Targeted disruption of Huntingtin-associated protein-1 (Hap1) results in postnatal death due to depressed feeding behavior

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HAP-1 is a huntingtin-associated protein that is enriched in the brain. To gain insight into the normal physiological role of HAP-1, mice were generated with homozygous disruption at the Hap1 locus. Loss of HAP-1 expression did not alter the gross brain expression levels of its interacting partners, huntingtin and p150glued. Newborn Hap1⁻/⁻ animals are observed at the expected Mendelian frequency suggesting a non-essential role of HAP-1 during embryogenesis. Postnatally, Hap1⁻/⁻ pups show decreased feeding behavior that ultimately leads to malnutrition, dehydration and premature death. Seventy percent of Hap1⁻/⁻ pups fail to survive past the second postnatal day (P2) and 100% of Hap1⁻/⁻ pups fail to survive past P9. From P2 until death, Hap1⁻/⁻ pups show markedly decreased amounts of ingested milk. Hap1⁻/⁻ pups that survive to P8 show signs of starvation including greatly decreased serum leptin levels, decreased brain weight and atrophy of the brain cortical mantle. HAP-1 is particularly enriched in the hypothalamus, which is well documented to regulate feeding behavior. Our results demonstrate that HAP-1 plays an essential role in regulating postnatal feeding.

INTRODUCTION

Huntingtin-associated protein-1 (HAP-1) was initially identified in the yeast two-hybrid system as an interacting partner for huntingtin, the protein encoded by the Huntington disease (HD) gene (1). The HAP-1–huntingtin interaction is increased by expansion of the polyglutamine tract in huntingtin, suggesting that HAP-1 function may be affected in HD (1).

Up to three transcripts differing at their 3' (Hap1A, 1B, and 1C) are expressed from the mouse Hap1 gene due to alternative splicing but only two distinct protein isoforms (HAP-1A and HAP-1B) are encoded (2–4). For simplicity, we usually refer to all Hap1 isoforms together. Northern blot and in situ hybridization analyses have demonstrated Hap1 transcript expression in the olfactory bulb, hypothalamus, brain stem, striatum, cerebellum, hippocampus and colliculi of mouse and rat (1,2,4–6). Expression of Hap1 has also been demonstrated in more defined human brain regions including the amygdala, caudate nucleus, corpus callosum, substantia nigra, subthalamic nucleus and thalamus (7). Hap1 transcripts are associated with neurons and not glia (5). Immunoblotting and immunohistochemistry have confirmed the expression of HAP-1 proteins in regions of the central nervous system (CNS) (1,7–9). The only peripheral organ with high Hap1 transcript expression is the testis (2,4) but HAP-1 protein expression in peripheral tissues has never been documented. Based on its restricted pattern of expression, HAP-1 appears to function solely in neurons of the brain. During mouse development, Hap1 transcripts are detected starting at embryonic day 8.5
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(A8.5) in the neuroepithelium and are enriched in the hypothalamus, superior colliculus and cerebellum by E17.5 (4).

The molecular function of HAP-1 remains poorly defined. HAP-1 has been shown to bind p150glued, an accessory protein for the microtubule motor dynein (10,11). HAP-1 also interacts with duo (alternatively named P-Clp10 or Kalirin-7), a brain specific rac1 guanyl nucleotide exchange factor that binds post synaptic density-associated proteins (12,13). Huntingtin is hypothesized to be involved in vesicle trafficking (14–18). Thus, HAP-1 may be involved in a number of cellular processes including vesicular transport, possibly along microtubules and rac signal transduction at the post-synaptic density.

The proposed molecular functions of HAP-1 have been supported by data on its subcellular localization. HAP-1 has been observed in association with subcellular fractions representing crude synaptic vesicles (5,8,10). Both rat HAP-1 isoforms co-sediment with polymerized endogenous microtubules (11). HAP-1 is also transported along rat sciatic nerve axons in a retrograde direction (19). In immunocytochemical studies, HAP-1 was localized primarily in the cytoplasm and in association with axon terminals and post-synaptic dendritic spines (8,9). Transfection of Hap1A cDNA into PC12 cells stimulated neurite extension suggesting an additional role in neuronal differentiation (20).

To gain insight into the normal in vivo role of HAP-1, we generated mice with homozygous disruption at the Hap1 locus. Hap1−/− mice were born with a normal Mendelian ratio (Table 1). Newborn pups failed to thrive after birth and did not survive past the 9th postnatal day (P9). Decreased feeding after P2 was determined to be the primary cause of death in Hap1−/− pups. Malnourished Hap1−/− pups that survived to P8 showed signs of severe starvation including atrophy in the brain cortical region and decreased serum leptin levels. The effects of Hap1 homozygous disruption on feeding behavior are likely due to Hap1−/− loss of function in the hypothalamus. These unexpected findings establish Hap1 in a crucial role regulating rodent postnatal feeding behavior.

RESULTS

Targeted disruption of murine Hap1

A replacement vector was generated by placing a Neo gene cassette (PGKNeoBpA) 3’ to the ATG translation initiation codon in exon 1 of murine Hap1 as detailed under Materials and Methods (Fig. 1A). We have previously described a similar strategy (21). Correct integration of the selectable marker into the endogenous Hap1 locus of the embryonic stem (ES) cells was detected by long range polymerase chain reaction (PCR) in which one of the primers (A) is anchored in the Neo sequence (Fig. 1B). Integration in several targeted ES cell clones was confirmed by Southern blotting. EcoRI digested DNA of three independent clones were hybridized with a 3’ external probe that binds sequences outside the targeting region. This probe detected two EcoRI fragments, corresponding to the wild-type (10.3 kb) and targeted alleles (7.7 kb) (Fig. 1C). The insertion of the Neo cassette introduced an additional internal EcoRI site, producing a shorter fragment in the targeted allele. Correct targeting was further confirmed using a 5’ probe and Neo specific probe (data not shown). Correctly targeted ES cells were used to derive chimeric mice that transmitted the mutation through their germ line.

Mice heterozygous for targeted disruption in Hap1 were of normal size and fertile. Progeny from matings were genotyped using two PCR reactions (Fig. 1D). Firstly, the long range PCR described above recognizes the Neo cassette in Hap1+/− and Hap1+/− mice. A second PCR reaction recognizes the undisrupted Hap1 genomic region of Hap1+/+ and Hap1+/− mice. We confirmed using quantitative PCR that Hap1 transcript levels were decreased approximately 50% in brains of Hap1+/− E18.5 embryos relative to wild-type (Fig. 1E).

Ablation of HAP-1 expression but normal expression of p150glued and huntingtin in brains of Hap1−/− mice at P1

To confirm loss of HAP-1 expression, we prepared extracts from whole brains of Hap1+/+, +/− and −/− mice at P1. Proteins resolved on a SDS-polyacrylamide gel were immunoblotted with a monoclonal antibody that detects both HAP-1A and HAP-1B isoforms at approximately 100 kDa (Fig. 2A). Equivalent high levels of HAP-1A and HAP-1B expression were observed in brain extracts of Hap1+/+ and +/− mice. In contrast, expression of both HAP-1 isoforms was not detectable in brains of Hap1−/− mice. Immunoblotting for actin expression on the same membrane confirmed equal loading.

We determined whether the complete absence of HAP-1 resulted in grossly altered expression of its interacting partners. Brain extracts from P1 mice were immunoblotted using a monoclonal antibody specific for p150glued, the dynein accessory protein that interacts with HAP-1 proteins (10,11). Equivalent high expression levels of p150glued were detected in the brains of Hap1+/+, +/− and −/− mice (Fig. 2A). Brain extracts were also immunoblotted with monoclonal antibody 2166 to study huntingtin expression (Fig. 2B). We observed that huntingtin expression levels in the brains of Hap1−/− mice are similar to those in Hap1+/− and wild-type mice. When studying p150glued and huntingtin expression, actin immunoblotting confirmed equal loading of protein.

Smaller size of Hap1−/− newborn pups in spite of grossly normal development

Since HAP-1 is expressed in the brain during development, we next determined whether loss of HAP-1 function is associated with embryonic lethality. We observed that the frequency of Hap1+/+, +/− and −/− embryos was similar to the expected 1:2:1 Mendelian ratio at E17.5–19.5 (Table 1). Hap1−/− embryos between E17.5–19.5, once removed from their amniotic sacs, had normal exterior appearances. In accord with normal development, Hap1+/+, +/− and −/− pups were born with a normal Mendelian ratio (Table 1). Newborn Hap1−/− mice displayed normal proportions of head and body size in addition to normal pink colored skin, which indicated proper circulatory and respiratory function. However, Hap1−/− mice at P1 were slightly smaller than Hap1+/+ and +/−.
Figure 1. Homozygous disruption at the mouse Hap1 locus. (A) Top: Schematic showing genomic structure and exons 1–11 of the mouse Hap1 locus. The positions of the TATA box transcription start, ATG initiation codon and TGA termination codon are indicated. The relative positions of recognition sites for EcoRI (E), HindIII (H) and XhoI (X) are shown above. Bottom: To generate the targeting construct, a 900 base HindIII–XhoI fragment of Hap1 was replaced with the 1.6 kb pgk Neo bpa selectable marker. The Neo cassette contains an EcoRI site at its 5' end that becomes incorporated into the targeted Hap1 allele. The targeting vector contains 3.3 kb of 5' and 3.4 kb of 3' homologous sequence. PCR using primer A, which is anchored in the Neo sequence and primer B amplifies from disrupted Hap1 loci in +/− and −/− animals. PCR using primers C and D amplifies only from intron 1 sequences in the endogenous Hap1 loci of +/+, +/− and −/− animals. The position of the 3' Hap1 probe (3' pr) employed during Southern analysis is shown as a black box below the sequence. We previously determined the complete genomic organization of the mouse Hap1 locus (ref. 3). Positions of restriction digest sites (using numbering system of AJ003128): EcoRI-1021; HindIII-2607; EcoRI-2696; HindIII-5989; XhoI 6894; EcoRI-13009. (B) Representative PCR from DNA of 7 independent ES cell clones using primers A and B that recognizes insertion of the Neo cassette. The expected 3.3 kb amplicon is detected in 5 of 7 clones. (C) Southern analysis using a probe hybridising to the 3' portion of Hap1 on EcoRI digested DNA from 3 representative ES cell clones. A strong 7.7 kb signal, corresponding to the targeted Hap1 allele, was detected in clones 22, 29 and 48 but not in untransfected ES cells (WT). The probe also detects a strong 10.3 kb signal representing the wild-type Hap1 allele. (D) Representative PCR data on DNA from Hap1+/+, +/− and −/− mice. Top: PCR employing primers C and D only amplifies the 500 bp product from DNA of Hap1+/+ and +/− mice. Bottom: Long range PCR employing primers A and B only amplifies the 3.3 kb product from disrupted loci of Hap1+/− and −/− mice. (E) Relative expression levels of Hap1 mRNA were quantified in brain total RNA extracts obtained from Hap1+/+, +/− and −/− E18.5 embryos using real time quantitative PCR (n=3 embryos per group). Hap1 expression levels from each embryo were normalized to actin mRNA levels quantified in the same sample. The average Hap1 normalized expression levels within each group± standard deviations are indicated. Normalized Hap1 expression levels were corrected by subtracting background PCR signals detected in this assay from Hap1−/− samples. The relative Hap1 expression levels in Hap1+/− embryo brain expresses approximately 50% less Hap1 mRNA than wild-type embryo brain.
littermate controls. The body weights of n = 172 animals at P1 from 13 litters are shown in Figure 3A. Almost all (>94%) of Hap1<sup>+/−</sup> pups weighed less than 1.60 g. In contrast, 44% of Hap1<sup>+/+</sup> and 48% of Hap1<sup>−/−</sup> animals weighed more than 1.60 g. The mean body weight of Hap1<sup>−/−</sup> P1 animals was significantly lower than either Hap1<sup>+/+</sup> or Hap1<sup>+/−</sup> animals (Hap1<sup>−/−</sup> versus Hap1<sup>+/+</sup>; P < 0.0005).

To determine whether development of internal anatomy was normal in P1 Hap1<sup>−/−</sup> mice, 8 µm sagittal whole body sections were stained with haematoxylin and eosin (H&E) and analyzed by light microscopy (Fig. 3B). Qualitative assessment of Hap1<sup>−/−</sup> mice revealed that all organs were present and similar in appearance to those of wild-type littermates. Proper formation of the heart, lung, liver, stomach, kidneys and adrenal glands could be observed. Hap1<sup>−/−</sup> newborn mice are smaller than most control wild-type littermates and this is reflected in a proportionately smaller stomach containing milk.

A higher magnification (40 ×), the cyto-architectures of the heart, thymus, lungs, liver, kidneys, stomach lining, small-intestine and large-intestine were normal as compared to the control littermates.

Since HAP-1 proteins are restricted to the CNS, we investigated whether brain development was normal in newborn Hap1<sup>−/−</sup> mice. We observed that homozygous disruption of Hap1 did not affect the general exterior appearance or size of the brain at P1. To search for brain defects at P1, a complete series of 8 µm coronal sections were prepared from Hap1<sup>−/−</sup> and control wild-type littermate pups followed by staining with cresyl violet or H&E. The gross anatomy and neuronal density within the cortex was observed to be normal in brains of Hap1<sup>−/−</sup> mice as compared to wild-type (Fig. 3C). In addition the neuronal density and organization within the hypothalamus and surrounding region were identical between Hap1<sup>−/−</sup> and wild-type mice despite the normal enrichment of HAP-1 within this area (Fig. 3D and Fig. 8). In Hap1<sup>−/−</sup> mice, all major hypothalamic nuclei were present and of normal size including the ventromedial hypothalasus (VMH) and dorsomedial hypothalamus (DMH). In addition, the 3rd ventricle was not enlarged. The arcuate nucleus (ARC) and paraventricular nuclei (PVN) were also normally formed in Hap1<sup>−/−</sup> P1 pups (data not shown). We confirmed that the developing olfactory bulb and main olfactory epithelium were normal in Hap1<sup>−/−</sup> mice at P1 (Fig. 3E). Our investigation, which included detailed characterization of the olfactory bulb subventricular zone, granule cell layer, mitral cell layer and external plexiform layer, indicated no abnormalities.

Postnatal death in Hap1<sup>−/−</sup> mice

We initially observed that no Hap1<sup>−/−</sup> animals could be detected at weaning age (P21) in multiple matings of Hap1<sup>+/−</sup> mice. Since Hap1<sup>−/−</sup> pups survived development and were born at the expected frequency, we investigated their natural history on a daily basis after birth. Greater than 70% of Hap1<sup>−/−</sup> pups were dead by P2 and 100% of Hap1<sup>−/−</sup> pups failed to survive past P8 (Fig. 4A). These data are based on analyses of n = 19 Hap1<sup>−/−</sup> pups derived from 7 litters. Hap1<sup>−/−</sup> mice weighed slightly less than control littermates at P1 but this difference becomes more dramatic by P3 (Fig. 4B). Hap1<sup>+/+</sup> and Hap1<sup>+/−</sup> pups gained weight daily with a markedly higher rate than Hap1<sup>−/−</sup> pups. By P5, Hap1<sup>−/−</sup> pups weighed on average 30-40% less than their

### Table 1. Normal Mendelian frequency of Hap1<sup>+/+</sup>, +/−, and −/− mice at E17.5–E19.5 and P1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E17.5–E19.5</th>
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<tr>
<td>+/+</td>
<td>11</td>
<td>47</td>
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<td>19</td>
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<td>+/−</td>
<td>13</td>
<td>50</td>
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<td>total</td>
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<td>205</td>
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Hap1<sup>+/−</sup> × Hap1<sup>+/−</sup> matings were established. To study frequencies in utero, embryos from n = 3 litters were isolated between E17.5–E19.5 and genotyped. To study Hap1<sup>−/−</sup> mice at birth, the progeny from n = 15 litters were isolated and genotyped.
Figure 3. Decreased body weights in Hap1<sup>−/−</sup> P1 pups despite grossly normal development of brain and internal anatomy. (A) Distribution of body weights in n = 172 Hap1<sup>+/−</sup>, +/- and −/− P1 animals as observed in n = 13 litters. Hap1<sup>−/−</sup> pups weighing more than 1.60 g are very rarely observed in contrast with Hap1<sup>+/−</sup> and +/- animals. The mean body weight ± standard deviation is indicated to the right of scatter plots. (*) The mean body weight of Hap1<sup>−/−</sup> animals is significantly different from the mean weights of Hap1<sup>+/−</sup> and +/- pups as determined by two-sample, two-tailed Student’s t-tests (on data with equal variances): Hap1<sup>−/−</sup> versus +/-, P < 0.005; Hap1<sup>−/−</sup> versus +/−, P < 0.00005; Hap1<sup>−/−</sup> versus −/−, P = 0.25. (B) Photographs showing sagittal sections through whole bodies of Hap1<sup>+/−</sup> and −/− P1 pups stained with H&E. At P1, no major differences were observed in the gross anatomy of major organs including the heart (H), liver (Li), lungs (Lu), adrenal gland (A) and kidney (K). Less milk could be observed in the stomach (S) of the Hap1<sup>−/−</sup> pup. The enlarged space around the caudal brain of the Hap1<sup>+/−</sup> animal is an artifact from tissue processing. Scale bar: 2 mm. (C) Photomicrographs of H&E stained dorsal cortex from Hap1<sup>+/−</sup> and −/− pups at P1. No differences were observed between the cortex of Hap1<sup>+/−</sup> and −/− pups in the marginal zone, pyramidal cell layers, corpus callosum and sub-ventricular zone (SVZ). Scale bar: 100 μm. (D) Photomicrographs of H&E stained hypothalamic region at the level of the ventromedial hypothalamus from P1 Hap1<sup>+/−</sup> and −/− pups. No gross differences could be observed in the formation of the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). The size of the 3rd ventricle (3v) was normal in the Hap1<sup>−/−</sup> brain. Scale bar: 100 μm. (E) Photomicrograph showing cresyl violet stained section of the olfactory bulb and surrounding region of Hap1<sup>−/−</sup> pups. Scale bar: 350 μm. Bottom panels show a more detailed view of the olfactory bulb. No abnormalities in Hap1<sup>−/−</sup> pups could be detected at the formation of the subventricular zone (SVZ), granule cell layer, mitral cell layer and external plexiform layer. Scale bar: 70 μm.
Failure to thrive in Hap-1\textsuperscript{+/+} newborn mice. (A) Plot showing survival profiles of Hap1\textsuperscript{+/+}, Hap1\textsuperscript{+/−}, and Hap1\textsuperscript{−/−} mice from the 1st to 8th postnatal day. Data are representative of \(n = 22\) Hap1\textsuperscript{+/+}, \(n = 53\) Hap1\textsuperscript{+/−}, and \(n = 19\) Hap1\textsuperscript{−/−} animals from \(n = 7\) litters. Greater than 70% of Hap1\textsuperscript{−/−} pups are found dead by P2 and no Hap1\textsuperscript{−/−} pups have survived past P8. Significantly decreased survival is not observed in Hap1\textsuperscript{+/−} pups. Data analysis from \(n = 14\) litters. Each Hap1\textsuperscript{+/+} data point represents 10–36 pups; each Hap1\textsuperscript{+/−} data point represents 14–99 pups; each Hap1\textsuperscript{−/−} data point represents 3–37 pups. Standard deviations of weights are indicated. (*) Hap1\textsuperscript{−/−} pups weigh significantly less than control pups by P5 (60% the weight of Hap1\textsuperscript{+/+}, \(P < 0.00001\); 66% the weight of Hap1\textsuperscript{+/−}, \(P < 0.001\); \(P\) values determined by two-sample, two-tailed Student's t-tests on data with unequal variances). (B) Plot showing average weights daily after birth in Hap1\textsuperscript{+/+}, Hap1\textsuperscript{+/−}, and Hap1\textsuperscript{−/−} pups. Data analysis from \(n = 14\) litters. Each Hap1\textsuperscript{+/+} data point represents 10–36 pups; each Hap1\textsuperscript{+/−} data point represents 14–99 pups; each Hap1\textsuperscript{−/−} data point represents 3–37 pups. Standard deviations of weights are indicated. (*) Hap1\textsuperscript{−/−} pups weigh significantly less than control pups by P5 (60% the weight of Hap1\textsuperscript{+/+}, \(P < 0.00001\); 66% the weight of Hap1\textsuperscript{+/−}, \(P < 0.001\); \(P\) values determined by two-sample, two-tailed Student's t-tests on data with unequal variances). (C) Photo of a live Hap1\textsuperscript{−/−} and Hap1\textsuperscript{+/−} pup at P3. The Hap1\textsuperscript{−/−} pup is dramatically smaller, dehydrated and malnourished. Little to no milk is observed in the stomachs of Hap1\textsuperscript{−/−} animals at P3 in contrast with control Hap1\textsuperscript{+/+} littermates. The scale in millimeters is indicated. (D) Top view of live Hap1\textsuperscript{−/−} and Hap1\textsuperscript{+/−} at P3. Note dramatically smaller size and loose skin observed in Hap1\textsuperscript{−/−} animals.
control littermates. By appearance, Hap1<sup>1+/−</sup> mice were obviously not as healthy compared to control littermates by P2 and could be readily distinguished by animal technical staff blinded to the pup genotypes. Hap1<sup>1+/−</sup> pups appeared dehydrated and malnourished (Fig. 4C, D). The skin of Hap1<sup>1+/−</sup> pups was dry, pale and present in loose folds around the body. Despite these, many other physiological processes were normal in Hap1<sup>1+/−</sup> mice including sensitivity to tactile stimulation and mild pain. By P8, hair and melanin were observed to form on Hap1<sup>1+/−</sup> mice similar to control littermates.

In contrast to mice with homozygous disruption, Hap1<sup>1+/−</sup> mice displayed normal feeding behavior and could survive for at least 8 months without a detectable phenotype. In Hap1<sup>1+/−</sup> adult mice, body size and mating activity were normal. The average litter sizes from Hap1<sup>1+/−</sup> × Hap1<sup>1+/−</sup> matings were normal (13.1±3.4 as observed in n=15 litters).

Central cause for diminished feeding

The inability to thrive has been observed in 100% (n=43) of Hap1<sup>1+/−</sup> animals from 13 litters. At P1, less milk can often be observed in stomachs of Hap1<sup>1+/−</sup> pups as compared to littermates (see Fig. 3B). However, decreased feeding is obvious by P2 when little to no milk was observed in stomachs of Hap1<sup>1+/−</sup> pups.

Hap1<sup>1+/−</sup> pups were always observed to be grouped together with control littermates under the nursing mother ruling out the possibility that they were selectively ignored. We investigated whether the diminished feeding behavior of Hap1<sup>1+/−</sup> mice was caused by a physical disability. Hap1<sup>1+/−</sup> pups initially appeared lively, similar to control littermates. Hap1<sup>1+/−</sup> mice ranging from P1–P6 were able to right themselves properly after being placed on their backs demonstrating normal balance and motor coordination. Suckling in neonatal mice is a complex process involving both coordinated rhythmic mouth movements and swallowing. Like control littermates, Hap1<sup>1+/−</sup> pups between P1–P4 demonstrated rhythmic jaw movements upon manual stimulation of the mouth region indicating that decreased feeding was not due to a gross facial structural defect.

We investigated whether diminished feeding was due to a failure of Hap1<sup>1+/−</sup> mice to compete with more fit littermates. Newborn pups were genotyped at birth and the litter was trimmed between P1–P2 to contain 5 Hap1<sup>1+/−</sup> pups in addition to 3 control Hap1<sup>1+/−</sup> littermates that would stimulate lactation in the nursing mother. Hap1<sup>1+/−</sup> animals did not thrive even in this context that favored their access to feeding. Despite the absence of competition, less milk could be observed in the stomachs of Hap1<sup>1+/−</sup> animals as compared to controls (Fig. 5). Two of five Hap1<sup>1+/−</sup> pups died by P2. Four of five Hap1<sup>1+/−</sup> mice failed to survive to P5. The single Hap1<sup>1+/−</sup> animal that survived to P7 weighed 40% less than the Hap1<sup>1+/−</sup> control littermates and appeared dehydrated.

Decreased leptin levels in malnourished Hap1<sup>1+/−</sup> mice

In mammals, energy homeostasis is tightly regulated in part by leptin, an endocrine peptide signal produced by peripheral adipocytes that ultimately enters the brain to inhibit feeding behavior (22). Previously, mouse pups with the anorexia mutation have been shown to display preweaning starvation, growth defects and low levels of serum leptin (23). We investigated whether symptomatic Hap1<sup>1+/−</sup> mice similarly displayed decreased leptin levels. Serum was obtained from malnourished Hap1<sup>1+/−</sup> pups at P8 and from healthy Hap1<sup>1+/−</sup> control pups. Leptin levels in the control Hap1<sup>1+/−</sup> pups were 17–22 ng/ml, which falls in the expected range for wild-type postnatal mice at this age (Fig. 6) (24–26). In contrast, leptin levels in Hap1<sup>1+/−</sup> pups were approximately 7-fold lower and detectable only at background levels (2–3 ng/ml). These results suggest that Hap1<sup>1+/−</sup> mice may share some of the pathogenic mechanisms with the anorexia mouse model.
Malformation of cortical neurons in Hap1<sup>−/−</sup> pups

Proper development of the brain, in particular the cortical layers, is highly dependent on nutrition during the postnatal period (27). We observed that brains of Hap1<sup>−/−</sup> mice at P8 weighed 30% less than controls (Hap1<sup>+/−</sup>: 218 and 221 mg; Hap1<sup>+/+</sup>: 304 and 325 mg). The brains of P8 Hap1<sup>−/−</sup> pups appeared proportionately smaller than those of Hap1<sup>+/−</sup>-littermate controls (Fig. 7A). The brains of P8 Hap1<sup>−/−</sup> pups also displayed striking inward depressions in both hemispheres of the cortical mantle that extended from the somatosensory cortex to the parietal associated cortex, auditory cortex and visual cortex.

To study the cortical malformation in higher detail, 8 μm thick coronal sections from Hap1<sup>−/−</sup> and control Hap1<sup>+/+</sup>-P8 brains were stained with cresyl violet and analysed. The thickness of the cortical mantle as measured from pia to white matter was reduced by up to 34% in the affected areas of the Hap1<sup>−/−</sup> brain as compared to controls (Fig. 7B). The morphologic alteration appeared to take place throughout cortical neuronal layers II through VI. In layer V, which appeared most affected, neuronal density was 1.75-fold higher.

![Figure 7. Decreased brain size and malformation of cortical layers in Hap1<sup>−/−</sup> pups at P8. (A) Dorsal views of fixed brains from Hap1<sup>+/−</sup> and Hap1<sup>−/−</sup> pups at P8. The brains of Hap1<sup>−/−</sup> animals at this age appear proportionately smaller and weigh 30% less than controls. (B) Photomicrographs of 8 μm thick coronal sections stained with cresyl violet showing the cortical mantle in brains of Hap1<sup>+/−</sup> and Hap1<sup>−/−</sup> pups at P8. A 34.3% reduction in total cortical thickness from the white mater (WM) to pia is observed in the Hap1<sup>−/−</sup> brain. Proper formation of cortical layers I to VI are indicated in the Hap1<sup>+/−</sup> brain. Scale bar 150 μm. (C) High magnification view of cortical layer V in Hap1<sup>+/−</sup> and Hap1<sup>−/−</sup> brains at P8. The cell bodies in Hap1<sup>−/−</sup> brains are smaller, compressed and slightly hyperchromatic as compared to control brains. Increased neuronal density can also be observed in the brains of Hap1<sup>−/−</sup> pups. Scale bar: 25 μm.](https://academic.oup.com/hmg/article-abstract/11/8/945/638548)
in the Hap1−/− brain as compared to the Hap1+/− littermate control brain. Similarly, a 1.4-fold increase in neuronal density was observed in cortical layer II of the Hap1−/− brain compared to controls. The thickness of cortical layer I, which contains axons from pyramidal neurons, was not altered in Hap1−/− animals. As observed in cortical layer V, cell bodies were smaller and shrunken in Hap1−/− brains (Fig. 7C).

Within this cortical layer, we determined that the cell body cross sectional area was decreased 37% in Hap1−/− brains as compared to control brains (Hap1+/−: 172.1 ± 60.4 μm², n = 157 neurons; Hap1+/−: 274.1 ± 89.3 μm², n = 122 neurons; P < 0.0001 by one-sided, non-paired and unequal variance
HAP-1 protein expression in the hypothalamus during embryogenesis

Hap1 mRNA is enriched in the developing mouse hypothalamus at E17.5 (4). In light of the feeding abnormality in postnatal Hap1+/−/ mice and the known critical role of the hypothalamus in feeding behavior regulation (for review see refs 28 and 29), we wished to characterize the normal developmental HAP-1 expression levels in the hypothalamus relative to other brain regions. In coronal brain sections prepared from wild-type embryos at E17.5, we observed strong HAP-1 immunoreactivity within the hypothalamus (Fig. 8A). Only moderate levels of HAP-1 immunoreactivity were observed in the ventromedial thalamic nucleus and habenula. By comparison, very low HAP-1 immunoreactivity was observed in the surrounding striatum, cortex and globus pallidus. Significant HAP-1 immunoreactivity could also not be detected in the embryonic olfactory bulb (data not shown).

A more detailed characterization of the hypothalamus from wild-type E17.5 embryos revealed high levels of HAP-1 immunoreactivity in the dorsomedial nucleus (DMN) and ARC (Fig. 8B). In contrast, the ventromedial hypothalamic nucleus (VMH) displayed lower levels of HAP-1 immunoreactivity. Interestingly, the ARC is known to play a critical role regulating feeding behavior in response to the endocrine signal leptin (28,29). The hypothalamus of Hap1−/−/ embryos contained no HAP-1 immunoreactivity, confirming loss of protein expression.

Within the ARC and DMN, levels of diffuse neuronal cytoplasmic HAP-1 immunoreactivity were equivalent between the Hap1+/+/ and Hap1+/−/ mice (Fig. 8C). In the hypothalamus of Hap1+/+/ mice, HAP-1 immunoreactivity was also observed in many of the neurons as large cytoplasmic puncta corresponding to stigmoid bodies. We have previously described these large HAP-1 immunopositive structures of undefined function which reside in the neuronal perikarya (8). Fewer neurons in brains of Hap1−/−/ animals contained HAP-1 positive stigmoid bodies. Consistent with previous findings, we observed that the diffuse HAP-1 immunoreactivity is mostly cytoplasmic and largely excluded from the nucleus (Fig. 8D).

DISCUSSION

HAP-1 was the first protein shown to interact with huntingtin (1). Despite its description 6 years ago, little is known about its normal physiological role. We set out to derive insights into the in vivo function of the protein by generating mice with homozygous disruption at the Hap1 locus. As expected, expression of HAP-1 protein in the brain was completely ablated by the genetic targeting strategy.

All of our findings indicate that prenatal development is normal in mice without HAP-1. Evidence in support of this conclusion includes the normal frequency, exterior appearance and internal organ structures of Hap1+/−/ newborn pups. Development of the brain, including the hypothalamus, where HAP-1 proteins are predominantly expressed, appeared unaffected at birth. As such, HAP-1 appears to be playing a non-critical role during in utero development.

In contrast with the findings from prenatal development, HAP-1 was essential in early postnatal life. Hap1−/−/ newborn mice initially are healthy but the majority of Hap1−/−/ pups are dead by P2 and no Hap1−/−/ pup has survived past P8. The fraction of Hap1−/−/ mice that survive past P2 gain weight poorly and show clear signs of malnutrition and dehydration. Early during P1 (6–12 hours after birth), the amounts of milk in stomachs of Hap1−/−/ pups were significant but slightly lower than in control littermates. The slightly decreased amounts of ingested milk likely underlie the lower body weights of P1 Hap1−/−/ pups. Importantly, little to no milk was present in Hap1−/−/ mice by P2 indicating that feeding sharply decreases during the early postnatal period. We conclude that diminished feeding and malnutrition are the primary causes of death in newborn Hap1−/−/ animals.

Malnourished Hap1−/−/ animals that survive to P8 display profoundly altered brain morphology and neuronal architecture. The abnormalities included decreased overall brain weight, decreased overall cortical mantle thickness, malformation of the cortical layers and shrunken cell bodies. We interpret these pathological changes to be a secondary consequence of prolonged starvation as opposed to a direct effect of HAP-1 loss of function. HAP-1 is not well expressed in the cortex (where the most drastic brain atrophy occurred). In addition, controlled malnutrition postnatally in wild-type mice has been previously documented to result in decreased overall cortex thickness and increased cell density within cortical layers II and V that closely resemble the changes observed in Hap1−/−/ mice (27).

Malnutrition could conceivably be due to a physical obstruction of feeding. Contrary to this idea, newborn Hap1+/−/ pups feed initially relatively well indicating that facial structures required for suckling and swallowing were normal at birth. The stomach lining structure in Hap1+/−/ pups appears intact suggesting that nutrient absorption in downstream portions of the gastrointestinal tract was normal. Hap1+/−/ pups also possessed functional righting reflexes thereby demonstrating proper strength, balance, and limb coordination. These findings distinguish the Hap1+/−/ mouse model from mice with homozygous disruption in the POU domain gene Brn-3a, which show suckling defects in addition to uncoordinated limb and trunk movements (30). Hap1+/−/ mice also demonstrate normal rhythmic jaw movements upon mouth stimulation in contrast to mice with homozygous disruption of the N-methyl-D-aspartate receptor α2 subunit, which show abnormal suckling and defective mouth reflexes (31). Taken together, the decreased feeding of Hap1−/−/ pups does not appear to be caused by a peripheral physical impediment.

Olfaction cues are an important component of rodent neonatal suckling (32,33) and mouse models have been generated which display defective suckling and postnatal death due to abnormal olfaction (34,35). Functionally normal olfaction in Hap1−/−/ mice remains to be conclusively demonstrated. However, in Hap1−/−/ pups at P1, the olfactory bulb cellular organization was normal and the pups fed relatively well. In addition, HAP-1 is weakly expressed in the olfactory bulb at this age compared with the hypothalamus (C.-A.G., E.Y.C. and M.R.H., unpublished). Based on these
findings, we conclude that the suckling defect in Hap1−/− pups is likely not due to defective olfaction.

Feeding, weight gain and survival of Hap1−/− pups could not be improved by trimming the litter to remove almost all of the competing control littersmates. This observation, along with our other data, suggests that Hap1−/− mice possess a central depression of postnatal appetite that progresses in severity until premature death 2–8 days after birth. This idea is interesting in light of the well-documented role of the hypothalamus in feeding and energy balance regulation (for review see refs 28 and 29). The endocrine peptide leptin, which is generated and secreted by peripheral adipocytes, signals to the hypothalamus and ultimately inhibits feeding behavior (22). In particular, leptin is known to bind its receptor on a sub-population of neurons within the ARC and up-regulate the expression of alpha-melanocyte-stimulating hormone (α-MSH) and the peptide encoded by cocaine- and amphetamine-regulated transcript (CART). α-MSH is subsequently released at synapses within the PVN of the hypothalamus to stimulate additional neuronal circuits that result in feeding behavior inhibition (36). Leptin also binds receptors on a distinct ARC neuronal sub-population to inhibit expression of the orexigenic signals neuropeptide Y (NPY) and agouti-related protein (AGRP), which are both also released at synapses within the PVN (36). Strikingly, direct injection of NPY into the PVN stimulates robust feeding behavior within minutes (37).

Here we have shown that HAP-1 is expressed in the embryonic brain of wild-type mice with particular enrichment in the ARC and DMN of the hypothalamus. Other brain regions such as the striatum, cortex, and olfactory bulb contained very low levels of HAP-1. In wild-type mice at P2, HAP-1 levels were also relatively low in the olfactory bulb in contrast with high HAP-1 levels within the hypothalamus (C.-A.G., E.Y.C. and M.R.H., unpublished). Neuronal dysfunction in the hypothalamus, particularly within the ARC, due to HAP-1 ablation could possibly underlie the observed decreased appetite of Hap1−/− mice.

Serum leptin levels were drastically reduced to background levels in malnourished P8 Hap1−/− mice. In adults, leptin levels correlate with amounts of body fat (38) but this tight correlation is not well conserved in early postnatal mice (24,26). However, the decreased leptin levels in Hap1−/− mice are most likely a secondary effect of prolonged starvation and the resulting absence of fat depots. Leptin was only poorly detected in serum of Hap1−/− and control pups at P2 (E.Y.C. and M.R.H., unpublished) as observed by others in wild-type mouse pups at this age (23). Since decreased feeding in Hap1−/− mice is obvious by P2, abnormalities in leptin signaling likely cannot explain the feeding disorder. In addition, signaling mechanisms that respond to serum leptin do not form in the mouse until P17 (39).

Regardless of its role or etiology, decreased leptin levels are also observed in mice homozygous for the recessive anorexia (anx) mutation, which display decreased feeding, starvation and premature death. Thus, Hap1−/− mice may share some of the pathogenic mechanisms involved in anx/anx mice. Indeed, abnormalities in NPY, AGRP and CART levels or localization have been documented in anx/anx mice (23,40,41). To investigate further a possible hypothalamic disturbance, we measured transcript levels of NPY and AGRP in extracts prepared from dissected hypothalami of P1.5 Hap1−/− mice. We did not detect significantly altered expression of NPY or AGRP mRNA in the hypothalamus of Hap1−/− pups as compared to controls (E.Y.C. and M.R.H., unpublished). These preliminary findings, however, do not rule out alterations of neuropeptide transcript levels in more defined areas of the hypothalamus or alterations in neuropeptide or receptor protein levels.

The direct cause of decreased feeding in Hap1−/− mice remains to be demonstrated but we speculate that hypothalamic dysfunction is the primary cause. In one possible scenario, HAP-1 loss of function in hypothalamic neurons could impair vesicle trafficking and disturb the synthesis of neuropeptides or receptors critical for appetite regulation. Alternatively, HAP-1 ablation could result in abnormal rac1 signal transduction and cytoskeleton dysregulation via altered function of duo, the rac1 regulatory protein that binds HAP-1 (12,13).

The levels of p150glued and huntingtin were unaltered in the brains of Hap1−/− mice. As such, there does not appear to be a cellular pathway to link HAP-1 expression and the steady state levels of at least these two HAP-1 interacting proteins. HAP-1 proteins have been hypothesized to function in vesicle trafficking involving microtubules within neurons and this proposed role is supported by its interaction with p150glued and huntingtin. Further studies are required on the functional effects of Hap1 homoygous disruption in vesicle trafficking.

Consistent with our previous data gathered in adult rat brain (8), HAP-1 was primarily cytoplasmic in the mouse embryonic brain and could be detected in large structures corresponding to stigmoid bodies. These data indicate that stigmoid bodies form during gestation and may have a role in early brain development. The function of stigmoid bodies remains undefined but some findings have suggested a role in the ARC involving sex-hormone response (42,43).

Mice with heterozygous disruption in Hap1 were confirmed to show a 50% decrease in Hap1 mRNA levels relative to wild-type. However, total HAP-1 protein levels in the brain did not correlate with decreased Hap1 mRNA levels. As follows, we predict that HAP-1 has a relatively long half-life that reflects a critical function in the cell. The unaltered HAP-1 levels in Hap1−/− mice could explain their lack of phenotype. We did observe slightly fewer HAP-1 immunoreactive stigmoid bodies in the hypothalamus of Hap1−/− mice but it remains unclear whether this reflects decreased overall stigmoid body numbers or decreased HAP-1 content within each stigmoid body.

The precise role of HAP-1 in HD pathogenesis still remains unresolved but our findings from Hap1−/− mice may have relevance. Abnormalities have been observed in the hypothalamus of HD patients such as neuronal degeneration and enhanced dopaminergic activity with altered hormonal release (44,45). Altered HAP-1 localization or function in the hypothalamus resulting from increased interaction with mutant huntingtin could be involved in these findings. In addition, it has been well recognized that HD patients often display weight loss in spite of excellent appetites (46,47). In light of our results on decreased feeding in response to altered brain HAP-1 levels, we speculate that abnormal HAP-1 function may be involved in the overall weight loss of HD patients.

In summary, we have defined a novel unexpected role for HAP-1 in the regulation of postnatal mouse feeding behavior.
This finding raises the general question on the role of HAP-1 in influencing feeding of mammals including humans. Further studies of the Hap1−/− mouse model could provide additional insights into the neural circuits regulating feeding behavior.

MATERIALS AND METHODS

Construction of Hap1 gene targeting replacement vector

A 7.5 kb EcoRI-Xbal genomic fragment encompassing exons 1–8 of murine Hap1 (3) was derived from a phage clone isolated using a 129/Sv mouse genomic library (Stratagene, La Jolla, California, USA). This genomic sequence was subcloned into pBluescriptII (Stratagene). A 1.6 kb PGKNeo+pA cassette was introduced into exon 1, downstream of the ATG translation initiation codon, by replacing a 905 basepair (bp) HindIII-XhoI fragment encompassing both exon 1 and intron 1 (Fig. 1A).

Transfection of embryonic stem cells

The targeting vector was linearized with NotI and introduced into R1 ES cells (48) by electroporation at a concentration of 10 μg DNA per 1 × 10⁶ cells. The cells were cultured on gelatinized dishes in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with knockout serum replacement (Gibco BRL, Burlington, Ontario, Canada) and leukemia inhibitory factor (LIF), essentially as previously described (21). Colonies that survived G418 selection (180 μg/ml) were picked and tested for homologous recombination by PCR using a Neo specific primer (5′-GGG CTC TAT GGC TTC TGA GGC G-3′; primer A) and an external primer (5′-CTG CTC CCA TAA TCC TGC ATG-3′; primer B) (Fig. 1A). Positive clones were expanded and genomic DNA tested by Southern blotting using a 5′ external probe, 3′ external probe and Neo specific probe.

Generation of aggregation chimeras

Correctly targeted ES cells were used to derive chimeras using the aggregation procedure with ICR cleavage stage embryos (49). Following aggregation, embryos were cultured overnight until the blastocyst stage and transferred into ICR pseudopregnant females. Chimeras were bred to ICR mice to follow germline transmission of the targeted allele. Chimeric mice derived from ES cell clone #22 resulted in germline transmission. Mice used for analysis were on a mixed 129/C57BL6 background.

Genotyping of progeny

Heterozygous × heterozygous matings were established and the resulting progeny were genotyped using DNA isolated from tails (or clipped fingers when studying P1 animals). Heterozygous and homozygous knockout offspring were identified by PCR using the Neo specific primer set described above. Homozygous knockout animals were identified using PCR primers specific for the targeted Hap1 sequences (forward 5′-ATC CCG CAT TGG GCA CTA TTT G-3′ (primer C) and reverse 5′-CCC ACT CCT CCC TCT TCA TTC CAG-3′ (primer D) (Fig. 1A).

Assessment of Hap1 mRNA levels

Brains from mouse embryos at E18.5 were isolated and stored in RNA later reagent (Ambion Inc., Austin, Texas, USA) at 4°C. Total RNA was isolated from brain tissues using Trizol reagent (Gibco BRL) according to manufacturer’s protocol. cDNA was produced for each mouse from 2 μg of total RNA using superscript II reverse transcriptase (Gibco BRL). Hap1 mRNA was quantified using the SYBR green Lightcycler real-time PCR system (Roche Molecular Biochemicals, Laval, Quebec, Canada) according to manufacturer’s protocols. Hap1 primers (forward: 5′-ACA TAG TTG CCT CCA GTC CCC-3′; reverse: 5′-GAT TCC CAG TTC AGC CCC CC-3′) are specific for a 120 base region (bases 1909–2028 of mRNA sequence NM_010404) in the 3′ untranslated region of transcript Hap1A (3). Hap1 mRNA levels were normalized to actin mRNA levels measured in the same sample using primers (forward: 5′-CCA TGT ACC CAG GCA TTG C-3′; reverse: 5′-ATG GTG CTA GGA GCC AGA GC-3′). Actin levels per μg RNA were virtually identical between mice of all genotypes.

Antibodies

Polyclonal antiserum specific for HAP-1B (CAG1-3) was raised in rabbits against a synthetic peptide (EQQPIVPTQD-SQRLE) corresponding to residues 595–609 deduced from the rat Hap1B cDNA sequence. Briefly, the peptide was coupled to keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, Illinois, USA) according to the manufacturer’s instructions. Immunization of rabbits with conjugated peptides and production of antisera were performed by Pel Freeze. Antibodies were affinity purified and characterized by immunocytochemistry and immunoblotting.

A monoclonal antibody specific for both HAP-1A and -1B was developed as follows. To generate a Hap-1-glutathione-S-transferase (GST) fusion protein, a PCR-amplified fragment corresponding to residues 290–426 of rat Hap-1 cDNA was subcloned into BamHI/EcoRI digested pgEx-2T. The construct was transformed into E. coli DH5alpha. The expressed fusion protein was purified and used for immunization of several BALB/c female mice. B cells from drained lymph nodes were fused with PUA1 (American Type Culture Collection, Manassas, Virginia, USA) fusion partner cells and anti-fusion protein monoclonal antibody secreting cells were cloned by a dilution method. One clone, 1B6 showed specific immunoreactivity for both HAP-1 isoforms by immunoblotting.

Immunoblotting of brain extracts

Total brain was dissected from P1 pups (taking care to exclude the olfactory bulb and brainstem) and the tissue was snap frozen at −80°C. Frozen tissues were homogenized in buffer A LB (20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol vol/vol, 1% Triton-X-100 vol/vol) containing protease inhibitors 20 μg/ml Aprotinin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), 2 μg/ml leupeptin (Roche Biochemical) and 1 mM Pefabloc SC (Roche Biochemical). Lysates were cleared by centrifugation at 4°C for 10 minutes and protein concentrations were measured using the Bradford-type protein assay kit.
Immunohistochemistry

Heads of embryos were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS) for 48 h at 4°C. Sets of brains from Hap1+/+, +/−, and −/− animals were embedded into gelatin and processed in parallel. Parasagittal, corona or horizontal sections were cut at 50 μm thickness throughout the brain using a freezing microtome. Free-floating sections were rinsed in PBS and processed for immunofluorescence. Sections were blocked and permeabilized in PBS containing 5% normal goat serum (NGS) and 0.1% triton-X-100 for 30 minutes at room temperature (RT). Following several rinses in PBS, sections were incubated in PBS containing 1% NGS and polyclonal antibodies against HAP-1 (CAG1-3; 1:200) for 12 h at 4°C. Following PBS rinses, sections were incubated in rhodamine conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) for one hour at RT. Sections were rinsed in PBS and incubated in 10 nM CuSO4 pH 5.0 for 30 minutes at RT. For visualization of nuclei, sections were rinsed in PBS, incubated in PBS containing bis-benzimide (1:10 000) for 5 minutes, rinsed further and mounted onto glass slides using Gel/Mount (Biomeda Corp., Hayward, California, USA). Confocal fluorescence images were acquired on a Zeiss LSM 510 NLO microscope using 543 nm excitation and LP 560 nm emission for rhodamine and 780 nm two-photon excitation and 390–465 nm emission for bis-benzimide.

Histology

Whole embryos and P1 pups were fixed by immersion in 3% paraformaldehyde/2.5% gluteraldehyde in PBS. Several sets of embryos and P1 pups containing 1 homozygous knockout, one heterozygote and one wild-type littermate were paraffin embedded for haematoxylin and eosin (H&E) staining using standard procedures.

Neuronal densities and size

Neuronal densities and size were obtained from coronal 8 μm thick paraffin sections stained with cresyl violet. Counts were performed in layer II and layer V of cortex at the level of the parietal associated cortex. For each cortical layer analysed, five random 220 × 170 μm² windows were visualized at 40× using a Zeiss Axioskop and captured with an AxioCam camera using AxioVision software. All neurons with visible nuclei were counted. Cross sectional areas (including nuclei and surrounding perikarya) were measured on the same window frames using NIH Image software.

Assessment of serum leptin levels

Truncal blood was collected from Hap1+/− and control animals at P8. Cleared serum was produced by centrifugation at 14 000 g at 4°C for 10 minutes and samples were stored at −80°C until analysis. Serum leptin levels were analysed using a commercially available immunoassay ELIZA kit (Assay Designs Inc., Ann Arbor, Michigan, USA) as per manufacturer’s protocol. Serum samples were diluted 50-fold prior to analysis. Purified leptin standards provided with kit were used for calibration.

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