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Adiposity and serum leptin increase in fatty (*fa*/*fa*) BNZ neonates without decreased VMH serotonergic activity

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Horwitz, Barbara A., Jock S. Hamilton, Vanessa H. Routh, Kerri Green, Peter Havel, and Albert Chan. Adiposity and serum leptin increase in fatty (*fa*/*fa*) BNZ neonates without decreased VMH serotonergic activity. *Am. J. Physiol.* 274 (*Endocrinol. Metab.* 37): E1009–E1017, 1998.— Decreased ventromedial hypothalamic (VMH) serotonergic activity occurs in genetic and diet-induced animal models of obesity. We previously found that this activity was lower in adult and in 12-day-old Zucker *fa*/*fa* vs. *Fa*/*Fa* pups, the *fa*/*fa* animals being identified by their greater adiposity. In the present study, we evaluated *fa*/*fa* rats (Brown Norway-Zucker hybrids) at ages 2, 4, 7, and 12 days to test the hypothesis that lower VMH serotonergic activity occurs before increased adiposity and/or attenuated energy expenditure. Our results negate this hypothesis. VMH serotonergic activity showed no consistent genotype differences even at 12 days of age. In contrast, by *day 7*, *fa*/*fa* vs. *Fa*/*Fa* pups had higher serum leptin concentrations, greater percent body fat, lower resting and cold-induced energy expenditure, and lower activity of brown fat thyroxine 5′-deiodinase, an enzyme that converts thyroxine to triiodothyronine. We conclude that the onset of increased adiposity induced by the *fa* gene does not require decreased VMH serotonergic activity and that the lower serotonergic activity seen in older *fa*/*fa* pups may be secondary to metabolic consequences of the disruption of the leptin regulatory pathway.

genetic obesity; energy expenditure; brown adipose tissue; uncoupling protein; Brown Norway-Zucker rats; thyroxine 5′-deiodinase; ventromedial hypothalamus

HYPOTHALAMIC SEROTONERGIC activity appears to contribute significantly to central regulation of energy balance (10, 12, 21). This view is supported by evidence from studies of rodents and humans, including the following observations: metabolic rate is increased and food intake is decreased in humans and rodents after systemically administered fenfluramine [which elevates brain levels of serotonin (5-HT); see Refs. 19, 26, 31]; similar decreases in food intake occur after peripheral administration of fluoxetine and sertraline (19, 39), both of which elevate brain 5-HT, and after microinjection of 5-HT into the paraventricular nucleus (PVN; see Ref. 21); microiontophoresis of 5-HT excites thoracic sympathetic preganglionic neurons (24); injection of 5-HT into the PVN or the ventromedial hypothalamic nucleus (VMH) results in increased firing rates of sympathetic nerves to brown adipose tissue (35); and intracerebral ventricular injection of *p*-chlorophenylalanine (which decreases brain 5-HT levels) reduces brown fat thermogenic capacity (10). In addition, injection of 8-hydroxy-dipropylamino-tetralin (a 5-HT_{1A} agonist) into the medial and dorsal raphe, where 5-HT cell bodies are located, results in increased food intake (12). Because 5-HT_{1A} receptors in the raphe are inhibitory autoreceptors (7, 15), their activation would decrease serotonergic transmission from the raphe. All of these data, as well as the fact that Zucker rats with genetic or diet-induced obesity exhibit lower 5-HT turnover/release in their VMH than do their lean counterparts (32, 33), are consistent with a role for 5-HT in inhibiting food intake and stimulating sympathetic activity.

The finding that adult obese Zucker rats have blunted VMH serotonergic activity raised the question of whether this attenuation is secondary to the rats' obesity and/or associated metabolic disturbances (e.g., hyperinsulinemia and hyperphagia) or whether it occurs earlier in the development of obesity. We addressed this issue in a previous study in which we measured indexes of serotonergic activity in the VMH of 12-day-old Zucker pups that were presumed to be genetically obese (*fa*/*fa*) by virtue of their high levels of body fat (34). In comparison with known genetically lean (*Fa*/*Fa*) pups, the presumptive *fa*/*fa* pups were fatter, had higher serum insulin levels, and had lower VMH serotonergic activity than did the *Fa*/*Fa* pups. However, there was no linear relationship between VMH serotonergic activity and serum insulin or carcass fat. These data led to the conclusion that the lower serotonergic activity of the 12-day-old Zucker *fa*/*fa* pups was not likely to be secondary to their hyperinsulinemia or to their elevated body fat content (34). Nonetheless, because these variables were elevated in the *fa*/*fa* pups studied, it was not possible to determine whether the blunted serotonergic activity occurred before these elevations or before the attenuated energy expenditure of Zucker *fa*/*fa* pups, which occurs by *day 2* of age (2, 25, 29).

The present study was undertaken to examine this issue, i.e., to test the hypothesis that the lower VMH serotonergic activity observed in rats homozygous for the fatty (*fa*) mutation occurs before the onset of increased adiposity, increased serum insulin, and/or blunted energy expenditure. For this, we utilized 2-, 4-, 7-, and 12-day-old Brown Norway-Zucker (BNZ) hybrids, in which we could detect the *fa* gene by using molecular probes and thus distinguish homozygous lean (+/+) and obese (*fa*/*fa*) littermates at any age. We measured VMH levels of 5-HT and its metabolite, 5-hydroxy-3-indoleacetic acid (5-HIAA), circulating concentrations of insulin, and resting and cold-induced energy expenditure. In addition, we assayed serum leptin as an indicator of the disrupted leptin-adipose feedback system and as an additional index of adiposity (1), brown fat uncoupling protein (UCP)-1 content as an index of

brown fat sympathetically stimulated thermogenic capacity, and brown fat thyroxine 5′-deiodinase activity, which converts thyroxine (T_4) to triiodothyronine (T_3). The latter acts in conjunction with the sympathetic neurotransmitter, norepinephrine, to regulate expression of UCP1.

METHODS

Animal Breeding/Care

Brown Norway lean (+/+) female rats obtained from Charles River were mated with Zucker obese (*fa/fa*) male rats from the Animal Models Core of the University of California Davis Clinical Nutrition Research Unit. The resulting F_1 pups (+/*fa*) were crossed to each other to yield F_2 pups. These pups carry the *fa* and/or wild-type (+) allele on a background that is ~50% Zucker and 50% Brown Norway. All cages were provided with rat chow (Purina 5008) and water ad libitum. At *day 1* of age (*day 0* being the date of birth), each litter was culled to 10 pups. The afternoon before the experimental day, the dam and litter were transferred from the vivarium into the laboratory where they continued to be housed at 22–25°C on a 14:10-h light-dark cycle (lights on at 0600; off at 2000). The exception to this was the 2-day-old pups used in *Expt. 1*, which were brought into the laboratory on the morning of the experiment. Although pups of three genotypes were generated in these crosses, this paper compares the results from the homozygous lean (+/+) and obese (*fa/fa*) pups.

Genotyping

Pups were genotyped using DNA isolated from spleen via a method similar to that described by Truett et al. (42). Frozen spleens were ground with a mortar and pestle and incubated at 65°C for 2.5–3 h (or until completely digested) in a buffer containing (final concentration) 20 mg pancreatic RNase/ml, 0.1 mg proteinase K/ml, 10 mM Tris, 0.1 mM EDTA, and 0.5% SDS, pH 8. DNA was extracted using phenol-chloroform followed by ethanol precipitation. This DNA, resuspended in 10 mM Tris plus 1 mM EDTA, was used to genotype the pups either by restriction fragment-linked polymorphism (RFLP) analysis or by single sequence length repeat (SSR) analysis. For the RFLP analysis (40), genomic DNA was digested with *Taq* I, electrophoresed through a 1% agarose gel, transferred to nylon membrane (Duralon, Stratagene, LaJolla, CA) by capillary action with 15× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and fixed by ultraviolet cross-linking (UVP, San Gabriel, CA). These Southern blots were prehybridized at 60°C for ~60 min in 5× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1 mM EDTA disodium salt, pH 7.4), 5× Denhardt's (1× Denhardt's = 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA), 0.5% SDS, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. Approximately 50 ng of the human DNA fragment VC85 (American Type Culture Collection, Rockville, MD) were radioactively labeled with [α-^{32}P]ATP (Amersham, Arlington Heights, IL) via random priming, isolated in low-melting-temperature agarose (9), and used as a probe. Blots were washed two times with 2× SSC, 0.5% SDS at 65°C for 5 min each wash, one time with 1× SSC, 0.5% SDS at 65°C for 60 min, and finally with 0.5× SSC, 0.5% SDS at 65°C for 60 min. Obese (*fa/fa*) animals showed a distinctly different banding pattern when compared with +/+ animals (40). SSR analysis was performed according to the method of Kershaw et al. (18), which utilizes the polymerase chain reaction (PCR) with genomic DNA as a template. Primers based on sequences from the phosphoglucomutase locus, 1.25 cM from the *fa* gene, and the complement component 8 polypeptide locus, 3.74 cM from the *fa* gene, were used to amplify microsatellite polymorphisms that exist between the Zucker and Brown Norway strains. The resulting PCR products were run on 6% nondenaturing polyacrylamide gels at 350 V overnight and stained with ethidium bromide (0.5 μg/ml, 30 min). Pups showing recombination were excluded from the study.

Body Composition; Serum Insulin, Glucose, and Leptin; Tissue Protein

Composition of the eviscerated carcasses (minus the head and interscapular and cervical brown fat depots) was determined gravimetrically following the method of Bell and Stern (3). Serum glucose was measured by the glucose oxidase method using a Beckman Glucose Analyzer (Beckman Instruments, Irvine, CA). Serum leptin (*Expt. 1*) was analyzed by radioimmunoassay using mouse anti-leptin sera (Linco, St. Charles, MO; see Ref. 1). Serum insulin (*Expt. 2*) was also measured by radioimmunoassay as previously described (34). Purified rat insulin served as the reference standard (21.3 U/mg; Novo Biolab, Wilton, CT), porcine insulin antisera was purchased from ICN Diagnostics (Costa Mesa, CA), and ^{125}I-labeled insulin (1,800–2,100 Ci/nmol) was obtained from Amersham. Protein was measured using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL).

Experiment 1

Oxygen consumption. Pups were removed from the dam between 0700 and 0800, weighed, and placed into individual Plexiglas metabolic chambers submerged in a water bath for which the temperature was initially set at thermoneutrality. In preliminary experiments, we determined thermoneutrality for each age by ascertaining the temperature range at which there was minimal oxygen consumption (age 2 days: 32–34°C; 4 days: 31–33°C; 7 days: 28–30°C; 12 days: 27–29°C). Ambient temperature in each of the nine metabolic chambers was monitored with a YSI (Yellow Springs) thermister. Oxygen consumption was measured in an open system using an Ametek S3-A oxygen analyzer and a Houston strip-chart recorder. The S3-A can detect oxygen differences of 0.01%. In this experiment, the dried airflow rate was 200 ml/min for the 2-, 4-, and 7-day-old pups and 400 ml/min for the 12-day-old pups; the chamber size was ~200 ml; and the time for 99% washout of the system was ~4.9 min at 200 ml/min flow and 2.5 min at 400 ml/min flow. Data were corrected to standard temperature and pressure, and oxygen volume was calculated on the basis of *Eq. 4* in the study by Hill (13).

Using three separate oxygen analyzer-recorder systems, we measured up to nine pups per litter simultaneously over a range of temperatures by use of the following protocol. Pups were allowed to acclimate to the chamber for at least 30 min at thermoneutrality. Resting rates were then recorded over an additional 30–60 min such that, for every pup, we obtained three to four values, each recording period lasting at least 5 min. After this, the chamber temperature was lowered in steps of 1°C every 30 min until oxygen consumption passed the maximum for each pup. As each pup reached this point, it was removed from the chamber, its rectal temperature was measured, and it was decapitated. Because not all pups reached maximal oxygen consumption at the same temperature, pups were removed and killed at varying times. Generally, all nine pups were killed and tissues harvested within the span of 1 h. Immediately after decapitation, brown adipose tissue from the interscapular and cervical regions was removed, frozen in liquid nitrogen, and stored at −70°C

until preparation for UCP1 and deiodinase analyses; spleen was removed, similarly frozen, and stored at −70°C until used for genotyping; trunk blood was collected and centrifuged at 16,000 *g* for 20 min at 4°C, and the resulting serum was stored at −70°C until measurement of leptin; and the eviscerated carcass was stored at −20°C until body composition measurement.

UCP1. UCP1 was measured in brown fat homogenates by immunoassay using a modification of the method of Lean et al. (20). Homogenate aliquots (2–12 μg protein/well) were separated by SDS-PAGE (3% acrylamide stacking gel with an 11% lower gel). The resulting protein bands were transferred to nitrocellulose membranes, which were then blocked with nonfat dry milk, and probed with rabbit anti-rat UCP1 sera. UCP1 was detected using goat anti-rabbit antibody coupled to alkaline phosphatase (Bio-Rad, Richmond, CA) and quantified via scanning densitometry. Rat UCP, purified by the method of Lin and Klingenberg (22), served as the standard. The anti-rat UCP1 sera did not react with liver or muscle homogenates, indicating little, if any, cross-reactivity with UCP2 or UCP3.

Thyroxine 5′-deiodinase activity. Brown fat was thawed and homogenized in a buffer (9 vol/wt) containing (final concentration, in mM) 250 sucrose, 1.0 HEPES, 1.0 tetrasodium EDTA, and 5.0 dithiothreitol, pH 7.0. A sample was taken for measurement of protein (via the BCA assay), and the remaining portion of the homogenate was stored at −70°C until the day of assay when it was thawed, sonicated, and diluted to 5 μg protein/μl. At least three concentrations of the homogenate were incubated for 60 min at 37°C in a shaking water bath with 3 nM T_4 containing trace amounts of ^{125}I-labeled T_4 (~50,000 counts/min per incubation vial of 100 μl). The ^{125}I-T_4, which was labeled at the 5′ and 3′ positions (New England Nuclear Labs; specific activity, 4,400 Ci/mmol; 320 μC/ml), was purified of free ^{125}I on a column containing Sephadex LH-20 (25–100 μm particle size). After the 1-h incubation, vials were removed from the bath, bovine serum albumin (50 μl of 8% BSA) was added to bind T_4 and T_3, and trichloroacetic acid (350 μl of 10% trichloroacetic acid) was added to precipitate the protein. The incubation vials were then centrifuged for 3 min at 16,000 *g*, the protein pellet was discarded, and the supernatant was passed over a column of Dowex 50W-X2 (dry mesh 100–200) to remove any residual T_3 and T_4. The ^{125}I in the eluant was quantified using a Packard 5360 Auto-Gamma counter, and the amount of ^{125}I present in control vials (no homogenate) was subtracted from that in each experimental vial.

Experiment 2

General protocol. A second group of 12-day-old F_2 pups was generated, housed, and brought into the laboratory as described above. However, these pups were not cold exposed. Rather, the pups were separated from their dams at 0600 (immediately after lights on) and placed in individual cardboard boxes containing a layer of Kimwipes. The top of the box was covered loosely with a Kimwipe to reduce effects of air currents, and the box was positioned on a surgical heating pad (37°C) such that a thermal gradient was established and the pups could move to the thermal region of their choice. Using this protocol, we have found the air temperature in the boxes to be between 27 and 29°C and the P_{O_2} and P_{CO_2} to be similar to those in uncovered boxes. Pups were decapitated between 0800 and 0830, and their brains were rapidly removed, frozen on dry ice, and stored at −70°C until prepared for assay. Blood was collected, and serum was frozen and stored at −70°C until assayed for insulin. The carcasses were eviscerated and frozen until analysis of carcass composition as described above.

Brain dissections and analysis of 5-HT and 5-HIAA. The VMH was dissected according to the method of Palkovits (27). Brains were sectioned into 300-μm slices from which tissue punches were taken bilaterally in the VMH using a blunt 21-gauge needle. The starting points for coordinates were those from Sherwood and Timiras (37) for 10-day-old rat pups. These were then compared with our own atlas generated using 4-, 7-, and 12-day-old BNZ brains that were frozen, sliced into 80-μm sections, and stained for Nissl substance. Landmarks were distinguished by vascularity because white matter was not completely formed at 12 days of age. Anterior-posterior location was determined by the lateral ventricles, hippocampus, interpeduncular nucleus, and the pontine nuclei; and the location of the VMH was determined by its relationship to the third and fourth ventricles and its distance from the ventral border of the brain. Samples were placed in 210-μl cold mobile phase containing dihydroxybenzylamine as an internal standard, sonicated for 5–10 s, and centrifuged for 30 min at 19,000 *g*. The supernatant was stored at −70°C until analysis of 5-HT and its metabolite 5-HIAA. The pellet was stored at −70°C until determination of protein using the BCA assay method.

5-HT and 5-HIAA were analyzed using HPLC with electrochemical detection as described by Routh et al. (33). Peaks were quantified by area and compared with a regression line fitted to a series of standards analyzed throughout the day. Correlation coefficients for the standard lines always exceeded 0.97. The mobile phase consisted of 0.106 M chloroacetic acid buffer containing 0.45 mM EDTA, 0.40 mM 1-octanesulfonic acid, and 6.5% methanol, pH 2.9. Standards were solubilized in 0.131 M acetic acid containing 4.8 mM sodium bisulfate and 0.67 mM disodium EDTA. Ultrapure water (18 MΩ × cm resistance) was used for all solutions.

Levels of 5-HT and 5-HIAA are indexes of the activity of the serotonergic system in the VMH. The amount of 5-HT was taken to represent the 5-HT pool available under steady-state conditions (41); the amount of 5-HIAA was considered to be an index of 5-HT release; and the ratio of 5-HIAA to 5-HT was taken to be an index of turnover (11).

Statistical Analysis

All data (except for that from selected subsets of *fa/fa* and *Fa/Fa* pups in *Expt. 2*) were analyzed by multifactorial ANOVA with main effects of genotype and gender within age groups. Where significant differences occurred, a one-way ANOVA with a protected Fisher's least significant difference post hoc test was used for specific group comparisons. In *Expt. 2*, in addition to analyzing the data from all pups as indicated above, several variables were also analyzed in subsets of the two genotypes. This analysis was done by the Mann-Whitney rank-sum test. For all analyses, differences were considered significant at $P \leq 0.05$.

RESULTS

Experiment 1

Body mass, carcass mass, body composition, and brown fat thermogenic capacity. Although the *fa/fa* males were heavier than the *fa/fa* females at 2 days of age (7.05 ± 0.16 vs. 6.00 ± 0.20 g, respectively), this was not true of the lean pups. Additionally, there were no significant gender or genotype differences in body composition at this age (data not shown). Similarly, at 4 days of age, no significant effects of genotype or gender were observed for body mass, carcass mass, fat-free dry

mass (g or percent), or body fat (g or percent; Table 1). However, at *day 7*, percent carcass fat of the *fa/fa* pups was significantly greater than that of the +/+ pups ($P = 0.005$; Table 1) despite the absence of significant differences in body mass ($P = 0.48$), carcass mass ($P = 0.73$), or absolute amount of fat (g; $P = 0.06$). By *day 12*, both absolute and relative (percent) amounts of carcass fat were greater in the *fa/fa* vs. +/+ pups, with no gender differences in fat content (Table 1). Thus, between ages 4 and 7 days, increased carcass adiposity in the *fa/fa* genotype became detectable.

In contrast, there were few significant genotype or gender differences in brown fat mass, protein, or UCP1 content at the four ages studied. The exceptions were at *day 4* when brown fat mass (g) and total protein (mg) were higher in the *fa/fa* vs. +/+ pups. At no age did we observe significant genotype differences in UCP1 (either total or body mass corrected) despite increases with age (data for 4, 7, and 12 days shown in Table 2).

Thyroxine 5′-deiodinase activity. Genotype differences in the thyroxine 5′-deiodinase activity of brown fat removed from the cold-exposed pups were present at 7 and 12 days but not at 2 and 4 days (Table 2; 2-day-old data not shown). At both of the older ages, activity (expressed in terms of mg protein, total, or total corrected for body mass) was significantly lower in the *fa/fa* vs. +/+ animals. For example, at 7 days of age, the body mass-adjusted activity in males was 1.25-fold higher in +/+ vs. *fa/fa* rats, and in the females, it was 2.5-fold higher. At 12 days of age, these differences had risen to 2.6 and 5.8, respectively.

Resting (minimal) and cold-exposed (maximal) rates of oxygen consumption. Minimal values of oxygen consumption (Table 3) showed no genotype or gender differences at *days 2* (data not shown) or *4* of age. By *day 7*, the *fa/fa* pups exhibited lower minimal rates of oxygen consumption than did the +/+ pups, this effect being more pronounced in females than in males (Table 3). The genotype difference was present in metabolic rates adjusted for body mass (ml oxygen/h × g body mass$^{0.67}$) as well as in unadjusted rates (i.e., ml oxygen/h). This genotype difference was also present at 12 days of age (Table 3).

Cold exposure increased the oxygen consumption of all pups (Table 3), with genotype differences in the response to cold occurring in 7- and 12-day-old pups but not in the 2- and 4-day-old animals. In general, the lean 7- and 12-day-old pups had greater maximal rates of oxygen consumption (body mass adjusted; Table 3) and greater cold-induced rates of oxygen consumption than did the *fa/fa* pups. Associated with this blunted thermogenic response of the *fa/fa* pups was the greater degree of hypothermia observed at the cessation of the cold exposure (Table 3). For example, at 7 days of age, the colonic temperatures of female +/+ pups averaged 23.9 ± 0.6°C when pups were removed from the chamber, whereas those of their *fa/fa* littermates averaged 21.2 ± 1.1°C. Similar patterns were observed in the 12-day-old females as well as in the males at both ages. Because the *fa/fa* pups generally reached their maximal oxygen consumption sooner than did the +/+ pups, they tended to spend less time in the cold. Thus the

Table 1. *Body mass, adiposity, and leptin concentrations in neonatal BNZ pups*

	Body Mass, g	Carcass Mass, g	White Fat Mass, g	White Fat, %carcass mass	Leptin, ng/ml
			4 Day old		
FL	8.20 ± 0.36 (11)	4.00 ± 0.21 (11)	0.21 ± 0.03 (11)	4.90 ± 0.49 (11)	1.43 ± 0.55 (8)
FO	8.52 ± 0.32 (11)	4.17 ± 0.17 (11)	0.24 ± 0.04 (11)	5.64 ± 0.70 (11)	5.14 ± 2.04 (10)
ML	8.65 ± 0.33 (14)	4.28 ± 0.20 (14)	0.22 ± 0.03 (14)	4.90 ± 0.40 (14)	0.56 ± 0.04 (5)
MO	8.59 ± 0.35 (16)	4.29 ± 0.20 (16)	0.24 ± 0.03 (15)	5.53 ± 0.68 (15)	2.04 ± 0.40 (12)
Gend	0.457	0.329	0.899	0.927	0.138
Geno	0.711	0.679	0.360	0.257	0.055
G × G	0.600	0.693	0.809	0.919	0.397
			7 Day old		
FL	12.32 ± 0.39 (19)	6.23 ± 0.23 (19)	0.46 ± 0.04 (18)	7.20 ± 0.44^{b} (18)	1.75 ± 0.50 (9)
FO	12.23 ± 0.67 (11)	6.29 ± 0.41 (11)	0.57 ± 0.07 (11)	8.90 ± 0.59^{a} (11)	2.83 ± 0.60 (9)
ML	13.31 ± 0.57 (12)	6.76 ± 0.31 (12)	0.44 ± 0.05 (11)	6.25 ± 0.60^{b} (11)	1.23 ± 0.23 (10)
MO	12.51 ± 0.82 (14)	6.45 ± 0.47 (14)	0.54 ± 0.06 (12)	7.76 ± 0.54^{a,b} (12)	2.63 ± 0.65 (10)
Gend	0.309	0.344	0.603	0.060	0.490
Geno	0.477	0.726	0.059	**0.005**	**0.023**
G × G	0.565	0.611	0.887	0.863	0.762
			12 Day old		
FL	20.34 ± 0.71^{b} (11)	10.84 ± 0.48 (11)	0.74 ± 0.14^{b} (10)	6.65 ± 1.02^{b} (10)	1.45 ± 0.36^{b,c} (6)
FO	21.07 ± 0.83^{a,b} (10)	12.01 ± 0.67 (10)	1.24 ± 0.16^{a} (10)	10.08 ± 0.97^{a} (10)	2.45 ± 0.58^{a,b} (5)
ML	21.65 ± 0.38^{a,b} (17)	11.75 ± 0.24 (17)	0.72 ± 0.07^{b} (16)	6.00 ± 0.57^{b} (16)	0.91 + 0.07^{c} (17)
MO	22.80 ± 0.58^{a} (23)	12.41 ± 0.38 (23)	1.29 ± 0.10^{a} (22)	10.06 ± 0.57^{a} (22)	2.85 ± 0.29^{a} (21)
Gend	**0.023**	0.147	0.923	0.667	0.851
Geno	0.155	**0.044**	**<0.001**	**<0.001**	**<0.001**
G × G	0.753	0.567	0.754	0.681	0.192

Values are means ± SE; no. of animals in parentheses. BNZ, Brown Norway-Zucker; F, female; M, male; L, lean (+/+); O, obese (*fa/fa*). All variables, except body mass, were measured in cold-exposed pups. For each age and variable, means with no superscripts or sharing a common superscript do not differ significantly. In the two-way ANOVAs: Gend, gender; Geno, genotype; G × G, gender × genotype. Significant *P* values are in bold type.

Table 2. *Brown fat mass, protein, thyroxine 5′-deiodinase activity, and UCP in neonatal BNZ pups*

	BAT Mass, mg	BAT Protein, mg	Thyroxine 5′-Deiodinase Activity			UCP	
			fmol/(mg protein × h)	fmol/h	fmol/(g $BM^{0.67}$ × h)	μg	μg/g $BM^{0.67}$
				4 Day old			
FL	92 ± 6 (11)	12.9 ± 0.6 (11)	56.3 ± 12.8 (10)	696 ± 57 (10)	178 ± 41 (10)	959 ± 129 (11)	236 ± 31 (11)
FO	123 ± 12 (11)	17.1 ± 1.8 (11)	39.9 ± 4.0 (9)	634 ± 100 (9)	151 ± 20 (9)	1,270 ± 212 (11)	301 ± 46 (11)
ML	99 ± 8 (14)	13.4 ± 0.9 (14)	51.6 ± 10.3 (12)	716 ± 155 (12)	161 ± 32 (12)	1,162 ± 190 (13)	272 ± 40 (13)
MO	105 ± 6 (16)	15.3 ± 1.0 (16)	55.4 ± 13.1 (12)	891 ± 228 (12)	206 ± 52 (12)	967 ± 108 (16)	235 ± 28 (16)
Gend	0.660	0.588	0.634	0.439	0.633	0.759	0.694
Geno	0.829	**0.011**	0.581	0.751	0.817	0.721	0.702
G × G	0.064	0.314	0.375	0.351	0.383	0.124	0.166
				7 Day old			
FL	127 ± 5 (19)	19.0 ± 0.8 (19)	51.2 ± 4.6^{a} (10)	917 ± 94^{a} (10)	176 ± 16^{a} (10)	1,910 ± 190 (19)	366 ± 40 (19)
FO	117 ± 6 (11)	17.6 ± 1.1 (11)	14.7 ± 6.8^{b} (8)	234 ± 93^{c} (8)	46 ± 18^{b} (8)	2,300 ± 320 (11)	435 ± 56 (11)
ML	118 ± 7 (12)	19.7 ± 1.4 (12)	32.6 ± 7.8^{a} (10)	704 ± 191^{a,b} (10)	120 ± 30^{a,b} (10)	2,140 ± 250 (12)	384 ± 44 (12)
MO	132 ± 7 (14)	21.1 ± 1.2 (14)	19.1 ± 6.7^{b} (9)	409 ± 145^{b,c} (9)	83 ± 32^{b} (9)	2,570 ± 350 (14)	466 ± 54 (14)
Gend	0.66	0.055	0.333	0.896	0.845	0.383	0.623
Geno	0.829	0.978	**0.007**	**0.008**	**0.024**	0.148	0.130
G × G	0.064	0.206	0.331	0.436	0.165	0.946	0.887
				12 Day old			
FL	144 ± 8 (11)	25.4 ± 2.7 (11)	35.6 ± 12.7 (8)	996 ± 371 (8)	141 ± 54 (8)	3,815 ± 429 (10)	513 ± 59 (10)
FO	149 ± 8 (10)	24.2 ± 1.7 (10)	8.1 ± 3.1 (6)	180 ± 66 (6)	24 ± 9 (6)	3,344 ± 472 (9)	444 ± 63 (9)
ML	136 ± 6 (17)	23.4 ± 1.2 (17)	32.2 ± 15.1 (8)	735 ± 376 (8)	93 ± 47 (8)	3,626 ± 393 (16)	467 ± 49 (16)
MO	159 ± 7 (23)	25.3 ± 1.0 (23)	11.7 ± 3.9 (10)	284 ± 95 (10)	35 ± 11 (10)	3,862 ± 321 (23)	476 ± 38 (23)
Gend	0.891	0.773	0.991	0.778	0.624	0.699	0.900
Geno	0.079	0.820	**0.028**	**0.030**	**0.028**	0.782	0.568
G × G	0.214	0.335	0.733	0.514	0.439	0.407	0.460

Values are means ± SE; no. of animals in parentheses. BM, body mass; BAT, brown adipose tissue; UCP, uncoupling protein. All variables were measured in cold-exposed pups. For each age and variable, means with no superscripts or sharing a common superscript do not differ significantly. In the two-way ANOVAs, significant *P* values are in bold.

Table 3. *Oxygen consumption [resting (minimal) and cold exposed (maximal)] and colonic temperature in neonatal BNZ pups*

	$\dot{V}_{O_2}$, ml·h^{-1}·g $BM^{-0.67}$		$\dot{V}_{O_2}$, ml/h		Colonic Temperature, °C
	Resting	Cold exposed	Resting	Cold exposed	
			4 Day old		
FL	3.18 ± 0.09 (10)	7.32 ± 0.27 (10)	13.10 ± 0.50 (10)	30.33 ± 1.81 (10)	26.6 ± 0.5 (11)
FO	3.06 ± 0.15 (10)	6.78 ± 0.40 (10)	12.73 ± 0.74 (10)	28.31 ± 2.03 (10)	26.3 ± 0.2 (12)
ML	3.46 ± 0.21 (13)	8.12 ± 0.46 (13)	14.59 ± 0.90 (13)	34.70 ± 2.57 (13)	27.4 ± 0.5 (14)
MO	3.39 ± 0.24 (15)	7.80 ± 0.79 (15)	14.46 ± 1.17 (15)	34.32 ± 3.77 (15)	26.3 ± 0.3 (16)
Gend	0.146	0.099	0.105	0.093	0.334
Geno	0.649	0.581	0.795	0.693	0.072
G × G	0.893	0.735	0.903	0.787	0.872
			7 Day old		
FL	3.66 ± 0.22^{a} (15)	9.30 ± 0.56^{a} (15)	19.75 ± 1.20^{a} (15)	49.87 ± 1.72^{a} (15)	23.9 ± 0.6^{a} (19)
FO	2.48 ± 0.25^{c} (10)	5.03 ± 0.71^{b} (10)	13.25 ± 1.31^{b} (10)	26.76 ± 3.40^{b} (10)	21.2 ± 1.1^{b} (11)
ML	3.40 ± 0.29^{a,b} (12)	8.37 ± 0.96^{a} (12)	19.22 ± 1.80^{a} (12)	47.55 ± 6.16^{a} (12)	23.7 ± 0.6^{a} (12)
MO	2.83 ± 0.19^{b,c} (11)	4.81 ± 0.46^{b} (11)	15.93 ± 1.21^{a,b} (11)	27.33 ± 3.04^{b} (11)	20.4 ± 0.7^{b} (13)
Gend	0.844	0.423	0.454	0.832	0.494
Geno	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**
G × G	0.213	0.624	0.266	0.726	0.681
			12 Day old		
FL	3.93 ± 0.18 (11)	10.32 ± 0.39^{a} (11)	29.25 ± 1.58 (11)	76.82 ± 3.52 (11)	24.7 ± 1.5^{a} (11)
FO	3.35 ± 0.16 (10)	8.82 ± 0.99^{a,b} (10)	25.47 ± 1.32 (10)	67.75 ± 7.95 (10)	19.7 ± 1.3^{b} (10)
ML	3.83 ± 0.14 (17)	9.87 ± 0.45^{a} (17)	29.80 ± 1.26 (17)	77.07 ± 4.14 (17)	22.1 ± 1.1^{a,b} (17)
MO	3.61 ± 0.15 (23)	7.99 ± 0.47^{b} (23)	29.16 ± 1.47 (23)	64.60 ± 4.28 (23)	20.8 ± 0.6^{b} (23)
Gend	0.625	0.283	0.187	0.781	0.505
Geno	**0.022**	**0.006**	0.169	**0.040**	**0.006**
G × G	0.295	0.743	0.328	0.744	0.100

Values are means ± SE; no. of animals in parentheses. For each age and variable, means with no superscripts or sharing a common superscript do not differ significantly. In the 2-way ANOVAs, significant *P* values are in bold.

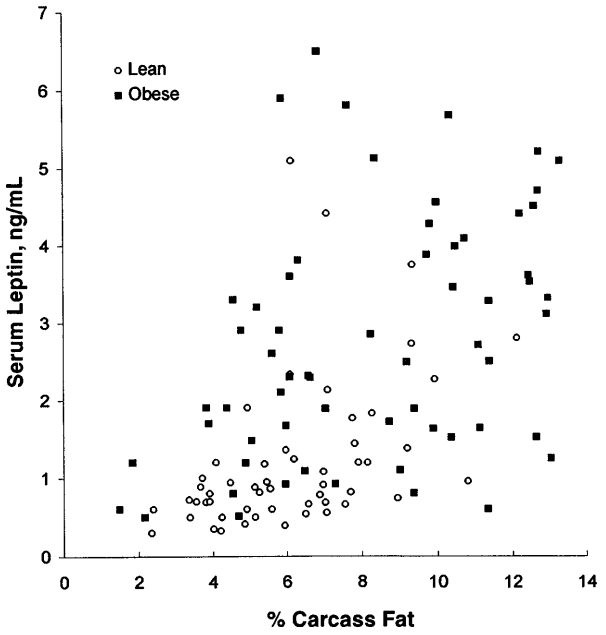

Fig. 1. Scatter diagram of serum leptin levels plotted against percent carcass fat for 4-, 7-, and 12-day-old +/+ (○) and *fa/fa* (■) Brown Norway-Zucker pups. With the use of linear regression analysis, the best-fit line is described by $y = 0.259x + 0.156$; $r^2 = 0.254$, $F = 38.17$, $P < 0.001$. Rats are from *Expt. 1*.

genotype differences in colonic temperature that we noted are probably an underestimate.

Serum leptin concentrations. Serum leptin concentrations (ng/ml) were significantly higher in *fa/fa* than in +/+ pups at 7 and 12 days of age (Table 1). Although leptin levels were also higher in the *fa/fa* pups at 4 days of age, the variability in the values precluded reaching significance ($P = 0.055$). There were no significant gender differences in serum leptin concentrations at any age. When leptin concentration was expressed in terms of relative adiposity (ng/ml per %carcass fat), no genotype or gender differences were observed at any of the ages studied (data not shown), suggesting that the amount of carcass fat was a significant factor determining the leptin concentration in these pups. Linear regression analysis ascribes ~25% of the variation in the leptin levels of the pups to percent body fat (Fig. 1).

Experiment 2

Body composition, serum insulin levels, and VMH concentrations of 5-HT and 5-HIAA. As was the case with the animals in *Expt. 1*, the 12-day-old *fa/fa* pups were significantly fatter than were the +/+ pups, both in absolute (g) and relative (%) terms. This was accompanied by larger carcass mass in the *fa/fa* pups (Table 4). Neither gender or genotype differences were found in the VMH concentrations of 5-HT, the concentrations of 5-HIAA, or the ratio of 5-HIAA to 5-HT, indicating no blunting of VMH 5-HT release. There was no linear relationship between the indexes of serotonergic activity and percent body fat ($r^2 = 0.04$; data not shown). Insulin values were quite variable, so much so that means 42–158% higher in the *fa/fa* vs. +/+ pups did not differ significantly (Table 4).

To facilitate comparison of the data from the BNZ pups with those previously obtained on Zucker pups (34), we also analyzed values of 5-HT, 5-HIAA, the ratio of 5-HIAA to 5-HT, and insulin from subsets of each BNZ genotype, namely, *fa/fa* pups with carcass fat of 11.0–14.6% ($n = 12$) and *Fa/Fa* pups with carcass fat of 7% or below ($n = 8$). Data from the 12-day-old males and females were combined. For these two subsets of pups, there were no significant genotype differences in values of 5-HT (fm/μg protein: *fa/fa* = 30.8 ± 2.0; +/+ = 29.2 ± 3.1), 5-HIAA (fm/μg protein: *fa/fa* = 29.2 ± 1.5; +/+ = 26.9 ± 2.4), or the ratio of 5-HIAA to 5-HT (*fa/fa* = 0.972 ± 0.071; +/+ = 0.944 ± 0.063). However, insulin values of the selected *fa/fa* pups (36.6 ± 9.8 μU/ml) were significantly higher ($P = 0.015$) than those of the selected +/+ pups (10.6 ± 3.3 μU/ml).

DISCUSSION

The major finding in this study is the observation that genetically obese (*fa/fa*) BNZ pups exhibited increased serum leptin levels, increased adiposity, and decreased energy expenditure before any detectable

Table 4. *Ventromedial hypothalamic serotonergic activity and body composition of 12-day-old BNZ pups*

	Body Mass, g	Carcass Mass, g	BAT Mass, mg	Carcass Fat		Ventromedial Hypothalamus			
				g	%	Insulin, μU/ml	5-HIAA, fm/μg protein	5-HT, fm/μg protein	5-HIAA/5-HT
FL	20.31 ± 0.65 (6)	10.75 ± 0.31 (6)	143 ± 11[c] (6)	0.74 ± 0.08[b] (6)	6.85 ± 0.56[b] (6)	21.2 ± 8.1 (6)	28.15 ± 2.77 (6)	33.54 ± 3.77 (6)	0.87 ± 0.08 (6)
FO	22.38 ± 0.38 (9)	11.97 ± 0.16 (9)	187 ± 7[a] (9)	1.23 ± 0.09[a] (9)	10.17 ± 0.64[a] (9)	30.2 ± 6.2 (9)	28.97 ± 3.58 (9)	29.32 ± 2.29 (9)	1.03 ± 0.08 (9)
ML	20.38 ± 0.83 (5)	10.86 ± 0.52 (5)	144 ± 11[b,c] (5)	0.67 ± 0.13[b] (5)	5.96 ± 0.91[b] (5)	8.1 ± 2.1 (5)	26.07 ± 2.27 (5)	26.54 ± 2.00 (5)	0.98 ± 0.04 (5)
MO	21.40 ± 0.53 (14)	11.47 ± 0.37 (14)	167 ± 5[b] (14)	1.17 ± 0.13[a] (11)	9.67 ± 0.73[a] (11)	24.0 ± 2.2 (11)	29.21 ± 1.38 (12)	28.90 ± 1.52 (12)	1.02 ± 0.03 (12)
Gend	0.438	0.624	0.236	0.587	0.375	0.146	0.622	0.14	0.417
Geno	**0.018**	**0.029**	**<0.001**	**<0.001**	**<0.001**	0.156	0.294	0.707	0.121
G×G	0.375	0.448	0.193	0.944	0.808	0.799	0.535	0.188	0.326

Values are means ± SE; no. of animals in parentheses. 5-HIAA, 5-hydroxy-3-indoleacetic acid; 5-HT, serotonin. All variables were measured in non-cold-exposed pups. For each age and variable, means with no superscripts or sharing a common superscript do not differ significantly. In the two-way ANOVA, significant *P* values are in bold.

decrease in VMH serotonergic activity. Our previous analysis of 12-day-old Zucker pups indicated that VMH serotonergic activity (as indexed by 5-HIAA levels) was in fact lower in the obese (*fa/fa*) vs. homozygous lean (*Fa/Fa*) pups (45), but it was not possible to determine whether this change occurred before the onset of increased fat accretion. To address this, the present study was designed using BNZ *fa/fa* and +/+ littermates that could be distinguished at any age. Our data demonstrate that the *fa/fa* pups increased their adiposity before any measurable decrement in VMH serotonergic activity. A significantly greater percent body fat was detectable in 7-day-old *fa/fa* vs. +/+ pups, indicating that increased accretion of triacylglycerol in the adipocytes begins before *day 7*. In contrast, no significant genotype differences in any of the measured indexes of VMH serotonergic activity were observed at *day 12*, even when the "fattest" *fa/fa* pups were compared with the "leanest" +/+ pups. Thus, although decreased VMH serotonergic activity may occur in older *fa/fa* pups and may contribute to the degree of obesity, data from the present study indicate that it is not essential for the obesity to be manifested.

Similarly, decreased VMH serotonergic activity does not appear to be necessary for the manifestation of blunted resting or cold-induced energy expenditure, both of which were detectable in the BNZ *fa/fa* pups by *day 7*. One potential contributor to the attenuated resting oxygen consumption is lower availability/effectiveness of thyroid hormone, a major regulator of basal metabolism and a hormone that is lower in adult Zucker *fa/fa* vs. lean rats (8). Although this has not been measured in neonatal *fa/fa* pups, the decreased activity of thyroxine 5′-deiodinase that we observed in brown fat from these animals is consistent with lower levels of circulating T_3. That is, this deiodinase converts T_4 to T_3, the active form of thyroid hormone, and available evidence suggests that a significant portion of the T_3 generated in brown fat may be released into the circulation during acute (and prolonged) cold exposure (38). Another potential contributor to the lower minimal oxygen consumption of the *fa/fa* pups is diminished thermic effects of feeding. Although the minimal oxygen consumption was measured at rest and at thermoneutrality, the pups were not postabsorptive. Thus we cannot rule out the possibility of blunted diet-induced thermogenesis (i.e., sympathetically mediated increase in oxygen consumption after feeding) or blunted obligatory metabolism (that associated with nutrient processing) in these pups as can occur in adult *fa/fa* rats (e.g., Refs. 23 and 30).

The attenuated energy expenditure of the *fa/fa* pups that occurred during cold exposure (maximal oxygen consumption) is most likely due to blunted thermogenesis in brown adipose tissue, the major site of cold-induced heat production at this age (6). Activation of this heat production occurs in response to sympathetic stimulation of the adipocytes and is mediated by norepinephrine interacting primarily with β-adrenergic receptors in the brown adipocyte plasma membrane. This is followed by activation of adenylyl cyclase, generation of cAMP, activation of protein kinase A, phosphorylation (and activation) of hormone-sensitive lipase, and hydrolysis of triacylglycerol. The resulting fatty acids bind to UCP1 in the mitochondrial membrane and there follows an increase in proton translocation back into the mitochondrial matrix. This dissipation of the proton gradient across the inner mitochondrial membrane results in uncoupling of oxidative phosphorylation from the electron transport system and greatly elevated rates of substrate (fatty acid) oxidation. Heat is released as a by-product of the substrate oxidation (cf. Ref. 14).

The blunted cold-induced thermogenesis could involve several mechanisms. Among these is decreased thermogenic capacity of the tissue resulting from decreased UCP1 content. That this is not the case is demonstrated by the fact that both the concentration and the total amount of UCP1 were not significantly lower in the obese vs. lean genotype at any of the four ages examined. This does not, however, preclude the possibility of some other factor in the thermogenic pathway being limiting. Another potential explanation is that cold exposure elicits more robust signaling to and/or more effective signal transduction in brown adipocytes in the lean vs. obese pups. Consistent with these latter possibilities is the fact that the thyroxine 5′-deiodinase activity in brown fat from cold-exposed pups, an activity that is also stimulated by norepinephrine (17), was significantly greater in lean than in obese pups by *day 7* of age. We are currently measuring urinary norepinephrine to evaluate the effects of cold exposure on the sympathetic activity of BNZ pups.

The fact that genotype differences in energy expenditure were not detectable at *days 2* or *4* in the BNZ pups but were in Zucker pups (25, 29) most likely reflects the influence of the Brown Norway background on the temporal expression of the fatty phenotype. That is, although the fatty phenotype appears to be comparable in 9- to 10-wk-old BNZ and Zucker *fa/fa* rats (4), attenuation of energy expenditure was delayed in the *fa/fa* pups. A similar pattern was observed in VMH serotonergic activity and serum insulin levels. In both cases, statistically significant genotype differences were present in 12-day-old Zucker rats (lower serotonergic activity and higher circulating insulin in the *fa/fa* pups) but not in 12-day-old BNZ pups.

One potential confounding factor in evaluating the strain differences in insulin and serotonergic activity is the methodology used to categorize the 12-day-old *fa/fa* pups in the two studies. In the Zucker study (34), we selected the fattest as presumptive *fa/fa* pups because molecular methods were not yet available for genotyping. These presumptive Zucker *fa/fa* pups had relative carcass fat values that ranged from 11.5 to 14% (34). In contrast, the carcass fat of the 12-day-old BNZ *fa/fa* pups in the present study ranged from 6.1 to 14.6%.

To eliminate this methodological difference, we analyzed a subset of the 12-day-old BNZ pups, applying selection criteria similar to those used for the Zucker pups. That is, we compared BNZ *fa/fa* pups with carcass fat above 11.0% with +/+ pups with carcass fat

of 7% and below. The fact that there were still no genotype differences in any of the measured indexes of serotonergic activity supports the conclusion that Brown Norway genes can modulate the time course of the expression of early events associated with the fatty phenotype. On the other hand, serum insulin values were significantly higher in the selected BNZ *fa*/*fa* vs. +/+ pups, suggesting minimal, if any, effects of the Brown Norway background on the expression of this variable in the *fa*/*fa* pups.

The higher levels of serum leptin observed in the *fa*/*fa* pups are consistent with the nature of the fatty mutation. This mutation involves substitution of the amino acid proline for glutamine at residue 269 in the leptin receptor (16, 28), which renders the receptor dysfunctional (36, 43), disrupts the leptin-adiposity feedback pathway that is hypothesized to play a major role in regulating energy balance (e.g., see Ref. 5), and initiates the sequence of events culminating in the metabolic changes associated with the development of obesity.

In summary, evaluation of several metabolic characteristics of F_2 BNZ neonates suggests the following chronology for the obesity-related variables we measured in this study. Among the first of the measured metabolic variables to show genotype differences in the pups were circulating levels of leptin, which tended to be higher in the *fa*/*fa* vs. +/+ pups at *day 4* of age ($P = 0.055$). By *day 7*, this difference was statistically significant as were genotype differences in resting and cold-induced oxygen consumption, percent body fat, and brown fat thyroxine 5′-deiodinase activity. Thus, between *days 4* and *7*, the *fa*/*fa* pups in this study began to exhibit blunted energy expenditure, increased fat deposition, and most likely attenuated sympathetic activity. Although hyperinsulinemia was not a consistent characteristic of the *fa*/*fa* pups even at *day 12*, the fattest *fa*/*fa* pups (those above 11% carcass fat) did have higher insulin values than did the leanest +/+ pups (carcass fat of 7% and below).

The absence of genotype differences in steady-state levels of VMH 5-HT, 5-HIAA, and the ratio of 5-HIAA to 5-HT at 12 days of age (even in the fattest pups) indicates that the *fa* gene-induced early metabolic changes leading to the increased accretion of fat do not require altered VMH serotonergic activity. It also implies that the altered serotonergic activity seen in older *fa*/*fa* pups is most likely a secondary, rather than a primary, effect of the disruption of the leptin regulatory pathway.

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