A potential role for hypothalamosomedullary POMC projections in leptin-induced suppression of food intake

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Zheng H, Patterson LM, Rhodes CJ, Louis GW, Skibicka KP, Grill HJ, Myers MG, J, Berthoud HR. A potential role for hypothalamosomedullary POMC projections in leptin-induced suppression of food intake. Am J Physiol Regul Integr Comp Physiol 298: R720–R728, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00619.2009.—Melanocortin-3/4 receptor ligands administered to the caudal brain stem potently modulate food intake by changing meal size. The origin of the endogenous ligands is unclear, because the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract (NTS) harbor populations of proopiomelanocortin (POMC)-expressing neurons. Here we demonstrate that activation of hypothalamic POMC neurons leads to suppression of food intake and that this suppression is prevented by administration of a melanocortin-3/4 receptor antagonist to the NTS and its vicinity. Bilateral leptin injections into the rat arcuate nucleus produced long-lasting suppression of meal size and total chow intake. These effects were significantly blunted by injection of SHU-9119 into the fourth ventricle, although SHU-9119 increased meal size and food intake during the first, but not the second, 14-h observation period. Leptin effects on meal size and food intake were abolished throughout the 40-h observation period by injection of SHU-9119 into the NTS at a dose that by itself had no effect. Neuron-specific tracing from the arcuate nucleus with a Cre-inducible tract-tracing adenovirus found retrograde labeling in the NTS at all times that the animal was alive. The tracer was also found in the NTS of animals that had received bilateral injections of leptin into the arcuate nucleus. These data support the hypothesis that the arcuate nucleus is the origin of POMC projections into the NTS and that the NTS integrates changes in peripheral signals with central inputs to modulate feeding behavior. Evidence from human genetic studies (40) suggests that a portion of the population may lack these projections, as indicated by obesity susceptibility. These findings provide important insights for treatments of leptin-resistant obesity. Am J Physiol Regul Integr Comp Physiol 298: R720–R728, 2010. First published January 13, 2010. © 2010 American Physiological Society.
To verify injection sites in the arcuate nucleus, 300 nl of a 1% Chicago Blue (Sigma, St. Louis, MO) solution was injected 5 min before perfusion, and 30-μm-thick frontal sections were examined under a microscope. Injection sites were mapped on the nearest plates from the stereotaxic atlas of Paxinos and Watson (27).

Selective Anterograde Tracing of POMC Neurons in POMC-Cre Mice

We utilized a modified pShuttle-based (20) adenoviral transfer vector containing the cytomegalovirus vector promoter upstream of a transcription-blocking cassette followed by sequences encoding far-nesylated enhanced green fluorescent protein (EGFP) to generate Ad-iZ/EGFP, which mediates the expression of EGFP only when transduced into Cre-expressing cells (24). For injections of Ad-iZ/EGFP into Pomc<sup>Cre</sup> mice, 300 nl of anterograde viral tracer from a 5.1 × 10<sup>11</sup> plaque-forming units/ml stock were unilaterally infused into two rostrocaudal locations of the arcuate nucleus at a rate of 60 nl/min through a 33-gauge stainless steel injector connected to a 1.0-μl Hamilton syringe with a short piece of PE-10 tubing (antero-posterior −1.6 and −1.8 mm, mediolateral 0.20 mm, dorsoventral 5.8 mm) in seven mice (26). To minimize backflow of the tracer, the injector was left in place for another 10 min after the injection before the wound was closed. Ten days were allowed for uptake and transport.

Tissue Preparation and Immunohistochemistry

Rats or mice were deeply anesthetized with pentobarbital sodium (90 mg/kg ip) and transcardially perfused with heparinized saline (20 U/ml) followed by ice-cold 4% phosphate-buffered (pH 7.4) paraformaldehyde. Brains were extracted, blocked, and postfixed in the same fixative overnight. Tissue was immersed for 24 h in 18% sucrose-0.05% sodium azide in 0.1 M PBS. Coronal sections (20–25 μm thick) of forebrain or caudal brain stem were cut in a cryostat and divided into five series. For immediate processing, sections were held in PBS (4°C); for long-term storage (−20°C), a cryoprotectant solution (50% PBS, 30% ethylene glycol, and 20% glycerol) was used. Free-floating sections were pretreated with 0.5% sodium borohydride in PBS to minimize aldehyde cross-linking of the fixative and blocked with donkey normal serum. Appropriate washing in PBS followed all incubations; after the last wash, sections were mounted in 100% glycerol with 5% n-propyl gallate as an antifade agent. Negative controls (without the primary antibody) or preadsorbed controls were used to establish the lack of nonspecific staining.

Visualization of far-nesylated green fluorescent protein-labeled POMC projections. Sections were double-labeled with an immunofluorescent method using simultaneous incubation in two primary antibodies, chicken anti-green fluorescent protein (anti-GFP, 1:1,000 dilution; Abcam, Cambridge, MA) and sheep anti-α-MSH (1:40,000 dilution; Millipore-Chemicon, Temecula, CA), overnight at room temperature or for 72 h at 4°C. Secondary antibodies, Cy3-conjugated donkey anti-chicken (1:200 dilution) and Cy2-conjugated donkey anti-sheep (1:600 dilution; both from Jackson ImmunoResearch, West Grove, PA), were applied for 2 h at room temperature.

α-MSH-immunoreactive axon profiles in chronic supracollicular decerebrate rats. The brain stem of rats that were subjected to a two-stage complete brain transection [dorsally at the superior colliculus and ventrally at the diencephalic-mesencephalic border (18)] ≥4 wk before death were compared with the caudal brain stems of intact rats that were maintained identically to the decerebrate rats. For mapping α-MSH-immunoreactive axon profiles in the dorsal vagal complex, the primary antibody was the sheep polyclonal anti-α-MSH diluted to 1:40,000 (Millipore-Chemicon). Sections were labeled with Cy3-conjugated donkey anti-sheep (Jackson ImmunoResearch) and mounted as described above.
Quantification of α-MSH Immunoreactivity in the Brain Stem

Confocal image stacks (10 scans, 2 μm apart, obtained with an ×20 lens; Zeiss LSM 310) of α-MSH-immunoreactive axon profiles in the dorsal vagal complex of decerebrate (n = 7) and control rats (n = 7) were used for densitometric analysis. For each rat, stacks from three representative rostrocaudal levels of the dorsal vagal complex [−13.24 mm from bregma (rostral to area postrema), −13.68 mm (full area postrema), and −14.30 mm (caudal to area postrema)] were selected for analysis (27). Images were densitometrically analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD) by determination of the relative total area (combined length and diameter of fibers) of pixels above a threshold brightness value that was held constant for all sections from all animals. Staining artifacts were eliminated by use of size and shape criteria.

Statistical Analysis

Cumulative food intakes measured at thirteen 3-h intervals were analyzed using separate two-way repeated-measures ANOVA, with arcuate nucleus injection (saline or leptin) and fourth ventricle or NTS injection (saline or SHU-9119) as between-subjects factors and with time as within-subjects repeated factor (SPSS Statistics, version 17.0). Food intake, meal size, and meal frequency for the two dark periods after the last injection were analyzed using separate two-way ANOVAs, with arcuate nucleus injection (saline or leptin) as one factor and fourth ventricle/NTS injection (saline or SHU-9119) as within-subjects repeated factor or with fourth ventricle/NTS injection as one factor and arcuate nucleus injection as repeated factor. Thus, pairwise comparisons of the individual differences between saline and leptin for conditions with and without SHU-9119 pretreatment or differences between saline and SHU-9119 pretreatment for conditions with and without arcuate leptin injection were computed separately for each dark period. Densities of α-MSH fibers in the caudal brain stem were analyzed using two-way ANOVA, with surgical manipulation (decerebration or intact) as between-subjects factor and rostrocaudal NTS levels (obex, full area postrema, rostral to area postrema) as within-subjects repeated factor and Bonferroni’s-adjusted post hoc comparisons.

RESULTS

Caudal Brain Stem SHU-9119 Attenuates Food Intake Suppression Induced by Arcuate Nucleus Leptin Injection

Two separate experiments were carried out to test the ability of the MC3R/MC4R antagonist SHU-9119 administered to the fourth ventricle or directly to the NTS to block feeding suppression by leptin administered at the level of the hypothalamus.

SHU-9119 injections into the fourth ventricle. As shown in Fig. 1A, in the absence of SHU-9119, leptin significantly suppressed cumulative food intake compared with saline control starting 9 h after injection and lasting throughout the 40-h observation period. In the presence of SHU-9119, leptin was unable to significantly suppress food intake throughout the observation period, although there was a tendency toward the end of the 40-h observation period. Two-way ANOVA over

Fig. 1. Suppression of food intake and meal size by leptin administration to the arcuate nucleus is attenuated by injection of the melanocortin 3/4 receptor (MC3R/MC4R) antagonist SHU-9119 (SHU) into the 4th ventricle (4V). Leptin (30 pmol/side) or saline (Sal) was injected bilaterally into the arcuate nucleus 1 h after administration of SHU-9119 (100 pmol) or saline into the 4th ventricle. A: cumulative food intake throughout the 40-h observation period. Horizontal gray bars indicate dark periods. B and C: food intake during the 2 dark periods (2–14 h and 26–38 h, including 2 h before dark onset). D and E: meal size and meal frequency during the 2 dark periods. *P < 0.05, based on multiple comparisons after ANOVA (A) or ANOVA with pairwise comparisons of differences between saline and leptin with and without SHU-9119 pretreatment (B–E). #P < 0.05, based on ANOVA with pairwise comparisons of differences between saline and SHU-9119 pretreatment with and without arcuate leptin injection.

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of SHU-9119 in the absence of leptin for the 0- to 14-h period. SHU-9119 showed a significant food intake-enhancing effect compared with saline control starting 9 h after injection and lasting throughout the 40-h observation period. Two-way ANOVA yielded a significant effect of arcuate nucleus injection \([F(1,36) = 9.7, P = 0.004]\) and a significant interaction \([F(1,36) = 4.8, P = 0.035]\), but no significant effect of fourth ventricular injection \([F(1,36) = 1.6, P = 0.23]\).

Because food intake was lower and may have been affected by housekeeping during the intervening 12-h light period, further analysis was performed separately on food intake during the two dark periods, including 2 h before dark onset (Fig. 1, B and C). Pairwise comparisons of the individual differences between saline and leptin showed significant food intake-suppressing effects of leptin in the absence of SHU-9119 \([t(9) = 2.23, P = 0.026\) and \(t(9) = 3.91, P < 0.001]\) but no effect in the presence of SHU-9119 \([t(9) = 0.51, P = 0.96\) and \(t(9) = 1.69, P = 0.11]\) for the 0- to 14-h period (Fig. 1B) and the 24- to 38-h period, respectively (Fig. 1C). However, pairwise comparisons of the individual differences between saline and SHU-9119 showed a significant food intake-enhancing effect of SHU-9119 in the absence of leptin for the 0- to 14-h period \([t(9) = 3.36, P = 0.004]\), but not for the 24- to 38-h period.

Meal pattern analysis applied to the two 12-h dark periods (without the 2 h before dark onset) in the same manner as for food intake (see above) revealed that, during the first night, leptin significantly decreased meal size in the absence \([t(9) = 2.14, P = 0.047]\), but not in the presence, of SHU-9119 and had no effect on meal frequency (Fig. 1D). However, as with food intake, meal size was significantly increased by fourth ventricular SHU-9119 treatment alone during this period \([t(9) = 3.92, P < 0.01]\). During the second night, leptin again significantly suppressed meal size in the absence \([t(9) = 2.62, P = 0.017]\), but not in the presence, of SHU-9119 and also significantly decreased meal frequency in the absence of SHU-9119 (Fig. 1E). During this period, SHU-9119 by itself did not significantly increase meal size. Leptin also significantly decreased meal frequency in the absence of SHU-9119 pretreatment and had a similar effect in the presence of SHU-9119 that was, however, not significant.

**SHU-9119 injections into the NTS.** As shown in Fig. 2A, in the absence of SHU-9119, leptin significantly suppressed cumulative food intake compared with saline control starting 9 h after injection and lasting throughout the 40-h observation period. ANOVA was applied to the entire 40-h period in leptin-responsive rats only, there were significant effects of arcuate nucleus injection \([F(1,27) = 4.4, P = 0.046]\).

Because food intake was lower and may have been affected by housekeeping during the intervening 12-h light period, further analysis was performed separately on food intake during the two dark periods, including 2 h before dark onset (Fig. 1, B and C). Pairwise comparisons of the individual differences between saline and leptin showed significant food intake-suppressing effects of leptin in the absence of SHU-9119 \([t(9) = 2.23, P = 0.026\) and \(t(9) = 3.91, P < 0.001]\) but no effect in the presence of SHU-9119 \([t(9) = 0.51, P = 0.96\) and \(t(9) = 1.69, P = 0.11]\) for the 0- to 14-h period (Fig. 1B) and the 24- to 38-h period, respectively (Fig. 1C). However, pairwise comparisons of the individual differences between saline and SHU-9119 showed a significant food intake-enhancing effect of SHU-9119 in the absence of leptin for the 0- to 14-h period \([t(9) = 3.36, P = 0.004]\), but not for the 24- to 38-h period.

**Fig. 2. Suppression of food intake and meal size by leptin administration to the arcuate nucleus is abolished by injection of the MC3R/MC4R antagonist SHU-9119 into the nucleus of the solitary tract (NTS).** A: cumulative food intake throughout the 40-h observation period. Horizontal gray bars indicate dark periods. B and C: food intake during the 2 dark periods (0–14 h and 24–38 h, including 2 h before dark onset). D and E: meal size and meal frequency during the 2 dark periods. *P < 0.05, based on multiple comparisons after ANOVA (A) or pairwise comparisons of mean differences between saline and leptin with and without SHU-9119 pretreatment (B–E). #P < 0.05, based on ANOVA with pairwise comparisons of mean differences between saline and SHU-9119 pretreatment with and without arcuate leptin injection.
Fig. 3. Leptin injection sites in the arcuate nucleus. A: histological verification of typical bilateral cannula tracks in the arcuate nucleus used to determine injector tip location. B: injection sites (●) for bilateral arcuate nucleus injections in rats with 4th ventricular administration of SHU-9119. C: injection sites for bilateral arcuate nucleus injections in rats with NTS administration of SHU-9119. Sites are superimposed on images from the stereotaxic atlas of Paxinos (26).
profiles and a moderate number of relatively large-caliber, varicose axon profiles were present throughout the NTS and dorsal motor nucleus (Fig. 5). In decerebrate rats, most of the larger-caliber fibers were missing, and only the fine, dustlike immunoreactivity remained in this area. Quantitative analysis revealed that the density of α-MSH-immunoreactive fibers was significantly decreased in decerebrate compared with intact rats at all three rostrocaudal levels of the dorsal vagal complex (Fig. 6). ANOVA yielded significant effects of neurological status \( F(1,12) = 14.6, P = 0.002 \) and rostrocaudal location \( F(2,11) = 6.57, P = 0.013 \) but no significant interaction \( F(2,11) = 3.32, P = 0.074 \). About 70% of α-MSH immunoreactivity was absent after decerebration, reaffirming that these fibers originate from above the supracollicular transection.

DISCUSSION

Although progress has been made in identifying cellular neural mechanisms crucial for the control of food intake and regulation of energy balance, we are relatively ignorant regarding the cross talk between major brain areas orchestrating behavioral, endocrine, and autonomic output. Descending projections from the hypothalamus to the caudal brain stem are thought to play an important role in the modulation of basic brain stem reflex activity by information regarding overall energy status, other competing motivated behaviors, and cognition and emotion. Long before discovery of the hypothalamic “feeding” peptides, it was shown that hypothalamic activity modulates taste and gastrointestinal sensory functions at the level of the dorsal vagal complex (3, 30).

More recently, melanocortin signaling in the paraventricular nucleus of the hypothalamus and projections of leptin-activated oxytocin neurons from this nucleus to the dorsal vagal complex have been implicated in modulation of CCK-induced satiation and food intake (7–9). Oxytocin’s inhibitory effect on food intake may be mediated by oxytocinergic projections abutting on glucagon-like peptide (GLP-1) neurons in the caudal NTS (28, 29). Hypothalamic neurons expressing POMC and other “feeding” peptides, such as gastrin-releasing peptide, corticotropin-releasing hormone, orexin, and melanin-concentrating hormone, also project to the dorsal vagal complex and, thus, are in a position to modulate feeding-related brain stem activity (38). Specifically, POMC neurons in the arcuate nucleus are known to be primary recipients of circulating nutritional information, and their output via the MC4R can strongly inhibit food intake (2, 11, 13), but the critical site(s) of signaling has not been clearly identified. One possible site is the paraventricular nucleus of the hypothalamus (2), where medulla-projecting oxytocin neurons might relay the signal to the caudal brain stem, as mentioned above (9). Another possible site is the caudal brain stem directly, where a subset of arcuate POMC neurons projects (38) and MC3R/MC4R ligands powerfully modulate meal size and food intake (6, 33, 37, 38). In contrast to oxytocin neurons, which are only found in the hypothalamus, POMC neurons are also found in the commissural NTS (15, 21, 25), which could be the source of α-MSH, the natural ligand for MC3R/MC4R expressed in relevant brain stem circuits for physiological suppression of food intake (14, 15). Here we provide evidence that axon terminals of arcuate-to-NTS-projecting POMC neurons are at least one source of the natural ligand.

To activate hypothalamic POMC neurons, we injected small doses of leptin directly into the arcuate nucleus, because it was shown that at least a subset of arcuate POMC neurons expresses the long-form leptin receptor lepr\( ^b \) (10) and that POMC neurons increase firing rate in response to leptin (13). This
localized leptin administration produced a long-lasting suppression of food intake, as has previously been demonstrated with intraventricular administration of much higher doses of leptin (31) and after selective restoration of leptin receptor signaling in transgenic leptin receptor-deficient mice (12). Given the small doses used in our experiment, it is unlikely that leptin leaked out of the injection site and acted at a distant site, such as the caudal brain stem. This is important, because leptin delivered directly to, or near, the caudal brain stem has also been shown to decrease food intake (19). In those experiments, the lowest dose of leptin administered into the fourth ventricle to produce a significant suppression of food intake was 150 pmol (830 ng), 2.5-fold higher than the bilateral dose injected into the arcuate nucleus in the present experiment (60 pmol or 330 ng). Although this strongly supports a hypothalamic site of action in the present experiment, tagging leptin with a radioactive marker will be necessary to provide a definitive answer.

Most importantly, when the rats were pretreated with fourth ventricular or direct NTS administration of the MC3R/MC4R antagonist SHU-9119, leptin’s capacity to suppress meal size and food intake was greatly diminished, supporting the view that the effect initiated in the hypothalamus is mediated by melanocortin signaling at the level of the caudal brain stem. Because SHU-9119 delivered at these sites can powerfully stimulate food intake (17, 38), we needed to find a dose that by itself has little effect (32). With direct NTS injections, we found a dose of SHU-9119 (20 pmol) that did not significantly increase food intake by itself but did completely prevent the arcuate leptin-induced decrease of 14-h food intake. With fourth ventricular injections, we did not completely succeed in finding that dose, inasmuch as SHU-9119 did increase food intake.

Fig. 5. Transection of all neural connections at midbrain level abolishes the majority of α-melanocytostimulating hormone (α-MSH) immunoreactive axon profiles in the NTS. α-MSH-immunoreactive fibers at 3 rostrocaudal levels of the NTS are shown in intact (A–C) and midcollicular decerebrate (D–F) rats 8 wk after decerebration. Large-caliber, beaded, varicose axon profiles in intact rats are completely absent in decerebrate rats. Only very fine, dustlike labeling was found in decerebrate rats. Scale bar: 150 μm for all images. See Fig. 4 legend for definition of abbreviations.

Fig. 6. Quantitative assessment of relative contribution of forebrain and caudal brain stem to α-MSH innervation of the NTS. Densitometric analysis of α-MSH immunoreactivity at 3 rostrocaudal levels of the NTS of intact and decerebrate rats. α-MSH immunoreactivity is significantly reduced (*P < 0.01, based on ANOVA) by >70% at all 3 levels. AP, area postrema.
intake during the first 14 h after treatment. However, during the second 12 h of leptin-induced suppression of food intake, SHU-9119 alone did not significantly change food intake, but it still largely prevented leptin’s suppressive effects on meal size and food intake. Effects on total food intake were paralleled by effects on meal size, with little effect on meal frequency. Specifically, during the second nighttime feeding period, fourth ventricular SHU-9119 pretreatment completely eliminated leptin’s suppressive effect on meal size without itself having a significant effect. Thus the meal pattern data lend additional support for the conclusion that arcuate leptin acts via direct melanocortin projections to the caudal brain stem.

With our leptin injections into the arcuate nucleus, we can neither rule out activation of leptin-sensitive neurons in the adjacent ventromedial, premamillary, dorsomedial, and even perifornical hypothalamus nor activation of POMC and other neurons that, via projections to the paraventricular nucleus or the lateral hypothalamic area, could indirectly affect melanocortin signaling in the caudal brain stem. However, because the leptin-induced suppression of food intake and meal size was almost completely prevented by fourth ventricular and NTS administration of SHU-9119, activation of these other hypothalamic sites could only be involved if it would lead to enhanced α-MSH signaling in the caudal brain stem. Because there are only two sources of endogenous neuronal α-MSH in the caudal brain stem, POMC neurons in the arcuate nucleus with projections to the NTS and POMC neurons in the NTS, these other leptin-activated sites would have to gain access to one of these sources.

Our anatomic results show clearly that some arcuate POMC neurons send axonal projections to the dorsal vagal complex, including the NTS. Because Ad-iZ/EGFPf-mediated expression of EGFPf selectively targets membranes of cre-expressing neurons, it effectively labels even the long projections of arcuate POMC neurons, but not projections of other than POMC neurons located near the injection site. These findings confirm and extend earlier results with a retrograde tracer (38). Tracer injections that covered the entire dorsal vagal complex and some of the surrounding brain stem structures retrogradely labeled ~15% of POMC neurons in the anterior half of the arcuate nucleus, including the retrochiasmatic area (38). However, retrograde tracing does not reveal the distribution of POMC/α-MSH nerve fibers and terminals in the dorsal vagal complex, and immunohistochemistry for such fibers is inconclusive regarding their origin because of interference with the local population of POMC neurons within the NTS (21, 22). The neuron-specific anterograde tracing strategy used here unambiguously demonstrates innervation of the NTS by arcuate POMC neurons. However, two injections of anterograde tracer into the elongated arcuate nucleus do not label all brain stem-projecting POMC neurons, and labeling in the NTS is not quantitative, as indicated by the relative scarcity of GFP-positive fibers and terminals. For a more quantitative assessment of the distribution of origin-specific GFP/α-MSH in the NTS, we used an additional approach, the decerebrate rat model.

Our analysis of the brain stems of rats with complete surgical disconnection of forebrain-brain stem connections shows that the contribution of the forebrain to α-MSH immunoreactivity observed in the NTS is as much as 70%, leaving only 30% to originate from local medullary POMC neurons. These observations largely confirm earlier observations of Palkovits et al. (25) using electrolytic lesions in the midbrain. In that study, the forebrain contribution was mainly noted in the rostral NTS. In our analysis, a similar high contribition by the forebrain was seen throughout the rostrocaudal extent of the NTS. One possible explanation for this difference is the difficulty of completely interrupting descending connections with midbrain lesions compared with the total decerebration procedure used in the present experiment. This would lead to an overestimation of the local brain stem contribution and an underestimation of the forebrain contribution.

**Perspectives**

Despite intensive research, the roles of the caudal brain stem and hypothalamus in the control of food intake are not completely clear. Studies in the decerebrate rat model have demonstrated that neural circuitry in the brain stem is sufficient to organize the oromotor and autonomic controls necessary for ingestion of food placed in the mouth, but the extent to which caudal brain stem mechanisms act autonomously or are under modulatory influence from the hypothalamus and other forebrain areas in the intact animal is not clear. Here we confirm the existence of POMC projections from the arcuate nucleus of the hypothalamus to the dorsal vagal complex that are in a position to modulate meal size and total food intake. Together with oxytocin projections from the paraventricular nucleus (9), they provide a pathway by which leptin, acting at the level of the hypothalamus, may modulate the strength of vagal afferent input to the NTS. Leptin, acting at the level of the caudal brain stem, can also enhance the anorectic response to exogenous CCK, but by a different mechanism; apparently, it does not enhance vagal afferent input and does not increase c-Fos activity in NTS neurons (36). Together, these findings suggest a control system with considerable redundancy. Future research will have to more fully elucidate anatomic and signaling characteristics of the underlying neural circuitry in the caudal brain stem and hypothalamus.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

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