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RESEARCH ARTICLE

Seasonal oscillation of liver-derived hibernation protein complex in the central nervous system of non-hibernating mammals

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ABSTRACT

Mammalian hibernation elicits profound changes in whole-body physiology. The liver-derived hibernation protein (HP) complex, consisting of HP-20, HP-25 and HP-27, was shown to oscillate circannually, and this oscillation in the central nervous system (CNS) was suggested to play a role in hibernation. The HP complex has been found in hibernating chipmunks but not in related non-hibernating tree squirrels, leading to the suggestion that hibernation-specific genes may underlie the origin of hibernation. Here, we show that non-hibernating mammals express and regulate the conserved homologous HP complex in a seasonal manner, independent of hibernation. Comparative analyses of cow and chipmunk HPs revealed extensive biochemical and structural conservations. These include liver-specific expression, assembly of distinct heteromeric complexes that circulate in the blood and cerebrospinal fluid, and the striking seasonal oscillation of the HP levels in the blood and CNS. Central administration of recombinant HPs affected food intake in mice, without altering body temperature, physical activity levels or energy expenditure. Our results demonstrate that HP complex is not unique to the hibernators and suggest that the HP-regulated liver–brain circuit may couple seasonal changes in the environment to alterations in physiology.

KEY WORDS: CTRP, Hibernation, Metabolism, Plasma proteins, Cerebrospinal fluids

INTRODUCTION

Seasonal variation in physiology (e.g. reproduction, metabolism and migration) occurs widely in the animal kingdom (Farner, 1985), yet the underlying control mechanisms remain largely unknown. Hibernation represents one of the most extreme examples of seasonal physiological adaption, which is employed by a selective subset of mammals to conserve resources in cold or inhospitable environments (Andrews, 2007; Carey et al., 2003; Drew et al., 2007; Storey, 2010). Profound and precisely-controlled physiological changes occur during hibernation, enabling these mammals to endure dramatic drops in whole-body metabolism, heart and respiratory rates, and body temperature (Carey et al., 2003; Drew et al., 2007; Hampton et al., 2010; Heldmaier et al., 2004; Storey, 2010).

Attemping to identify molecular probes that closely track the hibernation process, Kondo and co-workers have uncovered a 140 kDa protein complex in the plasma of hibernating chipmunks (*Tamias asiaticus*) that is absent from the closely related non-hibernating tree squirrel (*Callosciurus caniceps*) (Kondo and Kondo, 1992; Takamatsu et al., 1993). This protein complex consists of four components – three closely related hibernation-specific proteins HP-20, HP-25 and HP-27, and a 55 kDa proteinase inhibitor (HP-55) belonging to the serpin superfamily (Kondo and Kondo, 1992; Takamatsu et al., 1993). HP-20, HP-25 and HP-27 share similar protein domain structures, consisting of a signal peptide, an N-terminal collagen domain with 13–14 G-x-Y repeats and a C-terminal globular domain homologous to the classical immune complement C1q (Takamatsu et al., 1993). These proteins belong to the C1q and tumor necrosis factor (TNF) superfamily (Kishore et al., 2004). All components of the hibernation protein (HP) complex are expressed exclusively and at high levels by the liver and circulate in the blood as a hetero-oligomeric complex. HP-20, HP-25 and HP-27 associate to form heterotrimers, whereas HP-55 associates in a non-covalent manner with the HP complex (Kondo and Kondo, 1992; Takamatsu et al., 1993).

Liver mRNAs and plasma levels of the chipmunk HP complex decrease dramatically in hibernating chipmunks (Kondo and Kondo, 1992; Takamatsu et al., 1993). The transcript and circulating levels of the complex are tightly regulated throughout the entire hibernation process. It has been shown that the plasma HP complex levels begin to drop before the onset of hibernation, that they remain low (approximately one tenth of its euthermic levels) during hibernation and then increase to its euthermic levels concomitant with the termination of hibernation (Kondo and Kondo, 1992). Previously, Kondo and co-workers have shown that an endogenously generated circannual output dictates the oscillating levels of plasma HP complex between summer euthermia and winter hibernation (Kondo et al., 2006). Importantly, HP complex levels in the cerebrospinal fluid (CSF) oscillate in an inverse manner relative to its plasma levels (Kondo et al., 2006). Further, the proteinase inhibitor HP-55 dissociates from the HP complex in CSF, leading to the current model that the HP-20–HP-25–HP-27 heterotrimer is the biologically active HP complex that may convey a hormonal signal from the peripheral tissue to the CNS in preparation for hibernation (Kondo, 2007; Kondo et al., 2006). In support of this model, disrupting the hormonal circuit that is regulated by the HP complex, using a neutralizing antibody, terminated or shortened the duration of hibernation (Kondo et al., 2006). However, a direct action of the HP complex in regulating hibernation has yet to be demonstrated.

The HP complex was found only in hibernating sciurids like the chipmunk and the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (Kondo and Kondo, 1992; Takamatsu et al., 1993). Despite the presence of HP genes in the closely related non-hibernating tree squirrel (*Callosciurus caniceps*), these genes are either not expressed (e.g. *HP-27*) due to mutations in the promoter or they exist as pseudogenes (e.g. *HP-25*) due to frame-shift or

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nonsense mutations (Kojima et al., 2001; Ono et al., 2003). Thus, expression of genes that control hibernation appears to have been determined based on comparison of only three species. The generality of this assumption with regard to the genetic control and origin of hibernation has not been fully established. Here, we show that the HP genes are indeed conserved in non-hibernating mammals and probably regulate physiological functions that are distinct from hibernation. We discuss the broader implications of our study with regard to the acquisition of a novel physiological control mechanism via rewiring of existing endocrine circuits.

RESULTS

Identification of HP-20, HP-25 and HP-27 homologs in non-hibernating mammals

We identified HP homologs from the draft genome sequences of thirteen-lined ground squirrel, cow, pig, European rabbit, gray mouse lemur, greater galago, lesser hedgehog tenrec, sheep, elephant, nine-banded armadillo and bottlenose dolphin (Fig. 1A and Table 1). In all cases, the HP genes cluster on the same chromosome and lie 5–30 kb from each other, indicating conservation of the gene locus throughout mammalian evolution. No predicted gene resides between the intervening sequences that separate one HP gene from another. However, the relative orientation of HP genes with respect to each other varies between mammalian species, indicative of active recombination within the HP gene locus. Genomic analyses also revealed the presence of two additional HP-like genes in the pig genome, presumably resulting from gene duplication events. One such gene (designated as ψ) is probably a pseudogene resulting from the presence of a nonsense mutation in the third exon of the gene that encodes the functional globular C1q domain. Similarly, one and two HP-like genes found in the European rabbit and nine-banded armadillo, respectively, also accumulated nonsense mutations that are likely to render the transcribed protein non-functional (Fig. 1A). These data suggest a high evolutionary rate within the HP gene locus. Of the sequenced primate genomes, gray mouse lemur and greater galago possess an intact HP gene locus. In contrast, chimpanzee (*Pan troglodytes*) and humans (*Homo sapiens*) possess only remnants of the HP genes (only exon 3 can be unambiguously identified) due to the accumulation of nonsense mutations and insertions (Fig. 1B). HP genes were not detected in mouse, rat, dog, cat, chicken, opossum, duck-billed platypus, frog, zebrafish and puffer fish genomes.

Cloning and analysis of cow HPs

Here, we focus our molecular, biochemical and functional characterizations on cow (*Bos taurus*; Linnaeus 1758) HPs. Cow HP genes consist of three small exons and share similar exon and intron organization (Fig. 2). The encoded cow HP cDNAs were cloned from liver using PCR (Fig. 2). The deduced amino acid sequences of cow HP-20, HP-25 and HP-27 consist of four domains: a signal peptide for protein secretion based on SignalP prediction (Dyrløv Bendtsen et al., 2004), a short N-terminal region with one conserved cysteine residue, a collagen domain with 12–13 G-x-Y repeats, and a C-terminal globular domain homologous to the immune complement protein C1q (Kishore and Reid, 2000). Mammalian HP-20, HP-25 and HP-27 are widespread among placenta mammals (Fig. 3; Table 1), with amino acid identity ranging from 33–71% among HP orthologs. Structure-based alignment between adiponectin, complement C1q and TNF family members (TNF-α, TNF-β and CD40L) reveals highly conserved residues (G159, Y161, F237 and L241 in adiponectin) that are important in the packing of the protomer's hydrophobic core (Shapiro and Scherer, 1998). These residues are also conserved in cow HP proteins (Fig. 3, arrows). Hence, HP proteins

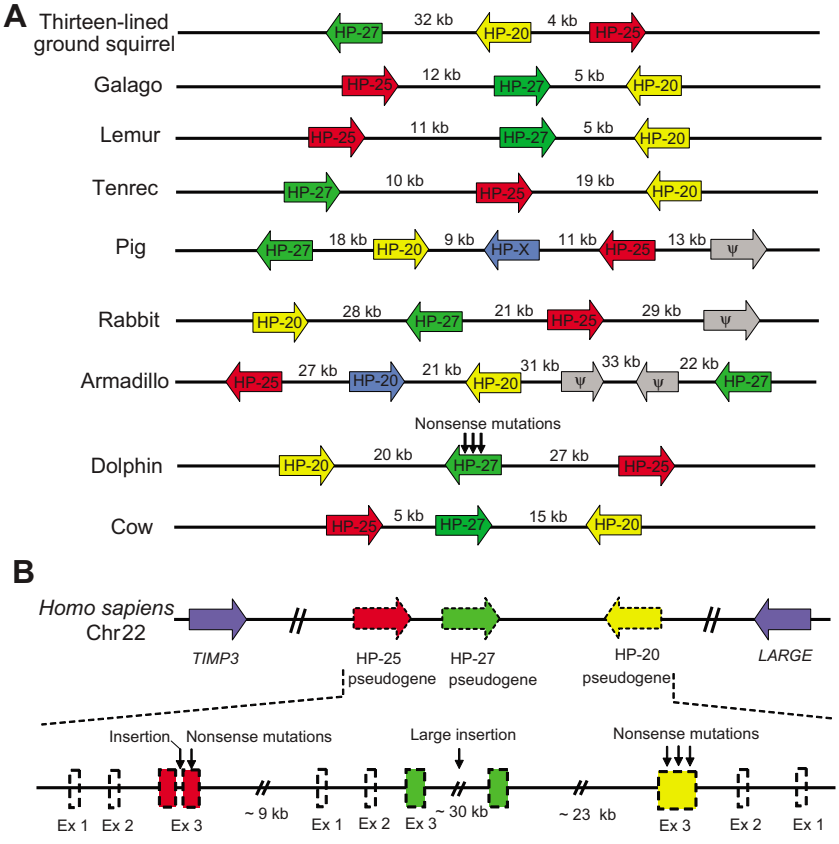

Fig. 1. Identification of chipmunk HP homologs in non-hibernating mammals. (A) Homologs of chipmunk HPs found in other vertebrate species. HP gene loci are found in thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*; scaffold JH393302), pig (*Sus scrofa*; contig FP104567), European rabbit (*Oryctolagus cuniculus*; contig NC_013672), gray mouse lemur (*Microcebus murinus*; contig NT_165971), greater galago (*Otolemur garnettii*; contig NT_091708), lesser hedgehog tenrec (*Echinops telfairi*; contig NT_166802), nine-banded armadillo (*Dasypus novemcintus*; contig NW_00492307), bottlenose dolphin (*Tursiops truncate*; contig NW_004200895) and cow (*Bos taurus*; contig NC_007303) genomes. The transcriptional orientations of HP genes are indicated by the direction of the arrow. ψ, a possible HP-like pseudogene due to nonsense mutation in an exon that encodes the functional C1q domain. (B) Human HP pseudogenes are located on chromosome 22 between the *TIMP3* and *LARGE* genes. Depicted is exon (Ex) 3 of human HP pseudogenes, which encodes the C-terminal globular C1q domain. Nonsense mutations and insertions are indicated with arrows. Exons 1 and 2 of human HP pseudogenes have degenerated beyond recognition.

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Table 1. Percentage amino acid identity at the globular C1q domain between chipmunk and other mammalian HP proteins

	Percentage amino acid identity		
C1q domain	Chipmunk HP-20	Chipmunk HP-25	Chipmunk HP-27
Thirteen-lined ground squirrel HP-20	81	43	39
Thirteen-lined ground squirrel HP-25	43	81	50
Thirteen-lined ground squirrel HP-27	43	54	85
Cow HP-20	64	38	40
Cow HP-25	47	63	56
Cow HP-27	44	58	57
Pig HP-20	65	41	39
Pig HP-25	46	54	54
Pig HP-27	38	50	62
Pig HP-X	40	52	52
Sheep HP-20	64	46	43
Sheep HP-25	38	62	57
Sheep HP-27	39	56	55
Lemur HP-20	66	37	38
Lemur HP-25	47	57	57
Lemur HP-27	42	53	53
Galago HP-20	62	33	35
Galago HP-25	46	61	57
Galago HP-27	43	57	57
Tenrec HP-20	57	33	35
Tenrec HP-25	42	57	48
Tenrec HP-25-like	43	52	52
Rabbit HP-20	64	37	40
Rabbit HP-25	44	61	59
Rabbit HP-27	43	57	55
Elephant HP-25	49	64	57
Elephant HP-25-like	43	59	52
Elephant HP-27	42	57	65
Armadillo HP-20	71	45	40
Armadillo HP-20-like	66	41	41
Armadillo HP-25	45	58	55
Armadillo HP-27	41	55	64
Dolphin HP-20	56	34	37
Dolphin HP-25	43	66	50
Dolphin HP-27	48	59	56

belong to the C1q/TNF superfamily of proteins (Kishore et al., 2004). Phylogenetic analysis of cow C1q family members revealed that HPs form a separate clade (Fig. 4A). Phylogenetic analysis of mammalian HPs also revealed three distinct families of HP in which closely related members with shared evolutionary history are clustered together (Fig. 4B).

Liver-specific expression of cow HP mRNAs

Chipmunk HP-20, HP-25 and HP-27 are expressed exclusively, and at high levels, by the liver (Takamatsu et al., 1993). Similarly, quantitative real-time PCR analyses revealed that cow HPs are also expressed specifically, and at high levels, by the liver (Fig. 5A). All other tissues except the kidney express non-detectable levels of the HP transcripts. Consistent with liver-specific expression, the ~1 kb promoter region of cow *HP-20* and *HP-27* genes drove the expression of a luciferase reporter in a human hepatocyte cell line (HepG2) but not in a human kidney (HEK 293) cell line (Fig. 5B). Moreover, we observed significant suppression of *HP-20* and *HP-27* luciferase reporter expression in HEK 293 cells relative to cells that had been transfected with the control pGL3-Basic vector, suggesting that the promoters contain elements that actively suppressed *HP-20* and *HP-27* transcription in non-liver cells. The presence of multiple hepatocyte nuclear factor (HNF) binding sites on the *HP-20* and *HP-27* promoters suggest that cow HP genes may be regulated by this class of transcription factors, as has been shown for chipmunk HP genes (Kojima et al., 2000; Ono et al., 2001; Ono et al., 2004).

Cow HPs are secreted multimeric glycoproteins

Cow HPs are secreted proteins when expressed in heterologous HEK 293 cells (Fig. 6A). The differences in the apparent molecular mass of the HPs found in the cell lysates versus conditioned medium suggest the presence of post-translational modification. Cow HP-20, HP-25 and HP-27 are predicted to have zero, one and two *N*-linked glycosylation sites, respectively, based on the presence of an N-x-S/T motif (Gavel and von Heijne, 1990). Consistent with this, *N*-glycanase (PNGase F) digestion altered the apparent molecular mass of the secreted cow HP-25 and HP-27 on immunoblots, confirming the presence of *N*-linked glycans (Fig. 6B). All C1q family members characterized to date form trimers as their basic structural unit (Kishore et al., 2004; Seldin et al., 2012; Tsao et al., 2003; Wei et al., 2012a; Wei et al., 2011; Wei et al., 2013; Wong et al., 2008). Some trimers are further assembled into intermolecular disulfide-linked hexamers and octadecamers (Briggs et al., 2009; Suzuki et al., 2007; Tsao et al., 2003). Similarly, cow HPs also form disulfide-linked multimers, as revealed by non-reducing SDS-PAGE analysis (Fig. 6C). It has been shown that the conserved N-terminal cysteine residues in C1q family proteins are important for the formation of higher-order structures that are larger than a trimer (Pajvani et al., 2003; Tsao et al., 2003). Cow HPs contains at least two conserved cysteine residues in the N-terminal region preceding the globular C1q domain (Fig. 2B). Mutations of both cysteine residues were required to disrupt the formation of higher-order multimeric structures in HP-25 and HP-27 (Fig. 6D). Nonetheless, the N-

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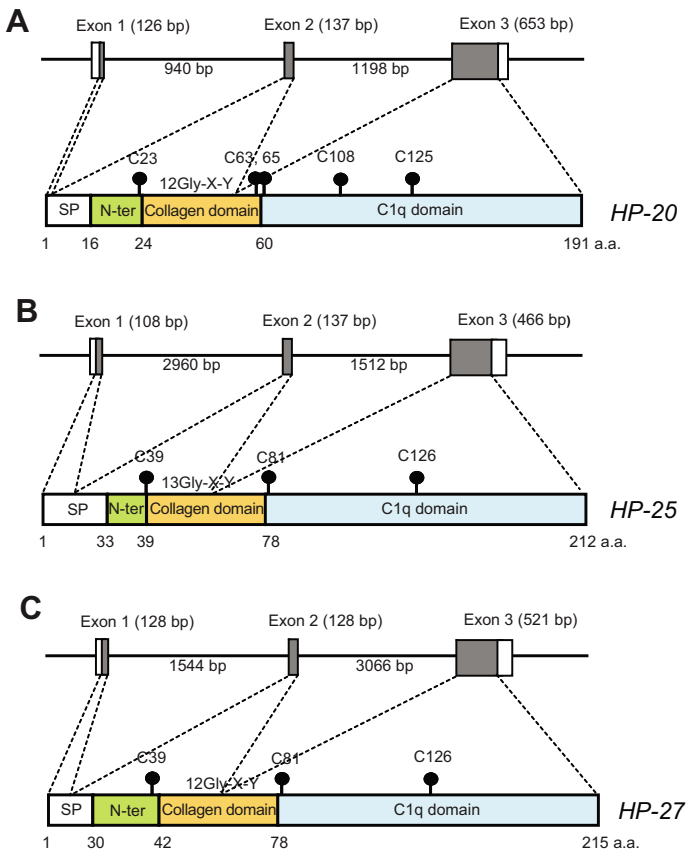

Fig. 2. Exon and intron and domain structure of cow HP genes and proteins. Cow HP genes are located on chromosome 5. The exon and intron and protein domain structures of cow *HP-20* (A), *HP-25* (B) and *HP-27* (C) genes. Gray bars indicate exons that code for protein, and white bars indicate exons that code for the 5′ and 3′ UTR of the transcript. Four major domains are found in cow *HP-20*, *HP-25* and *HP-27* – a signal peptide (SP), an N-terminus (N-ter) with one conserved cysteine residue, a collagen domain with 12 or 13 G-x-Y repeats, and a C-terminal globular C1q domain. All cysteine residues are indicated with a ball-and-stick. a.a., amino acid residue.

terminal cysteine mutants still formed dimers under non-reducing SDS-PAGE.

Cow HPs form heteromeric complexes via the C1q domain

Chipmunk HP-20, HP-25 and HP-27 form heterotrimeric complexes (Kondo and Kondo, 1992; Kondo et al., 2006). To address whether cow HPs also physically associate with each other to form heteromeric complexes, we expressed epitope-tagged proteins in different combinations in HEK 293 cells and performed coimmunoprecipitations on the secreted proteins. We observed physical association between HP-20–HP-25, HP-20–HP-27 and HP-25–HP-27 (Fig. 6E, lanes 6–9). However, we failed to observe the formation of HP-20–HP-25–HP-27 heterotrimeric complexes when all three proteins were coexpressed (Fig. 6E, lane 9). The formation of heteromeric complexes among C1q family proteins is mediated by the globular C1q domain (Bao et al., 2006; Iijima et al., 2007; Wong et al., 2008). To map the interaction domain, we generated constructs containing only sequences that correspond to the signal peptide and the C1q domain (g-HPs; Fig. 6F). The expressed g-HPs lacked the entire collagen domain and the variable-length N-terminus. As shown in Fig. 6G, deletion of the N-terminal region and collagen domain did not affect protein expression and secretion. Coimmunoprecipitation of the epitope-tagged g-HPs confirmed that the C1q domain is sufficient for heteromeric complex formation between different HP proteins (Fig. 6G, lanes 5–7).

Cow HPs circulate in the blood and CSF as distinct multimeric complexes

Chipmunk HPs are secreted from the liver and circulate in the blood (Kondo and Kondo, 1992; Takamatsu et al., 1993). To determine whether the cow HPs also circulate in plasma, we generated anti-peptide antibodies that specifically recognize either HP-25 or HP-27 (Fig. 7A). Using the HP-specific antibodies, we demonstrated that cow HP-25 and HP-27 are found in serum (Fig. 7B). Consistent with our inability to recognize HP genes in the mouse genome, cow HP-specific antibodies failed to detect the presence of HP-25 and HP-27 in mouse (*Mus musculus*; Linnaeus 1758) serum samples (Fig. 7B). The apparent molecular mass of serum HP-25 and HP-27 were consistent with the size of the recombinant proteins secreted from transfected cells (Fig. 6). The doublet bands of serum HP-25 on immunoblots probably represent different glycosylated forms of the protein. As with the chipmunk HPs, cow HP-25 and HP-27 found in the calf serum also exist as higher-order oligomers, as revealed by non-reducing, non-denaturing Tris-Glycine native gel electrophoresis (Fig. 7C). We detected two distinct HP complexes (~750 kDa complex 1 and ~600 kDa complex 2) in serum. In contrast, we detected only the ~600 kDa complex 2 in cow CSF (Fig. 7D). It has been shown that, in hibernating chipmunk, the HP complex that circulates in plasma contains additional HP-55 α1-antitrypsin inhibitor (also known as serpinA1) (Kondo and Kondo, 1992; Takamatsu et al., 1997). It is thought that when the chipmunk HP complex gets into the CNS shortly before the onset of hibernation, the HP-55 α1-antitrypsin inhibitor dissociates from the heterotrimeric complex, enabling the active heterotrimer to exert its biological effects in the CNS (Kondo et al., 2006). The studies on chipmunk HPs suggest that the larger cow HP complex 1 may contain additional homologous HP-55-like α1-antitrypsin inhibitor (Fig. 7C,D). In contrast, the smaller cow HP complex 2 found in the CSF probably does not contain the homologous HP-55-like α1-antitrypsin inhibitor.

Circannual oscillation of cow HPs in the CNS

Remarkably, circulating levels of HP-25 and HP-27 changed seasonally in CSF (Fig. 7E,G), reminiscent of the circannual oscillation of chipmunk HPs in the CNS (Kondo et al., 2006). Cow CSF was collected on three separate but consistent times of year, roughly corresponding to winter (February), summer (June) and autumn (October). We quantified HP-25 and HP-27 levels in CSF from three adult cows over a 4 year period and determined that HP levels indeed oscillated in a cyclical manner, with a periodicity of 1 year (Fig. 7G). The annual peak levels of HPs in the CNS occurred in winter month (February). Although less pronounced, serum HP levels also oscillated circannually, in a roughly reciprocal manner (Fig. 7F,H). The levels of cow HPs were ~100 times higher in serum than in CSF, as has been shown previously for chipmunk HPs (Kondo et al., 2006).

Cow HPs act in the CNS to modulate food intake

We next investigated whether the oscillating cow HPs might regulate distinct physiological processes through pathways in the CNS. Given the difficulty of performing functional studies in cows, mice were used instead. Despite the absence of HP genes in mice, intracerebroventricular (i.c.v.) administration of recombinant cow HPs to mice decreased food intake over a 24 h period relative

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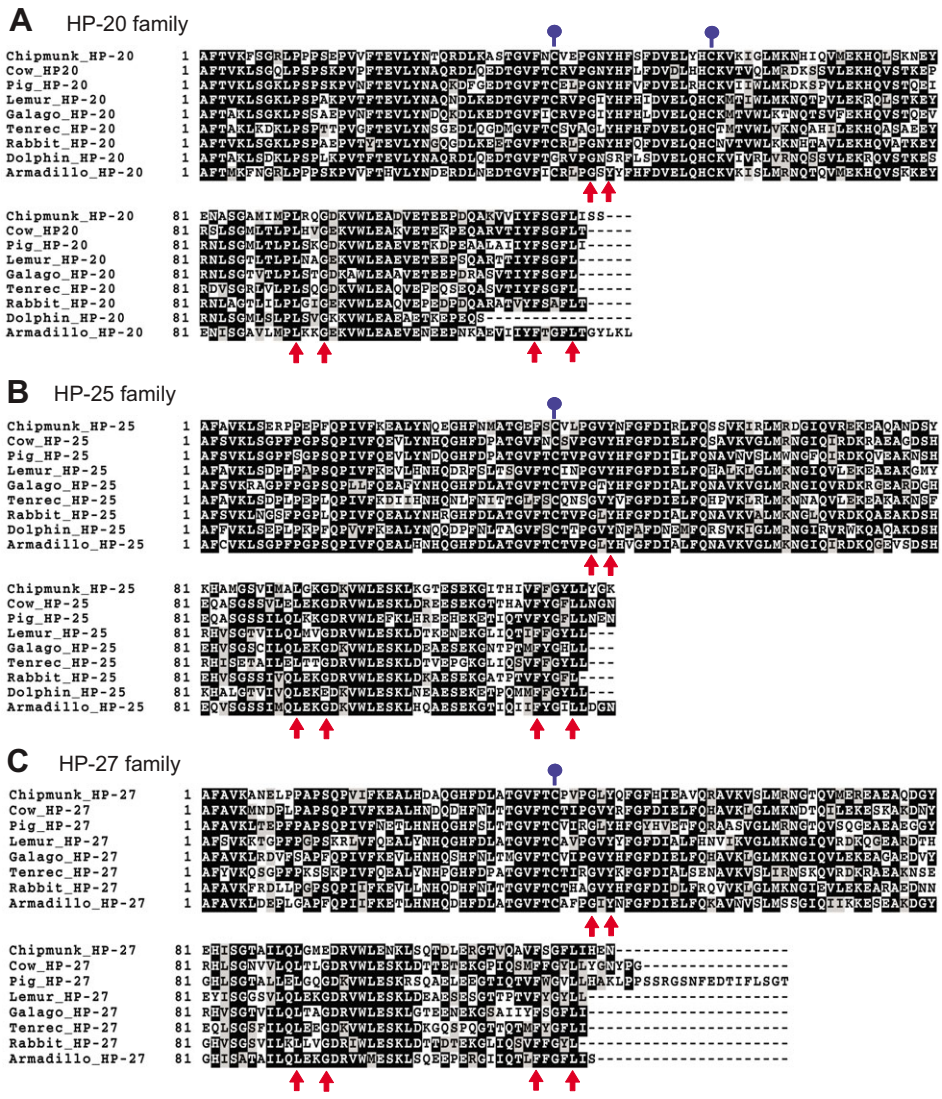

Fig. 3. Sequence alignment of vertebrate HPs. (A–C) ClustalW alignments of the C1q domain of HP proteins from Siberian chipmunk, cow, pig, gray mouse lemur, greater galago, lesser hedgehog tenrec, European rabbit, bottlenose dolphin and nine-banded armadillo. Identical amino acids are shaded and gaps are indicated by a dashed line. All conserved cysteine residues are indicated with a ball-and-stick. Arrows indicate the highly conserved residues found in all C1q/TNF superfamily members (Shapiro and Scherer, 1998).

to vehicle-injected controls (Fig. 8A). Similar results were obtained in three independent experiments using separate cohorts of mice. We evaluated possible changes in whole-body energy balance following i.c.v. injection of recombinant proteins. Central administration of cow HPs did not alter locomotor activity in either the light or dark phase of the photocycles, body temperature, oxygen consumption in vehicle- or recombinant protein-injected mice (Fig. 8B–D). Mice injected with recombinant HPs also showed no difference in energy expenditure compared with vehicle-injected controls (Fig. 8E).

DISCUSSION

Chipmunk and cow HPs share extensive and striking molecular, structural and biochemical conservation. The chipmunk HP complex was reported to play a role in the hibernation process via a poorly understood central mechanism (Kondo et al., 2006), whereas cow HPs can potentially act in the CNS to modulate physiological processes unrelated to hibernation. Owing to the size of cattle, the quantity of recombinant cow HPs needed for i.c.v. injection is orders of magnitude greater compared with that administered centrally to mice. Owing to this and other technical difficulties of studying the central action of cow HPs in cattle, mice were used in our functional study, despite the absence of HP genes in this species. Cow HPs are part of the C1q protein family (Kishore et al., 2004) and consist of over 30 members, all of which possess the signature C1q domain at the C-terminus. Several conserved members of this family, such as adiponectin and C1q/TNF-related proteins are involved in modulating food intake (Byerly et al., 2014; Byerly et al., 2013a; Kubota et al., 2007; Qi et al., 2004) and energy metabolism (Berg et al., 2001; Peterson et al., 2012; Peterson et al., 2013; Peterson et al., 2010; Seldin et al., 2012; Wei et al., 2014; Wei et al., 2012b; Wei et al., 2011; Wei et al., 2013; Wong et al., 2009; Wong et al., 2008; Yamauchi et al., 2002). Thus, in mice, the functional homolog of cow HPs may be subsumed by other related C1q family members. Given that mouse lacks the HP genes, the reduction in food intake in response to central delivery of recombinant cow HPs needs to be interpreted with caution.

HP genes are found in greater galago, gray mouse lemur, lesser hedgehog tenrec and nine-banded armadillo (Fig. 1). The basal placental mammal (lesser hedgehog tenrec) in Madagascar has recently been shown to undergo daily torpor to conserve energy during the winter months where the body temperature is maintained between 18 and 25°C (Lovegrove and Génin, 2008). The reddish-gray mouse lemur (*Microcebus griseorufus*), a species closely related to the gray mouse lemur (*Microcebus murinus*), has been shown to hibernate during the dry season in Madagascar (Kobbe and

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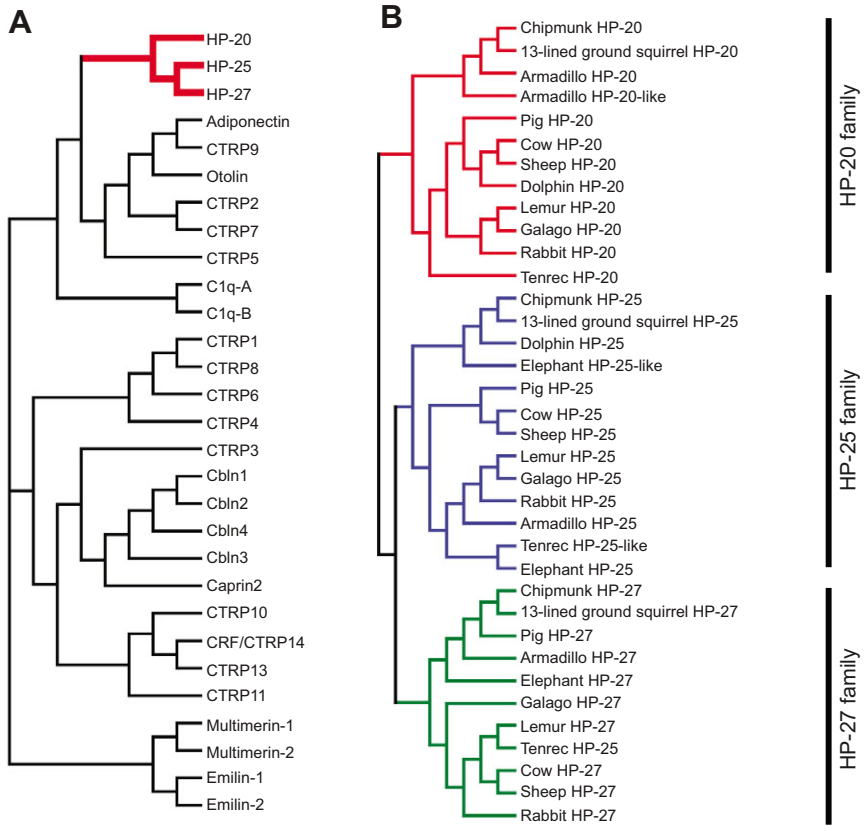

Fig. 4. Phylogenetic analysis of cow HPs.
(A) Phylogenetic tree of cow C1q family members. GenBank accession number for each of the cow proteins: C1q-A chain (NP_001014945), C1q-B chain (NP_001040064), Otolin (NP_001179533), multimerin-1 (Ensembl; Acc:ENSBTAP00000013581), multimerin-2 (XP_589798.3), emilin-1 (XP_594120), emilin-2 (XP_592120.3), CTRP1 (NP_001076878), CTRP2 (XP_589781), CTRP3 (NP_001094608), CTRP4 (XP_001788795), CTRP5 (NP_001092608), CTRP6 (NP_001095342), CTRP7 (NP_001069669), CTRP8 (XP_602976.2), CTRP9 (NP_001069314), CTRP10 (XP_595145.3), CTRP11 (Swiss-Prot;Acc:Q86Z23), CTRP13 (XP_870462.2), CRF (XP_597900.3), adiponectin (NP_777167), caprin-2 (XP_876944.2), cerebellin-1 (Cbln1; XP_876031.2), Cbln2 (NP_001092389), Cbln3 (NP_001073071), Cbln4 (NP_001075006), cow HP-20 (FJ645734), cow HP-25 (FJ645735) and cow HP-27 (FJ645736).
(B) Phylogenetic tree of mammalian HPs. The cladogram is based on the sequence alignment of the C1q domain and it is different from the species tree.

Dausmann, 2009). Although direct functional evidence is lacking, the HP complex may function to regulate seasonal physiology in these mammalian species. In contrast, pig, rabbit, sheep, dolphin and elephant do not hibernate or undergo seasonal torpor. The presence of conserved HP genes implies a physiological function, unrelated to hibernation, for HPs in these mammalian species. It is known that animals in the wild regulate their physiology (e.g. reproduction, metabolism and migration) in a seasonal manner (Farner, 1985; Gwinner, 2003). For instance, circannual reproductive rhythm has been documented for sheep (Karsch et al., 1989), cattle (Philo and Reiter, 1980), European rabbit (Ben Saad and Baylé, 1985) and pig (Love et al., 1993). The annual reproductive rhythm is linked to the circannual oscillation of reproductive hormones, such as gonadotrophin, prolactin, luteinizing hormone, pineal serotonin (5-HT) and testosterone. Based on the distribution of HP genes in both hibernators and non-hibernators (Fig. 1), and our limited functional study of cow HPs (Fig. 8), we speculate that the conserved HP complex may represent a novel class of oscillatory proteins (Fig. 7)

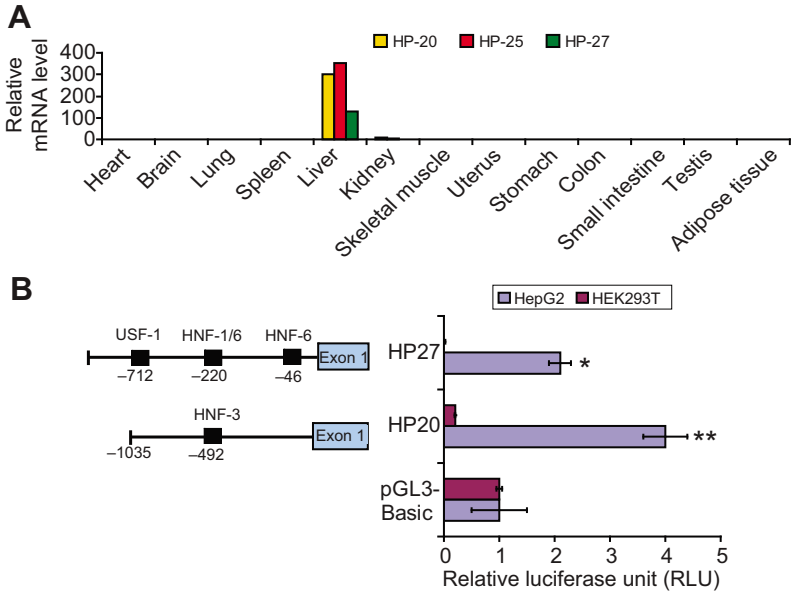

Fig. 5. Liver-specific expression of cow HPs mRNAs.
(A) Quantitative real-time PCR analyses of *HP-20*, *HP-25* and *HP-27* mRNA expression in cow tissues. Transcript abundance was normalized to *18S* rRNA. (B) HEK 293 and HepG2 cell lines were transfected with control pGL3-Basic vector or pGL3-Basic vector containing an *HP-20* (−1035 bp) or *HP-27* (−1348 bp) promoter region upstream from the transcriptional start site (left panel). Luciferase activity was assayed and values were normalized against total protein content. Values were reported as fold increase of relative luminescence when compared with cells that expressed the control plasmid pGL3-Basic (right panel). Means ± s.e.m. are shown. **P*<0.05 and ***P*<0.005 compared with cells transfected with control pGL3-Basic vector.

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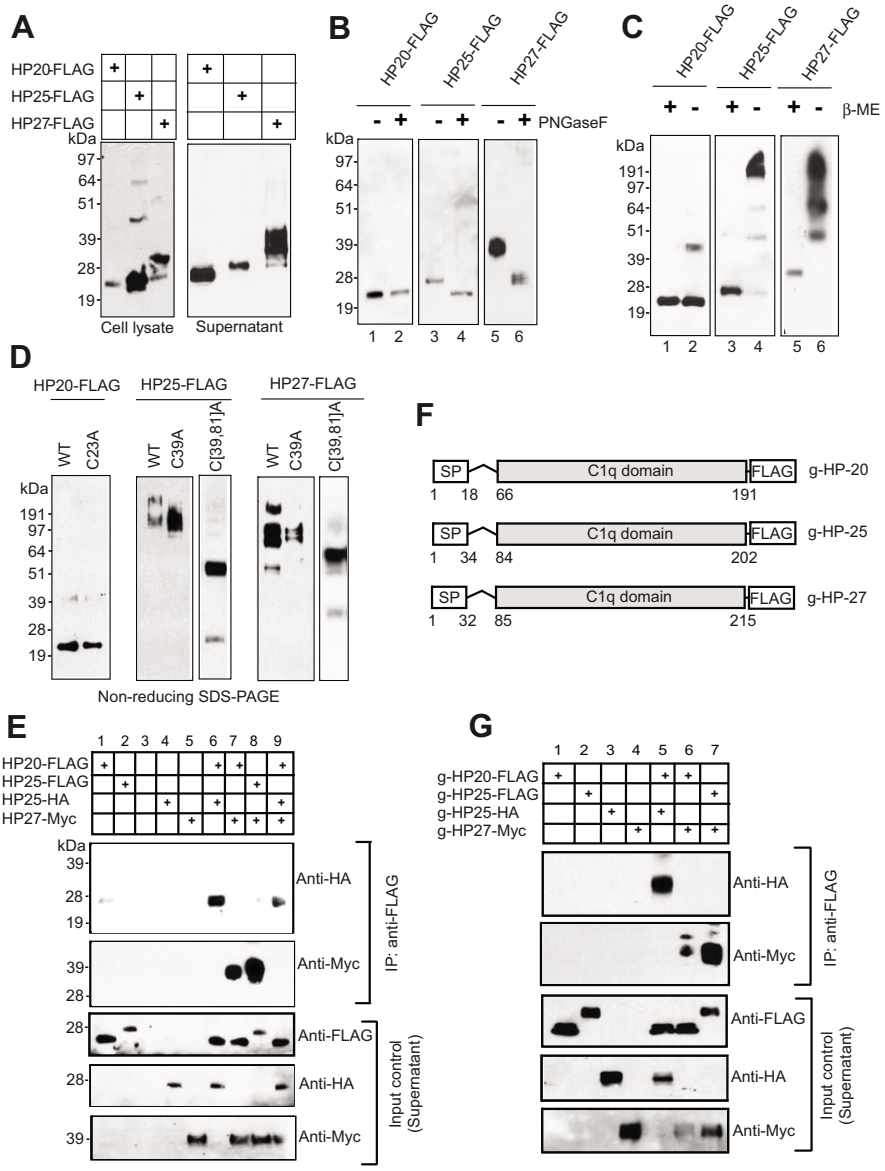

Fig. 6. Cow HPs are secreted multimeric glycoproteins. (A) Immunoblot analyses of the cell lysate and supernatant harvested from transfected HEK 293 cells expressing FLAG-tagged cow HPs using the antibody against FLAG. (B) Immunoblot analyses of recombinant cow HPs treated with (+) or without (–) *N*-glycanase (PNGase F). (C) Immunoblot analyses of recombinant cow HPs treated with (+) or without (–) reducing agent (β-mercaptoethanol, β-ME). (D) Native gel immunoblot analyses of wild-type (WT) and cysteine mutants of cow HPs. (E) Supernatants from transfected cells expressing different combinations of epitope-tagged cow HPs were subjected to immunoprecipitation (IP) and immunoblot analyses with the indicated antibodies (the first two panels). The bottom three panels indicate the presence of FLAG-, HA- and Myc-tagged input proteins. (F) Diagram showing the globular-domain-only constructs of cow HPs (g-HP). SP, signal peptide. (G) Immunoprecipitation and immunoblot analyses with the indicated antibodies of supernatants harvested from transfected cells that coexpressed different combinations of epitope-tagged cow g-HPs (first two panels). The bottom three panels indicate the presence of FLAG-, HA- and Myc-tagged input proteins.

that carry hormonal signals from the periphery (i.e. liver) to the CNS to regulate circannual physiology.

Although the core molecular components underlying circadian rhythm in mammals have been elucidated (Reppert and Weaver, 2002), much less is known about the molecular components that underlie the endogenously generated circannual rhythm and the seasonal physiology that this rhythm regulates (Lincoln et al., 2003). A previous study in the Soay sheep suggests that oscillating melatonin signals, generated by the pineal gland in response to photic input, act on melatonin receptor-expressing calendar cells in the par tubelaris of the pituitary gland to entrain a circannual clock that dictates endogenous circannual output (Lincoln et al., 2006). This circannual output, in turn, gives rise to seasonal oscillation of prolactin secretion by the adjacent lactotroph cells in the pituitary gland. It remains to be determined whether the same circannual output generated by the pituitary calendar cells dictates the oscillating levels of HPs in the CNS. In the chipmunk, expression of HP mRNAs in the liver is dramatically reduced before the onset of hibernation (Kondo and Kondo, 1992; Takamatsu et al., 1993). Coincidentally, there is a rise in the circulating levels of HPs in the CSF (Kondo et al., 2006). How chipmunk and cow HP complexes get transported, via passive or active mechanisms, from the plasma into the CSF in a seasonal manner is not known. To establish that cow HPs indeed oscillate in a circannual fashion, it must be shown that an endogenously generated circannual output (independent of the environment) dictates the circannual oscillation; to do so would require exposing the cattle to constant light or constant dark cycle for two consecutive years, a technically difficult, if not impossible, task to accomplish for a large animal.

Although much is known about the molecular mechanisms that generate diverse morphological phenotypes seen in the animal kingdom (True and Carroll, 2002), much less is known about how different physiological control mechanisms evolved, especially in the context of existing mechanisms. The evolutionary origin of hibernation in mammals is not known. Previously, the HP complex had been found only in hibernating sciurids like the chipmunk and thirteen-lined ground squirrel and had not been found in the related non-hibernating tree squirrel, suggesting that genes that control hibernation are specific to the hibernators (Kondo and Kondo, 1992; Takamatsu et al., 1993). However, this model is based entirely on the presence or absence of the HP complex in sciurid species. The generality and validity of the model proposed by Kondo and co-

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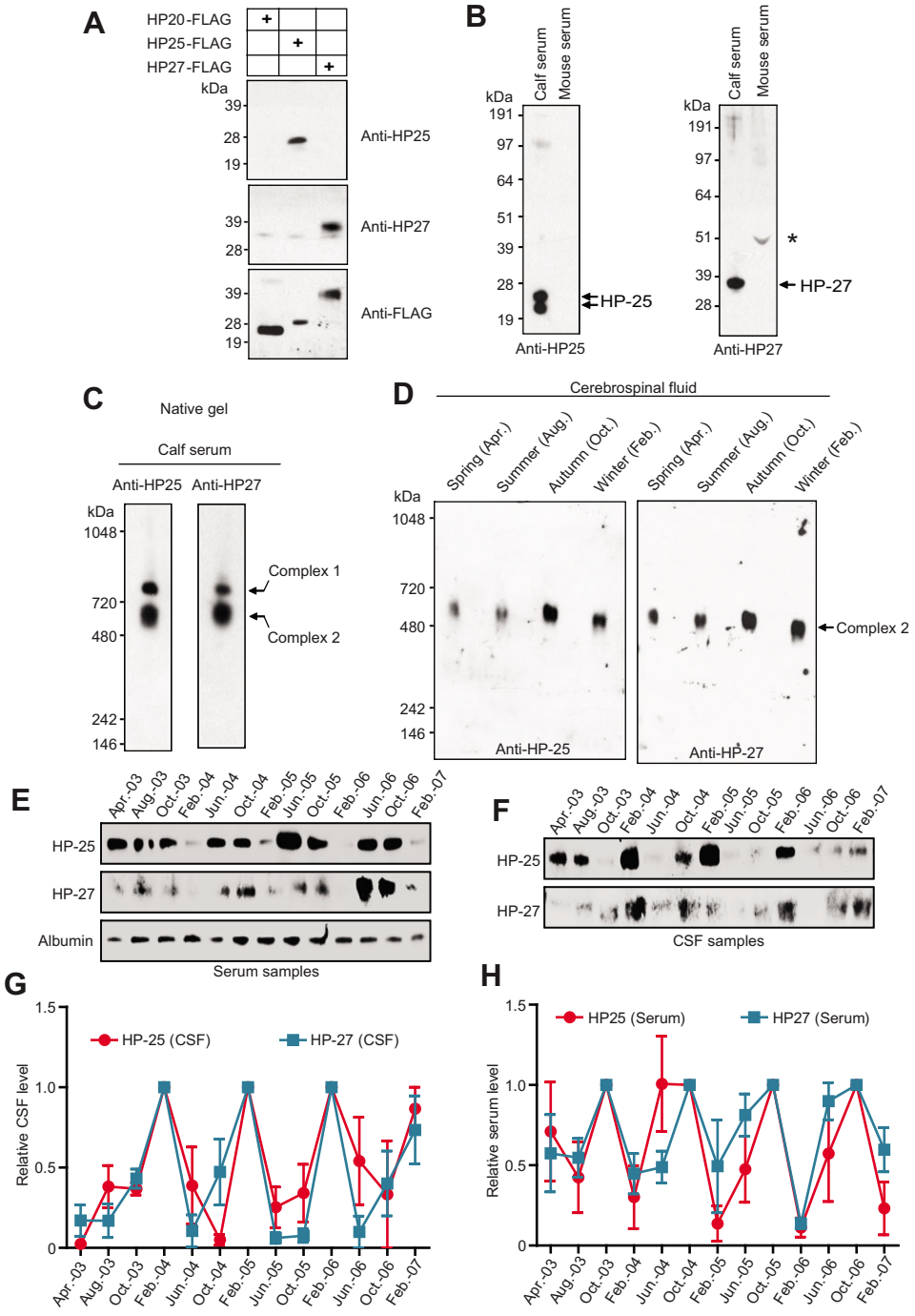

Fig. 7. Cow HP levels oscillate in serum and CSF as distinct heteromeric complexes. (A) Immunoblot analysis was performed to demonstrate the specificity of antibodies against HP-25 (top panel) and HP-27 (middle panel). The bottom panel shows the presence of epitope-tagged HPs. (B) Immunoblot analyses revealed the presence (indicated by arrows) of HP-25 and HP-27 in cow, but not mouse, serum. *Non-specific band. (C) Native gel immunoblot analyses revealed the existence of two distinct heteromeric complexes of cow HPs in serum. A representative result from one of the cow samples is shown. (D) Native gel immunoblot analyses of cow HPs in CSF collected at different times of the year. A representative result from one of the cow samples is shown. Serum from the same cow was used in C and D. (E,F) Reducing SDS-PAGE immunoblot analyses of cow HP-25, HP-27, and albumin (loading control) in serum (E) and CSF (F) at different times of the year. A representative result from one of the cow samples is shown. (G,H) Seasonal oscillation of cow HPs (N=3) in CSF (E) and serum (F) as quantified by reducing SDS-PAGE immunoblot analyses. For each separate year, serum or CSF levels of HP-25 and HP-27 were normalized to their peak level (set at 1).

workers has not been tested in other mammalian species. Further, all functional data pertaining to the role of chipmunk HP complex in hibernation are based on the use of a neutralizing antibody (Kondo et al., 2006); evidence for a direct role of the chipmunk HP complex in regulating hibernation is currently lacking. Thus, it is possible that the chipmunk HP complex may regulate seasonal physiology unrelated to hibernation. In contrast, other studies that examined the expression of α2-macroglobulin by liver in hibernating Richardson's ground squirrels (*Spermophilus richardsonii*) and Columbian ground squirrels (*Spermophilus columbianus*) suggest that differential expression of existing genes rather than the acquisition of hibernation-specific genes underlies the origin of hibernation (Srere et al., 1992; Van Breukelen and Martin, 2002). Differential gene expression of pancreatic lipase probably accounts for the efficient utilization of lipids in the heart of hibernating thirteen-lined ground squirrels (Andrews et al., 1998). In hibernating arctic ground squirrels (*Spermophilus parryii*), uncoupling protein genes (*Ucp-2* and *Ucp-3*) are differentially upregulated in the white adipose tissue and skeletal muscle (Boyer et al., 1998). In hibernating jerboa (*Jaculus orientalis*), the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene has also been shown to be differentially regulated at the transcriptional level in skeletal muscle and at the post-translational level in liver (Soukri et al., 1996a; Soukri et al., 1996b). Using a mouse microarray, Yan et al. have shown that significant numbers of genes are indeed differentially regulated in the brown adipose tissue of hibernating arctic ground squirrels (Yan et al.,

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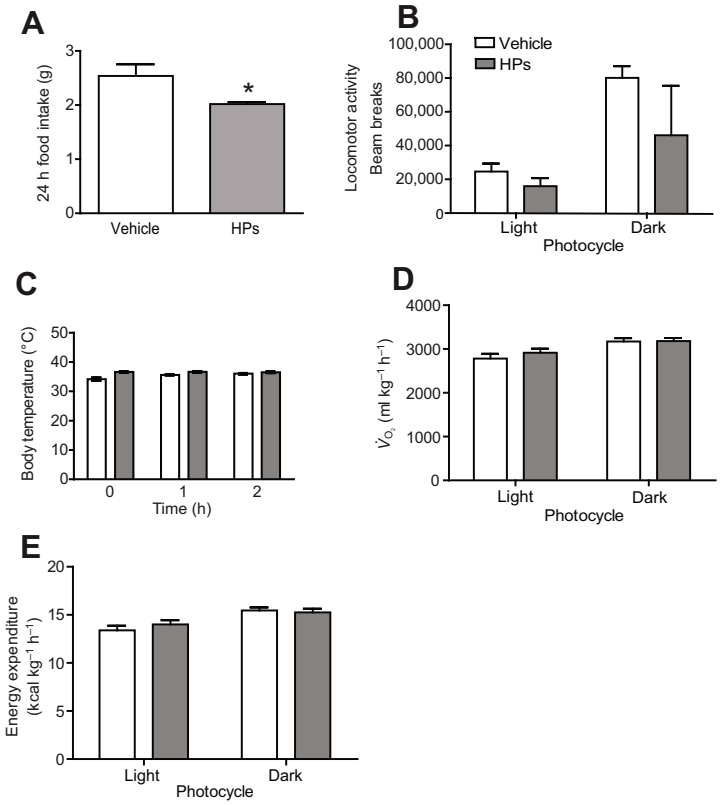

Fig. 8. Cow HPs act in the CNS to modulate food intake. Vehicle control or recombinant HPs (equal mixture of the three proteins at 0.1 µg µl^{-1}) was injected into the lateral ventricle of cannulated mice (*N*=6 per group); multiple measurements followed. (A) Food intake over a 24 h period. (B) Locomotor activity during light (12 h) and dark cycles (12 h). (C) Body temperature before (0 h) and after i.c.v. injection of recombinant HPs. (D–F) Indirect calorimetry measurements of $\dot{V}_{O_2}$ (D) and energy expenditure (E). **P*<0.05 (between vehicle and recombinant HPs).

2006). It is clear from the present study that HP genes are found in divergent mammalian species that do not hibernate. Previous studies and our data (Figs 1 and 2) thus support differential gene expression as a more plausible model for the origin of hibernation.

Levels of the HP complex oscillate in the CNS of both chipmunk (a hibernator) and cow (a non-hibernator) in a seasonal manner, with peak levels seen in winter (Fig. 7). Assuming a hibernation role for the chipmunk HP complex (Kondo et al., 2006), the potential functional divergence of the HP complex between hibernators and non-hibernators, despite its striking molecular, structural and biochemical conservation (Figs 3, 5 and 6), provides a novel example that links how the CNS interprets changes in the peripheral tissue-derived hormonal signals to alterations in complex physiology. The broader implication of our study suggests the differential wiring of an existing endocrine circuit as a potential general mechanism to generate novel physiological phenotypes. Many important and conserved developmental genes (e.g. *Hox* and *Pax*), considered as an evolutionary 'toolkit', are repeatedly deployed in different organisms across divergent taxa, as well as in different cellular and physiological contexts, to sculpt complex morphological phenotypes (True and Carroll, 2002). We propose here that the 'endocrine circuit' could also be considered as a modular 'toolkit' potentially co-opted, tinkered and rewired by organisms to acquire novel physiological functions.

MATERIALS AND METHODS

Mice

C57BL/6 male mice (~8 weeks old) were purchased from The Jackson Laboratory and allowed to acclimatize to the animal facility for 1–2 weeks. Mice were provided with standard chow diet (no. 5001, Lab Diet, St Louis, MO, USA) and water *ad libitum* and housed in polycarbonate cages on a 12 h light:12 h dark photocycle. Animal experiments were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine.

In silico identification of chipmunk HP-20, HP-25 and HP-27 homologs in sequenced vertebrate genomes

Chipmunk HP-20, HP-25 and HP-27 cDNAs (GenBank accession: D12974, D12975 and D12976, respectively) and their deduced protein sequences were used to screen databases containing available vertebrate genomes for homologous genes using bioinformatic resources found at the National Center for Biotechnology Information (NCBI) and Ensembl databases. We used the annotated genomes (based on gene prediction) or the tblastn program of the Basic Local Alignment Search Tool (BLAST) to query all the available high throughput genome sequences in the NCBI GenBank databases in September of 2009 and March of 2014. In addition, we also queried selected genomes using the tblastn program for the presence of genes that encode HP-like proteins. We manually examined each candidate HP homolog by pair-wise comparison to ensure that the putative HP homolog was most closely related in amino acid sequence (in the C1q domain) to the chipmunk HPs and not to any other C1q family members. The genomes examined included human (*Homo sapiens*), rhesus macaque (*Macaca mulata*), chimpanzee (*Pan troglodytes*), orangutan (*Pongo abelii*), galago (*Otolemur garnettii*), lemur (*Microcebus murinus*), dog (*Canis lupus familiaris*), cat (*Felis catus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), cow (*Bos taurus*), pig (*Sus scrofa*), sheep (*Ovis aries*), rabbit (*Oraryctolagus cuniculus*), opossum (*Monodelphis domestica*), horse (*Equus cabalus*; incomplete), tenrec (*Echinops telfairi*), elephant (*Loxodonta africana*), bottlenose dolphin (*Tursiops truncatus*), nine-banded armadillo (*Dasypus novemcintus*), duck-billed platypus (*Ornithorhynchus anatinus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), puffer fish (*Tetraodon nigroviridis*) and frog (*Xenopus tropicalis*).

Cloning of cow HP-20, HP-25 and HP-27 cDNA

Cow (*Bos taurus*) *HP-20*, *HP-25* and *HP-27* cDNAs were cloned from total liver cDNAs (Clontech) using the following primers: *HP-20*, (80F1) 5′-ATGGCTGATCTCCGGATACTGGTC-3′ and (80R1) 5′-AGAACTATGT-CAGGAAACCAGAG-3′; *HP-25*, (81F1) 5′-GAGGAGGTGGTCAACG-AAAGCGC-3′ and (81R1) 5′-CTAGCTGTCTTAATTTCCATTGAGC-3′; *HP-27*, (82F1) 5′-ATGCCTGGGAGAGGAGGACAATC-3′ and (82R1) 5′-CTTAGCCAGGATAATTTCCATAGAG-3′. The cycling conditions

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comprised 35 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 50 s. PCR products were fractionated in 1% agarose gel, purified and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA). The entire cDNA insert was sequenced. Cow *HP-20*, *HP-25* and *HP-27* cDNA sequences were deposited in the GenBank database and assigned the accession numbers FJ645734, FJ645735 and FJ645736, respectively.

Sequence alignment and phylogenetic analysis of cow HP-20, HP-25 and HP-27

We performed pairwise sequence alignment of cow HP-20, HP-25 and HP-27 with other cow C1q family members using the 'Blast 2 sequence' option at the NCBI website. Multiple sequence alignments were performed using ClustalW (Larkin et al., 2007). A phylogenetic tree was generated using *MEGA* program version 4 (Tamura et al., 2007) based on ClustalW alignments of all cow C1q-domain-containing protein sequences.

Cloning of cow HP-20 and HP-27 promoter

The 1025 bp and 1348 bp promoter regions of cow *HP-20* and *HP-27* genes, respectively, were amplified from cow genomic DNA (Novagen, Madison, WI, USA) using the QIAGEN LongRange PCR kit according to the manufacturer's instructions. The primers included *HP-20*: 5′-CTTAAT-TGTGTTCAGTGCTGAGCTG-3′ and 5′-CTTAGCTAAATGATATGAAG-CTGCCTG-3′ and *HP-27*: 5′-CAAAGGCTACAACATCTAGTCCTATG-3′ and 5′-AACATATGGAATCCAATGCTCTAGTG-3′. All PCR products were fractionated in 1% agarose gel, purified and cloned into the pCR2.1 TOPO vector (Invitrogen). Cloned promoter sequences were verified by DNA sequencing. Inserts were further excised and subcloned into the *SacI* and *XhoI* restriction sites of the pGL3-basic vector (Promega, Madison, WI, USA).

Luciferase assay

The Luciferase Assay System (Promega) was used according to the manufacturer's protocol. HEK 293T and HepG2 cell lines (American Tissue Culture Collection) were cultured in 24 well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal cow serum and antibiotics (penicillin and streptomycin). Cells were transfected with 50 ng per well of control plasmid (pGL3-Basic), pGL3-HP-20, pGL3-HP-25 or pGL3-HP-27. Cell lysates were harvested 48 h post-transfection. Luciferase activity was assayed using the Lumat LB 9507 luminometer (Berthold), and values were normalized against total protein content. Values were reported as fold increase of relative luminescence when compared with cells expressing control plasmid pGL3-Basic.

Epitope-tagged cDNA constructs

The C-terminal FLAG- or hemagglutinin (HA)-tagged cow HP-20, HP-25 and HP-27 constructs were generated by PCR. Constructs containing only the leader peptide and the C-terminal globular domain of cow HP-20, HP-25 and HP-27 were also generated. g-HP-20 encompassed amino acid residues 1–18 and 66–191; g-HP-25 encompassed residues 1–34 and 84–212; and g-HP-27 encompassed residues 1–32 and 85–215. All constructs were verified by DNA sequencing.

Site-directed mutagenesis

A PCR-based method using a Stratagene kit was used to mutate C23 (cow HP-20), or C23, C39 and C81 (cow HP-25 and HP-27) to alanine. The following primers were used in the mutagenesis: *HP-20* (C23A) forward primer, 5′-CCTGGAGAAAGGAGGGGCTACCGGACCTCCGGGGC-3′ and reverse primer, 5′-GCCCCGGAGGTCCGGTAGCCCCTCCTT-TCTCCAGG-3′; *HP-25* (C39A) forward primer, 5′-CAGCAGACTCT-GAGCTGGCTGGACCTCGTGGAGCCCG-3′ and reverse primer, 5′-CGGGCTCCACGAGGTCCAGCCAGCTCAGAGTCTGCTG-3′; *HP-27* (C39A) forward primer, 5′-ACCAGTTACCGAGTCCGCTGATTCTCA-AGGACCTC-3′ and reverse primer, 5′-GAGGTCCTTGAGAATCAGC-GGACTCGGTAACTGGT-3′. *HP-25* (C23A,C81A) forward primer, 5′-TTCCTGGACTGGTTGAGAAGGCCCCACCCCTGCCTCAGTCTGC-3′ and reverse primer, 5′-GCAGACTGAGGCAGGGGTGGGGCCTTCTCA-ACCAGTCCAGGAA-3′. *HP-27* (C23A,C81A) forward primer, 5′-TTCCGGGAGACATTGAGAGTGCCTTGTCTCCCCCTAAATCTGC-3′ and reverse primer, 5′-GCAGATTTAGGGGGAGACAAGGCACTCT-CAATGTCTCCCGGAA-3′. All mutant constructs were verified by DNA sequencing.

Generation of cow HP-25- and HP-27-specific antibodies

Polyclonal antibodies specific to peptide epitopes (cow HP-25, RNGIQIRDKRAEAGDSHEQ; HP-27, KNDTQILEKESKAKDNYRH) were generated by YenZym Antibodies (Burlingame, CA, USA). Sera from rabbits were collected and tested against FLAG-tagged cow HP-25 and HP-27 proteins. Attempts to generate an anti-peptide antibody that can specifically recognize cow HP-20 were not successful.

Quantitative real-time PCR analysis of HP-20, HP-25 and HP-27 expression in cow tissues

Cow heart, brain, lung, spleen, liver, kidney, skeletal muscle, uterus, stomach, colon, small intestine (Biochain Institute, Newark, CA, USA), and testis and adipose tissue (Zyagen, San Diego, CA, USA) total RNAs were reverse-transcribed using random primers and the Superscript II system (Invitrogen). The default PCR protocol was used on an Applied Biosystems Prism 7500 Sequence Detection System. Quantitative real-time PCR analysis was performed as previously described (Peterson et al., 2012). All data were normalized to *18S* rRNA. The PCR primers used were: *HP-20* forward, 5′-CTGGAGAAAGGAGGGTGTACCG-3′ and reverse, 5′-GTGG-CATGGGCATTTTACACTTG-3′; *HP-25* forward, 5′-CAGACTCTGAGC-TGTGTGGACCTCG-3′ and reverse, 5′-GTGGGCACTTCTCAACCA-GTCCAG-3′; *HP-27* forward, 5′-CCGAGTCCTGTGATTCTCAAGG-3′ and reverse, 5′-GGGAGACAAGCAACTCTCAATG-3′; cow *18S* rRNA forward, 5′-GAGAAACGGCTACCACATCC-3′ and reverse, 5′-GACA-CTCAGCTAAGAGCATCG-3′.

Recombinant protein production and purification

C-terminal FLAG-tagged cow HP-20, HP-25 and HP-27 constructs were used to express secreted recombinant proteins in mammalian HEK 293 GripTite™ cells (Invitrogen) as described previously (Peterson et al., 2010). Purified proteins were dialysed against 20 mmol l^{-1} Hepes buffer (pH 8.0) containing 135 mmol l^{-1} NaCl in a 10 kDa cut-off Slide-A-Lyzer dialysis cassette. Dialyzed recombinant proteins were concentrated in 10 kDa cut-off Amicon Ultra centrifugal filters (Millipore, Temecula, CA, USA), quantified by Coomassie Plus™ protein assay (Thermo Scientific, Carlsbad, CA, USA), aliquoted and stored at −80°C.

Cow serum and CSF

All samples were collected from healthy cows. Three animals per age group (between 6 and 63 months old) were sampled. Cow serum was taken by blood draw of 100 ml whole blood. Blood serum was retrieved by incubation of the whole blood for 4 h at room temperature, followed by centrifugation at 1000 ***g*** for 10 min and careful extraction of the supernatant. CSF (6–7 ml) was retrieved by lumbar puncture of animals under sedation (0.04 mg xylazine kg^{-1} body weight by intramuscular injection), collected in 10 ml vials and centrifuged at 1500 ***g*** for 15 min at room temperature to eliminate cells and insoluble material. Samples were stored in aliquots at −70°C until analysis. Sera and CSF samples were taken every 4 months over a 4 year period. For CSF samples, Bradford protein assay was used to quantify the total protein content. Five micrograms from each CSF sample were then loaded onto a reducing 4–12% NuPAGE gel (Invitrogen) or a non-reducing Tris-Glycine gel and immunoblotted for cow HP-25 and HP-27. For cow serum samples, we first performed a quantitative western blot analysis for albumin using an antibody against albumin (Sigma, St Louis, MO, USA). Using serum albumin level for normalization, we then loaded an equal amount of protein from each cow sample onto a reducing 4–12% NuPAGE gel and immunoblotted for cow HP-25 and HP-27. All cow experiments were approved by the Institutional Animal Care and Use Committee at the Federal Research Institute for Animal Health (Germany).

Immunoblot analysis

Supernatants and cell pellets were resuspended in 60 µl NuPAGE LDS sample buffer (Invitrogen) containing reducing agent (β-mercaptoethanol),

heated at 90°C for 10 min and separated on a 10% Bis-Tris NuPAGE gel (Invitrogen). For non-reducing gel electrophoresis, protein samples were resuspended in NuPAGE sample buffer (no reducing agent was added) and separated on a 3–8% Tris-Acetate gel (Invitrogen). Proteins from gels were transferred to 0.2 µm Protran BA83 nitrocellulose membrane (Whatman), blocked in 2% non-fat milk for 1 h and probed with the M2 antibody against FLAG (Sigma) or an antibody against HA (high affinity clone 3F10, Roche Applied Science, Branford, CT, USA) in the presence of 2% non-fat milk overnight. Immunoblots were washed three times (5–10 min each) in PBS containing 0.1% Tween-20 and incubated with anti-mouse IgG horseradish-peroxidase-conjugated or anti-rat IgG horseradish-peroxidase-conjugated antibodies (Amersham Biosciences, Piscataway, NJ, USA) (1:5000) for 1 h. Blots were washed three times, developed in enhanced chemiluminescence reagent (Millipore) and visualized with MultiImage III FluorChem® Q (Alpha Innotech, San Leandro, CA, USA). Quantification of signal intensities was performed using Alphaview Software (Alpha Innotech).

Native Tris-Glycine gel electrophoresis

Protein samples in 2× Tris-Glycine native gel buffer (Invitrogen) were separated in 4% Tris-Glycine gels (Invitrogen) at 125 V for 3–4 h, transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA) and subjected to immunoblot analysis using the M2 antibody against FLAG. The NativeMark protein standard (Invitrogen) was used in native gel electrophoresis to estimate the apparent molecular weight of native cow HP-20, HP-25 and HP-27.

Coimmunoprecipitation

Supernatants (300 µl) from transfected cells were combined with 500 µl of immunoprecipitation buffer (150 mmol l^{-1} Tris-HCL, pH 7.4, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA and 1% Triton X-100) and subjected to immunoprecipitation using the anti-FLAG affinity gel (Sigma) or anti-HA affinity resin (clone 3F10, Roche). Samples were rotated for 4 hours at 4°C, washed four times with 1 ml immunoprecipitation buffer and subjected to western blot analysis using the antibodies against FLAG or HA.

Stereotaxic cannulation

100 mg kg^{-1} ketamine and 0.5 mg kg^{-1} dexmedetomidine were administered via the intra-peritoneal (i.p.) route to allow mice to reach a surgical plane of anesthesia (no response to tail-pinch) within 5 min. Stereotaxic brain surgery was performed as described previously (Byerly et al., 2013b), guided by a digital stereotaxic alignment system (David Kopf Instruments, Tujunga, CA, USA). After surgery, mice were given Antisedan® (atipamezole hydrochloride) at a total dose of 2 mg kg^{-1} i.p. in three boluses 5–10 min apart. During the recovery period, mice were kept in a plastic container over a heating pad set at 40°C to prevent hypothermia and facilitate recovery. Once mice were ambulating, we administered flunixin as an analgesic drug (2.5 mg kg^{-1} i.p.).

Recombinant protein administration

Cannulated mice were given i.c.v. injections of 2 µl of vehicle buffer or an equal mixture of recombinant cow HP-20, HP-25 and HP-27 (0.15 µg $µl^{-1}$) with the use of a disinfected stainless steel injector connected by polyethylene tubing to a Hamilton syringe. Vehicle buffer [20 mmol l^{-1} Hepes buffer (pH 8.0) containing 135 mmol l^{-1} NaCl] was the same buffer in which recombinant HPs were suspended; hence, this served as control in i.c.v. injection. However, we did not administer a non-specific protein as additional control. At the end of the experiment, cannula patency was confirmed by injecting neuropeptide Y i.c.v. (0.25 nmoles) and measuring hyperphagic responses at 1 h intervals as a positive behavioral test. Animals retaining functional cannulas were included in the final data set. Injections for behavioral studies were given within 30 min of the onset of the dark photocycle. Body weight and food intake were measured every day.

Food intake measurement

Ad libitum intakes of 1 g chow pellets were measured at 0.5, 1, 2, 4 and 24 h time points after vehicle or recombinant protein injections, and daily intake was monitored for at least two additional days. Food intake measurements were facilitated by the use of wire-bottom cages.

Locomotor activity measurement

Mice were tested in plexiglass open-field chambers equipped with panels of infrared lights that formed a grid of beams across the field. When beams are interrupted by the animal, the information is stored in a computer for later analysis for patterns of locomotor activity (the light beams are not harmful to the animals). Mice were first acclimated to the chambers for 1 day to achieve stable baseline locomotor activity, then injected with vehicle or recombinant HPs just before the onset of the dark period, and allowed *ad libitum* access to standard rodent chow and water within the chamber during the 24 h testing period. Locomotor activity data were collected 2 h after injection to eliminate potential effects of stress due to central administration of recombinant proteins.

Analysis of whole-body energy metabolism

Cannulated C57BL/6 mice were housed in ventilated caging (Innovive, San Diego, CA, USA) to adapt to the local 12 h light:12 h dark cycle and other environmental conditions, including individual housing. Mice were weighed, and their *ad libitum* food and water were checked every day. Mice that had adapted to the animal facility were then moved to individual chambers of an Oxymax indirect calorimeter (Columbus Instruments), an open-circuit, positive-ventilation system that controls and monitors airflow, and monitors the percentages of O_2 and CO_2 at both inlet and outlet ports. The chambers were Lucite, with stainless steel or plastic floors (perforated, for exit of feces, urine and spilled food onto a piece of cage paper within the oxy chamber). Daily body weight and food intake were monitored while the mice remained in the Oxymax. This involved replacement of the soiled cage paper (for spillage data) and cage wipe-out with a paper towel. The duration of experiments for each cohort was 4 days. These studies measured rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$). Energy expenditure was calculated as $EE=\dot{V}_{O_2}\times[3.815+(1.232\times RER)]$ (Lusk, 1928), where RER is the respiratory exchange ratio.

Statistical analysis

All results are expressed as mean ± s.e.m. Statistical analysis was performed using Prism 5 software (GraphPad). A two-tailed Student's *t*-test was performed on all *in vivo* mouse experiments. All metabolic data were normalized to baseline (baseline=2 days acclimation in the Oxymax apparatus), with baseline day being the third day of data acquisition with no protein or vehicle injected. The analysis then compared the vehicle-injected and the recombinant protein-injected group, with each animal normalized to their own specific baseline.

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Competing interests

The authors declare no competing financial interests.

Author contributions

G.W.W. conceived the project; M.M.S., M.S.B., P.S.P., R.S. and G.W.W. designed and executed the experiments; A.B.-B. and M.H.G. provided critical reagents; M.M.S., M.S.B., P.S.P. and G.W.W. drafted and revised the manuscript.

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