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Conditional Deletion Of Brain-Derived Neurotrophic Factor in the Postnatal Brain Leads to Obesity and Hyperactivity

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Brain-derived neurotrophic factor has been associated previously with the regulation of food intake. To help elucidate the role of this neurotrophin in weight regulation, we have generated conditional mutants in which brain-derived neurotrophic factor has been eliminated from the brain after birth through the use of the cre-loxP recombination system. Brain-derived neurotrophic factor conditional mutants were hyperactive after exposure to stressors and had higher levels of anxiety when evaluated in the light/dark exploration test. They also had mature onset obesity characterized by a dramatic 80–150% increase in body weight, increased linear growth, and elevated serum levels of leptin, insulin, glucose, and cholesterol. In addition, the mutants had an abnormal starvation response and elevated basal levels of POMC, an anorexigenic factor and the precursor for α-MSH. Our results demonstrate that brain derived neurotrophic factor has an essential maintenance function in the regulation of anxiety-related behavior and in food intake through central mediators in both the basal and fasted state. (*Molecular Endocrinology* 15: 1748–1757, 2001)

BRAIN-DERIVED NEUROTROPHIC factor (BDNF), a member of the family of neurotrophins, is essential for the survival and maintenance of peripheral sensory neurons (1, 2). Because most *Bdnf*$^{-/-}$ mutants die during the second postnatal week, less is known about the *in vivo* role of this neurotrophin in the postnatal brain. Previous findings implicated BDNF in weight regulation as exogenous BDNF treatment was demonstrated to cause a reduction in weight and *Bdnf*$^{+/-}$ animals show an age-related increase in body weight (3, 4). However, the mode of action of BDNF in weight regulation remains elusive.

Through the generation and characterization of several mouse genetic obesity models, a number of central and peripheral factors and their mechanism of action in food intake and metabolic function have been identified. Central regulation of food intake is generally associated with the hypothalamus, where orexigenic factors such as NPY, agouti related protein (AGRP), orexin, and melanin concentrating hormone (MCH) and anorexigenic factors such as cocaine and amphetamine-related transcript, serotonin, TRH, and α-MSH are present (5–12). BDNF and its receptor, TrkB, are expressed in various hypothalamic nuclei associated with eating behavior and obesity (3). They are present in neurons in the lateral hypothalamus (feeding center), the ventromedial nucleus (satiety center), and the paraventricular and arcuate nuclei, both of which are required for maintenance of normal body weight.

Conclusive evidence that BDNF acts through a central mechanism to regulate weight is still lacking. In addition, it is necessary to establish whether this neurotrophin has a developmental or a maintenance role in weight regulation. To answer some of these questions and to circumvent the problem of early mortality associated with global ablation of the BDNF gene, conditional mutant mice were generated in which BDNF was eliminated in a tissue- and temporal-specific manner using the cre-loxP recombination system (13). Analysis of these mice revealed that BDNF affects locomotor behavior and regulates food intake through a central maintenance mechanism that is independent from its developmental function.

Abbreviations: AGRP, Agouti-related protein; BDNF, brain-derived neurotrophic factor; CamK, α-calcium/calmodulin-dependent protein kinase II; ES, embryonic stem; MC4-R, melanocortin 4 receptor; MCH, melanin-concentrating hormone.

RESULTS

Generation of Conditional BDNF Mutants

LoxP sites were inserted around the single coding exon of BDNF by standard gene targeting techniques

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(14) (Fig. 1A). Mice carrying the floxed BDNF allele (*Bdnf* ^{2lox}) were generated using targeted ES cell clones and homozygous mice for this allele were normal and fertile. Excision of the BDNF coding sequence in the brain was accomplished by crossing the *Bdnf* ^{2lox} allele with two different lines of mice expressing cre recombinase under the direction of the α-calcium/calmodulin-dependent protein kinase II (CamK) promoter, which drives expression in postmitotic neurons (15). To assess temporal and spatial activity of cre recombinase, a LacZ reporter transgene that is activated by cre-mediated recombination (16) was crossed with the CamK-cre93 strain. We detected only a few blue cells in various regions of the newborn brain (Fig. 1B). However, the cre transgene became widely activated at P21 in the cortex, hippocampus, hypothalamus, and brainstem with no recombinase activity detected in glia (Fig. 1B and data not shown). In the CamK-cre159 transgenic line, cre recombinase activity began at P15, and the final level of recombination was reached by the fourth postnatal week (data not shown). Both lines of conditional mutants showed a similar spatial pattern of recombination. BDNF conditional mutants obtained by crossing the floxed BDNF allele with CamK-cre93 and CamK-cre159 transgenic mice are referred to as *Bdnf* $^{2lox/2lox}$/93 and *Bdnf* $^{2lox/2lox}$/159, respectively.

Elimination of the BDNF coding sequence in both lines of conditional mutants was restricted to the brain (Fig. 1C and data not shown). Northern blot analysis showed that BDNF was substantially reduced in the hypothalamus, hippocampus, and cortex of adult *Bdnf* $^{2lox/2lox}$/93 mice (Fig. 1D and data not shown). We conclude that the CamK-cre93 and 159 transgenes lead to the deletion of BDNF postnatally and thus provide a genetic tool to assess the role of this neurotrophin in the postnatal brain.

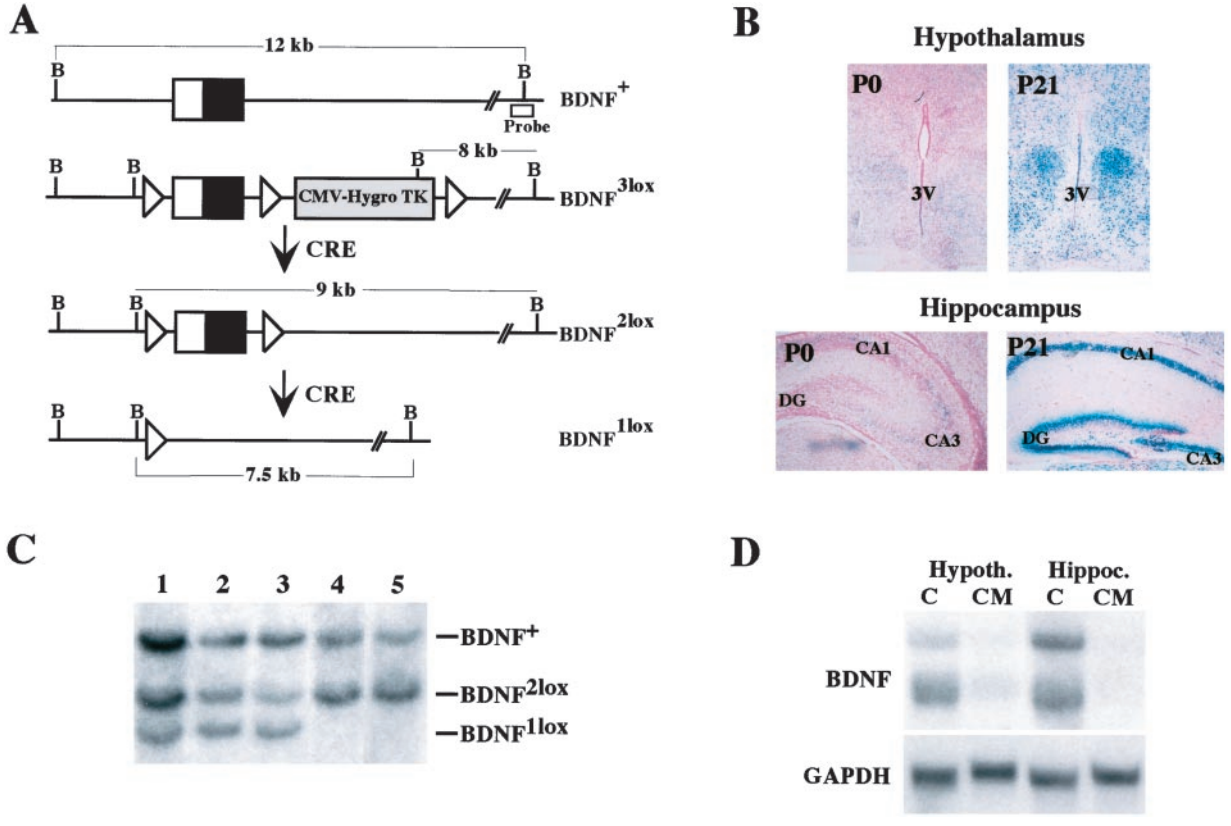

Fig. 1. Generation and CamK-cre-Mediated Recombination of the Floxed *Bdnf* Allele

A, Three lox P sites (*triangles*) and a selection cassette (*gray box*) were introduced into the *Bdnf* wild-type allele (*Bdnf* $^{+}$) to generate the *Bdnf* ^{3lox} allele. The *Bndf* ^{2lox} allele was produced by removal of the selection cassette *in vitro* by cre recombinase (*vertical arrows*). *Bdnf* ^{2lox} carriers were crossed to mice expressing the cre recombinase under the CamK-cre promoter, which resulted in the generation of the *Bdnf* ^{1lox} allele in the brain. The *white and black rectangle* represents the single coding exon of *Bdnf* and the *black* portion of the box represents sequence coding for the mature form of BDNF. B, X-gal staining of coronal sections of hypothalami and hippocampi obtained from P0 and P21 CamK-cre93/lac Z reporter mice. Lac Z expression requires cre-mediated recombination. C, Representative Southern blot containing DNA samples extracted from hypothalamus (1), hippocampus (2), cortex (3), kidney (4), and heart (5) from a *Bdnf* $^{2lox/+}$/93 mouse, 12 wk of age. D, Representative Northern blot containing RNA extracted from adult wild-type and *Bdnf* $^{2lox/2lox}$/93 hypothalami and hippocampi probed with BDNF and GAPDH. Abbreviations: B, *Bgl*II; CMV, cytomegalovirus; Hypoth, hypothalamus; Hippoc, hippocampus; 3V, third ventricle; DG, dentate gyrus; C, control; CM, conditional mutant.

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BDNF Conditional Mutant Mice Are Anxiety Prone and Obese

Bdnf $^{2lox/2lox}$/93 mice were viable, hyperactive when stressed (Fig. 2), and displayed increased intermale aggression (data not shown). When we examined baseline activity during the light cycle, BDNF conditional mutants appeared to be marginally more active than the controls but this was not statistically significant (Fig. 2A). However, there was a clear difference in the locomotor behavior of the mutants compared with the controls when any attempt was made to handle them or their cages. They became very agitated and active and appeared stressed, and this behavior was already apparent by the fourth postnatal week. To investigate this further, mice were individually placed in fresh cages and their locomotor activity monitored during and after a 1-h habituation period. Exposure to a novel cage is a mild stressor, which initially causes an increase in activity in normal mice that is reduced substantially after a period of habituation. Whereas control mice appeared relaxed after the habituation hour, both female and male mutants continued to appear agitated. In normal mice, activity was reduced by 72% after the habituation hour compared with a 40% reduction in activity observed in the mutants. We found that mutant mice were 66% and 250% more active than the controls during the habituation period and the posthabituation hour, respectively (Fig. 2A). These results suggested that the absence of central BDNF caused an increase in anxiety levels in the mutants.

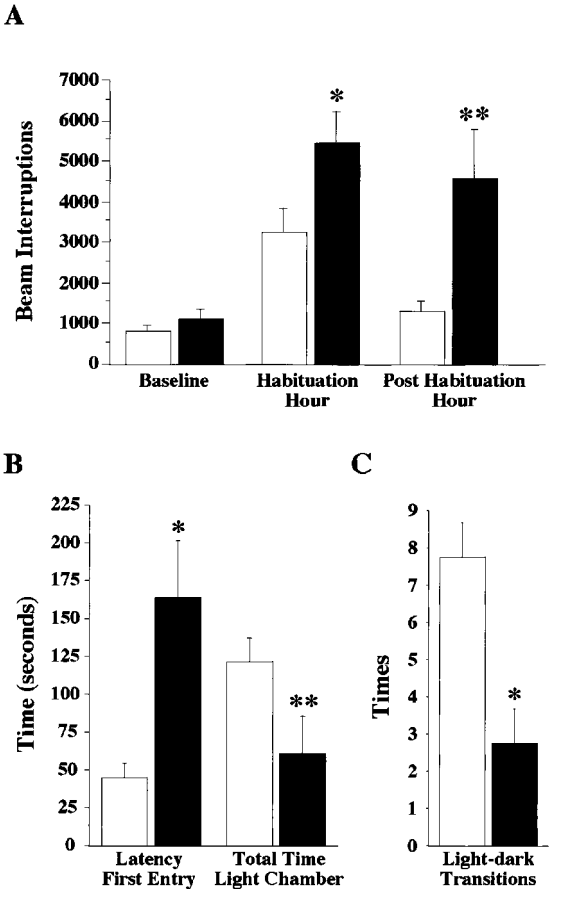

Fig. 2. BDNF Conditional Mutants Have Increased Levels of Anxiety

A, Locomotor activity of control (*white columns*) and BDNF conditional mutant (*black columns*) mice at baseline (n = 9) and after exposure to a novel chamber (n = 6) was monitored. Baseline activity was measured for 1 h during the light cycle, and activity after exposure to a novel chamber was measured during the first hour immediately after placement into a fresh cage and for an additional hour after the animals were allowed to habituate for 1 h to the new chamber. *, $P < 0.05$; **, $P < 0.03$. B, Latency of first entry into the light zone and total time spent in the light chamber during the dark/light exploration test. Each control (*white columns*) and conditional mutant (*black columns*) mouse (n = 12) was tested for a period of 5 min. *, $P < 0.004$; **, $P < 0.044$. C, Number of dark-light compartment transitions during the dark/light exploration test (n = 12). *, $P < 0.001$.

To further investigate the anxiety-related behavior of the mutants, the light/dark exploration test was performed. For this test, animals were placed in a box consisting of a dark chamber and a larger brightly illuminated second chamber. The latency for the first entry into the light compartment, total time spent in the light compartment, and the number of dark-to-light chamber transitions were monitored for each animal for a period of 5 min. Because mice have a natural aversion to open and bright spaces, animals that are more anxiety prone will have longer latency times for the first transition into the light zone, will spend less total time in the light chamber, and will make less dark-to-light zone transitions. Conditional mutants exhibited a reduction in exploratory behavior and an increase in anxiety-like behavior during the light/dark exploration test (Fig. 2, B and C). It took mutants 3.6 times longer to make the first transition from the dark to the light compartment, and they spent half as much time in the light zone compared with the controls (Fig. 2B). In addition, mutants only made 35% the number of dark-to-light zone transitions compared with the control animals (Fig. 2C). These results show that the absence of normal levels of central BDNF has an anxiolytic effect.

In addition to changes in locomotor activity and anxiety-related behavior, conditional mutants had increased body weights compared with littermate controls, and this difference reached statistical significance at 8 wk of age (Fig. 3, A and B). By 30 wk of age, mutant males and females were 80 and 150% heavier than age-matched controls, respectively. Weight of mutant females at 30 weeks was 64.6 g ± 3.8 compared with 25.8 g ± 1.5 for the controls ($P < 0.0004$) (Fig. 3A). Conditional mutant males had a weight mean value of 56.5 g ± 1.3 compared with 31.4 g ± 2.9 for the controls ($P < 0.002$) (Fig. 3B).

To assess whether the increased body weight in the mutants was associated with hyperphagia, food consumption was monitored in control and mutant ani-

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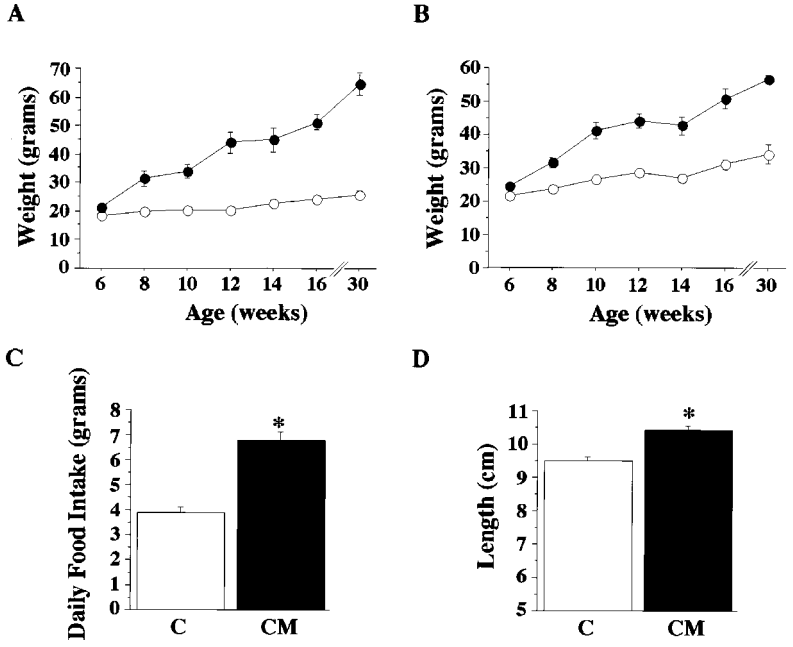

Fig. 3. Increased Body Weight, Food Intake, and Body Length of the BDNF Conditional Mutants

A, Growth curve of control (*open circles*) and *Bdnf* $^{2lox/2lox}$/93 (*solid circles*) females (n = 15). B, Growth curve of control (*open circles*) and *Bdnf* $^{2lox/2lox}$/93 (*filled circles*) males (n = 14). C, Measurement of daily food intake by controls and conditional mutant mice (n = 15). *, $P < 0.0003$. D, Measurement of body length of control and *Bdnf* $^{2lox/2lox}$/93 mice (n = 11). *, $P < 0.01$. Abbreviations: C, controls; CM, conditional mutants.

mals fed a standard chow diet *ad libitum.* We found that food intake in *Bdnf* $^{2lox/2lox}$/93 mice was 74% higher than that of the controls ($P < 0.0003$) (Fig. 3C). Furthermore, when *Bdnf* $^{2lox/2lox}$/93 mutants were pair fed with control littermates, they lost 19.3% of their weight in a period of 9 d. Alterations in linear growth were also examined in the mutants. They had a 10% increase in naso-anal length compared with the controls ($P < 0.01$) (Fig. 3D).

BDNF is an important survival and differentiation factor during development of the nervous system (1, 2). As BDNF was removed from the brain postnatally in BDNF conditional mutants, it was unlikely that the phenotypes observed were the result of the disruption of developmental functions normally carried by this neurotrophin. To confirm that the obesity and hyperactivity in the mutants arose from the lack of a maintenance function of BDNF in the brain, the weights of *Bdnf* $^{2lox/2lox}$/159 mice were measured and their locomotor activity monitored. Recombination of the floxed BDNF allele in this line of mice begins at P15 and is completed during the fourth postnatal week. Similar to *Bdnf* $^{2lox/2lox}$/93 mice, *Bdnf* $^{2lox/2lox}$/159 mutants were obese and hyperactive (data not shown), confirming that the phenotype was caused by the loss of BDNF as a maintenance factor for central neurons involved in food intake and locomotor behavior.

Finally, to ascertain if the reproductive capability of conditional mutants was compromised, vaginal smears were performed to determine whether females were cycling and mutant animals were bred to wild-type controls. After examining vaginal smears from two lean and three obese mutants, we found that only the former were cycling normally. Moreover, when three lean and five obese mutant females were bred to wild-type males, only the lean mutants became pregnant and produced progeny. These lean mutants were young animals that subsequently became obese and sterile. These data show that sterility in the obese female mutants is a secondary effect of the obesity. Mutant males were also examined and found to be fertile independently of obesity (data not shown). We conclude that BDNF has a maintenance function in weight regulation through a central mechanism.

BDNF Conditional Mutants Are Hyperleptinemic, Hyperinsulinemic, and Hyperglycemic

Leptin, a satiety signal produced in adipose tissue, and insulin, an important factor in regulation of glucose homeostasis, lipid metabolism, and energy balance, are often altered as a response to feeding, fasting, and obesity (17–21). Elevated levels of these hormones were associated with the obesity observed in BDNF conditional mutants. Serum levels of leptin and insulin were 15-fold and 6-fold higher, respectively, in obese mutants as compared with controls (Fig. 4, A and B). Serum levels of leptin and insulin in mutants that were not yet obese were determined to be normal (data not shown), indicating that the elevated levels detected in the obese mutants were secondary effects of obesity.

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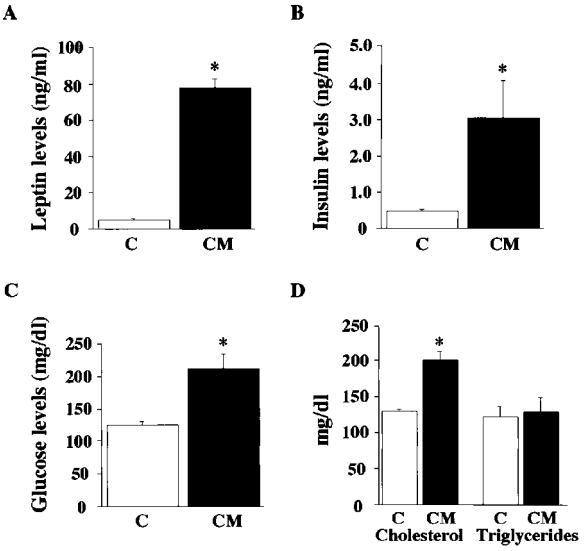

Fig. 4. BDNF Conditional Mutant Mice Are Hyperleptinemic, Hyperinsulinemic, and Hyperglycemic

A, A 15-fold elevation in the serum levels of leptin was detected in *Bdnf* $^{2lox/2lox}$/93 mice compared with the controls (n = 10). *, $P < 0.0001$. B, Fasting insulin levels were increased by 600% in the conditional mutants (n = 6). *, $P < 0.05$. C, A 70% elevation in the fasting levels of serum glucose was detected in the conditional mutants (n = 8). *, $P < 0.02$. D, Cholesterol levels were increased by 54% in the mutants, and triglyceride levels were similar to those of the controls (n = 8). *, $P < 0.005$. Abbreviations: C, controls; CM, conditional mutants.

Glucose levels in the conditional mutants were 70% higher than in the controls (Fig. 4C). As changes in lipid metabolism and synthesis have been associated with obesity, we investigated whether levels of cholesterol and triglycerides were altered in the conditional mutants. Cholesterol levels in control mice were 130.3 ± 3.3 mg/dl and 200.5 ± 11.4 mg/dl in the mutants, representing a 54% increase in the latter (Fig. 4D). There was no significant difference in triglyceride levels in the conditional mutant serum compared with that from the controls (Fig. 4D). These findings indicate that BDNF conditional mutants are leptin and insulin resistant and that resistance is a secondary effect of the obesity.

Expression of Hypothalamic Weight-Regulating Factors Is Normal in BDNF Conditional Mutants

The hypothalamus is a known center for the regulation of food intake and metabolic function (22). To examine the overall organization of the mutant hypothalamus, brains from BDNF conditional mutants were obtained for histological and immunohistochemical examination. Brain sections stained with cresyl violet failed to uncover any gross morphological abnormalities (data not shown).

Expression of hypothalamic orexigenic and anorexigenic factors known to regulate eating behavior and metabolic function were also examined in control and mutant mice 14 to 16 wk of age. Expression levels and the pattern of distribution of NPY, MCH, orexin, AGRP, α-MSH, serotonin, and TRH appeared normal in all of the mutants examined (Fig. 5 and data not shown), suggesting that BDNF does not affect expression of these factors.

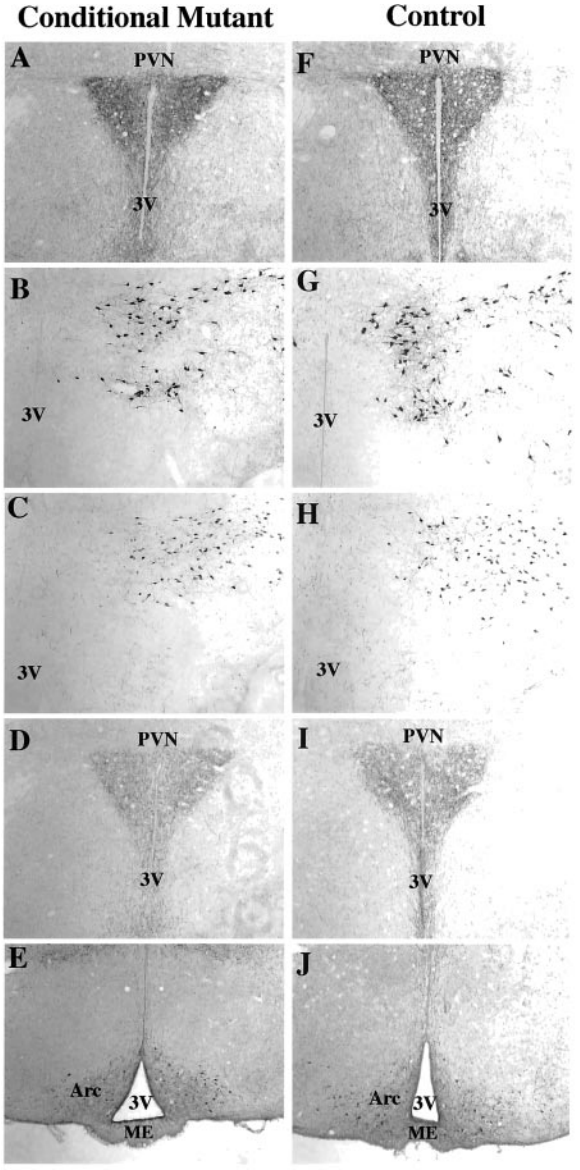

Fig. 5. Immunohistochemical Analysis of the BDNF Conditional Mutant Hypothalamus

Immunohistochemical delineation of NPY (A and F), MCH (B and G), orexin (C and H), AGRP (D and I) and α-MSH (E and J) in the hypothalamus of conditional mutant (A–E) and control (F–J) mice. No differences could be distinguished between the animal groups (n = 3). PVN, Paraventricular nucleus; 3V, third ventricle; Arc, arcuate nucleus; ME, median eminence.

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Enhancing Levels of Serotonin Does Not Rescue the Obesity Phenotype of BDNF Conditional Mutant Mice

Serotonin has been associated previously with BDNF regulation of food intake (4). To examine this possibility, we treated control and BDNF conditional mutants with fluoxetine, a serotonin reuptake inhibitor (5 mg/kg of body weight) once daily for 20 d. After 7 d of treatment, mutants appeared less hyperactive than vehicle-treated mutants when handled, indicating that the fluoxetine treatment was effective. Monitoring of food intake and weight revealed that fluoxetine treatment did not significantly decrease the amount of food consumed by conditional mutants after 20 d of treatment (Fig. 6A). Furthermore, the weight of vehicle-treated mutants increased by 4.0% during the course of the treatment and that of the fluoxetine-treated mutants increased by 6.6% (Fig. 6B). These results suggest that factors other than, or in addition to, serotonin are components of the mechanism through which BDNF regulates weight.

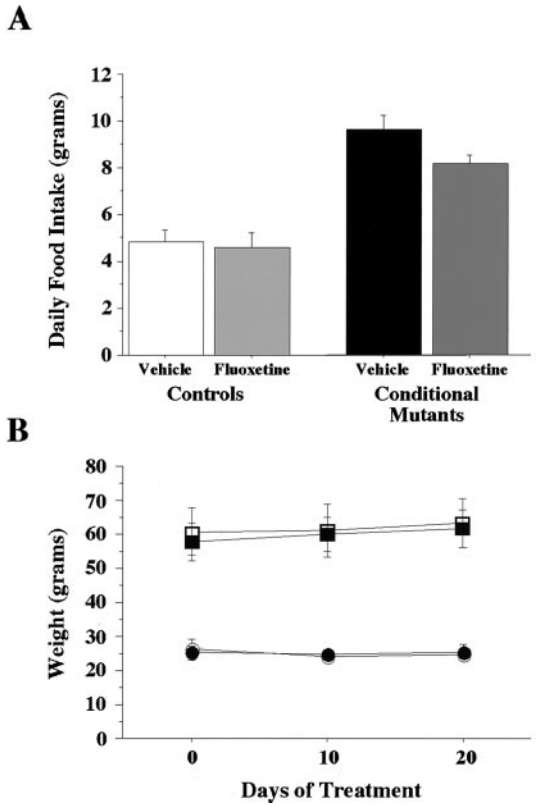

Fig. 6. Treatment of Control and BDNF Conditional Mutant Mice with Fluoxetine

A, Measurement of daily food intake of control and conditional mutant mice that were treated with vehicle or with fluoxetine (5 mg/kg of body weight) once daily for 20 days (n = 4). B, Measurement of body weight of control (*open circles*) and mutant (*open squares*) treated with vehicle and control (*solid circles*) and mutant (*solid squares*) treated with fluoxetine at days 0, 10, and 20 of treatment (n = 4).

The Starvation Response in BDNF Conditional Mutants Is Abnormal

The starvation response triggered by extended fasting is characterized by a decrease in serum levels of leptin and insulin in normal animals (21, 23, 24). Fasting also elicits a dramatic elevation in the levels of NPY, an orexigenic factor, and a decrease in the levels of POMC, the precursor for the anorexigenic factor α-MSH, in the normal hypothalamus (25, 26). All of these responses signal the animal to eat. Because BDNF appeared to have an anorexigenic effect, we decided to examine whether its expression levels were reduced during the starvation response. To accomplish this, the levels of expression of BDNF mRNA were measured in hypothalami obtained from wild-type mice fasted for 68 h and compared with those of control mice fed *ad libitum*. We found that fasting did not dramatically alter the hypothalamic levels of BDNF (data not shown), suggesting that changes in the expression of this neurotrophin were not involved in the starvation response.

We also investigated whether in the absence of BDNF in the brain, the starvation response was normal. Wild-type and BDNF conditional mutant mice were fasted for 48 h, and the serum levels of leptin, insulin, and glucose and the hypothalamic levels of NPY and POMC mRNA were measured. Whereas fasting serum levels of insulin in the mutants were similar to those of the controls, their levels of leptin remained elevated (data not shown). In addition, glucose levels were reduced to 3 mg/dl (97% reduction) and 86 mg/dl (60% reduction) in control and mutant mice, respectively. Basal levels of NPY mRNA in the hypothalamus were comparable in the controls and the conditional mutants, confirming the findings of the immunohistochemical analysis (Fig. 7). As expected, fasting induced a 3-fold increase in the levels of NPY mRNA in the control hypothalamus. In contrast, conditional mutants had an attenuated response to fasting, their levels of NPY mRNA being increased only by 30% (Fig.

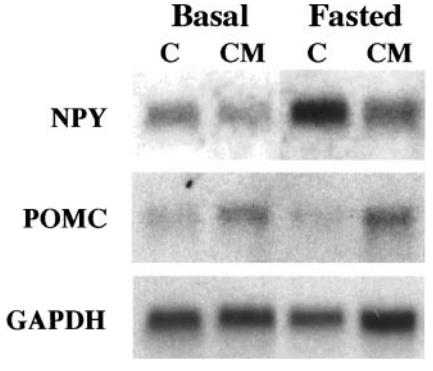

Fig. 7. The Starvation Response Is Not Normal in BDNF Conditional Mutants

Representative Northern blot showing levels of hypothalamic NPY and POMC mRNA in mice fed *ad libitum* or fasted for 48 h. C, Control; CM, conditional mutant.

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7). However, the feeding behavior of the mutants after fasting was comparable to that of the controls, albeit containing lower fasting levels of hypothalamic NPY mRNA (data not shown). These data show that BDNF is an important regulatory signal for NPY expression during the starvation response.

POMC mRNA levels in the mutant hypothalamus were also altered. A 50–100% increase in basal levels of POMC mRNA was detected in 75% of the BDNF conditional mutants examined when compared with controls (Fig. 7). As in wild-type mice, POMC levels were slightly reduced after a 48-h fast in the conditional mutants. The anorexigenic effect of POMC is mediated by its processed derivative, α-MSH, acting through the melanocortin-4 receptor (MC4-R) (27). BDNF conditional mutants are hyperphagic in spite of an increase in POMC, suggesting that MC4-R signaling might be compromised. To investigate whether POMC mRNA levels were elevated in the mutants due to a reduction in the expression levels of MC4-R, hypothalamic samples obtained from control and mutant mice were subjected to Western blot analysis. We found that expression levels of the melanocortic receptor in the hypothalamus were not dramatically changed in the conditional mutants (data not shown). These results suggest that if melanocortin signaling is abnormal in the mutants, the defect is not due to an absence of MC4-R protein.

DISCUSSION

BDNF conditional mutants are hyperaggressive and hyperphagic, have increased levels of anxiety, and are substantially more obese than previously described for *Bdnf* $^{+/-}$ mice. As BDNF was removed after birth, subsequent to the migration and differentiation of most neurons in the central nervous system, we conclude that BDNF has a maintenance function in the modulation of anxiety-related behavior and of food intake. In addition, because BDNF was present at normal levels in all tissues except the brains of the mutants, we can infer that the obesity arose from the lack of a central function of this neurotrophin.

BDNF has been shown previously to affect behavior. *Bdnf* $^{+/-}$ and BDNF conditional mutant males have increased intermale aggression (Ref. 4 and our unpublished observations) that can be attenuated with fluoxetine, a serotonin reuptake inhibitor. Locomotor behavior was also examined in *Bdnf* $^{+/-}$ mice but the results reported are conflicting. One group found no differences in horizontal activity between control and *Bdnf* $^{+/-}$ mutant mice and a significant reduction in vertical activity in the latter (4). Another group reported that only a subset of *Bdnf* $^{+/-}$ mice displayed hyperactivity that inversely correlated with obesity (3). BDNF conditional mutants examined in this study had a substantial increase in total locomotor activity when stressed but not at baseline, suggesting a role for this neurotrophin in the regulation of anxiety-related behavior. This finding is supported by results obtained from the light/dark exploration test, which demonstrated that the mutants were anxiety prone. As BDNF has been suggested to be important for proper serotonergic neurotransmission (4, 28, 29), a careful examination of this system in the mutants could help explain the behavior observed.

Unraveling the mechanism through which BDNF regulates food intake has proven challenging. Previous studies examining factors relevant in weight regulation found that expression levels of cocaine and amphetamine-related transcript and leptin receptors were normal in *Bdnf* $^{+/-}$ mice (3). Another report suggested that a reduction in serotonin in *Bdnf* $^{+/-}$ mice induced obesity (4). However, reduction in serotonin levels was not detected until 18 months of age even though weight increase was already detected by 8 wk of age. The authors argued that serotonergic neurotransmission was possibly defective in the mutants, preceding a substantial decrease in serotonin levels. Here, we show that treatment with fluoxetine, a serotonin reuptake inhibitor, did not significantly reduce food intake or body weight in the conditional mutants. The treatment, however, was effective in curtailing the hyperactivity of the mutants. Our results suggest that a reduction in serotonin could be partly responsible for the obesity observed in the mutants but that other factors must also be involved.

It has been suggested that BDNF acts peripherally to regulate weight (30). Moreover, in addition to being present in the central nervous system, BDNF and Trk B expression have been detected in peripheral tissues involved in metabolic function such as exocrine and endocrine pancreas (31) and adrenal gland (32). Previous results based upon *Bdnf* $^{+/-}$ mutant mice could not distinguish between a peripheral and a central effect of BDNF on obesity. Because BDNF depletion was restricted to the brain in the conditional mutant mice described here, we can definitively conclude that the dramatic increase in body weight observed was due to the disruption of a central function of BDNF.

A thorough study of the BDNF conditional mutant hypothalamus determined that expression levels of NPY, AGRP, TRH, α-MSH, MCH, orexin, and serotonin, all factors involved in regulation of food intake and metabolic function, were not dramatically changed. Although expression of some of these factors is regulated by leptin and insulin and BDNF conditional mutants had elevated levels of these hormones, previous reports show that changes in expression of these factors are not obligatory under conditions of leptin and insulin resistance (33, 34).

Even though α-MSH expression appeared normal in the mutants, mRNA levels of its precursor, POMC, were increased by 50–100% in most of the mutants examined. This discrepancy could be due to the fact that α-MSH expression was examined by immunohistochemistry, which is not a quantitative assay, and subtle differences in α-MSH expression could have gone undetected. The elevated serum levels of leptin, an inducer of POMC (26), are unlikely to be the cause

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of the POMC mRNA up-regulation observed in the mutants because increased levels of this anorexigenic factor were also detected in lean mutants with normal levels of leptin. It is also improbable that BDNF acts directly to inhibit expression of POMC, as this would result in an increase in food intake, and this and other reports show that BDNF inhibits eating (3, 4, 35). Alternatively, the elevated levels of POMC mRNA in the mutant hypothalamus could be indicative of defective melanocortin signaling. In such a scenario, where factors downstream of POMC could potentially be absent or defective, increased levels of POMC would not be expected to antagonize the hyperphagic behavior induced by the absence of BDNF. Curiously, linear growth was significantly increased in BDNF conditional mutants, reminiscent of other obesity models involving melanocortin signaling such as the POMC and MC4-R-null mice (12, 36). Those mutants have a 5–11% increase in body length, which is not characteristic of other obesity models such as the leptin and leptin receptor-deficient mice (37, 38). In addition, like POMC and MC4-R-null mice, BDNF conditional mutants display late-onset obesity. The fact that expression levels of MC4-R protein in the mutant hypothalamus appeared normal suggests that if melanocortin signaling was perturbed, it was not at the level of receptor expression but rather somewhere downstream of MC4-R activation.

BDNF conditional mutants also failed to exhibit the dramatic elevation in hypothalamic NPY normally induced by extended fasting. Leptin, a negative regulator of NPY, was substantially elevated in the mutants even under fasting conditions and may have prevented the up-regulation of NPY or other orexigenic factors. However, this seems unlikely as conditional mutants appeared to be leptin resistant, as demonstrated by the fact that their NPY basal levels remained normal in the presence of a 15-fold excess of leptin. An alternative explanation is that BDNF is a required inductive signal for increased NPY expression during the starvation response. The ability of BDNF to induce NPY expression in other neuronal cell populations during development is well documented (39, 40). Thus, together these data show that in addition to having a developmental role in the differentiation of certain NPY-containing neurons, BDNF has a maintenance role in the hypothalamus, facilitating expression of NPY during fasting.

In conclusion, our results show that BDNF is an essential factor in the central regulation of locomotor behavior and food intake. As BDNF conditional mutants are viable and have a postnatal depletion of BDNF exclusively in the brain, they provide a genetic model with which to investigate mood disorders and central mediators of obesity. Because the phenotype of the mutants is easy to assess, these animals should provide a valuable tool by which to examine the efficacy of treatments aimed at activating the TrkB receptor signaling pathway. Such strategies may be beneficial in ameliorating these debilitating conditions.

MATERIALS AND METHODS

Generation of *Bdnf* ^{2lox} Allele and of Conditional Mutants

For the generation of floxed BDNF mice, a targeting construct was designed in which exon 5, the single coding exon in the BDNF gene, was flanked by lox P sites (Fig. 1a). A cytomegalovirus-hygromycin-thymidine kinase selection cassette flanked by lox P sites was also introduced downstream of exon 5. This targeting construct was introduced into J1 embryonic stem (ES) cells by electroporation, and selected homologous integrant clones were transiently transfected with a cre recombinase-containing vector to remove the selection cassette and generate *Bdnf* $^{2lox/+}$ ES cell clones. These were used for the generation of *Bdnf* ^{2lox} allele carrier mice. *Bdnf* $^{2lox/2lox}$ and *Bdnf* $^{2lox/-}$ mice were crossed to mice expressing the cre recombinase under the direction of cam kinase-cre promoter. All the animals used in these studies were of mixed background, and all studies were conducted in accord with the principles outlined in "Guidelines for Care and Use of Experimental Animals."

Southern and Northern Blot Analysis

To determine cre-mediated recombination levels of the floxed BDNF allele, DNA was extracted from cerebral cortex, cerebellum, hypothalamus, hippocampus, kidney, and heart from *Bdnf* $^{2lox/2lox}$/93 and *Bdnf* $^{2lox/2lox}$/159 mice. DNA samples were digested with *Bgl*II and subjected to Southern blot analysis using a 3′-probe (Fig. 1). Bands 12, 9, and 7.5 kb in size were detected from *Bdnf* $^{+}$, *Bdnf* ^{2lox}, and *Bdnf* ^{1lox} alleles, respectively. For Northern blot analysis, hypothalamic tissue samples were obtained from control and *Bdnf* $^{2lox/2lox}$/93 mice that were fed *ad libitum* or fasted for 48 or 68 h. RNA extracted from those samples was subjected to Northern blot analysis to examine NPY, POMC, and BDNF mRNA expression. Two or three independent experiments were performed for NPY and POMC mRNA measurements, respectively. A total of five individual hypothalamic samples from each experimental group were examined for NPY mRNA expression and eight for POMC mRNA expression. Quantification of the Southern and Northern blots was done using a BAS 2000 phosphoimager (Fuji Photo Film Co., Ltd.) and the Image Gauge (Fuji Photo Film Co., Ltd.) v3.3 computer software.

X-gal Staining

To examine cre-mediated recombination in the brain, CamK-cre93 and 159 mice were crossed to the ROSA26-lacZ reporter mice (16). Brains obtained from their progeny were processed for X-gal staining, which was performed as described previously (16).

Measurement of Locomotor Activity

Differences in locomotor activity were assessed during the light cycle by placing mutants 2–6 months of age and sex and age-matched controls individually into cages and monitoring locomotor activity at baseline and after exposure to a novel chamber. Some of the mutants used in this study were not yet obese. Baseline activity was measured for 1 h subsequent to allowing animals to habituate to the activity monitor for 3 h. Activity was also monitored for 1 h (habituation period) immediately after placement into a fresh cage and for 1 h subsequent to habituation. Exposure to a novel cage has been used previously as a mild stressor (41). Total activity was quantified using the Opto-Varimex-Mini infrared photocell activity monitor (Columbus Instruments, Columbus, OH). Statistical significance was determined using a unpaired *t* test and values represent mean ± SEM.

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Light/Dark Exploration Test

The light/dark exploration test is an accepted and frequently used anxiety test (42, 43). To test anxiety behavior, control and BDNF conditional mutant mice (n = 12), 8–10 wk of age, were placed in a box (20 × 20 × 45 cm) containing a light and dark chamber. The light chamber constructed of clear plastic material was two-thirds the size of the box and was brightly illuminated by a 150-W lamp. The dark compartment occupied the remaining third part of the box and was constructed of black plastic material that prevented the entrance of light. The two chambers were separated by a black plastic wall with a doorway (7 × 7 cm) to allow passage from one chamber to the other. Animals were placed in the dark compartment, and the latency for the first transition to the light compartment, total time spent in the light compartment, and number of transitions from the dark compartment to the light compartment were monitored for a period of 5 min. The box was cleaned after testing each animal. Statistical significance was determined using an unpaired *t* test and values represent mean ± SEM.

Body Weight, Monitoring of Food Intake, and Linear Growth

Control and mutant mice were maintained in a 12-h light/12-h dark cycle and fed a standard chow diet and water *ad libitum*. Growth curves for males and females were obtained by measuring body weight at 6, 8, 10, 12, 14, 16, and 30 wk of age. Food intake was determined by individually caging animals between 12 and 16 wk of age that were fed *ad libitum* and weighing their food every 3 d for a total of 9 d. Food restriction experiments were performed by feeding individually caged mice 4 g of food daily and measuring their body weight every 3 d. For determination of linear growth, mice that were 20 wk old were fully extended in order to measure the nasoanal distance. Statistical significance was determined using a paired *t* test and all values represent mean ± SEM.

Analysis of Serum

For determination of insulin, glucose, cholesterol, and triglyceride levels, blood samples were collected at 1100 h from conditional mutant and control mice that had been fasted for the previous 17 h. Leptin levels were measured in serum samples obtained at 1100 h from animals fed *ad libitum*. For analysis of the serum, RIAs were performed in duplicates using leptin and insulin RIA kits (Linco Research, Inc., St. Charles, MO). To examine levels of glucose, cholesterol, and triglycerides, colorimetric kit assays were performed and analyzed using a 747 spectrophotometer (Hitachi, Mountain View, CA) Statistical significance was determined using a paired *t* test, and all values represent mean ± SEM.

Immunohistochemistry

Mice between 14 and 16 wk of age were anesthetized with Nembutal (50 mg/kg), and blood was taken from the inferior vena cava and perfused transcardially with 10 ml 0.01 M PBS, pH 7.4, containing 15,000 U/liter heparin sulfate, followed by 30 ml 2% paraformaldehyde/4% acrolein in 0.1 M phosphate buffer (PS), pH 7.4, and 10 ml 2% paraformaldehyde in the same buffer. Brains were cryoprotected in a 20% sucrose solution, snap frozen on dry ice, and sectioned in a cryostat. Free floating sections (20-μm) through the rostral-caudal extent of the hypothalamus were preincubated with 1% sodium borohydride in distilled water followed by 0.5% H_2O_2 in PBS for 15 min, and then permeabilized with 0.5% Triton X-100 in PBS for 20 min. To reduce nonspecific antibody binding, the sections were treated with 2.5% normal horse serum in PBS for 20 min. Every fourth section through the hypothalamus was incubated for 2 d at 4 C in one of the following antibodies: rabbit anti-NPY (1:10,000, Peninsula Laboratories, Inc. Belmont, CA), rabbit anti-AGRP (1:16,000, Phoenix Pharmaceuticals, Inc., Mountain View, CA), rabbit anti-TRH (1:25,000) (44), sheep anti-α-MSH (1:40,000) (45), rabbit anti-MCH (1:12,000, a gift of E. Flier) (9), orexin (1:6,000, a gift of M. Yanagisawa) (8), and serotonin (1:1000, DiaSorin, Inc., Stillwater, MN). After washes in PBS, the sections were incubated in biotinylated donkey antirabbit IgG or biotinylated donkey antisheep IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:500 for 2 h at room temperature and developed using Vectastain detection system (Vector Elite Kit, Vector Laboratories, Inc., Burlingame, CA) using 3′,3-diamino benzidine HCl as the chromagen.

Fluoxetine Treatment

Control and conditional mutant mice between 18 and 20 wk of age that were individually caged, received ip injections of fluoxetine at a dose of 5 mg/kg of body weight once daily for 20 d. Food intake and weight were monitored during the course of the treatment.

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