Huntingtin-associated protein 1 (Hap1) mutant mice bypassing the early postnatal lethality are neuroanatomically normal and fertile but display growth retardation

Ioannis Dragatsis1,*, Scott Zeitlin2 and Paula Dietrich1

1Department of Physiology, College of Medicine, The University of Tennessee, Health Science Center, Memphis, TN 38163, USA and 2Department of Neuroscience, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

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Huntingtin-associated protein 1 (HAP1) is the first huntingtin interacting protein identified in a yeast two-hybrid screen. Although Hap1 expression has been demonstrated in neuronal and non-neuronal tissues, its molecular role is poorly understood. Recently, it has been shown that targeted disruption of Hap1 in mice results in early postnatal death as a result of depressed feeding behavior. Although this result clearly demonstrates an essential role of Hap1 in postnatal feeding, the mechanisms leading to this deficiency, as well as the role of Hap1 in adults, remain unclear. Here we show that Hap1 null mutants display suckling defects and die within the first days after birth due to starvation. Upon reduction of the litter size, some mutants survive into adulthood and display growth retardation with no apparent brain or behavioral abnormalities, suggesting that Hap1 function is essential only for early postnatal feeding behavior. Using a conditional gene repair strategy, we also show that the early lethality can be rescued if Hap1 expression is restored in neuronal cells before birth. Furthermore, no synergism was observed between Hap1 and huntingtin mutation during mouse development. Our results demonstrate that Hap1 has a fundamental role in regulating postnatal feeding in the first 2 weeks after birth and a non-essential role in the adult mouse.

INTRODUCTION

Huntingtin-associated protein 1 (HAP1) was the first protein identified in a yeast two-hybrid screen for huntingtin-interacting polypeptides (1). Hap1 (the mouse homolog) maps to mouse chromosome 11 (band D) (2) and encodes a cytoplasmic protein associated with microtubules and membrane organelles, and is enriched in a nerve terminal vesicle-rich fraction. Analysis of axonal transport has shown that the rapid anterograde accumulation of both huntingtin (htt) and Hap1 is compatible with their transport on vesicular membranes (3). Stigmoid bodies in the cytoplasm of neurons also contain Hap1 (4,5). In addition to its interaction with htt, Hap1 also interacts with Duo, a Trio-like protein with a rac guanine nucleotide exchange factor domain potentially involved in cytoskeletal functions (6), and with the p150 glued component of dynactin, a dynein accessory protein that participates in microtubule-based transport (7,8). Recently, it was found that htt and Hap1 may function as a scaffold for the activation of NeuroD by mixed-lineage kinase 2 (MLK2) (9) and that htt enhances vesicular transport of brain-derived neurotrophic factor (BDNF) along microtubules, a transport that involves Hap1 (10).

Hap1 gene in mouse consists of 11 exons and can generate at least three RNA transcripts by alternative splicing (Hap1A, Hap1B and Hap1C) (2,11). In the adult mouse brain abundant Hap1A and Hap1B transcripts are easily detected (12). In situ hybridization analyses have demonstrated Hap1 mRNA expression in the adult mouse and rat olfactory bulb, hypothalamus, brain stem, striatum, cerebellum, hippocampus and colliculi; in the mouse, pituitary, ovary and testes were the only peripheral organs showing Hap1 expression (12). During mouse development, Hap1 transcripts are first detected at E8.5 in the neuroepithelium and are enriched in the...
generation of targeted disruptions of the Hap1 and htt mutations in mouse development. of this analysis indicate that there is no synergism between analyzed embryos and pups carrying both mutations. The results of this analysis demonstrate an essential role of Hap1 in postnatal feeding behavior, the mechanisms leading to this deficiency, as well as the role of Hap1 in adults, remain unclear.

Here we show that Hap1 null mutants display suckling defects and die within the first days after birth due to starvation. In addition, we show that by reducing the litter size, some of the mutants can survive into adulthood with no apparent brain or behavioral abnormalities, suggesting that Hap1 function is essential only for early postnatal feeding behavior. Using a conditional gene repair strategy, we also show that the early lethality can be rescued if Hap1 expression is restored in neuronal cells before birth. Furthermore, in an attempt to clarify the biological significance of the interaction between Hap1 and htt, we generated and analyzed embryos and pups carrying both mutations. The results of this analysis indicate that there is no synergism between Hap1 and htt mutations in mouse development.

RESULTS

Generation of targeted disruptions of the Hap1 gene

In mice, the Hap1 gene is organized in 11 exons and is flanked by exons from potentially one or more genes (11). We therefore inactivated Hap1 by homologous recombination using two different approaches: (1) replacing the promoter region, exon 1 and part of the first intron with a neomycin-resistance cassette (Fig. 1A). This approach might disrupt other genes that are in the vicinity (see Discussion); and (2) inserting a floxed neostop cassette (17,22,23) in the first intron in such a way that a conditional gene repair allele (Hap1stop) is generated (Fig. 1B). The second scheme should create a functionally null allele (17), should not interfere with the expression of the alleged neighboring genes and should enable rescue the Hap1 null phenotype with the appropriate Cre expression.

Mutant embryonic stem cell clones were obtained by homologous recombination for both replacement vectors and inserted into blastocysts (see Materials and Methods). Chimeric mice were mated to C57BI/6 mice to establish transmission, and progeny were intercrossed.

Neonatal death of Hap1 mutants

Genotyping of litters from Hap1 (Fig. 1C) and Hap1stop heterozygous intercrosses demonstrated that Hap1 mutants die some time before 2 weeks of age. Close examination of litters at birth revealed that Hap1 mutants were born at the expected frequency of 25% and were indistinguishable from their littermates. As anticipated, Hap1 transcripts were detected by northern blot analysis of RNA from wild-type (WT) and Hap1 heterozygotes but were absent in samples from Hap1−/− and Hap1stop/− mutants (Fig. 1D). No differences in phenotypic features of Hap1−/− and Hap1stop/stop or of the compound Hap1stop/− mice were observed, and unless specified in the text, we use Hap1 mutants in general for any of the earlier-mentioned combinations.

Suckling defects in Hap1 mutant pups

To determine the cause of neonatal lethality in the Hap1 mutants, litters from Hap1 heterozygous mice were monitored from birth (P0) until weaning age (P21). Hap1 mutants at birth had normal body weight and normal respiratory rate, were well oxygenated and responded to touch with vocalization, righting and rooting reflexes. Homozygous mutant mice 12 h after birth could be spotted as the pups with no milk in their stomachs (Fig. 2A) and by P3 most of the homozygous mutants were dead (Table 1). To determine whether impaired suckling phenotype was present in Hap1 mutant pups, milk quantity was correlated with genotype. The majority of Hap1−/− pups had little or no milk in their stomachs (Table 2). Hap1 mutant pups could open and close their mouths, and anatomical examination revealed that the palate, mouth and esophagus were intact.

Olfactants/pheromones present on the nipple are the primary sensory cue used in orientation and localization, although tactile sensation is also required. Once a nipple is located, a rooting reflex program is activated and involves rhythmic suckling mouth movements and swallowing. Structures involved in the overall behavior include the olfactory epithelia, olfactory bulb, various cranial ganglia, the nerves/muscles involved in rhythmic contractions and swallowing and perhaps the vomeronasal organ and olfactory cortex (entorhinal/amygdala) (24,25). To determine more precisely the behavioral defect that impaired suckling in Hap1−/− pups, we observed their tactile reflexes and analyzed their capacity in attaching to the nipple of a lactating female. All Hap1−/− pups exhibited a normal rooting reflex in response to manual stimulation of their mouth region, indicating normal tactile sensation and motor control.

Attachment to the nipple was recorded (see Materials and Methods): 18 of 19 WT or heterozygous pups attached to the mother’s nipples as opposed to only 3 of 6 mutants. Furthermore, Hap1 mutant pups had a latency time for attachment that was on average four times longer than that of WT pups (257 ± 37 and 62 ± 33 s, respectively; data are expressed as mean ± SD, P < 0.0001 Student’s t-test).

Analyses of mutant brains

The brains of newborn Hap1 mutant mice and two WT littermates were serially sectioned in the coronal plane and stained. No obvious abnormalities were observed in Hap1 mutant brains, including the hypothalamus and the olfactory bulb, where Hap1 is predominantly expressed. The hindbrain-located trigeminal motor neurons, which are affected in
Figure 1. Targeted disruption of the Hap1 locus. (A) Schematic of the Hap1 knockout vector (V), wild-type Hap1 allele (WT) and the targeted Hap1 null allele (−). Exons are depicted as black rectangles. (B) Schematic of the Hap1stop gene repair targeting vector (V), showing the location of the neostop cassette within intron 1 near Hap1 exon 1 (black rectangle, containing the translation initiation site), together with the wild-type Hap1 allele (WT), the product of homologous recombination (STOP) and the product of Cre-mediated recombination (ΔSTOP). The position of neo cassette (neo) and the transcriptional/translational stop sequences (HIS3-stop) are indicated along with as the positions of the loxP sites (gray ovals). The position of a 3′ flanking probe along with the sizes of the diagnostic restriction fragments identifying the wild-type, Hap1−/−, Hap1stop alleles are indicated. The transcriptional orientations of the Hap1 gene and the neo cassette are indicated with arrows, and wavy lines indicate plasmid vector sequence. The NotI site that was used to linearize the vectors before electroporation to ES cells is depicted with two vertical lines. The termination of the Hapstop transcript within the neostop cassette is indicated with two dashed gray lines. Following Cre-mediated recombination, the neostop cassette is excised leaving a single loxP site (gray oval) within the Hap1 intron 1 that does not interfere with expression (black arrow). Some of the restriction sites present in the sequence are EcoRI (R), KpnI (K), HindIII (H) and XhoI (X). (X) symbolizes the site used to clone the neostop cassette. (C) Southern blot showing DNA prepared from pups digested with EcoRI and hybridized with the 3′ flanking probe is shown. The probe distinguishes between a 10.3 kb wild-type and 8.2 kb Hap1 targeted genomic fragments. (D) Northern analysis of total RNA from neonatal mouse brains obtained from Hap1−/−, Hap1stop/− and wild-type (WT) mice. The upper panel is hybridized with a probe containing exons 2–7 of the Hap1 gene. The middle panel was hybridized with a probe containing exon 1 of the Hap1 gene. Note that after a long exposure there is a faint small transcript present in the Stop/− lane (white arrowhead). The two major Hap1 transcripts are depicted (Hap1A, Hap1B). A region of the RNA gel containing the 18S rRNA species (stained with ethidium bromide to control for sample loading) is shown in the bottom panel.
lurcher mice (26), also appeared normal in all newborn mice analyzed (data not shown). Gross anatomical and histological examination of other organs from mutant mice did not reveal overt abnormalities. In situ hybridization confirmed that Hap1 mutant mice lack detectable Hap1 expression in brain (data not shown; Fig. 3C and D).

Recordings from the olfactory bulb showed spontaneous synaptic currents and action potentials fired by the mitral cells, both actions indicative of the ability of the mutant mouse to respond to odors (data not shown). To assess the state of hypothalamic compensation in response to Hap1 deficiency, we studied hypothalamic expression of mRNAs encoding three appetite-regulating neuropeptides, namely neuropeptide Y (NPY), agouti-related protein (AGRP) and pro-opiomelanocortin (POMC). Expression of NPY, AGRP and POMC in the arcuate nucleus remained unchanged in Hap1−/− mice (Fig. 3E–H).

Surviving Hap1 mutant mice

About 8% of the homozygous mutant mice ultimately began to feed, but by 2 weeks these homozygotes showed a 70% reduction in body weight and remained at a competitive disadvantage for feeding when compared with their heterozygous and WT littermates. They were recognizable by their smaller size, poorly groomed coats and hunched posture. The vast majority of the surviving homozygotes were malnourished and succumbed to death before weaning.

Weight measures of different organs of Hap1 mutants at P3 and P15 revealed that at both ages brain and spleen were significantly smaller in Hap1 mutants than in WT littermates (Table 3). This reduction in organ weight is not to be attributed to any specific function of the mutation but rather represents a secondary effect of starvation. Similar results are found in anorexia mutant mice (27), although anx/anx mutants die around weaning age.

Reducing the litter size by removing WT and heterozygous pups enhanced the survival of homozygous mutant animals. Usually, two litters born the same day and the two lactating females were kept together while the litters size was reduced so that only three or four Hap1−/− pups and an equal number of WT pups remained. At 3 weeks, when WT and heterozygous mice were weaned, homozygous mutants continued to nurse. In these conditions, one of the homozygous mutants from every two to three litters survived to sexual maturity. The surviving male and female homozygotes appear normal, they mate and are fertile. No obvious brain abnormalities were seen in hematoxylin and eosin (H&E) stained coronal sections of brains of three surviving adult mice (Fig. 3A and B).

Survival frequency of Hap1 mutant mice

<table>
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<tr>
<th>Genotyping</th>
<th>Number of pups surviving postnatal day</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT, Hap+/−</td>
<td>90</td>
</tr>
<tr>
<td>Hap1−/−</td>
<td>32</td>
</tr>
</tbody>
</table>

*One pup was found dead. No obvious abnormality was found.

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A comparison of the postnatal growth curves of WT and surviving Hap1 mutants showed that the rate of growth of the mutants was slower than that of WT during the first 3 weeks after birth. Thus, their relative size decreased progressively and by P21 Hap1 mutants were 30% the weight of their heterozygous littermates. Surviving mice at P15 are littermates and photographed at the same magnification (B and C).

Table 1. Survival frequency of Hap1 mutant mice

<table>
<thead>
<tr>
<th>Genotyping</th>
<th>Number of pups surviving postnatal day</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT, Hap+/−</td>
<td>90</td>
</tr>
<tr>
<td>Hap1−/−</td>
<td>32</td>
</tr>
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</table>

*One pup was found dead. No obvious abnormality was found.

One pup was found dead and partially cannibalized the second week after birth.

One pup was dead at the time of observation.

Table 2. Milk score at P1 in control and Hap1 mutant pups

<table>
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<th>Genotyping</th>
<th>Milk score</th>
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<tr>
<td>WT, Hap1+/−</td>
<td>71</td>
</tr>
<tr>
<td>Hap1−/−</td>
<td>0</td>
</tr>
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</table>

++, fully distended stomach (milk obviously visible through the skin); +, partially distended stomach; 0, no milk visible in the stomach.
Rescue of early postnatal lethality by conditional gene repair

Hap1 lethality can be bypassed in Hap1stop mice by using a tissue-specific Cre expression. For the rescue experiments, we used a transgenic line expressing Cre under control of the alpha subunit of the calcium/calmodulin-dependent protein kinase II gene (CamKII) promoter (19). The Cre transgenic line employed, R1ag#5, expresses Cre in the brain as early as E15.5, and by P1 extensive recombination...

Figure 3. Histological and in situ hybridization analysis of Hap1 mutant mice. H&E stained coronal sections through a 12-month-old WT (A) and Hap1−/− surviving littermate (B). Note that despite the smaller size of the Hap1−/− mutant brain, no other abnormalities are evident. In situ hybridizations of coronal sections through a P1 WT (C and E) and Hap1−/− brain (D and F–H). In situ hybridization for Hap1 expression (C, D, see Material and Methods, dark purple/brown is the positive signal). Hap1 is highly expressed in the arcuate hypothalamic nucleus, ventromedial hypothalamic nucleus (VMH), amygdaloid nucleus (AN), paraventricular thalamic nucleus (PVT), paraventricular hypothalamic nucleus (PVHT) and zona incerta (ZI) in the wild-type brain (C). As expected, no Hap1 expression can be seen in a Hap1−/− brain (D). In situ hybridization analyses revealed no difference in expression of POMC (E, F), AGRP (G) and NPY (H) between WT (E) and Hap1−/− P1 brains (F–H). Median eminence (ME) and the third ventricle (3v) are indicated.
Table 3. Organ weights from Hap1−/− and control littermate pups

<table>
<thead>
<tr>
<th>Tissue weights</th>
<th>Control (mg)</th>
<th>Hap1−/− (mg)</th>
<th>Control (mg/g body weight)</th>
<th>Hap1−/− (mg/g body weight)</th>
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<tbody>
<tr>
<td>Age = 3 days</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Body weight (g)</td>
<td>2.62 ± 0.21</td>
<td>2.06 ± 0.12</td>
<td>3.81 ± 2.04</td>
<td>21.9 ± 3.2*</td>
</tr>
<tr>
<td>Heart, lung</td>
<td>91.6 ± 5.3</td>
<td>93.7 ± 4.2</td>
<td>5.7 ± 0.4</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>87.0 ± 9.7</td>
<td>85.9 ± 8.7</td>
<td>39 ± 3.4</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>9.9 ± 0.9</td>
<td>9.6 ± 0.8</td>
<td>3.6 ± 0.3</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.7 ± 0.6</td>
<td>7.5 ± 0.5</td>
<td>3.9 ± 0.3</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>74.0 ± 11.3</td>
<td>73.2 ± 10.5</td>
<td>25.3 ± 6.9</td>
<td>16.7 ± 4.0</td>
</tr>
<tr>
<td>Intestines</td>
<td>130.2 ± 12.1</td>
<td>128.9 ± 11.9</td>
<td>48.0 ± 4.9</td>
<td>46.2 ± 5.1</td>
</tr>
<tr>
<td>Brain</td>
<td>107.3 ± 3.7</td>
<td>105.5 ± 3.2</td>
<td>41.1 ± 1.2</td>
<td>60.0 ± 1.7***</td>
</tr>
<tr>
<td>Age = 15 days</td>
<td></td>
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</tr>
<tr>
<td>Body weight (g)</td>
<td>11.1 ± 0.91</td>
<td>11.1 ± 0.89</td>
<td>5.5 ± 0.5</td>
<td>5.9 ± 0.5</td>
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<tr>
<td>Heart</td>
<td>59.1 ± 3.2</td>
<td>57.9 ± 3.1</td>
<td>5.5 ± 0.4</td>
<td>5.9 ± 0.5</td>
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<tr>
<td>Lung</td>
<td>188.2 ± 17.3</td>
<td>188.2 ± 17.2</td>
<td>12.3 ± 1.9</td>
<td>22.5 ± 4.2</td>
</tr>
<tr>
<td>Liver</td>
<td>394.0 ± 35.6</td>
<td>393.8 ± 35.5</td>
<td>13.5 ± 1.3</td>
<td>23.0 ± 5.1*</td>
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<tr>
<td>Kidney</td>
<td>57.4 ± 3.2</td>
<td>57.2 ± 3.1</td>
<td>5.9 ± 0.7</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>46.6 ± 0.7</td>
<td>46.4 ± 0.6</td>
<td>4.1 ± 0.1</td>
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<tr>
<td>Stomach</td>
<td>139.9 ± 28.1</td>
<td>139.7 ± 28.0</td>
<td>22.6 ± 2.2</td>
<td>12.3 ± 2.6</td>
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<tr>
<td>Intestines</td>
<td>583.3 ± 11.5</td>
<td>583.2 ± 11.4</td>
<td>52.3 ± 1.2</td>
<td>51.1 ± 4.0</td>
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<tr>
<td>Brain</td>
<td>369.0 ± 12.3</td>
<td>369.0 ± 12.2</td>
<td>251.8 ± 8.2</td>
<td>109.8 ± 3.2***</td>
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Statistical test for significant differences were carried out only on normalized data.

*P < 0.05, ***P < 0.001 value significantly different from control mice, n = 4–6.

is observed in many areas of the brain, including the hypothalamus (28, and data not shown). We observed 100% rescue of the early postnatal lethality in mutants with the R1ag+/5 line. However, although rescued mice survived to adulthood, they still exhibited a slight growth deficiency when compared with their WT littermates and by P21 they were ~70% the size of their WT counterparts (Fig. 4B).

**DISCUSSION**

Although Hap1 was the first protein identified to interact with htt (1), very little is known about its physiological role. Two reports have shown that Hap1 is essential in early postnatal life for feeding behavior, and according to these reports, the majority of Hap1 mutant mice die at P2 and none survive past P15 (15,16). Here, we confirm these findings and extend our analysis of Hap1 function in newborns and adult mice.

We also found that in the first days after birth Hap1 mutants display an abnormal feeding behavior that leads to death. Although normal at birth, newborn mutant pups can be easily distinguished from their WT littermates a few hours later by the lack of milk in their stomachs, and ~80% die before P3. All Hap1 mutant pups exhibited a normal rooting reflex in response to manual stimulation of their mouth region, indicating normal tactile sensation and motor control.

We extended the existing information by observing that half of the mutant mice failed to attach to the mother’s nipple, and the remaining half had a lag time four times longer than their...
Nipple-searching behavior and attachment to the nipple, in newborns lead to a reduction in nipple attachment efficiency in Pdn mutants) or surgical lesions in the olfactory system which its role is mediated.

In newborns. In lurcher mice, suckling deficiency appears to be caused by loss of trigeminal ganglia neurons, resulting in a sensory defect on the face and mouth, which impairs the sucking response (26). Mice homozygous for a null mutation in the NMDA receptor ε2 subunit do not display suckling movement upon mechanical stimulation, and exhibit abnormal neuronal pattern formation in the trigeminal complex (38).

Sacculating defects in Hap1 mutants are not attributed to abnormal trigeminal neuronal pattern formation or neuronal loss, since we did not detect reduced neuronal cell numbers in the trigeminal ganglion (data not shown) and Hap1 mutants exhibited normal rooting reflex in response to manual stimulation of their mouth region, indicating normal tactile sensation and motor control.

Hypothalamic dysfunction has also been implicated in altered feeding behavior (39). Many studies have shown that a number of hypothalamic neuropeptides either stimulate or inhibit feeding actions. Overexpression of AGRP in transgenic mice leads to obesity (40), and fasting increases the levels of both AGRP and NPY (41). Furthermore, mice homozygous for the anorexia mutation (anx) are hypophagic (27), and exhibit altered histochemistry of NPY–AGRP in the arcuate nucleus and reduced POMC mRNA levels (42,43). Although we did not observe any abnormal morphology or changes in expression of these feeding peptides within the hypothalamus of P1 Hap1 mutants, our analysis does not preclude that expression of other feeding peptides may be altered.

However, the fact that Hap1 mutants surviving to adulthood display normal food intake suggests that hypothalamic function in regulating appetite may not be significantly impaired.

We cannot rule out at the moment that other abnormalities in hypothalamic function may be present. For instance, gustatory coding could be affected in Hap1 mice, and could be at least in part responsible for the delay in nipple attachment observed in Hap1 mutants. In newborn rats, as early as a few hours after birth, mechanisms of gustatory detection have control over attachment to the nipple and suckling behavior (44). Similarly, gustation is also used by piglets (together with tactile sense and olfaction) for nipple attachment and ingestive behavior (45). The process of gustatory coding consists of neural responses that provide information about the quality and quantity of food and whether it should be swallowed. The lateral hypothalamus (among other centers) which expresses high levels of Hap1, exerts an influence on taste and ingestive behavior (46). The hypothesis that Hap1 mutants may have altered gustatory responses still remains to be tested.

Neurotransmitters such as dopamine, norepinephrine and serotonin have been implicated in controlling feeding behavior in rats and mice. However, dopamine-deficient mice display aphagia only around weaning age (47,48). Furthermore,
dopamine and norepinephrine have no effect in suckling response in the neonate (49). In contrast, suckling response in neonate rats has been shown to be stimulated by serotonin (5-HT) agonists, and disrupted by serotonin antagonists (50,51), while in weanling rats and adults serotonin action is inverted, and appears to inhibit attachment and food intake (49,51,52). On the other hand, disruption of serotonin receptors failed to reveal an essential role of serotonin in feeding behavior in neonates (53,54). However, keeping in mind that serotonin exerts its multiple actions through at least 14 known serotonin receptor subtypes (55,52) and only a fraction of these have been inactivated in mice (52), it is reasonable to speculate that disruption of Hap1 may alter serotonin function through the hypothalamic–pituitary–adrenal axis.

Histological analysis of adult Hap1−/− brains did not reveal any obvious abnormality. In adults, cortical layers were normal in thickness and architecture, further confirming the initial assumption that the cortical atrophy observed in Hap1−/− pups that die prior to P8 results solely from malnutrition (15). The absence of any gross brain malformation and abnormal behavior suggest that Hap1 does not play an essential role in the adult brain. Moreover if Hap1 is required for the initial assumption that the cortical atrophy observed in Hap1 mutants could be rescued from feeding behavior. The utilization of other Cre lines with more restricted expression will provide this information.

Construction of the Hap1 conditional gene repair vector

We also generated a conditional gene repair allele of the Hap1 mutation by inserting a floxed neostop sequence in the first intron of the Hap1 gene. Hap1 transcripts could not be detected in Hap1stop−/− mice (Fig. 1D). Furthermore, mice homozygous for the deletion/replacement allele (Hap1−/−), the gene repair allele (Hap1stop/stop) and the compound alleles (Hap1stop/−) displayed identical phenotypes, further corroborating that the gene repair allele functions as a null allele. This analysis also indicates that exons in the 5′ end of the Hap1 gene (11), identified as putative open reading frame (ORF) by a sequence analysis program, are either computer generated ORFs that do not code for any protein or belong to a protein that does not have any essential function.

The conditional gene repair strategy also allows the rescue of the mutant phenotype by appropriate expression of Cre recombinase. To prove the concept and try to deduce more information about the Hap1 mutation, we used a transgenic line that expresses Cre in neuronal cells. We found that we could achieve 100% rescue of the postnatal lethality using the Cre line R1ag#5, which results in extensive recombination in the central nervous system, suggesting that neuronal dysfunction underlies the abnormal feeding behavior of Hap1 mutants. Unfortunately, the broad spectrum of Cre expression precluded identifying the exact neuronal population in which Hap1 is essential for early postnatal feeding behavior. The utilization of other Cre lines with more restricted expression will provide this information. Interestingly, although Hap1 mutants could be rescued from the early postnatal lethality with this approach, they still exhibited a decreased growth rate between P10 and P21. The incomplete rescue of the growth rate could be potentially attributed to incomplete restoration of expression of Hap1 in the CNS. Alternatively, Hap1 may also function outside the CNS.

With regard to Hap1–htt interactions, several lines of evidence suggest that both proteins exert their essential functions independently from each other: (1) in contrast to Hap1 mutants, inactivation of htt in the CNS does not lead to feeding abnormalities (28); (2) inactivation of Hap1 does not lead to neurodegeneration in the adult brains (Fig. 3B), whereas inactivation of htt in the forebrain with the R1ag#5 line leads to massive neuronal cell loss (28); and (3) Hap1;htt double homozygotes are indistinguishable from htt null mutants, and Hap1−/−;Hdh+/− mutants are indistinguishable from Hap1 null mutants (Table 4 and data not shown), suggesting that there is no synergistic interaction between the two proteins, at the genetic level.

In conclusion, we have shown that although Hap1 has an important role for feeding in the early postnatal period, its function does not appear to be essential in the adult mouse. Although adult Hap1 mutant mice exhibit growth retardation, they are fertile, have normal life span and are neuroanatomically normal. Also, our analyses of Hap1 and Hdh double mutants suggest that these two proteins do not interact, or act synergistically, at least during embryonic development. Further analysis of Hap1 mutants may provide insights into the mechanism controlling early postnatal feeding behavior.

MATERIALS AND METHODS

Construction of the Hap1 deletion/replacement vector

We constructed a targeting vector by replacing 5 kb of mouse sequence containing the Hap1 promoter, exon 1 and a portion of the first intron with a neo cassette, placed opposite to the Hap1 transcriptional orientation. The final product [cloned into pBluescript SK+ (pBSK+)] consisted of a 5′ genomic fragment (4.55 kb) from a Not1 site in the phage polylinker to a blunted Kpn1 site, the pgk-neo cassette and the downstream Hap1 genomic fragment (5.8 kb) from a Xho1 site to a blunted HindIII site (Fig. 1A).

Construction of the Hap1 conditional gene repair vector

We assembled a neo stop cassette as previously described (17). Briefly, we combined a pgk-neo gene cassette with the His3-SV40 pA sequences from a Cre-mediated recombination reporter plasmid (pEF1α–lox–STOP–lox–LacZ). We then inserted two loxP sequences in the same orientation flanking the neo and STOP sequences (Fig. 1B). The cassette was cloned into pBSK+ and can be excised with Xba1 and Kpn1.

For the construction of the Hap1 conditional gene repair vector, we inserted the floxed neo stop cassette into an Xho1 site at the first intron of the Hap1 gene in such a way that the orientation of the neo cassette is the same as the Hap1 gene (Fig. 1B).

Gene targeting in ES cells and generation of mice

We linearized the targeting vectors with Not1 digest, and we introduced them by electroporation into W9.5 ES cells grown on mitomycin C-treated G418-resistant primary mouse fibroblasts. ES cell DNA purified from G418-resistant clones was analyzed by Southern blotting using a probe that
distinguishes between the targeted and wild-type Hap1 alleles. We then used a neo probe to verify that a single copy integration occurred in the targeted ES cells (data not shown).

We injected targeted ES cells into the blastocoel cavity of E3.5 C57BL/6 embryos using standard procedures (18). Germline chimeras were produced with three independent Hap1 ES clones from each vector. Chimeras were backcrossed to C57BL/6 females that yielded heterozygous progeny from the Hap1 deletion/replacement allele (Hap1+/−) and the Hap1 conditional gene repair allele (Hap1stop/+).

In all experiments performed, Hap1 mutants were in a mixed C57BL/6 × 129 genetic background.

CamK2α–cre transgene and genetic crosses

For conditional activation of the Hap1 gene repair allele, we used the R1ag5 transgenic line (19) that expresses cre under the control of the calcium/calmodulin–dependent protein kinase II gene (CamK2α) promoter. CamK2α–cre transgenic mice have been backcrossed six times in C57BL/6 129 genetic background. To obtain rescued mutants, we first crossed mice harboring the cre transgene with mice heterozygous for the Hap1 deletion. We then crossed the Hap1+/−;cre mice with mice heterozygous for the conditional gene repair allele (Hap1stop/+).

Genetic analysis of Hap1 and Hdh interaction

Hdh+/− mice (31) were crossed with Hap1+/− mice, and litters were analyzed at birth or at E8.5. Hdh+/− mice were in a mixed C57BL/6 × 129 genetic background.

Genotyping of mice

For routine genotyping of mice we used PCR analysis (30 cycles consisting of 45 s denaturation at 94°C, 45 s annealing at 61°C and 1 min extension at 72°C). We detected the Hap1 deletion allele using the primers Hap12: 5′-GACTGCACTCTCATCTGGCCC-3′ and PGK: 5′-AGCGCATGCTCAGACTGCC-3′ to generate a 170 bp product. For the conditional gene repair allele, we used the primers PGK and Hap11: 5′-GGTTAGGATGGTGACTGCAT-3′ to generate a 200 bp product. The cre transgene was detected using the primers CRE-1: 5′-CTGCCACGACCAAGTGACAGC-3′ and CRE-2: 5′-CTTCTCTACACCTGCGGTGCT-3′ to generate a 325 bp product corresponding to a portion of the cre coding region. To discriminate between the recombinant conditional gene repair Hap1 allele and the wild-type allele, PCR amplification across the single loxP site remaining following Cre-mediated recombination was performed using the primers Hap11 and Hap12 to generate 265 and 315 bp products corresponding to the wild-type and recombinant allele, respectively. Genotyping for the Hdh allele was performed as described (31).

Northern analysis

Total RNA was isolated from adult brain tissue using Trizol reagent according to the protocol provided by the manufacturer. RNA (15 μg) was fractionated on 1% agarose MOPS/sodium acetate/EDTA gels containing formaldehyde. RNA was then transferred to nylon membranes in 10× SSC and the blots hybridized with 32P-labeled random primed DNA probes corresponding to nucleotides 28–237 (exon 1) and nucleotides 408–1053 exons 2–7 (GenBank accession no. AJ000262 for RNA and AJ003128 for the gene). Uniform loading of RNA was monitored by ethidium bromide staining of ribosomal RNA (Fig. 1D).

Histological and anatomical analyses

For histology, we fixed embryos or tissues overnight in Z-fix solution (buffered zinc formalin; Analtech). Embryos were then incubated for 24 h at 4°C in 0.25 M sucrose, 0.2 M glycine, 0.1 M phosphate buffer (pH 7.3); dehydrated; cleared with toluene; and embedded in paraffin. Paraffin blocks were sectioned at 7 μm and stained with H&E. For immunohistochemistry and in situ hybridization analyses, we fixed tissues in 4% paraformaldehyde in PBS and cryopreserved the specimens in 30% sucrose/PBS. We used OCT freezing compound (Tissue Tek) for embedding. Frozen tissues were sectioned at 9 μm in a cryostat and thaw-mounted on SuperFrost slides (Fisher Scientific).

In situ hybridization analyses

In situ hybridization on sections was performed as described using digoxigenin-labeled antisense probes. Probes used were Hap1 (exon 2–7), NPY, AGRP and POMC.

Attachment to the nipple behavior of wild-type and Hap1 mutant mice

Dams, 12–24 h after delivering their newborns, were anesthetized and laid on a paper towel in a 35°C incubator. Wild-type, heterozygous and Hap1 mutant pups (P0, P1) were placed on their mother’s nipple. Each pup received help until the moment it was successfully attached to the nipple (holding the nipple in their mouth to start suckling was defined as successful performance for the test). The actual time needed was recorded. Pups that needed more than 5 min to attach were considered to have failed the test.

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