Supplemental information

Huntingtin-Associated Protein 1 is essential for postnatal growth by regulating neurogenesis

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, 615 Michael Street, Atlanta, GA 30322, USA.

[2]The Institute of Neuroscience, Soochow University, 199 Ren-Ai Road, Suzhou City, Jiangsu 215123, China

[3]School of Pharmacy and Medical Sciences, Faculty of Health Sciences, Sansom Institute, University of South Australia, Adelaide, SA 5000, Australia

[4]State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.

Correspondence should be addressed to: Xiao-Jiang Li or Shi-Hua Li, Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322. Phone: (404) 727-3290; Fax: (404) 727-3949; E-mail: xli2@emory.edu or sli@emory.edu.

* The first two authors contributed equally to this work.

Short title: Hap1 in postnatal neurogenesis and development

Supplemental Methods and Materials

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), D/F12 and Neurobasal with high glucose, B-27 supplement, N-2, D-Hank's, HEPES, collagenase, and fetal calf serum (FCS) were obtained from Invitrogen. Penicillin G, streptomycin, glutamine, trypsin, poly-D-lysine, papain, EDTA, basic bFGF, EGF, retinoic acid (RA), laminin, and BSA, BrdU, lactacystin, leupeptin and pepstatin A were from Sigma. Human recombinant BDNF was obtained from Rockland Immunochemicals. Cell culture dishes, coverslips, plates, and flasks were purchased from Corning and Nunc, Inc. Fluor 594-conjugated goat anti-guinea pig, Fluor 594-conjugated goat anti-rabbit, and Fluor 488-conjugated donkey anti-mouse IgG were purchased from Invitrogen and Molecular Probes. Guinea pig antibody (EM77) to Hap1 was generated in our laboratory (Li et al., 1996; 2003). Antibodies against nestin and GFAP (Chemicon), β-tubulin III (sigma), neuronal-specific nuclear protein (NeuN, Millipore), sox2 (Cell signaling), Ki67 (Thermo Fisher Scientific), DCX (Santa Cruz, CA), BrdU (Sigma and Accurate Chemical & Scientific), MBP (Chemicon), Gapdh (Millipore), TrkB (BD Biosciences), sortilin (Abcam), KHC (Millipore), and alpha-tubulin (Santa Cruz, CA) were used in Western blotting and immunocytochemistry.

Primary neuronal culture

Primary cultures from the hypothalamus of E18 mouse embryos were prepared as previously described (Sheng et al., 2008). The hypothalamus was dissected in a sterile

35-mm petri dish containing ice-cold Hanks' balanced salt solution (HBSS, Ca^{2+} -and Mg^{2+}-free), chopped into 1-mm^3 pieces by microscissors, and digested with 2 ml 0.125% (w/v) trypsin and 0.05% collagenase at 37°C for 25 min. After the activity of enzymes was terminated by transferring tissue pieces into 10 ml DMEM-supplemented 200 U/ml DNaseI and 20% heat-inactivated FCS, the tissue was subsequently dissociated by triturating mechanically 10 times through a fire-polished Pasteur pipette. The cell suspension was filtered through a 60-μm metal mesh, spun down, and washed twice with culture medium. The cell pellet was resuspended in DMEM containing 10% FCS culture medium, diluted to an approximated plating density of 2×10^5 cells/cm^2 with neurobasal medium containing 2% B-27, and plated into either 24-well plates or coverslips coated with PDL and laminin. Finally, cultures were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2. To enhance the growth of neuronal cells, astrocyte-conditioned medium was added to the cultures on day 2 after plating.

For in vitro BDNF rescue assay, primary hypothalamic neurons from WT or Hap1-null mice were cultured as above with or without the addition of recombinant human BDNF (20 ng/ml) in the culture medium. Cells were cultured for 5-6 days in vitro before fixation and immunostaining.

Neural stem cell culture expansion

Neurospheres were isolated and cultured from the brain tissues from the specified embryonic day 11 mice as previously described (Tropepe et al 1999; Brewer and Torricelli, 2007). Briefly, dissections from WT, Het, or Hap1 KO mice were pooled in D-Hank's solution, washed, chopped into 1 mm^3 and then transferred to media containing

0.125% (w/v) trypsin and 0.001% (v/v) DNaseI for 15 min at 37°C. After enzymatic dissociation, the tissues were washed in D-Hank's and subsequently transferred to media containing 0.7 mg/ml trypsin inhibitor, mechanically dissociated into single cells, and plated in uncoated 35-mm dishes at equal density (1×10^4/ml). Neurosphere cultures were maintained in DMEM/F12 (1:1) medium supplemented with 2% N2, 25 ng/ml bFGF, 20 ng/nl EGF, 2 mM glutamine, and 2 mg/ml heparin in an incubator at 37°C in a humidified atmosphere of 5% CO_2. Half of the culture medium was replaced twice a week. Five to seven days after plating, floating cell spheres were collected and passaged. Secondary spheres following 5-7 passages were counted for assessment of the self-renewal, neurosphere formation, and multi-potential capacity of neural stem cells.

Immunofluorescence and microscopy

Cultured cells were fixed in 4% paraformaldehyde in 0.01 MPBS for 15 min at room temperature, permeabilized and blocked with 0.1% Triton X-100/3% BSA /3% normal donkey (or goat) serum/PBS for 1 h, and incubated with primary antibodies to Hap1, nestin, or β tubulin-III in 3% BSA/PBS overnight at 4° C. Cells were washed 3 times with PBS and then incubated with species-specific fluorophore-conjugated secondary antibodies (Alexa 488- or 594-conjugated) and nuclear dye Hoechst diluted in 0.01 MPBS for 1 h at room temperature.

Immunofluorescent staining of brain sections was performed using the method described previously (Sheng et al., 2008; Xu et al., 2010). Briefly, mice were deeply anesthetized, perfused with 4% paraformaldehyde, postfixed for an additional 10 h in the same fixative, and switched to 30% sucrose at 4°C. After completely sank, brains were sectioned at 15 μm (40 μm for BrdU staining) with a cryostat at −19°C and mounted

onto gelatin-coated slides. The tissues on slides were washed and blocked with a buffer containing 3% bovine serum albumin and PBST (0.2% Triton X-100 in PBS) for 1 h at room temperature. Primary guinea pig antibody against Hap1 and goat antibody against β-tubulin III were incubated with the tissue at 4°C overnight, followed by incubation with Alexa 488- or rhodamine-conjugated secondary antibodies and nuclear Hoechst dye. For BrdU and GFAP double immunostaining, sections were first treated with 2 N HCl for 30 min at 37°C and then neutralized with 0.1 M sodium borate (pH 8.5) for 15 min at room temperature. The brain sections were examined using a Zeiss (Axiovert 200M, Germany) microscope with a digital camera (Orca-100; Hamamatsu Photonics, Bridgewater, NJ) and the Openlab software (Improvision, Lexington, MA). Brain sections of WT and Hap1 KO mice containing approximately the same brain regions were compared for neuronal or other staining.

Northern blotting and Western blotting

Northern blotting was performed using the embryonic, postnatal, and adult brain tissues from WT mice. The blots were probed with a HAP1 cDNA probe encoding 278-445 amino acids of HAP1 and also probed with a cDNA probe for Gapdh. For Western blotting of cultured cells, the cells were extracted in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors. Mouse brain tissues were homogenized and then extracted with the lysis buffer. The extracts were subjected to SDS-PAGE. The nitrocellulose membranes containing transferred proteins were blocked with 5% non-fat dry milk/PBS for 1 h at room temperature and incubated with primary antibodies in 3% BSA/PBS overnight at 4°C. Secondary antibodies conjugated with HRP were incubated with the blot in 5% milk/PBS for 1 h at room temperature. ECL-plus (GE Healthcare) was

then used to reveal immunoreactive bands on the blots. Hypothalamus of adult P1 Hap1 KO mice and littermate controls were isolated using Lysosome Isolation Kit (Sigma Aldrich), samples from total and lysosome fractions were then loaded for western blot analysis, in which TrkB, Hap1 and Lamp1 were probed. 6 mice per group were used for the quantification.

TrkB half-life assay

HEK 293 cells were transfected with TrkB-GFP and either PRK-Hap1A or a control vector. 24 hours after transfection, cells were treated with BDNF (100 ng/ml) or DMSO, together with cycloheximide (20ug/ml) to inhibit protein synthesis and induce TrkB internalization. Cells were then collected after the indicated times and lysed for western blot analysis.

For live-cell imaging, PC12 cells were transfected and treated the same way as in HEK 293 cells. Images of TrkB-GFP transfected cells from each group were taken every 10 minutes for a total of 3 hours by Zeiss Observer A1 microscope coupled with XLmulti SI incubator, and GFP fluorescence was quantified for at least 8 cells per group using AxioVision Rel. 4.8 software.

For inhibitor studies, 293 cells were transfected for 24 hours Cells were then serum starved, pre-treated with lactacystin (10 μM), leupeptin/pepstatin (10 μg/ml each) or DMSO for 30 min, and then incubated with medium containing BDNF, cycloheximide, and respective inhibitors for 3 hours before being collected.

Immunoprecipitation

For Immunoprecipitation of sortilin in 293 cells, cells were grown in 10-cm plates and were co-transfected with TrkB-GFP and either PRK-Hap1A or a control vector. 24 hours after transfection, cells were treated with BDNF (100ng/ml) for 15 min and then lysed with 1 ml NP40 buffer (50mM Tris pH7.4, 50mM NaCl, 0.1% Triton X-100, 0.3% NP40) containing Halt protease inhibitor cocktail (Thermol Scientific). The lysate was centrifuged at 15,000 rpm at 4°C for 5 minutes, and 250 ul of the supernatant from each sample was used for immunoprecipitation. Samples were adjusted to 500 ul in volume and preabsorbed by 50 ul protein A agarose beads (Sigma-Aldrich) at 4°C for 2 hours with gentle rocking. Supernatants were then collected and incubated with anti-sortilin antibody (Abcam) at 4 °C overnight. Next, 15 ul of protein A beads were added for an additional hour to pull down the endogenous sortilin and associated proteins. Beads were spun down at 2000xg at room temperature for 30s and were washed 3 times with lysis buffer. After final wash, SDS loading buffer was added to the samples and the immunoprecipitated proteins were boiled and resolved by electrophoresis for western blot analysis. For immunoprecipitation of Trk in mouse brain, hypothalamic tissues from P1 Hap1 KO and control mice were collected and homogenized with NP40 buffer. Brain lysate was used for immunoprecipitation as described above. Anti-Trk (C14, Santa Cruz) was used in this study.

Supplemental figure legends

Figure S1. Body weight and food intake of adult Hap1 KO mice. (**A**) Western blot analysis of expression of Hap1 in the brains of WT, floxed Hap1 (loxp/loxp, control) mice and Hap1 KO mice in which Cre expression is induced by tamoxifen injection. (**B**) Body

weight (g) of adult Hap1 KO (n=10) and control (n=10) mice that were injected with tamoxifen at the age of 3 months. (**C**) Food intake (g/d/g body weight) of adult Hap1 KO and control mice.

Figure S2. Lack of Hap1 does not affect astrocyte differentiation but neurogenesis in the mouse neurospheres. Cultured mouse neurospheres following differentiation at 7 days of culture were stained by antibodies to the neuronal protein β-tubulin III and glial protein GFAP. No significant difference in GFAP staining was seen between Hap1-null (KO) neurospheres and WT mice despite the decreased number of β-tubulin III-positive cells in Hap1-null neurospheres. Scale bars: 20 μm.

Figure S3. Lack of Hap1 decreases the proliferation of neurons in mouse neurospheres. (A) BrdU incorporations were measured in the WT, Het, and Hap1 KO mouse neurosphere cells, nestin (green) served as marker for neural stem cells (upper panel). (**B**) Quantifications of BrdU positive cells in the images from (A) (n=15 images per group, lower panel). Note that there are more BrdU positive cells in WT and Het groups than in the KO group. * $P<0.05$. Error bars represent SD. (**C**) Western blot of the mouse neurospheres showing that loss of Hap1 reduces the level of Ki67 (a marker for proliferating cells) and DCX (a marker for neuroblasts) in Hap1-null (KO) neurospheres**.** Scale bar: 15 μm.

Figure S4. Lack of Hap1 does not affect neurogenesis in embryonic mouse brain. (A) Western blot analysis of E14.5 whole brain and E18 hypothalamic tissues from WT

and Hap1-null (KO) mouse fetuses. (B) Immunofluorescent staining of E18 hypothalamus of WT and KO mouse fetuses. Immunostaining with antibody to DCX (green) and nuclear staining (blue) are shown in the merged images. Scale bar: 10 μm.

Figure S5. Lack of Hap1 reduces the number of cultured neurons from the brains of Hap1-null mice. Hypothalamic neurons cultured from WT and Hap1 null (KO) mouse embryos (E18-19). The neurons were stained with antibodies to Hap1 and β-tubulin III (upper panel) and counted for the percentage of neuronal cells (lower panel). * $P<0.05$ (n=15). Scale bar: 10 μm. Error bars represent SD.

Figure S6. Loss of Hap1 in adult mice does not reduce neurons expressing NPYY1R in the hypothalamus. (**A**) Immunostaining of the hypothalamus of Hap1 adult KO and control mice with antibodies to NeuN and NPYY1R. (**B**) Western blot analysis of hypothalamic tissues from P1 KO mice at 15 and 30 days and the cortex and hypothalamus of adult KO mice. WT mice served as control. The blots were probed with antibodies to Hap1, NPYY1R, and NeuN. Scale bar: 10 μm.

Figure S7. **Lack of Hap1 does not affect the number of calbindin cells in the hypothalamus.** Immunostaining of hypothalamic neurons with the antibodies to NeuN and calbindin (CBD) (left panel) and counting of CBD-positive neurons (% of total cells in right panel, n=15) revealed no significant difference between WT and P1 Hap1 KO hypothalamus. Scale bar: 15 μm. Error bars represent SD.

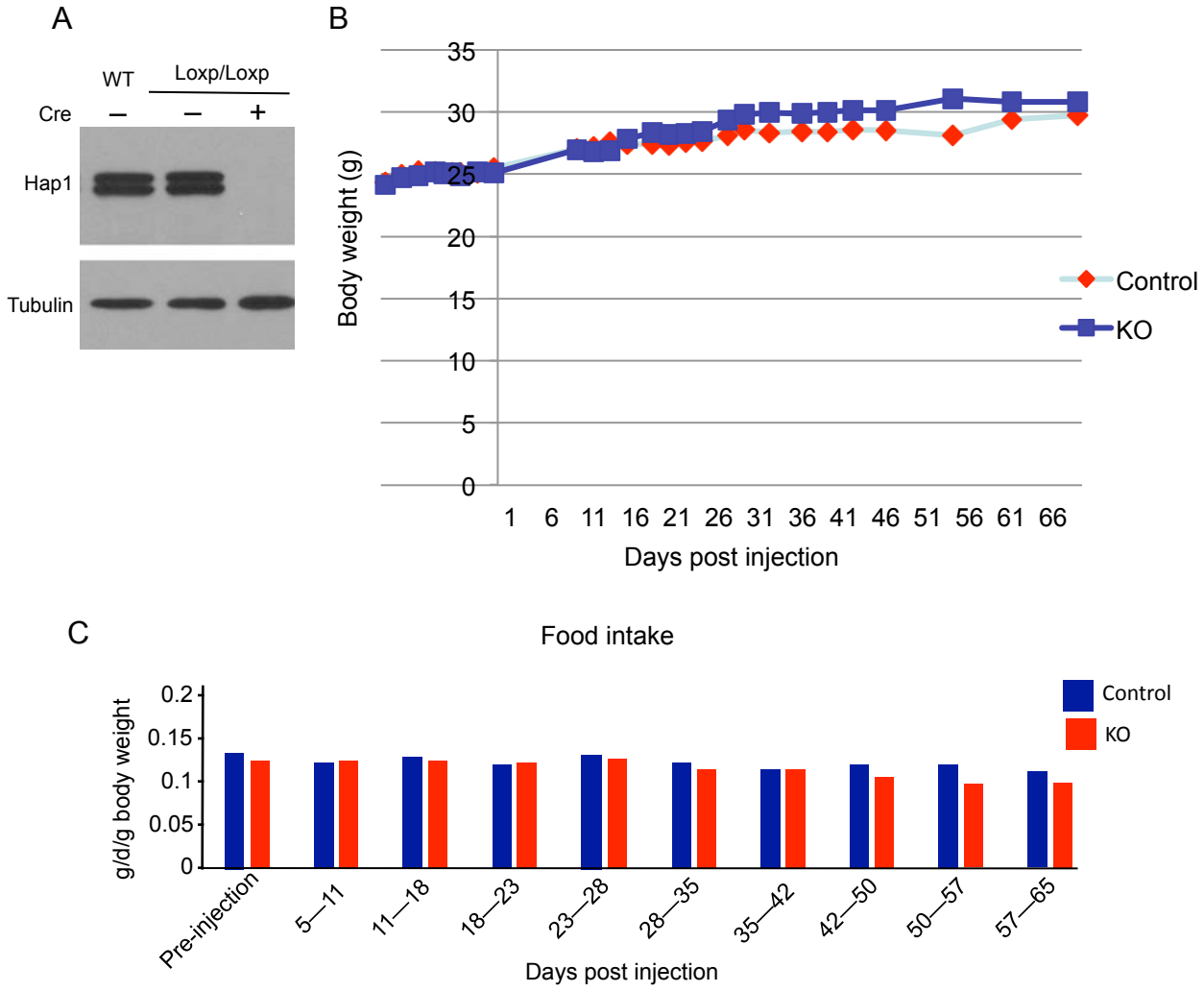
Figure S8. **BDNF increases the number of NPYY1R expressing cultured hypothalamic neurons from Hap1-null mice.** (**A**) The cultured hypothalamic neurons were treated with BDNF and stained with the antibodies to NPYY1R (red) and β-tubulin III (green, in merged images), revealing that the number of these neurons in Hap1-null (KO) hypothalamic cultures was increased by BDNF. Scale bar: 10 μm. (**B**) The number of neurons (left panel) and the relative number of NPYY1R-containing neurons (right panel, % of total cells) were counted in 15 images per group, revealing an increased number of Hap1-null (KO) neurons by BDNF treatment. * $P<0.05$. Error bars represent SD.

Figure S9. Double immunostaining of BrdU with nestin and sox2. Hypothalamic tissues of Hap1-null mice at P1, which had been treated with BDNF for 2 hours before BrdU injection, were immunostained with antibodies against BrdU (green), and NPC markers nestin or sox2 (red). Scale bar: 10 μm. Co-expression of BrdU with nestin and sox2 suggests that these cells (arrows) labeled with BrdU were NPCs.

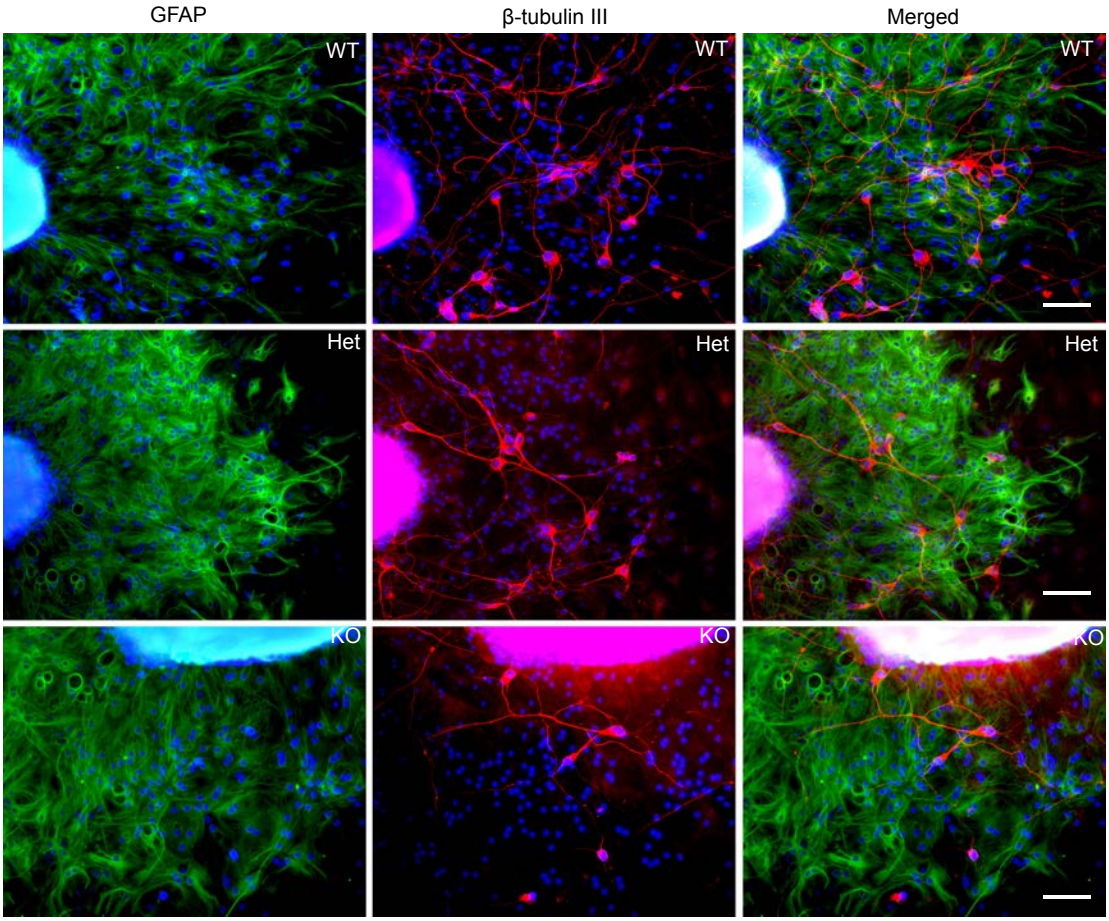
Figure S10. Co-localization of Hap1A with sortilin and TrkB. (A) HEK293 cells were transfected with TrkB-EGFP and Hap1A. The cells were stained with anti-Hap1 (red) and examined under a fluorescent scope to reveal the expression of TrkB (green), Hap1A (red), and nuclei staining (blue). Scale bar: 10 μm. (**B**) HEK293 cells were co-transfected with sortilin and Hap1A or TrkB. The transfected proteins were colocalized in intracellular

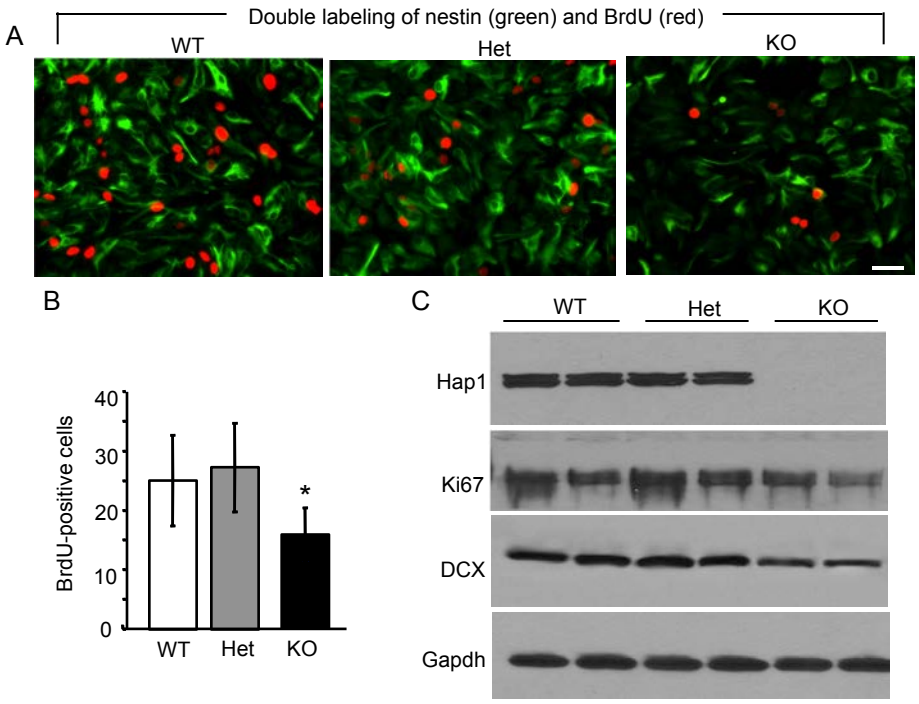
puncta formed by Hap1A or sortilin. Scale bar: 5 μm. (**C**) Colocalization of endogenous Hap1 and sortilin in the hypothalamus of mouse brain. Scale bar: 5 μm.

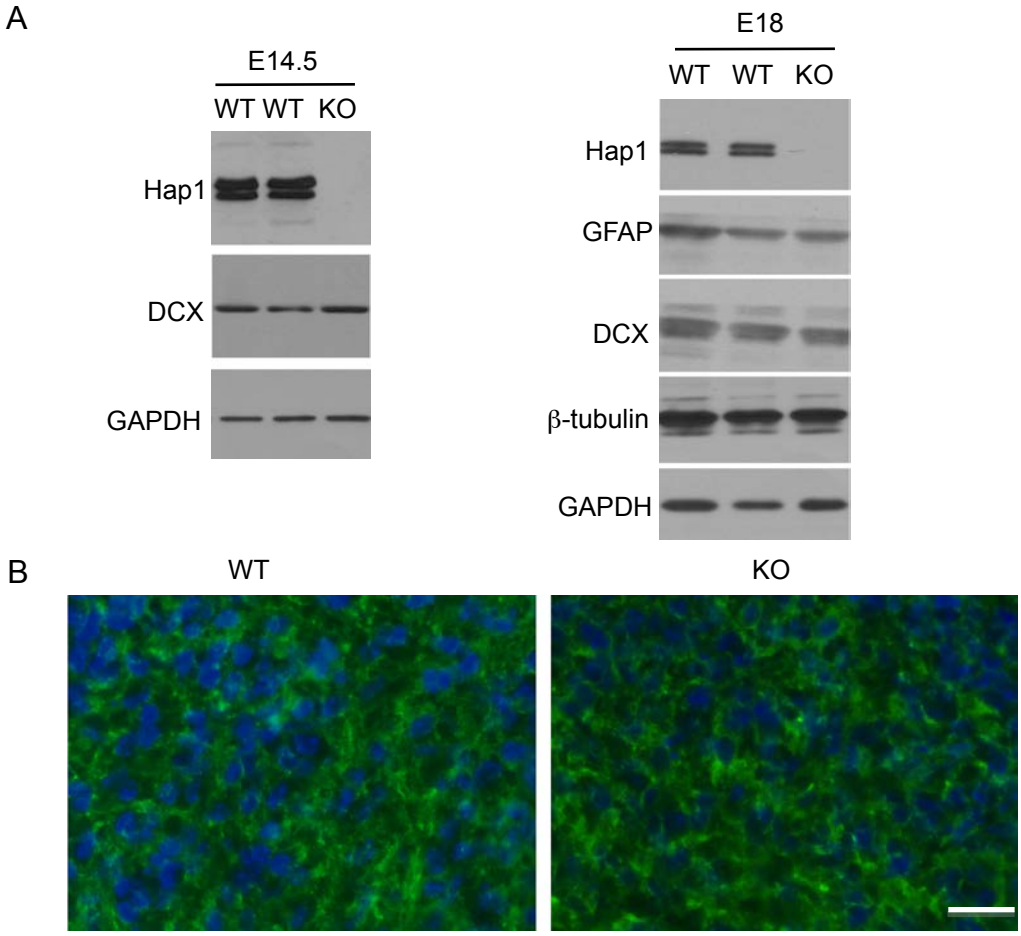
Figure S11. Expression of Hap1 in cultured olfactory neurons. Olfactory neurons from WT (upper panel) and Hap1-null (KO, lower panel) mice at DIV4 were stained with guinea pig antibody to Hap1 and rabbit antibody to kinesin heavy chain. The nucleus was stained with DAPI. Scale bar: 10 μm.

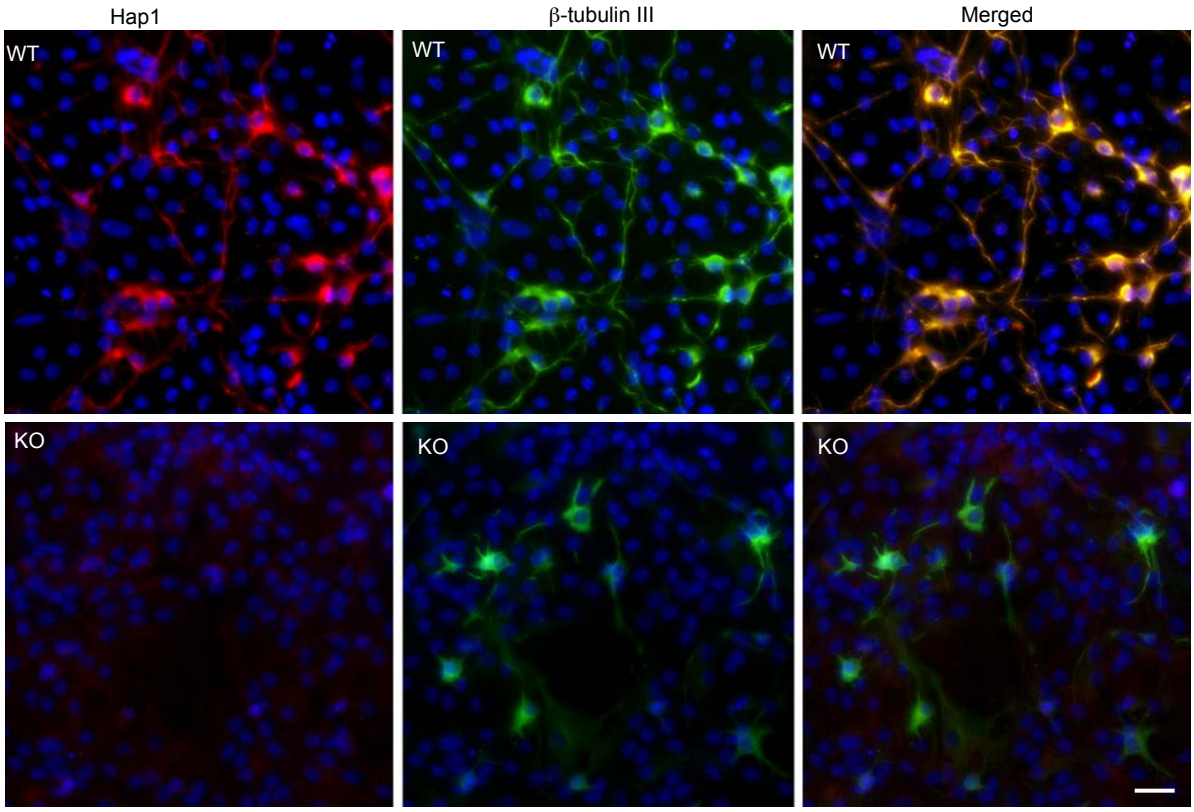

A
WT
Loxp/Loxp
Cre
–
–
+
Hap1
Tubulin
B
Body weight (g)
35
30
25
20
15
10
5
0
1 6 11 16 21 26 31 36 41 46 51 56 61 66
Days post injection
Control
KO
C
Food intake
g/d/g body weight
0.2
0.15
0.1
0.05
0
Pre-injection
5—11
11—18
18—23
23—28
28—35
35—42
42—50
50—57
57—65
Days post injection
Control
KO

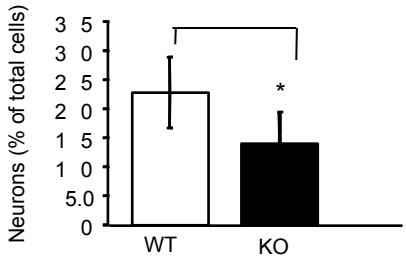
Xiang_Supplemental Fig.S2

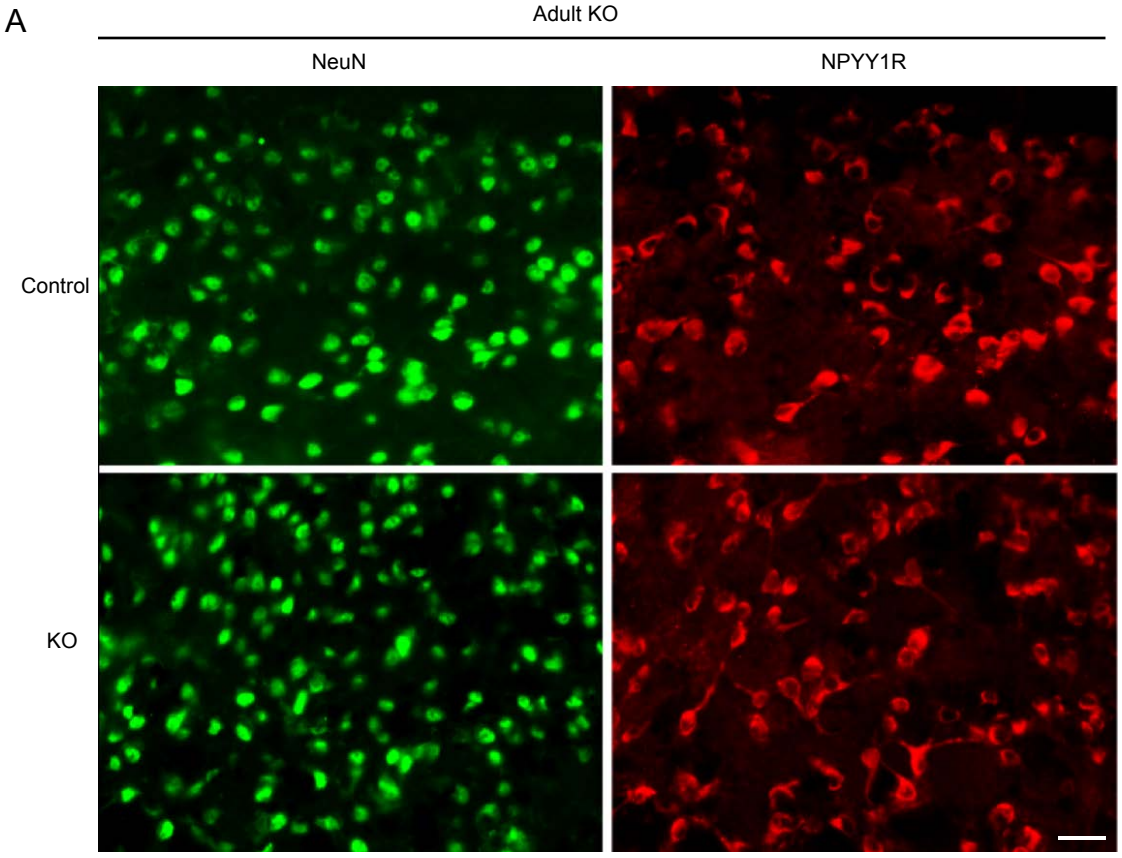



A
Double labeling of nestin (green) and BrdU (red)
WT
Het
KO
B
BrdU-positive cells
40
30
20
10
0
*
WT
Het
KO
C
WT
Het
KO
Hap1
Ki67
DCX
Gapdh

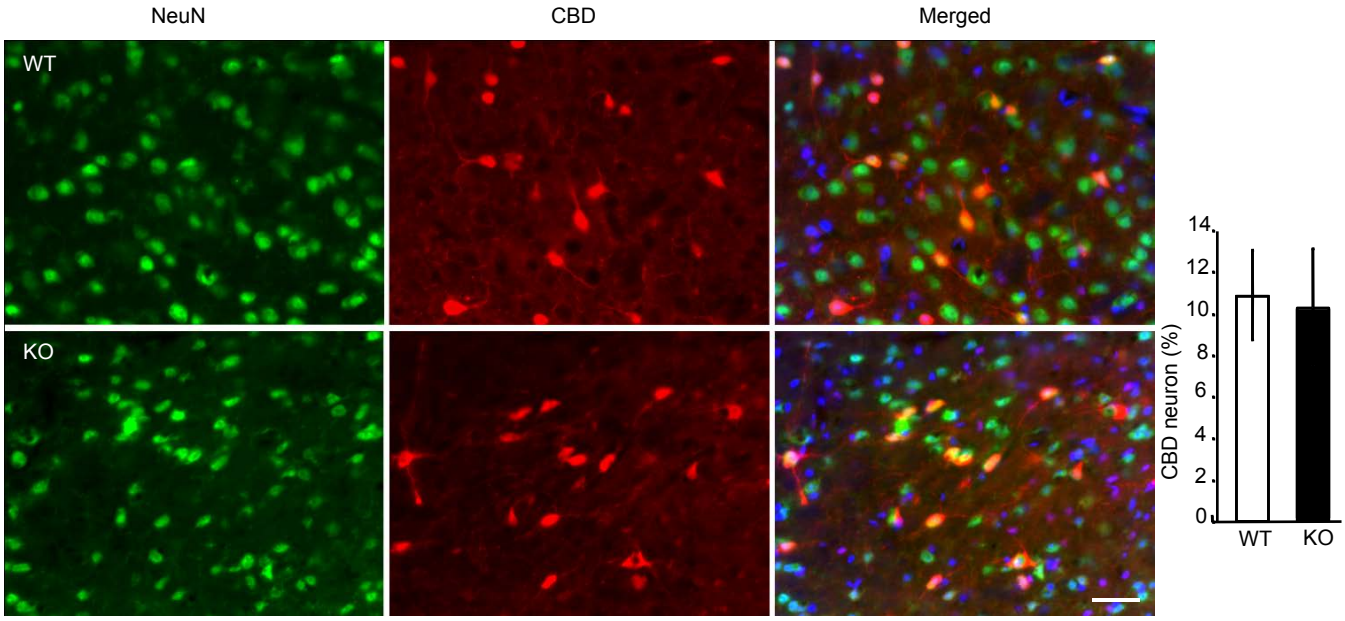

A
E14.5
WT WT KO
Hap1
DCX
GAPDH
E18
WT WT KO
Hap1
GFAP
DCX
β-tubulin
GAPDH
B
WT
KO

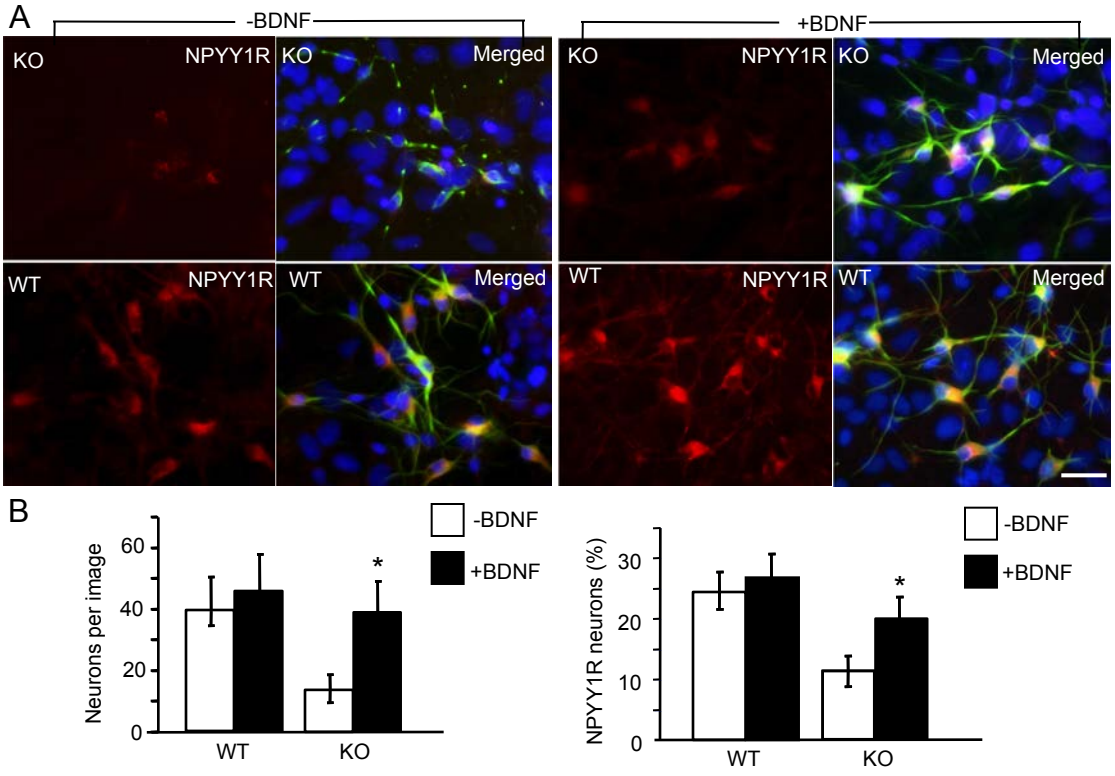

Hap1
β-tubulin III
Merged
WT
WT
WT
KO
KO
KO

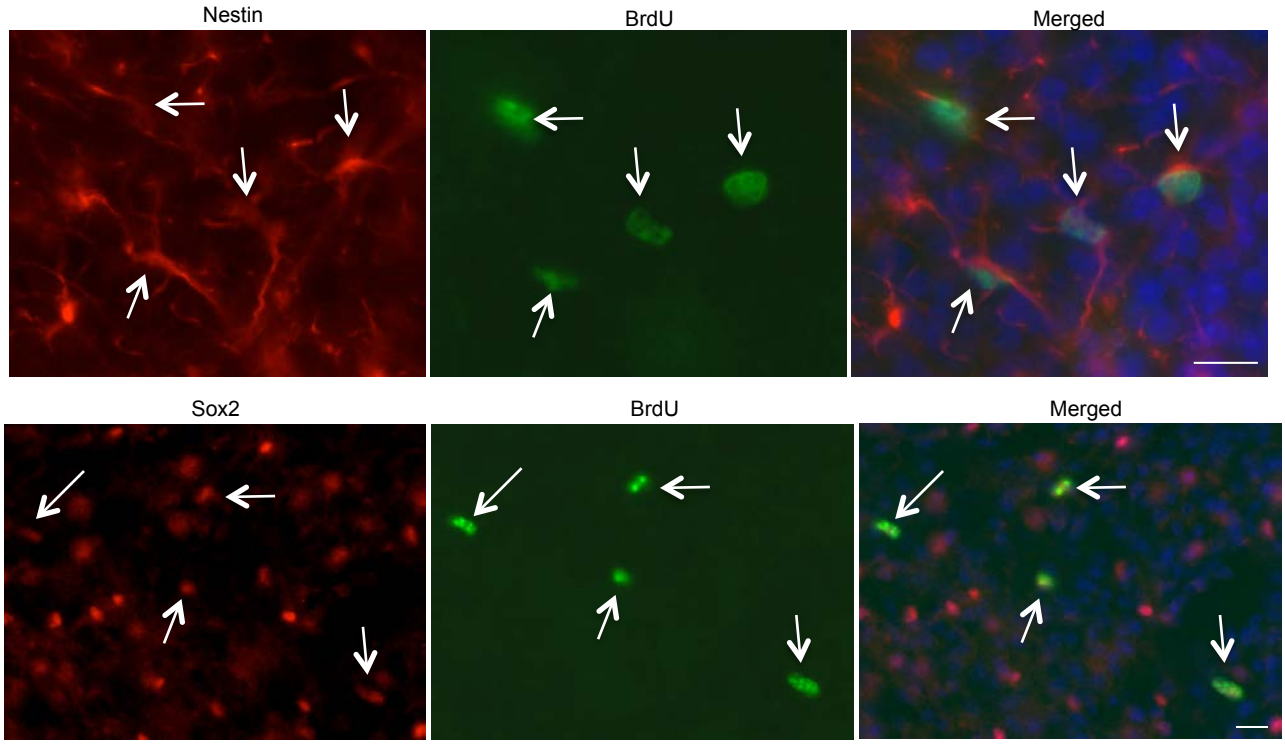

Neurons (% of total cells)
35
30
25
20
15
10
5.0
0
*
WT
KO

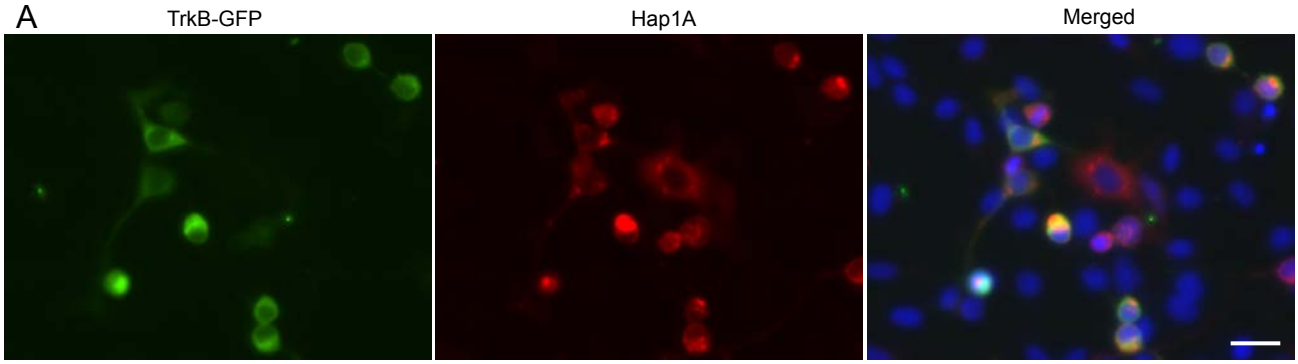

A
Adult KO
NeuN
NPYY1R
Control
KO
B
P1 KO
Adult KO
15 days
30 days
Cortex
Hypothalamus
Control
KO
Control
KO
Control
KO
Control
KO
Hap1
NPYY1R
NeuN

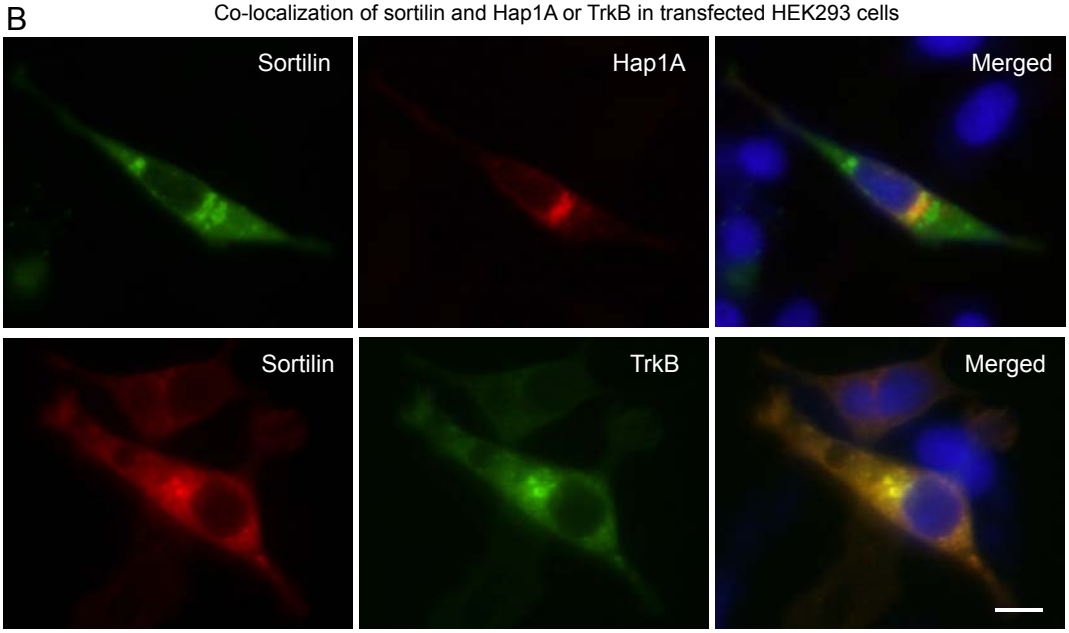
Xiang_Supplemental Fig.S7

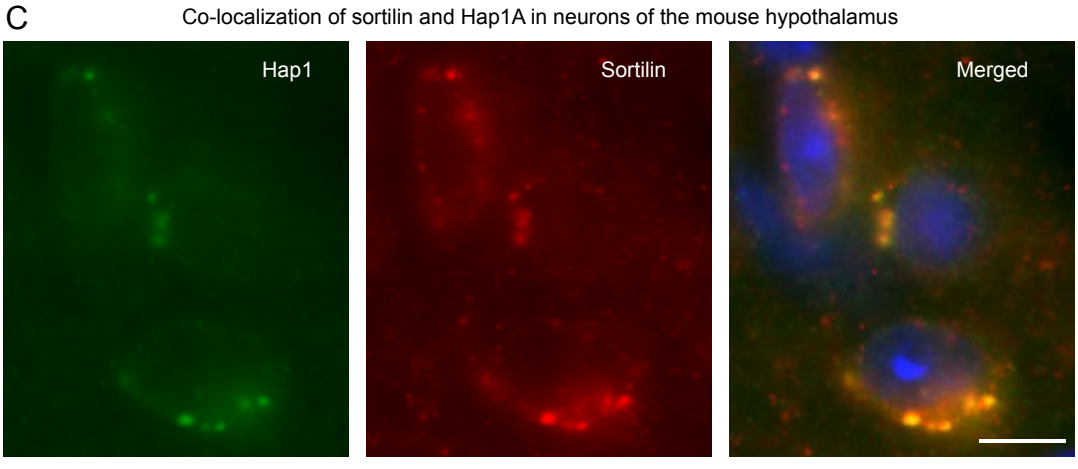


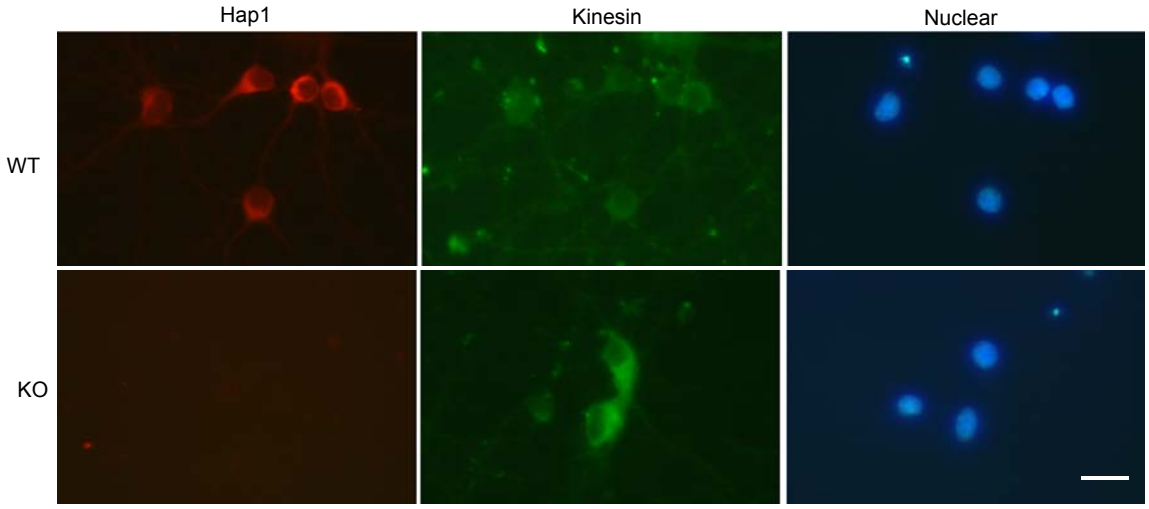

A
-BDNF
+BDNF
KO
NPYY1R
KO
Merged
WT
NPYY1R
WT
Merged
B
Neurons per image
60
40
20
0
WT
KO
-BDNF
+BDNF
*
NPYY1R neurons (%)
30
20
10
0
WT
KO
-BDNF
+BDNF
*


Nestin
BrdU
Merged
Sox2
BrdU
Merged


A
TrkB-GFP
Hap1A
Merged


B
Co-localization of sortilin and Hap1A or TrkB in transfected HEK293 cells
Sortilin
Hap1A
Merged
Sortilin
TrkB
Merged


C
Co-localization of sortilin and Hap1A in neurons of the mouse hypothalamus
Hap1
Sortilin
Merged


Hap1
Kinesin
Nuclear
WT
KO