Leptin in the hindbrain facilitates phosphorylation of STAT3 in the hypothalamus

Bhavna N. Desai and Ruth B. S. Harris

Department of Physiology, Medical College of Georgia, Georgia Regents University, Augusta, Georgia

Address for reprint requests and other correspondence: R. B. S. Harris, Dept. of Physiology, Georgia Regents Univ., 1120 15th St., Augusta, GA 30912 (e-mail: ruharris@gru.edu).

ObRbs are expressed in multiple areas of the brain, with a high level of expression in hypothalamic nuclei, including the arcuate nucleus (Arc), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and lateral hypothalamus and moderate levels of expression in other midbrain and hindbrain nuclei, including the ventral tegmental area, dorsal raphae, nucleus accumbens, nucleus of the solitary tract (NTS), and area postrema, among others (10, 27). The Arc has received the most attention as a nucleus critical to the control of energy balance by leptin and other peripheral signals (4). In the last decade, however, a number of studies have shown that extrahypothalamic nuclei also play a major role in the lepin-mediated control of energy balance (16, 20, 21). As a result, the idea that neural regulation of energy balance may be distributed among several brain nuclei in the forebrain and hindbrain has been proposed (15).

In support of the distributed model of energy balance regulation, we have recently shown that the chronic activation of both forebrain and hindbrain ObRs is required to produce a leptin response comparable with that seen with acute high-dose leptin injections into either the third or fourth ventricle (6). Our results show that subthreshold doses of leptin, too low to influence energy balance when infused separately into either the third or fourth ventricle, reduce body fat by 50%, reduce lean body mass by 16%, and cause a transient 60% reduction in food intake when infused simultaneously for 12 days (6).

This suggests that under near physiological conditions, the forebrain and hindbrain integrate their leptin signals to either potentiate each other or to inhibit feeding neurons in other areas of the brain via neuronal projections to a common site. The exact nature of the communication between the forebrain and hindbrain is unknown. To understand the mechanism by which neurons in the forebrain and hindbrain interact, it is important to first determine which brain nuclei are involved in this integrated response. Therefore, the objective of the present study was to identify brain nuclei that are selectively activated during the simultaneous infusion of low-dose leptin into both the third and fourth ventricles.

We determined which nuclei were chronically activated due to the continuous infusion by localizing transcription factor ΔFosB levels within the brain. The acute transcription factor FosB is induced within 1–3 h after the acute administration of drugs and other stimuli and degraded to basal levels within hours of the end of drug administration (47). In contrast, ΔFosB, a truncated splice variant of FosB, accumulates during chronic stimulation by drugs or other stimuli with a stable expression for 2–4 wk after withdrawal of the stimulus. Therefore, ΔFosB has been established as a marker for chronic
neuronal activation (24). We also determined which brain nuclei were activated directly by leptin by localizing phosphorylated (p)STAT3, an accepted marker of ObRb activation, within the brain (2). The experiments in the present study provide new information on potential brain nuclei that may communicate with each other to produce a leptin-mediated reduction in body fat and body weight.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 275–300 g (Harlan Laboratories, Indianapolis, IN) were housed individually in hanging wire mesh cages in a room maintained at 20–23°C with lights on for 12 h each day from 7:00 AM. They had free access to chow (Harlan Teklad Rodent Diet 8604) and water unless otherwise stated. Each rat had a Nylabone (Nylabone Products, Neptune, NJ) in their cage for enrichment. All animal procedures were approved by the Institutional Animal Care and Use Committee of Georgia Regents University. For each experiment, rats were allowed to adapt to their environment for 1 wk before surgery. Body weight was recorded daily.

Experiments

**Experiment 1.** We have previously demonstrated that rats infused with low-doses of leptin into both the third and fourth ventricle for 6 days show a reduction in food intake, body weight, and body fat (6). This experiment was a pilot study designed to test for the presence of ΔFosB in our rats, to test commercially available ΔFosB antibodies in our system to identify the best antibody for immunohistochemistry, and to confirm changes in pSTAT3 expression in rats. Eighteen male Sprague-Dawley rats were fitted with third and fourth ventricle infusion cannulas (Plastics One, Roanoake, VA) using previously described coordinates (6). Rats were given 4 days to recover from surgery, after which baseline food intakes and body weights were recorded for 4 days and the rats were then divided into two weight-matched groups. An Alzet mini-osmotic pump (model 2002, DURECT) with an infusion rate of 0.25 μl/h was connected to each cannula to deliver either saline in both ventricles or 0.1 μg leptin in the third ventricle or 0.6 μg leptin in the fourth ventricle such that there were two treatment groups, as follows: saline-saline (SS) and leptin-leptin (LL) (the first letter indicates infusion in the third ventricle and the second letter indicates infusion in the fourth ventricle). The doses of leptin were based on those used in a previous study (6). Daily food intake and body weight were recorded for 6 days after the pumps were attached. On the sixth day, rats were euthanized, and tissue blocks of the hypothalamus and hindbrain were collected (19). The purpose of this experiment was to localize brain nuclei showing increased activation due to the chronic infusion of low-dose leptin simultaneously into both the third and fourth ventricles. We used pSTAT3 as a marker for nuclei that were activated directly by leptin (2) and ΔFosB as a marker for chronic neuronal activation that was not limited to leptin (1). A total of 30 male Sprague-Dawley rats in 2 cohorts of 20 and 10 rats with all groups represented in each cohort were housed and maintained in wire mesh cages throughout the study. Surgical procedures were the same as in experiment 1. After 1 wk of recovery from cannula placement, baseline food intakes and body weights were recorded for 3 days. Alzet mini-osmotic pumps were attached to infusion cannula such that there were four treatment groups: saline-saline (SS), saline-leptin (SL), leptin-saline (LS), and leptin-leptin (LL) (first letter indicates infusion in the third ventricle and the second letter indicates infusion in the fourth ventricle). On the sixth day, rats were anesthetized with ketamine-xylazine (100 mg/kg-10 mg/kg) and then perfused pericardially with 0.9% saline (with 10 mM HEPES) followed by 4% paraformaldehyde. Brains were collected, stored in 4% paraformaldehyde at 4°C overnight, transferred to 25% sucrose and 0.01% sodium azide solution, and stored at 4°C. A microtome was used to make 30-μm serial coronal sections throughout the brain, and every fourth section was used for immunohistochemistry.

CHRONIC ACTIVATION OF BRAIN NUCLEI. Commercially available antibodies reported to be selective for ΔFosB were inconsistent in their immunostaining pattern. The results of experiment 1 showed no detectable expression of acute FosB in either the hypothalamic or hindbrain in LL rats. Therefore, we used Fos B/ΔFosB (sc-48) antibody for immunostaining because any changes in immunoreactivity were due to differences in chronically accumulated ΔFosB. As a result, we denoted FosB/ΔFosB immunoreactivity as ΔFosB immunoreactivity. Free floating brain sections were washed three times for 10 min each in 0.1 M PBS and treated with 0.3% H2O2 for 10 min to destroy endogenous peroxidases. After a second set of three PBS washes, sections were blocked with 2% normal goat serum in 0.1 M PBS for 1 h at room temperature to prevent nonspecific labeling. Sections were then incubated for 72 h at 4°C in 2% normal goat serum, 0.1 M PBS, and FosB/ΔFosB antibody (1:700). Sections were washed in 0.1 M PBS and placed for 1.5 h in biotinylated goat anti-rabbit antibody from a Vector Lab Elite ABC Kit (Vector Laboratories, Burlingame CA). After three more PBS washes, sections were incubated for 1 h in ABC reagent (Vector Laboratories). Sections were washed again in 0.1 M PBS, and peroxidase activity was detected using diaminobenzidine with nickel (Vector Laboratories). Images were captured using DP2-BSW (version 2.2) software connected to a DP72 Olympus upright microscope (Olympus America, Center Valley, PA) and adjusted for autocontrast before quantification using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Sections from the entire brain were scanned to detect ΔFosB-immunoreactive nuclei. Owing to the dense ΔFosB immunoreactivity observed in several positive nuclei, immunoreactive cells in the Arc were counted both manually as well as with ImageJ software (National Institutes of Health, Bethesda, MD) to test for differences in counting methods. No significant differences were observed in the results, and, therefore, ImageJ was used to count ΔFosB-immunoreactive cells in all other nuclei. The experiment was coded, each slide was counted twice using ImageJ, and the code was broken at the end of the experiment. The Paxinos Rat Brain Atlas (35) was used to reference plate numbers for the brain nuclei.

**DIRECT LEPTIN-MEDIATED ACTIVATION.** Leptin-mediated activation of brain nuclei was determined by immunostaining for pSTAT3 as previously described (19). Every fourth 30-μm section of the entire brain was processed. Sections from the entire brain were scanned to detected pSTAT3-immunoreactive nuclei. Quantification of pSTAT3-immunoreactive cells was carried out manually. The experiment was coded, each slide was counted twice, and the code was broken at the end of the experiment. The Paxinos Rat Brain Atlas (35) was used to reference plate numbers for the brain nuclei. Representative fluorescent images for pSTAT3 were captured using Zen 2009 software.
connected to a Zeiss LSM 510 upright confocal microscope (Carl Zeiss Microscopy, Cambridge, MA).

**Data Analysis**

Statistically significant differences between treatment groups were determined using Statistica software (version 9.0, StatSoft, Tulsa, OK). Differences were considered significant at $P < 0.05$. Single end-point measures were compared by an unpaired $t$-test, one-way ANOVA, or two-way ANOVA depending on the experimental design. Daily measures of food intake and body weight were compared by repeated-measures ANOVA. Post hoc differences were determined using Duncan’s multiple-range test or an unpaired $t$-test.

**RESULTS**

**Experiment 1: Low Doses of Leptin Produce a Significant Leptin Response When Infused Centrally Into Both the Third and Fourth Ventricles for 6 Days**

Rats that received low doses of leptin infusions into both the third and fourth ventricles (LL rats) reduced their energy intake during the 5 days of the experiment ($P < 0.05$) compared with saline-infused control rats (SS rats; Fig. 1A). LL rats also lost weight on days 4–6 of the infusion ($P < 0.05$; Fig. 1B). The weight loss was accompanied by a reduction in all fat depots weighed in LL rats (data not shown) and in total dissected fat (Fig. 1C). Hypothalamic pSTAT3 was significantly increased in LL rats after 6 days ($P < 0.05$; Fig. 1D), but there was no difference in hindbrain pSTAT3 (Fig. 1E).

When probed with the antibody selective for chronically accumulating ΔFosB (9890S), there was a significant increase in hypothalamic ΔFosB expression in LL rats ($P < 0.03$) compared with both SS rats and the 2-DG-injected rat, which was a negative control for ΔFosB expression (Fig. 2A). In contrast, the hindbrain showed no differences between groups (Fig. 2C). When probed with the acute FosB antibody that also detects truncated ΔFosB (FosB/ΔFosB, sc-48), there was very low expression of acute FosB in SS and LL rats and no detectable expression of FosB in the hindbrain of either SS or LL rats (Fig. 2, B and D). There was a measurable expression of acute FosB in the hypothalamus but not the hindbrain of the rat injected with 2-DG, which was a positive control for FosB expression (Fig. 2B).

**Experiment 2: Rats Infused Centrally With Low Doses of Leptin Into Both the Third and Fourth Ventricles for 6 Days Show Differential Activation of Brain Nuclei**

Rats that received low-dose leptin infusions into either the third ventricle (LS rats) or fourth ventricle (SL rats) showed no differences in body weight or energy intake compared with saline-infused control rats (SS rats) during the 6 days of the experiment (Fig. 3, A and B). LL rats showed a significant reduction in energy intake for all days of leptin infusion compared with the other three treatment groups ($P < 0.05$; Fig. 3A). LL rats weighed significantly less than SS rats on day 6 ($P < 0.05$; Fig. 3B), and this was reflected in a significant reduction in both inguinal and retroperitoneal fat pad weights (data not shown).

On day 6, pSTAT3 immunoreactivity was detected in the hypothalamic and hindbrain nuclei of rats infused with leptin. pSTAT3 was significantly increased in the medial and medial posterior Arc, VMH, DMH, and posterior hypothalamus (PH) of SL and LS rats compared with SS control rats ($P < 0.01$); however, LL rats showed significantly higher pSTAT3 immunoreactivity than SL, LS, and SS rats ($P < 0.001$; Fig. 4, A and B). pSTAT3 immunoreactivity was detected in the lateral hypothalamus and midbrain nuclei such as the ventral tegmental area, but there were no differences between treatment groups (data not shown). In the hindbrain, pSTAT3 immunoreactivity was detected in the area postrema and NTS, but there were no differences observed between treatments despite the infusion of a sixfold higher dose of leptin into the fourth ventricle than in the third ventricle (Fig. 5, A and B). There was no detectable pSTAT3 immunoreactivity in nuclei rostral to the
hypothalamus, such as limbic nuclei. The relative expression of pSTAT3 in the different brain nuclei of each treatment group is shown in Table 1 and denoted with plus signs in increasing order of significance, with each significantly different from the next at \( P < 0.05 \).

The FosB antibody was used to detect changes in FosB immunoreactivity, a marker of chronic neuronal activation. The 6-day infusion of either saline or leptin resulted in the activation of neurons in multiple nuclei in the brain (see Table 2). Leptin infusion in LS and SL rats significantly increased FosB expression in the Arc (Fig. 6, A and E), VMH (Fig. 6, B and F), and DMH (Fig. 6C) compared with SS control rats (\( P < 0.05 \)), and the increase was exaggerated in LL rats (\( P < 0.001 \); Fig. 6, A–C, E, and F). LL rats also showed a significant increase in FosB in the ventral tuberomamillary nucleus (VTM) compared with the other three groups (\( P < 0.05 \); Fig. 6D). There was no detectable FosB immunoreactivity in nuclei rostral to the hypothalamus, such as limbic nuclei. In the midbrain and hindbrain, there were no differences in FosB immunoreactivity across the different treatment groups. Relative levels of expression in the different nuclei are shown in Table 2.

**DISCUSSION**

We have previously reported that under near physiological conditions, leptin is required to chronically activate ObRs in both the hypothalamus and hindbrain to inhibit food intake and reduce body fat (6). The objective of the experiments described here was to identify which brain nuclei are activated by these low-dose chronic infusions. The results confirm the previous finding that subthreshold doses of leptin are required/necessary to be infused into both the forebrain and hindbrain to produce an inhibition of food intake and body fat and also demonstrate that despite the simultaneous infusion into both regions of the brain, only select brain nuclei in areas of the hypothalamus known to respond to leptin are activated.

LL rats demonstrated a sharp decrease in food intake compared with single ventricle-infused rats (LS and SL rats) and SS control rats during the first 5 days of infusions, and a significant reduction in body weight and body fat was observed on day 6. This was consistent with a previous study (6) where we found that subthreshold doses of leptin, when infused into both the forebrain and hindbrain for 12 days, reduce body fat by 50%. The increased pSTAT3 in the

---

**Fig. 2.** Western blot analysis of transcription factors FosB (A and C) and FosB/FosB (B and D) in hypothalamic (A and B) and hindbrain (C and D) tissues from rats in experiment 1. A rat injected intraperitoneally with 400 mg/kg 2-deoxyglucose (2DG) 2 h before euthanization was used as a positive control for the expression of the acute transcription factor FosB. Data are means ± SE for 8–9 rats.

*Significant difference between LL and SS rats at \( P < 0.05 \).

**Fig. 3.** Food (energy) intake (A) and body weight (B) of rats from experiment 2 infused with saline or low-dose leptin in either the third or fourth ventricles (LS and SL rats) or both ventricles (LL rats) for 6 days (the first letter indicates infusion in the third ventricle and the second letter indicates infusion in the fourth ventricle). Data are means ± SE for 7–8 rats. *Significant difference between LL rats and all other groups at \( P < 0.05 \).
hypothalamus of LL rats that was associated with the behavioral changes was consistent with the role of hypothalamic leptin signaling in the regulation of energy balance (42). No differences in pSTAT3 were observed in the hindbrain of LL rats despite a sixfold higher concentration of leptin being infused into the fourth ventricle. This suggests that the inhibition of food intake and decrease in body fat were due to an activation of hypothalamic OBRs with a requirement of the simultaneous presence of subthreshold doses of leptin in the hindbrain.

Fig. 4. Immunohistochemistry of rat hypothalamus brain sections from experiment 2 probed for phosphorylated (p)STAT3 immunoreactivity (ir). Data and images represent pSTAT3 immunoreactivity in the arcuate nucleus (Arc), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and posterior hypothalamus (PH). Images have been adjusted for brightness and contrast. Representative images are at ×10 magnification. Groups that do not share a common superscript are significantly different at P < 0.05. Representative images are at ×10 magnification.
Immunohistochemistry data support this observation, and increased pSTAT3 immunoreactivity was localized to the Arc, VMH, DMH, and PH in LL rats. All these nuclei have been implicated in leptin-induced changes in energy balance. The Arc, an essential nucleus in the control of energy balance (4), has two main ObRb-expressing neuronal populations: agouti related peptide (AgRP) neurons, which coexpress orexigenic peptides AgRP and neuropeptide Y (17), and proopiomelanocortin (POMC) neurons, which coexpress anorexigenic peptides POMC and cocaine- and amphetamine-regulated transcript (9). Leptin has been shown to act in the Arc to suppress expression of AgRP and neuropeptide Y and/or increase expression of POMC and cocaine- and amphetamine-regulated transcript, thereby inhibiting food intake and reducing body weight (26, 34). Neuroanatomic evidence shows that AgRP neurons are primarily localized in the medial Arc and that POMC neurons are localized in the lateral Arc (9, 17). Bates et al. (2) reported that leptin-dependent inhibition of AgRP is only partially mediated by pSTAT3. In the experiments described here, we found that pSTAT3 and ΔFosB were both increased in the medial and lateral Arc of LL rats, suggesting that ObRbs on both AgRP and POMC neurons are activated in LL rats and that these different populations of neurons contribute to the changes in food intake and body composition of the animals.

Deletion of ObRbs from both AgRP and POMC neurons in the Arc results in an obese phenotype that is modest compared with that of ObRb-depleted mice (46). This suggests that other populations of neurons expressing ObRbs are also involved in the control of energy balance (27), and our data showing increased pSTAT3 immunoreactivity in the DMH, VMH, and PH of LL rats are consistent with that idea. The increase in pSTAT3 immunoreactivity in the DMH of LL rats is consistent with previous findings that ObRb-expressing neurons within the DMH increase sympathetic nervous system outflow to brown adipose tissue, thereby increasing thermogenesis (11). We have previously shown that brown adipose tissue uncoupling protein-1 expression, a marker of thermogenesis, is increased in LL rats (6).

Leptin administration activates steroidogenic factor-1-expressing neurons in the dorsomedial VMH (7, 8) to control food intake, and obesity has been observed in transgenic mice lacking the steroidogenic factor-1 gene (30). More recently, it has been reported that neurons in the ventrolateral VMH expressing brain-derived neurotrophic factor-1 are leptin responsive and also respond to changes in energy status (12, 28, 44). Consistent with these findings, our results show an increase in pSTAT3 immunoreactivity in both dorsomedial and ventrolateral VMH in LL rats, suggesting that the activation of both these areas is important for the leptin-mediated reduction in body fat. The increase in pSTAT3 immunoreactivity in the PH of LL rats is also not surprising because the PH has been implicated in the integration of autonomic responses, including thermogenesis (18).

Although pSTAT3 is an accepted marker of ObRb activation, there are other factors, such as serotonin and proinflammatory cytokines (IL-1, IL-6, and TNF), present in the brain.
that can also activate STAT3 (13, 38). Our results show that the pSTAT3 immunoreactivity distribution associated with chronic leptin infusion is restricted to nuclei expressing Ob-RBs, and, thus, we assume that it is most likely an effect of ObRb activation. However, additional experiments must be performed to demonstrate that the increased hypothalamic pSTAT3 immunoreactivity in LL rats is limited to cells that express ObRb. ObRb are also distributed across midbrain and hindbrain nuclei, such as the ventral tegmental area, NTS, and area postrema. Although our results showed no differences in pSTAT3 immunoreactivity in these nuclei, infusion of leptin into the forebrain and saline in the hindbrain in LS rats did not cause a decrease in body weight or food intake, suggesting that basal activation of neurons in the hindbrain in addition to activation of hypothalamic nuclei was required for the reduction in body fat and food intake observed in LL rats.

Immediate early genes have commonly been used as markers for determining neuronal activation and can be described as inducible transcription factors that control the expression of essential genes involved in neuronal responses to stimuli (31). A number of studies have used proteins coded by c-Fos and FosB genes to localize brain nuclei activated by different types of acute stimuli (25). c-Fos protein (55 kDa) and FosB protein (46/48 kDa) are induced between 1 and 3 h after acute stimulation, and their half-lives are ~2 and 9 h, respectively, after which a desensitization of gene expression is observed (33, 37). In contrast, the FosB gene codes for a splice variant called ΔFosB (33 kDa), which is induced ~6 h after stimulation (32). ΔFosB protein accumulates with chronic exposure to stimuli such as drugs of abuse, stressors, or antipsychotic drugs and is stably expressed in cells for up to 1 mo after withdrawal of the stimulus (1, 32, 37). Therefore, we used ΔFosB as an independent marker of neuronal activation to determine whether there were sites other than those expressing pSTAT3 that were selectively activated in LL rats.

Basal ΔFosB immunoreactivity was found in multiple brain nuclei, including the hippocampus, amygdala, dorsal raphae, ventral tegmental area, and thalamus. Six days of saline infusion induced levels of ΔFosB immunoreactivity similar to those reported by others (37, 39). Basal neuronal activation in these nuclei may be due to the process of infusion itself or another common stimulus from the environment. Increased ΔFosB immunoreactivity in the Arc, VMH, and DMH after leptin infusion into both the third and fourth ventricles, but no change in ΔFosB immunoreactivity in midbrain or hindbrain nuclei, correlates closely with the pSTAT3 data, suggesting that chronic infusion of leptin in both ventricles stimulates only hypothalamic nuclei that are leptin responsive.

Table 1. Relative pSTAT3 immunoreactivity localized in the brain nuclei of rats infused with saline and leptin into the third and fourth ventricles in different combinations

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Brain Nucleus</th>
<th>SS group</th>
<th>LS group</th>
<th>SL group</th>
<th>LL group</th>
</tr>
</thead>
<tbody>
<tr>
<td>26–28</td>
<td>Paraventricular nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30–37</td>
<td>Arcuate nucleus, medial</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>30–37</td>
<td>Arcuate nucleus, lateral</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>31–37</td>
<td>Ventromedial nucleus, dorsomedial</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>31–37</td>
<td>Ventromedial nucleus, dorso lateral</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>30–35</td>
<td>Dorsomedial nucleus, dorsal</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>33–37</td>
<td>Lateral hypothalamus</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>35–37</td>
<td>Posterior hypothalamus</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>52–55</td>
<td>Dorsal raphae</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>75–77</td>
<td>Nucleus of the solitary tract, central</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>75–77</td>
<td>Nucleus of the solitary tract, medial</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>75–76</td>
<td>Area postrema</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Rats were divided into the following groups based on infusions: saline-saline (SS), leptin-saline (LS), saline-leptin (SL), and leptin-leptin (LL), where the first letter indicates infusion in the third ventricle and the second letter indicates infusion in the fourth ventricle. pSTAT3, phosphorylated STAT3; +, basal expression; ++, increased expression (significantly different from + at P < 0.05); ++++, highest expression (significantly different from + at P < 0.01 and ++ at P < 0.05).

Table 2. Relative ΔFosB immunoreactivity localized in the brain nuclei of rats infused with saline and leptin into the third and fourth ventricles in different combinations

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>Brain Nucleus</th>
<th>SS group</th>
<th>LS group</th>
<th>SL group</th>
<th>LL group</th>
</tr>
</thead>
<tbody>
<tr>
<td>26–28</td>
<td>Paraventricular nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29–37</td>
<td>Arcuate nucleus (medial, lateral, and posterior)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>29–34</td>
<td>Ventromedial hypothalamus (central, dorsomedial, and ventrolateral)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>29–34</td>
<td>Dorsomedial hypothalamus</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>34–35</td>
<td>Lateral hypothalamus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>35–38</td>
<td>Ventral tuberomammillary nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34–37</td>
<td>Posterior hypothalamus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31–35</td>
<td>CA1, CA2, and CA3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31–35</td>
<td>Dentate gyrus (hilus)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31–36</td>
<td>Habenular nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31–36</td>
<td>Lateral thalamic nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>43–45</td>
<td>Red nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>43–45</td>
<td>Occulomotor nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>52–55</td>
<td>Dorsal raphae</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>68–71</td>
<td>Lateral paragigantocellular nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>68–71</td>
<td>Medial vestibular nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>70–77</td>
<td>Hypoglossal nucleus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>74–77</td>
<td>Nucleus of the solitary tract</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Basal expression; ++, increased expression (significantly different from + at P < 0.05); ++++, highest expression (significantly different from + at P < 0.01, and ++ at P < 0.05).
There were no treatment effects on ΔFosB immunoreactivity in the PH even though this area showed increase in pSTAT3 in LL rats. ΔFosB is a marker of chronic neuronal activation, and, therefore, an increase in pSTAT3 immunoreactivity was expected to correlate with an increase in ΔFosB. It is possible that, although leptin activated STAT3, there was a simultaneous down-regulation of another stimulatory signal in the PH. This would have resulted in no net change in ΔFosB immunoreactivity. Others have shown that the VTM is an important regulator of food intake and that lesions of the VTM result in hyperphagia (29). The increased ΔFosB immunoreactivity in the VTM of LL rats compared with all other groups suggests that this nucleus may be indirectly activated by leptin and contributes to some aspect of the metabolic phenotype observed in LL rats. A recent study (40) has shown that overexpression of ΔFosB in the ventral hypothalamus of wild-type mice is sufficient to significantly increase energy expenditure, and this supports our finding of increased ΔFosB immunoreactivity being restricted only to hypothalamic nuclei and within the VTM of LL rats. It has been established that catecholaminergic projections ascend directly from the NTS and indirectly from the dorsal vagal complex through the ventral medulla to the VTM (14). Activity of these neurons has been studied in the context of fatigue and hypoarousal behaviors associated with sickness, but it would be interesting to investigate the importance of leptin-mediated activation of these ascending pathways and their contribution to the control of energy balance.

The main role of ΔFosB is to mediate long-term adaptations in the brain (33); therefore, our results suggest that hypothalamic brain nuclei important for integrating leptin signals from both the forebrain and hindbrain have the potential to cause long-term adaptations in the brain that subsequently lead to sustained changes in metabolism. It is important to note that the distribution of ΔFosB in the brain only highlights nuclei that are activated chronically and will not provide any indication of nuclei that are chronically inhibited by leptin. The possibility that leptin in the hindbrain may be lowering the threshold for hypothalamic ObRb activation through an inhibition of an inhibitory input from the hindbrain to the hypothalamus cannot be excluded, and this could explain the lack of increased pSTAT3 and ΔFosB in the hindbrain of LL rats.

The results of experiment 2 imply that the chronic infusion of subthreshold doses of leptin into both the third and fourth ventricles of rats lowers the threshold for a direct leptin-mediated activation of neurons expressing ObRbs in the Arc, VMH, DMH, and PH and an indirect activation of neurons in the VTM in the forebrain, which, in turn, are responsible for inducing a state of negative energy balance in these animals. We have previously demonstrated that infusion of 0.3 μg leptin/day for 7 days into the third ventricle alone is sufficient to induce weight loss and that the blockade of the cerebral aqueduct, preventing humoral communication between the forebrain and hindbrain, enhances the response to high concentrations of leptin in the forebrain when peripheral and hindbrain leptin concentrations are normal (45). Here, we show that in conditions where both hindbrain and forebrain are able to detect changes in leptin, a dose of leptin as low as 0.1 μg leptin/day in the third ventricle is sufficient to produce a similar weight loss to that produced by higher doses infused into the third ventricle alone. Therefore, we propose that activation of leptin receptors in the hindbrain lowers the threshold for activation of hypothalamic ObRbs by leptin. This would increase the sensitivity of leptin-responsive pathways that are initiated in the forebrain and result in a more precise control of energy balance. Although infusion of low doses of leptin into either the third or fourth ventricle alone (SL and LS rats) had no effect on food intake and energy balance, there was a significant increase in pSTAT3 and/or ΔFosB in some areas of the hypothalamus. It is not clear from our results whether this partial activation of pSTAT3 represents a subthreshold activation of neurons or whether it represents activation of pathways that result in a response that is unrelated to energy balance.

The exact mechanism of how leptin in the fourth ventricle potentiates the activation of ObRbs in the hypothalamus is unclear. There are at least three potential explanations. First, it is possible that leptin in the hindbrain reduces the threshold of ObRb activation in the hypothalamus through a neural mechanism. This is supported by the pSTAT3 and ΔFosB data, which show that the chronic infusion of leptin in both forebrain and hindbrain leads to an increased activation of hypothalamic nuclei with no changes in activation of hindbrain nuclei. Similar findings have been reported by Ritter and colleagues (41). Although we did not find an increase in either pSTAT3 or ΔFosB in the hindbrain, the distribution of ΔFosB only highlights nuclei that are chronically activated and will not provide any indication of nuclei that are chronically inhibited by leptin. Thus, leptin in the hindbrain may be lowering the threshold for hypothalamic ObRb activation through inhibition of an inhibitory input from the hindbrain to the hypothalamus.

Second, leptin in the fourth ventricle could potentially diffuse from the fourth ventricle into the third ventricle and activate hypothalamic ObRbs; however, this is unlikely as it has been established that cerebrospinal fluid only flows from the third to fourth ventricle and not from the fourth ventricle to the third ventricle (5). In contrast, leptin in the hindbrain could slowly drain into the subarachnoid space adjacent to the hypothalamus and facilitate activation of hypothalamic ObRbs. The above data do not provide any evidence for or against this mechanism, and, therefore, this possibility cannot be excluded. The integrated response to leptin could be an additive or a synergistic effect, and further experiments are required to determine the exact mechanism of action.

Finally, it is possible that forebrain and hindbrain leptin responses are integrated through the process of volume transmission, which is diffusion through the cerebrospinal fluid, of neurotransmitters released at points anatomically distant from target cells and results in activation of extrasynaptic receptors (3). In the context of energy balance, volume transmission has been shown to be important for glucagon-like peptide-1 sig-

---

Fig. 6. Immunohistochemistry of rat hypothalamus and extrahypothalamic sections from experiment 2 showing differences in activation of chronically accumulating transcription factor ΔFosB. Data and images represent ΔFosB immunoreactivity in the Arc (A and E), VMH (B and F), DMH (C), and ventral tuberomammillary nucleus (VTM; D and G). ^-C Groups that do not share a common superscript are significantly different at \( P < 0.05 \). Representative images are at \( \times 10 \) magnification. Images have been adjusted for brightness and contrast.
naling across the blood-cerebrospinal fluid-brain and blood-brain barrier (22); however, for hormones such as leptin, the importance of volume transmission still needs to be established. Our current data provide no evidence either for or against this mechanism, and additional experiments are needed in order to include or exclude the probability of this mechanism.

In summary, the experiments described in this report show that under near physiological conditions, leptin signals from both the forebrain and hindbrain integrate to induce a state of negative energy balance, and it appears that this is facilitated by leptin in the hindbrain lowering the threshold for activation of ObRbs in the hypothalamus, specifically the Arc, VMH, DMH, and PH, and through an indirect activation of VTM. Future experiments will be designed to confirm whether this response is neural or humoral. Examination of the neuroanatomic evidence for connections between the hypothalamus and hindbrain (43) and immunohistochemical evidence for sites that express ObRbs (10) combined with results from our experiments provide obvious candidates for sites of neural communication between the forebrain and hindbrain that include the NTS, Arc, and VMH.

GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-053903 (to R. B. S. Harris).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: B.N.D. and R.B.H. conception and design of research; B.N.D. performed experiments; B.N.D. analyzed data; B.N.D. interpreted results of experiments; B.N.D. prepared figures; B.N.D. drafted manuscript; B.N.D. and R.B.H. edited and revised manuscript; B.N.D. and R.B.H. approved final version of manuscript.

REFERENCES


