

Bsx, a Novel Hypothalamic Factor Linking Feeding with Locomotor Activity, Is Regulated by Energy Availability

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We recently reported that the hypothalamic homeobox domain transcription factor *Bsx* plays an essential role in the central nervous system control of spontaneous physical activity and the generation of hyperphagic responses. Moreover, we found *Bsx* to be a master regulator for the hypothalamic expression of key orexigenic neuropeptide Y and agouti gene-related protein. We now hypothesized that *Bsx*, which is expressed in the dorsomedial and arcuate nucleus (ARC) of the hypothalamus, is regulated by afferent signals in response to peripheral energy balance. *Bsx* expression was analyzed using *in situ* hybridization in fed vs. fasted (24 h) and ghrelin vs. leptin-treated rats, as well as in mice deficient for leptin or the ghrelin signaling. Ghrelin administration increased, whereas ghrelin receptor antagonist decreased ARC *Bsx* expression. Leptin injection attenuated the fasting-induced in-

crease in ARC *Bsx* levels but had no effect in fed rats. Dorsomedial hypothalamic nucleus *Bsx* expression was unaffected by pharmacological modifications of leptin or ghrelin signaling. Obese leptin-deficient (*ob/ob*) mice, but not obese melanocortin 4 receptor-knockout mice, showed higher expression of *Bsx*, consistent with dependency from afferent leptin rather than increased adiposity *per se*. Interestingly, exposure to a high-fat diet triggered *Bsx* expression, consistent with the concept that decreased leptin signaling due to a high-fat diet induced leptin resistance. Our data indicate that ARC *Bsx* expression is specifically regulated by afferent energy balance signals, including input from leptin and ghrelin. Future studies will be necessary to test if *Bsx* may be involved in the pathogenesis of leptin resistance. (*Endocrinology* 149: 3009–3015, 2008)

ENERGY BALANCE IS regulated by close interaction among subpopulations of neurons located in several distinct areas of the brain. The currently accepted model suggests that the hypothalamus, and more specifically the arcuate nucleus (ARC), is playing a major role by connecting afferent signals with central circuitries orchestrating efferent commands to govern food intake, motor activity, and peripheral cell metabolism. Neurons in the hypothalamic ARC are expressing specific neuropeptides with orexigenic or anorexigenic effects. One particularly important neuronal population contains neurons coexpressing neuropeptide Y (NPY) and agouti gene-related protein (AgRP), potent stim-

ulators of food intake. An adjacent set of ARC neurons co-expresses proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript, which suppress food intake (1, 2). These cells sense and respond to circulating hormones providing important information related to peripheral energy homeostasis. Leptin, a hormone secreted by white adipose tissue in levels proportional to fat mass, signals the status of energy stores by activating POMC/cocaine- and amphetamine-regulated transcript neurons and inhibiting NPY/AgRP neurons (1, 2), therefore, reducing feeding and increasing energy expenditure. A lack of leptin signaling results in the development of morbid obesity in rodents and humans (3, 4). Ghrelin, an endogenous leptin opponent secreted mainly by the stomach, directly activates NPY/AgRP neurons (5) and indirectly inhibits POMC neurons (6), thereby stimulating feeding and decreasing energy expenditure (7). Mice lacking ghrelin signaling are partially protected from high-fat diet (HFD) induced obesity (8, 9). The integrated character of these ARC neuron populations is illustrated by the fact that NPY/AgRP neurons inhibit POMC neurons, and AgRP competes with the POMC product α -MSH at melanocortin 4 receptors (MC4Rs) (6). The

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Abbreviations: AgRP, Agouti gene-related protein; ARC, arcuate nucleus; CNS, central nervous system; DMH, dorsomedial hypothalamic nucleus; GHS-R, GH secretagogue receptor; HFD, high-fat diet; icv, intracerebroventricular; KO, knockout; LFD, low-fat diet; MC4R, melanocortin 4 receptor; *ob/ob*, leptin deficient; NPY, neuropeptide Y; POMC, proopiomelanocortin; SSC, standard saline citrate.

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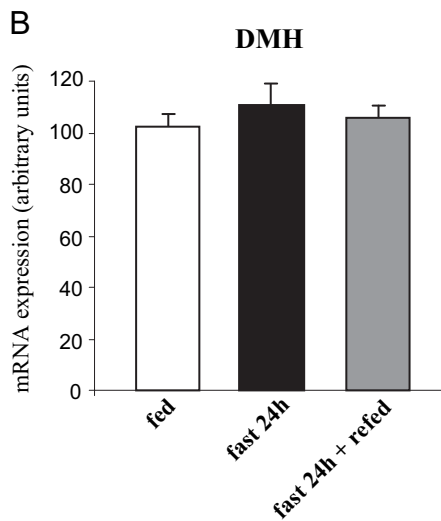
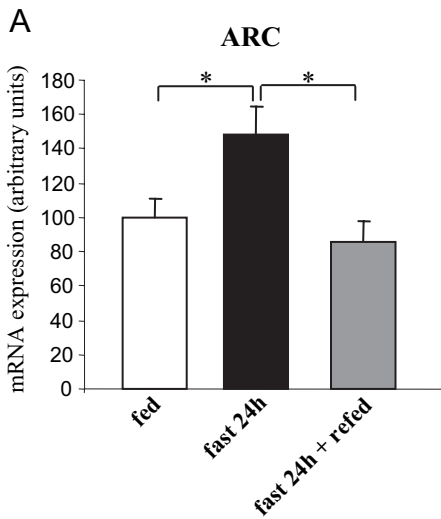
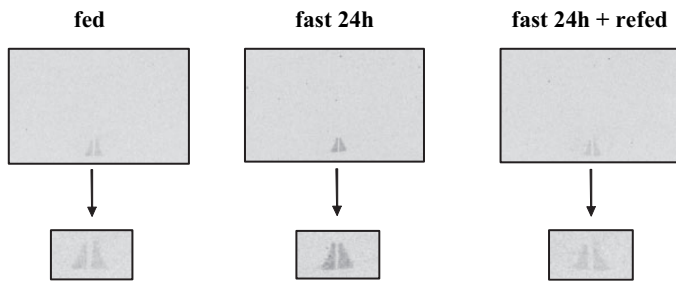


FIG. 1. A, Representative pictures of Bsx mRNA levels in the ARC of fasted rats measured by *in situ* hybridization. Effect of 24-h fasting and refeeding on Bsx mRNA expression in the ARC. B, Effect of 24-h fasting and refeeding on Bsx mRNA expression in the DMH. *n* = 6–7 animals per group. *, *P* < 0.05.

crucial role of NPY/AgRP neurons in the regulation of feeding behavior and body weight was recently highlighted when the selective ablation of AgRP expressing neurons in adult mice led to reduced food intake and body weight (10–12).

The novel evolutionary conserved and brain-specific homeobox transcription factor Bsx was identified in vertebrates and is widely expressed throughout species (13). We have

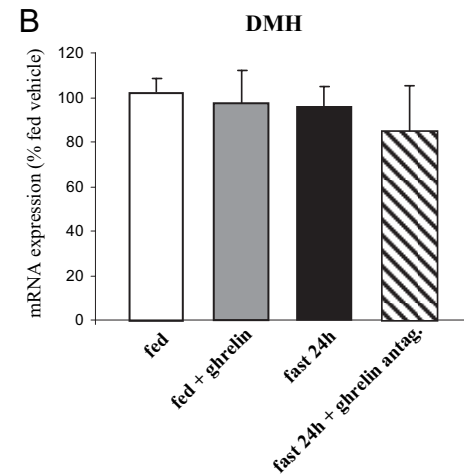
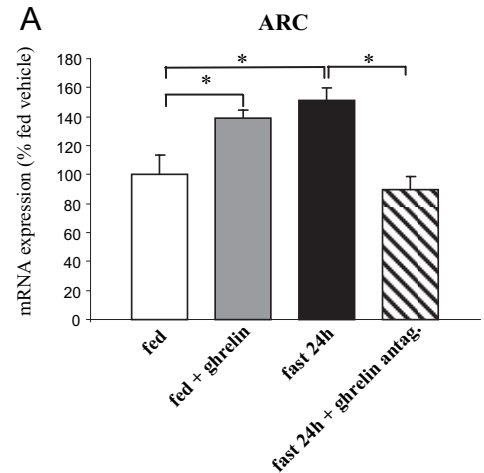
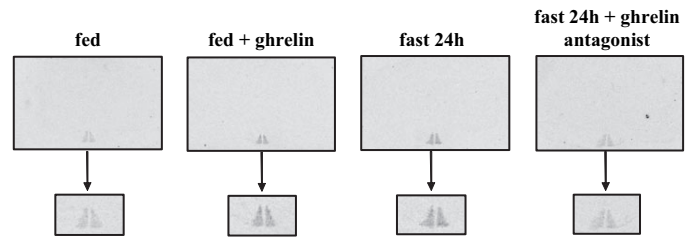


FIG. 2. A, Representative pictures of Bsx mRNA expression in the ARC of *ad libitum*-fed and fasted rats after ghrelin injection. Effect of ghrelin and ghrelin antagonist on Bsx expression in the ARC. B, Effect of ghrelin and ghrelin antagonist on Bsx expression in the DMH. *n* = 6–7 animals per group. *, *P* < 0.05.

recently demonstrated that Bsx is in hypothalamic NPY/AgRP neurons of the ARC, as well as in the dorsomedial hypothalamic nucleus (DMH) (14). Based on the genetic loss of function studies in wild-type and leptin-deficient (*ob/ob*) mice, Bsx is required for the physiological expression of NPY/AgRP, normal locomotor behavior patterns, and the appropriate generation of hyperphagic responses.

Here, we have tested the hypothesis that hypothalamic Bsx mRNA expression may be regulated by nutritional status, exposure to dietary lipids, or input from afferent endocrine signals reflecting acute or chronic changes in energy balance.

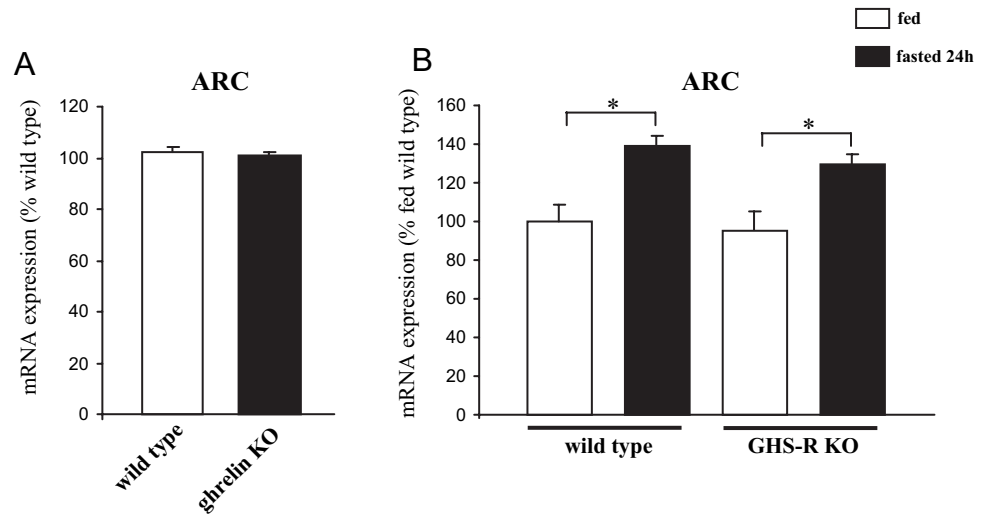


FIG. 3. A, Bsx mRNA expression in the ARC of ghrelin KO mice. B, Bsx mRNA expression in the ARC of *ad libitum*-fed and 24-h fasted GHS-R KO. $n = 5-7$ animals per group. *, $P < 0.05$.

Materials and Methods

Animal models

Male Sprague Dawley rats (200–250 g) were housed in air-conditioned rooms (22–24 C) under a 12-h light, 12-h dark cycle and fed a standard chow, low-fat diet (LFD) (D12450B, 10 kcal percent fat, 70 kcal percent carbohydrates, and 20 kcal percent protein; 3.85 kcal/g) or a HFD (D12451, 45 kcal percent fat, 35 kcal percent carbohydrates, and 20 kcal percent protein; 4.73 kcal/g; Research Diets, Inc., New Brunswick, NJ) during 12 wk. All mice were fed a standard chow diet. Adult ghrelin gene disrupted (knockout) mice, GH secretagogue receptor (GHS-R) disrupted mice, which were originally kindly provided from Regeneron Pharm. Inc., originated from an in-house breeding colony of our laboratory at the University of Cincinnati. *ob/ob* mice were obtained from our breeding colony at the University of Cambridge. MC4R knockout (KO) mice were kindly provided by N. Balthasar, J. Elmquist, and B. Lowell (all from the University of Texas Southwestern Medical Center, Dallas, TX). Mice were killed when they were 12–14 wk old. Animal experiments were conducted in accordance with the standards approved by the University of Cincinnati Institutional Animal Care and Use Committee and the Faculty Animal Committee at the University of Santiago de Compostela.

Implantation of intracerebroventricular (icv) cannulas

Rats were anesthetized by an ip injection of ketamine/xylazine (ketamine 100 mg/kg plus xylazine 15 mg/kg). Chronic icv cannulas were implanted stereotaxically as described previously (15).

Ghrelin and leptin challenge

Rats received two icv injections of saline, ghrelin, or ghrelin antagonist: one before the beginning of the dark phase and a second one in the beginning of the light phase. To prevent increased nutrient supply due to overfeeding, the ghrelin-treated group was pair fed with the fed *ad libitum* group. Ghrelin (Bachem, Bubendorf, Switzerland) and ghrelin antagonist (16), BIM-28163, were injected in the third ventricle and administered at equimolar doses (10 nmol/injection dissolved in 3 μ l saline), and rats were killed 8 h after the second injection. The effect of leptin on fasting-induced increase of BSX levels was measured in rats fed *ad libitum* or fasted for 24 h. Two ip injections of recombinant human leptin (Sigma-Aldrich, St. Louis, MO; 20 μ g/rat) or vehicle were given (12-h difference).

In situ hybridization

Coronal hypothalamic sections (16 μ m) were cut on a cryostat and immediately stored at -80 C until hybridization. For Bsx mRNA detection, we used a specific antisense oligodeoxynucleotide: 5'-CCTCAA CCGCTTGGGCTTGTGTAGCAG AATGTC C-3' (GenBank accession

no.: XM_001064837; 5' position: 147). *In situ* hybridizations were performed as previously reported (17). The frozen sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30 min. They were then dehydrated using 70, 80, 90, and 95%, and absolute ethanol (5 min each). The hybridization was performed overnight at 37 C in a moist chamber. Hybridization solution contained 5×10^5 cpm per slide of the labeled probe, 4 \times standard saline citrate (SSC), 50% deionized formamide, 1 \times Denhardt's solution, 10% dextran sulfate, and 10 μ g/ml sheared, single-stranded salmon sperm DNA. Afterward, the hybridization sections were sequentially washed in 1 \times SSC at room temperature, four times in 1 \times SSC at 42 C (30 min/wash), and once in 1 \times SSC at room temperature (1 h), and then rinsed in water and ethanol. Finally, the sections were air-dried and exposed to Hyperfilm β -Max (Amersham Intl., Little Chalfont, UK) at room temperature for 3 wk. The slides were then developed in Kodak D-19 developer (Eastman Kodak Co., Rochester, NY) and fixed (Kodak fixer).

To compare anatomically similar regions, the slides were matched according to the rat or mouse brain atlas of Paxinos and Watson (29). The slides from control and treated animals at each treatment time were always exposed to the same autoradiographic film. All sections were scanned, and the specific hybridization signal was quantified by densitometry using a digital imaging system (ImageJ; Institutes of Health, Bethesda, MD). The OD of the hybridization signal was determined and subsequently corrected by the OD of its adjacent background value. For this reason a rectangle, with the same dimensions in each case, was drawn enclosing the hybridization signal over each nucleus and over adjacent brain areas of each section (background). We used 16–20 sections for each animal (four to five slides, four sections per slide). The mean of these 16–20 values was used as the densitometry value for each animal.

Levels of plasma hormones

Plasma leptin, insulin, and ghrelin levels were measured by RIA as described previously (15, 17) using reagents provided in commercial kits (rat leptin RIA, rat ghrelin RIA, and rat insulin RIA; LINCO Research, Inc., St. Charles, MO).

Statistical analysis and data presentation

Data are expressed as having a mean of \pm SEM and analyzed using a computerized package for statistical analysis. A statistically significant difference was determined by the Student's *t* test when only two groups were compared or ANOVA, followed by a *post hoc* multiple comparison test (Tukey's test). A *P* value less than 0.05 was considered significant.

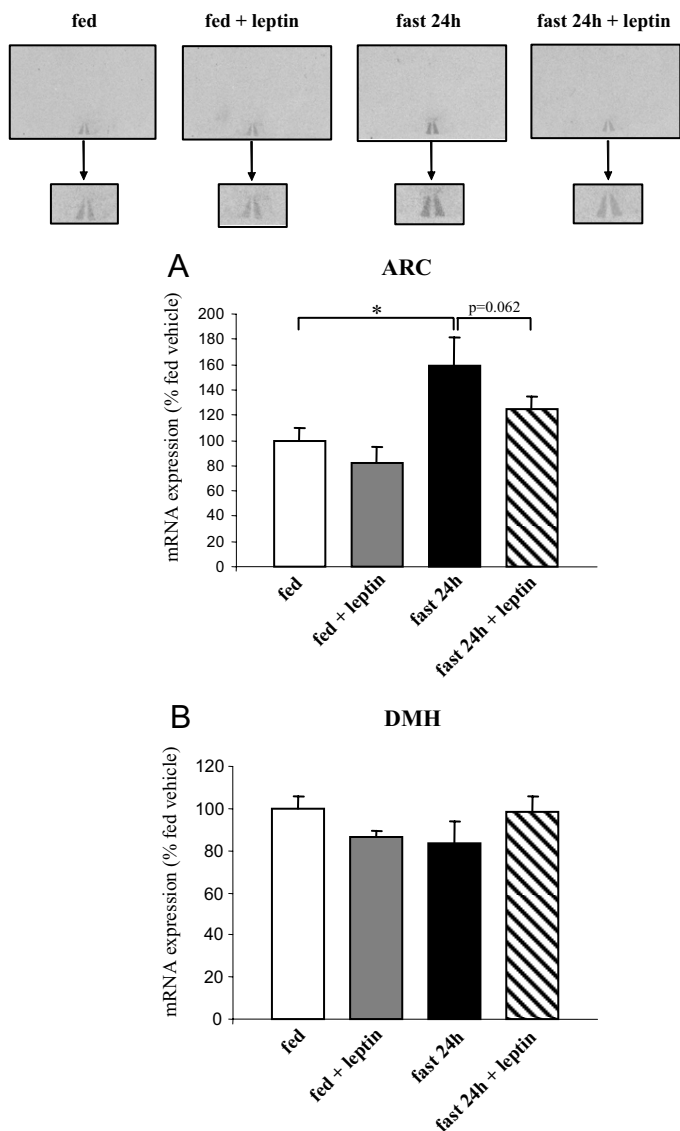


FIG. 4. A, Representative pictures of Bsx mRNA expression in the ARC of *ad libitum*-fed and fasted rats after leptin injection. Effect of leptin on rats fed *ad libitum* and fasted rats in the ARC. B, Effect of leptin on rats fed *ad libitum* and fasted rats in the DMH. $n = 6-7$ animals per group. *, $P < 0.05$.

Results

Fasting increases and refeeding decreases Bsx mRNA levels specifically in the ARC

Bsx mRNA content in the ARC of 24-h fasted rats was significantly higher ($P < 0.05$) than in *ad libitum*-fed rats. When fasted rats were refed for 12 h, Bsx mRNA expression in the ARC was reduced to a level similar to fed animals. However, in the DMH, Bsx expression did not differ between fasted and fed rats (Fig. 1). Because Bsx expression was triggered during fasting, we next tested if ghrelin, as the predominant hunger hormone and as the only circulating hormone known to stimulate the activity of AgRP neurons, would affect hypothalamic Bsx levels.

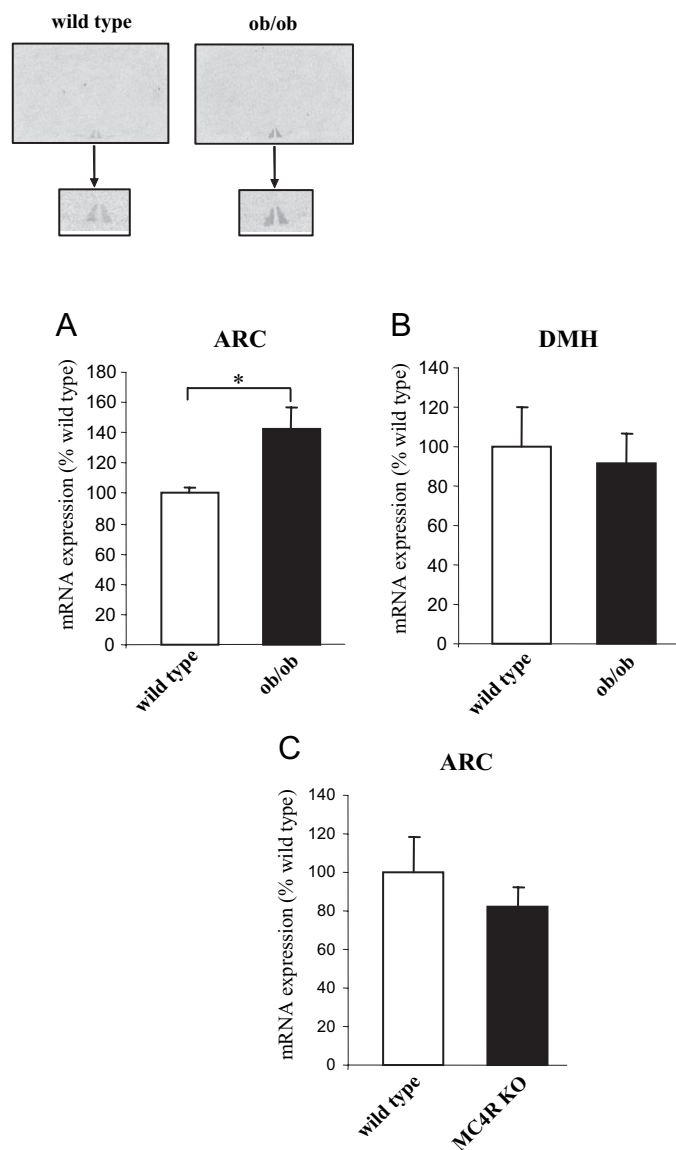


FIG. 5. A, Bsx mRNA expression in the ARC of ob/ob mice. B, Bsx mRNA expression in the DMH of ob/ob mice. C, Bsx mRNA expression in the ARC of MC4-R KO mice. $n = 6-7$ animals per group. *, $P < 0.05$.

Ghrelin promotes but is not essential for ARC Bsx expression

We observed a significant increase in Bsx mRNA in the ARC of fed rats after treatment with ghrelin (Fig. 2A). Bsx mRNA expression was increased to an extent comparable with that in the ARC of fasted rats (in which endogenous ghrelin is high) compared with rats fed *ad libitum*. The fasting induced increase in Bsx gene expression was reversed by administration of a ghrelin receptor antagonist (Fig. 2A). No change in Bsx gene expression was seen in the DMH (Fig. 2B). To test if endogenous ghrelin signaling was essential for Bsx regulation, we next analyzed ARC Bsx mRNA expression in ghrelin KO and GHS-R KO mice. Consistent with their normal ARC NPY/AgRP levels (10, 11), we did not observe any differences between these animal models with deficient ghrelin signaling and their respective wild-type controls (Fig. 3). Interestingly, the lack of

ghrelin signaling did not affect the Bsx response to fasting because GHS-R deficient mice showed an up-regulation of Bsx in the ARC after 24-h fasting. We conclude that ghrelin modulates Bsx expression, but endogenous ghrelin signaling is not essential to regulate ARC Bsx.

Leptin administration reverses fasting-induced Bsx levels

Leptin administration did not affect ARC Bsx levels in fed rats, but it did partially reverse the fasting-induced Bsx increase in Bsx (Fig. 4A). Again, neither fasting nor leptin administration had any effect on Bsx expression in the DMH (Fig. 4B). We had shown before that Bsx expression is increased in *ob/ob* mice (14). Here, we confirmed those findings (Fig. 5A) but also show that this phenomenon appears to be limited to the ARC because no differences were observed in DMH Bsx expression (Fig. 5B). To test whether increased Bsx expression in *ob/ob* mice is a specific consequence of leptin deficiency or a general phenomenon in morbid genetic obesity, we quantified hypothalamic Bsx expression in mice with a deletion of the gene for MC4R. However, ARC and DMH Bsx expression levels of MC4R KO mice did not differ from lean littermate controls (Fig. 5C).

A HFD increases Bsx mRNA expression in the ARC

Rats fed a HFD gained more weight than rats fed a LFD (Table 1). Increased hypothalamic levels of AgRP and NPY mRNA are known to occur in rodents upon exposure to a HFD and are often interpreted as a consequence of HFD-induced leptin resistance (18). To test the hypothesis that Bsx could be an important upstream player in hypothalamic leptin resistance, we fed adult male Sprague Dawley rats a HFD or LFD for 12 wk. Intriguingly, ARC Bsx levels were increased in rats on a HFD compared with low-fat fed rats (Fig. 6), and no differences were observed in DMH Bsx expression (data not shown).

Discussion

The hypothalamic ARC is believed to contain the most important neuronal circuits for sensing metabolic energy availability. Afferent information informing the central nervous system (CNS) about peripheral energy status is communicated through hormones such as ghrelin and leptin, neuronal input from afferent vagus via hindbrain areas, and direct signaling by circulating nutrients, just to name a few major examples. Functional components of this CNS “sensor” region appear to be rendered at least partially dysfunctional upon exposure to a HFD, an observation that has been closely linked with the phenomenon of leptin resistance, as well as with the difficulties to successfully prevent or cure obesity and the metabolic syndrome.

TABLE 1. Effect of a HFD on body weight, and plasma leptin, insulin, and ghrelin levels

| | LFD | HFD |
|-----------------|-------------|--------------------------|
| Body weight (g) | 440.3 ± 5.4 | 502.3 ± 7.6 ^a |
| Leptin (ng/ml) | 8.37 ± 0.81 | 8.31 ± 0.68 |
| Insulin (ng/ml) | 1.66 ± 0.21 | 1.96 ± 0.53 |
| Ghrelin (pg/ml) | 32.0 ± 4.1 | 14.9 ± 3.69 ^a |

^a $P < 0.01$.

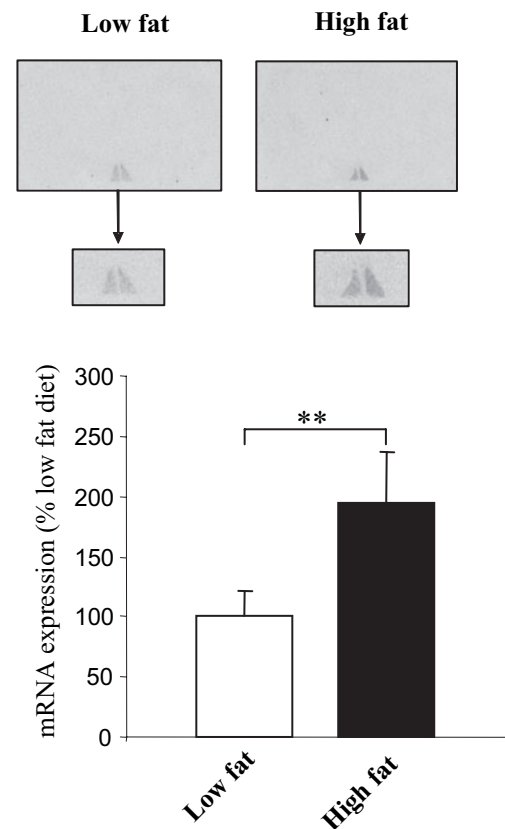


FIG. 6. Effect of a 12-wk HFD on Bsx expression in the ARC. $n = 6-7$ animals per group. **, $P < 0.01$.

Bsx is a novel transcription factor that is located precisely at the interface between the incoming afferent signals reflecting nutritional status and the hypothalamic neuroendocrine circuits governing energy homeostasis. Therefore, Bsx represents a prime candidate for a potentially important mechanistic player in the pathogenesis of leptin resistance. We have recently shown that Bsx is an essential regulator of NPY and AgRP expression in the ARC. Moreover, Bsx is required for normal locomotor activity patterns, as well as for the generation of physiological hyperphagia in response to prefasting, decreased leptin, or increased ghrelin signaling.

Here, we show that Bsx is not only regulating other important integral CNS factors controlling energy balance and metabolism but is also regulated by changes in energy availability. Consistent with that observation, we report that afferent endocrine factors that communicate nutrient availability in the gastrointestinal tract (ghrelin) or the size of accumulated fat stores (leptin) to the CNS are up- and down-regulating ARC Bsx expression, respectively. Our findings also support the notion that Bsx function is closely connected with NPY/AgRP expression. Bsx is regulated by conditions that are associated with NPY/AgRP changes, such as fasting and refeeding, leptin or ghrelin administration, or leptin deficiency (19–23), whereas Bsx was unchanged in the absence of endogenous ghrelin signaling, in which NPY/AgRP expression levels are normal (Table 2) (8, 9). The interaction of AgRP- and NPY-expressing neurons with POMC-expressing neurons is currently believed to be crucial in the regu-

TABLE 2. NPY, AgRP, and Bsx regulation in the hypothalamic ARC

| | NPY | AgRP | Bsx |
|--|-----|------|-----|
| Fast | ↑ | ↑ | ↑ |
| Refed | ↓ | ↓ | ↓ |
| Ghrelin administration | ↑ | ↑ | ↑ |
| Ghrelin KO | ↔ | ↔ | ↔ |
| GHS-R KO | ↔ | ↔ | ↔ |
| Leptin administration (fed <i>ad libitum</i>) | ↓ | ↓ | ↔ |
| Leptin administration (fasting) | ↓ | ↓ | ↓ |
| ob/ob | ↑ | ↑ | ↑ |
| MC4-R KO | ↔ | ↔ | ↔ |

lation of energy homeostasis (1, 6). However, mice lacking NPY and AgRP genes either alone or in combination do not exhibit relevant changes in energy homeostasis (24, 25). Results from a different approach recently indicated that ablation of entire neurons coexpressing *Agrp/Npy* results in anorexia and substantial weight loss (10–12). We also found earlier that similarly to NPY and AgRP KOs, the lack of Bsx does not alter basal body weight or food intake significantly (14), although those mice showed a decreased locomotor activity. Interestingly, deficiency for Bsx rescues the hyperphagia of leptin-deficient mice (14) similar to what has been observed in ob/ob mice without NPY (26). A difference between these potentially relevant models may be that Bsx mutant mice (but not NPY KO mice) showed a reduction in AgRP expression. Therefore, there are substantial similarities among NPY, AgRP, and Bsx KO models, but there are also differences with the Bsx KO mouse.

Although acute changes in energy balance, such as fasting and refeeding, are clearly regulating Bsx expression, chronic changes such as obesity *per se* do not seem to have any major impact on Bsx as we conclude from unchanged hypothalamic Bsx levels in morbidly obese MC4R KO mice. On the other hand, ob/ob mice show increased Bsx expression, however, in combination with our findings from MC4R KO mice and leptin administration studies, we conclude that this difference is likely a consequence of leptin deficiency, rather than of increased fat mass. However, Bsx also appears to represent a target of dietary lipids because chronic exposure to a HFD triggered an increase in Bsx expression. This finding is particularly intriguing because it would be consistent with a role for Bsx in the development of leptin resistance, a condition that is signified by the failure of the energy balance regulatory system to down-regulate orexigenic neuropeptide circuits in a hypercaloric environment.

A very consistent finding in our studies is represented by the fact that Bsx is specifically regulated in the ARC, but not in the DMH. The current model of central energy balance regulation suggests that parts of the medio-basal hypothalamus are less protected from blood-borne factors and, therefore, are more likely to sense changes in peripheral metabolism. Other hypothalamic nuclei, such as the DMH, are better protected by the blood-brain barrier and less accessible from the periphery. Therefore, DMH Bsx might be involved in other important functions, such as the control of circadian rhythms (27).

Initial reports had demonstrated that the orexigenic properties of ghrelin require the presence of Bsx (14). Here, we

further corroborate the importance of Bsx for ghrelin action by showing that ghrelin administration increases, whereas GHS-R blockade decreases, ARC Bsx expression.

Overall, our results show that Bsx is regulated by leptin and ghrelin, but the exact signal transduction mechanisms regulating Bsx are unknown. It seems reasonable to speculate that leptin might exert its actions on Bsx through the known intracellular pathways activated by the leptin receptor such as the Janus kinase-signal transducer and activator of transcription 3 pathway and/or the phosphatidylinositol 3-kinase pathway (28). It is well known that ghrelin plays an opposite role to leptin in the regulation of energy balance (1). However, the ghrelin signal-dependent transcriptional regulation is still poorly understood, and so far there are no solid data connecting the ghrelin receptor to Janus kinase-signal transducer and activator of transcription 3 or phosphatidylinositol 3-kinase pathways. Our and other laboratories are currently working on a better understanding of the exact signal transduction mechanisms regulating Bsx.

In summary, we conclude that Bsx likely represents a physiologically relevant part of energy balance control systems. Its expression is regulated by changes in energy balance, by nutrient signals such as ghrelin and leptin, and by exposure to dietary lipids. Because Bsx function includes governing hyperphagic responses and controlling spontaneous physical activity patterns (14), we propose that Bsx is a crucial molecular link at the interface between peripheral energy metabolism and the hypothalamic control of feeding and motor activity. Further studies will be necessary to dissect if Bsx is a relevant pathogenetic component in the development of HFD-induced leptin resistance.

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