Peripheral ghrelin deepens torpor bouts in mice through the arcuate nucleus neuropeptide Y signaling pathway

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Peripheral ghrelin deepens torpor bouts in mice through the arcuate nucleus neuropeptide Y signaling pathway. Am J Physiol Regul Integr Comp Physiol 291: R1303–R1309, 2006. First published July 6, 2006; doi:10.1152/ajpregu.00232.2006.—Many small mammals have the ability to enter torpor, characterized by a controlled drop in body temperature (Tb). We hypothesized that ghrelin would modulate torpor bouts, because torpor is induced by fasting in mice coincident with elevated circulating ghrelin. Female National Institutes of Health (NIH) Swiss mice were implanted with a Tb telemeter and housed at an ambient temperature (Ta) of 18°C. On fasting, all mice entered a bout of torpor (minimum Tb: 23.8 ± 2.0°C). Peripheral ghrelin administration (100 µg) during fasting significantly deepened the bout of torpor (Tb minimum: 19.4 ± 0.5°C). When the arcuate nucleus (ARC) of the hypothalamus, a ghrelin receptor-rich region of the brain, was chemically ablated with monosodium glutamate (MSG), fasted mice failed to enter torpor (minimum Tb: 31.6 ± 0.6°C). Furthermore, ghrelin administration had no effect on the Tb minimum of ARC-ablated mice (31.8 ± 0.8°C). Two major pathways that regulate food intake resiude in the ARC, the anorexigenic α-melanocyte stimulating hormone (α-MSH) pathway and the orexigenic neuropeptide Y (NPY) signaling pathway. Both Arc mice, which have the α-MSH pathway blocked, and Npy−/− mice exhibited shallow, aborted torpor bouts in response to fasting (Tb minimum: 29.1 ± 0.6°C and 29.9 ± 1.2°C, respectively). Ghrelin deepened torpor in Arc mice (Tb minimum: 22.8 ± 1.3°C), but had no effect in Npy−/− mice (Tb minimum: 29.5 ± 0.8°C). Collectively, these data suggest that ghrelin’s actions on torpor are mediated via NPY neurons within the ARC.

body temperature; leptin

SMALL MAMMALS HAVE THERMOREGULATORY challenges not seen with larger mammals like humans. The small size of mammals like mice, chipmunks, and hamsters necessitates a limited capacity for storage of fuels. In addition, the increased surface area-to-volume ratio of these animals requires a much higher metabolic rate relative to body size. In cold environments, the cost of thermoregulation may become prohibitively expensive. As such, some small mammals enter the hypometabolic state of torpor (18, 21, 54), generally defined as a regulated reduction in Tb below 31°C (26). By entering a state of torpor, whether daily or longer bouts of hibernation, energy expenditure is reduced 50 to 90% (18, 27). Regulation of metabolic rate is not abandoned during a bout of torpor, as body temperature (Tb) is well regulated, albeit at a much lower set point (18). While low ambient temperature (Ta) contributes toward the torpor response, the neurophysiological signaling mechanisms that lead to the onset of torpor are not well defined, although recently it has become clear that the adipocyte-derived hormone leptin may play a role in mice, hamsters, and a marsupial (16, 17, 19).

Within the hypothalamus, the arcuate nucleus (ARC) plays a key role in the integration of signals for nutrient availability and energetic balance, including metabolic and feeding control (6). The ARC has access to both the third ventricle and portal vasculature of the median eminence, allowing it to sample physiological factors in both blood and cerebrospinal fluid (6, 46). Previous investigations have shown that perinatal treatment of rodent pups with monosodium glutamate (MSG) ablates the ARC (2, 9, 11, 12, 25, 30, 38, 49, 51), resulting in adult-onset obesity. The two most prolific types of neurons in the ARC are proopiomelanocortin and cocaine-and amphetamine-regulated transcript (POMC/CART) and neuropeptide Y/agouti-related protein (NPY/AgRP) neurons (44). POMC neurons produce a number of peptides that are derived from a common precursor, POMC. The POMC neurons are part of an anorexigenic pathway that originates in the ARC whose activity is decreased during fasting (14). The peptide most relevant to appetite regulation appears to be α-melanocyte-stimulating hormone (α-MSH). The POMC neurons release α-MSH, which activates melanocortin 4 receptors (MC4Rs) initiating a satiation response (5). The agouti protein is an endogenous antagonist of melanocortin receptors, and its ectopic expression outside of the hair follicles and in the hypothalamus, as seen in the Ag mouse, blocks the action of α-MSH at the MC4R (3). This action can lead to hyperphagia, reduced energy expenditure, hyperinsulinemia, yellow coat color in mice, and obesity. The second neuron type within the ARC that plays an important role in energy balance is the orexigenic NPY/AgRP neuron (14, 35). These neurons are activated during fasting to initiate the hunger response (48). When injected centrally, NPY is among the most potent orexigenic substances identified (28). However, Npy−/− mice and the double knockout of NPY and AgRP results in a relatively normal phenotype, including the response to fasting (13, 41), suggesting the presence of multiple redundant pathways to initiate the hunger response.

Energy balance is regulated centrally through many known peripherally secreted hormones, including insulin, leptin, and the stomach-derived hormone ghrelin (reviewed in Ref. 32). Ghrelin, a 28-amino acid peptide, is an appetite-stimulating hormone that is released in response to food deprivation (52, 58). It was discovered as the first endogenous ligand for the growth hormone secretagogue receptor. These receptors are found throughout the brain, including the ARC, as well as on...
the vagus nerve (8, 20, 24). Recently, it has been discovered that in addition to its synthesis and release from the gut, ghrelin-producing neurons are also found within the ARC (7). Within the ARC, it appears that the action of ghrelin is to activate NPY/AgRP neurons (4, 7, 23, 31, 37, 50, 55, 57). Peripheral and central injection of ghrelin increases food intake and hypothalamic NPY expression (29, 45); blocking the action of NPY blocks ghrelin-induced food intake (33). Because one prerequisite for torpor in mice is caloric restriction, it is of interest that short-term fasting elevates circulating ghrelin levels, whereas feeding reduces circulating ghrelin (52, 58).

Because ghrelin is likely a short-acting hormone that is responsive to the fed state of an animal, we sought to determine whether ghrelin can impact fasting-induced torpor in mice. Furthermore, the integral role that caloric restriction plays in the onset of torpor in laboratory mice suggests that the hypothalamic ARC, an area of the brain that acts as a conduit for hormonal and neuronal signals relevant to energy homeostasis, may be a critical component of torpor. To test the role of ghrelin and the ARC in torpor, we utilized peripheral ghrelin injections into ARC-ablated mice, as well as mice with blocked melanocortin signaling or deficient in NPY signaling.

**MATERIALS AND METHODS**

*Animals.* Female National Institutes of Health (NIH) Swiss, Ay mice, aa mice, and Npy−/− mice were housed individually in a 12:12-h light-dark cycle and fed ad libitum. On receipt of the animals, the mice were housed at a Ta of 28°C. The NIH Swiss mice were ordered from Harlan Sprague-Dawley, and all other strains were ordered from Jackson Labs, Bar Harbor, ME. All studies were approved by the Williams College Institutional Animal Care and Use Committee.

**MSG treatment.** To ablate the ARC, postnatal mouse pups were treated with MSG while a second cohort of mice was injected with vehicle (saline) and served as controls. Seven pregnant NIH Swiss mice were purchased and monitored until pups were born. The day of delivery was considered postnatal day 0. These mouse pups were injected subcutaneously with either 1) vehicle on postnatal days 1–5 (3 litters), or 2) 2 mg MSG/g of body wt on postnatal days 1 and 2, followed by 5 mg MSG/g on postnatal days 3–5 (4 litters). Approximately 35% of the pups injected with MSG died within the first week. None of the vehicle-treated pups died. Of the 15 surviving female MSG-treated mice and 18 saline-treated, 8 from each group were implanted with temperature telemeters at 6 wk of age and assessed for the torpor response to fasting and ghrelin administration at 7 wk of age.

**Implantation.** Mice were anesthetized initially with 5% isoflurane in an oxygen stream, and maintained on 1–2% isoflurane. The animals were transcardially perfused with 20 ml of heparinized saline (1,000 U heparin/ml) followed by 80 ml of 4% paraformaldehyde in saline (PFA). The brain was removed and placed in 4% PFA overnight at 4°C. After 24 h, the PFA was removed, and the brains were immersed in 3a 30% sucrose solution for 48 h at 4°C. Forty-micrometer coronal sections were taken through the region of the brain that includes the ARC and moved to a 1× PBS. Brain sections were washed three times in PBS. The sections were incubated in 0.2% Triton-X 100 in PBS for 5 min and again washed three times in PBS. The sections were blocked in 10 ml mg/ml BSA in PBS at room temperature for 15 min. A polyclonal antibody directed against tyrosine hydroxylase (Chemicon International, Temecula, CA) was diluted to 1:5,000 in BSA and incubated at 4°C overnight on a rocking platform. Sections were washed three times with PBS and incubated with a Cy3-labeled goat α-rabbit IgG antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) at a dilution of 1:1,000 in BSA at 4°C overnight. The sections were then washed three times in PBS, stained for 10 min with 0.67 µg/ml 4,6-diamidino-2-phenylindole (Sigma-Aldrich; St. Louis, MO), washed three times in PBS, and mounted on a slide. Brain images were captured with a Zeiss Axioscope equipped with a ×10 objective.

**Statistical analysis.** Data are reported as means ± SE. Tb minimum was analyzed with two-factor repeated-measures ANOVA with group as a between-subjects factor and treatment as a repeated measure, followed by a Bonferroni test. Body weights, average Tb, and maximum Tb were compared between control and experimental groups using Student’s t-tests. The 0.05 level of confidence was accepted for statistical significance.

**RESULTS**

In response to fasting at 18°C, all of the NIH Swiss mice (n = 6; body wt = 23.5 ± 0.7 g) injected with saline entered a torpid state late in the dark cycle (Fig. 1, top), consistent with noninjected fasted mice (Stephens N, Swoap SJ, unpublished observations). The Tb minimum of fasted mice injected with saline was 23.8 ± 2.0°C. When injected with ghrelin during the fast, these mice exhibited significantly deeper bouts of torpor (Fig. 1, bottom) and with Tb minimum (19.4°C ± 0.5), only 1.4°C above Tb at 18°C). To determine whether ghrelin could induce torpor in the fed state, ghrelin (100 µg) or saline was injected into these same mice 2 wk later under ad libitum conditions, 4.5 h into the dark cycle. The Tb minimum of fed mice injected with ghrelin was not significantly different from that of saline-injected fed mice (34.9 ± 0.3 vs. 35.2 ± 0.2°C, respectively).

MSG-treated female pups (Fig. 2A) and male pups (data not shown) were smaller than saline-treated pups, until about the seventh week of age. MSG-treated mice continued to grow,
whereas the body weight of saline-treated mice remained stable during weeks 8–14 (Fig. 2B). A subset of the female mice (n = 8 for each group) was used for implantation at 6 wk of age and for torpor analysis at 7 wk of age. This time point was chosen as a window where control and MSG-treated mice were relatively close in body weight (body weights at 6 wk of age: saline-treated = 22.3 ± 0.3 g; MSG-treated body weights = 20.2 ± 0.6 g). The body weights of the remaining littermates (n = 10 for saline-treated, n = 7 for MSG-treated) continued to be monitored until 14 wk of age (Fig. 2B). In the 14-wk-old saline-treated mice, we found tyrosine hydroxylase immunoreactive neurons present in the ARC (Fig. 2C). In contrast, MSG-treated mice displayed little or no staining of this same area, consistent with others that show postnatal MSG treatment ablates the ARC (2, 9, 11, 12, 25, 30, 49, 51).

The average Tb for MSG-treated mice housed at 18°C was significantly cooler by 1°C than saline-treated mice under fed conditions (35.5 ± 0.2°C vs. 36.6 ± 0.2°C, respectively: P < 0.05). Whereas these two groups of mice had similar maximum Tb (MSG-treated = 37.8 ± 0.2°C; saline-treated = 38.0 ± 0.1°C), the MSG-treated mice had a lower Tb minimum, whereas fed (34.2 ± 0.2°C vs. 35.2 ± 0.2°C, respectively: P < 0.05). Figure 3, top shows typical tracings of Tb of MSG-treated mice under three conditions: 1) fed, 2) fasted plus saline injection, and 3) fasted plus ghrelin injection. When MSG-treated mice were fasted and injected with saline or ghrelin, none exhibited the steep fall in Tb as seen in torpor of control mice. Fasted MSG-treated mice injected with saline exhibited some level of hypothermia (Tb minimum = 31.6 ± 0.6°C), significantly higher than fasted control mice from the same cohort (Tb minimum = 24.7 ± 1.3°C). As Fig. 3, bottom shows, ghrelin had no effect on the Tb response to fasting in MSG-treated mice (Tb minimum = 31.8 ± 0.8°C), whereas ghrelin injections into the saline-treated mice deepened the torpor bouts (19.7 ± 0.6°C), as we observed in the previous experiment with ghrelin injections (Fig. 1).

**Fig. 1.** Effect of fasting and ghrelin on body temperature (Tb) of National Institutes of Health (NIH) Swiss mice. *Top:* the Tb of 6 female NIH Swiss mice was telemetrically monitored during 1) ad libitum feeding, 2) fasting with a 0.1 cc saline ip injection at 4.5 h after initiation of the fast, or 3) fasting with a 0.1 cc ghrelin injection (100 μg) ip at 4.5 h after initiation of the fast. Typical tracings of a mouse in each condition are shown. Fasting was initiated at the start of the dark cycle. Fasted mice entered bouts of torpor late in the dark cycle. *Bottom:* the Tb minimum over the 24-h period was calculated. Peripheral ghrelin injection induced a significantly deeper bout of torpor. Data are shown as means ± SE. *P < 0.05 vs. fasted + saline injection.

**Fig. 2.** Perinatal monosodium glutamate (MSG) treatment ablates the arcuate nucleus and induces obesity. NIH Swiss pups were treated with either saline or MSG during postnatal days 1–5 (see MATERIALS AND METHODS for dosage). A: the body weight for females from each litter was monitored from weaning (3 wk of age) until 14 wk of age. The arrow indicates the time when mice were implanted with Tb telemeters for torpor analysis. B: a photo of 2 saline-treated female mice and 2 MSG-treated female mice at 14 wk of age. C: 40-μm coronal section shows tyrosine hydroxylase immunoreactive neurons in the arcuate nucleus (ARC) of a control mouse. These neurons are completely absent in this section from an MSG-treated mouse brain. ME, median eminence; III, third ventricle; white lines, approximate boundary of the ARC.
As the ARC is important for the torpor response, we next examined the Tb response to fasting in mice with deficient signaling in one of the two of the major neuron types within the ARC. Six-week-old female Ay mice (n = 18) and littermate controls (n = 8; genotype = aa) had similar body weights at this age (21.2 ± 0.6 g vs. 21.6 ± 0.8 g, respectively). Ay mice exhibited a lower maximum Tb than their littermate controls (37.7 ± 0.1 vs. 38.4 ± 0.1 °C, respectively; P < 0.05). However, Ay mice had the same mean Tb as aa mice over a 24-h period (37.0 ± 0.1 vs. 36.8 ± 0.2°C, respectively). The Ay mice also had the same Tb minimum as aa mice (35.4 ± 0.1 vs. 35.3 ± 0.1 °C, respectively). When fasted and injected with saline, Ay mice exhibited two very different responses in Tb. Of the 18 Ay mice fasted and injected with saline, eight entered a typical torpor bout (as seen in Fig. 4, top). The Tb minimum of these eight mice that exhibited a torpor bout was 27.1 ± 0.6°C, which is significantly (P < 0.05) warmer than the Tb minimum of fasted aa mice (24.6 ± 0.4 °C). The other 10 Ay mice exhibited aborted torpor bouts. Here, the mouse initiated a steep drop in Tb, indicative of torpor but quickly returned to a normal Tb (Fig. 4, top). As such, the Tb minimum achieved by this group of mice was only 30.0 ± 1.2°C. When injected with ghrelin, all fasted Ay mice exhibited a torpor bout, with a Tb minimum of 22.8 ± 3.3°C.

The other major neuron type within the ARC is the orexigenic NPY/AgRP neurons. Fed Npy−/− mice (n = 6; body weight = 23.1 ± 0.7 g) exhibited normal maximum Tb (38.1 ± 0.1°C), average Tb (36.9 ± 0.1°C) and Tb minimum (36.0 ± 0.1°C). In response to fasting, none of the saline-injected Npy−/− mice entered a typical torpor bout (Fig. 5, top). Rather, each mouse exhibited aborted torpor bouts (minimum Tb: 29.5 ± 0.8°C). In contrast to the Ay mice, none of the six Npy−/− mice responded to the injection of ghrelin (minimum Tb: 29.1 ± 0.6°C).

**Discussion**

In response to cool Ta and calorie restriction, laboratory mice enter a state of torpor (17, 22, 39, 47, 56). The principle finding in these studies is that peripheral ghrelin deepens the Tb minimum of a torpor bout. Furthermore, our data suggest that ghrelin acts within the ARC of the hypothalamus, specifically within NPY/AgRP neurons that reside in the ARC, to modulate torpor.

In addition to ghrelin’s known orexigenic effects (reviewed in Ref. 32), we show here that ghrelin can deepen the Tb minimum of a torpor bout. It is of interest to note that circulating ghrelin reaches a peak near the end of the dark cycle (36). This timing is coincident with the onset of torpor in fasting mice (see Fig. 1), which is typically 8 h after initiation of the fast. It is currently not clear whether the actions of peripherally administered ghrelin impact Tb during a torpor bout via the vagus nerve, direct actions on the ARC itself, or both. Some evidence exists that suggests ghrelin does not cross the blood-brain barrier very well in the blood-to-brain direction (1). Ghrelin may influence torpor bouts via the vagus nerve, as others have shown that vagal afferents contain ghrelin recep-
Fig. 5. Effect of fasting and ghrelin on \(T_b\) of \(Npy^{-/-}\) mice. Top: the \(T_b\) of 6 female \(Npy^{-/-}\) mice, missing the orexigenic neuropeptide Y neurotransmitter, was monitored during 1) ad libitum feeding, 2) fasting with a 0.1 cc saline ip injection at 4.5 h after initiation of the fast, or 3) fasting with a 0.1 cc ghrelin ip injection (100 \(\mu g\)) at 4.5 h after initiation of the fast. Fasting was initiated at the start of the dark cycle. Typical tracings of an \(Npy^{-/-}\) mouse in each condition are shown. None of the fasted mice exhibited typical torpor bouts (compare to tracings in Fig. 1). Bottom: the \(T_b\) minimum over the 24-h period was calculated. Peripheral ghrelin injection had no effect on this parameter. Data shown as means ± SE. *P < 0.05 vs. fasted + saline injection.

The lack of a \(T_b\) response to ghrelin in \(Npy^{-/-}\) suggests that ghrelin does require NPY neurons to exert its physiological effects on \(T_b\). Indeed, multiple lines of evidence from other studies support the concept that ghrelin exerts its physiological effects through NPY/AgRP neurons. First, peripherally administered ghrelin increases the c-fos immunoreactivity within NPY neurons (37, 55). Second, mice deficient in both NPY and AgRP do not exhibit the normal increase in feeding in response to ghrelin (4). Finally, ghrelin directly increases the physiological activity of NPY neurons in the ARC (7, 31). This is in agreement with our data that show that peripherally administrated ghrelin had no effect on the \(T_b\) of \(Npy^{-/-}\) mice (Fig. 5) but deepened the torpor bouts of normal and \(A_\alpha\) mice (Figs. 1 and 4). Given that \(Npy^{-/-}\) mice were able to initiate torpor bouts but were unable to maintain them independent of ghrelin administration, we conclude that the NPY pathway is critical for both the full torpor phenotype and the modulation of torpor by ghrelin in mice.

Whereas ghrelin was important in modulating fasting-induced torpor bouts in mice, ghrelin may not play a role in animals that depend largely on photoperiod changes for a signal for torpor bouts. The Siberian hamster can enter torpor when maintained for extended periods of time in a shortened photoperiod (16, 40), although torpor entry occurs only after significant fat mass loss (43). Both circulating ghrelin levels and ghrelin receptor expression in the brain of Siberian hamsters are relatively unchanged between long photoperiods (non-

... tors and transmit a starvation signal to the nucleus of the solitary tract (8, 20, 24). Indeed, blockade of gastric vagal afferents eliminates ghrelin-induced feeding (8). However, as stated earlier, the ARC is situated such that its neurons likely sample physiological factors (ghrelin, insulin, leptin, glucose) directly in the circulation (6, 46). The ghrelin receptor colocalizes within NPY neurons (57), although these receptors may be of little significance, as implied by our data that show that peripherally administered ghrelin has no effect on the \(T_b\) of \(Npy^{-/-}\) mice.

...ghrelin ip injection (100 \(\mu g\)) at 4.5 h after initiation of the fast. Fasting was initiated at the start of the dark cycle. Typical tracings of an \(Npy^{-/-}\) mouse in each condition are shown. None of the fasted mice exhibited typical torpor bouts (compare to tracings in Fig. 1). Bottom: the \(T_b\) minimum over the 24-h period was calculated. Peripheral ghrelin injection had no effect on this parameter. Data shown as means ± SE. *P < 0.05 vs. fasted + saline injection.

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torpor season) vs. short photoperiods (torpor season; Ref. 53). These hamsters can enter torpor in a long photoperiod if calorically restricted long enough, presumably to reduce body fat stores and circulating leptin (16, 42). This dependence on low circulating leptin in these hamsters may be mediated via NPY, as intracerebroventricular delivery of NPY induces torpor-like bouts of hypothermia, with a drop in Tb of 10°C (40).

The complex interplay between circulating hormones that relay nutritional status like insulin, leptin, and ghrelin and the neurons residing in the hypothalamus that respond to these hormones likely set the stage for entrance into torpor. We show here that ghrelin can deepen torpor bouts in fasting mice, and its likely mechanism of action is through NPY/AgRP neurons that reside within the ARC.

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