Huntingtin-associated protein 1 regulates postnatal neurogenesis and neurotrophin receptor sorting

Jianxing Xiang,[1] Hao Yang,[1] Ting Zhao,[1] Miao Sun,[1] Xingshun Xu,[2]
Xin-Fu Zhou,[3] Shi-Hua Li,[1] and Xiao-Jiang Li[1,4]

[1]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USA. [2]The Institute of Neuroscience,
Soochow University, Suzhou City, Jiangsu, China. [3]School of Pharmacy and Medical Sciences, Faculty of Health Sciences,
Sansom Institute, University of South Australia, Adelaide, Australia. [4]State Key Laboratory of Molecular Developmental Biology,
Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.

Defective neurogenesis in the postnatal brain can lead to many neurological and psychiatric disorders, yet the mechanism behind postnatal neurogenesis remains to be investigated. Huntingtin-associated protein 1 (HAP1) participates in intracellular trafficking in neurons, and its absence leads to postnatal death in mice. Here, we used tamoxifen-induced (TM-induced) Cre recombination to deplete HAP1 in mice at different ages. We found that HAP1 reduction selectively affects survival and growth of postnatal mice, but not adults. Neurogenesis, but not gliogenesis, was affected in HAP1-null neurospheres and mouse brain. In the absence of HAP1, postnatal hypothalamic neurons exhibited reduced receptor tropomyosin-related kinase B (TRKB) levels and decreased survival. HAP1 stabilized the association of TRKB with the intracellular sorting protein sortilin, prevented TRKB degradation, and promoted its anterograde transport. Our findings indicate that intracellular sorting of neurotrophin receptors is critical for postnatal neurogenesis and could provide a therapeutic target for defective postnatal neurogenesis.

Introduction

Neurogenesis is the process by which neurons are generated from neural stem and progenitor cells. Unlike embryonic neurogenesis, which is largely controlled by transcription factors, and adult neurogenesis, which is restricted to a few brain regions, postnatal neurogenesis is critical for the maturation of neuronal connections in the central nervous system and is profoundly influenced by environmental factors after birth. Because aberrant postnatal brain maturation can be caused by multiple mechanisms and leads to a variety of neurological and psychiatric disorders such as schizophrenia (1), it is important to understand the role of postnatal neurogenesis and find out important molecules that regulate this process.

Huntingtin-associated protein 1 (Hap1), originally identified as a neuronal protein that interacts with the Huntington disease (HD) protein, huntingtin (htt), is critical for postnatal development, as *Hap1*-KO mice often die before P3 due to inhibited feeding behavior (2, 3). Mounting evidence has shown that both htt and Hap1 participate in intracellular trafficking of membrane receptors (4, 5). Consistent with its potential involvement in neuropathology, Hap1 is enriched in neuronal cells (6). However, the expression of Hap1 in mouse brain is developmentally regulated (7, 8) and varies in different regions, with the highest level in the hypothalamus that is regulated by feeding behavior (9). Thus, Hap1's function may be cell-type dependent and is critical for hypothalamic function, which regulates the growth and energy balance of animals.

Despite the essential role of Hap1 in early development, some important issues remain unclear. First, we do not know whether Hap1 functions differentially in early development versus adulthood or whether loss of Hap1 also affects the survival and growth of adult animals. Second, Hap1 is important for the endocytosis of several membrane receptors, including those for EGF, GABA, and neurotrophins (10–14). How Hap1 is involved in the intracellular trafficking of different receptors remains to be investigated. More importantly, how this trafficking function is related to the role of Hap1 in early development needs to be elucidated. Understanding these issues could help us unravel the pathogenesis of a number of neurological disorders. For example, Hap1 interacts with the N-terminal fragments of mutant htt, which may accumulate in aged neurons to affect Hap1's function in the adult brain. Hap1 also binds tightly to Ahi1 (13), a protein whose depletion results in an early brain development disorder, Joubert syndrome (15, 16). Thus, loss of Hap1 or its dysfunction at different ages or in different types of cells can contribute to various pathological conditions. Furthermore, unraveling the mechanism by which Hap1 maintains the postnatal survival of animals is also important for understanding an important biological process in early animal development.

In the current study, we investigated conditional *Hap1*-KO mice by depleting Hap1 expression in mice at different ages via tamoxifen-induced (TM-induced) Cre recombination. Depletion of Hap1 in late embryos or early postnatal stage (P1) mice can cause early death and retarded growth by reducing neurogenesis. In contrast, depletion of Hap1 in adult mice does not lead to these phenotypes. Moreover, we found that Hap1 is important for endocytic receptor tropomyosin-related kinase B (TrkB) signaling in developing hypothalamic neurons via its association with the intracellular sorting protein, sortilin, which prevents lysosomal degradation of TrkB and promotes TrkB association with kinesin for its anterograde transport. These findings suggest that intracellular sorting of neurotrophin receptors is important for postnatal neurogenesis and can be a therapeutic target when this neurogenesis is aberrant.
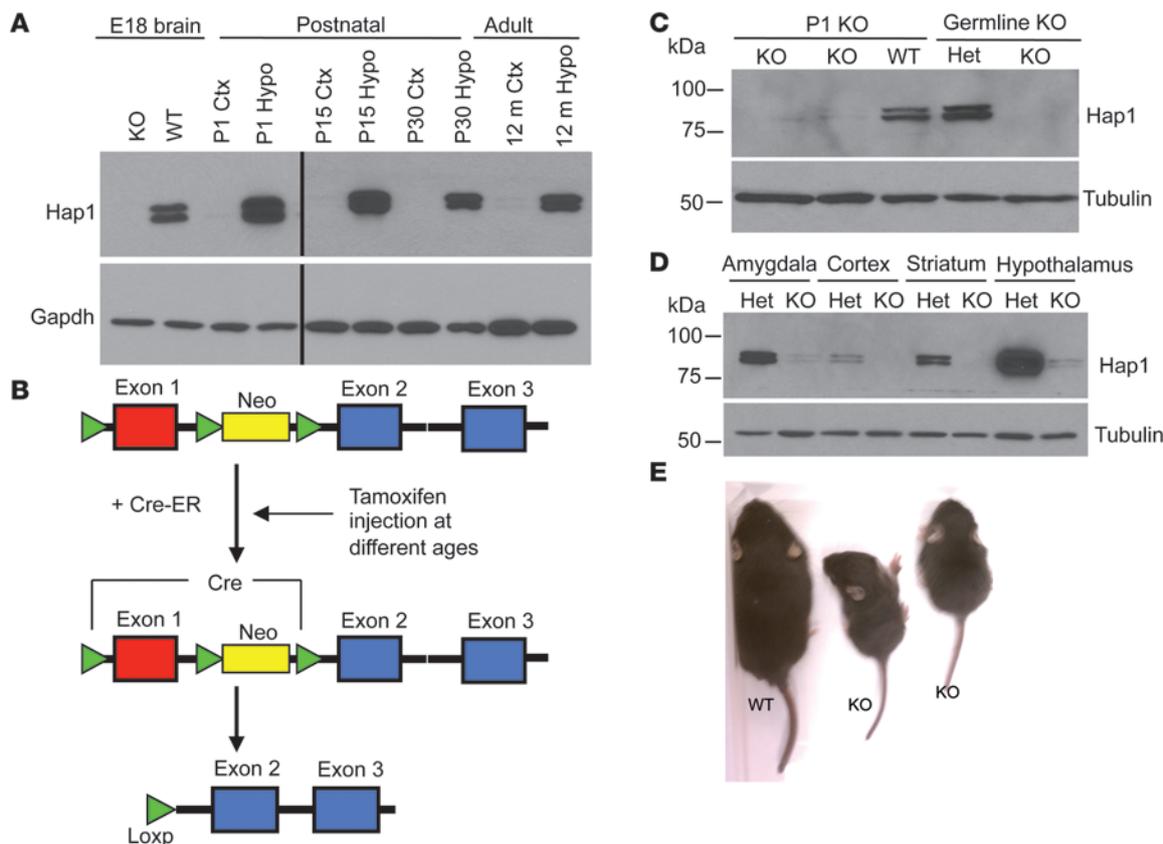
Figure 1
Generation of conditional *Hap1*-KO mice. (**A**) Western blot analysis of Hap1 expression in developing and adult WT mouse brains. Ctx, cortex; Hypo, hypothalamus. The lanes were run on the same gel but were noncontiguous. (**B**) The exon 1 of the mouse *Hap1* gene was flanked by loxP sites and the neomycin-resistant (neo) gene for generating floxed Hap1 mice. The floxed Hap1 mice were crossed with transgenic mice expressing Cre-ER, resulting in disruption of the *Hap1* gene. (**C**) Western blot analysis of the whole-brain tissues of WT and homozygous (KO) floxed Hap1 mice 15 days after TM injection at P1. The brain tissues from germline KO and heterozygous mice (het) were also included. (**D**) Western blots of brain regional tissues of *Hap1* adult KO mice. The brain tissues of 3-month-old mice were isolated 10 days after TM injection. Note that Hap1 consists of 2 isoforms (Hap1A and Hap1B) and is markedly reduced in homozygous (KO) floxed Hap1 mouse brain as compared with Het mouse brain. (**E**) Reduced body size of *Hap1* P1 KO mice 15 days after TM injection at P1 compared with a WT mouse that had also been injected with TM.

Results

Depletion of Hap1 expression affects the postnatal survival and growth of mice. Analysis of *Hap1* RNA expression in mouse brain showed that Hap1 expression is not detectable until E8.5–E12 and becomes prominent in the developing hypothalamus and limbic system at E15 (7, 8). Because, in the mouse brain, neurogenesis peaks between E10 and E13 and the central nervous system is continuously developed from E14 to P30 (17–19), Hap1 may regulate brain development during the late embryonic or postnatal period. By comparing the relative levels of Hap1 in the mouse brain at different ages via Western blotting, we found that the highest level of Hap1 occurs in the postnatal brain (Figure 1A), which suggests that Hap1 is important for brain formation and maturation after birth. To investigate the role of Hap1 in early development and adulthood, we generated conditional *Hap1*-KO mice in which exon 1 of the *Hap1* mouse gene is flanked by 2 loxP sites and can be depleted by Cre recombination at different ages (Figure 1B). These mice were crossed with transgenic mice in which Cre begins to express at E11 under the neuronal nestin promoter (20). Like *Hap1*-null mice generated from germline gene targeting, the crossed nestin–*Hap1*-KO mice also died

before P3. Thus, reducing Hap1 expression in neuronal cells from E11 caused postnatal death, leading us to investigate the effect of *Hap1* deficiency in postnatal mice.

The floxed Hap1 mice were next crossed with transgenic mice that express Cre-ER ubiquitously. The crossed offspring carrying the floxed Hap1 and Cre-ER were i.p. injected with TM, which binds the cytoplasmic Cre-ER and directs it to the nucleus to remove the floxed exon 1 of the *Hap1* gene, leading to the disruption of the *Hap1* gene at postnatal or adult ages (Figure 1B).

Our first experiment was to inject TM into floxed Hap1/Cre-ER mice at P1. Western blotting showed that Hap1 expression was dramatically reduced in postnatal brains 15 days after TM injection (Figure 1C and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI69206DS1). Similarly, after injecting TM into the floxed Hap1/Cre-ER mice at 2 to 3 months of age (adult KO), we also saw a dramatic reduction of Hap1 in various brain regions, including the amygdala, cortex, striatum, and hypothalamus (Figure 1D). Importantly, TM-induced *Hap1* P1 KO (P1 KO) mice showed early postnatal death and reduced body size (Figure 1E); however, embryonic Hap1 depletion led to the postnatal death of all nestin-Cre *Hap1*-KO
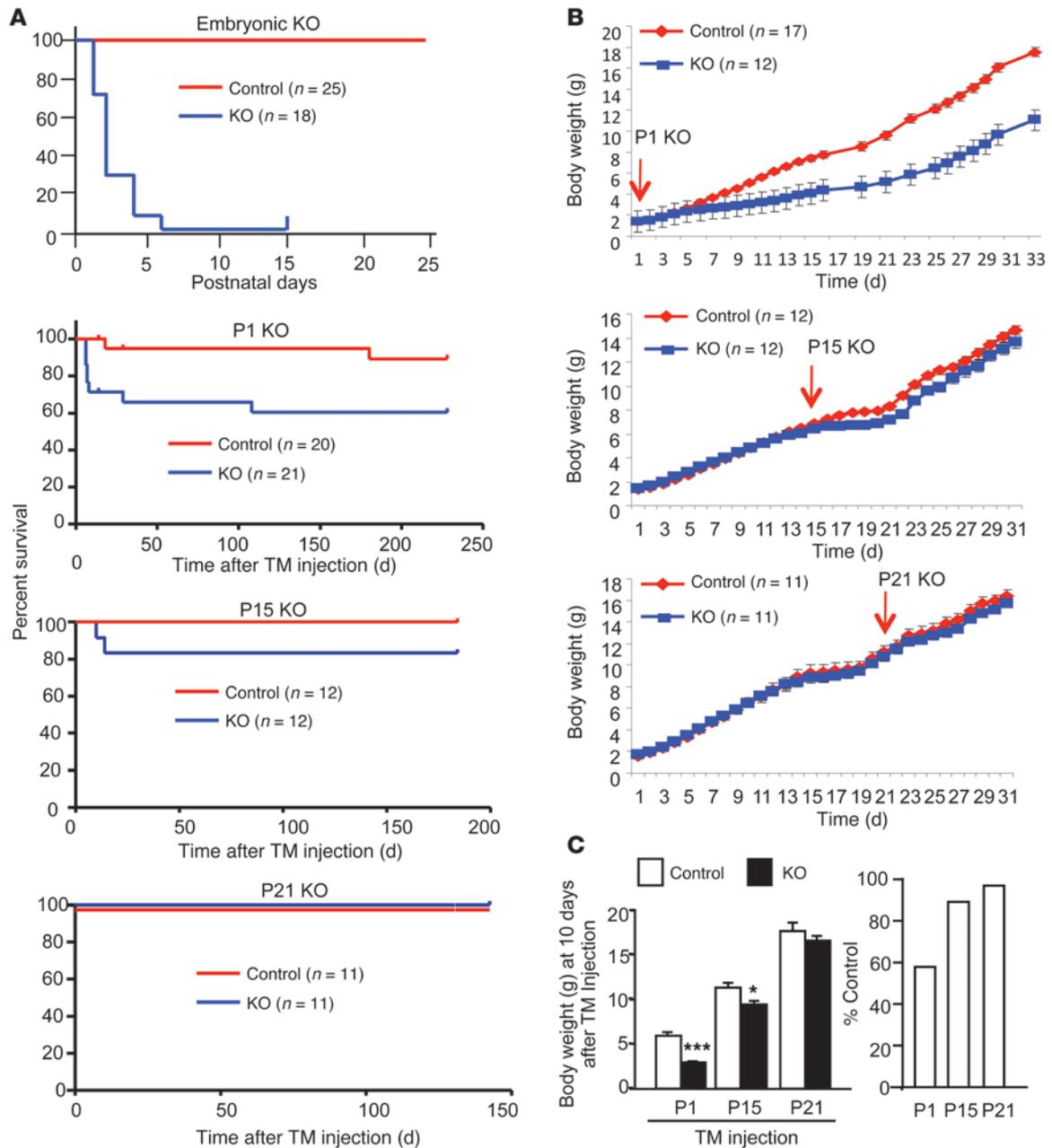
Figure 2
Reduced survival and growth of mice when *Hap1*-KO occurs at embryonic or early postnatal days. (**A**) The survival of *Hap1*-KO mice when the *Hap1* gene is depleted from E11 in nestin-Cre *Hap1*-KO mice or from P1, P15, or P21 via TM injection. The control mice are heterozygous floxed Hap1 mice that had also been injected with TM. (**B**) Body weights of the control and *Hap1*-KO mice induced by TM at P1, P15, and P21. Arrows indicate the day when TM injections began. (**C**) Body weights of mice 10 days after TM injection at P1, P15, or P21. *$P < 0.05$; ***$P < 0.001$. The percentages of the control mouse body weight are also presented. Error bars represent SEM.

mice within 15 days, whereas about 25% of *Hap1* P1 KO mice died after 5 days of TM injection, and 60% of mice survived to adulthood (Figure 2A). When TM was injected into floxed Hap1/Cre-ER mice at P15 (P15 KO), more mice (>80%) survived, and fewer than 20% of mice died. More importantly, when the floxed Hap1/Cre-ER mice were injected with TM at P21 (P21 KO), after the organization of the hypothalamus was basically completed at P19 (21), or at 2 to 3 months (adult KO), all mice could live as normally as the control mice that were heterozygous floxed

Hap1 mice injected with TM (Figure 2A). Thus, postnatal death certainly depends on the age at which Hap1 expression is reduced.

Retarded growth is another remarkable phenotype of *Hap1*-null mice (2, 3). We therefore monitored the body weight of mice when their Hap1 expression was suppressed at P1, P15, and P21 via TM induction (Figure 2B). As expected, P1 KO mice began gaining less body weight 5 days after TM injection, the time when Hap1 expression had been markedly decreased. When Hap1 expression was reduced from P15, there was only a slight decrease in the body
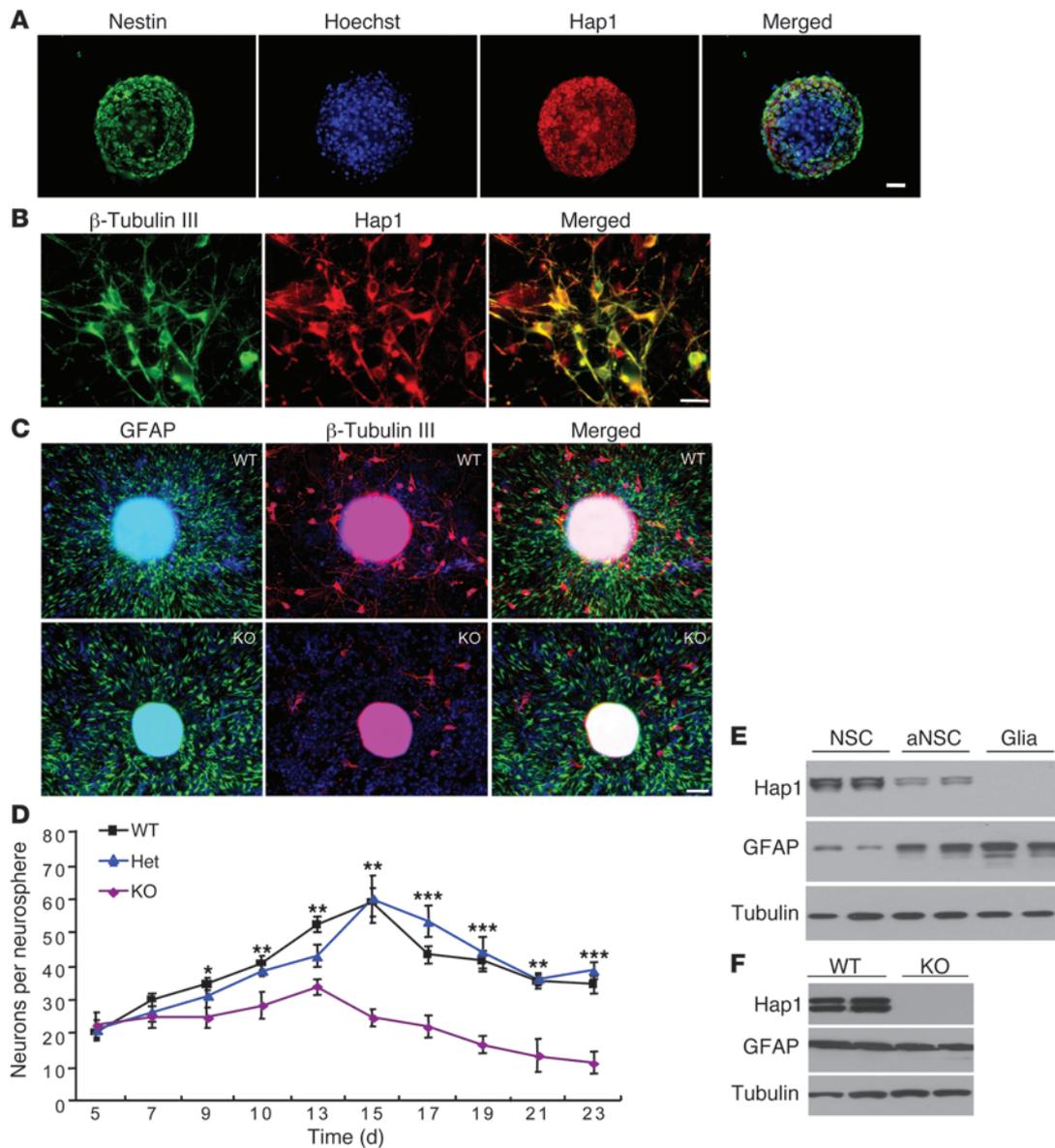
Figure 3
Lack of Hap1 does not affect astrocyte differentiation in the mouse neurospheres. (**A**) Low-magnification micrograph (×10) showing that neurospheres from WT mouse brain express Hap1 (red) and nestin (green). The cellular nuclei were labeled by Hoechst dye (blue). Scale bar: 20 μm. (**B**) High-magnification micrograph (×40) showing the coexpression of β-tubulin III (green) and Hap1 (red) in differentiated neuronal cells in the neurospheres. Scale bar: 10 μm. (**C**) Immunostaining of the mouse neurospheres, which had been differentiated for 5 days in culture, with antibodies to the neuronal protein β-tubulin III and glial protein GFAP (upper panel). Scale bar: 40 μm. (**D**) The relative numbers of neuronal (β-tubulin III–positive) cells were counted at different time points after induction with retinoic acid and 2% serum (lower panel). Note that there are more neuronal cells in WT and Het groups than in the KO group. In each group, the value represents mean ± SD obtained from 20 neurospheres from each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with *Hap1*-KO neurospheres. (**E**) Western blot analysis of cultured NSC, aNSCs, and mature astrocytes showing the absence of Hap1 in the mature glial cells. (**F**) Western blots of WT and *Hap1*-null (KO) mouse hypothalamus showing that the absence of Hap1 does not affect the level of GFAP. Two different samples of each genotype were analyzed via Western blotting.

weight gain of P15 KO mice. Moreover, depletion of Hap1 at P21 (P21 KO in Figure 2B) or at 3 months (Supplemental Figure 1B) did not cause any significant decrease in body weight, though Hap1 deficiency in adult mice slightly reduced food intake, which was more obvious 42 to 50 days after TM injection (Supplemental Figure 1C). To better compare the growth of mice that had depleted Hap1 expression for the same period of time, we compared the body weights of mice 10 days after TM injection. The

results clearly showed an age-dependent decrease in body weight gain compared with the control mice, as P1 KO and P15 KO mice showed 57% ($P < 0.001$) and 88% ($P < 0.05$) of the control mouse body weight, while P21 KO mice were not significantly different from the control mice (Figure 2C).

Lack of Hap1 selectively reduces postnatal neurogenesis. The postnatal death of conditional *Hap1*-KO mice led us to investigate whether Hap1 affects neurogenesis during early brain development. Neu-
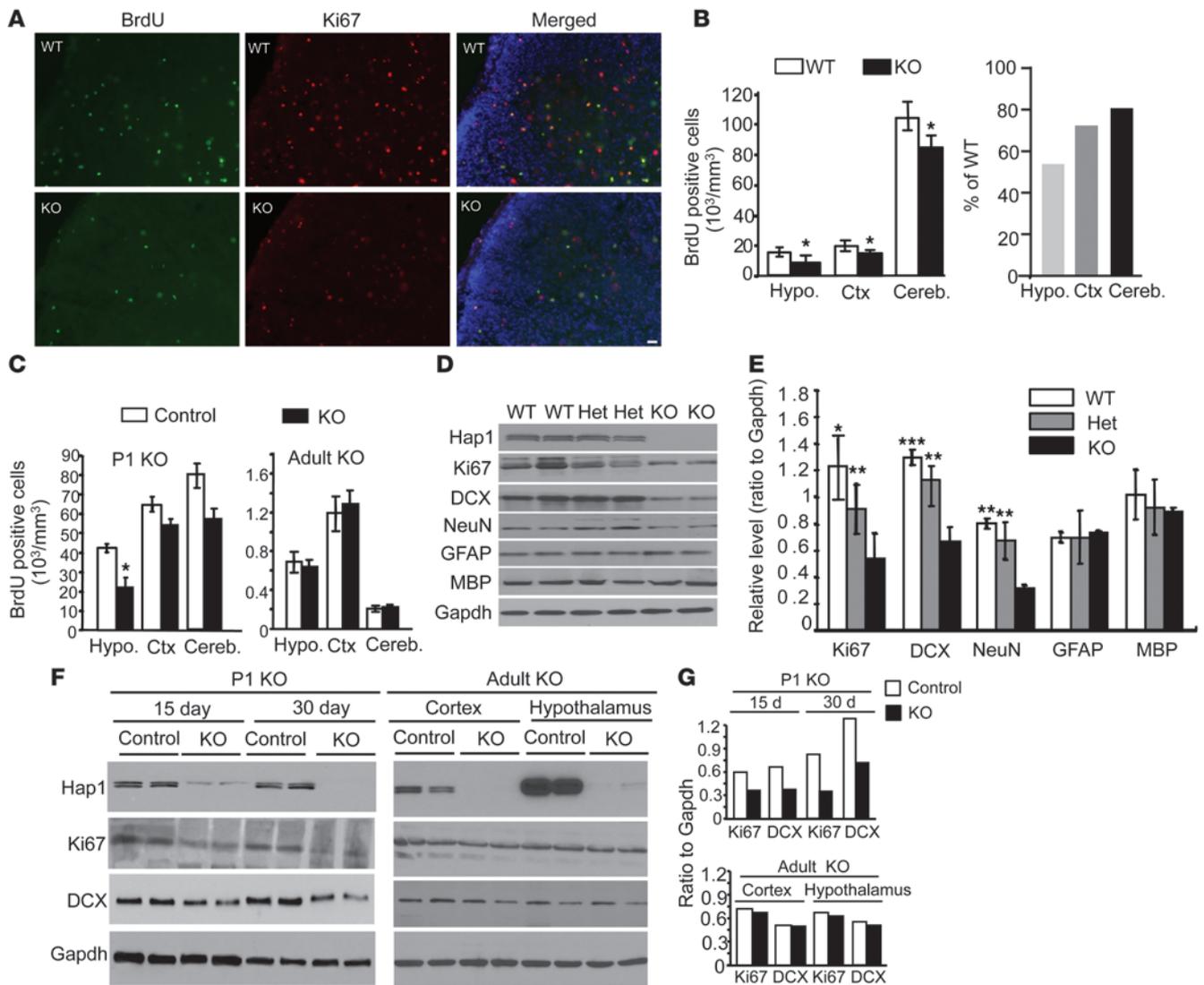
Figure 4

Lack of Hap1 reduces neuronal proliferation in the brains of *Hap1*-null mice. (**A**) Immunofluorescent staining of BrdU and Ki67 in the hypothalamus of WT (upper panels) and *Hap1*-null (KO, lower panels) mice that had been injected with BrdU at P1 for 2 hours. Scale bar: 10 μm. (**B**) Stereological quantification of the numbers of BrdU-positive cells in the brain regions of WT and *Hap1*-null mice (KO, left), and the ratio of these between KO and WT mice (right). *n* = 3 for each group, *P < 0.05. (**C**) Quantitative analysis of the number of BrdU-positive cells via stereology in *Hap1* P1 KO and adult KO mice showing that loss of *Hap1* only reduces neurogenesis in P1 KO mice. *n* = 3 for each genotype, *P < 0.05. (**D**) Western blots of the hypothalamic tissues of WT, heterozygous (Het), and homozygous (KO) *Hap1*-KO mice (P2) with antibodies to Hap1, Ki67, DCX, NeuN, GFAP, MBP, and Gapdh. Two different samples of each genotype are presented. (**E**) The ratios of proteins indicated in **D** to Gapdh were obtained from 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the KO sample. (**F**) Western blots of brain tissues of control, *Hap1* P1 KO, and adult KO mice with antibodies to Hap1, Ki67, DCX, and Gapdh. Samples were analyzed after TM injection for 15 (P1 KO) or 30 (P1 and adult KO) days. (**G**) Relative levels of proteins (ratio to Gapdh) on the Western blots were presented. All error bars represent SEM.

rospheres can be obtained from a single-cell suspension of neural stem as well as progenitor cells isolated from the fetal brain and have been an extremely useful tool for analyzing the proliferation and differentiation of neural stem and progenitor cells. We therefore cultured neurospheres from the E11 mouse brain tissues as described previously (22, 23). We first verified the expression of Hap1 in neurospheres from WT mouse brain and saw that cells in the neurospheres, which expressed the marker protein nestin for neural stem and progenitor cells, also expressed Hap1 (Figure 3A). After induction of differentiation of the neurosphere cells, immunofluorescent

double labeling with antibodies to Hap1 and the neuronal protein β-tubulin III confirmed that Hap1 was expressed in differentiated neuronal cells in the neurospheres (Figure 3B).

We also needed to find out whether Hap1 is required for glial differentiation, as glial progenitor migration to differentiate into astrocytes and oligodendrocytes is a major event in the postnatal brain (24). Since neurons and glia arise from neural progenitor cells (NPCs), we next examined whether lack of Hap1 affects neuronal or glial differentiation in neurospheres. Double staining of neurospheres with antibodies to neuronal (β-tubulin III)
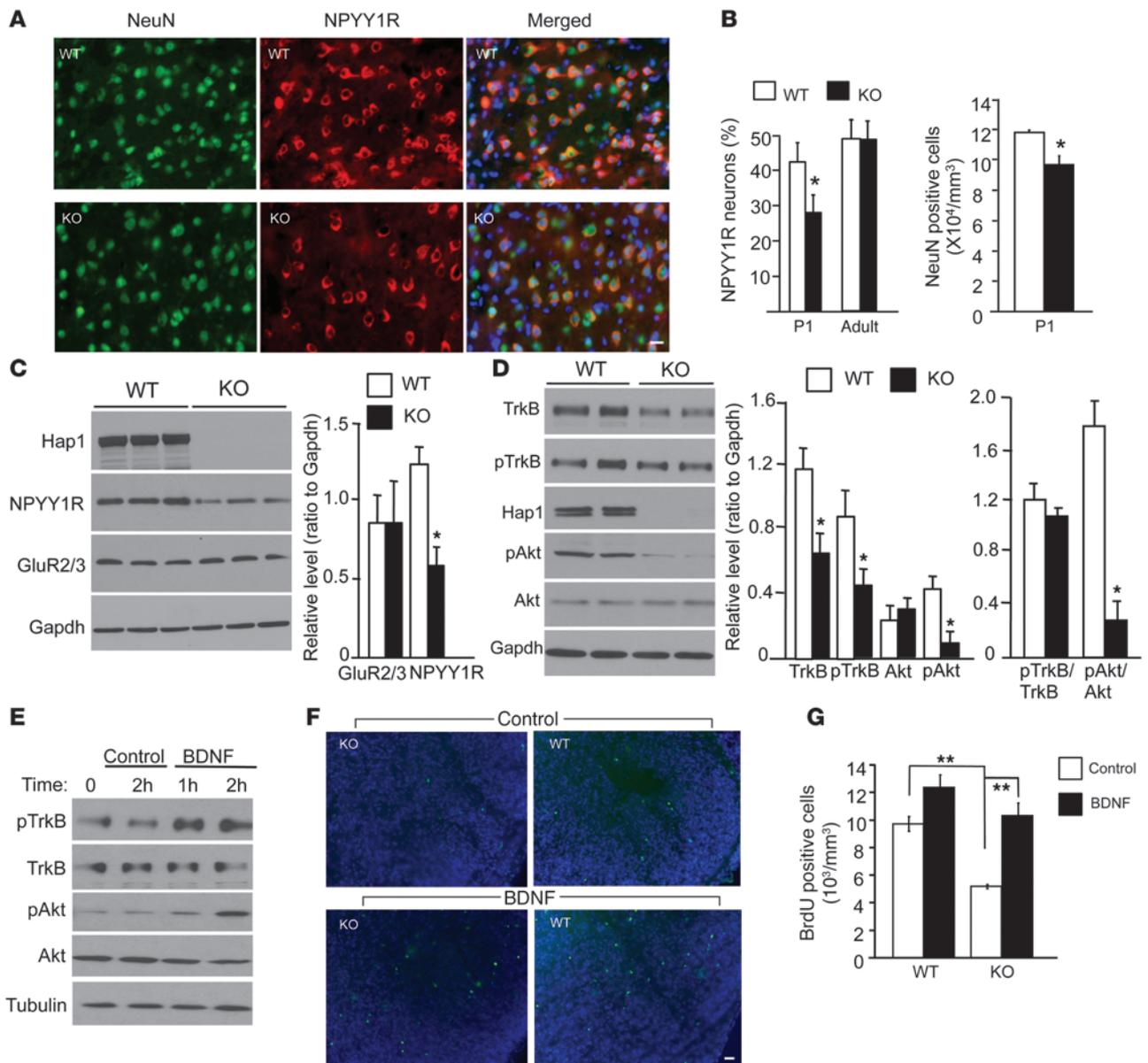
Figure 5
Loss of *Hap1* reduces neurons expressing NPYY1R in the mouse hypothalamus. (**A**) Immunostaining of the hypothalamus of *Hap1* P1 KO mouse with antibodies to NeuN and NPYY1R. Scale bar: 10 μm. (**B**) Quantitative analysis (*n* = 15 images per group) of the numbers (% of total cells) of NPYY1R-positive cells in WT, P1 KO, and adult KO (see Supplemental Figure 6) mouse hypothalamus (upper panel) and the stereology analysis of the numbers of NeuN-positive cells in *Hap1* P1 KO and WT mouse hypothalamus (lower panel, *n* = 3 per genotype). (**C**) Western blot analysis of the hypothalamic tissues of WT and *Hap1* P1 KO mice and quantifications. (**D**) Western blot analysis (left panel) and quantification (middle panel) of WT and *Hap1*-null (KO) mouse hypothalamic tissues showing decreased TrkB and its signaling (pTrkB, pAkt) when *Hap1* is absent. The ratios of pTrkB to TrkB and pAkt to Akt between WT and KO mice were also shown (right panel). *P < 0.05. (**E**) Western blot of hypothalamic tissues from WT P1 mice that were injected into the third ventricle with either control (sterile PBS) for 2 hours, or 5 μg BDNF for 1 or 2 hours. Noninjected littermate served as control. (**F**) BrdU immunostaining of hypothalamus from P1 WT and *Hap1*-null (KO) mice that had been injected with either control or BDNF for 4 hours. Scale bar: 10 μm. (**G**) Quantification of BrdU-positive cells in the hypothalamus of control or BDNF-treated P1 WT or KO mice (*n* = 3 mice per genotype). **P < 0.01. All error bars represent SEM.

and glial (GFAP) proteins showed a marked reduction in neuronal staining in *Hap1*-null (Hom) neurospheres, while GFAP labeling was unchanged (Figure 3C and Supplemental Figure 2). Quantification of the relative numbers of neuronal (β-tubulin III positive) cells verified that lack of Hap1 reduced the numbers of neuronal cells after inducing neurosphere differentiation for more than

9 days (Figure 3D). We also performed Western blot analysis of neural stem cells (NSC), adult neural stem cells (aNSC), and mature glial cells. The level of Hap1 was decreased in aNSCs and disappeared in mature glial cells (Figure 3E), suggesting that Hap1 is not required for glial differentiation. To confirm this idea, we performed Western blotting of brain tissues from WT and *Hap1*-
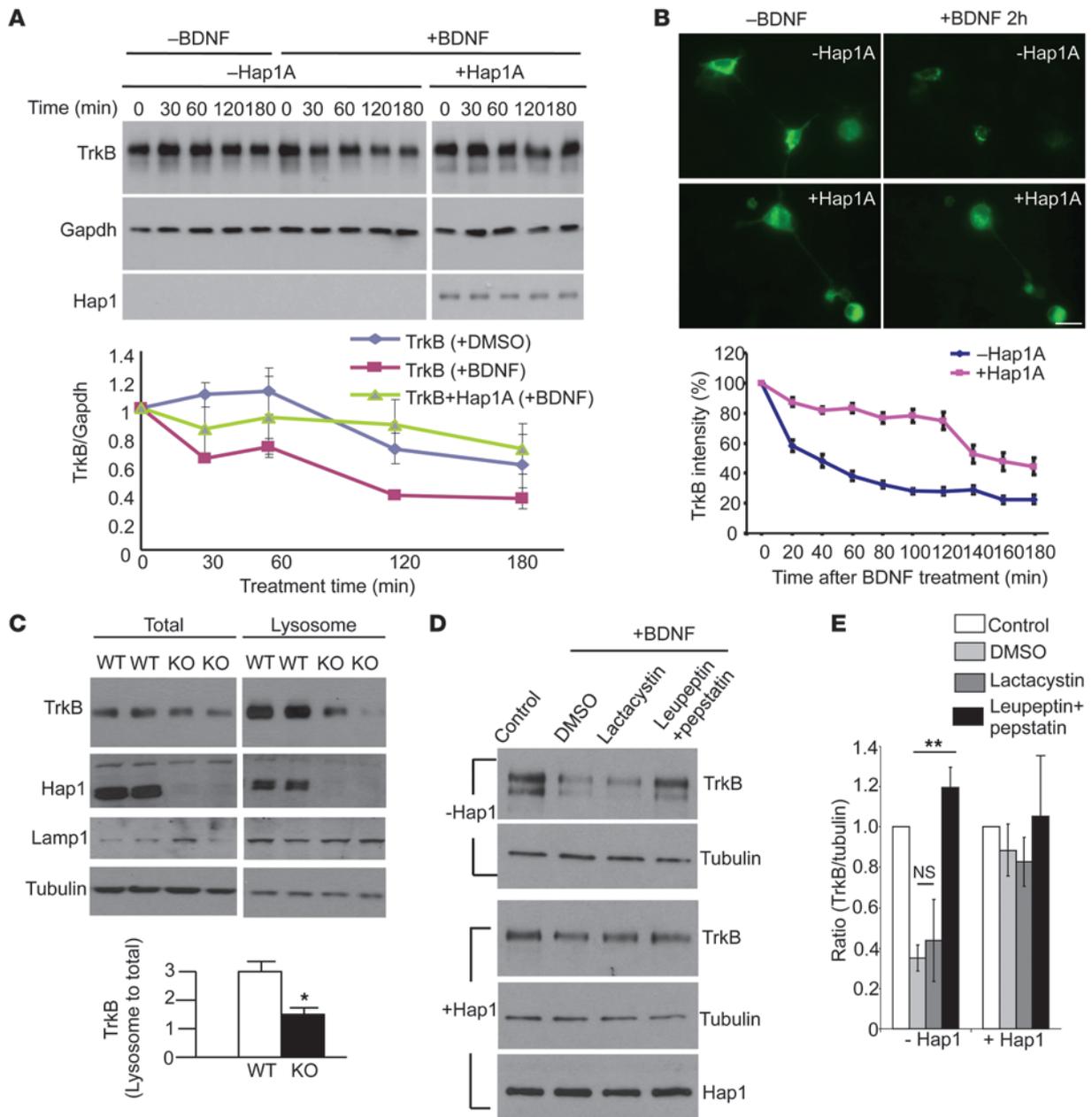
Figure 6
Hap1 reduces lysosomal degradation of TrkB. (**A**) Western blot analysis of degradation of TrkB in the presence of Hap1A in transfected HEK293 cells that were treated without or with BDNF (100 ng/ml). (**B**) Fluorescent images of transfected cells expressing TrkB-GFP after BDNF treatment in the absence or presence of Hap1A. Quantification data are also present beneath **A** ($n = 3$) and **B** ($n = 8$). Scale bar: 10 μm. (**C**) Lysosome fractions isolated from the hypothalamic regions in WT and *Hap1*-KO mice showing a decreased level of TrkB in the absence of *Hap1*. Blots were probed with antibodies to the lysosome protein, Lamp1, Hap1, TrkB, and tubulin. The relative level of TrkB (lysosome to total, $n = 6$) was quantified. *$P < 0.05$. (**D**) Degradation of transfected TrkB is reduced by the lysosome enzyme inhibitors leupeptin/pepstatin (10 μg/ml), but not the proteasome inhibitor lacacystin (10 μM). DMSO served as a control. (**E**) Quantification of the ratio of TrkB to tubulin ($n = 3$) is shown. **$P < 0.01$. All error bars represent SEM.

null (KO) mice at P1 and found that lack of *Hap1* indeed did not reduce the GFAP level in the *Hap1*-KO brain (Figure 3F). Thus, lack of *Hap1* appears to selectively impair neurogenesis.

We used a BrdU incorporation assay to measure the proliferation of neurosphere cells. As expected, lack of Hap1 reduced BrdU incorporation or cell proliferation in the neurospheres (Sup-

plemental Figure 3, A and B) as well as the levels of both double-cortin (DCX), an immature neuronal or neuroblast marker, and Ki67, a mitotic marker or proliferating cell marker (Supplemental Figure 3C). To examine whether loss of Hap1 affects neurogenesis in vivo, we injected BrdU into P1 mouse pups and measured BrdU incorporation in the mouse brains. Significant ($P < 0.05$)
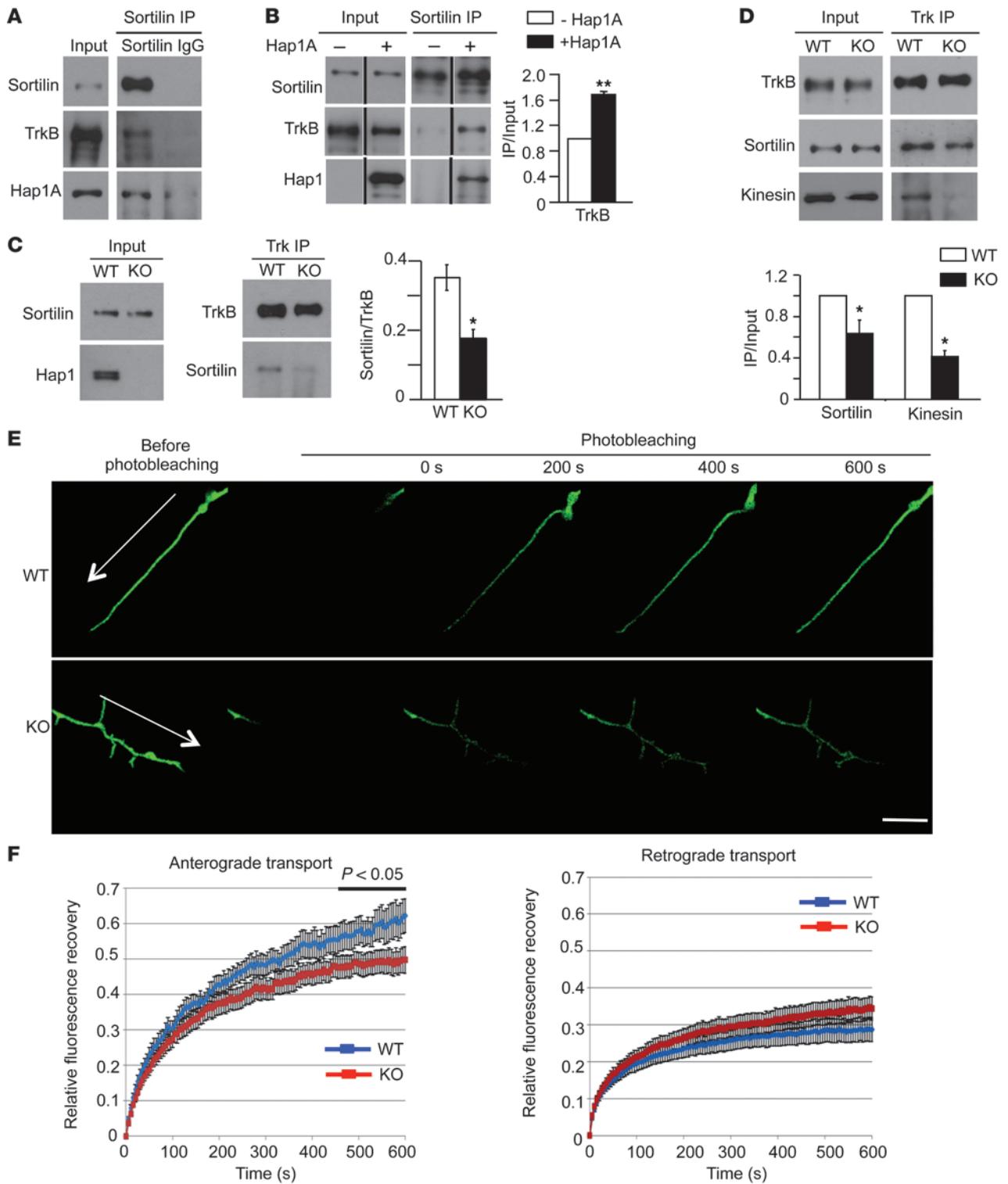
Figure 7

Hap1 stabilizes sortilin association with TrkB and promotes TrkB transport. (**A**) Immunoprecipitation of endogenous sortilin in HEK293 cells showing the coprecipitation of transfected TrkB and Hap1A. (**B**) Coexpression of Hap1A increased the association of TrkB with sortilin after cells were treated with BDNF (100 ng/ml for 30 minutes). The lanes were run on the same gel but were not contiguous. The ratio of precipitated to input (IP/input) is shown in the right panel (*n* = 3). (**C**) Immunoprecipitation of TrkB from the hypothalamic tissues of WT or *Hap1* P1 KO mice showing a reduced precipitation of sortilin when *Hap1* is absent. The ratios of precipitated sortilin to TrkB are also presented (*n* = 3, right panel). *P < 0.05. (**D**) Immunoprecipitation of TrkB from P1 KO mouse hypothalamic tissues showing reduced association of sortilin and kinesin with TrkB. In **B** and **D**, IP/input is normalized to control (*n* = 3). *P < 0.05; **P < 0.01. (**E**) Loss of *Hap1* reduces the anterograde transport of TrkB in cultured olfactory neuronal cells at DIV4. The neuronal tips were photobleached and then examined for the recovery of TrkB-GFP levels, which represent the anterograde transport of TrkB-GFP from the cell body to neurite tips. Arrows indicate anterograde transport directions. Scale bar: 5 μm. (**F**) Quantitation of anterograde and retrograde transport of TrkB-GFP in cultured WT and KO neurons via FRAP. For studying anterograde (WT = 9, KO = 10) and retrograde (WT = 7, KO = 9) trafficking of TrkB, fluorescence in neurites near tips and soma was photobleached, respectively. All error bars represent SEM.

decreases in the numbers of BrdU-positive cells were seen in the cortex, hypothalamus, and cerebellum in *Hap1*-KO mice with more decrease seen in the *Hap1*-KO hypothalamus compared with WT mice at P1. Ki67 immunostaining also showed similar decrease in the *Hap1*-KO brains (Figure 4, A and B). To verify that lack of *Hap1* indeed affects neurogenesis after birth, we measured BrdU incorporation in the brains of P7 mice that depleted Hap1 expression via TM at P1 (P1 KO). P1 KO mice also showed a decrease in BrdU incorporation in the hypothalamus, and again, this decrease was greater than in the cortex and the cerebellum (Figure 4C). However, when *Hap1* was depleted via TM injection at 2 months of age (adult KO), we saw no significant difference in the number of BrdU-positive cells in the cortex, hypothalamus, and cerebellum between adult control and adult KO mice (Figure 4C).

We also performed Western blot analysis of the hypothalamic tissues from WT, Het, and Hom *Hap1*-KO mice at P2 and found that levels of Ki67, DCX, and NeuN, but not the glial proteins GFAP (astrocyte marker) and MBP (myelin basic protein), were reduced in *Hap1*-null hypothalamus (Figure 4D). These differences were further verified by quantification of the ratios of these proteins to Gapdh on the same blots (Figure 4E). Similar reductions in Ki67 and DCX were also seen in *Hap1*-null neurospheres (Supplemental Figure 3B). It is important to know whether neurogenesis is affected in embryonic or postnatal brains of *Hap1*-null mice. We thus examined E14.5 and E18 mouse brains, but did not find significant differences in the levels of DCX and β-tubulin between WT and *Hap1*-null brains (Supplemental Figure 4). We then examined whether decreases in Ki67 and DCX after birth are age dependent by performing Western blotting on brain tissues from control and *Hap1* P1 KO mice at days 15 and 30 after TM injection at P1. The results revealed that both Ki67 and DCX were reduced in the hypothalamic tissues from P1 KO mice (Figure 4, F and G). However, in neither the cortex nor hypothalamic tissues from *Hap1* adult KO mice that had depleted the Hap1 expression via TM at age of 2 month for 30 days was there any obvious change in Ki67 and DCX (Figure 4, F and G). Together, these results suggest that Hap1 is important for neurogenesis in postnatal brain.

Hap1 is important for the neurogenesis of hypothalamic neurons. Given that Hap1 is abundantly expressed in the hypothalamus and that the hypothalamic function is critical for postnatal growth, we focused on the role of Hap1 in hypothalamic neurogenesis. We first examined whether cultured hypothalamic neurons from *Hap1*-null embryos (E18–E19) can grow in vitro. We found that lack of Hap1 decreased the number of cultured hypothalamic neurons that were labeled by anti-β-tubulin III (Supplemental Figure 5). To identify which types of hypothalamic neurons are affected in *Hap1*-null mice, we performed immunostaining with several antibodies to the proteins that are expressed in hypothalamic neurons and found that only neuropeptide Y Y1 receptor (NPYY1R) was reduced (Figure 5A). NPYY1R is critical for energy homeostasis (25, 26) and is selectively expressed in certain types of hypothalamic neurons, such as nitric oxide synthase–positive neurons (27). However, as compared with controls, NPYY1R is not reduced in *Hap1* adult KO mouse hypothalamus (Supplemental Figure 6A) while *Hap1* P1 KO mouse hypothalamic tissues showed a reduction in NPYY1R (Figure 5B). The number of NeuN-positive cells was also reduced in *Hap1* P1 KO hypothalamus (Figure 5B), which could reflect the loss of NPYY1R-positive cells. Western blotting confirmed that the decrease of NPYY1R selectively occurred in conditional *Hap1*-KO mice that deplete the *Hap1* gene at P1 (Figure 5C), but not in adult mice (Supplemental Figure 6B). Western blotting also showed that another membrane receptor, GluR2/3, which is widely expressed in neuronal cells (28, 29), did not significantly change (Figure 5C). In addition, immunostaining revealed that the numbers of calbindin-labeled neurons were similar in WT and *Hap1* P1 KO mouse hypothalamic tissues (Supplemental Figure 7). Thus, loss of *Hap1* may selectively reduce the number of differentiated neurons that express NPYY1R in the hypothalamus of postnatal mouse brain.

Lack of Hap1 reduces TrkB in hypothalamic neurons. As brain-derived neurotrophic factor (BDNF)/TrkB signaling is known to maintain neurogenesis (30–32) and Hap1 deficiency impairs Trk signaling and reduces the survival of cultured neurons (12, 13), it would be important to know whether impaired TrkB signaling might affect hypothalamic neurogenesis during early development. Western blots confirmed that there was a decrease in TrkB and phosphorylated TrkB (pTrkB) in *Hap1*-null hypothalamic tissues of P1 mice. The ratio between pTrkB and TrkB showed only a slight but not significant decrease in *Hap1*-null hypothalamus (Figure 5D), indicating that Hap1 acts mainly on maintaining the TrkB level rather than the phosphorylation of TrkB. More importantly, the phosphorylation of Akt, downstream of BDNF/TrkB signaling, was also reduced in *Hap1*-null hypothalamic tissues (Figure 5D). The downregulation of Akt activation was more obvious than that of TrkB, probably due to signal magnification through multiple signaling cascades into downstream effectors. In addition, other receptors that signal to activate Akt might also be downregulated by the loss of Hap1.

It is known that BDNF/TrkB signaling is required for neuronal survival and growth in cultured neurons (33, 34). Thus, if Hap1 deficiency affects BDNF/TrkB signaling, this deficiency should affect the survival of cultured hypothalamic neurons and BDNF should rescue the defective survival. We therefore cultured hypothalamic neurons from *Hap1*-null mice and found that BDNF indeed increased the number of NPYY1R-positive hypothalamic neurons in culture (Supplemental Figure 8A). Double staining with the antibody to β-tubulin III confirmed that these hypothalamic

cells were neuronal cells (Supplemental Figure 8A). Quantification of β-tubulin III and NPYY1R-containing cells also showed that BDNF significantly ($P < 0.05$) increased the number of neurons and NPYY1R-positive cells in *Hap1*-null hypothalamic cultures (Supplemental Figure 8B). To determine whether Hap1 indeed regulates early postnatal neurogenesis via BDNF/TrkB signaling in vivo, we injected BDNF into the third ventricle of P1 WT or *Hap1*-null mice. The BDNF administration led to robust activation of TrkB and downstream effectors, such as Akt phosphorylation (Figure 5E). Analysis of BrdU-positive cells 4 hours after BDNF injection revealed that BDNF treatment doubled neurogenesis in the hypothalamus of *Hap1*-null mice while only a trend of increase was observed for WT mice (Figure 5, F and G). Double immunostaining of BrdU and NPC markers nestin and sox2 verified that the increased BrdU-positive cells were indeed NPCs (Supplemental Figure 9). There are abundant NPCs in the P1 hypothalamus, and BDNF may regulate the proliferation and differentiation of NPCs by accelerating the completion of S-phase in the cell cycle (35). It is possible that in the absence of Hap1, there is a large population of NPCs that are quiescent due to the suppressed BDNF/TrkB signaling, and when the signaling is restored, a portion of these cells might quickly exit dormancy and start to proliferate, resulting in increased BrdU staining. Taken together, we find that loss of Hap1 can reduce TrkB levels to affect the neurogenesis of hypothalamic NPYY1R neurons, a defect that can be rescued by BDNF.

Hap1 determines TrkB level via stabilizing the complex of TrkB and sortilin. Although Hap1 has been found to stabilize internalized receptors, the mechanism by which loss of Hap1 reduces TrkB levels and impairs TrkB signaling remains to be investigated. To this end, we first examined the effect of Hap1 on the half-life of TrkB by coexpressing TrkB with Hap1A in HEK293 cells, because HEK293 cells do not express endogenous Hap1. We confirmed that this cotransfection led to the coexpression of both TrkB and Hap1 in the majority of transfected cells (Supplemental Figure 10A). The transfected cells were treated with BDNF to trigger the endocytosis of TrkB and its degradation. Coexpression of Hap1 apparently increased the level of TrkB and its half-life after BDNF stimulation (Figure 6A). Since transfected TrkB is tagged with GFP, fluorescent signals of TrkB could be quantified to assess the half-life of TrkB. This assay also confirmed the protective effect of Hap1 on TrkB degradation (Figure 6B). To further examine whether lack of Hap1 promotes the degradation of TrkB in the lysosomes, we isolated lysosome-enriched fractions from WT and *Hap1*-KO mouse hypothalamus and observed a reduction of TrkB in the lysosome-enriched fraction from *Hap1*-KO mouse brains (Figure 6C). Since this lysosome-enriched fraction also contains other organelles such as endosomes that associate with Hap1, the decreased level of TrkB is likely due to an increased lysosome degradation of TrkB. To verify that TrkB is indeed degraded by the lysosomes, TrkB-transfected cells were treated with the proteasome inhibitor lactacystin or the lysosome enzyme inhibitors leupeptin/pepstatin simultaneously with BDNF induction of TrkB degradation. Inhibition of the lysosomes enzymes apparently stabilized more TrkB than inhibiting the proteasome (Figure 6D).

It is known that endocytic receptors are targeted to the lysosome for degradation, recycled to the plasma membrane, or transported to other compartments to mediate signaling pathways. Sortilin, which mediates intracellular sorting of endocytic proteins and also modulates the intracellular trafficking of receptors (36), was recently found to interact with Hap1 (37) and to enhance TrkB trafficking

and signaling (38). We coexpressed TrkB with Hap1A in HEK293 cells and then immunoprecipitated the endogenous sortilin. We observed the coprecipitation of TrkB and Hap1A (Figure 7A), which suggests that sortilin, TrkB, and Hap1 form a protein complex. By comparing the coprecipitation of TrkB with sortilin in the presence and absence of Hap1A, we found that Hap1A could increase the association of TrkB and sortilin (Figure 7B).

To further validate that Hap1 associates with TrkB and sortilin, we coexpressed Hap1A or TrkB with transfected sortilin in HEK293 cells and observed their colocalization with sortilin (Supplemental Figure 10B). More importantly, sortilin is colocalized with Hap1 in the cytoplasmic puncta in mouse hypothalamic neurons (Supplemental Figure 10C), providing evidence for their association in the brain. To examine the effect of Hap1 on the association of TrkB with sortilin, we performed in vivo immunoprecipitation of sortilin from the hypothalamic tissues of WT and KO mice. In the absence of Hap1, there was a reduced amount of precipitated sortilin with TrkB compared with that from WT mouse hypothalamic tissues (Figure 7C). Sortilin has been found to facilitate the anterograde transport of TrkA and enhances its signaling (36). Immunoprecipitation of TrkB from P1 KO mouse hypothalamic tissues revealed that lack of Hap1 reduced the association of TrkB with sortilin and kinesin heavy chain, an anterograde transporter motor protein (Figure 7D). We then performed fluorescence recovery after photobleaching (FRAP) assay to measure the recovery of fluorescence in neurite parts near tips or soma in cultured neuronal cells, which reflects anterograde or retrograde transport of TrkB-GFP in cultured neurons. *Hap1*-null hypothalamic neurons do not grow well and cannot develop long processes in culture, whereas olfactory neurons, which normally express abundant Hap1, can still grow in culture in the absence of Hap1 (Supplemental Figure 11). Thus, we cultured olfactory neurons from WT mice and *Hap1*-null mice to examine the influence of Hap1 on TrkB transport. Consistent with the reduced association of kinesin with TrkB in the absence of Hap1, loss of Hap1 reduces the anterograde, but not retrograde, transport of TrkB in cultured primary neuronal cells (Figure 7, E and F). Taken together, our findings suggest that Hap1 stabilizes the association of TrkB with sortilin to prevent its lysosomal degradation, leading to enhanced anterograde transport and intracellular signaling of TrkB.

Discussion

Although neurogenesis occurs throughout life in vertebrates, this important process plays different roles during early development and adulthood. Embryonic neurogenesis defines the neuronal architecture, subtypes of neurons, and brain patterning, whereas adult neurogenesis, which is restricted to a few small brain regions, largely affects the function and plasticity of existing neuronal circuitry to regulate learning, memory, and mood (39, 40). In the postnatal stage, major changes in the connectivity and organization of neural networks take place, and brain maturation is particularly sensitive to external stimuli (41–43). However, little is known about the role of neurogenesis in postnatal brain development. Our studies demonstrate that Hap1 plays an important role in postnatal neurogenesis by maintaining the intracellular sorting of TrkB and its anterograde transport, providing mechanistic insight into postnatal neurogenesis and development.

The critical role of Hap1 in postnatal growth is supported by its developmentally regulated expression, which peaks during early postnatal days. Whether Hap1 plays a similar role in postnatal

and adult animals has been an unresolved question. Understanding this issue is important for elucidating the function of Hap1 in animal development, and also in other pathological conditions. For example, Hap1 was originally found to interact with htt, the HD protein (6) that is essential for early embryonic development (44). Later studies showed that mutant htt could affect the intracellular trafficking of various cargos via its avid binding to Hap1 (14, 45–50). However, the pathological features of HD are age dependent and are characterized by progressive neurodegeneration, suggesting that a gain of toxic function plays a predominant role in HD pathology. Such a gain of toxic function may also affect the normal function of Hap1. For example, the hypothalamic dysfunction and metabolic abnormalities were seen in HD transgenic mice that express mutant htt specifically in the hypothalamus (51). However, after inducing Hap1 depletion in mice via TM injection, we saw none of the typical HD symptoms in adult mice. Because mutant htt affects intracellular trafficking via its abnormal interaction with Hap1 (14, 45–50), the loss of Hap1 in the absence of mutant htt may be unable to mimic the pathological changes in HD. Rather, the consequences of the loss of Hap1 are more likely to reveal the fundamental function of Hap1. Hap1 also binds tightly to Ahi1, which is found to be involved in ciliogenesis (13). In fact, ciliogenesis has been shown to be regulated by a protein complex consisting of htt, Hap1, and PCM1 (48); therefore, the loss of Hap1 may have profound implications for a number of ciliopathies. The regulatory role of Hap1 in ciliogenesis also supports the role of Hap1 in early brain development. Ciliogenesis is important for the building of the cell's antenna or extracellular fluid mediation. In our study, we did not examine whether a ciliogenesis defect is involved in hypothalamic neurogenesis. Since Hap1 is abundant in the cell bodies of hypothalamic neurons and since TrkB acts intracellularly, our findings support the idea that Hap1-associated intracellular transport of growth factor receptors is important for neurogenesis.

The discovery from our studies is that loss of Hap1 selectively inhibits the growth of postnatal mice, but not adult mice. Although germline *Hap1*-KO mice (2, 3) and nestin-Cre *Hap1*-KO mice could not survive into adulthood, Dragatsis et al. found that reducing the litter size could enable a very few *Hap1*-null mice to escape the early postnatal lethality, though these mice still display growth retardation (52). This finding suggests that genetic background and environmental factors also influence the postnatal function of Hap1. However, adult mice with Hap1 deficiency induced by TM do not display obvious defects in feeding and growth. This fact also indicates that the vital role of Hap1 in postnatal neurogenesis is critical for early development. In addition, because Hap1 is present in endocrine cells (53), a systemic reduction in Hap1 in adult mice might lead to some compensatory effects. Similarly, systemically knocking out NPYYR1 causes obesity (54), though intracerebroventricular administration of NPYYR1 antagonist inhibits rodent feeding behavior (25, 55).

The role of Hap1 in early brain development is also supported by its formation of a stable protein complex with Ahi1, a protein whose depletion causes the brain developmental disorder Joubert syndrome (15, 16). Because Hap1 is more restricted to neuronal cells, it may possess a distinguished role or function in neuronal cells, even though it forms a stable complex with Ahi1. In support of this idea, we found that Hap1 specifically regulates the proliferation and differentiation of NPCs into neuronal cells, but not glial cells, in early postnatal developing mouse brains. The

loss of hypothalamic neurons containing NPYY1 receptors may critically contribute to the phenotypes of *Hap1*-null mice, because these neurons are important for normal feeding, metabolism, and growth via NPYY1R signaling, which can regulate energy balance and feeding behavior (25, 55–57). The selective effect of Hap1 on the neurogenesis of NPYY1R-containing neurons in the hypothalamus may be related to its differentiated expression in different types of developing neurons. Evidence has emerged that Hap1 can stabilize internalized membrane receptors (10–13, 58), but how this function is related to early development remains unclear. In the current study, we provide new mechanistic insight into the specific functions of Hap1 on TrkB, suggesting that Hap1 associates with sortilin to prevent the lysosomal degradation of TrkB and to promote its anterograde transport. Sortilin is an intracellular transport protein for neurotrophins and their receptors; it also associates with other proteins and mediates multiple functions, including targeting receptors to the lysosome or transporting them to different compartments (36, 38, 59). Such diverse functions are likely modulated and specified by proteins that associate with the sortilin-receptor complex. Hap1 has been found to colocalize with sortilin in the cytoplasmic stigmoid bodies in adult hypothalamic neurons (60), and our findings suggest that association of Hap1 with the sortilin-TrkB complex plays an important role in postnatal hypothalamic neurogenesis by regulating TrkB level and its anterograde trafficking. Hap1 is known to interact with kinesin and dynactin p150 (14, 61–63), which are involved in anterograde and retrograde transport, respectively. We found that lack of Hap1 only affects the anterograde transport of TrkB in cultured neuronal cells. Because Hap1 is also found to associate with sortilin, the anterograde transport of TrkB is likely dependent on the protein complex consisting of Hap1 and sortilin while its retrograde transport may require different proteins to target to retrograde transporters. In addition, the role of Hap1 might be different depending on the cell types and brain regions. Whether the same regulatory roles of Hap1 in intracellular trafficking seen in olfactory neurons also exist in hypothalamic neurons remains to be investigated. Given that Hap1 is abundant in hypothalamic neurons and BDNF/TrkB signaling is important for neurogenesis, it is possible that loss of Hap1 may particularly affect TrkB signaling in NPYY1R-containing neurons during early brain development, thereby impairing their proliferation and maturation. However, whether this impaired neurogenesis directly leads to the growth retardation and death in our animal models requires further investigations.

Postnatal development is profoundly affected by environmental factors as well as neurotrophins and the neuroendocrine signals. For example, nociceptive sensory neuron loss is found to occur in BDNF-deficient mice during the postnatal period, suggesting that neurotrophins are important for developing neurons to cope with environmental stimuli (64). Neurotrophins act on their membrane receptors to trigger receptor endocytosis, and the function of endocytic receptors largely relies on their intracellular sorting, which can be degraded by the lysosomes, recycled to the plasma membrane, or transported to different cellular compartments to elicit distinct signaling and function. Our findings suggest that intracellular sorting of neurotrophin receptor is important for postnatal neurogenesis, a mechanism that is different from that for embryonic neurogenesis, which is largely mediated by transcription factors and secreted signaling molecules (41). Recent studies revealed that both genetic and environmental risk factors

for schizophrenia disturb not only embryonic, but also postnatal neurogenesis, possibly contributing to neurochemical alterations associated with schizophrenia (65). Since pharmacological manipulation of Trk signaling is possible, our studies indicate that intracellular sorting neurotrophin receptors could make a therapeutic target for those neurological disorders that are caused by defective postnatal neurogenesis.

Methods

Animals. Mice were housed in the Division of Animal Resources at Emory University on a 12-hour light/12-hour dark cycle. Germline *Hap1*-KO mice were generated in our early study (3). Generation of conditional *Hap1*-KO mice, in which exon 1 of the mouse *Hap1* gene is flanked by 2 loxP sites to allow the Cre-mediated deletion of exon 1, was described in our recent study (66). Transgenic mice expressing Cre under the control of the rat neuronal nestin promoter (B6.Cg[SJL]-Tg[Nes-cre]1Kln/J) were obtained from The Jackson Laboratory. Conditional *Hap1*-KO mice were generated by crossing the floxed Hap1 mice with Cre-ER transgenic mice (B6.Cg-Tg[CAG-cre/Esr1]5Amc/J; The Jackson Laboratory), which have a TM-inducible Cre-mediated recombination system driven by the chicken β actin promoter/enhancer coupled with the CMV immediate-early enhancer. Restricted to the cytoplasm, the Cre/Esr1 (Cre-ER) protein can only gain access to the nuclear compartment after exposure to TM.

TM induction in mice. TM (T5648; Sigma-Aldrich) was first dissolved in 100% ethanol as stock solution (20 mg/ml) and stored at –20°C before use. On the day of induction, a calculated amount of TM was mixed with corn oil, and ethanol was removed by Vacufuge plus (Eppendorf). To induce *Hap1*-KO in mice, P1 pups were injected s.c. with 1.1 mg TM per 40 g body weight for 3 consecutive days. Mice at P15 or older were i.p. injected with 4 mg TM per 40 g body weight for 5 consecutive days. Genotyping of these mice was performed with genomic DNA extracted from the tails; we used PCR to amplify the mouse Hap1 DNA fragment (from 4929 nt to 5003 nt) using forward (5′-TTTTTCTGGGGAGCATACGTC-3′) and reverse (5′-ATCCGTTATCCCAGGGTCTGA-3′) primers. Primers (forward: 5′- GCG-GTC GGCAGTAAAAACTATC-3' and reverse: 5′-TGTTTCACTATCCAG-GTTACGG-3′) that amplify Cre recombinase were also used to determine the presence of Cre.

Neurosphere proliferation, identification, and differentiation. Neurospheres were isolated and cultured from the brain tissues from the specified E11 mice as previously described (22, 23). Briefly, cultured neurospheres at passage 2 were first dissociated using trypsin-EDTA and a mechanical triturating by a fire-polished Pasteur pipette. Single-cell suspensions were collected after being filtered through a 60-μm mesh. Viable cells were assessed using Trypan blue exclusion. Subsequently, cells were plated in 24-well plates coated with PDL or uncoated at equal density (1×10^4/ml) and cultured for 24 hours before examination. Cells plated into the uncoated well were continuously maintained for different days.

For investigation of the differentiation of NSCs, the neurospheres were seeded on PDL-coated glass coverslips at 20 neurospheres/cm² in DMEM containing 2% FCS and 4 μM retinoic acid. The cultures for NSC differentiation were maintained for 10 different durations (5, 7, 9, 10, 13, 15, 17, 19, 21, and 23 days). At each indicated time point, morphologies of cultured neurospheres were observed under a phase-contrast microscope (Axiovert 200 M). Characterization of the plasticity of these NSCs was identified by immunocytochemistry with anti–β-tubulin III and anti-GFAP.

BrdU incorporation assay. For the in vitro study of NSC proliferation, BrdU (5 μM) was added to the culture medium for 18 hours. After removal of the supernatant, cells were fixed with 4% paraformaldehyde and treated with 2 N hydrochloric acid for 30 minutes at 37°C to denature DNA for further immunostaining.

For BrdU injection into P1 *Hap1*-null mice and controls, BrdU (50 mg/kg body weight) was prepared in saline and injected s.c. (3 mice per group). Two hours later, mice were perfused and fixed as described in Supplemental Methods. For BrdU injection into *Hap1* P1 KO mice and controls, mice at P6 were i.p. injected with 50 mg/kg body weight BrdU (3 mice per group). 24 hours later, animals were perfused and fixed as above. For BrdU injection into *Hap1* adult KO mice and controls, 6-month-old mice that had been injected with TM at 2 month of age were i.p. injected with 50 mg/kg body weight BrdU twice a day for 3 days (3 mice per group). 24 hours after the final injection, mice were perfused and fixed as above.

BDNF treatment. Surgical procedures were conducted as previously described with modifications (67). In brief, P1 mouse pups were anesthetized on wet ice for 3 minutes before being secured to a surgical platform. 1 μl of human recombinant BDNF (5 mg/ml in sterile PBS) was injected into the third ventricle (at the midline coordinates of 0.5 mm rostral to the lambda and 3.0 mm below the skull, as determined in pilot experiments) over 2 minutes from a 5 μl Hamilton syringe. The needle was kept still for another 2 minutes before withdrawal. The pups were then left to recover under a heat lamp and returned to the mother. Two hours after BDNF treatment, BrdU was injected s.c., and the pups were perfused 2 hours later for neurogenesis analysis.

Stereology and quantification. We examined at least 3 control mice and 3 *Hap1*-KO mice for each comparison. To quantify BrdU-positive cells, the optical-fractionator method was used, as implemented in the semi-automatic stereology system StereoInvestigator 5.4.3 (MicroBrightField). Target brain regions were cut in 40-μm serial sections, every fifth section of which was used for analysis. BrdU-positive cells were counted on each section. StereoInvestigator software was used to calculate the volume of the target region based on the traced target area for each section and the distance between sections sampled. The total number of BrdU-positive cells in the target region calculated by the software was then divided by the volume to yield BrdU-positive cell density presented as number of BrdU-positive cells in a cubic mm of the target region. Quantification of cell number within the different brain regions was performed at ×40 using a Zeiss AX10 microscope by an observer blind to experimental groups.

Quantification of cultured cells was performed with a Zeiss (Axiovert 200 M) inverted phase-contrast microscope (×40). For neurosphere production, all neurospheres were counted under inverted phase-contrast microscopic observation at ×20 magnification, and the observation fields of the culture dishes or plates were chosen randomly. The examination was conducted according to the same criteria used for counting: the same amplification, the same batch cell, the same number of fields, and the same number of cultures. Each dish or well of a plate was analyzed by counting 15 randomly chosen fields.

Quantification of BrdU-positive cells, GFAP, and β-tubulin III double-staining, or Hap1 and β-tubulin III double-staining was performed via the optical fractionator method, as described above by an observer blind to experimental group. The images were taken under a fluorescence microscope with a Zeiss digital camera controlled by Openlab software (Improvision) at the same setting. β-tubulin III–positive cells with long neurites (over twice the body size) were counted and calculated.

FRAP. Olfactory bulb neurons were transfected with 1 μg TrkB-GFP constructs at DIV4. Eighteen hours after transfection, the glass coverslip was transferred in a chamber of Nikon A1R confocal microscope, which kept cells at 37°C, 5% CO_2. For visualizing GFP signal, images were acquired utilizing a ×63 oil immersion objective lens, and 488 nm laser. For studying anterograde trafficking of TrkB, fluorescence in neuritic tips was photobleached. For retrograde study, somatic fluorescence was photobleached. Full-power 488 nm laser was used to photobleach the targets. After photobleaching, images were collected every 5 seconds over a

period of 10 minutes. Fluorescent intensity in photobleached regions was quantified with Nikon Element Software.

Statistics. All data are expressed as mean ± SD or SEM. The statistical significance was determined using 1-way ANOVA between groups with SPSS 10.0 software. $P < 0.05$ was considered statistically significant.

Study approval. All procedures and husbandry were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academies Press. 8th edition. Revised 2011). All animal studies were approved by the IACUC of Emory University.

Address correspondence to: Xiao-Jiang Li or Shi-Hua Li, Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Atlanta, Georgia 30322, USA. Phone: 404.727.3290; Fax: 404.727.3949; E-mail: xli2@emory.edu (X.J. Li), sli@emory.edu (S.H. Li).

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