



# Hypothalamic neurogenesis persists in the aging brain and is controlled by energy-sensing IGF-I pathway



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## ABSTRACT

Hypothalamic tanycytes are specialized glial cells lining the third ventricle. They are recently identified as adult stem and/or progenitor cells, able to self-renew and give rise to new neurons postnatally. However, the long-term neurogenic potential of tanycytes and the pathways regulating lifelong cell replacement in the adult hypothalamus are largely unexplored. Using inducible nestin-CreER<sup>T2</sup> for conditional mutagenesis, we performed lineage tracing of adult hypothalamic stem and/or progenitor cells (HySC) and demonstrated that new neurons continue to be born throughout adult life. This neurogenesis was targeted to numerous hypothalamic nuclei and produced different types of neurons in the dorsal periventricular regions. Some adult-born neurons integrated the median eminence and arcuate nucleus during aging and produced growth hormone releasing hormone. We showed that adult hypothalamic neurogenesis was tightly controlled by insulin-like growth factors (IGF). Knockout of IGF-1 receptor from hypothalamic stem and/or progenitor cells increased neuronal production and enhanced  $\alpha$ -tanycyte self-renewal, preserving this stem cell-like population from age-related attrition. Our data indicate that adult hypothalamus retains the capacity of cell renewal, and thus, a substantial degree of structural plasticity throughout lifespan.

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## 1. Introduction

The hypothalamus is an important structure of the forebrain that integrates and responds to a variety of hormonal and metabolic signals (Coll and Yeo, 2013; Williams et al., 2000). The recent discovery that production of new neurons persists in the postnatal hypothalamus has opened promising lines of research, promoting neurogenesis as potential modulator of adult hypothalamic plasticity and function. However, little is known about the extent and regulation of hypothalamic neurogenesis throughout lifespan, as well as on its possible impact on physiology later in life. Recently, proliferating glial-like cells have been detected lining the paraventricular zone of the adult hypothalamus in rodents (Xu et al., 2005). Although the cell of origin of adult-born hypothalamic neurons is still a matter of debate,  $\alpha$ -tanycytes are thought to represent the stem cell-like pool, whereas  $\beta$ -tanycytes, which form

a barrier between median eminence (ME) milieu and ventricular cerebrospinal fluid, show only limited self-renewal (Robins et al., 2013; Rodríguez et al., 2005). In this study, we used conditional mutagenesis driven by a nestin-CreER<sup>T2</sup> transgene (Lagace et al., 2007) to perform long-term tanycyte lineage tracing in vivo. We detected significant production of new neurons in the hypothalamus between 3 and 16 months of age, contributing to cell replacement in various hypothalamic nuclei. Interestingly, several studies suggest that the postnatal dynamics of hypothalamic neurogenesis impacts feeding regulation, but it remains unclear whether increased or decreased neurogenesis is beneficial for long-term energy balance (Kokoeva et al., 2005; Lee et al., 2002; Pierce and Xu, 2010). Conversely, tanycytes are known to respond to a range of trophic factors and energy-sensing pathways (Pérez-Martín et al., 2010), suggesting that environmental changes in diet or activity may influence neurogenesis. Circulating IGF-I is a major molecular integrator of nutrition and exercise that differentially modulates short-versus long-term dynamics of adult neurogenesis. Although acute administration of IGFs promotes stem cell proliferation and differentiation (Aberg et al., 2000; Llorens-Martín et al., 2010), lifelong suppression of IGF-1 receptor (IGF-1R) from

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adult neural stem cells in the subventricular zone (SVZ) significantly delays age-related decline of olfactory bulb neurogenesis (Chaker et al., 2015). Intriguingly, long-term inhibition of IGF signals in neuronal regenerative compartments also leads to physiological changes in olfaction and energy metabolism (Chaker et al., 2015). Here, we asked whether somatotrophic signaling in adult hypothalamic stem and/or progenitor cells (HySC) regulates neurogenesis in the hypothalamic niche. To test this hypothesis, we induced conditional knockout (KO) of IGF-1R specifically in adult nestin<sup>+</sup> cells, an intervention that mimics food scarcity by genetically lowering cell sensitivity to IGF-I and quantified short- and long-term effects of this mutation on hypothalamic neurogenesis. Results support the idea that IGF signaling pathways coordinate environmental changes and neuronal replacement in an important energy-sensing region of the adult mammalian brain.

## 2. Materials and methods

### 2.1. Mouse models

Experimental protocols were approved by *Comité d'éthique pour l'Expérimentation Animale* "Charles Darwin" (approval N°Ce5/2012/074). All analyses were performed on males. We backcrossed CAG-tdTomato<sup>+/0</sup> mice (<http://jaxmice.jax.org/strain/007908.html>) to 129/SvPas (129) genetic background. Founder group A was produced crossing CAG-tdTomato<sup>+/0</sup> with knock-in IGF-1R<sup>lox/lox</sup> mice on 129 background. Founder group B was obtained crossing nestin-CreER<sup>T2</sup> transgenic mice with C57BL/6 (B6) background (provided by Dr. Amelia J. Eisch) with IGF-1R<sup>lox/lox</sup> (B6) mice. The final triple transgenic mutants (nestin-CreER<sup>T2</sup>;CAG-tdTomato<sup>+/0</sup>; IGF-1R<sup>lox/lox</sup>) were generated by crossing founders A and B, resulting in fully reproducible F1 hybrid B6/129 background. In parallel, age-matched control animals were produced on the same F1 hybrid background by crossing nestin-CreER<sup>T2</sup> (B6) with CAG-TdTomato<sup>+/0</sup> (129) mice (nestin-CreER<sup>T2</sup>;CAG-TdTomato<sup>+/0</sup>; IGF-1R<sup>wt/wt</sup>). All mice were housed in individually ventilated cages under specific pathogen-free conditions at 21 °C–23 °C, 12 hours/12 hours light/dark cycle, and food and water ad libitum. Control and mutant mice received tamoxifen (Tam; T5648, Sigma–Aldrich) at 12 weeks of age (84 mg kg<sup>−1</sup> in 2 intraperitoneal injections per day, for 5 consecutive days). Experimental groups of KO and control animals were analyzed 1, 6, and 13 months after induction of Cre recombination.

### 2.2. Tissue sampling

Mice were anesthetized and perfused transcardially with cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Brains were postfixed overnight at 4 °C in 4% paraformaldehyde, and then incubated in 0.1-M phosphate-buffered saline with 30% sucrose until they sank. Brains were snap-frozen in isopentane at −45 °C and sectioned into 30-μm slices on a freezing microtome in coronal plane from Bregma −1.22 to −2.54. Coordinates were determined using a mouse brain atlas (Paxinos and Franklin, 2001). Sections were collected in 6 parallel sets of 10, with sections being 180 μm apart. All 6 sets represented comparable samples of the entire arcuate nucleus (ARC) and ME region, and each cell type-specific staining was performed on a given set.

### 2.3. Immunohistochemistry

Cell type-specific antibody staining was performed on free-floating sections and combined with Tom<sup>+</sup> newborn cell lineage tracing. Antibodies used were: rabbit polyclonal anti-IGF-1Rβ subunit antibody (sc-713, 1:200, Santa-Cruz Biotechnology), rabbit polyclonal

anti-glia fibrillary acidic protein antibody (GFAP 1:2000; Dako), anti-nuclear Ki67 antibody (1:250; Millipore), mouse monoclonal anti-neuronal marker antibody (NeuN 1:500; Millipore), rabbit polyclonal anti-P-STAT3 antibody (1:200; Abcam), rabbit polyclonal anti-Neuropeptide Y antibody (1:500; Abcam), mouse monoclonal anti-GABA-B receptor 1 antibody (1:1000; Abcam), rabbit polyclonal anti-GHRH antibody (1:200; Abcam), and rabbit polyclonal anti-glutamate receptor 1 antibody (AMPA subtype; 1:200; Abcam).

### 2.4. Cell quantification

All tissues used for quantification were imaged using a spinning disc microscope (Yokogawa spinning head, Roper/Leica). Cell densities and absolute number of neurons were measured on Z-stacks composed of 2–4 μm optical slices. Cells were manually counted using Z-stack treatment options of Fiji software (<http://fiji.sc/Fiji>). Tanycytes were identified by their highly specific morphology, and α and β subtypes discriminated based on their anatomical position with respect to the 3rd ventricle (Robins et al., 2013). To assess neuronal morphology, about 20 cells per group were analyzed by acquisition of Z-stacks of 15–20 optical sections per neuron at 0.5-μm intervals. 3D-reconstructions of glial cells and neurons were performed using the 3D Project Fiji plug-in.

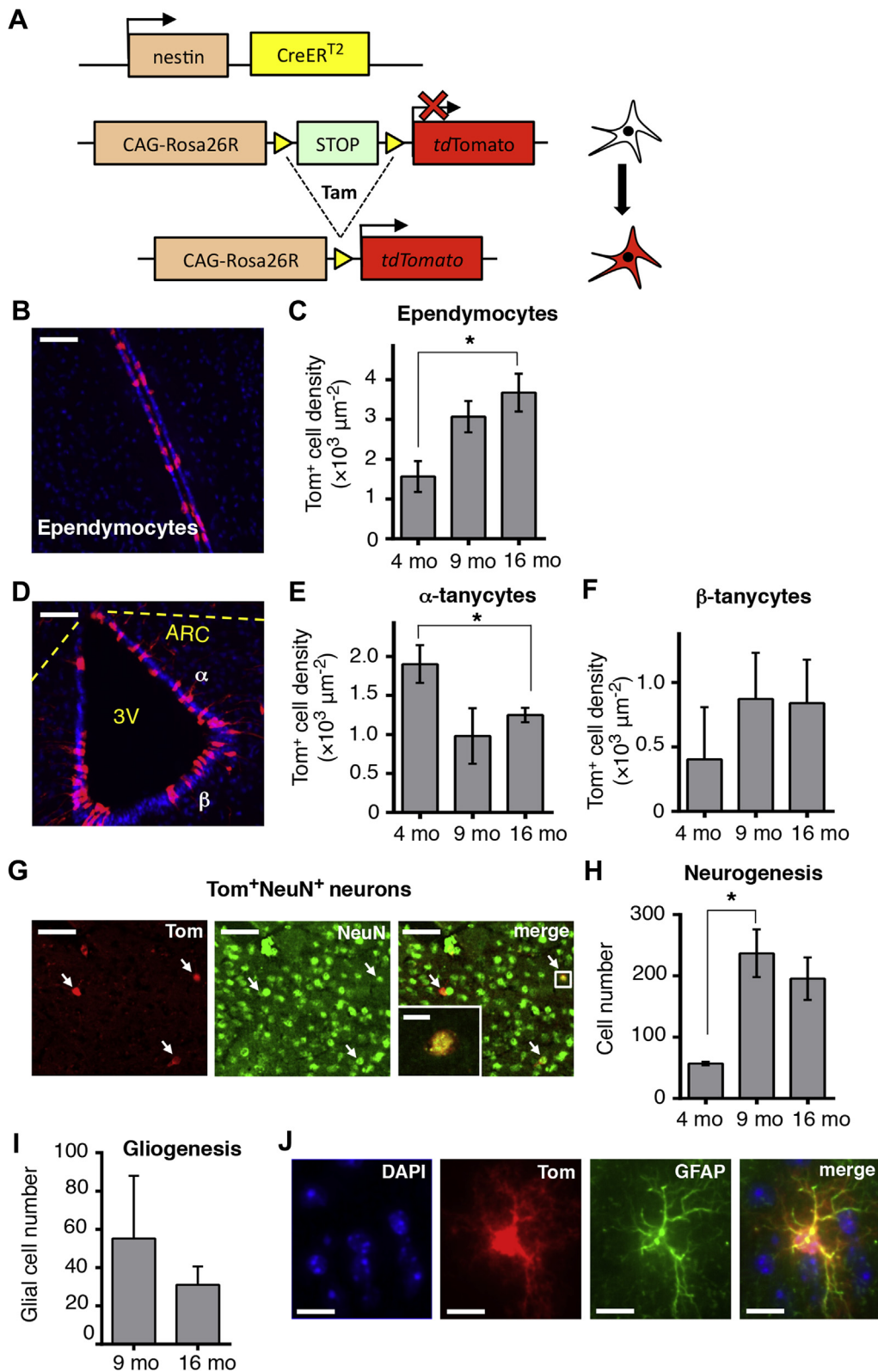
### 2.5. Statistical analyses

All data are reported as mean ± standard error of the mean. The threshold of statistical significance was defined as  $p < 0.05$ . Sample size  $n$  is the number of mice per experimental group. Student's  $t$  test was used to compare groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## 3. Results

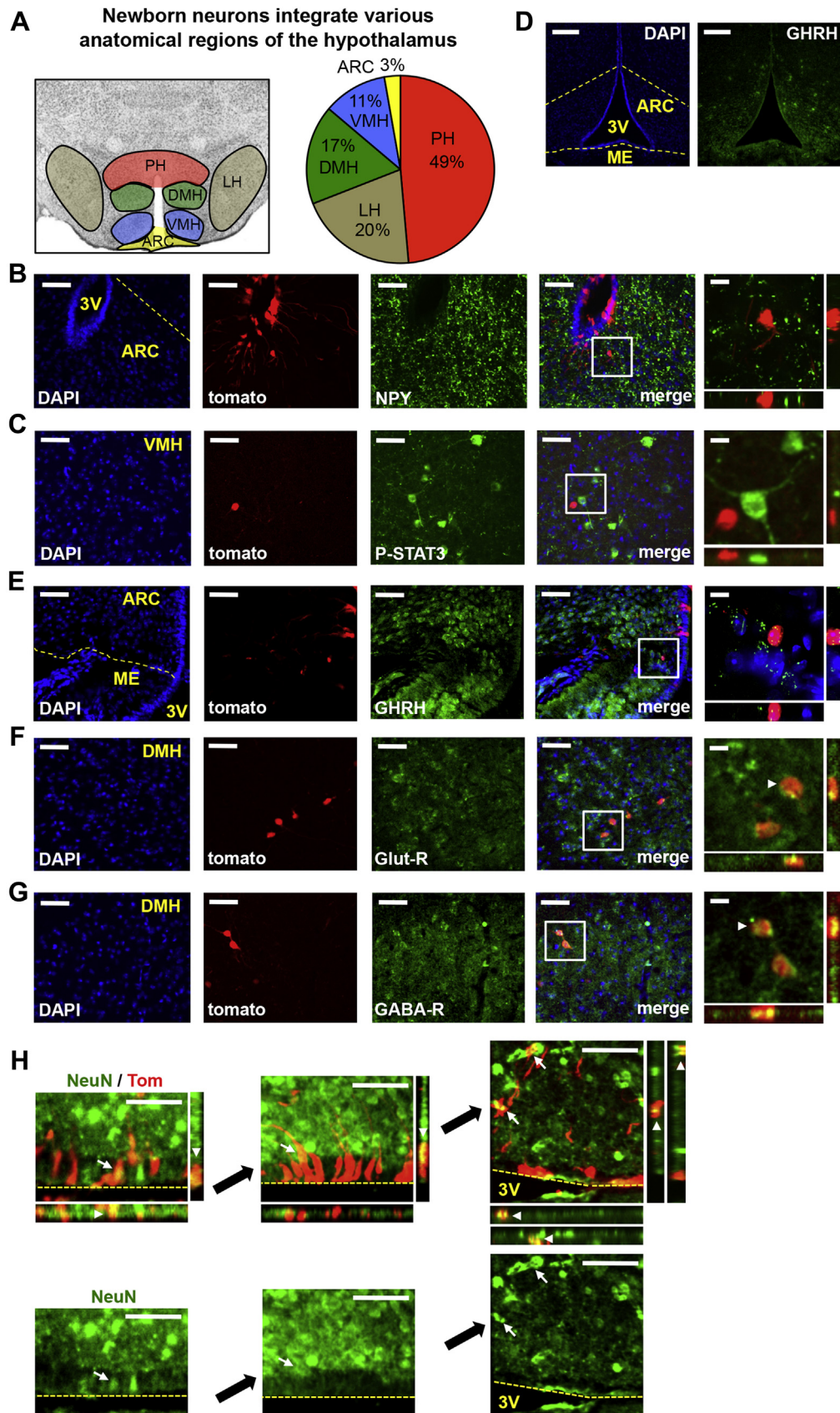
### 3.1. Neurogenesis and gliogenesis occur beyond postnatal periods in the aging hypothalamic niche

To perform long-term tanycyte lineage tracing, we combined the nestin-CreER<sup>T2</sup> transgene (Lagace et al., 2007) with the Cre-inducible tdTomato knock-in reporter (Madisen et al., 2010). We injected nestin-CreER<sup>T2</sup>;CAG-tdTomato<sup>+/0</sup> mice with Tam at 3 months of age to exclude all developmental neurogenesis (Fig. 1A). Red fluorescence was efficiently induced in ependymocytes (Fig. 1B–C) and in α- and β-tanycytes (Fig. 1D–F). This new mouse model allowed cumulative recording of neurogenesis at different time points during adult life and aging. We observed that ependymal cell density increased significantly between 4 and 16 months of age (Fig. 1C;  $1565 \pm 387$  vs.  $3676 \pm 476$ ,  $p < 0.05$ ). Because Tam-inducibility ensured that all increases in Tom<sup>+</sup> cell number in this transgenic mouse were due to proliferation, these data support the still debated idea that ependymocytes keep proliferating in the aging brain. We also demonstrated for the first time a significant age-related attrition of the α-tanycyte compartment (Fig. 1E; −34%,  $p = 0.02$ ). In contrast, β-tanycytes were substantially less abundant than α-tanycytes, and their number remained unchanged with age (Fig. 1F), corroborating the hypothesis that this cell compartment was rather transitory in the adult neurogenic process. Interestingly, the number of Tom<sup>+</sup>NeuN<sup>+</sup> cells (Fig. 1G) increased dramatically between 4 and 9 months, reaching a plateau between 9 and 16 months (Fig. 1H;  $57 \pm 3$ ,  $237 \pm 39$ , and  $196 \pm 35$  cells, respectively,  $p_{4-9\text{ mo}} = 0.04$ ). This result clearly demonstrates that production of new neurons continues in the adult hypothalamus. As some postnatal gliogenesis has been reported in this region (Migaud et al., 2010), we quantitatively compared neurogenic and gliogenic fate over age. At 9 and 16 months, we found few newborn cells presenting glial



**Fig. 1.** Dynamics of neurogenesis in the aging hypothalamic niche. (A) Schematics of inducible transgenic approach used for in vivo lineage tracing. Administration of Tam-activated Cre recombinase in all nestin<sup>+</sup> cells, inducing intense red label in these cells and their progeny. All mice were injected at 3 months of age and analyzed 1, 6, and 13 months after Tam-induced labeling. (B) Representative micrograph of recombined ependymal cells (red); section counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). (C) Age-dependent dynamics of ependymocyte population;  $p = 0.05$ . (D) Representative micrograph of recombined  $\alpha$ - and  $\beta$ -tanycytes (red) lining the 3rd ventricle (3V); section counterstained with DAPI (blue). (E and F) Age-dependent dynamics of  $\alpha$ -tanycytes (E) ( $p = 0.02$ ) and  $\beta$ -tanycytes (F). (G) Sections of dorsomedial hypothalamus stained with neuronal marker NeuN. Arrows indicate adult-born neurons. Scale bars: 50 μm in large micrographs, 10 μm in inset. (H) Age-dependent evolution of neuronal population;  $p = 0.04$ . (I) Absolute number of Tom<sup>+</sup> glial cells detected in the hypothalamic niche at 9 and 16 months. Quantifications were performed on 10 sections per animal and  $n_{4\text{ mo}} = 2$ ,  $n_{9\text{ mo}} = 6$ , and  $n_{16\text{ mo}} = 5$  animals per group. (J) 3D Z-projection image representing newborn glial cells (astrocytes colabeled with GFAP). Scale bar: 10 μm. \*  $p < 0.05$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)





**Fig. 2.** Identity of newborn hypothalamic neurons. All results are from 9-mo-old animals. High magnification micrographs are presented with orthogonal views of corresponding confocal Z-stacks. (A) Hypothalamic integration sites of adult newborn neurons. (B–C) Double labeling of Tom<sup>+</sup> neurons in the ARC-ME region with functional marker NPY (B), and in the VMH-DMH region with P-STAT3 (C), showing no colocalization with Tom<sup>+</sup> cells. (D) GHRH immunostaining is restricted to the ARC-ME region around the third ventricle; scale bar: 100  $\mu$ m. (E) GHRH staining colocalizing with Tom<sup>+</sup> cells in the ARC-ME region. (F and G) Tissue sections representing DMH nuclei stained with anti-glutamate receptor (F) and

morphology (oligodendrocytes, microglia, and astrocytes) in the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH), as compared to the number of newborn neurons (Fig. 1I and J). Importantly, we could not detect any gliogenic activity at 4 months, suggesting that limited gliogenesis developed predominantly during late adult life.

### 3.2. Adult neurogenesis produces specific neuronal cell-types in functionally different regions of the hypothalamus

Adult neurogenesis in the hypothalamic niche was widespread and concerned multiple nuclei located in the posterior hypothalamus (PH), DMH, VMH, mediobasal, and lateral hypothalamus (LH) (Fig. 2A). At 9 months of age, the vast majority of newborn neurons integrated into dorsomedial hypothalamus (PH, DMH, and VMH), whereas the ARC and ME also received some  $\text{Tom}^+$  neurons (Fig. 2A; 49% in PH vs. 3% in ARC). The long-term integration pattern that we observed differed substantially from the neurogenesis reported from early-postnatal periods, confined predominantly to ARC and ME (Lee et al., 2012). Because of this intriguing discrepancy, we aimed at characterizing which types of neuron are born in the aging hypothalamus. We checked whether neurons contributing to the regulation of food satiety were replaced in ARC and DMH but did not find tangible evidence for colocalization of  $\text{Tom}^+$  cells with neuropeptide Y (NPY; Fig. 2B;  $n = 6$  animals,  $> 250$   $\text{Tom}^+$  cells screened). Similarly, newborn hypothalamic cells were all negative for P-STAT3, indicating that none of them pertained to the populations of leptin-sensitive, orexigenic NPY (AgRP) or to anorexigenic (proopiomelanocortin) neurons (Fig. 2C;  $n = 6$ ,  $> 250$  cells screened). Interestingly, we found that some  $\text{Tom}^+$  adult-born cells in the aging ARC-ME were growth hormone releasing hormone (GHRH) producing neurons (Fig. 2D–E). In contrast to the situation in the most ventral hypothalamus, DMH and PH contained a substantial proportion of total newborn neurons. These cells were expressing receptors for either glutamate or GABA, the 2 most abundant neurotransmitters in the hypothalamus (Fig. 2F–G; 59% were glutamate receptor-positive and 23% GABA receptor-positive,  $n = 6$ ,  $> 250$   $\text{Tom}^+$  cells screened). This result suggested that adult-born neurons were able to functionally integrate into preexisting neurocircuitry, possibly as relay interneurons. Although the cell of origin and the differentiation steps of newborn hypothalamic neurons are still a matter of debate, the anatomical disposition of  $\text{Tom}^+\text{NeuN}^+$  cells around the 3rd ventricle suggested that they were potentially stemming from  $\alpha$ -tanyocytes and progressively migrated into the hypothalamic parenchyma (Fig. 2H).

### 3.3. Suppression of IGF-I signaling in adult HySCs delays age-related decline of hypothalamic neurogenesis

To study the role of IGF-I signaling in hypothalamic neurogenesis, we used conditional gene targeting of IGF-1R. Nestin-CreER<sup>T2</sup>;CAG-tdTomato<sup>+0</sup>;IGF-1R<sup>flox/flox</sup> mutants and nestin-CreER<sup>T2</sup>;CAG-tdTomato<sup>+0</sup> control mice were injected with Tam at 3 months of age to induce HySC-specific IGF-1R KO. This provoked both red fluorescent label and loss of IGF-1R in all tanyocytes and

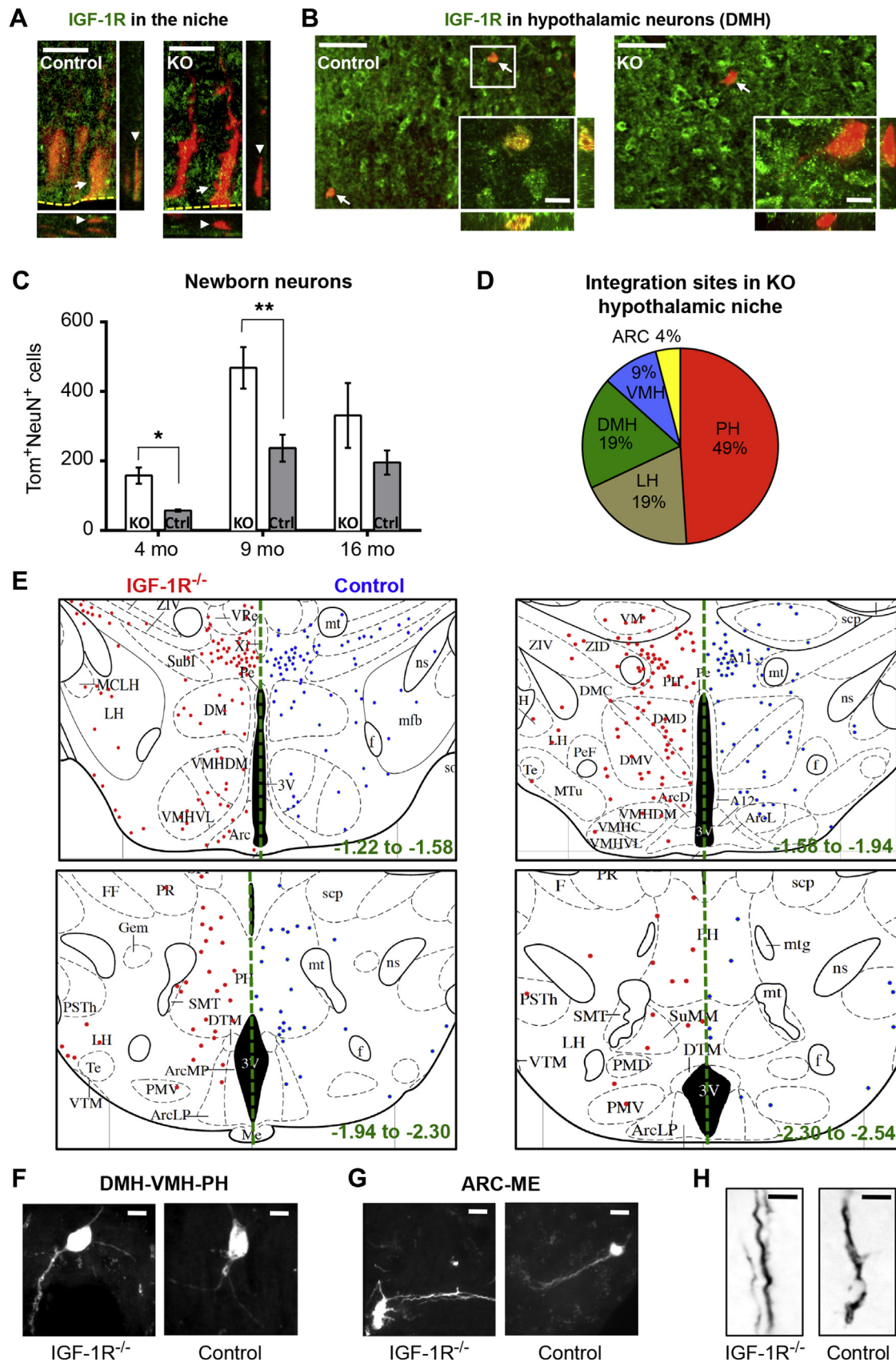
tanyocyte progeny in mutants, and red fluorescence in the corresponding cells in control mice, allowing quantitative comparison of IGF-1R<sup>-/-</sup> and IGF-1R<sup>wt/wt</sup> newborn cell lineages. We confirmed that tanyocytes and neurons all express high levels of IGF-1R in wild type animals (Fig. 3A–B, left panels). In contrast, adult-born  $\text{Tom}^+$  neurons and tanyocytes in mutants were completely devoid of IGF-1R (Fig. 3A–B, right panels).

Using the neuronal marker NeuN, we quantified  $\text{Tom}^+$  newborn mature neurons in the hypothalamic niche over age, and observed significantly increased neuronal production in mutants compared to controls at 4 months (Fig. 3C;  $158 \pm 23$  vs.  $57 \pm 3$ ,  $p_{4\text{ mo}} = 0.04$ ), and at 9 months (Fig. 3C;  $468 \pm 60$  vs.  $237 \pm 39$ ,  $p_{9\text{ mo}} = 0.008$ ). Despite noticeable age-related decline of neurogenesis in both groups between 9 and 16 months ( $-29\%$  in KO vs.  $-17\%$  in controls), mutants tended to preserve more adult-born neurons at long-term (Fig. 3C;  $331 \pm 93$  vs.  $196 \pm 35$  cells,  $p_{16\text{ mo}} = 0.24$ ). Of critical note, complete suppression of IGF-I signaling in adult tanyocytes did not affect gliogenesis in the aging hypothalamus (9 months: KO  $48 \pm 11$  vs. control  $57 \pm 27$ , and 16 months: KO  $28 \pm 12$  vs. control  $31 \pm 10$ , non-significant results). Interestingly, the cumulative integration in mutants (Fig. 3D) was similar in proportion to controls (Fig. 2A), confirming that the absolute number of newborn neurons was significantly increased in KO animals in all hypothalamic nuclei and also along the antero-posterior axis (Fig. 3E). Of important note, most newborn neurons originating from the 3rd ventricle seemed to have migrated long distances before integrating into hypothalamic nuclei in KO and control animals (Fig. 3E). These data clearly demonstrate that the deletion of IGF-1R in adult HySC dramatically enhanced hypothalamic neurogenesis, both at short and at long-term. Using Kaplan–Meier analysis, we found longevity of KO mice unchanged (Supplementary Fig. 1), excluding the possibility that increased neurogenesis in old animals was due to a systemically modified aging process. Thus, IGF-1R KO in neural stem cells and their progeny maintained youthful neurogenesis in the brain of mice with normal aging trajectory.

In addition to the impact on neurogenesis, IGF-I signaling also affected the morphology of newborn neuron selectively in the ARC-ME region (Fig. 3F–G). Whether KO or control, adult-born neurons newly integrated into the ARC-ME had significantly smaller soma than those integrated into VMH, DMH, and PH (at 16 months of age, KO: ARC-ME neurons  $178 \pm 28 \mu\text{m}^3$  vs. VMH-DMH-PH neurons  $522 \pm 50 \mu\text{m}^3$  and controls: ARC-ME neurons  $193 \pm 61 \mu\text{m}^3$  vs. VMH-DMH-PH neurons  $482 \pm 81 \mu\text{m}^3$ ;  $n = 5$ –7 cells for ARC-ME and  $n = 18$  cells for posterior regions;  $p < 0.001$ ). Interestingly, extensions of IGF-1R<sup>-/-</sup> nerve cells in the ARC-ME region appeared to be conspicuously more tortuous than in controls at 9 months (not shown) and 16 months of age (Fig. 3G). To quantify tortuosity, we defined dendritic linearity index as curvilinear length divided by the straight line connecting the ends of the measured segment (Irintchev et al., 2005). We found that dendrites of IGF-1R<sup>-/-</sup> neurons were significantly more tortuous than controls (Fig. 3H; KO  $1.09 \pm 0.01$  vs. control  $1.04 \pm 0.01$ ,  $n = 4$ –5,  $p = 0.04$ ). Moreover, IGF-1R null neurons also displayed significantly more dendrites sprouting from the soma (Fig. 3G; KO  $4.6 \pm 0.5$  vs. control  $2.0 \pm 0.2$  dendrites per cell,  $n = 4$ –5,  $p = 0.012$ ). These results suggest that outgrowth is strongly dependent on IGF-I signaling also in the adult

anti-GABA receptor (G) antibodies. Arrowheads indicate neurons observed in orthogonal views. (H) Adult-born neurons (white arrows) identified as  $\text{Tom}^+$  (red) and  $\text{NeuN}^+$  (green) cells were found in variable distance to the third ventricle wall (3V; delimited with dashed line). Spatial arrangement of these new neurons relative to  $\text{Tom}^+$  tanyocytes is compatible with the idea of neurogenic tanyocytes. Depicted events could, from left to right (black arrows), represent successive stages in adult hypothalamic neurogenesis. Although no direct proof, positions of new neurons are suggestive of swift migration away from the ventricle walls into the brain parenchyma. Scale bars: 50  $\mu\text{m}$  and 10  $\mu\text{m}$  for high magnification micrographs. Abbreviations: ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; GHRH, growth hormone releasing hormone; LH, lateral; ME, median eminence; NPY, neuropeptide Y; PH, posterior; VMH, ventromedial hypothalamus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)





**Fig. 3.** IGF-I signaling and lifelong regulation of hypothalamic neurogenesis. (A) Adult-born tanycytes residing in the hypothalamic niche express IGF-1R in control animals (left; red, and green label), whereas in mutants they do not (right; red label only). The wall of the third ventricle (3V) is delimited with a dashed line. Arrows indicate Tom<sup>+</sup>IGF-1R<sup>+</sup> and Tom<sup>+</sup>IGF-1R<sup>-</sup> cells; corresponding orthogonal views confirm colocalization. Scale bars: 20  $\mu$ m. (B) Tom<sup>+</sup> newborn neurons express IGF-1R in controls (left), whereas in mutants they

brain and that these effects might be specific to neuroendocrine neurons in ARC and ME.

### 3.4. IGF-I signaling controls $\alpha$ -tanycyte maintenance by modulating their division pattern over age

We next asked whether enhanced neurogenesis in mutants was accompanied by stem/progenitor cell depletion with age. To address this point, we determined  $\alpha$ - and  $\beta$ -tanycyte density in vicinity of the 3rd ventricle. Importantly, at 4 months, KO and controls started with the same number of  $\alpha$ -tanycytes (Fig. 4A). In control animals, we observed a marked attrition of this cell compartment between 4 and 16 months (−48%). In contrast, mutants maintained and even increased the number of  $\alpha$ -tanycytes with age (+33%). These differences resulted in twice as many  $\alpha$ -tanycytes in mutants compared to controls, both at 9 and 16 months of age (Fig. 4A–B; +132% at 9 months,  $p_{9\text{ mo}} = 0.02$ , and +108% at 16 months,  $p_{16\text{ mo}} < 0.0001$ ). However, we did not observe such age-dependent differences in  $\beta$ -tanycyte density comparing KO and controls (Fig. 4C). Starting with the same number of cells at 4 months, mutants tended to have higher  $\beta$ -tanycyte density at 9 months of age (+78%,  $p = 0.24$ ), but this trend disappeared at age 16 months. Similarly, nonneurogenic ependymal cells seemed unaffected by lifelong suppression of IGF-I signaling (Fig. 4D). Thus, inhibition of IGF signaling in adult HySC prevented age-related depletion selectively in  $\alpha$ -tanycytes.

Intriguingly, mutants maintained the pool of  $\alpha$ -tanycytes between 9 and 16 months, but produced substantially less neurons at 16 months compared to 9 months. In fact,  $\alpha$ -tanycyte density (Fig. 4A) and number of newborn neurons (Fig. 3C) were no longer correlated in the 16-month-old mutants. To explain this discrepancy, we checked whether proliferation propensity decreased in IGF-1R<sup>−/−</sup>  $\alpha$ -tanycytes between 9 and 16 months of age. We found that the proportion of Ki67<sup>+</sup>  $\alpha$ -tanycytes was similar at both ages in mutants (Fig. 4E), indicating that IGF-1R<sup>−/−</sup>  $\alpha$ -tanycytes at 16 months were preferentially self-renewing and less differentiating. This result strongly points to a late-life shift in cell fate choice induced by suppression of IGF-I signaling in HySC. Collectively, these data demonstrate that deletion of IGF-1R from nestin<sup>+</sup> cells in the adult hypothalamus allows better maintenance of  $\alpha$ -tanycytes specifically with age, possibly through lifelong control of their division pattern.

## 4. Discussion

### 4.1. Neurogenesis in aging versus early-postnatal hypothalamus

Using a conditional genetic marker, we performed long-term lineage tracing of nestin<sup>+</sup> neuronal progenitors in the mammalian hypothalamus. This allowed recording neurogenesis over extended periods of adult life and revealed for the first time quantitative aspects of neuronal replacement in this adult stem cell niche. Importantly, all previous studies reported lineage-tracing starting P28 or younger (Haan et al., 2013; Lee et al., 2012; Robins et al., 2013), thus encompassing significant developmental effects. In contrast, the present study focuses on fully adult animals, in which

hypothalamic neurogenesis has long been thought negligible. Published data on SC-dependent maintenance of other adult tissues show that these processes generally decline with increasing age. Therefore, it is very likely that our findings comprise most if not all of adult neurogenesis after 3 months, and that the bulk of this neurogenesis occurred between 3 and 9 months of age. We demonstrated that hypothalamic neurogenesis generated several types of neurons. Interestingly, identity as well as integration sites of these neurons differed markedly from what has been described for hypothalamic neurons born during postnatal periods (Kokoeva et al., 2005; Rojczyk-Gołębiewska et al., 2014). In the present study, adult-born neurons mostly integrated into PH, DMH, and VMH nuclei, and a smaller proportion was found in the ARC and ME. Moreover, most of these new neurons were interneurons receiving GABA and glutamate afferences in the posterior hypothalamus, and some were GHRH neurons, in the most ventral regions. We did not find any anorexigenic or orexigenic leptin-sensitive newborn neurons during aging, contrasting with what has been documented for early-postnatal hypothalamic neurogenesis (Kokoeva et al., 2005; Rojczyk-Gołębiewska et al., 2014). This intriguing observation suggests that early and late adult neurogenesis of the hypothalamus might contribute to different neurophysiological functions. Several reports show that postnatal neurogenesis in the hypothalamus is controlling energy homeostasis during the first 2 months of adulthood (Sousa-Ferreira et al., 2014), and accordingly concerns NPY, proopiomelanocortin, and P-STAT3 neurons. We propose that the here observed late-life hypothalamic neurogenesis is mainly targeting relay interneurons that might be important for tuning the activity of specific neuronal circuits in selected nuclei of the hypothalamus.

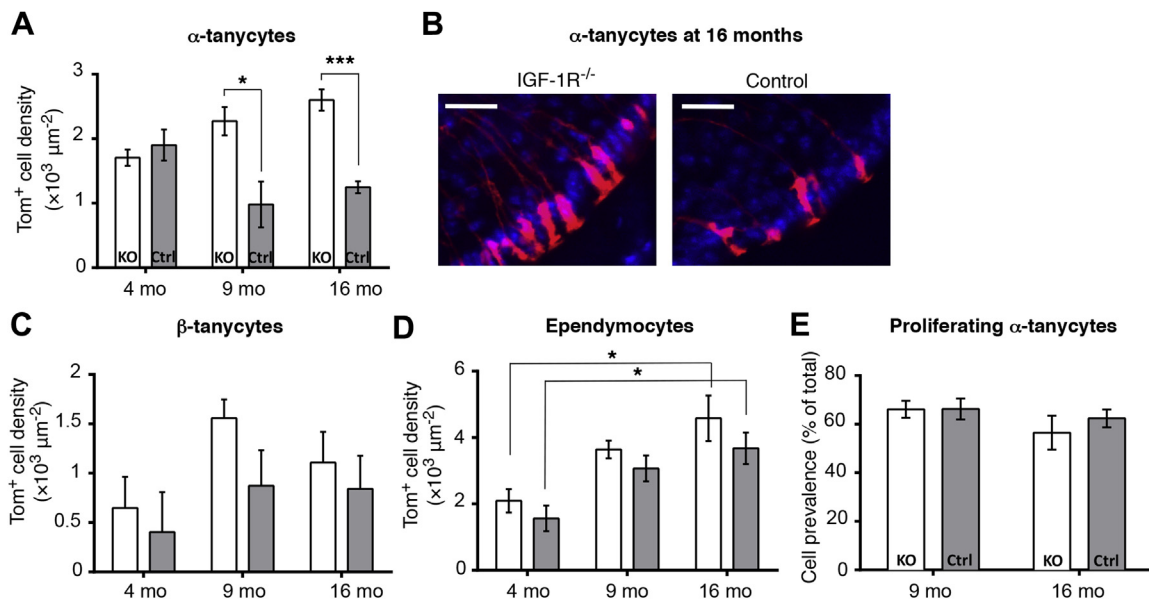
### 4.2. Adult-born GHRH-producing neurons

Another surprising observation was that GHRH neurons continued to be produced in the brain throughout adult life. These neurons are important elements in the forward regulation and retroregulation of the somatotrophic neuroendocrine axis. Their nerve endings extend to the ME, and GHRH secretion eventually induces growth hormone release from the pituitary into the peripheral blood. It is well-documented that GHRH neurons decrease in number with age (Kuwahara et al., 2004). Thus, de novo neurogenesis of specialized neuroendocrine neurons in the adult brain as we demonstrated here may open promising avenues of research for regenerative medicine and novel therapeutic strategies targeting replacement of neuropeptide and hormone-producing cells in the central nervous system.

### 4.3. IGF-I signaling, neurogenesis, and metabolism

In this study, we demonstrated that hypothalamic neurogenesis continues in the adult and aging brain, which implies that it is controlled by specific molecular pathways throughout lifespan. Previous studies have shown that cell replacement in this new niche is tightly regulated by nutrient-sensing factors, such as brain-derived neurotrophic factor, ciliary neurotrophic factor, and fibroblast growth factors FGF-2, -10, and -18 (Kokoeva et al., 2005;

do not (right). Scale bars: 50  $\mu$ m; 10  $\mu$ m in insets. Orthogonal views corresponding to insets confirm IGF-1R immunostaining throughout Z-stacks. (C) Number of newborn neurons in the adult hypothalamus over age.  $n_{4\text{ mo}} = 2\text{--}3$  and  $n_{9\text{--}16\text{ mo}} = 5\text{--}6$ ,  $p = 0.04$  at 4 months and  $p = 0.008$  at 16 months. \* $p < 0.05$ , \*\* $p < 0.01$ . (D) Proportion of newborn neurons integrated in each hypothalamic nucleus (% of total Tom<sup>+</sup> neurons in the niche). (E) Neuronal integration map in controls versus mutants reported on representative coronal sections of the brain, from Bregma −1.22 to −2.54. The map was built by compiling all Tom<sup>+</sup> cells from 5 animals for each group, using the same number of sections per genotype. (F and G) Representative micrographs of newborn neurons integrated into DMH-VMH-PH (F) and ARC-ME (G) regions of 16-month-old animals. Scale bars: 10  $\mu$ m. (H) Dendrite morphology of ARC-ME neurons at 16 months of age. Scale bar: 5  $\mu$ m. Abbreviations: ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; GHRH, growth hormone releasing hormone; IGF-I, insulin-like growth factor; IGF-1R, insulin-like growth factor type 1 receptor; ME, median eminence; NPY, neuropeptide Y; PH, posterior hypothalamus; VMH, ventromedial hypothalamus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** IGF-I controls cell fate choice of  $\alpha$ -tanycytes throughout lifespan. (A–D) Tanycyte and ependymal cell densities in mutant and control mice over age. (A)  $\alpha$ -tanycyte density;  $n = 5–6$ ,  $p_{9\text{ mo}} = 0.017$ ,  $p_{16\text{ mo}} < 0.0001$ . (B) Micrographs illustrating  $\alpha$ -tanycyte abundance in knockout (KO) versus control animals. Scale bars: 50  $\mu\text{m}$ . (C)  $\beta$ -tanycyte density. (D) Ependymal cell density. (E) Proportion of Tom<sup>+</sup>Ki67<sup>+</sup>  $\alpha$ -tanycytes at 9 and 16 months of age in mutants and controls. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .

Robins et al., 2013). Moreover, hypothalamic neurogenesis is known to be highly sensitive to nutrition and metabolic status of the animal. For instance, models of rodent obesity display a significant decrease of postnatal neurogenesis in the arcuate nucleus and ME (McNay et al., 2012). Inversely, inhibiting hypothalamic neurogenesis is sufficient to alter weight and metabolic balance at short-term (Lee et al., 2012). Here, we propose that IGF-I signaling, a potent energy-sensing pathway, could serve as a molecular bridge connecting nutrition and cell replacement during adult life and aging. Constitutive downregulation of IGF-I signaling, which is a genetic intervention mimicking conditions of low environmental energy resources, prolongs individual lifespan and preserves regenerative potential of stem cells (Ratajczak et al., 2011). Of note, our experimental model does not allow to distinguish whether the proneurogenic phenotype that we observed in the hypothalamus is a direct consequence of IGF-1R deletion in tanycytes or an indirect effect of IGF-1R KO occurring simultaneously in nestin-expressing cells in the subventricular niche. Thus, non-cell autonomous mechanisms involving olfactory circuitry may well play a role in this regulation of adult hypothalamic neurogenesis. Intriguingly, mutant mice used for the present study also develop a metabolic phenotype at 16 months of age, becoming leaner and more insulin-sensitive with age (Chaker et al., 2015). Although primarily explained by important changes in olfactory bulb neurogenesis and olfactory sensory function, this phenotype potentially also depends on the fact that aged mutants produce more relay interneurons in the hypothalamus, and/or that these neurons are IGF-1R deficient. Collectively, our work suggests that the dynamics of neurogenesis in the hypothalamic niche adapts itself to changes in environmental resources and chemosensory signals. This plasticity seems to be controlled lifelong by IGF signals and might in turn exert a feed-forward effect on regulation of individual metabolic homeostasis. Interestingly, in mutants and controls, the number of tanycytes far exceeded neurons, raising the issue of whether only a subset of tanycytes has the capacity to generate new neurons. Similarly, as the number of tanycytes was the same in mutants and controls at 4 months, it will be important to parse whether the increase in lineage-traced neurons at all ages

in mutant mice stems from increased proliferation rates alone or depends also on increased survival of adult-born neurons.

#### 4.4. IGF signals and $\alpha$ -tanycyte self-renewal during aging

Downregulation of IGF signals is known to prolong lifespan in an evolutionary conserved manner (Clancy et al., 2001; Holzenberger et al., 2003; Kenyon et al., 1993). One cellular mechanism underlying this longevity effect is preservation of long-term tissue maintenance, as has been reported for SVZ neurogenesis as a paradigm (Chaker et al., 2015). In the present study, we demonstrated that inhibition of IGF signals in HySCs also delays age-related decline of hypothalamic neurogenesis, regardless of the much lower cell turnover compared to olfactory bulb neurogenesis. Two major mechanisms could give rise to this proneurogenic effect: (1) enhanced migration, integration, and survival of newborn KO neurons; and (2) altered proliferation dynamics, and better maintenance of the HySC pool.

Further studies are needed to test relative contributions and also to identify the precise origin of adult hypothalamic neurogenesis. Of note, this nestin-CreER<sup>T2</sup> causes neither tdTomato recombination in peripheral tissues nor does it produce any recombination in the brain in the absence of Tam (Chaker et al., 2015). Moreover, upon Tam administration, cell lineage label is switched on in just a few days. Nevertheless, we cannot exclude that the lineages that we detected, sporadically express CreER<sup>T2</sup> further down the differentiation pathway. Thus, we cannot make a definitive statement on proliferation dynamics involving tanycytes as the only cells of origin. Migration of neuroblasts in adult brain can be as fast as 100  $\mu\text{m}/\text{h}$  in the olfactory bulb (Bakhshetyan and Saghatelian, 2015), and therefore, even if one started looking only a few days after Tam administration, Tom<sup>+</sup> cells in the parenchyma may still have originated from the walls of the 3rd ventricle. Notwithstanding the previously mentioned, based on the present data, we may conclude that deletion of IGF-1R shifted the  $\alpha$ -tanycyte division pattern towards more self-renewal at long-term, with no effect on  $\beta$ -tanycyte or ependymal cell number. This selective action on the  $\alpha$ -tanycyte subset is concordant with IGF-I having more



dramatic effects on neurogenesis occurring in the medial periventricular region of the niche, as demonstrated at short-term in 8-week-old rats (Pérez-Martín et al., 2010). One important pending question is whether the cellular mechanism by which IGF-I signaling controls neurogenesis in the hypothalamus is cell autonomous or not. Indeed, ependymocytes, which are an important component of the hypothalamic niche, are also KO for IGF-1R. This could be sufficient to modify neurogenesis independently of direct effects on stem/progenitor cells, as demonstrated for other genes in subependymal cells of the SVZ (Lim et al., 2000).

## 5. Conclusions

This work constitutes a first proof that highly specific neurogenesis exists in the aging hypothalamus and that its dynamics depends on IGF-I signaling. The discovery of lifelong cellular plasticity provides novel insights and perspectives for neuroendocrinology and for the development of innovative hormone replacement therapies.

## Disclosure statement

The authors declare they have no conflict of interests.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neurobiolaging.2016.02.008>.

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