IKKβ/NF-κB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes

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Adult neural stem cells (NSCs) are known to exist in a few regions of the brain; however, the entity and physiological/disease relevance of adult hypothalamic NSCs (htNSCs) remain unclear. This work shows that adult htNSCs are multipotent and predominantly present in the mediobasal hypothalamus of adult mice. Chronic high-fat-diet feeding led to not only depletion but also neurogenic impairment of htNSCs associated with IKKβ/NF-κB activation. In vitro htNSC models demonstrated that their survival and neurogenesis markedly decreased on IKKβ/NF-κB activation but increased on IKKβ/NF-κB inhibition, mechanistically mediated by IKKβ/NF-κB-controlled apoptosis and Notch signalling. Mouse studies revealed that htNSC-specific IKKβ/NF-κB activation led to depletion and impaired neuronal differentiation of htNSCs, and ultimately the development of obesity and pre-diabetes. In conclusion, adult htNSCs are important for the central regulation of metabolic physiology, and IKKβ/NF-κB-mediated impairment of adult htNSCs is a critical neurodegenerative mechanism for obesity and related diabetes.

Adult neurogenesis, a once unconventional concept, is now an impelling topic in neuroscience. Recent research has shown that the adult central nervous system contains NSCs that generate neural cells, including neurons, astrocytes and oligodendrocytes. Adult NSCs are present predominantly in the sub-ventricular zone (SVZ) of the forebrain and the sub-granular zone (SGZ) of the hippocampal dentate gyrus. With regard to the hypothalamus, 5-bromodeoxyuridine (BrdU) labelling showed postnatal neurogenic activities in the hypothalamus of mice in CNTF-stimulated or basal conditions, and cell proliferation was reported in the hypothalamus of mutant mice with agouti-related peptide (AGRP) neuronal degeneration. More recently, postnatal turnover of arcuate neurons and its impairment in obesity conditions was demonstrated, and, on the basis of observations of newborn pups or pre-adult young mice, it was reported that tanycytes in the median eminence are important for postnatal hypothalamic neurogenesis. We are interested in understanding the identity and characteristics of adult htNSCs at post-development ages and their physiological actions and especially their disease relevance. It is known that environmental changes such as chronic high-fat-diet (HFD) feeding induce hypothalamic dysfunctions causing and promoting obesity and diabetes. Recent research revealed that the proinflammatory pathway involving IkB kinase β (IKKβ) and the downstream nuclear factor-κB (NF-κB) mediates HFD-induced hypothalamic inflammation, causing a metabolic syndrome. It is of note that in addition to being an inflammatory regulator, IKKβ/NF-κB controls cell survival, growth, apoptosis and differentiation in a cell-specific manner. Whereas IKKβ/NF-κB can be either pro-survival or anti-survival depending on the cell type and conditions, inflammatory changes such as those induced by brain microglia have been found to inhibit neurogenesis.

Metabolic physiological activities including feeding, body weight and glucose homeostasis are critically regulated by the mediobasal hypothalamus (MBH), which comprises the arcuate nucleus (ARC) and the ventral medial hypothalamic region. This regulation is primarily mediated by the balance between anorexigenic neurons expressing proopiomelanocortin (POMC) and orexigenic neurons expressing neuropeptide Y (NPY) and AGRP. In normal physiological conditions, increased systemic levels of nutrients and related hormones can act in the hypothalamus to activate the POMC neurons but inhibit the NPY/AGRP neurons. As a result, appetite is suppressed and energy expenditure is enhanced to maintain body weight and metabolic balance. However, under an obesity-prone environment such as chronic HFD feeding, POMC neurons are significantly impaired, exhibiting reduced sensitivities to hormones such as insulin and leptin, leading to the onset of central insulin and leptin resistance, which is known to be a critical neural mechanism for obesity and related diabetes.
Figure 1 In vivo and in vitro definition of hNSCs in adult mice. (a,b) Brain sections across the arcuate nucleus (ARC) of C57BL/6 mice (a) or nestin-Cre mice (b) were used for immunostaining. Brain sections across the dentate gyrus are included in a. The mice used were chow-fed 3-month-old males. Nuclear staining by DAPI (blue) revealed all cells in the sections. Merged images show the co-distribution of the indicated molecular markers. (c) Numbers of nestin\(^{+}\), Sox2\(^{+}\) or nestin\(^{-}\)Sox2\(^{+}\) cells in the mediobasal hypothalamus (MBH; comprising the ARC and the ventral medial hypothalamic region) were compared with numbers of tanycytes in the lateral third-ventricle wall (L3V, T) versus the median eminence (ME, T). \(n=6\) mice. The y-axis values represent the total number of cells in the indicated regions (±s.e.m.). Representative immunostaining images for cell count analysis are shown in b. (d,e) Hypothalamic tissues were sampled from normal C57BL/6 mice (chow-fed, 3-month-old) for neurosphere culture as described in Methods. Neurospheres were formed and passaged in growth medium containing bFGF and EGF. Neurospheres at various passages were attached to slides and immunostained for Sox2 (d), nestin and Blbp (e). Images were merged with DAPI staining to reveal the nuclear distribution of Sox2 and the cytoplasmic distribution of nestin and Blbp. (f) The hypothalamus and various other brain components were sampled from normal C57BL/6 mice (chow-fed, 3-month-old) for the neurosphere (NS) assay. The data show the total number of primary neurospheres (without passage) normalized by the mass (mg) of brain tissue from which neurospheres were derived. Hy, hypothalamus; Co, cortex; Po, pons; Th, thalamus; Ce, cerebellum; DG, dentate gyrus. **\(P<0.01\), ***\(P<0.001\), \(n=4\) mice per group; error bars reflect means ± s.e.m. (g) Neurospheres were derived from the hypothalamus of normal mice (chow-fed, 3-month-old). Dissociated neurospheric cells at the same passage were induced to differentiate as described in the Methods. Following 7-day differentiation, cells were immunostained for the neuronal marker Tuj1, the astrocyte marker GFAP and the oligodendrocyte marker O4. Nuclear staining of DAPI revealed all cells in the slides. Scale bars, 50 μm.
We also revealed that hypothalamic neurospheric cells at various (Fig. 1b and Supplementary Fig. S2); nonetheless, tanycytes in the (Fig. 1g). Together, these results show that Sox2/nestin-co-expressing (Fig. 1a and Supplementary Fig. S1). Indeed, various neuronal markers with the third-ventricle lateral walls (Fig. 1c). By definition, NSCs contains more than 10 times the number of htNSCs when compared to the MBH neural cells, including neurons, in post-development adult mice (Supplementary Fig. S2). In contrast, htNSCs in the MBH expressed both Sox2 and nestin strongly (Fig. 1b and Supplementary Fig. S2); nonetheless, tanycytes in the upper portion of the third-ventricle lateral walls still shared this pattern (Supplementary Fig. S2). As the MBH is much bigger, it contains more than 10 times the number of htNSCs when compared with the median eminence, and ~3 times more when compared with the third-ventricle lateral walls (Fig. 1c). By definition, NSCs are self-renewing, multipotent cells and can give rise to neurons, astrocytes and oligodendrocytes. Using in vitro neurosphere assays, we assessed whether adult htNSCs possess these characteristics. The results revealed that hypothalami of adult mice produced in vitro neurospheres that expressed the NSC biomarkers Sox2, nestin and Blbp (Fig. 1d,e), and this characteristic was maintained for >15 passages. The numbers of primary neurospheres obtained from the hypothalamus were comparable to those for the dentate gyrus (Fig. 1f). We also revealed that hypothalamic neurospheric cells at various passages can differentiate into neurons, astrocytes and oligodendrocytes (Fig. 1g). Together, these results show that Sox2/nestin-co-expressing htNSCs with full NSC functions are enriched in the MBH and the third-ventricle walls of adult mice at post-development ages, and thus, in addition to SVZ and SGZ, the hypothalamus is another critical adult NSC-containing region of the brain.

RESULTS

Enrichment, stemness and multipotency of adult htNSCs

Here, we aimed to investigate the entity and characteristics of htNSCs in adult mice. Using immunostaining for Sox2, an authentic biomarker of NSCs (ref. 44), we found that Sox2-positive cells were enriched in the MBH and the adjacent third-ventricle wall of adult hypothalami (Fig. 1a and Supplementary Fig. S1). Indeed, various neuronal markers such as NeuN were undetectable in these cells (Fig. 1a). Other regions of the brain were also analysed, and Sox2-positive cells were similarly found in the hippocampal dentate gyrus (Fig. 1a) and SVZ. In addition to Sox2, nestin has often been used to report neural stem cells and also neural progenitors and precursors.55 Owing to the poor quality of nestin immunostaining, we used nestin–Cre mice to report nestin activities based on nestin-promoter-controlled Cre expression. Indeed, Sox2 significantly co-exists with nestin in adult htNSCs (Fig. 1b). We observed strong nestin activities in tanycytes located in the median eminence as well as the third-ventricle lateral walls, as recently reported in newborn pups or pre-adult young mice. Interestingly, tanycytes in the median eminence had weak or absent Sox2 expression despite strong nestin expression in post-development, adult mice (Supplementary Fig. S2). In contrast, htNSCs in the MBH expressed both Sox2 and nestin strongly (Fig. 1b and Supplementary Fig. S2); nonetheless, tanycytes in the upper portion of the third-ventricle lateral walls still shared this pattern (Supplementary Fig. S2). As the MBH is much bigger, it contains more than 10 times the number of htNSCs when compared with the median eminence, and ~3 times more when compared with the third-ventricle lateral walls (Fig. 1c). By definition, NSCs are self-renewing, multipotent cells and can give rise to neurons, astrocytes and oligodendrocytes. Using in vitro neurosphere assays, we assessed whether adult htNSCs possess these characteristics. The results revealed that hypothalami of adult mice produced in vitro neurospheres that expressed the NSC biomarkers Sox2, nestin and Blbp (Fig. 1d,e), and this characteristic was maintained for >15 passages. The numbers of primary neurospheres obtained from the hypothalamus were comparable to those for the dentate gyrus (Fig. 1f). We also revealed that hypothalamic neurospheric cells at various passages can differentiate into neurons, astrocytes and oligodendrocytes (Fig. 1g). Together, these results show that Sox2/nestin-co-expressing htNSCs with full NSC functions are enriched in the MBH and the third-ventricle walls of adult mice at post-development ages, and thus, in addition to SVZ and SGZ, the hypothalamus is another critical adult NSC-containing region of the brain.

In vivo neurogenesis of adult htNSCs leads to new MBH neurons

Subsequently, we employed BrdU labelling to determine whether adult-onset htNSCs can lead to new neurons in post-development, adult mice. Through a pre-implemented intracerebroventricular (i.c.v.) cannula, normal adult C57BL/6 mice received a single BrdU injection for the proliferation analysis of BrdU-labelled cells. The results revealed that the number of BrdU-labelled cells doubled at 7 days post-labelling (Fig. 2a). To study the survival rates of BrdU-labelled cells, we employed daily i.c.v. BrdU injections for 7 consecutive days. By comparing the numbers of BrdU-labelled cells at day 10 versus 30, we found that the survival rate of these cells over 30 days was about 70% (Fig. 2b). During this period, a fraction of these BrdU-labelled cells differentiated into neurons (Fig. 2c). Using immunostaining for MBH neuropeptides including POMC (Fig. 2d) and NPY, we found that among the newly generated neurons (Fig. 2e), 8% of them were POMC neurons (Fig. 2f), and 4% were NPY neurons (Fig. 2g). A small number of BrdU-labelled cells differentiated into S100B-expressing astrocytes at day 10, and these astrocytes seemed to undergo a turnover process because the total number dropped by 30% at day 30 (Fig. 2h). In addition, a few BrdU-labelled RIP-expressing oligodendrocytes were detected (Fig. 2i). Overall, when compared with the whole population of mature neural cells in the ARC, the numbers of new neural cells generated through adult htNSCs-directed neurogenesis revealed by BrdU labelling was rather small. On the other hand, these small increments suggest that htNSC-mediated neurogenesis is slow, including neuronal generation in mice at post-development adult ages.

In vivo neurogenesis of adult htNSCs is slow in physiology

In addition to BrdU labelling, we developed an alternative approach by which we permanently labelled htNSCs with fluorescent YFP for long-term fate mapping. Briefly, we delivered Sox2-promoter-directed lentiviral Cre or control lentivirus to the MBH of ROSA-lox–STOP–lox–YFP mice. Cre-dependent removal of the lox–STOP–lox cassette enables the ROSA promoter to induce YFP in Sox2-expressing htNSCs in the MBH (Fig. 3a). Using this tracking system, we confirmed that YFP was expressed in Sox2-positive htNSCs at day 5 post lentiviral Cre delivery (Fig. 3b). At this time point, none of the YFP-expressing cells expressed the neuronal marker NeuN. However, after an 80-day follow-up, the MBH of mice clearly showed increased numbers of YFP-labelled cells (Fig. 3b), and a significant number of these YFP-labelled cells were neurons (Fig. 3c). Using neuropeptide immunostaining for POMC (Fig. 3d) and NPY, we detected ~1,000 new neurons generated in the ARC at day 80 (Fig. 3e)—which account for 6% of the neuronal population in this region, and 10% of the new neurons were POMC neurons (Fig. 3f) and 3% were NPY neurons (Fig. 3g). We also detected some YFP-positive S100B-expressing astrocytes (Fig. 3h) and a few YFP-positive RIP-expressing oligodendrocytes (Fig. 3i). Therefore, when compared with the short-term BrdU tracking, neuronal differentiation revealed by long-term YFP tracking was more appreciable. By comparing these two neurogenic tracking methods, it can be further deduced that neuronal differentiation by adult htNSCs is rather slow in normal physiological conditions. Taken together, these results show that htNSCs slowly and cumulatively lead to significant numbers of new MBH neural cells, including neurons, in post-development adult mice under physiological conditions.
Figure 2 BrdU tracking of adult htNSCs-mediated neurogenesis in mice. (a) C57BL/6 mice (chow-fed males, 4-month-old) received a single i.c.v. injection of BrdU. The brains were fixed at day 1 versus 7 and sectioned for BrdU staining. Total numbers of BrdU-labelled cells in serial ARC sections were counted. (b–i) C57BL/6 mice (chow-fed males, 4-month-old) received daily i.c.v. injections of BrdU consecutively for 7 days. The brains were fixed at day 10 versus 30 and then sectioned for BrdU staining (b) or co-immunostaining with the indicated markers (c–i). (b) Total numbers of BrdU-labelled cells in serial ARC sections were counted. (e–i) Total numbers of BrdU-labelled cells co-immunostained with NeuN (e), POMC (f), NPY (g), S100B (h) and RIP (i) in serial ARC sections were counted. **P < 0.01, ***P < 0.001, n = 6 mice (a,b,g,i), n = 4 mice (e,h) and n = 5 mice (f) per group. Error bars reflect means ± s.e.m. Scale bars, 50 μm.

Long-term HFD feeding impairs survival and neurogenesis of adult htNSCs

To investigate the disease relevance of htNSCs, we focused on HFD-induced obesity and pre-diabetes. Using BrdU labelling, we found that when compared with chow-fed mice, mice with long-term (4-month) HFD feeding had much fewer BrdU-labelled cells in the MBH (Supplementary Fig. S3a). Moreover, these HFD-fed mice suffered from severe decreases in the proliferation and survival rates of BrdU-labelled cells (Supplementary Fig. S3b,c). Using co-immunostaining with NeuN or neuropeptides, we found that BrdU-labelled cells in HFD-fed mice showed impaired differentiation into neurons, including POMC and NPY neurons (Supplementary Fig. S3d–f), astrocytes...
Figure 3 Fate mapping of adult htNSCs in mice under normal physiological conditions. ROSA-lox-STOP-lox-YFP mice (chow-fed males, 3-month-old) were bilaterally injected in the mediobasal hypothalamus with lentiviruses that directed Cre expression under the control of the Sox2 promoter. Following the indicated days post viral injection, hypothalamus sections were prepared to track the neural differentiation of YFP-labelled cells. (a) Schematic of the lentiviral vector and the genetic mouse model. Lentivirus expressing Cre under the control of the Sox2 promoter was termed P_{sox2}-Cre lentivirus. The control was a lentiviral vector without Cre (schematic not shown). (b-d) Co-imaging of YFP (green) with immunostaining (red) of Sox2 (b), NeuN (c) or POMC (d) at indicated days post viral injection. Cell nuclear staining (blue) by DAPI revealed all cells in the sections. (e-i) YFP-labelled NeuN-positive cells (YFP+C_NeuN+C; e); POMC-positive cells (YFP+C_POMC+C; f); NPY-positive cells (YFP+C_NPY+C; g); S100B-positive cells (YFP+C_S100B+C; h) and RIP-positive cells (YFP+C_RIP+C; i) in serial ARC sections were counted. ***P < 0.001, n = 5 mice (e,f), n = 6 mice (g,i) and n = 4 mice (h) per group. Error bars represent mean ± s.e.m. Scale bars, 50 μm.
the MBH of HFD-fed mice (Fig. 4a,b). On the basis of these findings, we predicted that impaired hypothalamic neurogenesis by HFD feeding could result in a decrease in the number of certain types of neuron over an extra-long HFD feeding period. Indeed, following an 8-month HFD feeding period, the number of POMC neurons in the ARC decreased by ~12% when compared with chow-fed mice (Fig. 4c), in agreement with recent observations. Using *in vitro* neurosphere assays, we observed that neurospheres derived from the hypothalamus of HFD-fed mice were not only fewer but also smaller than those derived from chow-fed mice (Fig. 4d–f). *In vitro* proliferation rate analysis showed that hNSCs derived from HFD-fed mice proliferated poorly (Fig. 4g). *In vitro* differentiation analysis

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**Figure 4** Impaired survival and neurogenesis of hNSCs derived from mice with dietary obesity. (**a–c**) Adult male C57BL/6 mice were maintained under chow versus HFD feeding for 4 months (**a,b**) or 8 months (**c**). Hypothalamic sections were immunostained for Sox2 (**a**) or POMC (image not shown). DAPI nuclear staining revealed all cells in the section. Sox2-immunoreactive (Sox2+) cells (**b**) and POMC neurons (**c**) were counted in the ARC. 3V, third ventricle. (**d–f**) C57BL/6 mice were maintained under normal chow versus HFD feeding for 4 months, and the hypothalami of these mice were removed to generate neurospheres for *in vitro* assays. (**d–f**) Morphology (**d**), number (**e**), and size (**f**) of primary neurospheres. (**g**) Primary neurospheres were passaged with the same initial number (10⁴ cells per group), and cell outputs were followed for 5 passages. (**h,i**) Neurospheric cells at the same passage (representing passages 5–10) were induced to differentiate for 7 days, cells were fixed and immunostained for Tuj1 (**h**), and Tuj1-positive (Tuj1+) cells were counted in the slides (**i**). *P < 0.05, **P < 0.01, ***P < 0.001, comparisons between chow and HFD at indicated points, n = 5 mice (**b**), n = 6 mice (**c**) and n = 4 mice (**e,f**) per group, and n = 4 per group (**g,i**); error bars reflect mean ± s.e.m. Scale bars, 50 µm.
further revealed that htNSCs derived from HFD-fed mice showed a striking impairment in neuronal differentiation (Fig. 4h,i). Cultured NSCs isolated from the SVZ and SGZ of these HFD-fed mice were also impaired in terms of proliferation and neuronal differentiation, but the impairments were less severe when compared with the changes in htNSCs (Supplementary Fig. S4a–c). Taken together, these results show that chronic HFD feeding causes neurogenic impairment in the mediobasal hypothalamus.

**Adult htNSCs are vulnerable to micro-environmental inflammation**

In our studies, we noted that htNSCs derived from obese mice exhibited impaired proliferation and differentiation even after being passaged to subsequent generations. In exploring the underlying basis, we found that excessive cytokines TNFα and IL-1β were produced in the medium of these htNSCs over culture passages (Supplementary Fig. S5a,b). As cytokines TNFα and IL-1β are produced by NF-κB activation and are also known to potently activate IKKβ/NF-κB, a positive feed-forward loop consisting of TNFα/IL-1β and IKKβ/NF-κB may work as a molecular basis for the transgenerational defects in these cells. To test this idea, using short interfering RNAs (siRNAs), we co-inhibited the gene expression of TNFα and IL-1β in htNSCs derived from obese mice (Supplementary Fig. S5c), and then examined the proliferation and differentiation of these cells. BrdU pulse labelling revealed that co-inhibition of TNFα and IL-1β improved the proliferation of these htNSCs (Supplementary Fig. S5d,e). In addition, the level of neuronal differentiation of these cells was markedly increased by the co-inhibition of TNFα and IL-1β (Supplementary Fig. S5f,g). In this context, we directly measured NF-κB signalling, and found that NF-κB activation was upregulated in htNSCs derived from obese mice (Fig. 5a). We then examined whether hypothalamic inflammation is important for HFD-feeding-induced defects of htNSCs, given that we also observed that HFD feeding increased the number of MBH microglia cells and the level of production of TNFα in these cells (Supplementary Fig. S6A). To test this hypothesis, we inflicted IKKβ/NF-κB in microglia using microglia-specific IKKβ gene ablation. Briefly, IKKβlox/lox mice received an MBH injection of microglia-specific (CD11b-promoter-driven) lentiviral Cre. We confirmed that the level of HFD-feeding-induced microglial TNFα expression decreased in these Cre-delivered mice but not in the control mice (Supplementary Fig. S6b). Subsequently, htNSCs from these mice were isolated for in vitro analyses, showing that HFD-feeding-induced impairments of htNSC proliferation and differentiation were significantly attenuated in htNSCs isolated from Cre-delivered mice (Supplementary Fig. S6c–e). Hence, adult htNSCs are vulnerable to inflammation, and hypothalamic microglia are involved in this inflammatory mechanism.

**IKKβ/NF-κB inhibits survival and proliferation of htNSCs through apoptosis**

Given the significant relevance of IKKβ/NF-κB in htNSCs, we directly studied whether and how IKKβ/NF-κB activation or inhibition affected adult htNSCs. To facilitate this study, we established stable cell lines of htNSCs with genetically induced IKKβ/NF-κB activation or inhibition. Using a lentiviral system to transfer complementary DNA into the genome of infected cells (Supplementary Fig. S7a), htNSCs derived from normal mice were stably transduced with the cDNA of constitutively active IKKβ(GFP-conjugated) to activate NF-κB, termed IKKβ-htNSCs. In parallel, htNSCs stably transduced with cDNA encoding dominant-negative IκBα (GFP-conjugated) to inhibit NF-κB were generated, termed IκBα-htNSCs. Control htNSC lines were stably transduced with GFP cDNA, termed GFP-htNSCs. Using antibiotic selection, lentivirus-transduced htNSCs survived and were passaged in blasticidin-containing medium, as verified by the presence of GFP in individual cells over serial passages (Supplementary Fig. S7b–d). Western blots confirmed that the level of NF-κB activities increased in IKKβ-htNSCs (Fig. 5b) and decreased in IκBα-htNSCs (Fig. 5c). These cells were subjected to BrdU pulse labelling for proliferation assay. As shown in Fig. 5d,e, the level of proliferation of IKKβ-htNSCs significantly decreased when compared with the control cells. We further analysed the total cell numbers over 4 passages, and found that the total proliferation output of IKKβ-htNSCs was only ~1% of the control (Fig. 5f). We then used a TdT-mediated dUTP nick end labelling (TUNEL) assay to assess whether the growth defect in IKKβ-htNSCs was the result of enhanced apoptosis. The results showed that when compared with control cells, there was a ~9-fold induction of apoptosis in IKKβ-htNSCs (Fig. 5g,h). At the same time, IκBα-htNSCs were analysed for proliferation and apoptosis. We found that the proliferation rates of IκBα-htNSCs and control cells were comparable (Fig. 5e,f), consistent with the observation that both groups exhibited barely any apoptosis (Fig. 5h). Furthermore, to explore the molecular basis that underlies the apoptosis of IKKβ-htNSCs, we analysed a series of apoptotic and anti-apoptotic genes that are known as NF-κB target genes. The results revealed that apoptotic genes Bim, Bax, Bnip3 and caspase-3 were upregulated in IKKβ-htNSCs but downregulated in IκBα-htNSCs (Supplementary Fig. S8a). Upregulation of the anti-apoptotic genes Bcl-2, Bcl-xl and Traf2 by IKKβ/NF-κB was also observed, but these changes were relatively modest. Therefore, IKKβ/NF-κB overactivation uses the apoptotic program to significantly reduce the survival of adult htNSCs.

We next examined whether IKKβ/NF-κB mediates the proliferation defect in htNSCs derived from obese mice. To do this, htNSCs derived from mice with HFD-induced obesity were stably transduced with dominant-negative IκBα versus GFP, termed IκBα-htNSCs and GFP-htNSCs, respectively. To provide a normal reference, htNSCs derived from chow-fed mice were stably transduced with GFP, termed GFP-htNSCsshow. BrdU pulse labelling verified that GFP-htNSCs show proliferated poorly with only a small number of BrdU-positive cells (Fig. 5i,j). Strikingly, we found that IκBα-htNSCs proliferated as normally as the reference control (Fig. 5i,j). Furthermore, proliferation outputs over 4 passages revealed that whereas GFP-htNSCs show exhibited a severe proliferative defect, IκBα-htNSCs had as normal a proliferation as GFP-htNSCs show (Fig. 5k). The TUNEL assay was also performed to assess apoptosis of these cells, and showed that apoptosis was evident in GFP-htNSCs show, but rarely seen in IκBα-htNSCs show, which was similar to the normal reference GFP-htNSCs show (Fig. 5l,m). In sum, IKKβ/NF-κB mediates the proliferation defect in htNSCs derived from obese mice.
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Figure 5 Impaired in vitro proliferation of hNSCs with IKKβ/NF-κB activation. (a) Adult C57BL/6 mice were fed on a chow versus HFD for 4 months. Hypothalamic neurospheres were generated from these mice, cultured and passed in vitro. Western blotting was performed for cultured neurosphere cells to measure phosphorylated RelA (pRelA). RelA and β-actin were analysed as controls. (b,c) Neurospheres were derived from the hypothalamus of chow-fed C57BL/6 mice (3-month-old). Dissociated neurospheric cells were infected with lentiviruses expressing C4IKKβ (GFP-conjugated), DNIkBα (GFP-conjugated) and GFP. In vitro models of hNSCs with stable transduction of GFP, C4IKKβ and DNIkBα were established and maintained in the selection medium, and named GFP-htNSCs, IKKβ-htNSCs and IxBα-htNSCs (Supplementary Fig. S7), respectively. Cells were analysed by western blotting to analyse pRelA. Total protein levels of RelA and β-actin were analysed as controls. (d–f) Cells (passage 6) were labelled with BrdU and analysed for BrdU-positive (BrdU+) cells. (g) Cells (passage 6) were subjected to TUNEL staining, and TUNEL-staining-positive cells were counted. (h–m) Neurospheres derived from the hypothalamus of C57BL/6 mice that received a normal chow versus HFD for 4 months. Dissociated neurospheric cells were infected with lentiviruses expressing DNIkBα (GFP-conjugated) or GFP to establish IxBα-htNSCsHFD, GFP-htNSCsHFD and GFP-htNSCsHFD models of htNSCs with stable transduction of GFP, C4IKKβ and DNIkBα were established and maintained in the growth medium. (i) Cells (passage 6) were pulse labelled with BrdU, and BrdU-positive (BrdU+) cells were analysed. (j) Cells (passage 6) were pulse labelled with BrdU, and BrdU-positive (BrdU+) cells were analysed. (k) Cell outputs from the same initial number at passage 6 were followed for 4 passages. (l,m) A TUNEL assay was performed for cells (passage 6, day 2) and analysed using TUNEL staining. (b,c,e,f,h) IKKβ: IKKβ-htNSCs; IxBα: IxBα-htNSCs; GFP: GFP-htNSCs. *P < 0.05, **P < 0.01, n = 4 (e,f,h,k), n = 5 (j) and n = 6 (m) per group. Error bars reflect mean ± s.e.m. Statistics in (f,k): data points in red or blue lines were compared to the corresponding points in green line. Scale bars, 50 μm. Uncropped images of blots are shown in Supplementary Fig. S9.
IKKβ/NF-κB inhibits neuronal differentiation of htNSCs through Notch signalling

IKKβ-htNSCs, IxBt-htNSCs and control GFP-htNSCs were subjected to neuronal differentiation analysis. GFP-htNSCs exhibited ~17% of neuronal differentiation (Fig. 6a,b). However, neuronal differentiation completely stopped in IKKβ-htNSCs, as we barely detected any neuronal morphology or neuronal markers in these cells (Fig. 6a,b). This change recapitulated the defect of neuronal differentiation in htNSCs derived from obese mice shown in Fig. 4h.i. Conversely, IxBt-htNSCs showed a pronounced increase in the level of neuronal differentiation, which was threefold higher when compared with GFP-htNSCs (Fig. 6a,b). We next explored how IKKβ/NF-κB affected the neuronal differentiation of htNSCs. Through gene expression profiling, we found that messenger RNA levels for Notch isoforms including Notch1, 3 and 4 increased on IKKβ/NF-κB activation but decreased on IKKβ/NF-κB inhibition (Fig. 6c). Gene expression of Notch ligands including delta-like ligand-1 (DLL1), DLL4 and Jagged2 was similarly affected by IKKβ/NF-κB (Fig. 6d). Notch signalling, reflected by Notch protein cleavage, was upregulated by IKKβ/NF-κB activation but downregulated by NF-κB inhibition (Fig. 6e). To further examine the relationship between IKKβ/NF-κB and Notch signalling, we examined the promoter regions of genes that encode Notch signalling components, and found that the canonical NF-κB-DNA-binding motif 5’-GGRNNYYCC-3’ exists in the promoters of DLL4, Notch1 and Notch4 genes (Fig. 6f). Thus, we examined whether IKKβ/NF-κB can control the promoter activities of these genes. To do this, we created luciferase plasmids controlled by each of these promoters, and found that IKKβ/NF-κB increased the transcriptional activities of these promoters when transfected into HEK293 cells (Fig. 6g–i). On the other hand, mutagenesis of the NF-κB-DNA-binding motif abrogated the effect of IKKβ/NF-κB in activating the transcriptional activities of these promoters (Fig. 6g–i). To further examine the IKKβ/NF-κB–Notch connection in htNSCs, we ablated Notch genes using co-transduction of lentiviral short hairpin RNA (shRNA) against individual Notch isoforms (Supplementary Fig. S8b–e). Notch inhibition completely reversed the differentiation defect in IKKβ-htNSCs (Fig. 6j,k). Notch inhibition also promoted neuronal differentiation in GFP-htNSCs (data not shown), which is similar to the effect of IKKβ/NF-κB inhibition shown in IxBt-htNSCs (Fig. 6a,b). Together, these results show that IKKβ/NF-κB employs Notch signalling to inhibit the neuronal differentiation of htNSCs. In this context, we employed IxBt-htNSCs(HP), GFP-htNSCs(HD) and GFP-htNSCs(chow) (Fig. 5i–m) to determine whether IKKβ/NF-κB inhibition can reverse the neuronal differentiation defect in htNSCs derived from obese mice. The results revealed that neuronal differentiation was impaired in GFP-htNSCs(HP), but was substantially improved in IxBt-htNSCs(HD) (Fig. 7a,b). In parallel, we investigated whether Notch inhibition could similarly improve the neuronal differentiation of htNSCs derived from obese mice, as indeed these cells showed upregulation of Notch signalling (Fig. 7c). We ablated Notch isoforms in these cells by co-infection with lentiviral shRNAs against Notch1–4, as used for Fig. 6j,k. With Notch inhibition, GFP-htNSCs(HD) exhibited an enhanced level of neuronal differentiation (Fig. 7d,e). In sum, the neuronal differentiation defect in htNSCs derived from obese mice can be reversed by inhibition of NF-κB or downstream Notch signalling.

IKKβ/NF-κB in htNSCs mediates obesity and pre-diabetes in adult mice

To investigate whether IKKβ/NF-κB in htNSCs is important for disease, we developed a mouse model with IKKβ/NF-κB gain-of-function in MBH htNSCs of adult mice. These mice were obtained through MBH injection of Sox2-promoter-driven lentiviruses expressing constitutively active IKKβ (Fig. 8a). We assessed IxBt degradation using immunostaining to report IKKβ/NF-κB activation in htNSCs of these mice. Indeed, IxBt degradation occurred in Sox2-positive cells but not other cells in the ARC of IKKβ-injected mice (Fig. 8b). In contrast to the control group, the proliferation and survival of BrdU-labelled cells over a 14-day tracking period were poor in IKKβ-injected mice (Fig. 8c), and BrdU-labelled cells in IKKβ-injected mice showed impaired neuronal differentiation (Fig. 8d). Interestingly, over this 14-day period, IKKβ didn’t result in a significant number of S100B-expressing astrocytes (Fig. 8e). Over a 3-month tracking period, the number of Sox2-positive htNSCs decreased by 60% in IKKβ-injected mice when compared with the control mice (Fig. 8f). As a result, the total number of POMC neurons in the ARC of IKKβ-injected mice decreased by ~10% (Fig. 8g). NPY neurons in the ARC were not significantly affected over this 3-month period (Fig. 8h). In physiological studies, we maintained IKKβ-injected mice and the controls under normal chow feeding and monitored their glucose and insulin levels as well as food intake and body weight profiles. As shown in Fig. 8i,j, IKKβ-injected mice manifested glucose intolerance and hyperinsulinaemia at ~3 months post gene delivery. These mice also exhibited overeating and weight gain, and these effects cumulatively resulted in the onset of severe obesity at ~10 months post gene delivery despite normal chow feeding (Fig. 8k,l). The findings here explicitly demonstrate that IKKβ/NF-κB overactivation mediates the effect of chronic HFD feeding in impairing htNSC-directed neurogenesis, causing obesity and pre-diabetic disorders.

DISCUSSION

Adult htNSCs are multipotent but vulnerable to inflammation

The existence of adult NSCs in the brain was not a well accepted notion until recently, and so far, research on adult NSCs has been mainly based on the SVZ and SGZ in the brain14–15. Recently, several studies have reported observations of postnatal hypothalamic neurogenesis in mice16–19. Of note, in a recent study20, it was reported that median eminence tanyocytes led to newborn neurons in mouse pups at ages of postnatal day (P)10 to P19, an observation based on BrdU labelling and nestin-based fate mapping. Furthermore, this study showed that tanyocyte-directed neuronal generation can be enhanced by short-term (30-day) HFD feeding starting at either a pre-weaning (P15) or a pre-adult (P45) age21. Recently, several studies in the mouse have shown that tanycyt-mediated neurogenesis is important for physiology or diseases. Using Sox2, which is known as an authentic NSC marker44, we found that Sox2-expressing htNSCs are enriched in the MBH and surrounding third-ventricle ependyma. These htNSCs can be isolated and maintained in vitro in the form of neurospheres for >15 passages, and dissociated neurosphere cells at any generation express NSC/progenitor biomarkers, and can differentiate into neurons, astrocytes and oligodendrocytes. Our findings are in line with recent literature that reported observations of postnatal hypothalamic neurogenesis16–20. Therefore, these Sox2-expressing htNSCs could be used as a model system to study the role of Notch in hypothalamic neurogenesis.
Figure 6 In vitro effect of IKKβ/NF-κB activation on neuronal differentiation of hTNSCs. (a–e) Dissociated IKKβ-hTNSCs, IκBα-hTNSCs and GFP-hTNSCs from passage 6 were induced to differentiate. (a,b) Immunostaining of neuronal marker Tuj1. (b) The percentage of Tuj1-positive (Tuj1+) cells. IKKβ, IKKβ-hTNSCs; IκBα, IκBα-hTNSCs; GFP, GFP-hTNSCs. (c,d) mRNA levels of genes encoding Notch isoforms (c) and Notch ligands (d). (e) Analysis of Notch signalling by western blot measurement of the cleaved Notch1 protein. (f–i) NF-κB controls genes that encode Notch signalling proteins. (f) NF-κB-DNA-binding motif in the promoter regions of murine DLL4, Notch1 and Notch4 genes. (g–i) Gene promoter activities of wild-type (WT) versus mutant (Mut) murine DLL4 (g), Notch1 (h) and Notch4 (i) in HEK293 cells in the presence or absence of IKKβ/NF-κB activation, induced by transfection of pcDNA expressing constitutively active IKKβ or a control. (j,k) IKKβ-hTNSCs were co-infected with lentiviral shRNAs against Notch1-4, as evaluated in Supplementary Fig. S8. Cells were induced to differentiate and analysed for neuronal marker Tuj1 using immunostaining (j) and quantitatively analysed for Tuj1-positive (Tuj1+) cells (k). *P < 0.05, **P < 0.01, ***P < 0.001, n = 4 (b–d,g–i) and n = 6 (k) per group; error bars reflect mean ± s.e.m. Scale bars, 50 μm. Uncropped images of blots are shown in Supplementary Fig. S9.
Figure 7 Effect of NF-κB inhibition on the differentiation of htNSCs derived from obese mice. (a, b) Dissociated IκB-htNSCsHFD, GFP-htNSCsHFD and GFP-htNSCsChow (established in Supplementary Fig. S7) at passage 6 were induced to differentiate. Cells were then immunostained for the neuronal marker Tuj1. (b) Percentage of Tuj1-positive (Tuj1⁺) cells. (c) Western blotting of cleaved Notch1. β-actin was used as an internal control. (d, e) GFP-htNSCsHFD expressing lentiviral Notch1−4 shRNAs versus control shRNA were induced to differentiate for 7 days. Cells were then fixed and analysed for Tuj1 immunostaining (d). (e) Percentage of Tuj1-positive (Tuj1⁺) cells. **P < 0.01, ***P < 0.001, n = 4 per group (b, e); error bars reflect means ± s.e.m. Scale bars, 50 μm. Uncropped images of blots are shown in Supplementary Fig. S9.

Adult cells are a bona fide population of htNSCs. Interestingly, in contrast to the previous findings, our data showed that tanyocytes constitute only a small fraction of htNSCs, whereas most htNSCs exist in the MBH in post-development adult mice. This difference may reflect a developmental dynamic of htNSCs. In our research, we further examined the effect of chronic HFD feeding on htNSCs. Differing from the short-term HFD feeding condition that was reported to promote hypothalamic neurogenesis in pre-adult ages, we found that long-term (4-month) HFD feeding remarkably depleted htNSCs and also severely impaired their neuronal differentiation, as assessed using both in vivo and in vitro paradigms. We postulate that whereas neurogenic upregulation by short-term HFD feeding may represent a compensatory reaction, long-term HFD feeding is detrimental for the cell fate of adult htNSCs. As we further uncovered, the neurodegenerative actions of chronic HFD feeding are attributed to IKKβ/NF-κB overactivation that involves the inflammatory paracrine actions of microglia. In sum, our findings provide conclusive in vivo and in vitro evidence of the critical existence of multipotent adult htNSCs, and also reveal that these cells are vulnerable to hypothalamic inflammation induced by chronic HFD feeding.
Figure 8  Mouse model of htNSCs-specific IKKβ activation and metabolic phenotypes. C57BL/6 mice (3-month-old, chow-fed males) bilaterally received intra-MBH injections of PSox2–CAIKKβ versus PSox2–control lentiviruses. All mice were maintained under normal chow feeding throughout the experiments. (a) Schematic of lentiviral vector that expressed CAIKKβ under the control of the Sox2 promoter (PSox2–CAIKKβ). The same vector without CAIKKβ was used as the matched control (PSox2–control). (b) Hypothalamic sections were prepared from mice at 2 weeks post-injection and co-immunostained for Sox2 (green) and IκBα (red). (c–e) Mice with PSox2–CAIKKβ versus PSox2–control received a single i.c.v. injection of BrdU. The brains were fixed at day 1, 7 or 14 post BrdU injection. Brain sections across the ARC were processed with BrdU staining or co-immunostaining with NeuN or S100B, and analysed for total BrdU-labelled cells (c) and BrdU-labelled cells positive for NeuN (d) or S100B (e). (f–h) Brain sections across the ARC were prepared from mice at ~3 months (3 M) post lentiviral injection, subjected to Sox2 and POMC immunostaining, and counted for Sox2-positive cells (f), POMC neurons (g) and NPY neurons (h) in serial ARC sections. (i–l) Data show glucose tolerance (i) and fasting insulin levels (j) of mice at 3 M post lentiviral injection, and food intake (k) and body weight (l) of mice at 10 months (10 M) post lentiviral injection. Baseline body weight levels of mice before lentiviral injections are also included in l. Glucose tolerance test, GTT. *P < 0.05, **P < 0.01, ***P < 0.001, n = 5 mice (c,e) and n = 4 mice (f–h) per group; n = 6 mice per group (d,i–k) and n = 10 mice per group (l). Error bars reflect mean ± s.e.m. Scale bars, 50 μm (b).
IKK\(\beta\)/NF-\(\kappa\)B impairs survival and differentiation of adult htNSCs

Given the significant link between hypothalamic inflammation and htNSCs, we comparatively examined IKK\(\beta\)/NF-\(\kappa\)B in htNSCs derived from normal versus obese animals. It was uncovered that IKK\(\beta\)/NF-\(\kappa\)B is overactivated in htNSCs derived from obese mice. IKK\(\beta\)/NF-\(\kappa\)B activation in htNSCs derived from obese mice was transferable over generations, and the underlying basis was revealed to be attributed to the paracrine release of cytokines such as TNF\(\alpha\) and IL-1\(\beta\) — both of which are not only two classical IKK\(\beta\)/NF-\(\kappa\)B products but also potent inducers of IKK\(\beta\)/NF-\(\kappa\)B activation. Indeed, inhibition of both cytokines blocked the paracrine activation loop of IKK\(\beta\)/NF-\(\kappa\)B to improve the proliferation and neuronal differentiation of htNSCs derived from obese mice. To better decipher the role of IKK\(\beta\)/NF-\(\kappa\)B in the cell biology of htNSCs, we developed in vitro models of htNSCs with genetically induced IKK\(\beta\)/NF-\(\kappa\)B activation or inhibition. Using cell output and BrdU labelling analyses, we found that the survival and proliferation of htNSCs were reduced by IKK\(\beta\)/NF-\(\kappa\)B activation but improved by IKK\(\beta\)/NF-\(\kappa\)B inhibition, and both effects were remarkable. In-depth analyses revealed that IKK\(\beta\)/NF-\(\kappa\)B activation upregulates apoptotic genes to cause apoptosis of htNSCs, and these defects are reversible by IKK\(\beta\)/NF-\(\kappa\)B inhibition. In parallel, we importantly discovered that IKK\(\beta\)/NF-\(\kappa\)B strongly suppresses neural differentiation of htNSCs, and identified that upregulation of Notch signalling by IKK\(\beta\)/NF-\(\kappa\)B is responsible. These findings are in agreement with recent literature showing that Notch signalling correlates with NF-\(\kappa\)B in various other cell types\(^{46-49}\), and also support the literature reports that Notch signalling inhibits neuronal differentiation and neurogenesis of NSCs (refs 50–56). Thus, IKK\(\beta\)/NF-\(\kappa\)B negatively affects the survival and neurogenesis of adult htNSCs through apoptotic and Notch molecular programs.

Adult neurogenesis through htNSCs is important for physiology and disease

Adult NSCs belong to a small population of cells with a slow dividing rate in the brain, and because of this nature, knowledge regarding the biological characteristics and physiological functions of these cells is still limited. In this work, using two in vivo tracking approaches, we found that adult htNSCs contribute to the generation of new MBH neurons in normal mice at post-development ages. Indeed, the neurogenic activities of adult htNSCs are slow and thus modest during short-term periods, as revealed by the BrdU-based pharmacological approach. With genetic YFP labelling, htNSC-induced neuronal generation over a long period was more evident. Strikingly, chronic HFD feeding can markedly decrease the number htNSCs and inhibit neuronal differentiation of these cells in the MBH of adult mice. When compared with the entire population of mature neurons, the fraction of neuronal loss due to htNSC defects is relatively modest and does not affect the vitality of the animals in general. However, for certain types of neuron, such as POMC neurons, that have rather small population numbers by nature, even a modest loss can have major impacts on physiology. In this context, we studied whether adult htNSCs are important for physiology using mice with adult-onset htNSC ablation induced by IKK\(\beta\)/NF-\(\kappa\)B activation in the MBH. These mice developed overeating, glucose disorder, insulin resistance and obesity. The collective development of these metabolic diseases was associated with a fractional loss of neurons including POMC neurons. These findings, while aligning with the critical role of POMC neurons in metabolic regulation, indicate that adult htNSC-directed neurogenesis is required for the central regulation of physiology. These metabolic derangements induced by htNSCs-specific IKK\(\beta\)/NF-\(\kappa\)B activation contribute, to a certain extent, to the disease outcomes of chronic HFD feeding—which indeed similarly inhibits the survival and neurogenesis of adult htNSCs. In support of this, recent research reported that chronic HFD feeding leads to a loss of POMC neurons in the MBH (ref. 43), a phenomenon that we similarly observed in this work. Together, these results indicate that dietary obesity represents a mild version of neurodegenerative disease, and neural regeneration can be a basis for developing new strategies to counteract obesity and related co-morbidities.

METHODS

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

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

The authors sincerely thank F. H. Gage (The Salk Institute for Biological Studies, USA) for the Sox2-promoter-containing lentiviral vector, D. Hickstein (National Cancer Institute, National Institutes of Health, USA) for the CD11b-promoter cDNA, and the members of the Cai laboratory for general technical assistance. This study was supported by Albert Einstein College of Medicine internal start-up funds and NIH R01 DK078750 and R01 AG031774 (all to D. Cai).

AUTHOR CONTRIBUTIONS

D.C. conceived the project and designed the study. J.L. carried out all experiments and participated in the experimental design. Y.T. generated luciferase constructs of wild-type and mutant Notch signalling element gene promoters. J.L. and D.C. carried out data interpretation and discussion. D.C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doi/10.1038/ncb2562
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METHODS

Animals, biochemical treatments and phenotyping. C57BL/6, nestin-Cre and ROSA-lox–STOP–lox–YFP mice were from Jackson Laboratory. IkKβ−/− mice were used in our previous research[22] and maintained under standard conditions. All mice in this study were males. The high-fat diet was obtained from Research Diets. The body weight of individually housed mice was measured twice a week and food intake was recorded daily. For the glucose tolerance test, overnight fasted mice were injected with glucose (2 mg kg−1 body weight) intraperitoneally, and blood glucose levels at various time points were measured using a Glucometer Elite (Bayer). Fasting insulin levels were measured using ELISA kits (Linco). For BrdU labeling, mice were pre-implemented with a cannula into the lateral ventricle, and after surgical recovery, they were injected a single i.c.v. injection of 10 μg BrdU (Sigma) for proliferation rate analysis or daily i.c.v. injections of 10 μg BrdU consecutively for 7 days for survival rate analysis. The mice were perfused with 4% PFA at the indicated days post-injection, and their brains were removed, post-fixed and sectioned for BrdU staining. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Lentiviruses and MBH injections. Using the Sox2-promoter-controlled lentiviral system[4] (provided by F. H. Gage), Sox2-promoter-controlled lentivectors were created to direct the expression of Cre, C3IKKβ or control GFP. Cre (GFP-conjugated) was cloned into a lentiviral vector controlled by the CD11b promoter (provided by D. Hickstein). Lentiviruses were produced by co-transfecting viral expression plasmids with the packaged plasmids into HEK293 FT cells, as described previously[22,27]. Bilateral injections of mediobasal hypothalamus were described previously[22,27]. Briefly, anesthetized mice under an ultraprecise stereotactic unit (resolution: 10 μm, David Kopf Instruments) were injected with purified lentiviruses in the vehicle (PBS) into each side of the mediobasal hypothalamus through a guide cannula directed to the coordinates at 0.17 mm posterior to the bregma, 0.03 mm lateral to the middle line and 0.50 mm below the skull surface.

Neurosphere culture and analysis. The hypothalamus was dissected from adult mice as described previously[22,27]. Tissues were cut into small pieces (~1 mm3), digested with 0.25% papain (Worthington) for 30 min at 30 °C and gently triturated approximately 10 times using fire-polished tips. The desired cell population was separated by density gradient centrifugation. After washing twice with Hibernate-A medium (BrainBits LLC), the cells were suspended in growth medium containing Neurobasal-A (Invitrogen), 2% B27 without vitamin A (Invitrogen), 10 ng ml−1 EGF (Sigma) and 10 ng ml−1 BFGF (Invitrogen), seeded in ultralow adhesion 6-well plates (Corning) at a density of 100,000 cells per well and incubated in 5% CO2 at 37 °C. On day 7, the neurospheres were collected through centrifugation, dissociated into single cells by trypsinization using TrypLE express media and passed in suspension culture to form further generations of neurospheres. For neurosphere counting, the neurospheres prepared in 24-well plates were counted under a microscope. For quantification of the neurosphere size, the neurospheres were photographed microscopically and the diameters were measured using the software Image J.

NSC proliferation and differentiation assays. For the NSC proliferation output assay, the neurospheres were dissociated into single cells and plated in ultralow adhesion 6-well culture plates at a density of 104 cells in 1 ml of growth medium. Cells were passaged every 5 days at a density of 105 cells in 1 ml of growth medium. The viable cells in each passage were evaluated by trypsin blue staining. The accumulated total cell number for each passage was calculated on the assumption that the total cells from the previous passage were replated. For the BrdU incorporation assay, the neurospheres were dissociated into single cells and plated on poly-n-lysine (Sigma) and laminin (Roche)-coated coverslips at a density of 105 ml−1 in growth medium. Following 24-h culturing, cells were treated with BrdU (Sigma) at a final concentration of 10 μM for 2 h. Subsequently, the cells were washed and fixed with 4% PFA for BrdU staining. For the TUNEL assay, the samples were washed and fixed with 4% PFA for BrdU staining. For the TUNEL assay, the apoptosis detection kit (Upstate). Images were captured from multiple areas in each Notch isoform including Notch1, 2, 3 and 4 and matching control lentiviral vectors were purchased from Sigma (see detailed information in Supplementary Information). Lentiviruses were produced by co-transfecting viral expression vectors with the packaged plasmids into HEK293 FT cells (ATCC), as described previously[22,27]. For NSC transduction, the NSCs were maintained in growth medium with lentiviruses for 3 days, followed by a cell selection process through adding nucleoside antibiotic blasticidin (1 μg ml−1) to the culture medium. Cells transduced with lentiviral DNAs were resistant to blasticidin, and were monitored for the induction of GFP using a fluorescence microscope. Transduced cells were stably passaged in blasticidin-containing selection growth medium, and the presence of GFP in all cells was monitored over passages.

Promoter assays. The promoter sequences of mouse DLL4, Notch1 and Notch4 genes were PCR amplified (~1987 to +41 for DLL4, −717 to +92 for Notch1, and −708 to +16 for Notch4), and sub-cloned into the pGL3 luciferase vector (Promega) using standard strategies. Point mutations were made on 5′-GGRNNYYYCC-3′ decanucleotides present in the promoter sequences to disrupt the NF-κB-DNA binding base pairs (5′-GGGNNCTCC-3′ was replaced with 5′-CCTTTAGTGA-3′ for DLL4, 5′-GGGAGTTCGCC-3′ was replaced with 5′-GTGAGGGAGA-3′ for Notch1, and 5′-GGGGAGTTCGCC-3′ was replaced with 5′-TTCGAGAAAG-3′ for Notch4). HEK293 cells (ATCC) were cultured in standard conditions at 37 °C and 5% CO2 with DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and PenStrep, and transfected with luciferase and expression plasmids using Lipofectamine 2000 (Invitrogen). The Dual Luciferase Reporter Assay (Promega) was performed according to the manufacturer’s instructions, and co-transfection of pRL-TK was used to internally control firefly activity. Empty plasmids pGL3 and pCDNA3.1 were used as negative controls.

Heart perfusion, tissue or cell immunostaining and imaging. Mice under anesthesia were trans-heart perfused with 4% PFA, and the brains were removed, post-fixed for 4% PFA for 4 h and infiltrated with 20–30% sucrose. Brain sections (20 μm) were made using a cryostat at −20 °C. Cultured cells on coverslips were fixed with 4% PFA for 10 min at room temperature. For BrdU staining, the samples were pre-treated with 2 M HCI for 20 min followed by a 2-min incubation with 0.1 M sodium borate (pH8.5). Fixed tissue sections or cells were blocked with the serum of appropriate species, penetrated with 0.2% Triton X-100, and treated with primary antibodies, followed by reaction with Alexa Fluor 488 or 555 or horseradish peroxidase-conjugated antibodies (Pierce). The TNF-α-actin (1:1,000, Cell Signaling, #4967). Secondary antibodies were HRP-conjugated antibodies (Pierce). Using the Sox2-promoter-controlled lentiviral vector, the NSCs were maintained in growth medium containing Neurobasal-A (Invitrogen), 2% B27 without vitamin A (Invitrogen), 10 ng ml−1 EGF (Sigma) and 10 ng ml−1 BFGF (Invitrogen), seeded in ultralow adhesion 6-well plates (Corning) at a density of 100,000 cells per well and incubated in 5% CO2 at 37 °C. On day 7, the neurospheres were collected through centrifugation, dissociated into single cells by trypsinization using TrypLE express media and passed in suspension culture to form further generations of neurospheres. For neurosphere counting, the neurosphere size was measured using the software Image J.

Statistical analysis. The data are presented as mean ± s.e.m. Statistical differences were evaluated using Student’s t-test for comparisons of two groups or analysis of variance (ANOVA) and appropriate post hoc analyses for comparisons of more than two groups. P < 0.05 was considered significant.

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**Figure S1** In vivo characterization of hNSCs in adult mice. Brain sections across the hypothalamus of normal C57BL/6 mice (chow-fed males, 3 months old) were co-immunostained with Sox2 (green) and NeuN (red). DAPI staining revealed the nuclei (blue) of all cells in the sections. (a) Low-magnification images comprise the ARC and VMH. (b) High-magnification images show cell morphology in the regions outlined by dash lines. Images were merged to show the co-localization of Sox2 with DAPI but not with NeuN. 3V: third ventricle; ARC: arcuate nucleus; VMH: ventral medial hypothalamic nucleus; L3V, T: Lateral third-ventricle wall tanyctyes; ME, T: median eminence tanyctyes. Scale bar = 50 µm (a,b).
Figure S2 Sox2 and nestin expression in tanycytes. Brain sections across the MBH were prepared from Nestin-Cre mice (chow-fed males, 3-month-old) and were subjected to co-immunostaining of Sox2 (green) with Cre (red). L3V, T: Lateral third-ventricle wall tanycytes; ME, T: median eminence tanycytes. Scale bar = 50 µm.
Figure S3 Chronic HFD feeding impairs in vivo survival and neurogenesis of htNSCs. (a) Adult male C57BL/6 mice were maintained under chow vs. HFD feeding for 4 months. Mice then received icv injections of Brdu for 7 days and subsequently fixed, and brains were removed and sectioned across the indicated regions for Brdu staining. Images were merged with DAPI nuclear staining to show the localization of cellular nuclei. (b) Adult male C57BL/6 mice were maintained under chow vs. HFD feeding for 4 months. Mice then received a single-day icv injection of Brdu, and brains were fixed at Day 1 vs. 7, and sectioned for Brdu staining and counting analysis of Brdu-positive cells in serial ARC sections. (c–h) Adult male C57BL/6 mice were maintained under chow vs. HFD feeding for 4 months. Mice then received daily icv injections of Brdu consecutively for 7 days. Mice were fixed at Day 7 vs. 28, and brain sections across the ARC were made for Brdu labeling or co-immunostaining with NeuN, POMC, NPY, S100B and RIP. Bar graphs showed the numbers of Brdu-labeled cells (c) and the Brdu-labeled cells positive for NeuN (d), POMC (e), NPY (f), S100B (g), and RIP (h) in serial ARC sections. ** P < 0.01, *** P < 0.001, n = 5 mice (b,d,g), n = 6 mice (c,f,h) and n = 4 mice (e) per group. Error bars reflect means ± s.e.m. Scale bar = 50 µm (a).
Figure S4 In vitro analyses for NSCs derived various brain regions of obese mice. Male C57BL/6 mice were maintained under normal chow vs. HFD feeding for 4 months (~16 weeks, 16W). Primary neurospheres were isolated from the hypothalamus, SGZ and SVZ of these mice and cultured in vitro. (a) Cultured cells were labeled with Brdu (image not shown) and analyzed for the percentage of Brdu-positive (Brdu+) cells. (b) Neurospheric cells at the same number were induced to differentiate for 7 days, and cells were fixed and immunostained for TuJ1. DAPI staining reveals all the cells in the slides. (c) TuJ1-positive (TuJ1+) cells were counted in the slides. * P < 0.05, ** P < 0.01, comparisons between indicated groups, n = 4 per group (a,c); error bars reflect means ± s.e.m. Scale bar = 50 µm (b).
Figure S5 Cytokines and the effects on proliferation and differentiation of htNSCs. Male C57BL/6 mice were on a chow vs. HFD for 4 months, and neurospheres were derived from the hypothalamus of these mice and passaged in vitro. (a,b) Media from cultured neurospheres at various passages were collected for measuring TNF-α (a) and IL-1β (b) levels. Data presented show the levels measured for the media of cells at Passage 3 (P3) and Passage 6 (P6). * P < 0.05, ** P < 0.01, n = 6 per group; error bars reflect means ± s.e.m. (c–g) Obese mice-derived htNSCs were co-transfected with siRNAs against TNF-α and IL-1β. Irrelevant siRNA was used in the control group. (c) TNF-α and IL-1β mRNA levels in these cells were measured. (d,e) Cells labeled with Brdu were analyzed by Brdu immunostaining (d) and counted (e). (f,g) Cells were induced to differentiate, and following a 7-day differentiation, immunostaining of neuronal marker Tuj1 was performed (f) and analyzed for Tuj1-positive cells (g). ** P < 0.01, n = 4 per group (a,b,c,e,g); error bars reflect means ± s.e.m. Scale bar = 50 µm (d,f). si: siRNA.
Figure S6 Involvement of microglia in obesity-associated htNSCs impairment. (a) Adult male C57BL/6 mice received chronic HFD feeding vs. normal chow feeding, and brain sections were then prepared for co-immunostaining of microglia marker Iba1 and cytokine TNF-α. DAPI staining reveals all the cells. Images show the staining in the arcuate nucleus (ARC). (b–e) Chow-fed IKKβlox/lox mice bilaterally received intra-MBH injections of CD11b promoter-driven lentiviral Cre or matched lentiviral control, and then maintained under chronic HFD vs. normal chow feeding. (b) Co-immunostaining of Iba1 with TNF-α in the ARC sections. (c–e) Primary neurospheres isolated from the hypothalami of these mice were subjected to Brdu labeling to reveal proliferation rate (c) and Tuj1 immunostaining (d) to reveal the efficiency of neuronal differentiation (e). * P < 0.05, ** P < 0.01, *** P < 0.001, comparisons between chow and HFD at indicated points, n = 4 per group (c,e); error bars reflect means ± s.e.m. Scale bar = 50 µm (a,b,d).
**Figure S7** Assessment of htNSCs with IKKβ/NF-κB manipulations. (a) Schematic of lentiviral vector that employs CMV promoter to express CaIKKβ, DNIκBα, and GFP. (b–d) Immunostaining assessments of the model of GFP-htNSCs (b), DNIκBα-htNSCs (c) and CaIKKβ-htNSCs (d) for various stem cells markers. Data presented in (b–d) represent the analyses of cell models at various passages. Musa-1: Musashi-1. Scale bar = 50 μm (b,c,d).
**Figure S8** mRNA analyses of apoptotic genes and Notch isoforms. (a) Dissociated IKKβ-htNSCs, IκBα-htNSCs, and GFP-htNSCs with same initial cell numbers at Passage 6 were maintained in growth medium. The mRNA levels of apoptotic and anti-apoptotic genes in these cells at Passage 6 were assessed. (b–e) IKKβ-htNSCs co-infected with lentiviral shRNAs against Notch1–4 vs. lentiviral control shRNA were analyzed for mRNA levels of Notch1 (b), Notch2 (c), Notch3 (d), and Notch4 (e). *** P < 0.001, n = 4 per group (a–e); error bars reflect means ± s.e.m. au: arbitrary unit.
**Figure S8** full scans