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Jianxing Xiang Date

The role of huntingtin-associated protein 1 (Hap1) in postnatal development

By

Jianxing Xiang
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology

Xiao-Jiang Li, M.D., Ph.D.
Advisor

Guy Benian, M.D.
Committee Member

Andrew Escayg, Ph.D.
Committee Member

Victor Faundez, Ph.D.
Committee Member

Kerry Ressler, Ph.D.
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

The role of huntingtin-associated protein 1 (Hap1) in postnatal development

By

Jianxing Xiang
B.S., Zhejiang University, 2008

Advisor: Xiao-Jiang Li, M.D., Ph.D.

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Abstract

The role of huntingtin-associated protein 1 (Hap1) in postnatal development

By Jianxing Xiang

Huntingtin-associated protein 1 (Hap1) is an intriguing neuronal-enriched protein that interacts with several disease-causing proteins such as huntingtin (htt) and Ahi1, whose mutations lead to Huntington's disease (HD) and Joubert syndrome (JS), respectively. As Hap1 might be involved in the pathogenesis of both neurodegenerative and neurodevelopmental disorders, as HD and JS represent, a systematic study of the role of Hap1 at different ages could considerably help us unravel the mechanisms of a number of diseases as well as develop novel therapies. To achieve that, we generated an inducible Hap1 knockout (KO) mouse model in which Hap1 can be deleted via tamoxifen (TM) injection at different ages. By inducing Hap1 KO at early postnatal days, we observed growth retardation, a phenotype that is similar to that of germline Hap1 KO mice, and was not seen when Hap1 was depleted at postnatal day 21 (P21) or later. To look for possible mechanisms, we examined neurogenesis in the hypothalamus, a brain region critical for food intake and energy expenditure, and found that it was also impaired in early Hap1 KO mice. Moreover, tropomyosin-related kinase B (TrkB), a receptor for brain-derived neurotrophic factor (BDNF) is downregulated by the loss of Hap1, and acute treatment of BDNF in the ventricles of Hap1 KO mice rescued the impaired hypothalamic neurogenesis, indicating that BDNF/TrkB signaling, which is suppressed in the absence of Hap1, is critical for postnatal hypothalamic neurogenesis. Interestingly, early, but not late postnatal Hap1 KO mice when grown to adults exhibited depressive behavior, which was accompanied by the reduction of neurogenesis in the hippocampal dentate gyrus. Adult expressed Hap1, however, protects mice from stress induced depressive behavior by maintaining adult hippocampal neurogenesis. Together, these results reveal differential roles of Hap1 at different postnatal stages, and suggest that Hap1 is required for postnatal neurogenesis, a mechanism that may underlie the growth defect of Hap1 KO mice, and could contribute to the pathogenesis of a number of neurological diseases such as depression. The role of Hap1 in stress response could also have profound influence on the progression of certain diseases.

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Chapter 1

General Introduction

1.1 Huntingtin-associated protein 1 (Hap1)

Hap1 was initially discovered in a hunt for interacting proteins of huntingtin (htt) (Li et al., 1995). It was described as a brain enriched protein that binds to htt in a polyglutamine (polyQ) length dependent manner. Because in Huntington's disease (HD), the expansion of an N-terminal polyQ tract in htt protein triggers the disease when it reaches a critical point, and the longer the repeat length becomes, the earlier the age at onset tends to be (MacDonald et al., 1993), Hap1 was thought to be a disease modifying protein with its binding preference to mutant htt (mhtt). Another reason Hap1 might be involved in HD pathology is that although HD features neurodegeneration of the central nervous system (CNS), htt is expressed ubiquitously throughout the body (Trottier et al., 1995), this brain specific pathology could be explained by its binding with partners that are enriched in the brain, such as Hap1.

Unlike htt, which is expressed fairly uniformly across all the brain regions (Bhide et al., 1996), Hap1 exhibits quite a selective pattern of expression in the brain (Fujinaga et al., 2004; Gutekunst et al., 1998; Page et al., 1998; Sheng et al., 2008). Hap1-immunoreactive cells are predominantly found in the hypothalamic nuclei, which take part in the control of body metabolism and homeostasis. They are also highly abundant in the amygdala, brain stem, and accessory olfactory bulb. Moderate to low expression is detected in other brain regions including the hippocampus, striatum and cortex. Careful examination of the expression pattern of Hap1 revealed that while it is highly enriched in the brain, its expression can also be detected in certain peripheral tissues to varying extent (Cape et al., 2012; Dragatsis et al., 2000; Liao et al., 2005). Immunohistochemistry staining using a specific antibody against Hap1 showed that Hap1-immunoreactive cells are widely distributed in the anterior lobe of the pituitary gland, scattered or clustered in thyroid gland, medullae of adrenal glands, and selectively

found in the pancreatic β-cells. Hap1 expression could also be detected in the mucosa of the stomach and small intestine with a distribution pattern resembling that of gastrointestinal endocrine cells. Taken together, the selective expression pattern of Hap1 in the hormone-releasing hypothalamus as well as in many peripheral endocrine glands/cells suggests that it is important in the neuroendocrine system. Also, based on the functional similarity between neurons and endocrine cells in vesicular trafficking, it is very likely that Hap1 is involved in the intracellular trafficking and hormonal secretion in certain types of endocrine cells.

Hap1 consists of two isoforms named Hap1A and Hap1B that differ in C-terminal sequences in rodents (Li et al., 1995; Nasir et al., 1998). In humans, however, only one form of Hap1 has been identified, which shares more sequence homology with murine Hap1A (Li et al., 1998c; Li et al., 1995). Immunohistochemistry analysis revealed that Hap1 forms cytoplasmic inclusions called 'stigmoid bodies' whose formation could be initiated in vitro via expression of Hap1A, but not Hap1B, although both isoforms are found in the structure (Li et al., 1998a). In fact, the stigmoid body was already described in previous studies by Shinoda's group as a dot-like, non-membrane-bound cytoplasmic inclusion that is 1-3 microns in diameter with moderate to low electron density (Shinoda et al., 1992; Shinoda et al., 1993). The function of the stigmoid body remains mysterious, however, the discovery that Hap1 is present in this structure could certainly help unravel its biological role, which could also be critical to the function of Hap1.

Subcellular localization of Hap1 is very similar to that of htt. It associates with microtubules and many membranous structures such as mitochondria, endoplasmic reticulum (ER), tubulovesicles, synaptic vesicles, endosomes and lysosomes (Gutekunst et al., 1998). As quantitative comparison of organelle associations between Hap1 and htt showed them to be almost identical, it was hypothesized and later confirmed that Hap1

and htt are involved in intracellular transport of many proteins and organelles. However, unlike htt which is localized both in the cytoplasm and nucleus (De Rooij et al., 1996), Hap1 is only observed in the cytoplasm, which is consistent with a lack of a transmembrane domain and nuclear localization signal in its sequence, indicating that it localizes and functions in the cytoplasm (Gutekunst et al., 1998).

1.2 Hap1 in molecular trafficking

Previous work using rat sciatic nerve showed that both htt and Hap1 are rapidly transported in axons in a speed consistent with the movement of vesicle membranes, and they also move retrogradely in nerve fibers (Block-Galarza et al., 1997). This finding suggested that htt and Hap1 might function in vesicle trafficking as well as membrane recycling through microtubules in neurons. The first direct evidence that Hap1 participates in intracellular trafficking came from studies that showed its interaction with the microtubule transporter dynactin p150Glued (Engelender et al., 1997; Li et al., 1998b). Dynactin, or dynein activator complex is a multi-subunit protein that aids in bidirectional intracellular transport by actively linking microtubule motor proteins dynein and kinesin-2 to organelles or vesicles for microtubule-based trafficking (Berezuk and Schroer, 2007; Deacon et al., 2003; King and Schroer, 2000). Hap1 and dynactin P150Glued are able to be co-immunoprecipitated and co-migrate in a sucrose gradient. Upon co-transfection into HEK293 cells, P150Glued is recruited to the stigmoid bodies formed by Hap1A (Li et al., 1998b).

Hap1 was also found to associate with kinesin (McGuire et al., 2006; Twelvetrees et al., 2010), a motor protein responsible for anterograde transport (Vale et al., 1985). McGuire et al. demonstrated via the yeast two-hybrid system, glutathione S-transferase

(GST) pull down, and co-immunoprecipitation (co-IP) that Hap1 interacts with kinesin light chain, a subunit of the kinesin motor complex (McGuire et al., 2006). Knocking down Hap1 by small-interfering RNA (siRNA) inhibits neurite outgrowth in PC12 cells, and suppressing Hap1 expression in live neuronal cells attenuates kinesin-dependent anterograde transport of amyloid precursor protein vesicles. Twelvetrees et al. showed that Hap1 also interacts with KIF5, a kinesin family motor protein, via binding with its heavy chains in cortical neurons (Twelvetrees et al., 2010).

It was reported by Rong et al. that the phosphorylation of Hap1 decreases its association with dynactin p150Glued and kinesin light chain and reduces its localization in neurite tips (Rong et al., 2006). Later, they found that a versatile protein 14-3-3 (Aitken et al., 2002) also binds to Hap1, and the phosphorylation of Hap1 strengthens this binding which results in decreased association of Hap1 with kinesin light chain (Rong et al., 2007a).

Together, these studies suggest that Hap1 may bind to different microtubule motor proteins and play a role in either anterograde or retrograde trafficking of organelles or vesicles. The specific role of Hap1 could be pathway or cell type dependent. Also, Hap1 phosphorylation or its association with other regulatory proteins might modulate its function in trafficking.

One example of vesicles whose transport requires Hap1 is brain-derived neurotrophic factor (BDNF) vesicles. It was demonstrated in cultured neurons that normal transport of BDNF vesicles along microtubules requires both htt and Hap1 (Gauthier et al., 2004). Reducing Hap1 level via siRNA blocked BDNF vesicle transport. Furthermore, this transport is also markedly reduced by mhtt through its interaction with Hap1 and dynactin p150Glued, which leads to attenuated microtubule association of the Hap1-dynactin p150Glued complex (Gauthier et al., 2004). It was revealed that Hap1

also binds to pro-BDNF, a precursor of BDNF, and mediates its transport and release (Wu et al., 2010; Yang et al., 2011). The association of Hap1 with pro-BDNF is significantly decreased in mhtt-transfected PC12 cells, and more importantly, in HD patient brain homogenates (Wu et al., 2010). More recently, Wong and Holzbaur discovered that htt and Hap1 participate in the regulation of axonal transport of autophagosomes, which is disrupted by expression of mhtt (Wong and Holzbaur, 2014). The dysregulation of autophagosome dynamics could lead to mhtt aggregation and dysfunctional mitochondria which may contribute to neurodegeneration in HD. Collectively, these results suggest that the involvement of htt and Hap1 in microtubule-based intracellular trafficking might be relevant to HD pathology.

The role of Hap1 in molecular trafficking is also evidenced by its involvement in membrane receptor trafficking, which has major functions in the uptake of substances and in diverse signaling events (Goldstein et al., 1985; Stahl and Schwartz, 1986). Hap1 was shown to interact with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), an early endosome-associated protein involved in the endosome-to-lysosome trafficking and signal transduction (Bache et al., 2003; Bergeland et al., 2001; Li et al., 2002; Raiborg et al., 2001). Hap1 and Hrs are found to be co-localized on early endosomes by immunofluorescence and subcellular fractionation studies. Like Hrs, overexpressing Hap1 results in enlarged early endosomes and inhibition of the degradation of internalized epidermal growth factor receptors (EGFR) (Li et al., 2002), a receptor tyrosine kinase that is important for neuronal survival (Kornblum et al., 1998; Sibilia et al., 1998). The involvement of Hap1 in EGFR trafficking was further supported by the finding that EGFR protein levels are decreased in Hap1 KO mouse brain, suggesting that Hap1 plays an important role in internalized EGFR trafficking in a way that inhibits its lysosomal degradation and thus stabilizes its level and signaling (Li et al., 2003).

A more detailed look at Hap1 in the trafficking of an internalized receptor came from the work done by Kittler et al. (Kittler et al., 2004). They demonstrated that Hap1 binds directly to γ-Aminobutyric acid type A receptor (GABAAR) and regulates its endocytic trafficking. GABAARs are critical sites of synaptic inhibition in the brain, and when bound to GABA, the major inhibitory neurotransmitter, they undergo clathrin-dependent endocytosis, after which they can be either recycled back to the plasma membrane or targeted to the lysosomes for degradation (Jacob et al., 2008; Moss and Smart, 2001). This sorting decision is shown to be regulated by Hap1, which binds directly to internalized GABAAR, inhibits its lysosomal degradation and facilitates its recycling. A later study by Twelvetrees et al. found that KIF5 is responsible for delivering GABAARs to synapses and Hap1 is the adaptor linking KIF5 and GABAARs as disruption of HAP1-KIF5 complex by knocking down Hap1 or expressing mhtt decreases the number of synaptic GABAARs (Twelvetrees et al., 2010). Taken together, Hap1 stabilizes GABAARs level in the synapse by regulating its endocytic sorting and KIF5-mediated synaptic transport. Compromising this regulatory machinery by mhtt leads to reduced synaptic inhibition which may contribute to HD pathogenesis.

A similar regulatory mechanism by Hap1 was also found to apply to neurotrophin receptors. Receptor tropomyosin-related kinases (Trks) are receptors that bind neurotrophins and mediate signaling essential for neuronal survival and differentiation (Huang and Reichardt, 2003). There are three members in the Trk family: TrkA, TrkB, and TrkC, which are preferentially activated by nerve growth factor (NGF), BDNF, and neurotrophin-3 (NT-3), respectively (Barbacid, 1994). The endocytic sorting of internalized Trk receptors is critical in determining the surface membrane level of the receptors and the potency of continued signaling. Hap1 was found to maintain membrane TrkA level by preventing the degradation of internalized TrkA (Rong et al., 2006). As TrkA is important for neurite outgrowth, suppressing Hap1 expression via

siRNA in PC12 cells reduces TrkA and inhibits cells from growing long neurites after NGF treatment. Likewise, knocking down Hap1 expression also suppresses the level of membrane and internalized TrkB, suggesting that Hap1 maintains TrkB level by blocking its lysosomal degradation and thus enhancing its recycling back to the membrane (Sheng et al., 2008).

Besides receptors that are located in the plasma membrane, Hap1 was also shown to interact with intracellular membrane receptor inositol-(1,4,5) triphosphate receptor type 1 (InsP3R1), which controls calcium release from the ER (Tang et al., 2003; Tang et al., 2004). The ternary complex of InsP3R1-Hap1A-htt is formed not only in vitro, but also in vivo in medium spiny neurons (MSNs), a type of neuron preferentially degenerated in HD, to regulate calcium release and its mediated signaling. However, in the context of mhtt, the function of this complex is disturbed, resulting in supranormal calcium release into the cytoplasm which could lead to pathogenic downstream pathways and eventually degeneration of MSNs.

Furthermore, investigations on milton, the *Drosophila* Hap1 homolog confirmed a conserved role of Hap1 in intracellular trafficking (Glater et al., 2006; Stowers et al., 2002). It was found that axonal transport of mitochondria to synapses requires milton, the loss of which results in synaptic terminals and axons lacking mitochondria. Mechanistically, milton recruits kinesin heavy chain to mitochondria, which is essential for the anterograde transport of mitochondria along axons.

1.3 Animal models of Hap1 KO

Due to the well established interaction of htt and Hap1 and the close relationship between the two in molecular trafficking, it was believed that like htt, Hap1 might also be

an essential gene for development. Inactivation of htt gene in mice results in early embryonic lethality before embryonic day 8.5 (E8.5), indicating that htt is critical for early embryonic development even before the emergence of the nervous system (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Since Hap1 expression could only be detected at E8.5 at earliest (Dragatsis et al., 2000), it is not likely that its absence would lead to such an early death in mice as htt KO does. Its expression in the developing brain, however, continues to elevate in mid to late embryonic and early postnatal days (Dragatsis et al., 2000; Sheng et al., 2008), suggesting that its function might be critical in these developmental stages. Therefore, creating a germline Hap1 KO mouse model was extremely important to unravel the physiological role of Hap1, which would certainly add to our understanding about HD pathogenesis considering that Hap1 binds much more tightly to mhtt (Li et al., 1995).

Targeted deletion of the Hap1 gene results in early postnatal death of the mice (Chan et al., 2002; Dragatsis et al., 2004; Li et al., 2003). Hap1 KO pups are born in expected Mendelian ratio with no apparent defects. However, they could only ingest a markedly-below-normal amount of milk which quickly led to malnutrition, dehydration and premature death. Despite grossly normal development, close examination of the Hap1 KO brain using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining and electron microscopy revealed the degeneration of hypothalamic regions that control feeding behavior (Li et al., 2003). The hypothalamic degeneration not only explains the depressed feeding of Hap1 KO pups, but also has relevance to HD pathology as HD patients often show a loss of body weight with marked hypothalamic degeneration in late stages of the disease (Kremer et al., 1990; Kremer et al., 1991; Pratley et al., 2000; Sanberg et al., 1981). Dragatsis et al. reported that reducing litter size and putting two lactating females in the same cage allowed very few Hap1 KO mice to survive to sexual maturity without obvious brain abnormalities (Dragatsis et al.,

2004). This result indicates that when given maximal nurturing environment, it is possible for a very few number of Hap1 KO pups to live past critical early growth period. It is also possible that genetic background has some influence on the postnatal function of Hap1 as Dragatsis et al used mice on a mixed C57BL/6 x 129 background whereas Hap1 KO mice Chan et al and Li et al created were on a mix background of 129 and ICR, black swiss and 129, respectively.

Since Hap1 is particularly abundant in the hypothalamus which controls feeding behavior (Bouret et al., 2004) and ablating Hap1 causes hypothalamic degeneration, it would be very interesting to see the effect of depleting Hap1 specifically in the hypothalamus or in selected hypothalamic nuclei. Orexin (hypocretin)-producing neurons (orexin neurons) are a small group of neurons located in the lateral hypothalamus (LH), and have been shown to regulate feeding behavior (Sakurai et al., 1998). Using Cre-loxp system, Lin et al. were able to selectively delete Hap1 in orexin neurons (Lin et al., 2010). Orexin-Hap1 KO mice show reductions in food intake, body weight and locomotor activity. Close examination of orexin neurons reveals neuritic fragmentation and redistribution of synaptic proteins. Since orexin neurons project widely throughout the brain (Date et al., 1999; Peyron et al., 1998), findings from this study suggest that the depressed feeding and malnutrition seen in Hap1 KO mice can be partially contributed to the disruption of the processes of orexin neurons and their synaptic formation with other neurons. More importantly, it might represent a common mechanism of action by which Hap1 maintains the physiological integrity of many types of neurons and their networking.

Previous work demonstrated that Hap1 is also present in peripheral tissues (Liao et al., 2005). Most of Hap1-immunoreactive cells found outside the brain are endocrine cells including the pancreatic β-cells. Due to the similar functionality between neurons

and endocrine cells, it would be interesting to examine the phenotypes of mice upon Hap1 deletion in these cells. Therefore, Cape et al. generated conditional KO mice in which Hap1 is selectively ablated the pancreatic β-cells (Cape et al., 2012). These mice display impaired glucose tolerance and decreased insulin release in response to glucose, a phenomenon that is also seen in the R6/2 HD mouse model (Bjorkqvist et al., 2005; Hurlbert et al., 1999; Smith et al., 2009). In addition, HD patients have been observed to have a higher prevalence of diabetes and defective glucose tolerance (Kremer et al., 1989; Podolsky and Leopold, 1977; Podolsky et al., 1972). It remains to be uncovered whether Hap1 dysfunction is involved in the abnormal metabolism found in HD patients and mouse model. Importantly, this study verifies a common function of Hap1 in regulating vesicle secretion both in neurons and endocrine cells.

1.4 Hap1 and HD

HD is an inherited neurodegenerative disorder caused by an abnormal expansion of the N-terminal polyQ tract in the protein htt (MacDonald et al., 1993). It belongs to the family of so-called polyQ diseases that include 8 other neurodegenerative disorders (Shao and Diamond, 2007). The hallmarks of polyQ diseases feature expanded polyQ tract (>36 glutamines), formation of misfolded protein aggregates and inclusion bodies, and neuropathology in distinct brain regions. In HD, the striatum and neocortex are the brain areas preferentially degenerated. The degeneration as the disease progresses also extends to other parts of the brain including the hypothalamus and hippocampus (Vonsattel et al., 1985). As a result, HD is clinically characterized by a triad of motor, cognitive and psychiatric symptoms. One major puzzle to the field of HD research is that since the disease protein htt is ubiquitously expressed throughout the whole body, what is the molecular basis for the selective neuropathology in certain parts of the brain? One

possibility is that the selective neurodegeneration occurs through the interaction between htt and its brain-specific associated proteins. Hap1 is the first known htt associated protein that is predominantly, though not exclusively, expressed in the CNS neuronal cells (Li et al., 1995; Li et al., 1996). Its interaction with htt increases with increasing size of the polyQ expansion, therefore Hap1 was considered as a promising candidate to be involved in HD pathogenesis.

One theory that Hap1 could contribute to HD neuropathology is that it forms a complex with htt and other proteins to carry out important cellular functions, which could be disrupted in the context of mhtt. It was described above that both htt and Hap1 participate in intracellular trafficking, and in many cases, e.g., the transport of BDNF vesicles, the complex of htt, Hap1 and other trafficking proteins and cargoes is perturbed when mhtt is also expressed as in HD (Gauthier et al., 2004; Tang et al., 2003; Twelvetrees et al., 2010; Wong and Holzbaur, 2014; Wu et al., 2010; Yang et al., 2011). Since mhtt binds more tightly to Hap1 than normal htt does, and oftentimes it alters the association of Hap1 and microtubule motor proteins, it is plausible to think that the binding between mhtt and Hap1 may reduce the formation and functionality of htt-Hap1-motor protein-cargo complex, resulting in impaired microtubule-based cargo transport, cellular toxicity and neurodegeneration.

Besides the scenario in which mhtt causes neuropathology by affecting the physiological function of Hap1, it is also conceivable that Hap1 may protect neurons from mhtt toxic gain of function in certain brain regions by binding and sequestering mhtt. The evidence came from the relationship between the pattern of Hap1 expression and that of neurodegeneration in HD. In most affected areas of HD, i.e., the striatal and cortical regions, Hap1 expression is fairly low. However, in areas that do not show obvious neurodegeneration such as the hypothalamus, brainstem and olfactory bulb,

Hap1 shows robust expression (Gutekunst et al., 1998; Li et al., 1996). Therefore, it is reasonable to think that abundant Hap1 expression in these regions protects them from degeneration mediated by mhtt, which is expressed in similar levels throughout the brain. Multiple lines of evidence suggest that Hap1 can indeed protect mhtt induced toxicity. For example, Hap1 prohibits mhtt transfected cells from undergoing apoptosis when EGFR signaling is downregulated (Li et al., 2003). Also, in primary hypothalamic neurons, overexpressing Hap1 significantly reduces mhtt aggregation and subsequent cell death (Li et al., 2003). Furthermore, Zucker et al. microdissected out medium spiny neurons (MSNs), which selectively degenerate in HD and neuronal nitric-oxide-synthase-positive interneurons (nNOS-INs), which survive in HD from the striatum, and found that among others, Hap1 mRNA shows striking enrichment in nNOS-INs as compared to MSNs, suggesting that Hap1 might protect nNOS-INs from degeneration (Zucker et al., 2005). In later stages of HD, patients often develop weight loss, sleep disturbance and metabolic alterations, which are signs of hypothalamic dysfunction (Aziz et al., 2007; Morton et al., 2005; Petersen and Bjorkqvist, 2006; Politis et al., 2008). This could be explained by an age-related decrease in cellular capacity to clear misfolded proteins like mhtt (Carrard et al., 2002; Rubinsztein et al., 2011). As a result, mhtt level gradually builds up as patients age and eventually exceeds the pace that it can be sequestered by Hap1 even in Hap1-enriched hypothalamus. To see if Hap1 can really slow down the disease progression, it would be interesting to assess the beneficial effect of overexpressing Hap1 in predominantly affected regions in HD.

The two scenarios that Hap1 could be involved in HD as discussed above are by no means exclusive to each other. In fact, they may very likely work simultaneously, and concertedly contribute to HD pathogenesis. More detailed investigation on the role of Hap1 in physiological as well as pathological conditions would significantly improve our understanding of HD, and possibly lead to better therapeutic advances in HD.

A few years ago, an association study that aimed to investigate Hap1 and HD age-at-onset (AAO) uncovered among 980 European HD patients that those patients with homozygous Hap1-T441M polymorphism show an 8-year delay in the AAO (Metzger et al., 2008). Functional studies showed that M441-Hap1 interacts with mhtt more strongly than T441-Hap1 does, resulting in fewer htt fragments and reduced toxicity (Metzger et al., 2008). This was the first genetic evidence indicating that Hap1 might indeed modify HD AAO. However, it must be noted that two other association studies with smaller sample sizes tried to replicate this finding but came up with different conclusions. The first one looked at 419 unrelated German HD patients, and found only modest association of Hap1-T441M polymorphism with HD motor AAO (Taherzadeh-Fard et al., 2010). The second study, however, failed to demonstrate an association between this polymorphism and HD AAO in 298 Greek HD patients (Karadima et al., 2012). Therefore, the role of Hap1 as a genetic modifier of HD AAO remains to be clarified.

1.5 Hap1 and other neurological diseases

Besides HD, Hap1 has also been reported to associate with many other disease-related proteins, implicating a potential role of Hap1 in the neuropathology of these diseases (Table 1.1). Among the identified proteins, Hap1-Ahi1 interaction is arguably the best studied and established. Ahi1 is a neuronal-enriched protein whose mutations lead to Joubert syndrome (JS) (Dixon-Salazar et al., 2004; Ferland et al., 2004), which is an autosomal recessive neurodevelopmental disorder marked by a congenital brain malformation of the cerebellar vermis and brainstem (Maria et al., 1997). Patients of JS develop hypotonia, ataxia, developmental delay and other motor and behavioral abnormalities (Maria et al., 1999). Through immunoprecipitation-mass spectrometry (IP-MS) analysis, Sheng et al. found that Ahi1 is abundant in Hap1 immunoprecipitates

from the WT brain (Sheng et al., 2008). Closer examination discovered that Hap1 and Ahi1 form a stable complex and substantially stabilize each other both in vitro and in vivo. In fact, they display essentially identical patterns of expression in the brain. Sheng et al. went on to find that truncated Ahi1, corresponding to mutations in JS, fails to stabilize Hap1, and the loss of Hap1 in mice leads to abnormal cerebellar development and defective axonal decussation (or cross over), resembling JS neuropathology. Due to the extremely close association of Hap1 and Ahi1, dysfunction of Hap1 caused by Ahi1 mutations could critically contribute to the disease pathogenesis of JS. Furthermore, JS belongs to a class of diseases called ciliopathies, in which structural and functional defects in the primary cilia are known to be an underlying cause (Badano et al., 2006). Several JS-related proteins including Ahi1 have been shown to regulate ciliogenesis and vesicle trafficking (Cantagrel et al., 2008; Hsiao et al., 2009), and Keryer et al. found that htt-Hap1 complex regulates ciliogenesis by assisting the retrograde transport of pericentriolar material 1 protein (PCM1) (Keryer et al., 2011), a major component of the centriolar satellites that form the structural basis for ciliogenesis (Dammermann and Merdes, 2002). Therefore, the interaction between Ahi1, Hap1 and htt may be critical for ciliogenesis and the normal brain development. Together, the results highly support the involvement of Hap1 in JS neuropathology, suggesting that targeting pathways regulated by Hap1-Ahi1 could provide therapeutic options for JS.

Apart from JS that mutations of Ahi1 directly lead to, it was reported that neuronal deficiency of Ahi1 causes depressive phenotypes in mice (Xu et al., 2010). Major depression disorder is a leading cause of disability worldwide that affects hundreds of millions of people in many different ways (Tanti and Belzung, 2010; Vos et al., 2012). To date, we have no clear clues as what causes depression, although it is believed that biological, psychological and social factors all play a role (Blazer and Hybels, 2005). As a result, there is still no effective treatment for it. Xu et al. found that

Ahi1 deficiency in neuronal cells significantly impairs the endocytic trafficking of TrkB, which has been shown to be important in depression and other neuropsychiatric disorders (Angelucci et al., 2005; Martinowich et al., 2007), resulting in reduced TrkB level and signaling. Overexpressing TrkB in the amygdala is able to alleviate the depressive phenotypes, further supporting the involvement of TrkB in depressive like behaviors caused by the loss of Ahi1. Since Ahi1 and Hap1 form stable complex in the brain and stabilize the level of each other, the depletion of Ahi1 also leads to remarkable downregulation of Hap1. Moreover, it has already been proposed that Hap1 maintains TrkB protein level by inhibiting its lysosomal degradation (Sheng et al., 2008). Therefore, the behavioral phenotypes seen in the Ahi1-deficient mice might be attributable to Hap1 dysfunction. More comprehensive studies on the role of Hap1 in depression such as the one we did in chapter 4 are needed to clarify this relationship, which could enrich our knowledge about the genetic underpinnings of depression, and potentially advance the diagnostic and therapeutic approaches for this disorder.

Spinal and bulbar muscular atrophy (SBMA), also called Kennedy's disease is a member of the polyQ neurodegenerative disease family that results in muscle cramps and progressive weakness due to motor neuron degeneration in the brainstem and spinal cord (Rhodes et al., 2009). In an in vitro study, Hap1 was found to associate with androgen receptor (AR) (Takeshita et al., 2006), the protein whose expansion in polyQ tract leads to SBMA (La Spada et al., 1991). Takeshita et al. demonstrated that Hap1 interacts with AR in a polyQ-length-dependent manner and is able to sequester polyQ-AR into the stigmoid bodies formed by Hap1 in HEp-2 cells, which results in significantly decreased polyQ-AR induced cell apoptosis. This finding suggests that Hap1 might interact with AR and protect neurons in certain brain regions from polyQ-AR mediated neurodegeneration in similar ways as it does with htt in HD. Despite clear in vitro interaction, evidence for an in vivo interaction between Hap1 and AR is still missing

possibly due to a much weaker binding of Hap1 and normal AR. Therefore, examination in a polyQ-AR context, such as using SBMA patient brain tissues might be a better choice. If the presence of polyQ-AR in the stigmoid bodies can be truly identified in vivo, it would significantly add to our current understanding on the pathogenensis of SBMA, and shed light on the diagnostic and therapeutic applications for the disease.

Hap1 was also shown to be related to two other polyQ diseases, spinocerebellar ataxia 3 and 17 (SCA3 and SCA17), that are caused by polyQ expansion in ataxin-3 and TATA-box binding protein (TBP), respectively (Kawaguchi et al., 1994; Nakamura et al., 2001). In the case of SCA3, both normal and polyQ expanded ataxin-3 proteins are found in the stigmoid bodies when co-transfected with Hap1 in N2a cells (Takeshita et al., 2011). The in vitro association between the two can also be verified by co-IP. Using unbiased yeast two-hybrid screens for interacting proteins of SCA17 disease protein TBP, Hap1 was isolated as a binding partner of TBP (Prigge and Schmidt, 2007). It was found that Hap1 can sequester a subset of both normal and polyQ-TBP into the stigmoid bodies in transfected cells, while a significant portion of TBP still goes into the nucleus. Together, these results suggest that besides HD and SBMA, Hap1 could also provide protection from SCA3 and SCA17 neuropathology.

Alzheimer's disease (AD) is the most common form of dementia. Although the pathogenesis of AD is not well understood, it is generally believed to be driven by the production and deposition of amyloid-beta (Aβ) peptide, which forms the amyloid plaques that can be readily observed in the brains of AD patients and are recognized as a hallmark feature of AD (Murphy and LeVine, 2010). Mutations in amyloid precursor protein (APP) may lead to overproduction of Aβ, and are associated with familial forms of AD (Citron et al., 1992). A recent report by Yang et al. suggests that Hap1 may also affect AD pathogenesis by regulating APP trafficking (Yang et al., 2012). The association

of Hap1 and APP in a same protein complex is demonstrated by co-immunofluorescent staining of both proteins in co-transfected HEK293 cells as well as co-IP in human brain lysate. They found in Hap1 KO cortical neurons that APP is more retained in ER-cis Golgi intermediate compartments, trans-Golgi network, and early endosomes. APP endocytic transport and re-insertion into the plasma membrane are also reduced in Hap1 KO neurons. More importantly, knocking down Hap1 expression in cultured cortical neurons from AD mouse model increases Aβ levels. The results suggest that Hap1 may negatively regulate Aβ production in neurons by directing APP intracellular trafficking into a non-amyloidogenic pathway and Hap1 may also shield neurons from developing AD neuropathology.

More recently, Hap1 was found to interact with tuberous sclerosis complex (TSC) disease protein TSC-1 (Mejia et al., 2013). Although TSC is a multi-system disorder that affects not just CNS, but a variety of organs (Orlova and Crino, 2010), its neurological manifestations, which include seizures, intellectual disability (ID), developmental delay and autistic disorder, might be a cause of disrupted complex formation between TSC-1 and its neural specific interacting proteins. Using IP-MS analysis, Mejia et al. discovered that Hap1 is a high-score parter of TSC-1 in Neuro2a cells and mouse primary cortical neurons. The binding can also be confirmed in E18 mouse brain through co-IP. Functional data suggest that Hap1 and TSC-1 regulate hippocampal pyramidal neuron morphogenesis and positioning through mTORC1 signaling during early development as knocking down either Hap1 or TSC-1 in cultured hippocampal neurons leads to specification of supernumerary axons, which can be suppressed by inhibiting mTORC1, and reducing Hap1 or TSC-1 from E15-P3 mouse brains causes defective stratum oriens-stratum pyramidale migration of the pyramidal neurons. Since Hap1 is preponderantly a neuronal specific protein, the uncovered association between Hap1 and TSC-1 might offer novel clues for the neurological basis of TSC. It was reported that between 25% and

61% of individuals with TSC meet the diagnostic criteria for autism (Harrison and Bolton, 1997), which affects 1-2 per 1000 individuals in the general population (Newschaffer et al., 2007), making TSC the leading genetic cause of autism. Therefore, the complex of Hap1 and TSC-1 might have potential roles in the pathogenesis of autism, or broader autistic spectrum disorder (ASD) as well. In fact, recent whole-exome sequencing of ASD cases and controls identified a homozygous Hap1 nonsense mutation in one of the patients (Lim et al., 2013), further suggesting the relevance of Hap1 in ASD. The same study also identified a complete knockout in the gene Mecp2, which when mutated leads to Rett syndrome, the most physically disabling ASD (Amir et al., 1999). Interestingly, Hap1 and Mecp2 are both predicted to be targeted by mir-328, and Mcep2 KO mice have significantly decreased levels of Hap1 and htt in the brain (Roux et al., 2012). Because these mice show altered axonal trafficking of vesicles such as BDNF (Roux et al., 2012), and display neurological symptoms that mimic Rett syndrome (Guy et al., 2001), it is therefore reasonable to think that molecular trafficking mediated by Hap1 is disturbed in Rett syndrome patients, which could contribute to the neurological features of the disease.

Taken together, the above evidence suggests a role of Hap1 in a number of neurological disorders. Hap1 might modulate the pathogenesis and progression of these diseases by interacting with disease related proteins directly or indirectly in complexes. It may form a complex with these other proteins to regulate important cellular processes, e.g., axonal trafficking or ciliogenesis, which are perturbed in disease contexts. Alternatively, it may also sequester the mutant forms of the disease proteins into the stigmoid bodies to provide protection from specific neuropathologies. As these common pathways might underlie a variety of neurological disorders including both neurodevelopmental and neurodegenerative disorders, understanding the biological role

of Hap1 at different ages may substantially contribute to the current knowledge about these diseases.

1.6 Dissertation goals

Both cellular and animal studies have demonstrated that Hap1 is an important intracellular trafficking protein whose function is particularly essential for the feeding and survival of the neonatal mice. However, there were a few critical questions remaining about the role of Hap1 in mouse development. First of all, we did not know if Hap1 regulates the animal growth and survival only during early postnatal life or in a similar manner throughout the entire postnatal development or even the whole life. Secondly, we had yet found a molecular mechanism that could explain the phenotype of Hap1 KO mice. Moreover, we had not generated conditional Hap1 KO mice that could be examined for disease-related phenotypes in adults. Therefore, the main goals of my dissertation can be summarized as follows:

1. Create a conditional Hap1 KO mouse model in which Hap1 deletion can be induced at different ages upon chemical treatment (described in chapter 2). Characterization of the induced Hap1 KO mice would allow us to find out the postnatal developmental time frame during which Hap1 critically controls animal growth and survival.

2. Look for molecular mechanisms underlying the phenotypes observed in Hap1 KO mice. The role of Hap1 in molecular trafficking especially that of important membrane receptors is a promising candidate to explain the early developmental phenotype of Hap1 KO mice. We will combine cell biology and mouse genetic tools to test this and other hypotheses.

3. Examine adult behavioral phenotypes of conditional Hap1 KO mice. Inducing Hap1 KO at different ages would allow us to have Hap1 KO mice grown into adults. Examination of disease-related behavioral phenotypes of these mice would give us better ideas on the function of Hap1 as well as its potential involvement in neurological disorders.

This dissertation offers systematic and comprehensive analyses on the role of Hap1 in mouse postnatal development, which could significantly improve our current understanding of the biological function of Hap1. Our mechanistic study could also uncover important molecules and pathways regulated by Hap1 that critically involve in the control of mouse postnatal feeding and growth.

Table 1.1

Disease-related proteins that are associated with Hap1		
Protein	**Detection method**	**Related disease**
Huntingtin (htt)	In vitro and in vivo (Y2H, Co-IP, …)	Huntington's disease (HD)
Ahi1	In vitro and in vivo (Co-IP, GST pull-down IF, …)	Joubert syndrome (JS)
Androgen receptor (AR)	In vitro (IF, Co-IP)	Spinal and bulbar muscular atrophy (SBMA)
Ataxin-3	In vitro (IF, Co-IP)	Spinocerebellar ataxia type 3 (SCA3)
TATA-box binding protein (TBP)	In vitro (Y2H, Co-IP, IF)	Spinocerebellar ataxia type 17 (SCA17)
Amyloid precursor protein (APP)	In vitro and in vivo (IF, Co-IP)	Alzheimer's disease (AD)
TSC-1	In vitro and in vivo (Co-IP)	Tuberous Sclerosis complex (TSC)
Y2H: Yeast two hybrid; IF: Immunofluorescence; Co-IP: Co-immunoprecipitation.		

Chapter 2

Essential role of Hap1 in mouse early postnatal survival and growth

This chapter represents part of work published as: Jianxing Xiang, Hao Yang, Ting Zhao, Miao Sun, Xingshun Xu, Xin-Fu Zhou, Shi-Hua Li and Xiao-Jiang Li. Huntingtin-associated protein 1 regulates postnatal neurogenesis and neurotrophin receptor sorting. J Clin Invest. 2014;124(1):85–98. doi:10.1172/JCI69206. J.X. performed all the experiments described in this chapter. X.J.L. and S.H.L. participated in writing and editing the manuscript.

Abstract

Huntingtin-associated protein 1 (Hap1) has been shown to interact with several disease-causing proteins, and therefore could be involved in the pathogenesis of these diseases. However, the biological function of Hap1 remains largely unknown. Since germline Hap1 knockout (KO) leads to early postnatal lethality of the mice, we proposed that Hap1 is essential for the postnatal growth of the animals. However, whether this function of Hap1 persists throughout the lifespan, or changes when animals become older needs to be investigated using a different system. Here, we generated an inducible Hap1 KO mouse model in which Hap1 can be deleted via tamoxifen (TM) injection. We confirmed the successful depletion of Hap1 expression by western blotting analysis. By inducing Hap1 KO at early postnatal days (P1 to P15), we observed growth retardation and early death, phenotypes that are similar to those of germline Hap1 KO mice, and were not seen when Hap1 was depleted later in the development or in the adults (P21 and later). These effects only occurred during early development, as the surviving Hap1 P1 KO mice resumed normal rate of growth. The results suggest that Hap1 is essential for early postnatal growth of the mice, but might be dispensable for their growth after that period.

Introduction

Huntingtin-associated protein 1 (Hap1), originally identified as a neuronal protein that interacts with the Huntington's disease (HD) protein, huntingtin (htt), is critical for postnatal development, as Hap1 KO mice often die before P3 due to inhibited feeding behavior (Chan et al., 2002; Li et al., 2003). The expression of Hap1 in mouse brain is developmentally regulated (Dragatsis et al., 2000; Page et al., 1998). It is first detected in E8.5 neuroepithelium and its expression continues to rise in late embryonic and early

postnatal stages. The highest expression of Hap1 appears in the early postnatal period while its level goes down when the animals become older. Its expression also varies in different regions, with the highest level in the hypothalamus that regulates feeding behavior (Sheng et al., 2006). 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, many important issues regarding its function remain unclear. The first one we would like to address is whether Hap1 functions differentially in early development versus adulthood, or whether loss of Hap1 also affects the survival and growth of adult animals. Understanding this issue 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 (Sheng et al., 2008), a protein whose depletion results in an early brain development disorder, Joubert syndrome (Dixon-Salazar et al., 2004; Ferland et al., 2004). Thus, loss of Hap1 or its dysfunction at different ages or in different types of cells can contribute to various pathological conditions.

However, the germline KO model could not serve this purpose as almost all KO mice die before P3, which makes it impossible to study the role of Hap1 in later stages. To circumvent this obstacle, a different system has to be used, and the Tamoxifen (TM)-induced Cre recombination system happens to be an excellent model for this. The beauty of this model is that a specific gene is only deleted after TM injection, in other words, the deletion can be controlled temporally (Hayashi and McMahon, 2002). The mechanism for this is that Cre in this system is fused to a mutated form of estrogen receptor (Cre-ER), which does not bind to its natural ligand and is therefore kept in the cytoplasm.

However, upon delivery of TM into the cells, TM binds to the mutated estrogen receptor, and directs its translocation to the nucleus, where Cre-mediated recombination takes place.

With this powerful tool, we were able to delete Hap1 in Cre-ER/Hap1 double floxed mice via TM injection at P1, P15, P21, or 2-month of age. The successful deletion of Hap1 was verified by western blotting analysis. We discovered that Hap1 is essential for animal survival and growth only in the early postnatal stage, while after P21, its absence does not lead to any apparent phenotypes. The severity of the phenotypes correlated with the time when TM was injected, with the P1 KO mice showing the most severe phenotypes. A considerable portion of the P1 KO mice died before P10, and those that survived past P21 regained normal rate of growth, indicating that growth after early postnatal period is independent of Hap1.

Results

Generation of conditional Hap1 KO mice. Analysis of Hap1 RNA expression in mouse brain showed that Hap1 expression is not detectable until embryonic day E8.5-E12 and becomes prominent in the developing hypothalamus and limbic system at E15 (Dragatsis et al., 2000; Page et al., 1998). Because in the mouse brain, neurogenesis peaks between E10 and E13 and the central nervous system is continuously developed from E14 to P30 (Farinas et al., 2002; Finlay and Darlington, 1995; Lawson and Biscoe, 1979), 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 2.1 A), 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 exon1 of the Hap1 mouse gene is flanked by two loxP sites and can be depleted by Cre recombination at different ages (Figure 2.1 B). These mice were crossed with transgenic mice in which Cre begins to express at E11 under the neuronal nestin promoter (Tronche et al., 1999). 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 injected with TM, which binds the cytoplasmic Cre-ER and directs it to the nucleus to remove the floxed exon1 of the Hap1 gene, leading to the disruption of the Hap1 gene at postnatal or adult ages (Figure 2.1 B).

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 2.1 C). Similarly, after injecting TM into the floxed Hap1/Cre-ER mice at 2-3 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 2.1 D).

Depletion of Hap1 expression affects the postnatal survival and growth of mice. Importantly, TM-induced Hap1 P1 KO mice showed early postnatal death and reduced body size (Figure 2.1 E); however, embryonic Hap1 depletion led to the postnatal death of all nestin-Cre Hap1 KO 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 2.2 A). When TM was injected into floxed Hap1/Cre-ER mice at P15 (P15 KO), more mice (>80%) could survive, 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 (Ifft, 1972), or at 2-3 months (adult KO), all mice could live as normally as the control mice that were heterozygous floxed Hap1 mice injected with TM (Figure 2.2 A). Thus, postnatal death certainly depends on the age at which Hap1 expression is reduced.

Retarded growth is another remarkable phenotype of Hap1-null mice (Chan et al., 2002; Li et al., 2003). We therefore monitored the body weight of mice when their Hap1 expression was suppressed at P1, P15, and P21 via TM induction (Figure 2.2 B). As expected, P1 KO mice began gaining less body weight 5 days after TM injection, the time when Hap1 expression had been decreased significantly. When Hap1 expression was reduced from P15, there was only a slight decrease in the body weight gain of P15 KO mice. Moreover, depletion of Hap1 at P21 (P21 KO in Figure 2.2 B) or at 3 months (Figure 2.3 A) did not cause any significant decrease in body weight, though Hap1 deficiency in adult mice slightly reduced food intake, which was more obvious 42-50 days after TM injection (Figure 2.3 B). 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 to 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 2.2 C).

Discussion

The critical role of Hap1 in postnatal growth is suggested by its developmentally regulated expression, which peaks during early postnatal days. Whether Hap1 plays a similar role in postnatal and adult animals had been an unresolved question. Understanding this issue was 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 (Li et al., 1996) that is essential for early embryonic development (Cattaneo et al., 2005). Later studies showed that mutant htt could affect the intracellular trafficking of various cargos via its avid binding to Hap1 (Gauthier et al., 2004; Keryer et al., 2011; Ma et al., 2011; Mandal et al., 2011; Tang et al., 2003; Tang et al., 2004; Twelvetrees et al., 2010). However, the pathological features of HD are age-dependent and are characterized by progressive neurodegeneration, suggesting that a toxic gain of function plays a predominant role in HD pathology. Such a toxic gain of 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 (Hult et al., 2011). 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 (Gauthier et al., 2004; Keryer et al., 2011; Ma et al., 2011; Mandal et al., 2011; Tang et al., 2003; Tang et al., 2004; Twelvetrees et al., 2010), 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 (Sheng et al., 2008). In fact, ciliogenesis has been shown to be regulated by a protein complex consisting of htt, Hap1 and PCM1 (Keryer et al., 2011), and 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. The fact that Hap1 KO mice also develop cerebellar defects which mimic those seen in Joubert syndrome (JS) patients (Sheng et al., 2008) indicates that neurodevelopmental disorders such as JS could be caused by downregulation or dysfunction of Hap1. Whether ciliogenesis defects occur in the cerebellum of Hap1 KO mice, or even represent a common mechanism by which Hap1 affects early brain development remains to be investigated.

The new 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 (Chan et al., 2002; Li et al., 2003) and nestin-Cre Hap1 KO mice could not survive into adulthood, Dragatisis 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 (Dragatsis et al., 2004). 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. In addition, because Hap1 is present in endocrine cells (Liao et al., 2005), a systemic reduction in Hap1 in adult mice might lead to some compensatory effects. Similarly, systemically knocking out NPYYR1 causes obesity (Kushi et al., 1998), though intracerebroventricular administration of NPYYR1 antagonist inhibits rodent feeding behavior (Kanatani et al., 2001; Kanatani et al., 2000). Thus, the use of Cre-ER under a neuronal specific or brain region specific promoter may provide a more definitive answer to whether adult deletion of Hap1 in neuronal cells or a specific brain region can cause any feeding or growth defect.

To explain the differential role of Hap1 in the body weight gain of mice before and after P21, it is possible that reduced expression of Hap1 after early postnatal stage renders it less critical in regulating food intake and body growth. However, it is more likely that different molecular pathways are responsible for the early vs. later stage animal growth. While fewer pathways may be active for controlling early phase growth, more pathways may become activated after a certain time point, e.g., around P21, since at that time, the brain has become mature, and signaling pathways that control a certain biological process show greater complexity than when mice are first born, and oftentimes, these pathways show functional redundancy, which makes the system more robust and less vulnerable to lesions and mutations. Thus, when Hap1 is absent, other pathways that are not dependent on Hap1 might still function to keep the normal growth of animals. Since feeding is a complicated behavior which is controlled by different molecules at different stages (Saper et al., 2002), our results clearly identify Hap1 as an essential regulator of feeding behavior in early postnatal life.

Materials and Methods

Animals. Mice were housed in the Division of Animal Resources at Emory University on a 12-h light/dark cycle. All procedures and husbandry were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Germline Hap1 KO mice were generated in our early study (Li et al., 2003). Generation of conditional Hap1 KO mice, in which exon1 of the mouse *Hap1* gene is flanked by two loxP sites to allow the Cre-mediated deletion of exon1, was described in our recent study (Lin et al., 2010). 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 (The Jackson Lab, B6.Cg-Tg(CAG-cre/Esr1)5Amc/J), which have a TM-inducible cre-mediated recombination system driven by the chicken beta actin promoter/enhancer coupled with the cytomegalovirus (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 (Sigma T5648) 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 subcutaneously 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 4929nt to 5003nt) using the forward (5'- TTT TTC TGG GGA GCA TAC GTC-3') and reverse (5'- ATC CGT TAT CCC AGG GTC TGA-3') primers. Primers (forward: 5'- GCG GTC TGG CAG TAA AAA CTA TC -3' and reverse: 5'- TGT TTC ACT ATC CAG GTT ACG G -3') that amplify Cre recombinase were also used to determine the presence of Cre.

Western blotting. Mouse brain tissues were homogenized and then extracted with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC, and 1% Triton X-100) with Halt protease inhibitor cocktail (Thermol Scientific) and phosphatase inhibitors. 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. Guinea pig antibody (EM77) to Hap1 (Li et al., 2003; Li et al., 1996), Gapdh (Millipore), alpha-tubulin (Santa Cruz, CA) were the primary antibodies used.

Statistical analysis. All data are expressed as mean ± SD or SEM. The statistical significance was determined using one-way analysis of variance between groups (ANOVA) with SPSS 10.0 software. A value of $P<0.05$ was considered statistically significant.

Figure 2.1

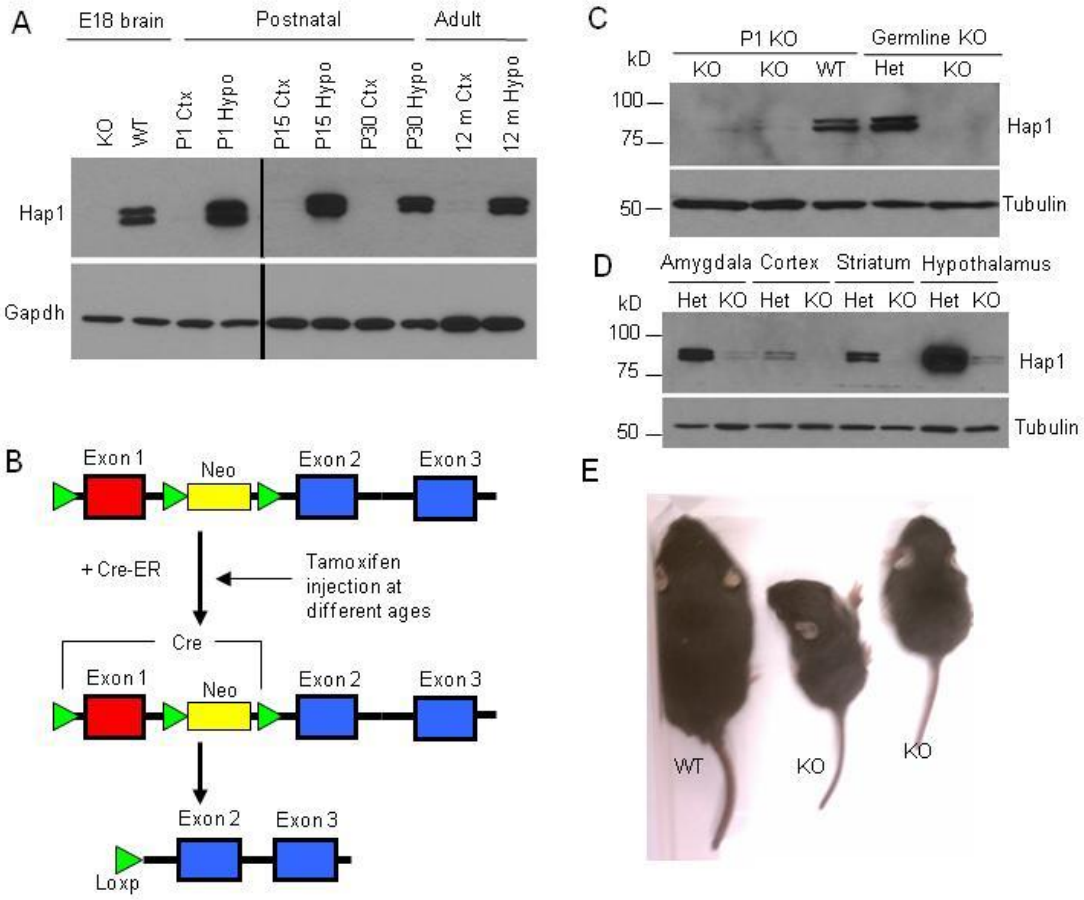
A
E18 brain
Postnatal
Adult
KO
WT
P1 Ctx
P1 Hypo
P15 Ctx
P15 Hypo
P30 Ctx
P30 Hypo
12 m Ctx
12 m Hypo
Hap1
Gapdh
B
Exon 1
Neo
Exon 2
Exon 3
+ Cre-ER
Tamoxifen injection at different ages
Cre
Loxp
C
P1 KO
Germline KO
kD
100
75
50
Tubulin
D
Amygdala
Cortex
Striatum
Hypothalamus
Het
E
WT
KO

Figure 2.1

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 exon1 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 (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.

Figure 2.2

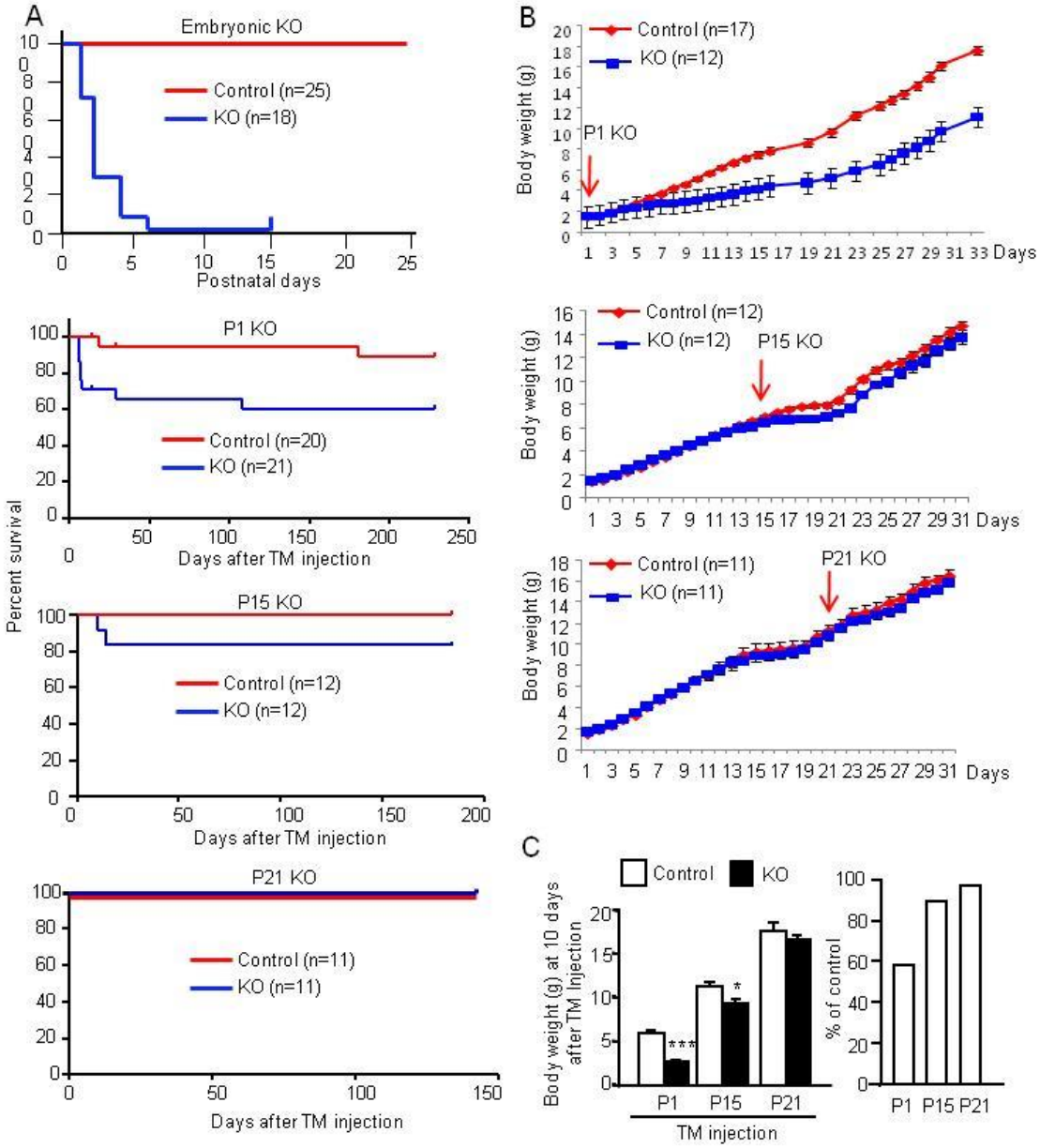
A
Embryonic KO
Control (n=25)
KO (n=18)
Postnatal days
P1 KO
Control (n=20)
KO (n=21)
Days after TM injection
P15 KO
Control (n=12)
KO (n=12)
Days after TM injection
P21 KO
Control (n=11)
KO (n=11)
Days after TM injection
Percent survival
B
Control (n=17)
KO (n=12)
P1 KO
Body weight (g)
Days
Control (n=12)
KO (n=12)
P15 KO
Body weight (g)
Days
Control (n=11)
KO (n=11)
P21 KO
Body weight (g)
Days
C
Control
KO
Body weight (g) at 10 days after TM Injection
P1
P15
P21
TM injection
% of control
P1 P15 P21

Figure 2.2

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, 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 injection was started. (**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.

Figure 2.3

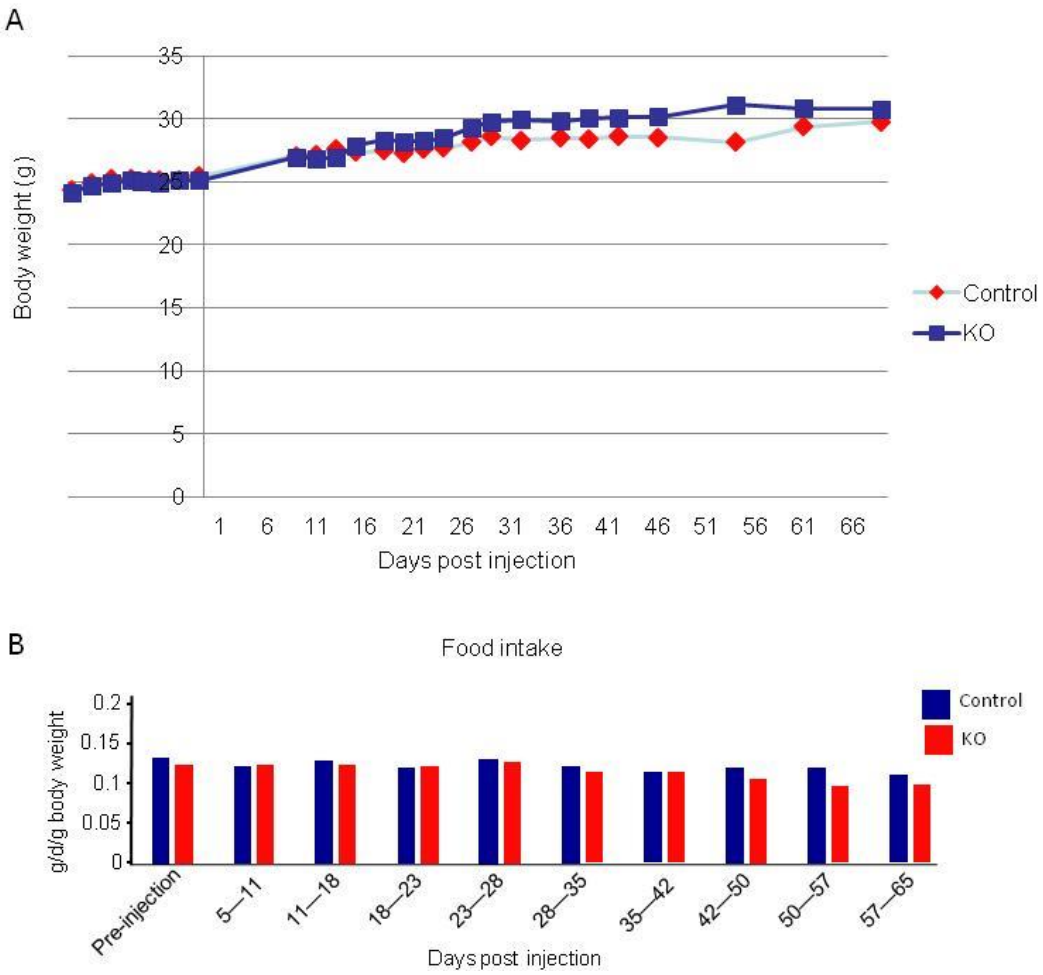
A
Body weight (g)
35
30
25
20
15
10
5
0
1 6 11 16 21 26 31 36 41 46 51 56 61 66
Days post injection
Control
KO
B
Food intake
g/d/g body weight
0.2
0.15
0.1
0.05
0
Pre-injection
5—11
11—18
18—23
23—28
28—35
35—42
42—50
50—57
57—65
Days post injection
Control
KO

Figure 2.3

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

Chapter 3

Hap1 regulates postnatal neurogenesis and neurotrophin receptor sorting

This chapter represents part of work published as: Jianxing Xiang, Hao Yang, Ting Zhao, Miao Sun, Xingshun Xu, Xin-Fu Zhou, Shi-Hua Li and Xiao-Jiang Li. Huntingtin-associated protein 1 regulates postnatal neurogenesis and neurotrophin receptor sorting. J Clin Invest. 2014;124(1):85–98. doi:10.1172/JCI69206. X.F.Z. provided the sortilin plasmid, and commented on the work; X.X. performed experiments described in Figure 3.13 C; M.S. performed experiments described in Figure 3.5; T.Z. performed fluorescence recovery after photobleaching (FRAP) analysis; H.Y. performed majority of the primary culture work and analyses, and helped with other experiments. J.X. performed all the other experiments described in this chapter. X.J.L. and S.H.L. participated in writing and editing the manuscript.

Abstract

Postnatal neurogenesis is critical for the maturation of the brain after birth, and therefore its impairment can lead to growth retardation and many neurological and psychiatric disorders, yet the mechanism behind postnatal neurogenesis remains to be investigated. Using tamoxifen (TM)-induced Cre recombination to deplete Hap1 in mice at different ages, we have found that reducing Hap1 expression selectively affects survival and growth of postnatal mice, but not adults. In this study, we aimed to examine if the above observation is linked to a reduced postnatal neurogenesis, especially in the hypothalamus, which controls feeding behavior, and how postnatal neurogenesis is regulated by Hap1. Our results indicate that neurogenesis, but not gliogenesis, was affected in Hap1-null neurospheres and mouse brains. This decreased neurogenesis was also found in Hap1 P1 KO, but not adult KO mouse brains. In the absence of Hap1, postnatal hypothalamic neurons exhibited reduced receptor tropomyosin-related kinase B (TrkB) levels and decreased survival. Acute intraventricular injection of brain-derived neurtrophic factor (BDNF), the ligand for TrkB, rescued hypothalamic neurogenesis in Hap1-null mice. Mechanistic study revealed that hap1 stabilized the association of TrkB with the intracellular sorting protein sortilin, prevented TrkB degradation, and promoted its anterograde transport. Our findings suggest 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 (Jaaro-Peled et al., 2009), 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 neuronally-enriched protein that interacts with the Huntington's disease (HD) protein, huntingtin (htt), is critical for postnatal development, as Hap1 KO mice often die before P3 due to inhibited feeding behavior (Chan et al., 2002; Li et al., 2003). Unraveling the mechanism by which Hap1 maintains the postnatal survival of animals is important for understanding a key biological process in early animal development. Since robust neurogenesis occurs in the early postnatal brain including hypothalamus, which controls growth and energy balance, we were interested to examine if Hap1 regulates postnatal neurogenesis, especially in the hypothalamus. We also aimed to identify specific types of neurons that are affected by the loss of Hap1, as Hap1 function might be cell-type specific, and neurons that control feeding and body growth might be especially vulnerable in the absence of Hap1.

Mounting evidence has shown that both htt and Hap1 participate in intracellular trafficking of membrane receptors (Caviston and Holzbaur, 2009; Rong et al., 2007b). More specifically, Hap1 is important for the endocytosis of several membrane receptors, including those for EGF, GABA, and neurotrophins (Kittler et al., 2004; Li et al., 2002;

Rong et al., 2006; Sheng et al., 2008; Twelvetrees et al., 2010). 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. Neurotrophin receptors, such as TrkB have been implicated in the control of adult neurogenesis (Bergami et al., 2009; Bergami et al., 2008; Li et al., 2008), however, whether they also play an important role in postnatal neurogenesis, especially hypothalamic neurogenesis is still unknown. We would like to test the idea that Hap1 regulates the endocytic trafficking of TrkB, which serves as an important signaling molecule that mediates postnatal hypothalamic neurogenesis.

In the current study, we investigated neurogenesis in both germline Hap1 KO mice and TM induced Hap1 KO mice. Depletion of Hap1 in germline as well as early postnatal stage (P1) mice can cause reduced neurogenesis in many different brain regions, with the hypothalamus being the most affected region, consistent with its most abundant level of Hap1 expression. In contrast, depletion of Hap1 in adult mice does not lead to this phenotype. 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. The importance of TrkB in postnatal hypothalamic neurogenesis was further verified by acute injecting its ligand, brain-derived neurotrophic factor (BDNF) into the ventricles of the Hap1 KO mice. We found that BDNF treatment rapidly rescued the neurogenesis defect in these mice, indicating indeed, Hap1 regulates postnatal hypothalamic neurogenesis via maintaining TrkB level and signaling. 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.

Results

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. Neurospheres 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 whole brain tissues as described previously (Brewer and Torricelli, 2007; Tropepe et al., 1999). 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 3.1 A). 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 3.1 B).

We also needed to find out whether Hap1 is required for glial differentiation, as glial progenitor migration and differentiation into astrocytes and oligodendrocytes are major events in the postnatal brain (Cayre et al., 2009). 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) and glial (GFAP) proteins showed a marked reduction in neuronal staining in Hap1-null (Hom) neurospheres, while GFAP labeling was unchanged (Figure 3.1 C, Figure 3.2). 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 3.1 D). 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 3.1 E), suggesting that Hap1 is not required for glial differentiation. To confirm this idea, we performed Western blotting of brain tissues from WT and Hap1-null (KO) mice at P1 and found that lack of Hap1 indeed did not reduce the GFAP level in Hap1 KO brain (Figure 3.1 F). Thus, lack of Hap1 appears to selectively impair neurogenesis.

We then 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 (Figure 3.3 A,B) as well as the levels of both doublecortin (DCX), an immature neuronal or neuroblast marker, and Ki67, a mitotic marker or proliferating cell marker (Figure 3.3 C). 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 decreases in the numbers of BrdU-positive cells were seen in the cortex, hypothalamus, and cerebellum in Hap1 KO mice with a greater decrease in the Hap1 KO hypothalamus compared with WT mice at P1. Ki67 immunostaining also showed a similar decrease in the Hap1 KO brains (Figure 3.4 A,B). To verify that lack of Hap1 indeed affects neurogenesis after birth, we measured BrdU incorporation in the brains of P7 mice that was depleted for 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 3.4 C). 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 3.4 C).

We also performed Western blot analysis of the hypothalamic tissues from WT, heterozygous (Het), and homozygous (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, oligodendrocyte marker), were reduced in Hap1-null hypothalamus (Figure 3.4 D). These differences were further verified by quantification of the ratios of these proteins to Gapdh on the same blots (Figure 3.4 E). Similar reductions in Ki67 and DCX were also seen in Hap1 null neurospheres (Figure 3.3 B). 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 brain (Figure 3.5). We then examined whether decrease in Ki67 and DCX after birth is 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 3.4 F,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 3.4 F,G). All 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 (E18-19) 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 (Figure 3.6). 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), which is normally expressed in about 40%of hypothalamic cells, is reduced (Figure 3.7 A). NPYY1R is critical for energy homeostasis (Kanatani et al., 2000; Pralong et al., 2002) and is selectively expressed in certain types of hypothalamic neurons, such as nitric oxide synthase-positive neurons (Fetissov et al., 2003). However, as compared to controls, NPYY1R is not reduced in Hap1 adult KO mouse hypothalamus (Figure 3.8 A) while Hap1 P1 KO mouse hypothalamic tissues showed a reduction in NPYY1R (Figure 3.7 B). The number of NeuN-positive cells (mature neurons) is also reduced in P1 KO hypothalamus (Figure 3.7 B), 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 3.7 C), but not in adult (Figure 3.8 B). Western blotting also showed that another membrane receptor, GluR2/3, which is widely expressed in neuronal cells (Ginsberg et al., 1995; Peytevin et al., 2000), did not significantly change (Figure 3.7 C). In addition, immunostaining revealed that the numbers of the calcium-binding protein calbindin-labeled neurons, which account for around 10% of all hypothalamic cells, were similar in WT and Hap1 P1 KO mouse hypothalamic tissues (Figure 3.9). 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 (Castren and Rantamaki, 2010; Kozisek et al., 2008; Moyse et al., 2006) and Hap1 deficiency impairs

Trk signaling and reduces the survival of cultured neurons (Rong et al., 2006; Sheng et al., 2008), 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, activated form of TrkB) 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 3.7 D), 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 3.7D). The down-regulation 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 down-regulated by the loss of Hap1.

It is known that BDNF/TrkB signaling is required for neuronal survival and growth in cultured neurons (Barde, 1989; Meyer-Franke et al., 1995). 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 (Figure 3.10 A). Double staining with the antibody to β-tubulin-III confirmed that these hypothalamic cells were neuronal cells (Figure 3.10 A). Quantification of β-tubulin-III and NPYY1R-containing cells also showed that BDNF significantly increased the number of neurons and NPYY1R positive cells in Hap1-null hypothalamic cultures (Figure 3.10 B). To determine if 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 3.7 E). Analysis of BrdU-positive cells 4 h post 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 3.7 F,G). Double immunostaining of BrdU and NPC markers nestin and sox2 verified that the increased BrdU-positive cells are indeed NPCs (Figure 3.11). 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 cell cycle (Fukumitsu et al., 2006). 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 level 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 co-expressing TrkB with Hap1A in HEK293 cells, because HEK293 cells do not express endogenous Hap1. We confirmed that this co-transfection led to the co-expression of both TrkB and Hap1 in the majority of transfected cells (Figure 3.12 A). The transfected cells were treated with BDNF to trigger the endocytosis of TrkB and its degradation. Co-expression of Hap1 apparently increased the level of TrkB and its half-life after BDNF stimulation (Figure 3.13 A). 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 3.13 B). To further examine whether lack of Hap1 promotes the degradation of TrkB in the lysosomes, we isolated a lysosome-enriched fraction from WT and Hap1 KO mouse hypothalamus and observed a reduction of TrkB in the lysosome-enriched fraction from Hap1 KO mouse brains (Figure 3.13 C). Since this lysosome-enriched fraction also contains other organelles such as endosomes that associate with Hap1, the decreased level of TrkB could be attributable to increased degradation in lysosomes or by other mechanisms. To address this issue, 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 3.13 D).

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 (Nykjaer and Willnow, 2012), has been recently found to interact with Hap1 (Yang et al., 2011) and to enhance TrkB trafficking and signaling (Vaegter et al., 2011). We co-expressed TrkB with Hap1A in HEK293 cells and then examined the immunoprecipitates for endogenous sortilin. We observed the co-precipitation of TrkB and Hap1A (Figure 3.14 A), which suggests that sortilin, TrkB, and Hap1 form a protein complex. By comparing the co-precipitation of TrkB with sortilin in the presence and absence of Hap1A, we found that Hap1A could increase the association of TrkB and sortilin (Figure 3.14 B).

To further validate that Hap1 associates with TrkB and sortilin, we co-expressed Hap1A or TrkB with transfected sortilin in HEK293 cells and observed their

colocalization with sortilin (Figure 3.12 B). More importantly, sortilin is colocalized with Hap1 in the cytoplasmic puncta in mouse hypothalamic neurons (Figure 3.12 C), 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 immunopreciptiation of sortilin from the hypothalamic tissues of WT and KO mice. In the absence of Hap1, there is a reduced amount of precipitated sortilin with TrkB compared to that from WT mouse hypothalamic tissues (Figure 3.14 C). Sortilin is found to facilitate the anterograde transport of TrkA and enhances its signaling (Nykjaer and Willnow, 2012). 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 3.14 D). We then performed the 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 (Figure 3.15). 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 3.14 E,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 impacts the function and plasticity of existing neuronal circuitry to regulate learning, memory, and mood (Ming and Song, 2011; Zhao et al., 2008). 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 (Berardi et al., 2000; Blackshaw et al., 2010; Hua and Smith, 2004). 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.

In the previous chapter, I have shown that Hap1 specifically regulates postnatal survival and growth. Since neuronal nestin-Cre Hap1 KO mice showed the exact same lethal phenotype as the germline KO mice, we believe that despite some expression in the peripheral tissues, Hap1's main function as an essential early postnatal protein is in the maturation of the nervous system. 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 (Dixon-Salazar et al., 2004; Ferland et al., 2004). Because Hap1 is more restricted to neuronal cells, it may possess a distinctive 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 the energy balance and feeding behavior (Eva et al., 2006; Gehlert, 1999; Kanatani et al., 2001; Kanatani et al., 2000). 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 (Kittler et al., 2004; Li et al., 2002; Rong et al., 2006; Sheng et al., 2008; Xu et al., 2010), 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 (Chen et al., 2005; Nykjaer and Willnow, 2012; Vaegter et al., 2011). 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 (Gutekunst et al., 2003), 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 (Engelender et al., 1997; Li et al., 1998b; McGuire et al., 2006; Twelvetrees et al., 2010), 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 a 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 impacted 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 (Valdes-Sanchez et al., 2010). 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 receptors 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 (Berardi et al., 2000). 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 (Inta et al., 2011). Since pharmacological manipulation of Trk signaling is possible, our studies indicate that intracellular sorting of neurotrophin

receptors could make a novel therapeutic target for those neurological disorders that are caused by defective postnatal neurogenesis.

Materials and Methods

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., 2003; Li et al., 1996). 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 E11 mice as previously described (Brewer and Torricelli, 2007; Tropepe et al., 1999). 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.

Neurosphere proliferation, identification, and differentiation. 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 h before examination. As for cells plated into the uncoated well, they were continuously maintained for different days.

For investigation of the differentiation of neural stem cells, the neurospheres were seeded on PDL-coated glass coverslips at 20 neurospheres/cm^2 in DMEM containing 2% FCS and 4 μM retinoic acid. The cultures for neural stem cell 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 neural stem cells was identified by immunocytochemistry with anti-β-tubulin III and anti-GFAP.

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 sunk, 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.

Western blotting. For Western blotting of cultured cells, the cells were extracted in ice-cold RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC, and 1% Triton X-100) containing Halt protease inhibitor cocktail (Thermol Scientific) 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 Hap1 P1 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.

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.

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 (20 ug/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, HEK293 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.

BrdU incorporation assay. For the in vitro study of neural stem cell proliferation, BrdU (5 μM) was added to the culture medium for 18 h. After removal of the supernatant, cells were fixed with 4% paraformaldehyde and treated with 2 N hydrochloric acid for 30 min 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 subcutaneously (3 mice per group). Two hours later, the mice were perfused and fixed as described in the supplemental methods. For BrdU injection into Hap1 P1 KO mice and controls, mice at P6 were i.p. injected with 50mg/kg body weight BrdU (3 mice per group). 24 hours later, the 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 50mg/kg body weight BrdU, twice a day for three days (3 mice per group). 24 hours after the final injection, the mice were perfused and fixed as above.

BDNF treatment. Surgical procedures were conducted as previously described with modifications (Nawa et al., 1994). In brief, P1 mouse pups were anesthetized on wet ice for 3 min 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 min from a 5 μl Hamilton syringe. The needle was kept still for another 2 min 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 subcutaneously, 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 semiautomatic stereology system StereoInvestigator 5.4.3 (MicroBrightField). Target brain regions were cut in 40 μm serial sections in which every fifth section was used for analysis. BrdU-positive cells were counted on each section. The volume of the target region was determined by the StereoInvestigator software by using 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 millimeter 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 200M, Germany) inverted phase-contrast microscope (40X). 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 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 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 the

Openlab software (Improvision, Lexington, MA) at the same setting. β-tubulin III-positive cells with long neurites (over twice the body size) were counted and calculated.

Fluorescence Recovery after Photobleaching (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% CO2. For visualizing GFP signal, images were acquired utilizing a 63X 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 488nm 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.

Statistical analysis. All data are expressed as mean ± SD or SEM. The statistical significance was determined using one-way analysis of variance between groups (ANOVA) with SPSS 10.0 software. A value of $P<0.05$ was considered statistically significant.

Figure 3.1

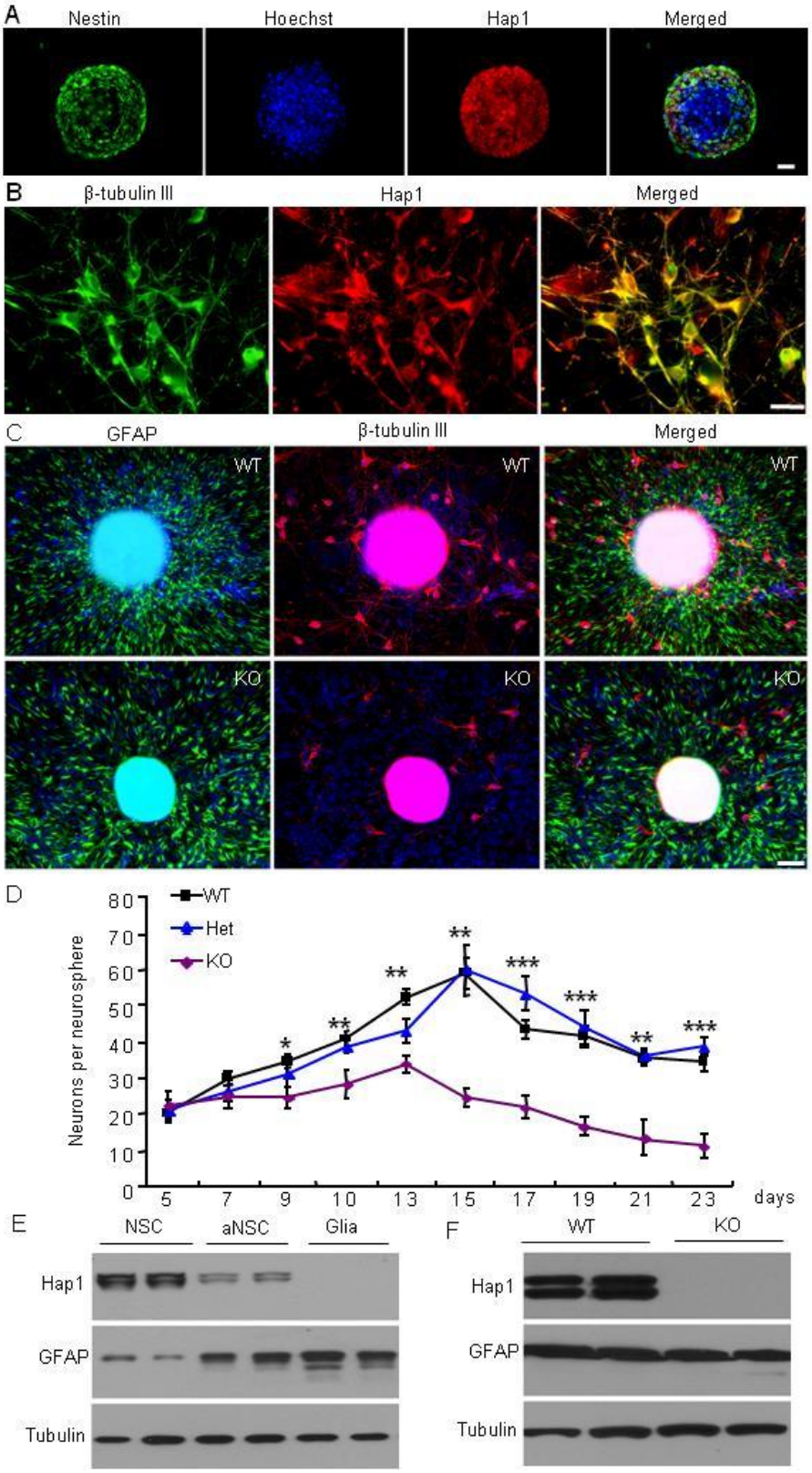
A
Nestin
Hoechst
Hap1
Merged
B
β-tubulin III
Hap1
Merged
C
GFAP
β-tubulin III
Merged
WT
KO
D
WT
Het
KO
Neurons per neurosphere
days
E
NSC
aNSC
Glia
Hap1
GFAP
Tubulin
F
WT
KO
Hap1
GFAP
Tubulin

Figure 3.1

Lack of Hap1 does not affect astrocyte differentiation in the mouse neurospheres. (**A**) Low-magnification micrograph (10x objective) 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 (40x objective) showing the co-expression 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 neural stem cell (NSCs), adult neural stem cells (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.

Figure 3.2

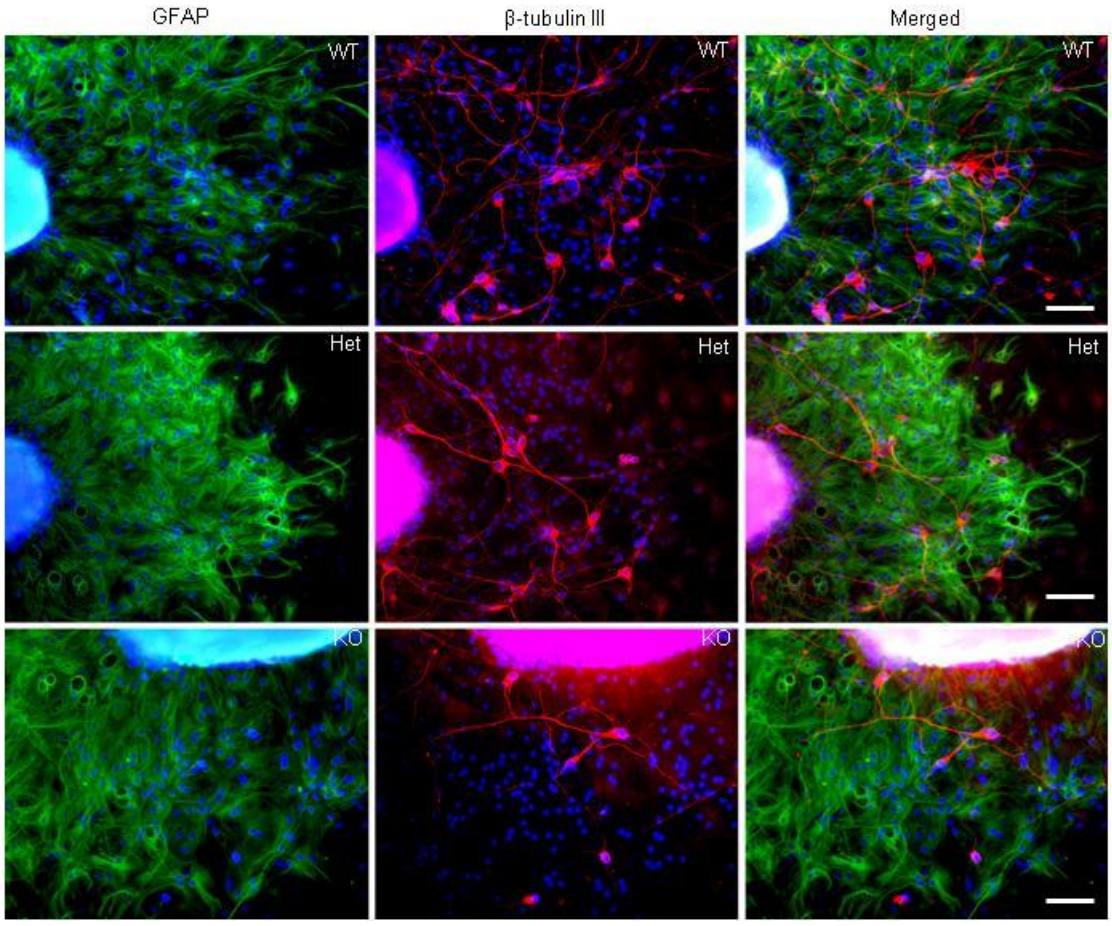

Figure 3.2

Lack of Hap1 affects neurogenesis but not astrocyte differentiation 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 3.3

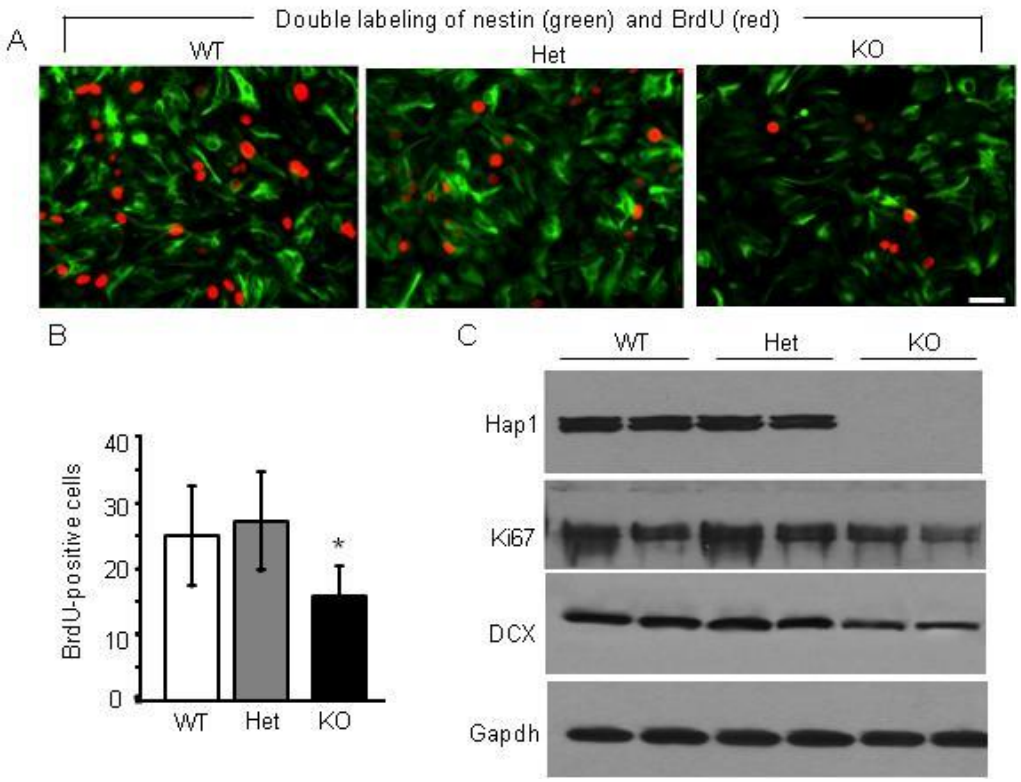
A
Double labeling of nestin (green) and BrdU (red)
WT
Het
KO
B
BrdU-positive cells
40
30
20
10
0
*
WT
Het
KO
C
WT
Het
KO
Hap1
Ki67
DCX
Gapdh

Figure 3.3

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. (**B**) Quantifications of BrdU positive cells in the images from **A** (n=15 images per group). 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 3.4

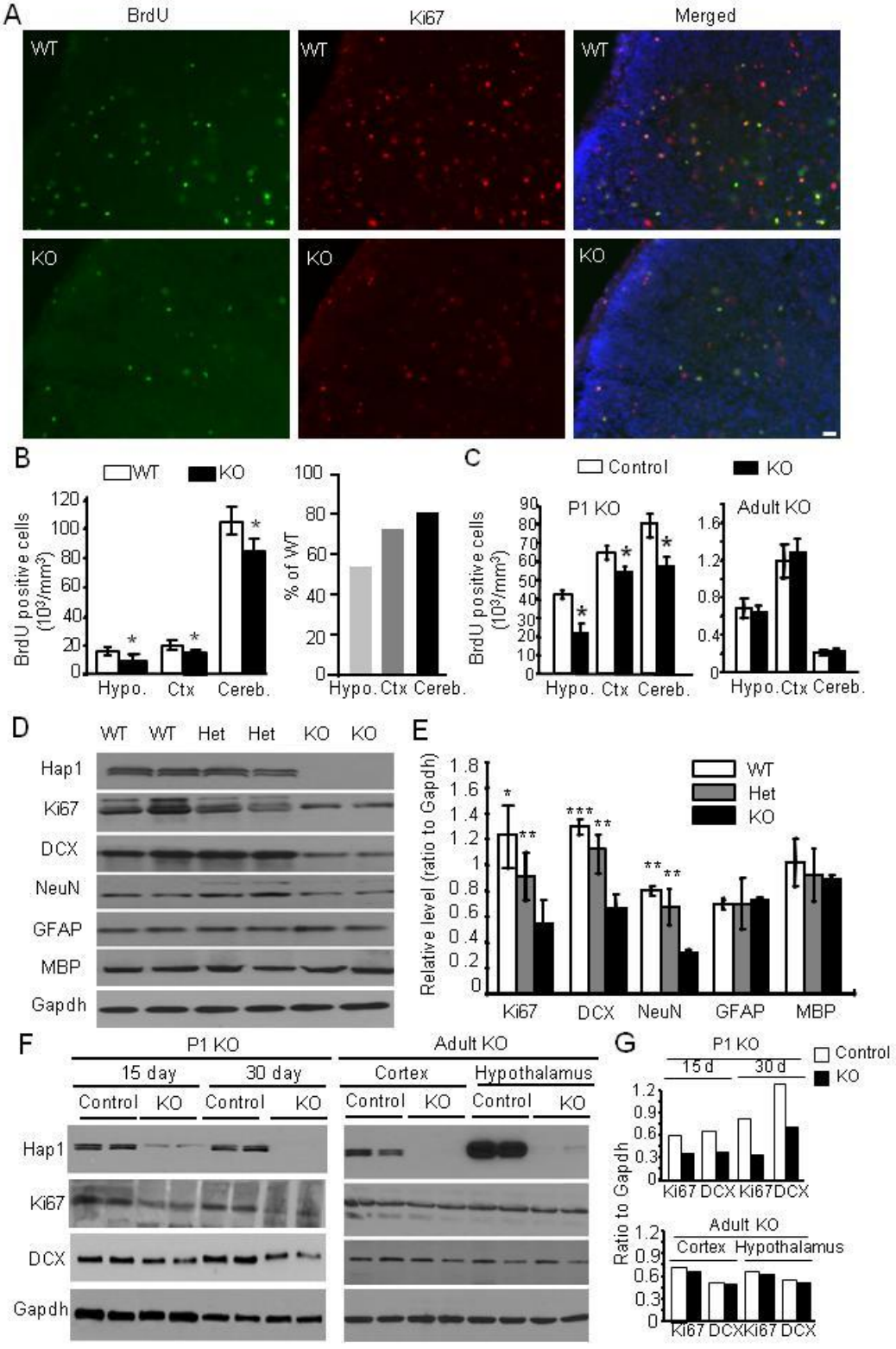
A
BrdU
Ki67
Merged
WT
KO
B
WT
KO
BrdU positive cells (10³/mm³)
% of WT
Hypo.
Ctx
Cereb.
C
Control
KO
P1 KO
Adult KO
BrdU positive cells (10³/mm³)
D
WT WT Het Het KO KO
Hap1
Ki67
DCX
NeuN
GFAP
MBP
Gapdh
E
WT
Het
KO
Relative level (ratio to Gapdh)
Ki67
DCX
NeuN
GFAP
MBP
F
P1 KO
Adult KO
15 day
30 day
Cortex
Hypothalamus
Control
KO
Hap1
Ki67
DCX
Gapdh
G
P1 KO
15 d
30 d
Control
KO
Ratio to Gapdh
Ki67 DCX Ki67 DCX
Adult KO
Cortex
Hypothalamus
Ki67 DCX Ki67 DCX

Figure 3.4

Lack of Hap1 reduces the neuronal proliferation in the brains of Hap1-null mice. (**A**) Immunofluorescent staining of BrdU and Ki67 in the hypothalamus of WT (upper panel) and Hap1-null (KO, lower panel) 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. All error bars represent SEM. (**F**) Western blots of the brain tissues of control, Hap1 P1 KO, and adult KO mice with antibodies to Hap1, Ki67, DCX, and Gapdh. The samples were analyzed after TM injection for 15 (P1 KO) or 30 (P1 and adult KO) days. (**G**) The relative levels of proteins (ratio to Gapdh) on the western blots were presented.

Figure 3.5

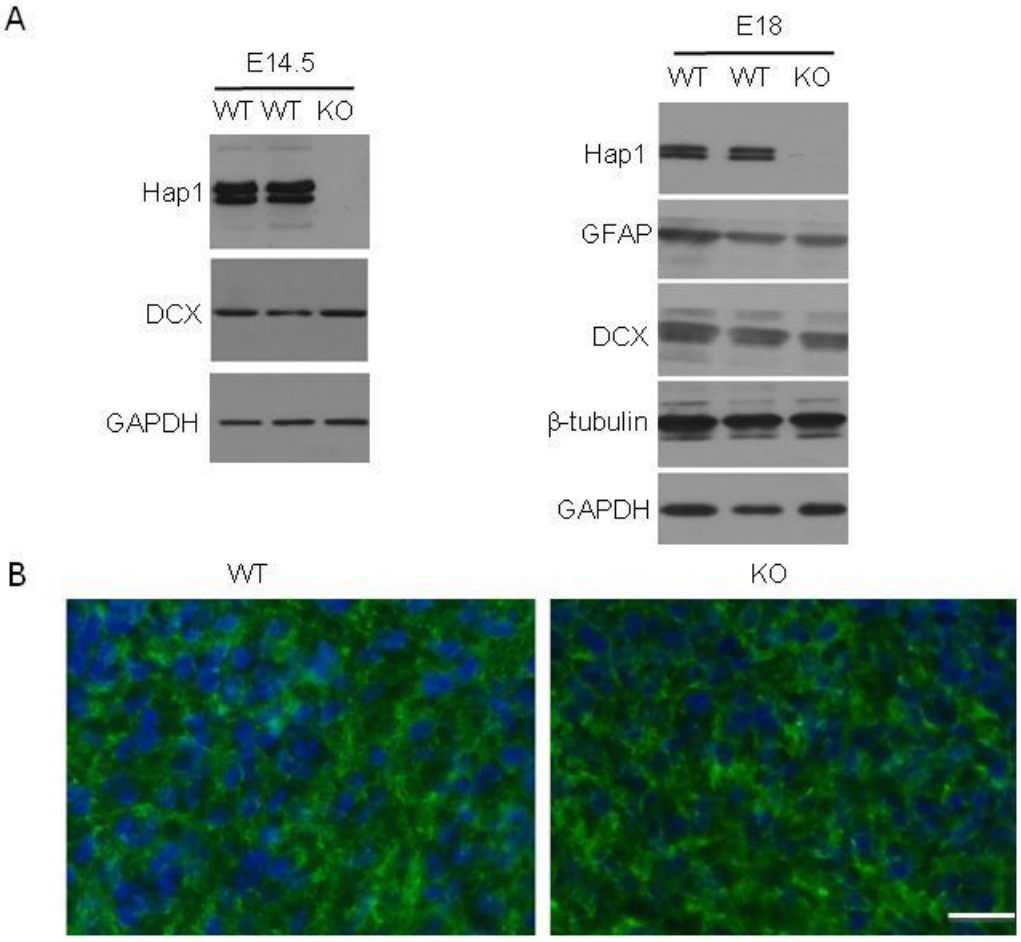
A
E14.5
WT WT KO
Hap1
DCX
GAPDH
E18
WT WT KO
Hap1
GFAP
DCX
β-tubulin
GAPDH
B
WT
KO

Figure 3.5

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 3.6

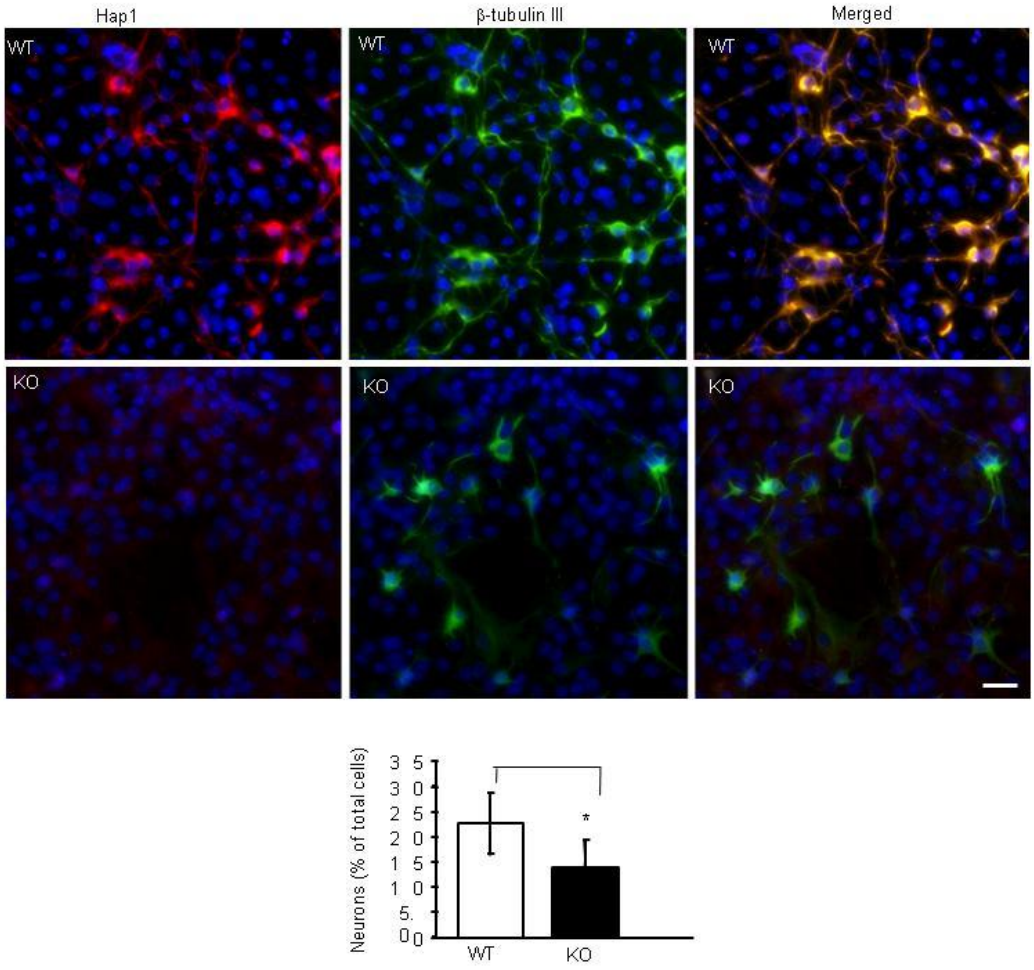
Hap1
β-tubulin III
Merged
WT
WT
WT
KO
KO
KO
Neurons (% of total cells)
3 5
3 0
2 5
2 0
1 5
1 0
5.
0 0
*
WT
KO

Figure 3.6

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 3.7

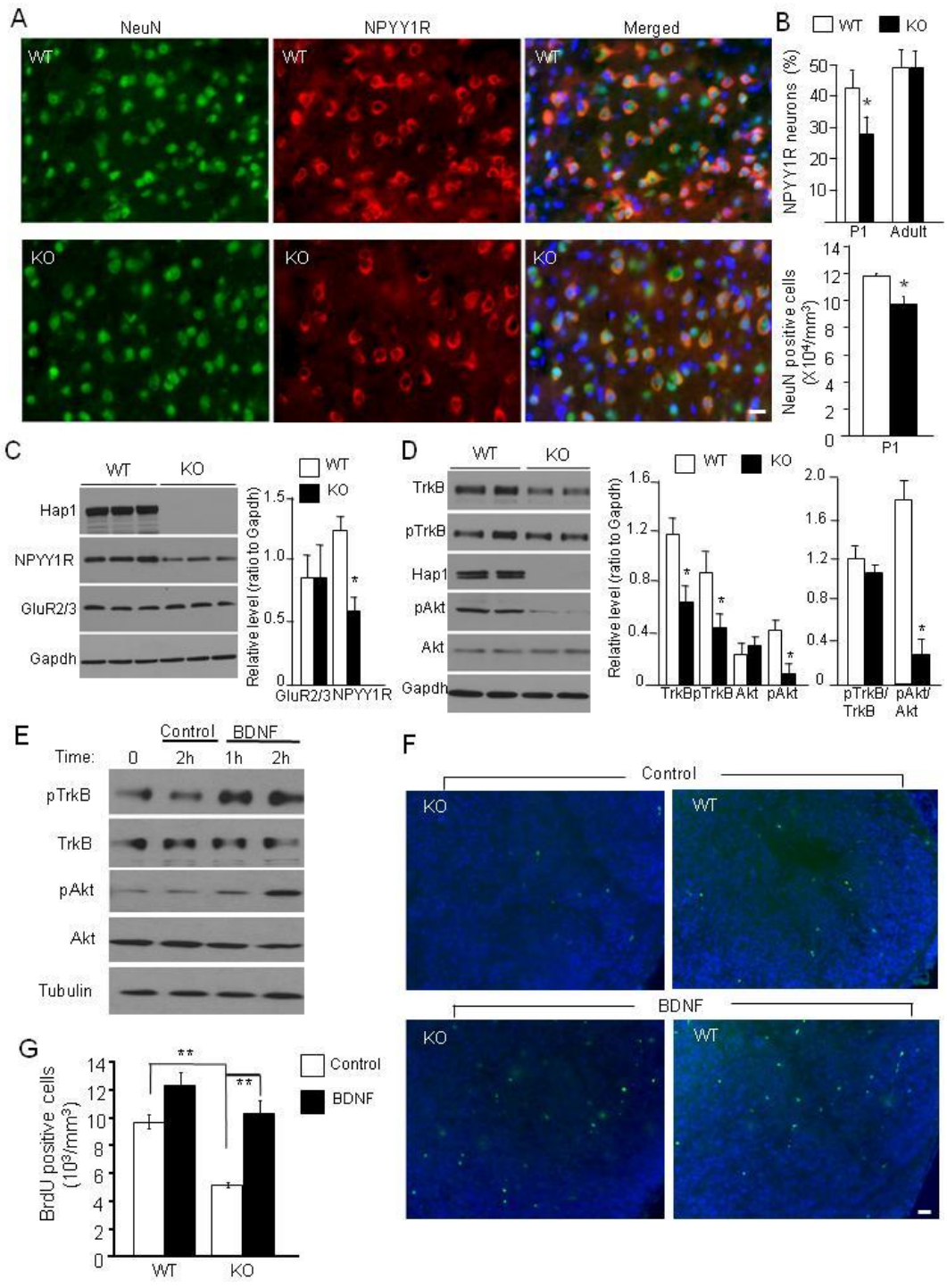
A
NeuN
NPYY1R
Merged
WT
KO
B
WT
KO
NPYY1R neurons (%)
P1
Adult
NeuN positive cells (X 10⁴/mm³)
C
Hap1
NPYY1R
GluR2/3
Gapdh
Relative level (ratio to Gapdh)
GluR2/3
NPYY1R
D
TrkB
pTrkB
Hap1
pAkt
Akt
Gapdh
TrkB
pTrkB
Akt
pAkt
pTrkB/ TrkB
pAkt/ Akt
E
Control
BDNF
Time:
0
2h
1h
2h
pTrkB
TrkB
pAkt
Akt
Tubulin
F
Control
BDNF
G
BrdU positive cells (10³/mm³)
Control
BDNF

Figure 3.7

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 (Figure 2.11) 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 2h, or 5 μg BDNF for 1 or 2h. Non-injected 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 4h. 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.

Figure 3.8

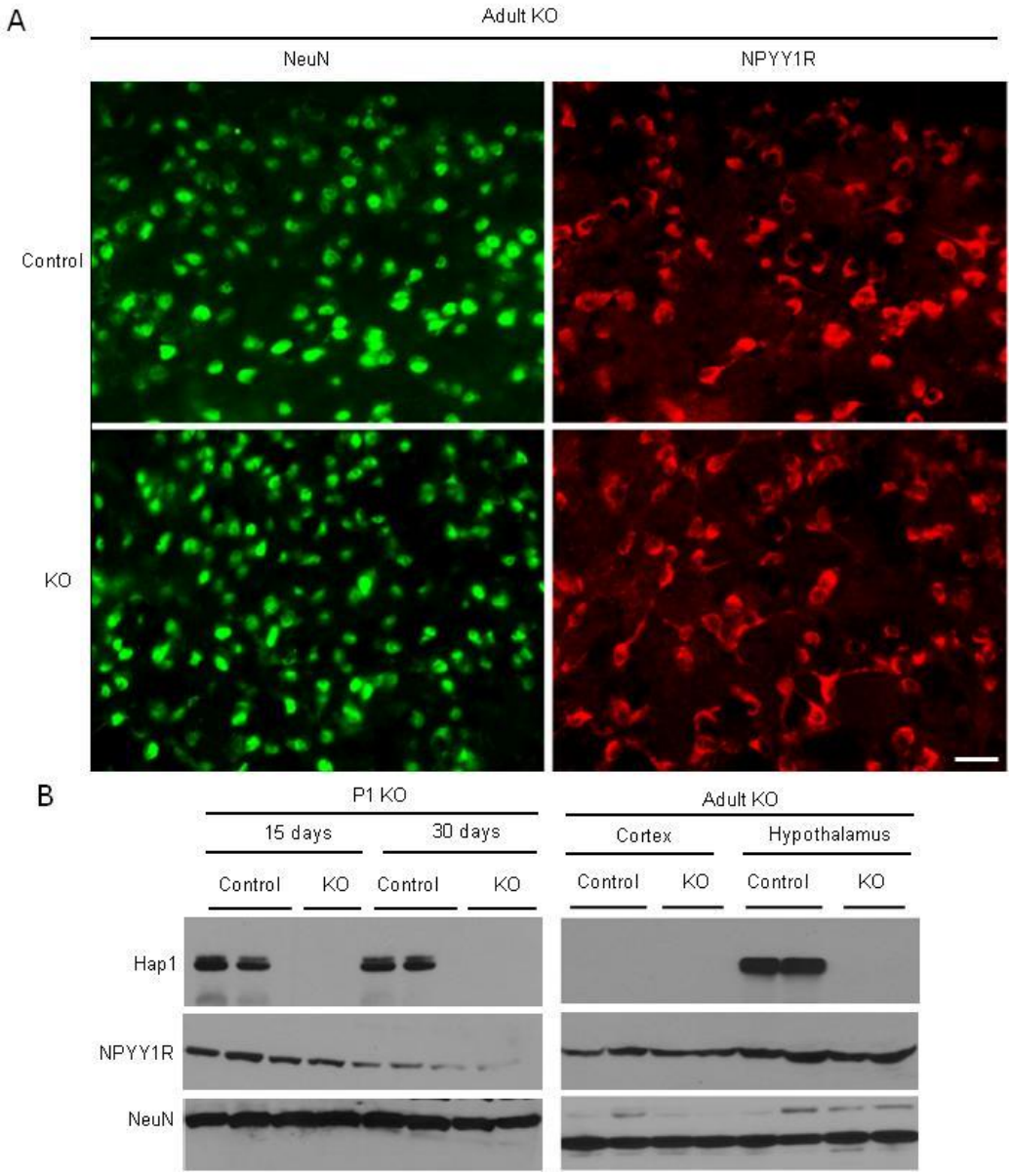
A
Adult KO
NeuN
NPYY1R
Control
KO
B
P1 KO
15 days
30 days
Adult KO
Cortex
Hypothalamus
Control
KO
Control
KO
Control
KO
Control
KO
Hap1
NPYY1R
NeuN

Figure 3.8

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 3.9

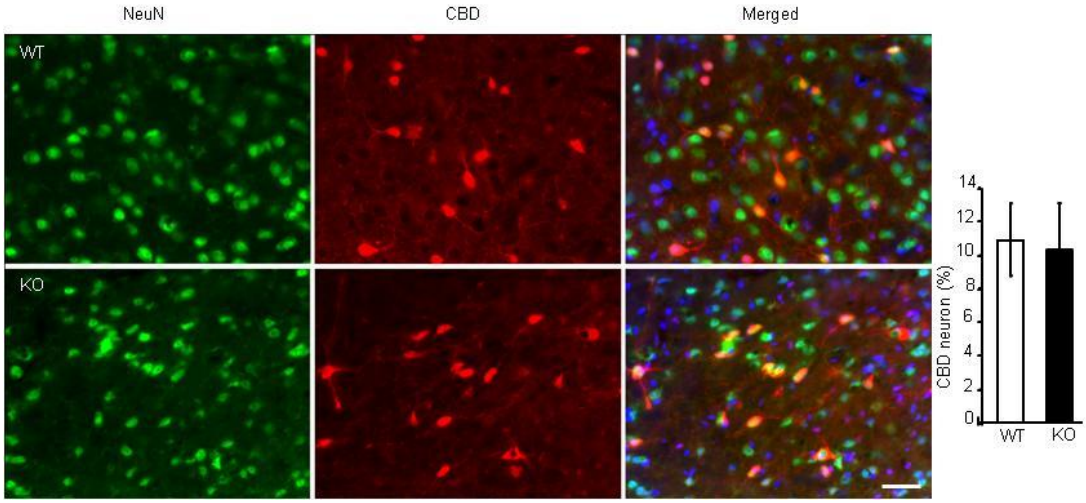

Figure 3.9

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 3.10

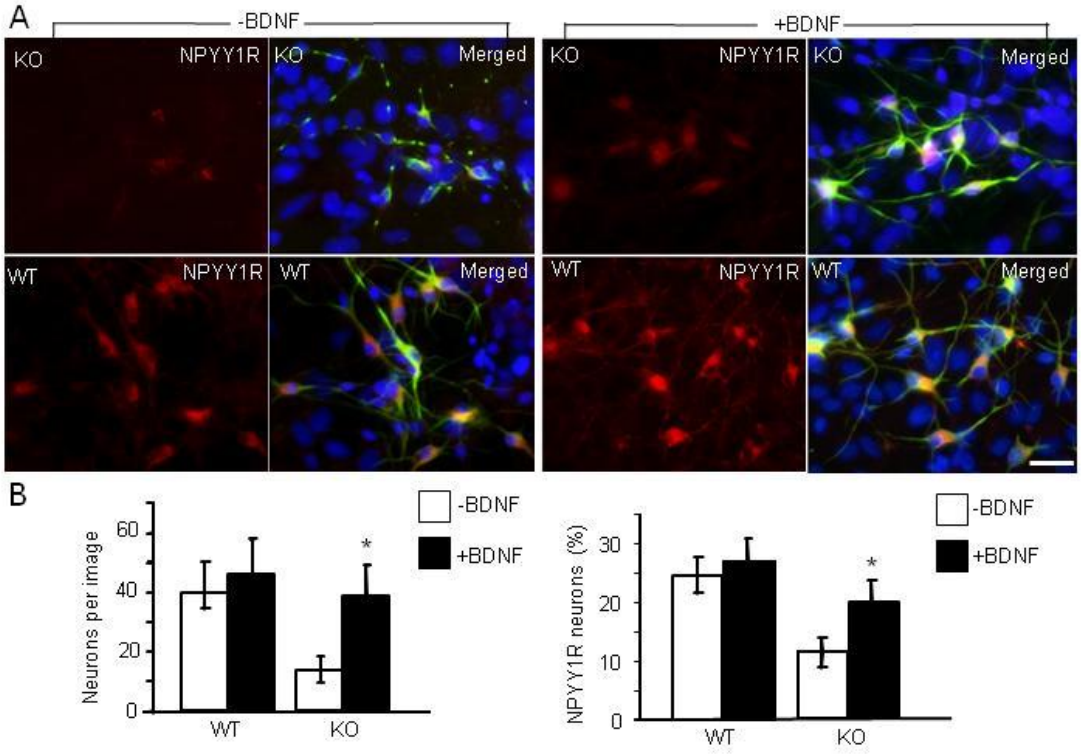
A
-BDNF
+BDNF
KO
NPYY1R
KO
Merged
WT
NPYY1R
WT
Merged
B
Neurons per image
60
40
20
0
WT
KO
-BDNF
+BDNF
*
NPYY1R neurons (%)
30
20
10
0
WT
KO
-BDNF
+BDNF
*

Figure 3.10

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 3.11

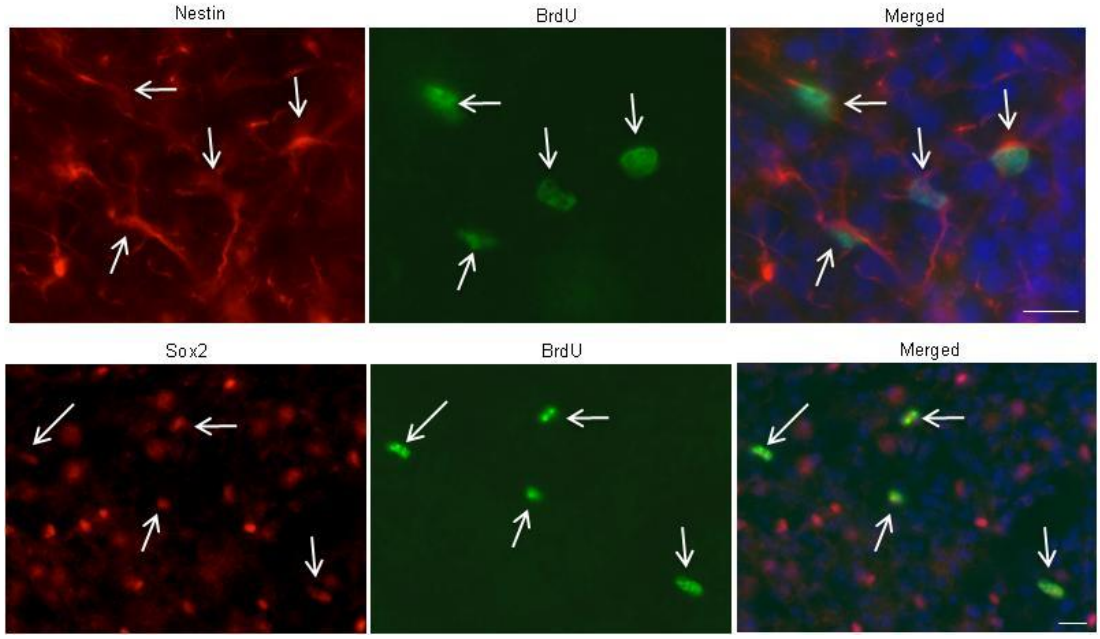

Figure 3.11

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 3.12

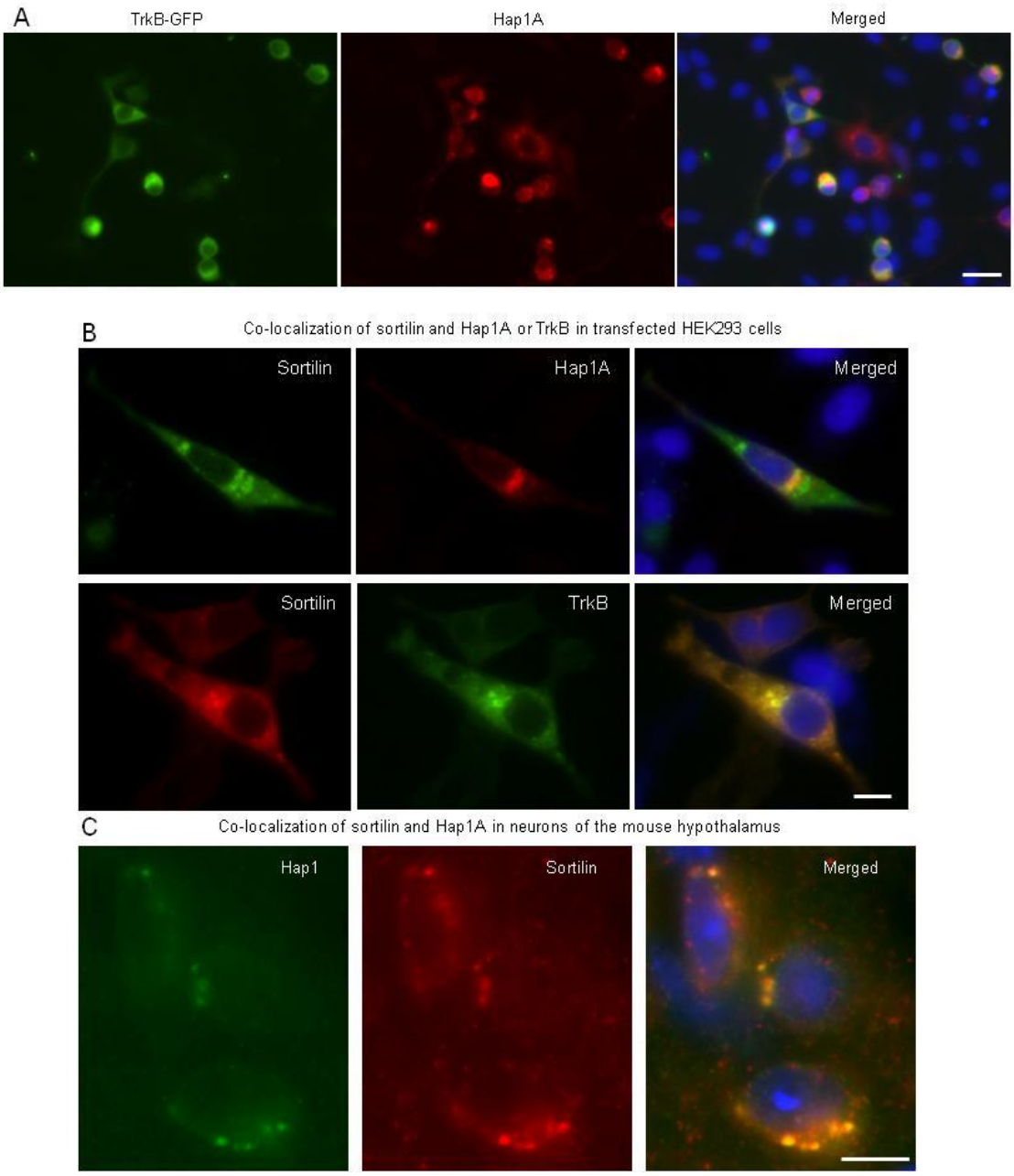
A
TrkB-GFP
Hap1A
Merged
B
Co-localization of sortilin and Hap1A or TrkB in transfected HEK293 cells
Sortilin
Hap1A
Merged
Sortilin
TrkB
Merged
C
Co-localization of sortilin and Hap1A in neurons of the mouse hypothalamus
Hap1
Sortilin
Merged

Figure 3.12

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**) Co-localization of endogenous Hap1 and sortilin in the hypothalamus of mouse brain. Scale bar: 5 μm.

Figure 3.13

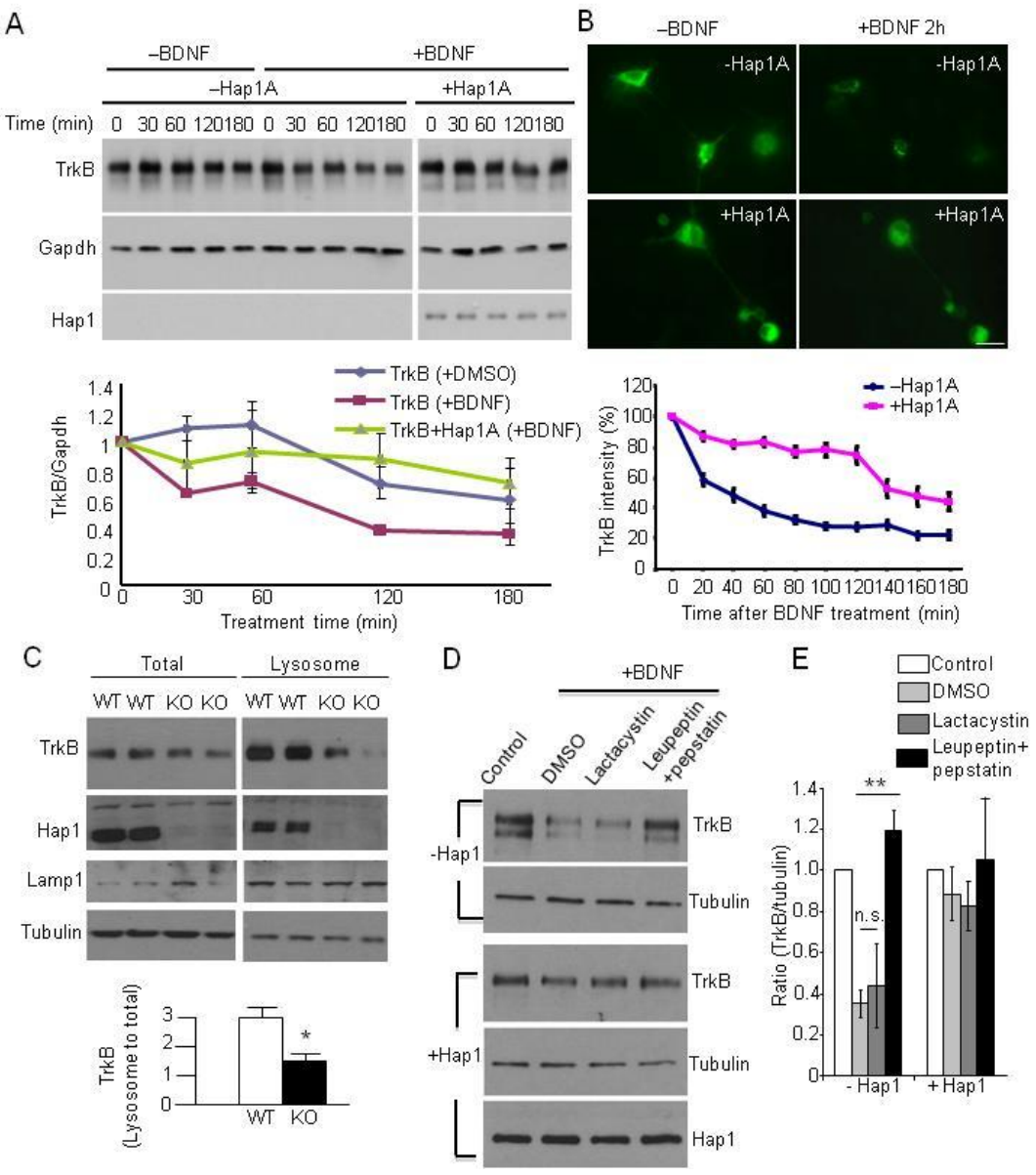
A
–BDNF
+BDNF
–Hap1A
+Hap1A
Time (min) 0 30 60 120180 0 30 60 120180 0 30 60 120180
TrkB
Gapdh
Hap1
B
–BDNF
+BDNF 2h
-Hap1A
+Hap1A
TrkB (+DMSO)
TrkB (+BDNF)
TrkB+Hap1A (+BDNF)
TrkB/Gapdh
Treatment time (min)
–Hap1A
+Hap1A
TrkB intensity (%)
Time after BDNF treatment (min)
C
Total
Lysosome
WT WT KO KO WT WT KO KO
TrkB
Hap1
Lamp1
Tubulin
TrkB (Lysosome to total)
WT KO
D
+BDNF
Control
DMSO
Lactacystin
Leupeptin +pepstatin
-Hap1
+Hap1
TrkB
Tubulin
Hap1
E
Control
DMSO
Lactacystin
Leupeptin+ pepstatin
Ratio (TrkB/tubulin)
**
n.s.
- Hap1
+ Hap1

Figure 3.13

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. The 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.

Figure 3.14

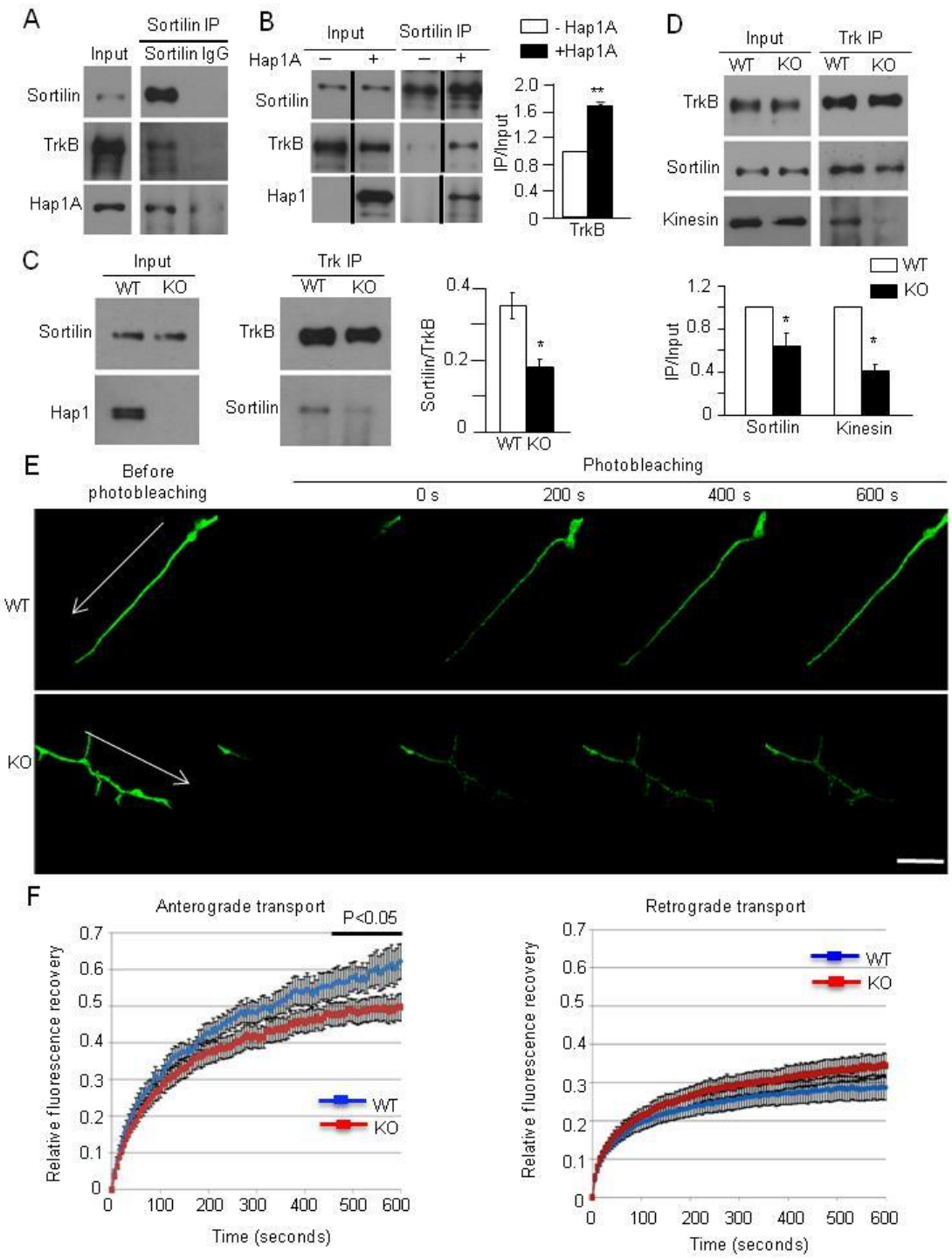
A
Sortilin IP
Input
Sortilin IgG
Sortilin
TrkB
Hap1A
B
Input
Sortilin IP
Hap1A
Sortilin
TrkB
Hap1
- Hap1A
+Hap1A
IP/Input
TrkB
D
Input
Trk IP
WT KO WT KO
TrkB
Sortilin
Kinesin
C
Input
Trk IP
WT KO
Sortilin
Hap1
TrkB
Sortilin
Sortilin/TrkB
WT KO
WT
KO
IP/Input
Sortilin
Kinesin
E
Before photobleaching
Photobleaching
0 s
200 s
400 s
600 s
WT
KO
F
Anterograde transport
P<0.05
Relative fluorescence recovery
Time (seconds)
WT
KO
Retrograde transport
Relative fluorescence recovery
Time (seconds)
WT
KO

Figure 3.14

Hap1 stabilizes sortilin association with TrkB and promotes TrkB transport. (**A**) Immunoprecipitation of endogenous sortilin in HEK293 cells showing the co-precipitation of transfected TrkB and Hap1A. (**B**) Co-expression of Hap1A increased the association of TrkB with sortilin after cells treated with BDNF (100 ng/ml for 30 min). The lanes were run on the same gel but were not contiguous. The ratio of precipitated to input (IP/input) is shown in 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 fluorescence recovery after photobleaching (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.

Figure 3.15

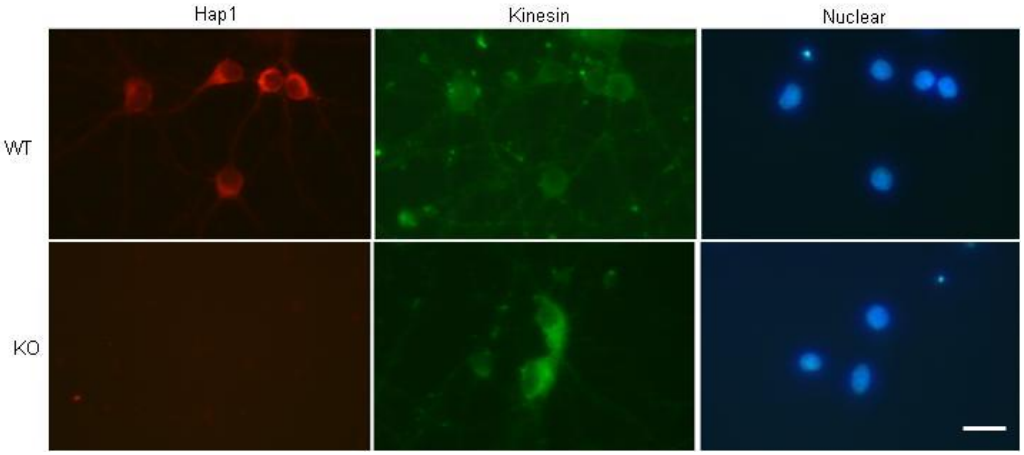

Figure 3.15

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.

Chapter 4

Differential roles of early postnatal and later stage Hap1 expression in hippocampal neurogenesis and depression

Abstract

It is known that Hap1 and Ahi1 stabilize each other by binding tightly together and forming the stigmoid bodies. Previous study revealed that mice with neuronal Ahi1 deficiency displayed depressive phenotypes. Because of the close relationship between Hap1 and Ahi1, we would like to investigate the effect of Hap1 KO at different ages on mouse depressive behaviors. Interestingly, we found that early postnatal, but not later stage Hap1 depletion led to depressive phenotypes when these mice became adults. The phenotypes can be recapitulated in adult camk2a-Hap1 conditional KO mice, in which Hap1 deletion occurs early postnatally in the forebrain. To further examine the mechanism underlying this phenomenon, we tested hippocampal neurogenesis after Hap1 depletion, and found that early Hap1 KO resulted in a reduction in BrdU incorporation as well as the ratio of newborn cells that differentiate into mature neurons in the dentate gyrus. Furthermore, although Hap1 deletion after early postnatal stage did not seem to affect neurogenesis and cause depressive behaviors under normal conditions, we showed that it could become detrimental under certain stress situations as Hap1 P21 KO mice showed reduced hippocampal neurogenesis and displayed depressive behaviors after repeated restraint stress. These results suggest that while early postnatal Hap1 expression is required for hippocampal neurogenesis, which may underlie depressive phenotypes of Hap1 KO mice, late postnatal and adult Hap1 expression might serve as a buffer mechanism to protect animals against stress induced depressive behaviors by maintaining hippocampal neurogenesis.

Introduction

Major depressive disorder is a leading cause of disability around the world (Tanti and Belzung, 2010). In the USA, the lifetime prevalence for major depression was estimated to be as high as 16.2% (Greenberg et al., 2003). Various symptoms are associated with depression including anhedonia, depressed mood, fatigue, increased stress sensitivity, thoughts of worthlessness, inappropriate guilt, helplessness, and other cognitive and metabolic abnormalities. According to Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-V), either depressed mood or anhedonia must be present to propose the diagnosis of depression (APA, 2013). Despite its wide influence, the cause of depression has not been made clear, nor have we come up with an effective treatment for it. To gain insight into its etiology, scientists asked if genetics could be a factor leading to depression. Twin Studies revealed that a patient's identical twin has a much higher risk than a patient's non-identical twin, a risk that was estimated to be about 40%, with the remaining 60% being due to environmental factors (Lesch, 2004). In light of this, several genome-wide linkage studies of major depression have been carried out in an effort to find the "depression genes" (Boomsma et al., 2008), however, the replication of candidate genes among many such studies was poor, and only a few genes were found to be truly associated with major depression, including *NPY* and *TNF* (Bosker et al., 2011), possibly due to the heterogeneity of the phenotype as well as the contextual genetic or environmental factors.

To better study the cause and the treatment of depression, animal models have been created to model the disease, and different behavioral tests have been used for assessing depressive phenotypes as well as the efficacy of drug treatment. Two most widely used behavioral tests are the forced swimming test (FST) and the tail suspension test (TST), both of which score the animal's passive response to a threat as being depressive-like behaviors. Previous work by Xu et al. found that neuronal deficiency of *Ahi1*, a disease gene for Joubert syndrome, led to a depressive-like phenotype in mice

(Xu et al., 2010). Due to the close relationship between Ahi1 and Hap1, we hypothesized that Hap1 deletion might also cause similar behavioral changes in mice. While both germline and neuronal Hap1 KO are lethal, our TM-induced Hap1 KO model not only allows us to test this hypothesis, but also brings another layer to it, which is to examine the effect of Hap1 KO at different ages on mouse depressive behaviors. This is also an important question to ask since many genes function differently in development and adulthood, and we have already discovered that early postnatal Hap1 depletion led to growth retardation and reduced survival, while late postnatal and adult depletion did not, indicating Hap1 has distinct roles in early vs. later stages.

Another question we would like to ask is whether Hap1 deletion affects hippocampal neurogenesis, which has emerged as the most popular theory of depression, largely due to the fact that many antidepressants work by enhancing hippocampal neurogenesis (Malberg, 2004; Malberg et al., 2000) and ablating adult neurogenesis reduces some of the behavioral effects of antidepressants (Santarelli et al., 2003). In chapter 3, we found that Hap1 regulates early postnatal hypothalamic neurogenesis, however, whether it also plays a similar role in hippocampal neurogenesis remains to be investigated. The expression level of Hap1 in the hippocampus is relatively low compared to the hypothalamus, but it has been found to be present in the hippocampal dentate gyrus (DG) where neurogenesis is actively taking place (Islam et al., 2012; Page et al., 1998), suggesting a possible link between Hap1 and hippocampal neurogenesis. Although adult neurogenesis is the primary focus of studies concerning depression, the importance of postnatal neurogenesis has also been raised, as it is capable of affecting depressive behaviors in adulthood (Brummelte et al., 2006; Hayashi et al., 2008; Lajud et al., 2013; Lajud et al., 2012). However, all these studies used either chemicals or maternal separation approach to model the relationship between postnatal neurogenesis and adult depression, and to our knowledge, a genetic model of this is still lacking.

In the current study, we tested behavioral phenotypes of depression in mice that were depleted of Hap1 at different ages. We found that only early postnatal Hap1 KO led to depressive like behaviors, which are also associated with reduced postnatal hippocampal neurogenesis. Moreover, although later stage Hap1 KO did not cause a depressive phenotype or neurogenesis defect under normal laboratory settings, it increased the susceptibility of stress induced depression later in life. The results suggest that Hap1 expression in early and later stages has differential roles in hippocampal neurogenesis and depression, and while Hap1 conditional KO mice might be useful as genetic models to study neurogenesis and depression, manipulating Hap1 level could also be considered for the treatment of depression.

Results

Early postnatal depletion of Hap1 leads to depressive behaviors in adult mice. Since mice with neuronal deficiency of Ahi1 showed depressive phenotypes as determined by FST and TST (Xu et al., 2010), we were interested to see if Hap1 KO mice display similar phenotypes, and how Hap1 deletion at different ages affects the behavioral outcomes. To test these ideas, we used TM to induce Hap1 KO at different postnatal stages, and when these mice grew to 2- to 3-month old, we subjected them to FST and TST. These two tests showed very similar results as early postnatal (P1, Figure 4.1 A, and P10, Figure 4.1 B), but not late postnatal (P15, Figure 4.1 C, and P21, Figure 4.1 D) or adult (Figure 4.1 E) depletion of Hap1 resulted in significant increases in immobility during the tests, which is considered depressive-like behavior. To further strengthen the idea that early postnatal Hap1 depletion leads to adulthood depressive phenotypes, we crossed Hap1 floxed mice with camk2a-cre transgenic mice and got Hap1 conditional KO mice in which Hap1 is deleted in camk2a expressing cells. Since camk2a is expressed early

postnatally to varying extents in the forebrain areas including the cortex, hippocampus, and hypothalamus, we expected these mice to behave in a somewhat similar way as the P1 or P10 KO mice. FST and TST confirmed this thought as camk2a-Hap1 KO mice at 2- to 3-month old indeed displayed greater immobility as compared to controls (Figure 4.1 F).

We also tested locomotor activity since the relationship between decreased or altered locomotion and depression was suggested in previous clinical studies (Aronen et al., 1996; Finazzi et al., 2010; Kim et al., 2013) and is well within the general conception of the manifestation of depression. Consistent with the previous findings, we showed that adult Hap1 P1 KO in general displayed lower locomotor activity as compared to controls (Figure 4.2 A). To rule out the possibility that our Hap1 KO mice had physical impairment, which could result in poor performances in depression tests as well as locomotor activity assay, we did rotarod test on these P1 KO mice, and found instead they performed significantly better than the controls (Figure 4.2 B). The enhanced performance in this demanding test could very likely be the result of the smaller body size of the KO mice, which allowed them to more readily balance and cling onto the rod. More importantly, it argues against the possibility that these KO mice were physically challenged to perform in the depression tests.

Imipramine, a tricyclic antidepressant, is used in the treatment of major depression, and can rapidly reduce depressive phenotypes in mice (Saarelainen et al., 2003; Xu et al., 2010). To examine if it can rescue the depressive phenotype in our mice, we delivered imipramine via i.p. and found that it substantially increased the mobility of camk2a-Hap1 KO mice in both FST and TST, more so than it did to the control mice (Figure 4.3 A,B). The mobility of mice after the treatment was not significantly different between the genotypes (Figure 4.3 A,B). To further validate the depressive phenotype,

we assessed anhedonia in camk2a-Hap1 KO mice by sucrose preference test. Anhedonia, or inability to experience pleasure is considered the core feature of major depression (Gorwood, 2008). In rodents, sucrose preference test is widely used to assess anhedonia based on the finding that depressed human patients had higher hedonic responses to sucrose solutions than controls (Amsterdam et al., 1987). We found in this test that camk2a-Hap1 KO mice displayed lower preference to 1% sucrose solution than controls (Figure 4.3 C). Measurements of fluid intake indicated that although water intake was not different between the genotypes, camk2a-Hap1 KO mice consumed significantly less sucrose solution and total fluid (Figure 4.3 C). Collectively, these results support a depressive phenotype caused by the early loss of Hap1.

Early postnatal Hap1 depletion affects hippocampal neurogenesis. Although many theories of depression exist, the neurogenesis theory has gained more and more popularity in recent years (Eisch and Petrik, 2012). A great number of studies have focused on the relationship between depression and hippocampal neurogenesis (Sahay and Hen, 2007), due to the fact that the hippocampus is a brain region involved in the control of mood and memory, and remains one of only a few sites active for adult neurogenesis. Since we have shown in the previous chapter that Hap1 regulates early postnatal hypothalamic neurogenesis, we would also like to examine if Hap1 also plays a role in hippocampal neurogenesis. Thus, we first injected BrdU into P6 Hap1 P1 KO and controls, and analyzed BrdU incorporation 24 hours later at P7. BrdU immunostaining and stereology quantification clearly showed that there was a decrease in proliferating cells in the hippocampal DG (Figure 4.4 A,B). We then performed the same assay on P34 Hap1 P1 KO and P21 KO mice, and found that decreased BrdU incorporation still existed in P1 KO DG, but was not seen in P21 KO DG as compared to controls (Figure 4.4 C,D),

suggesting that late postnatal depletion of Hap1 does not lead to impaired hippocampal neurogenesis. Since camk2a-Hap1 KO mice started to delete Hap1 in camk2a expressing cells in the early postnatal stage, and showed depressive phenotypes in adulthood, we went on to test these mice for BrdU incorporation. We found that as in the case for P1 KO mice, camk2a-Hap1 KO mice also had reduced BrdU+ cells in the DG (Figure 4.4 E), further supporting the important role of early postnatal Hap1 expression in hippocampal neurogenesis.

Neural progenitor cells (NPCs) when undergoing differentiation can give rise to both neuronal and glial cells. Besides a decreased number of proliferating NPCs as the number of BrdU+ cells indicated, we would also like to know if the differentiation of NPCs is also perturbed by the early postnatal loss of Hap1. Therefore, we injected BrdU into P6 Hap1 P1 KO and control mice, and sacrificed them 4 weeks later so that NPCs that incorporated BrdU at P6 would differentiate into either mature neurons or glia. By co-immunostaining BrdU with NeuN, a marker for mature neurons, or GFAP, a marker for mature astrocytes revealed that P1 deletion of Hap1 significantly affected neuronal differentiation, as the ratio of NeuN+ cells that were co-stained with BrdU dropped from about 70% in controls to 53% in P1 KO mice (Figure 4.4 F, left panel). In contrast, the ratio of GFAP+ cells that were co-stained with BrdU was increased in P1 KO mice (Figure 4.4 F, right panel). We think that this increase is not likely due to the direct effect of Hap1 in glial cell differentiation since Hap1 is not found to be expressed in glia (Gutekunst et al., 1998; Li et al., 1996), but rather a result of decreased neuronal differentiation that leads to a higher ratio of glial cell population in P1 KO mice.

Later stage Hap1 expression protects animals against stress induced depression by maintaining hippocampal neurogenesis. Since neural activities and signal transduction

are very different between developing brain and mature brain, it is not strange that many genes play different roles in these two stages. For example, Pax-genes play important roles in brain regionalization during development, but in the adult brain, they are expressed in discreet areas, and involved in the commitment of the precursor cells to different neuronal cell fates (Stoykova and Gruss, 1994). The role of Hap1 in animal growth and neurogenesis supports the notion that Hap1 is an essential gene for early postnatal development partly through regulating neurogenesis, but might play a different role in the adult brain. We wanted to test the idea that adult expressed Hap1 responds to stress in the regulation of hippocampal neurogenesis and animal behaviors since the hippocampus is a brain region known to be highly responsive to stress (Snyder et al., 2011). To this end, we used Hap1 P21 KO mice, which did not present gross phenotypes including impaired neurogenesis, and subjected them to 7-day repeated restraint stress. Consistent with our previous results, P21 KO mice under normal conditions did not have impaired hippocampal neurogenesis, however, after repeated stress, they showed significantly reduced hippocampal neurogenesis, much greater than that of the control mice (Figure 4.5 A,B). It has been known that repeated restraint stress suppresses hippocampal neurogenesis, but does not lead to depressive-like behaviors in WT rodents (Dunn and Swiergiel, 2008; Gregus et al., 2005; Luo et al., 2005; Pham et al., 2003). We believe that a certain level of neurogenesis has to be maintained in order for the animals to battle against potential behavioral changes caused by stress. While the deletion of Hap1 reduced hippocampal neurogenesis to a remarkably low level following stress, it is possible that it might have reached a point that animal behaviors could be affected. Assessed by FST, we found that after repeated restraint stress, Hap1 P21 KO mice exhibited a marked increase in immobility as compared to non-stressed KO and stressed control mice (Figure 4.5 C), suggesting that indeed adult expressed Hap1 participates in the maintenance of hippocampal neurogenesis in response to certain

types of stress, such as restraint stress, and thereby protects the animals against stress induced depressive like behaviors.

Discussion

Since its identification as the first known interacting protein of Htt, a number of studies have tried to reveal Hap1's role both physiologically as well as in the disease context. It has become more evident that neuronal expression of Hap1 is essential for the early postnatal survival of the mice, as both germline and neuronal Hap1 KO result in an early postnatal lethal phenotype (Chan et al., 2002; Li et al., 2003). However, whether the loss of Hap1 has any detrimental effect on adult mice was unclear. More importantly, whether Hap1 depletions induced at different ages have differential effects on adult animal behaviors remains to be investigated. The induced Hap1 KO model allowed us to examine this question by providing a sufficient number of adult mice that had Hap1 gene deleted at various stages. We were able to demonstrate that early postnatal Hap1 depletion leads to depressive like behaviors in the adult mice, a phenomenon that was also observed in neuronal Ahi1 KO mice (Xu et al., 2010), whereas later stage Hap1 depletion has no obvious effect on these behaviors.

Depression is a major mental disorder that affects hundreds of millions of people worldwide (Vos et al., 2012). Due to its wide spectrum of symptoms, many genetic and environmental factors may trigger the onset of a depressive episode. These episodes are normally observed in adults, but could be found as early as in childhood and adolescence, although in those cases, they are more difficult to diagnose, and oftentimes overlooked. However, the importance of early life experience to adult depression is accentuated by the fact that in the most significant cases of adult depression, some form of abuse was

experienced in the childhood (Springer et al., 2003). It is therefore apparent that environmental factors in early life could contribute significantly to adult depression, but whether genetic mutations also play a role in predisposing people with unpleasant early life experience, or even otherwise 'healthy' individuals to depression remains largely unknown. Our results using induced Hap1 KO mice demonstrated that early loss of a gene could indeed contribute to the etiology of depression, suggesting that early genetic diagnosis could potentially help with the prediction and early intervention of certain forms of depression.

Despite various known or unknown genetic and environmental causes of depression, there might be some common pathways that these factors work on to cause neurological symptoms. In recent years, neurogenesis has quickly become the favorite theory of depression due to the findings that many antidepressants function through enhancing adult hippocampal neurogenesis (Malberg, 2004; Malberg et al., 2000). The course of neurogenesis peaks at embryonic stage, and continues to occur actively in the postnatal brain. However, in the adult brain, neurogenesis is limited to a few discrete loci, and occurs far less actively (Ming and Song, 2011). Although adult hippocampal neurogenesis mediates the effect of many antidepressants, its ablation or reduction does not directly lead to depressive like behaviors in rodents (Jayatissa et al., 2010; Surget et al., 2008), suggesting that adult neurogenesis might not be the actual or sole cause of depression. However, a relationship between decreased postnatal neurogenesis and adult depression has been suggested by several studies using either chemicals or maternal separation as their approaches (Brummelte et al., 2006; Hayashi et al., 2008; Lajud et al., 2013; Lajud et al., 2012). The combined results may indicate that since postnatal neurogenesis is required for the maturation of the CNS after birth, its reduction might affect the connectivity of certain critical neural circuitries, which renders the animals susceptible to depression later in their lives. We showed in our study

that Hap1 P1 deletion significantly reduced hippocampal neurogenesis at an early postnatal stage, which then led to depressive behaviors in adults. Since these P1 KO mice also showed severe growth retardation and reduced survival, it was not known if factors other than hippocampal neurogenesis contributed to the observed phenotypes. Therefore, we used camk2a-Hap1 KO mice as another model for the study, which depletes Hap1 early postnatally, mainly in the cortex and hippocampus. These mice as compared to P1 KO mice, had substantially less severe growth and survival phenotypes, yet exhibited a postnatal neurogenesis defect as well as adult depressive behaviors, indicating that reduced postnatal neurogenesis is more likely to be the factor leading to the depressive phenotypes. In conclusion, we provided by far the first genetic model that links postnatal neurogenesis to adult depression, which has great influence on our knowledge about depression as well as its potential therapeutic interventions.

The data from both previous and current chapters indicate that Hap1 is an essential gene for early postnatal neurogenesis, but seems to be dispensable in adults. However, mice used for experiments were raised in standard housing conditions, which are far different from all the stress and threats animals would experience in the wild. Therefore, a lot of conclusions made with these types of settings need to be interpreted with caution, and may not reflect the real function of the genes studied. In our case, although Hap1 depletion in adult did not lead to overt phenotypes, its expression might be required under certain stress conditions. Also, despite a lack of a depressive phenotype caused by ablation of adult hippocampal neurogenesis, it has been found that stress downregulates adult hippocampal neurogenesis, and adult-born hippocampal neurons are required for the regulation of the hypothalamic–pituitary–adrenal (HPA) axis in buffering stress induced depressive behaviors (Eisch and Petrik, 2012; Mirescu and Gould, 2006; Snyder et al., 2011). Thus, an investigation of whether adult Hap1 expression influences hippocampal neurogenesis and depressive behaviors under stress

situation was of great interest to us. We chose Hap1 P21 KO mice to study this question as the background influence of early Hap1 loss on adult hippocampal neurogenesis and depressive phenotype is eliminated in these mice, therefore the differences seen between genotypes would be a sole result of their differential responses to stress. We found that 7-day repeated restraint stress was able to diminish adult hippocampal neurogenesis in P21 KO mice more dramatically than the controls, indicating that Hap1 expression is required for maintaining the level of hippocampal neurogenesis in response to stress. Moreover, P21 KO mice exhibited significantly elevated immobility in FST after stress as compared to non-stressed KO and stressed control mice, suggesting that adult expressed Hap1 serves an important role in maintaining a proper level of hippocampal neurogenesis needed for the animals to buffer against stress induced changes in behaviors.

In conclusion, our study revealed differential roles of Hap1 expression in early postnatal vs. later stage. While early loss of Hap1 remarkably affected postnatal hippocampal neurogenesis, and led to adult depressive phenotypes, later stage Hap1 depletion diminished hippocampal neurogenesis in response to stress, which then caused weakened ability of the animals to maintain normal behaviors. Together, we provided a novel genetic model to study the relationship between postnatal neurogenesis and depression, as well as stress and its induced neurogenesis and behavioral deficits. Furthermore, it is worth considering the idea that upregulating Hap1 level or its signaling might have a beneficial effect for the treatment of depression.

Materials and Methods

Animals. Mice were housed in the Division of Animal Resources at Emory University on a 12-h light/dark cycle. All procedures and husbandry were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Generation of conditional Hap1 KO mice, in which exon1 of the mouse *Hap1* gene is flanked by two loxP sites to allow the Cre-mediated deletion of exon1, was described in our recent study (Lin et al., 2010). Transgenic mice expressing Cre under the control of the mouse calcium/calmodulin-dependent protein kinase II alpha (camk2a) promoter were obtained from Dr. Stephen Warren's lab (Emory University). Camk2a-Hap1 KO mice were generated by crossing the floxed Hap1 mice with camk2a-Cre transgenic mice. Conditional Hap1 KO mice were generated by crossing the floxed Hap1 mice with Cre-ER transgenic mice (The Jackson Lab, B6.Cg-Tg(CAG-cre/Esr1)5Amc/J), which have a tamoxifen-inducible cre-mediated recombination system driven by the chicken beta actin promoter/enhancer coupled with the cytomegalovirus (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 tamoxifen.

Tamoxifen (TM) induction in mice. TM (Sigma T5648) 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 or P10 pups were injected subcutaneously with 1.1 mg or 2.2 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 4929nt to 5003nt) using the forward (5'- TTT TTC TGG GGA GCA TAC GTC-3') and reverse (5'-

ATC CGT TAT CCC AGG GTC TGA-3') primers. Primers (forward: 5'- GCG GTC TGG CAG TAA AAA CTA TC -3' and reverse: 5'- TGT TTC ACT ATC CAG GTT ACG G -3') that amplify Cre recombinase were also used to determine the presence of Cre.

BrdU incorporation assay. For BrdU injection into Hap1 P1 KO mice and controls, mice at P6 were i.p. injected with 50 mg/kg body weight BrdU. The animals were perfused and fixed 24 hours later for the analysis of the number of proliferating cells, or 4 weeks later for the analysis of differentiation into neuronal or glial cells. For BrdU injection into camk2a-Hap1 KO or P21 KO mice and controls, mice at P33 were i.p. injected with 50mg/kg body weight BrdU. 24 hours later, the mice were perfused and fixed for analyzing the number of proliferating cells.

Immunofluorescence and microscopy. 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 sunk, 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 antibodies against BrdU (Accurate Chemical), NeuN (Chemicon), or GFAP (Millipore) 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 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.

Stereology and quantification. We examined at least 3 control mice and 3 Hap1 KO mice for each comparison. To quantify BrdU+cells, the optical-fractionator method was used, as implemented in the semiautomatic stereology system StereoInvestigator 5.4.3 (MicroBrightField). Target brain regions were cut in 40 μm serial sections in which every fifth section was used for analysis. BrdU+ cells were counted on each section. The volume of the target region was determined by the StereoInvestigator software by using traced target area for each section and the distance between sections sampled. The total number of BrdU+ cells in the target region calculated by the software was then divided by the volume to yield BrdU+ cell density presented as number of BrdU+ cells in a cubic millimeter 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.

Forced swimming test (FST). Mice were placed individually into a round plastic cylinder (18 cm in height, 15 cm in diameter) filled with water (25 °C) at a depth of 10 cm. Immobility time, defined as floating or the absence of active behaviors such as swimming or struggling to escape, was measured. Slight movements of the feet and tail necessary to keep the head above water were excluded as mobility. Each mouse was measured for 6

min by an investigator who was kept blind to the genotypes of the mice and drug treatment. No pretest training of mice was performed.

Tail suspension test (TST). Briefly, mice were suspended by taping the tail (~1 cm from tip of tail) to a horizontal bar at a height of 40 cm from the table surface for 6 min. The trial was conducted for a duration of 6 min and the immobility time was recorded manually via stopwatch by a trained observer who was blind to the genotypes of the mice examined. Mice were considered immobile when hung passively and motionlessly without escape-oriented behaviors.

Locomotor activity. Locomotor activity was measured using an automated system (San Diego Instruments, La Jolla, CA) with photobeams that record ambulations (consecutive beam breaks). Mice were individually placed in the chambers under 12-h light-dark cycle with free access to food and water. Mice were allowed 4 h to acclimate to the new environment before recording. Activities were recorded every 30 min for 24 h.

Rotarod test. Motor activity was evaluated using Rotamex (Columbus Instruments). Mice were trained on a rotating rod at a speed of 5 rpm for three 5-min trials on 3 consecutive days. Testing was performed on the fourth day. During the test, the rotating rod was gradually accelerated to 40 rpm over 5 min. Latency to fall from the rotarod was recorded in 3 trials, and the average of the 3 trials was used for each mouse.

Imipramine treatment. Tricyclic antidepressant imipramine (30 mg/kg, sigma) was freshly made in saline and i.p. injected into the mice 30 min before the FST or TST. Saline injection was used as vehicle treatment.

Sucrose preference test. Sucrose preference test was done as previously described (Snyder et al., 2011), with modification. Briefly, mice were individually housed with free access to food and two weighed bottles of liquid: one filled with water, the other with 1% sucrose solution. The positions of the two bottles were balanced across animals. After 3 days of acclimation, both bottles were removed and weighed at 12 pm, and put back in reversed positions at 7 pm. The bottles were weighed again in 1 h for an acute test, and again on the ensuing morning for overnight test. Sucrose preference was calculated as $(\Delta weight_{sucrose})/(\Delta weight_{sucrose} + \Delta weight_{water}) \times 100$.

Restraint stress. Mice were subjected to repeated restraint stress by placement for 4 h per day for 7 consecutive days in ventilated 50 ml conical tubes. After each stress session, mice will be immediately returned to their home cages. Control mice will be housed in separate cages from the stressed mice, and will be deprived of food and water but otherwise untouched during each session. 24 h after the final session, mice were evaluated by FST. For neurogenesis analysis, BrdU (100mg/kg body weight) was i.p. injected before the stress session on each of the last 3 days. 24 h after the final session, mice were perfused and fixed, the brains were then sectioned for BrdU immunostaining.

Statistical analysis. All data are expressed as mean ±SEM. The statistical significance was determined using one-way analysis of variance between groups (ANOVA) with SPSS 10.0 software. A value of $P<0.05$ was considered statistically significant.

Figure 4.1

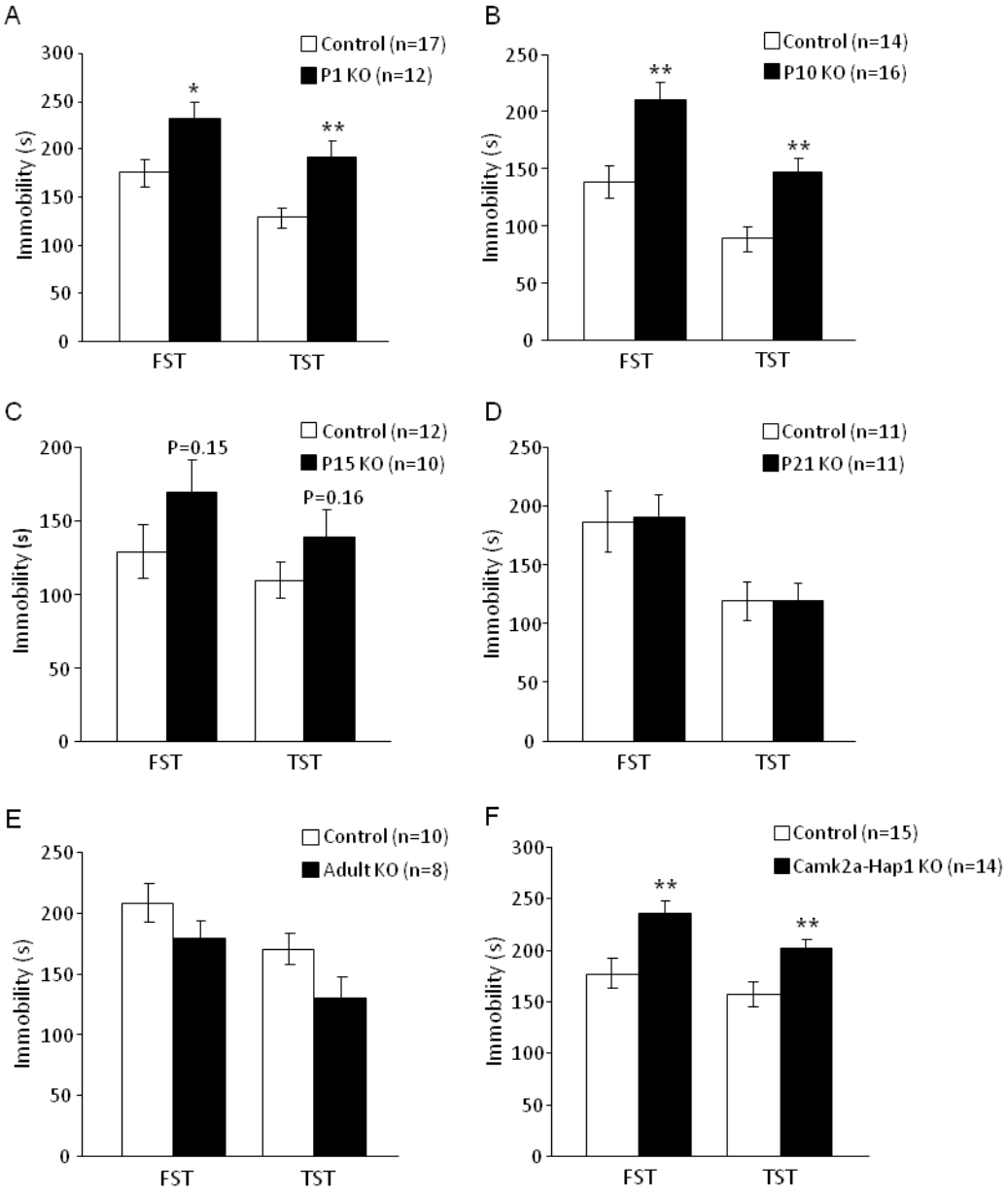
A
Control (n=17)
P1 KO (n=12)
Immobility (s)
300
250
200
150
100
50
0
*
**
FST
TST
B
Control (n=14)
P10 KO (n=16)
Immobility (s)
250
200
150
100
50
0
**
**
FST
TST
C
Control (n=12)
P15 KO (n=10)
Immobility (s)
200
150
100
50
0
P=0.15
P=0.16
FST
TST
D
Control (n=11)
P21 KO (n=11)
Immobility (s)
250
200
150
100
50
0
FST
TST
E
Control (n=10)
Adult KO (n=8)
Immobility (s)
250
200
150
100
50
0
FST
TST
F
Control (n=15)
Camk2a-Hap1 KO (n=14)
Immobility (s)
300
250
200
150
100
50
0
**
**
FST
TST

Figure 4.1

Early postnatal depletion of Hap1 leads to depressive behaviors in adult mice. Forced swimming test (FST) and tail suspension test (TST) were performed on 2- to 3-month old Hap1 P1 KO mice (**A**), P10 KO mice (**B**), P15 KO mice (**C**), P21 KO mice (**D**), adult KO mice (**E**), and camk2a-Hap1 KO mice (**F**). Note that early postnatal Hap1 depletion, but not late postnatal or adult depletion led to increased immobility in FST and TST. *P<0.05; **P<0.01.

Figure 4.2

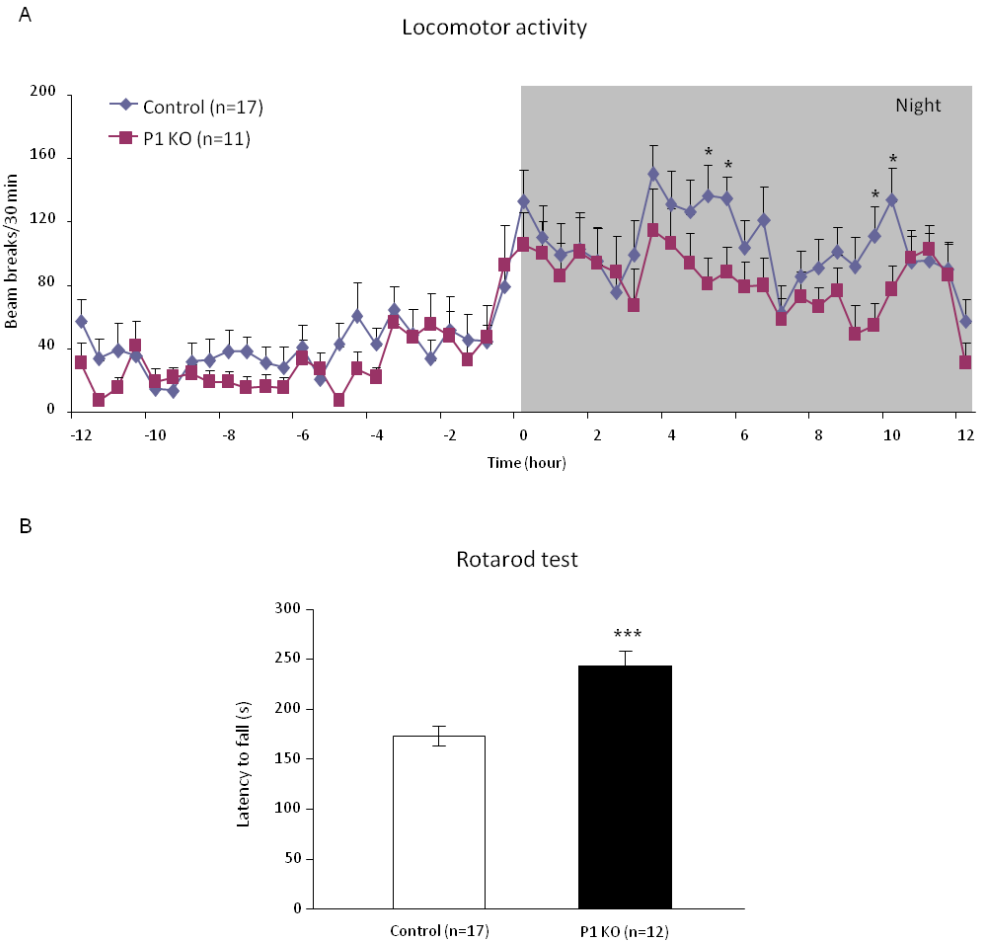
A
Locomotor activity
Control (n=17)
P1 KO (n=11)
Night
Beam breaks/30 min
Time (hour)
B
Rotarod test
Latency to fall (s)
***
Control (n=17)
P1 KO (n=12)

Figure 4.2

Decreased locomotor activity and increased rotarod performance of Hap1 P1 KO mice. (**A**) locomotor activities of 2-month old Hap1 P1 KO and control mice were monitored and recorded for 24 h. The KO mice showed decreased locomotor activity. *P<0.05. (**B**) Rotarod analysis of 2-month old Hap1 P1 KO and control mice. The KO mice showed increased rotarod performance. ***P<0.001.

Figure 4.3

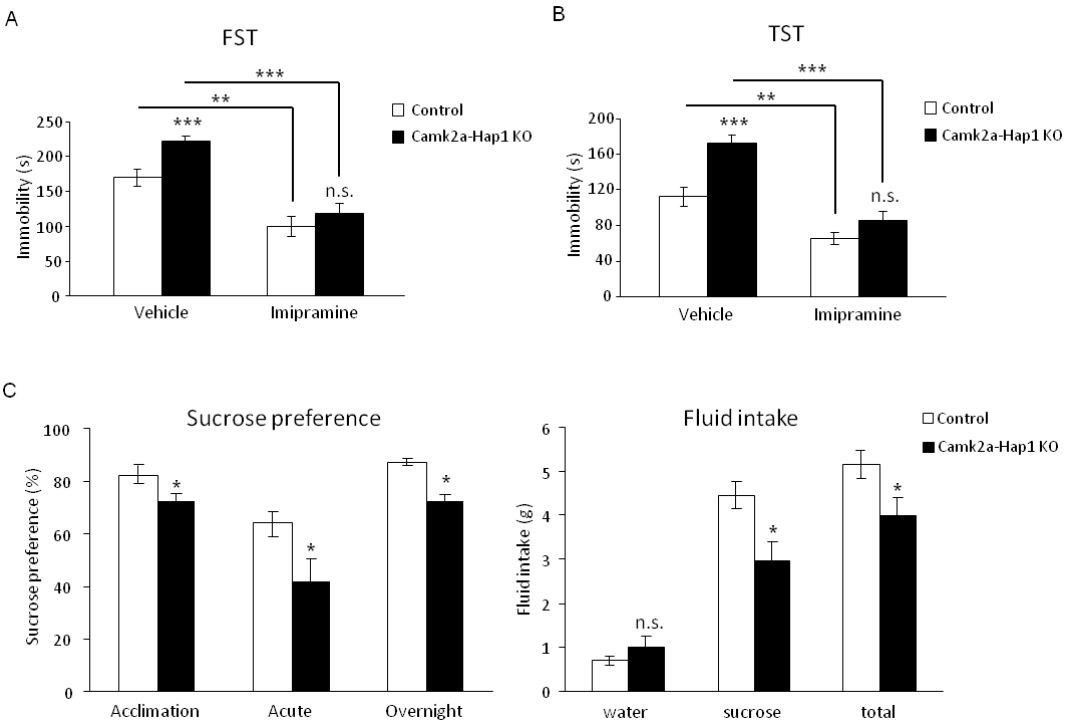
A
FST
***
**
***
n.s.
Immobility (s)
250
200
150
100
50
0
Vehicle
Imipramine
Control
Camk2a-Hap1 KO
B
TST
***
**
***
n.s.
Immobility (s)
200
160
120
80
40
0
Vehicle
Imipramine
Control
Camk2a-Hap1 KO
C
Sucrose preference
Sucrose preference (%)
100
80
60
40
20
0
*
*
*
Acclimation
Acute
Overnight
Fluid intake
Fluid intake (g)
6
5
4
3
2
1
0
n.s.
*
*
water
sucrose
total
Control
Camk2a-Hap1 KO

Figure 4.3

Validation of depressive phenotypes. Imipramine (30mg/kg) was i.p. injected 30 min before forced swimming test (**A**) and tail suspension test (**B**) on 4-month old camk2a-Hap1 KO mice and controls. n= 10-13 per group. The depressive phenotypes of the KO mice were rescued by the drug treatment. (**C**) Sucrose preference was assessed during acclimation and in acute and overnight tests. 2-month old camk2a-Hap1 KO mice showed reduced preference as compared to controls. Fluid intake was also measured for overnight test. Camk2a-Hap1 KO mice consumed less sucrose solution than controls. n=11-12 per group. n.s., not significant; *P<0.05; **P<0.01; ***P<0.001.

Figure 4.4

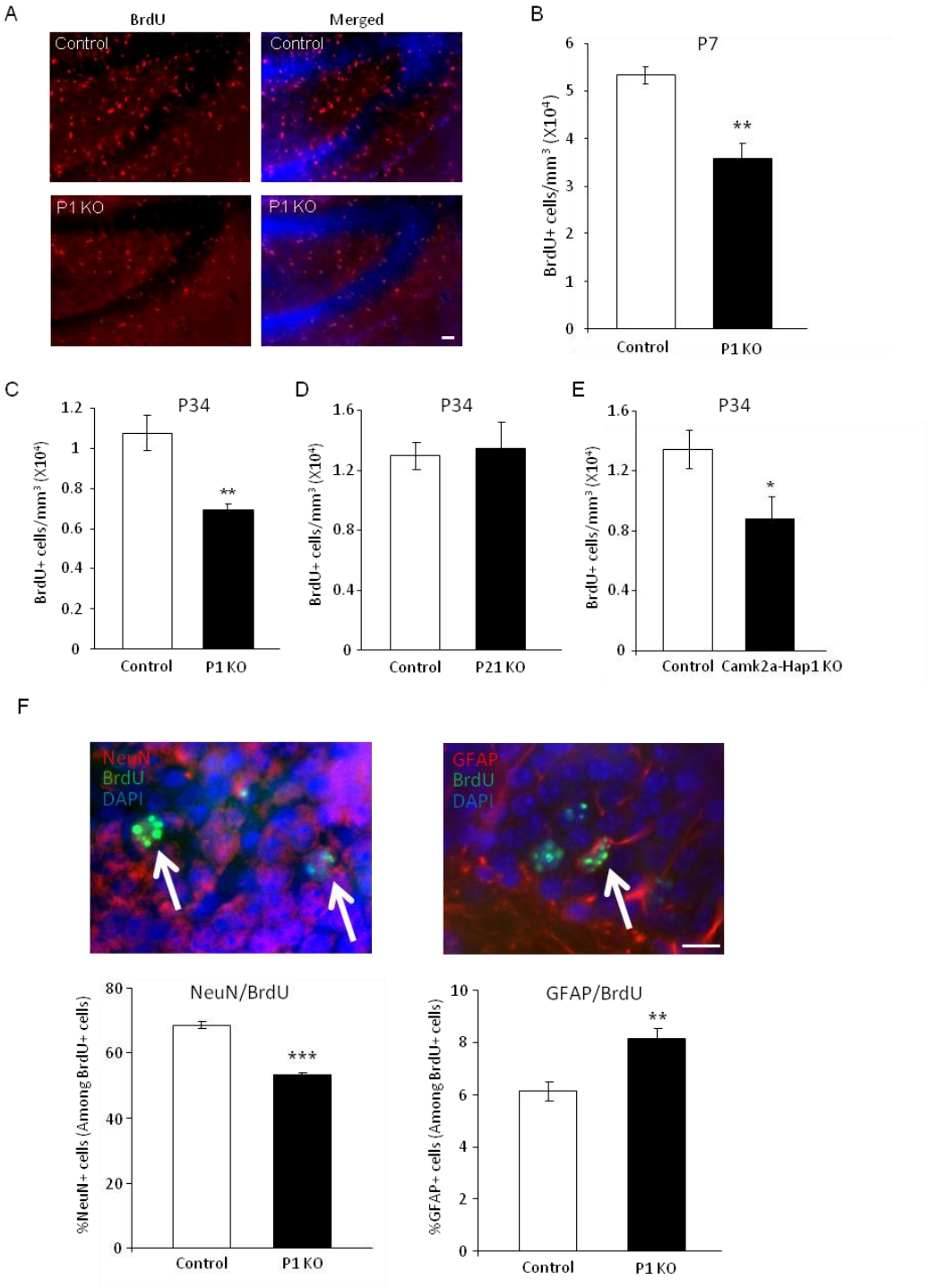
A
BrdU
Merged
Control
Control
P1 KO
P1 KO
B
P7
BrdU+ cells/mm³ (X10⁴)
Control
P1 KO
C
P34
BrdU+ cells/mm³ (X10⁴)
Control
P1 KO
D
P34
BrdU+ cells/mm³ (X10⁴)
Control
P21 KO
E
P34
BrdU+ cells/mm³ (X10⁴)
Control
Camk2a-Hap1 KO
F
NeuN
BrdU
DAPI
GFAP
BrdU
DAPI
NeuN/BrdU
%NeuN+ cells (Among BrdU+ cells)
Control
P1 KO
GFAP/BrdU
%GFAP+ cells (Among BrdU+ cells)
Control
P1 KO

Figure 4.4

Early postnatal Hap1 depletion leads to reduced hippocampal neurogenesis. (**A**) Hap1 P1 KO and control mice were injected with BrdU at P6, and perfused at P7 for immunostaining of BrdU in the DG of the hippocampus. P1 KO mice showed significantly fewer BrdU+ cells in the DG. Scale bar: 10 μm. (**B**) Stereological quantification of BrdU+ cells in **A**. n=4 per genotype. (**C**) Stereological quantification of BrdU+ cells in the DG of P34 Hap1 P1 KO and control mice that were injected with BrdU at P33. n=4 for control, n=5 for KO. (**D**) Stereological quantification of BrdU+ cells in the DG of P34 Hap1 P21 KO and control mice that were injected with BrdU at P33. n=4 per genotype. (**E**) Stereological quantification of BrdU+ cells in the DG of P34 Camk2a-Hap1 KO and control mice that were injected with BrdU at P33. n=5 for control, n=4 for KO. (**F**) Quantification of the ratios of NeuN+ (left panel) and GFAP+ cells (right panel) in BrdU+ cells. n=4 per genotype. Double staining images of NeuN+/BrdU+ and GFAP+/BrdU+ cells are also presented. Arrows indicate double positive cells. Scale bar: 10 μm. *P<0.05; **P<0.01; ***P<0.001.

Figure 4.5

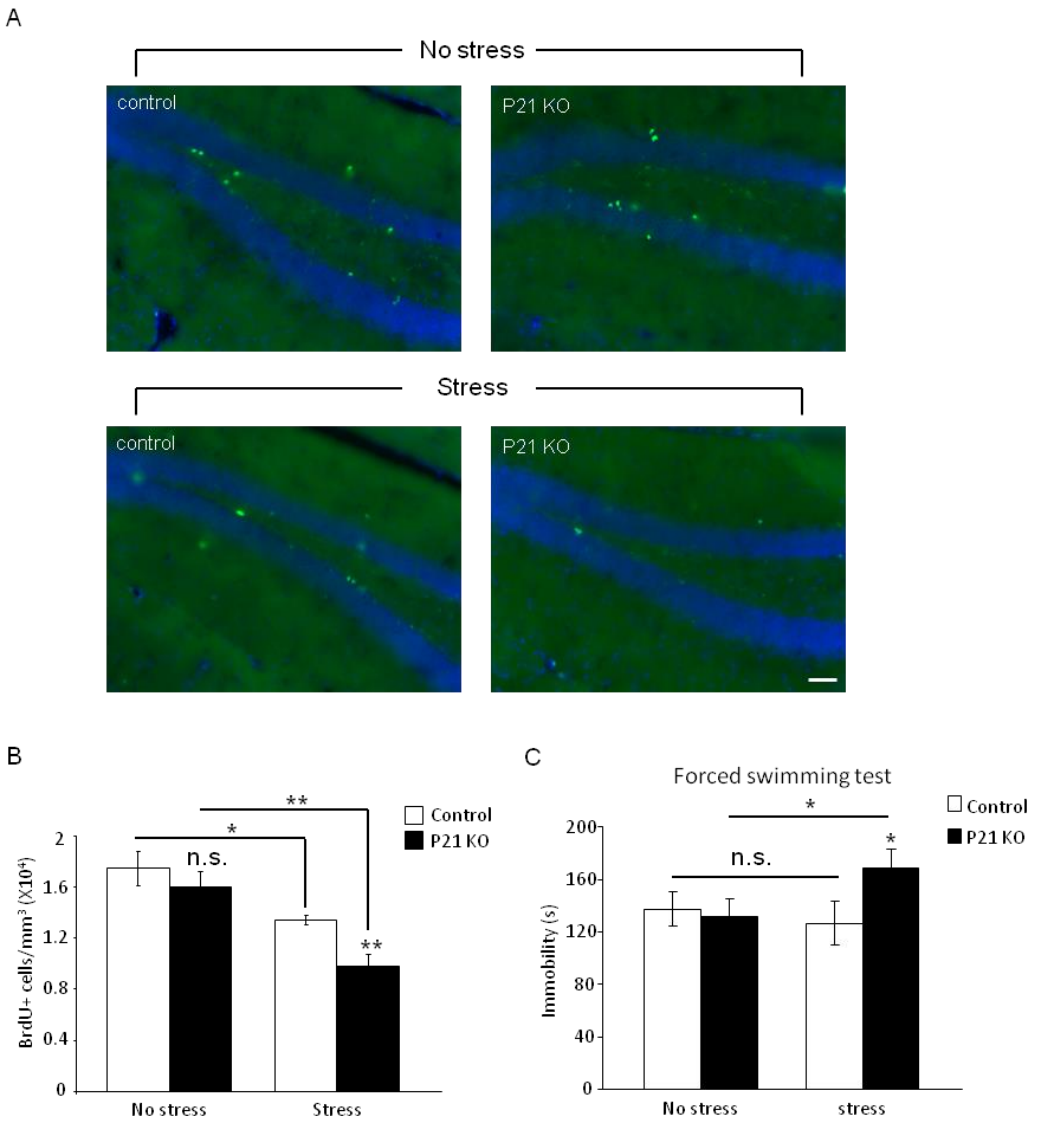
A
No stress
control
P21 KO
Stress
control
P21 KO
B
Control
P21 KO
n.s.
*
**
**
BrdU+ cells/mm³ (X10⁴)
2
1.6
1.2
0.8
0.4
0
No stress
Stress
C
Forced swimming test
Control
P21 KO
n.s.
*
*
Immobility (s)
200
160
120
80
40
0
No stress
stress

Figure 4.5

Adult Hap1 expression protects mice from stress induced depressive behavior by maintaining hippocampal neurogenesis. (**A**) 1-year old Hap1 P21 KO and control mice were subjected to 4 h restraint stress for 7 consecutive days. BrdU was i.p. injected in the final 3 days of the treatment. Immunostaining of BrdU in the DG was performed 1 day after the final treatment. Scale bar: 20 μm. The stressed groups showed decreased BrdU+ cells as compared to non-stressed groups, and the decrease was more dramatic in stressed KO mice than controls. (**B**) Stereological quantification of BrdU+ cells in **A**. n=3-4 per group. (**C**) Forced swimming test was conducted 1 day after the final treatment. n=9-11 per group. Note, while stressed control mice did not exhibit depressive behavior as compared to non-stressed controls, stressed KO mice displayed significant depressive behavior as compared to stressed control and non-stressed KO mice, a phenomenon that might be explained by the reduced hippocampal neurogenesis. *P<0.05; **P<0.01.

Chapter 5

General conclusions and future directions

General conclusions

In this study, we set out to define the role of Hap1 in different ages of mice. Previous results using germline and neuronal Hap1 KO suggested that Hap1 is essential for mouse early postnatal development (Chan et al., 2002; Li et al., 2003). However, the lack of these KO mice surviving into adults impeded a complete examination of Hap1's role at all stages including adulthood. In our study, we utilized TM inducible Cre-ER recombination system to precisely control the time that Hap1 gene will be deleted. This system allowed Hap1-floxed mice with Cre to express Hap1 as the WT mice, and thus live normally until they were given TM to delete Hap1 from the genome. The successful depletion of Hap1 upon TM delivery via i.p. was verified by western blotting. These mice that had Hap1 deleted at different ages were characterized for gross phenotypes as well as neurological and behavioral phenotypes.

Retarded growth and early death were two remarkable phenotypes observed in germline Hap1 KO mice. Since these mice were born in consistent with Mendelian ratio, and appeared to be just like WT at the time of birth, we believed that early postnatal Hap1 expression is critical for feeding and growth after birth, and thus knocking out Hap1 early postnatally would somehow mimic what we had seen in the germline KO mice. The results we got supported our hypothesis as we were able to show that Hap1 P1 KO mice exhibited both severe retarded growth and early death although some of them managed to survive into adults probably due to the fact that complete deletion of Hap1 and turnover of pre-existing Hap1 mRNA and protein take a few days after TM injection, thus the residual Hap1 might just be enough to help them live past early postnatal days, which is the most critical stage for their survival.

To answer whether this critical role in animal growth and survival is also assumed by Hap1 at late postnatal and adult stages, we deleted Hap1 at P15, P21 and 2-month of

age. We found that P15 KO led to a modest retardation in body weight gain, and a much lower lethality as compared to P1 KO mice. However, P21 KO and adult KO did not present any noticeable phenotypes in either growth or survival. These results strongly indicate that Hap1 is essentially and selectively involved in the regulation of animal growth and survival at an early postnatal stage.

The selective effect of Hap1 KO led us to investigate whether Hap1 participates in postnatal neurogenesis, which is an active process that affects the maturation and functionality of the brain. Neurospheres derived from Hap1 KO mouse brains gave rise to fewer neurons upon differentiation as compared to those from WT mouse brains. The number of astrocytes, however, was not changed between the two genotypes, indicating a selective defect of neuronal differentiation in Hap1 KO brains. To test this in vivo, we injected BrdU subcutaneously, and found that the incorporation of BrdU was considerably reduced in Hap1 germline or P1 KO brains, especially in the hypothalamus, which expresses Hap1 most abundantly. Western blotting analysis also confirmed the defective neuronal differentiation in the KO hypothalamus, as markers of neuronal lineage showed reduced expression, whereas those of glial lineage did not show any difference. In contrast, depleting Hap1 in adults did not cause any reduction in neurogenesis, which is consistent with a lack of an obvious phenotype in adult KO mice. Together, these data indicate that neurogenesis, but not gliogenesis, was affected upon early loss of Hap1, suggesting that Hap1 is a key regulator for early postnatal neurogenesis of the brain, especially the hypothalamus.

We next tried to identify what types of neurons were affected in Hap1 KO hypothalamus. Since Hap1 is involved in the trafficking and stabilization of a number of membrane receptors (Kittler et al., 2004; Li et al., 2002; Rong et al., 2006; Sheng et al., 2008; Xu et al., 2010), we examined the level of several receptors that are highly

expressed in the brain. Interestingly, we found that NPYY1R, but not GluR2/3 was reduced in Hap1 KO hypothalamus. Another type of neuronal cells that express calbindin was also unaffected. These results suggest that Hap1 selectively affects the neuronal differentiation of NPCs into certain types of neurons, e.g., NPYY1R+ cells. Because NPY/NPYY1R signaling is critical for energy balance and feeding behavior (Eva et al., 2006; Gehlert, 1999; Kanatani et al., 2001; Kanatani et al., 2000), the loss of NPYY1R+ cells may greatly contribute to the phenotypes of Hap1 KO mice.

As BDNF/TrkB signaling is important for neurogenesis and cell survival (Castren and Rantamaki, 2010; Kozisek et al., 2008; Moyse et al., 2006), and Hap1 deficiency affects Trk signaling and reduces the survival of cultured hypothalamic neurons (Rong et al., 2006; Sheng et al., 2008), we were interested to see if impaired TrkB signaling would lead to a neurogenesis defect in the hypothalamus. We first determined by western blotting that TrkB, its activated form, pTrkB, and the downstream signaling molecule pAKT were all reduced in Hap1 KO hypothalamus. To confirm that this reduced TrkB signaling indeed causes neurogenesis defect, we injected recombinant human BDNF, the ligand for TrkB, directly into the third ventricle, which is adjacent to the hypothalamus. Surprisingly, an acute treatment of BDNF was able to completely rescue neurogenesis in Hap1 KO hypothamus as determined by BrdU incorporation assay, which strongly suggests that Hap1 maintains hypothalamic neurogenesis through stabilizing TrkB level and its signaling.

To gain more mechanistic insight into how Hap1 stabilizes TrkB level, we did series of in vitro and in vivo experiments, and found that Hap1 stabilizes endocytic TrkB by inhibiting its lysosomal degradation. This is achieved through another molecule, sortilin, which is an intracellular sorting protein that has multiple functions. We demonstrated that Hap1 stabilizes the complex of TrkB and sortilin, which keeps TrkB

from being sent to the lysosomes for degradation. In addition, the interaction between TrkB and kinesin was also disrupted in Hap1 KO hypothalamus, and as a result, we were able to show an impaired anterograde, but not retrograde trafficking of TrkB in cultured olfactory bulb neurons derived from Hap1 KO mice. The in vitro culture of Hap1 KO hypothalamic neurons is very difficult, and it is hard to obtain Hap1 KO hypothalamic neurons with long neurites for trafficking study, therefore, whether the same results can be observed in hypothalamic neurons remains unknown. However, the above data suggest that upon ligand binding, internalized TrkB can be bound to a complex consisting of Hap1, sortilin and kinesin, which directs anterograde TrkB trafficking to the postsynaptic membrane for continued signaling events (Figure 5.1). In the absence of Hap1, the formation of the above complex is attenuated, thus more TrkB would be delivered to lysosomes for degradation, which results in less membrane bound TrkB and diminished signaling (Figure 5.1). Nevertheless, the complex nature and regional differences of the brain made us believe that Hap1's function regarding TrkB could be brain region and cell type specific. In brain areas or cells that express less Hap1, other molecules might take on the role that Hap1 plays in the hypothalamus and direct TrkB intracellular trafficking.

To sum up, we demonstrated that Hap1 regulates the proliferation of NPCs and their differentiation into certain types of neurons, e.g., NPYY1R+ cells through stabilizing BDNF/TrkB signaling in the hypothalamus. However, whether in Hap1 KO mice, the growth retardation and early death phenotypes can be attributed to the decreased hypothalamic BDNF/TrkB signaling, and the consequent loss of NPYY1R+ neurons still requires further investigation. Previous studies, however, suggest that this might be quite a novel finding if proved to be the case. Although complete deletion of BDNF or TrkB is early postnatal lethal (Ernfors et al., 1994; Klein et al., 1993), multiple lines of evidence indicated that reducing BDNF or TrkB expression leads to hyperphagia

and obesity in adult mice. Their approaches include generating heterozygous BDNF mice (Kernie et al., 2000; Lyons et al., 1999), mice that express TrkB at a quarter of the normal amount (Xu et al., 2003), conditional knockout of BDNF in the forebrain (Rios et al., 2001), specific deletion of BDNF in hypothalamic nuclei of adult mice (Unger et al., 2007), and ablation of TrkB in certain brain regions including multiple nuclei of the hypothalamus (Liao et al., 2013). Despite all the above evidence, we have so far yet to have a mouse model that reduces BDNF or TrkB level specifically in the hypothalamus from an early postnatal stage. We believe that BDNF/TrkB in early postnatal hypothalamus participates in the regulation of neurogenesis, which has profound influence on feeding and growth. Since BDNF has been reported to be highly expressed in the hypothalamus during early postnatal days and needed for postnatal maturation of cholinergic neurons (Katoh-Semba et al., 1997; Kim et al., 2007; Ward and Hagg, 2000), and knocking out either BDNF or TrkB resulted in a loss of sensory neurons (Ernfors et al., 1994; Perez-Pinera et al., 2008), it is very likely that BDNF/TrkB has a distinctive and essential role during early postnatal hypothalamic development, much like Hap1. Deletion of BDNF or TrkB in broader brain regions might have compensatory effects that somehow block the regional specific consequences of hypothalamic BDNF/TrkB KO. Similarly, a lack of early growth phenotype in NPYY1R deficient mice (Kushi et al., 1998) could also be a result of compensatory effects of the systemic loss of NPYY1R. Since Hap1 is highly enriched in the hypothalamus, Hap1 KO mice might turn out to be a better model to study the specific function of BDNF/TrkB and NPY/NPYY1R signaling in early postnatal hypothalamus.

To find out what could happen in adult if Hap1 is deleted at different ages, we tested depressive behaviors in adult mice that had Hap1 deleted at various postnatal days. What we found was an age dependant effect of Hap1 depletion. Knocking out Hap1 in early postnatal days led to dramatic increase in adult depressive behaviors as assessed by

FST and TST, whereas depleting Hap1 after P21 has no effect on these behaviors. The depressive phenotype was also observed in mice with neuronal Ahi1 deficiency (Xu et al., 2010), since Hap1 and Ahi1 bind tightly together and regulate the level of each other, we think that they might function together in the developing brain, which strengthens the mental solidity of the animals to fend off adult depression.

Hippocampal neurogenesis has emerged as the favorite theory to explain depression, however, a causal relationship between adult hippocampal neurogenesis and depression has not been established. Since neurogenesis happens more dynamically in early postnatal brain, and we have shown that Hap1 regulates postnatal hypothalamic neurogenesis, we hypothesized that the reduced postnatal hippocampal neurogenesis could contribute to adult depressive behaviors. Our results clearly suggest a correlation between early postnatal hippocampal neurogenesis and adult depression as early postnatal deletion of Hap1 reduced hippocampal neurogenesis and led to adult depressive behaviors while later stage Hap1 deletion did not. Because Hap1 KO affects neurogenesis in many brain regions as our results have already demonstrated, it is possible that the effect of Hap1 deletion in other parts of the brain, e.g., the hypothalamus also contributes to the depressive phenotype. To address this, we used another conditional Hap1 KO mouse model, camk2a-Hap1 KO which deletes Hap1 from an early postnatal stage mainly in the cortex and hippocampus. Analyses of behavioral phenotypes and hippocampal neurogenesis revealed that these mice had decreased hippocampal neurogenesis and developed adult depressive behaviors without experiencing severe growth retardation. These results suggest that hippocampal neurogenesis defect caused by the early loss of Hap1 may indeed lead to depressive behaviors in adult mice.

The obvious differences between wild and laboratory mice led us to investigate whether Hap1 may regulate adult hippocampal neurogenesis in response to stress. To this end, we subjected Hap1 P21 KO mice to 7-day repeated restraint stress, and found that although both P21 KO and control mice showed decreased hippocampal neurogenesis after stress, the reduction in P21 KO mice was far more dramatic. It is known that under certain stress conditions, adult-born hippocampal neurons become critical in buffering stress induced behavioral changes (Eisch and Petrik, 2012; Mirescu and Gould, 2006; Snyder et al., 2011). Therefore, we hypothesized that the unusually suppressed hippocampal neurogenesis following stress in P21 KO mice may compromise their ability to buffer stress, which could result in depressive behaviors. FST revealed that while control mice remained capable of behaving normally in this test after 7-day stress, P21 KO mice displayed much increased immobility, suggesting a declined ability to cope with stress. These results suggest that adult expressed Hap1 may not be dispensable as it seems, its role shifts from being essential regulator of early postnatal brain development to a buffer mechanism against stress by maintaining neurogenesis in the adult brain.

In summary, we have demonstrated that Hap1 plays critical role in regulating early postnatal neurogenesis of many brain regions, which is essential for the normal growth and survival of the mice, and could also affect animal behaviors in the adult stage. Although it might seem that Hap1 has a much diminished role after early postnatal days, we argued that its absence in adult hippocampus weakens animals' ability to maintain proper level of hippocampal neurogenesis in stress conditions and thus renders them vulnerable to stress induced depressive behaviors. Results from our studies greatly improved our understanding of the biological function of Hap1 as well as the significance of postnatal neurogenesis in many different aspects. Manipulating the level of Hap1 or its

target molecules, e.g., TrkB, could be considered as novel therapeutic options for those neurological disorders that are caused by defective postnatal neurogenesis.

Future directions

In the current study, we aimed to investigate the role of Hap1 at various ages in mice. We discovered that BDNF/TrkB signaling is a critical pathway in mediating Hap1's function in the regulation of early postnatal neurogenesis, and possibly body growth and survival. We have shown that an acute treatment of BDNF in the third ventricle rapidly induced TrkB and AKT activation and completely restored hypothalamic neurogenesis to WT level in Hap1 KO mice, however, it was unable to rescue the lethal phenotype caused by malnutrition. The acute nature of the treatment allowed only transient increase in the BDNF/TrkB signaling, which declined in just 4 hours post treatment, however, it was enough to stimulate a short burst of milk drinking behavior in these KO mice (Figure 5.2). It would be interesting to look if persistent increase in BDNF level could rescue the inhibited feeding behavior and thus early death in Hap1 KO animals. Long term administration of BDNF in neonatal mice such as through infusion was extremely difficult due to the small size of the brain. To persistently upregulate BDNF level in Hap1 KO mouse brain, I propose two ways. The first way it can be done is through in utero intraventricular injection of BDNF plasmid or viral construct. The BDNF construct will be made under the control of a ubiquitous promoter, such as CAG promoter, which should turn on the expression of BDNF in the brain by the time the mice are born. The second way is to cross the Hap1 KO mice with a BDNF-overexpressing transgenic mouse line which expresses BDNF under a ubiquitous promoter, such as CAG promoter. This would be a more direct way to test the effect of early postnatal BDNF on the rescue of Hap1 KO lethal phenotype.

The reverse way to the above strategy would be to eliminate BDNF/TrkB signaling in the hypothalamus of WT mice and see if this causes neurogenesis defect and growth retardation. Since BDNF is a secreted protein that can be transported long distance, it would be difficult to delete both expressed and internalized BDNF specifically in hypothalamic nuclei. Therefore, a more logic way would be to knock out TrkB in these selective areas. Previous studies using TrkB conditional KO mice did not reveal early postnatal growth retardation (Liao et al., 2013; Xu et al., 2003). In contrast, these mice displayed hyperphagia and obesity when they became adults. We argued that BDNF/TrkB could have differential functions in different brain regions, and knocking out TrkB in multiple brain areas might elicit compensatory effects. Therefore, I propose that ablation of TrkB in Hap1-expressing cells could be an ideal model to study the role of hypothalamic TrkB in early postnatal animal growth because Hap1 is highly enriched in the hypothalamus and by doing so, it would be a direct approach to test if the compromised BDNF/TrkB signaling in Hap1 KO mice indeed contributes to the growth and lethal phenotypes. This conditional TrkB deletion can be easily done by crossing floxed TrkB mice with transgenic mice expressing Cre under Hap1 promoter. Also, to assess the role of NPYY1R+ cells in the neurogenesis and growth defects of Hap1 KO mice, we can also specifically delete NPYY1R in Hap1+ cells using the same strategy.

It has been thought that Hap1 is neuroprotective for HD as htt and Hap1 interact with each other and can be often seen in the same complex. It was hypothesized that because mhtt binds more tightly with Hap1, its accumulation in the aged brain might affect Hap1's normal function which may contribute to HD pathology. However, our results indicate that the loss of Hap1 alone in the adult brain does not lead to any of the HD phenotypes such as weight loss, motor deficit and early death. Therefore, it is more likely that Hap1 binds to and sequester mhtt from damaging the brain, and in the absence of Hap1, mhtt level builds up more quickly and the neurodegeneration could

occur earlier and progress more dramatically. To test this idea, we can cross Hap1 floxed mice expressing Cre-ER with HD transgenic lines such as N171-82Q mice that overexpress the first 171 amino acids of htt protein with 82 polyQs. The offspring that have floxed Hap1 and express both Cre-ER and N171-82Q mhtt would be injected with TM in the pre-symptomatic late postnatal or adult stage (P21 to 2-month of age) to delete Hap1, and these mice would be compared with N171-82Q mice that have normal Hap1 expression to see if Hap1 deletion could accelerate the disease progression in this HD model. We would examine mhtt level and aggregation, batteries of behavioral readouts, and ultimately survival between Hap1 deleted N171-82Q mice and controls for the conclusion. If the results suggest that Hap1 loss indeed exacerbates HD phenotypes, it would make Hap1 a therapeutic target for HD.

Reversely, since Hap1 expression is considerably low in MSNs that are preferentially degenerated in HD (Ehrlich, 2012; Zucker et al., 2005), it would be riveting to see if overexpressing Hap1 in MSNs could ameliorate the disease progression. For example, we can generate DARPP32-Hap1 transgenic mice which overexpress Hap1 specifically in MSNs, and cross them with HD transgenic mice. This double transgenic mouse line would allow us to determine if Hap1 can really protect MSNs from mhtt-mediated toxicity and consequently slow down the disease progression. Likewise, Hap1 has been reported to interact with many other disease related proteins, it would also be interesting to use the same strategy to assess Hap1's roles in these other diseases.

We have shown that Hap1 in adult mice regulates hippocampal neurogenesis in response to restraint stress. This could prove vital for our understanding of Hap1's function in adult mice because Hap1 is highly expressed in the hypothalamus, which is a pivotal brain region in stress response and homeostatic control. It is therefore important to test if Hap1 also plays a role in response to other forms of stress. For example, the

hypothalamus controls body temperature and circadian circle, we can subject Hap1 adult KO mice and controls to heat or cold stress, or irregular circadian circle, and evaluate whether the adult KO mice have impairment in maintaining homeostasis. Previous work discovered that Hap1 forms a unique cytoplasmic structure called the stigmoid body with unknown function (Li et al., 1998a). We speculated that stigmoid bodies might be involved in stress response by quickly releasing key stress proteins that are conserved in this structure. It would also be interesting to test this hypothesis. Since the isolation of stigmoid bodies from the brain is possible (Torre et al., 2003), we may use mass-spectrometry to identify novel components of the stigmoid body, which could reveal some stress related proteins and lead to new directions of this study.

Our results indicate that early postnatal Hap1 deletion reduces hippocampal neurogenesis, and leads to adult depressive phenotype. To find a molecular target for this, we prepared WT and Hap1 KO mouse hippocampal lysate and employed mass-spectrometry to look for any interesting proteins whose levels are significantly altered between the two samples. Among all the proteins identified, we found c-kit, or mast/stem cell growth factor receptor, which is downregulated in Hap1 KO hippocampus to be quite an interesting target. Previous work has suggested that c-kit is expressed in the neuroproliferative zones of the rat brain, and in vivo administration of its ligand, stem cell factor (SCF) increased BrdU staining in these regions (Jin et al., 2002). Thus, we think that c-kit could be involved in the regulation of hippocampal neurogenesis in the postnatal brain. Our recent data validated the decreased c-kit level in Hap1 KO hippocampus (Figure 5.3). We also showed that Hap1 and c-kit are co-expressed in the same cells both in cultured hippocampal neurons and in mouse hippocampal DG, and Hap1 stabilized transfected c-kit protein level in N2a cells (Figure 5.4). The future of this study would be to inject viral construct of c-kit to the hippocampus of the neonatal

camk2a-Hap1 KO mice and assess if overexpressing c-kit can rescue the neurogenesis defect and the depressive phenotype.

Figure 5.1

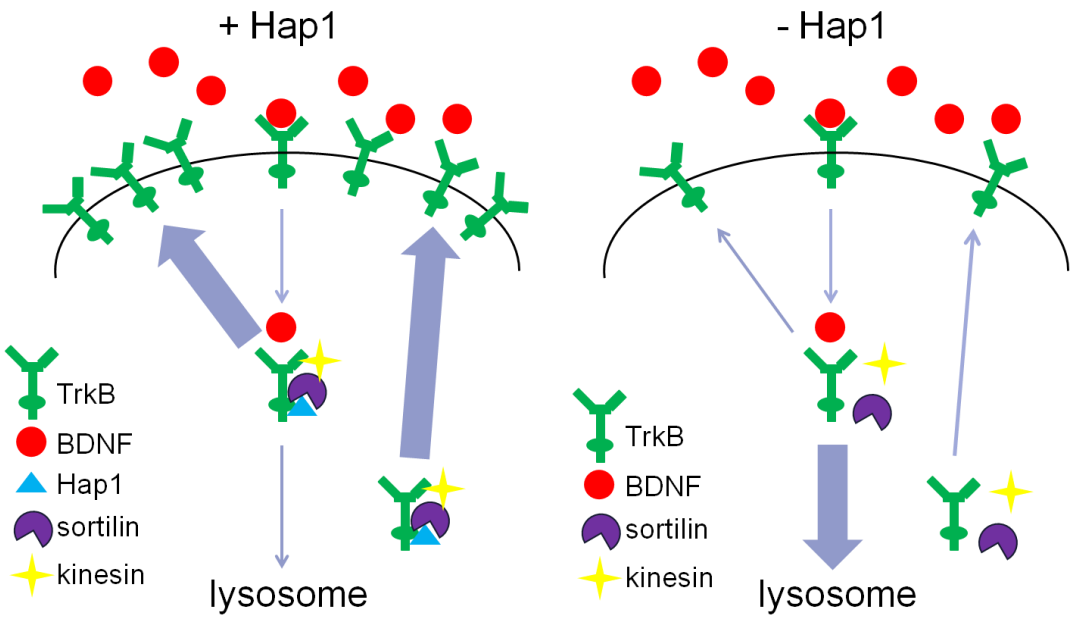
+ Hap1
- Hap1
TrkB
BDNF
Hap1
sortilin
kinesin
lysosome
TrkB
BDNF
sortilin
kinesin
lysosome

Figure 5.1

Working model of how Hap1 regulates TrkB endocytic trafficking. In the presence of Hap1 (left panel), extracellular BDNF binds to postsynaptic TrkB, which is then internalized into the neuron. The internalized TrkB associates with Hap1, which then recruits sortilin and kinesin to facilitate TrkB recycling to the postsynaptic membrane for continued signaling events. The newly synthesized TrkB may also require the complex of Hap1-sortilin-kinesin for effective anterograde trafficking to the postsynaptic membrane. In the absence of Hap1 (right panel), the association of internalized TrkB with sortilin and kinesin is significantly decreased, which leads to increased TrkB lysosomal degradation and reduced membrane recycling. The anterograde trafficking of newly synthesized TrkB may also be affected. Together, BDNF/TrkB signaling in the postsynaptic membrane is significantly diminished without Hap1.

Figure 5.2

Figure 5.2

Acute BDNF treatment stimulated feeding behavior of Hap1 KO mice. Hap1 KO pup was injected with 5 μg BDNF into the third ventricle. Pictures of the same pup were taken immediately before (left panel) and 2 hours after the treatment (right panel). Note that after the acute treatment, more milk could be observed in the stomach of the pup indicating that BDNF stimulated its feeding behavior.

Figure 5.3

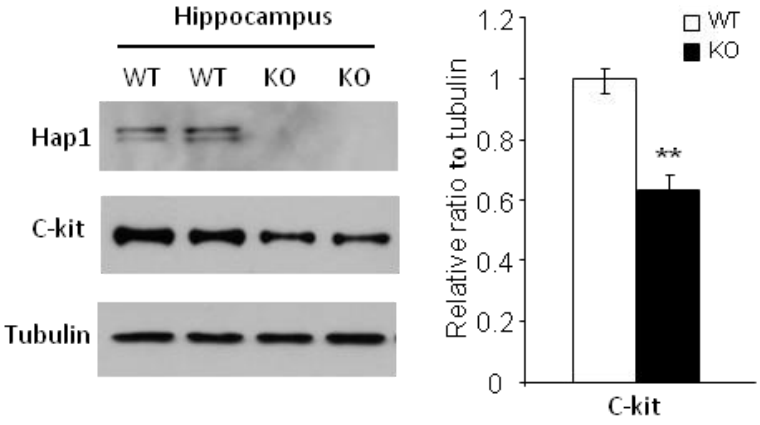
Hippocampus
WT
WT
KO
KO
Hap1
C-kit
Tubulin
1.2
1
0.8
0.6
0.4
0.2
0
Relative ratio to tubulin
WT
KO
**
C-kit

Figure 5.3

C-kit level is decreased in Hap1 KO hippocampus. Western blot analysis (left panel) of WT and Hap1 KO hippocampal homogenate showed decreased c-kit protein level in KO samples. Quantification of c-kit to tubulin ratio is normalized to WT and is shown in the right panel. n=4 per genotype. **P<0.01.

Figure 5.4

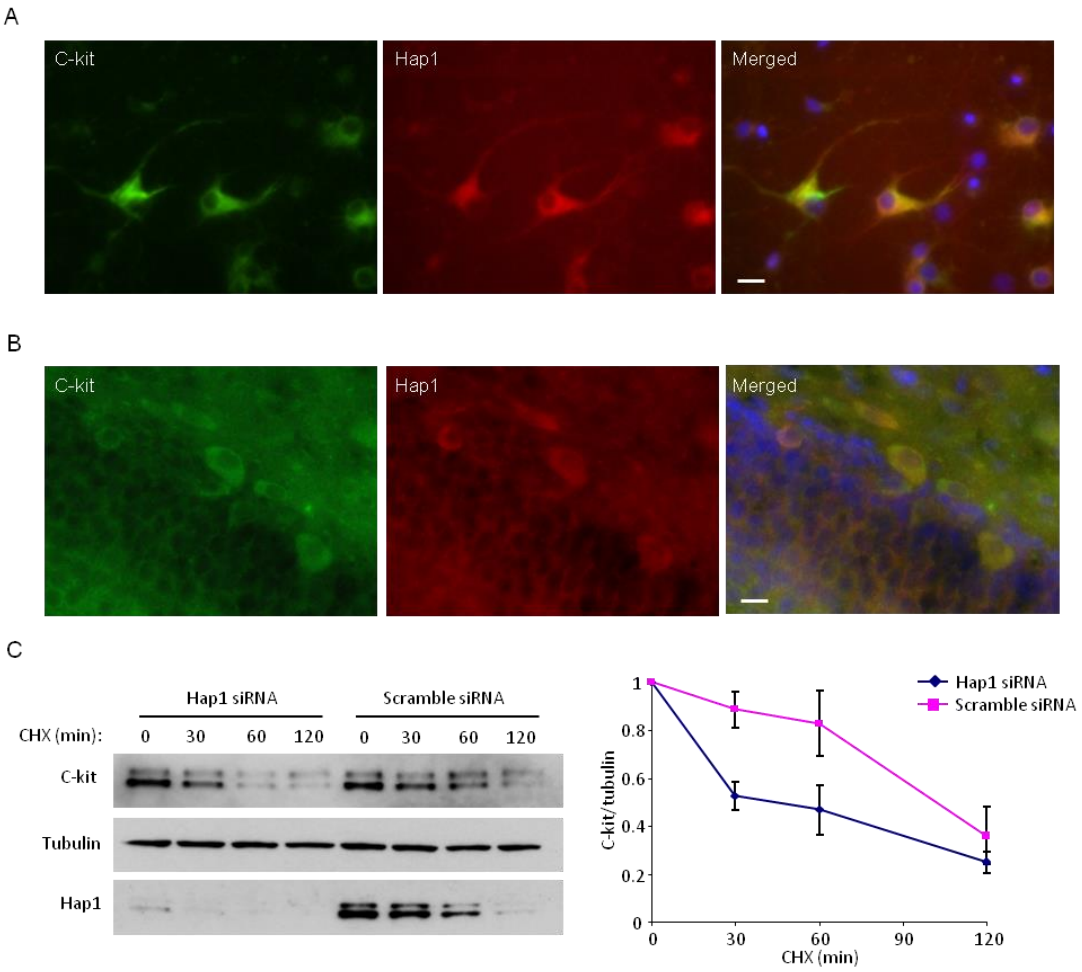
A
C-kit
Hap1
Merged
B
C-kit
Hap1
Merged
C
Hap1 siRNA
Scramble siRNA
CHX (min):
0
30
60
120
0
30
60
120
C-kit
Tubulin
Hap1
Hap1 siRNA
Scramble siRNA
C-kit/tubulin
1
0.8
0.6
0.4
0.2
0
0
30
60
90
120
CHX (min)

Figure 5.4

Hap1 is co-expressed with c-kit and stabilizes its level. (**A**) Hippocampal neuronal culture in DIV5 was immunostained with antibodies against Hap1 and c-kit. Both proteins are co-expressed in the same neurons. Scale bar: 10 μm. (**B**) Brain sections from P15 mouse were immunostained with antibodies against Hap1 and c-kit. Co-expression of the two proteins was found in the hippocampal DG. Scale bar: 10 μm. (**C**) Plasmid construct expressing c-kit was transfected into N2a cells. At the same time, the cells were treated with either scramble or Hap1 siRNA. Protein stability of c-kit was then assessed by western blotting (left panel). Quantification of 3 independent experiments is shown on the right panel. Hap1 knockdown markedly decreased the stability of c-kit in N2a cells.

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