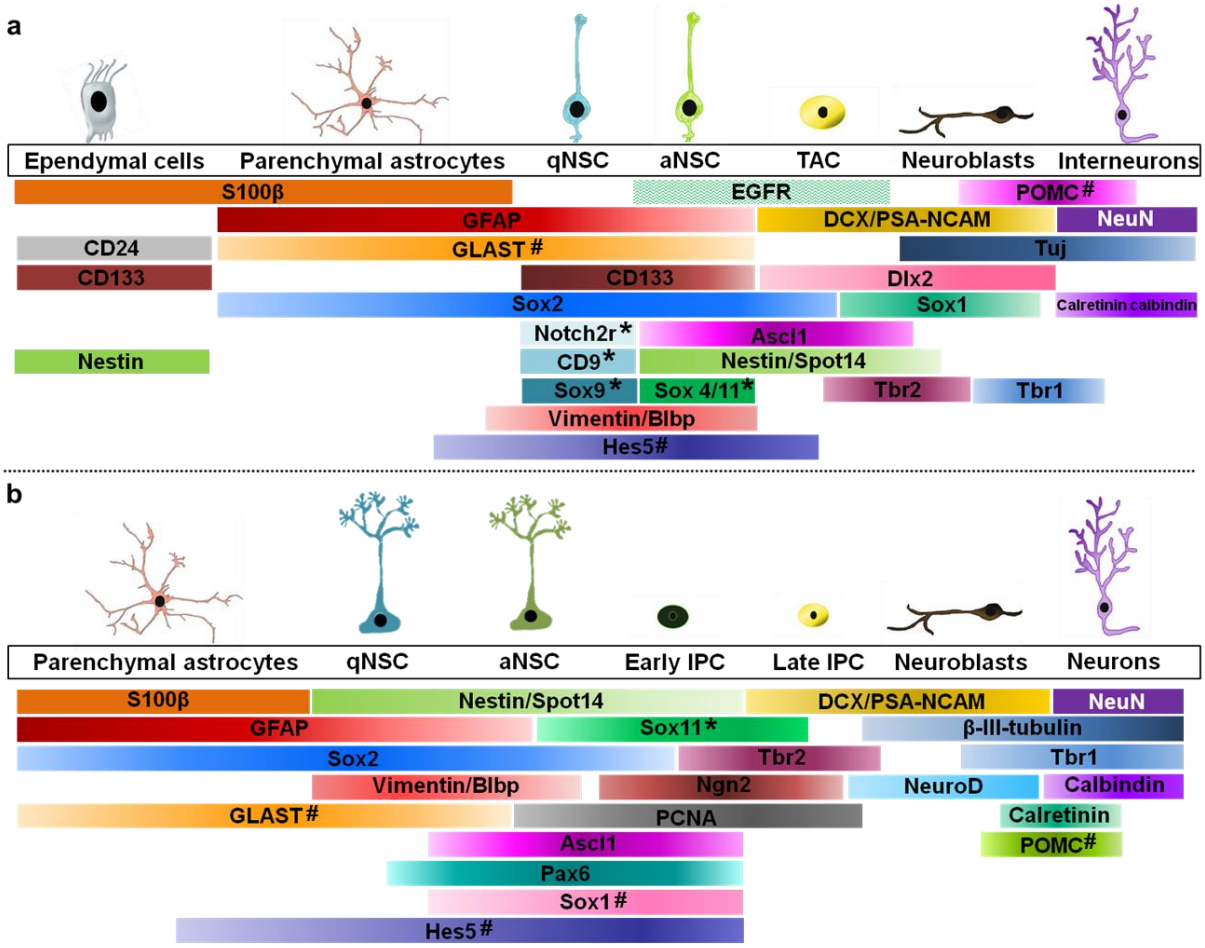


Figure 2: Summary of the expression of markers used to identify different cell types of the adult subventricular zone **(a)** and subgranular zone **(b)** neurogenic niches. aNSC, active neural stem cells; IPC, intermediate progenitor cells; qNSC, quiescent neural stem cells; TAC, transit amplifying cells. *data obtained from transcriptomic analyses only; #data obtained with the use of transgenic reporter lines only. (Scheme modified from Semerci & Maletic-Savatic 2016, Frontiers in Biology; Codega et al. 2014, Neuron; Llorens-Bobadilla et al. 2015, Cell Stem Cell; Shin et al. 2015, Cell Stem Cell).

1.4 A NICHE OF ONE'S OWN

In contrast to embryonic development, when stem cells are surrounded by a continuously changing environment, aNSCs are stable and housed in a well-defined niche that supports their lifelong maintenance and that includes ependymal cells (in the case of the SVZ), organised vasculature, extracellular matrix, immune cells and axonal terminals. (Lim & Alvarez-Buylla, 2014; Magnusson & Frisen, 2016). Moreover, aNSCs are anchored to surrounding endothelial and ependymal cells, a state seen to promote quiescence (Ottone et al., 2014).

Stem cell niches express a plethora of different factors and signals deriving from their different constitutive elements: the local vasculature is a source of factors coming from the blood as well as from the endothelial cells themselves; neurotransmitters, such as glutamate, GABA, serotonin, dopamine, are derived from both local neurons and external innervations (Chaker et al., 2016; Fuentealba et al., 2012). Also the immature neuroblasts, found in intimate contact with NSCs, are able to regulate their own production through different pathways. For instance, they express high levels of Ascl1, known to promote the expression of Notch ligands; as previously said, Notch signalling induces a more quiescent state, suggesting a possible feedback mechanism by direct cell-cell contact (Chaker et al., 2016; Fuentealba et al., 2012). Furthermore, immature neuroblasts can release GABA, thus activating $GABA_A$ receptors on precursor cells inducing their depolarisation with consequent increased Ca^{2+} levels and enhanced NeuroD expression (Liu et al., 2005; Tozuka et al., 2005). This, in turn, inhibits progenitor proliferation and promotes neuronal differentiation (Liu et al., 2005; Tozuka et al., 2005), acting again as a negative feedback apt to downregulate their own production (Chaker et al., 2016; Fuentealba et al., 2012). High levels of BMP signalling promote glial over neuronal differentiation. This signalling is at least partially controlled by the niche as ependymal cells in the SVZ, secrete the BMP antagonist Noggin, and express LRP2, a receptor that sequesters BMP, both of which are required for neurogenesis *in vivo*. In the SGZ, radial astrocytes express Noggin, whose levels increase with physical exercise and which promotes neurogenesis (Fuentealba et al., 2012; Gajera et al., 2010).

1.4.1 The SVZ niche

The SVZ is a thin layer of dividing cells located along the walls of the lateral ventricles between the multiciliated ependymal layer and a planar vascular plexus. It is the largest germinal region in the adult rodent brain (Alvarez-Buylla & Lim, 2004; Lim & Alvarez-Buylla, 2014; Tong & Alvarez-Buylla, 2014). Stem cells in this region are called type B cells. They are characterised by astrocytic features, and give rise to rapidly dividing transit amplifying cells, called type C cells, that will differentiate into neuroblasts, called type A cells, characterised by expression of markers typically associated to young/immature neurons such as doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM) (Fig. 2 and 3). These neuroblasts then migrate along the rostral migratory stream (RMS) to reach the OBs where they mature into different subpopulations of inhibitory interneurons of both the

granular and periglomerular layers where they will contribute to olfactory learning and discrimination (Alvarez-Buylla & Lim, 2004; Lim & Alvarez-Buylla, 2014; Tong & Alvarez-Buylla, 2014).

The population of type B cells is not homogeneous. A viral lineage tracing experiment targeting different areas of the adult SVZ showed a strong regional heterogeneity: each region is characterised by the expression of specific TFs, reminiscent of the pattern seen during embryonic development, and cells of each TF domain give rise to specific subtypes of interneurons integrated in different regions of the OB; more importantly, this regionality is already acquired during embryonic development (Fuentealba et al., 2015). Type B cells, mainly of the dorsal SVZ, are also able to produce astrocytes of both the corpus callosum and RMS (Sohn et al., 2015) and oligodendrocytes of the corpus callosum (Ortega et al., 2013), in a Sonic hedgehog (Shh)- and Wnt-dependent manner (Ortega et al., 2013).

Type B cells are found in a very specific and well characterised niche with a peculiar pinwheel organisation at the ventricular surface. The pinwheel structure is given by the large apical surfaces of the ependymal cells (E1), always containing at least 50 motile cilia, packed around B cells clustered together in the centre with a small apical surface where a short single primary cilium, characterised by the expression of CD133, is found (Mirzadeh et al., 2008) (Fig. 3). This cilium allows B cells to remain in contact with the lumen of the ventricles, filled with the CSF, whose production and composition are dynamically regulated by the choroid plexus, therefore exposing B cells to a constantly changing flux of factors (Silva-Vargas et al., 2016). Intermingled between E1 and B cells, rare biciliated ependymal cells (E2) were also described, however their function has not been understood yet. Both E1 and E2 cells could be recognised by their expression of CD24, never found in B cells (Fig. 2). Moreover, E1 and E2 were never found to divide as they were never seen to express Ki67, a marker of cell division expressed throughout the cell cycle, or to incorporate bromodeoxyuridine (BrdU), an analog of thymidine incorporated into the DNA during its synthesis, that is extensively used to label dividing cells (Mirzadeh et al., 2008; Taupin, 2007).

On the other side, B cells present long $GFAP^+$ basal processes, running tangential to the wall of the lateral ventricle, almost always terminating in specialised endfeet on a blood vessel (Fig. 3). Moreover, these processes contribute to form the gliotubes along which the

immature neuroblasts migrate (Mirzadeh et al., 2008). While the cilium at the apical surface lets B cells sense factors of the CSF, their endfeet give them access to blood-derived factors. As a matter of fact, clusters of dividing cells are associated with blood vessels where the blood brain barrier (BBB) appears to be leaky, due to the lack of astrocytic or pericytic coverage, therefore allowing entrance of small molecules from the circulation (Tavazoie et al., 2008).

The choroid plexus, a vascularized epithelial structure floating within the brain ventricles, has been recently identified as an active part of the SVZ niche (silva-vargas 2016). This complex structure, able to sense signals coming from the blood and the niche itself and to modulate its secretome accordingly, is essential for physiological brain homeostasis, immune surveillance and inflammatory and repair processes. It has been now shown that it can also affect quiescent and active NSCs as well as intermediate progenitors and neuroblasts within the SVZ niche (Silva-Vargas et al., 2016). BMPs, Wnts (which support oligodendrogenesis), Shh are just some of the factors that can be found in the CSF and that modulate the behaviour of SVZ aNSCs. With aging, the secretome of the choroid plexus is subjected to important modifications, to which only active aNSCs appear to be sensitive, suggesting how it may contribute to the decline in neurogenesis that occurs in older animals (Silva-Vargas et al., 2016).

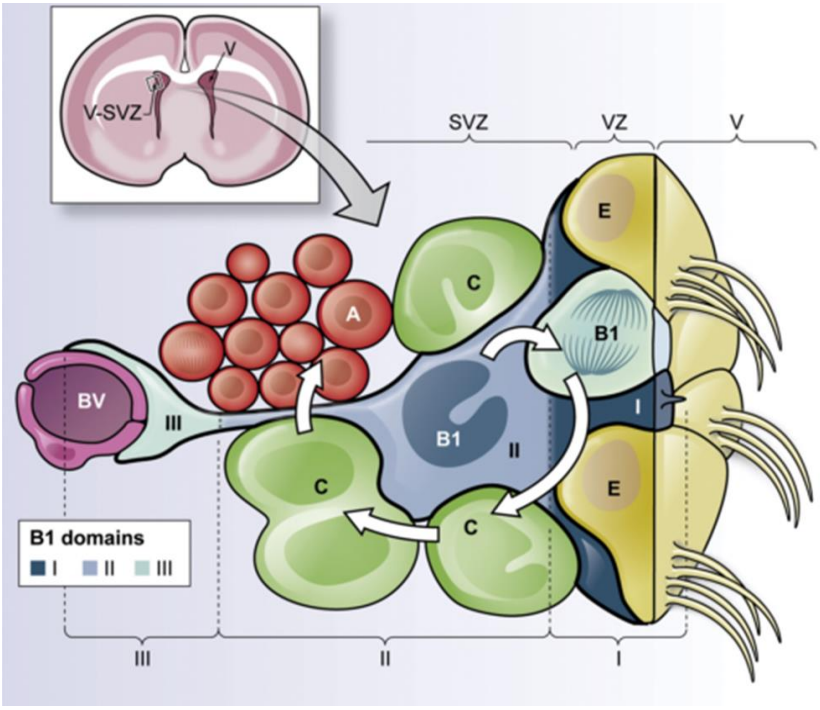


Figure 3. Schematic of the Different Domains of the Adult SVZ niche. (Upper left panel) A frontal cross-section of the adult mouse brain showing the location of the SVZ. **(Lower panel)** Cellular composition of the adult SVZ niche. Neural stem cells (NSCs) correspond to type B1 cells (blue), surrounded by multi-ciliated ependymal cells (E) forming pinwheel-like structures. B1 cells give rise to transit-amplifying cells, C cells (green), which divide to generate neuroblasts (type A cells, red). B1 present a thin apical process containing a primary cilium that contacts the lateral ventricle (V), being in direct contact with the CSF, and a long basal process ending on blood vessels (BV, purple). (From Fuentealba et al. 2012, Cell stem Cell).

1.4.2 The SGZ niche

The SGZ is located at the interface between the granule cell layer and the hilus of the DG, therefore the niche is not in contact with the ventricles (Fig. 4). Here the local aNSCs, also referred to as type I progenitors, exhibit a radial process spanning the granule cell layer and arborizing profusely in the molecular layer and express nestin, GFAP, blbp and Sox2 (Fig 2 and 4). They give rise to intermediate progenitors, type II progenitors or D cells, characterised by a high proliferative activity, short processes and expression of early neuronal markers (DCX, PSA-NCAM) while they stop expressing GFAP, as they are already committed to a neuronal fate (Fig 2 and 4). D cells produce type III cells, which do not show proliferation capacity and will mature into glutamatergic granule cells of the DG (G cells) (Kempermann et al., 2015) (Fig. 4). Moreover, astrocytes, but not oligodendrocytes, were found to be generated in the SGZ, from which they migrate into the hilus, the granule cell layer and the molecular layer (Bonaguidi et al., 2011).

Just like B cells, type I cells are highly polarised (Fig. 4); the side facing the hilus presents a primary cilium that has been shown to be necessary for the expansion and establishment of postnatal hippocampal progenitors through activation of Shh signalling (Breunig et al., 2008; Y.-G. Han et al., 2008). Dividing type I progenitors in the SGZ are in close proximity to an extensive network of interconnected blood vessels (Fig. 4). Unlike the SVZ, where endothelial cells are very rarely seen to divide, in the SGZ active angiogenesis is associated with neurogenesis (Palmer et al., 2000). Increased angiogenesis results from increased expression of the vascular endothelial growth factor (VEGF), which is also associated with increased progenitor proliferation and neurogenesis, even though its action has not been fully understood (Fuentealba et al., 2012). Furthermore, type I progenitors present an elaborate and extensive set of thin branches in the inner molecular layer of the DG where GABAergic and glutamatergic inputs arrive, as well as dopaminergic, serotonergic or

acetylcholinergic projections from other brain regions (Zhao, 2006). Disruption of these different neurotransmitter and input systems perturbs adult neurogenesis; however, the specific contribution of each individual system has not been yet identified (Kempermann et al., 2015).

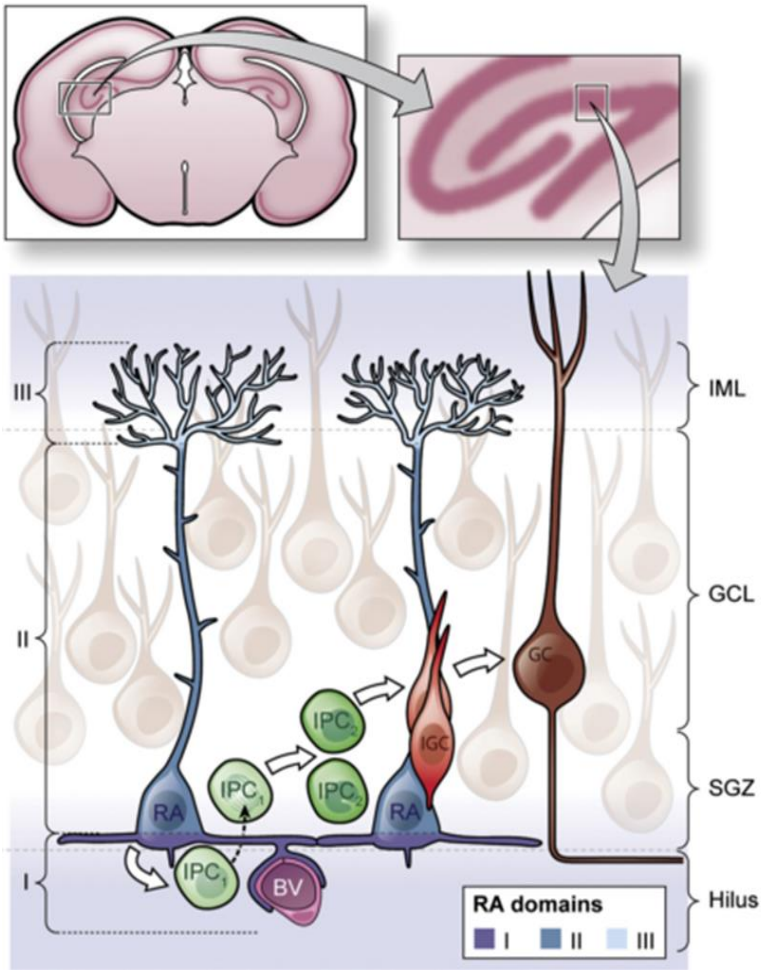


Figure 4. Schematic of the Different Domains of adult SGZ niche. (Upper panel) A frontal cross-section of the adult mouse brain showing the hippocampal formation (left). The insert shows a higher magnification indicating the location of the dentate gyrus (DG) (right). **(Lower panel)** Cellular composition of the adult DG domains. Radial astrocytes (RA), also known as type I cells (blue) give rise to intermediate progenitor cells (IPCs, green), or type II cells, which progressively (via IPC1, light green and IPC2, dark green) differentiate into immature granule cells (IGCs, or type III cells, red). Mature granule cells (GCs, brown) send an axon parallel to the SGZ into the hilus, whereas their dendrites branch into the molecular layer (ML). Radial astrocytes are polarized cells with their cell body in the SGZ, where they contact blood vessels (BV, purple) and neighboring radial astrocytes, a long main process that traverses through the granule cell layer (GCL) and then branches diffusely in the inner molecular layer (IML). (From Fuentealba et al. 2012, Cell stem Cell).

1.5 PHYSIOLOGICAL RELEVANCE OF ADULT NEUROGENESIS

Neurogenesis increases plasticity on multiple levels; while adding new cells to existing circuits it causes structural remodelling, synaptogenesis, and changes in synaptic strength. Neurogenesis encompasses different stages: from cell birth through fate determination, survival, until integration and acquisition of functional properties. Of particular relevance is the selection occurring through modulation of the survival rate of the newborn neuroblasts both in the SVZ and the DG that ensures at any moment a readily available pool of cells. All these processes are sensitive to environmental signals and are subject to reduction with aging, possibly through the activation of the hypothalamic–pituitary–adrenal axis (Danzer, 2012; Fuchs & Flügge, 2014; Gonçalves et al., 2016; Lledo et al., 2006; van Praag et al., 2002).

1.5.1 A role for adult neurogenesis in the SVZ

As previously mentioned, neuroblasts deriving from the SVZ migrate along the RMS, closely associated with each other along a tube-like structure formed by glial cells (Peretto et al., 1996), to reach the OBs where, as early as two weeks later, granule and periglomerular cells can be found in their respective OB layers. These cells make only local contacts in the bulb, directly or indirectly modulating the processing of sensory information (Lledo et al., 2006; Lledo & Valley, 2016).

An enriched odour exposure increases the SVZ but not SGZ neurogenesis. The adult-born neurons indeed are more inclined to respond to novel odours and are preferentially activated by the recollection of an odour-reward memory. Therefore, the main role of neurogenesis in the adult SVZ seems to be that to mediate odour-associated learning, allowing an adaptable consolidation of odour-associated memories. This plasticity appears to be achieved by a highly dynamic formation and elimination of dendrodendritic synapses with principal neurons of the OB (mitral and tufted cells) (Sakamoto et al., 2014).

The situation changes in pathological conditions. For instance, ischemic brain insults in the cortex stimulate progenitor proliferation in the SVZ of adult rodents (Buffo et al., 2008). In an experimental stroke model, immature neurons also migrated from the SVZ to the damaged striatal area where they expressed markers for striatal spiny neurons, as to replace the damaged ones. None of these neurons however survive in the environment of the striatum (Arvidsson et al., 2003; Magnusson et al., 2014). Seizure is also known to stimulate

proliferation in the SVZ and to alter neuronal precursor migration in the rat brain by increasing the number of neuroblasts found in the RMS and by inducing a portion of them to exit the RMS prematurely or to migrate ectopically even though the majority of them will fail to survive (Parent et al., 2002).

1.5.2 A role for adult neurogenesis in the SGZ

Newborn neurons of the DG migrate only a short distance to reach the inner granule cell layer from where they rapidly extend long axonal projections to the CA3 pyramidal cell layer within 4 to 10 days after division (Markakis & Gage, 1999), while their dendrites grow in the opposite direction, reaching the molecular layer within two weeks (Ming & Song, 2005; Whitman & Greer, 2007). Nevertheless, as early as 7 days after division, they begin to show GABAergic inputs, followed by glutamatergic ones around two weeks after cell birth (Ming & Song, 2005; Whitman & Greer, 2007).

Neurogenesis in the DG is influenced by many signals including adrenal steroids, seizure, exercise, inflammation and antidepressants (Kriegstein & Alvarez-Buylla, 2009). The role of these newborn neurons once integrated in the hippocampal circuits has been deeply studied and mainly linked to spatial learning, memory and possibly mood. Neurogenic activity and survival rate of newborn neurons in the SGZ are increased by physical activity and hippocampus-dependent learning tasks respectively (Aimone et al., 2014). Moreover, the ablation of adult neurogenesis impairs the ability of the mice to discriminate stimuli with little spatial separation (Clelland et al., 2009). Hippocampal neurogenesis also exerts a role in the stress response: when this process is inhibited, a greater stress response has been observed in different behavioural tests in response to non-stressful stimuli (Schloesser et al., 2009). Furthermore, blockade of adult neurogenesis affects the response to antidepressants; in fact, antidepressant drugs recruit newborn neurons to re-establish hippocampal regulation of the HPA axis after chronic stress (Surget et al., 2011).

Adult neurogenesis in the hippocampus is altered in various pathological conditions including epilepsy, stroke, degenerative neurological disorders, neuropsychiatric disorders, as well as in the case of alcohol intoxication (Danzer, 2012). Ischemic injury increases neurogenesis in the SGZ. Most of the newborn cells differentiate into granule neurons, some of which mislocate in the hilus region for example. Also during epilepsy an increased however disrupted neurogenesis activity characterises the hippocampus with large numbers

of adult-generated granule cells integrating abnormally into the DG participating in aberrant network reorganizations (Walter et al., 2007). On the opposite side, stressful stimuli such as sleep deprivation, social isolation, exposure to predator odour, as well as a condition of depression, have all been found to reduce granule cell neurogenesis, most probably through mediation of corticosterone since adrenalectomy blocks the anti-neurogenic effects of each of these stimuli (Danzer, 2012).

1.5.3 The interplay with the reproductive function

1.5.3.1 The SVZ

The olfactory system is implicated in the control of reproductive behaviour as it plays crucial roles in mating behaviour or interaction with the offspring.

Oestradiol influences SVZ cell proliferation in a dose- and time-dependent manner. If administered in an acute dose, it decreases cell proliferation, leading to a lower number of adult-born neurons in the OB of ovariectomised female mice (Brock et al., 2010); chronic administration has no effect on the proliferative activity of the SVZ while it reduces the survival of newborn neurons in the main OB (Veyrac & Bakker, 2011) (Fig. 5). Pregnancy also affects adult neurogenesis in the SVZ; it has been shown that during this period, an increase in proliferation occurs in the SVZ, with newborn neurons found to be integrated in the circuits of the OB (Shingo, 2003). In studies where pseudopregnancy was induced, it was shown that embryo implantation was not necessary for these effects, therefore pointing to a role for circulating maternal hormones in stimulating forebrain neurogenesis. In particular, these effects depend upon the action of prolactin, and appear specific to the SVZ/OB system (Shingo, 2003) (Fig. 5). Prolactin has been shown to mediate increased neurogenesis in both the SVZ and SGZ of male mice that physically interacted with their pups. Indeed, prolactin receptor null mice, albeit showing normal olfaction, do not recognise their own pups toward which they display aggression, as with cage intruders, behaviour that is associated to impaired neurogenesis in the two niches (Mak & Weiss, 2010).

1.5.3.2 The SGZ

Fluctuations in gonadal hormones can also modulate hippocampal neurogenesis in mice and rats. However, the effects of oestrogens are difficult to summarise as they are extremely

variable depending on the species, age, sex, timing of BrdU injections, timing after ovariectomy, dose and type of exogenous oestrogens (Galea et al., 2013). Anyhow, effects in males are less robust than in females.

Pregnancy and lactation are characterised by neural and behavioural changes which require a high degree of plasticity for the maternal brain. Besides, the hippocampus controls different components of maternal behaviour, including pup retrieval and nest building (Galea et al., 2013). As a general effect, we can say that acutely administered oestradiol enhances proliferation within the SGZ, in agreement with the findings from Tanapat et al. who showed an increased proliferation in the SGZ of female rats during proestrus, when the levels of oestrogens are higher, compared to diestrus or male animals (Tanapat et al., 1999). Progenitor cells of the hippocampus express ERs and respond to agonists of both Erα and β, with an increase in proliferation, while ER antagonists partially block oestrogen effects (Galea et al., 2013) (Fig. 5).

Progesterone receptors are found in the hippocampus and treatment with exogenous progesterone increases cell proliferation in ovariectomised female and male rats, and cell survival in male mice. However, when combined with oestrogens, progesterone blocks oestrogen-dependent increase in cell proliferation (Liu et al., 2010).

Finally, androgen receptors (ARs) are also expressed in the hippocampus (Xiao & Jordan, 2002). An upregulation in cell survival with no significant effects on cell proliferation has been shown in male rodents in response to testosterone or dihydrotestosterone, a result that can be blocked by an AR antagonist (Hamson et al., 2013).

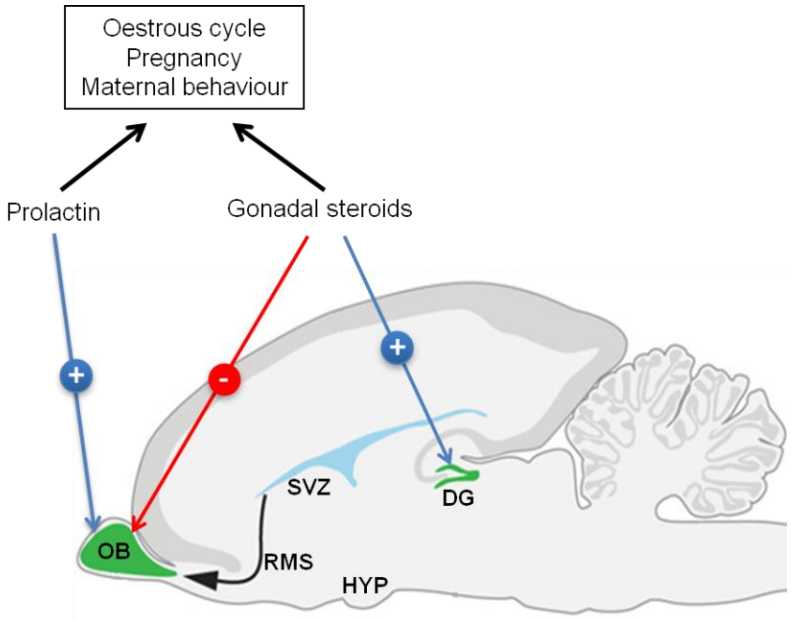


Figure 5: Effects of reproduction hormones on adult neurogenesis. Gonadal steroids negatively affect cell proliferation in the OB, and enhance it in the DG. Prolactine is known to stimulate neurongenesis in the OB. DG, dentate gyrus; HYP, hypothalamus; OB, olfactory bulbs; RMS, rostral migratory stream; SVZ, subventricular zone.

1.6 aNSC AND ADULT NEUROGENESIS IN HUMANS

Data regarding adult neurogenesis in human are more controversial than those obtained from rodents due for instance to difficulties in obtaining human specimens and limits in the experimental strategies that can be applied (Bergmann et al., 2015).

1.6.1 The human SVZ

The human SVZ niche has been detailed and astrocytes with proliferating features have been described (Sanai et al., 2004). During the first 6 months of postnatal life, robust streams of immature DCX/PSA-NCAM expressing neurons tangentially migrate just underneath the ependymal layer of the wall of the lateral ventricle and along the RMS. Between 6 and 18 months, the cytoarchitecture of the SVZ deeply changes until resembling that observed in adults (Sanai et al., 2011). After the sixth month of life, this dense network of putative migratory neurons is completely depleted leaving a hypocellular gap, also observed in macaques and marmosets (Gil-Perotín et al., 2013), between the ependymal layer and a ribbon of $GFAP^+$ and $vimentin^+$ astrocytes (Fig. 6a,c). Some of these astrocytes were seen to coexpress the proliferation marker Ki67 and they were able to incorporate BrdU in organotypic slices. Moreover, when these astrocytes were dissected and cultured *in vitro*, they were able to form neurospheres with the ability to self-renew and differentiate into the three neural cell types. All these data suggest the presence of a subpopulation of mitotically active NSCs (Sanai et al., 2004). In adults, no RMS can be observed, maybe due to the long distance that separates the SVZ from the OBs in humans as well as our microsmatic capabilities.

In 2005, the group of Frisén developed an unusual technique to address the issue of studying adult neurogenesis in humans. They took advantage of the dramatic increase in atmospheric ^{14}C levels resulting from the tests of nuclear weapons in the 1960's followed by its exponential decline; since ^{14}C is integrated in the DNA during its synthesis, they showed that the level of ^{14}C in genomic DNA closely parallels atmospheric levels so it could be used to establish the time point when the DNA was synthesized and cells were born during the life of a person (Spalding et al., 2005). Following this rationale, they showed that in the human OB no significant postnatal neuronal turnover was observed (Bergmann et al., 2012; Spalding et al., 2013) (Fig. 6e).

Nevertheless, the presence of proliferating astrocytes in the SVZ and the absence of migrating neuroblasts in the RMS posed the question of the fate of the putative newborn cells. A study showed the generation of new interneurons in the human striatum both through the detection of neurons positive for iododeoxyuridine (which incorporates into the DNA of dividing cells similarly to BrdU) in post-mortem tissues from cancer patients who had received IdU as an anticancer drug (Ernst et al., 2014), and through ^{14}C dating approaches. This suggested that a subpopulation of striatal interneurons was subjected to turnover (Ernst et al. 2014). Thus, it may be speculated that neuroblasts born in the adult human SVZ migrate to the striatum and contribute to the neuronal turnover observed in this region (Bergmann et al., 2015) (Fig. 6e).

1.6.2 The human DG

The first experiments able to prove that adult neurogenesis persists within the human hippocampus took advantage of the use of BrdU in cancer patients who had received one intravenous injection for diagnostic purposes. In this study, BrdU-positive cells were found to express a neuronal phenotype in the DG but not in the SVZ (Eriksson et al., 1998). Moreover, DCX and PSA-NCAM-expressing putative neuroblasts were found in the DG (Knoth et al., 2010). Finally, the group of Frisén showed that a subpopulation corresponding to one third of hippocampal neurons was subjected to turnover during human's adult life, with a less pronounced age-dependent decline than rodents, since hippocampal neurons had ^{14}C levels that corresponded to times after the individual's birth year, unlike neurons from the cortex or the cerebellum (Bergmann et al., 2012; Spalding et al., 2013).

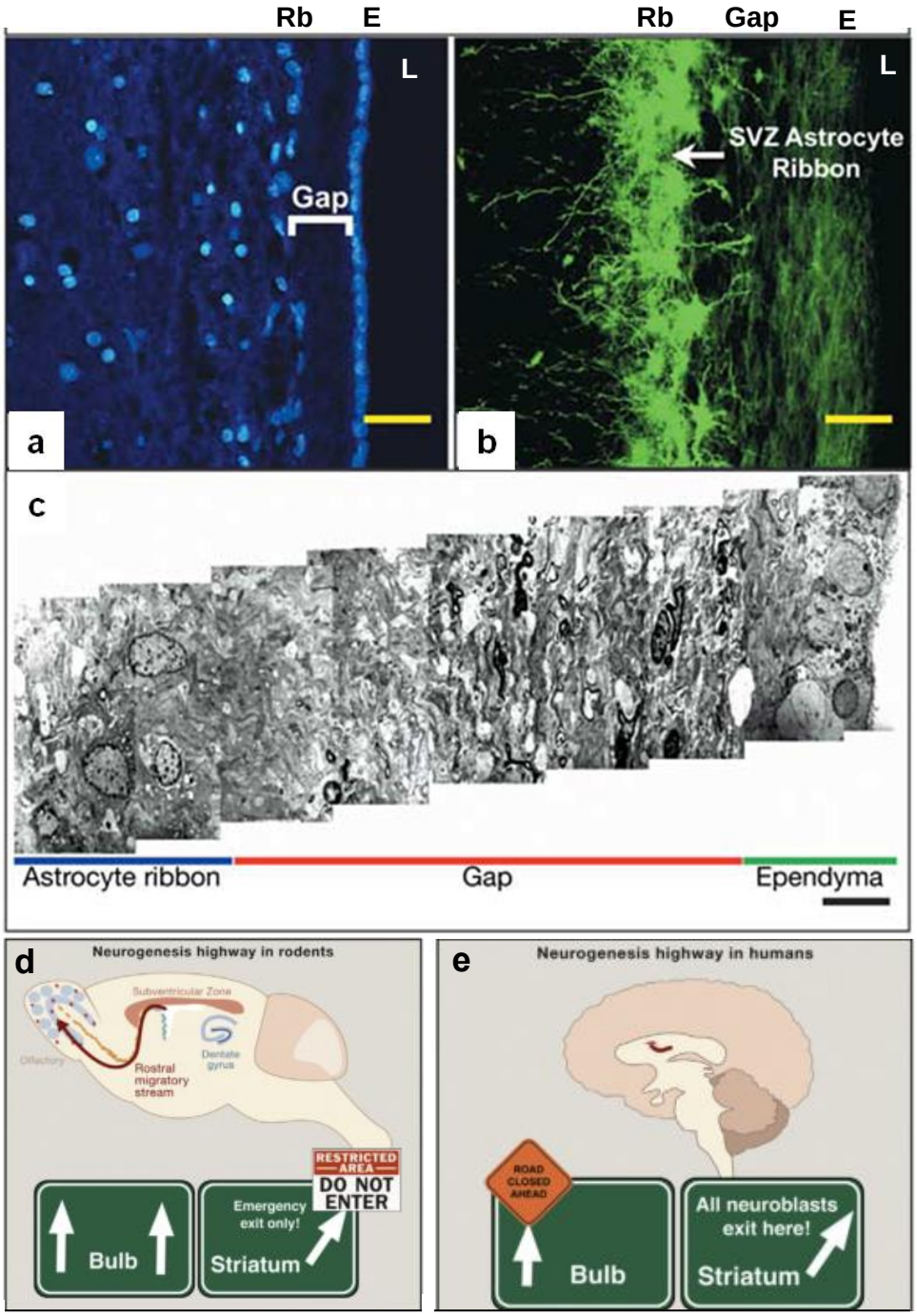


Figure 6: The SVZ niche in the adult human brain. **(a)** Coronal section revealing a region of high cellularity (Rb) that is separated from the ependymal (Ep) by a hypocellular gap. Nuclei were counterstained with DAPI (blue). **(b)** Vibratome section showing GFAP expression in SVZ astrocyte ribbon and GFAP-positive fibre bundles filling the subependymal gap. **(c)** Panoramic electron micrograph of a postmortem adult human SVZ, showing the ependymal lining, gap region and astrocyte ribbon. **(d,e)** Whereas neuroblasts from the adult SVZ in rodents migrate to the olfactory bulb to become interneurons, and enter the striatum only under pathological conditions such as ischemia **(d)**, in humans hardly any adult neurogenesis can be found in the adult OB, but it occurs to a substantial degree in the striatum **(e)**. Scale bars, 40 µm (a,b); 10 µm (c). LV, lateral ventricle; SVZ, subventricular zone (a-c are modified from Sanai 2004, Nature; d,e are from Kempermann 2014, Cell).

1.7 CELL NEOGENESIS IN THE HYPOTHALAMUS

1.7.1 Structural features of the hypothalamus

Due to its role in regulating essential physiological processes for the maintenance of life, the hypothalamus is a conserved structure among vertebrates (Xie & Dorsky, 2017).

This small region, located in the most ventral part of the forebrain, can be divided both rostro-caudally and medio-laterally. From rostral to caudal, we can find four zones: preoptic (Fig. 7b,f), anterior (Fig. 7c,g), tuberal (Fig. 7d,h) and mammillary (Fig. 7e,i); each of them can be then divided in three levels: periventricular, medial and lateral. However, the embryonic origin of the preoptic region has been subject of debate as it is not clear if it origins from the telencephalon or diencephalon (Markakis, 2002). These four zones are nevertheless composed by many nuclei containing specific neuronal cell types. Each of these nuclei is involved in controlling a different aspect of physiological homeostasis and behaviour (Markakis, 2002). For instance, the preoptic area (POA) (Fig. 7b,f) controls reproduction, sleep, thermoregulation and electrolyte balance. The anterior hypothalamus, which includes the supraoptic (SON), suprachiasmatic (SCN), paraventricular (PVN), anteroventral periventricular (AVPV) and anteroventral (AVP) nuclei (Fig. 7b,c,f,g), regulates circadian rhythms, feeding and other homeostatic functions. The tuberal hypothalamus, where we find the arcuate (ARC), ventromedial (VMH) and dorsomedial (DMH) nuclei (Fig. 7d,h), is involved in the control of energy balance, stress and aggression (Markakis, 2002). Finally, the mammillary hypothalamus, which includes the mammillary bodies (Fig. 7e,i), plays a role in arousal, stress response together with spatial and episodic memory (Vann & Nelson, 2015). Each of the roles here listed never depends on the action of a single nucleus. The different hypothalamic regions are in fact strongly interconnected between each other and with different areas of the brain, in order to be able to adjust their function according to the physiological status of the organism as well as inputs coming from the surrounding environment.

In order to play its many roles, the hypothalamus links the nervous system to the endocrine system, working in concert with the pituitary gland to which it is morphologically linked. The pituitary gland, or hypophysis, is an endocrine gland composed by two regions with distinct embryological origins. The anterior region, or adenohypophysis, is a neuroepithelial structure composed by different types of endocrine cells defined by the hormones they produce; the posterior region, or neurohypophysis, has a neural nature and is composed by

multiple axonal projections arriving from the hypothalamus via the infundibular stalk (Cattaneo, 2005).

The hypothalamus contains two different types of neuroendocrine neurons: the magnocellular neurons and the parvocellular neurons. The first ones project into the posterior lobe of the pituitary where they directly release oxytocin and vasopressin; the second ones release their releasing or inhibiting hormones into a small portal blood system directly connected with the anterior pituitary (the hypophyseal portal system) where they act on their specific target to stimulate or inhibit the release of hypophyseal hormones into the systemic circulation (Maggi et al., 2015). We can distinguish three main pathways with which the hypothalamus and pituitary exert their neuroendocrine function and whose functions are in many ways entangled: the hypothalamic–pituitary–gonadal (HPG) axis, which regulates the reproductive function; the hypothalamic–pituitary–adrenal (HPA) axis, which regulates the response to stress and the hypothalamic–pituitary–thyroid (HPT) axis, which regulates metabolism.

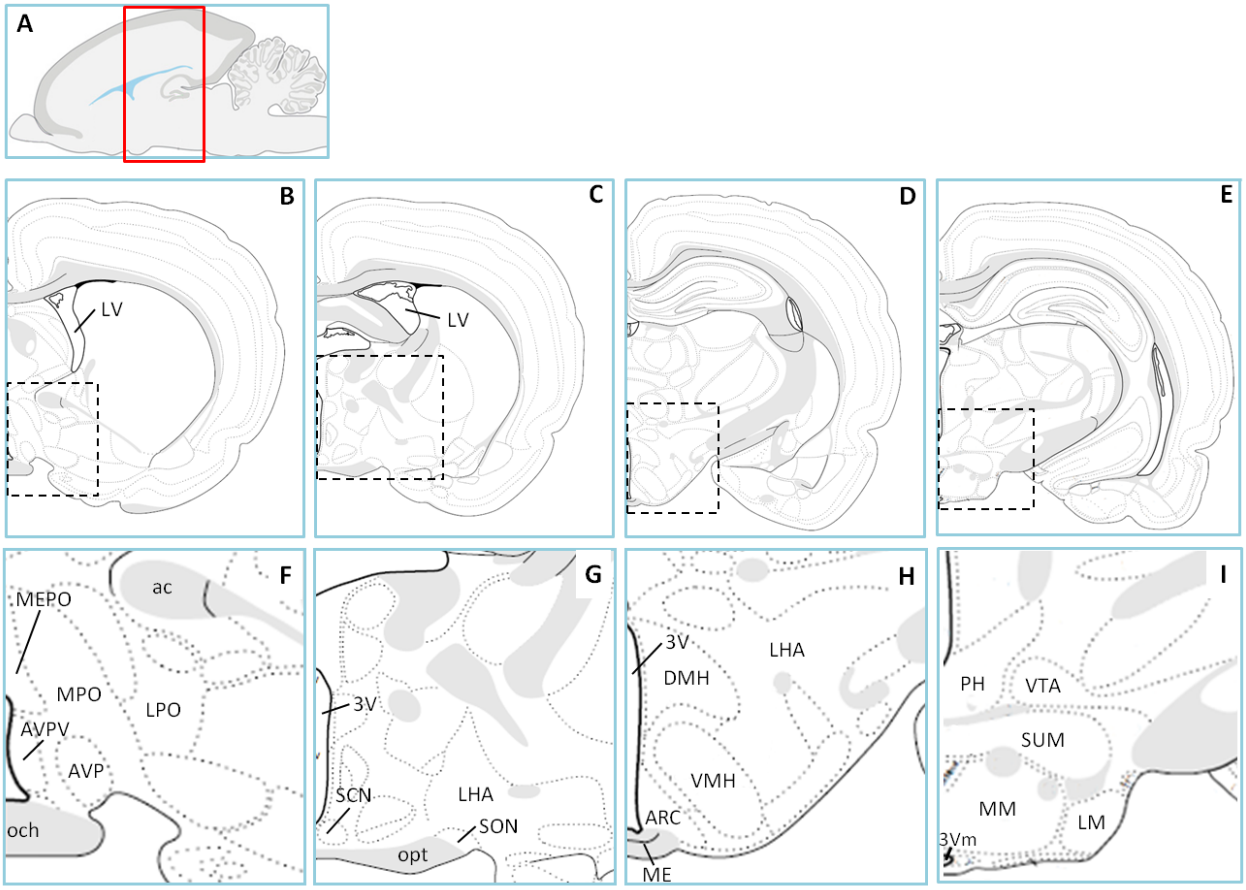


Figure 7: The hypothalamic nuclei. (A) Sagittal view of a mouse brain, the red square corresponds to the region shown in B-I. **(B-E)** Coronal view of the four different levels of the hypothalamus showing from rostral to caudal the preoptic (b), anterior (c), tuberal (d) and mammillary (e) regions. **(F-I)** The boxed regions in B-E are shown at higher magnification. 3V, third ventricle; 3Vm, 3V mammillary recess; ac, anterior commissure; ARC, arcuate nucleus; AVP, anteroventral nucleus; AVPV, anteroventral periventricular nucleus; DMH, dorsomedial hypothalamus; LHA, lateral hypothalamus; LM, lateral mammillary nucleus; LPO, lateral preoptic area; LV, lateral ventricle; ME, median eminence; MEPO, median preoptic nucleus; MM, medial mammillary nucleus; MPO, medial preoptic nucleus; och, optic chiasm; opt, optic tract; PH, posterior hypothalamic nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; SUM, supramamillary nucleus; VMH, ventromedial hupothalamus; VTA, ventral tegmental area (Swanson Atlas).

1.7.2 Building the hypothalamus

1.7.2.1 Embryonic life

The hypothalamus derives from the forebrain, which further subdivides into the telencephalon and the diencephalon. While the telencephalon gives rise to the cerebral cortex and basal ganglia, the diencephalon is at the origin of the thalamus, the hypothalamus and the epithalamus (Xie & Dorsky, 2017; Ware et al., 2014).

Specific patterning of the hypothalamus begins around E8 in mice and is strictly dependent on Shh, which is secreted by the axial mesoderm underlying the neural plate to ventralise the CNS (Lupo et al., 2006). Sonic hedgehog acts in concert with other morphogens such as Wnt, secreted by the posterior neurectoderm and somites, and BMP, secreted by the prechordal mesoderm (Barth et al., 1999; Braun, 2003; Kapsimali et al., 2004). At E9.5, the hypothalamic primordium is morphologically evident and at this point, Shh is down-regulated by local production of BMPs (Patten & Placzek, 2002). This leads to the subdivision of the primordial hypothalamus into three regions, the anterior hypothalamus, the tuberal hypothalamus and the mammillary hypothalamus (Ware et al., 2014). The preoptic neuroepithelial patch gives rise to the medial POA between E12 and E16. The medial preoptic nucleus is generated in two waves, the lateral part first and the central one later, between E14 and E18 while the nuclei of the tuberal region (DMH, VMH and ARC), are generated between E12 and E17 (Markakis, 2002).

Hypothalamic neurogenesis in the mouse begins at E10.5 (Shimada & Nakamura, 1973). As for other brain regions, dividing progenitors reside in the ventricular zone. After a rapid proliferative expansion, they undergo asymmetric divisions to produce both neuronal and

glial precursors that migrate to the adjacent parenchyma where they differentiate (Xie & Dorsky, 2017; Ware et al., 2014). Gliogenesis begins just after neurogenesis ends, around E15.5. Production of the correct number of neurons and maintenance of the progenitor population depend upon induction of a Notch/proneural loop (Ware et al., 2014). The axons of the newborn hypothalamic neurons project through the medial forebrain bundle; from here, these axons progress medially to reach the median eminence (ME), depending upon mechanisms that remain unknown (Markakis, 2002).

1.7.2.2 Post-natal life

Not a lot is known regarding cell neogenesis in the postnatal hypothalamus. As for the rest of the brain, this is definitely the moment when most of the astrocytes are produced. However, the exact origin of these cells is not clear. The first weeks of postnatal life are also the moment when tanycytes, glioependymal specialised cells that line the floor of the third ventricle, differentiate (Mirzadeh et al., 2017; Rodríguez et al., 2005).

Neurogenesis has also been reported in the hypothalamus at this stage. For instance, new neurons are added postnatally in the AVPV and in the sexually dimorphic nucleus (SDN) of the POA, two sexually dimorphic nuclei, implicated in female ovulation and male sexual behaviour respectively (Ahmed et al., 2008; Mohr et al., 2016). The addition of new neurons during postnatal development is sexually dimorphic and reflects the differences that will be seen during adulthood: more neurons are added in the female AVPV compared to males, while the opposite is seen for the SDN. These processes depend upon gonadal hormones, as differences are lost in gonadectomised animals. This suggests that gonadal steroids modulate the addition of new cells during puberty to maintain and accentuate sexual dimorphisms in the adult brain (Ahmed et al., 2008; Mohr et al., 2016).

1.7.3 Cell neogenesis in the adult hypothalamus

In order to properly fulfil their role of integration of signals from other brain nuclei, the periphery and the environment, the different hypothalamic nuclei need to display a remarkable degree of structural plasticity, hence structural alterations, correlated with changes in the animal's physiological state to deliver an adequate response.

During the past years, different studies have identified several mechanisms through which this adaptation process is accomplished, such as changes in the relationships between neurons and their associated astrocytes, as well as synaptic remodelling, which modifies the type and the efficacy of synaptic inputs and the extent of tonic coupling among different neurons (Hatton & Ellisman, 1982; Theodosis & Poulain, 1993). These mechanisms have been observed in different contexts. For instance, a modification in the number of dendritic spines in the DMH and VMH neurons is observed in response to variations of gonadal hormones throughout pubertal maturation or across the oestrus cycle (Frankfurt, 1994). Plasticity is necessary for the function of the circadian clock, which adapts to variations in the light and dark cycles during the day and throughout the year (Bosler et al., 2015; Coomans et al., 2015); this is examplified by the increase in synaptic innervation of the SCN by glutamatergic fibers at daytime compared to night-time, or by the transitions of chromatin between condensed and decondensed states over the 24-hour cycle. All the mechanisms described so far are rapid and reversible. In addition, an increasing number of studies have described neurogenesis and gliogenesis in the adult hypothalamus across different species suggesting that longer-term plastic remodelling involving the integration of newborn cells into existing circuits may also be at work in the hypothalamus. Newborn neurons and glial cells have been reported postnatally and during adulthood in the hypothalamus of rat, mouse, hamsters, sheep, meadow vole and pig. Even though these reports present many differences, probably due to their use of different experimental strategies, species, sex, and genetic background of the animals as well as quantification methods, they all suggest a role for this process in the physiopathology of the hypothalamus (Djogo et al., 2016; Fowler et al., 2002; Haan et al., 2013; Huang et al., 1998; Kokoeva et al., 2005; Lee et al., 2012; Li et al., 2012; Mcnay et al., 2012; Migaud et al., 2015; Pencea et al., 2001; Robins et al., 2013a; Sharif et al., 2014).

1.7.4 Identity of hypothalamic NSCs

Adult hypothalamic neuro and gliogenesis have been mainly studied in the tuberal region of the hypothalamus, i.e. in the ARC, DMH and VMH. Despite the number of works reporting the presence of neural stem/progenitor cells in this region, their precise identity and location remain controversial. Three main and nonexclusive hypotheses have being proposed: (i) the population of stem cells is located in the hypothalamic parenchyma, (ii)

they correspond to subependymal astrocytes, or (iii) they correspond to tanycytes (Sharif et al., 2014).

1.7.4.1 The parenchymal hypothesis

The data pushing towards this hypothesis are based on the observation that shortly after BrdU administration, dividing cells can be found at considerable distance from the third ventricle, with sometimes closely apposed pairs of BrdU-labeled cells that are reminiscent of a recent mitotic event (Pencea et al., 2001; Pérez-Martín et al., 2010; Kokoeva et al., 2007; Haan et al., 2013; Robins et al., 2013b). Moreover, lineage tracing studies showed that NG2 glial cells, found in the hypothalamic parenchyma, are able to proliferate both symmetrically to self-renew and asymmetrically to give rise to oligodendrocytes and a smaller population of neurons (Robins et al., 2013b); and that $Sox2^{+}$/$Nestin^{+}$ cells, found in the mediobasal hypothalamus, can give rise to new neurons and, to a lesser extent, glial cells, in adult mice (Li et al., 2012).

However, when hypothalamic NSCs have been successfully isolated *in vitro*, the dissected tissue included the third ventricle wall. Whenever cultures were prepared from regions distant from the third ventricle, NSCs were not obtained (Robins et al., 2013a). These results may implicate that, while real stem cells reside within the ventricular wall, multipotent neural progenitor cells, which retain dividing ability, are found in the parenchyma (Sharif et al., 2014). Nonetheless, considering the impossibility to isolate the ventricular lining from the adjacent parenchyma in microdissection procedures, one cannot exclude the possibility that parenchymal cells located in the proximity of the third ventricle could be stem cells.

1.7.4.2 The subependymal astrocytes hypothesis

Evindences for this hypothesis come from a BrdU-based study. Perez-Martin and collegues (2010) described a population of subependymal GFAP-positive astrocytes located in the middle part of the rat third ventricle that proliferated in response to insulin-like growth factor I (IGF-I). Structural features of this population resemble that of the SVZ neurogenic niche with the GFAP-positive cells contacting the ventricle through an apical process bearing a single cilium and showing many labyrinthine extensions of their basal membrane (Pérez-

Martín et al., 2010). However, lineage-tracing experiments will be needed to determine the fate potentiality of these cells.

1.7.4.3 The tanycyte hypothesis

The ependymal layer of the third ventricle is lined by a single cell layer of multiciliated cubic ependymal cells found in the more dorsal part, an overlapping zone with both ependymal cells and tanycytes, and a ventral zone with non-ciliated tanycytes which retain characteristics of RG cells (Pérez-Martín et al., 2010; Rodríguez et al., 2005) (Fig. 8). Tanycytes are considered as RG-like cells as they are in contact with the CSF on their apical face while their basal long extensions project into different nuclei of the hypothalamus or into the ME (Rodríguez et al., 2005). They express many different neural stem/progenitor cell markers such as nestin, Sox2, GFAP, GLAST, vimentin, BLBP and components of the Notch or fibroblast growth factor (FGF) signalling pathways (Sharif et al., 2014).

Tanycytes are not a homogeneous cell population; they have been classically subdivided, with respect to their location, spatial relationships, morphology, cytochemistry and ultrastructure, into four groups: α1, α2, β1 and β2 tanycytes (Rodríguez et al., 2005). Alpha tanycytes are located along the lateral walls of the third ventricle: α1 tanycytes send their processes into the VMH and part of the DMH, while α2 tanycytes project to the ARC; β1 tanycytes line the lateral evaginations of the third ventricle in the infundibular recess and project to the latero-external region of the ME; β2 tanycytes line the floor of the third ventricle and extend their projections into the ME to reach the perivascular space of the capillaries of the hypothalamo–hypophysial portal system (Recabal et al., 2017; Rizzoti & Lovell-Badge, 2016; Robins et al., 2013a; Rodríguez et al., 2005; Sharif et al., 2014) (Fig. 8). While they all express nestin, Sox2 and vimentin, only α tanycytes express GFAP, and only β tanycytes express BLBP and Fgf10 (Haan et al., 2013; Robins et al., 2013a).

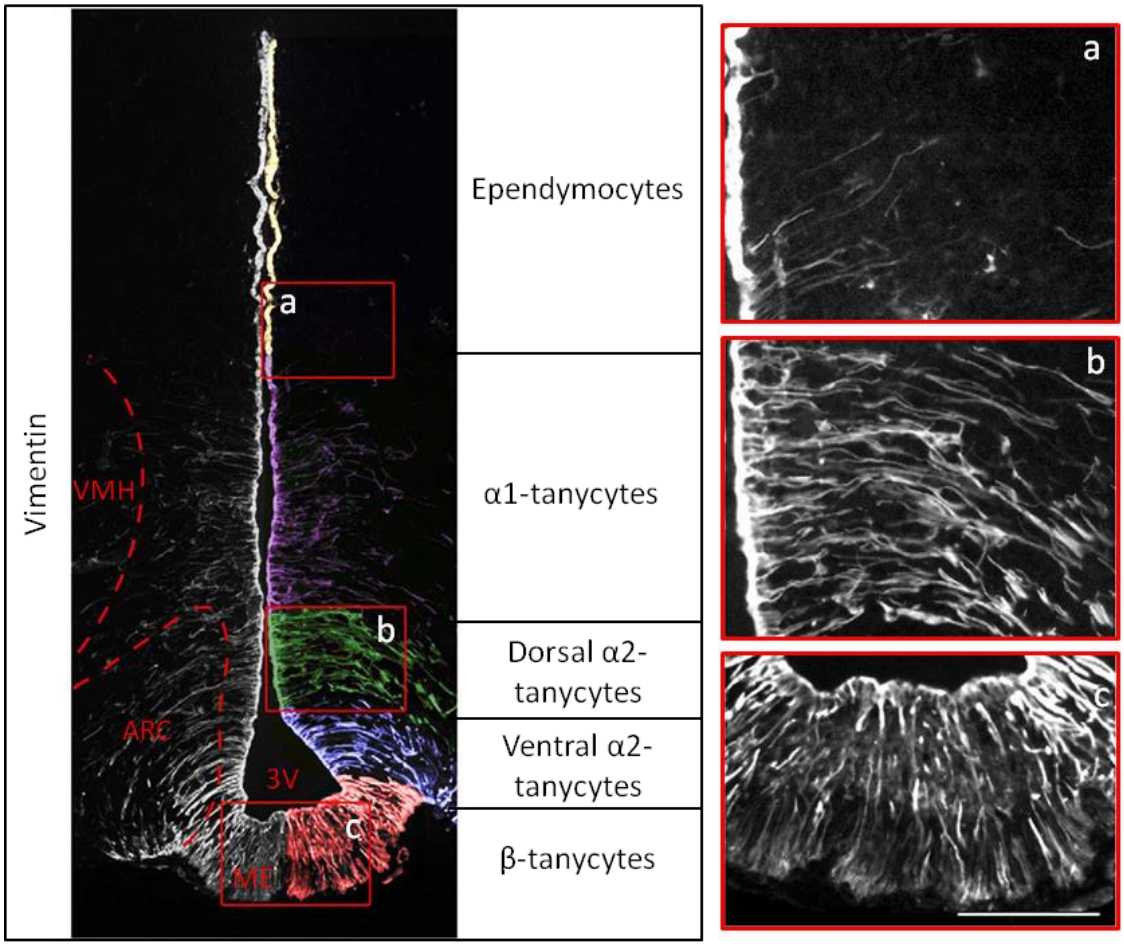


Figure 8: The tanycytes of the third ventricle. Left hand panel: coronal section of the adult mouse third ventricle (3V), immunolabelled with vimentin (white). Tanycytes were false-coloured to distinguish them from ependymocytes. Right-hand panels: high-power magnifications of boxed regions in the left panel (Modified from Robins 2013, Nature Communications).

BrdU-based studies have always found dividing cells within the tanycytic pool, however, with a much lower frequency than in the parenchyma (Pencea et al., 2001; Kokoeva et al., 2007; Haan et al., 2013), a behaviour that may be compared to the quiescent state of SVZ and SGZ NSCs (see chapter 1.3.1). This rather quiescent state is also evidenced by the observation that tanycytes labelled with BrdU during early postnatal development retain BrdU expression into adulthood but can also show co-expression of the cell cycle marker Ki67 (Sharif et al., 2014).

Lineage-tracing studies have been used to explore tanycyte stem cell properties by following their progeny. Even though all studies showed that tanycytes are neurogenic and gliogenic *in vivo*, results were not homogeneous when different tanycytic subtypes were traced. For instance, a few hours after intracerebroventricular (i.c.v.) injection of a GFP-expressing

adenovirus in the third ventricle, only tanycytes were labelled while, 4 weeks later, GFP-expressing neurons were found in the parenchyma of the rat hypothalamus (Xu et al., 2005). In young postnatal mice (P4), fate mapping using Nestin:CreER^{T2} driver animals induces reporter gene expression in tanycytes within a few hours, while reporter-expressing neurons are observed after one month, mainly in the ME (Lee & Blackshaw, 2012).

In the study from Robins et al (2013), a GLAST:: CreER^{T2} model was used to specifically trace adult dorsal α tanycytes. These cells were able to give rise to other tanycytes, including those of the more ventral region of the third ventricle, as well as astrocytes and sparse numbers of neurons (Robins et al., 2013a). Moreover, when microdissecting the region containing β tanycytes only, they were not able to isolate NSCs in *in vitro* neurosphere experiments. When Haan and collegues (2013) fate-mapped β tanycytes using a FGF10-promoter-driven reporter gene, they identified only neuronal progeny in the parenchyma (Haan et al., 2013). Thus, from these results, it may be speculated that GLAST^{+} dorsal α tanycytes are neural stem cells, able to give rise to other tanycytes, as well as both parenchymal astrocytes and neurons, while β tanycytes are intermediate progenitors with a commetted neuronal fate (Sharif et al., 2014) (Fig. 9).

1.7.5 Modulators of hypothalamic neurogenesis

Most of the studies on hypothalamic neurogenesis have been performed in non-physiological conditions where proliferation was either stimulated or inhibited. Many different factors have been tested for their ability to influence hypothalamic adult neurogenesis.

1.7.5.1 Growth factors

An initial work performed in rats showed that infusion of brain derived neurotrophic factor (BDNF) in the lateral ventricles stimulated proliferation and neurogenesis in the hypothalamus (Fig. 9). Interestingly, the BrdU-positive cells did not express the BDNF receptor, TrkB, which was nevertheless found at the level of their neighbouring cells (Pencea et al., 2001). Intracerebroventricular administration of ciliary neurotrophic factor (CNTF) increases proliferation in the hypothalamus at the level of the DMH, VMH, ARC and ME, all regions known to be involved in the control of energy homeostasis (Fig. 9). CNTF induces

weight loss in obese animals and humans, and its effect is retained even after termination of the treatment. It appears that this long-term effect depends on the stimulation of hypothalamic neurogenesis, which increases the number of leptin-sensitive neurons in the region. Unlike for the case of BDNF, the newborn cells are directly responsive to CNTF since they express CNTF receptor (Kokoeva et al., 2005). Moreover tanycytes of different subpopulations have been shown to differently respond to stimulatory cues. For instance, FGF2 stimulates proliferation of rat α tanycytes and mouse α2 tanycytes (Xu et al., 2005, Robins et al., 2013), and IGF-I, whose receptor is expressed by both proliferating and non-proliferating cells, stimulates proliferation of $GFAP^+$ α tanycytes in rats (Perez-Martin et al., 2010) (Fig. 9).

1.7.5.2 Diet

Adult hypothalamic neurogenesis can also be affected by diet (Fig. 9). In the study from McNay et al. (2012), they showed a decrease in adult hypothalamic neurogenesis due to high fat diet (HFD), as well as an increase in apoptosis of the newborn cells, an effect that was amplified in ob/ob leptin-deficient mice (Mcnay et al., 2012). Li et al. (2012) reported impairment in hypothalamic cell proliferation and differentiation, both *in vivo* and *in vitro*, together with a decrease in Sox2 expression in responce to chronic HFD. It is known that the environment of the hypothalamus in HFD or obese conditions is strongly inflammatory and the proinflammatory pathway of nuclear factor-κB (NF-κB) and its upstream IκB kinase-β (IKKβ) is a key mediator of hypothalamic inflammation. Accordingly, NSCs derived from HFD-treated animals were found to display an overactivation of this signalling pathway, which caused both the increased cell death, via the stimulation of pro-apoptotic cytokine secretion, and decreased neuronal differentiation, via activation of the Notch signalling (Li et al., 2012).

Another study showed different outcomes of HFD on hypothalamic neurogenesis according to the region and sex of the animals. Indeed, HFD inhibited neurogenesis in the ARC of males and females. However, it increased the proliferative activity in the ME of female mice only (Lee et al., 2014). In the study of Gouazé et al. (2013), HFD was shown to acutely increase cell proliferation in the hypothalamus, without affecting cell survival, and to induce a higher percentage of newborn neurons to adopt a proopiomelanocortin (POMC) phenotype in the ARC. Blocking cell proliferation by chronic i.c.v. infusion of the antimitotic drug AraC

accelerated the onset of diet-induced obesity in mice, suggesting that cell renewal in the adult hypothalamus participates in the long-term regulation of food intake (Gouazé et al., 2013).

1.7.5.3 Other modulators

Adult neurogenesis in the hypothalamic neurogenic niche is not only modulated by diet but also by the hormonal status in female mice. Shortly after HFD initiation in ovariectomised animals, an increase in cell proliferation and in the number of newborn oestrogen receptor (ER)-expressing neurons in the ARC, VMH and DMH was seen. Oestradiol administration however, prevented the increase proliferation and the weight gain induced by HFD. On the contrary, oestradiol increased cell proliferation in a status of normal diet. This shows that hypothalamic cell proliferation, as previously seen in the SVZ and SGZ, can be influenced by the action of gonadal hormones (Bless et al., 2016; Bless et al., 2014)

Voluntary exercise stimulates hypothalamic neurogenesis in the adult rat brain after stroke, improving recovery of metabolic homeostatic functions in these animals (Niwa et al., 2016).

Finally, the proliferative capacity of the hypothalamus is seasonally regulated (Migaud et al., 2015). As shown in sheep and hamsters, two seasonal mammalian models, proliferation in the hypothalamus is stimulated during the short photoperiod, when these animals are sexually active (Migaud et al., 2015).

1.7.6 Functional implications of hypothalamic neurogenesis

After successive divisions, newborn cells are found to express proteins characteristic of migrating, immature neurons, including PSA-NCAM and DCX, which have also been found in the human hypothalamus (Batailler et al., 2014). Later on, newly generated cells differentiate into neurons that integrate within the hypothalamic circuits involved in energy homeostasis as shown by their expression of markers of terminally differentiated hypothalamic neuronal subtypes, such as POMC or neuropeptide Y/agouti-related protein (NPY/AgRP) neurons (Lee & Blackshaw, 2012) and their responsiveness to leptin (Kokoeva et al., 2005). For this reason, virtually all the studies on this subject have focused on the involvement of adut hypothalamic neurogenesis in metabolism.

A BrdU-based study suggested a high degree of neuronal turnover in the ARC. 63% of POMC and 34% of NPY neurons born during embryogenesis are lost between 4 and 12 weeks of age, with no alterations in the total number of ARC neurons, suggesting that this neuronal loss must be balanced by a process of adult neurogenesis, which contributes to remodelling of the network (Mcnay et al., 2012). Adult neurogenesis seems to be able to reshape the feeding hypothalamic circuits to maintain homeostasis in deleterious conditions. Specific deletion of the mitochondrial transcription factor A gene in AgRP neurons, results in progressive neurodegeneration of these neurons due to depletion of mitochondria over a period of several months, which in turn results in increased cell proliferation and generation of new AgRP leptin-sensitive neurons. This compensatory neurogenesis counteracts the deleterious effects of AgRP neuron loss on body weight and food intake (Pierce & Xu, 2010).

To understand the role of adult hypothalamic neurogenesis experiments have been performed to evaluate the effects of its inhibition on metabolic homeostasis. Inhibition of cell proliferation in the hypothalamus has been linked to increased body weight and fat mass. For instance, a study performed specific ablation of NSCs in the mediobasal hypothalamus of adult mice via injection of lentiviruses expressing the Herpes simplex virus thymidine kinase under the Sox2 promoter. This enzyme converts ganciclovir, a non-toxic nucleoside analog into a toxic product that kills cells during DNA replication, hence dividing Sox2-positive cells. This procedure caused increased food intake, weight gain and glucose intolerance (Li et al., 2012). When hypothalamic NSCs were implanted in obese animals, with the purpose of understanding whether they could produce a beneficial effect to these animals, they failed to survive due to the inflammatory state of the hypothalamic parenchyma in obese animals. However, when hypothalamic inflammation was prevented by NF-κB inhibition, grafted hypothalamic NSCs survived, proliferated and counteracted obesity and glucose intolerance (Li et al., 2014). In another study, proliferation blockade through i.c.v. infusion of AraC into the third ventricle, led to increased food intake and body weight. Moreover, this weight gain was preceded by a condition of leptin resistance due to lack of responsiveness of ARC leptin receptor-expressing neurons without affecting downstream effector cells (Djogo et al., 2016). From these data it seems that cell renewal in the adult hypothalamus exerts a protective role against excessive body weight gain under detrimental nutritional conditions.

Interestingly, when cell proliferation was inhibited in peripubertal mice at the level of the ME using focal X-ray radiation, HFD-fed animals gained significantly less weight and fat mass compared to non-irradiated animals (Lee et al., 2012). These results may reflect a role for overfeeding-induced neurogenesis, specifically at the level of the ME before establishment of puberty, in reducing baseline energy consumption and promoting energy storage in the form of fat; a response that would optimise adaptation of animals in the wild, where sources of food are rare (Lee & Blackshaw, 2012).

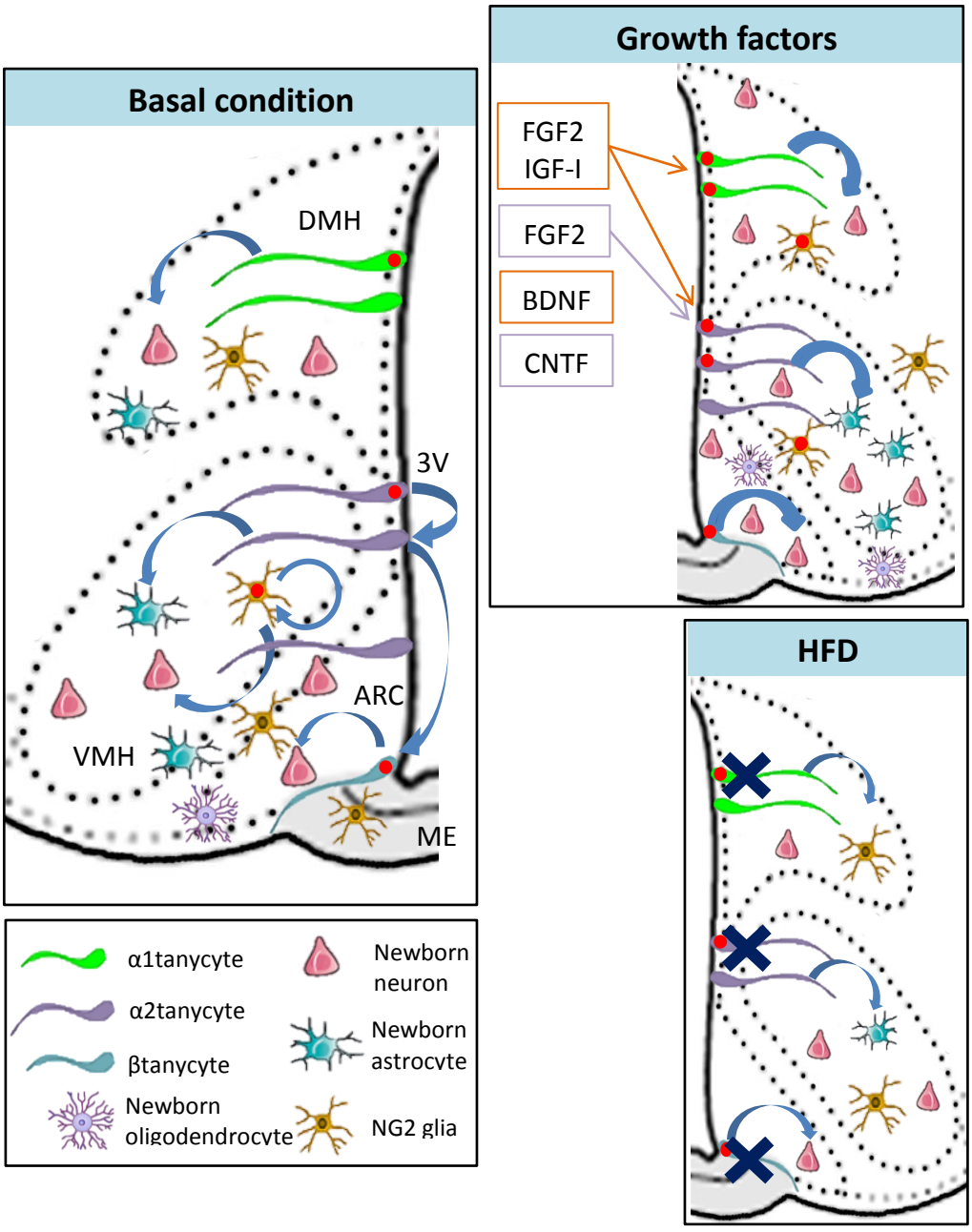


Figure 9: Summary of our current knowledge on cell neogenesis in the adult rodent hypothalamus. Left hand panel: Tanycytes of the different regions differentially give rise to new tanycytes, glial cells and neurons. Proliferating cells, indicated by a red nucleus, are also found at the level of the

parenchyma (e.g. NG2 glial cells), which can self-renew and give rise to newborn neurons. **Right hand panels:** representation of the effects of growth factors (upper panel) or high fat diet (HFD) (lower panel) on cell neogenesis. FGF2 and IGF-I increase cell proliferation in rat α tanycytes (orange arrows), FGF2 increases cell proliferation in mouse α2 tanycytes (purple arrow). BDNF and CNTF stimulate proliferation in the parenchyma in rat and mouse respectively. Chronic HFD inhibits cell proliferation in the hypothalamus. 3V: third ventricle; ARC: arcuate nucleus; DMH: dorsomedial hypothalamus; ME: median eminence; VMH: ventromedial hypothalamus. See main text for details and references.

1.7.6.1 Beyond energy homeostasis

Most of the studies have focused on the role of hypothalamic adult cell neogenesis on energy metabolism and data concerning other hypothalamic-controlled functions are still scares.

Nonetheless, a recent study showed that Gonadotropin releasing hormone (GnRH), the master regulator of the reproductive function (see chapter 2), can stimulate cell proliferation in the hypothalamus of old mice (Zhang et al., 2013). Besides, GnRH treatment decelerates ageing, as attested by improvement of different age-related markers, such as cognitive functions, muscle fibre size and skin thickness (Zhang et al., 2013). Another interesting study regarding the role of hypothalamic cell proliferation in aging came earlier this year from the same group. They showed that hypothalamic NSCs decrease in number with age and that they exert a positive effect on the process of aging. They implanted hypothalamic NSCs derived from newborn mice, in the mediobasal hypothalamus of old animals. The implanted NSCs had been previously modified to survive in the proinflammatory environment of the aged hypothalamus. This procedure caused ageing retardation and lifespan extension (Zhang et al., 2017), suggesting that hypothalamic NSCs actively regulate systemic aging.

The role of adult hypothalamic neurogenesis has also started to be evaluated in another region of the hypothalamus, the POA, in relation to thermogenesis: the ability of the organism to adapt to changes of the external temperature. It has been shown that in rats, heat exposure stimulates proliferation of neuronal progenitor cells in the hypothalamus, a process that is strongly impaired with aging, and it promotes differentiation of these cells into GABAergic neurons. Moreover, some of the newborn neurons were activated by heat exposure. Inhibiting cell proliferation through infusion of the animitotic drug AraC within the lateral ventricle, the ability of heat tolerance was impaired, therefore suggesting that heat

exposure-induced hypothalamic cell proliferation plays a role in the process of thermoregulation (Matsuzaki et al., 2009, 2015, 2017).

At the level of the POA, another group explored the neogenic potential of the organum vasculosum of the lamina terminalis (OVLT), one of the circumventricular organs of the CNS, meaning those regions lacking a BBB, therefore able to sense signals found in the circulatory system through fenestrated capillaries. This region was shown to contain proliferative cells able to differentiate into both glial cells and neurons *in vitro* whereas only gave rise to astrocytes *in vivo* (Bennett et al., 2009). Interestingly, when nestin-positive cells were isolated from the OVLT and ectopically transplanted into the SVZ of recipient animals, these cells migrated along the RMS and integrated into the OB where they expressed neuronal markers, showing that the OVLT contains multipotent cells whose fate is determined by the environment (Bennett et al., 2010).

The POA, and expecially the OVLT region, is critical for the regulation of the reproductive function (see next chapter). The identification of a putative niche of neural stem/progenitor cells in this region during adulthood raises the possibility that newborn cells may be implicated in the central control of reproduction.

Chapter 2

The central control of reproduction

2.1 THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

The HPG axis is an organized set of neural and endocrine tissues dedicated to the control and regulation of the reproductive function; this means to allow correct development of the gametes, release of gonadal hormones, fertilization and gestation, together with reproductive and parental behaviours (Herbison, 2016).

The axis is composed by: the GnRH neurons of the hypothalamus, the gonadotrope cells of the anterior pituitary and the gonads, testes in males and ovaries in females (Herbison, 2016) (Fig. 10).

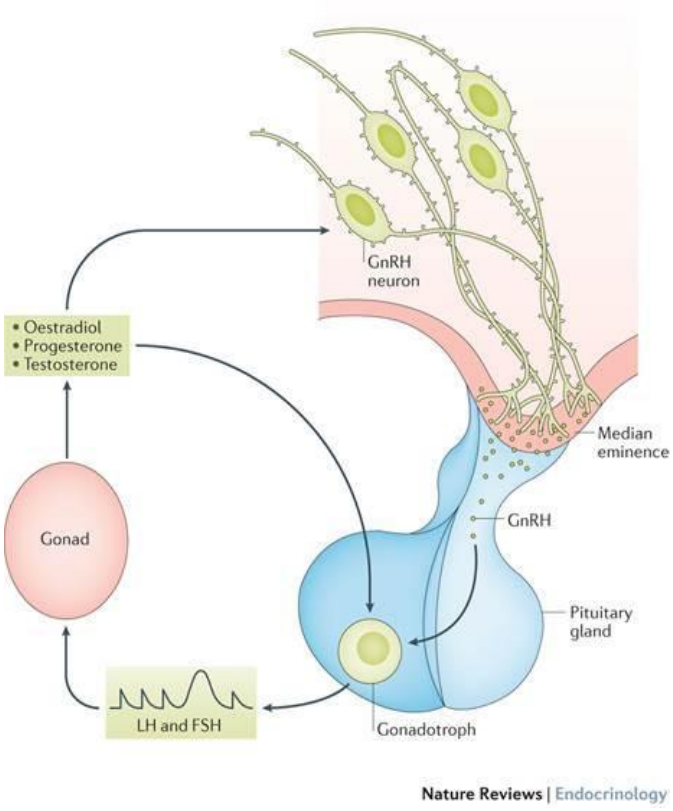


Figure 10: The hypothalamic–pituitary–gonadal axis. Schematic of the three components of the HPG axis. The GnRH neurons project their terminals to the median eminence portal system; the GnRH decapeptide then acts on anterior pituitary gonadotrophs to control the release of gonadotropins that regulate gonadal function. Gonadal steroid hormones will then feedback on the brain and pituitary gland. GnRH: Gonadotropin-releasing hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone (From Herbison A, Nature reviews Endocrinology 2016).

The GnRH neurons are a small population of neurons dispersed between the OB and the mediobasal hypothalamus (Cariboni et al., 2007) with the majority of the cell bodies being found in the medial septum, POA and anterior hypothalamus (Herbison, 2015). These neurons have a peculiar bipolar morphology as they present two processes originating from the opposite sides of their cell body (Jennes et al., 1985). Independently from their location, most of the GnRH neurons project their terminals and release the decapeptide GnRH in a synchronized manner at the level of the ME (Herbison, 2016). This interesting structure at the base of the hypothalamus is one of eight circumventricular organs of the central nervous

system (Johnson & Gross, 1993). Furthermore the ME is part of the hypophyseal portal system, one of the three portal systems in the human body, from which blood is drained directly into another capillary bed through veins instead of passing through the heart (Green & Harris, 1949). These characteristics make the ME a strategic interface between the hypothalamus and the peripheral endocrine system (Yin & Gore, 2010).

From the ME, GnRH reaches the anterior pituitary and acts on the gonadotrope cells to induce the synthesis and release of the gonadotropins: the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), which act at the level of the gonads via specific transmembrane receptors. In order to be effective, the GnRH needs to be secreted in a pulsatile manner since continuous administration down regulates the activity of the gonadotropes, leading to infertility (Belchetz et al., 1978; Christian & Moenter, 2010).

The gonads, testes and ovaries, are the primary reproductive organs, responsible for the gametogenesis - spermatogenesis and oogenesis respectively - and for the release of gonadal hormones, which regulate development and maintenance of sexual characteristics and function. The cells directly responsible for the gametogenesis are the germ cells, from which the gametes are obtained following different mitotic and meiotic divisions. However, these are not the only players involved as during this process, specialized cells provide structural and metabolic support to the developing gametes.

In the testes, the Leydig cells are responsible for the release of androgens: testosterone, dihydrotestosterone and androstenedione while the Sertoli cells support and nourish the developing sperm cells (Jin & Yang, 2014; Tremblay, 2015). In males, LH stimulates the secretion of testosterone from the Leydig cells while FSH regulates the maturation of sperm cells (Bliss et al., 2010; Jin & Yang, 2014).

In the ovaries, each single germ cell, or oocyte, is contained within an ovarian follicle, the functional unit of the ovary (Levine, 2015). In this structure, we can distinguish granulosa and outer theca cells. The theca cells provide structure and support to the follicle as it matures and are responsible for the production of androstenedione. The granulosa cells are mainly responsible for the production of oestrogens (oestrone, E1 and oestradiol, E2), which are produced via aromatization of the androgens secreted by the theca cells and, after ovulation occurs, progesterone, which is involved in pregnancy maintenance. Maturation of the follicles goes through primordial, primary, secondary and antral stages. From this stage,

only a single follicle in humans, and a few in rodents, will reach the preovulatory stage (Graafian follicles), under cyclic gonadotropin stimulation, while most of them will undergo degeneration (Levine, 2015). These Graafian follicles, during each reproductive cycle, ovulate to release the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum (McGee & Hsueh, 2000). FSH stimulates follicular growth, with a consequent increase in estradiol secretion, while LH induces ovulation of the mature follicles and their consequent transformation into corpora lutea (Bliss et al., 2010; Levine, 2015; Simonneaux & Bahougne, 2015).

The gonadal hormones promote the growth of the secondary sex organs and also form important positive and negative feedback loops that act centrally on the GnRH system and at the level of the pituitary (Christian & Moenter, 2010) to modulate gonadotropin output (see chapter 3.3).

2.2 THE RAT OVARIAN CYCLE

The role of the HPG axis is that of ensuring that reproduction occurs; this means optimising the process based on the animal's characteristics and ecosystem; being ovulation and pregnancy very costly in terms of energy demand for females, these processes are finely regulated while spermatogenesis occurs as a continuum throughout adult life in males. Ovulation is regulated in a cyclic manner with a follicular and a luteal phase: during follicular phase, a follicle develops and produces increasing amounts of oestrogens until ovulation occurs. Then, the follicle turns into the corpus luteum (luteal phase). If the ovum is not fertilised menstruation is induced and a new follicular phase begins (Levine, 2015). Animals differ for the duration of their cycles. For instance primate follicular phase lasts at least 2 weeks and they also present a long luteal phase. Domestic animals such as cows, sheep and pigs have a very short follicular phase but a quite long luteal phase with a 17-21-day long cycle. Rats, mice and hamsters have a very short cycle and ovulate every 4-5 days. Finally, a group of mammals that includes rabbits, domestic cats and ferrets, does not ovulate unless coitus occurs (reflex ovulators). It is also possible to distinguish between seasonal and nonseasonal breeders with the former ovulating only during a specific season, like sheep or horses, and the latter cycling throughout the year, as in the case of primates, mice or rats (Levine, 2015).

Female rats have been extensively used as a model to study reproductive physiology due to their regular and short cycle. As mentioned before, rats ovulate every 4-5 days and it is possible to distinguish four phases that can easily be determined by analysis of vaginal smear: Proestrus, characterised by the presence of nucleated epithelial cells, is the phase that precedes ovulation; in Estrus, when ovulation occurs, cornified squamous epithelial cells are mainly found; Metestrus (or diestrus-1) and Diestrus (or diestrus-2) occur in absence of fertilisation - they correspond to the lutheal phase in primates - and are characterised by predominant presence of leukocytes together with nucleated epithelial cells (Levine, 2015; Westwood, 2008) (Fig. 11b).

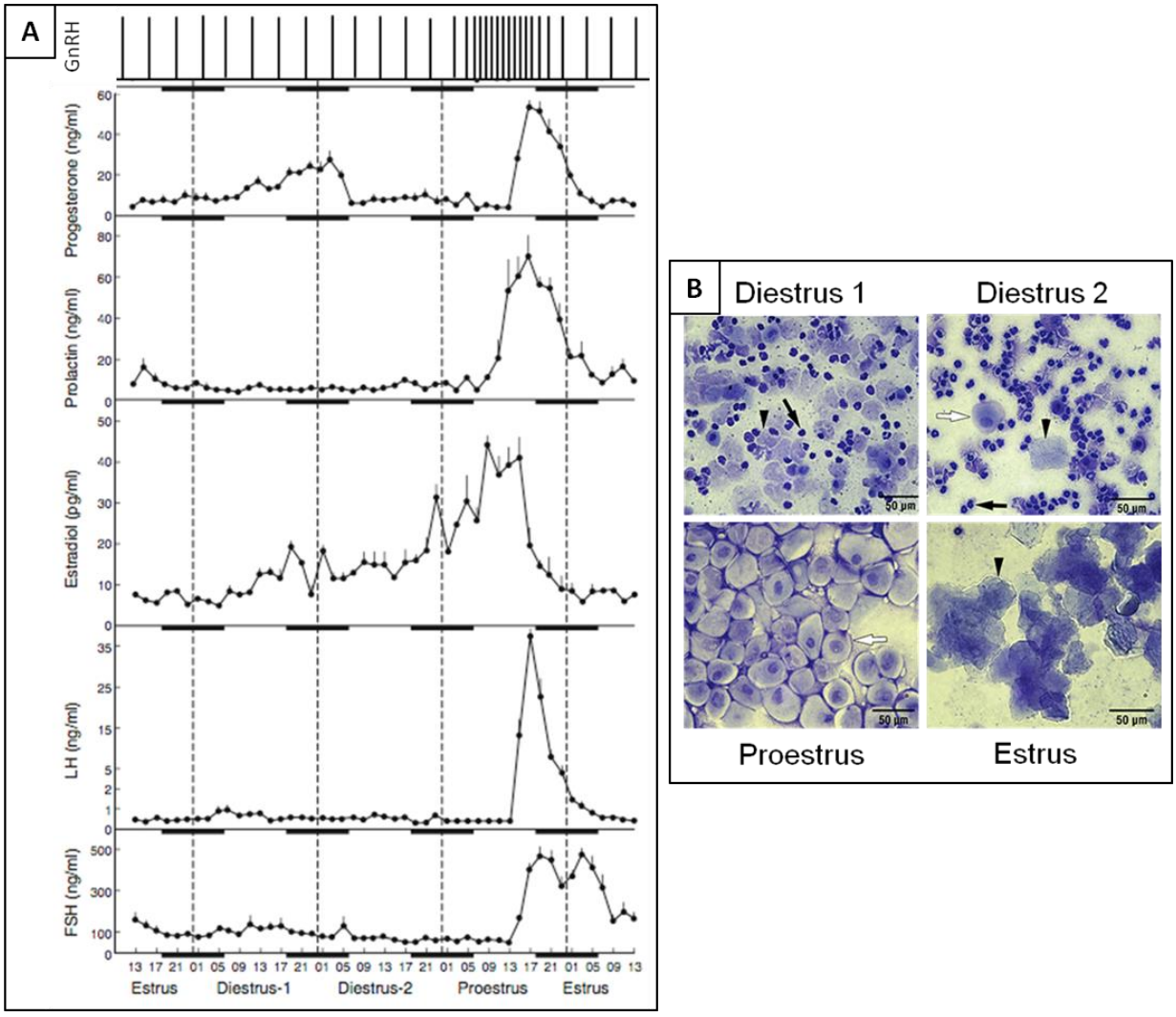


Figure 11: The rat's oestrus cycle. (A) The pattern of GnRH, progesterone, prolactin, oestradiol, LH and FSH release throughout the 4-day oestrous cycle. The concentrations were obtained at 2-h intervals from five to six different rats. The numbers below represent the time-of-day in terms of the 24-h clock. **(B)** Vaginal smear from rat, stained with crystal violet throughout the stages of the oestrus cycle. The relative enrichment in the different cell types allows to identify the oestrus cycle stage. The two days of diestrus are characterised by a predominance of leukocytes (black arrows); in proestrus, only nucleated epithelial cells are found (white arrows); estrus is characterised by an

elevated number of cornified squamous epithelial cells (black arrowheads) (Modified from Smith et al. 1975, Endocrinology; and from McLean et al. 2012, Journal of Visualized Experiments).

2.2.1 Patterns of hormonal secretion

2.2.1.1 GnRH

While GnRH basal secretion is pulsatile and induces a pulsatile release of LH, a surge of GnRH and therefore LH occurs on the afternoon of proestrus to induce ovulation (Herbison, 2016). After the surge occurs, GnRH activity declines and basal pulsatility is restored. A peculiar characteristic of the GnRH neurons is their range of different spontaneous firing patterns: in several electrophysiological experiments, neurons were found silent, continuously active, or irregularly active, with bursting activity (Constantin et al., 2013; Herbison, 2015; Sabatier et al., 2004). This heterogeneity of firing activity has been found both *in vitro* and *in vivo*, therefore raising the question of whether different firing patterns represent different GnRH neuronal populations with different roles in the generation of a GnRH pulse. For instance, studies performed on animals presenting a mutation resulting in the absence of GnRH expression have shown that after ventricular transplantation of GnRH neurons, as little as 20 of these cells were sufficient to generate pulsatile LH secretion (Kokoris et al., 1988). What appears to be constant is that in order to obtain GnRH pulses, at least a subpopulation of GnRH neurons must be synchronized, but how this synchronization occurs remains still unknown. Two models have been proposed to explain synchronization at the level of GnRH neuron cell bodies: an intrinsic pulse generation, which involves only GnRH neurons, and an extrinsic pulse generation, which involves a separate group of neurons within the GnRH neuronal network. A third hypothesis however, would be that the process of synchronisation only occurs at the level of GnRH neuron terminals in the ME, thus regulating the release of the decapeptide rather than its synthesis (Herbison, 2015).

2.2.1.2 Gonadotropins

The LH surge on the afternoon of proestrus induces ovulation by rupture of the follicle, and formation of the corpora lutea; LH then decreases to basal levels on the morning of estrus (Gallo, 1981; Levine, 2015) (Fig. 11a). FSH levels remain low during late estrus until afternoon of proestrus when there is a rapid increase, in parallel with the increase in LH release, which peaks on the evening of proestrus after which it begins to decline. However,

on the morning of estrus, a second peak of FSH occurs, independently from the hypothalamic control, after which basal levels are reached again (Butcher et al., 1974; Gay et al., 1970; Levine, 2015) (Fig. 11a). Unlike LH, FSH in not normally released in a pulsatile manner. The release of LH and FSH is regulated by the frequency of GnRH pulses: a higher pulse frequency increases LH secretion over FSH, whereas low GnRH pulse frequency favours FSH secretion (Kaiser et al., 1997; Levine, 2015). This regulation appears to occur at the level of gene transcription in the gonadotropes as the expression of the GnRH receptor is increased by high frequency pulses of GnRH itself, probably increasing the responsiveness of the pituitary to GnRH to switch towards LH release (Kaiser et al., 1997).

2.2.1.3 Prolactin

Prolactin is a hormone produced by the lactotrophs of the anterior pituitary whose release is mainly regulated by the action of three populations of hypothalamic dopaminergic neurons that inhibit its release: the tuberoinfundibular (TIDA), the tuberohypophyseal (THDA) and the periventricular hypophyseal (PHDA) dopaminergic neurons (Grattan, 2015). While TIDA neurons project to the ME from where dopamine is transported to the anterior pituitary, THDA and PHDA neurons project to the neurohypophysis from where dopamine reaches the anterior pituitary through short portal vessels (Grattan, 2015). The main role of prolactin, as suggested by its name, is to regulate lactation although many other adaptive roles have been found to be regulated by this hormone in a non-irreplaceable way (see chapter 3.6) (Grattan, 2015). For instance, prolactin acts on nutrient uptake and metabolism to increase body weight in pregnant females. Prolactin also reduces anxiety and favours establishment of maternal behaviour.

During the oestrous cycle, prolactin secretion is regulated with a peak of release overlapping with the LH surge during afternoon of proestrus (Fig. 11a); however, its role in the events of the oestrus cycle have not been clearly established yet (Butcher et al., 1974; Smith et al., 1975).

2.2.1.4 Ovarian steroids

During the development of the ovarian follicle increasing amounts of oestrogens are released by granulosa cells until reaching a peak at the beginning of the afternoon of proestrus (Fig. 11a). Two peaks of release have been described for progesterone, one from

the preovulatory follicle, on the afternoon of proestrus, and the second from the corpora lutea, in the afternoon of metestrus, independent from the pituitary (Butcher et al., 1974; Levine, 2015; Yoshinaga et al., 1969) (Fig 11a).

2.3 REGULATION OF THE GNRH SYSTEM

GnRH neurons are the master regulators of reproduction and therefore their activity is finely modulated by the action of different neuronal populations, which convey different internal and external information to which GnRH activity adapts.

For instance, in order for GnRH surge to occur at the right moment of the day and of the stage of follicle maturation, GnRH neurons receive signals from both the SCN, which conveys signals from the circadian clock (Palm et al., 1999), and the AVPV, which integrates oestrogens signals coming from the growing follicle (Wiegand et al., 1978).

Most importantly, GnRH neurons receive positive and negative feedbacks from the gonads through the action of the gonadal hormones that act via multiple neuronal and glial mediators.

Tonic levels of LH and FSH are required for the release of oestrogens; increasing oestrogens and progesterone levels, secreted from metestrus through diestrus, exert a negative feedback, restraining the hypothalamo–pituitary axis and leading to basal secretion of LH during this time. When critical levels are reached, a switch to a positive feedback occurs, therefore stimulating the LH and FSH surge on the afternoon of proestrus (Levine, 2014). Generally, the action of progesterone on the secretion of LH and FSH seems to be that of potentiating oestrogen's effects. The release of FSH does not only depend on ovarian steroids: the gonads in both males and females also produce inhibins and activins, proteic complex that respectively inhibit and stimulate FSH release at the level of the anterior pituitary. Moreover, a third protein produced by the ovaries, follistatin, has the ability to bind and block the effect of activins, therefore contributing to the inhibition of FSH release (Hoffmann et al., 1979; Shander et al., 1980). While there seems to be no effect of progesterone on prolactin release (Levine, 2015), oestrogen is able to induce the proestrus peak of prolactin by acting both at the level of the lactotrophs, where it increases prolactin gene expression (Avtanski et al., 2014), and at the level of the hypothalamus, where it decreases the inhibitory effect of dopamine on prolactin release (Labrie et al., 1989).

ER and progesterone receptor (PR) are both classic nuclear receptors, able to modulate transcription of different genes by binding at the level of specific sequences found in the

promoter of target genes. ER also modulates transcription indirectly as the receptor can also be found at the cell membrane and in this case, it does not translocate into the nucleus as a result of its stimulation (Glidewell-Kenney et al., 2007). Moreover, it appears that during induction of the LH surge, oestrogens can also act through PR in a ligand-independent manner (Levine et al., 2001). Mice lacking ERα or PR, but not ERβ isoform, exhibit hypergonadotropism and an absence of estrous cyclicity, thus suggesting that only the former is involved in conveying the negative and positive feedback actions of oestradiol during the estrous cycle (Chappell et al., 1997; Couse et al., 2003). Both positive and negative feedbacks act at many different levels: by modulating GnRH neuron pulsatility indirectly, through action on different neuronal populations, by increasing the sensitivity of the pituitary gland to GnRH via modulation of GnRH receptor expression, and by regulating gonadotropin gene expression (Levine, 2015).

Despite the importance of gonadal hormones in regulating GnRH neuron activity, GnRH neurons are not able to directly sense changing levels of oestrogens as they lack ERα. Therefore, both positive and negative feedbacks on GnRH release must be exerted through the action of intermediaries (Cheong et al., 2014; Levine, 2015).

2.3.1 Neurotransmitters and neuropeptides

GnRH neurons receive synaptic contacts from both GABAergic and glutamatergic neurons. Moreover, the vast majority of GnRH neurons express postsynaptic ionotropic $GABA_A$ receptors and pre- or postsynaptic metabotropic $GABA_B$ receptors as well as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and kainate receptors. The sources of GABA and glutamate inputs to GnRH neurons, which concern not only the GnRH neuron cell bodies but also their terminals at the level of the ME, have not all been clearly identified (Herbison & Moenter, 2011; Spergel et al., 1999).

2.3.1.1 GABA

It appears that GABA neurons located in the AVPV, lateral POA and SCN directly project to GnRH neurons. The general action of GABA seems to be inhibitory over GnRH tone. GABA levels in the POA are elevated at times of negative feedback and then fall just before the GnRH/LH surge (Herbison & Deyer, 1991; Robinson et al., 1991; Rotsztejn et al., 1982).

Besides, during proestrus, a time when positive feedbacks are conveyed towards GnRH neurons, a strong reduction in presynaptic $GABA_B$ receptor signalling by AVPV afferents occurs compared with other stages of the cycle. This reduction may result from a lower expression of presynaptic $GABA_B$ receptors or a change in their efficacy (Liu & Herbison, 2011). Moreover, GABA neurons of the AVPV and ARC express ERs (Cheong et al., 2015; Van Vugt et al., 2004). The role of these neurons in regulating oestrogen positive and negative feedback has been investigated in mouse lines with selective KO of ERα in GABAergic neurons. The results showed that while functional ERα in GABA neurons was not necessary for the negative feedback, it was critical for the positive feedback, through mechanisms that remain to be elucidated (Cheong et al., 2015).

2.3.1.2 Glutamate

Even though we can speculate that glutamatergic inputs arrive from many different brain regions, the only documented input is that of the AVPV. The role of glutamate has not been fully characterised yet. Glutamate levels in the POA increase during the surge of LH (Jarry et al., 1992) and it has stimulatory effects over GnRH tone. This increase is in association with the estradiol-induced LH surge, wich can be blocked with the use of NMDA or AMPA antagonists (Ping et al., 1997). In accordance, glutamatergic neurons have been shown to express ERα, and this signalling appears to be essential for the establishment of the positive feedback (Cheong et al., 2015). Furthermore, it has been shown that oestradiol can increase the GnRH response to NMDA receptors stimulation (Arias et al., 1993).

2.3.1.3 Kisspeptin

It is now well known that the strongest activators of GnRH activity are the kisspeptin neurons, which are found in the ARC and in the AVPV.

The first suggestions for a role of kisspeptin neurons in regulation of the reproductive function came from the discovery that inactivating mutations of the kisspeptin receptor,

Kiss1r (or Gpr54), are associated with hypogonadotropic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2004). From there, the role of kisspeptin has been described in a variety of other species and transgenic lines have been developed such as mice lacking kisspeptin or Kiss1r, which show total infertility associated with hypogonadotropism and absent ovarian cyclicity (Colledge, 2009). Moreover, restoring Kiss1r expression specifically in the GnRH neurons has been proven sufficient to totally recover fertility, therefore placing kisspeptin neurons as the master afferents directly controlling GnRH neuron activity (Kirilov et al., 2013).

More than 90% of kisspeptin neurons express ERα (Lehman et al., 2010). For this reason, their role in mediating oestrogen feedback has been extensively studied. Firstly, mice with specific ablation of ERα in kisspeptin neurons showed total acyclicity. It has been shown that oestrogens increase kisspeptin mRNA expression in the AVPV and decrease it in the ARC (Gottsch et al., 2009), and that levels in the AVPV vary at time of LH surge. Besides, infusion of kisspeptin in the ARC affects GnRH pulse generation (Levine, 2015). It is now well established that neurons of the AVPV mediate the oestrogen positive feedback and are therefore necessary for the surge of GnRH and LH hence for ovulation to occur (Kirilov et al., 2013). Moreover, the population of kisspeptin neurons of the AVPV is sexually dimorphic, with female animals displaying a higher number of neurons compared to males (Ahmed et al., 1990). Kisspeptin neurons of the ARC have been suggested to be involved in mediating oestrogen negative feedback, modulating basal pulsatility of GnRH and LH even though this has not been fully demonstrated yet (Levine, 2015).

It has also been shown that kisspeptin is able to modulate GABA and glutamate transmission to GnRH neurons according to oestrogens levels. For instance, kisspeptin is able to decrease the hyperpolarising effects of $GABA_B$ receptor activation, therefore attenuating their inhibitory tone during the stage of oestrogen's positive feedback (Zhang et al., 2009). This suggests that GABAergic and glutamatergic tones are also involved in mediating, at least in part, kisspeptin action on GnRH neurons (Levine, 2015).

2.3.2 Orexigenic and anorexigenic modulators

The metabolic status of the organism is critical for maintenance of ovarian cycles as a status of nutrient deficit could impair pregnancy or lactation, hence decrease the probability of

offspring's survival. At the same time, excessive fat accumulation, as in the case of obesity, negatively affects the general health condition of the organism.

Therefore, important modulators of GnRH activity are factors that inform on the energy status of the animal such as leptin, insulin and ghrelin. Leptin is a hormone secreted by the adipocytes, whose levels directly relate to the amount of body fat mass (Casanueva & Dieguez, 1999), being an indicator of energy sufficiency. Animals which are congenitally deficient for leptin (ob/ob) or leptin receptor (LepR) (db/db) are obese and sterile (Swerdloff et al., 1978). Insulin is a pancreatic hormone released according to glucose levels in the blood, which can act as a modulator of leptin synthesis itself and accordingly stimulate the HPG axis (Sonksen & Sonksen, 2000); however, the mechanisms involved are not as well characterised as those for leptin (Tena-Sempere, 2015). Ghrelin is an orexigenic circulating factor produced by the stomach (Dickson et al., 2011). In accordance to its role as a signal for energy insufficiency, ghrelin has an inhibitory effect over the HPG axis both during puberty and adulthood (Tena-Sempere, 2015). Nevertheless, as for oestrogens, these hormones do not act directly on GnRH neurons, even though they are the final output of their actions to regulate reproduction. Different neuronal populations are therefore required in this cross-talk between reproduction and energy homeostasis. Anorexigenic POMC and orexigenic NPY/AgRP neurons of the ARC regulate appetite according to the energy status of the organism, the former by suppressing and the latter by promoting food intake. Because of their ability to sense levels of metabolic hormones and to respond to gonadal hormones, these cells play a crucial role in regulating reproductive function according to the energy status of the animal (Manfredi-Lonzano, 2017; Tena-Sempere, 2015).

2.3.2.1 POMC neurons

POMC neurons are able to sense leptin, insulin, kisspeptin and NPY. POMC is the precursor for different neuropeptides such as β-endorphin and α-melanocyte stimulating hormone (α-MSH) but these neurons also co-express other neurotransmitters including GABA and glutamate (Manfredi-Lonzano et al., 2017). Leptin acts directly on POMC neurons to regulate energy homeostasis: α-MSH is a signal of energy sufficiency that appears to mediate leptin effects on adult fertility (Maria Manfredi-Lozano et al., 2016) since restoring melanocortin signalling in animals lacking leptin receptor rescues fertility (Israel et al., 2012). B-endorphin

is an endogenous opioid indicating a condition of energy insufficiency that both inhibits basal LH release and blocks LH surge (Manfredi-Lozano, et al., 2017; Nestor et al., 2014). Moreover, a subset of POMC neurons express ERα and are therefore capable of responding to oestrogens, which increase POMC activity (Gao et al., 2007). Loss of ERα from these neurons causes increased weight together with abnormal cyclicity pointing towards a possible role of these cells in mediating oestrogen's negative feedback (Xu et al., 2011).

Finally, a possible crosstalk between POMC and kisspeptin neurons is suggested by the findings that inhibition of kisspeptin neurons or ablation of Kiss1r weaken LH response to α-MSH (Manfredi-Lonzano et al., 2017).

2.3.2.2 NPY/AgRP neurons

NPY/AgRP neurons act by sensing decrease in leptin levels and therefore stimulating food intake by antagonising the anorexigenic action of α-MSH since AgRP acts as an endogenous antagonist of α-MSH receptors. Food restriction increases NPY expression in the hypothalamus and as a signal of adverse metabolic condition, this has been linked to decreased GnRH and LH secretion. A subset of NPY/AgRP neurons express ERα (Sar et al., 1990) and oestrogens have been shown to decrease NPY/AgRP expression (Pelletier et al., 2007) and to hyperpolarise these neurons (Roepke et al., 2011).

2.3.3 Nitric oxide

Nitric oxide (NO) is a gaseous messenger discovered as a signalling molecule in the brain in the 1980's (Garthwaite et al., 1988). NO is produced by NO synthase (NOS); it is not stored in vesicles but it rapidly diffuses and induces formation of cyclic GMP (cGMP) by direct stimulation of soluble guanylate cyclase. Three isoforms of NOS have been identified: an endothelial form (eNOS), an inducible form (iNOS) and a neuronal one (nNOS). This last isoform is considered the main source of NO in the brain and it is indeed widely distributed in many regions with a particularly dense distribution at the level of the OVLT, where most GnRH neuron cell bodies are found, and of the ARC (Chachlaki et al., 2017a). Recent studies from our laboratory have shown the phenotype of these nNOS expressing neurons to be mainly glutamatergic at the level of the OVLT and GABAergic in the ARC (Chachlaki et al., 2017a). Early studies with total KO animals for nNOS showed as their most striking

phenotype impairment in the reproductive activity and an increasing number of studies are investigating the role of nNOS neurons within GnRH neuron network. NNOS neurons have been shown to express ERα (Chachlaki et al., 2017a), kisspeptin receptor (Hanchate et al., 2012) and leptin receptor (Bellefontaine et al., 2014), putting them at the centre of these entangled circuits and giving them the ability to contribute to the action of these messengers on GnRH release.

For instance, kisspeptin stimulates NO release in the OVLT (Hanchate et al., 2012). It has also been shown that inhibition of nNOS activity at the level of the POA ablates the stimulatory action of leptin on LH surge (Bellefontaine et al., 2014). Besides, nNOS activity is strictly dependant on its coupling with the NMDA receptor, as the Ca^{2+} influx caused by the binding of glutamate favours nNOS phosphorylation, hence activation and therefore linking NO levels to glutamate, another well-known GnRH activator. Moreover, this process appears to be depended on ERα activation, as this can promote the association between nNOS and the NMDA receptor, and nNOS phosphorylation is highest on the day of proestrus, when oestrogen levels peak (Chachlaki et al., 2017b). Even though the absence of nNOS in the brain impairs GnRH function, the direct effect of NO on GnRH neuron electrical activity has been shown to be inhibitory (Clasadonte et al., 2008). Due to its half-life and diffusion constant, NO works as a volume transmitter capable of coordinating neuronal activity in a restricted brain volume. Hence, the main hypothesis on a role for NO is that of being involved in synchronizing the activity of different GnRH neurons to finally promote the release of GnRH in the ME (Chachlaki et al., 2017b).

2.3.4 Autocrine control of the GnRH neurons

Over the years, many neurotransmitters and neuropeptides have been reported to be expressed in GnRH neurons. However their nature and mode of action have not been entirely characterised yet. Among them, we can mention galanin (Rossmanith et al., 1996) and cholecystokinin (CCK) (Ciofi, 2000; Giacobini & Wray, 2007), which work as autocrine factors acting on the GnRH neurons themselves; and neurotensin (NT) (Ciofi, 2000), GABA (Zhu et al., 2015) and glutamate (Chachlaki et al., 2017a; Hrabovszky et al., 2004), whose possible autocrine role has not been demonstrated yet.

Galanin: Galanin is a neuropeptide widely expressed in the CNS, released by both GnRH neurons, that also express galanin receptor (Rossmanith et al., 1996; Todman et al., 2005), and the anterior pituitary, under the control of oestrogens (Kaplan et al., 1988). During the estrous cycle of the rat, galanin and GnRH contents in the ME show an identical profile (López et al., 1991). Galanin's expression by GnRH neurons increases gradually during pubertal maturation and presents a sexually dimorphic pattern, being higher in females (Rossmanith et al., 1994). Galanin is able to increase LH levels (Sahu et al., 1987), and its levels are higher during the LH surge in response to gonadal steroids (Rossmanith et al., 1996). It also enhances the ability of GnRH to stimulate LH release (López et al., 1991). It has been hypothesised that galanin works as an autocrine modulator of GnRH neurons: galanin, co-secreted with GnRH at the level of the ME, would inhibit GnRH "leakage" between consecutive pulses, therefore increasing the efficacy of each pulse (Rossmanith et al., 1996).

Cholecystokinin: CCK is a neuropeptide extensively expressed throughout the CNS, with high concentrations in the limbic system. Its levels in several brain nuclei, at the level of the MPO for instance, have been shown to change according to circulating gonadal steroids (Oro et al., 1988). CKK is expressed by at least a subpopulation of GnRH neurons in adult female but not male rats (Ciofi, 2000). A strong sexual dimorphism has also been reported in mice, with adult males showing a much lower level of GnRH/CCK coexpression than females. Moreover, CCK inhibits GnRH neuronal activity by acting directly through its receptor (Giacobini & Wray, 2007).

Glutamate and GABA: Even though some evidences show that the vast majority of GnRH neurons are glutamatergic (Chachlaki et al., 2017a; Hrabovszky et al., 2004), there is still no consensus over the actual release of glutamate by these neurons and a possible auto-excitatory action of glutamate on GnRH neuron activity (Herbison, 2015).

A subpopulation of GnRH neurons in the mouse forebrain has been found to be GABAergic (Zhu et al., 2015). The number of these neurons increases during estrus in female mice compared to the diestrus phase or male mice, a sexual dimorphism that becomes evident only after sexual maturation is attained (Zhu et al., 2015).

Neurotensin: NT is a neuropeptide distributed throughout the CNS, with highest levels in the hypothalamus, amygdala and nucleus accumbens. It induces a variety of effects, including analgesia or hypothermia. NT-expressing cells in the AVPV and MPO express ERα and their

number increases in the presence of oestrogens, reaching a peak at the moment of the preovulatory LH surge in female mice (Dungan Lemko et al., 2009). NT has also been detected in adult female but not male rats (Ciofi, 2000). However, the number of GnRH neurons co-expressing only NT was quite low with most of them found to also co-express CKK (Ciofi, 2000). GnRH neurons express NT receptor (Dungan Lemko et al., 2009), but a direct role for this peptide on the LH surge has not been clearly demonstrated yet (Dungan Lemko et al., 2009).

2.3.5 Glial cells

After many years being neglected as the "glue" of the CNS, there is now a rising number of studies acknowledging the actual importance of glial cells in regulating neuronal activity.

For instance, glial cells of the hypothalamus are critical modulators of the GnRH neurons through different mechanisms: release of paracrine factors, juxtacrine interactions and structural plasticity (Sharif et al., 2013). These cells are mainly astrocytes, both at the level of GnRH cell bodies and terminals, and tanycytes, which interact with GnRH fibers at the level of the ME.

2.3.5.1 Paracrine factors

The paracrine factors that have been most extensively studied in relation to this matter are prostaglandin E2 (PGE2) and transforming growth factor (TGF) β.

PGE2 is released by both astrocytes and tanycytes in response to erbB receptor signalling activation. It derives from the arachidonic acid through the sequential activity of cyclooxygenases (COX) and a specific synthase (Fig. 12) (Crofford, 2001). The erbB receptor family is a group of four tyrosine kinase receptors named erbB1 (or EGF receptor, EGFR), erbB2 (or Neu), erbB3 and erbB4. While erbB1 and 4 are fully functional receptors, with an extracellular ligand binding domain and a cytoplasmic tyrosine kinase domain, erbB2 and 3 lack the extracellular or cytoplasmic domain respectively, and act in the hypothalamus as coreceptor, in heterodimers always including erbB1 or erbB4 (Hynes & Lane, 2005). These receptors can differentially bind different growth factors of the EGF family such as EGF, TGFα and neuregulins (NRGs). Hypothalamic astrocytes express erbB1, 2 and 4 (Sharif et al., 2009)

and tanycytes express erbB1, 2 and 3, while none of these receptors is expressed in GnRH neurons (Voigt et al., 1996).

Activation of erbB heterodimers induces production and release of PGE2 which binds to specific E-prostanoid receptors (EPs) (Fig. 12) (Woodward et al., 2011). Different subclasses of EPs exist (EP1-4) (Woodward et al., 2011) (Fig. 12) and GnRH neurons express both EP1 and 2, both at the level of nerve terminals and cell bodies. While activation of EP2 stimulates GnRH firing by acting at the level of the cell body, activation of EP1 induces mobilisation of intracellular calcium stores (Fig. 12) that is involved in the release of GnRH at the level of the ME (Clasadonte et al., 2011).

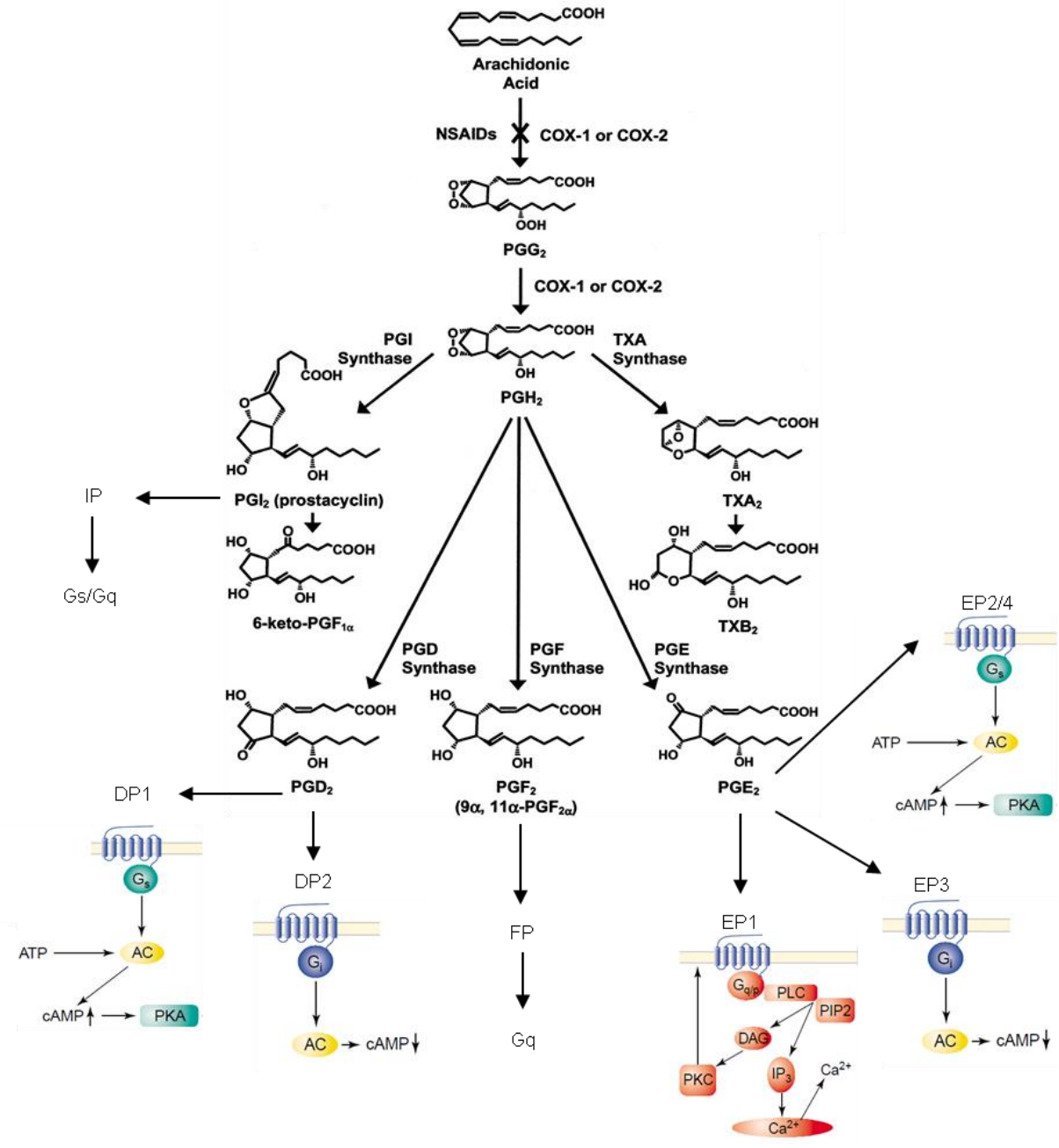


Figure 12: The arachidonic acid cascade and PGs signalling. Arachidonic acid is metabolized by cyclooxygenases (COX-1 and 2) to prostaglandin (PG)H2 via the short-lived intermediate PGG2. PGH2 then spontaneously rearranges or is enzymatically isomerized, oxidized, or reduced to yield bioactive prostaglandin isomers. Each PG binds to its specific receptor, which are all transmembrane receptors coupled to different G-proteins. Gq/p signalling results in an increase in the level of intracellular calcium. Gs proteins induce the expression of cAMP. Gi proteins are most often inhibitory to cAMP. AC, adenylate cyclase; DAG, diacylglycerol; IP3, inositol triphosphate; PIP2, phosphatidylinositol diphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (Modified from Simmons D. L. 2004, Pharmacological reviews and Harris S. G. et al. 2002, Trends in Immunology).

Release of PGE2 in astrocytes depends upon activation of ionotropic and metabotropic glutamate receptors on glial cells (Fig. 13). This results in a cascade of events including recruitment of erbB1 and erbB4 receptors and their pro-ligands, pro-TGFα and pro-NRG, to the cell membrane, and transactivation of these receptors through the action of metalloproteinases such as TGF-α-converting-enzyme (TACE), which cleaves the pro-ligands into their mature forms that will activate erbB receptors (Dziedzic et al., 2003) (Fig. 13).

Oxytocin, as mentioned before, is produced by magnocellular neurosecretory cells of the supraoptic and paraventricular nuclei. It is able to accelerate GnRH release pulsatility, however, oxytocin receptors are not expressed by GnRH neurons themselves but by adjacent astrocytes. Moreover, its action is abolished if PGE2 synthesis is inhibited. Therefore, it seems that oxytocin exerts its role on GnRH neurons, just like EGF ligands, through the mediation of astrocytes (Parent et al., 2008).

In tanycytes, the activity of COX is stimulated by the TGFα-induced activation of erbB1/erbB2 receptors but also by NO released by adjacent endothelial cells (De Seranno et al., 2010).

All these mechanisms may be regulated by the action of gonadal steroids as oestrogens are known to stimulate TGFα release and COX activity (Ma et al., 1992).

The importance of this pathway is shown in animals with disrupted erbB signalling such as those expressing a dominant negative erbB4 in astrocytes which show decreased GnRH release and impaired reproductive function (Prevot, et al., 2003). Moreover, blocking either astrocyte metabolism or COX activity suppresses the spontaneous firing activity of GnRH neurons in brain slices, an effect that can be rescued by administration of exogenous PGE2 (Clasadonte et al., 2011).

TGFβ1, produced by astrocytes in accordance to oestrogen levels throughout the oestrus cycle (Galbiati et al., 2001), is able to regulate GnRH expression and release *in vitro* in the immortalized hypothalamic cell line GT1. Besides, the expression of its receptors has been demonstrated *in vivo* at the level of GnRH neuron cell bodies, while it is absent in the ME. Whether TGFβ1 modulates GnRH neuron activity in the POA remains to be explored (Sharif et al., 2013).

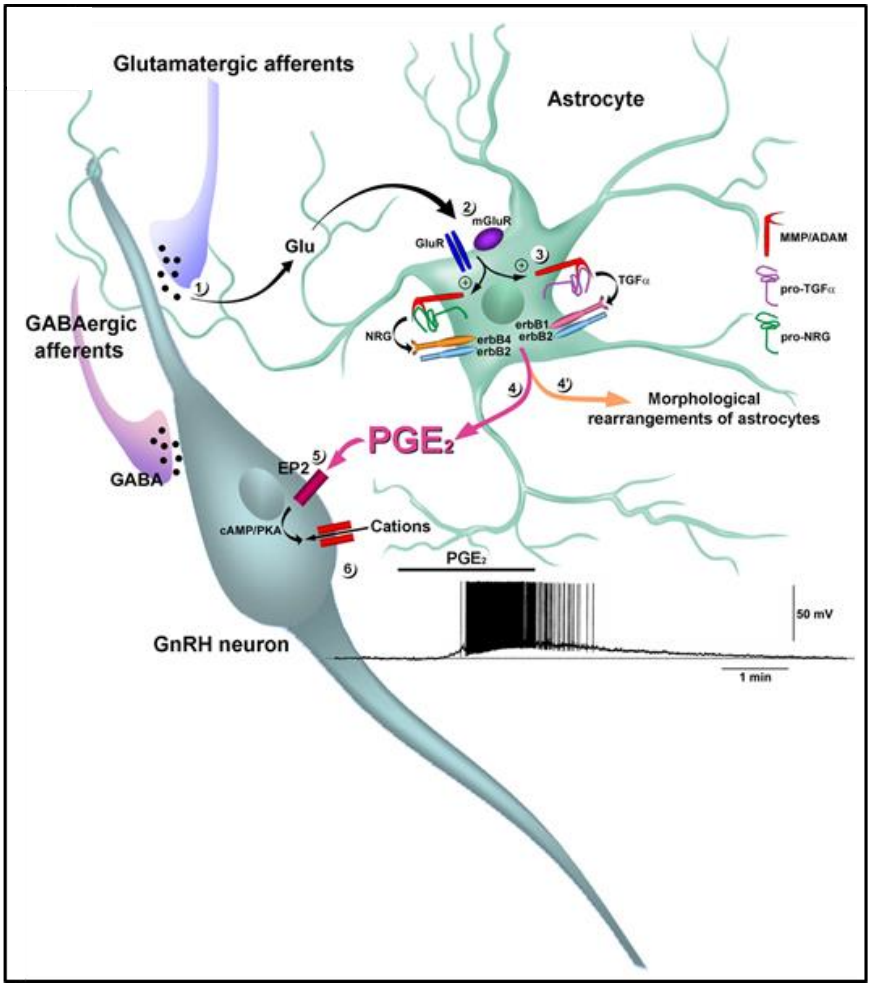


Figure 13: Schematic representation of GnRH neurons-astrocytes interactions in the POA. Neuronal glutamate (Glu) co-activates metabotropic glutamatergic (mGluR) and AMPA glutamatergic receptors (GluR) in astrocytes, stimulating the activity of metalloproteinases of the ADAM family that subsequently release mature TGFα and NRG, which activate erbB1/erbB2 and erbB4/erbB2 heterodimers, respectively. Activation of erbB receptors promotes the release of PGE2, which binds to EP2 receptors in GnRH neurons and induces a cAMP/PKA pathway that leads to a reversible membrane depolarization and initiation of spike firing through the activation of a nonselective cation current (From Clasadonte J. 2011, Frontiers in Neuroendocrinology).

2.3.5.2 Juxtacrine interactions

Three major protein complexes have been involved in GnRH neuron-glial cell juxtacrine interactions: the PSA-NCAM, the receptor protein tyrosine phosphatase-β/ contactin/contactin-associated protein-1 (RPTPβ/contactin/CASPR1) complex and synaptic cell adhesion molecule 1 (SynCAM1) (Sharif et al., 2013). Although their specific roles have not been fully clarified yet, they have all been found to be expressed by astrocytes and/or GnRH neurons with expression profiles changing according to reproductive maturation status or oestrus cycle phase. Furthermore, SynCAM1 appears to be necessary for activation of erbB4-dependent PGE2 release (Sandau et al., 2011). The expression of SynCAM1 in the POA decreases in mice with impaired erbB4 signalling; NRG treatment, hence activation of erbB4, results in rapid association of erbB4 with SynCAM1 and activation of SynCAM1 transcription. The importance of this interaction in the astrocytic modulation of GnRH activity was shown in animals with impaired SynCAM1 activity in GFAP-expressing astrocytes, which presented disrupted reproductive capacities as a result of defective astrocytic PGE2 response to NRGs (Sandau et al., 2011).

2.3.5.3 Structural plasticity

As already noticed by Ramon y Cajal in his first description of the CNS, astrocytes enwrap neuronal cell bodies and terminals and these contacts are subjected to profound remodelling in response to different stimuli (Theodosis & Poulain, 2008). Astrocytes are found to contact GnRH neuron cell bodies and blood vessels at the same time in a tripartite complex sensitive to changing levels of gonadal hormones.

A very specific and peculiar example of how these cytoskeletal remodellings can affect GnRH release is represented by the role of tanycytes in the ME. More specifically, it appears that during periods of low oestrogen levels, such as during diestrus, tanycytic end-feet engulf GnRH terminals separating them from the perivascular space of the ME (Fig. 14). However, during proestrus, when oestrogen levels are high, tanycytes retract their end-feet therefore allowing GnRH terminals to reach the basal lamina of the perivascular space hence favouring the release of GnRH into the portal blood (Prevot et al., 1998) (Fig. 14). The factors involved in this process have not been all identified. Nevertheless, the same factors involved in the paracrine crosstalk between tanycytes and GnRH neurons appear to have a role in their structural remodelling, as in the case of EGF, TGFβ1, PGE2 or endothelial NO (Sharif et al.,

2013). For instance, TGFα, produced by both tanycytes and astrocytes, binds and activates erbB1/B2 heterodimers in both cell types, inducing the release of PGE2. PGE2 then stimulates the release of TGFβ1 from tanycytes, which acts as an autocrine factor to induce endfeet retraction through the activity of metalloproteinases (Prevot et al., 2003) (Fig. 14). Moreover, as previously said, NO produced from endothelial cells stimulates COX activity and therefore PGE2 production (De Seranno et al., 2010). Recent works from our laboratory have also identified semaphorin7A (sema7A) as a tanycytic factor released according to progesterone levels, being maximal on the afternoon of diestrus-2 and able to stimulate GnRH terminal retraction from the capillary space as well as tanycytes' end-feet expansion (Parkash et al., 2015).

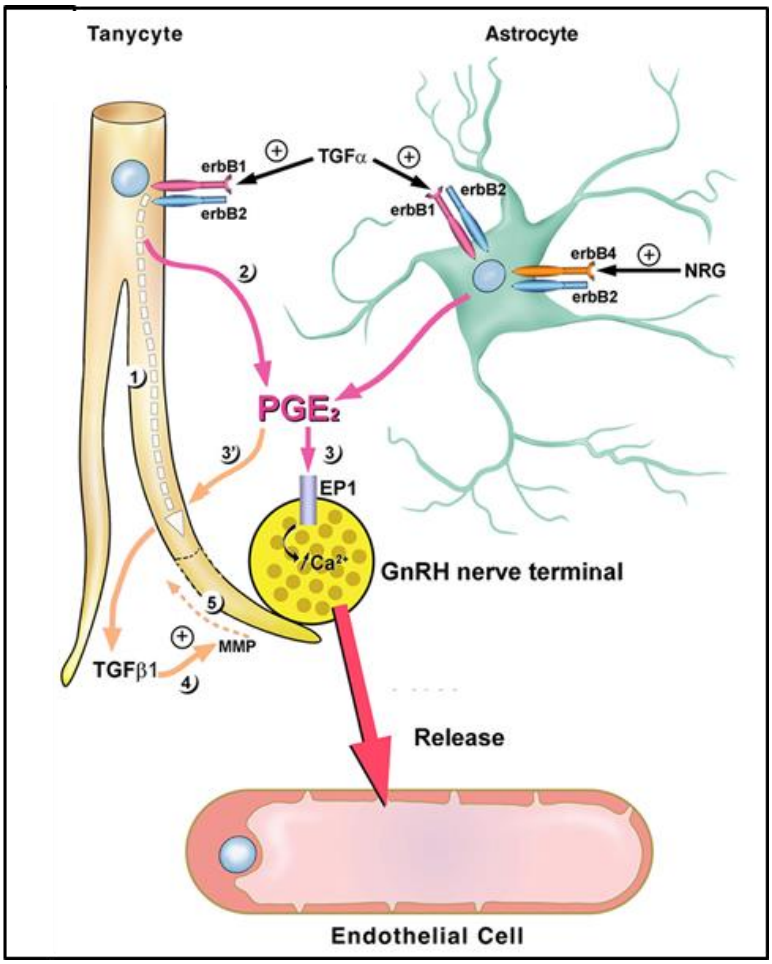


Figure 14: Schematic representation of GnRH neuron–glial cell interactions in the ME. The ligand-dependent activation of erbB1 receptors in tanycytes results in plastic changes characterized by a phase of retraction of their endfeet, which requires PGE2 synthesis, production of TGFβ1 and metalloproteinase activity. In addition, PGE2 released by tanycytes and astrocytes is able to directly stimulate GnRH release at nerve endings via binding to EP1 receptors (Modified by Clasadonte J. 2011, Frontiers in Endocrinology).

Together with these main modes of action, it is important to mention that hypothalamic astrocytes are not only able to sense gonadal hormones, as they express androgen, oestrogen and progesterone receptors (Melcangi et al., 2001), but they are also able to produce neurosteroid hormones, which can act in a paracrine way to modulate different functions such as anxiety, cognition, sleep and aggression (Garcia-Segura & McCarthy, 2004). An interesting example is the ability of astrocytes to respond to oestrogen signalling by producing neuroprogesterone, which is involved in the pre-ovulatory LH surge, as demonstrated by the inability of oestrogens to induce an LH surge in ovariectomised rats treated with a blocker of progesterone synthesis (Micevych et al., 2003; Micevych et al., 2007; Terasawa & Kenealy, 2012). Intriguingly, astrocytes from neonatal rats, even though able to sense oestrogen levels, do not respond by releasing progesterone, in agreement with hypothalamic non responsiveness to oestradiol-positive feedback in neonatal animals (Micevych et al., 2007).

2.3.5.4 Human findings

Due to the difficulties in performing human studies, it has not been easy to prove the role of glial cells in the regulation of GnRH function in our species. Nonetheless, a tight interaction between GnRH neurons, astrocytes and tanycytes has been described both at the level of their cell body and terminals (Baroncini et al., 2007), as found in other species (Fig. 15). Moreover, magnetic resonance imaging (MRI) studies have been performed in young women to study differences in the hypothalamus throughout the oestrous cycle. Diffusion MRI experiments showed that when the early follicular phase LH pulsatile release occurs, hence the hypothalamus is more active, a higher rate of water molecule diffusion is observed compared to the pill-supplemented period, when the hypothalamus is fully inhibited, or compared to males. Moreover, these changes were not observed in the thalamus, a non-neuroendocrine region (Baroncini et al., 2010). Modifications of water diffusion can be linked to shifting of water from the intracellular to the extracellular space, changes in membrane permeability or a decrease in the tortuosity of the extracellular space. Interestingly, changes in the astrocytic coverage of neurons modify the extracellular space geometry and diffusion parameters during physiological activation (Piet et al., 2004). Hence suggesting that the glial coverage of hypothalamic neurons changes according to the activity of the hypothalamus.

Proton MR spectroscopy experiments, which measure freely mobile molecules, performed in the same groups, showed reduced choline levels in the hypothalamus, but not the thalamus, during the pill-free period when compared to the period of oral contraceptive use or to males. Choline is especially concentrated in astrocytes and alterations in choline levels have been linked to disturbances in the formation and degradation of cell membranes (Manganas et al., 2007).

Altogether these data lead to the speculation that activation of the gonadal axis in women is associated to microstructural changes that cause a reduction in the astroglial coverage of GnRH neurons (Baroncini et al., 2010).

Finally, insights on the glial control of GnRH neurons in humans come from studies performed on hypothalamic hamartomas (HH), which are rare congenital tumour-like malformations sometimes associated with precocious puberty. It was therefore hypothesised that this effect was due to the ectopic expression of GnRH in the HHs. Nevertheless, some HHs associated with precocious puberty were shown not to express GnRH, while they were enriched in TGFα- and erbB1 receptor-expressing astrocytes (Jung et al., 1999). These findings suggest that the glial erbB signalling system may also exist in humans and stimulate the GnRH neurons in a similar way to what has been observed in rodents (Sharif et al. 2013).

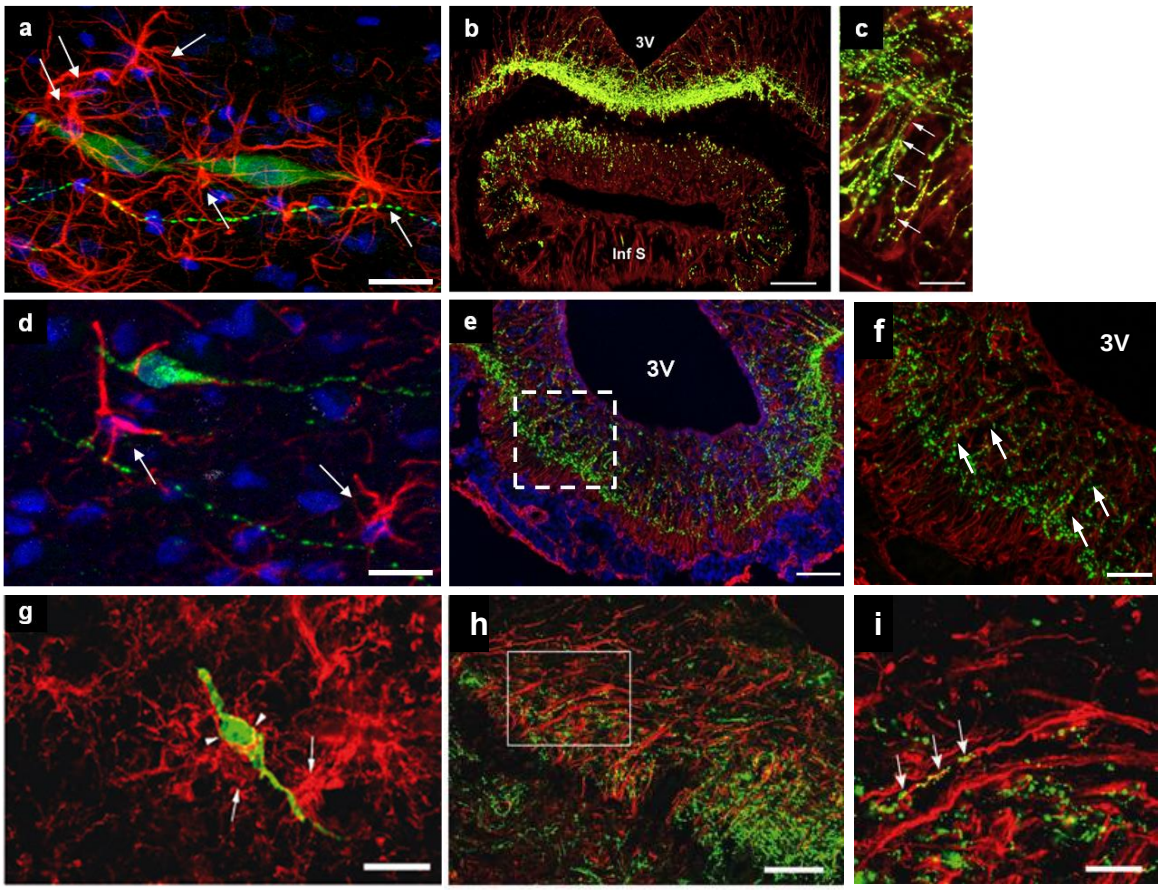


Figure 15: Close relationship between GnRH neurons and glial cells in mammals. (a, d, g) Microphotographs representing GFAP-immunoreactive astrocytes (red, arrows) enwrapping the cell bodies and processes of GnRH neurons (green) in the mouse **(a)**, microcebus murinus (a non-human primate) **(d)**, and human **(g)** hypothalamus. **(b, e, h)** Microphotographs the median eminence showing interaction between dense GnRH-terminals (green) and vimentin-immunoreactive **(b,e)** or nestin-immunoreactive **(h)** tanycytic processes in the mouse **(b)**, microcebus murinus **(e)** and human **(h)**. c, f and i show higher magnification views of b, e and h respectively. Arrows point to GnRH terminals (green) closely apposed to tanycytic processes (red). 3V, third ventricle; Inf S, infundibular stem. Scale bars: 120 µm in h; 100 µm in b and e, 50 µm in c anf f; 30 µm in i; 20 µm in a, d and g. (a panel from Ojeda et al. 1999; b-c panels from Prevot 2010, Frontiers in Neuroendocrinology; g-I panels from Baroncini 2007, Journal of Neuroendocrinology; d-f panels, personal observation).

2.4 EMBRYONIC DEVELOPMENT OF THE GNRH SYSTEM

The correct functioning of the HPG axis is tightly dependent on the proper development and establishment of GnRH circuits and correct signalling pattern.

The story of GnRH neurons begins during embryogenesis, more precisely around embryonic day 10.5 (E10.5) in mice and rats (S. Wray, 2010) and gestational week 6.5 (GW 6.5) in human (Casoni et al., 2016). Unlike the other parvocellular neuroendocrine neurons, which derive from the proliferative neuroepithelium of the third ventricle (Forni et al., 2011;

Markakis, 2002), the GnRH neurons arise from the nasal placode with a single peak of proliferation. However, the identity of their progenitors still remains to be elucidated (Markakis, 2002; Wray, 2010) as their molecular profile within the placode is not homogeneous as previously thought (Forni et al., 2011). In fact, together with a placode origin a putative neural crest origin has also been proposed to contribute to the GnRH neuron cell lineage (Forni et al., 2011).

Being born outside from the brain, these neurons migrate through the nasal mesenchyme, cribriform plate and the OB to finally reach the basal forebrain. This journey ends around E18.5 in mice and rats (Cariboni et al., 2011; S. Wray, 2010) and GW20 in humans (Kim et al., 1999) when all the cells have left the nasal compartment and have reached their final destination.

The GnRH neurons migrate along a migratory route formed by the terminal and vomeronasal nerves emerging from the vomeronasal epithelium before migration begins (Casoni et al., 2016; Wray et al., 1989). Interaction with these axons appears to be fundamental for facilitating correct migration, as proved by the observation that disruption of axonal growth and/or of direct interactions between the fibers and migrating GnRH neurons results into migration defects and consequent deficits in reproduction (Cariboni et al., 2007; Wray, 2010). Nevertheless, vomeronasal sensory axons and terminal nerve axons are not the only components of these migratory scaffold as non GnRH neuronal cells, glial cells and blood vessels have also been shown to bundle together in a structure whose exact nature remains poorly understood (Forni & Wray, 2012). One of the components of this path are the olfactory ensheathing cells (OECs), which are glial cells that normally ensheath the olfactory, vomeronasal and terminal nerves and that are involved in their growth and guidance. Accordingly, mice lacking OECs as a result of a mutation in the Sox10 gene, which is involved in the development of the neural crests, from which OECs derive, present misrouted and defasciculated vomeronasal and terminal nerves and show an abnormal migration of GnRH neurons since these cells fail to enter the brain at E14.5. However, due to the mortality of the mutants, data following this age were impossible to obtain (Pingault et al., 2013).

The transition between the nasal mesenchyme and the developing CNS tissue represents a dramatic change in the environment surrounding the migrating GnRH neurons. This transition occurs at the level of the nasal-forebrain junction where the cells appear to pause

before entering the brain. The reasons for this delay are not clear but it is hypothesized that it could allow maturation of GnRH neurons or axons before they reach the final appropriate brain regions (Wray, 2010).

This quite peculiar and long migratory journey traversing different and still developing tissues means that GnRH neurons face a spatio-temporally changing combination of molecules that act as soluble or membrane-bound guidance cues as well as repellent or attractant molecules forming chemoattractive gradients. Several classes of factors have been studied in relation to this process and different mutations have been associated with disrupted migration and therefore reproductive capacity (Wray, 2010). These factors include, but are not limited to: guidance molecules such as slits and semaphorins, growth factors, cytokines, chemokines, a variety of neurotransmitters and neuromodulators such as GABA, glutamate and CCK, and even transcription factors. It is important to point out that among the plethora of factors that have been shown to have an impact on the migration of GnRH neurons, a notable absent in rodents is GnRH itself (Wray, 2010) as demonstrated by studies on two hypogonadal mouse strains, the Gnrh mutant (hpg) mice and the Gnrh receptor mutant mice, that have a normal GnRH neuron population in terms of size, distribution and projection to the ME (Gill et al., 2008).

Once their final destination is reached, GnRH neurons detach from their guiding fibers and spread to subsequently extend their terminals to the ME. In adults, GnRH neurons are found in different regions along their main migratory pathway from the OB to the mediobasal hypothalamus. However, this is not the only path they undertake. The existence of a dorsal migratory route has been shown both in mice and humans, from where GnRH neurons reach extrahypothalamic areas such as the hippocampus, piriform cortex and amygdala. The function of these neurons has not been clarified yet (Casoni et al., 2016; Schwanzel et al., 1987).

Regardless of their location, most GnRH neurons project their terminals to the ME where they are found in rodents from E15-E16 (Eurenius & Jarskar, 1979). However, the first release of the neurohormone coincides with the last two or three days of embryonic development (Aubert et al., 1985). This precocious release of GnRH seems to be important to prime the HPG axis and more specifically to favour the formation of cell networks in the

anterior pituitary, being this the moment when the gonadotroph cells begin to organise (Le Tissier et al., 2012; Prevot, 2015).

2.5 POSTNATAL DEVELOPMENT OF THE GNRH SYSTEM

The capacity for sexual reproduction is not a characteristic of newborn mammals even though at birth, both GnRH cell bodies and fibers are in place. This means that at this point the system still needs to undergo a postnatal period of maturation in order to achieve sexual competence corresponding to initiation of puberty.

The onset of puberty implies a switch from a low prepubertal GnRH release pattern to an adult phasic and synchronous activation, characterized by GnRH pulses. Puberty is the result of complex interactions between different regions of the hypothalamus as well as genetic and environmental inputs. Considering that GnRH neurons are able to release GnRH way before the onset of puberty, it is clear that the gatekeepers of the pubertal process' initiation are transsynaptic and glial inputs able to modulate GnRH neurons' activity. Importantly, initiation of the processes that will lead to puberty does not need an influence from the gonads but depends upon events that occur within the brain. However, puberty itself results from the action of gonadal hormones on GnRH neurons (Ojeda et al., 1980; Prevot, 2015).

When trying to understand a process as long and complex as the establishment of puberty onset (which takes weeks for rodents and years for primates), it is important to take into account not only the process of maturation of the GnRH neurons themselves but also the concomitant development of the surrounding CNS as well as that of the gonads. The postnatal period is in fact a critical moment for the shaping of neural circuits and it is at this moment that the GnRH neural network is established with excitatory and inhibitory transsynaptic inputs, converging from different nuclei of the hypothalamus, as well as nonsynaptic inputs.

Postnatal development in female mice and rats can be divided in four periods: neonatal, infantile, juvenile and peripubertal (Prevot, 2015).

The **neonatal period** goes from P0 to P7. During the first week of life, the ovarian development occurs independently from the hypothalamic-pituitary axis and by day 7,

developed secondary follicles with functional FSH and LH receptors are found in the ovaries (McGee & Hsueh, 2000) (Fig. 16).

The **infantile period** goes from P8 to P21, when pups are weaned from their mothers. The most striking event during this period is a peak in the FSH levels seen at P12 in both mice and rats, which corresponds to the first centrally-driven activation of the HPG axis and may correspond to minipuberty, a phase in the human pubertal maturation when an activation of the HPG axis occurs in the early postnatal life to return quiescent again until time of puberty; in girls, FSH levels increase during the first 3 months and remain high even until 3-4 years of age (Kuiri-Hänninen et al., 2014). This event is gonadal independent but it is critical for the development of the ovarian follicles being FSH a survival factor for early antral follicles that would otherwise undergo apoptosis (McGee & Hsueh, 2000). On the contrary, LH levels remain very low during this period, probably due to the fact that GnRH pulsatile release is at very low frequencies (Kaiser et al., 1997) (Fig. 16).

The **juvenile period** lasts until P30 in rats and between P25 and P40 in mice. During the juvenile period, both levels of FSH and LH remain very low, while GnRH pulse frequency increases. Meanwhile, oestrogens begin to exert their negative feedback on the GnRH neurons through ARC kisspeptin neuron inputs; however, oestrogen levels are still too low to achieve a positive feedback effect (White & Ojeda, 1981) (Fig. 16).

The **peripubertal period** culminates with the first ovulation, which occurs around P34-P38 in rats and 6-12 months after the first menstruation in humans. Approaching puberty, GnRH pulses keep increasing in frequency and LH levels acquire a nocturnal pattern with afternoon pulses being higher in amplitude but not in frequency compared to morning pulses (Urbanski & Ojeda, 1985). While the events described so far are gonad-independent, puberty relies on the ability of rising oestrogen levels to trigger a GnRH/LH/FSH surge which will result in the first ovulation (Ojeda et al., 1976) (Fig. 16).

2.5.1 Establishment of the GnRH neural network

These first weeks of postnatal life are important for maturation of hypothalamic axonal projections and synapses. GnRH neurons gradually receive synaptic inputs during development seen as an increase in synaptophysin punctae found in close apposition with GnRH neurons' proximal dendrites (Xue et al., 2014). These axonal inputs arrive from

different hypothalamic nuclei such as the ARC, VMH and DMH (Bouret, 2004), as well as the AVPV (Polston & Simerly, 2006) (Fig. 16).

2.5.1.1 Inhibitory inputs

The progressive changes in the secretion of GnRH seen throughout postnatal development are believed to be due to a gradual decrease in inhibitory and increase in excitatory inputs to the GnRH neurons.

An initial "restraint" is necessary to avoid precocious activation of GnRH neurons and precocious puberty. The main neurotransmitters involved are GABA and opioid peptides (Ojeda et al., 2003).

Even though the general effect of **GABA** on GnRH neuron activity is inhibitory both before and after puberty, it is important to point that its role on the progression of puberty onset does not imply only a direct action on GnRH neurons themselves. While, intuitively, blockade of $GABA_A$ receptors advances puberty (Donoso et al., 1990), the direct activation of these receptors in GnRH neurons results in an excitatory effect due to the high intracellular concentration of chloride present in these cells before puberty (Gore, 2001). Therefore, its role in preventing precocious puberty must be exerted indirectly by inhibiting different GnRH excitatory systems. On the other hand, a direct activation of $GABA_B$ receptors induces inhibition of GnRH neuron activity (Lagrange et al., 1995) maybe contrasting the excitatory tone mediated by $GABA_A$ receptors. It appears that the GABAergic innervation of GnRH neurons does not change throughout postnatal development (Cottrell et al., 2006). However, the effects of GABAergic signalling do not remain constant across development: GnRH neurons' intracellular content of chloride decreases while the expression profile of the different $GABA_A$ receptor subunits, known to affect sensitivity to GABA itself, is subject to variation (Temple & Wray, 2005).

The role of **endogenous opioids** in this picture has not been fully examined yet; however, it has been shown that opioid peptides can inhibit gonadotropin secretion during postnatal development and this effect decreases around the time of puberty (Bezzi et al., 1998; Nakahara et al., 2013; Parpura et al., 1994).

2.5.1.2 Excitatory inputs

While inhibitory signals are necessary to prevent an early puberty, increasing excitatory signals are required to induce an adult pulsatile GnRH secretion pattern (Ojeda et al., 2003).

The main excitatory inputs come from **glutamatergic neurons** that stimulate GnRH neurons and other components of the GnRH neuronal network by activating both NMDA and AMPA receptors, two classes of ionotropic glutamate receptors (Eyigor & Jennes, 1997). During postnatal development, the hypothalamic glutamate content rises while glutamatergic projections to the POA increase (Goroll et al., 1994; Prevot, 2014). It is important to point that hypothalamic glutamate does not only derive from neurons but also from astrocytes, which have been shown to be able to release glutamate in response to calcium waves (Charles et al., 1991; Haydon, 2001).

Hypothalamic glutamatergic projections do not only reach GnRH neurons but also the neighbouring GABAergic neurons. It was shown that, during postnatal development, glutamate acts on GABAergic synapses both at a postsynaptic level, by inhibiting the calcium increase induced by GABA-mediated depolarization, and at a presynaptic level by reducing GABA release (van den Pol et al., 1998). Therefore, it has been hypothesised that during the postnatal development an increase in glutamatergic inputs precedes and causes the decline of the inhibitory GABAergic tone, leading to the final activation of GnRH neurons (Ojeda et al., 2003).

A further point in favour of the gradual increase in glutamatergic inputs is the evidence that during postnatal development, GnRH neurons undergo a remodelling of their dendritic tree structure and spine density (Cottrell et al., 2006; Xue et al., 2014). Dendritic spines can be used as an index for excitatory inputs and their number dramatically increases between the pre- and the post-pubertal period in male mice, corroborating the idea that GnRH neurons receive increasing excitatory inputs (Cottrell et al., 2006). A peculiarity of these neurons is also the fact that many spines are found at the level of their cell body and not only on their dendrites.

2.5.1.3 Neuropeptidergic inputs

AVPV neurons extend and develop their projections from embryonic stage until well after birth. Projections contacting the GnRH neurons have been found as early as E19, while

caudal projections to the PVH develop during the first postnatal week (Polston & Simerly, 2006). Projections from the ARC are immature at birth and only develop during the second week of life in mice (Bouret, 2004). A mature innervation pattern is reached around P12 with fibers reaching the DMH, VMH, lateral hypothalamus (LHA) and PVN (Bouret, 2004).

Kisspeptin neurons are found in both the AVPV and the ARC and, as already mentioned, are responsible for mediating oestrogen's feedbacks to the GnRH neurons. It has been shown that in mice lacking Kiss1r, puberty is totally absent (Seminara et al., 2004) while mice with an activating mutation of Kiss1r show an early puberty onset (Teles et al., 2008). The sensitivity of GnRH neurons to kisspeptin increases during postnatal development with only 25% of them being responsive in juveniles, 50% in prepubertal and up to 90% in adult mice (Han, 2005). However, these changes do not depend on differences in Kiss1r expression in GnRH neurons (Han, 2005). At the same time, during late infantile period, kisspeptin fibers from the ARC reach the POA and mediate oestrogen's negative feedback, avoiding a premature activation of GnRH neurons (Caron et al., 2012; Mayer et al., 2010). Kisspeptin neurons in the AVPV on the other hand are critical for ovulation since they mediate oestrogen's positive feedback; therefore when maturation is completed, their signalling results in the GnRH and therefore LH surge necessary for the release of the oocyte from the follicle (Kirilov et al., 2013).

In the ARC, anorexigenic **POMC** and orexigenic **NPY/AgRP** neurons inhibit or favour the onset of puberty depending on the energy status of the animal. POMC and NPY/AgRP neurons are able to, respectively, inhibit or stimulate GnRH release both directly and indirectly, probably in concert with kisspeptin neurons (Manfredi-Lozano et al., 2017; Nestor et al., 2014). For instance leptin levels, which can be sensed by both these neuronal populations as a signal of energy adequacy, have a permissive role over puberty with leptin deficiency resulting in delayed or absent puberty (Vázquez et al., 2015). Moreover, blocking α-MSH signalling delays puberty in female rats and abolishes the permissive effect of leptin (Manfredi-Lozano et al., 2016) while deletion of NPY signalling in leptin-deficient animals restores the onset of puberty (Sainsbury et al., 2002).

2.5.1.4 nNOS neurons

Unlike other neuronal populations, nNOS neurons do not send projections to GnRH neurons, as NO is a gas that freely diffuses through biological membranes. Nonetheless, unpublished data from our laboratory show that activation of nNOS neurons in the OVLT occurs concomitantly with the peak of FSH observed at P12 in female mice. Moreover, inhibition of nNOS signalling specifically during the infantile period delays puberty onset and perturbs fertility in adult animals (Konstantina Chachlaki and Vincent Prevot, unpublished data). As previously mentioned, nNOS neurons are also able to convey metabolic signals to GnRH neurons. A study performed in leptin-deficient obese mice showed that nNOS inhibition prevented exogenous leptin administration to induce normal sexual maturation even though a normal body weight was rescued (Bellefontaine et al., 2014).

2.5.2 Glial cells

Alongside the events involved in the establishment of the neuronal connectivity, postnatal development is characterised by the birth and maturation of most glial cells. In addition to their role in regulating GnRH neurons during adulthood, glial cells are involved in the maturation of the GnRH system.

As seen in a previous chapter (see chapter 3.3.5), glial cells use different strategies to modulate GnRH neuron activity during adulthood. The main factors involved are TGFβ1, EGF, TGFα, NRGs growth factors and the erbB tyrosine kinase receptors (Sharif et al., 2013). The expression of TGFα and erbB receptors increases in concomitance with puberty onset (Ma et al., 1994). Moreover, animals with disrupted erbB signalling, as a result of a mutation in erbB1 receptor or the expression of a dominant-negative form of erbB4 receptor specifically in astrocytes, have delayed puberty, and this delay is exacerbated when both signalling are disrupted (Prevot et al., 2003). On the contrary, transplantation of TGF-overexpressing cells in the vicinity of GnRH neuron cell bodies and terminals induces precocious puberty (Rage et al., 1997).

Adhesion molecules, which are involved in the crosstalk between glial cells and GnRH neurons (see chapter 3.3.5.2), also participate in the maturation of the system. In particular, SynCAM1, whose levels in the hypothalamus increase during sexual maturation, is required

for sexual maturation since transgenic mice with an astrocyte-specific disruption of SynCAM1 function show delayed puberty (Sandau et al., 2011).

These are only some examples of the molecules involved in the tight interactions between GnRH neurons and glial cells. However, many others still need to be characterised for their possible contribution to the initiation of puberty.

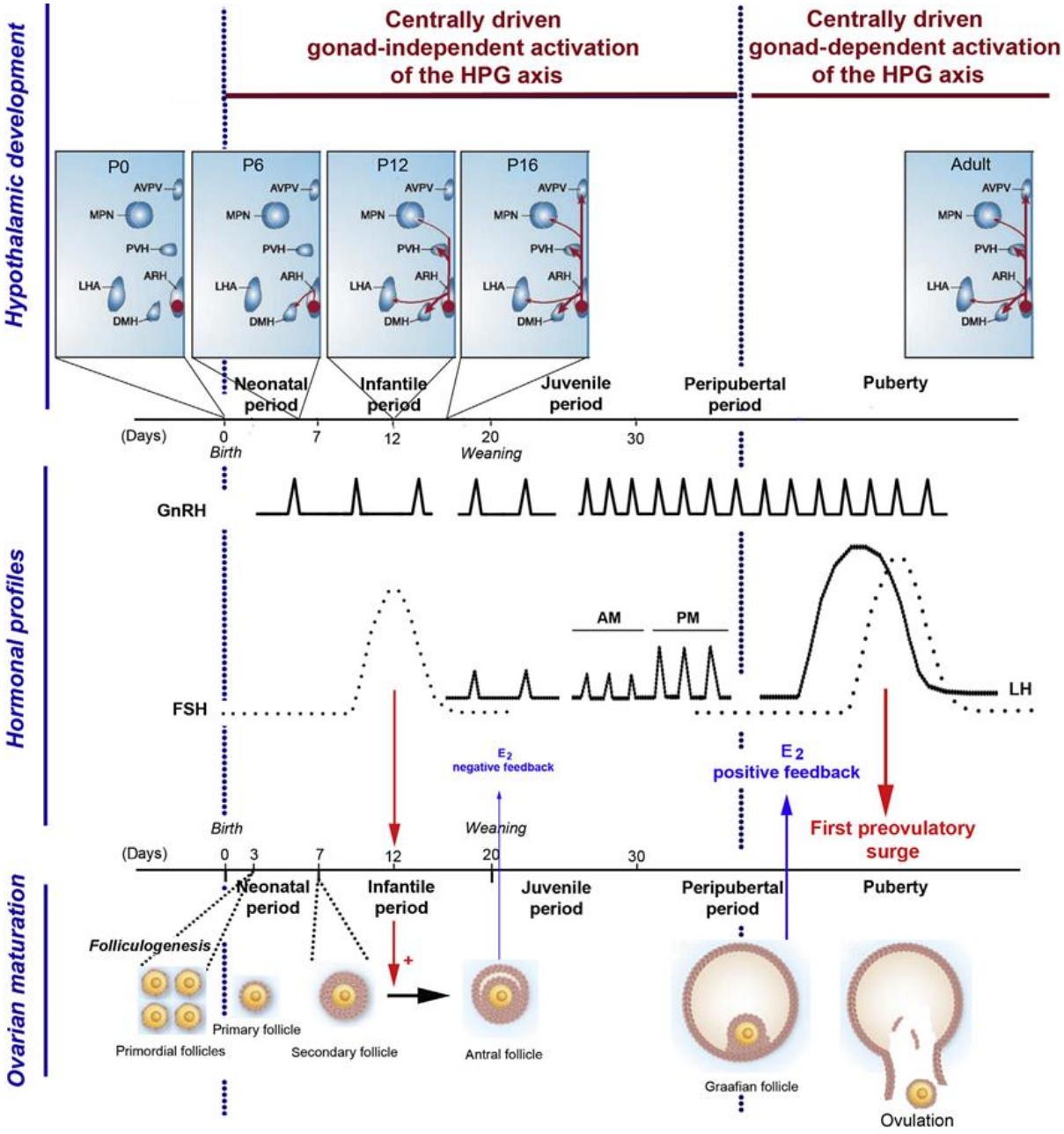


Figure 16: Centrally driven activation of the female HPG axis during postnatal development. Ovarian maturation: Scheme of follicules and consequent E2 secretion during postnatal development. **Hormonal profiles:** Schematic diagrams illustrating changes in GnRH, FSH and LH profiles during female postnatal development. **Hypothalamic development:** Gradual establishment

of hypothalamic neuronal inputs to GnRH neurons. AVPV: anteroventral periventricular nucleus; ARH: arcuate nucleus of the hypothalamus; DMH: dorsomedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; MEPO: median preoptic nucleus; MPN: medial preoptic nucleus; PVH: paraventricular nucleus (Modified from Prevot V. 2014, Physiology of reproduction 4th edition).

2.6 PREGNANCY AND BEYOND

2.6.1 Pregnancy

The ultimate purpose of the finely regulated process of ovulation is to ensure fertilisation of the ovum following insemination. As previously said, if fertilisation does not occur the corpus luteum formed from residual follicular granulosal and thecal cells degenerates and a new cycle begins.

However, if fertilisation does occur the corpus luteum keeps producing progesterone, which is essential for maintenance of normal pregnancy in mammals, until the placenta begins to take over (Pate et al., 2012). Circulating levels of progesterone are maintained very high throughout almost the entire course of pregnancy until they drop in concomitance with parturition. The corpus luteum, however, does not only produce progesterone but is also important for the production of oestrogens, either by a direct release or by aromatisation of placenta-derived androgens. Oestrogen levels increase gradually during pregnancy until reaching a peak at parturition that drops soon afterwards until oestrus cycle is resumed. At the beginning of pregnancy, prolactin is synthesised by the anterior pituitary in two daily surges, one diurnal and one nocturnal (Butcher et al., 1972); these two surges are gonadal independent as they are initiated even in the absence of gonadal hormones but the latter seem to be required for termination of the surges. The two daily surges of prolactin are responsible for the increased life span of the corpus luteum and its secretion of progesterone early in pregnancy and are thus critical for the maintenance of pregnancy (Smith et al., 1975). Maintenance of the corpus luteum is achieved through two actions of prolactin: increased expression of the LH receptor, leading to increased progesterone synthesis, and repression of the enzymes responsible for progesterone metabolism in the ovary. In the second half of pregnancy, pituitary secretion of prolactin is inhibited by the high release of placental lactogens, which stimulate the activity of the dopaminergic hypothalamic neurons that in turn inhibit the release of prolactin from the anterior pituitary

(Tonkowicz & Voogt, 1983; Grattan & Tissier, 2015). During pregnancy, prolactin also prepares the mammary glands in view of lactation.

2.6.2 Lactation

After parturition, the process of suckling mediates important features, necessary for successful nourishment of the offspring themselves: it increases prolactin secretion, which controls milk production; it inhibits GnRH and consequently LH pulsatility; it increases orexigenic and decreases anorexigenic neuronal activity in order to increase maternal food intake (Grattan, 2015).

Prolactin is released in response to a neuroendocrine reflex set in motion by suckling of the pups (Selmanoff & Gregerson, 1985). This secretion, unlike the surge release, is tightly linked to the stimulus itself as introduction of pups immediately increases prolactin levels and each episode of secretion requires reapplication of the stimulus. During lactation, in many species, there is a suppression of ovarian function meant to avoid overlapping of this process with a new pregnancy due to the high level of energy expenditure that accompanies both these events. However, while some species undergo a period of anovulation (e.g. ewes), others still ovulate but they either have a much longer gestation than lactation so that the end of pregnancy will not overlap with lactation of the previous offspring (as in horses), or lactation can induce a late implantation (as in the case of rats) (Goodman 2015).

Arcuate NPY and kisspeptin neurons express prolactin receptors and prolactin can be at least partially responsible for NPY neuron activation (Li et al., 1999), which increases food intake, and kisspeptin neuron inhibition, which decrease GnRH neuron activity. This increase in food intake, or hyperphagia, is necessary to avoid exhaustion of maternal energy stores. During gestation, leptin levels are significantly increased due to a hyper production of leptin by the placenta and adipose tissue; therefore, to explain the increase in food intake, a physiological state of leptin resistance seems to be necessary (Grattan et al., 2007). Two mechanisms have been proposed to underlie this process: a reduction in the transport of leptin through the BBB, assessed by measuring leptin levels in the CSF of pregnant rats, and an alteration in the hypothalamic response to leptin (Trujillo et al., 2011). In particular, a decrease in leptin receptor levels in the VMH together with a decrease in the number of leptin-responsive cells

occurs during pregnancy (Ladyman & Grattan, 2005), allowing hyperphagia even in a situation of hyperleptinemia.

2.6.3 Maternal behaviour

Parental care is indispensable for survival of the offspring and is therefore finely regulated. Parenting behaviour is usually sexually dimorphic and most of mammalian species display maternal but not paternal behaviour. Maternal behaviour includes many different components such as: nest building, care of the young, recognition and bonding, nursing and defensive behaviour as well as aggression against intruders.

For maternal behaviour to be initiated, adaptation of different neural circuitries of the maternal brain needs to occur prior to parturition. The pregnancy hormones prolactin, oestrogens and progesterone are the main responsible for this process of reorganisation. The main brain circuits involved are the OB, the medial POA (MPO) and the mesolimbic dopaminergic motivation system. The OB circuitry is critical for recognition of the offspring as olfaction is the primary sensory mediator of social information in rodents (Lonstein et al., 2014). In relation to this, it has been shown that during pregnancy, prolactin stimulates the birth of new olfactory interneurons with important functional consequences for the associated maternal behaviour (Shingo, 2003).

The MPO is the key hypothalamic area for the establishment of maternal behaviour (Fisher, 1956). A number of studies have shown that functional impairment of the MPO through different approaches, such as wounds, pharmacological or electrolytic injuries, lesion of the axonal projections emanating from this area, all result in disrupted maternal behaviour (Lonstein et al., 2014). This region is able to sense ovarian and pituitary hormones, thanks to a widespread expression of different receptors whose levels are increased at the end of pregnancy, and then send the information to the motivation system (Brunton & Russell, 2008). This crosstalk is necessary for the newly mothers to become attracted by newborns, through activation of the reward dopaminergic circuitry (Carlson et al., 2006), and to suppress their fear response to pups, through active GABAergic inhibition of the periaqueductal gray (Brooks et al., 2005). Nonetheless, the role of the MPO in regulating maternal behaviour does not rely on activation of endocrine pathways but rather on its electrical activity. Brain adaptation to motherhood depends on the structural plasticity

displayed by this region. Even though most of these events, i.e. changes in the size of neuronal cell bodies or in the number of dendritic branches, appear to be temporary, the remodelling of MPO circuits is in part permanent since after the first pregnancy, female rats are more prone to act maternally in the future (Bridges, 2016; Brunton & Russell, 2008; Champagne & Curley, 2016). One example is an increase in GFAP-immunoreactive astrocytes found in the MPO after the first pregnancy, which is dependent on the direct interaction with the offspring (Keyser-Marcus et al., 2001).

Chapter 3

Aims

The hypothalamus is the centre of control of all vegetative functions in the vertebrate brain. According to signals informing on the physiological state of the organism, as well as information from the environment, this small region of the ventral forebrain adapts its response to coordinate the action of different organs via the intermediary of the pituitary gland. Changes in synaptic inputs, as well as neuron-glia interactions have been identified as important plasticity mechanisms used by the hypothalamus to exert its roles. Moreover, accumulating data suggest that the adult hypothalamus hosts a niche of neural stem cells that give birth to new neurons and glial cells involved in the control of energy metabolism. This suggests that the hypothalamus retains an even higher level of plasticity than once thought and opens many questions on whether the post-natal generation of new cells contributes to the regulation of hypothalamic functions others than the control of metabolism.

During my Ph.D., I have explored the possible contribution of hypothalamic cell neogenesis to the control of reproduction, a function governed by the hypothalamus and known to require a high degree of plasticity. I have addressed this question in two contexts: the post-natal maturation of the GnRH system and the adaptation of the maternal brain to the occurrence of a pregnancy.

Moreover, while the existence of a neural stem cell niche in the adult hypothalamus has been postulated from studies performed in different mammalian species, mostly rodents, it remained to be determined whether a niche also exists in the human hypothalamus.

The specific aims of my work were:

- to explore the interactions between GnRH neurons and cells born in their environment during the post-natal period, and the possible contribution of this dialogue to the maturation of the GnRH system.

- to map cell proliferation in hypothalamic regions involved in the control of reproduction during adulthood to explore whether such a process may be relevant for this function.

- to evaluate whether the adult human hypothalamus contains cells with molecular properties of neural stem/progenitor cells and address this question in the frame of a comparative approach with rodents and a non-human primate.

Chapter 4

Materials and Methods

4.1 ANIMALS

Animal studies were approved by the Institutional Ethics Committee 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). Tissues were obtained from female Sprague-Dawley rats (Janvier Laboratories, Saint Berthevin, France). GnRH neurons were sorted from transgenic female Wistar rats, kindly provided by Prof. A. Herbison (University of Otago, Dunedin, New Zealand), in which EGFP expression is driven by the rat GnRH promoter (Fujioka et al., 2003). All animals were maintained at 23-25°C on a 12-hour (h) light/12-h dark cycle (lights on at 07:00 a.m.) with food and water available ad libitum.

4.2 BRDU INJECTIONS

Postnatal animals received a single intraperitoneal (i.p.) injection of BrdU at a dose of 300 mg/kg body weight (Sigma) at different postnatal ages and were allowed to survive for 2 hr, 24 hr, 7 days or ≥ 60 days.

Adult animals received either a single i.p. injection of BrdU at a dose of 300 mg/kg body weight or 3 injections of 100 mg/kg body weight each at 9 am, 1 pm and 5 pm on one day of the estrus cycle and were sacrificed 2 hr later. Animals receiving 3 injections in diestrus 2 were mated or not on the following day and then sacrificed 7, 14 or 21 days later.

4.3 PUBERTY ONSET AND ESTROUS CYCLICITY

Animals were inspected daily for imperforation of the vaginal membrane (i.e. vaginal opening). Thereafter, oestrous cycle was monitored by daily inspection of vaginal smears under an inverted microscope: diestrus 1 and 2 were defined by the presence of a predominance of leukocytes in the vaginal lavage, proestrus was characterized by a predominance of nucleated rounded epithelial cells, and oestrous was distinctively characterized by large numbers of cornified squamous epithelial cells forming clusters.

4.4 TISSUE PREPARATION

Animals were anesthetized by i.p. injection of pentobarbital (70 mg/kg). They were perfused transcardially with a rinse of saline solution (0.9% NaCl), followed by 100 ml (postnatal animals) or 400 ml (adult animals) of 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4). The

brains were removed and immersed in the same fixative for 2-3 hours at 4°C. They were then transferred to PB containing 20% sucrose until they had sunk. After embedding in OCT embedding matrix (CellPath, Newtown, UK), brains were frozen in liquid nitrogen and stored at -80°C. Frontal coronal sections (14 µm thick) containing the preoptic region only (0.20 mm to -0.30 mm relative to bregma) or the full extent of the hypothalamus, were cut on a cryostat (CM3050S, Leica, Nussloch, Germany) and mounted on chrom-alum-gelatin-coated glass slides. Sections were collected in five series with 6 sections per slide and stored at -80°C until use. Anatomical landmarks were determined using the rat brain atlas of Swanson (Swanson, 2004).

4.5 IMMUNOFLUORESCENCE

4.5.1 Antibodies

Immunofluorescent stainings were performed using the following primary antibodies and working concentrations: rat monoclonal anti-BrdU (1:100, AbD Serotec, Oxford, UK), rabbit polyclonal anti-GnRH (1:3,000), mouse monoclonal anti-PCNA (1:10,000, Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-GFAP (1:1,000, DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-GFAP (clone G-A-5, 1:500; Sigma, St. Louis, MO), mouse monoclonal anti-HuC/D (1:500, Molecular Probes, Eugene, OR), goat polyclonal anti-Olig2 (1:100, RnD Systems, Minneapolis, MN), mouse monoclonal anti-O4 (1:300, RnD Systems), mouse monoclonal anti-APC (clone CC-1, 1:100, Merck Millipore, Darmstadt, Germany), mouse monoclonal anti-MAP2 (1:500, Sigma), goat polyclonal anti-Sox2 (1:500, Santa Cruz Biotechnology), rabbit polyclonal anti-ki67 (1:2,000, Abcam), mouse monoclonal anti-Nestin (clone rat-401, 1:300, Merck Millipore, Darmstadt, Germany), rabbit polyclonal anti-Blbp (1:500, Merck Millipore, Darmstadt, Germany). The secondary antibodies used were Alexa Fluor 488-, Alexa Fluor 568-, Alexa Fluor 647-, Alexa Fluor 350-conjugated anti-IgG (1:400, Molecular Probes) and fluorescein-conjugated anti-mouse IgM (1:400, Vector Laboratories, Burlingame, CA).

4.5.2 Immunohistochemistry

Sections were washed with 0.1 M phosphate-buffered saline (PB). For BrdU immunodetection, the sections were submitted to microwave pretreatment (4 minutes at 800 W followed by 2 cycles 5 minutes each at 400 W in 0.1 mol/L sodium citrate buffer, pH

6) before the immunolabeling procedure. Sections were then rinsed and incubated with antibodies diluted in PB containing 0.3% Triton X-100 and 10% normal goat or donkey serum (Sigma).

The primary antibodies were applied for 1 or 2 nights at 4°C and the secondary antibodies for 1 hour at room temperature. Cell nuclei were stained with Hoechst 33258 bis-Benzimide (1:10,000; Molecular Probes). After washing, slides were coverslipped with Mowiol mounting medium. Control sections were incubated in the absence of primary antibodies.

4.5.3 Immunocytochemistry

Coverslips were quickly rinsed in phosphate-buffered saline (PBS) and fixed in 4% PFA for 20 minutes before being processed for immunolabelling.

Nonspecific sites were blocked with PBS 0.1 M containing 0.1% Triton X-100 and 0.3% normal goat or donkey serum (Sigma) for 30 minutes at room temperature. Sections were then incubated with primary antibodies diluted in the same solution overnight at 4°C. Secondary antibodies were applied for 1 hour at room temperature. Cell nuclei were stained with Hoechst 33258 bis-Benzimide (1:10,000; Molecular Probes). After washing, coverslips were mounted on slides using Mowiol mounting medium. For O4 immunodetection, the anti-O4 antibody was applied to living cells for 2 hr at 37°C before fixation.

4.6 MICROSCOPIC ANALYSIS

4.6.1 Postnatal animals

Immunofluorescence was examined on a fluorescent microscope (Imager.Z1, Zeiss or DMRB, Leica) with appropriate filters. Images were acquired using an Axio Imager.Z1 ApoTome microscope (AxioCam MRm camera, AxioVision 4.6 software system; Zeiss). Illustrations were prepared with the help of Photoshop CS5 software (Adobe).

All analyses were made in the preoptic region, as defined in the Swanson stereotaxic brain atlas, between plate 17 (i.e. beginning of the OVLT) and plate 20 (i.e., crossing of the anterior commissure), a region where the GnRH neurons that stimulate the LH surge are located. This region extended over 840 µm anteroposteriorally, which corresponded to 60 coronal sections. Analyses were made on one section every 70 µm (i.e. one out of every 5 sections) with a mean of 8.8 ± 0.3 sections analyzed per animal. Estimation of total cell populations in the preoptic region was obtained by multiplying the number of cells per

section by the total number of sections per preoptic region, and then correcting for split nuclei. BrdU+ cells were counted at the x40 objective with the help of an optical grid; 2,654.9 ± 725.1 BrdU+ cells were counted per group (n = 3 to 4 animals per group).

4.6.1.1 Morphological association between GnRH neurons and $BrdU^+$ cells

Quantification was made under the fluorescent microscope at the x40 objective. A BrdU+ cell was considered morphologically associated with a GnRH neuron when it was located at a distance corresponding to the size of a $BrdU^+$ nucleus (i.e., 10 µm) from the GnRH neuron cell body with no interposed cell nucleus in between. To characterize the morphological association during postnatal development in rats (n = 3 to 10 animals per group), 163.6 ± 13.0 GnRH neurons were analyzed per animal. For the group of rats that received multiple injections of BrdU (n = 4), 194.8 ± 8.3 GnRH neurons were analyzed per animal. For microsphere-injected rats (n = 3 animals per group), 125.3 ± 24.1 GnRH neurons were analyzed per animal.

4.6.1.2 Phenotypic identity of $BrdU^+$ cells

To determine the phenotype of BrdU+ cells in the preoptic region, double immunofluorescent labeling of BrdU and GFAP, APC or HuC/D at the four postnatal ages were analysed on photomontages of the whole preoptic region taken at the x20 objective. 257.8 ± 25.3 $BrdU^+$ cells were examined per animal (n = 3 animals per group). Results are displayed as the percentage number of co-labeled cells over the total number of BrdU+ cells analyzed. To determine the phenotype of BrdU+ cells morphologically associated with GnRH neurons, triple immunofluorescent labelings of GnRH, BrdU and GFAP, APC or HuC/D were analyzed under the microscope at the x40 objective. 124.8 ± 12.0 GnRH neurons were analyzed and 23.2 ± 3.9 $BrdU^+$ cells have been identified at their neighbourhood per animal (n = 3 to 4 animals per group). Results are displayed as the percentage of co-labeled cells over the total number of $BrdU^+$ cells associated with GnRH neurons analyzed.

4.6.2 Adult animals

Analysis of sections and acquisition of images were done using an Axio Imager Z2 Apo-Tome microscope equipped with a motorized stage (Zeiss, Germany) and an ORCAFlash 4.0 V2 camera (Hamamatsu, Japan) driven by the Zen imaging software (Zeiss).

Specific filter cubes were used for the visualization of green (Alexa 488 fluorochrome) (Excitation wavelength: 475/40 nm, beam splitter: 500 nm, emission wavelength: 530/50 nm), red (Alexa 568 fluorochrome) (excitation wavelength: 550/25 nm, beam splitter: 570 nm, emission wavelength: 605/70 nm), far red (Alexa 647 fluorochrome) (excitation wavelength: 640/30 nm, beam splitter: 660 nm, emission wavelength: 690/50 nm) and blue fluorescence (Hoechst) (excitation wavelength: 365 nm, beam splitter: 395 nm, emission wavelength: 445/50 nm). High magnification photomicrographs represent maximal intensity projections derived from 9-14 triple or quadruple-ApoTome images collected using the z-stack module of the Zen imaging software and a Zeiss 20x (numerical aperture NA = 0.8). All images were captured in a stepwise fashion over a defined z-focus range corresponding to all visible fluorescence within the section and consistent with the optimum step size for the corresponding objective and the wavelength (500 nm). To create photomontages, ApoTome images were captured using the z-stack module coupled to the MosaiX module of the Zen imaging software and a Zeiss 20x objective for each filter sequentially. Images to be used for figures were pseudocolored, adjusted for brightness and contrast and merged using Photoshop (Adobe Systems, San Jose, CA).

4.6.2.1 Analysis at the level of the POA

Analysis was performed on 10 sections per POA (n = 4 to 5 animals per group), spanning between plate 16 and plate 21 of the Swanson stereotaxic brain atlas, covering a region of 700 µm anteroposteriorally. Analyses were made using a 20x objective, on one section every 70 µm (i.e. one out of every 5 sections). The different nuclei of the POA were delimited by Hoechst nuclear staining on the Zen imaging software. Estimation of total cell populations was obtained by multiplying the number of cells per section by the total number of sections per preoptic region, and then correcting for split nuclei. Morphological association with different neuronal populations was evaluated as described above.

4.6.2.2 Analysis at the level of the ME

Analysis was performed from the beginning to the end of GnRH terminal signal at the level of the ME, between plate 26 and plate 31 of the Swanson stereotaxic brain atlas. Between 11 and 27 sections were counted per animal (n = 3 to 4 animals per group). Analyses were made using a 20x objective, on one section every 70 µm (i.e. one out of every 5 sections).

The number of BrdU+ cells within the ME was evaluated together with the interaction of these cells with the GnRH fibers as described above.

4.6.2.3 Analysis at the level of the DG

In the DG of the hippocampus, 27 to 40 sections were counted per animal in a one-in-five series of sections (14 µm thickness) using a 20x objective, throughout the rostrocaudal extent of the granule cell layer. The number of BrdU-positive cells was calculated as described above.

4.7 STATISTICAL ANALYSIS

The data are presented as mean ± s.e.m. Statistical differences were evaluated using Student t-test for comparisons of two groups or one-way analysis of variance (ANOVA) with Tukey post-hoc test for comparisons of more than two groups. When the criterion for normality or equal variances was not met, the Mann-Whitney Rank Sum Test or the Kruskal-Wallis One Way ANOVA on Ranks Test was used to compare two or more groups, respectively. $P < 0.05$ was considered significant. All statistics were performed using SigmaStat software.

4.8 HYPOTHALAMIC PROGENITOR CULTURES

The preoptic region of rats aged 1 or 2 days was dissected and minced on 20 µm nylon mesh (Buisine, Clermont de l'Oise, France). The dissection excluded the subventricular zone of the lateral ventricle. The cell suspension was transferred to a culture flask (BD Biosciences, San Jose, CA) coated with Poly-D-lysine (10 µg/ml, Sigma) and laminin (10 µg/ml, Sigma) and cultured in "neurosphere medium" composed of Dulbecco's Modified Eagle's Medium (DMEM)/F-12 with L-glutamine, 15 mM HEPES supplemented with 2% (v/v) B27, 20 ng/ml EGF (human recombinant), 20 ng/ml FGF2 (human recombinant), 2 mM L-glutamine, and 100 units of penicillin and 100 µg of streptomycin/ml (all from Gibco, Carlsbad, CA) under a humid atmosphere of 5% CO2-95% air at 37°C. Cell culture medium was changed every 3 to 4 days. Cells were passaged once before performing the experiments, when 70-80% confluence was reached. Cells were used for migration or proliferation assays (see below) or were snap frozen in dry ice for gene expression analysis (see chapter 4.13). The phenotype of the cells was characterized by immunocytochemistry, as described above.

4.9 *IN VITRO* MIGRATION ASSAY

Boyden microchemotaxis chambers were used according to manufacturer's instructions (Neuro Probe, Gaithersburg MD, USA). In brief, the cells grown in complete neurosphere medium were harvested and the suspension (500 cells/µl in control medium: neurobasal medium (Gibco, Carlsbad, CA) with L-glutamine, 15 mM HEPES supplemented with 2% (v/v) B27, 2 mM L-glutamine, and 100 units of penicillin and 100 µg of streptomycin/ml) plated on the open-bottom wells of the upper compartment, previously coated with poly-D-lysine and laminin. The two compartments of each well were separated by a polyvinylpyrrolidone-free polycarbonate porous membrane (8 µm pore size). The lower chamber was filled with control medium containing one of the following treatment: GnRH 100 ng/ml (Genecust), prostaglandin D2 (PGD2) 1 µM (Sigma), galanin 2 µM (Tocris), glutamate 10 µM (Sigma), GABA 2 µM (Sigma), EGF 100 ng/ml (Gibco) (positive control). Four different experiments were run, with 3 to 6 wells per condition in each of them.

For the experiments with BWA868C (Cayman Chemical), this compound was added to the cell suspension in the upper compartment to block DP1 receptor, at either 10 nM or 1 µM in the absence or presence of PGD2 in the lower chamber. Three to six wells per condition were analysed in three different experiments.

After 18 hours of incubation, cells attached to the upper side of the filter were mechanically removed with a cotton swab. Cells that had migrated to the lower side were fixed with 4% PFA (30 minutes at room temperature) and stained with Hoechst (1:10,000). The stained cells were photographed at 20X magnification and counted on ImageJ.

4.10 *IN VITRO* PROLIFERATION ASSAY

For proliferation experiments, cells were transferred to 24-well plates on poly-D-lysine/laminin-coated glass coverslips and grown in DMEM/F-12 with L-glutamine, 15 mM HEPES supplemented with 2% (v/v) B27, 20 ng/ml EGF (human recombinant), 20 ng/ml FGF2 (human recombinant), 2 mM L-glutamine, and 100 units of penicillin and 100 µg of streptomycin/ml. Once cells were 70-80% confluent, they were starved in growth medium devoid of FGF2 (starvation medium) for 24 hours prior to the treatment with GnRH, PGD2, Galanin, glutamate or GABA (same concentration as in chapter 4.10) in starvation medium for 24 hours. FGF2 was added to starvation medium at a concentration of 100 ng/ml as a positive control. Cells were then labelled for Ki67, as described above, to mark cycling cells.

Six coverslips per condition were analyzed in 3 different experiments. Results are presented as the percentage of $Ki67^+$ cells over the total number of Hoechst-stained nuclei; 663 ± 36.7 nuclei were counted in each coverslip.

4.11 IMMUNOBLOTTING

Three different hypothalamic progenitor cultures were plated in 12-well-plates coated with poly-D-lysine and laminin. Cells were starved for 24 hours in DMEM/F-12 with L-glutamine, 15 mM HEPES supplemented with 2 mM L-glutamine, 100 units of penicillin and 100 µg of streptomycin/ml, 5 µg/ml insulin (Sigma) and 16 µg/ml putrescine (Sigma). On the day after, new starvation medium was added to all wells and the cells were treated 1 hour later for 7 minutes at room temperature with PGD2 1 µM and/or BWA868C (10 nM or 1 µM). Cells were then snap frozen in dry ice and stored at -80°C until use.

Protein extracts were prepared in 60 µl of lysis buffer per well (pH 7.4, 25 mM Tris, 50 mM b-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mg/ml leupeptin and pepstatin, 10 mg/ml aprotinin, 100 mg/ml phenylmethyl sulfonyl fluoride and 1% Triton X-100) by trituration through a 26-G needle. The cell lysates were cleared by centrifugation at 12,000 g for 15 min and protein content was determined using the Bradford method (BioRad, Hercules, CA). The samples were stored at –80°C until use. After thawing, 4X sample buffer (Invitrogen) and 10X reducing agent (Invitrogen) were added and the samples were boiled for 5 min before electrophoresis at 150 V for 60 min in precast 3%–8% SDS–polyacrylamide Tris-acetate gels according to the protocol supplied with the NuPAGE system (Invitrogen, Carlsbald, CA). After size-fractionation, the proteins were transferred onto Nitrocellulose membranes (0.2 mm pore-size membranes; Invitrogen) in the blot module of the NuPAGE system (Invitrogen) for 90 min at room temperature. Membranes were blocked for 1 h in TBS containing 0.05% Tween 20 (TBST) and 5% non-fat milk at room temperature, incubated overnight at 4°C with primary antibodies and washed four times with TBST before being exposed to horseradish peroxidase-conjugated secondary antibodies diluted in 5% non-fat milk-TBST for 1 h at room temperature. The immunoreactions were detected with enhanced chemiluminescence (PerkinElmer, Boston, MA). The rabbit polyclonal anti-phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204; 1:1,000) and the rabbit polyclonal anti-p44/42 MAPK (Erk1/2) (1:1,000) were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal

anti-actin (1:1,000) was purchased from Sigma. The secondary antibodies used were anti-mouse (1:2,000) and anti-rabbit (1:2,000) horseradish peroxidase-conjugated antibodies purchased from Sigma (Saint-Quentin Fallavier, France).

4.12 ISOLATION OF HYPOTHALAMIC GNRH NEURONS USING FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

The preoptic region of Gnrh::EGFP rats was 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 EGFP fluorescence (excitation: 488 nm, 50 mW; detection: GFP bandpass 530/30 nm, autofluorescence bandpass 695/40 nm) by comparing cell suspensions from Gnrh::Egfp and wild-type animals. For each animal, 80 to 300 EGFP-positive cells were sorted directly into 10 µl of extraction buffer containing 0.1% Triton X-100 (Sigma) and 0.4 unit/µl RNaseOUT (Life technologies).

4.13 QUANTITATIVE REAL TIME-PCR ANALYSES

Gene expression analyses were performed on FACS-sorted GnRH neurons and hypothalamic progenitor cultures. For the latter, RNA was extracted with phenol-chloroform and precipitated with ethanol. Briefly, 100 µl of Trizol (Life Scientific, Carlsbad CA, USA) was used to dissociate cells before adding 100 µl of chloroform (Merck, Darmstadt, Germany) and centrifuging at 12,000 g for 15 minutes at 4°C. The aqueous phase was carefully collected and added to isopropanol at 1:1 before centrifuging at 12,000 g for 10 minutes at 4°C. The aqueous phase was then discarded and the pellet washed in 70% ethanol.

After centrifugation at 12,000 g for 5 minutes at 4°C, the pellet was left to air-dry and then diluted in 10 µl DEPC water. The purity and quantity of RNA was determined by UV spectroscopy (Nanodrop 1000. Thermo Scientific, Waltham MA, USA).

Messenger-RNAs obtained from FACS-sorted GnRH neurons or hypothalamic progenitor cell cultures were reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies). In the case of sorted cells, a linear preamplification step was performed using the TaqMan PreAmp Master Mix Kit protocol (Applied Biosystems). Real time-qPCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System using exon-boundary-specific TaqMan Gene Expression Assays (Applied Biosystems): *Gnrh1* (Rn00562754_m1);

Gpr44 (or *PtgdrII*) (Rn02349421_s1); PtgdrI (Rn00824628_m1); Ptgds (Rn00564605_m1). Control housekeeping genes: Actb (Rn00667869_m1) and *Rn45s;H* (Rn03928990_g1).
Gene expression data were analyzed using SDS 2.4.1 and Data Assist 3.0.1 software (Applied Biosystems) with R45S and actin as control housekeeping mRNAs following a standardized procedure.

4.14 HIGH-RESOLUTION FLUORESCENT *IN SITU* HYBRIDISATION (FISH) BY RNASCOPE

FISH was performed on frozen brain sections of the POA of female rats at different ages following the RNAscope version 2 procedures (Advanced Cell Diagnostics, Inc., Newark, CA, USA). Specific probes were used to detect: *Ptgds* in Channel 1 (Accession No NM_013015.2 Target region 2 - 702); *PtgdrI* in Channel 1 (Accession No M_022241.1 Target region 224 - 1157); *Gnrh1* in Channel 2 (Accession No NM_012767.2 Target region 2 - 454); *Sox2* in Channel 3 (Accession No NM_001109181.1 Target region 51 - 2079). Hybridization of a probe against the Bacillus subtilis dihydrodipicolinate reductase (dapB) gene was used as negative control.

4.15 STEREOTACTIC BRAIN INFUSION OF BWA868C

4.15.1 Acute injections

Female rats were i.p. injected with BrdU at P8. A group (n = 4) was sacrificed 2 hours after the injection. The other animals were placed in a stereotaxic frame (Kopf Instruments, California) under anesthesia (isoflurane), and 2 burr holes were drilled 0.5mm apart from the bregma, according to a rat brain atlas (Paxinos and Franklin, 1987). A 10 µl Hamilton syringe was slowly inserted into the POA (5.2 mm deep relative to the dura), and 1 µl of solution containing either vehicle (saline solution 0.9%) either BWA868C 10 nM was injected bilaterally using an infusion pump (KD Scientific, Holliston, MA) over 5 min. Animals were killed 24 hours later and analysed for morphological association between GnRH neurons and $BrdU^+$ cells (see chapeter 4.6.1.1).

4.15.2 Long-term injections

Female rats were i.p. injected with BrdU at P8, placed in a stereotaxic frame (Kopf Instruments, California) under anesthesia (isoflurane), and one burr hole was drilled at the

level of the bregma, according to a rat brain atlas (Paxinos and Franklin, 1987). A stainless steel 5 mm cannula filled with BWA868C mixed with melted cocoa butter (10 nM) or cocoa butter alone was stereotaxically implanted at the level of the bregma (as described in Ojeda and Ramirez, 1969). After implantation, the cannulas were fixed to the skull with superglue, also used to suture the skin wound. Blood was collected from animals at P12 for plasma FSH measurements (see chapter 4.18). Four controls and 5 BWA-injected animals were sacrificed 7 days after the surgery and analysed for morphological association between GnRH neurons and $BrdU^+$ cells (see chapter 4.6.1.1). Other animals were followed for puberty onset and oestrous cyclicity (described in chapter 4.3), and blood was collected on the day prior to vaginal opening as well as on the day of proestrus.

4.16 HORMONE LEVELS MEASUREMENT

Plasma LH was measured using a high sensitivity Enzyme-Linked Immunosorbent Assay (ELISA) as described elsewhere (Steyn et al., 2013). Serum FSH levels were measured using radioimmunoassay as previously described (Martini et al., 2006). The accuracy of hormone measurements was confirmed by the assessment of rodent serum samples of known concentration (external controls).

RESULTS

Chapter 5

Cell neogenesis in the postnatal hypothalamus in the female rat: involvement in sexual maturation

5.1 INTRODUCTION

The most important hypothalamic function for a species is that of controlling the reproductive physiology and behaviour. The master regulators of reproduction are the GnRH neurons, a small population of neurons found at the level of the POA. Following embryonic migration from the olfactory placode, where these neurons are born, they settle down in the hypothalamus where they are found at birth. However, they require postnatal maturation to determine the timely onset of puberty and subsequent oestrous cyclicity. The postnatal development of GnRH neurons in female rats can be divided into 4 stages, a neonatal period, from birth to P7; an infantile period, from P8 to weaning, during which a peak of FSH occurs at P12, corresponding to the first centrally-driven activation of the HPG axis; a juvenile period, from P21 to P30; and a peripubertal period. During this postnatal period, GnRH release gradually increases and culminates in the first LH surge, causing the first ovulation, which in rats corresponds to the day of vaginal opening (Prevot, 2015). Meanwhile, GnRH neurons receive neuronal inputs from their close environment as well as from other hypothalamic nuclei, such as those controlling the metabolic state or the circadian clock, which contribute to the fine tuning of the maturation of the GnRH system. In addition to neurons, astrocytes play an important role in regulating GnRH neuron activity, as well as ensuring correct onset of puberty and establishment of oestrous cyclicity.

Considering that the postnatal period is the moment when most glial cells are born in the brain, a project has been initiated in the laboratory with the aim to explore whether the birth of new cells in the environment of GnRH neurons was involved in their postnatal maturation. Previous results obtained by a former Ph.D. student (Cécile Allet), which I will briefly summarise, showed that the birth of new astrocytes morphologically associated with GnRH neurons during the infantile period was important for sexual maturation of female rats.

Starting from here, the question I addressed concerned the mechanisms involved in the establishment of the crosstalk between maturating GnRH neurons and newborn astrocytes.

Our findings identify a signalling pathway involving PGD2 and its receptor DP1, responsible for the morphological interactions between GnRH neurons and newborn cells during the infantile period in female rats and suggest a role for this interaction in the correct onset of sexual maturation.

5.2 PREVIOUS RESULTS

Proliferation was analysed in the POA of female rats at different postnatal ages through i.p. injections of BrdU (Fig. 1a,b). The proliferative activity was highest in the infantile period (P8, P12) compared to the juvenile period (P20, P24) (Fig. 1c). The BrdU-positive cells were multipotent progenitors, as attested by their ability to differentiate into the three neural lineages *in vivo* (Fig. 1d-g). However, most newborn cells differentiated into astrocytes at all ages studied (Fig. 1d,g). *In vitro* experiments confirmed the presence of neural progenitors in the postnatal POA since neurospheres that proliferated, self-renewed and differentiated in all neural lineages could be obtained from microdissected postnatal POA (not shown).

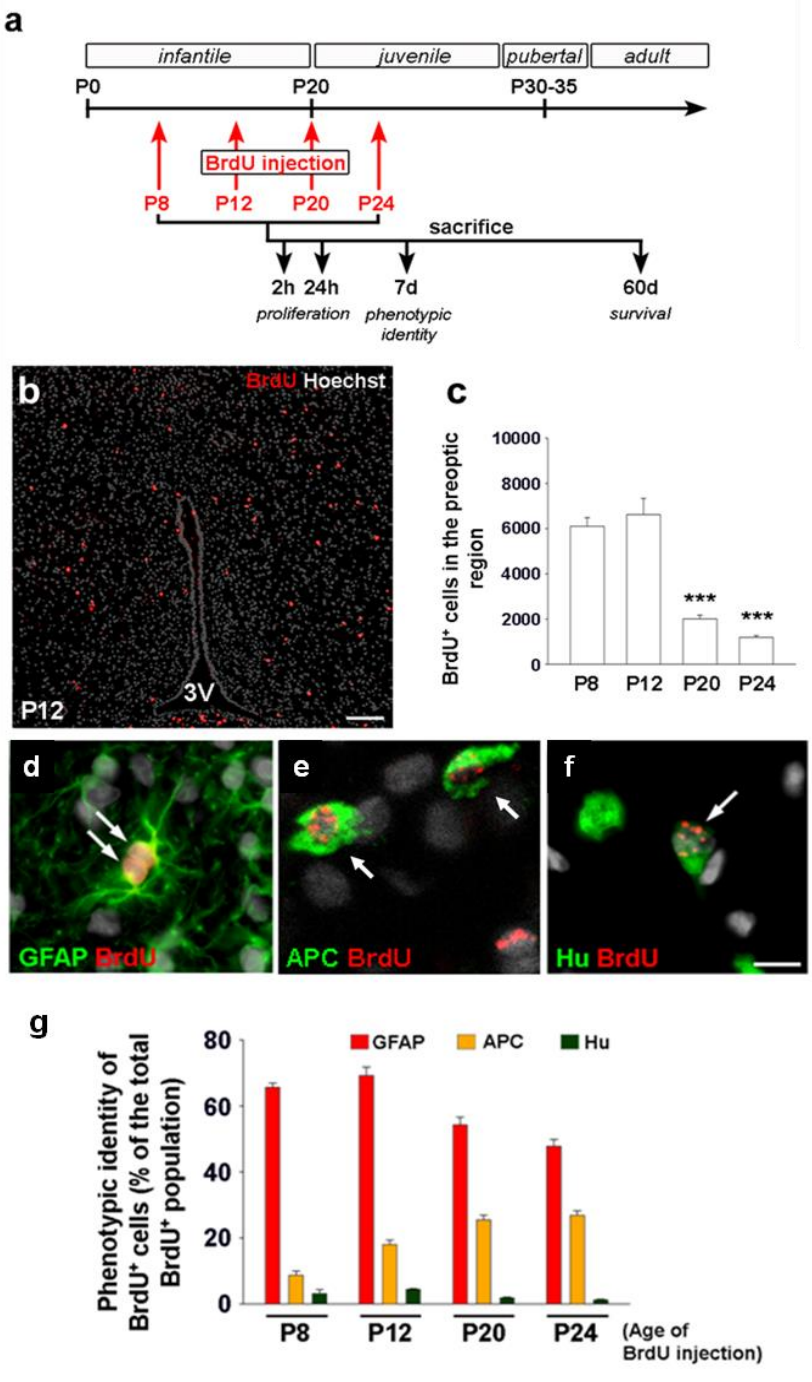


Figure 1. The postnatal POA contains multipotent progenitors that mainly differentiate into astrocytes. (a) Diagram of the experimental protocol. Female rats received a single intraperitoneal injection of BrdU (300 mg/kg) at P8 (early infantile), P12 (infantile), P20 (early juvenile) or P24 (juvenile). Animals were perfused at different survival times: 2 hr and 24 hr to study proliferation, 7 d to evaluate the phenotypic identity of newborn cells and 60 d to monitor the long-term survival of newborn cells. **(b)** Coronal section of the preoptic region of a P12-injected animal immunolabeled for BrdU (red). **(c)** Quantification of the total number of BrdU+ cells in the preoptic region of animals injected with BrdU at P8, P12, P20 or P24 and analysed 2 hr later. Note the drop between the infantile and the juvenile period (n = 3-4 animals per group). **(d-g)** Female rats were injected with BrdU at P8, P12, P20 or P24. Brains were collected 7 d later and processed for immunodetection of BrdU (red) and markers (green) of the astroglial (GFAP, **d**), oligodendroglial (APC, **e**) or neuronal (Hu, **f**) lineages. Arrows point to co-labeled cells. **(g)** Quantification of the phenotypic identity of newborn cells calculated as the percentage of cells co-labeled for BrdU and GFAP, APC or Hu over the total number of BrdU+ cells in the preoptic region (n = 3 animals per group). Sections were counterstained with the nuclear marker Hoechst (white). Scale bars: 100 µm (in b); 10 µm (d-f). Mean ± s.e.m.; *** P < 0.001 relative to P8 or P12. 3V, third ventricle.

When co-immunolabelling for BrdU and GnRH was performed at different postnatal ages, close morphological interactions were seen between some GnRH neuronal cell bodies and newborn cells (Fig. 2a). The association was most prominent during the infantile period (Fig. 2b) and was preferential towards GnRH neurons compared to the whole neuronal population of the POA (not shown). When animals were injected with BrdU at P8 and sacrificed 7 days later, the association between GnRH neurons and newborn cells was found to increase (Fig. 2b), while the total number of GnRH neurons remained unchanged (not shown), suggesting that GnRH neurons were able to recruit newborn cells in their vicinity. Moreover, this partnership was still observed in adult animals (P8+60d, Fig 2b). This suggests that during a specific window of the infantile period, newborn cells preferentially form close morphological associations with GnRH neuronal cell bodies, and that GnRH neurons retain their newborn companion cells into adulthood (Fig 2c).

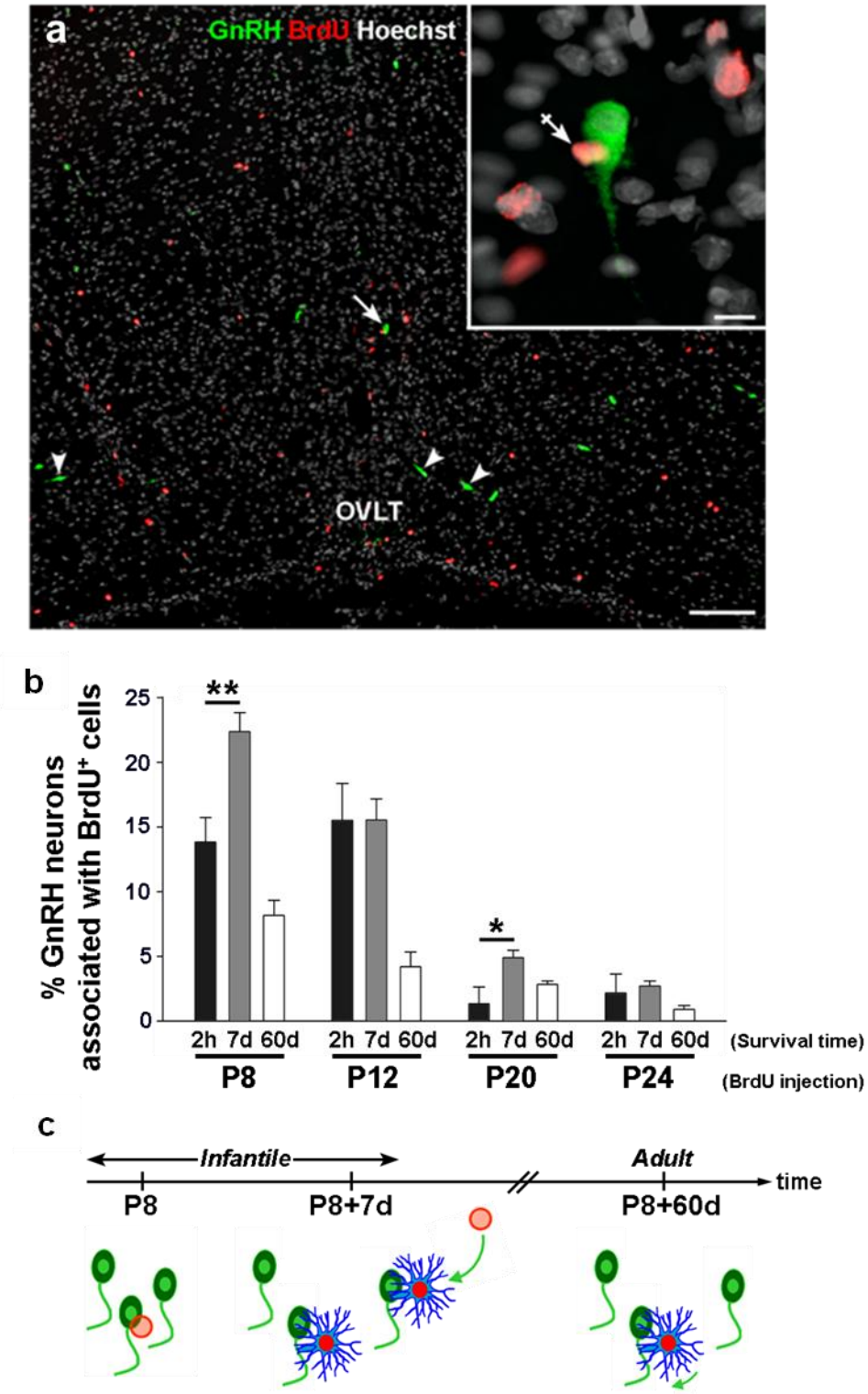


Figure 2. Morphological interactions between GnRH neurons and newborn cells in the postnatal POA. (a) Coronal section of the preoptic region immunolabeled for GnRH (green) and BrdU (red). Arrow and arrowheads point to GnRH neuron cell bodies. Inset, higher magnification view of the GnRH neuron pointed by the arrow, showing the close morphological interaction with a BrdU+ cell (crossed arrow). **(b)** Female rats received a single injection of BrdU at P8, P12, P20 or P24 and the proportion of GnRH neurons associated with $BrdU^+$ cells was quantified 2 hr, 7 d or 60 d later (see Fig. 1a) (n = 3-10 animals per group). **(c)** Schematic of the proposed model. At P8, cells are generated (red) in the vicinity of GnRH neurons (green). Within the following week, additional GnRH neurons

recruit in their surroundings cells born at P8, most of which differentiate into astrocytes (blue, see Fig. 3). At adulthood (P8 + 60 d), some GnRH neurons are still associated with P8-newborn cells. Scale bars: 100 µm (a main panel), 10 µm (inset in a). Mean ± s.e.m.; * $P < 0.05$; ** $P < 0.01$. OVLT, organum vasculosum of the lamina terminalis.

To study the fate of the population of cells born on P8 and found in close association with GnRH neurons, triple immunolabelling were performed for GnRH, BrdU and markers of the astroglial (GFAP) or neuronal (HuC/D) lineages (Fig. 3). Seven days after the BrdU injection, 69.3 ± 7.1% of these cells expressed GFAP (Fig. 3a,d), a proportion that was similar to that observed in the whole preoptic region (compare with Fig. 1g; Student t-test, $p = 0.681$). Only rare neurons were observed (Fig. 3c). I completed this characterisation by evaluating the oligodendroglial differentiation, and showed that 26.7 ± 14.5% of $BrdU^+$ cells expressed the oligodendroglial marker APC (Fig. 3b). Moreover, I analysed the phenotype of BrdU+ cells associated with GnRH neurons at the moment of their birth, i.e. 2 hours after the injection of BrdU, by performing immunolabelings for Sox2, a marker of immature undifferentiated cells, or GFAP. Notably, most cells born on P8 in the vicinity of GnRH neurons did not express GFAP at the moment of their birth (7.14 ± 7.14%; 2 hr vs. 7d, $P < 0.001$) (Fig. 3d), while most of them expressed Sox2 (66.33 ± 5.71%) (Fig. 3d), suggesting they were progenitors that subsequently differentiated into astrocytes.

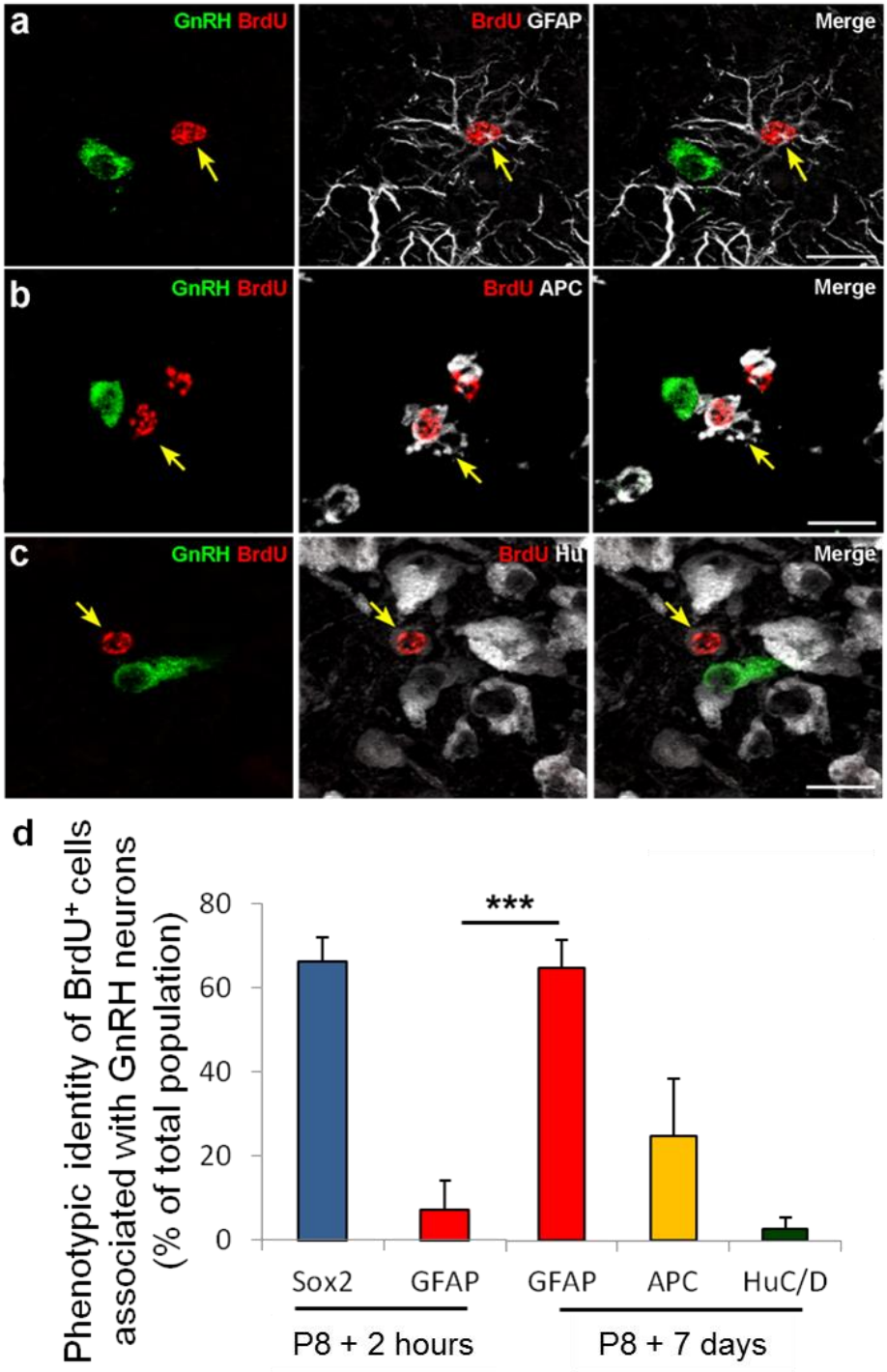


Figure 3. Phenotypic identity of newborn cells morphologically associated with GnRH neurons. (a-c) Triple immunolabeling for GnRH (green), BrdU (red) and GFAP (**a**, white), APC (**b**, white) or HuC/D (**c**, white) of sections from the preoptic region of female rats injected with BrdU at P8 and sacrificed 7 d later. Arrows point to BrdU+ cells morphologically associated with GnRH neurons. **(d)** Quantification of the phenotypic identity of newborn cells associated with GnRH neurons 2hr or 7 d after an injection of BrdU at P8, calculated as the percentage of cells co-labeled for BrdU and Sox2, GFAP, APC or HuC/D over the total number of BrdU+ cells (n = 3-4 animals per group). Scale bars: 20 µm. *** $P < 0.001$.

To address the functional role of this infantile wave of astrogenesis in the central control of reproduction, proliferation in the POA was selectively inhibited in infantile female rats by stereotaxic injection of microparticles loaded with the antimitotic drug paclitaxel (Fig. 4a-c). Paclitaxel-treated animals exhibited a marked delay in the onset of puberty, as attested by late vaginal opening and first estrus compared to control animals (Fig. 4d-f). These data suggest that astrogenesis in the POA during the infantile period is required for the timely onset of puberty.

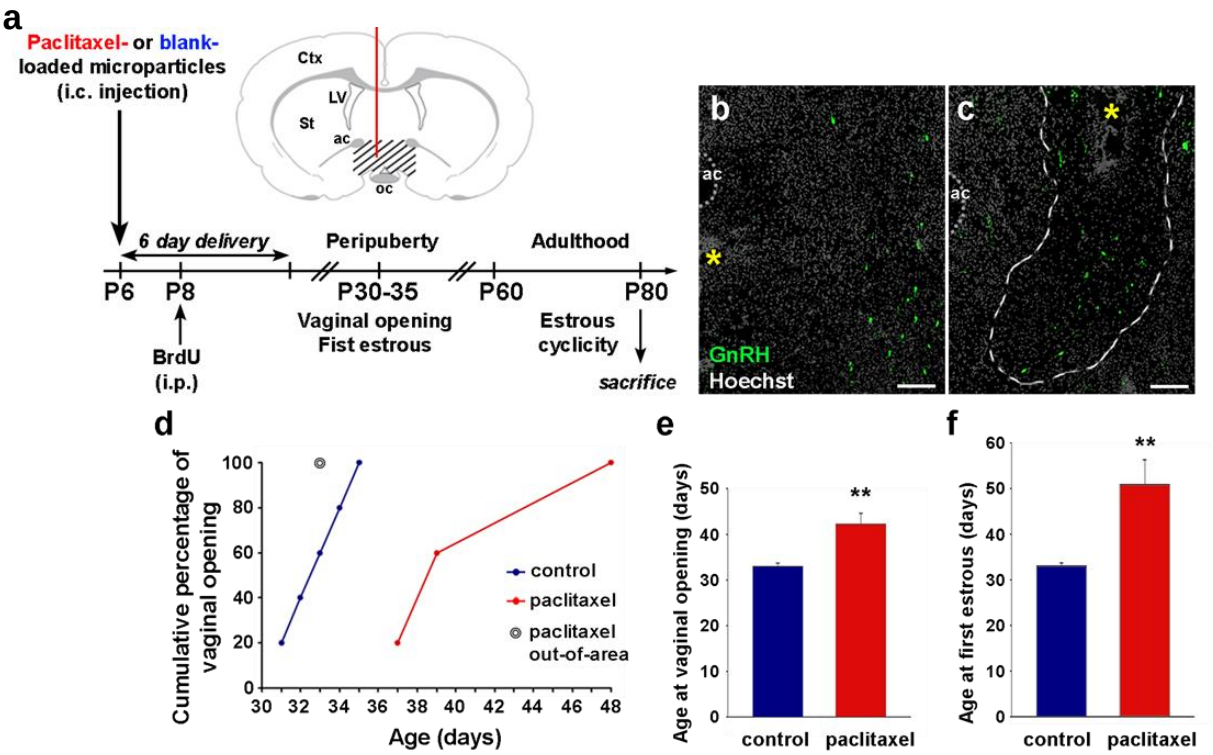


Figure 4. Selective inhibition of proliferation in the preoptic region of infantile female rats delays the onset of puberty. (a) Diagram of the experimental protocol. The drawing is a coronal view of the rat brain showing the anatomical localization of the implantation site of microparticles (red line) into the hypothalamic preoptic region (dashed area). i.c., intracerebral; i.p., intraperitoneal. **(b,c)** Coronal sections from P8 animals injected with blank- **(b)** or paclitaxel-loaded microparticles **(c)** immunolabeled for GnRH (green). The star indicates the injection site. Nuclei were counterstained with Hoechst (white). Paclitaxel-treated animals showed reduced cell density at the injection site (c, region delimited by the white dashed line). **(d)** Cumulative percentage of vaginal opening in animals injected with blank- (control, n = 5) or paclitaxel-loaded microparticles (n = 5). The two animals injected with paclitaxel outside the preoptic region (paclitaxel out-of-area) showed normal vaginal opening. **(e)** Paclitaxel-treated animals exhibited a significant delay in vaginal opening compared to controls. **(f)** Paclitaxel-treated animals showed a significant delay in first estrous compared to controls. Mean ± s.e.m.; ** $P < 0.01$. ac, anterior commissure; Ctx, cerebral cortex; LV, lateral ventricle; oc, optic chiasm; St, striatum. Scale bars: 200 µm.

5.3 RESULTS

The previous results suggest that astrogenesis in the environment of GnRH neurons during the infantile period is important for the onset of puberty. GnRH neurons appear to establish preferential and long-lasting partnerships with glial progenitors. Moreover, the increase in the proportion of GnRH neurons associated with newborn cells seen between P8 and P15 (Fig. 2b) suggests that GnRH neurons are able to attract local progenitors in their vicinity. However, the molecular determinants involved in these interactions remained to be explored.

5.3.1 Identification of candidate factors

In order to identify factors produced by GnRH neurons and able to affect their neighbouring glial progenitors, we used a candidate approach. In addition to GnRH itself, we selected from the literature a series of factors reported to be expressed in GnRH neurons: NT, CCK, galanin, glutamate and GABA (Herbison, 2015; Ciofi, 2000). However, since these factors were identified in different contexts (mouse or rat, embryonic or adult life), we first evaluated their expression in GnRH neurons of infantile rats. Double immunostainings showed that GnRH neuron cell bodies express galanin but lack CCK and NT during the infantile period (Fig. 5). To determine whether GnRH neurons produce glutamate or GABA, we evaluated the expression of v-Glut and v-GAT, the vesicular transporters of glutamate and GABA respectively, found in nerve terminals. We observed the co-localisation of GnRH with v-Glut or v-GAT at the level of their terminals in the OVLT and most prominently in the ME (Fig. 6).

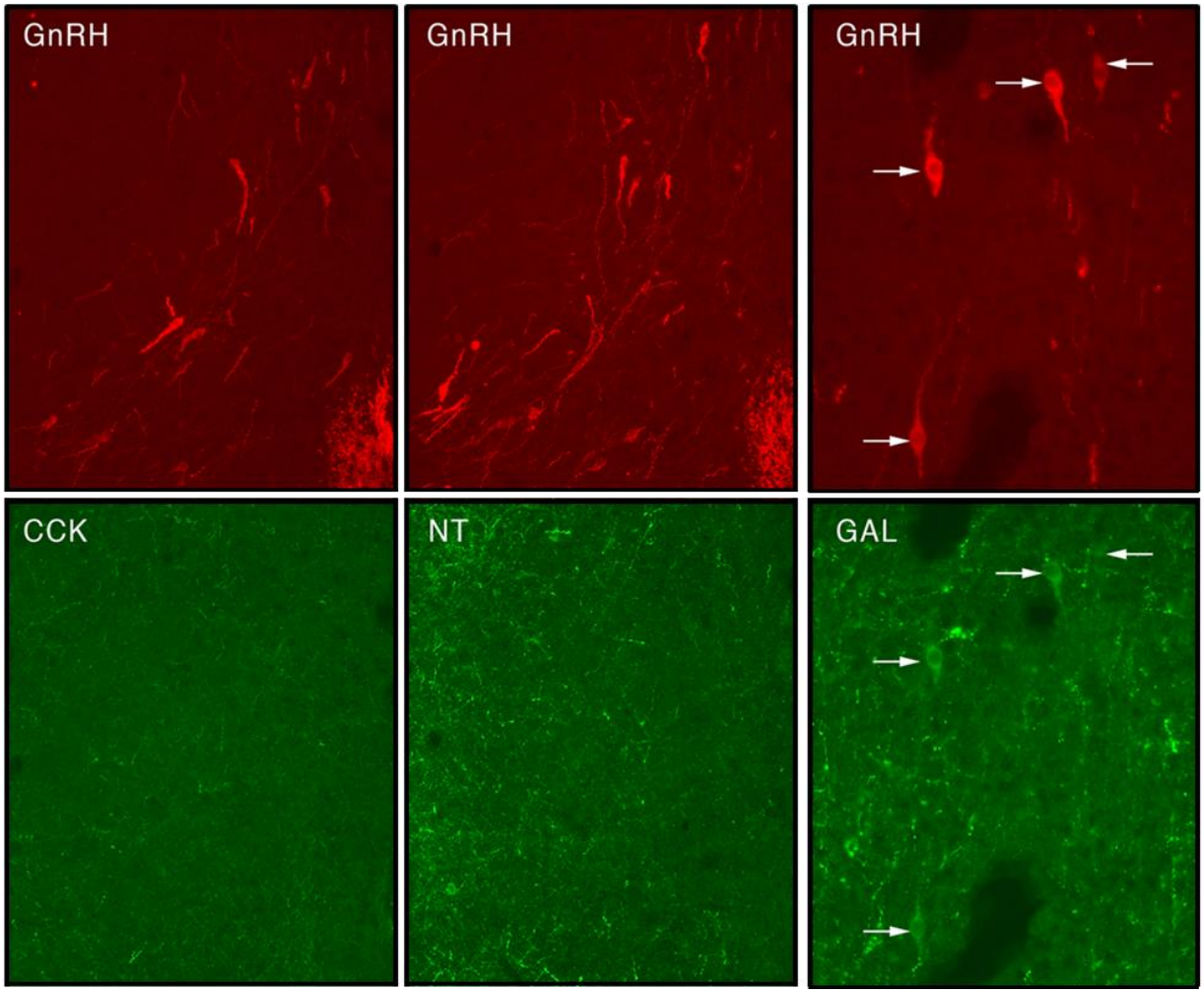


Figure 5. GnRH neurons express galanin but lack cholecystokinin (CKK) or neurotensin (NT) during the infantile period. Double immunolabelings of GnRH (red, upper panels) and CCK, NT or galanin (GAL) (green, lower panels) in P11 rats. Arrows point to GnRH neuron cell bodies that co-express galanin (Philippe Ciofi, U862, Bordeaux).

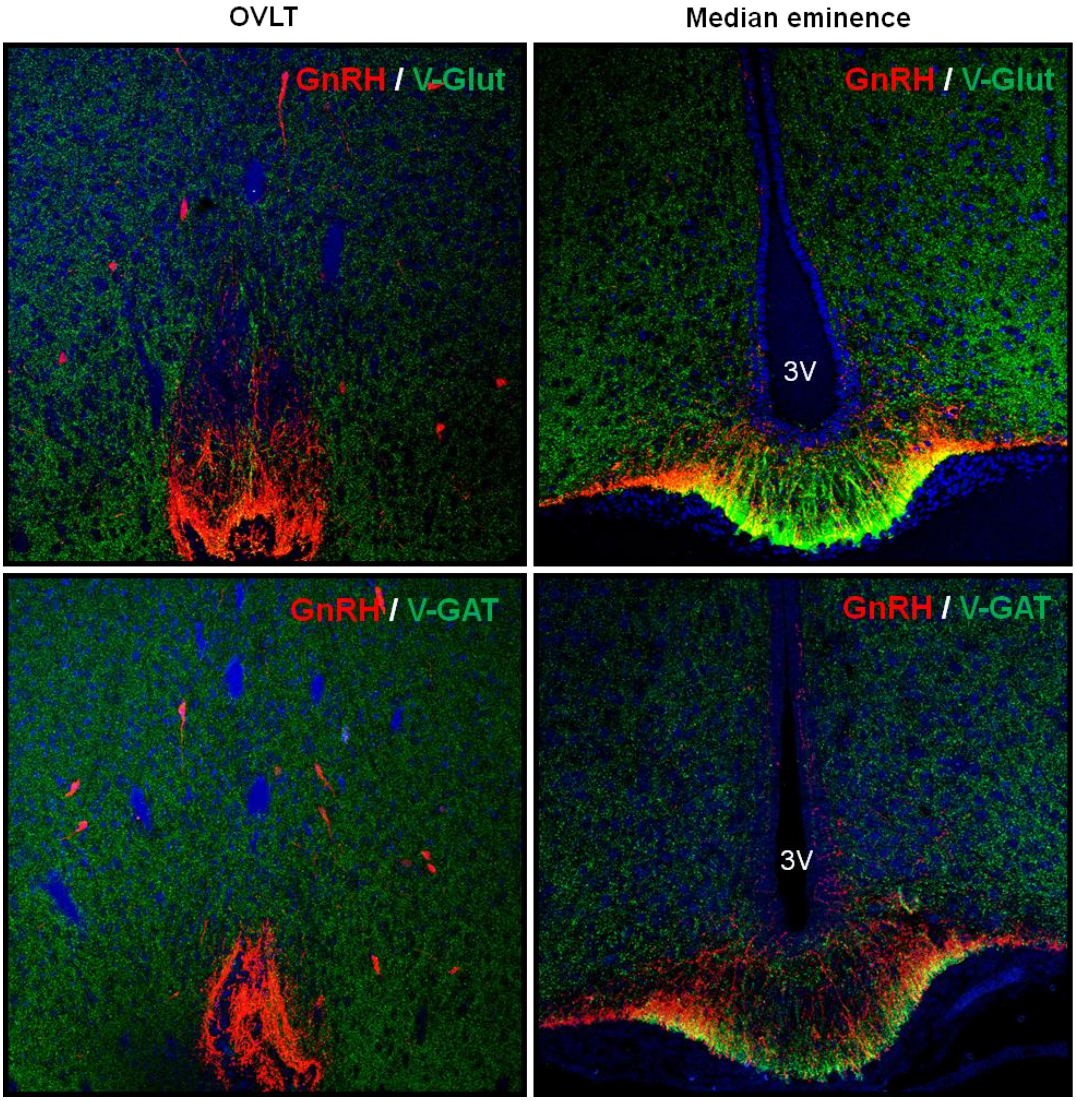


Figure 6. GnRH neurons express v-Glut and v-GAT during the infantile period. Double immunolabelings showing the co-expression of GnRH (red) and the vesicular glutamate transporter (v-Glut) (green, upper panels) or the vesicular GABA transporter (v-GAT) (green, lower panels) in P8 female rats at the level of the organum vasculosum of the lamina terminalis (OVLT) (left) and the median eminence (right). Nuclei were counterstained with Hoechst (blue). 3V, third ventricle.

Moreover, in collaboration with the laboratory of Dr. Francois Pralong in Switzerland, a transcriptomic analysis was performed on GnV3 cells, an *in vitro* model of mature rat GnRH neurons (Mansuy et al., 2011), exposed or not to hypothalamic astrocyte-conditioned medium. A series of genes were found to be over- or underexpressed in GnV3 cells treated with astrocyte factors compared to untreated GnV3 cells. The most upregulated gene was Ptgds, the gene coding for the brain-type prostaglandin D2 (PGD2) synthase, which showed a 40.6-fold increase in the presence of astrocyte factors, suggesting that GnRH neurons

increase their capacity of PGD2 synthesis in a context of gliogenesis. We therefore added PGD2 to the panel of candidate factors.

5.3.2 Development and characterisation of hypothalamic progenitor cultures

In order to evaluate whether the selected factors are able to influence the motility and/or the proliferation of progenitor cells, we developed primary cultures of hypothalamic progenitor cells from microdissections of the POA of P1-P2 rats (Fig. 7a).

The cultures obtained were characterised for the expression of different markers expressed by immature cells (Nestin, Sox2, Blbp), glial (Olig2, GFAP) or neuronal lineages (HuC/D, Tuj), as well as the proliferation marker Ki67 (Fig. 7b-e). Almost all the cells expressed Blbp (98.53 ± 0.32%), Nestin (95.47 ± 0.79%) and Sox2 (88.83 ± 1.75%), as expected for immature progenitors. A smaller percentage of cells expressed Olig2 (42.44 ± 2.88%) and GFAP (25.77 ± 2.86%). While very few cells expressed HuC/D (7.25 ± 3.1%), none were positive for Tuj. Ki67 was expressed by 7.74 ± 2.06% of cells (Fig. 7e). This suggested that our primary cultures have an antigenic profile of neural progenitors and proliferate at a low level.

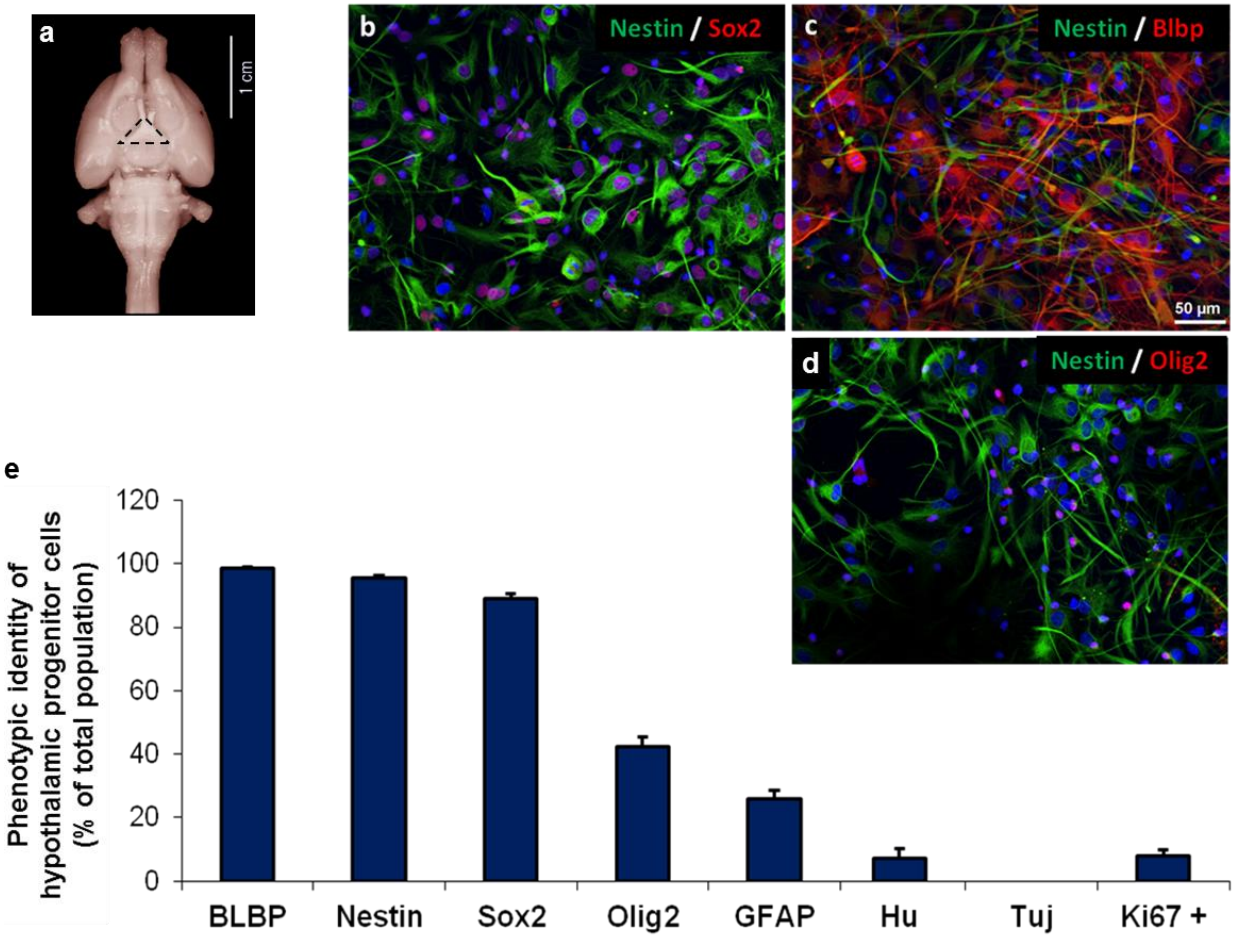


Figure 7. Characterization of the primary cultures of rat POA progenitors. The preoptic region of P1-P2 rats was dissected **(a)**, mechanically dissociated and grown in DMEM/F12 + EGF + FGF2. Adherent cells were labelled for Nestin (**b, c, d** green), Sox2 (**b**, red), Blbp (**c**, red), Olig2 (**d**, red), GFAP, HuC/D, Tuj and Ki67 (not shown). The percentage of cells expressing each marker was calculated over the total number of Hoechst-positive cells **(e)**. Nuclei were counterstained with Hoechst (blue). Mean ± s.e.m.

5.3.3 PGD2 attracts POA progenitors *in vitro* via DP1 signalling

To explore whether GnRH neurons produce factors able to recruit POA progenitors, we used the transwell assay (Fig. 8a) to evaluate the ability of GnRH, PGD2, galanin, glutamate and GABA to attract hypothalamic progenitors. EGF was used as a positive control, as it has been previously shown to stimulate the migration of neural stem and progenitor cells (Sun et al., 2005; Zhang et al., 2011). Out of all the factors tested, only PGD2 showed the ability to attract progenitor cells (Fig. 8b, n = 4 experiments).

In order to show that PGD2 was acting through a direct action on progenitor cells, we used RT-qPCR to evaluate the expression of the 2 receptors known to bind PGD2, DP1 and DP2, in hypothalamic progenitor cultures. Both receptors were found to be expressed by these cells *in vitro* (Fig. 8c). Since DP1 has been reported to be the most abundant PGD2 receptor in the brain (Mizoguchi et al., 2001), we subsequently focused on this receptor. In order to evaluate whether the migratory effect of PGD2 involves DP1, we used BWA868C, a specific antagonist of the DP1 receptor extensively used in the literature (Kumar et al., 2015; Ohinata et al., 2008; Schratl et al., 2007). To test the efficacy of BWA868C in blunting PGD2 signalling, hypothalamic progenitor cultures were treated with PGD2 alone or in the presence of BWA868C and activation of the mitogen activated protein kinase (MAPK) pathway was analysed by western blot analysis (Fig. 8d). Treatment with BWA868C inhibited the PGD2-induced phosphorylation of ERK (Fig. 8d).

BWA868C was then tested in a transwell assay for its ability to block PGD2-mediated attraction. While the presence of BWA868C alone did not affect the motility of progenitor cells, it totally blocked the stimulatory effect of PGD2 on cell migration (Fig. 8e), suggesting that PGD2 attracts progenitor cells *in vitro* via the activation of the DP1 receptor.

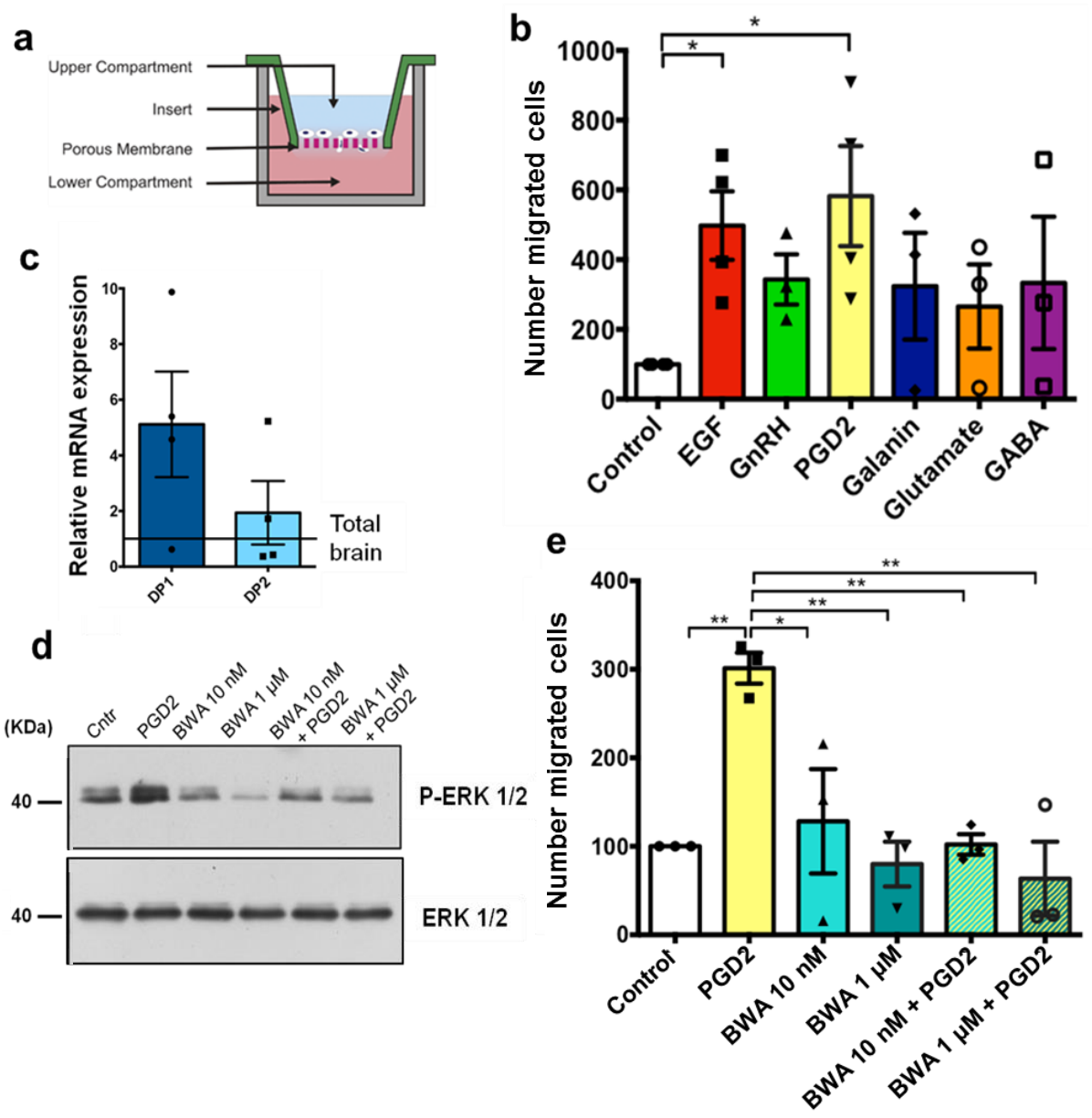


Figure 8. PGD2 attracts POA progenitor cells in vitro. (a) Schematic of the transwell assay; cells are seeded on a porous membrane placed in a well containing the factor to test. If cells are attracted by this factor, they migrate through the membrane to the bottom surface, where they can be subsequently fixed, stained, and counted. **(b)** Quantification of transwell assays showing that PGD2 attracts progenitor cells. EGF was used as a positive control (n = 4 experiments). **(c)** Real-time PCR analysis of DP1 and DP2 mRNA levels in progenitor cultures (n = 4 cultures). **(d)** Western blot experiment showing that progenitors respond to PGD2 treatment with increased phosphorylation of the MAPK ERK1/2, an effect that is inhibited by treatment with the DP1 receptor antagonist BWA868C (n = 3). **(e)** Quantification of transwell assays showing that treatment with BWA868C inhibits the PGD2-dependent migration of POA progenitors (n = 3 experiments).

5.3.4 The candidate factors do not affect hypothalamic progenitor proliferation *in vitro*

To explore whether factors produced by GnRH neurons create a micro-environment that could also regulate the proliferation of progenitor cells, the same factors were tested *in vitro* for their ability to affect proliferation of POA progenitor cultures. In this experiment, we used FGF2 as a positive control since it has been shown to be a more powerful stimulator of proliferation of hypothalamic progenitor cells than EGF (Xu et al., 2005).

Cultures were starved for 24 hours, treated for 24 hours with the different factors and proliferative cells were identified by their expression of the cell cycle marker Ki67 (Fig 9a). The percentage of cells expressing Ki67 was not affected by any of the treatments (Fig. 9b), suggesting that none of the candidate factors regulates the proliferation of POA progenitors.

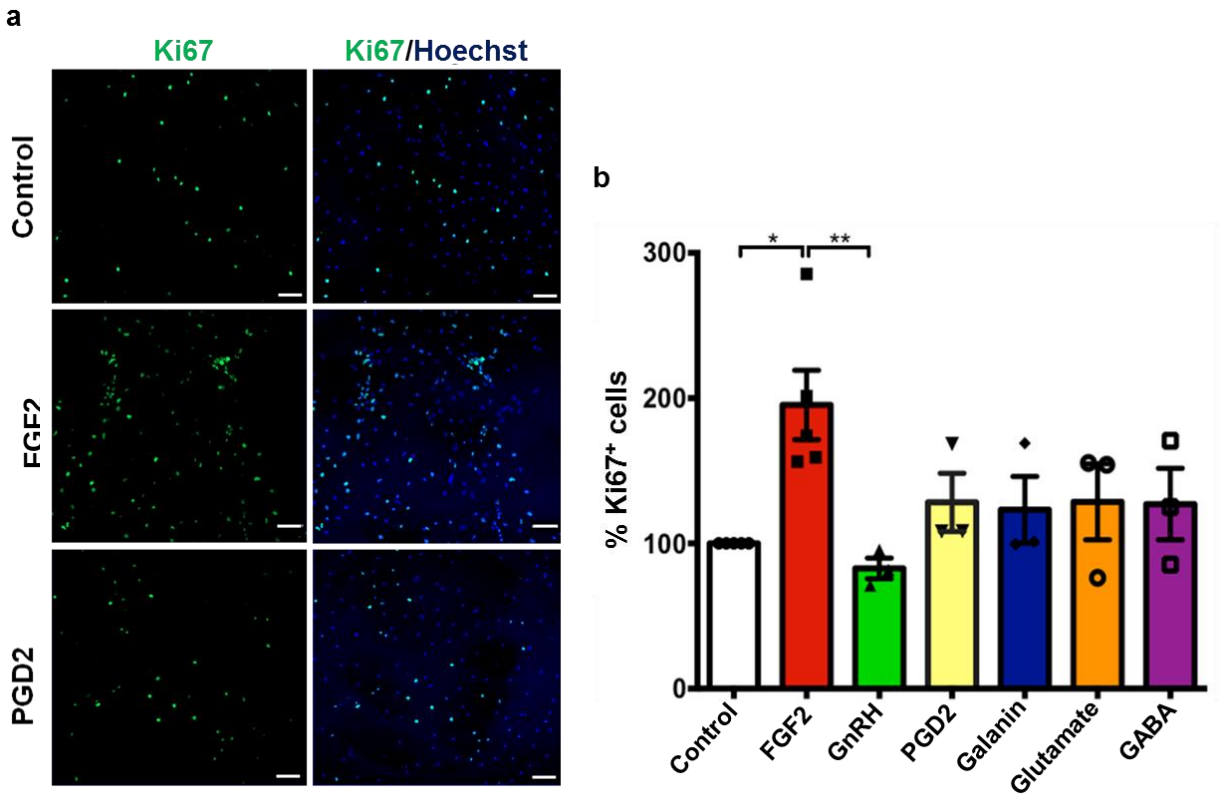


Figure 9. The candidate factors do not affect the proliferation of POA progenitors *in vitro* (a) Immunolabelling of Ki67 (green) in POA progenitor cultures untreated (control) or treated with FGF2 or PGD2. Nuclei were counterstained with Hoechst (blue). Scale bar: 100 µm. Mean ± s.e.m.; * $P < 0.05$; ** $P < 0.01$. **. (b)** Quantification of the percentage of Ki67+ cells in response to GnRH, PGD2, Galanin, Glutamate and GABA. FGF2 was used as a positive control (n = 3 experiments).

5.3.5 GnRH neurons recruit newborn cells through the PGD2/DP1 signalling *in vivo*

5.3.5.1 *In vivo* expression of PGDS and DP1

To address whether the PGD2/DP1 signalling pathway also regulates the migration of POA progenitors *in vivo*, the expression of PGDS and DP1 in the postnatal POA was first analysed using two approaches.

a. In situ hybridization

The RNAscope technique was used to perform FISH on the POA of female rats at P4, P8 and P12. Triple *in situ* hybridisation was performed with three different probes directed against GnRH, the progenitor marker Sox2 and either PGDS (Fig. 10a) or DP1 (Fig. 10b).

The distribution of each staining was coherent with what is expected from the literature (Allen brain atlas, http://mouse.brain-map.org/experiment/show/77280331; Herbison, 2015; Urade et al.,1987). The signals obtained with the GnRH and the Sox2 probes were comparable to immunohistochemistry results; Sox2 was, for example, enriched at the level of the SVZ. PGDS and DP1 were, as expected, more abundant at the level of the meninges. PGDS was also strongly expressed at the level of the choroid plexus. Together with the observation that no signal was observed when a negative control probe was used, these data support the specificity of the hybridisation probes.

In the POA, the PGDS signal was enriched at the level of the OVLT and could be observed in GnRH neurons as well as non-GnRH cells (Fig. 10a). The signal for DP1 was detected in Sox2-expressing cells, as well as in GnRH neurons and other cells in the parenchyma (Fig. 10b).

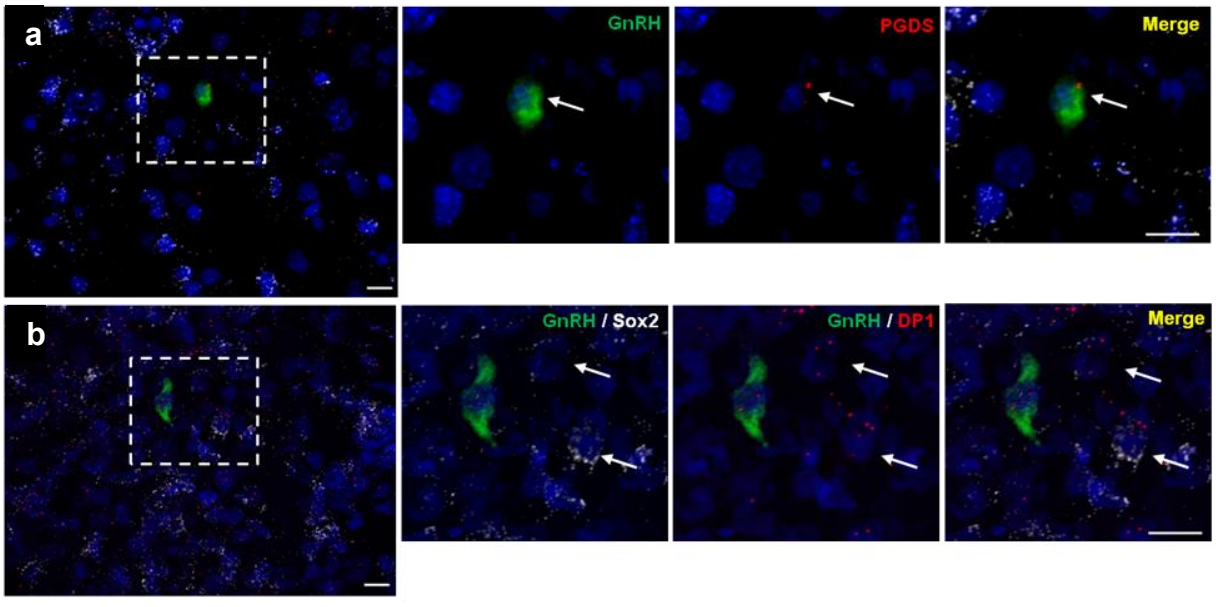


Figure 10. In situ hybridisation experiment showing that GnRH neurons express PGDS and progenitors express DP1 in the postnatal POA *in vivo*. (a,b) Representative confocal images of fluorescent *in* situ hybridization experiments using the RNAscope technology on brain sections of P8 female rats, showing the presence of GnRH (a, b, green), Sox2 (a, b, white), PGDS (a, red) and DP1 (b, red) mRNAs. The boxed panels in a and b are shown at higher magnification in the right panels. Arrows point to a GnRH/PGDS co-expressing cell (a) and Sox2/DP1 co-expressing cells next to a GnRH neuron (b). Scale bars: 10 µm in a and b panels; 20 µm in high-magnification views.

b. Cell sorting

In a second approach, GnRH neurons were isolated from the POA of GnRH::EGFP female rats at P8, P12 and P20 (Fig. 11a). EGFP-positive cells were compared to negative ones, showing at each age a strong enrichment in the expression of GnRH (Fig. 11b-d). The levels of GnRH mRNA in the EGFP-positive cells were highest at P8 and P12, and then decreased at P20 (Fig. 11e). The expression of PGDS was then compared among the different ages both within the sorted GnRH neurons (Fig. 11f) and within the negative cells (Fig. 11g) of the POA. An increase in the expression of PGDS in GnRH neurons was observed between P8 and P20 (Fig. 11f), while the levels of PGDS mRNA in the negative cells remained unchanged during the same period (Fig. 11g).

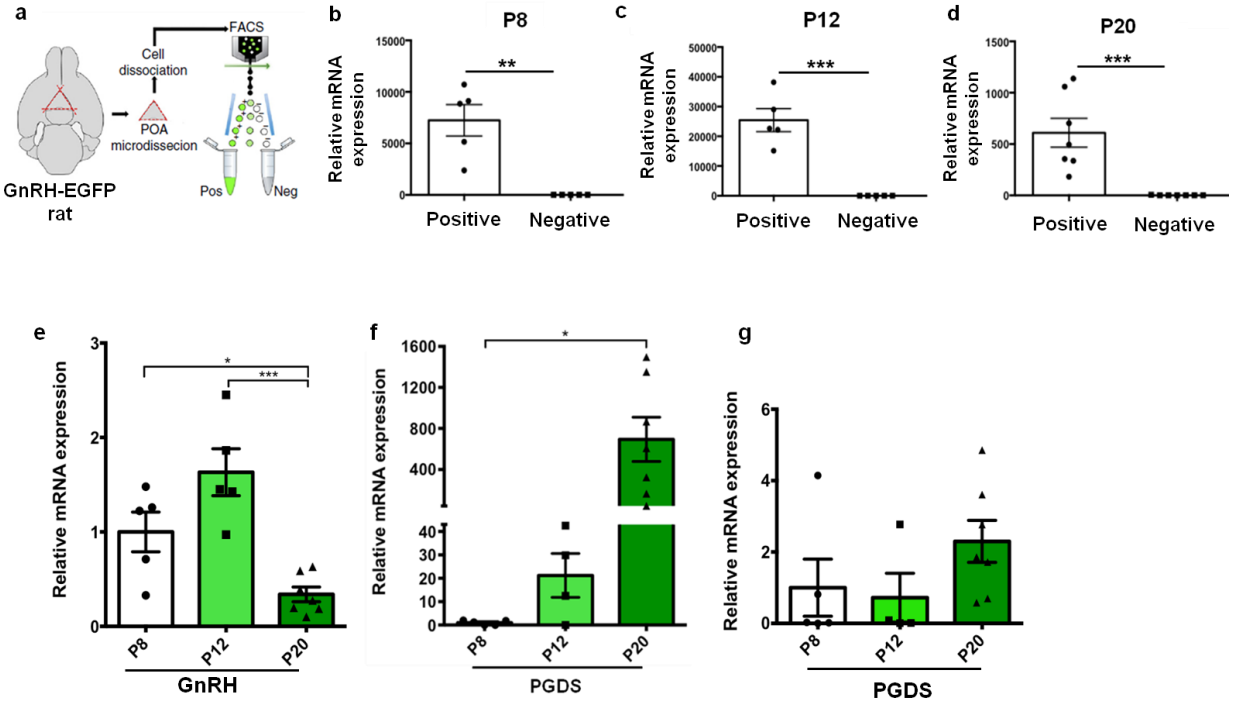


Figure 11. Cell sorting experiment showing that postnatal GnRH neurons express PGDS. (a) Schematic of the cell sorting procedure used to sort GnRH neurons from the POA of Gnrh::EGFP rats. **(b-d)** Real-time PCR analysis of the expression of GnRH in EGFP-positive versus negative FACS-sorted

cells from the POA of Gnrh::EGFP rats at P8 (b), P12 (c) and P20 (d). **(e-g)** Real-time PCR analysis of the expression of GnRH (e) or PGDS (f-g) mRNA in EGFP-positive cells (e,f) or negative cells (g) compared among ages. Mean ± s.e.m.; * P < 0.05; ** P < 0.01; *** P < 0.001.

Altogether, these results show that GnRH neurons express PGDS and nearby Sox2-positive progenitors express DP1 in the POA *in vivo*. Moreover, our RT-qPCR analysis shows that the expression of PGDS specifically increases within GnRH neurons during the postnatal period.

5.3.5.2 PGD2/DP1 signalling regulates the recruitment of $BrdU^+$ cells in the vicinity of GnRH neurons *in vivo*

Having shown that PGDS and DP1 receptors were expressed in the postnatal POA, we next addressed whether the PGD2/DP1 signalling was involved in the recruitment of newborn cells in the vicinity of GnRH neurons *in vivo*.

To this end, BWA868C was stereotaxically injected in the POA of P7 female rats that had just received an injection of BrdU (Fig. 12a). Three experimental groups were analysed: a group of animals sacrificed 2 hours after the BrdU injection (no stereotaxic injection), a group of animals stereotaxically injected with saline and sacrificed 24 hours later, and a group of animals stereotaxically injected with 10 nM BWA868C and sacrificed 24 hours later. We then quantified the morphological association between GnRH neurons and $BrdU^+$ cells in the three conditions.

Two hours after the BrdU injection, 11.79 ± 0.89% (n = 4 animals) GnRH neurons were associated with $BrdU^+$ cells (Fig. 12b). This proportion increased to 27.16 ± 3.14% (n = 5 animals) in animals injected with saline and sacrificed 24 hours later. However, this increase was not seen in animals injected with BWA868C (15.79 ± 1.48% GnRH neurons associated with $BrdU^+$ cells; n = 5 animals).

Quantification of the total number of $BrdU^+$ cells in the POA showed that the population of $BrdU^+$ cells increased between 2 hours and 24 hours (Fig. 12c). Treatment with BWA868C did not affect the total number of $BrdU^+$ in the POA, suggesting that the decreased association between GnRH neurons and $BrdU^+$ cells was not due to a general depletion of the $BrdU^+$ cell population but rather to an inhibition of the recruitment of $BrdU^+$ cells in the vicinity of GnRH neurons.

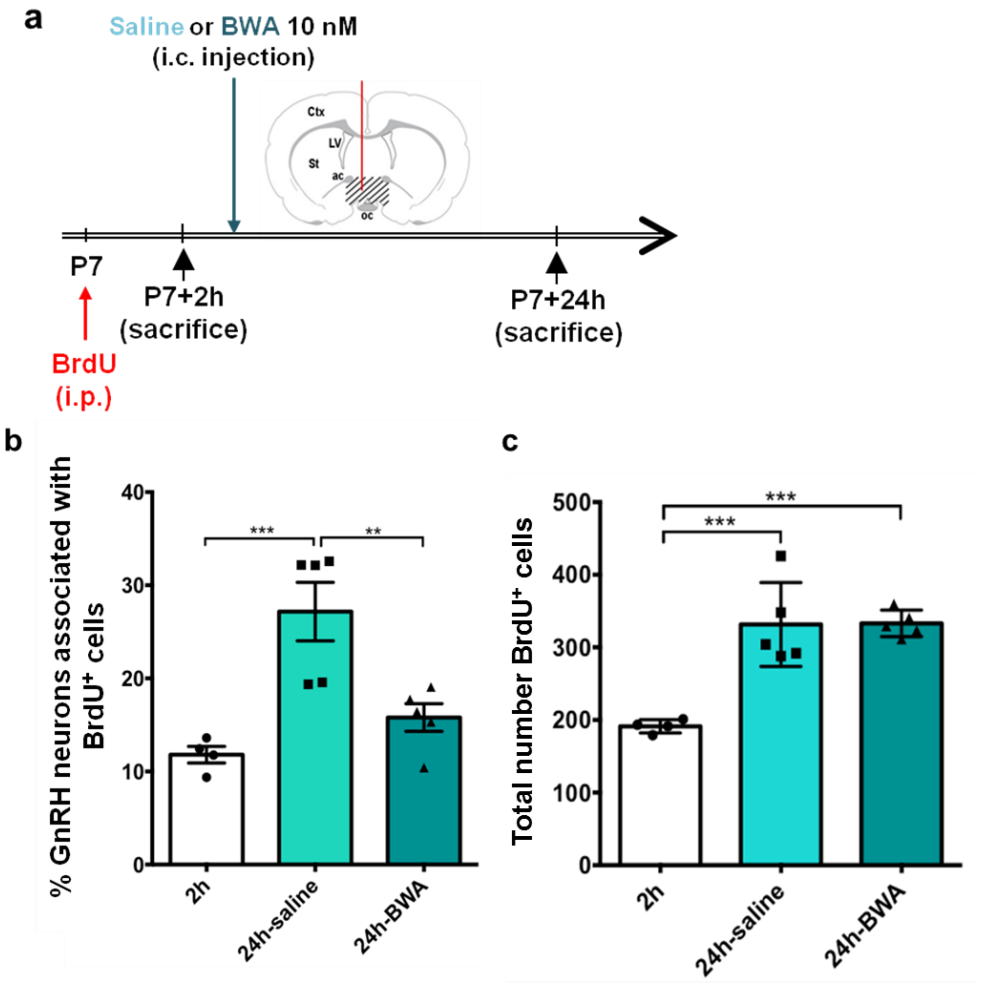


Figure 12. The DP1 signalling promotes the recruitment of newborn cells in the vicinity of GnRH neurons. (a) Schematic of the experimental protocol. The drawing is a coronal view of the rat brain showing the anatomical localization of the injection site, i.e. the POA (dashed area). i.c., intracerebral; i.p., intraperitoneal. (**b**) Quantification of the proportion of GnRH neurons associated with BrdU+ cells, showing that treatment with the DP1 antagonist BWA868C prevents the increased association seen between 2 hours and 24 hours. (**c**) Quantification of the total number of BrdU+ cells in the POA showing that BWA868C treatment does not affect cell proliferation. Mean ± s.e.m.; ** $P < 0.01$; *** $P < 0.001$.

5.3.6 Impairment of DP1 signalling in the POA during the infantile period perturbs the onset of oestrous cyclicity

Finally, whether activation of the DP1 signalling during the infantile period was functionally important for sexual maturation was evaluated.

BWA868C was delivered to the POA of P8 female rats who had just received a single injection of BrdU. A modified protocol was used to allow longer term delivery of the compound (as described in chapter 4.15.2). Animals were monitored for FSH levels at P12, when a peak is known to occur, puberty onset and oestrous cyclicity (Fig. 13a).

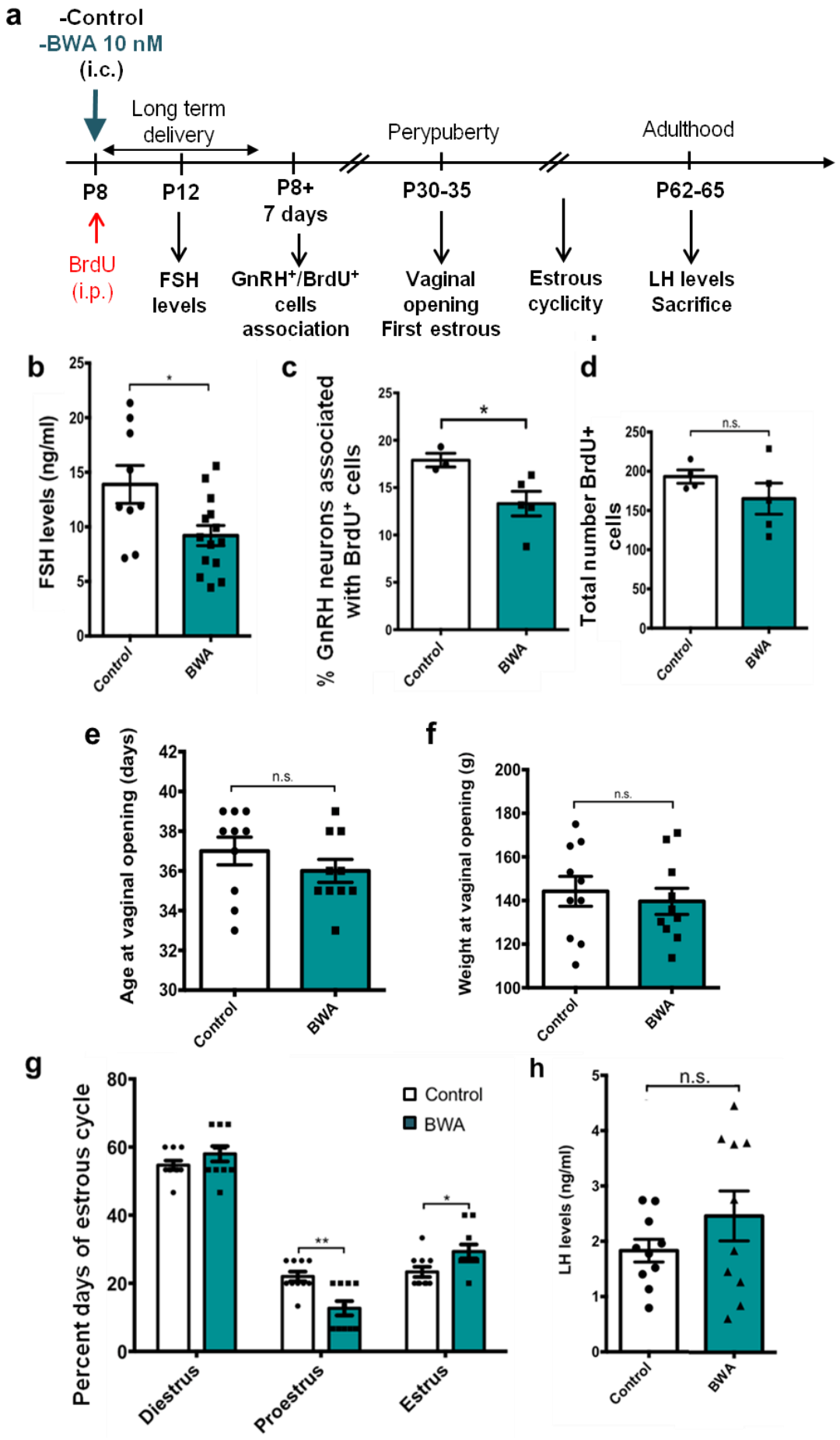
Animals that had received BWA868C showed a decrease in the levels of FSH compared to control animals (Fig. 13b).

In a group of animals sacrificed 7 days after the injection of BrdU (i.e. P15), we evaluated the association between GnRH neurons and $BrdU^+$ cells and observed a decreased proportion of GnRH neurons associated with $BrdU^+$ cells in BWA868C-treated animals compared to controls (Fig. 13c), while the total number of $BrdU^+$ cells in the POA was not affected (Fig. 13d).

When animals were analysed for puberty onset, no difference was seen in the day of vaginal opening or first estrus (Fig. 13e), as well as in the weight on the day of vaginal opening (Fig. 13f).

Monitoring of the oestrus cycle showed that BWA868C-treated animals spent more time in estrus at the expense of the time spent in proestrus during the first 15 days after the onset of puberty compared to control animals (Fig. 13g). After this time window, the cycle became regular. Animals were sacrificed on the day of proestrus (between P62-65) and LH levels were measured (Fig. 13a), confirming no differences between the two groups (Fig. 13h).

Altogether, these results suggest that integrity of the DP1 signalling during the infantile period is important for the recruitment of glial progenitors in the environment of GnRH neurons and for the correct initiation of estrous cyclicity.


a
-Control
-BWA 10 nM
(i.c.)
Long term delivery
Perypuberty
Adulthood
P8
P12
P8+ 7 days
P30-35
P62-65
BrdU (i.p.)
FSH levels
GnRH+/BrdU+ cells association
Vaginal opening First estrous
Estrous cyclicity
LH levels Sacrifice
b
FSH levels (ng/ml)
c
% GnRH neurons associated with BrdU+ cells
d
Total number BrdU+ cells
n.s.
e
Age at vaginal opening (days)
f
Weight at vaginal opening (g)
g
Percent days of estrous cycle
Control
BWA
Diestrus
Proestrus
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h
LH levels (ng/ml)
Control
BWA

Figure 13. Integrity of DP1 signalling during the infantile period is necessary for the correct establishment of the estrus cycle. (a) Schematic of the experimental protocol. **(b)** Circulating FSH levels at P12 are lower in BWA868C-injected animals compared to controls. **(c)** The percentage of GnRH neurons associated with newborn cells 7 days after the injection of BrdU is lower in BWA868C-injected animals, while the total number of BrdU+ cells in the POA is not affected **(d)** compared to control animals. **(e)** The day of vaginal opening and **(f)** the weight on the day of vaginal opening do not change between the two conditions. **(g)** During the first 15 days after vaginal opening, BWA-injected animals spend more time in estrus and less in proestrus compared to controls. After this time window, the cycle goes back to normal, as also seen by LH levels on the day of proestrus, which do not show any difference between BWA868C-treated and contro animals **(h)**.

Chapter 6

Cell neogenesis in the hypothalamus of the adult female rat

6.1 INTRODUCTION

A growing number of studies conducted in various mammalian species have documented both neurogenesis and gliogenesis in the postnatal hypothalamus and have linked them to the regulation of energy homeostasis (Lee et al., 2012; Li et al., 2012; Kokoeva et al., 2005). However, this is only one of the many critical physiological functions regulated by the hypothalamus.

The orchestration of the reproductive axis is another key hypothalamic function, which requires a high degree of structural plasticity between GnRH neurons, the master regulators of reproduction, and their neuronal and non-neuronal partners (Prevot et al., 2010; Ojeda et al., 2010).

Reproduction depends upon female ovulation, which occurs in a cyclic manner. Rats (as well as mice) present a very short cycle that can be divided into 4 stages: 2 days of diestrus, one day of proestrus and a day of estrus. During these stages, ovarian follicles gradually mature and secrete increasing levels of gonadal hormones, which feedback onto the hypothalamus. Between proestrus and estrus, ovulation is induced by a surge of GnRH and LH, which depends on the positive feedback exerted by high levels of ovarian oestrogens.

Neurogenesis and gliogenesis have been previously described in hypothalamic regions controlling reproduction, such as the OVLT, which is found in the POA, where most GnRH neuron cell bodies are found in rodents, or the ME, where GnRH nerve terminals release the GnRH neurohormone into the pituitary portal blood vessels for delivery to the anterior pituitary (Lee et al., 2012; Bennett et al., 2009). These observations raised the possibility that cell neogenesis might participate in mechanisms of plasticity involved in the neuroendocrine regulation of reproduction.

The second aim of my Ph.D. was to map cell proliferation in the adult hypothalamus of female rats at the level of the two main regions involved in the control of GnRH neuron activity, the POA and the ME, throughout the different stages of the estrous cycle, and to explore whether the reproductive status influences cell proliferation.

6.2 RESULTS

6.2.1 Cell proliferation throughout the estrous cycle

In order to evaluate whether cell proliferation is physiologically regulated according to the reproductive status, cell proliferation was studied across the estrous cycle in adult female rats in different regions. Animals (n = 5 per group) received a single injection of BrdU (300 mg/kg body weight) at each stage of the cycle (i.e. diestrus 1, diestrus 2, proestrus and estrus) and were sacrificed 2 hours later to analyse cell proliferation (Fig. 1a).

6.2.1.1 Cell proliferation at the level of the POA

Cell proliferation occurs in the POA (Fig. 1b) of adult female rats. It reaches its peak on the day of diestrus 2, and its lowest levels on the day of estrus (Fig. 1c).

Due to the observation that newborn cells are found in association with GnRH neurons during the infantile period in the POA, this relationship was also evaluated during adulthood. The percentage of $BrdU^+$ cells associated with GnRH neurons was evaluated at each stage of the cycle; however, the values were extremely low and did not show variation throughout the cycle (Fig. 1d).

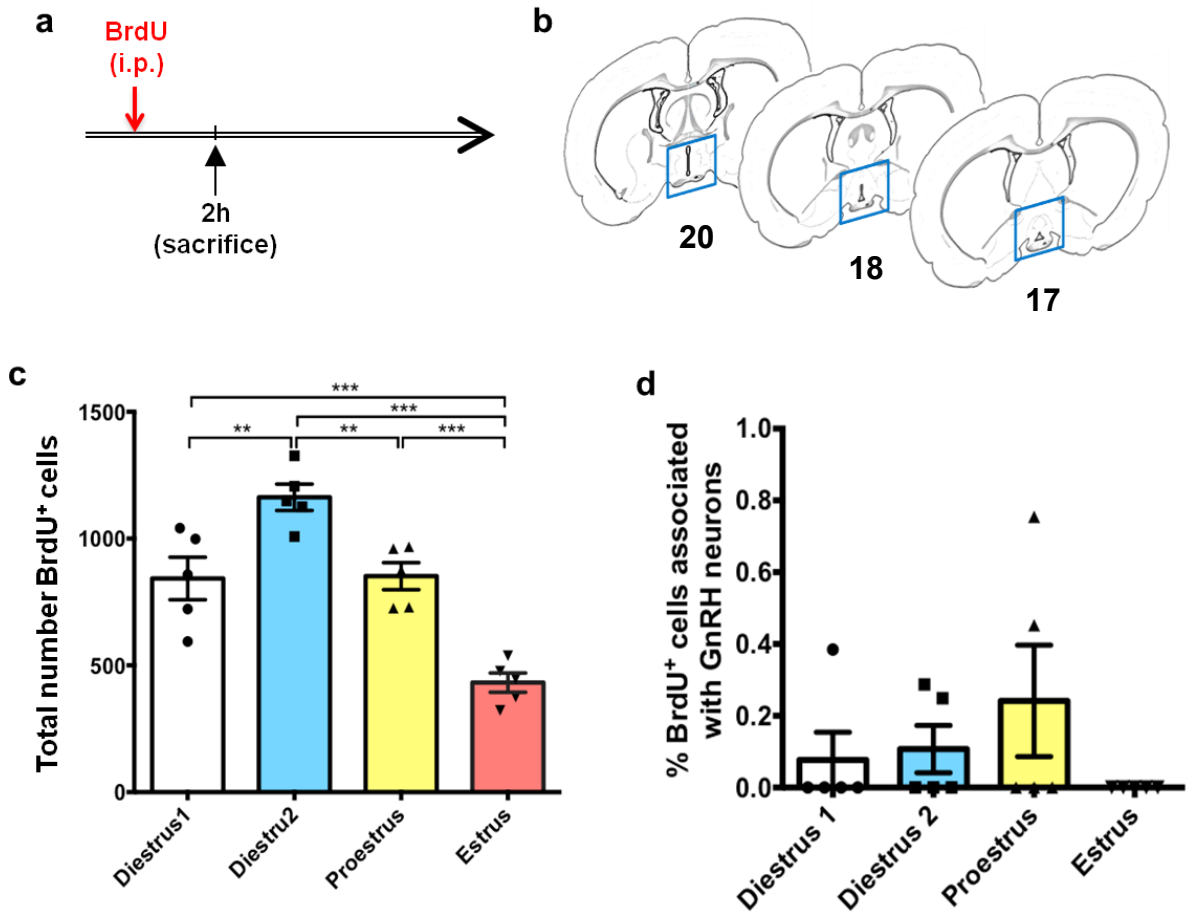

Figure 1. A peak in cell proliferation occurs in the POA of adult female rats on the day of diestrus 2. **(a)** Schematic of the protocol. **(b)** Representative coronal sections of the rat brain, corresponding to levels 17, 18 and 20 of the Swanson brain atlas, to show the area analysed for the quantification. **(c)** The number of BrdU-expressing cells in the POA of adult female rats is highest on the day of diestrus 2 and lowest on the day of estrus. **(d)** $BrdU^+$ cells born during adulthood are not associated with GnRH neurons (n = 5 animals per group). i.p., intraperitoneal. Mean ± s.e.m.; ** $P < 0.01$; *** $P < 0.001$.

6.2.1.2 Cell proliferation at the level of the ME

The GnRH neurons project their terminals to the ME, a region where adult neurogenesis has been previously described in relation to energy homeostasis (Lee et al., 2012).

Analysis of cell proliferation in the ME (Fig. 2a) across the estrous cycle revealed a higher level of proliferation during the day of diestrus 2 when compared to estrus (Fig. 2b).

The association of newborn cells with GnRH neuron terminals in the ME was then evaluated (Fig. 2c). The percentage of $BrdU^+$ cells found associated with GnRH fibers was higher in the

two days of diestrus (27.26 ± 2.49% in diestrus 1 and 29.96 ± 2.76% in diestrus 2) compared to estrus (16.87 ± 1.96%) (Fig. 2d).

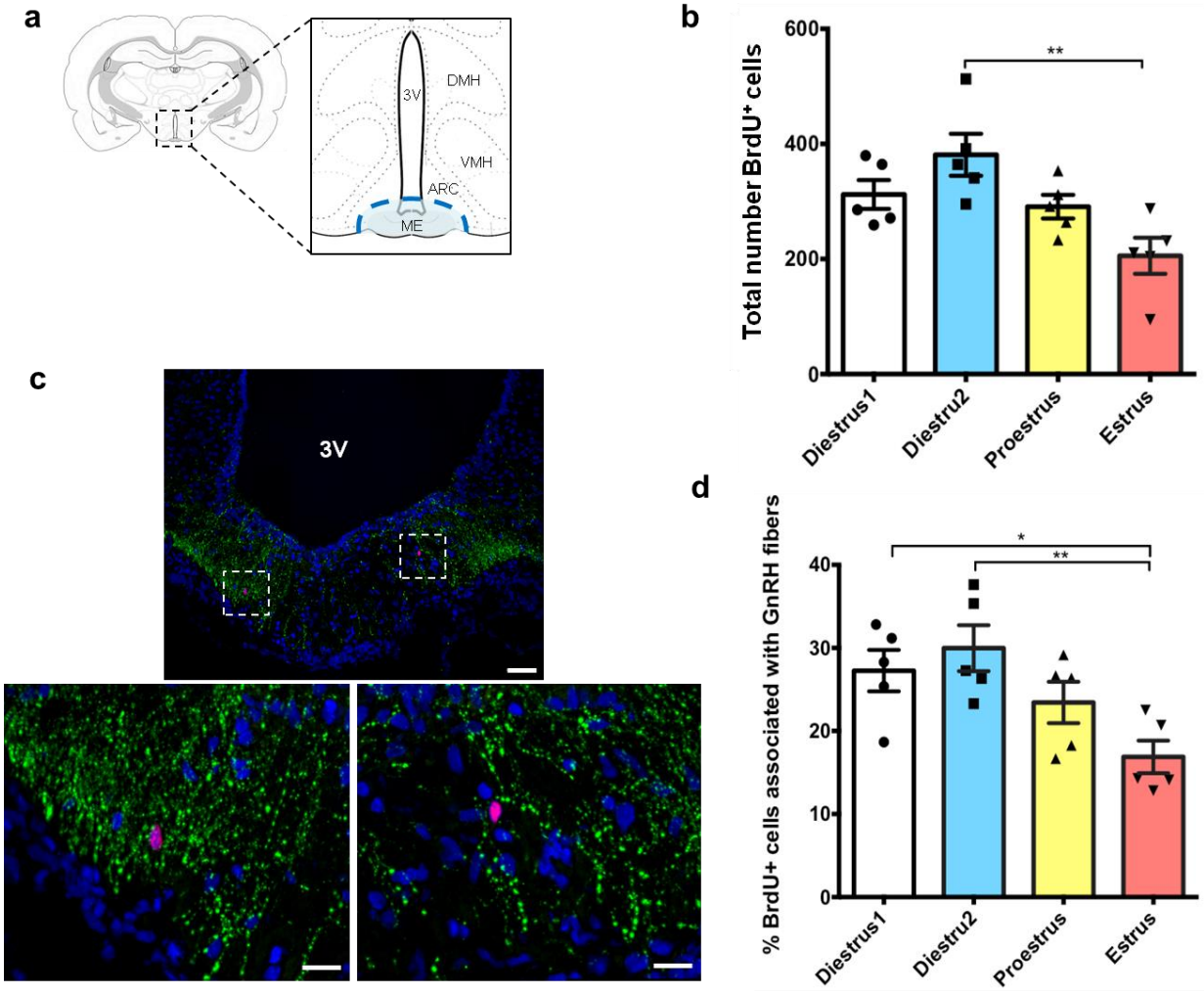

Figure 2. Cell proliferation in the ME of adult female rats across the estrous cycle. (a) Representative coronal section of the rat brain, corresponding to level 29 of the Swanson brain atlas, to show the area analysed for the quantification. **(b)** Proliferation in the ME is higher in diestrus 2 compared to estrus (n = 5 animals per group). **(c)** Coronal section of an animal injected with BrdU and labelled for GnRH (green) and BrdU (red). The lower panels are high-magnification views of the boxed areas in the upper panel. Nuclei were counterstained with Hoechst (blue). **(d)** The percentage of BrdU+ cells associated with GnRH fibers within the ME is higher during the two days of diestrus compared to estrus. Scale bars: 50 µm in c top panel, 20 µm in c bottom panels. Mean ± s.e.m.; * $P < 0.05$; ** $P < 0.01$. ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; 3V, third ventricle.

6.2.1.3 Cell proliferation at the level of the DG

As previously said, the two regions where adult neurogenesis has been widely accepted are the SVZ of the lateral ventricles and the DG of the hippocampus. A previous study reported variations in cell proliferation in the DG of adult female rats across the estrous cycle (Tanapat et al., 1999), therefore this region (Fig. 3a) was also included to the analysis.

As in the ME, proliferation was higher on the day of diestrus 2 compared to the day of estrus (Fig. 3b). These results are discordant with those of Tanapat et al. (1999) who found a peak of proliferation on the day of proestrus. However, this difference could be explained by differences in the quantification method used. In particular, we restricted our quantification to the subgranular zone and quantified the full rostro-caudal extent of the dentate gyrus while Tanapat and colleagues quantified proliferation in the subgranular, granular and hilus regions and they did not specify the extent of the region quantified.

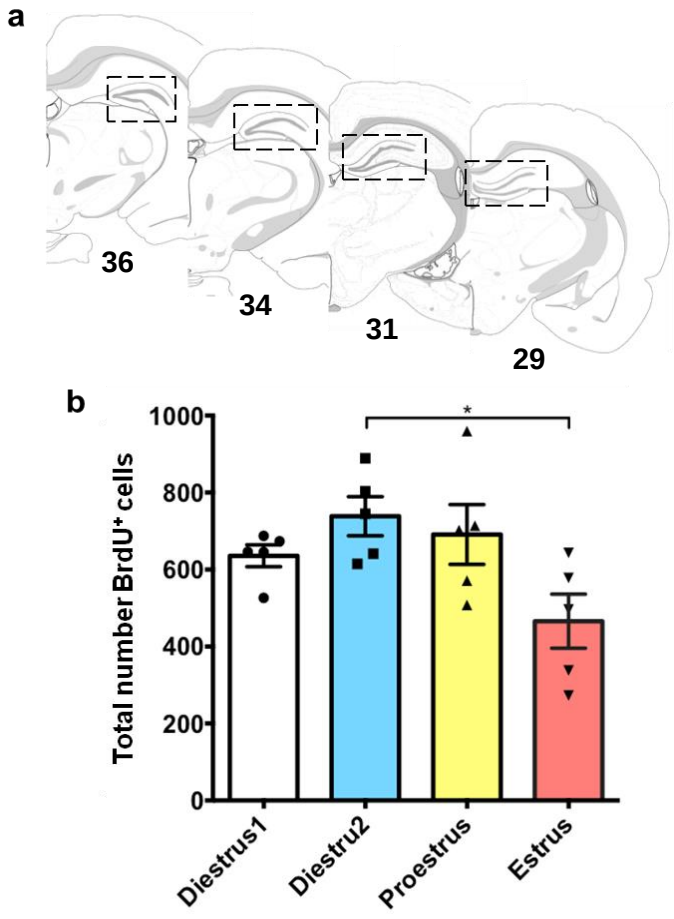

Figure 3. Cell proliferation in the hippocampal dentate gyrus of adult female rats across the estrous cycle. (a) Representative coronal sections of the rat brain, corresponding to levels 29, 31, 34 and 36 of the Swanson brain atlas to show the area analysed for the quantification. **(b)** Proliferation in the DG is higher in diestrus 2 compared to estrus (n = 5 animals per group). Mean ± s.e.m.; * $P < 0.05$.

6.2.2 Pregnancy increases survival of cells born before mating

To analyse the evolution over time of the population of cells born in diestrus 2, animals were injected with BrdU and sacrificed 2 hours, 7, 14 and 21 days later (Fig. 4a). In order to label the entire population of cells born in diestrus 2, the animals received 3 injections of BrdU across the day (100 mg/kg body weight each, at 9 am, 1 pm and 5 pm).

The POA is a complex region composed by different nuclei where different neuronal populations can be found. For instance, postnatal neurogenesis and gliogenesis have been described in hypothalamic regions controlling GnRH function, such as the organum vasculosum of the lamina terminalis (OVLT), where GnRH neurons' cell bodies are found, together with nNOS neurons, known to regulate their activity; as well as at the level of the anteroventral periventricular nucleus (AVPV), which contains kisspeptin neurons responsible for mediating the oestrogen positive feedback onto GnRH neurons (Bennett et al., 2009; Ahmed et al., 2008).

Therefore, cell proliferation was evaluated in the different nuclei of the POA, i.e. the OVLT, the median preoptic nucleus (MEPO), the medial preoptic nucleus (MPO), the AVPV and the anteroventral nucleus (AVP), using the Swanson atlas for anatomical landmarks (Fig. 4b).

At the level of the MPO, the number of newborn cells increased 7 days after the BrdU injections, to drastically decrease during the following two weeks, suggesting that after a period of proliferation, only a fraction of newborn cells survives in the region (Fig. 4c). In the other POA nuclei, the population of $BrdU^+$ cells did not show significant variations over time.

Noteworthy, a decrease in the number of $BrdU^+$ cells can result from a dilution of the BrdU content over successive divisions rather than cell death. However, the BrdU staining in cell nuclei was as intense and homogeneous in animals analysed at 14 and 21 days compared to 7 days. Moreover, mitotic figures were not observed in animals analysed at 14 and 21 days, suggesting that the disappearance of $BrdU^+$ cells in these animals is a consequence of cell death.

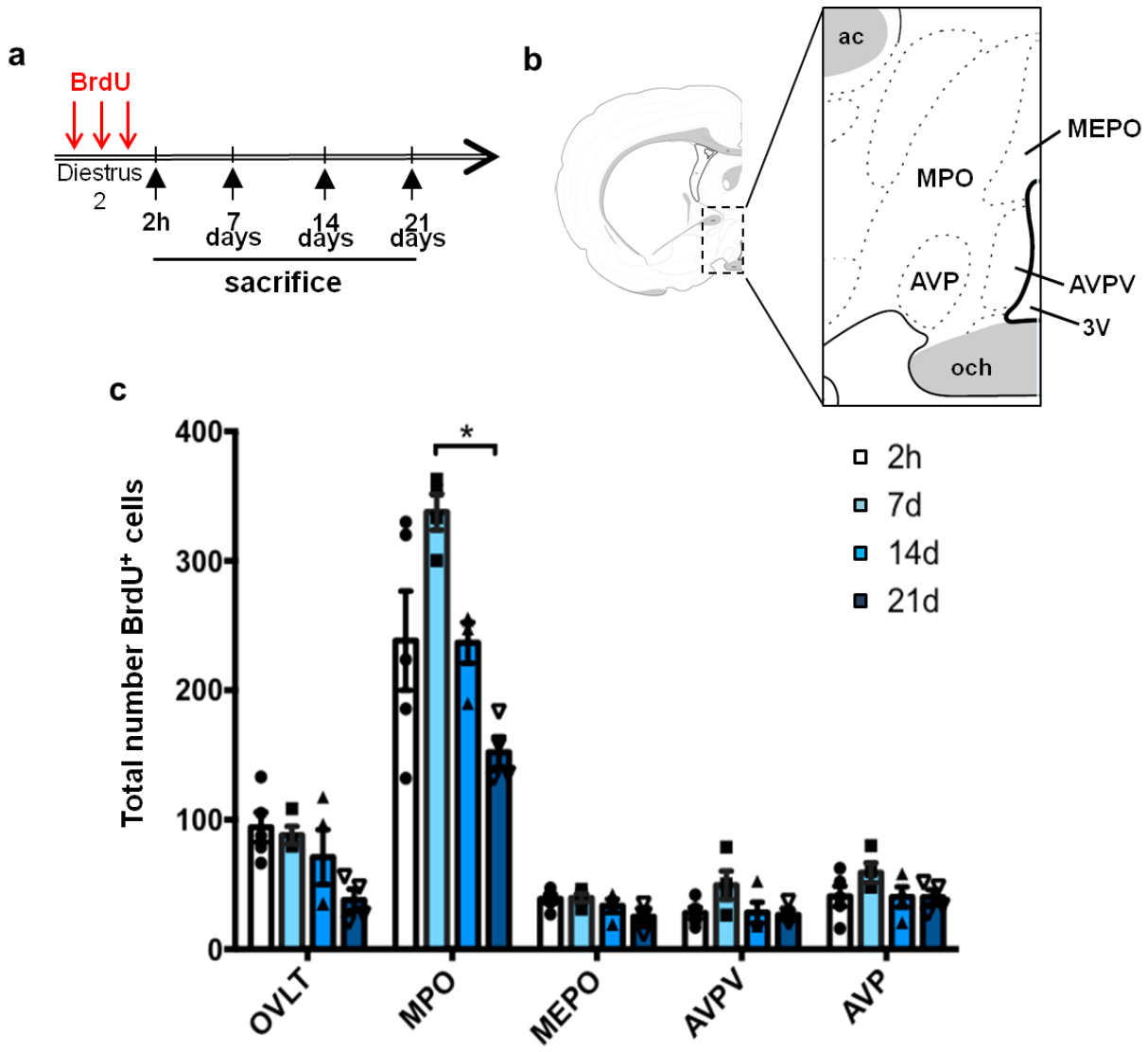

Figure 4. Follow-up of the population of cells born in diestrus 2 in the different POA nuclei. (a) Schematic of the protocol. **(b)** Representative coronal section of the rat brain showing the different nuclei of the POA according to the Swanson brain atlas. **(c)** Quantification of the total number of $BrdU^+$ cells in the different POA nuclei 2 hours, 7, 14 and 21 days after a triple injection of BrdU on the day of diestrus 2. Ac, anterior commissure; AVP, anteroventral nucleus; AVPV, anteroventral periventricular nucleus; MEPO: median preoptic nucleus; MPO, medial preoptic nucleus; och, optic chiasm; OVLT, Organum vasculosum of the lamina terminalis; 3V, third ventricle. Mean ± s.e.m.; * $P < 0.05$.

Among its functions, the MPO is the region involved in the control of maternal behaviour, a process that is established during pregnancy and is indispensable for survival of the offspring. The possibility that cell survival may be influenced by the occurrence of a pregnancy was then addressed.

Animals received 3 injections of BrdU on the day of diestrus 2 and were put in the presence of a male rat on the next day (proestrus). Animals were separated on the day after to make sure that mating would occur directly following the BrdU injections. Animals were then sacrificed 14 or 21 days later and the number of $BrdU^+$ cells within the different nuclei of the POA were compared to non-pregnant animals at the same time points (Fig. 5a).

No differences were observed at the level of the OVLT, MEPO, AVPV and AVP. However, a higher number of cells born on the day of diestrus 2 survived in the MPO when pregnancy occurred, as observed both 14 (Fig. 5b) and 21 days (Fig. 5c) after the BrdU injections. The observation that pregnancy enhances survival of newborn cells selectively in the MPO raises the possibility that these cells may be involved in the plasticity processes necessary for the establishment of maternal behaviour, which are known to develop during pregnancy until birth of the offspring.

One of the neuronal populations found in the MPO and that have been linked to one of the components of maternal behaviour, i.e. maternal aggression, are the nNOS neurons (Gammie & Nelson, 1999), which are also involved in the regulation of GnRH neuron activity (see chapter 2.3.3). For this reason, the association between $BrdU^+$ cells and nNOS neurons was analysed 14 days after BrdU injection. Due to the low number of $BrdU^+$ cells and the high number of nNOS neurons in the POA, only 0.92% ± 0.13 (n = 3 animals) of nNOS neurons were found associated with $BrdU^+$ cells in non-pregnant animals. This proportion was not affected by pregnancy (1.12% ± 0.14 nNOS neurons associated with $BrdU^+$ cells in pregnant animals (n = 3); P = 0.35 vs non-pregnant rats). The percentage of newborn cells associated with nNOS neurons in the MPO was also evaluated. In non-pregnant animals, 12.19% ± 2.57 (n = 3 animals) of $BrdU^+$ cells were associated with nNOS neurons. This proportion was not affected by pregnancy (11.23% ± 2.72 $BrdU^+$ cells associated with nNOS neurons in pregnant animals (n = 3); P = 0.81 vs non-pregnant rats).

These results suggest that if newborn cells regulate the activity of MPO neuronal circuits, they do so by interacting with neurons other than the nNOS neurons.

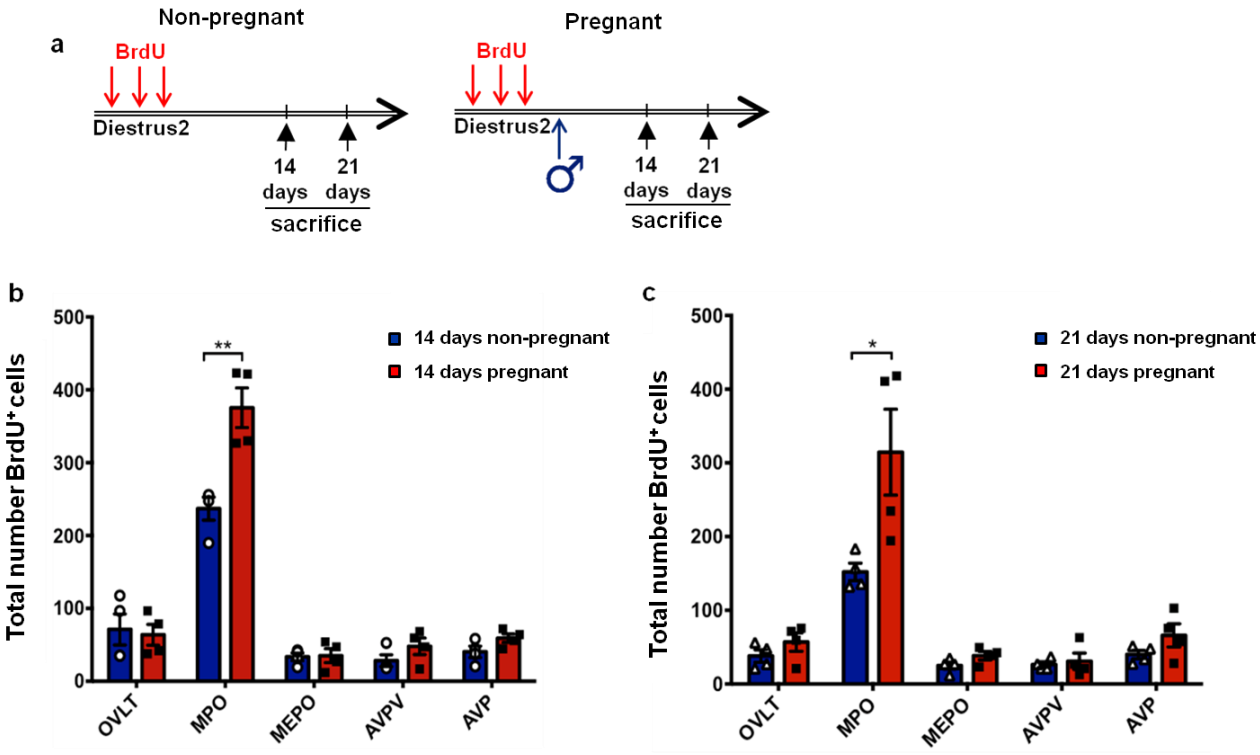

Figure 5. Pregnancy increases survival of newborn cells in the MPO. (a) Schematic of the protocol. 14 **(b)** and 21 **(c)** days after the injections, the total number of $BrdU^+$ cells is higher in the MPO of pregnant rats compared to non-pregnant animals. Mean ± s.e.m.; * $P < 0.05$; ** $P < 0.01$.

Chapter 7

The stem/progenitor cell niche in the adult human hypothalamus

The Journal of Comparative Neurology

A comparative study of the neural stem cell niche in the adult hypothalamus of human, mouse, rat and grey mouse lemur (*Microcebus murinus*)

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Abstract

The adult brain contains niches of neural stem cells that continuously add new neurons to selected circuits throughout life. Two niches have been extensively studied in various mammalian species including humans, the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. Recently, studies conducted mainly in rodents have identified a third neurogenic niche in the adult hypothalamus. In order to evaluate whether a neural stem cell niche also exists in the adult hypothalamus in humans, we performed multiple immunofluorescence labeling to assess the expression of a panel of neural stem/progenitor cell (NPC) markers (Sox2, nestin, vimentin, GLAST, GFAP) in the human hypothalamus and compared them with the mouse, rat and a non-human primate species, the grey mouse lemur (*Microcebus murinus*). Our results show that the adult human hypothalamus contains four distinct populations of cells that express the five NPC markers: i) a ribbon of small stellate cells that lines the third ventricular wall behind a hypocellular gap, similar to that found along the lateral ventricles, ii) ependymal cells, iii) tanycytes, which line the floor of the third ventricle in the tuberal region, and iv) a population of small stellate cells in the suprachiasmatic nucleus. In the mouse, rat and mouse lemur hypothalamus, co-expression of NPC markers is primarily restricted to tanycytes, and these species lack a ventricular ribbon. Our work thus identifies four cell populations with the antigenic profile of NPCs in the adult human hypothalamus, of which three appear specific to humans.

7.1 INTRODUCTION

Neural stem cells persist in the brain of vertebrates beyond development (Doetsch, 2003). In adult mammals, the two best-described neural stem cell niches are the subventricular zone (SVZ) that lines the wall of the lateral ventricles, and the subgranular zone (SGZ) at the base of the hippocampal dentate gyrus (Alvarez-Buylla & Lim, 2004; Ming & Song, 2005). These niches harbor neural stem cells that self-renew throughout life and generate new neurons and glial cells that are functionally integrated into selected circuits and participate in specific brain functions such as learning, memory and odor discrimination (Gonçalves, Schafer, & Gage, 2016; Lledo & Valley, 2016). Almost two decades ago, a study performed in the golden hamster reported for the first time the generation of new neurons in the adult hypothalamus (Huang, DeVries, & Bittman, 1998), a small diencephalic region surrounding the third ventricle and involved in the control of vital processes such as energy metabolism, reproduction, biological rhythms and other homeostatic processes. Since then, a number of studies have accumulated suggesting that neurons and glial cells are generated from local neural stem/progenitor cells (NPCs) in the hypothalamus throughout life (Batailler et al., 2014; Chaker et al., 2016; Gouazé et al., 2013; Haan et al., 2013; Jourdon et al., 2015; Kokoeva, Yin, & Flier, 2005, 2007; Lee et al., 2012; Li, Tang, & Cai, 2012; McNay, Briançon, Kokoeva, Maratos-Flier, & Flier, 2012; Pencea, Bingaman, Wiegand, & Luskin, 2001; Pérez-Martín et al., 2010; Pierce & Xu, 2010; S. C. Robins et al., 2013; Sarah C. Robins et al., 2013). Hypothalamic NPCs are regulated by various physiological factors and pathophysiological conditions (Rojczyk-Gołębiewska, Pałasz, & Wiaderkiewicz, 2014; Sharif, Ojeda, & Prevot, 2014), and hypothalamic neurogenesis, while it occurs at a lower rate than in the two classic niches (Migaud et al., 2010), has functional significance for the control of energy metabolism (Djogo et al., 2016; Gouazé et al., 2013; Kokoeva et al., 2005; Lee et al., 2012, 2014; Li et al., 2012; Li, Tang, Purkayastha, Yan, & Cai, 2014; Pierce & Xu, 2010). Several different, and non-exclusive, hypotheses have been proposed concerning the identity and location of hypothalamic NPCs: some studies suggest that they reside within the hypothalamic parenchyma (Kokoeva et al., 2007; Li et al., 2012; Sarah C. Robins et al., 2013), some that they are subependymal astrocytes located in a region of the mid third ventricle (Pérez-Martín et al., 2010), and others that they are a subpopulation of tanycytes, ependymoglial

cells that line the ventral portion of the third ventricle (Chaker et al., 2016; Haan et al., 2013; Jourdon et al., 2015; Lee et al., 2012; S. C. Robins et al., 2013).

Most of our knowledge of adult neural stem and progenitor cells comes from rodent models, and few studies have been conducted in humans. Neuroanatomical studies and the recently developed technique of ^{14}C dating have confirmed that cells exhibiting neural stem cell properties and neurogenesis persist in the adult human brain (Bergmann, Spalding, & Frisén, 2015). While the hippocampal neurogenic system in adult humans exhibits features similar to that of rodents, i.e. the local generation of dentate granule neurons from nearby neural stem cells (Eriksson et al., 1998; Ernst et al., 2014; Knoth et al., 2010; Spalding et al., 2013), the SVZ neurogenic system appears to differ significantly between human and rodents. Not only does the cellular organization of the niche differ (Quiñones-Hinojosa et al., 2006; Sanai et al., 2004), but newborn neurons adopt a different fate, migrating into the adjacent striatum to become medium spiny neurons (Ernst et al., 2014) instead of migrating long distances along the rostral migratory stream to become part of the olfactory bulb circuit (Bergmann et al., 2012) as in rodents (Ming & Song, 2005).

A recent neuroanatomical study has revealed the presence of cells expressing the neuroblast marker doublecortin (DCX) in the adult human hypothalamus (Batailler et al., 2014), raising the possibility that neurogenesis, and a neural stem cell niche, may exist in this region in humans. While the expression of the NPC markers nestin and vimentin has been reported in the adult human hypothalamus (Baroncini et al., 2007; Dahiya, Lee, & Gutmann, 2011; Nogueira et al., 2014), there has been no detailed mapping of the expression of a repertoire of NPC markers in the adult human hypothalamus as yet.

Here, we carried out a neuroanatomical characterization of the expression of a combination of NPC markers in the adult human hypothalamus, along with the SVZ and SGZ. To evaluate potential inter-species variability, the expression of NPC markers in the adult hypothalamus was concomitantly analyzed in the mouse, rat and a non-human primate species, the grey mouse lemur (*Microcebus murinus*).

7.2 MATERIALS AND METHODS

7.2.1 Tissue

Human

Tissues were made available in accordance with French bylaws (Good Practice Concerning the Conservation, Transformation and Transportation of Human Tissue to be Used Therapeutically, published on December 29, 1998). Permission to use human tissues was obtained from the French Agency for Biomedical Research (Agence de la Biomédecine, Saint-Denis la Plaine, France, protocol no. PFS16-002).

A total of nine brains were obtained from autopsies (average age 81 years, range 60–92 years; average postmortem delay 23 hours, range 8–41.5 hours). The brain samples were taken from patients that donated bodies to Science in compliance with the French laws on bioethics. Structures were identified by reference to an atlas of the human brain (Mai, Paxinos, & Voss, 2008).

One 11-week old (menstrual age) human fetus was obtained after elective abortion from the Gynaecology Department of the University Hospital of Lille. Written informed consent had been previously obtained from the parent.

One *de novo* glioblastoma multiforme (GBM) sample was obtained from the Neurosurgery Clinic of the University Hospital of Lille. The GBM specimen was obtained at initial surgery of a 65-year old male. The tumor was classified and graded by a neuropathologist according to the World Health Organization system (Louis et al., 2007). Written informed consent had been previously obtained from the patient and/or his family members. The tissue was processed as described previously (Duhem-Tonnelle et al., 2010).

Rodents

Animal studies were approved by the Institutional Ethics Committee 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). Tissues were obtained from 7 adult female Sprague-Dawley rats (2-3 months, Janvier Laboratories, Saint Berthevin, France) and 3 adult female mice (3-4 months, Charles River, L'Arbresle, France). The animals were maintained on a 12-hour light/12-hour dark cycle (lights on at 07.00 h), with food and water available *ad*

libitum.

Grey mouse lemur (Microcebus murinus)

Tissues were obtained from one male (1.9 year-old) and one female (5.9 year-old) grey mouse lemur (*Microcebus murinus*) bred in the laboratory breeding colony of Brunoy (MNHN, France, license approval N° A91.114.1). The two animals were sacrificed during the long day period (14-hours of light/day), which corresponds to the period of reproductive activity. Mouse lemur studies were approved by the Cuvier Ethics Committee for the Care and Use of Experimental Animals of the Muséum National d'Histoire Naturelle (n°2083-2015090311335786).

7.2.2 Fluorescent immunostaining

Human

After whole brain removal, blocks of 20 mm per side encompassing the hypothalamus were harvested with the optic chiasm as the anterior limit and the mammillary bodies as the posterior limit. The hippocampus was collected from a cut posterior to the mammillary bodies. The dorso-lateral and ventral walls of the body of the lateral ventricles were collected on a width of about 5 mm towards the parenchyma. Tissue blocks were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 1 week at 4°C, cryoprotected in PB containing 0.9% sodium chloride (PBS) and sucrose at 10% for 48 hours followed by 20% for 3 days, embedded in Tissue Tek® (Sakura Finetek, Villeneuve d'Ascq, France) and frozen in isopentane cooled with liquid nitrogen. Coronal cryostat sections (14 or 20 μm) were mounted on chrome-alum-gelatin coated slides, air-dried, and subjected to immunofluorescent stainings. After rehydration in a solution of sodium citrate 0.01 M (pH 6) (citrate buffer), the sections were subjected to microwave pretreatment in citrate buffer for 4 minutes at 800 W followed by 2 cycles 4 minutes each at 400 W for antigen retrieval. After cooling at room temperature and washing in PB, sections were incubated for 15 minutes in PBS containing 0.3% Triton X-100 and 2% normal donkey serum (PBSTS) at room temperature to block the nonspecific sites. Sections were then incubated with primary antibodies diluted in PBSTS for one to two nights at 4°C in a humid chamber. The characteristics of the primary antibodies used are provided in Table 1. After rinsing in PB, secondary antibodies diluted in PBSTS were deposited on sections and incubated at room

temperature for 1 hour. The characteristics of the secondary antibodies used are provided in Table 2. After washing in PB, cell nuclei were stained with Hoechst 33258 bis-Benzimide (Molecular Probes, Eugene, OR, USA) diluted at 1/1000 in PB for 2 minutes and then washed in PB. To avoid the strong autofluorescence caused by lipofuscin granules usually present in adult human brain tissue, sections were immersed in a solution of 0.3% Sudan Black B (Sigma, St Quentin Fallavier, France) in 70% ethanol for 5 minutes (Romijn et al., 1999). This treatment completely blocked autofluorescence. After washing in PB, sections were coverslipped in Mowiol mounting medium (20% Mowiol, 2.5% DABCO in Tris 0.2 M pH 8.5). Control sections were incubated in the absence of primary antibody. Omission of the primary antibody resulted in no staining.

The human fetus was immersion fixed in 4% paraformaldehyde for 4 days at 4°C, cryoprotected in 30% sucrose/PBS overnight at 4°C, and then processed and subjected to immunofluorescent stainings as described above.

Rat

Animals were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg). They were perfused transcardially with a rinse of saline solution (0.9% NaCl), followed by 500 ml of 4% paraformaldehyde in 0.1 M PB (pH 7.4). The brains were removed and immersed in the same fixative for 2-3 hours at 4°C. They were then transferred to PB containing 20% sucrose until they had sunk, embedded in Tissue Tek® (Sakura Finetek, Villeneuve d'Ascq, France), and frozen in liquid nitrogen. Frozen 14-µm coronal sections were prepared using a cryostat (CM3050, Leica) and collected throughout the full extent of the third ventricle. The sections were mounted onto chrome-alum-gelatin coated slides, air-dried and subjected to immunofluorescent stainings as described above. Anatomical landmarks were determined using the rat brain atlas of Swanson (Swanson, 2004).

Mouse

Animals were anesthetized by intraperitoneal injection of xylazine 10 mg/kg (Rompun 2%, Bayer) and ketamine 200 mg/kg (Ketalar, Parke-Davis), perfused transcardially with a rinse of saline solution, followed by 100 ml of 4% paraformaldehyde in 0.1 M PB (pH 7.4). After brain removal, post-fixation and freezing, 30 µm free-floating coronal sections encompassing the full extent of the third ventricle were prepared using a cryostat and collected in ice-cold PB.

Free-floating sections were subjected to immunofluorescent stainings following the above-described procedure, mounted on chrome-alum-gelatin coated slides, air-dried and coverslipped in Mowiol mounting medium. Anatomical landmarks were determined using the mouse brain atlas from Paxinos and Franklin (Paxinos & Franklin, 2001).

Grey mouse lemur (Microcebus murinus)

Animals were anesthetized by intraperitoneal injection of pentobarbital 5 mg/kg, perfused transcardially with a rinse of saline solution, followed by 120 ml of 4% paraformaldehyde in 0.1 M PB (pH 7.4). After brain removal, post-fixation and freezing, frozen 14-μm coronal sections were prepared using a cryostat and collected throughout the full extent of the third ventricle. The sections were mounted onto chrome-alum-gelatin coated slides, air-dried and subjected to immunofluorescent stainings as described above. Anatomical landmarks were determined using the *Microcebus murinus* brain atlas of Bons (Bons, Silhol, Barbié, Mestre-Francés, & Albe-Fessard, 1998).

7.2.3 Antibody characterization

Human

We used human fetal cerebral cortex and GBM as positive control tissues to verify the specificity of primary antibodies in human tissues.

In the fetal cerebral cortex, the SRY (sex determining region Y)-box 2 (Sox2) antibody (goat polyclonal # sc-17320 from Santa Cruz) yielded a nuclear staining with decreasing density from the ventricular to the pial surface (Fig. 1a), as reported by others (Vinci et al., 2016). This antibody detects a single band of 34 kDa on Western blots of human neural progenitor and GBM stem-like cell lysates (Guichet et al., 2013). Moreover, immunoreactivity is lost when human embryonic stem cells are treated with a small interfering RNA (siRNA) directed against Sox2 (Adachi, Suemori, Yasuda, Nakatsuji, & Kawase, 2010). In the adult hypothalamus, a double labeling with another Sox2 antibody (mouse monoclonal # ab79351 from Abcam, 1/300) showed an overlap of the two stainings (Fig. 1i, j).

In agreement with previous reports, the nestin antibody stained radial glial cells in the fetal cerebral cortex (Fig. 1e) (Vinci et al., 2016), tumoral cells in the GBM specimen (Fig. 1h) (Ludwig & Kornblum, 2017) and hypothalamic adult tanycytes (Fig. 6) (Baroncini et al., 2007).

This antibody detects a band at 177 kDa, the expected molecular weight, on Western blots of human GBM stem-like cell lysates (Ciechomska, Przanowski, Jackl, Wojtas, & Kaminska, 2016). Moreover, inhibition of nestin expression using short hairpin RNA or siRNA transfection in a human lung adenocarcinoma cell line results in decreased immunolabeling (Narita et al., 2014).

Vimentin was detected in radial glial cells in the fetal cerebral cortex (Fig. 1c), as described elsewhere (Howard, Chen, & Zecevic, 2006; Vinci et al., 2016). It was strongly expressed in adult tanycytes (Fig. 6b) in agreement with other reports (Baroncini et al., 2007; Koopman, Taziaux, & Bakker, 2017). The M0725 antibody from Dako detects a band at 54 kDa on Western blots of human mesenchymal stromal cell lysates (Talele, Fradette, Davies, Kapus, & Hinz, 2015). Treatment of a human cholangiocarcinoma cell line with siRNA against vimentin suppressed immunoblot and immunofluorescent signals with this antibody (Saentaweesuk et al., 2017). The two anti-vimentin antibodies used yielded a similar expression profile.

We had previously used the Glial Fibrillary Acidic Protein (GFAP) antibody (rabbit polyclonal Z0334 from Dako) to stain cerebro-cortical (Duhem-Tonnelle et al., 2010) and hypothalamic (Baroncini et al., 2007) astrocytes in the adult human brain, and tumoral cells and reactive astrocytes in human GBM (Duhem-Tonnelle et al., 2010). This antibody stained radial glial cells in the fetal cerebral cortex (Fig. 1f, g), as described by others (Howard et al., 2006) and it detects a single band at 50 kDa on Western blots of human GBM cell lysates (Tardito et al., 2015). A co-immunolabeling with another anti-GFAP antibody (mouse monoclonal MAB3402 from Chemicon) previously used by others to stain the ribbon of SVZ astrocytes (Quiñones-Hinojosa et al., 2006; Sanai et al., 2004) yielded a strict co-localization (Fig. 1k, l). The expression profile observed with the two antibodies along the lateral ventricles replicates the pattern of mRNA expression determined by *in situ* hybridization (Allen brain atlas; http://human.brain-map.org/ish/experiment/show/159107343), with a labeling of ependymal, subependymal and small parenchymal cells.

The Glutamate-Aspartate Transporter (GLAST) antibody labeled radial glial cells in the fetal cerebral cortex (Fig. 1d) following an expression profile described elsewhere (Howard et al., 2006). The expression profile observed along the lateral ventricles replicates the pattern of mRNA expression determined by *in situ* hybridization, with a strong enrichment in ependymal and ribbon cells, as well as scattered parenchymal cells (Allen brain atlas;

http://human.brain-map.org/ish/experiment/show/160133315). This antibody detects a single band at 65 kDa on Western blots of human cerebrocortical homogenates (Roberts, Roche, & McCullumsmith, 2014). Based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), we determined the GLAST immunogen peptide (ab127026, Abcam) sequence to be from amino acid 522-541 (data not shown).

The Ki67 antibody has been used previously to stain proliferative cells in human GBM (Duhem-Tonnelle et al., 2010). It yielded a dense nuclear staining in the fetal cerebral cortex following a gradient with the highest density of positive cells in the ventricular and subventricular zones (Fig. 1b), as described elsewhere (Bayatti et al., 2008; Harkin et al., 2016). In the adult human brain, the Ki67 antibody produced a patchy staining in the nucleus of cells, some of which exhibited mitotic figures (Fig. 8). This antibody detects a band at 360 kDa on Western blots of primary human dermal fibroblast cell lysates (Chierico et al., 2017). The immunoblot signal is lost following siRNA-mediated knock-down of Ki67 in various human cell lines (Sun et al., 2017).

The Neuronal Nuclei (NeuN) antibody has been used previously to stain mature neurons in the adult human cerebral cortex (Duhem-Tonnelle et al., 2010) and hippocampus (Knoth et al., 2010). This antibody detects a doublet at 46-50 kDa, the expected apparent molecular weight, in Western blots of human hippocampus autopsy homogenates (Engel et al., 2011).

The Vasoactive intestinal peptide (VIP) antibody has been used previously to immunostain neurons in the monkey and human enteric nervous system (Noorian, Taylor, Annerino, & Greene, 2011) and produced a staining pattern in the suprachiasmatic nucleus (Fig. 7a) similar to that described in the literature with other antibodies (Hofman, Zhou, & Swaab, 1996; Wang et al., 2015) or using *in situ* hybridization (van Wamelen et al., 2013).

The CD68 antibody has been used previously by others to stain microglia in the human brain (Monier, Evrard, Gressens, & Verney, 2006). As expected for a lysosomal membrane protein, it yielded a cytoplasmic punctuate staining (Fig. 8g). This antibody detects bands at 110, 70, 40 kDa in Western blots of human lung, spleen and U937 monocytic cell line extracts using reducing conditions. An additional band is detected at 220 kDa in spleen extracts using non-reducing conditions (Pulford et al., 1989).

Rodent

The Sox2 antibody (goat polyclonal # sc-17320 from Santa Cruz) yielded a strong staining in the SVZ (not shown) and in the hypothalamus (Fig. 9a, e, i, Fig. 10a, e) that recapitulated expression patterns previously described with other antibodies in the mouse (Haan et al., 2013; Li et al., 2012) and rat (Bennett, Yang, Enikolopov, & Iacovitti, 2009; Hoefflin & Carter, 2014). It also replicates the pattern of mRNA expression determined by *in situ* hybridization in the adult mouse brain (Allen brain atlas; http://mouse.brain-map.org/experiment/show/77280331). This antibody detects a single band at 34 kDa in Western blots of mouse embryonic stem cell lysates (manufacturer's technical information). Moreover, it yields an immunostaining in a mouse mesenchymal stem cell line stably overexpressing Sox2 by retroviral infection while no staining is observed in non-infected cells (Ding et al., 2012).

The nestin antibody stained tanycytes and capillaries (Fig. 9b, f, j, Fig. 10f), as previously reported in the mouse (Alliot, Rutin, Leenen, & Pessac, 1999; Haan et al., 2013; Lee et al., 2012) and rat (Bennett et al., 2009; Takamori et al., 2009). This expression profile replicates the pattern of mRNA expression determined by *in situ* hybridization in the adult mouse brain (Allen brain atlas; http://mouse.brain-map.org/experiment/show/1387). This antibody detects a doublet in the 200-220 kDa range on Western blots of rat brain homogenates (Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014). Moreover, rat cardiac ventricular fibroblasts infected with a lentivirus containing a shRNAmir directed against nestin show reduced immunocytofluorescent and immunoblot signals (Béguin, Gosselin, Mamarbachi, & Calderone, 2012).

Vimentin was detected in ependymal cells, tanycytes and capillaries (Fig. 9c, g, inset in j, Fig. 10b, h), as previously reported in the mouse (Langlet, Mullier, Bouret, Prevot, & Dehouck, 2013; Mullier, Bouret, Prevot, & Dehouck, 2010) and rat (Pérez-Martín et al., 2010). The two anti-vimentin antibodies used yielded a similar expression profile. This expression profile replicates the pattern of mRNA expression determined by *in situ* hybridization in the adult mouse (Allen brain atlas; http://mouse.brain-map.org/experiment/show/79907904) and rat brain (Helfer et al., 2016). The Dako antibody detects a single band at 54 kDa on Western blots of rat hepatic stellate cell lysates (Mezaki et al., 2013).

Microcebus murinus

The specificity of the Sox2 and vimentin antibodies was inferred from the similarity in the expression pattern with rodents. Noteworthy, we tested 2 anti-nestin antibodies, MAB1259 from R&D Systems and MAB353 clone rat-401 from Millipore, recognizing human and rodent nestin, respectively, but none of them yielded any staining in the grey mouse lemur.

7.2.4 Histological staining

For anatomical identification of hypothalamic regions and nuclei, coronal cryostat sections were immersed in a solution of Toluidine Blue dissolved at 0.5% in a solution of 1% sodium borate for 6 minutes at room temperature. After three rinses in water, sections were dehydrated in successive alcoholic solutions: 2 times 5 minutes in ethanol 70°, 7 minutes in ethanol 70° containing 0.2% acetic acid, 2 times 5 minutes in ethanol 100° and 2 times 3 minutes in xylene. Sections were coverslipped in Eukitt mounting medium (Sigma).

7.2.5 Microscopy

Analysis of sections and acquisition of images were done using an Axio Imager Z2 Apo-Tome microscope equipped with a motorized stage (Zeiss, Germany) and an ORCA-Flash 4.0 V2 camera (Hamamatsu, Japan) driven by the Zen imaging software (Zeiss). Specific filter cubes were used for the visualization of green (Alexa 488 fluorochrome) (Excitation wavelength: 475/40 nm, beam splitter: 500 nm, emission wavelength: 530/50 nm), red (Alexa 568 fluorochrome) (excitation wavelength: 550/25 nm, beam splitter: 570 nm, emission wavelength: 605/70 nm), far red (Alexa 647 fluorochrome) (excitation wavelength: 640/30 nm, beam splitter: 660 nm, emission wavelength: 690/50 nm) and blue fluorescence (Hoechst) (excitation wavelength: 365 nm, beam splitter: 395 nm, emission wavelength: 445/50 nm). High magnification photomicrographs represent maximal intensity projections derived from 15–52 triple or quadruple-ApoTome images collected using the z-stack module of the Zen imaging software and a Zeiss 20x (numerical aperture NA = 0.8) or 40x objective (NA = 0.75). All images were captured in a stepwise fashion over a defined z-focus range corresponding to all visible fluorescence within the section and consistent with the optimum step size for the corresponding objective and the wavelength (500 nm). To create photomontages, ApoTome images were captured using the z-stack module coupled to the MosaiX module of the Zen imaging software and a Zeiss 5x (NA = 0.15) or 20x objective for

each filter sequentially. Images to be used for figures were pseudocolored, adjusted for brightness and contrast and merged using Photoshop (Adobe Systems, San Jose, CA).

7.2.6 Analysis

Human

Co-expression of nestin and Sox2 was systematically analyzed on sections distributed across the antero-posterior extent of seven hypothalami. Then, triple stainings for nestin/Sox2/vimentin, nestin/Sox2/GLAST, nestin/Sox2/GFAP, nestin/Sox2/Ki67 and vimentin/Sox2/NeuN were performed on representative sections. Double and triple immunostainings were performed on a total of 73 ± 6 (mean ± SEM) (range, 47 – 94) sections per specimen.

Quantification of stainings in the hypothalamic ependymal layer, ribbon cells and buds was performed on the seven specimens. A total of 2044 ± 226 (mean ± SEM) ependymal cells, 2117 ± 423 (mean ± SEM) ribbon cells and 836 ± 260 (mean ± SEM) bud cells were counted per specimen. Noteworthy, the border of the third ventricle was usually less well preserved at its floor, where tanycyte cell bodies are found, thus precluding the quantification of stainings in this cell type. Quantification of stainings in the suprachiasmatic nucleus was made on three hypothalami. While the rodent suprachiasmatic nucleus appears as a high cell density spherical structure clearly delimited after counterstaining for cell nuclei, it is not the case in the human. The localisation of the human suprachiasmatic nucleus was determined using an atlas (Mai et al., 2008) and further verified by an immunostaining for VIP, which is produced by a subpopulation of suprachiasmatic neurons (Hofman et al., 1996). For quantitative analysis of the stainings, a total of 2931 ± 235 (mean ± SEM) cells were counted on five to seven sections per suprachiasmatic nucleus.

Expression of NPC markers in the dentate gyrus of the hippocampus was analyzed on specimens collected from three brains. Double and triple immunostainings were performed on a total of 20 ± 4 (mean ± SEM) (range, 12 – 26) sections per specimen.

The SVZ was analysed on samples collected from six brains. Three samples were collected in the dorsal portion of the body of the lateral ventricle, 4 in its ventral portion and 3 in the temporal horn. Notably, three ventral SVZ samples were present in the tissue block containing the third ventricle, enabling comparison between these regions on the same

sections. Double and triple immunostainings were performed on a total of 33 ± 10 (mean ± SEM) (range, 12 – 94) sections per specimen.

Rat

Co-immunolabelings of nestin, Sox2 and vimentin were performed and analysed on 42-204 sections per animal regularly spaced between plate 17 (i.e. the anterior-most part of the third ventricle above the optic chiasm) and plate 38 (i.e., posterior-most part of the third ventricle and end of mammillary nuclei) of the rat brain atlas from Swanson (Swanson, 2004).

Mouse

Triple nestin/Sox2/vimentin immunostainings were performed and analysed on 9-12 sections per animal regularly spaced between plate 34 (i.e. a region containing the optic chiasm and the anterior part of the suprachiasmatic nucleus) and plate 53 (i.e., posterior-most part of the third ventricle and mammillary nuclei) of the mouse brain atlas from Paxinos and Franklin (Paxinos & Franklin, 2001).

Microcebus murinus

Co-expression of vimentin and Sox2 was analysed on the male and female specimens on a total of 78 and 60 sections, respectively, encompassing the rostro-caudal extent of the third ventricle, i.e. plate 39 to 51 of the *Microcebus murinus* brain atlas of Bons (Bons et al., 1998).

7.2.7 Statistics

Differences in the percentage numbers of NPC marker-expressing cells between the different cell populations were analyzed by One Way Analysis of Variance (ANOVA) using the SigmaStat software. The level of significance was set at $p<0.05$.

7.3 RESULT

7.3.1 The human hypothalamus

For the neuroanatomical analysis, the hypothalamus was antero-caudally subdivided into four regions: the anterior-most region surrounding the preoptic recess (Fig. 2a), the anterior hypothalamic area containing the optic chiasm, the anterior commissure, the paraventricular and supraoptic nuclei (Fig. 2b), the tuberal region containing the ventromedial, dorsomedial, infundibular nuclei and the median eminence (Fig. 2c, d), and the mammillary region containing the mammillary bodies and the posterior hypothalamic nucleus (Fig. 2e) (Mai et al., 2008).

In order to identify putative NPCs, we first mapped the co-expression of the NPC markers nestin and Sox2 in the whole hypothalamus (Fig. 3) and then performed triple stainings for nestin, Sox2 and vimentin, GLAST or GFAP in representative regions (Fig. 4, 5, 6, 7 and Table 3). A similar expression profile was found in the seven hypothalami analyzed. Expression of nestin and Sox2 was primarily detected in a region bordering the third ventricle (Fig. 3). Deeper in the parenchyma, labeling for Sox2 was absent while labeling for nestin was only seen in capillary vessels (Fig. 3, asterisks). Ependymal cells, which line the ventricle, showed intense Sox2 (Fig. 3, 4), low to high vimentin (Fig. 4a, c) and intense GLAST (Fig. 4h, j) immunoreactivity in their vast majority, while expression of nestin (Fig. 4b, i, p) and GFAP (Fig. 4o, q) was less ubiquitously detected (Table 3). Co-expression of nestin and Sox2 was detected in 32.1 ± 4.0% of ependymal cells. Among the population of nestin/Sox2 co-expressing ependymal cells, 96.8 ± 1.4% expressed vimentin, 98.9 ± 0.3% expressed GLAST and 81.1 ± 7.1% expressed GFAP, implying that at least 77% of nestin/Sox2-positive cells also expressed vimentin, GLAST and GFAP. Ependymal cells often invaginated into the underlying parenchyma to form crowns (Fig. 3f) or rosettes (Fig. 3b-e). Heterogeneity in the antigenic profile and organization of ependymal cells was not specific to a particular area of the third ventricle.

A ribbon of small process-bearing cells expressing nestin, Sox2, vimentin, GLAST and GFAP consistently lined the border of the ventricle, separated from it by a hypocellular gap (Fig. 3a_1, a_3, b, c, d, Fig. 4a, h, o, table 3). The ribbon was variable in thickness, from one (Fig. 3a_1) to multiple cell layers (Fig. 3a_3), as well as the gap that ranged from ~20 µm (Fig. 3a_1) to ~100-150 µm (Fig. 3d). Noteworthy, the hypocellular gap was consistently at its largest

extent in the mammillary region (Fig. 3d). Co-expression of nestin and Sox2 was detected in 18.0 ± 3.7% of ribbon cells. Nestin/Sox2 co-expressing ribbon cells frequently sent long processes contacting the ependymal layer (Fig. 3b, inset) and/or capillaries (Fig. 3g). Triple co-immunolabelings showed that nestin/Sox2 co-expressing ribbon cells also expressed vimentin (Fig. 4e-g), GLAST (Fig. 4l-n) and GFAP (Fig. 4s-u) in 83.8 ± 4.7%, 87.7 ± 4.2% and 80.9 ± 9.4% of them, respectively, implying that at least 52% of nestin/Sox2-positive cells also express vimentin, GLAST and GFAP. The hypocellular gap contained nestin- and vimentin-expressing processes (Fig. 3 and 4), presumably corresponding to ependymal and/or ribbon cell processes, and was enriched in GLAST (Fig. 4h) and GFAP (Fig. 4o) immunoreactivity. The gap and ribbon were devoid of NeuN-immunoreactive neurons. NeuN-immunoreactive cells were primarily found in the parenchyma at distance from the subependymal region (not shown) and occasionally in the proximal parenchyma underlying the subependymal region (Fig. 5j-l).

Notably, some portions of the third ventricular wall were devoid of the gap-and-ribbon organization but exhibited a thick border enriched in intermingled nestin/Sox2-positive cells (Fig. $3a_2$) sometimes bulging into the ventricle (Fig. $3a_4$, g, h) and showing systematic interruption of the ependymal layer. These buds were highly variable in size and shape, from long and flat (Fig. $3a_2$, a_4) to ovoid and protuberant (Fig. 3g, h), with frequent strengthening in nestin staining (Fig. 3h). They contained a rich network of NPC marker-expressing cells (Table 3) but were devoid of NeuN-immunoreactive cells (Fig. 5j-l). Co-expression of nestin and Sox2 was detected in 46.8 ± 5.5% of bud cells. Among the population of nestin/Sox2 co-expressing cells, 72.3 ± 7.8% expressed vimentin (Fig. 5a-c), 98.5 ± 1.3% expressed GLAST (Fig. 5d-f) and 95.5 ± 2.1% expressed GFAP (Fig. 5g-i), implying that at least 66% of nestin/Sox2-positive cells also express vimentin, GLAST and GFAP. These structures were found anywhere along the ventricular wall of all hypothalami examined.

In the tuberal region, the floor of the third ventricle does not exhibit the gap-and-ribbon organization and is lined by tanycytes whose processes extend into the median eminence (Fig. 6a). Co-immunolabelings showed that Sox2, nestin, vimentin, GLAST and GFAP were expressed by tanycytes (Fig. 6). While nestin and vimentin, GLAST or GFAP were frequently co-expressed in tanycyte processes, long processes expressing each marker separately were also seen in variable proportions between the different specimens.

While nestin and Sox2 expression was mainly restricted to the proximal border of the third ventricle, an exception was seen in the suprachiasmatic nucleus (Fig. 2a, b, Fig. 7a), which contained a population of small stellate nestin/Sox2 co-expressing cells at distance from the ventricle (Fig. 7b). The five NPC markers were detected in the suprachiasmatic nucleus (Table 3). Eleven ± 2.0% of suprachiasmatic cells co-expressed nestin and Sox2, and among this population, 74.7 ± 10.4% expressed vimentin (Fig. 7c-e), 80.9 ± 5.5% expressed GLAST (Fig. 7f-h) and 65.6 ± 10.6% expressed GFAP (Fig. 7i-k), implying that at least 21% of nestin/Sox2-positive cells also express vimentin, GLAST and GFAP. Since Sox2 expression was previously reported to co-localise with neuronal markers in the rodent suprachiasmatic nucleus (Hoefflin & Carter, 2014), we performed a triple staining for Sox2, GLAST and NeuN to evaluate whether NPC markers could be found in NeuN-expressing neurons. NeuN was only expressed in a small fraction of suprachiasmatic neurons (Fig. 7m), as already observed in rats (Geoghegan & Carter, 2008). We found no evidence of GLAST/Sox2 co-expression in NeuN-positive neurons (Fig. 7l, m).

Proliferative cells, identified by their expression of the cell cycle marker Ki67, were detected near the third ventricle border in variable proportions in 6/7 hypothalami. We identified only two nestin/Sox2-positive cells that co-expressed Ki67 in one specimen in the ribbon and at the level of a bud (Fig. 8a-e). Most Ki67-labeled cells co-expressed the microglia marker CD68 (Fig. 8f-h).

Comparison of the stainings between the different cell populations showed that the fraction of Sox2-positive cells that co-expressed nestin was higher in the ribbon (63.2 ± 3.4%) compared to ependymal (39.3 ± 4.1%) and suprachiasmatic cells (37.3 ± 4.8%) (ANOVA with Tukey post-hoc test, $p<0.01$). However, when the population of nestin/Sox2-positive cells was considered, the fraction that co-expressed vimentin, GFAP or GLAST was not different between the ribbon, the ependymal layer and the suprachiasmatic nucleus (Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's post-hoc test, $p>0.05$). In buds, the population of nestin/Sox2-positive cells was enriched (ANOVA with Tukey post-hoc test, $p<0.001$) and the fraction of nestin/Sox2-positive cells that also expressed GLAST was higher compared to the ribbon and the suprachiasmatic nucleus (Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's post-hoc test, $p<0.05$).

7.3.2 The rodent and grey mouse lemur hypothalamus

The organization of the third ventricular border significantly differed between human and rodents. The rodent third ventricle wall did not exhibit the hypocellular gap and ribbon of NPC marker co-expressing cells (Fig. 9a-k). Co-expression of nestin, Sox2 and vimentin was essentially restricted to the tanycytes with the exception of very occasional ependymal cells (Fig. 9a-k and Table 4). Noteworthy, significant differences were seen between the mouse and rat third ventricle wall. While the mouse ventricular border was composed of a regular layer of ependymal cells (Fig. 9a, e), the rat one was much more irregular with thickenings and evaginations of the ependymal layer frequently protruding in the dorsal part of the ventricle (Fig. 9i). The mouse (Fig. 10a-d) and rat (Fig. 10e-h) suprachiasmatic nucleus contained a population of Sox2-positive cells that did not co-express nestin nor vimentin.

The organization and expression profile of the grey mouse lemur third ventricle region was reminiscent of that found in mice. The third ventricle wall was composed of a regular layer of ependymal cells devoid of a gap-and-ribbon organization (Fig. 9l, o). Co-expression of Sox2 and vimentin was detected in ependymal cells (Fig. 9l-n) and tanycytes (Fig. 9o-q and Table 4). The suprachiasmatic nucleus contained Sox2-positive cells that did not co-express vimentin (Fig. 10i-k).

7.3.3 The human SVZ and the dentate gyrus of the hippocampus

In parallel with the analysis of the human hypothalamus, we evaluated the expression of NPC markers in the two classical neurogenic niches, the SVZ and the hippocampal dentate gyrus.

The SVZ exhibited a similar pattern of NPC marker expression as the third ventricle, with a ribbon of small process-bearing cells co-expressing nestin, Sox2, vimentin, GLAST and GFAP (Fig. 11 and Table 3), separated from the ependymal layer by a hypocellular gap enriched in GLAST (Fig. 11i, k) and GFAP immunoreactivity (Fig. 11m, o). Ependymal cells expressed Sox2, GLAST, and, at varying levels, vimentin, nestin and GFAP (Fig. 11 and Table 3). This expression profile was observed at the different rostro-caudal and dorso-ventral levels of the lateral ventricle analyzed. However, differences were noted compared to the third ventricle. The SVZ exhibited a more uniform organization, with a more regular ribbon and a thinner gap compared to the third ventricle (e.g. compare Fig. 11a-d to Fig. 3b-d). While buds similar to those found in the third ventricle were observed (not shown), crowns

and rosettes of ependymal cells were not seen. With regard to the expression of NPC markers, detection of nestin in ependymal cells was much rarer than in the third ventricle while strong vimentin immunoreactivity was more consistently observed in these cells (Fig. 11e, g).

In the hippocampus, a population of radial stem cells has been described in the SGZ of the dentate gyrus in several species including rodents and non-human primates (Aizawa, Ageyama, Terao, & Hisatsune, 2011; Gonçalves et al., 2016). We failed to detect Sox2-expressing cells in the dentate gyrus and nestin immunoreactivity was restricted to capillaries (Fig. 12a). The lack of Sox2 staining was not due to a technical detection problem since Sox2 expression was readily observable on the same sections along the border of the temporal horn of the lateral ventricle in ependymal and ribbon cells (Fig. 12a, inset). No GLAST immunoreactivity was observed in the dentate gyrus region (not shown). Moreover, while GFAP and vimentin were detected in small stellate cells close to the dentate gyrus, they were not seen in subgranular cells with a radial morphology (Fig. 12b, c).

7.4 DISCUSSION

Studies conducted in rodents have shown that the adult hypothalamus contains a neurogenic niche, with local NPCs that give birth to glial and neuronal cells in the nearby hypothalamic parenchyma (Chaker et al., 2016; Haan et al., 2013; Li et al., 2012; S. C. Robins et al., 2013; Sarah C. Robins et al., 2013). In the adult human hypothalamus, the recent identification of cells expressing the neuroblasts marker DCX, some of which exhibit a morphology characteristic of very immature neuronal progenitors (Batailler et al., 2014), raised the possibility that a local niche of NPCs may exist within this structure. Here we identify in the adult human hypothalamus four populations of cells, ependymal cells, ventricular ribbon cells, tanycytes and suprachiasmatic small stellate cells, which exhibit an antigenic profile of NPCs. In rodents and grey mouse lemur, a similar antigenic profile is essentially restricted to tanycytes.

In order to identify NPCs in the human hypothalamus, we used a series of five markers known to be expressed in these cell types during embryogenesis and adulthood (Götz, Sirko, Beckers, & Irmler, 2015; Kriegstein & Alvarez-Buylla, 2009; Kuhn, Eisch, Spalding, & Peterson, 2016; Vinci et al., 2016). At present, a single marker specific for neural stem cells is not available since markers found in neural stem cells are also expressed in progenitors and/or mature cells. For instance, this is the case for GFAP, which is expressed both in neural stem cells and mature astrocytes, or nestin, which is expressed in neural stem cells, neural progenitors and endothelial cells (Kuhn et al., 2016; von Bohlen und Halbach, 2011). Higher specificity can however be obtained by using a combination rather than a single marker. In the two main neurogenic niches, the SVZ and the SGZ, neural stem cells have been shown to express a repertoire of markers including GFAP, nestin, Sox2, GLAST, vimentin, BLBP and Pax6 (Kriegstein & Alvarez-Buylla, 2009; von Bohlen und Halbach, 2011). Therefore, we performed triple immunostainings for nestin, Sox2 and vimentin, GLAST or GFAP and we considered that cell populations expressing all markers were the best candidates for being considered as neural stem or progenitor cells (Kuhn et al., 2016). Noteworthy, technical constraints preclude the concomitant detection of the five markers of interest at the same time. However, our quantitative analysis showed that at least a fraction of nestin/Sox2-positive cells also expressed vimentin, GLAST and GFAP. Interestingly, GFAP discriminates neural stem cells from neural progenitors in the two classical niches in rodents since neural

stem cells express GFAP but neural progenitors lose its expression (Doetsch, 2003). In addition, a recent study in mouse suggested that among tanycytes, a subpopulation of GFAP-positive alpha tanycytes exhibited neural stem cell properties (S. C. Robins et al., 2013). Therefore, if the repertoire of markers expressed by rodent neural stem cells is conserved in humans, it may be speculated that the nestin/Sox2/vimentin/GLAST/GFAP-immunoreactive ribbon, ependymal, suprachiasmatic cells and tanycytes are neural stem cells.

The ribbon of NPC marker-immunoreactive small stellate cells lining the human third ventricle is highly reminiscent of the ribbon of astrocytes described by Sanai and colleagues along the human lateral ventricles (Sanai et al., 2004). In their study, the authors identified a band of stellate cells separated from the ependyma by a hypocellular gap. SVZ ribbon cells expressed GFAP, vimentin and some of them were immunoreactive for Ki67. Importantly, by preparing astrocyte cultures from intraoperative specimens, they showed that SVZ astrocytes were capable of generating multipotent, self-renewing neurospheres, suggesting that they were *bona fide* neural stem cells. In the present study, and in agreement with Sanai and colleagues, the SVZ ribbon lining the lateral ventricles was found to express GFAP, vimentin as well as the other NPC markers Sox2, nestin and GLAST. Given the similar morphology and NPC marker expression profile between SVZ ribbon cells and hypothalamic ribbon cells, it may be speculated that hypothalamic ribbon cells exhibit neural stem cell properties as their SVZ counterpart. Noteworthy, the hypothalamic ribbon appears to be a characteristic feature of the human brain since it was not observed in the rodent or grey mouse lemur hypothalamus and has not been previously reported in the literature in other species. A striking difference between the lateral and the third ventricular border was the higher degree of irregularity in the aspect of the third ventricular border that exhibited frequent invaginations of ependymal cells, highly variable width of the gap and thickness of the ribbon. Such heterogeneity may explain why the hypothalamic ribbon was not previously identified from small samples of the human third ventricular wall (Sanai et al., 2004). In both the lateral and the third ventricular wall, we observed occasional bulges of the wall characterized by an interruption of the ependymal layer and enrichment in NPC markers. Such protrusions are described by neuropathologists as nodular gliosis (or ependymal granulations or nodular ependymitis). Even though they have been associated with central nervous system infection or ventriculomegaly (Johnson & Johnson, 1972; Shook et al., 2014), they are also observed in non pathological brains and occur at a similar rate in young (20-40

years) and aged brains (60 and over) (Gilmore & Bouldin, 2002; Johnson & Johnson, 1972). The suggested origin for these structures is a proliferation of subependymal glial cells (Gilmore & Bouldin, 2002) as a consequence of reactive gliosis. Reactive gliosis involves several characteristic features including cellular hypertrophy, up-regulation of GFAP expression (Sofroniew, 2005) and can be accompanied by the re-expression of NPC markers such as nestin and vimentin (Goc, Liu, Sisodiya, & Thom, 2014; Götz et al., 2015). While the ventricular protrusions were highly enriched in NPC marker-expressing cells, these cells did not exhibit signs of hypertrophy and the expression of GFAP in protrusions was as intense as in the nearby hypocellular gap lying behind an intact ependyma. Moreover, NPC marker-expressing cells found in protrusions appear to be in continuity with adjacent ribbon cells. Therefore, our observations raise the possibility that protrusions may originate from the local proliferation of ribbon cells rather than reactive astrocytes. Along the same line, ventricular protrusions are not observed in mice (Shook et al., 2014), which lack a ventricular ribbon.

Co-expression of NPC markers was also detected in a substantial fraction of human third ventricle ependymal cells. Whether ependymal cells have neural stem cell properties has been a matter of debate since almost two decades but recent rodent studies suggest that they do not (discussed in (Pastrana, Silva-Vargas, & Doetsch, 2011). Accordingly, and in agreement with *in situ* expression profile data (Allen brain atlas), rodent third ventricle ependymal cells showed ubiquitous and robust Sox2 and vimentin immunoreactivity, while most of them exhibited low to undetectable levels of nestin. Notably, studies using different expression profiling strategies and/or different antibodies (Chaker et al., 2016; Chen, Wu, Jiang, & Zhang, 2017; Lin et al., 2015; Mirzadeh et al., 2017; S. C. Robins et al., 2013) have reported variable results as to the presence of nestin in ependymal cells of the rodent third ventricle. Whether these apparently discrepant results reflect the existence of several alternative splicing-derived isoforms and/or post-translational modifications, some of them being more specific to stem cells, remains to be determined. Another population of NPC marker co-expressing cells seen in the human hypothalamus but not in the other species was found in the suprachiasmatic nucleus. In mammals, the suprachiasmatic nucleus is the endogenous masterclock that coordinates endogenous rhythms with the external light-dark cycle thereby controlling circadian physiology and behaviour. We also found a rich population of Sox2-expressing cells in the mouse, rat and grey mouse lemur suprachiasmatic

nucleus. However, these cells did not co-express nestin and/or vimentin, suggesting that they are not NPCs. Accordingly, a previous study showed that Sox2-positive cells in the rodent suprachiasmatic nucleus mostly co-expressed neuronal markers (Hoefflin & Carter, 2014). In the human suprachiasmatic nucleus, we did not find evidence for the expression of GLAST and Sox2 in NeuN-positive mature neurons. It must be noted that NeuN immunostaining is weak and only reveals a small fraction of suprachiasmatic nucleus neurons, as previously shown in the rat (Geoghegan & Carter, 2008), calling for caution in the interpretation of data. However, the morphological characteristics of NPC-marker expressing cells, ie, small stellate, argue in favour of a non-neuronal identity. Altogether, the observation of cells with the same NPC antigenic profile as ribbon cells in the human ependymal layer and suprachiasmatic nucleus raises the intriguing possibility that these regions may contain neural stem cells.

Tanycytes are highly specialized ependymoglial cells that line the floor and the baso-lateral walls of the third ventricle in the tuberal region of the hypothalamus. Tanycytes have recently emerged as hypothalamic neural stem cells (Goodman & Hajihosseini, 2015). In agreement with previous studies (Bennett et al., 2009; Haan et al., 2013; Lee et al., 2012; Pérez-Martín et al., 2010; S. C. Robins et al., 2013), we here confirm that rodent tanycytes express NPC markers and expand these results to the grey mouse lemur concerning the two markers analysed, Sox2 and vimentin. In contrast to the ventricular ribbon that is only seen in humans, our study and others show that mice, rats, sheep (Batailler et al., 2014), grey mouse lemur and humans (Baroncini et al., 2007; Koopman et al., 2017; Sidibe et al., 2010) share NPC marker-expressing tanycytes. In adult mice, lineage-tracing studies have shown that tanycytes give birth to neurons that populate nearby hypothalamic regions including the arcuate and ventromedial nuclei (Chaker et al., 2016; Haan et al., 2013; S. C. Robins et al., 2013). Interestingly, DCX-expressing cells are detected in the arcuate and ventromedial nuclei of the sheep and human hypothalamus (Batailler et al., 2014), raising the possibility that tanycytes may also be neurogenic in these species.

At present, the organization of the rodent hypothalamic stem cell niche remains vague. While we find in the human hypothalamus a gap-and-ribbon organization reminiscent of that found in the human SVZ (Sanai et al., 2004), the situation is different in rodents in which the architecture of the SVZ stem cell niche is not recapitulated in the hypothalamus (Mirzadeh, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008). Lineage-

tracing studies performed in adult mice have shown that new hypothalamic neurons can be produced not only from tanycytes but also from parenchymal cells that express Sox2 and/or NG2 (Li et al., 2012; Sarah C. Robins et al., 2013). Whether tanycytes and parenchymal neurogenic cells correspond to distinct NPC populations or whether they are linked by lineage relationship is currently unknown. However, if the combination of markers expressed in the adult rodent SVZ and SGZ (Semerci & Maletic-Savatic, 2016) is shared by the hypothalamic niche, our observation of NPC marker co-expression in tanycytes but not in parenchymal cells suggests that tanycytes are neural stem cells while parenchymal cells are more committed progenitors.

Neuroanatomical (Eriksson et al., 1998; Knoth et al., 2010) and, more recently, ^{14}C dating studies (Spalding et al., 2013), have brought evidence that a neurogenic niche exists in the human hippocampus. Here, we failed to detect a population of NPC marker-expressing cells in the dentate gyrus of the human hippocampus. In an extensive immunofluorescent study of neurogenesis-associated markers in the human hippocampus, Knoth and colleagues (2010) did not find radial cells, the putative neural stem cells in the rodent hippocampus (von Bohlen und Halbach, 2011) but detected Sox2/DCX and nestin/DCX co-expressing cells, i.e. early neuronal precursors, in humans up to 79 and 100 years of age, respectively. However, the expression of these markers declined with age. Since our study was conducted on samples from aged brains ($\geq$ 77 year-old), we cannot rule out the possibility that we missed these cells if present in very low number.

In the present study, our criteria for identifying NPCs were based on their molecular signature. However, proving that these cells are *bona fide* neural stem cells will require functional arguments, which is challenging in human studies. Neural stem cells are defined by two canonical properties, the ability to extensively self-renew and to differentiate into all three neural lineages, i.e. neuronal, astroglial and oligodendroglial. Even if it has some limitations, the neurosphere assay is commonly used to probe *in vitro* whether a tissue contains cells with neural stem cell properties (Pastrana et al., 2011). A study reported the possibility to grow neurospheres from rapid autopsy samples of SVZ from elderly human subjects (Leonard et al., 2009). A key to success certainly resides in the short post-mortem interval (less than 5 hours in the study by (Leonard et al., 2009) and may explain our inability to grow neurospheres from hypothalami with post-mortem intervals over 7 hours, the shortest post-mortem delay reached in our study (unpublished observations). Future

neuroimaging developments enabling to detect and measure levels of adult neurogenesis in living human brains (Ho, Hooker, Sahay, Holt, & Roffman, 2013) are eagerly awaited.

Comparative studies of adult neurogenesis in the two classical niches have revealed a great diversity in this process among species (Lindsey & Tropepe, 2006). The general view that adult neurogenesis has undergone a phylogenetic reduction has recently been re-evaluated to propose that this process may rather have evolved to optimize the functioning of neuronal networks that have to deal with new situations (Kempermann, 2012). Even though we need comparative data from many more species to start to draw a phylogenetic tree of adult hypothalamic neurogenesis, our results raise the intriguing possibility that the human brain may have expanded its hypothalamic neural stem cell niche compared to rodents and lemur primates. Whether this translates into higher functionally-relevant neurogenesis and a higher degree of plasticity and adaptability in hypothalamic circuits will be challenging future questions to address.

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Table 1. Primary antibodies used in the study.

Antigen	Host & Clonality	Dilution	Reference (Company)	Immunogen
Sox2	goat polyclonal	1/300	sc-17320 (Santa Cruz)	Peptide corresponding to amino acids 277-293 of human Sox2 (YLPGAEVPEPAAPSRLH), affinity-purified serum (Poché, Furuta, Chaboissier, Schedl, & Behringer, 2008).
Nestin †	mouse monoclonal	1/400	MAB1259 (R&D Systems)	NS0 mouse myeloma cell line transfected with human N estin
Nestin‡	mouse monoclonal	1/500	MAB353 clone rat-401 (Millipore)	Nestin purified from embryonic rat spinal cord
Vimentin	mouse monoclonal	1/300	M0725 clone V9 (Dako)	Purified vimentin from porcine eye lens
Vimentin	chicken polyclonal	1/500	AB5733 (Millipore)	Recombinant Golden Syrian hamster vimentin
GFAP	rabbit polyclonal	1/500	Z0334 (Dako)	GFAP isolated from cow spinal cord
GFAP	mouse monoclonal	1/500	MAB3402 (Chemicon)	Purified glial filament
GLAST	rabbit polyclonal	1/300	ab416 (Abcam)	Synthetic peptide corresponding to amino acids 522-541 of rat EAAT1 (PYQLIAQDNEPEKPVADSET)$.
Ki67	rabbit polyclonal	1/100	ab15580 (Abcam)	Synthetic peptide conjugated to KLH corresponding to amino acids 1222-1234 of human Ki67 (C-EDLAGFKELFQTP).
CD68	mouse monoclonal	1/300	M0814 clone KP1 (Dako)	Lysosomal fraction of human lung macrophages
NeuN	mouse monoclonal	1/300	MAB377 (Chemicon)	Purified cell nuclei from mouse brain
VIP	goat polyclonal	1/300	sc-21041 (Santa Cruz)	16-Residue synthetic peptide representing the carboxy-terminal domain of human VIP from amino acids 125–140

†antibody used for immunostainings on human tissue; ‡antibody used for immunostainings on rodent tissue. CD68, cluster of differentiation 68; EAAT1, Excitatory Amino Acid Transporter 1 (synonymous GLAST, Glutamate-Aspartate Transporter); GFAP, Glial Fibrillary Acidic Protein; NeuN, Neuronal Nuclei; Sox2, SRY (sex determining region Y)-box 2; VIP,

Vasoactive intestinal peptide. [$]Based on liquid chromatography coupled to tandem mass spectrometry performed in this study.

Table 2. Secondary antibodies used in the study.

Species	**Conjugated**	**Dilution**	**Reference (Company)**
Donkey anti-mouse IgG	Alexa 568	1/500	A10037 (Molecular Probes)
Donkey anti-mouse IgG	Alexa 488	1/500	A21202 (Molecular Probes)
Donkey anti-rabbit IgG	Alexa 568	1/500	A10042 (Molecular Probes)
Donkey anti-rabbit IgG	Alexa 488	1/500	A21206 (Molecular Probes)
Donkey anti-goat IgG	Alexa 568	1/500	A11057 (Molecular Probes)
Donkey anti-goat IgG	Alexa 647	1/500	A21447 (Molecular Probes)
Donkey anti-chicken IgG	Alexa 488	1/500	703-545-155 (Jackson Immunoresearch)

Table 3. Summary of the expression of NPC markers in the adult human hypothalamus, SVZ, parenchyma and hippocampal dentate gyrus.

	Hypothalamus						***SVZ***				***Parenchyma***		***Dentate gyrus***	
	ependymal cells	**gap**	**ribbon cells**	**buds**	**tanycytes**	**SCh**	**ependymal cells†**	**gap**	**ribbon cells**	**buds**	**endothelial cells**	**astrocytes ‡**	**endothelial cells**	**astrocytes ‡**
Sox2	+ (84.5 ± 2.3%)	+	+ (29.0 ± 5.4%)	+ (58.3 ± 6.1%)	+	+ (30.6 ± 4.0%)	+	+	+	+	-	-	-	-
nestin	+ (38.1 ± 4.1%)	+	+ (37.0 ± 3.7%)	+ (67.7 ± 4.9%)	+	+ (16.4 ± 1.3%)	+/--	+	+	+	+	-	+	-
vimentin	+ (83.6 ± 3.8%)	+	+ (49.0 ± 2.7%)	+ (57.6 ± 5.7%)	+	+ (38.8 ± 3.4%)	++/-	+	+	+	+	+/-	+	+/-
GLAST	+ (97.1 ± 0.7%)	++	+ (80.6 ± 3.6%)	+ (97.6 ± 1.1%)	+	+ (28.0 ± 7.5%)	+	++	+	+	-	+/-	-	-
GFAP	+ (60.9 ± 8.0%)	++	+ (78.0 ± 9.5%)	+ (92.6 ± 3.0%)	+	+ (42.7 ± 13.0%)	+/-	++	+	+	-	+	-	+

†Ependymal cells that line the lateral ventricles are more rarely nestin-immunoreactive (+/--) but show higher vimentin immunoreactivity (++/-) compared to ependymal cells of the third ventricle. ‡In the parenchyma lying behind the hypocellular gap of the third and lateral ventricles, and in the hippocampal dentate gyrus, vimentin and/or GLAST are detected in small stellate cells at a lower frequency than GFAP-positive cells (+/-), suggesting that they are expressed by subpopulations of astrocytes. SCh, suprachiasmatic nucleus; SVZ, subventricular zone of the lateral ventricles. Quantification of the stainings in the hypothalamus is given as mean ± SEM.

Table 4. Summary of the expression of NPC markers in the adult hypothalamus of the mouse, rat and grey mouse lemur.

	Mouse hypothalamus				***rat hypothalamus***				***Mouse lemur hypothalamus***			
	ependymal cells	**tanycytes**	**parenchymal cells**	**endothelial cells**	**ependymal cells**	**tanycytes**	**parenchymal cells**	**endothelial cells**	**ependymal cells**	**tanycytes**	**parenchymal cells**	**endothelial cells**
Sox2	+	+	+	-	+	+	+	-	+	+	+	-
nestin	+ rare[†]	+	-	+/-	+ rare[†]	+	-	+	NA	NA	NA	NA
vimentin	+	+	-	+/-	+	+	+ rare[‡]	+	+	+	+ rare[‡]	+

[†]Most ependymal cells showed low to undetectable nestin labeling. The rare ependymal cells with marked, unambiguous nestin immunoreactivity were scattered or gathered at varying dorso-ventral and antero-posterior locations along the third ventricular border. [‡]Occasional small vimentin-positive stellate cells were detected in the parenchyma surrounding the dorsal part of the third ventricle. NA, not available.

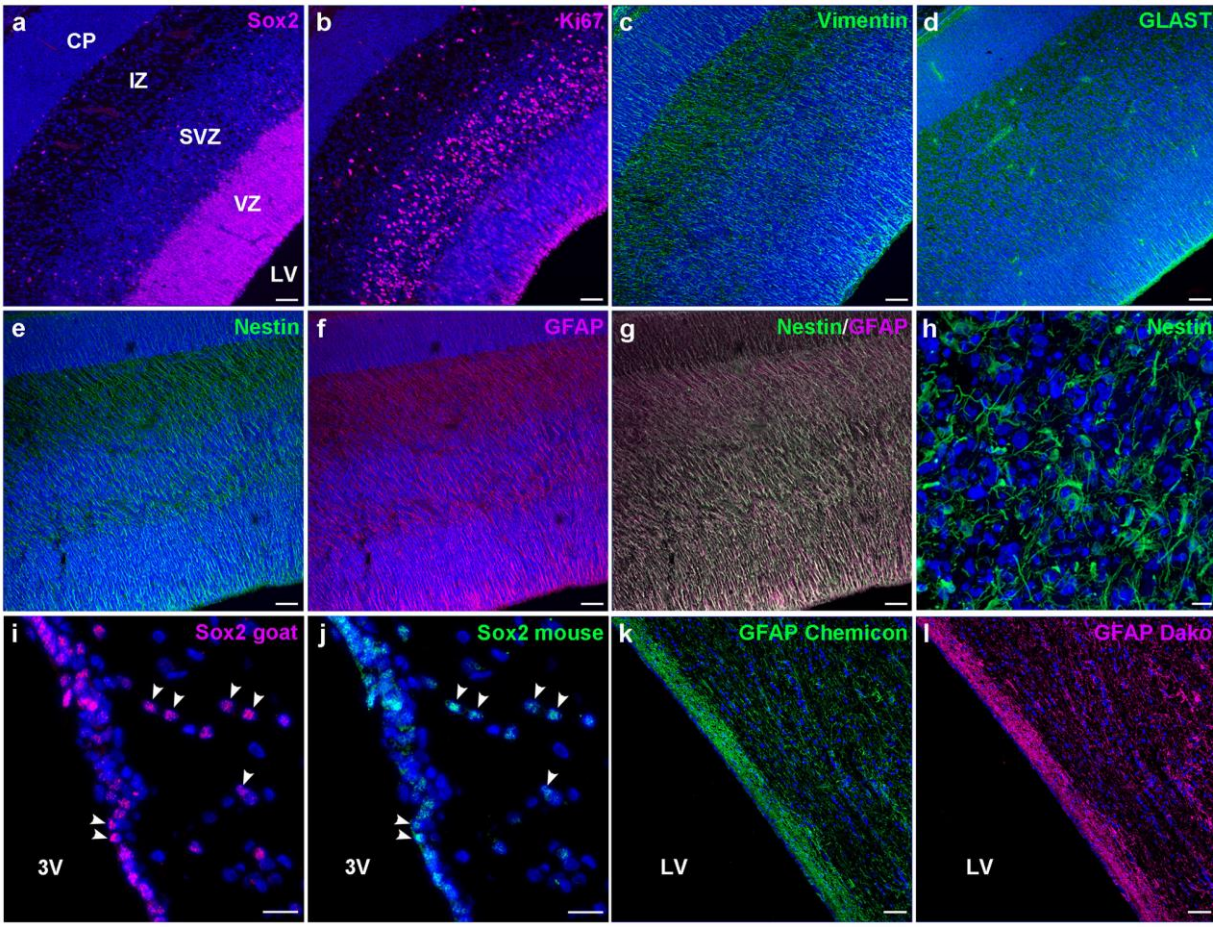

Figure 1. Characterization of the specificity of antibodies used to detect NPC markers in human tissues. **a-g**: Expression of Sox2 (a, magenta, stained with the goat polyclonal # sc-17320 from Santa Cruz), Ki67 (b, magenta), vimentin (c, green, stained with the mouse monoclonal M0725 clone V9 from Dako), GLAST (d, green), nestin (e, green) and GFAP (f, magenta, stained with the rabbit polyclonal Z0334 from Dako) in the cerebral cortex of a 11-week-old human fetus. All markers are detected in radial glial cells, which have their cell body lining the ventricle (LV) and extend a long process across the nervous tissue toward the pial surface (top left corner, not visible). Note the co-localisation of nestin and GFAP in radial glial cells (g, merge). **h**: Expression of nestin in an adult human GBM. **i, j**: Double immunofluorescent labeling using two different anti-Sox2 antibodies (i, magenta: goat polyclonal # sc-17320 from Santa Cruz; j, green: mouse monoclonal # ab79351 from Abcam) in the adult human hypothalamus yielded the same expression profile. Arrowheads point to examples of double-labeled cells. **k, l**: Double immunofluorescent labeling using two different anti-GFAP antibodies (k, green: mouse monoclonal MAB3402 from Chemicon; l, magenta: rabbit polyclonal Z0334 from Dako) in the adult human SVZ yielded the same expression profile. Nuclei were counterstained with Hoechst (all panels except g, blue). 3V, third ventricle; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; SVZ, subventricular zone; VZ, ventricular zone. Scale bars = 50 µm in a-g, k, l; 20 µm in h, i, j.

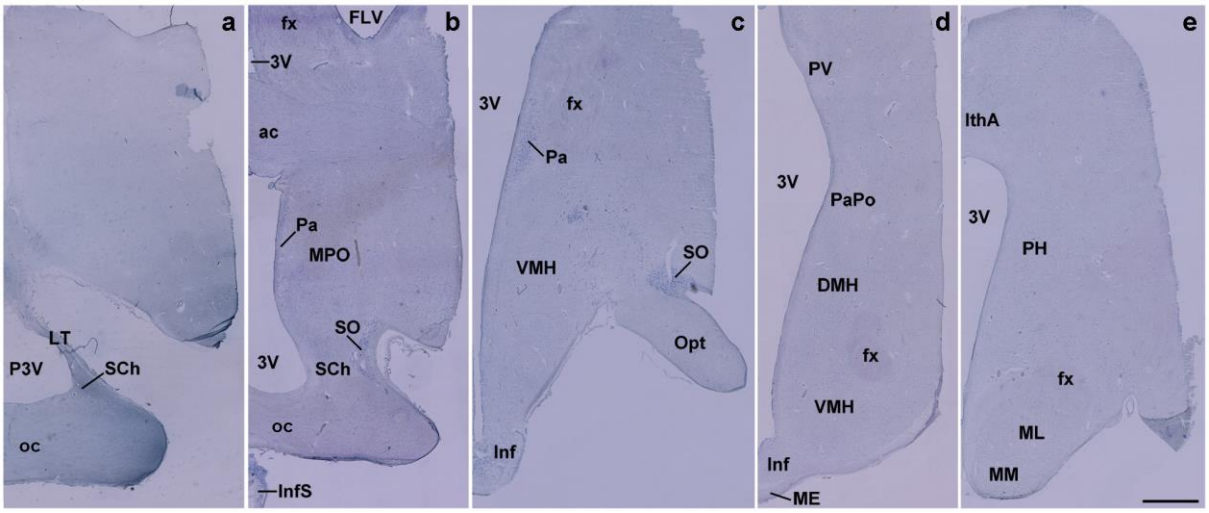

Figure 2. Representative coronal histological sections stained with Toluidine Blue across the rostro-caudal extent of the human hypothalamus. **a:** anterior-most region containing the preoptic recess of the third ventricle. **b:** anterior hypothalamic area. **c, d:** tuberal region. **e:** mammillary region. 3V, third ventricle; ac, anterior commissure; DMH, dorsomedial hypothalamic nucleus; FLV, frontal horn of lateral ventricle; fx, fornix; Inf, infundibular nucleus (or arcuate nucleus of the hypothalamus); InfS, infundibular stalk; IthA, interthalamic adhesion; LT, lamina terminalis; ME, median eminence; ML, medial mammillary nucleus, lateral part; MM, medial mammillary nucleus, medial part; MPO, medial preoptic nucleus; oc, optic chiasm; opt, optic tract; P3V, preoptic recess of the third ventricle; Pa, paraventricular nucleus; PaPo, paraventricular hypothalamic nucleus, posterior part; PH, posterior hypothalamic area; PV, paraventricular thalamic nucleus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; VMH, ventromedial hypothalamic nucleus. Scale bar = 2 mm.

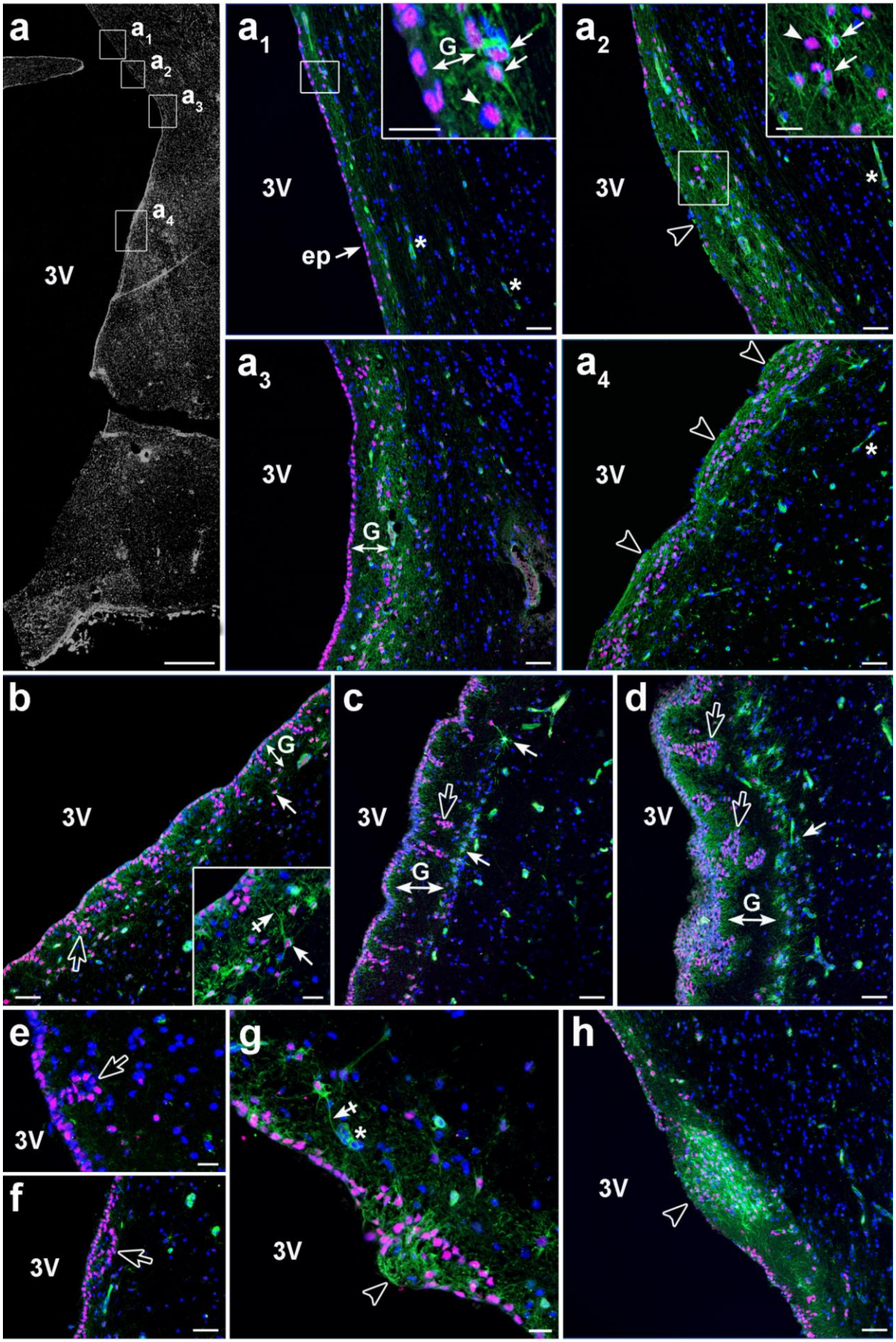
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Figure 3. Expression of nestin and Sox2 along the wall of the human third ventricle. **a**: Low-magnification photomontage of Hoechst counterstaining (grey) showing the third ventricle border at an antero-posterior level corresponding to the end of the anterior hypothalamic area and the beginning of the tuberal region. **a_1-h**: Double immunofluorescent labeling of nestin (green) and Sox2 (magenta). Nuclei were counterstained with Hoechst (blue). **a_1-a_4**: High-magnification images of the areas indicated in a. Insets in a_1, a_2: High-magnification images of the boxed areas. Most of the third ventricular border is lined by a ribbon of small nestin/Sox2 double-labeled cells with multiple processes (arrows in a_1, a_2, b, c, d) separated from the ependymal layer by a hypocellular gap (G, double arrow in a_1, a_3, b, c, d). These cells cohabit with Sox2-positive/nestin-negative cells (arrowheads in a_1, a_2). Some nestin/Sox2 double-labeled cells in the ribbon extend long processes (crossed arrow) to contact ependymal cells (inset in b) or capillaries (g). The nestin-positive subependymal area shows occasional thickenings (a_2, a_3) that can protrude into the ventricle (a_4, g, h). Protrusions are associated with an interruption of the ependymal layer (empty arrowheads). Empty arrows point to invaginations of Sox2-positive/nestin-negative ependymal cells in the underlying parenchyma forming rosettes (b, c, d, e) or crowns (f). Regular invaginations of ependymal cells associated with bulging of the wall gives the border a crenelated aspect (b, c). Asterisks indicate nestin-positive capillaries. 3V, third ventricle; ep, ependymal layer; G, hypocellular gap. Scale bars = 1 mm in a; 50 µm in a_1, a_2, a_3, a_4, b, c, d, f, h; 20 µm in e, g and insets.

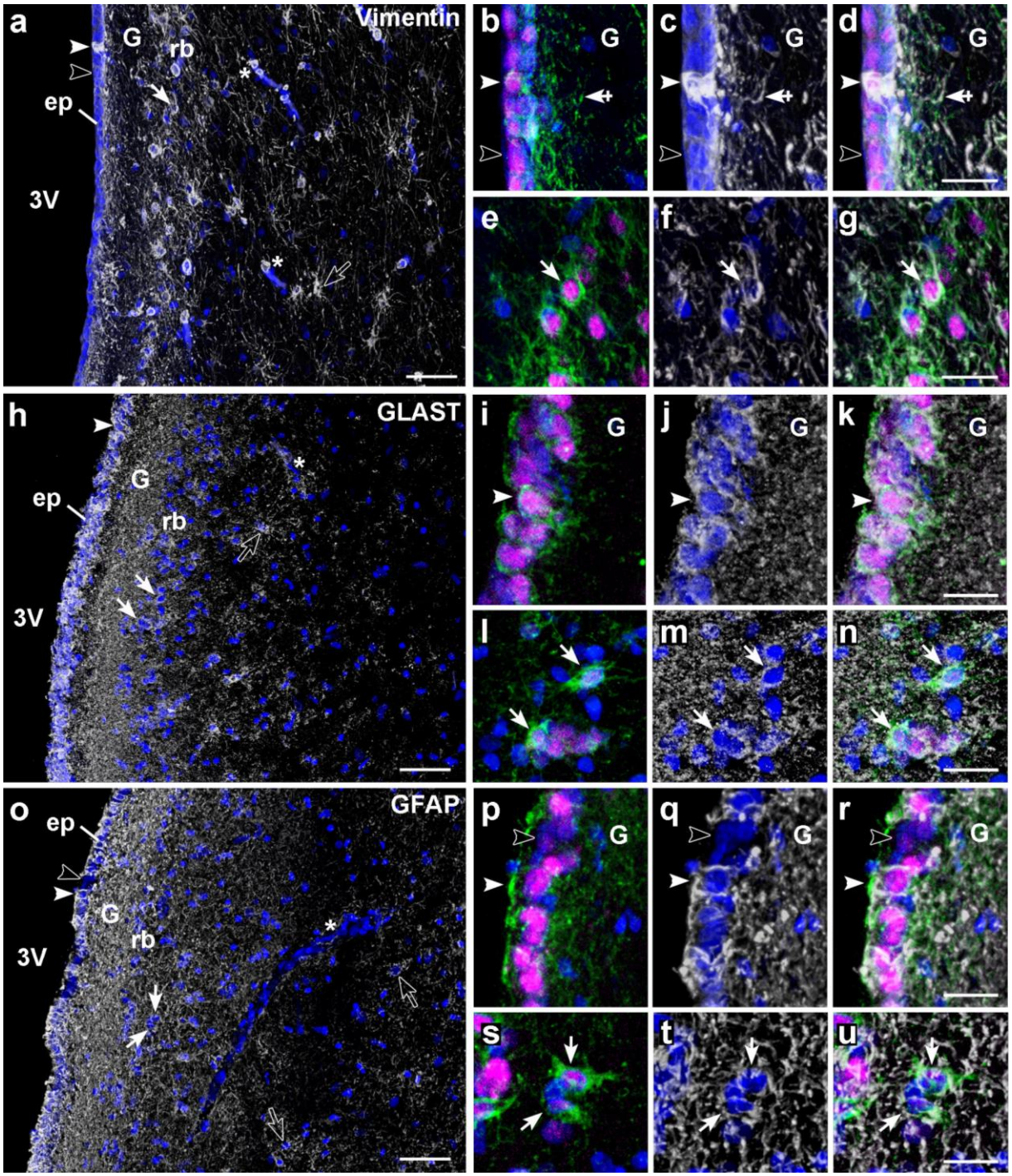


Figure 4. Expression of NPC markers in ependymal and ribbon cells of the human third ventricle. Nestin (green) and Sox2 (magenta) were co-immunodetected with vimentin (a, c, d, f, g, white), GLAST (h, j, k, m, n, white) or GFAP (o, q, r, t, u, white). The ependymal layer (ep) and gap (G) are shown at higher magnification in upper right panels (b-d, i-k, p-r) while the ribbon (rb) is shown at higher magnification in lower right panels (e-g, l-n, s-u). Plain arrowheads show ependymal cells that co-express nestin, Sox2 and vimentin, GLAST or GFAP. While GLAST was strongly detected in most ependymal cells (h), vimentin showed highly variable expression levels (a). Some ependymal cells showed low levels of vimentin in their soma (a-d, empty arrowheads) but prominent expression in their basal processes, some of which co-expressed nestin (crossed arrows in b-d). Ependymal cells were heterogeneous for the expression of GFAP, with GFAP-negative (q, empty arrowhead) next to

GFAP-positive cells (q, plain arrowhead). Note the strong expression of GLAST (h, j) and GFAP (o, q) in the hypocellular gap. Arrows point to ribbon cells that co-express nestin, Sox2 and vimentin, GLAST or GFAP. The same cells are pointed in main panels (a, h, o) and high-magnification views. Empty arrows in main panels point to vimentin- (a), GLAST- (h) or GFAP-expressing cells (o) in the parenchyma behind the ribbon. Stars indicate capillaries whose endothelial cells express vimentin (a) but lack GLAST (h) and GFAP (o). Nuclei were counterstained with Hoechst (blue). 3V, third ventricle; ep, ependymal layer; G, hypocellular gap; rb, ribbon. Scale bars = 50 µm in a, h, o; 20 µm in all other panels.

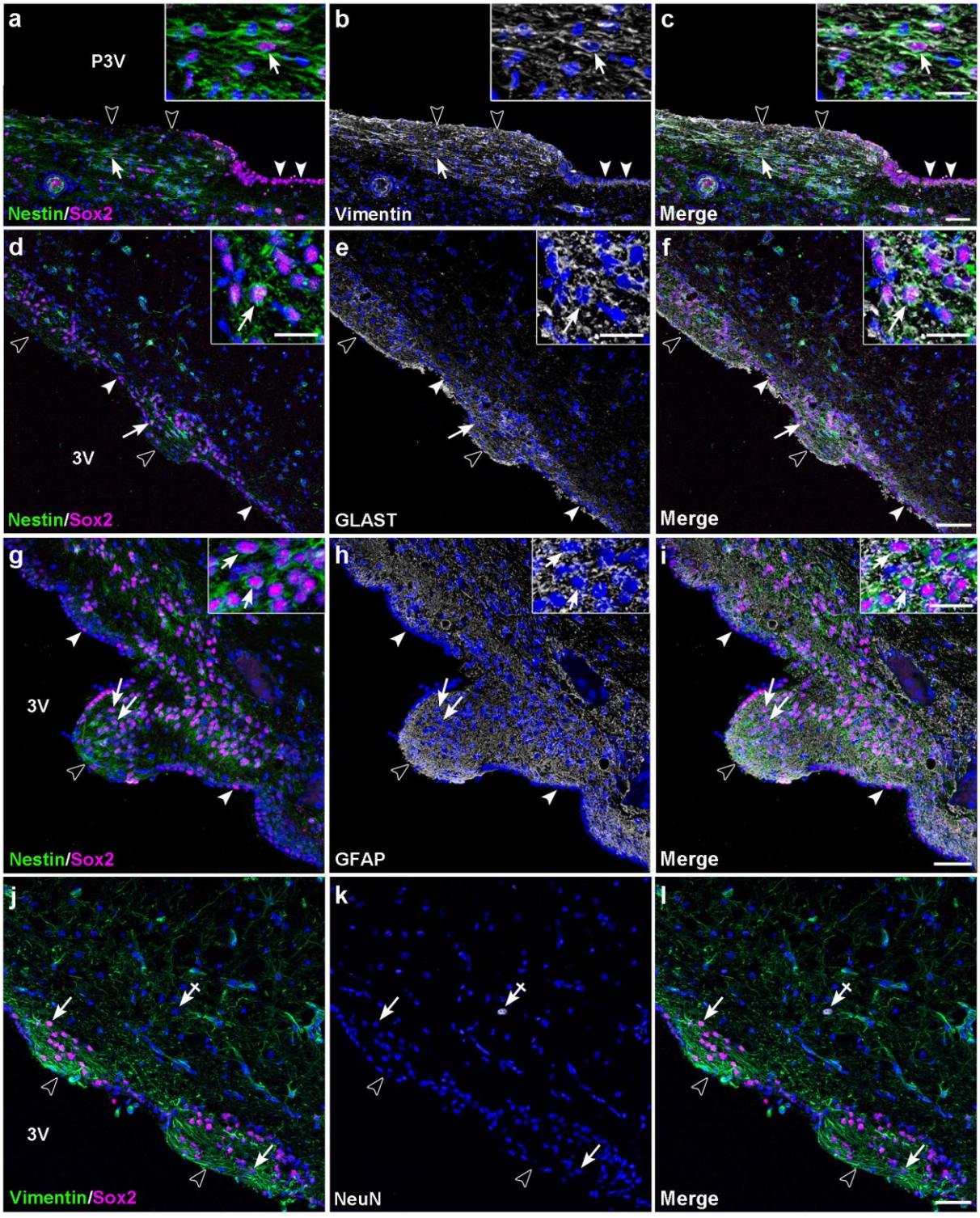


Figure 5. Expression of NPC markers in ventricular protrusions of the human third ventricle. Protrusions of various sizes and forms are found all along the third ventricle. The continuous ependymal layer (plain arrowheads) is interrupted on top of the protrusions (empty arrowheads). These structures contain nestin (a-i, green)/Sox2 (magenta)-immunoreactive cells that co-express vimentin (a-c, j-l), GLAST (d-f) and GFAP (g-i) (arrows, co-expressing cells are shown at higher magnification in insets). The subependymal region and buds are devoid of NeuN-immunoreactive cells, which are found in the deeper parenchyma (j-l, white, crossed arrow). Nuclei were counterstained with Hoechst (blue). 3V, third ventricle; P3V, preoptic recess of the third ventricle. Scale bars = 50 µm in main panels and 20 µm in insets.

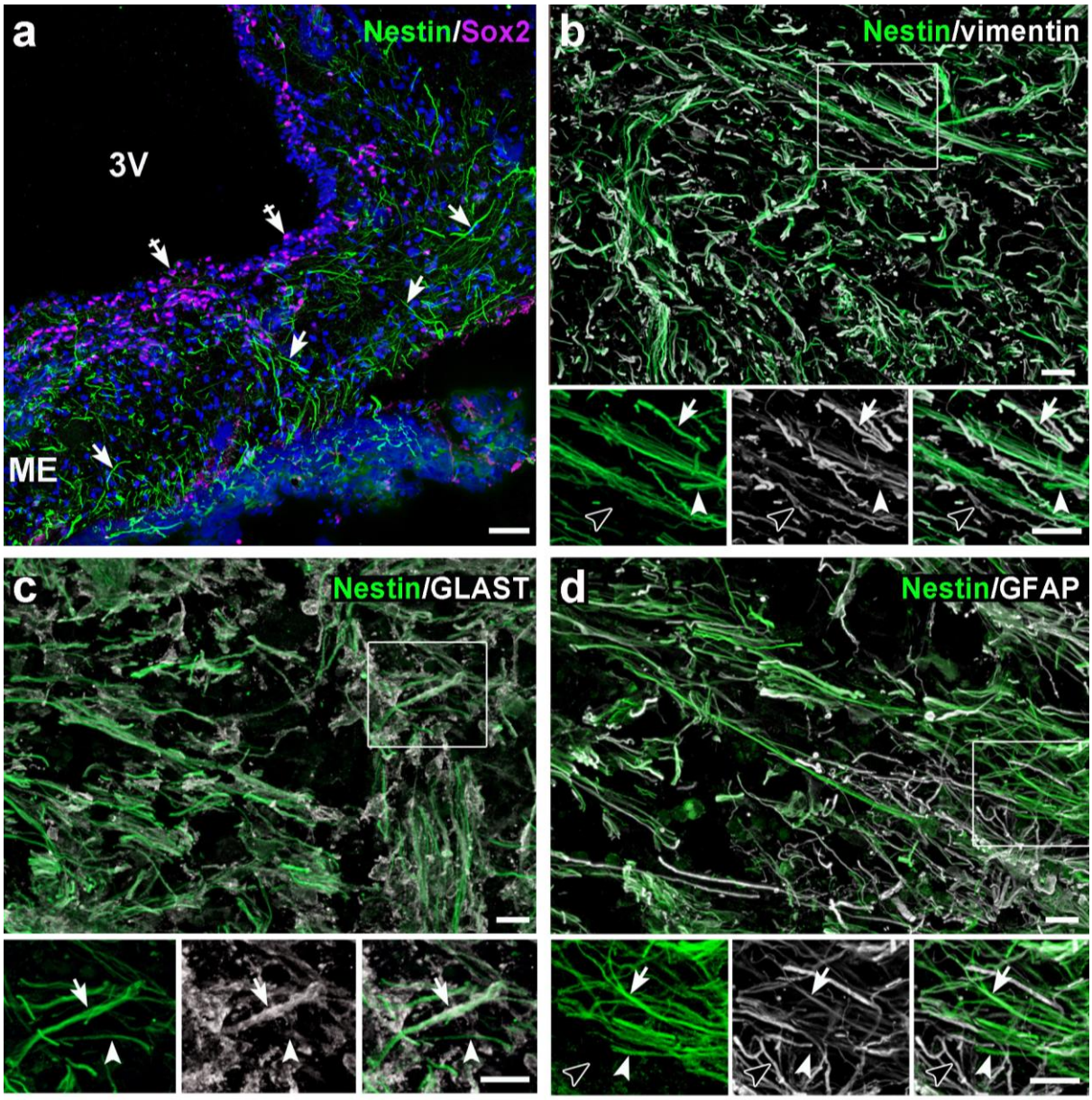


Figure 6. Expression of NPC markers in the median eminence of the human hypothalamus. **a**: Nestin (green) and Sox2 (magenta) are expressed in tanycytes, which line the floor of the third ventricle. Crossed arrows point to tanycyte nuclei and arrows show tanycyte processes. **b-d**: co-immunodetection of nestin (green) and vimentin (b, white), GLAST (c, white) or GFAP (d, white). The three panels below each main panel show a high magnification view of the boxed area. Nestin-immunoreactive tanycyte processes co-express vimentin (b, arrow), GLAST (c, arrow) and GFAP (d, arrow). Plain arrowheads point to nestin-positive processes devoid of vimentin (b), GLAST (c) or GFAP (d) immunoreactivity. Vimentin-positive (b, empty arrowhead) and GFAP-positive (d, empty arrowhead) processes devoid of nestin immunoreactivity were also seen. Nuclei were counterstained with Hoechst (blue, panel a). 3V, third ventricle, ME, median eminence. Scale bars = 50 µm in a, 20 µm in other panels.

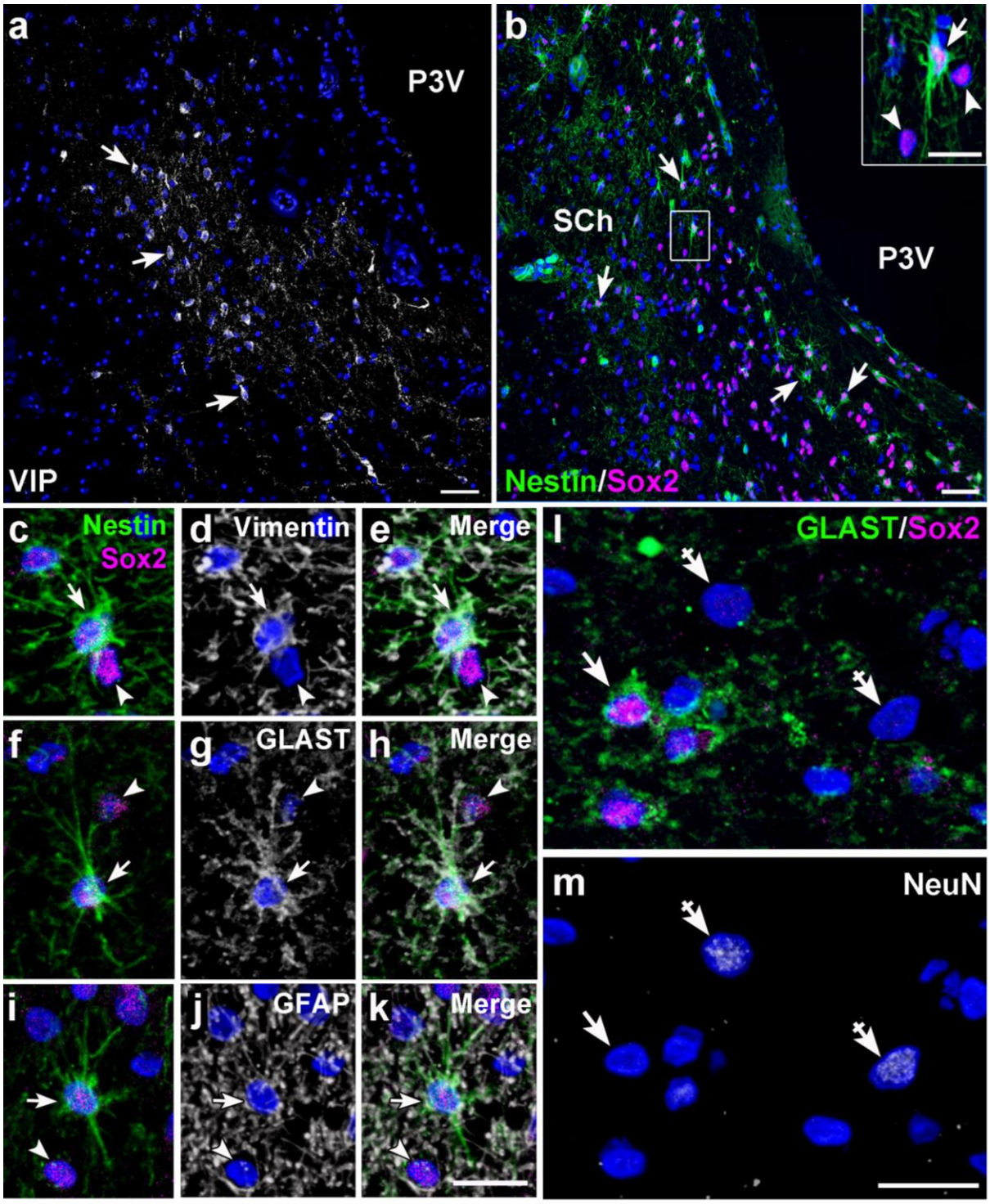


Figure 7. Expression of NPC markers in the human suprachiasmatic nucleus. **a**: Immunofluorescent staining of VIP (white) was performed to ascertain the correct localization of the suprachiasmatic nucleus on human hypothalamus sections. Crossed arrows point to VIP-immunoreactive neurons. **b**: Double immunofluorescent staining of nestin (green) and Sox2 (magenta). The inset is a high-magnification view of the boxed area in the main panel. Note the co-existence of small process-bearing nestin/Sox2 co-expressing cells (arrows) with Sox2-positive/nestin-negative cells (arrowheads). **c-k**: triple immunofluorescent stainings of nestin (green), Sox2 (magenta) and vimentin (white, **d, e**), GLAST (white, **g, h**) or GFAP (white, **j, k**). Arrows point to triple labelled cells and arrowheads show nestin-negative/Sox2-positive cells. **l, m**: Co-immunodetection of GLAST (green),

Sox2 (magenta) and NeuN (white) in the human suprachiasmatic nucleus. Empty arrows point to NeuN-positive neurons that lack GLAST and Sox2 expression. The arrow shows a GLAST/Sox2 co-expressing cell devoid of NeuN. Nuclei were counterstained with Hoechst (blue). P3V, preoptic recess of the third ventricle; SCh, suprachiasmatic nucleus. Scale bars = 50 µm in a, b; 20 µm in c-m and inset in b.

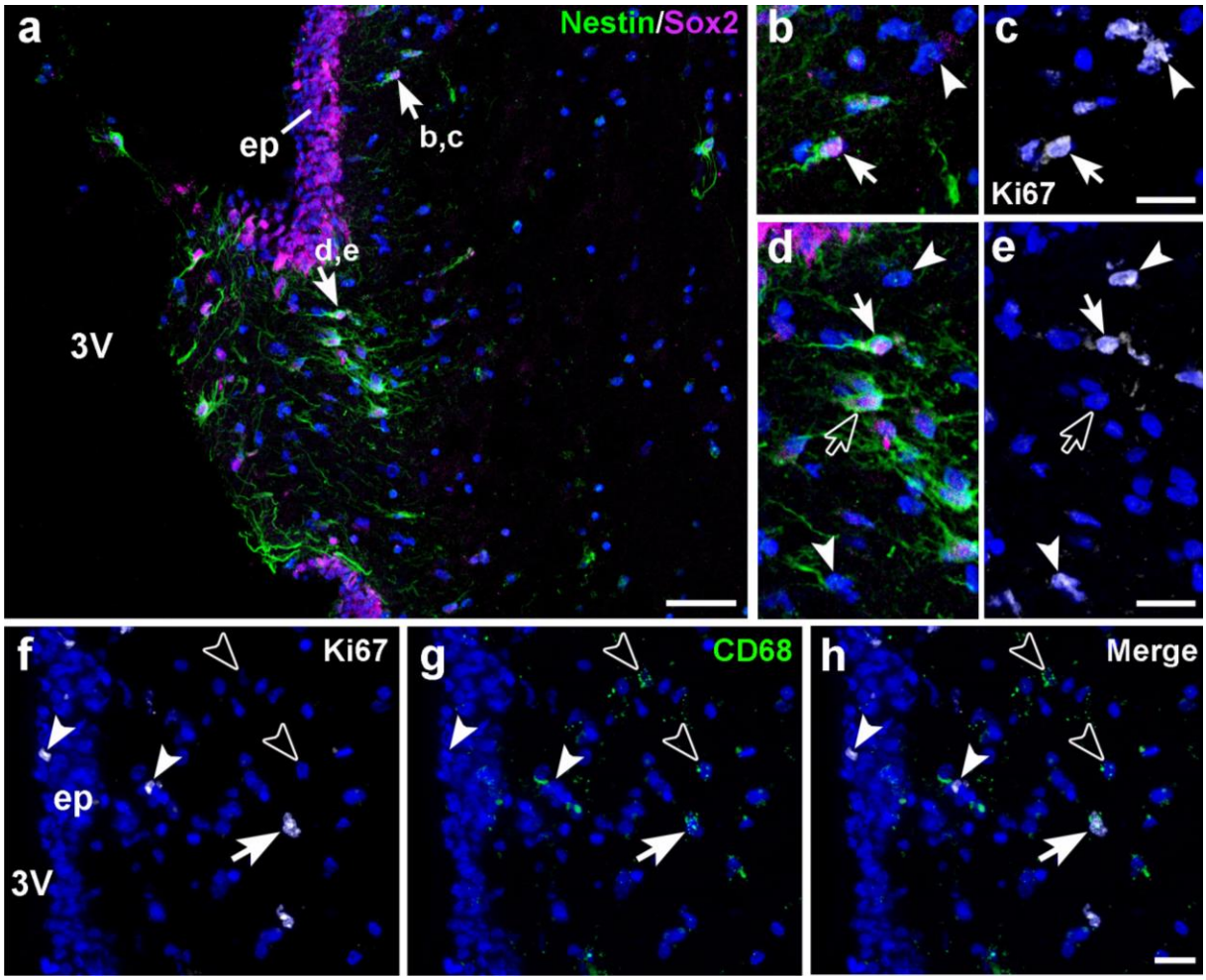
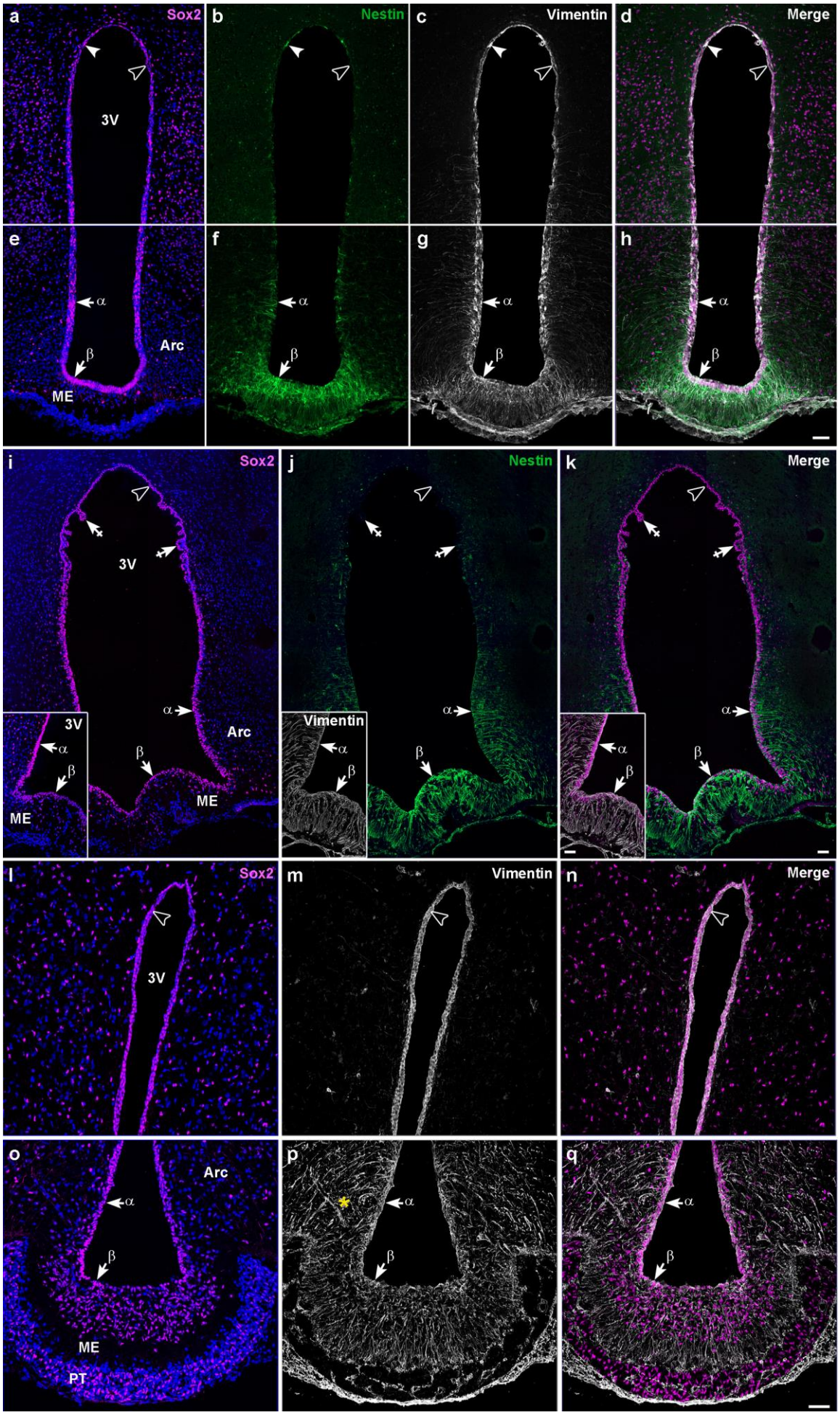


Figure 8. Proliferation along the wall of the human third ventricle. **a-e**: Co-immunofluorescent staining of nestin (green), Sox2 (magenta) (a, b, d) and Ki67 (c, e, white). The 2 cells pointed by the arrows in the main panel (a) are shown at higher magnification in the panels b-e. Very rare nestin/Sox2-co-expressing cells were immunoreactive for Ki67 (arrows). The empty arrow in d-e shows a nestin/Sox2-co-expressing cell devoid of Ki67 immunolabeling. Arrowheads in b-e point to Ki67-positive cells that do not co-express nestin and Sox2. **f-h**: Co-immunofluorescent staining of Ki67 (white) and CD68 (green) reveals the presence of double-labeled cells (arrow) in addition to Ki67-positive/CD68-negative cells (plain arrowheads) and Ki67-negative/CD68-positive cells (empty arrowhead). Note that Ki67-positive cells often appear as pairs of closely apposed cells (arrow), a picture suggestive of a recent mitotic event. Nuclei were counterstained with Hoechst (blue). 3V, third ventricle; ep, ependymal layer. Scale bars = 50 µm in a, 20 µm in b-h.


a
Sox2
3V
b
Nestin
c
Vimentin
d
Merge
e
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Nestin
Vimentin
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Figure 9. Expression of NPC markers along the third ventricle of the mouse (a-h), rat (i-k) and grey mouse lemur (l-q). Representative photographs were taken at the level of the tuberal region of the hypothalamus. The rat third ventricle is shown as a whole while the third ventricle from the mouse and grey mouse lemur is shown in its most dorsal (a-d, l-n) and ventral (e-h, o-q) portions. Co-immunostainings of Sox2 (magenta, a, e, i, l, o), nestin (green, b, f, j) and vimentin (white, c, g, inset in j, m, p). Merge images of all but hoechst stainings are shown in d, h, k, n, q. In the dorsal part of the third ventricle, ependymal cells co-express Sox2 and vimentin (empty arrowheads, a-d, i-k, l-n) with very occasional nestin co-labeling (a-d, plain arrowhead). In the ventral part of the third ventricle, dorsal (arrow ⍰) and ventral (arrow ⍰) tanycytes co-express Sox2, nestin and vimentin. While the third ventricle wall is composed by a regular layer of ependymal cells in the mouse (a, e) and grey mouse lemur (l, o), that of the rat shows thickenings frequently protruding inside the ventricle (crossed arrows in i). The yellow asterisk in p shows a capillary wrapped by tanycyte processes. Note that both tanycyte processes and endothelial cells are vimentin-immunoreactive (see Fig. 10j for examples of vimentin-positive capillaries). Nuclei were counterstained with Hoechst (blue, a, e, i, l, o). 3V, third ventricle; Arc, arcuate nucleus; ME, median eminence; PT, pars tuberalis. Scale bars = 50 µm.

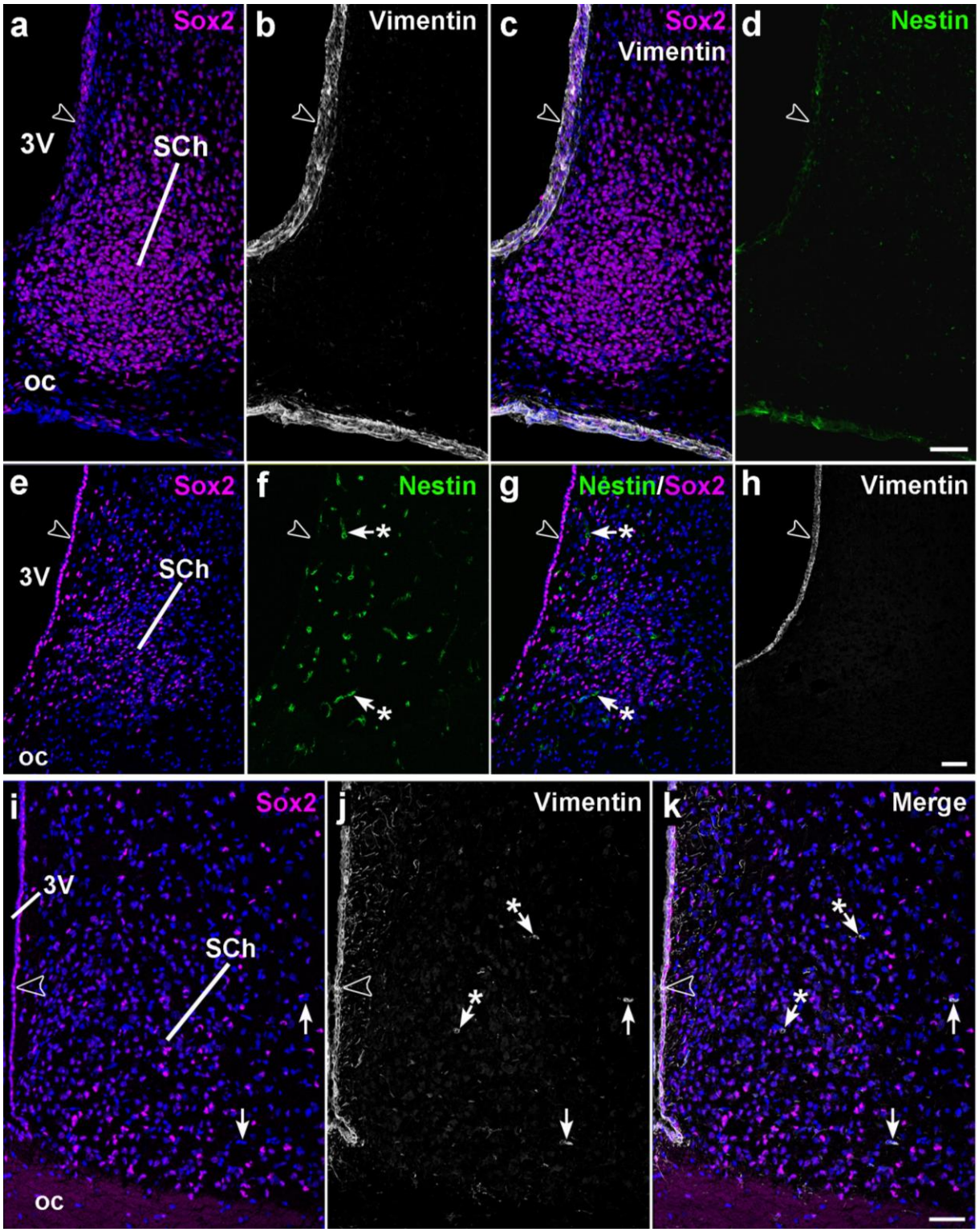

Figure 10. Expression of NPC markers in the suprachiasmatic nucleus of the mouse (a-d), rat (e-h) and grey mouse lemur (i-k). A high density of Sox2-expressing cells (magenta) is detected in the suprachiasmatic nucleus of all three species (a, e, i). In the mouse, the suprachiasmatic nucleus is devoid of vimentin (b) and nestin (d) immunoreactivity. In the rat, it also lacks vimentin expression (h) but contains nestin-immunoreactive capillaries (f, g, arrows with stars). In both species, the third ventricle ependymal layer (empty arrowheads) expresses Sox2, vimentin but shows low to undetectable levels of nestin. In the grey mouse lemur, the suprachiasmatic nucleus contains rare

vimentin-immunoreactive elements that do not co-express Sox2 (arrows with stars in j, k point to capillary sections; arrows in i-k show Sox2-immunonegative/vimentin-positive cells). The mouse lemur third ventricle is lined by Sox2 and vimentin-immunoreactive ependymal cells (i-k, empty arrowheads). Nuclei were counterstained with Hoechst (a, c, e, g, i, k, blue). 3V, third ventricle; oc, optic chiasm; SCh, suprachiasmatic nucleus. Scale bars = 50 µm.

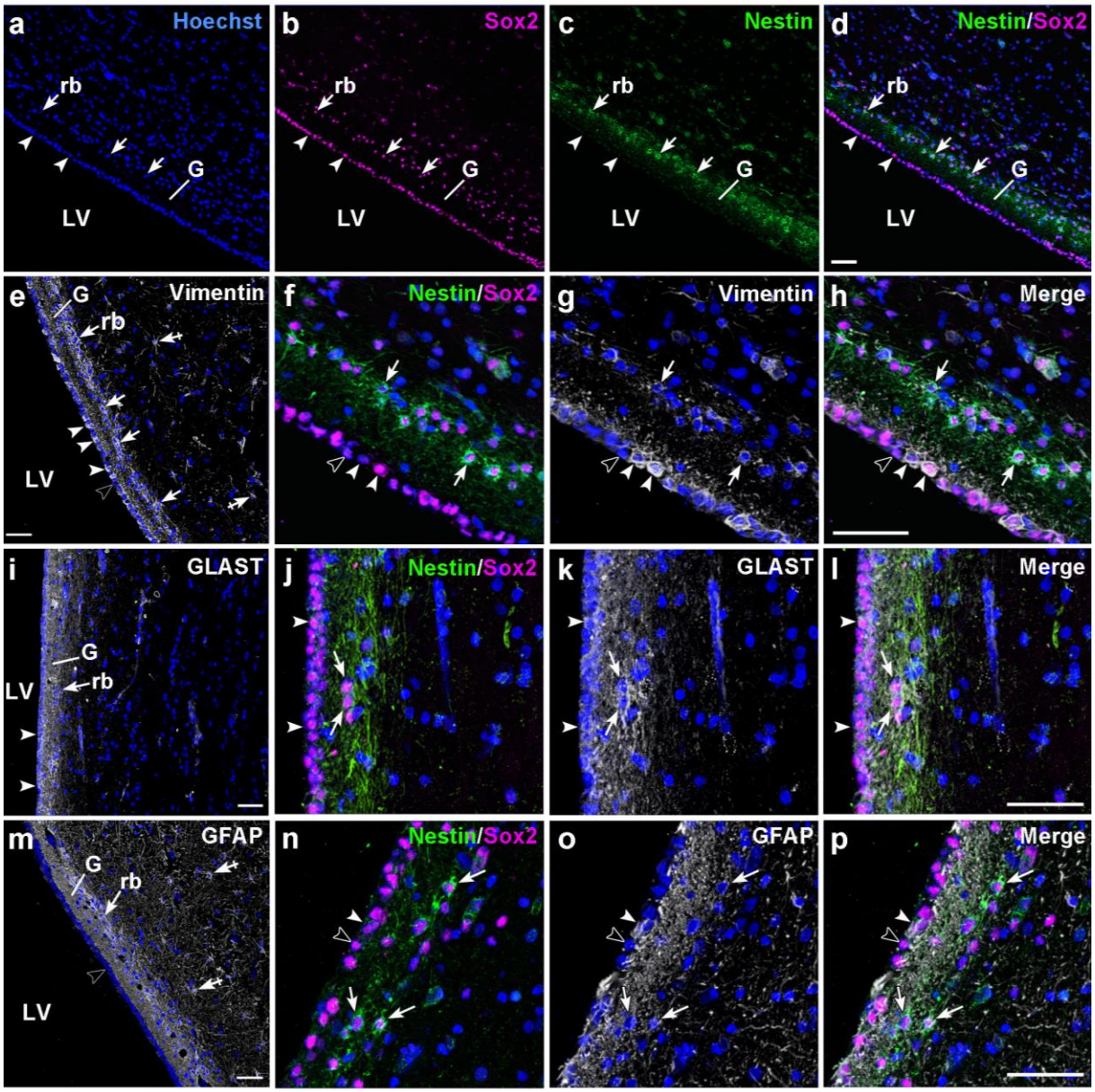

Figure 11. Expression of NPC markers in the human SVZ. **a-d**: Double immunofluorescent staining of nestin (green) and Sox2 (magenta) in the SVZ showing the ribbon (rb) of nestin/Sox2-co-expressing cells (arrows), separated from the ependymal layer (arrowheads) by the hypocellular gap (G). **e-h**: Vimentin (white) is expressed in ependymal cells (plain arrowheads), ribbon cells (arrows) and parenchymal astrocytes (crossed arrows in e). Note the variable levels of vimentin expression in ependymal cells, some of which express low to undetectable levels of the protein (empty arrowheads). The ribbon contains a population of cells that co-express nestin, Sox2 and vimentin (arrows, f-h). **i-l**: GLAST (white) is expressed in ependymal cells (arrowheads) and is enriched in the gap and ribbon. The ribbon contains a population of cells that co-express nestin, Sox2 and GLAST (arrows, j-l). **m-p**: GFAP (white) is enriched in the gap and ribbon. The ribbon contains a population of cells that co-express nestin, Sox2 and GFAP (arrows, n-p). GFAP is also expressed in parenchymal astrocytes (crossed arrows in m) but is absent from most ependymal cells (empty arrowheads). Rare ependymal cells express GFAP (plain arrowhead, n-p). Nuclei were counterstained with Hoechst

(blue). Microphotographs representative of the ventral (a-d, f-h, i-l, n-p) and dorsal (e, m) SVZ. LV, lateral ventricle. Scale bars = 50 µm.

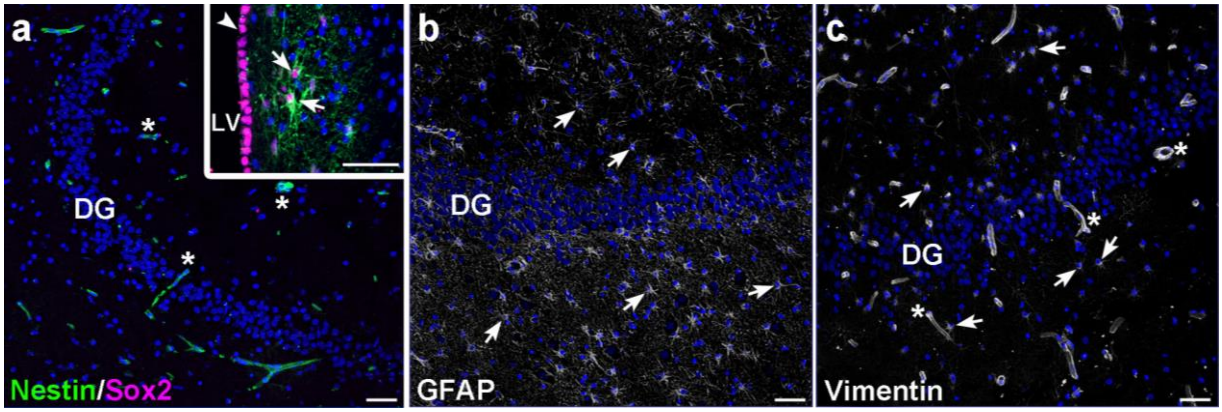

Figure 12. Expression of NPC markers in the dentate gyrus of the human hippocampus. **a**: Double immunofluorescent staining of nestin (green) and Sox2 (magenta). In the dentate gyrus, Sox2 was not detected and nestin was restricted to capillaries (asterisks). Note that Sox2 and nestin were readily detectable along the ventricular border of the temporal horn of the lateral ventricle on the same section (inset). Arrows point to nestin/Sox2 co-expressing cells; the arrowhead points to Sox2-positive/nestin-negative ependymal cells. **b**: Immunodetection of GFAP (white) reveals the presence of numerous astrocytes (arrows). **c**: Vimentin immunoreactivity (white) was detected in capillaries (asterisks) and small stellate cells (arrows). Nuclei were counterstained with Hoechst (blue). DG, dentate gyrus. LV, lateral ventricle. Scale bars = 50 µm.

Chapter 8

Discussion and Conclusions

8.1 DISCUSSION

From the moment when adult cell proliferation in the mammalian brain has been considered possible, an increasing number of studies have tried to describe this process and its functions. While for many years the scientific community has focused on 2 brain regions only, the SVZ and the SGZ, different groups have now turned their attention towards the possibility that stem cell niches may reside in the postnatal mammalian brain, as for instance at the level of the striatum or the hypothalamus (Lin & Iacovitti, 2015).

The hypothalamus is a well-conserved structure in all mammalian species, devoted to the control of all vegetative functions, as metabolism, reproduction, circadian rhythms, control of sleep, thirst, fear, indispensable functions for survival of the individual and of the species itself. In order to fulfil its many roles, the hypothalamus relies on a surprisingly high number of different neuronal populations, for such a small region. These neurons, located in discrete nuclei, communicate with each other and with extra-hypothalamic brain regions. Cell proliferation in the hypothalamus is still a poorly studied subject both in relation to the different brain developmental stages and to the different hypothalamic functions (Sharif et al., 2014).

Here, we have explored how the birth of new cells in the postnatal and adult female rat hypothalamus may be relevant for the reproductive function, a function known to require a high level of plasticity. Moreover, we provide the first description of the neural stem cell niche in the adult human hypothalamus.

8.1.1 Cell proliferation in the female rat POA is maintained throughout postnatal development and during adulthood

Through the use of BrdU as a marker of dividing cells, we described cell proliferation in the POA of infantile, juvenile and adult female rats.

The highest levels of proliferation are observed during the infantile stage, when newborn cells mainly differentiate into astrocytes, in accordance with data obtained in other brain regions, such as the cerebral cortex, where most astrocytes are born at this stage. The drop in cell proliferation, which can already be seen during the juvenile period, is even more evident during adulthood, when the levels of cell neogenesis become very low.

The process of astrogenesis is still not entirely understood; the exact origin of hypothalamic astrocytes is still not clear. Recent works suggest that they derive from the same radial glial cells that give rise to neurons during embryonic development, which lately migrate into the parenchyma to give rise to astrocytes. Using the *in vitro* neurosphere assay, previous experiments performed in the lab showed the presence of neural stem/progenitor cells in the POA of infantile rats as neurospheres could be obtained, passaged up to 31 times (the longest time examined) and differentiated into the three neural lineages. Moreover, enrichment in Sox2-expressing progenitor cells was attested by both *in situ* hybridisation (Chapter 5.3.5.1) and immunohistochemistry (personal observations) in this region. Sox2 mRNA and protein levels were extremely high at the level of the SVZ, as expected, and were particularly enriched in the POA at the level of the OVLT.

8.1.2 Newborn cells are associated with GnRH neurons and are necessary for their postnatal development

Interestingly, during the infantile period, some of the newborn cells were found in a morphological association with the GnRH neurons that was preferential compared to the entire neuronal population of the POA. During the postnatal period, the GnRH neurons undergo a very specific and peculiar developmental process, as, differently from other neurons, they are not functional until puberty is established.

It is well known that the crosstalk between GnRH neurons and glial cells at the level of their cell body and terminals is important for the modulation of GnRH neuron activity and secretion. In the POA, tight morphological interactions occur between GnRH neurons and astrocytes. For instance, adhesion molecules regulating the adhesiveness between these 2 cell types have been shown to play an important role in the onset of puberty (Sharif et al., 2013). At the same time, astrocytes regulate GnRH neuron activity and secretion through the release of gliotransmitters, such as PGE2, in a crosstalk that is established during postnatal maturation.

When cell proliferation was inhibited specifically within this region during the infantile period, the maturation process of GnRH neurons was altered as attested by delayed puberty and perturbed oestrous cyclicity, proving that the birth of astrocytes in the region is necessary for the correct maturation of the system.

8.1.3 The recruitment of newborn cells by GnRH neurons is necessary for the correct maturation of the system

Given the observation that newborn cells were preferentially found in association with GnRH neurons and that the percentage of neurons associated with newborn cells increased over time during a 7-day-window of the infantile period starting at P8, we asked the question of whether GnRH neurons are able to create a microenvironment around them that would facilitate the proliferation and/or the recruitment of the surrounding progenitor cells. The possibility that GnRH neurons would also regulate the differentiation of the newborn cells was not analysed since the fate of the newborn cells found in association with the GnRH neurons was not different compared to that of the whole population of the POA.

We used a candidate approach to identify factors that could be released by GnRH neurons at the level of the POA and recruit progenitors. GnRH, galanin, glutamate, GABA and PGD2, a member of the prostaglandin family, were selected and were tested *in vitro*. Only PGD2 had an attractive effect on progenitor cultures derived from rat POA through its DP1 receptor. However, none of the factors tested was able to alter the proliferative activity of these cells.

Prostaglandins are a family of unsaturated fatty acids derived from the arachidonic acid, which are not stored intracellularly, but rather are synthesised and released immediately. DP1 is a transmembrane receptor coupled to a Gs protein that stimulates cAMP formation and subsequent activation of PKA and MAPK pathways. PGD2 has been already identified as a chemoattractant for eosinophils, through the activation of DP1, in processes involved in the allergic response (Schratl et al., 2007).

We showed that this pathway is active *in vivo*, as blocking DP1 receptor activation in the POA of infantile female rats decreases the association between GnRH neurons and newborn cells. Moreover, we saw that blocking this pathway altered FSH levels at P12 and affected the establishment of estrous cyclicity, although the animals did not show altered vaginal opening or disrupted fertility during adulthood. The absence of an effect on the age of puberty and fertility may be due to different reasons; first of all, we do not know for how long the DP1 receptor antagonist remains active within the brain and what percentage of newborn cells is affected. An effect of compensation can be easily imagined since cell proliferation continues over the juvenile period and the proliferation rate is not affected, even though we showed that at this stage the rate of cell proliferation is lower; nonetheless, GnRH neurons may still be able to attract cells born later on and manage to rescue normal

activity in adulthood. If this were the case, it would also explain why complete inhibition of cell proliferation in the entire region resulted in a stronger phenotype.

Moreover, it has been shown that the number of functional GnRH neurons necessary to induce ovulation is relatively small (Herbison et al., 2008), indicating that disrupting the process of recruitment of newborn astrocytes only in a percentage of neurons may not be sufficient to affect the onset of puberty.

8.1.4 Proposed model and future perspectives

Results from transcriptomic analyses showed that exposure to astrocyte conditioned medium increases the expression of the PGD2 synthase gene in GnV3 neurons, an *in vitro* model for mature GnRH neurons. Besides, the expression levels of PGDS within sorted GnRH neurons increase between P8 (early infantile) and P20 (late infantile). Therefore, we propose a model in which GnRH neurons at P8 attract, via PGD2/DP1 signalling, newborn immature cells, which subsequently differentiate into astrocytes. The recruited astrocytes would then be able to induce an increase in the expression levels of PGDS, probably causing the attraction of more newborn cells from the vicinity, establishing a positive loop that would help GnRH neurons establish their glial network before the onset of puberty, when astrocytes are known to regulate the activity of GnRH neurons via multiple mechanisms, including adhesive, juxtacrine and paracrine interactions (Sharif et al., 2013) (Fig. 1).

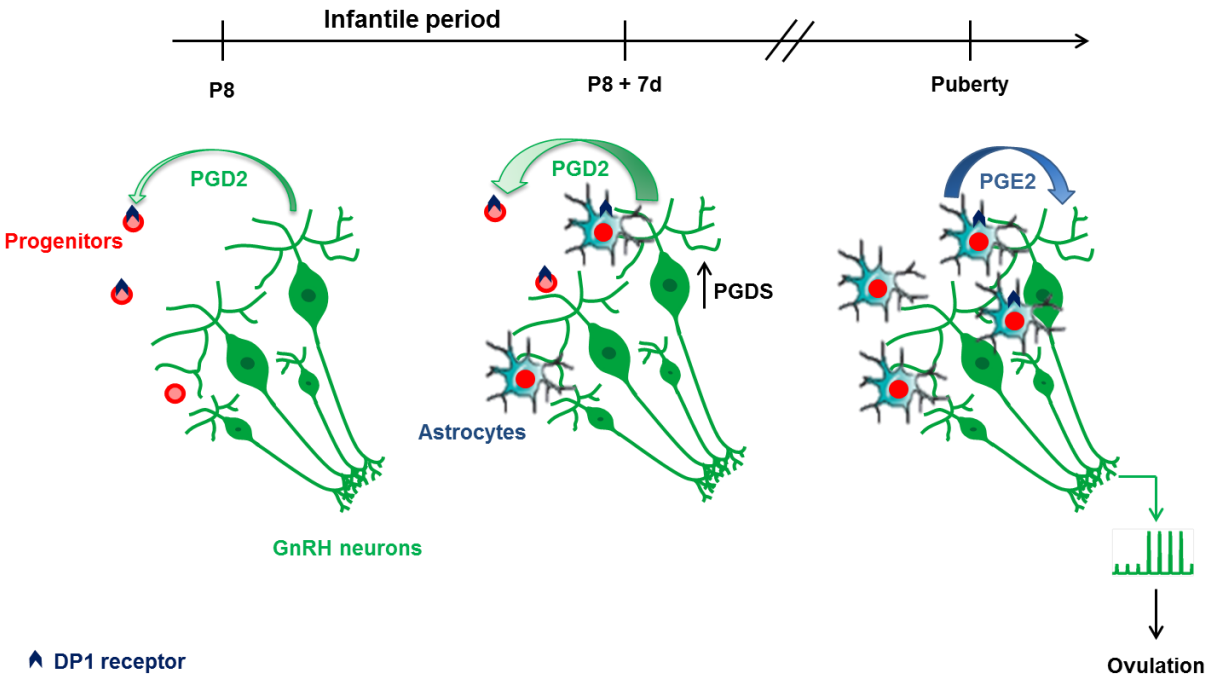

Figure 1: Proposed model. During the infantile period, GnRH neurons release PGD2 to attract DP1-expressing newborn cells from their vicinity. This period of intense recruitment is associated to increased expression of PGD2 synthase in GnRH neurons. The associated newborn cells then differentiate into astrocytes. At puberty, the astrocytes associated with GnRH neurons release PGE2 to stimulate their electrical and secretory activity, thus participating in the induction of the first ovulation that marks puberty onset. PGDS, PGD2 synthase.

While the role of glial-to-GnRH neuron interactions is well known, here for the first time we show that this interaction is not unidirectional. Our results show that the GnRH neurons actively shape their glial environment over their postnatal maturation, attracting the newborn astrocytes found in their vicinity. This would allow GnRH neurons to control the establishment of their interactions with glial cells, which are necessary for the regulation of GnRH function during adulthood. Furthermore, during postnatal maturation, GnRH neurons receive an increasing number of neuronal inputs coming from different nuclei of the hypothalamus. The ability to control the association with neighbouring astrocytes would also allow the GnRH neurons to modulate their level of glial coverage, which is known to affect accessibility to neuronal inputs (Clarke & Barres, 2013). Among the neurons that establish their projections over this time window, there are ARC neurons, known to regulate gonadal negative feedbacks (Bouret, 2004), necessary to restrain the system until proper maturation of the follicles is attained. An adequate balance over postnatal development, between the level of glial coverage and of the inputs driving negative feedbacks onto the GnRH neurons, may therefore be critical for the correct activation of the axis.

To overcome the difficulties encountered in inducing a longer blockage of DP1 signalling *in vivo*, different approaches could be used. For instance, an option may be that of injecting a virus in the POA of infantile female rats, carrying a siRNA against PGDS, under the control of the GnRH promoter, in the presence of a reporter gene. This would allow us not only to block this signalling in a more controlled manner, since expression of the siRNA could be checked through the presence of the reporter gene, but also to specifically knock it down in the GnRH neurons, avoiding possible interference with different hypothalamic functions exerted by PGD2, such as the control of sleep (Urade & Hayaishi, 2011). Moreover, it would be interesting to evaluate whether interactions with newborn cells over the infantile period

affect the electrophysiological properties of the GnRH neurons. Finally, to understand to which extent glial coverage changes over the postnatal maturation and its control by the GnRH neurons themselves, ultrastructural experiments using electron microscopy could be performed at different ages and after disruption of the PGDS/DP1 signalling.

8.1.5 Cell neogenesis in the hypothalamus of adult female rats during the estrous cycle

As previously mentioned, we observed cell proliferation in the POA of female rats during adulthood, even though at a lesser extent than during postnatal development. Moreover, we saw that the rate of cell birth is affected by estrous cyclicity. The highest levels of proliferation are found on the day before ovulation and the lowest ones on the day of estrus. In both the ME and the DG, we observed an increase in proliferation during the second day of diestrus when compared to the day of estrus. A higher percentage of newborn cells were found to be associated with GnRH terminals in the ME during the 2 days of diestrus compared to estrus, while no association was observed between GnRH neuron cell bodies and cells born during adulthood at the level of the POA.

After their birth, cells require a couple of weeks to differentiate and integrate within pre-existing circuits (Carlén et al., 2002; Ramirez-Amaya et al., 2006). Considering that rats ovulate every four days, it is improbable that the variations in cell proliferation over the estrous cycle regulate the cycle itself. We therefore searched for an explanation that would take into account the need for a long-term plasticity process, such as pregnancy.

8.1.6 Pregnancy increases cell survival in the MPO

The POA contains different nuclei with different roles and functions; when we focused on each nucleus separately, we observed an initial increase in the number of $BrdU^+$ cells in the MPO, followed by a drop 2 weeks afterwards. The final goal of ovulation is that of ensuring pregnancy; it has already been shown by Shingo et al. (2003), that during pregnancy prolactin enhances cell proliferation at the level of the SVZ, and this is required for normal postpartum maternal interactions with pups (Shingo et al., 2003; Larsen et al., 2008; Larsen and Grattan, 2010). Indeed the success of pregnancy requires profound modifications in brain functioning to trigger the cerebral and physiological adaptations necessary for pregnancy completion, parturition, preparation of lactation and onset of maternal

behaviour. We therefore explored whether cells born just before ovulation may serve a role in this process. We evaluated how cell survival may be influenced in case of pregnancy. We could indeed observe that more cells born before ovulation in the MPO survive if a pregnancy occurs.

The MPO is the region involved in the control of maternal behaviour; this develops around parturition and involves recognition, emotionality and attachment, necessary to provide nutrition and protection to the pups and hence allow their survival. In this complex picture, the MPO represents the central hub in the neural networks that organises the different components of maternal behaviour in response to pregnancy hormones (gonadal steroids, prolactin…) (Brunton & Russel, 2015). Expression of maternal behaviour is triggered by restructuring of hypothalamic neurons. This process is quite dynamic as mothers need to rapidly adapt to the changing developmental needs of the offspring. To this aim, at the level of the MPO there is an increased neural complexity by the end of pregnancy: MPO neurons are activated in order to signal to dopaminergic neurons of the ventral tegmental area, involved in the reward circuitry; neuronal connectivity and primary dendritic branches increase, as well as neuronal firing activity; at the same time the expression of receptors for prolactin and oestrogens, crucial hormones for regulating the progress and success of pregnancy, is upregulated (Bridges, 2016; Brunton & Russell, 2008; Champagne & Curley, 2016). In such a complex context, our results raise the possibility that addition of new cells to the MPO may be an additional mechanism of plasticity involved in the maternal brain adaptation to pregnancy.

8.1.7 Open questions

Which factors influence cell proliferation in the POA during the oestrous cycle and pregnancy?

The oestrous cycle is characterised by specific fluctuations in hormonal levels including gonadal steroids and pituitary hormones. These same hormones are then finely modulated to ensure pregnancy. Therefore, one of the next steps to better understand the process of adult cell neogenesis in the rat MPO will be to characterise which of these hormones is

responsible for regulating the peak in proliferation that occurs before ovulation as well as the increase in cell survival in case of pregnancy.

What do these cells become?

Another important point that needs to be elucidated is the phenotypical fate of the newborn cells that survive during pregnancy. Given the gliogenic nature of the POA described during postnatal development, our hypothesis points towards an astrocytic differentiation fate. Besides, astrocytes could efficiently play a role in supporting the changes in pre-existing neuronal circuits described in the MPO at the time of parturition. Indeed, astrocytes are able to modulate synaptic activity, dendritic spine number, as well as extracellular levels of ions and neurotransmitters (Clarke & Barres, 2013; Oliveira et al., 2015). Moreover, a recent work has shown how hypothalamic astrocytes are involved in the regulation of energy homeostasis through plastic changes in the morphology of their processes that depend on the feeding state and modulate extracellular levels of GABA with functional consequences on the activity of anorexigenic and orexigenic neurons (Zhang et al., 2017). It has been estimated that one astrocyte can contact up to 100,000 synapses (Halassa et al., 2007). Therefore, even considering that the total number of new cells in the MPO is not as elevated as in regions such as the OB, it is clear that even few astrocytes can affect the activity of thousands of neurons in the region.

For these reasons, our preliminary results brought us to hypothesise that cell neogenesis in the MPO, at the onset of gestation, participates to the adaptive changes that enable development of the maternal behaviour.

Where do these newborn cells come from?

An important question that remains unanswered concerns the origin of newborn cells. The existence of adult neural stem cell niches outside of the SVZ and SGZ is still a matter of debate and new regions are not easily accepted. A growing number of studies conducted in both mice and rats show that cell proliferation persists during adulthood in the tuberal region of the hypothalamus, is influenced by diet and affects metabolic homeostasis. By

analogy to what has been proposed in the tuberal region, newborn cells of the POA may derive from progenitor cells located within the parenchyma or migrate from a neighbouring stem cell niche. The proposed stem cell niche for the tuberal region of the hypothalamus resides within the wall of the third ventricle, where stem cells could correspond to tanycytes, specialised ependymoglial cells that exhibit morphological, molecular and functional characteristics of radial glia (Rodríguez et al., 2005). Tanycytes can also be found at the level of the POA along the beginning of the third ventricle. Recent works have shown that also at this level, these cells express stem and progenitor cell markers such as nestin, Sox2, GFAP, Vimentin, as well as the proliferation marker Ki67 (Bennett et al., 2009; Furube et al., 2015; Langlet et al., 2013). Moreover, neurospheres were obtained from this region, and could proliferate and differentiate into both glial and neuronal cells (Bennet et al., 2009). While these *in vitro* data show that stem/progenitor cells can indeed be found at the level of the POA, they do not clarify their location. To address the hypothesis that tanycytes may be the candidate stem cells of the POA, it would be interesting to perform fate mapping experiments of tanycytes of the OVLT, as previously done for tanycytes of the ME, to see if they are able to give rise to parenchymal glial and/or neuronal cells.

What is the role of these newborn cells?

To answer this question, cell proliferation could be selectively inhibited at the level of the MPO, before or after mating and the consequences over maternal behaviour evaluated.

8.1.8 The adult human hypothalamus likely contains neural stem/progenitor cells

Different findings suggest that the process of adult neurogenesis has not disappeared throughout evolution but that it has rather adapted to larger structures and different necessities. The third ventricular niche remains a subject of debate among scientists, due to the variability of the data collected at present. The recent identification of cells expressing the neuroblast marker DCX in the human hypothalamus raised the possibility that a local niche may exist in this region (Batailler et al., 2014); however, the hypothalamus had never been characterised in human for the expression of markers used to identify stem/progenitor cells.

Due to the absence of a single marker able to unambiguously label neural stem cells, we decided to use a series of five markers known to be expressed in these cells during embryogenesis and adulthood (Kriegstein & Alvarez-Buylla, 2009; Götz et al., 2015). We identified in the adult human hypothalamus four populations of cells, ependymal cells, ventricular ribbon cells, tanycytes and suprachiasmatic small stellate cells, which exhibit an antigenic profile of neural stem cells (NSCs). Tanycytes was the only population showing a similar antigenic profile between rodents, grey mouse lemur and human.

A very interesting feature was the description of a ribbon of cells expressing NPCs markers, separated from the ependymal layer by a hypocellular gap, which was highly reminiscent of the astrocyte ribbon described along the lateral ventricles by Sanai and colleagues (2004). The SVZ is the largest germinal zone in the adult brain. From this region, a chain of migrating newborn neurons, identified by their expression of DCX, is seen to travel to the OB in rodents and in the non-human primates rhesus monkey (Whang et al., 2011) and grey mouse lemur (personal observations). However, when adult human brains were analysed for the persistence of neurogenesis in the OB, such a phenomenon could not be observed using either neuroanatomical approaches (Sanai et al., 2011) or ^{14}C dating (Spalding et al., 2013).

Nevertheless, when Sanai and colleagues (2011) described the SVZ in newborn children, they surprisingly saw a very strong staining for DCX and a high number of migrating neurons reaching the OB, as well as a subregion of the prefrontal cortex, along a route never seen before in other species. These chains of migrating cells were found between the ependymal layer and the ribbon of cells, before entering the RMS, and had completely disappeared as early as the second year of life, leaving in their place a void corresponding to the hypocellular gap seen along the adult lateral ventricles (Sanai et al., 2011). The observation of a similar gap along the adult third ventricle raises really interesting questions regarding the possibility that the gap could serve during early postnatal development as a path for migrating neurons reaching different regions of the hypothalamus or adjacent structures, therefore contributing to the establishment of networks involved in the maturation of different systems of the neonatal brain. Due to obvious difficulties in obtaining specimens at these young ages, the role of newborn neurons at the level of the OB and of the prefrontal cortex in neonatal human brain is still not known. In the same way, the characteristics of the postnatal development of the human hypothalamus remain obscure.

The presence of engorged areas, referred to as buds, along the ventricle, where the expression of the stem/progenitor markers was strengthened was quite intriguing. Together with the striking difference between the lateral and the third ventricular border in regards of the much higher degree of irregularity in the aspect of the latter, it may be speculated that this area is indeed quite plastic and actively shaping its niche. Once again, it would be very interesting to study the differences between specimens from young and old persons, to understand if this morphological variability can be observed already during postnatal development or only during adult life, maybe being the result of enhanced or erroneous proliferation.

Finally, we estimated the percentage of cells co-expressing the whole panel of NPC markers in the four populations of cells identified in the human hypothalamus. These values were extremely variable among the same population in different specimens, as well as among different regions of the same hypothalamus. For this reason, another point that could be interesting to develop would be that to understand if this variability reflects differences in the neurogenic potential of these different populations; if this was the case, the next question that naturally arises is whether any of this variability could be explained by the age or the presence of different clinical conditions in the patients, such as obesity or neurodegenerative diseases. For example, high fat diet has been shown to cause a decrease in the levels of cell proliferation within the hypothalamus in rodents (Li et al., 2012; McNay et al., 2012), while aging (Zhang et al., 2013) and neurodegenerative diseases are known to affect proliferation within the DG both in rodents and humans.

8.2 CONCLUDING REMARKS

When Ramòn y Cajal's most famous proclamation is cited to introduce the topic of neural plasticity ("*In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated*"), we tend to forget mentioning the second part of his statement: "*It is for the scientists of the future to change, if possible, this harsh decree*". Since then, many scientists have accepted his challenge and the view of the brain as a static structure has now been definitely abandoned in favor of that of a system actively adapting to the different inputs that it constantly receives. The new questions now concern to which extent this happens, and which are the regions that have not been found yet.

The work of my thesis suggests that cell neogenesis during postnatal development and adulthood in the female rat preoptic region of the hypothalamus may be involved in the control of the reproductive function.

The process of postnatal maturation that leads to puberty is still a subject that has not been fully understood. Deviations in the age at which the onset of puberty occurs are becoming more and more common in young girls, due in part to environmental factors such as nutrition, stress and exposure to endocrine-disrupting chemicals. The consequences of this disruption tend to be underestimated but have been associated to psychological and behavioural disorders, increased risk of obesity, type 2 diabetes and cardiovascular diseases in adulthood (Leka-Emiri et al., 2017). This highlights the importance of gaining new insights on the mechanisms involved in pubertal maturation. Our results show that postnatal astrogenesis is involved in the onset of puberty, and the ability of GnRH neurons to attract these newborn cells from their neighbourhood seems to be necessary for their proper maturation, therefore adding postnatal cell proliferation to the processes involved in this complex puzzle.

Moreover, we observe that during adulthood cell proliferation in the POA is influenced by both estrous cyclicity and pregnancy, suggesting that this process may play a role also in the plastic adaptation of the mother brain during and/or after pregnancy.

Finally, we show for the first time the presence of different cell populations expressing a repertoire of stem cell markers in the adult human hypothalamus, suggesting the existence of a hypothalamic neurogenic niche and opening interesting questions on the possible roles played by these putative stem cells in regards to the different hypothalamic functions in humans.

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