Disruption of a novel regulatory locus results in decreased Bdnf expression, obesity, and type 2 diabetes in mice

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First published July 24, 2007; doi:10.1152/physiolgenomics.00093.2007.—Mutants of brain-derived neurotrophic factor (BDNF) are associated with obesity. However, the regulatory mechanism of BDNF expression is still unclear. We developed a novel mutant mouse line, transgenic insertional mutants with obesity, named Timo, in which a potential regulatory locus of Bdnf was disrupted by transgene insertion. The insertion site was identified and lies 857 kb upstream of the Bdnf gene. The disrupted genomic locus is conserved across the mouse, rat, dog, and human genome and contains several highly conserved elements that are able to upregulate reporter gene expression in vitro. Along with downregulation of BDNF to ~30% of wild-type animals, Timo/Timo mice exhibited increased body weight and fat content with hepatic steatosis and elevated serum levels of leptin, cholesterol, and LDL cholesterol. These mutant mice also showed obesity-independent insulin resistance, hyperinsulinemia, impaired glucose tolerance, age-dependent hyperglycemia, and shortened life span.

Molecular and phenotype analysis of Timo/Timo mice indicated the existence of a genome locus, lying 857 kb upstream of the Bdnf gene, that regulates BDNF expression, body weight, and glucose homeostasis.

SEVERAL LINES OF EVIDENCE indicated that brain-derived neurotrophic factor (BDNF) is crucial for glucose homeostasis and food intake regulation, in addition to its roles in survival, synaptic activity, and plasticity of neurons through tropomyosin-related kinase B (TrkB) receptor. Lapchak and Hefti (11) first reported that central administration of BDNF attenuated food intake regulation, in addition to its roles in survival, synaptic activity, and plasticity of neurons through tropomyosin-related kinase B (TrkB) receptor. Lapchak and Hefti (11) first reported that central administration of BDNF attenuated food intake suppression and weight loss. The TrkB hypomorphic mouse, in which the receptor of BDNF was reduced to one-quarter of the normal expression level in the brain, also demonstrated obesity and increased linear growth, indicating that BDNF function is mediated by the TrkB receptor (34). This is consistent with the report that a patient harboring a de novo missense mutation on TrkB displayed severe obesity (37). Moreover, peripheral injections of BDNF decreased food intake and lowered blood glucose level in db/db mice (21, 28).

Hyperphagia, obesity, hyperleptinemia, hyperinsulinemia, and hyperglycemia in Bdnf heterozygous mice and brain-specific conditional knockout mice proved the physiological role of BDNF in body weight control (8, 14, 25). Xu et al. (34) further suggested that BDNF in the ventromedial hypothalamic neurons may serve as an effector in the melanocortin-4 receptor signaling pathway controlling energy balance.

The Bdnf gene is composed of a complex structure of multiple regulatory elements and four promoters that regulate differential expression in central or peripheral tissues (17, 26, 27). The multiple transcripts by alternative promoter usage encode the same BDNF protein (exon V). The transference and release of BDNF protein can be regulated in an activity-dependent manner that allows for “fine-tuning” (1, 10, 12, 13). Recently, a de novo paracenric inversion, 46,XX.inv(11)(p12p15.3), a region encompassing the BDNF locus, was reported in a patient with severe hyperphagia, obesity, and complex neurobehavioral phenotypes (4). One of the inversion break points was identified and lies ~800 kb upstream of the Bdnf transcript start point.

The random insertion of transgenic fragments into the mouse genome by pronuclear microinjection procedure is potentially mutagenic, and transgene-induced mutations were found in 5–10% of transgenic mice (16, 24, 33). There are examples of the accidental insertion of a transgene into a crucial locus of genome that yielded important information (2, 18). The present study began with the observation of the obesity phenotype in the descendants of an intercrossing of a transgenic mouse line in which the transgene itself is not expressed. We went on to fine-map the transgene integration site. Intriguingly, this integration caused a reduction in Bdnf expression and led to hyperphagia, obesity, and complex metabolism phenotypes.

MATERIALS AND METHODS

Animals. Mice were maintained on a normal 12:12-h light-dark cycle and provided regular mouse chow and water ad libitum at an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited specific pathogen-free facility. C57BL/6J mice and CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animal welfare and experimental procedures were carried out strictly in accordance with National Research Council guidelines for the care and use of laboratory animals (1996) and were reviewed and approved by the Model Animal Research Center (Nanjing University) Institutional Animal Care and Use Committee. The transgenic mice were generated by microinjection into pronuclei of single cell (C57BL/6J × CBA/CaJ) F1 mouse embryos with 2.5-kb transgene DNA. The transgene construct contains a mouse Fk1-1 promoter (AF153057), cDNA of the Rage gene (NM_007425), and an SV40 polyadenylation signal from the pEGFP-C3 vector (Clontech, Mountain View, CA) (Fig. 1A). Using RT-PCR and Western blot analysis, we found that the
transgene *Rage* is not detected in the transgenic insertional mutants with obesity, named *Timo*, line. Then the mouse line with the *Timo* allele was backcrossed to C57BL/6J mice to at least the N4 generation for these studies. For the data in Fig. 6, mice of the N8 generation were used.

**Genome walking.** Genome walking was performed using LA PCR In Vitro Cloning Kit, following the protocol provided by the manufacturer (TaKaRa, Shiga, Japan). To identify the 3'-flanking sequence of the transgene, three gene-specific primers, S1 crossing over Flk-1 promoter and *Rage* cDNA (5'-GGC GGT GTC AGG TCT AGA ATG CCA G-3'), S2 in *Rage* cDNA (5'-GAC AAC ACC CAC CCC ACC TCC CCC TGA ACC-3'), and S3 in SV40 polyadenylation signal (5'-TCC GAC ACC CCC ACC TGC CAG TG-3'), were designed for nested PCR. Briefly, genome DNA extracted from the liver of the obese mice was digested by *Sal* I and ligated with provided *Sal* I cassettes. The digested fragments with ligated cassettes were used as the first template for the following nested PCR. Nested PCR was performed using primer pairs (S1, C1), (S2, C2), and (S3, C2) in turn. C1 and C2 were universe primers provided with the kit. Luckily, we got the 3'-flanking sequence (FS1) as a result of a mispairing of primers. For identifying the 5'-flanking sequence of the transgene, the two primers, Ch2S1 (5'-ATC CTC AAA CCC ACA AAT GCT GAC TCC-3') and Ch2S2 (5'-TTG AAA GCC ATG CTG AAG AAC ACT GC-3'), complimentary to the genome sequences at 108617659–108617825, were designed. The genome DNA of the obese mice was digested by *Bam* HI, ligated with *Bam* HI cassettes, and used as the template of the PCR. The 5'-flanking sequence of transgene was named flanking sequence-2 (FS2). Using this strategy, we found that the transgenic fragments inserted with inverse repeats and the insertion also caused a 260-bp deletion.

**Genotyping.** Primers fRage-f (5'-CAG GAC CCC ACA AAT GCT GAT CCC-3') in Flk-1 promoter and fRage-r (5'-CCT CAT CAG A AA AAG AG CACT GC-3') in *Rage* coding sequence were designed for transgene genotyping. Primers pA-f (5'-TCA CTG CAT TCT AGT TGT G-3') located in SV40 polyadenylation signal and Ch2-r (5'-GGA AAA GAA CAA TGG ATG TAG C-3') in FS1 were designed for genotyping of the right integration site. Primers Ch2S1 and S2, mentioned above, were used for genotyping of the left integration site. The three kinds of genotyping methods above will all give positive results in hemizygous or homozygous transgenic mice. PCR with

![Fig. 1. Identification of the transgene integration site in *Timo* mice. A: structure of the *Rage* transgene. The EcoR I/Mlu I fragment consists of a 0.9-kb Flk-1 promoter, a 1.2-kb mouse *Rage* coding sequence, and a 0.3-kb fragment containing SV40 early mRNA polyadenylation signal. B: diagram of the flanking sequences identified by genome walking, genetic markers used for linkage analysis, and *agouti* gene in chromosome 2 (Ch2). FS1, flanking sequence-1 at 108618086–108618208; FS2, flanking sequence-2 at 108617659–108617825; D2Mit249, marker at 101684575–101684721; D2Mit481, marker at 112973603–112973714; *agouti*, 154482843–154742453. C: diagram of the transgene integration in *Timo* mice. The *Bam* HI digest sites and the probe for Southern blots are indicated. Bdf1 gene is 857 kb apart from FS1. TG, transgene integration allele; WT, wild-type allele; BDNF, brain-derived neurotrophic factor. D: a PCR-based analysis of genomic DNA from interbred transgenic mice, which can be used for genotyping, indicating the PCR products specific for the presence of the transgene (transgene specific) and those specific for the endogenous sequence disrupted by the transgene (integration site specific). E: representative Southern blot of *Bam* HI-digested genomic DNA with a 5'-integration site-specific probe. TG, transgene-specific bands from disrupted allele; WT, endogenous bands from wild-type allele.](http://physiolgenomics.physiology.org/)

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primer Ch2-f (5’-GAG TCA CCA TAA AGG AGG AG-3’) and primer Ch2-r was used to detect endogenous intact sequence at the integration site and gave positive results in wild-type, or hemizygous, mice but negative results in homozygous mice.

**Linkage analysis.** Two microsatellite markers, D2Mit249 (DNA segment, Chr 2, Massachusetts Institute of Technology 249) and D2Mit481, the lengths of which are different in C57BL/6J and CBA/Caj backgrounds, were used for linkage analysis to map the insertion sites. The mice in which the marker showed both bands from CBA/Caj and C57BL/6J backgrounds were selected as parents. Genotyping was performed on all of the descendants of these mice, backcrossed with C57BL/6J. Almost all of the descendants showed both bands when genotyped, indicating that the transgene integrated into the CBA/Caj allele. So, the marker in the descendants will show two bands if it linked to the integrated transgene and only one band if the separation happened. The linkage ratio of the marker with the transgene is the portion of mice with two bands relative to total mice.

**Southern blot.** Genome DNA extracted from mouse liver was digested by BamH I, separated by agarose gel, and transferred to Zeta-Probe blotting membrane (Bio-Rad, Hercules, CA). The blot was hybridized with DNA probes prepared by Rediprime II Random Hybridization Solution (Clontech), washed, and developed following the protocol provided by the manufacturer.

**BDNF measurements.** Transcripts of Bdnf were determined by Northern blot and quantitative real-time RT-PCR. Total RNA isolated from brain, hypothalamus, and heart by TRIzol reagent (Invitrogen, Carlsbad, CA) was separated by denaturing agarose gel and transferred to Zeta-Probe blotting membrane. DNA probe containing Bdnf coding sequence (GenBank accession no. X55573) was used for hybridization. Primers targeting specific Bdnf transcripts and β-actin as the reference gene were designed for real-time RT-PCr (SYBR): Bdnf IV (forward, ACA GCA AAG CAA CTA TGT GCC; reverse, AGG CCA AGT CTC CCT GTC C), Bdnf IV' (forward, AGC GTG ACA ACA ATG TGA CTC C; reverse, TAG GCC AAG TGG CCT TGT CC), Bdnf all (forward, CCC ATG AAA GAA GAA GTA GTC CCT; reverse, GTC GTC AGA CCT CTC GAA CC), and β-actin (forward, GAG AAG ATC TGG CAC ACC; reverse, GCA TAC AGC GAC AGC ACA GC), BDNF protein levels were measured by Western blot. Tissue lysates from well-isolated hypothalamus were obtained in a buffer containing 50 mM Tris, pH 7.4, 5 mM NaCl, 1% Triton X-100, 1 mM DTT, and 15 mM EGTA supplemented with 1:100 Protease Inhibitor Cocktail (Sigma, St. Louis, MO). The blotted proteins were exposed to antibody to BDNF (N20); sc546 (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), following conventional protocols. Serum BDNF was measured after an overnight fast using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

**Food intake, linear growth, growth curve, and life span.** Before measurement of daily food intake, seven pairs of 12-wk-old female mice fed ad libitum were individually housed for 3 days. Food was measured at 12 PM each day for 7 consecutive days. For determination of linear growth, mice aged 24 wk were anesthetized and fully measured at 12 PM each day for 7 consecutive days. For determination of daily food intake, seven pairs of 12-wk-old female mice were obtained by measuring body weight once a week following conventional protocols. Serum BDNF was measured after an overnight fast using an unrelated research project, in which the mouse birth and females were obtained by measuring body weight once a week during 5-min exposures to wild-type male intruder mice. The latency to first biting was monitored during 5-min exposures to wild-type male intruder mice. Five tests were performed, one trial per day. The latency to first biting attack and the total time of biting attacks were recorded. For mice that failed to attack, the latency was recorded as 5 min.

**Resident-intruder aggression assay.** Male resident test mice 14–25 wk old were isolated for 1 wk. Aggressive behaviors in test mice were monitored during 5-min exposures to wild-type male intruder mice. Five tests were performed, one trial per day. The latency to first biting attack and the total time of biting attacks were recorded. For mice that failed to attack, the latency was recorded as 5 min.

**Body composition.** Uterine, perirenal, and mesenteric fat pads were harvested from 40-wk-old female mice and weighed. Another set of 40-wk-old male mice were anesthetized after 16 h of fasting by intraperitoneal injection with Avertin (Sigma Aldrich, St. Louis, MO), and fat and lean contents were measured by dual-energy X-ray absorptiometry (DEXA) (PIXImus, GE Medical Systems Lunar, Madison, WI).

**Metabolic measurements.** Blood was collected by cardiac puncture after an overnight fast, and serum was assayed with commercially available ELISA kits for leptin (R&D Systems) or insulin (Shibayagi, Gunma, Japan). Blood glucose was measured by Glucocard II Series of Test Meter (Arkray, Kyoto, Japan) from cut tail tips. Serum cholesterol, HDL cholesterol, and LDL cholesterol were examined by colorimetric kit assays and analyzed using a spectrophotometer (Hitachi, Mountain View, CA).

**Insulin and glucose tolerance test.** For insulin tolerance test, the mice were fasted for 4 h and received a single intraperitoneal injection of 0.05 U/ml insulin (0.5 U/kg body wt at time 0). Blood glucose measurements were taken at 30, 15, 30, and 60 min from cut tail tips. The intraperitoneal glucose tolerance tests were performed after 16 h of fasting. Mice received a single intraperitoneal injection of 20% glucose solution (2 g/kg body wt) at time 0. Blood glucose levels were measured at 30, 60, 90, 120, and 180 min.

**Sequence conservation analysis and cloning of the conserved elements.** Vertebre Multiz Alignment and Conservation was performed using the Blat tool of the University of California Santa Cruz (UCSC) genome bioinformatics site (http://genome.ucsc.edu/). The genome positions of the conserved regions by Blat were compared with the locations of BDNF gene in different species to verify whether they are orthologs. Primers were designed for cloning of the Bdnf promoters and the conserved elements: P1 (forward, GAA GAT CTA AAC CTG AAA AGG AGC TTC; reverse, GAA GAT CTA AAC CTG AAA AGG AGC TTC; containing Bgl II digestion site (underlined); reverse, CCT CGC AAT ATC CGC AAA GA), P3 (forward, GAA GAT CTC ACT TTG TTA ACC ATC AGC CC; containing Bgl II digestion site; reverse, CCT CGA AGC TTA AGT AAA CAT CAA GAC GC; containing Hind III digestion site), left conservation (LC) (forward, GAG AAT TGG CAC ACC; reverse, GCA TAC AGC GAC AGC ACA GC), BDNF protein levels were measured by Western blot. Tissue lysates from well-isolated hypothalamus were obtained in a buffer containing 50 mM Tris, pH 7.4, 5 mM NaCl, 1% Triton X-100, 1 mM DTT, and 15 mM EGTA supplemented with 1:100 Protease Inhibitor Cocktail (Sigma, St. Louis, MO). The blotted proteins were exposed to antibody to BDNF (N20); sc546 (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), following conventional protocols. Serum BDNF was measured after an overnight fast using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

**RESULTS**

*Generation of Timo transgenic mice. The Timo mouse strain was generated by an unrelated research project, in which the transgene was integrated into the CBA/Caj allele. So, the marker in the descendants will show two bands if it linked to the integrated transgene and only one band if the separation happened. The linkage ratio of the marker with the transgene is the portion of mice with two bands relative to total mice.**
transgenic mice were generated with transgene construct containing mouse Flk-1 promoter, mouse Rage cDNA, and an SV40 early mRNA polyadenylation signal (Fig. 1A) by microinjection of one cell embryo from the F1 mouse (C57BL/6J × CBA/CaJ). Among all the transgenic lines, five lines did not show Rage transgene expression by RT-PCR (data not shown). Because the transgenic procedure is potentially mutagenic (16), these five transgenic lines were intercrossed separately to screen the potential recessive phenotype. Interestingly, one of them had descendants with the obesity phenotype (see Fig. 4A).

We hypothesized that the obesity phenotype was caused by transgenic insertional mutagenesis because the Rage transgene is not expressed in this line, and previously generated Rage transgenic mice were not obese (35). This line of mice was identified by the integration site and was repeatedly backcrossed to the C57BL/6J genetic background by only choosing the mouse that could generate obese mice when backcrossed with parent mouse.

Identification of the transgene integration site. About one-fourth (43 obese mice of a total of 161 descendants) of the progeny from nonobese intercrossed Timo mice exhibited the obesity phenotype, indicating that the recessive Timo phenotype is caused by disruption of a single locus in the genome with transgene insertion. We adopted a genome walking strategy for quick cloning and sequencing of the adjacent genomic flanking transgene insertion (9). Two flanking genomic sequences, FS1 and FS2, were identified in the obese Rage transgenic mice. Using basic local alignment search tool (BLAST) with mouse genome, we determined that FS1 was a 3′-flanking sequence in the forward direction (108618086–108618208) in chromosome 2; FS2 was a 5′-flanking sequence in the reverse direction (108617659–108617825) in chromosome 2 (Fig. 1, B and C). We found that these two flanking sequences were 260 bp apart (Fig. 1B), suggesting that the transgene integration caused the deletion of a 260-bp DNA fragment between FS1 and FS2.

To confirm that the transgene integration site was in chromosome 2, linkage analysis was performed. Two markers, D2Mit249 (101684575–101684721) at the left of the integration site and D2Mit481 (112973603–112973714) at the right of the integration site, were selected for linkage analysis during backcross to C57BL/6J background (Fig. 1B). The linkage ratios of these two markers with Rage transgene were 97.4% (74/76) and 100% (67/67), respectively, indicating that the markers were closely linked with the transgene. Moreover, 87.6% (120/137) of the Timo+/ mice descendants showed grey coat color when the grey transgenic mice were backcrossed with black C57BL/6J mice, so the transgene was also linked with the dominant agouti gene, which controls coat color, at a distance of 46 Mb away from the flanking sequences in chromosome 2 (Fig. 1B). With the use of PCR primers based on the inserted transgene and the flanking endogenous genomic DNA, the wild-type (+/+), and transgene integration alleles (Timo+/ for integration of 1 allele; Timo/Timo for integration of both alleles) could be distinguished (Fig. 1D). Furthermore, Southern blot was also attempted to confirm the integration site. The probe beyond FS2 detected both the wild-type band and the transgenic band in Timo+/ mice but only the transgenic band in Timo/Timo mice (Fig. 1, C and E). These results are consistent with the genome walking data.

**Decreased Bdnf expression caused by transgene integration.** We next asked which gene was affected by the transgene integration. The transgene integration site was not located in any known or predicted genes. The nearest well-known gene to the integration site is Bdnf, haploinsufficiency of which causes obesity in both mice and humans (4, 8, 14). Therefore, we examined the expression of Bdnf in Timo/Timo mice, although the transgene integration site is 857 kb upstream from the Bdnf transcription start site (Fig. 1C). Northern blots detected two transcripts of Bdnf as previously described (6). Interestingly, Bdnf mRNA was found reduced in the brain and hypothalamus of Timo/Timo mice compared with wild-type controls aged 4 wk (obesity is not developed at this stage), 17 wk, and 35 wk (Fig. 2, A and B). Bdnf mRNA was also decreased in the heart of Timo/Timo mice (Fig. 2C). Reduced protein levels of BDNF in the hippocampus and the hypothalamus of Timo/Timo mice were evident by Western blot compared with wild-type controls (Fig. 2, D and E). Moreover, serum BDNF was decreased significantly to ~30% of the normal level in Timo/Timo mice (Fig. 2D).

Driven by four promoters, the Bdnf gene has at least eight known transcripts with four different 5′-ends, referred to as I, II, III and IV, and two alternative polyadenylation sites (27, 29). Real-time RT-PCR was performed to determine the expression levels of Bdnf transcripts I/′ and IV/IV′ in Timo mice. The former are the prominent transcripts of Bdnf expressed in hypothalamus, and the latter are the prominent transcripts of Bdnf expressed in lung and heart (27). Bdnf transcripts I/′ decreased to 35% in the hypothalamus (Fig. 3A) and to 55% in the hippocampus (Fig. 3B) compared with wild-type controls. Bdnf transcripts IV/IV′ decreased to 36% in the hypothalamus (Fig. 3C) and to 77% in the hippocampus (Fig. 3D). Bdnf transcripts IV/IV′ also decreased (76%, P = 0.065) in the lung of Timo/Timo mice, although the difference was not significant (Fig. 3E). The levels of all Bdnf transcripts were also determined and found to decrease to 44% in the hypothalamus (Fig. 3F) and to 77% in the hippocampus (Fig. 3G). Interestingly, the expression of Bdnf transcripts is reduced by the greatest amount in the hypothalamus, less in the hippocampus, and not significantly in the lung.

**Hyperphagia and obesity of Timo mice.** The adult Timo Timo mice became severely obese, whereas the Timo/+ mice were moderately obese compared with the sex-matched wild-type littermates (Fig. 4A). Growth curves were measured, and the body weight of Timo/Timo mice compared with that of wild-type littermates reached statistical significance at 6 wk of age at N4 to N5 (Fig. 4, D and E) and at 7 wk of age at N8 (see Fig. 7E). Timo/+ mice showed relatively moderate but significant obesity compared with wild-type controls, starting at 10 wk in females and 18 wk in males. By 69 wk, the females and males were 119 and 37% heavier than the age-matched wild-type littermates in Timo/Timo mice and 57 and 31% heavier in Timo/+ mice, respectively.

The development of obesity in Timo mice may result from elevated energy intake and/or decreased energy expenditure. To assess whether the Timo mice were hyperphagic, daily food intake was monitored in animals fed a standard chow diet ad libitum for 7 consecutive days. The homozygotes consumed 57% more food than the controls, indicating marked hyperphagia (Fig. 4B). Energy expenditure was also determined by...
measuring rectal temperature under fast and cold challenge, and no significant difference was found between homozygotes and wild types in both conditions (data not shown). This result suggested that thermogenesis is unaffected in Timo mice, or, alternatively, that this effect is too small to be detected by the methods employed in this study.

By measuring body length (naso-anal) at 24 wk of age, altered linear growth was observed in the Timo mice. The mean lengths of female and male mice were increased 11% and 7% in Timo/Timo mice vs. 7% and 3% in Timo/+ mice, respectively, compared with the wild-type controls (Fig. 4C).

Because altered aggressiveness has been investigated in BDNF+/− mice (14), offensive intermale fighting was measured to investigate aggressive behavior in Timo mice by using a resident-intruder paradigm. The latencies to first biting attack were significantly shorter in Timo/Timo residents than in wild-type residents (Fig. 5A), and the duration of biting attacks was significantly longer in the Timo/Timo group (Fig. 5B). The results suggested that Timo/Timo mice are more aggressive than wild-type mice.

**Figure 2.** Decreased Bdnf expression caused by transgene integration. A and B: Northern blots of total RNA from the brain and the hypothalamus of mice at the ages of 4, 17, and 35 wk. Arrows indicate the detected Bdnf transcripts. 18S RNAs were used for loading control. C: representative Northern blot of total RNA from heart of wild-type and Timo/Timo mice. D and E: representative Western blots of the hippocampus and the hypothalamus protein from mice of +/+ and Timo/Timo genotypes. Arrow indicates the BDNF band. Bottom: Ponceau red-stained protein for loading control. F: serum levels of BDNF in +/+ and Timo/Timo mice. Error bars indicate SE (n = 7). *P < 0.05 vs. wild-type controls.

**Increased Bdnf expression results in increased fat stores.** To determine whether the increased weight of Timo mice reflected changed body composition, DEXA was performed on Timo mice at 40 wk of age. Distinct fat pads (uterine, perirenal, and mesenteric) were also dissected and weighed. As shown in Fig. 6, A and B, both fat mass and lean mass were increased in Timo/Timo mice, whereas only fat mass was elevated in Timol/+ mice. The percentages of fat were increased in both Timol+ and Timo/Timo mice (Fig. 6B). Consistent with increased fat mass, serum leptin levels were also increased ~10-fold in Timo/Timo mice compared with wild-type mice (Fig. 6C). In addition, cholesterol levels were increased by 95% in Timo/Timo mice compared with wild-type controls (Fig. 6D). HDL cholesterol levels in homozygous mice were similar to those in wild types, whereas LDL cholesterol levels were increased in both Timol+ and Timo/Timo mice (Fig. 6D). Accumulation of lipids (hepatic steatosis) was also found in the liver of Timo/Timo mice (Fig. 6E).

Timo/Timo mice exhibit phenotypes of type 2 diabetes. It has been demonstrated that exogenous BDNF enhances the hypo-
The glycemic effect of insulin (19). Obesity is always associated with insulin resistance, which may be mediated by inflammation or by some adipose-secreted molecules such as retinol-binding protein-4 (32, 36). In Timo/Timo mice older than 8 wk, insulin resistance was observed along with obesity by insulin tolerance test (data not shown), and plasma insulin levels were elevated (see Fig. 7C). To investigate whether BDNF insufficiency could induce insulin resistance independently of obesity, we performed insulin tolerance tests at the age of 6 wk, when the body weight of the homozygous mice showed no significant difference from that of controls at the N8 generation (Fig. 7A). These young Timo/Timo mice demonstrated significant insulin resistance compared with their wild-type littermates (Fig. 7B). Timo/Timo mice also displayed impaired glucose tolerance (Fig. 7D) along with elevated plasma insulin levels (Fig. 7C). However, the blood glucose level did not increase significantly until 9 wk of age, ~2 wk after the significant body weight gains (Fig. 7, E and F). These results suggest that later development of the diabetes phenotype may be dependent on a preexisting abnormal insulin sensitivity as well as obesity-related changes.

Because obesity and diabetes are both risk factors for cardiovascular disease and early mortality (31), we traced the life spans of Timo mice. By 67 wk of age, the mortality rate of
Timo/Timo mice was ~15% in both females and males, whereas the Timo/+ and wild-type mice all survived (Fig. 4, F and G).

Highly conserved elements in the transgene integration region. The decreased expression of the Bdnf gene caused by the transgene integration suggested the existence of a potential regulatory locus at the transgene integration site. The transgenic fragment integrated into the regulatory locus and interrupted its regulatory function, leading to the reduction in Bdnf expression. Comparison among the human, mouse, rat, and dog sequences revealed that the transgene-integrated genomic region is conserved throughout the potential regula-

![Diagram](image_url)
tory locus when using a 100-bp and 70% identity cutoff, as previously used in the identification of functional mammalian regulatory elements (23) (Fig. 8A). The conserved genome region lies ~900 kb upstream of the Bdnf orthologs in all investigated species except in rat, in which the position of the Bdnf locus in the genome is unclear (data not shown). Comparison of the mouse with opossum and chicken sequences shows a decrease in the conserved sequences but highlights four highly conserved elements, named LC, RC1, RC2, and RC3, according to their relative position to the transgene integration site, respectively (Fig. 8A). A longer fragment containing RC3 was named RC3-l (RC3, long form); the fragment containing the 260-bp DNA sequence deleted by the transgene integration was named DF (Fig. 8A).

Functional analysis was performed on the highly conserved elements and the DF sequence of mouse, respectively, by assaying their ability to upregulate luciferase reporter gene expression driven by Bdnf promoter I (P1) (5) and Bdnf promoter III (P3) (26) in mouse-derived NIH-3T3 cells and human-derived HEK293T cells, in both of which Bdnf expression was confirmed by RT-PCR (data not shown). As shown in Fig. 8, B–E, LC was not able to upregulate reporter gene expression, and neither was RC1, except when the reporter was driven by P1 in HEK293T cells; DF could upregulate reporter gene expression by ~50%, which was significant. RC2 enhanced reporter expression ~1.5- to 2.5-fold, but the difference was not significant when reporter was driven by P3 in HEK293T cells. RC3 could enhance reporter levels ~1.5- to 4-fold in every situation; RC3-l elevated luciferase reporter gene expression ~2.5- to 4.5-fold. These results suggest that the enhancer abilities of the conserved genomic sequences may account for the regulatory function of the novel regulatory locus for the Bdnf gene.

Fig. 5. Offensive intermale aggression in Timo mice as assessed by resident-intruder assay (n = 11/genotype; 25–33 wk of age). A: latency to first biting attack. B: total time of biting attacks measured during 5 consecutive trials. Repeated-measures ANOVA revealed significant differences between the Timo/Timo and wild-type (+/+ ) groups for both biting attack latency (F = 14.90, P < 0.001) and time (F = 10.75, P < 0.01).

Fig. 6. Effects of decreased Bdnf on fat stores and metabolism. A: fat pad weight of female mice. B: body fat and lean composition of male mice by dual-energy X-ray absorptiometry analysis. C: serum leptin levels of male mice. D: serum levels of cholesterol, HDL cholesterol (HDL-C), and LDL cholesterol (LDL-C) of male mice. All data are presented as means ± SE (40 wk of age, n = 6–8/group). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. +/+ controls. E: representative hematoxylin and eosin staining of hepatic sections from 28-wk-old male mice.
DISCUSSION

On the basis of the strong evidence of decreased expression of BDNF in Timo/Timo mice and the phenotype similarities between Timo/Timo mice and other previously reported mouse models with BDNF haploinsufficiency or conditional deletion in the postnatal brain or TrkB hypomorph (8, 14, 25, 34), it is highly plausible that the phenotypes in Timo mice have resulted from the reduction in BDNF, and Timo/Timo mice may serve as a novel model for type 2 diabetes and obesity.

The transgene integration site in Timo mice was precisely identified at 108,618 kb in chromosome 2 by genome walking, genetic mapping, and Southern blot assay. It is interesting that this insertion could reduce expression of the Bdnf gene, because it is located ∼110 kb upstream of the transcription start point. There are two putative genes, Mett5d1 and Kif18a, that lie between the transgene insertion site and BDNF gene. However, RT-PCR assay failed to detect any of these transcripts in the brain (data not shown), and inspection of the mouse transcript map revealed no other obvious candidate genes near the integration site. Therefore, it is more likely that the transgene insertion disrupted a novel regulatory genomic locus that regulates BDNF expression.

Such a long-distance gene regulation has been reported for the sonic hedgehog gene, although the molecular mechanism is completely unknown (15). The recent report of a human case of hyperphagia, severe obesity, impaired cognitive function, and hyperactivity induced by BDNF haploinsufficiency also supports this explanation (4). This patient carried a distinct chromosomal inversion containing the BDNF locus. One of the proximal break points was fine-mapped to ∼850 kb upstream of the BDNF gene in the human genome by fluorescence in situ hybridization (FISH). The corresponding position of this break point is only ∼17 kb downstream of the transgene integration site in Timo mice. By this distinct inversion, the expression of BDNF at the same allele was totally shut down, because the BDNF transcripts in the patient did not contain the single nucleotide polymorphism residing in the allele with chromosomal inversion. Although the authors (4) did not present any ideas about how the chromosome inversion affects BDNF expression, their data and our results all fit very well with the hypothesis that a regulatory locus upstream of the inversion break point is crucial for the expression of BDNF.

It is very interesting to identify the potential sequence for BDNF transcriptional regulation, although the present system makes it difficult to draw direct conclusions. Genomic sequence comparison confirmed that the locus is conserved across multiple species and contains several highly conserved elements, such as RC2 and RC3, that possess potential transcription factor-binding sites (Fig. 8). The luciferase assay with BDNF basic promoter and these conserved elements indicated...
that these elements can functionally enhance the activity of BDNF basic promoter. These results gave some explanation about why the transgene insertion could reduce BDNF expression, although more detailed analysis is required for the explanation of the 857-kb distance between these elements and the BDNF promoter in the genome. Finally, it is impossible to rule out the possibility that other genes may also be affected by the regulatory locus, although we are inclined to believe that BDNF is the key target gene for this regulatory locus.

The reduction of BDNF expression in Timo/Timo mice is different from the haploinsufficiency in Bdnf/H11001/H11002 mice. Bdnf transcripts in hypothalamus of Timo/Timo mice decreased to 35%, whereas, in other tissues such as hippocampus and heart, the decrease was much less significant. It is plausible that

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Fig. 8. Highly conserved elements with enhancer function in the regulatory locus. A: sequence conservation and definition of highly conserved elements in the regulatory locus around the transgene integration site. Sequence conservation levels for mouse-rat, mouse-human, mouse-dog, mouse-opossum, mouse-chicken, mouse-Xenopus tropicalis, and mouse-tetraodon are analyzed by the University of California Santa Cruz Blat tool (http://genome.ucsc.edu/). Arrow indicates the transgene integration site at 108617826–108618085. The highly conserved elements are defined as follows: LC, left conservation; RC1, right conservation-1; RC2, right conservation-2; RC3, right conservation-3; and RC3-I, right conservation-3, long form. DF, the genomic sequence containing the 260-bp deleted fragment induced by the transgene integration. B–E: luciferase assays for the ability of the conserved elements and DF in the regulatory locus to enhance reporter gene expression driven by Bdnf promoter I (P1) (B and D) or Bdnf promoter III (P3) (C and E) in NIH-3T3 cells (B and C) or HEK293T cells (D and E). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. P1 and P3 controls, respectively.
the greater reduction in BDNF levels in hypothalamus of Timo/Timo mice leads to more severe phenotypes than haploinsufficiency of BDNF in BDNF+/− mice. Actually, the increase in body weight of Timo/Timo mice reaches significance earlier than for BDNF+/− mice (7 vs. 10 wk, respectively) (Fig. 7E) (14). At 6 mo of age, Timo/Timo females are 117% heavier than littermates (Figs. 4D and 7E), whereas BDNF+/− females are 33% heavier than controls (8). The more severe obesity may contribute to increased mortality in Timo/Timo mice.

Hyperphagia has been observed in Timo/Timo mice (Fig. 4B) and other BDNF mice models (14, 25). This increased energy intake is likely the major cause of obesity, because restriction of food intake could decrease the body weight to normal levels in BDNF heterozygous mice (3). It is worthy to note that, unlike the db/db mice, therogenesis is normal in Timo/Timo mice, although administration of BDNF increased energy expenditure in db/db mice (20). This suggests that there are differences between physiological and pharmacological functions of BDNF and leptin.

Obesity has been closely associated with an increased risk of developing insulin resistance and type 2 diabetes (7). However, we think that the insulin resistance and diabetes in Timo/Timo mice are at least partially independent of obesity, because impaired insulin tolerance was detected before the onset of obesity in Timo/Timo mice (Fig. 7, A and B). This result is consistent with previous reports that peripheral or intracerebroventricular administration of BDNF enhances insulin signal transduction in the liver of diabetic mice (30), and exogenous BDNF exerts its hypoglycemic effect independently of its hypophagic effect in obese diabetic mice (20).

In conclusion, we discovered a novel regulatory locus that may be crucial for Bdnf expression, food intake regulation, insulin sensitivity, and glucose homeostasis in mice. In the future, it will be valuable to elucidate the molecular mechanism of this long-distance regulation by genome manipulation as well as the physiological significance of this regulation manner in energy homeostasis and body weight control.

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