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Energy Expenditure and Bone Formation Share a Common Sensitivity to AP-1 Transcription in the Hypothalamus

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Abstract

The regulation of bone and fat homeostasis and its relationship to energy expenditure has recently been the focus of increased attention due to its potential relevance to osteoporosis, obesity and diabetes. Although central effectors within the hypothalamus have been shown to contribute to the regulation of both energy balance and bone homeostasis, little is known of the underlying mechanisms, including the possible involvement of transcriptional factors within the hypothalamus. Transgenic mice overexpressing Δ FosB, a splice variant of the AP1 transcription factor FosB with mixed agonist-antagonistic properties, have increased energy expenditure and bone mass. Since these mice express Δ FosB in bone, fat and hypothalamus, we sought to determine 1) whether overexpression of Δ FosB within the hypothalamus was sufficient to regulate energy expenditure and whether it would also regulate bone mass, and 2) whether these effects were due to antagonism to AP1. Our results show that stereotactic injection of an adeno-associated virus vector to restrict overexpression of Δ FosB to the ventral hypothalamus of wildtype mice induced a profound increase in both energy expenditure and bone formation and bone mass. This effect was phenocopied, at an even stronger level, by overexpressing of a dominant-negative DNJunD, a pure AP1 antagonist. Taken together these results suggest that downregulation of AP1 activity in the hypothalamus profoundly increases energy expenditure and bone formation, leading to both a decrease in adipose mass and an increase in bone mass. These findings may have physiological implications since Δ FosB is expressed and regulated in the hypothalamus.

Keywords

AP-1; hypothalamus; energy expenditure; bone formation

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INTRODUCTION

Osteoporosis and obesity, two widespread diseases with major social and economic impact, have long been considered as independent clinical entities. In recent years, a large body of work has however indicated not only that there is abundant cross-talk between bone and fat cells, but also that they share common precursors and a link to the regulation of energy and metabolism by the central nervous system (1,2). Whole body adiposity is affected predominantly by changes in the size of individual adipocytes, which is influenced by the balance between food intake and energy requirements. Skeletal homeostasis is maintained by the balance between the resorptive osteoclasts and the bone forming osteoblasts, which is influenced by many factors, including Ca^{2+} and phosphate metabolism and insulin. An increasing body of evidence suggests that both of these tissues can also be regulated by neuronal influences arising from the hypothalamus, a potent regulator of food intake and energy expenditure, via the sympathetic nervous system (SNS) (3–5) (6–10).

While this work has clearly established that the regulation of on the one hand bone and on the other energy metabolism and fat can be linked, the exact nature of this link and the relative roles of peripheral vs central pathways remain elusive. Insight into these pathways may also be gleaned from observations made in mouse models exhibiting altered homeostasis of both bone and fat. One such model, the ENO2- Δ FosB mice, in which the AP-1 transcription factor Δ FosB is overexpressed in several tissues including adipose, bone and brain, exhibit a striking decrease in fat mass and increase in bone formation rates and bone mass (11,12).

Δ FosB is a naturally occurring spliced variant of FosB, and it acts as a mixed agonist-antagonist of AP1 *in vitro* and *in vivo* (13,14). We have previously shown that targeted expression of Δ FosB to the osteoblast was able to cell-autonomously increase bone formation and bone mass, albeit less than in the ENO2- Δ FosB model, but had no effect on adipose mass, indicating that the decrease in fat is not osteoblast-dependent (15). In contrast, targeted expression of Δ FosB to the adipocyte failed to recapitulate either the bone or the adipose phenotype (16), demonstrating that neither the decreased adipose mass nor the increased bone mass is the result of a cell-autonomous effect of Δ FosB within adipocytes. Detailed metabolic analysis of the ENO2- Δ FosB mice revealed that these mice display an increase in energy expenditure due to increased fatty-acid oxidation within skeletal muscle (16). Taken together, these observations suggested that the two phenotypes might not be linked at the peripheral level and that the changes in energy expenditure, which led to the decrease in adipose mass, were the result of changes in another organ, independent of bone and adipose tissue. Whether the changes in bone and/or fat were the result of AP1 activity in the brain and whether they were mechanistically linked remained to be determined.

In the present study we sought to determine if the expression of Δ FosB or a pure AP1 antagonist (an N-terminal Δ 1–149aa truncation of JunD termed DNJunD) within the hypothalamus was able to affect energy expenditure, fat mass, or bone formation and thereby reproduce the changes we observed in the ENO2- Δ FosB mice. Our results reveal that targeted expression of either Δ FosB or DNJunD to the ventral hypothalamus was sufficient to give rise to an increase in energy expenditure. In addition, we observed a profound increase in bone formation and bone mass in these mice. These results demonstrate that repression of AP1 transcriptional activity within the hypothalamus centrally regulates both energy expenditure and bone formation, opening new avenues for the treatment of both obesity and osteoporosis.

METHODS AND MATERIALS

Animals

All animals were maintained on normal chow (60% carbohydrate/10% fat/30% protein calories). 8 to 12 week old male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The ENO2- Δ FosB mice were generated as previously described (11). All animal protocols were approved by the Harvard University Institutional Animal Care and Use Committee.

In situ hybridization of brain sections

Animals were anesthetized and brains were removed and snap frozen in dry ice slurry. Sections were prepared as previously described (17) briefly, antisense and sense riboprobes corresponding to a 715 bp of Δ FosB was amplified and labeled with 35 S-CTP (Amersham Biosciences, Piscataway, NJ). *In situ* hybridization of Δ FosB mRNA with cRNA probes were performed on brain sections anterior-posterior (-1.7 – -2.2 from Bregma) (17). Sections were developed with Emulsion NTB (Kodak, Rochester NY) for 1 week. (1 week in emulsion gives a signal intensity similar to 1 day exposed to film).

Immunostaining of brain sections

Animals were anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. For immunostaining, brains were removed and incubated in 4% paraformaldehyde for 2 hrs, then placed in 20% glycerol in PBS for 6 hrs. 10 μ m sections were prepared and labeled with antibodies to FosB, JunD or GFP at 1:1000 (Santa Cruz) and visualized with CY fluorophore-labeled secondary antibodies at 1:200 (Jackson Immunolabs).

RNA isolation and real-time PCR analysis

Total RNA was isolated from tissue samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. mRNA was converted to cDNA using Superscript II and an oligo-dT primer (Invitrogen, Carlsbad, CA), following manufacturer's instructions. Transcripts were quantified by real-time PCR using an iCycler (Bio-Rad), with the iQ SYBR Green Super Mix (Bio-Rad) and primers to FosB and normalized to actin as previously described (15).

Stereotaxic injections into of the ventral hypothalamus

Adeno-associated viruses (AAV) encoding either Δ FosB-GFP, DNJunD-GFP or GFP alone (previously described in (18)), were injected bilaterally into the ventral hypothalamus of C57BL/6 mice. Stereotaxic coordinates of the injection site were anterior-posterior -2.1 mm, lateral \pm 1.3 mm, and dorsoventral -5.7 mm at an angle of 10 degrees from Bregma. Animals were analyzed 6 weeks after injections.

CLAMS metabolic study

Mouse energy expenditure, food intake, and locomotor activity were measured using the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Food intake (Kcal/g/hr) was calculated from food consumption. Energy expenditure was calculated using indirect calorimetry as a measure of heat [(3.815 + 1.232 \times RQ) \times VO₂] (Kcal/kg/hr). All data was normalized to the body weight during analysis.

GST pull-down

Glutathione-S-transferase (GST) fusion proteins were produced and purified by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech.) as previously described

(15). For interaction studies, DNJunD protein was produced by *in vitro* transcription-translation and incubated with GST fusion proteins bound to glutathione-Sepharose beads overnight at 4 °C. The samples were washed three times in mRIPA, the beads were sedimented and boiled in 10 µl of loading buffer, and the complexes were analyzed by Western blotting.

Electromobility Shift Assay

AP-1-binding double-stranded probes (forward oligonucleotide – GTC GAC GTG AGT CAG CGC GC; reverse oligonucleotide – GGG CGC GCT GAC TCA CGT) were labeled with 15 µCi [³²P]-dCTP using the Amersham Random Prime kit and purified on NICK G50 Sephadex columns. Approximately equal amounts of *in vitro*-transcribed FosB, ΔFosB, Δ2ΔFosB, JunD and DNJunD proteins were incubated with labeled probe (25,000 cpm) in 340 mM KCl, 50 mM MgCl₂, 1 mM DTT and 3 µg/ml poly-dIdC (Sigma) at room temperature for 30 minutes. Samples were separated on 6% non-denaturing polyacrylamide gels at 200 volts for 2 hrs. Gels were dried and autoradiographed.

Luciferase Reporter Assay

HEK-293 cells (ATCC) were plated at a density of 1×10⁵ in 6-well plates and transfected using FuGENE 6 (Roche) with constructs encoding a 6X-TRE-luciferase reporter (6X-TRE-luc, Clontech) to measure AP-1 activity. Cells were also transfected with either pcJunD or pcNDJunD and cDNAs encoding the FosB isoforms (pcFosB, pcΔFosB2i3i and pcΔ2ΔFosB) at the indicated concentrations. Transfection efficiency was assessed by co-transfecting an SV40 Renilla luciferase construct (Promega); the total amount of transfected DNA was maintained at 1.25 µg per well by adding empty vector (pcDNA3.1). Dual-Luciferase assay (Promega) was performed according to the manufacturer's instructions.

Bone histomorphometry of the tibial metaphysis and calvarial segments

All mice were injected with calcein (Sigma, 40 mg/kg) and demeclocycline (Sigma, 20 mg/kg) at 10 and 3 days prior to sacrifice to label mineralizing fronts. Tibiae and calvaria were removed and fixed in 3.7% formaldehyde then infiltrated with a mixture of 90% methyl methacrylate (Sigma), 10% dibutyl phthalate (Sigma), and 0.15% benzoyl peroxide (Polyscience) at 4 °C and then embedded in a mixture composed of 85% methyl methacrylate (Sigma), 15% dibutyl phthalate (Sigma), and 0.05% benzoyl peroxide (Polyscience). Polymerization was performed at 37 °C. Standard undecalcified sections (4 µm) were prepared using a Reichert-Jung microtome (Cambridge Instrument), and were stained with toluidine blue or left unstained. A standard histomorphometric analysis of the tibial metaphysis and calvaria were performed using the Osteomeasure analyzing system (Osteometrics, Inc.) Tibial measurements were performed in a 1.28 mm² area starting 0.3 mm from the proximal growth plate.

Statistical analysis

The data are presented as means ± standard error of the mean (SEM), statistical analysis was performed with Student's t test for all *in vitro* experiments and 2-way ANOVAs for all *in vivo* experiments. P values of less than 0.05 were considered statistically significant.

RESULTS

Hypothalamic Expression of ΔFosB in the ENO2-ΔFosB mice

We have previously shown that targeted overexpression of ΔFosB to the osteoblastic or the adipocytic lineage had no effect on adipose mass and energy expenditure (15,16). We therefore sought to determine whether ΔFosB expression within the brain could be

responsible for the increased energy expenditure observed in the ENO2- Δ FosB mice. Given the well-established role of the hypothalamus in maintaining energy homeostasis, we performed *in situ* hybridization and immunofluorescent staining of brain sections to determine whether Δ FosB was overexpressed in these neurons. Increased Δ FosB expression was observed in the ventral hypothalamus (black circle) (Figure 1A) and labeled neurons were identified in ventromedial hypothalamus (VMH) and to a greater extent in the arcuate nucleus (ARC) (Figure 1B). Given that our antibody detects both Δ FosB and full-length FosB, we confirmed the increased Δ FosB expression by both western blot analysis and real-time qPCR using primers which can discriminate between Δ FosB and FosB. We observed a significant increase in Δ FosB expression at both the protein (10 fold) and mRNA level in the hypothalamus of the ENO2- Δ FosB mice (Figure 1C and D). These data show that Δ FosB is expressed in the hypothalamus in wildtype mice and markedly overexpressed in the ENO2- Δ FosB mice, supporting the hypothesis that Δ FosB may be playing a role within this brain region.

Central expression of Δ FosB reduces body weight and increases energy expenditure

Given the increased expression of Δ FosB within the hypothalamus of the ENO2- Δ FosB mice and the role of the hypothalamus in regulating energy expenditure, we sought to determine if increased expression of Δ FosB in the ventral hypothalamus was sufficient to alter the regulation of energy expenditure in wild-type mice. An adeno-associated virus (AAV) vector encoding either GFP alone or Δ FosB-GFP were stereotactically injected bilaterally in the ventral hypothalamus of wildtype mice (Figure 2A). After 6 weeks, when the transgene effects become maximal (18), the mice were subjected to metabolic analysis; mice in which the injection had not been successful, as determined by post-mortem immunofluorescence analysis of brain sections (Figure 2A), were eliminated. Successfully injected AAV- Δ FosB animals exhibited a decrease in body weight, despite an apparent increase in food intake (Figure 2B and 2C). The AAV- Δ FosB injected animals also exhibited increased O₂ consumption, CO₂ production and energy expenditure, despite reduced locomotor activity (Figure 2D–G). These results demonstrated that specific overexpression of Δ FosB within the ventral hypothalamus is sufficient to decrease body weight and to increase energy expenditure in mice. Accordingly, histomorphometric analysis of adipocytes in visceral fat pads demonstrated a significant decrease ($p < 0.05$) in the size of individual adipocytes and trend towards a reduction in adipose mass compared with wildtype animals (Figure S1A and S1B). Histological analysis of brown adipose tissue from these mice did not reveal any significant difference in BAT morphology, despite a trend towards smaller droplet size in the AAV-DNJUN injected mice (Figure S1D). Thus, targeted overexpression of Δ FosB in the ventral hypothalamus is sufficient to increase energy expenditure, reduce body mass and reduce fat mass adipocyte size, phenocopying the fat phenotype of the ENO2- Δ FosB mice.

DNJunD binds Fos isoforms but has reduced transcriptional activity

We have previously shown that ENO2-driven overexpression of $\Delta 2\Delta$ FosB, a further truncated form of Δ FosB that has no AP1 transcriptional activity and behaves as a pure antagonist to AP1, was able to recapitulate the bone and fat phenotypes observed in the ENO2- Δ FosB mice (19). However, whether these effects could be attributed solely to repressed AP1 activity is unknown. To gain further insight into the possible mechanisms responsible for the phenotypes observed in the ENO2- Δ FosB mice and the hypothalamic expression of Δ FosB with AAV, we determined if the phenotype is the result of reduced AP1 activity within the hypothalamus. To test this possibility, we used DNJunD, which retains its DNA binding and dimerization ability similar to JunD (Figure 3A, 3B and 3C) but lacks the transactivation domain and therefore serves as an AP1 antagonist that reduces the transactivating potential of all complexes that incorporate a Jun protein as a binding partner

(Figure 2C)(18,20). DNJunD therefore acts as a dominant-negative for all AP1 transcriptional activity.

Hypothalamic expression of DNJunD reduces body weight and increases energy expenditure

Using DNJunD, we next tested the hypothesis that antagonizing AP1 activity within the hypothalamus would result in a decrease in adipose mass and an increase in energy expenditure. AAV vectors encoding DNJunD-GFP or GFP alone were stereotaxically injected within the ventral hypothalamus of wildtype mice similar to what was done for Δ FosB. Strikingly, targeted expression of DNJunD in the ventral hypothalamus was sufficient to induce a significant decrease in body weight and an increase in CO_2 production and energy expenditure (Figure 4A–F), similar to what was observed in the ENO2- Δ FosB mice (16) and in the AAV- Δ FosB injected mice (Figure 2). The AAV-DNJunD injected mice also exhibited a decrease in ambulatory activity and an increased food intake (Figure 4C and 4B). Histological analysis of the adipose tissue revealed a reduction in the size of the adipocytes over that of control-injected mice (Figure S1A). Since what Δ FosB, $\Delta 2\Delta$ FosB and DNJunD have in common is their ability to antagonize AP1 transcriptional activity, these observations strongly suggest that AP1 activity within the hypothalamus down-regulates energy expenditure, such that expression of an AP1 antagonist increases energy expenditure and reduces fat mass in wild-type mice, recapitulating the adipose and energy phenotype observed in the ENO2- Δ FosB mice.

Increased expression of Δ FosB or DNJunD in the ventral hypothalamus also increases bone formation

Having established that the energy expenditure and fat phenotype were the direct consequences of expression of Δ FosB and its antagonism of AP1 within the ventral region of the hypothalamus, we addressed the important question of whether this mechanism would also affect bone formation and bone mass, determining whether the bone phenotype observed in the ENO2- Δ FosB mice was also dependent on these properties. Strikingly, dynamic histomorphometric analysis of the bones from the ventral hypothalamus-targeted AAV- Δ FosB and AAV-DNJunD mice revealed a 3- and 5-fold increase in linear mineral apposition rates (MAR) resulting in a very pronounced increase in the bone formation rate (BFR) (3- and 7-fold, respectively). These observations demonstrate unequivocally an anabolic action on individual osteoblasts as well as marked increases in osteoblast numbers (Figure 5G). This massive increase in BFR was not associated with changes in bone resorption and resulted in a significant increase in bone volume (BV/TV) associated with significant changes in trabecular numbers (Figure 5A–F), despite the relatively short period of exposure to AAV-induced Δ FosB or DNJunD expression. Thus, restricted expression of Δ FosB or a pure AP1 antagonist in the ventral hypothalamus induces a massive increase in bone formation, and thereby recapitulates not only the energy and fat but also the bone phenotype observed in the ENO2- Δ FosB mice.

DISCUSSION

Our results demonstrate that Δ FosB, a member of the AP1 family of transcription factors, serves as a positive regulator of both energy expenditure and bone mass within the hypothalamus. This provides further support for the existence of a link between central regulation of energy expenditure and bone mass. Furthermore, our findings provide novel insight into the central transcriptional regulation of bone and fat. Independent of which neuronal circuitry is involved within, or downstream of, the ventral hypothalamus, these results provide the first direct evidence for a very potent and AP1-sensitive hypothalamic relay in the regulation of bone formation and skeletal homeostasis. Furthermore, these

results demonstrate that both energy expenditure and fat and bone homeostasis share a common sensitivity to AP1 activity within the hypothalamus, which appears to play a potent role in down-regulating both energy expenditure and bone formation.

Notwithstanding our previous demonstration that the bone anabolic effects of Δ FosB is in part due to a cell-autonomous mechanism within the osteoblast lineage (15), the data reported here demonstrate that the increased bone formation observed in the ENO2- Δ FosB mice is predominantly due to Δ FosB's activity in the hypothalamus. Although overexpression of Fra1 under the H2K promoter has recently been reported to reduce adipocytic differentiation and fat mass through cell-autonomous effects (21) we have not seen such effects in the ENO2- Δ FosB mice (16). Supporting this view, the results presented here demonstrate that the reduced adipose mass results exclusively from a centrally mediated increase in energy expenditure, without any detectable cell-autonomous effect of Δ FosB within the adipocyte lineage (16) and independent of the changes occurring in the osteoblasts and in bone formation (15). Indeed, this study demonstrates that expression of Δ FosB in the ventromedial region of the hypothalamus is sufficient to recapitulate the entire phenotype of the ENO2- Δ FosB mice, i.e. increased energy expenditure, decreased fat mass and increased bone formation and bone mass.

Thus, the expression levels of Δ FosB in the hypothalamus can simultaneously affect bone formation and energy expenditure in adult mice, leading to increases in bone formation and bone mass as well as a decrease in adipose mass. As the endogenous level of expression of Δ FosB within the hypothalamus has previously been shown to be regulated under several circumstances (22–24), our results raise the possibility that bone formation and energy expenditure may be physiologically co-regulated, at least in part, through this transcription factor.

Communication between the brain and adipose tissue has been well established (4). Adipose tissue secretes hormones such as leptin and adiponectin that are able to cross the blood-brain barrier and activate specific neurons in the arcuate nucleus of the hypothalamus, which in turn regulate food intake and lipid utilization (25–27). However, we do not observe any significant difference in adiponectin levels in our AAV-inject animals (Figure S1C). It is however only recently that studies have identified the potential importance of the central nervous system, and more specifically of the hypothalamus, in the regulation of bone mass. Signaling via hypothalamic-pituitary relays and the SNS are now recognized as contributors to the regulation of bone homeostasis (6,9,28–31). In addition, the hypothalamus controls the synthesis and secretion of pituitary hormones (FSH, TSH, GH and MSH), several of which affect bone homeostasis (29,30,32,33). We however did not find any significant alterations in these hormones in the ENO2- Δ FosB mice or AAV injected mice (Figure S2), indicating that the very pronounced increase in bone formation rates observed in both models cannot be explained by changes in pituitary hormones.

It is therefore possible that, independent of pituitary regulatory functions, one or more hypothalamic signaling pathways are regulated by Δ FosB to increase bone mass and energy expenditure, only secondarily affecting fat accumulation. As proposed by others, the relay to peripheral osteoblasts may operate via the SNS (6,34). If Δ FosB acts on the leptin/SNS pathway to induce the increase bone formation observed here, it would have to oppose the effects of leptin and/or NPY/PYY in the hypothalamus and/or the effects of beta-2-adrenergic receptor (β_2 AR) activation in osteoblasts. We have shown that restoring the normal levels of circulating leptin in the ENO2- Δ FosB mice, which are low due to the pronounced decrease in adipocyte size, failed to rescue the bone phenotype (35) and that leptin signaling in these mice is not altered (35). Thus, Δ FosB would have to act downstream of leptin, i.e. on NPY or β_2 AR signaling, to prevent their negative effects on

bone formation. Analysis of NPY expression levels in the hypothalamus of the ENO2- Δ FosB mice revealed, however, that they were unchanged from control levels and β_2 AR sensitivity in the bone target cells, i.e. osteoblasts was also identical to controls (Figure S3). We therefore consider it unlikely that Δ FosB simply antagonizes the leptin/NPY/ β_2 AR pathway.

In considering alternate possibilities, it is worth mentioning the fact that Δ FosB could be affecting bone and energy via two independent pathways that share only their hypothalamic localization and sensitivity to Δ FosB expression levels. Alternatively, although we have eliminated the possibility that changes in energy expenditure and fat are secondary to changes in bone (15), the reverse could be true, i.e. that the effects on bone formation could be secondary to the changes in energy and/or in fat. In this case, however, increased energy expenditure in muscle, as observed here, would affect bone formation directly or indirectly. Two findings contradict this hypothesis. First, we have found that increased UCP3 expression in muscle, which leads to massive increases in muscle energy expenditure, failed to alter bone formation or bone mass (Figure S4). Second, and although mechanical changes in the muscle could theoretically affect bone, the fact that we observe the same increase in bone formation in the calvaria, a non-weight bearing bone, in the ENO2- Δ FosB and AAV-infected mice makes it an unlikely explanation (Figure S5). Muscle could however exert its action on bone indirectly, via energy-dependent myokines which would induce an increase in bone formation, in addition to depleting fat from adipocytes. Alternatively, the fat depletion secondary to increased energy expenditure in the muscle could alter the levels of key adipokines, affecting positively bone formation. As leptin is not apparently a candidate in our model, the decrease in adiponectin that we observed in the ENO2- Δ FosB mice could be a good candidate since Cornish and colleagues have recently reported an increase in bone mass in adiponectin-null mice (36).

The detailed molecular mechanism by which Δ FosB affects transcriptional and signaling events in the hypothalamus to affect both energy and bone remains to be elucidated. The truncated Δ FosB splice variant lacks most of the C-terminal region of FosB, its major transcriptional domain, and therefore behaves as a relatively weak AP1 transcriptional activator that can also compete with normal FosB functions, leading to its activity as an AP1 antagonist as well (37). The weak AP-1 activity of Δ FosB is unlikely to play a role in the hypothalamic co-regulation described here, however. First, ubiquitous overexpression of FosB fails to induce changes in bone with no reported changes in fat (38 and unpublished results). Second, we have recently reported that expression of a further truncated isoform of Δ FosB ($\Delta 2\Delta$ FosB) under the control of the same ENO2 promoter induces the same bone and fat phenotypes as those of the ENO2- Δ FosB mice despite the fact that it has lost all intrinsic AP1 transcriptional activity (19). Third, the fact that DNJunD expression in the hypothalamus induced both a stronger bone formation phenotype and reduction in adipocyte size provides further support for the conclusion that it is not AP1 transactivation that is required for the co-regulation of energy and bone but rather that it is the AP1 antagonism common to all three proteins (Δ FosB, $\Delta 2\Delta$ FosB and DNJunD) that is essential in this co-regulatory activity. These observations suggest that the changes observed are most likely the result of either failure to induce or suppression of AP1 target genes within the hypothalamus. Further work will be required to test this hypothesis.

Importantly, changes in the expression of Δ FosB in the brain, which we show here affects both energy expenditure and bone formation in a coordinated manner, occur naturally as the result of *fosB* gene induction and alternative splicing as well as through stabilization of the FosB protein (13,39). Although the mechanisms that regulate the splicing event that generates the Δ FosB transcript are not elucidated, the relative stability of the FosB protein is a key mechanism regulating its accumulation in certain tissues (40). Moreover, both the

stability and transactivation capabilities of Δ FosB are regulated by phosphorylation on specific serine residues (41,42). The regulation of Δ FosB levels under physiological conditions has been extensively studied in the nucleus accumbens and dorsal striatum (18,43,44). Δ FosB's expression has been associated with changes in expression of particular neuropeptides, such as dynorphin, which is downregulated by Δ FosB in the nucleus accumbens (18). Interestingly, dynorphin-null mice have been recently reported as having a decrease in adipose mass and increased fatty acid oxidation, but no change in NPY expression within the hypothalamus (45). It is yet to be determined whether these mice also have an altered bone mass, given the recent reports on Nmu and signaling upstream of the Y2 receptors (7,8,10,46).

The expression of FosB and its shorter isoforms in specific regions of the brain is induced by many stimuli, including seizures, stress, drugs, and exposure to newborn pups (18,22,39,43,47,48). The temporal and spatial expression of FosB isoforms within the brain is markedly dependent on the stimulus (reviewed in (43)). Alteration in the relative levels of expression of the FosB isoforms results in changes in several specific behaviors in these animals, including locomotor activity and motivation for food (44,49). Expression of FosB/ Δ FosB in the preoptic area of the hypothalamus of mice has been related to the nurturing of their pups by the fact that FosB/ Δ FosB is induced in adult animals in this area upon exposure to pups, and FosB-null mice do not care for their pups appropriately. The adult mice had however no reported abnormality in eating behavior or body weight (22). Finally, recent studies have shown that FosB/ Δ FosB expression is induced within the paraventricular nucleus of the hypothalamus upon both acute and repeated administration of cocaine (13,23). Apart from these observations, little is known about the consequences of altering the expression of Δ FosB within hypothalamic nuclei. In this context, our demonstration that variations in the level of Δ FosB in the hypothalamus co-regulate energy expenditure and bone formation is both novel and important.

In conclusion, we have shown that downregulation of AP1 activity, via Δ FosB or DNJunD expression, in hypothalamus simultaneously reduces adipocyte size by increasing energy expenditure and increases bone mass by increasing bone formation. This provides strong evidence for common central mechanisms in the regulation of both energy expenditure and adipose and bone mass. Furthermore our study shows that whether common or separate, the hypothalamic neuronal circuitry that regulates energy/fat and bone share a common sensitivity to AP1 transcriptional activity. These findings may open novel exploratory avenues for the treatment of osteoporosis and/or obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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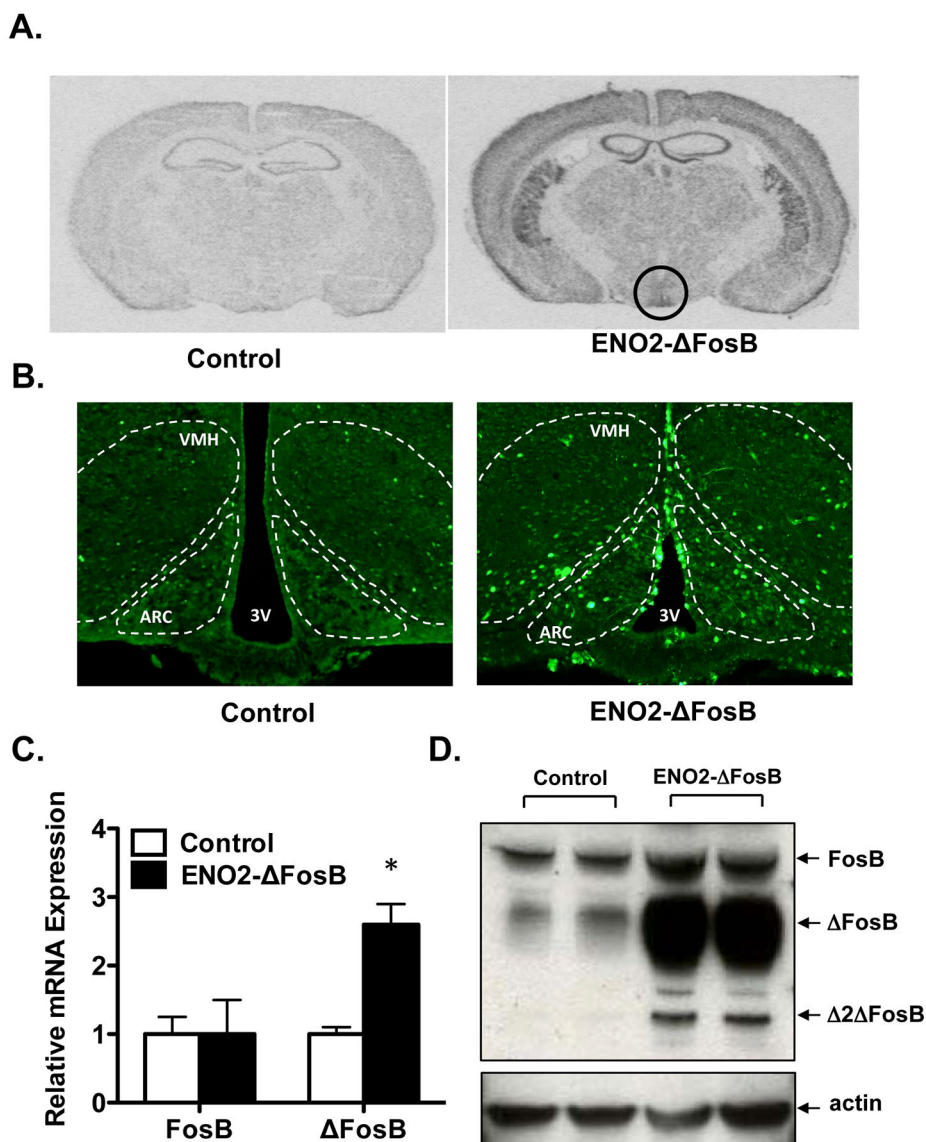


Figure 1. Increased Δ FosB expression in the hypothalamus of ENO2- Δ FosB mice
 A) *In situ* hybridization of Δ FosB probes in coronal sections 2.1 mm posterior of Bregma in brains from ENO2- Δ FosB mice and control littermates showing high levels of Δ FosB overexpression within the striatum and ventral hypothalamus, B) Immunofluorescent staining of coronal sections 2.1 mm posterior of Bregma in brains from ENO2- Δ FosB mice and control littermates for FosB, C) Real-time expression of FosB and Δ FosB of hypothalamic punches from ENO2- Δ FosB mice and control littermates, and D) Western blot analysis of Δ FosB of hypothalamic punches from ENO2- Δ FosB mice and control littermates. Actin was used as loading control.

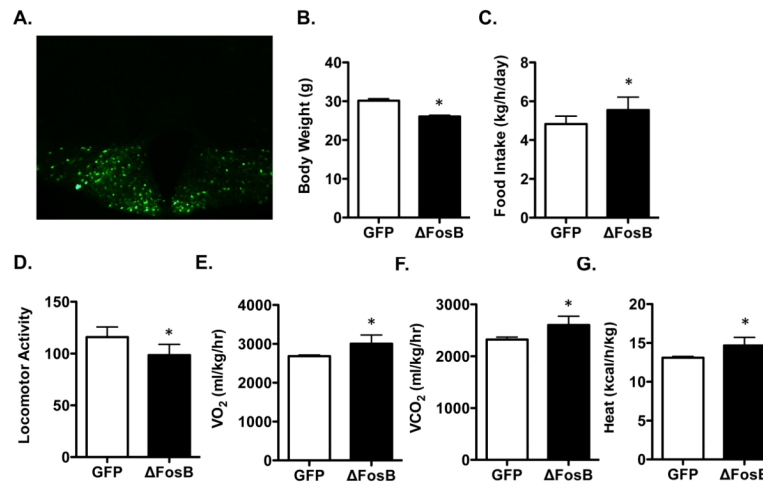


Figure 2. AAV- Δ FosB expression to hypothalamus increases energy expenditure
 A) Target validation of AAV-GFP injection B) Body weight, C) Food intake, D) Locomotor activity, E) Oxygen consumption (VO₂), F) Carbon dioxide production (VCO₂), and G) Energy expenditure (kcal/h/kg) in AAV-GFP and AAV- Δ FosB hypothalamic injected mice. (n = 8–10; * - p < 0.05 relative to AAV-GFP).

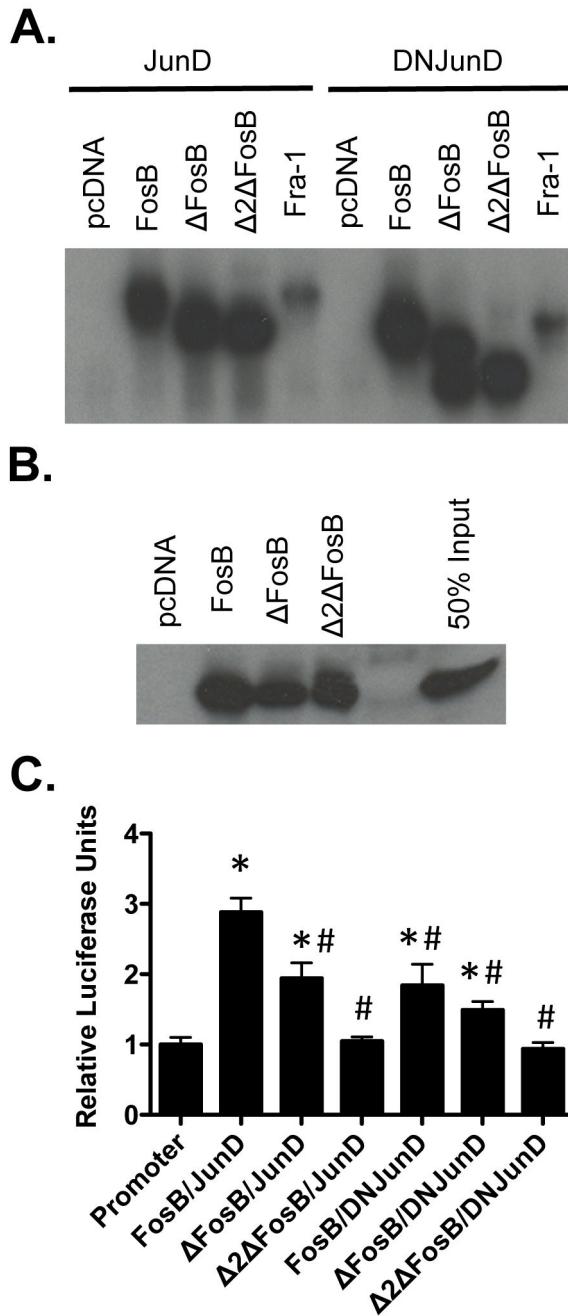


Figure 3. DNJunD acts as an antagonist of AP-1 activity

A) Gel shift of FosB isoforms with either JunD or DNJunD on AP1 DNA element, B) GST pull down of DNJunD by FosB and its truncated isoforms, C) AP1 reporter assay of FosB isoforms with either JunD or DNJunD on 6X TRE-luciferase (n = 3–4; * - p < 0.05 relative to promoter alone; # - p < 0.05 relative to FosB/JunD).

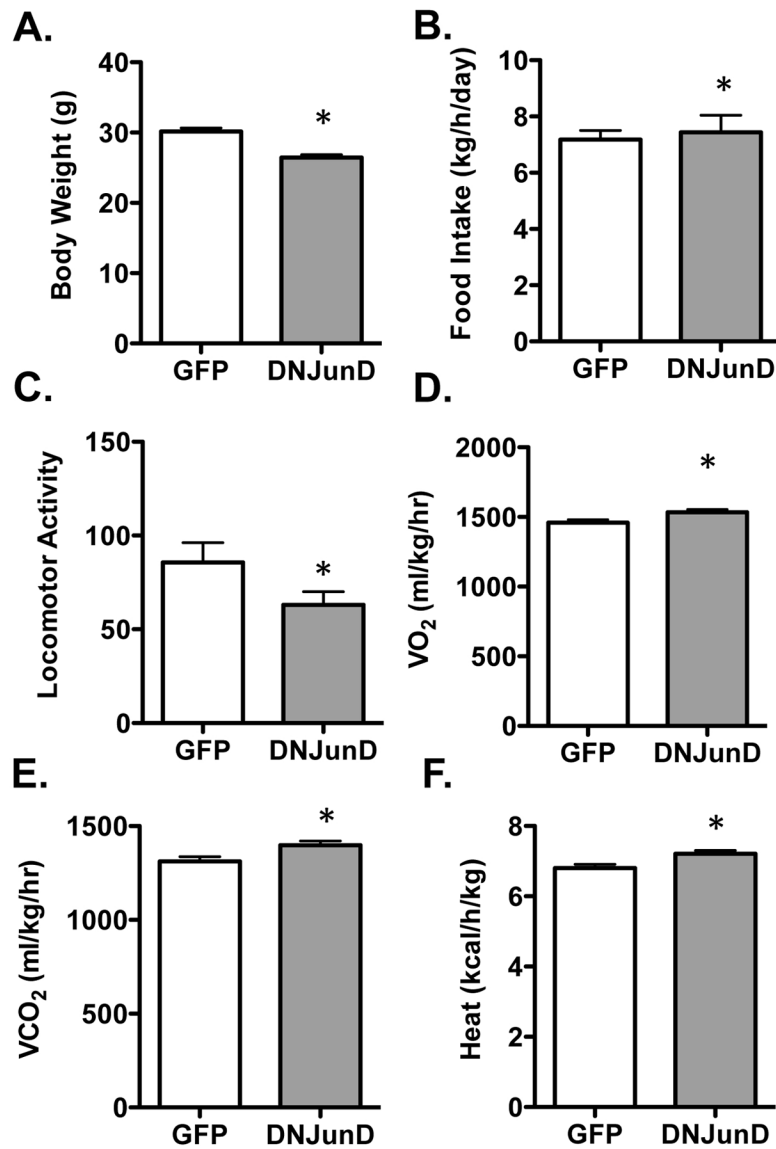


Figure 4. AAV-DNJunD expression in hypothalamus increases energy expenditure
 A) Body weight, B) Food intake, C) Locomotor activity, D) Oxygen consumption (VO_2), E) Carbon dioxide production (VCO_2), F) Energy expenditure (kcal/h/kg) in AAV-GFP and AAV-DNJunD hypothalamic injected mice. (n = 10–12; * - p < 0.05 relative to AAV-GFP).

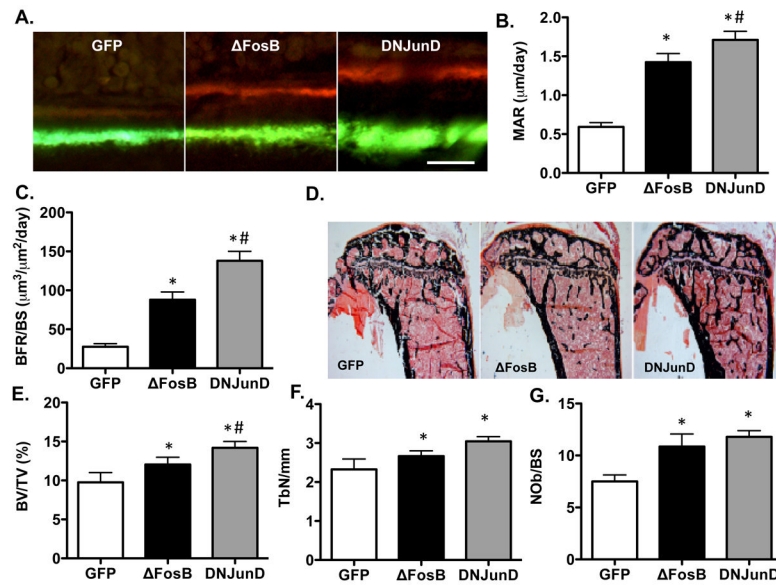


Figure 5. Antagonism of AP1 in the hypothalamus increases bone mass

A) Fluorescent double labeling of mineralizing front with calcein and demeclocycline in AAV-injected mice. B) mineral apposition rate (MAR), C) Bone formation rate (BFR/BS), D) von Kossa staining of proximal tibias, E) bone volume (BV/TV), F) trabecular number (TbN/mm) and G) osteoblast number (N.Ob/BS) in AAV-injected mice. (n = 10–12; * - p < 0.05; ** - p < 0.005 relative to AAV-GFP; # - p < 0.05; ## - p < 0.005 relative to AAV-ΔFosB).