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Anorexic behavior and elevation of hypothalamic malonyl-CoA in socially defeated rats

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ABSTRACT

Suppression of body weight and eating disorders, such as anorexia, are one of the major symptoms of psychiatric disorders such as depression. However, the mechanisms of weight loss and reduced appetite in depressive patients and in animal models of depression are largely unknown. In this study, we characterized the mechanism of anorexia resulting from depression using socially defeated rats as an animal model of depression. Socially defeated rats showed suppressed body weight gain, enlarged adrenal glands, decreased home cage activity, decreased food intake, and increased immobility in the forced swim test. These results are representative of some of the core symptoms of depression. Simultaneously, we observed decreased levels of phosphorylated AMP-activated protein kinase (AMPK) and acetyl-coenzyme A (CoA) carboxylase (ACC) and increased levels of malonyl-CoA in the hypothalamus of socially defeated rats. Hypothalamic malonyl-CoA controlled feeding behavior and elevation of malonyl-CoA in the hypothalamus induced inhibition of food intake. Our findings suggest that the suppression of body weight gain caused by social defeat stress is caused by anorexic feeding behavior via an increased concentration of malonyl-CoA in the hypothalamus.

1. Introduction

Depression is a major mental disorder; approximately 20% of the population is afflicted with depression [1]. Eating disorders, such as anorexia, are one of the major symptoms of depression [2,3]. Researchers have developed various types of depression-like animal models that are exposed to aversive psychological, physical or chemical stimuli, including social defeat, restraint, forced swimming, corticosterone administration, and mitogen-activated protein kinase inhibition [4–8]. The suppression of body weight gain has been reported in animal models of depression that are exposed to the stresses mentioned above [4–8]. Conversely, some animal models of depression showed unchanged or increased body weight gain [9,10]. Weight loss and reduced appetite are some of the criteria for diagnosing a major depression episode in DSM-IV [2]. However, the mechanisms of weight loss and reduced appetite in depressive patients and in animal models of depression are largely unknown.

Energy homeostasis is regulated by the central nervous system, in particular the hypothalamus. Many neuropeptides that control food intake are expressed in the hypothalamus, for example, orexigenic peptides (agouti-related protein (AgRP), melanin-concentrating hormone (MCH), and neuropeptide Y (NPY)) and anorexigenic peptides (proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)). Malonyl-coenzyme A (CoA) regulates food intake via the control of neuropeptides, including those described above, in the hypothalamus [11,12]. Malonyl-CoA is known to intermediate the fatty acid biosynthetic pathway and is synthesized from acetyl-CoA by acetyl-CoA carboxylase (ACC). ACC is phosphorylated and inactivated by phosphorylated AMP-activated protein kinase (AMPK) [11,12]. The injection of C75, a fatty acid synthase inhibitor, induced an elevation of malonyl-CoA in the hypothalamus and an inhibition of food intake [13]. Thus, malonyl-CoA functions as a master mediator in the hypothalamus that controls feeding behavior.

In this study, we investigate the mechanism of anorexia in depression using socially defeated rats, with a particular focus on the malonyl-CoA signaling pathway in the hypothalamus.

2. Materials and methods

2.1. Animals

Our detailed experimental design has been described previously [14]. Eight-week-old male Wistar rats were purchased from Charles River (Yokohama, Japan) and were housed individually at

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room temperature (22 ± 1 °C), with exposure to light from 6:00 to 18:00 and *ad libitum* access to food and water. After arrival, the rats were handled daily for 1 week to habituate them to the environment; they were then used as intruders. Twelve-week-old male Wistar rats from our colonies were used as residents. Each male was housed with a 12-week-old sterilized female in a large cage under the same conditions as described above. All experimental procedures followed the Guidelines of the Animal Care and Use Committee of Ibaraki University.

2.2. Stress exposure

The detailed experimental design has described previously [14]. Before the start of stress exposure, residents were paired with sterilized females for 3 weeks to establish territorial dominance. The intruder rat was introduced into each resident's home cage for up to 1 h. After the intruder displayed a submissive posture, the intruder was immediately removed and kept in a wire-mesh cage within the resident's home cage for the remainder of the hour. Rats from the stress group were subjected to this social defeat procedure on a daily basis for 5 weeks.

2.3. Behavioral analyzes

2.3.1. Open field test (OF)

The method for OF was described previously [14]. Each subject was placed in the same corner of the open field apparatus. The total distance traveled (in cm) during the 10-min session was recorded, and the result was analyzed on a Windows computer using Image J XX (O'Hara & Co., Ltd.), a modified software program based on the public domain Image J program.

2.3.2. Forced swim test (FS)

The method for FS was described previously [14]. Each rat was placed into an acrylic cylinder filled with water (24 ± 1 °C) to a height of 18 cm. After 15 min, the animal was transferred to a 35 °C environment for another 15 min (pre-test). Twenty-four hours later, the subject was placed into the cylinder again for 5 min (test). Prior to each test, the cylinder was cleaned and filled with fresh water.

2.4. Home cage activity and food intake

Home cage activity and food intake were measured using an activity sensor and a food intake monitor (O'Hara & Co., Ltd.).

2.5. Body and organ weights

Body weight was measured at the end of the control phase (baseline) and at weekly intervals during the stress phase. At the end of the experiment, the animals were anesthetized and decapitated. Adrenal glands were dissected quickly and were weighed, and their percentage of overall body weight was calculated.

2.6. Acyl-CoA cycling method

The concentration of malonyl-CoA was measured using the acyl-CoA cycling method [15]. Malonyl-CoA in hypothalamus was extracted by homogenization in 0.6 M H_2SO_4. After the neutralization, malonyl-CoA was measured by the acyl-CoA cycling method coupled with the citrate synthase treatment. First, acetyl-CoA in the extract was eliminated by the incubation at 25 °C for 20 min in the mixture containing 50 mM Tris–HCl (pH 7.2), 10 mM $MgSO_4$, 2 mM oxaloacetate, 1 U of citrate synthase from porcine heart (Roche Diagnostics GmbH, Mannheim, Germany) in 1 ml. An aliquot of the citrate synthase reaction mixture containing malonyl-CoA was transferred to the mixture for the acyl-CoA cycling method, that contained 50 mM Tris–HCl (pH 7.2), 1 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 50 mM malonate, 10 mM ATP, 1 U of malonate decarboxylase in 400 μl, and the reaction was carried out at 30 °C for 20 min, followed by the addition of 1 U of acetate kinase from *Escherichia coli* (Roche Diagnostics GmbH). After 20 min of incubation, 0.2 ml of 2.5 M neutralized hydroxylamine was added, and the incubation was continued for an additional 20 min. The reaction was terminated by adding 0.6 ml of 10 mM ferric chloride dissolved in 25 mM trichloroacetic acid-1 M HCl. The A_{540} of the acetohydroxamate formed was measured. Every assay was performed in duplicate.

2.7. Protein preparation and western blotting

The detailed procedures of protein preparation and western blotting have been described previously [14]. The rat's brains were rapidly removed and chilled on ice and the hypothalami were dissected out. The tissue was homogenized in ice-cold RIPA buffer with a Polytron homogenizer. The homogenate was centrifuged at 800*g* for 15 min at 4 °C and the supernatant was collected. The method of western blotting followed the protocols of the ECL plus western blotting detection reagents (GE Healthcare), except for the incubation time of the primary antibody. We changed the incubation time of the primary antibody from 1 h to overnight. Detection was performed using the ECL plus western blotting detection reagents and LAS-3000 mini (FUJIFILM).

The following primary antibodies were used as described below and diluted to 1:1000: anti-actin (Santa Cruz), anti-AMPKα (Cell Signaling (CS)), anti-phospho-AMPKα (CS), anti-ACC (CS) and anti-phospho-ACC (CS). The western blotting results were analyzed quantitatively using Image J.

2.8. Statistical analysis

Data were analyzed using Excel Toukei 2006 for Windows (Social Survey Research Information Co., Ltd. Tokyo, Japan). The weight of the adrenal glands, immobility time in FS, concentration of malonyl-CoA and western blotting data were analyzed using Student's *t*-test. The body weight gain, home cage activity, food intake and locomotor activity in the OF were analyzed using two-way repeated measures ANOVAs followed by Bonferroni post hoc tests.

3. Results

In this study, the stress group showed a suppression in body weight gain compared to the control group as our previous study (Fig. 1A) [14]. Prior to stress exposure, the body weights of the stress and the control groups were similar (294.8 ± 4.9 g vs. 301.2 ± 3.7 g, $p > 0.1$). A two-way repeated measures ANOVA revealed that stress had a significant effect on body weight gain (F (1, 78) = 33.78, $p < 0.001$), and the stress × time interaction was significant (F (6, 78) = 23.66, $p < 0.001$). Subsequently, Bonferroni post hoc tests revealed a significant difference in body weight gain between the stress and the control groups for each week tested ($p < 0.001$). The stress group had a significantly higher ratio of adrenal gland weight to body weight than the control group as our previous study (0.0187 ± 0.0010% vs. 0.0129 ± 0.0007%, $p < 0.001$; Fig. 1B) [14].

The locomotor activities in the OF were similar between the stress and the control groups (Fig. 2A). A two-way repeated measures ANOVA revealed that stress did not significantly affect locomotor activity in the OF (F (1, 26) = 1.31, $p > 0.1$), and the stress × time interaction was also not significant (F (2, 26) = 1.83,

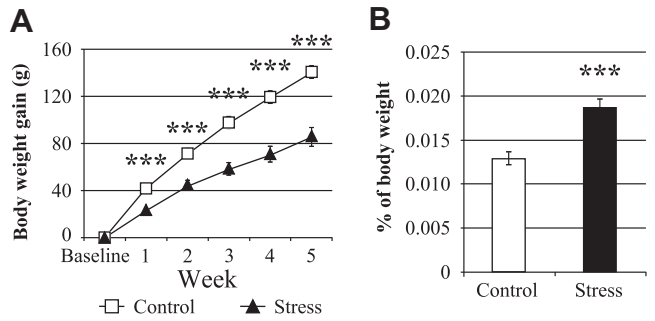

Fig. 1. The effects of chronic social stress on body weight gain and adrenal gland weight. (A) Body weight gain was calculated relative to the initial (baseline) body weight. Regarding body weight gain, a two-way repeated measures ANOVA showed that the main effect for stress ($p < 0.001$) and the stress × time interaction ($p < 0.001$) were significant. $^{***}p < 0.001$ (Bonferroni post hoc test). (B) The adrenal gland weight percentage was calculated from the total body weight at the end of the experiment. $^{***}p < 0.001$ (Student's *t*-test). Data represent the mean ± S.E.M. (Control, $n = 8$, Stress, $n = 7$).

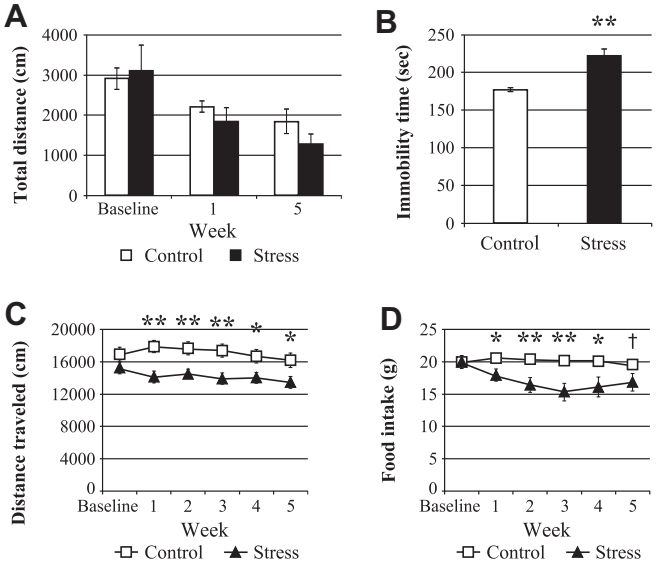

Fig. 2. The effects of chronic social stress on behavior. (A) The total distance traveled was measured during the 10-min period in the open field test. (B) The immobility time was measured during the 5-min periods in the forced swim test. $^{**}p < 0.01$ (Student's *t*-test). (C) The total distance in home cage during the dark phase. (D) Total food intake during the dark phase. $^{\dagger}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Bonferroni post hoc test). Data represent the means ± S.E.M. (Control, $n = 8$, Stress, $n = 7$).

$p > 0.1$) (Fig. 2A). In the FS, significantly prolonged immobility was observed in the stress group compared with the control group (223.2 ± 8.4 s vs. 177.3 ± 2.7 s, $p < 0.01$; Fig. 2B). Furthermore, we measured the spontaneous activities in the home cage and food intake in the rats exposed to social stress. During the dark phase, the home cage activities were significantly decreased in the stress group compared with the control group (Fig. 2C). A two-way repeated measures ANOVA revealed that stress ($F(1, 65) = 8.748$, $p < 0.05$) affects the activity in the home cage during the dark phase, but the stress × time interaction ($F(5, 65) = 2.059$, $p < 0.1$) was not significant affect for this activity. Subsequently, the Bonferroni post hoc tests revealed a significant difference between the stress and control groups for each week (Baseline, $p > 0.1$; 1–3 weeks, $p < 0.01$; 4–5 weeks, $p < 0.05$). Moreover, the intake of food during the dark phase was significantly decreased in the stress group compared with the control group (Fig. 2D). A

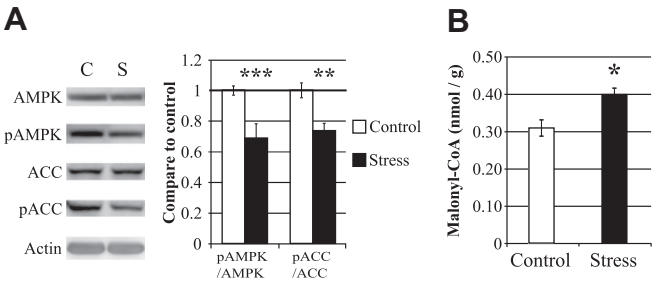

Fig. 3. The effects of chronic social stress on the malonyl-CoA signaling pathway in the hypothalamus. (A) The western blot analysis results in the hypothalamus. Bands were normalized to actin levels and compared quantitatively using Image J software ($n = 4$ / group). (B) The concentration of malonyl-CoA in the hypothalamus. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test); C, Control; S, Stress. Data represent means ± S.E.M. ($n = 3$ / group).

two-way repeated measures ANOVA showed that stress affected food intake during the dark phase ($F(1, 65) = 7.898$, $p < 0.05$), and the stress × time interaction was also significant ($F(5, 65) = 4.748$, $p < 0.001$). Subsequently, the Bonferroni post hoc tests revealed a significant difference between the stress and control groups for each week (Baseline, $p > 0.1$; 1 week, $p < 0.05$; 2–3 weeks, $p < 0.01$; 4 weeks, $p < 0.05$; 5 weeks, $p < 0.1$).

We observed the activities of AMPK and ACC and evaluated the concentration of malonyl-CoA in the hypothalamus. First, we evaluated phospho-AMPK and phospho-ACC by western blotting. The expression levels of actin, AMPK and ACC in the hypothalamus were similar between the stress and control groups (data not shown), whereas the ratios of phospho-AMPK/AMPK (0.693 ± 0.088 vs. 1.000 ± 0.029, $p < 0.001$) and phospho-ACC/ACC (0.739 ± 0.047 vs. 1.000 ± 0.049, $p < 0.01$) were both significantly lower in the stressed rats compared with the control rats (Fig. 3A). Second, we measured the concentration of malonyl-CoA and observed a significant increase in the hypothalamus of the stress group compared with the control group (0.401 ± 0.017 nmol/g vs. 0.310 ± 0.022 nmol/g, $p < 0.05$, Fig. 3B).

4. Discussion

In this study, we exposed rats daily to social defeat stress for 5 weeks. Our results demonstrated that chronic social defeat stress induced physiological, behavioral, and molecular changes. A suppression in body weight gain is one of the criteria used to diagnose major depression in humans and has been reported in previous studies using animal models of depression [2,4–8]. We observed that socially defeated rats also show a suppression in body weight gain compared to the control group as our previous study (Fig. 1A) [14]. This phenomenon may be related to feeding behaviors and metabolisms in socially defeated rats. Moreover, we also observed the hypertrophy of adrenal glands in socially defeated rats (Fig. 1B). Hypertrophy of the adrenal gland is a typical symptom of increased activity of the hypothalamic–pituitary–adrenal axis and a depression-like state in animal models [4]. Thus, this socially defeated rat has similar symptoms in the depressive patient.

We observed the spontaneous and depression-like behaviors of socially defeated rats using behavioral tests. Socially defeated rat showed similar locomotor activity compared to the control (Fig. 2A). Total duration of staying in the center area of was not different between two groups (data not shown). Prolonged immobility in the FS was used as a proxy for the symptoms of behavioral despair [1,16]. Socially defeated rats showed prolonged immobility in the FS (Fig. 2B). Thus, chronic social defeat stress induced depression-like behavior in rats, and these results were consistent with our previous study [14]. Furthermore, we observed decreases

in the home cage activity in socially stressed rats compared to control rats during the dark phase (Fig. 2C) but not during the light phase (data not shown). Decreased activity in the home cage is also one of the symptoms of depression in animal models [17]. Possibly the decrease of body weight gain in the stressed rats is not related to the elevated spontaneous activity. We observed decreases in the food intake in socially stressed rats compared to control rats during the dark phase (Fig. 2D) but not during the light phase (data not shown). Decreased food intake is another of the criteria that is used to diagnose major depression in humans [2]. These results indicate that the suppression of body weight gain in the socially defeated rats may be caused by decreased food intake during the dark phase.

Food intake is regulated by both orexigenic and anorexigenic neuropeptides in the hypothalamus. For example, the expression of AgRP, MCH, and NPY are increased in fasted mice and ob/ob mice; conversely, the expression of these neuropeptides is decreased in the hypothalamus of leptin-administrated fasted mice and −ob/ob mice [18,19]. These neuropeptides are also related to depressive disorders. Indeed, social isolation decreased the expression of α-MSH in the hypothalamus, and antidepressant treatment altered the expression of NPY in the brain [20,21]. Malonyl-CoA is known to regulate these neuropeptides in the hypothalamus and to control food intake. The administration of fatty acid synthase inhibitors, such as C75, decreased food intake following an elevation of malonyl-CoA in the hypothalamus along with the decreased expression of orexigenic peptides and a reciprocal increase in the expression of anorexigenic peptides [13,22]. ACC, which synthesizes malonyl-CoA from acetyl-CoA, is regulated by AMPK. Mice expressing a dominant negative form of AMPK in the hypothalamus and mice lacking AMPKα2 in AgRP neurons both showed a decrease in body weight gain and food intake, while mice lacking AMPKα2 in POMC neurons showed an increase in body weight gain and food intake [11,23]. Thus, we observed the activities of AMPK and ACC and evaluated the concentration of malonyl-CoA in the hypothalamus. Phosphorylation of AMPK and ACC in the socially defeated rats was decreased compared to the control (Fig. 3A). Therefore, social defeat stress induced the inactivation of AMPK and the activation of ACC in the hypothalamus. Furthermore, the concentration of hypothalamic malonyl-CoA in the socially defeated rats increased significantly (Fig. 3B). These results suggest that the decreased phosphorylation of AMPK and ACC in the hypothalamus of socially defeated rats facilitates the synthesis of malonyl-CoA. It is possible that elevated levels of malonyl-CoA in the hypothalamus led to a decrease in food intake in our chronic social defeat model. In the future, it is necessary to elucidate the relation between social stress and hypothalamic malonyl-CoA signaling pathway.

In conclusion, our data show that chronic social defeat stress induces depression-like physiological, behavioral and molecular changes. We observed decreased levels of phosphorylated AMPK and ACC and consequently increased levels of malonyl-CoA in the hypothalamus, decreased activity in the home cage and food intake during the dark phase, suppressed body weight gain, enlarged adrenal glands, and increased immobility in the FS in rats exposed to chronic social defeat stress. Because these changes represent several of the core symptoms of depression, our findings suggest that the suppression of body weight gain by exposure to chronic social defeat stress is induced by a suppression of food intake by elevated levels of malonyl-CoA in the hypothalamus.

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