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Effect of Food Deprivation or Short-term Western Diet Feeding on BDNF Protein Expression in the Hypothalamic Arcuate, Paraventricular, and Ventromedial Nuclei

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Running head: Fasting and western diet effects on hypothalamic BDNF

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ABSTRACT

Mutations in the brain-derived neurotrophic factor (BDNF) gene are associated with human obesity and BDNF has potent inhibitory effects on eating and body weight. Little is known about the effects of energy-balance manipulations on BDNF protein in the hypothalamus, though this brain region is critical for regulation of feeding and body weight and has high levels of BDNF. Here we investigated the effects of negative and positive energy status on BDNF protein levels in the arcuate (ARC), paraventricular (PVN), and ventromedial (VMH) hypothalamic nuclei, and the ectorhinal cortex. To achieve this, mice were food deprived for forty-eight hours or fed a western diet (WD), a restricted amount of WD, or chow for six hours, forty-eight hours, one week, or three weeks. BDNF protein levels were estimated as the number of neurons in each brain region that exhibited BDNF-like immunoreactivity (LIR). Food deprivation decreased BDNF protein (and mRNA) expression in the ARC compared with fed mice (32%). In contrast, one week of WD consumption increased BDNF protein expression in the VMH as compared with chow or restricted WD feeding (40%), and unexpectedly, increased BDNF protein in the ectorhinal cortex (20%). Furthermore, of the diet conditions and durations tested, only one week of WD consumption was associated with both hyperphagia and excess weight, suggesting effects of one or both contributed to the changes in BDNF levels. The decrease in ARC BDNF may support increased feeding in food-deprived mice, whereas, the increase in the VMH may moderate overeating in WD fed mice.

KEY WORDS: Obesity, BDNF, food intake

INTRODUCTION

Obesity has exceeded epidemic levels and become pandemic, expanding globally and thus directly impacting a large proportion of the human population (5, 68). More indirectly, the care and treatment of obese and overweight people, many of whom also suffer from secondary disorders, including diabetes, cardiovascular disease, and some forms of cancer (25), has resulted in an enormous economic burden (7, 18, 49). The BDNF locus is among the human chromosomal loci associated with susceptibility to obesity (66). Moreover, rare patients with Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation contiguous gene syndrome are obese and have chromosome deletions that include the BDNF locus (described in (31). Additionally, a variant of the BDNF receptor, tyrosine kinase B (trkB), has also been linked to human obesity (78).

A large body of evidence suggests BDNF is involved in feeding behavior and body weight regulation, specifically as a potent anorexigenic factor that acts at least in part by contributing to satiety. For instance, BDNF is expressed in several brain regions important for controlling feeding behavior, including the ventromedial hypothalamus (VMH), the dorsomedial nucleus of the hypothalamus (DMH), the paraventricular nucleus of the hypothalamus (PVN), and the arcuate nucleus of the hypothalamus (ARC; 10).

Increases in central BDNF levels produced by injecting BDNF into the third ventricle (35, 53) or into specific hypothalamic and brainstem areas, including the dorsal vagal complex (DVC), VMH and PVN, have all been associated with decreased food intake and body weight (1, 72, 74, 75). In contrast, decreases in BDNF levels produced by global heterozygous BDNF knockout (20, 31, 45), smooth muscle BDNF knockout combined with partial global BDNF knockout (19), forebrain-hypothalamus BDNF knockout (57), or virally targeted knockout of BDNF in the VMH (and adjacent DMH; 72) have all been associated with overeating and excess weight gain.

When BDNF binds to its high affinity receptor, trkB, it results in autophosphorylation of trkB's tyrosine residues, which activates several downstream signaling pathways (30). A mutation in humans that impairs this autophosphorylation and subsequent MAP kinase signaling resulted in hyperphagic obesity (78). This finding is consistent with the hypothesis that the effects of altered BDNF levels in the various hypothalamic regions on feeding behavior act through trkB receptor signaling.

An animal's nutrition or energy status can influence levels of BDNF protein expression. For example, in the hippocampus, exercise and caloric restriction increase BDNF protein levels, whereas, consumption of a western diet (WD) or other forms of a high-energy diet (HED; a diet high in carbohydrate and fat) reduce BDNF protein expression (14, 24, 48, 73). Despite high levels of BDNF protein in the hypothalamus and the significance of hypothalamic regions such as the ARC, PVN and VMH for regulation of food intake and body weight, little is known about the potential for nutrition or energy status to influence hypothalamic BDNF protein expression. Nevertheless, the potential for regulation of hypothalamic BDNF protein expression more generally is suggested by the observation of age-related changes in this BDNF (62). To date, and to our knowledge, the effect of nutrition or energy state on hypothalamic BDNF expression has only been examined at the messenger RNA (mRNA) level and only in the VMH (43, 71, 72, 77, 79). BDNF protein, however, not mRNA, is the functional molecule. Changes in protein and mRNA levels in general, and specifically for BDNF, do not always parallel one another, and may even be opposite in direction (e.g., 52, 62). Therefore, it is surprising that measurement of hypothalamic BDNF protein levels has been largely absent from studies of feeding and body weight. Since our ultimate interest is in BDNF protein effects on food intake and body weight, it was essential and most relevant to determine how hypothalamic BDNF protein levels respond to energy status.

The first aim of the present study was to investigate the effects of forty-eight hours of food deprivation on BDNF protein expression in the ARC, PVN, and VMH. Additionally, the

121 cerebral cortex was examined as an initial assessment of the effects of food deprivation on
122 BDNF protein expression outside the hypothalamus. Based on the outcomes of BDNF
123 manipulations described above we hypothesized that food deprivation, because it causes
124 increased food intake, will result in reduced BDNF protein levels in the VMH (reduced BDNF will
125 reduce inhibition of feeding). A similar effect may occur in the PVN and the ARC. The PVN is
126 also mainly inhibitory to feeding (34, 64, 70), although some neuropeptides instilled in the PVN
127 have stimulated feeding (e.g., 33, 67). The ARC, however, is mixed – it contains subpopulations
128 of neurons that are inhibitory or excitatory to feeding (61). The second aim was to examine the
129 effects of short-term consumption of a WD on BDNF protein in these brain regions. This is
130 crucial because changes in BDNF levels in the short-term could contribute to early stages of
131 hyperphagia and obesity. Understanding whether and how hypothalamic BDNF levels are
132 altered by early stages of positive energy balance will be important for designing studies that
133 test a causal role of these altered BDNF levels. Should BDNF play a causal role, this knowledge
134 could be valuable for devising a treatment strategy that takes advantage of BDNF's potent
135 anorexigenic effect to help prevent or moderate dietary obesity. A treatment that prevents
136 obesity would be a significant advance given the tremendous difficulties most obese people
137 have losing weight and maintaining weight loss (58). To capture potential changes in BDNF
138 protein expression in the ARC, VMH, and PVN, four different durations of WD exposure were
139 examined, six hours, forty-eight hours, one week and three weeks. In parallel, the cerebral
140 cortex was examined as an initial assessment of the effects of WD consumption on BDNF
141 protein expression outside the hypothalamus. Again, based on the effects of BDNF
142 manipulations described above, because overeating and obesity should inhibit food intake, we
143 hypothesized one or more of the durations of short-term WD exposure tested will result in
144 increased BDNF protein levels in the VMH and possibly in the ARC and PVN (increased BDNF
145 will increase inhibition of feeding).
146

MATERIALS AND METHODS

Animals. Male C57BL/6 mice three to five months of age were used. They were originally obtained from Harlen Industries (Indianapolis) and bred for several generations in our laboratory. Mice had ad libitum access to water and standard chow in pellet form (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO) and were bred and initially maintained at 22°C on a 14:10 hr light/dark cycle with lights on at 0500 and off at 1900. At a minimum of two weeks before beginning measurements to establish stable food intake (see below), mice were switched to a 12:12 hr light/dark cycle with lights on at 0200 and off at 1400. Optimal breeding conditions for our mice include a 14:10 hr light/dark cycle. The reason for the shift from a 14:10 hr to a 12:12 hr cycle is that mouse feeding behavior has typically been studied using a 12:12 hr or similar light cycle. Therefore, this shift in the light cycle made the conditions of our feeding experiments more comparable to those of other studies. The additional shift in the lights on phase was done so perfusion of mice in the 6 hr test diet duration groups could be done prior to 0000. The 2 wk duration of the adaptation phase for the shift in light cycle from 14:10 to 12:12 hr was based on previous experiments in which we adjusted the light cycle and found this duration of adaptation was sufficient time for mice to exhibit normal meal patterns, food intake and body weight (data not shown; e.g., 8). Consistent with our past experience, mice in the present study appeared to have normal food intake and body weight gain for their age after this adaptation phase. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

Experiment 1: Effects of food deprivation on BDNF protein expression in the ARC, VMH, and PVN

For all experimental runs, mice were housed individually in clear plastic cages. Control (FED) and food deprived (DEP) mice were run through the entire experiment in pairs, one mouse from each group, to minimize the contribution of any variation between runs due to unintended differences in procedures or laboratory environment to any group differences observed. Chow pellet intake and body weight of twenty animals were measured just before 1400 daily and stable food intake determined. Stable food intake was defined as consumption of $\pm$ 1 g each day, for five consecutive days, of the average intake for those five days (4). These mice were then divided into FED (n=10) and DEP (n=10) groups matched on food intake and body weight. Days 1-5 of the experiment were the five days of stable food intake followed by feeding or food deprivation on days 6-7. The FED group continued ad lib feeding of standard chow pellets for the final 48 hr, whereas, all food was removed from the DEP group at 1400 at the start of day 6. Forty-eight hours later at 1400 (end of day 7) mice were anesthetized and then fixed by cardiac perfusion.

Tissue Processing. Mice were given a lethal dose of Brevital Sodium (sodium methohexital; 100 mg/kg) and then perfused transcardially with saline at a rate of 4ml/min for ten minutes and then with chilled 4% paraformaldehyde dissolved in 0.1M sodium phosphate buffered saline (PBS) kept on ice for thirty minutes. Brains were removed and stored at 4˚C in 4% paraformaldehyde in PBS overnight and then incubated in 30% sucrose PBS overnight at 4˚C. Next, a block of brain containing the hypothalamus was excised, frozen in OCT (Tissue Tek) using liquid nitrogen, and stored at -80˚C until frozen sectioning.

Immunohistochemistry and nissl staining. Of the several potential approaches for assessing protein levels, we chose quantification of the cell numbers exhibiting BDNF– LIR and of these cells' staining intensity. Importantly, these approaches, as compared with ELISA, for example, ensure independent assessment of protein levels in the VMH, PVN, and ARC as

these nuclei lie in close apposition to one another.

First, a control brain was cross-sectioned at 30 μm through the VMH, PVN and ARC and stained with 0.02% cresyl violet to aid location of sections used for counting BDNF immunostained neurons and nucleus boundaries. Next, brains from WD and chow fed mice were cross-sectioned at 30 μm through the hypothalamus, which included approximately 50 coronal sections that contained the VMH, PVN and ARC in their entirety. All sections were immunostained for BDNF except every ninth section, which was stained with cresyl violet and used to help determine the shape and location of nucleus borders in each brain. The BDNF immunostaining protocol we employed was similar to that of Ewa et al. (16). Sections were stained using the free-floating technique with gentle agitation during the entire immunostaining procedure. Sections were first washed 3 x 15 min in PBS, incubated in goat block **(**8% normal goat serum, 0.5% Triton X-100, 2% bovine serum albumin) for 1 hr and then in primary antibody (1:1500; rabbit anti-BDNF polyclonal antibody, Millipore-Chemicon, AB1534SP) in diluent (0.3% triton X100, 1% bovine serum albumin, 0.08% soduim azide in PBS) at 4˚C for three days. Next, sections were washed 3 x 15 min in PBS and incubated in secondary antibody (1:600; Cy3-conjugated goat anti-rabbit, Jackson ImmunoResearch, 111-165-144) in diluent for 2 hr at 4˚C in the dark. Sections were then washed 3 x 15 min in PBS, mounted on slides with glycerol, and coverslips sealed with clear nail polish. Slides were stored at 4˚C in the dark until confocal imaging, which was done within 2 wk of staining. Ewa et al. (16) tested the specificity of this primary antibody for BDNF using western blots on hypothalamic and hippocampal tissues. Specific binding with bands at 18kDa for mature BDNF protein and at 30kDa for precursor BDNF was demonstrated. They also found no non-specific staining when primary or secondary antibodies were omitted. We also tested the specificity of the primary antibody using the same immunostaining procedure described above except no primary antibody was added to the goat diluent. No non-specific staining was observed.

Quantification of cells exhibiting BDNF-LIR. The ectorhinal cortex (referred to as CORTEX) was selected to assess whether food deprivation (or in Experiment 2, WD feeding) has global effects on BDNF protein expression (i.e. whether these manipulations have effects on BDNF protein outside the hypothalamus). The ectorhinal cortex was chosen for this purpose because it was present in sections used to count the ARC, VMH, and PVN and it could be readily identified. Slides were coded to permit blind quantification of labeled neurons in each section. Then sections were screened using several criteria to determine whether their quality was sufficient for counting. First, the cortex had to be intact and not folded in the areas counted. Second, the section had to be intact in areas counted and no areas of the section missing. Third, there had to be no obvious right to left or top to bottom staining gradients as a result of uneven staining. Three FED and two DEP animals were dropped because their brain sections did not meet these criteria, resulting in final group sizes of n = 7 (FED) and n = 8 (DEP).

An Olympus BX-DSU spinning disk confocal microscope and AL594 filter were used to image each nucleus using a Hamamatsu 1394 ORCA-ERA SIN 660671 camera at a magnification of 100X. Twenty-one optical sections were collected using 5000 ms exposure times and 1 μm steps between sections. Nuclei within each brain section from each animal were always scanned in the same order to minimize effects of fading on comparisons across animals: left ARC, right ARC, left VMH, right VMH, left PVN, right PVN, left CORTEX and right CORTEX (designation of right and left was based on the orientation of the section on the slide). The most clearly focused optical section collected from each half-nucleus (cells in sharpest focus across the entire imaged portion of the section) was identified and used for analysis. Examples of sections used for counting are shown in Figure 1.

Single plane images were then analyzed using Image J (National Institute of Health, Version 1.48 for Mac OSX 10.5.) according to methods adapted from “Image J: Area Measurements and Particle Counting Tutorial & Examples” (55). Images were converted to an

8-bit image and then inverted. Background was removed using a 50.0 pixel rolling ball radius. Images were then converted to a binary image and noise was removed using the despeckle feature. The border of each nucleus was outlined based on cresyl violet sections and The Mouse Brain Atlas (21). Cells that exhibited BDNF-LIR were counted using the analyze particle settings. Optical sections of the CORTEX were collected by lining up the bottom of the ectorhinal cortex with the bottom of the image frame. The outer most area of the second layer of the cortex was then aligned with the vertical image frame (vertical frame on the right side of ectorhinal cortex for the right side of the brain and on the left for the left side). The entire optical section obtained with this alignment was used for quantification. For each nucleus in each animal, three brain sections were scanned and used to quantify BDNF immunoreactive neurons. The number of BDNF-LIR cells were counted in both right and left sides of the brain and summed.

BDNF-LIR cell fluorescence intensity was quantified using the same images employed for quantification of cell number (e.g., 37). The consistent, but weak staining intensity encountered with commercial BDNF antibodies such as the one we employed resulted in an exceedingly small dynamic range in the staining intensity of our samples. Consequently, the magnitudes of group differences were less than 1%. Because meaningful comparisons of intensity could not be obtained, they are not discussed in the Results section.

Quantitative reverse transcriptase polymerase chain reaction (qPCR). To confirm the BDNF protein expression findings in this experiment with a complementary method, *BDNF* mRNA levels were measured using qPCR. Additional groups of FED (n=6) and DEP (n=6) mice treated the same as described above were used to examine BDNF mRNA expression. *BDNF* mRNA was quantified from RNA extracted from the ARC, VMH, PVN and CORTEX of FED and DEP. At the end of the 48 hr food deprivation period for the DEP group, all mice were sacrificed by cervical dislocation. Tissues were immediately dissected from 200 μm-thick coronal slices

through the hypothalamus, using a tissue punch (23 gauge needle), and then RNA was extracted using the RNAqueous(r)-Micro Total RNA Isolation Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Each RNA sample was incubated with DNase1 (Invitrogen) to remove genomic DNA. Then first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit in 30 µl PCR reactions according to the manufacturer's instructions (Thermo Fisher Scientific).

To quantify *BDNF* mRNA levels from the hypothalamic and cortical samples, qPCR was performed on the mRNA samples. Amplification of 4 ng cDNA from the first-strand reaction was performed in triplicate using a previously established protocol (3, 11, 19, 72). Based on this protocol, primer sequences employed were: *BDNF* forward: 5' GAA AGT CCC GGT ATC CAA AG 3', *BDNF* reverse: 5' CCA GCC AAT TCT CTT TTT 3', *β-actin* forward: 5' GGC TGT ATT CCCC TCC ATC G 3', *β-actin* reverse: 5' CCA GTT GGT AAC AAT GCC ATG T 3'. All primers were optimized such that the correlation coefficient was 0.99–1.0 and the PCR efficiency was 95–100%. Real-time PCR amplification was performed using an iCycler and PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences, MA).

Data Analysis. Values reported are means ± SEM. Graphs were made using Graphpad Prism 4.0 (Graphpad Software; San Diego, CA) and statistical analyses were performed using Statistica 6.0 (StatSoft; Tulsa, OK). In all statistical analyses, $p < 0.05$ was required for statistical significance. Body weight and food intake for FED and DEP groups were analyzed using an unpaired t-test. The independent variable was the diet condition (FED or DEP) and the dependent variable was the food intake in kilocalories (kcal), or body weight in grams. For comparisons of stable food intake and body weight across groups, the average daily food consumption and body weights, respectively, of each group were collapsed across the 5 days of stable intake and then compared using the unpaired t-test. The same test was employed to assess differences between the FED and DEP groups in the numbers of BDNF-LIR cells and

303 relative *BDNF* mRNA levels in the ARC, VMH, PVN, or CORTEX. The independent variable
304 was the diet condition (FED or DEP) and the dependent variable was the number of BDNF-LIR
305 cells within each brain region. Changes in *BDNF* mRNA levels between FED and DEP mice
306 were determined by comparing changes in *BDNF* with *β-actin*, a constitutively expressed
307 housekeeping gene. This was done using the Livak and Schmittgen method (44). Briefly, the
308 difference in CT values between *BDNF* and *β-actin* in each tissue was calculated and this
309 difference was compared in FED and DEP mice as determined previously (3, 19). Based on
310 studies that manipulated BDNF in the forebrain-hypothalamus, VMH, or PVN and suggested
311 BDNF inhibits feeding, we hypothesized BDNF-LIR cell number and *BDNF* mRNA would
312 decrease in the VMH, and possibly in the ARC and PVN of the DEP compared to the FED
313 group after 48 hr food deprivation. To our knowledge, no studies have measured the effects of
314 24-72 hour food deprivation on BDNF protein expression in this area of cortex. Chronic food
315 restriction (e.g., 3-4 weeks or longer) was found to have no effect on BDNF protein levels in the
316 prefrontal cortex, but 3 months of caloric restriction increased BDNF in an unspecified region of
317 cerebral cortex (14, 42, 51). This information is not sufficient to make a prediction about the
318 effect of 48-hour food deprivation on BDNF protein in the ectorhinal cortex. Therefore, we
319 predicted there would be no change.
320
321 **Experiment 2: Short-term effects of a WD on BDNF protein expression in the ARC, VMH,**
322 **and PVN**
323 *Diets.* Two diets were used throughout this experiment: (1) A powdered diet modeling
324 the “WD” (5TJN, catalog no.1810850, TestDiet, St. Louis, MO), which contains approximately
325 39.9% fat, 44.1% carbohydrate (sucrose) and 16.3% protein and has an energy density of 4.49
326 kcal/g. (2) A control diet, the standard chow maintenance diet, but in powdered form
327 (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO), which contains approximately 13.5% fat,
328 58% carbohydrates and 28.5% protein and has an energy density of 3.34 kcal/g. To model a

WD, a diet must contain high levels of fats and refined sugars and must produce dietary obesity – obesity due to increased food (calorie) intake (27). A WD is typically defined as a diet containing at least 35% fat, approximately 50% carbohydrates and 15% protein (22). The diet selected for the current experiment was similar to diets used in many studies of the effects of nutrition or energy status on BDNF expression in the brain (e.g., 48). The control diet chosen for this experiment was a standard laboratory chow rather than a diet with ingredients matched to the WD because chow has been the control diet in the majority of the studies of WD effects on central BDNF expression. Moreover, our mice were maintained on this diet since birth. Therefore, eliminating any changes in diet upon the start of the experiment reduced possible confounds.

Experimental design. Mice were housed individually and fed powdered standard chow in 2 oz. spill proof glass jars (Unifab, Kalamazoo, MI, USA; (65). Food intake and body weight were measured daily and stable food intake determined as defined in Experiment 1. After food intake stabilized, all animals were fed only powdered WD overnight (15 hr). This pre-exposure was done to prevent neophobia from occurring at the start of the test diet phase. Mice were then returned to powdered standard chow for two weeks to minimize any potential effects of WD pre-exposure on BDNF expression.

Animals were assigned a duration of test diet exposure of either 6 hr (n = 21), 48 hr (n = 29), 1 wk (n = 30) or 3 wk (n = 27). The groups of animals assigned to each duration were matched on average food intake and body weight as measured during the period of stable food intake. Each duration group was further divided into WD, WD-food restricted (WD-FR) and CHOW test diet groups except for the six-hour group, which was divided into WD and CHOW test diet groups. The WD-FR group was included to assess the role of the nutrients composing the WD themselves independent of hyperphagia associated with ad libitum consumption of a WD in any changes in BDNF protein expression observed. To achieve this, these mice were fed

daily an amount of the powdered WD equal to the average daily caloric consumption of chow by the CHOW group during the five-day period of stable food intake. For each of the durations, the WD, WD-FR and CHOW groups were also matched using food intake and body weight during the period of stable intake. This should have minimized the possibility differences in initial body weights confounded interpretation of group differences in the effect of the WD on BDNF expression. The original group sizes were: 6 hr WD (n=10), CHOW (n=10); 48 hr WD (n=10), WD-FR (n=10), CHOW (n=9); 1 wk WD (n=10), WD-FR (n=10), CHOW (n=10); 3wk WD (n=9), WD-FR (n=9), CHOW (n=9). The WD group received the powdered WD diet ad libitum during the test diet exposure. Fresh WD was given at a minimum of every 3 d. The WD-FR group was fed a pre-determined amount of the powdered WD that contained the average number of calories consumed by the CHOW group during the five-day period of stable food intake. The CHOW group received powdered chow ad libitum during the test diet exposure that was replaced every 3 days at a minimum.

Tissue preparation, immunohistochemistry, quantification and data analysis were performed as described for Experiment 1 unless stated otherwise. The brain regions examined for BDNF expression were also the same as in Experiment 1, including the ARC, PVN, VMH, and CORTEX. The CORTEX was included to gauge the effects of WD feeding on BDNF protein levels outside the hypothalamus, similar to Experiment 1.

Tissue processing. All animals were sacrificed and perfused with fixative at 1400, immediately prior to onset of the dark phase of the light cycle and within $\pm$ 1 hr of the end of their test diet exposure duration. Animals were processed in groups of three that included 1 WD, 1 WD-FR and 1 CHOW mouse from either the 6 hr, 48 hr, 1 wk, or the 3 wk group. Data from mice were not included in analysis if brain sections failed to meet the criteria described in the quantification section of Experiment 1 (mice dropped: 6 hr group n=2, 1 WD, 1 CHOW; 48 hr group n=3, 1 WD, 1 WD-FR, 1 CHOW; 1 wk group n=5, 1 WD, 2 WD-FR, 2 CHOW; 3 wk group

n=1, 1 WD). Data from mice with numbers of BDNF-LIR cells that were greater than 2 standard deviations from the mean of their group were also excluded from analysis (6 hr group n=2, 1 WD, 1 CHOW; 48 hr group n=2, 1 WD, 1 WD-FR; 1 wk group n=3, 1 WD, 1 WD-FR, 1 CHOW). The final group sizes were: 6 hr WD (n=8), CHOW (n=8); 48 hr WD (n=8), WD-FR (n=8), CHOW (n=8); 1 wk WD (n=8), WD-FR (n=7), CHOW (n=7); 3wk WD (n=8), WD-FR (n=9), CHOW (n=9).

Data analysis. For the 6 hr test diet exposure groups, differences in body weight and food intake were analyzed using a Student's unpaired t-test, whereas, for the 48 hr groups, one-way ANOVA with Bonferroni's Multiple Comparisons posthoc test was employed. The independent variable was the diet condition (WD, WD-FR and CHOW) and the dependent variable was either food intake in kcal, or body weight in grams. Repeated measures ANOVA across days was used to analyze group differences in food intake and body weight in the 1 and 3 wk test diet exposure groups. When the overall repeated measures ANOVA was significant, pairwise group comparisons were made between test diet groups using repeated measures ANOVA across days. Pairwise group comparisons of the numbers of cells exhibiting BDNF-LIR were made according to a priori hypotheses, using a Student's unpaired t-test. The independent variable was the time exposed to each diet condition and the dependent variable was the number of BDNF-LIR neurons within each brain region. Based on studies that manipulated BDNF in the forebrain-hypothalamus, VMH or PVN, and suggested BDNF inhibits feeding, we hypothesized BDNF-LIR cell number would increase in the VMH and possibly in the ARC, and PVN of WD mice compared to the WD-FR and CHOW group at all test diet durations. As there was no evidence for short-term effects of a WD on CORTEX BDNF protein levels, we predicted there would be no effect.

RESULTS

Experiment 1: Effect of food deprivation on BDNF protein expression in the ARC, VMH, and PVN

Food intake and body weight. ANOVA demonstrated there were no group differences in age and Bartlett's test showed there were no group differences in variances in age (data not shown). During the period of stable food intake, the FED and DEP groups exhibited similar average daily food intake ($t = 0.44$, $p = 0.33$; Fig. 2A) and body weight ($t = 0.64$, $p = 0.27$; Fig. 2B). Over the course of the 48 hr deprivation period, food intake for FED animals was greater than for DEP mice (Fig. 2A; no statistical test was done as no variance in DEP group). Body weight of DEP mice was significantly reduced after 24 (13%; 21.88 $\pm$ 0.45 g) and 48 (21%; 20.67 $\pm$ 0.52 g) hr compared with their initial weight (24.22 $\pm$ 0.43 g; $t = 4.17$, 6.47, respectively, both $p < 0.01$; Fig. 2B). Body weight for the FED group after 48 hr was 24.94 $\pm$ 0.52, which was similar to their weight at 0 hr (24.85 $\pm$ 0.54; $t = 0.13$, $p = 0.45$) and significantly greater than the weight of DEP mice at 48 hr (20.67 $\pm$ 0.52 g; $t = 6.66$, $p < 0.01$; Fig. 2B).

BDNF protein expression. The number of cells that exhibited BDNF-LIR in each brain region examined is shown in Fig. 2C. The number of BDNF-LIR cells in the ARC was significantly decreased in DEP as compared with FED mice (32%, $p < 0.05$). In the VMH there was a trend toward a decrease in BDNF-LIR cell number in DEP compared to FED mice, but it was not significant (27%; $p = 0.09$). There were no differences in BDNF-LIR cell numbers between FED and DEP groups in the PVN ($p = 0.12$) and CORTEX ($p = 0.27$).

BDNF mRNA expression. To confirm the effect of 48 hr food deprivation on BDNF protein expression in the ARC, we compared *BDNF* mRNA expression in the ARC as well as in

the, PVN, VMH, and CORTEX in the FED and DEP groups (Fig. 2D). Levels of *BDNF* mRNA normalized to *β-actin* mRNA in the brain regions of the FED group were set at 100%. Similar to BDNF protein expression, qPCR showed a significant decrease in *BDNF* mRNA levels only in the ARC (62%, $t = 2.57$, $p < 0.05$), a non-significant decreasing trend in the VMH (31%, $t = 1.12$, $p = 0.12$), no change in the PVN ($t = 0.49$, $p = 0.32$), and a non-significant increasing trend in the CORTEX ($t = 1.624$, $p = 0.07$).

Experiment 2: Short-term effects of a WD on BDNF protein expression in the ARC, VMH, and PVN

The effects of 6 hr, 48 hr, 1 wk, or 3 wk of WD consumption on BDNF protein expression in the ARC, VMH, and PVN were examined. Expression in the CORTEX was also investigated to gauge the potential for an effect of diet on brain regions outside the hypothalamus. The t and p values for comparisons of BDNF-LIR cell numbers are listed in Table 1. ANOVA demonstrated there were no group differences in age and Bartlett's test showed there were no group differences in variances in age (data not shown).

Six-hour test diet exposure.

Food intake and body weight. During the period of stable food intake, WD and CHOW animals consumed similar amounts of food ($t = 0.06$, $p = 0.47$; data not shown) and exhibited similar body weights ($t = 1.13$, $p = 0.14$; data not shown). The day prior to test diet exposure animals did not differ in body weight ($t = 0.13$, $p = 0.45$; data not shown) or food intake ($t = 0.01$, $p = 0.50$; data not shown). During the 6 hr test diet exposure, food intake was greater for WD than CHOW animals (48% increase; $t = 5.85$, $p < 0.01$; Fig. 3A). This group difference in test diet consumption did not produce a group difference in body weight ($t = 0.42$, $p = 0.34$; Fig. 3B).

460 *BDNF protein expression.* The measure used to estimate WD-induced changes in BDNF
461 protein levels was the number of cells exhibiting BDNF-LIR in each brain region (Fig. 3C).
462 Despite the increase in food intake in WD-fed mice, no differences in BDNF-LIR cell numbers
463 were observed in any of the brain areas examined between the WD and CHOW groups after 6
464 hr of test diet exposure. Mean cell counts in the WD and CHOW groups were similar in the
465 ARC, VMH, PVN, and CORTEX.
466
467 Forty-eight-hour test diet exposure.
468 *Food intake and body weight.* During the period of stable food intake, WD, WD-FR, and
469 CHOW animals consumed similar amounts of food ($F_{2,21} = 1.69$, $p = 0.21$; data not shown) and
470 exhibited similar body weights ($F_{2,21} = 1.20$, $p = 0.32$; data not shown). The day prior to test diet
471 exposure, animals did not differ in food intake ($F_{2,21} = 0.06$, $p = 0.95$; data not shown) or body
472 weight ($F_{2,21} = 0.96$, $p = 0.39$; data not shown). WD mice consumed significantly more food than
473 the WD-FR and CHOW groups during the test diet exposure phase (34 and 29% increases,
474 respectively; $F_{2,21} = 11.01$, both $p < 0.01$; Fig. 4A). No group differences in body weight
475 emerged by the end of the test diet exposure ($F_{2,21} = 2.33$, $p = 0.12$; Fig. 4B).
476
477 *BDNF protein expression.* There were no significant group differences in mean number
478 of BDNF-LIR cells in any of the brain areas examined after 48 hr test diet exposure (Fig. 4C). In
479 the VMH there was a non-significant trend towards an increase in BDNF-LIR cells in WD as
480 compared with CHOW mice (33%). In the CORTEX there were trends toward increases in
481 BDNF-LIR cells in WD (18.5%) and WD-FR (17%) compared with CHOW mice, but they were
482 not significant.
483
484
485 One-week test diet exposure.

Food intake and body weight. During the period of stable food intake, WD, WD-FR, and CHOW animals consumed similar amounts of food ($F_{2,19} = 0.08$, $p = 0.93$; data not shown) and exhibited similar body weights ($F_{2,19} = 1.19$, $p = 0.33$; data not shown). Moreover, these groups did not differ in body weight ($F_{2,19} = 0.55$, $p = 0.59$) or food intake ($F_{2,19} = 0.27$, $p = 0.77$) the day prior to test diet exposure (data not shown). Repeated measures ANOVA of group food intakes over days during test diet exposure showed significant main effects of diet and days ($F_{2,19} = 10.67$, $F_{7,133} = 2.80$, both $p < 0.01$) and a significant interaction (days x diet; $F_{14,133} = 3.55$, $p < 0.01$; Fig 5A). Pairwise comparisons between these same group food intakes using repeated measures ANOVA over days showed significant increases between WD and WD-FR and WD and CHOW groups (23 and 31% increases, respectively, based on averages over the 7 days; $F_{7,91} = 3.22, 4.86$, respectively, both $p < 0.01$), but no difference between WD-FR and CHOW mice ($F_{7,84} = 1.27$, $p = 0.27$). Repeated measures ANOVA of group body weights over days during test diet exposure demonstrated a significant main effect of days ($F_{7,133} = 16.80$, $p < 0.01$), but not of diet ($F_{2,19} = 2.28$, $p = 0.71$) and a significant interaction (diet x days; $F_{14,133} = 8.12$, $p < 0.01$; Fig 5B). Pairwise comparisons of these same group body weights using repeated measures ANOVA over days showed significant increases in WD compared to WD-FR and CHOW mice (both 11% based on averages over the 7 days; $F_{7,91} = 6.31, 2.49$, respectively, both $p < 0.01$), whereas, WD-FR and CHOW mice were not different ($F_{7,84} = 1.27$, $p = 0.27$).

BDNF protein expression. No group differences in the mean number of BDNF-LIR cell numbers were observed in the ARC (Fig. 5C). The increasing trend in the mean number of BDNF-LIR cells in the VMH of WD as compared with CHOW mice observed after 48 hr test diet exposure was further increased to 40% and became significant after 1 wk of exposure (Fig. 5C). No group differences in the mean number of BDNF-LIR cell numbers were observed in the PVN (Fig. 5C). In the CORTEX, similar to the VMH, the increasing trend in the mean number of BDNF-LIR cells of WD as compared with CHOW mice observed after 48 hr test diet exposure

was further increased to 20% and became significant after 1 wk of exposure (Fig. 5C). In contrast, the increasing trend of 13% in the WD compared to the WD-FR group was not significant (Fig. 5C).

Three-week test diet exposure.

Food intake and body weight. During the period of stable food intake, WD, WD-FR, and CHOW animals consumed similar amounts of food ($F_{2,23} = 3.34$, $p = 0.05$; data not shown) and exhibited similar body weights ($F_{2,23} = 0.34$, $p = 0.71$; data not shown). Further, these groups did not differ in body weight ($F_{2,23} = 0.09$, $p = 0.91$) or food intake ($F_{2,23} = 2.63$, $p = 0.09$) on the day prior to test diet exposure (data not shown). Repeated measures ANOVA of group food intakes over days during test diet exposure revealed significant main effects of days and diet ($F_{21,483} = 5.02$, $F_{2,23} = 5.74$, both $p < 0.01$), and a significant interaction (days x diet; $F_{42,483} = 2.75$, $p < 0.01$; Fig. 6A). Pairwise repeated measures ANOVA of these same group food intakes over days showed a significant decrease in WD-FR compared to CHOW mice (18% decrease based on averages over 3 wk; $F_{21,336} = 2.95$, $p < 0.01$). In contrast, an increasing trend in the WD compared to the WD-FR group failed to reach significance (20% increase based on averages over 3 wk; $F_{21,315} = 1.56$, $p = 0.06$). Some WD-FR mice did not consistently consume all of the food they were offered each day. The amount of food they were offered matched they average daily caloric intake of the CHOW group during the 5-day period of stable chow intake. This amount of food did not fully account for the slight increase in caloric consumption of the CHOW group with age from the 5 days of stable intake to the three-week test diet phase (an average of 1.5 kcal/day). Nevertheless, on average, the WD-FR mice ate even less (13.21 kcal/day) than they consumed during the stable intake phase (13.89 kcal/day) and CHOW mice ate during this phase (14.62 kcal/day). This suggests that their decreased intake relative to the other groups was mainly due to their failure to consistently consume all the restricted amount of food they were offered. Repeated measures ANOVA of group body weights over days during test diet

exposure showed a significant main effect for days ($F_{21,483} = 33.65$, $p < 0.01$) but not diet (Fig. 6B; $F_{2,23} = 1.04$, $p = 0.37$) and a significant interaction (days x diet; $F_{42,483} = 5.65$, $p < 0.01$). Pairwise comparisons of these same group body weights using repeated measures ANOVA over days demonstrated increases in the WD and WD-FR compared to CHOW mice (9 and 7% increases; $F_{21,315} = 9.99$, $F_{21,336} = 10.96$, respectively, both $p < 0.01$), whereas, the WD and WD-FR mice were similar ($F_{21,315} = 0.49$, $p = 0.97$).

BDNF protein expression. No significant differences in the numbers of cells exhibiting BDNF-LIR were observed between the WD, WD-FR, and CHOW groups in the ARC, VMH and PVN, although there were trends toward a decrease in the WD group compared to the WD-FR and CHOW groups in the PVN of 17 and 14.5%, respectively (Fig. 6C). In the CORTEX, however, there was a significant increase in the number of BDNF-LIR cells in the WD-FR compared to the CHOW group (12.5%). None of the other comparisons in the CORTEX were significant (Fig. 6C).

DISCUSSION

The goal of the first experiment was to determine the effect of food deprivation on BDNF protein in the ARC, VMH and PVN. The CORTEX was also examined to make an initial assessment of whether food deprivation altered BDNF levels outside the hypothalamus. BDNF protein levels were estimated by quantifying the number of neurons exhibiting BDNF-LIR in each of these brain areas. Forty-eight hours of food deprivation produced a decrease in BDNF protein expression in the ARC. A decreasing trend also occurred in the VMH, but it was not

significant. The aim of the second experiment was to determine the effect of short-term (six hour, forty-eight hour, one week, or three week) WD consumption on BDNF protein in the ARC, VMH and PVN, measured as in Experiment 1. Caloric intake was increased in WD compared to WD-FR and CHOW mice over the course of the six-hour, forty-eight hour and one week, but not the three-week duration of test diet consumption. In contrast, body weight increased significantly in WD compared to WD-FR and CHOW mice only after one week of test diet consumption and in WD and WD-FR compared to CHOW mice only after three weeks. WD consumption resulted in an increasing trend in BDNF protein expression in the VMH after forty-eight hours that became significant after one week and returned to control levels by three weeks. Within the hypothalamus, this effect appeared to be specific to the VMH as none of the durations of WD diet consumption tested altered BDNF expression in the ARC or PVN. BDNF protein in the CORTEX, however, was also increased after one week of WD exposure and three weeks of restricted WD consumption.

Effect of food deprivation on BDNF protein expression in the ARC, VMH, and PVN

In most areas of the brain that have been examined, BDNF protein expression has been observed to increase in response to chronic dietary restriction, appearing to have a protective effect against some insults to the brain (e.g., hippocampus, cerebral cortex and striatum; 14). To our knowledge, the effect of food deprivation on BDNF protein expression in the hypothalamus has not been examined. In the present study, there was a decrease in BDNF-LIR cell numbers in the ARC in response to forty-eight hour food deprivation. Similarly, *BDNF* mRNA in the ARC was reduced by this duration of food deprivation. There were trends toward decreases in the VMH for both protein and mRNA, but they did not reach significance. In contrast, three previous studies have found significant reductions of *BDNF* mRNA in the VMH (71, 72, 77). The failure for this decrease to reach significance in the present study could have been due to methodological differences between studies. Two of the previous studies used in situ

hybridization to detect *BDNF* mRNA (72, 77) and the third measured expression of only 2 of several different *BDNF* transcripts (71). Therefore, if some *BDNF* transcripts that were not measured did not decrease, or increased, and all transcripts were combined for measurement, it is possible the significant decreases would have washed out and been a non-significant trend as observed in the present study.

The degree to which this reduction of BDNF expression in the ARC contributes to satiety will depend on which ARC neurons are regulated by this BDNF and whether they are excited or inhibited. The ARC contains both POMC neurons that inhibit feeding and AGRP neurons that promote feeding. A small percentage of POMC and AGRP neurons express trkB receptors (39). These neurons account for only about 30% of ARC neurons that express trkB receptors (39). Consequently, a large number of neurons of unknown neurochemical identity in the ARC express trkB receptors and could therefore be regulated by BDNF. Ultimately, the present findings raise the possibility that reduced BDNF levels in the ARC following food deprivation may alter the sensitivity or activity of the AGRP-, POMC-, or unidentified ARC neurons that express trkB receptors to contribute to food deprivation-induced eating.

Effect of a WD on BDNF protein expression in the ARC, VMH, and PVN

Mice in the WD group showed an increase in BDNF-LIR cell number in the VMH compared to the WD-FR and CHOW groups after one week of consuming their test diets. Food intake was increased in WD animals after forty-eight hours and one week of test diet exposure, whereas, their body weight did not increase until one week of exposure. This could imply either the duration of overconsumption of the WD, the consequent increase in body weight, or the combination of both, contributed to the increase in VMH BDNF protein expression after one week of WD consumption. These group comparisons also identify some factors that are unlikely to have contributed to the increased VMH BDNF. In particular, the one-week WD-FR group consumed the same WD diet for the same duration as the one-week WD mice, but were not

permitted to overeat and did not exhibit any change in VMH BDNF protein levels. This observation suggests the one-week duration of WD consumption on its own could not have produced the increase in BDNF protein expression in the VMH. It further suggests the high-carbohydrate, high-fat macronutrient content of the WD on its own was not likely to have caused the increase in VMH BDNF. Thus, it appears overconsumption of the WD for one week was necessary to increase VMH BDNF protein.

The increase in VMH BDNF protein expression in the present study appeared to be selective within the hypothalamus to the VMH after one week of WD exposure, as no changes in BDNF protein levels were observed in the ARC or PVN after any of the durations of WD consumption tested. It remains possible, however, changes in BDNF expression within these nuclei could have been missed if they were associated with a subset of PVN or ARC neurons defined spatially, cytoarchitecturally, or neurochemically. Counts of these neurons would have been diluted by counts of the remaining subsets of neurons that did not exhibit a change.

The most parsimonious hypothesis to explain the increase in BDNF expression in the VMH after one week of WD consumption is that it was a compensatory response activated by the effects of the initial hyperphagia or excess weight gain to inhibit food intake and prevent or moderate excess weight gain. It remains to be demonstrated, however, whether this increase in BDNF is successful at inhibiting food intake, and if so to what degree. Little is known about how diet influences expression of BDNF or other molecules involved in neural function and communication. It was recently reported that forty-eight hours of hyperglycemia and hyperinsulinemia led to increased BDNF expression in the posterior VMH (50). Therefore, if one week of WD consumption caused similar metabolic changes, these could have contributed to the increased BDNF protein expression we observed in the VMH. It is also possible the increase in VMH BDNF was mediated by leptin. As animals develop dietary obesity circulating leptin levels typically increase (e.g., 43). Intravenous injection of leptin has been shown to increase BDNF protein in VMH neurons and in axons within the VMH and DMH that were closely

opposed to neurons expressing trkB (32). Moreover, C57Bl/6 mice consuming a WD for one week still respond to leptin by reducing food intake to a similar degree as controls, suggesting leptin resistance had not yet developed (40). Thus, if an increase in circulating leptin occurred by one week of WD consumption in the present study it could have led to an increase in VMH BDNF levels.

BDNF protein expression returned to control levels between one- and three weeks of WD feeding. Understanding the mechanism underlying this decrease could be valuable for identifying a means of maintaining increased VMH BDNF, which might moderate hyperphagia and weight gain. One process that could account for this decrease in VMH protein is the build-up of free radicals that lead to oxidative stress (OS). OS occurs in the hypothalamus of obese rodents and it decreases BDNF expression by reducing binding of a key transcription factor, CREB, to the BDNF promoters (17, 29, 41, 56, 69, 80). Another process that could contribute to the decrease in VMH BDNF protein that occurred between one- and three weeks of WD consumption is hypothalamic insulin resistance (9, 13), which could lead to reduced glucose uptake by glucose-sensitive VMH neurons, and therefore, result in decreased activity of these neurons (63). The probability such an effect could lead to reduced VMH BDNF levels is high given that a large proportion of VMH BDNF neurons have insulin receptors (38), and that BDNF expression is activity dependent, possibly involving epigenetic modification of the BDNF promoter (47). Finally, if leptin was involved in the increase in VMH BDNF protein, then leptin resistance could have contributed to the decrease of BDNF expression to control levels after three weeks of WD exposure. In rodents, leptin resistance appears to develop after a few weeks of HED or WD consumption (59, 60), and therefore, could have prevented leptin from maintaining an increase in VMH BDNF expression.

A potential alternative explanation for the increase in numbers of BDNF-LIR cells in the VMH after one week of WD consumption is that neurogenesis supplied additional BDNF neurons to the VMH. The available evidence, however, suggests it is unlikely neurogenesis

672 occurred in the VMH in the present study. First, hypothalamic neurogenesis associated with
673 consumption of a HED has only been observed in females – it was not detected in males (36)
674 and we only studied males. Second, only a small number of new neurons were generated in the
675 hypothalamus of females in this study. Moreover, the new neurons only occurred in the median
676 eminence, not in the VMH. Third, if neurogenesis accounted for the increased BDNF-LIR cell
677 numbers present after one week of WD consumption, then the cells generated should still be
678 present after three weeks of WD feeding. We found no increase, however, in BDNF-LIR cells in
679 the VMH in the mice that consumed the WD for this duration. Finally, in a preliminary analysis
680 we found no evidence for neurogenesis in the VMH. To examine whether total VMH neuron
681 number increased, as would be expected if WD-induced neurogenesis had occurred, we
682 performed total counts of cresyl violet-stained neurons in the ARC, VMH, PVN and CORTEX for
683 mice that consumed the WD for one week, using the sections from the BDNF immunostaining
684 that had been set aside for cresyl violet staining (see Methods section). No significant
685 differences in the total number of cresyl violet-stained neurons were found between the WD,
686 WD-FR, and CHOW one-week test diet groups for the ARC, VMH, PVN or CORTEX (data not
687 shown). These preliminary results are consistent with the interpretation the increase in number
688 of VMH cells exhibiting BDNF-LIR after one week of WD consumption was due mainly to
689 increased expression of BDNF from undetectable to detectable levels in some cells.
690
691 **Effect of a WD on BDNF expression in the CORTEX (echtorhinal cortex)**
692 The ectorhinal cortex was investigated as an initial assessment of whether food
693 deprivation (Experiment 1), or WD feeding (Experiment 2), has global effects on BDNF protein
694 expression (i.e. whether these manipulations have effects on BDNF protein outside the
695 hypothalamus). In a large number of previous studies, 16-21 weeks of HED (in some studies
696 these diets fit the criteria of a WD and in others they did not) feeding has typically reduced
697 BDNF protein expression in the prefrontal cortex (PFC), or an unspecified cortical region (e.g.,

6). Instances of decreased BDNF protein expression in the prefrontal cortex, typically about 35% in magnitude, were associated with impairment of certain types of memory such as recognition (e.g., 6).

In the present study, after much shorter durations of WD exposure, increased rather than decreased BDNF protein expression occurred in the CORTEX. These increases occurred after one week for the WD group (20%) and three weeks for WD-FR mice (12.5%). Because these increases were modest, their physiological relevance should be interpreted with caution. Moreover, the augmented BDNF expression in the CORTEX of the three week WD-FR group was confounded by a significant decrease in caloric intake compared with the three week CHOW mice. Thus, it is not possible to distinguish the contributions of WD consumption and reduced caloric intake to the increase in BDNF protein expression. The increase in BDNF protein in the ectorhinal cortex after one week of WD feeding suggests WD consumption can affect BDNF protein expression outside the hypothalamus. Similar to the argument presented above regarding increased VMH BDNF protein after one week of WD feeding, the hyperphagia or excess body weight gain associated with this duration of WD consumption probably contributed to the increased CORTEX BDNF protein in the WD group. The increase in CORTEX BDNF observed in the WD-FR group after three weeks of WD consumption raises the possibility that such a long exposure to the nutrient composition of the WD may produce a modest increase in CORTEX BDNF protein even without overeating. Interestingly, similar to the contrasting effects of WD vs. restricted WD feeding on VMH vs. CORTEX BDNF protein levels in the present study, altered BDNF mRNA expression was observed in the VMH and hippocampus of mice after long-term (fourteen weeks) ad libitum consumption of a WD, but occurred only in the hippocampus of mice pair fed the WD (79).

The scarcity of information on the ectorhinal cortex makes it difficult to envision what the potential significance of diet-induced increases in its BDNF levels might be. The ectorhinal cortex appears to regulate autonomic function, especially involving the sympathetic nervous

724 system (76). It receives input from the posterior basomedial amygdala, which is involved in
725 assigning emotional significance to sensory stimuli, especially those associated with stress,
726 anxiety and fear (23, 26, 46). The ectorhinal cortex projects to the amygdala transition area and
727 extended amygdala (54). These areas project to brainstem autonomic regions to regulate the
728 adrenal gland and peripheral sympathetic ganglia, including the stellate and celiac ganglia,
729 which innervate the heart and GI tract, respectively (54, 76). Thus, the ectorhinal cortex may be
730 involved in activation of sympathetic responses that contribute to emotion, anxiety and fear, and
731 increased BDNF levels might modulate this activation.
732
733 In conclusion, the effects of manipulations that produce negative- (food deprivation) and
734 positive (WD feeding) energy balance on BDNF protein expression in the hypothalamic ARC,
735 VMH, and PVN nuclei, key brain regions in the regulation of feeding and body weight, were
736 examined in normal mice. Considering the widespread expression of BDNF, we also examined
737 the ectorhinal cortex to begin to assess the potential for these energy-balance manipulations to
738 have global effects on BDNF protein. BDNF protein expression was estimated as the number of
739 cells exhibiting BDNF-LIR in each brain region examined. Compared with ad libitum CHOW
740 intake, forty-eight hour food deprivation caused a decrease in BDNF protein expression in the
741 ARC. In contrast, WD feeding increased BDNF protein expression in the VMH and CORTEX
742 after one week, but not after six hours, forty-eight hours, or three weeks and not in the ARC or
743 PVN. Additionally, three weeks of restricted WD food intake (WD-FR) produced a small,
744 selective increase in BDNF protein expression in the CORTEX. These results suggest opposite
745 manipulations of energy balance such as food deprivation vs. WD consumption can have
746 opposite effects on hypothalamic BDNF protein expression. These effects occurred, however, in
747 different hypothalamic nuclei (ARC vs. VMH) and over different time courses (forty-eight hours
748 vs. one week). These changes in BDNF levels are most probably part of the compensatory
749 homeostatic responses activated by the deviations from energy balance that result from food

deprivation and WD feeding. For example, one of the goals of responses to food deprivation would be to increase food intake, an effect that occurs in response to decreased BDNF. In contrast, one of the goals of responses to WD feeding would be to decrease eating, an effect that occurs in response to increased BDNF. The degree to which these altered BDNF levels contribute to changes in food intake remains to be established. The possible role of the effects of the WD and WD-FR manipulations on BDNF protein expression in the CORTEX is not clear, but may involve modulation of cortical regulation of autonomic, probably sympathetic, responses that accompany some emotional behaviors.

Perspectives and significance

Long-term (4-6 months) consumption of WD's have consistently reduced BDNF protein levels in the various brain areas that have been examined, including the hippocampus and prefrontal cortex (6, 48, 73). Given BDNF's potent anorexigenic activity, if a WD also reduced BDNF in the VMH, this would essentially decrease the baseline level of inhibition of feeding and thus could contribute to the WD-induced hyperphagia (1, 35, 53, 57, 72, 74, 75). Instead, we found short-term (one week) consumption of a WD increased VMH BDNF protein. This raises the possibility that the increase in VMH BDNF was part of a homeostatic response to counter the WD-induced overeating and excess weight gain. Consistent with this possibility, global BDNF heterozygous knockout mice that could not be distinguished from wild types based on food intake, body weight, meal size or meal frequency when fed a balanced diet, exhibited an exaggerated early hyperphagia compared to wild types when fed a WD (20). This could imply the knockout mice were not able to sufficiently increase BDNF levels to oppose the increase in food intake stimulated by the WD. In the present study, exposure to the WD for three weeks erased the increase in VMH BDNF that occurred after one week. Accordingly, prolonged WD feeding appears to neutralize this increase in VMH BDNF protein expression. If the increase in BDNF did comprise part of a compensatory response to oppose hyperphagia, by removing a

776 potentially inhibitory influence on feeding, this neutralization may have contributed to the
777 development of dietary obesity.

778 Similar to this pattern of reduced expression of VMH BDNF protein between one and
779 three weeks of WD consumption, dietary obesity is associated with altered expression of other
780 central and peripheral feeding regulatory molecules or their receptors (15, 28). The direction of
781 these changes in expression often suggested they would reduce the effectiveness of
782 anorexigens and increase the effectiveness of orexigens. In some instances, this has been
783 borne out. For example, in dietary obese animals, inhibition of feeding by CCK is less effective
784 than in chow fed controls (12). These changes would favor maintenance of obesity because
785 they reduce inhibitory influences on feeding or increase excitatory influences.

786 Importantly, if changes in the effectiveness of feeding signals occur before obesity
787 develops, they are more likely to play a causal role in the obesity than effects that occur after an
788 animal has become obese. Most of the research on the plasticity of feeding signals in dietary
789 obesity has been done in animals that are already obese, and consequently, little is known
790 about the timing of this plasticity relative to different stages of dietary obesity (e.g., 15), but see
791 (2). If the increase in VMH BDNF that occurs after one week of WD feeding is involved in a
792 response that limits hyperphagia and obesity, but subsequently falters, it may be valuable to
793 figure out why this reversal occurs and whether it is possible to prevent it, or even better how to
794 bolster the increase. Such knowledge could contribute to development of a strategy for
795 preventing or moderating dietary obesity. Thus, the finding of early BDNF responses to WD
796 exposure illustrates the importance of determining the timing and direction of changes to a
797 feeding signal in relation to the timeline of dietary obesity. In particular, determination of whether
798 any change in a signaling pathway in dietary obesity occurs prior to an animal becoming obese
799 identifies candidates that should be tested for a causal role. This strategy may be valuable to
800 aid identification of the signaling pathways that deserve the most attention.

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FIGURE LEGENDS

Figure 1. Digital images of the brain regions used for BDNF-LIR quantification are shown here stained with cresyl violet (photomicrographs; A,C,E) or immunohistochemistry using a BDNF antibody (confocal scans; B,D,F)**.** A,B. The VMH and ARC are illustrated. C,D. The PVN is shown. E,F. The ectorhinal cortex (CORTEX) is illustrated on one side of the brain. The region of the CORTEX shown in the confocal image in F is the entire region used to count cells with BDNF-LIR as defined in the Methods section. Scale bars = 100 μm. 3V, third ventricle.

Figure 2. Effect of 48-hour food deprivation on hypothalamic BDNF protein and mRNA levels. Food intake (A) and body weight (B) are plotted for the 5 days of stable chow consumption (day 1-5) and 2 days of food deprivation (DEP group) or continued chow feeding (FED group) (day 6 and 7). There were no differences in food intake or body weight before the start of food deprivation (day 1-5; $p = 0.33$ and 0.27, respectively). Body weight (both $p < 0.01$) and food intake (both $p < 0.01$) of the DEP group were significantly decreased compared with the FED group on days 6 and 7. C. The number of BDNF-LIR cells in the ARC, PVN, VMH and CORTEX are plotted. The number of BDNF-LIR cells were decreased in the VMH and ARC by 27 and 32%, respectively, after 48-hours of food deprivation, but only the decrease in the ARC was significant ($p < 0.05$). No changes in the number of BDNF-LIR cells occurred in the PVN ($p = 0.12$) or CORTEX ($p = 0.27$)**.** D. *BDNF* mRNA levels in the ARC, PVN, VMH and CORTEX are plotted. Bars represent relative percent *BDNF* ± SEM mRNA expression normalized to *β-actin* mRNA. Control values were set at 100%. *BDNF* mRNA levels were decreased in the VMH and ARC by 31% ($p = 0.14$) and 62% ($p < 0.05$), respectively, after 48-hours of food deprivation, but only the decrease in the ARC was significant. No change in *BDNF* mRNA expression was observed in the PVN ($p = 0.32$), and a non-significant increasing trend was observed in the CORTEX of DEP mice ($p = 0.07$)**.**

Figure 3. Food intake (A), body weight (B), and number of BDNF-LIR cells counted (C) are

plotted for the 6-hour test diet exposure groups. A. Food intake was increased by 48% in WD compared to CHOW mice during the 6 hours of test diet exposure ($p < 0.01$). B**.** In contrast, there were no differences in body weight between the WD and CHOW groups after 6 hours of test diet exposure ($p = 0.34$). C**.** There were no differences between the WD, WD-FR and CHOW groups in the BDNF-LIR cell counts in any of the brain areas examined after 6 hours of test diet consumption.

Figure 4. Food intake (A), body weight (B), and number of BDNF-LIR cells counted (C) are plotted for the 48-hour test diet exposure groups. A. Food intake was increased by 34 and 29% in the WD mice compared to the WD-FR and CHOW groups (both $p < 0.01$), respectively, after 48 hours of test diet consumption. B. In contrast, there were no differences in body weight between the WD, WD-FR and CHOW groups after 48 hours of test diet exposure ($p = 0.12$). C. There were no differences in the number of BDNF-LIR cells in any of the brain areas examined There were trends towards an increase in the VMH (WD vs. CHOW, 33% $p = 0.07$) and CORTEX (WD vs. CHOW, 18.5%, $p = 0.09$; WD-FR vs. CHOW, 17%, $p = 0.05$, but they were not significant. Note that BDNF-LIR cell counts for a given nucleus varied across test diet durations. This was due to differences in tissue processing as mice from each of the 2 or 3 groups within a given test diet duration were processed together, whereas, mice from different test diet durations were not processed together. Consistent with this interpretation, the relative differences in BDNF-LIR cell numbers between nuclei were maintained across test diet durations. In particular, BDNF-LIR cell numbers in a given nucleus in WD, WD-FR and CHOW groups changed in parallel from one test diet duration to another.

Figure 5. Food intake (A), body weight (B), and number of BDNF-LIR cells counted (C) are plotted for the 1-week test diet exposure groups. In A and B, Day 0 represents the day prior to test diet exposure. A**.** ANOVA with repeated measures over days showed significant main effects (diet and days) and interaction (days x diet) (all $p < 0.01$) for the food intake of the WD, WD-FR and CHOW groups. Repeated measures pairwise comparisons showed differences in

food intake between WD vs. WD-FR and WD vs. CHOW groups (23 and 31% increases, respectively; both $p < 0.01$), but no difference between WD-FR vs. CHOW mice ($p = 0.27$). B**.** ANOVA with repeated measures over days demonstrated a significant main effect of days ($p < 0.01$), but not diet ($p = 0.72$) and a significant interaction (days x diet) ($p < 0.01$) for the body weights of the WD, WD-FR and CHOW groups. Repeated measures pairwise comparisons of the body weights detected differences between WD vs. WD-FR mice and WD vs. CHOW groups (both 11% increases; both $p < 0.01$), but not between WD-FR vs. CHOW mice ($p = 0.27$). C**.** The number of cells that exhibited BDNF-LIR were increased in WD compared with CHOW mice in the VMH and CORTEX (40 and 20%, respectively; both $p < 0.05$), whereas a trend toward an increase in WD compared to WD-FR mice in the CORTEX was not significant (13% increase, $p = 0.13$). There were no differences in cell numbers showing BDNF-LIR between the WD, WD-FR, and CHOW groups in the ARC or PVN. In panels A and B, the same lower case letters placed to the right of each line in each graph indicate no significant difference between groups, whereas different letters indicate a significant difference between groups.

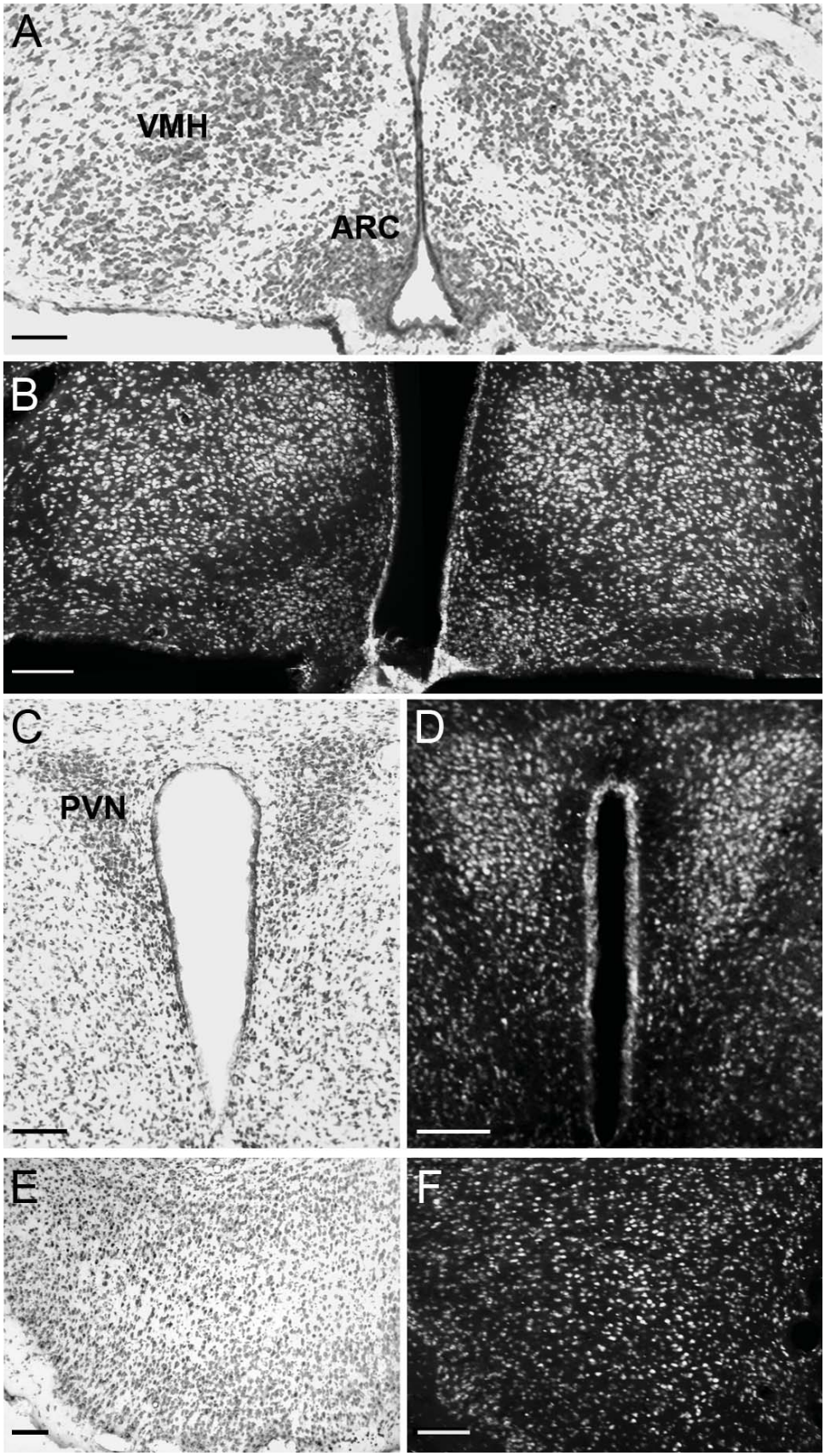
Figure 6. Food intake (A), body weight (B), and number of BDNF-LIR cells counted (C) are plotted for the 3-week test diet exposure groups. In A and B, Day 0 represents the day prior to test diet exposure. A. ANOVA with repeated measures over days showed significant main effects (diet and days) and interaction (days x diet) (all $p < 0.01$) for the food intake of the WD, WD-FR and CHOW groups. Repeated measures pairwise comparisons showed a decrease in food intake in the WD-FR vs. the CHOW group (18% decrease; $p < 0.01$) and an increasing trend in the WD vs. WD-FR mice (20% increase, $p = 0.06$). B**.** ANOVA with repeated measures over days demonstrated a significant main effect of days and diet, and a significant interaction (days x diet) (all $p < 0.01$) for the body weights of the WD, WD-FR and CHOW groups. Repeated measures pairwise comparisons of the body weights detected increases in WD vs. CHOW and WD-FR vs. CHOW mice (9 and 7% increases, respectively; both $p < 0.01$), but there was no difference between WD vs. WD-FR groups ($p = 0.97$). C**.** There were no

differences between the WD, WD-FR and CHOW groups in the BDNF-LIR cell counts in the ARC, or VMH after 3 weeks of test diet consumption. In the PVN there were trends toward decreases in the WD group compared to the WD-FR and CHOW groups, but they were not significant (17 and 14.5%, respectively; $p = 0.06$, $p = 0.07$). There was a significant increase in the WD-FR group compared to the CHOW group in the CORTEX (12.5%; $p < 0.05$). In panels A and B, the same lower case letters placed to the right of each line in each graph indicate no significant difference between groups, whereas different letters indicate a significant difference between groups. In panel A, to accurately represent each pairwise comparison, it was necessary to use 3 columns of letters, each using different letters to represent a distinct pairwise comparison.

Table 1. t and p Values for group comparisons of number of BDNF-LIR cells in Experiment 2

	WD vs. CHOW		WD vs. WD-FR		WD-FR vs. CHOW	
	t value	p value	t value	p value	t value	p value
6 hr Group						
ARC	1.12	0.14				
VMH	0.41	0.35				
PVN	0.60	0.28				
CORTEX	0.36	0.36				
48 hr Group						
ARC	0.30	0.38	0.34	0.37	0.60	0.28
VMH	1.60	0.67	1.62	0.13	0.38	0.36
PVN	0.72	0.24	0.02	0.49	0.52	0.31
CORTEX	1.34	0.10	0.01	0.45	1.70	0.05
1 wk Group						
ARC	0.44	0.34	0.62	0.27	0.87	0.20
VMH	2.27	0.02	2.23	0.03	0.26	0.40
PVN	0.49	0.32	0.43	0.34	0.14	0.44
CORTEX	1.81	0.04	1.16	0.13	0.91	0.19
3 wk Group						
ARC	0.78	0.23	0.92	0.19	0.26	0.40

VMH	0.44	0.33	0.07	0.47	0.47	0.32
PVN	1.54	0.07	1.68	0.06	0.47	0.32
CORTEX	0.46	0.32	1.22	0.12	1.73	0.05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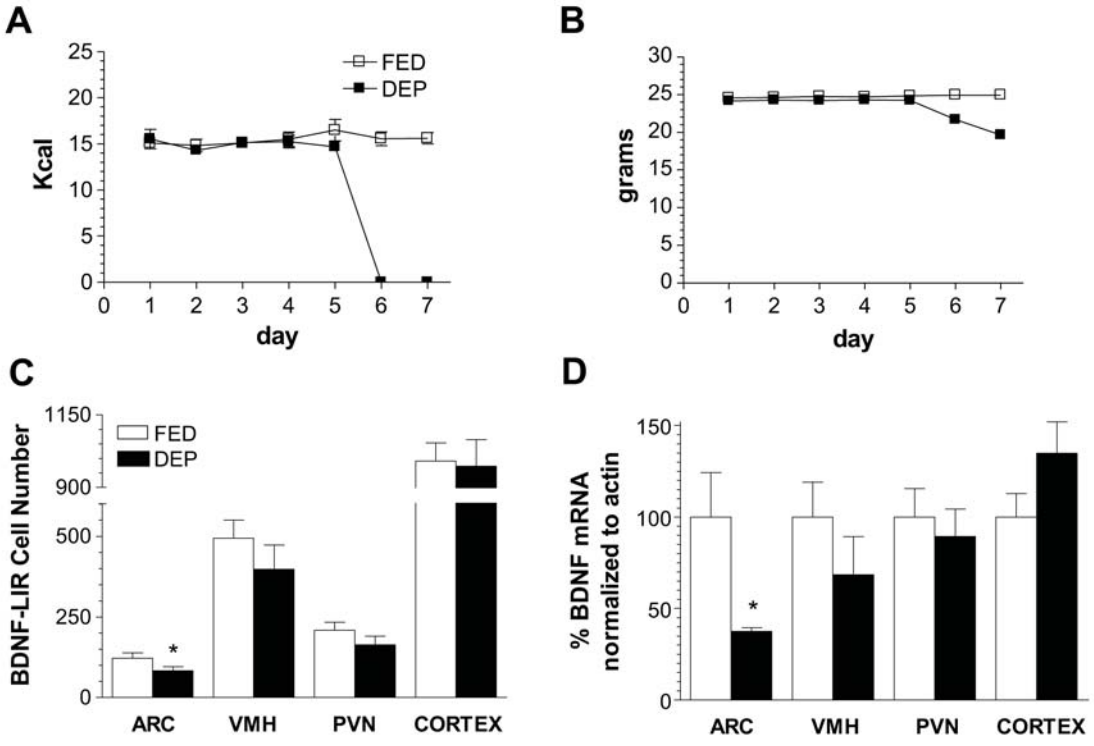
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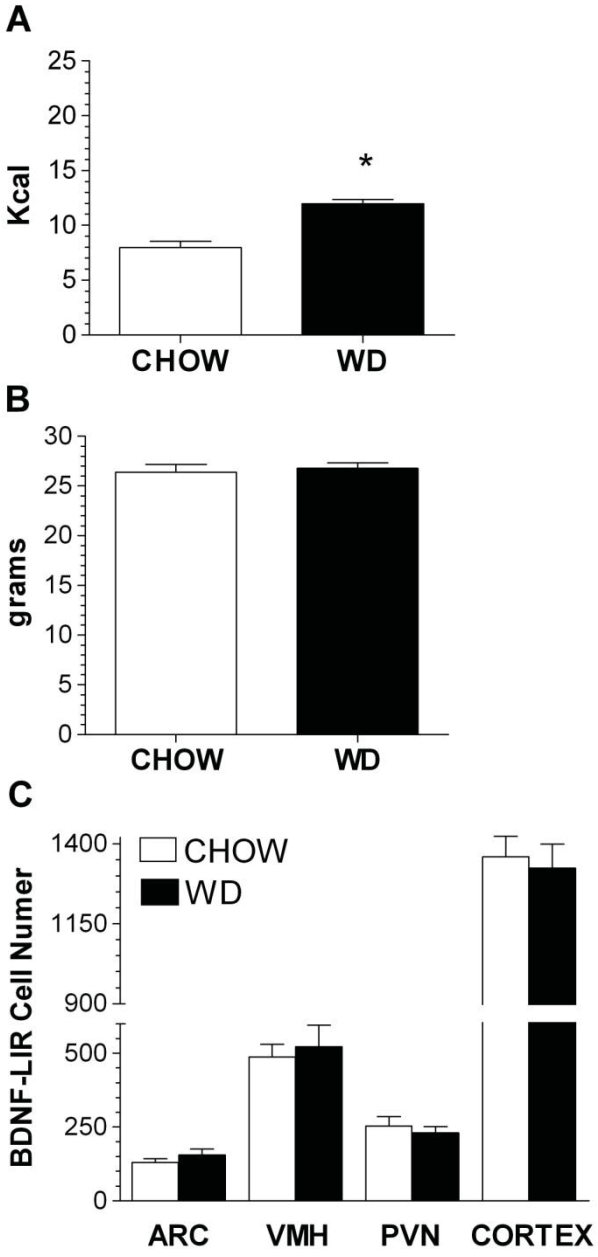
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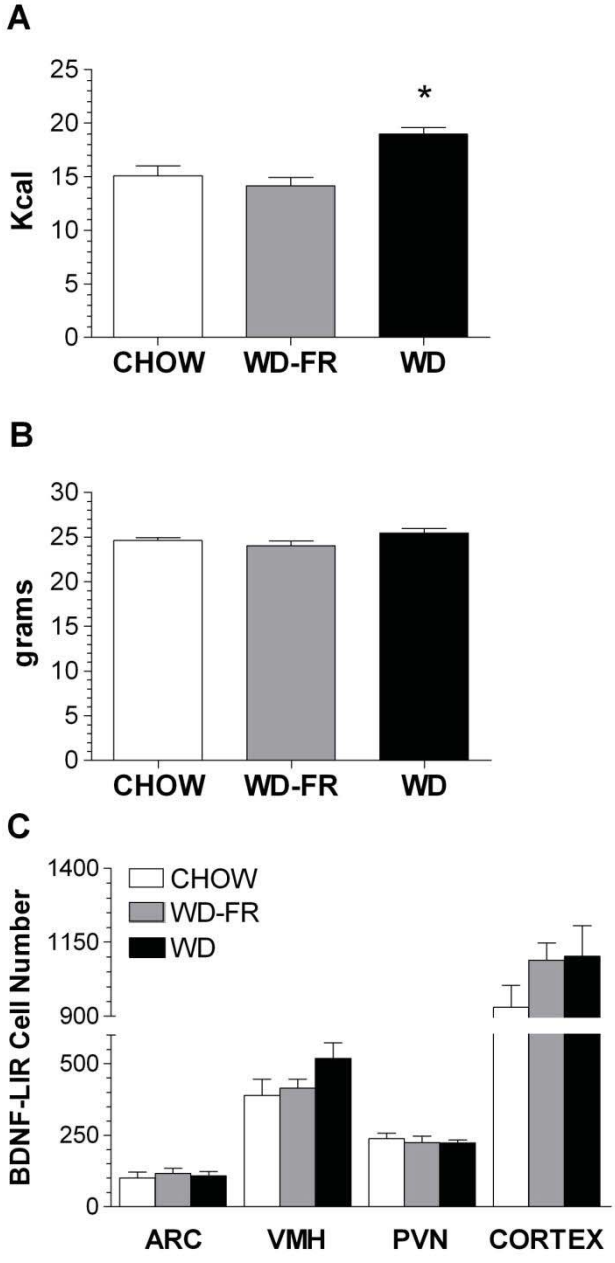
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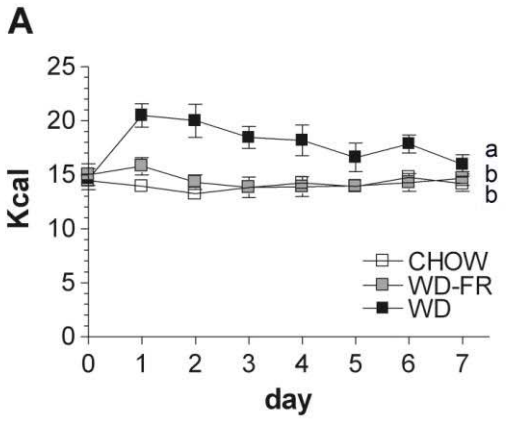
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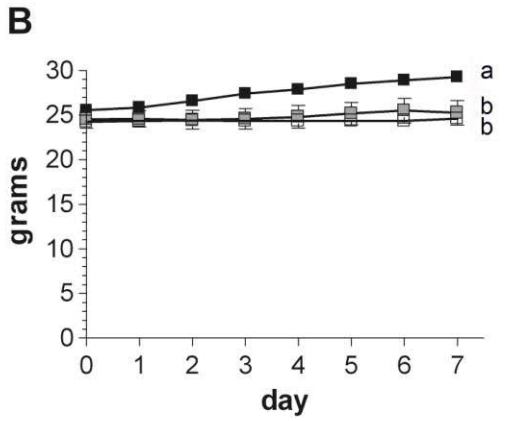
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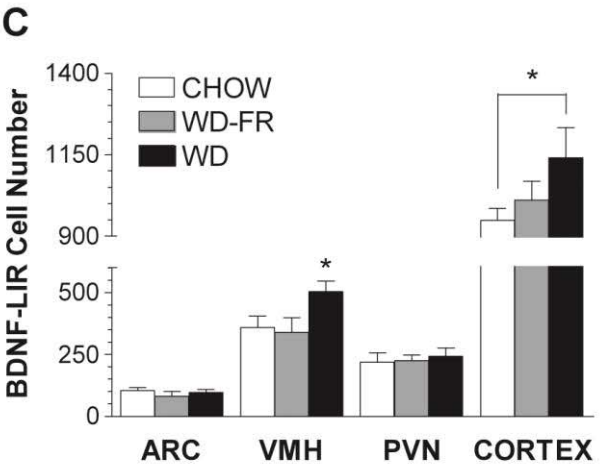
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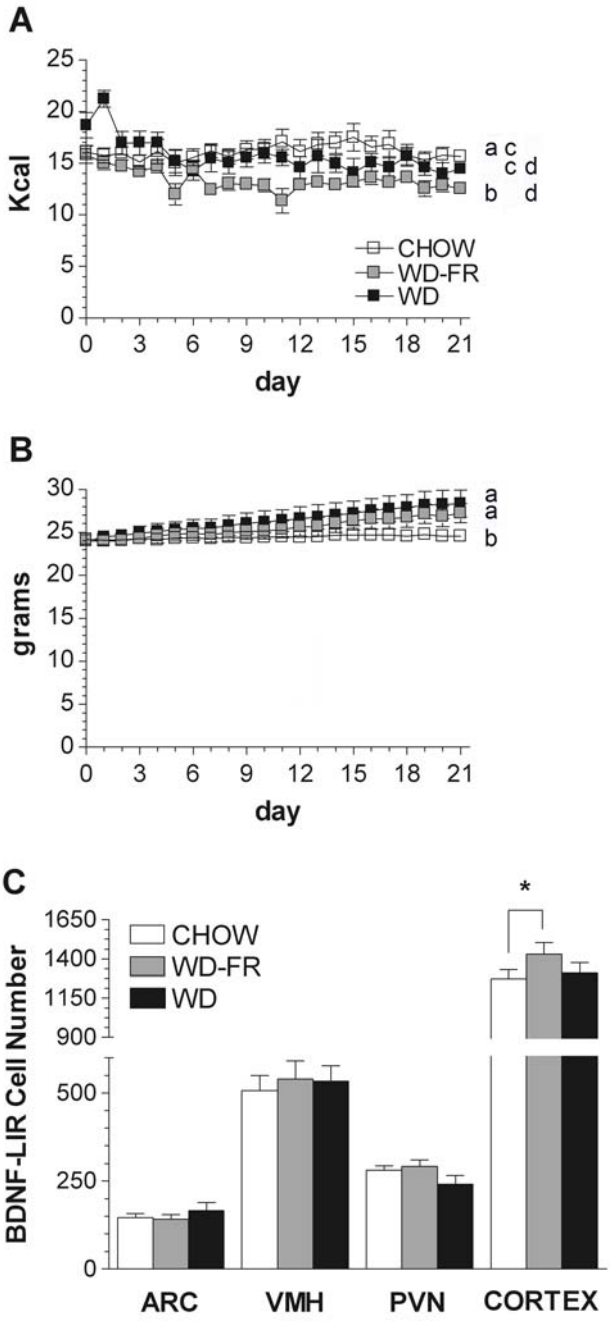
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