Changes in behavior and gene expression induced by caloric restriction in C57BL/6 mice

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Submitted 12 May 2009; accepted in final form 2 September 2009

Caloric restriction (CR) has been shown to suppress the development of age-associated diseases including cancer, atherosclerosis, and diabetes (23). Moderate CR and weight loss are now considered to be important for health promotion (3). In initial studies, CR was shown to cause negative emotional reactions, including depression, irritability, and anger in obese patients (37). Over the past 25 years, however, numerous studies have indicated that moderate CR combined with cognitive behavioral treatment reduced symptoms of anxiety and/or depression in overweight subjects (40, 41, 43, 44). The behavioral consequences of CR have also been investigated in animal models, and CR has been reported to decrease anxiety-like behavior in animals (18). However, another recent report showed that chronic CR in young rats can lead to the development of anxiety- and/or depressive-like behavior, likely through dysregulation of the brain serotonin system (16). Thus, the mechanisms through which CR affects anxiety- and depressive-like behaviors are not fully understood.

Repeated fasting and refeeding (RFR) has been used as another variant of CR, in which rodents are subjected to fasting every other day (1, 12, 20). Implementation of the RFR dietary regimen has been shown to result in an approximate 20–30% reduction in caloric intake over time (21). RFR has been shown to confer resistance to neurodegenerative diseases including Alzheimer’s disease (45) and Parkinson’s disease in mice (8). Marinković et al. (20) reported that CR and RFR differentially increased serum corticosterone levels. Despite having the same caloric intake, CR and RFR male rats have been shown to undergo unique hippocampal transcriptome responses to the different dietary regimens (22). These studies led us to speculate that these different feeding regimens may differentially induce the stress response and yield different behavioral consequences.

Microarrays have been widely used to measure gene expression profiles across different biological conditions. Because gene expression arrays allow rapid screening and quantification of differences in expression of large groups of functionally related genes, this technology is well suited for the systematic study of the complex, multigenic processes induced by CR (13). Previous microarray analysis has shown that chronic CR changes gene expression patterns in the mouse brain (32). However, there were no specific changes of gene expressions in CR, although biological functions were founded in the gene ontology analysis (13).

In this study, we assessed the effects of fasting and refeeding cycles on anxiety- and/or depressive-like behavior in RFR mice during a short dieting period of 16 days. We simultaneously analyzed the effects of simple food restriction in CR mice given half the daily amount of food consumed by RFR mice on feeding days. Using a whole mouse genome microarray, we examined how CR and RFR modified gene expression in brain regions involved in the regulation of psychological and physiological responses to stress: the prefrontal cortex, amygdala, and hypothalamus (15, 28).

METHODS

Animal care and feeding regimens. All mice were treated in accordance with the APS Guiding Principles for the Care and Use of Animals, and all procedures were approved by the Animal Care Committee of the University of Tokushima. Male C57BL/6 mice (8–9 wk) were purchased from CLEA Japan (Tokyo, Japan). They were maintained on a 12 h light-dark cycle (lights on at 9:00) at 24 ± 2°C and 50–60% humidity. Three mice were housed in each cage and
were allowed ad libitum access to tap water and laboratory animal chow (MF; Oriental Yeast, Tokyo, Japan) for 1 wk prior to experimentation.

We utilized three feeding regimens, and each group consisted of 36 mice (Supplemental Fig. S1).9 Nine mice in each feeding group were independently used for behavioral test each experimental point (a total of 108 mice were used: = 9 mice × 3 groups × 4 time points). Control mice had ad libitum (AL) access to food. The RFR mice were given access to food every other day, and CR mice were daily given half amount of chow consumed by the RFR group on the last feeding day. CR was started 2 days after RFR mice were subjected to fasting for the first time. These dietary regimens are illustrated in Supplemental Fig. S1. RFR and CR mice consumed the same total amount of food during the experimental period. We measured body weights and food intake at 20:00, at which point the day’s manipulated feeding or fasting was begun.

Behavioral tests. Nine mice were used in each feeding group and for each experimental period. They were subjected to four behavioral tests (the open field test, the light-dark transition test, the elevated plus maze test, and then the forced swimming test) at 14:00 on day 4, 8, 12, or 16. If a mouse could not complete a test (e.g., falling from the test apparatus), its behavioral data were omitted.

Locomotor activity and anxiety-like behavior were assessed by the open field test (33). In this test, a white acrylic chamber (45 cm long, 45 cm wide, and 30 cm high) was placed in the experimental room and illuminated by four indirect and homogenous lamps (150–200 lux). Each mouse was individually placed at a corner of the open field arena. Locomotor activity was then videotaped for 10 min, and the distance traveled, total time in the central area, and average movement speed were measured by an automated image analysis system (ImageJ OP; O’harra & Co., Tokyo, Japan) that was modified with the public domain ImageJ software (http://rsb.info.nih.gov/ij/).

Anxiety-like behavior was assessed by the light-dark transition test (7). A chamber was divided into two equal-sized compartments (22 cm long, 24 cm wide, and 30 cm high). The light compartment (450–500 lux) was composed of white flooring and transparent walls and lid. The dark compartment (0–5 lux) was composed of black flooring, walls, and lid. This compartment was completely enclosed except for a small opening (5 cm wide and 3.5 cm high) to allow movement between the dark and light compartments. Mice were placed in the dark compartment, and the latency to move to the light compartment for the first time was recorded. The time spent in each compartment was also recorded for 5 min. Increased time spent in the light compartment indicates reduced anxiety-like behavior (4).

Anxiety-like behavior was also examined with the elevated plus maze test (19). The plus maze consists of two connected runways. One runway has closed arms (30 cm long and 7 cm wide) protected by 15 cm high walls. Another runway has open arms (30 cm long and 7 cm wide) without walls. The four maze arms originated from a central platform (7 cm square). The maze was situated 70 cm above the floor. Each mouse was individually placed at the center of the maze facing the closed arm, and behavior in the maze was recorded for 5 min. The number of entries and time spent in the open and closed arms were measured automatically.

Depressive-like behavior was evaluated by the forced swimming test (31). Mice were individually placed in plastic cylinders (25 cm high and 20 cm diameter) containing water 13 cm deep at 22–23°C. Behavioral changes were recorded for 5 min. When a mouse was observed floating in the water in an upright position without moving, it was considered to be immobile.

RNA preparation. Another set of four mice in each group was subjected to the dietary regimen for 8 days and was used for RNA extraction. These mice did not undergo the behavioral tests. They were killed at 14:00 under general anesthesia with diethyl ether. After systemic perfusion with cold phosphate-buffered saline through the heart via a syringe attached to a 21-G needle, whole mouse brains were removed. Coronal brain sections (1 mm thick) were prepared on ice, with a brain slicer (Muromachi Kikai, Tokyo, Japan). The prefrontal cortex was sliced between 2.5 and 3.5 mm anterior to bregma. The hypothalamus and the amygdala were sliced between 1.5 and 2.5 mm posterior to bregma. We collected each region using an atlas of anatomy as a reference (9). Total RNA was immediately prepared from these samples using an RNeasy kit (Qiagen, Hilden, Germany). An equal amount of RNA from the four mice in each group was pooled and used for microarray analysis. The quality of purified RNA was assessed by an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Labchip kit (Agilent Technologies, Palo Alto, CA).

Microarray analysis and pathway analysis. Total RNA (400 ng) was first reverse transcribed using a T7 sequence-conjugated oligo dT primer. At the same time, we used an RNA Spike-In Kit One Color (Agilent) to adjust microarray data. Synthesis, amplification, and labeling of complementary RNA (cRNA) with Cy3 dye were performed according to the manufacturer’s protocols. Prepared cRNA was added to a whole mouse genome oligo DNA microarray (4 × 44 k; Agilent). Hybridization was performed at 65°C for 17 h. After washing, fluorescence intensity was assayed using a scanner (G2565BA: Agilent). The signal intensities of Cy3 were quantified and analyzed by subtracting background, using Feature Extraction software (Agilent). These data were normalized by GeneSpring 7.3 software (Agilent). We selected 21,851 genes having fluorescence intensities >100 for at least one RNA sample using GeneSpring 7.3. The complete datasets were deposited in the Gene Expression Omnibus database (accession number GSE15860).

We used Ingenuity Pathway Analysis (IPA) 7.1 (http://www.ingenuity.com) to determine functional pathways in the identified genes. IPA software contains most of the knowledge in the literature about biological interactions among genes and proteins, and we used it to calculate the probability of a relationship between each canonical pathway and the identified genes (5, 6).

Quantitative real-time RT-PCR. cDNA was prepared from total RNA (500 ng) using the oligo dT primer according to the instructions from the PrimeScript first strand cDNA synthesis kit (Takara, Shiga, Japan). The mRNA expression of eight genes was analyzed by quantitative real-time RT-PCR using an ABI-PRISM 7500 (Applied Biosystems, Foster City, CA). Primers were designed with the Primer 3 program (http://frodo.wi.mit.edu/primer3) (Table 1).

The PCR reaction was performed according to the Power SYBR Green PCR Master Mix (Applied Biosystems) protocol, and data were analyzed using SDS 1.2 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) or mitochondrial ribosomal proteins 6 (Mrps6) was used as an endogenous quantity control, and values were normalized to Gapdh or Mrps6 mRNA levels. The relative expression ratio of each mRNA comparing AD, RFR, and CR mice was analyzed, and statistical significance was calculated by Tukey-Kramer’s test after an ANOVA.

Western blot analysis. We also prepared protein samples from another set of four mice in each feeding group. The mouse amygdalas were collected by the same protocol as for RNA extraction. The collected samples were prepared using the Nuclear Extraction Kit (Cayman, Ann Arbor, MI) according to the manufacturer’s instructions, and the cytosolic fraction was isolated. Ten micrograms of the cytosolic fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. A 1:1,000 dilution of an antibody against mouse dopamine-activated cAMP-regulated phosphoprotein (Darrp-32) (#2302; Cell Signaling Technology, Danvers, MA) was used to measure protein expression. After the bound antibodies were detected with an enhanced chemiluminescence Western blot analysis detection kit (Amersham Pharmacia, Piscataway, NJ), the polyvinylidene difluoride membrane was reblotted using an anti-β-actin antibody (Abcam, Cambridge, MA), as a
loading control. Band intensity for each sample, determined as the brightness per area (pixel$^2$), was calculated using Image J software.

Statistical analysis. All data are presented as means ± SE. When three groups were compared, Tukey-Kramer’s post hoc test after one-way ANOVA was used for parametric analysis and Scheffe’s post hoc test after the Kruskal-Wallis test was used for nonparametric analysis (e.g., number of entries into the open arm). Repeated one-way ANOVA was used when the same mouse was measured repeatedly (e.g., travel distance every 2 min). For all comparisons, statistical significance was determined to be $P < 0.05$. These analyses were run using R programs (http://www.r-project.org/).

RESULTS

Food intake and changes in body weights. On day 0, the body weights (means ± SE) of nine AL (control), nine RFR, and nine CR mice were 23.5 ± 0.3, 23.9 ± 0.2, and 24.5 ± 0.2 g, respectively. After starting the dietary regimens, body weights were monitored daily for 16 days (Fig. 1A). In RFR mice, body weight fluctuated, and there was approximately a 4 g difference between fasting and feeding days. A repeated one-way ANOVA found significant main effects of day ($F_{16, 224} = 99.13, P < 0.05$) and group ($F_{2, 14} = 32.87, P < 0.05$), and an interaction of the two ($F_{32, 224} = 83.40, P < 0.05$). Tukey-Kramer’s post hoc test showed significant difference in body weight between CR and AL mice on all days between day 3 and day 16 ($P < 0.05$) (Fig. 1A). This post hoc test also indicated that the body weight of RFR mice was significantly lower on days after fasting compared with AL mice ($P < 0.05$) (Fig. 1A).

We also monitored the daily food intake in the three groups of mice for 16 days (Fig. 1B). The food intake of RFR mice was from 114 to 174% of the amount consumed by AL mice on feeding day. In CR mice, the daily food intake was accordingly from 57 to 87% of the amount consumed by AL mice.

Changes in behavior in RFR and CR mice. To assess behavioral changes in our mice, we first measured the distance traveled, movement speed, and time spent in the central area of the open field on day 4, 8, 12, or 16 (Fig. 2). In the analysis of the total distance traveled, one-way ANOVA indicated a significant difference on day 8 ($F_{2, 18} = 5.15, P < 0.05$) and day 12 ($F_{2, 18} = 4.37, P < 0.05$). Post hoc comparisons showed a significant difference between RFR and CR mice on day 8 ($P < 0.05$) and also on day 12 ($P < 0.05$) (Fig. 2A). For movement speed, a significant difference was observed between diet groups by one-way ANOVA ($F_{2, 18} = 7.48, P < 0.05$) (Fig. 2B). CR mice also had significantly slower movement speed compared with AL mice ($P < 0.05$) and RFR mice on day 8 ($P < 0.05$), but not on days 4, 12, or 16 (data not shown). In comparing the travel distance every 2 min over 10 min, a repeated one-way ANOVA showed significant main effects of time ($F_{4, 72} = 20.32, P < 0.05$) and group ($F_{2, 18} = 7.08, P < 0.05$), but no interaction between time and diet group ($F_{8, 72} = 1.90, P > 0.05$) (Fig. 2C). However, this effect was not found on the other days (data not shown). A one-way ANOVA could not detect any significant difference in time spent in the center area among the groups throughout the experimental period including on day 8 ($F_{2, 18} = 1.57, P > 0.05$) (Fig. 2D). These results indicate that CR mice showed behavioral changes with a reduction peak on day 8.

To assess anxiety-like behavior, we then subjected mice in the three diet groups to the light-dark transition test (Fig. 3). For the amount of time spent in the light compartment (Fig. 3A), a one-way ANOVA showed a significant difference between diet groups on day 8 ($F_{2, 18} = 3.65, P < 0.05$) and day 12 ($F_{2, 18} = 6.83, P < 0.05$). A post hoc test indicated that there was a significant difference between the RFR and CR mice on day 8 ($P < 0.05$). The RFR and CR mice stayed in the light compartment for a longer time on day 12 than the AL mice ($P < 0.05$). When latency to travel from the dark to the light compartment was compared (Fig. 3B), a significant difference between the groups was observed with a one-way ANOVA on day 8 ($F_{2, 18} = 8.51, P < 0.05$). A one-way ANOVA indicated no significant difference in the amount of time spent in the dark compartment on day 8 ($F_{2, 18} = 3.41, P > 0.05$) (Fig. 3C). On day 8, the number of transitions did not significantly differ among the three diet groups according to the Kruskal-Wallis test ($H_{2, 18} = 9.25,

Fig. 1. Body weight changes and food intake after beginning diets (AL, RFR, and CR). A: body weights of AL (n = 9), RFR (n = 9), and CR mice (n = 9) were measured at 20:00 before feeding manipulation for up to 16 days. B: daily food intakes of AL, RFR, and CR mice were measured in each cage, and an average of food intake per mouse was calculated from 3 cages of each group. The food intake of CR mice was the same as that of RFR mice, because CR mice were given half the amount of chow consumed by RFR mice on feeding days. Values are expressed as means ± SE. *Significant difference compared with the AL group ($P < 0.05$). Feeding groups: ad libitum (AL), RFR, repeated fasting and refeeding; CR, caloric restriction.

Physiol Genomics • VOL 39 • www.physiolgenomics.org
In the light-dark transition test, CR mice showed a reduction in anxiety-like behavior, with a peak difference on day 8.

The three groups were then subjected to the elevated plus maze test to further measure anxiety-like behavior (Fig. 4). There was no significant difference among the diet groups in the amount of time spent in the closed arms according to a one-way ANOVA (F2, 18 = 3.21, P = 0.05) (Fig. 4A) nor in the number of entries into the closed arms according to the Kruskal-Wallis test (H2, 18 = 0.47, P = 0.05) (Fig. 4B). For the time spent in the open arms (Fig. 4C), a one-way ANOVA revealed a significant difference among the diet groups (F2, 18 = 11.5, P < 0.001).

1.82, P > 0.05) (Fig. 3D). In the light-dark transition test, CR mice showed a reduction in anxiety-like behavior, with a peak difference on day 8.

The three groups were then subjected to the elevated plus maze test to further measure anxiety-like behavior (Fig. 4). There was no significant difference among the diet groups in
Post hoc comparisons showed that CR mice stayed in the open arms for longer time than the AL (P < 0.05) or RFR mice (P < 0.05). A significant difference was also seen in the number of entries into the open arms according to the Kruskal-Wallis test (H2, 18 = 6.96, P < 0.05) (Fig. 4D). CR mice entered the open arms more frequently compared with the AL (P < 0.05) or RFR mice (P < 0.05).

Finally, we analyzed depressive-like behavior with the forced swimming test on day 8 (Fig. 5). Among the three diet groups, a one-way ANOVA indicated a significant difference in immobility (F2, 18 = 12.99, P < 0.05) (Fig. 4D). CR mice revealed that immobility time was significantly less in CR mice than in the AL (P < 0.05) or RFR mice (P < 0.05).

RFR- and CR-induced changes in gene expression in specific brain regions. RNA was extracted from the mouse prefrontal cortex, amygdala, and hypothalamus, and gene expression was analyzed using a whole mouse genome microarray. Based on the results of the behavioral tests, we collected the brain RNA samples on day 8.

Among the 21,851 genes having fluorescence intensities >100 for at least one sample, we selected genes whose relative mRNA levels differed by more than twofold compared with those of AL mice for each brain region. As shown in Fig. 6, the most pronounced change was observed in the amygdala: a total of 1,354 genes were upregulated and 229 were downregulated by the food restriction regimens.

IPA was used to organize the differentially expressed genes into functionally annotated pathways and networks. We examined the canonical pathways modified by each gene group. Using IPA, we found nine canonical pathways to be significantly modified by the 884 upregulated genes in the amygdalas of CR mice (P < 1.00E-3) (Table 1). No pathways were found to be significantly modified by the others gene groups. Among these nine pathways, α-adrenergic signaling was the top-scoring pathway (P = 7.94E-06), and dopamine receptor signaling was the second (P = 2.51E-05). We also examined the biological functions modified by each gene group (Supplementary Tables S2 and S3). Many biological functions were changed through the differentially expressed genes in each region of three groups of mice. It was difficult to determine the CR-specific pathways. We focused on the top and second-highest scoring canonical pathways to explain the possible association between this gene expression signature in the amygdala and the behavioral changes seen in CR mice.

Confirmation by quantitative RT-PCR. Table 1 shows the canonical pathways significantly modified in the amygdalas of CR mice and a list of genes that are associated with each modified pathway. From the genes associated with α-adrenergic signaling and/or dopamine receptor signaling, we selected eight genes (Gys1, H2-bl, Mras, Prkar2a, Adcy2, Adcy5, Ppp1r1b, and Ppp1r10), and their mRNA expression was validated by quantitative RT-PCR. As shown in Fig. 7, we were able to confirm that expression of six of these genes (Gys1, Mras, Adcy2, Adcy5, Ppp1r1b, and Ppp1r10) was significantly upregulated in the amygdalas of CR mice.

Darpp-32 protein levels in mouse amygdalas. Although the expression of five genes was upregulated in CR mice compared with AL mice, expression of Ppp1r1b was higher in CR mice than in both AL and RFR mice (Fig. 7). We measured Darpp-32 protein, which is encoded by Ppp1r1b, in the amygdalas of the three groups of mice. Western blotting showed that the anti-Darpp-32 antibody recognizes 32-kDa protein (Fig. 8A). A one-way ANOVA indicated that there was a significant difference in Darpp-32 protein levels in the feeding groups (F2, 9 =
Fig. 6. Venn diagram of differentially expressed genes in 3 brain regions of RFR and CR mice. An equal amount of RNA prepared from the prefrontal cortex, the amygdala, or the hypothalamus of 4 mice in each group was pooled, and RNA samples were subjected to microarray analysis. A total of 21,851 genes having fluorescence intensities >100 in at least one RNA sample were selected. The number of upregulated (top) and downregulated (bottom) genes with changes >2-fold compared with AL mice is shown.

4.25, \( P < 0.05 \). A post hoc test showed that Darpp-32 protein was significantly increased in CR mice on day 8 compared with both AL and RFR mice (\( P < 0.05 \), Fig. 8B).

**DISCUSSION**

To examine the effects of food restriction on behavior we utilized two different feeding regimens, RFR and CR. RFR mice had free access to chow every other day, and CR mice were given half the amount of food consumed by the RFR mice on their feeding days on a daily basis. In a previous study, RFR mice were reported to consume roughly twice as much food as AL mice on their feeding days (1). Under our experimental conditions, RFR mice consumed \( \sim 160\% \) the amount of chow consumed by AL mice on their feeding days (Fig. 1B). It is possible that the differences in dieting regimens and in the amounts of food consumed may be responsible for differences between the previous and current studies.

Initially, we had expected to see more profound changes in anxiety- and depressive-like behavior in RFR mice than in CR mice. The reduction of anxiety-like behavior was supported by the results of the light-dark transition test (4). However, the elevated plus maze did not indicate any behavioral changes in RFR mice, whereas CR mice again showed a significant reduction in anxiety-like behavior (Fig. 5) (31). In the forced swimming test, immobility time was significantly shortened in CR mice on day 8, suggesting that CR mice are likely to display reduced depressive-like behaviors (Fig. 5) (31). In contrast, behavioral changes in CR mice were found to be consistent in several measures of anxiety-like and depressive-like behaviors. The light-dark transition test indicated a reduction in anxiety-like behavior in these mice (4). In the elevated plus maze test, CR mice again showed a significant reduction in anxiety-like behavior on day 8 (19). Of note, we found no reduction in anxiety-like behavior on day 16 in association with increased body weight. In a previous study (18), rats subjected to CR for 3 wk showed no significant behavioral changes. The previous report together with our findings suggest that the effects of CR on behavior may be transient. In the forced swimming test, immobility time was significantly shortened in CR mice on day 8, suggesting that CR mice are likely to display reduced depressive-like behaviors (Fig. 5) (31). In CR mice, anxiety- and depressive-like behaviors were observed even when they had the same caloric intake as RFR mice. These results suggest that the behavioral consequences of food restriction may be related to the way in which food restriction is implemented.

It has been proposed that some behavioral tests performed in a sequential series are sensitive to the effects of prior test experience. Paylor et al. (30) showed the effects of rapid

### Table 1. List of canonical pathways and related genes significantly upregulated by CR in the amygdala

<table>
<thead>
<tr>
<th>Canonical Pathway</th>
<th>Gene Symbol</th>
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<tbody>
<tr>
<td>α-Adrenergic signaling</td>
<td>Adcy2, Adcy3, Adcy5, Adra2a, Gnas7, Gys1, H2-bli, Mapk6, Mras, Prkar2a, Prkb1, Prkcg, Pygb, Pygm</td>
</tr>
<tr>
<td>Dopamine receptor signaling</td>
<td>Adcy2, Adcy3, Adcy5, Drd2, Freq, Ppm11, Ppp1r1b, Ppp1r10, Ppp2r5a, Ppp2r5e, Prkar2a, Slc18a3</td>
</tr>
<tr>
<td>PPARα/RXRα activation</td>
<td>Adcy2, Adcy3, Adcy5, Acrv2b, Adipor1, Apoai, Ckap5, Cyp2c44, Ins1, Mras, Nr2c2, Plcb1, Plcb3, Prkar2a, Prkcb1, Prkar2a, Rela</td>
</tr>
<tr>
<td>Synaptic long-term depression</td>
<td>Adcy2, Adcy3, Adcy5, Gna13, Mras, Npr1, Npr2, Plcb1, Plcb3, Ppm11, Ppp2r5a, Ppp2r5e, Prkar2b, Prkcb1, Prkcg2</td>
</tr>
<tr>
<td>O-glycan biosynthesis</td>
<td>Galnt2, Galnt6, Galnt10, Sstga1, Sstgalnac6</td>
</tr>
<tr>
<td>RAR activation</td>
<td>Adcy2, Adcy3, Adcy5, Akt2, Casn1, Mapkapt2, Pdkpl1, Pml, Prkar2a, Prkar2b, Prkcb1, Prkch, Rho13, Rela, Rurb, Smarca4, Sorbs3</td>
</tr>
<tr>
<td>Ceramide signaling</td>
<td>Akt2, Bad, Ctsd, Mras, Negr, Ppm11, Ppp2r5a, Ppp2r5e, Rela, Smad3</td>
</tr>
<tr>
<td>Insulin receptor signaling</td>
<td>Akt2, Bad, Crk, Gsy1, H2-bli, Ins1, Kiaa1303, Mras, Pdpk1, Ppm1r10, Prkch, Prkar2a, Sh2b2</td>
</tr>
<tr>
<td>G protein-coupled receptor signaling</td>
<td>Adcy2, Adcy3, Adcy5, Adra2a, Adra2c, Adora1, Akt2, Drd2, H1r7, Mras, Pdkpl1, Plcb1, Plcb3, Prkar2a, Prkcb1, Rap1 gap (includes EG5909), Rela</td>
</tr>
</tbody>
</table>

Canonical pathways with a \( P \) value < 1.00E-03 are listed.
sequential behavioral tests, in which mice were subjected to six behavioral tests within 1–2 days. They found that there was no major difference in performance between mice who underwent a standard test battery and those who underwent a rapid test battery (29). It is possible that exposure to prior behavioral tests may have affected our data; however, the reduction in anxiety-like behavior in CR mice and not in RFR mice was confirmed by the light-dark transition and elevated plus maze tests.

It is well known that CR has a profound effect on most tissues (10, 39) and alters the expression of a variety of genes that are thought to be responsible for the functional changes that occur in various tissues in response to CR. To understand the effects of CR, a number of studies have utilized the high-throughput analysis of gene expression by microarray to determine tissue-specific gene expression changes in CR (13). However, most studies have focused on the effects of CR on the liver. Fu et al. (10) reported that CR changed expression of a large number of genes in the hypothalamus as well as in the liver and heart, but changes in gene expression in different brain regions in response to CR have not previously been examined in detail.

To elucidate the mechanisms of CR-dependent changes in behavior, we analyzed gene expression in brain regions (pre-
frontal cortex, amygdala, and hypothalamus) that regulate adaptive responses to stress (15, 28). We analyzed gene expression data from day 8, because behavior in CR group was significantly different at this time point. Gene expression analysis of these regions revealed the most striking alterations in the amygdalas of CR mice (Fig. 6). IPA analysis indicated that the 884 upregulated genes in the amygdala significantly modified nine canonical pathways (Table 1), whereas the identified genes in the other regions did not modify any canonical pathways. The amygdala is the “nerve center” for the anxiety response and is involved in the development of anxiety disorders (11). In these nine modified canonical pathways, the top-two scoring pathways were α-adrenergic and dopamine receptor signalings. Serotonin and adrenaline signals are believed to be important for the pathogenesis of mood disorders, including major depression, and serotonin and norepinephrine reuptake inhibitors are now widely used as antidepressants (36). Furthermore, dysfunction of dopaminergic neurons has been shown to occur in mood disorders (25), and dopaminergic neurons are involved in the motivational aspects of feeding behavior (29). Therefore, these upregulated genes may be associated with the behavioral changes seen in CR mice.

Using Gapdh as a reference gene, we validated the changes in expression of eight genes that were identified as being significantly upregulated and involved in α-adrenergic and/or dopamine receptor signaling in the amygdala. Out of eight these genes, quantitative RT-PCR confirmed the upregulation of six (Gys1, Mras, Adcy2, Adcy5, Ppplr1b, and Ppplr10) in CR mice. We now recognize that there is a problem with using a single reference gene for normalization. In the microarray data, mitochondrial ribosomal proteins 6 (Mrps6) showed uniform expression in the three brain regions as well as Gapdh. However, the expression of Actb varied drastically, despite the fact that this gene is frequently used as a reference. We also validated gene expression using Mrps6 as a reference, and reconfirmed the upregulation of Gys1, Adcy5, Ppplr1b, Ppplr10 (Supplemental Fig. S2). Thus, the upregulation of these four genes appears to be more replicable.

Gys1 encodes glycogen synthase 1, which binds specifically to stress-activated protein kinase 2b/p38b (SAPK2b/p38b) and phosphorylates this molecule in the brain (17). Mras is a member of the ras-GTPase family and is activated by several trophic factors in astrocytes, including epidermal growth factor, basic fibroblast growth factor, and hepatocyte growth factor (27). Adenylate cyclases encoded by Adcy2 and Adcy 5 catalyze the formation of the secondary messenger cyclic adenosine monophosphate (cAMP) and can form a functional heterodimeric complex (2). Ppplr1b encodes a Darpp-32 protein that inhibits protein phosphatase 1 (PP-1) and dephosphorylates dopamine receptor signaling molecules (14) through dopamine receptor 1 signaling (42). As a result, Darpp-32 amplifies dopaminergic signaling (26). Ppplr10 encodes the PP-1 nuclear targeting subunit (PNUTS), which can bind to both PP-1 and GABA(C) receptors and cross-link them, suggesting that PNUTS may target PP-1 to the GABA(C) receptor (36). Based on previous knowledge, it is possible that these six upregulated genes implicated in α-adrenergic and dopaminergic receptor signaling may play a role in the behavioral changes seen in CR mice.

Of the six upregulated genes, Ppplr1b expression showed the greatest induction in CR mice compared with AL and RFR mice when tested the six candidate genes. We analyzed the protein levels of Darpp-32 encoded by Ppplr1b on day 8 and verified that the protein levels reflected both the gene expression data obtained using quantitative RT-PCR and microarray data. In Darpp-32 knockout mice, locomotor activity has been shown to be reduced by compounds that selectively enhance serotonin release (38). It has also been shown that a single nucleotide polymorphism (SNP) of the Ppplr1b gene affects the structural connection between the prefrontal cortex and striatum (24). A more recent report indicated a significant association between an SNP of the Ppplr1b gene and an index of anger traits (35). These findings highlight the importance of Darpp-32: it is a key regulatory molecule in dopamine receptor signaling and is involved in emotional reactions. We believe that increased Darpp-32 levels may contribute to the behavioral changes seen in CR mice, although we do not know whether increased expression of this protein has direct effects on reducing anxiety- and depressive-like behavior.

It has been suggested that RFR mice are physiologically and metabolically different on feeding and fasting days. We examined behavioral changes in RFR mice on fasting days using the four behavioral tests (Supplemental Fig. S3 and Supplemental Table S4). The results indicate that the RFR regimen mainly modifies locomotor activity possibly due to prolonged fasting; therefore, RFR mice showed no consistent changes in anxiety- and depressive-like behaviors under the fasting conditions.

In conclusion, we observed profound effects of food restriction on behavior in CR mice. To explain the presence of anxiety- and depressive-like behaviors in CR mice, we focused on the modulation of α-adrenergic and dopamine receptor signaling in the mouse amygdala through microarray techniques and pathway analysis. Considering the association of gene expression and protein production, these pathways, including Ppplr1b, may be partially responsible for the effects of CR on behavior. We believe our findings may contribute to an understanding of the regulation of the stress response to food restriction.

DISCLOSURES
No conflicts of interest are declared by the author(s).

REFERENCES


