Interaction of genetic and environmental programming of the leptin system and of obesity disposition

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Schmidt, Ingrid, Corinna Schoelch, Thomas Ziska, Darius Schneider, Eckhart Simon, and Andreas Plagemann. Interaction of genetic and environmental programming for disturbances of the leptin system and for obesity. Physiol Genomics 3: 113–120, 2000.—Possible adverse interactions between an usually inconspicuous genetic trait and early environmental factors favoring the development of obesity were investigated in rats heterozygous for the leptin receptor defect “fatty” (fa). Pups were exposed to early postnatal overfeeding by reducing litter size from normally 10–12 to only 4. Rearing +/+ and +/fa pups from day 3 to 21 in small litters increased fat-free dry mass and body fat, but only in the latter did a significant interaction with genotype occur. Pronounced differences in the responsiveness of +/+ and +/fa pups to “prophylactic” leptin treatment (from day 1 to 21) were observed, with +/fa females from small litters being nearly as fat and unresponsive as previously reported for normally reared fa/fa pups. Clear heterozygous differences in total hypothalamic leptin binding, but no litter size effect, paralleling the differences in leptin responsiveness, were observed. By early postnatal overfeeding an usually inconspicuous genetic trait may thus become etiologic for the development of obesity via physiological changes other than the decreased leptin binding characterizing the genetic defect.

Zucker rat; fatty gene; leptin receptor; neonatal programming; diet-induced obesity.

Environmental influences on the manifestation of genetic disorders are of great interest for basic research as well as for medical considerations of health risk factors. With special regard to obesity, genetic factors certainly contribute to the etiology but are unlikely to be solely responsible for the present worldwide obesity epidemic (5, 12). Because even in children, obesity prevalence has been continuously increasing in the past decades (37), predisposing for associated metabolic and cardiovascular diseases (4), and enlarging the risk for adult obesity (40), concepts for primary prevention are urgently needed. A major role of epigenetic environmental factors for the early onset of disturbances in body weight regulation is stressed by rapidly accumulating evidence (8, 9, 29). Especially, overweight occurring during critical periods of neonatal life was shown to persist into adult age (28, 40). Apart from the genotype, fetal and neonatal nutrition may, thus, play key roles in the development of obesity. For instance, metabolic disturbances during fetal life, like hyperglycemia and hyperinsulinemia in fetuses of diabetic mothers, were shown to favor persisting predispositions for overweight in humans (24, 30, 25). The question of whether and how a usually inconspicuous genotype may interact with epigenetic factors which can perinatally malprogram energy balance regulation was, however, barely considered experimentally until now (18).

Rats raised in small litters have proven to be an appropriate experimental model to study consequences of overnutrition during the critical perinatal period (1, 11, 26). Due to only partially identified mechanisms, rats overnourished during this period develop hyperphagia, overweight, and metabolic disturbances that persist throughout life (10, 39). These observations underscore the critical importance of early nutritional influences in “programming” the long-term inclination to become obese, irrespective of the genetic background or a specific genetic predisposition. To tackle, moreover, the question of how a normally inconspicuous genotype may interact with epigenetic factors perinatally programming an obesity disposition, it seems particularly helpful to evaluate the possible environmental impacts on well-defined rodent models, such as the leptin receptor mutation “fatty” (fa), which affects a gene that is crucial for normal body weight occurring during critical periods of neonatal life (11, 26). Due to only partially identified mechanisms, rats overnourished during this period develop hyperphagia, overweight, and metabolic disturbances that persist throughout life (10, 39). These observations underscore the critical importance of early nutritional influences in “programming” the long-term inclination to become obese, irrespective of the genetic background or a specific genetic predisposition. To tackle, moreover, the question of how a normally inconspicuous genotype may interact with epigenetic factors perinatally programming an obesity disposition, it seems particularly helpful to evaluate the possible environmental impacts on well-defined rodent models, such as the leptin receptor mutation “fatty” (fa), which affects a gene that is crucial for normal body weight regulation (7) but is classified as recessive under standard maintenance conditions (42).

Apart from medical considerations concerning the epidemic spreading of obesity in human populations, the analysis of the physiological changes provoked by the interaction of heterozygosity for the leptin receptor defect fa with the effect of rearing in small litters might also be generally helpful in identifying principal mechanisms by which the early environment might “program” the phenotypic penetrance of genetic traits. The leptin receptor defect fa has recently been demon-
strated to have a transient codominant influence on growth and body fat content during the first postnatal week (38, 33, 16). In the adult animal, however, the \( fa \) allele has been classified as recessive (42), although subtle differences in the body composition of adult \(+fa\) and \(+/+\) rats from the same breeding line were detected, particularly if the animals were subjected to a high-fat diet (19). In the present study we have tried to outline the interaction between heterozygosity for \( fa \) and early postnatal environmental influences by studying the consequences of access to an increased milk supply for body composition and the function of the leptin system.

**METHODS**

*Animals.* We used Zucker rat (13M) pups derived from \(+/+ \times +/fa\) matings from the outbred Zucker rat colony at the W. G. Kerckhoff-Institut founded in 1985 with breeding stock donated by Harry Carlisle (University of California, Santa Barbara). Apart from the brief treatment and weighing procedures, the pups were left undisturbed with their mothers until the end of the experiments at 21 days of age. Colonies were maintained at 22°C on a 12:12-h light-dark cycle. On the day of birth (day 0) each pup was marked with subcutaneous injections of India ink, and tail clips for genotyping were taken. When the litters (9–13 pups at birth) were 3 days old (that is, on the 4th postnatal day), pups were assigned either to litters containing 10–12 pups (normal litters) or to litters containing only 4 pups (small litters) with the average starting weights for each genotype and gender being matched as closely as possible for pups reared in normal and in small litters.

*Genotype identification.* Rat genomic DNA was isolated from tail clips following the protocol of a commercially available kit (QIAmp Tissue Kit; Qiagen, Hilden, Germany). Genotypes at the leptin receptor locus were then identified as described by Chua et al. (7). In short, a PCR was conducted in a reaction volume of 50 μl containing 200 μM dNTPs (Boehringer, Mannheim, Germany), 20 pmol of each primer (MWG, Ebersberg, Germany), 2.5 U Taq polymerase (Boehringer), and 10× PCR buffer containing 15 mM MgCl₂ (Boehringer) and 2 μl rat genomic DNA. Amplifications in a thermocycler (Gene Amp PCR System 2400; Perkin-Elmer, Weiterstadt, Germany) consisted of an initial denaturation at 94°C for 5 min; 35 cycles of 94°C, 55°C, and 72°C, each for 30 s; and a final extension at 72°C for 7 min. Then 15 μl of the resulting PCR product was digested by 5 U Msp I (Boehringer) for 4 h at 37°C. Digestion products were resolved by nondenaturating polyacrylamide gel electrophoresis and ethidium bromide staining.

*Leptin treatment.* For leptin treatment in some of the small litters, we used recombinant his-tagged murine leptin (17,600 Da) produced as described previously (21), because homology between rat and mouse leptin is greater 96% and no comparative data for the treatment with rat leptin would be available. Starting when the animals were 1 day old, two littersmates received subcutaneous injections of leptin (100 pmol·g⁻¹·day⁻¹) given in two doses daily at the end of the light and the dark phases until the end of the experiment, while two other pups of the same genotype received control injections (PBS). Within each litter, pups were randomly assigned to the treatment and control groups in such a way that the average body masses of the leptin-treated animals before the first injection was matched as closely as possible to that of their control littermates.

**Sample preparation and body composition analysis.** At the end of the light phase when pups were 21 days old, litters were removed from their mothers, pups were anesthetized by exposure to 100% CO₂ for 30 s, and decapitated. Blood was collected on ice in tubes containing heparin as anticoagulant. Dilution was determined by weighing, and the concentrations were corrected accordingly. Plasma was collected after centrifugation, and aliquots were stored at −80°C until leptin concentrations were determined with a commercial leptin radioimmunoassay (RIA) kit (Linco, St. Charles, MO). Brains were quickly dissected, frozen on dry ice, and stored at −50°C. Carcass mass was determined after stomach and bowels had been removed, and body composition [fat content and fat-free dry mass (FFDM)] were evaluated by drying to constant weight followed by whole body chloroform extraction in a Soxhlet apparatus (20).

*Plasma leptin.* Leptin concentrations were determined with a commercial RIA kit for murine leptin (Linco). From repeated measurements against a rat leptin standard (Linco), we determined a conversion equation for murine RIA-values between 1 and 5 mIU/ml. For samples measured above this concentration, aliquots were measured again after appropriate dilution. For each animal the results of two to four measurements within the calibrated range, carried out in different RIAs, were averaged. To decrease variability due to interassay differences, we measured pooled samples in each assay to standardize the results. Inter- and intra-assay coefficients of variance were 11% and 6%, respectively.

*Leptin receptor binding.* Firstly, we established the validity of the assay by determining leptin receptor binding in the brains of a group of pups reared in normal sized litters that contained all three genotypes (assay 1). Then we compared binding in the brains of the \(+/+\) and \(+/fa\) pups reared in small litters, which had been used for body composition analysis and plasma leptin determination (assay 2), and in the brains of the \(+/+\) and \(+/fa\) pups treated with leptin or PBS (assay 3). Because of the large interassay variability, a further group of \(+/+\) and \(+/fa\) pups that had been reared either in small or in normal litters was included in this part of the study, and their hypothalami were then analyzed together (assay 4) to permit intra-assay comparison of the litter size effect on leptin receptor binding. Inter- and intra-assay coefficients of variance were 14% and 8%, respectively.

The hypothalami were dissected out on a cryostat at −20°C, and each hypothalamic was homogenized separately in 6 ml of 1 mmol/l HEPES buffer containing 320 mmol/l sucrose (pH 7.2) at 4°C by use of a Potter-Elvehjem homogenizer for 10 strokes. These homogenates were centrifuged at 26,000 g for 25 min at 4°C. The resulting pellets were suspended in the calibrated range, carried out in the presence of 0.5 mmol/l unlabeled leptin (murine; R&D Systems, Wiesbaden, Germany) for one hypothalamic membrane protein suspension. At the end of incubation, 3 ml ice-cold washing buffer (25 mmol/l HEPES, 5 mmol/l MgCl₂, 0.1% BSA, and 0.01% Triton X-100) was added to the assay.
tube, and the samples were immediately filtered through Whatman GF/B filters (Adi Hassel, Munich, Germany) using a Brandel Harvester (Adi Hassel). The filters had been pre-soaked in assay buffer containing 2% BSA. The incubation tubes and filters were washed with 3 ml ice-cold washing buffer. The radioactivity of the filter was counted in the gamma counter (Wallace 1277 Gammanaster). Specific binding (fmol/mg membrane protein) was calculated as the difference between the total amount of bound tracer and the nonspecific binding for each hypothalamus.

**Statistical evaluation.** Growth of pups reared in different litters varies considerably (16, 38). Consequently, this factor has to be taken into account to enable the detection of subtle differences on body composition among suckling-age pups. As previously discussed in detail (16), regression analysis is one method that can be used efficiently to allow for the differences in growth when evaluating the statistical significance of differences in body fat content (and the body fat-dependent plasma leptin concentration) of pups reared in different litters. We therefore initially evaluated our data by regression analysis before determining mean values without consideration of litter effects.

The SigmaStat program was used to perform two-way ANOVAs (with litter size and genotype as the factors) for final body mass, body composition data, and plasma leptin concentrations, because this allows a coherent graphical presentation of mean values (as least-square means ± SE provided by ANOVA) in line with the outcome of the statistical analysis. To avoid distortion by small gender differences already present in suckling age pups (38), data of male and female pups were evaluated separately. For those litters in which leptin treatment was carried out, 2-way ANOVAs with litter and treatment (leptin vs. PBS) as grouping factors were performed.

The SPSS program was used to carry out multifactorial ANOVA on data for leptin receptor binding. Because visual inspection of data showed marked interassay variability, data obtained in different assays were first separately evaluated. For graphical presentation and further evaluation, data were then normalized for interassay variability. This was done by using the average of measurements carried out on all buffer controls (or PBS treated in assay 3) +/+ and +/fa pups within one assay as the basis and expressing individual data as percent deviations from the mean of the corresponding assay. These average values were 0.62 ± 0.32 (N = 31), 0.59 ± 0.39 (N = 20), 0.85 ± 0.07 (N = 16), and 1.13 ± 0.05 (N = 49) fmol/mg for assays 1, 2, 3, and 4, respectively.

**RESULTS**

**Effect of litter size on body fat content and growth of lean body mass.** In normal litters, fat mass at the same carcass mass was only minimally, but significantly, higher in +/fa than in +/+ pups (Fig. 1A). Rearing in small litters not only increased carcass and fat mass in both genotypes but markedly enlarged the genotype difference. This was demonstrated by reduction of litter size causing a significantly larger (P < 0.001) increase in the slope of the regression lines relating fat mass to carcass mass in +/fa pups (increase in slope from 0.07 to 0.24, P < 0.001) than in +/+ pups (increase in slope from 0.07 to 0.14, P < 0.05). Separate regression analysis for each gender (not shown) demonstrated that gender-related shifts were parallel and, thus, ascertained that the observed interaction between genotype effects and litter size effects on body fat mass were not distorted by gender differences.

Mean values of body composition data were separately determined for males and females, in order not to confound averages by gender differences (Fig. 2). For pups reared in normal litters a small but significant heterozygous difference was found only for body fat content of females. Rearing in small litters increased FFDM as well as body fat content in both genotypes and both genders (all P < 0.001), but significant interactions between litter size and genotype occurred only in body fat content. Thus, due to rearing in small litters, the heterozygous differences in total fat mass as well as percent body fat became more pronounced and differed significantly not only for females but also for males. Rearing in small litters thus precipitates or significantly enlarges genotype differences in body fat.
leptin levels varied remained rather small and the correlation loose.

In +/fa pups reared in normal litters, plasma leptin concentration increased much more steeply with fat mass than in +/+ pups \((P < 0.01\) for slope of regression lines). The +/fa pups reared in small litters showed a large increase in leptin concentration, which remained correlated to their disproportionally higher body fat. The slope of the +/fa regression line for small litters was, however, not increased in relation to that for +/fa pups reared in normal litters. This was also the case if plasma leptin concentration was not expressed on a logarithmic scale (Fig. 1B) but on a linear scale (not shown). Compared with the +/+ pups, the regression line for the +/fa pups reared in small nests displayed a marked parallel shift to higher values \((P < 0.001\) for \(y\)-intercept). The markedly higher plasma leptin levels in +/fa pups reared in small litters are thus related to their increased body fat content and are not due to a further increase of the enhanced proportionality in the relationship between plasma leptin and body fat seen under normal rearing conditions in +/fa relative to +/+ pups.

Genotype-specific differences in responsiveness to leptin treatment due to rearing in small litters. To find out to which degree the hyperleptinemia observed in small litters at the end of the experiment was associated with disturbances in leptin responsiveness, we treated pups during the entire suckling period with PBS or leptin in high doses, i.e., leptin treatment was started 2 days prior to the reduction of litter size. To assess the average degree of adiposity, we used percent body fat rather than fat mass in grams as the parameter to be considered, to take into account the differences in growth of lean body mass. Leptin effects on body composition were evaluated in females, because the heterozygous difference in fat content was largest in this gender (see Fig. 2). As Fig. 3 shows, treatment with recombinant leptin reduced the body fat content of +/+ females reared in small litters by one-third \((P < 0.01\) for difference to untreated littermates, 2-way ANOVA).

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**Fig. 2.** Least square means (±SE) of body mass, fat-free dry-mass (FFDM), total body fat mass, and percent body fat content of male and female Zucker rats reared in normal (light bars) or small (dark bars) litters. Cross-hatched bars = +/fa pups; unhatched bars = +/+ pups. Means were determined by 2-way-ANOVA with genotype and rearing procedure as the factors. The treatment effect was significant at \(P < 0.001\) for each of the displayed variables and is therefore not additionally indicated. Significant interactions between genotype and treatment are marked by crosses. Significant differences between +/+ and +/fa littersmates are marked by asterisks. \(* P < 0.05, ** P < 0.01, *** P < 0.001\).

**Fig. 3.** Percent body fat of female, leptin-treated (hatched bars) and control (solid bars) pups reared in small litters shown as deviation (±SE) from the percent body fat content of genotype-matched female pups reared in normal litters (i.e., the corresponding data from Fig. 2 are set as zero). For each treatment group, \(N = 5\) in +/+ and \(N = 8\) in +/fa pups.
binding was significantly (PBS) and genotype or between treatment and gender, no significant interaction between treatment (leptin vs. receptor binding (P < 0.001 for genotype in a 2-way ANOVA with gender and genotype as the factors). Leptin binding (least square mean ± SE, fmol/mg) in fa/fa pups was 0.36 ± 0.03 (N = 12), that in +/+ pups was 0.56 ± 0.03 (N = 19), and that in +/+ pups was 0.76 ± 0.04 (N = 12). The post hoc Student-Newman-Keuls t-test yielded significant differences (P < 0.001 for fa/fas vs. +/+ pups, as well as for fa/fas vs. +/+ pups). In the further analyses, only data of +/+ and +/+ pups were used.

Leptin treatment of pups reared in small litters (N = 16, each for +/+ and +/+ pups), had no effect on leptin receptor binding (P = 0.49) as determined in a three-way ANOVA with genotype, gender, and treatment (leptin vs. PBS) as the factors (assay 3). There was also no significant interaction between treatment (leptin vs. PBS) and genotype or between treatment and gender, but binding was significantly (P < 0.01) lower in +/+ than in +/+ pups.

Because visual inspection of data showed marked interassay differences, the effect of litter size was evaluated in an additional group of animals (assay 4), in which leptin receptor binding of pups reared in small and normal litters was commonly determined in one assay. Evaluation by 3-way ANOVA with genotype, gender, and litter size as the factors confirmed that leptin receptor binding did not change with litter size (P = 0.24) but differed clearly between +/+ and +/+ pups (P < 0.001) without showing an interaction between genotype and litter size (P = 0.43).

To allow for interassay differences when considering the entire set of data from +/+ and +/+ animals (with exclusion of the leptin-treated animals), we normalized data by relating them to the average for each assay as the basis. Three-way ANOVA on these data yielded P < 0.001 for genotype, P = 0.046 for gender, and P = 0.39 for litter size, with P > 0.05 for all interactions. Moreover, this kind of evaluation allowed to directly visualize these overall results of the binding studies (Fig. 4): leptin binding is much higher in +/+ pups (all above average line) than in +/+ pups (all below average line), but the difference between +/+ and +/+ pups was by no means larger in small than in normal litters. Figure 4 also demonstrates that despite considerable variability, the leptin receptor binding was slightly lower in females than in males, but that the genotype difference seemed to be rather smaller than larger in the females compared with the males. The heterozygous and gender differences in leptin receptor binding in the hypothalamus analyzed in total thus did not parallel the genotype × litter size interactions observed in the regulation of body fat content.

**DISCUSSION**

The salient point of this report is that the apparently minor change in the early environment established by the reduction of litter size triggers the obvious phenotypical manifestation of a genetic disorder, usually considered as a recessive trait. Whereas, compared with wild-type pups, the fat mass of heterozygous males and females reared in normal litters is slightly higher by 7 and 16%, respectively, the differences increase in small litters to 28 and 43%, respectively. With a body fat content of more than 14% (Fig. 2), the female +/+ weanlings from small litters were, therefore, nearly as fat as normally reared fa/fa pups, which have a body fat content of about 16% (22), i.e., about twice that of normally reared wild-type pups. Although rearing in small litters also increases lean body mass, statistical analysis shows that interaction of genotype with litter size is restricted to increased body fat content and reduced leptin responsiveness.

Physiological basis of acquired leptin resistance. When judging from the changes in body fat content induced by leptin treatment of pups in small litters, the
+/fa females were nearly as unresponsive as normally reared fa/fa pups (16, 35), whereas the +/+ females decreased their body fat content during the treatment to that of normally reared +/+ females. In litters of normal size, on the other hand, differences in leptin responsiveness between +/+ and +faa/pups were only detected in pups treated until 7 days of age (16) but not in pups treated until 16 days of age (34), an observation in accordance with a large heterozygous difference in fat deposition during the first and a lacking difference during the second postnatal week (22, 33, 38). In males and females alike, rearing +fa/pups in small litters causes a marked hyperleptinemia compared with +/+ pups. The relationship between leptin levels and body fat content, however, is generally maintained (see Fig. 1B). Due to the larger increase of fat mass in +fa than in +/+ pups reared in small litters, the average leptin levels of +faa/pups are, nevertheless, considerably higher than that of +/+ pups under this rearing condition, and sustained hyperleptinemia might be hypothesized to promote leptin resistance.

Since leptin actions on body fat content are mainly due to the action of the hormone on central nervous system, especially hypothalamic targets (2, 29), a change in hypothalamic leptin receptor binding would be a plausible explanation for the suppressed leptin responsiveness of +faa/pups reared in small litters. However, although decreased leptin responsiveness of +faa/pups from small litters, indeed, paralleled reduced hypothalamic leptin binding relative to equally reared +/+ pups, a similar heterozygous difference of leptin binding was also present among the pups reared in normal litters. Also, treatment with recombinant leptin did not result in reduced leptin binding compared with PBS-treated, sex- and gender-matched controls. Thus the functional disturbance of the leptin system demonstrated by the leptin resistance of the hyperleptinemic +faa females from small litters cannot be attributed to reduced leptin receptor binding at the end of the 21-day observation period, although their leptin resistance was nearly as pronounced as that of faa/pups (16, 35) in which leptin binding was much lower.

However, the results provide information about leptin differences only at the end of the study and are not necessarily representative of the entire treatment period. Furthermore, the small amount of tissue obtainable from pups only allowed measurement of total hypothalamic leptin binding. Thus possible changes of specific leptin receptor subtypes or differences in binding restricted to distinct hypothalamic nuclei cannot be excluded, and their elucidation would require histochemical approaches. For instance, specific regulation of leptin receptor binding depending on circulating leptin levels was described for the arcuate nucleus, whereas simultaneously the binding in other hypothalamic nuclei was unchanged (2, 3). Despite this limitation, our method was, however, sufficiently sensitive to reliably identify heterozygous differences in hypothalamic leptin binding. Functionally similar disturbances in leptin responsiveness, caused by the fa mutation and by early postnatal overfeeding, seem thus to be mediated via different mechanisms. Therefore, it has also to be considered that with increased fattening, factors other than changes in the function of central leptin receptors, for instance, changes in the fat cell itself, might contribute to the leptin resistance in +faa females from small litters.

Is overfeeding the cause for the genotype with litter size interaction of fat deposition? Rearing in small litters does not only change food availability but also changes in a complex manner the thermal conditions experienced during suckling age (31). Extensive studies have shown that the time mothers spent with their pups increases with decreasing litter size (17). This ensures not only high milk availability for pups reared in small litters but also decreases their thermoregulatory needs. And it is known that artificial rearing of +faa/pups at thermoneutrality markedly increases their percent body fat content above that of cold-reared pups even when food intake is kept constant (14, 20). Moreover, increases in body fat content due to rearing at ambient temperatures close to thermoneutrality have also been observed in mother-reared wild-type rats and mice (for review, see Ref. 31). On the other hand, pups huddling in small litters, in the first days will lose heat much more rapidly than pups from normal litters, whenever the mother leaves the nest, although huddling even in small groups significantly reduces the demand for autonomic cold defense (31). But because pups reared in small nests grow much more rapidly, they will soon develop a markedly increased resting heat production and a decreased heat loss due to a more favorable surface-to-volume ratio. Two-week-old pups reared in small litters thus show a strikingly decreased huddling activity compared with pups from normal litters (I. Schmidt, unpublished results), a typical indication for decreased thermoregulatory needs (32). Based on the rate of dilution of 3H2O in measurements carried out between 4 and 16 days of age, it was concluded for Sprague-Dawley rats reared in small litters that the entire excess energy storage was accounted for by their increased energy intake, whereas maintenance energy needs did not seem to differ from that of pups reared in litters of normal size (11). This suggests that the differences in energy expended for autonomic and behavioral thermoregulation and other activities average out to produce the same total daily energy expenditure in both rearing conditions. This leaves, however, unanswered the interesting question of whether different partitioning of energies among thermoregulatory thermogenesis, motoric activity, and digestion plus storage of ingested food might influence the later development. Moreover, the increased maternal attention and decreased competition for food experienced by pups in small litters might result in a multitude of behavioral and hormonal changes, possibly including altered stress levels. The question of to which degree increased milk availability, changes in milk composition, and changes in nonnutritional factors might contribute to the differences observed between pups reared in small and normal litters...
thus demands further thorough experimental attention (32).

No excessive milk intake compared with lean (+/?) litters occurs in suckling-age fa/fa pups reared in normal litters, but overfeeding on solid food is observed from the middle of the third postnatal week when pups start to consume pellets in addition to suckling (6, 36). Rather, the increased fat deposition of suckling-age fa/fa pups is energetically fueled mainly by their decreased thermoregulatory thermogenesis (14, 15, 20). On the other hand, while fa/fa pups become markedly hypothermic and hypometabolic, no differences in thermoregulatory thermogenesis occur between +/+ and fa/fa pups, when pups reared in normal litters are temporarily isolated for determination of autonomic cold defense abilities (33). It is therefore still unclear how +/+ pups reared in normal nests manage to accumulate 0.2–0.5 g more body fat than their wild-type littermates until day 21 (Ref. 22, and present study). Rearing in small litters increased the heterozygous difference in body fat mass to 1.2 g in males and 1.8 g in females, corresponding to energy accumulations higher by 50 and 70 kJ, respectively, than in gender-matched +/+ pups under the same rearing conditions. Based on previous findings (6, 36), overfeeding on pellets during the last days of the experiment rather than increased milk intake (relative to the +/+ pups in the same rearing regime) may have contributed to the enlarged body fat content of the heterozygous pups observed at 21 days of age. Another possibility in line with the previous findings would be that +/+ pups show a greater activity in thermoregulatory behavior (huddling), as demonstrated for fa/fa pups (32), and therefore are able to store more fat because they need to expend less energy for metabolic thermoregulatory activity. In view of the above-outlined complex changes of the mother-litter and pup-pup interactions caused by the change of litter size, the energetic changes underlying the enlargement of the heterozygous difference in body fat content of pups reared in small litters remain, however, highly speculative.

Genetic and early environmental factors causing susceptibility to metabolic disturbances in adult rats. When reared in litters of normal size and thereafter maintained on a standard pelleted diet, +/+ rats appear phenotypically normal. When, however, exposed to a high-fat diet after weaning, the +/+ animals develop more pronounced changes of metabolism than +/+ animals (19). The present study does not provide any information about the long-term effects of rearing in small litters and the associated early postnatal overfeeding on the high-fat rat milk. In Wistar rats, genetically not predisposed to develop obesity, extensive studies have shown, however, that neonatal overfeeding due to rearing in small litters may lead to a lasting inclination for obesity and associated metabolic and cardiovascular disturbances, like syndrome X, when giving these animals normal ad libitum access to a standard pelleted diet after weaning (26). These lasting changes induced by changes in litter size are accompanied by neuroendocrine changes in the hypothalamic neurons, which indicate disturbed leptin responsiveness, e.g., of the neuropeptide Y system, which is known to be decisively involved in the central control of food intake and energy expenditure (13, 27). Therefore, in recognition of our observation that changes in hypothalamic leptin binding could not be related directly to leptin resistance, it remains to be elucidated how the interactions between genotype and rearing conditions observed in the present study will project into adult age.

In conclusion, our data clearly demonstrate increased susceptibility to epigenetic “malprogramming” favoring the development of leptin resistance and obesity by reducing litter size in suckling rats heterozygous for an allele which normally results in an inconspicuous phenotype. Despite the apparent similarity of the functional disturbances induced by the fa mutation and by rearing heterozygous animals in small litters, the underlying mechanisms of the epigenetic malprogramming are different from the changes in total hypothalamic leptin binding that are caused by the defective allele.

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