Ontogeny of hyperphagia in the Zucker (fa/fa) rat

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Kowalski, Timothy J., Andrea M. Ster, and Gerard P. Smith. Ontogeny of hyperphagia in the Zucker (fa/fa) rat. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1106–R1109, 1998.—The ontogeny of hyperphagic behavior in the Zucker fatty (fa/fa) rat was examined. Wild-type, +/fa, and fa/fa pups aged postnatal day 5 (P5), P9, P12, P15, and P18 were evaluated using a test that measured ingestive behavior independent of the dam. The independent ingestive test consisted of giving pups access to a test solution [half-and-half (cream and milk)] on a tissue on the floor of a test chamber for 20 min. The latency to ingest and the intake (weight gain and percent weight gain) were measured and normalized to +/fa litters. Pups were tested once to eliminate any effects of test experience. fa/fa Pups ingested significantly more than lean pups (+/+ and +/fa) on P12, P15, and P18, but not on P5 or P9. The latencies of fa/fa pups did not differ significantly from the latencies of +/+ pups except on P18, when the latencies of fa/fa pups were significantly shorter. The latencies of +/fa pups were significantly longer than the latencies of fa/fa or +/+ pups on P5 and P12. These results demonstrate that hyperphagia in fa/fa rats emerges between the ages of P9 and P12 under the test conditions used.

independent ingestion; genetic obesity; appetitive behavior; development of food intake

The Zucker fatty (fa/fa) rat is an example of a genetic obesity with an autosomal recessive pattern of inheritance (27). The obesity in fa/fa animals is correlated with hyperphagia, decreased energy expenditure, compromised thermoregulatory thermogenesis, hyperinsulinemia, and hypercorticosteronemia (3). The fa mutation has recently been identified as an amino acid substitution in the extracellular domain of the receptor for leptin (7). As a consequence, the fafa animal has elevated plasma leptin levels and is resistant to exogenous leptin administration (10).

In adult fa/fa rats, the hyperphagia is characterized by a defect in the regulation of meal size (1, 6, 16), with some changes in meal frequency (1). Although the hyperphagia is not required for the development of obesity (2, 9), it is not known whether this behavior emerges before an increase in fat mass is detected [near postnatal day 7 (P7)] (17). Hyperphagia has been described as early as P17 in chow-fed fa/fa rats, near the time of weaning (22). Because P5–P20 fa/fa pups fail to ingest more milk than lean (+/+?) animals when suckling (4, 13), it is likely that hyperphagia is expressed when animals are engaged in adult-like eating behaviors.

One approach to define the onset of hyperphagia in fa/fa rats is to examine ingestion in preweaning rats independent of the dam. One such technique has been half-and-half devised by Hall and Bryan (15). This test of independent ingestion requires an isolated pup to ingest nutrients from the floor of a test chamber. The intake during the first experience in this test is a measure of the unconditioned controls of intake that are functioning on that postnatal day. Because the controls of this form of ingestive behavior are like adult eating and controls of suckling are not (14), it may be possible to use this technique to detect early defects in the control of meal size in fa/fa pups. In the present experiment, the intake of and latency to ingest independently half-and-half was measured in Zucker pups aged P5, P9, P12, P15, and P18. The results show that hyperphagia in fa/fa pups emerges between the ages of P9 and P12 when tested in these conditions.

METHODS

Animals. Lean (+/+ and +/fa) and obese (fa/fa) Zucker rats aged P5, P9, P12, P15, and P18 were used in the experiments. All animals were the progeny of primiparous and multiparous heterozygous females that were mated with heterozygous males in our laboratory. All breeders were derived from the Vassar College colony (Poughkeepsie, NY). Mating pairs and pregnant females were housed in Plexiglas containers with wood shavings as bedding. Rats received pelleted chow and water ad libitum and were maintained on a 12:12-h light-dark cycle (0700–1900) at 22 ± 2°C. Pregnant females were checked daily for pups, and the day pups were first seen was termed P0. On P3 or P4, animals were earclipped for identification and tissue collection (for genotyping; see below), and litters were culled to a maximum of 12 animals (range = 7–12 per litter). A total of 194 pups (+/+ n = 48; +/fa n = 90; fa/fa n = 56) from 31 litters were used, with pups derived from four to eight litters tested at each age. Many of the experimental trials (see below) were performed without knowledge of the genotype. Genotype was determined before testing, however, in two to four trials at each age; this did not affect the results. When genotype was not predetermined, all available pups in each litter were used. When the genotypes were known, however, only litters with fa fa pups were used. Pups with similar body weights were selected for testing, and +/fa and fa/fa pups from the same litter were always tested together.

Independent ingestion test. The procedure used was initially described by Hall and Bryan (15). Pups aged P5, P9, P12, P15, or P18 were removed from the dam 4 h before testing and were maintained at 33 ± 1°C in an incubator for 3.5 h. Thus each pup was mildly deprived of food, water, and maternal interaction, but not sibling interaction. Litters were tested together, in either one (litter n ≤ 8 pups) or two (litter n ≥ 8 pups) trials. Each pup was tested only once and was killed after the experimental trial with an overdose of pentobarbital sodium.

Thirty minutes before testing, the pups were brought into the testing area, where they were voided of urine and feces by
gently stroking the anogenital region using a cotton gauze pad. The urethra meatus and anus were sealed with cyanoacrylate (Krazy Glue, Columbus, OH) to prevent further excretion. Then pups were weighed to the nearest 0.01 g and placed onto a dry Kimwipe in 1-liter beakers within a test chamber. The test chamber was a 15-gallon glass aquarium with a Plexiglas top, which was maintained at 37 ± 1°C and 15–25% relative humidity. At the start of the 20-min test, the pups were lifted from the beaker and 4 ml of the prewarmed test solution [commercial half-and-half (milk and cream)] was squirted onto the Kimwipe. The pups were then placed onto the soaked Kimwipe and allowed to ingest the solution. The latency (seconds) to begin licking the Kimwipe was recorded. At the end of the test, each pup was removed from the test chamber, dried, and weighed again to the nearest 0.01 g. Intake was measured as the gain in body weight.

Determination of genotype at Lepr. Genotypes (+/+, +/fa, fa/fa) were determined according to a previously described method (8). Briefly, the A to C mutation at nucleotide 880 of Leprfa introduces an Msp1 restriction site, which can be used to detect the number of copies of the Leprfa allele. Tissue was digested with proteinase K, and genomic DNA was extracted using the Qiagen QIAamp Tissue Kit. Primers that flanked the Leprfa mutation (5′-TGAAGCCCGATCCACCGCTGG-3′ and 5′-CTCTCTTACGATTGTAGAATTCTC-3′) were used to generate a 143-bp PCR fragment. PCR was performed on a Perkin Elmer DNA Thermal Cycler using the following conditions: 92°C (2 min), 1 cycle; 92°C (30 s), 55°C (30 s), 72°C (1 min), 35 cycles; and 72°C (5 min), 1 cycle. The Advantage Genomic PCR Kit (Clontech) was used for PCR reactions. The PCR fragments were digested with Msp1 (80 mU/µl final concentration) for 1.5 h at 37°C and electrophoresed on a 2% agarose-2% low-melting point agarose gel. Digestion yields an uncut 143-bp product for the wild-type allele, whereas the mutant allele (fa) yields both a 106-bp and a 37-bp product.

Statistical analyses. A two-way ANOVA was used to assess significant differences among genotypes at each age. Genotype and litter were used as independent variables. If litter effects were not observed, results were reevaluated using a one-way ANOVA. Litter effects on intake were observed on P9 and P12, but no significant litter × genotype interaction on intake or latency was observed at any age. A one-way ANOVA was used to assess significant differences in intake and latency among ages within each genotype. Post hoc analyses were performed when appropriate using Fisher’s protected least-significant difference test. P values < 0.05 were considered significant.

RESULTS

Intake. fa/fa Pups ingested significantly more test solution than their +/+ littermates on P12 and P18, with a trend toward higher intake on P15 (P = 0.068) (Table 1). On P5, however, fa/fa pups ate significantly less than +/+ pups and ate equivalent amounts on P9. Compared with +/+ pups, fa/fa pups ate significantly more on P12, P15, and P18. On P5 no difference in intake between fa/fa and +/+ was observed; however, the intake of +/+ animals was significantly higher than +/+fa pups.

No significant difference in body weight among genotypes was observed at any age tested (Table 1); however, significant litter effects on body weight were observed at all ages and a significant litter × genotype interaction was seen on P5. Because litter effects on both grams of test solution ingested (on P9 and P12) and body weight (all ages) were seen, the intake was expressed as a percentage of body weight (Table 1). When intake was expressed in this manner, fa/fa animals ate significantly more than both +/+ and +/+fa pups on P15, as well as on P12 and P18 (Table 1). On P5, the intake of +/+fa pups was lower than +/+ pups, with a trend toward a lower intake than fa/fa pups (P = 0.058). The results on P9 were similar.

To correct for any differences in intake between trials, the intake as a percentage of body weight was normalized to the mean intake of the +/+fa pups within each trial (the +/+fa genotype was chosen because it was the most numerous in each trial). The normalized intake of test solution by fa/fa pups was significantly higher than +/+ and +/+fa pups on P12, P15, and P18 (Figure 1). No significant difference in intake between genotypes was observed on P9. On P5, however, there was a trend toward a main effect of genotype on intake [F(2,32) = 2.8, P = 0.073] due to a smaller intake of +/+fa pups than +/+ or fa/fa pups.

Although fa/fa pups on P12 ate more than lean littermates, the normalized intake on P12 was not significantly different from fa/fa pups on P5 or P9 (Figure 1). The magnitude of the hyperphagia (measured as normalized intake) in fa/fa animals on P15 and P18, however, was significantly different between these two ages and higher than the other ages tested.

Latency. The latencies of fa/fa pups were not significantly different from +/+ pups at any age tested. They were significantly shorter, however, than the latencies of +/+fa pups on P5 and P12 (Table 1). No significant

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Initial Weight, g</th>
<th>Intake, g</th>
<th>Intake, %Initial Wt</th>
<th>Latency, min</th>
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</thead>
<tbody>
<tr>
<td>P5</td>
<td>fa/fa (12)</td>
<td>8.2 ± 0.3</td>
<td>0.13 ± 0.02</td>
<td>1.64 ± 0.24</td>
<td>3.03 ± 0.58*</td>
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<td></td>
<td>+/+ (12)</td>
<td>8.8 ± 0.3</td>
<td>0.08 ± 0.01</td>
<td>0.94 ± 0.14</td>
<td>7.67 ± 1.44</td>
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<td>P9</td>
<td>fa/fa (11)</td>
<td>15.4 ± 0.9</td>
<td>0.39 ± 0.08</td>
<td>2.41 ± 0.32</td>
<td>2.55 ± 0.41</td>
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<tr>
<td></td>
<td>+/+ (14)</td>
<td>15.0 ± 0.7</td>
<td>0.33 ± 0.06</td>
<td>2.06 ± 0.37</td>
<td>3.15 ± 0.58</td>
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<tr>
<td>P12</td>
<td>fa/fa (10)</td>
<td>21.3 ± 0.6</td>
<td>1.17 ± 0.11</td>
<td>5.55 ± 0.56</td>
<td>1.80 ± 0.26*</td>
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<tr>
<td></td>
<td>+/+ (29)</td>
<td>18.7 ± 0.5</td>
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<td>3.42 ± 0.32</td>
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<td>+/+ (12)</td>
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<td>4.56 ± 0.38</td>
<td>2.23 ± 0.37*</td>
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<td>P15</td>
<td>fa/fa (12)</td>
<td>26.4 ± 1.2</td>
<td>0.73 ± 0.11</td>
<td>2.83 ± 0.39</td>
<td>1.98 ± 0.40</td>
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<td>+/+ (20)</td>
<td>26.3 ± 0.7</td>
<td>0.35 ± 0.06</td>
<td>1.37 ± 0.25</td>
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<td>P18</td>
<td>fa/fa (11)</td>
<td>30.8 ± 0.9</td>
<td>0.53 ± 0.07</td>
<td>1.75 ± 0.24</td>
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<td></td>
<td>+/+ (15)</td>
<td>30.6 ± 1.3</td>
<td>0.17 ± 0.03</td>
<td>0.57 ± 0.10</td>
<td>3.97 ± 1.03</td>
</tr>
</tbody>
</table>

Table 1. Initial body weight, intake, and latency of Zucker rat pups in the independent ingestive test.
differences in latency were seen across ages in fa/fa pups. The latency of +/fa pups on P5 was longer than +/fa at other ages, whereas the latency of +/+ pups on P18 was longer than +/+ at other ages. When litters were normalized to +/fa pups within each trial, the latency of +/fa pups was significantly longer than +/+ and fa/fa pups on P5 and longer than fa/fa pups on P12. Additionally, the normalized latency of +/+ pups was significantly longer than +/fa and fa/fa pups on P18.

**DISCUSSION**

This study demonstrates that fa/fa pups ate significantly more than +/+ or +/fa pups as early as P12. Furthermore, the magnitude of the hyperphagia in fa/fa pups (intake relative to +/fa littersmates) increases from ~1.6-fold more on P12 to 3.6-fold more on P18. Although there was a trend for the intake (as percent of body weight) of fa/fa rats to be higher than +/fa littersmates at P5, the intake of +/+ animals was significantly higher than +/fa animals, and no difference between +/+ and fa/fa animals was observed. This observation, the lack of an overall effect of genotype on P5 when the data were normalized to +/fa littersmates within trial, and no difference in intake between genotypes on P9 is interpreted as an absence of hyperphagia before P12 under these conditions.

The normalized latency of +/+ pups was significantly longer than +/fa and fa/fa pups on P18; however, this difference was not correlated with the difference in intake observed. It is possible that possessing either one or two copies of the fa allele at this age influences this aspect of behavior (i.e., increases the motivation to ingest in this paradigm). Interestingly, on P5 the lacies of +/+ and fa/fa pups were shorter than +/fa pups and the intakes were higher [significantly higher for +/+ pups, with a trend toward higher intake for fa/fa pups (see RESULTS)]. The significance of the differing performance of heterozygotes at this age, and the possible presence of this phenomenon at other ages, requires further investigation.

The independent ingestive test used in this study required the pups to identify the test solution, initiate ingestion by licking at the solution, and maintain ingestion until sated. This test therefore is a measure of both the appetitive and consummatory phases of ingestion. It is possible that evidence of hyperphagia in fa/fa rats may be detected earlier if only the consummatory behavior is examined. Experiments using intraoral infusions by anterior sublingual catheters are required to determine this.

The nature of the test substance on ingestive behavior in the independent ingestive test used can influence the amount consumed (15). It is possible, therefore, that the age at which the emergence of hyperphagia is observed may be different if another stimulus is used. The rationale for using half-and-half (cream and milk) in this study is that the fat content is similar in composition to the dams' milk (11% for half-and-half vs. 12% for dams' milk). Tests with novel stimuli are required to determine if emergence of hyperphagia in the fa/fa pups is dependent on the stimulus used.

The proximal lesion involved in the development of hyperphagia and metabolic abnormalities in the obese fa/ma rat is abnormal leptin signaling (19, 25). Recent work has demonstrated that the leptin endocrine system is functional in normal rats and dysregulated in fa/ma rats before weaning. This is evidenced by lower brown fat leptin mRNA levels in Wistar rat pups restricted from suckling for 18 or 24 h after birth (11). Additionally, inguinal adipose tissue leptin expression and plasma leptin levels are elevated in fa/ma pups as early as P10 (18, 26). Hypothalamic neuropeptide systems which are involved in the control of feeding and may be regulated by circulating leptin include neuropeptide Y (NPY) (20, 23), corticotropin-releasing hormone (CRH) (20), and the melanocortins (21). NPY has been shown to increase feeding in an independent ingestive paradigm as early as P2 (5); however, the ontogeny of the CRH and melanocortin systems in feeding behavior is not defined. Future work examining the ontogeny of these and other systems (such as serotonergic, opiodergic, and catecholaminergic systems) in preweaning fa/ma rats may provide a better understanding of the role of leptin in the control of feeding.

**Perspectives**

The findings of this study are important for several reasons. First, they demonstrate that fa/ma pups express hyperphagia when permitted to ingest independent of the dam at an age when they do not ingest more than lean littersmates while suckling (P5–P15; (4, 13)]. This observation further reinforces that suckling and independent ingestive behaviors at preweaning ages are controlled by different mechanisms (12, 14) and that the lesion(s) due to the fa mutation impinge on the control of adultlike ingestive behavior. Second, the isolation of the developmental period when the hyperphagic phenotype is expressed in the fa/ma rat allows the involvement of neural substrates implicated in the initiation of hyperphagia to be more easily tested. For example, it has been proposed that hyperphagia in the fa/ma rat is due in part to the elevated activity of the hypothalamic arcuaparaventricular NPY system (24).
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We have recently found that fa/fa pups overexpress arcuate preproNPY as early as P2, before the emergence of hyperphagia in this study (Kowalski et al., unpublished observations), supporting this hypothesis. Last, the identification and characterization of neural systems that are dysregulated during the emergence of hyperphagia in fa/fa rats will lead to a better understanding of mechanisms employed in the organization of normal feeding behavior.

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