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Social overcrowding as a chronic stress model that increases adiposity in mice

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Summary

Stress is a widely recognized risk factor for psychiatric and metabolic disorders. A number of animal models utilizing various stressors have been developed to facilitate our understanding in the pathophysiology of stress-related dysfunctions. The most commonly used chronic stress paradigms include the unpredictable chronic mild stress paradigm, the social defeat paradigm and the social deprivation paradigm. Here we assess the potential of social crowding as an alternative chronic stress model to study the effects on affective behaviors and metabolic disturbances. Ten-week-old male C57BL/6 mice were housed in groups of four (control) or eight (social crowding; SC) in standard cage for 9 weeks. Exploration, anxiety- and depressive-like behaviors were assessed in the open field test, the elevated T-maze, the novelty-suppressed test, and the forced swim test. SC mice exhibited a modest anxiety-like phenotype without change in depressive-like behaviors. Nine weeks of social crowding did not affect the body weight, but robustly increased adiposity as determined by increased mass of fat depots. Consistent with the increased fat content, serum leptin was markedly elevated in the SC mice. Specific changes in gene expression were also observed in the hypothalamus and the white adipose tissue following SC housing. Our study demonstrates the potential of social crowding as an alternative model for the study of stress-related metabolic and behavioral dysfunctions.

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Conflict of interest
All authors declare that they have no conflicts of interest.

Contributors
E.D. Lin designed the study, analyzed the data and wrote the manuscript. E.D. Lin, M. Sun, E. Choi, D. Magee and C. Stets conducted the experiments. M. J. During contributed to the preparation of the manuscript. All authors contributed to and have approved the final manuscript.

Keywords

C57BL/6 mice; social stress; obesity; anxiety; white adipose tissue (WAT); Crh; Npy; Sgk1; Ucp2; Adrb1

Introduction

Chronic stress has been shown to affect metabolic, endocrine and psychological functions both in animal studies and in human population-based and clinical studies (McEwen, 2000; de Kloet et al., 2005; Block et al., 2009; De Vriendt et al., 2009; Wallis and Hetherington, 2009; Koolhaas et al., 2011). Substantial evidence indicates that chronic stress is a risk factor for affective and metabolic disorders (McEwen et al., 2012; Hunter and McEwen, 2013; Sinha and Jastreboff, 2013). In a Finnish study by Marniemi et al. (2002), monozygotic twins discordant for obesity were assessed for differences in hormonal, physiological and psychological parameters. Notably, the obese co-twins showed higher index of psychosocial stress perception compared to their lean co-twins (Marniemi et al., 2002). In humans, the effect of stress on feeding and body weight appears to be bidirectional (Gibson, 2006; Serlachius et al., 2007; Torres and Nowson, 2007; Block et al., 2009). Why some people lose and others gain weight in response to stress is not fully understood and is likely to involve many factors (Stone and Brownell, 1994; Epel et al., 2004). One explanation is the balance between an increase in β-adrenergic activation, the body's main fat-burning mechanism (leading to weight loss), and the increased intake of sugar- and fat-rich comfort foods (resulting in weight gain) (Dallman et al., 2003; Dodt et al., 2003; Kuo et al., 2008). The observation that those who are initially overweight are more inclined to increase body weight when stressed whereas those who are of normal- or underweight do not led Dallman to propose that difference in metabolic outcomes might be the results of higher insulin concentration in people with higher body mass index (Dallman, 2010).

Similar to humans, animal models of chronic stress have produced variable and even opposite phenotypes of food intake, body weight gain and adiposity. Several studies described a pronounced anorexic phenotype following repeated stress exposure. Using an unpredictable chronic mild stress (UCMS) model, Michel and colleagues (2005) observed reduced weight gain and adiposity in stressed mice on a high-fat diet (Michel et al., 2005). A similar observation was made by Kim et al. (2003) with rats subjected to eight weeks of UCMS (Kim et al., 2003). Other types of chronic stressors, such as repetitive daily restraint, turpentine abscess, surgical stress and immobilization have also been reported to reduce food intake in rodents (Marti et al., 1994; Weninger et al., 1999). On the other hand, several studies also demonstrated that chronic psychosocial stress models such as the social defeat/sensory contact model, lead to hyperphagia and increased body weight gain and adiposity (Bhatnagar and Vining, 2003; Moles et al., 2006; Bartolomucci et al., 2009). In Syrian hamsters, social crowding (for female) and intermittent or chronic social defeat and footshock (for male) also lead to increases in food intake, body mass, and adiposity (Borer et al., 1988; Meisel et al., 1990; Foster et al., 2006; Solomon et al., 2007). Long-term social isolation has also been shown to accelerate body weight gain and adiposity in mice, although this effect appears to be strain-dependent (Nonogaki et al., 2007). Chronic stress was also

found to increase consumption of more palatable food, which are typically high in fat and/or sugar content (Gibson, 2006; Zellner et al., 2006; O'Connor et al., 2008; Roberts et al., 2013) and aggravate diet-induced obesity (Kuo et al., 2007; Kuo et al., 2008). Studies of mice subjected to chronic social defeat stress identified ghrelin signaling in catecholaminergic neurons as a critical mechanism for stress-induced food-reward behavior and the associated body weight gain (Lutter et al., 2008; Chuang et al., 2011; Patterson et al., 2013). However, there were also reports of a lack of effect or even reduced body weight gain in stressed mice fed high-fat diet (Michel et al., 2005; Bartolomucci et al., 2009; Finger et al., 2012).

The highly variable metabolic phenotypes observed in the different studies have been attributed to differences in the types of stressors, diets, protocol durations, strains of animals and stress intensities. The variability also reflects the complexity of the interaction between stress and metabolic processes and highlights the importance of the development and thorough characterization of different stress models. In this study, we used high-density living as a mild form of chronic social stress due to overcrowding. We assessed the affective behaviors and body composition changes following one and two months of social crowding, respectively.

Materials and Methods

Animals

Ten weeks old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were housed in groups of four (control; 3 cages) or eight (social crowding; 2 cages) in standard cages (31 cm × 17 cm × 14 cm) for 9 weeks. All mice were kept under a 12h light/dark cycle (lights on at 0600 hr), with free access to water and standard chow diet (11% fat, 28% protein, 61% carbohydrate, caloric density 3.4 kcal/g, Research Diets). Mice were transferred to clean cages when the bedding became too soiled, which were 1–2 weeks for the control group and twice weekly for the social crowding (SC) group. Mice were weighed at week 1, 4 and 8. All use of animals was approved by the Ohio State University Animal Care and Use Committee, and was in accordance with the NIH guidelines.

Behavioral analysis

After 1 month of housing in the different conditions, mice were subjected to behavioral testing in the following order: (1) open field test on day 30, (2) elevated T-maze on day 35, (3) forced swim test on day 37, and (4) novelty suppressed feeding on day 40. All tests were conducted during the light phase.

Open field test (OF)

To assess exploration and general motor activity, mice were placed individually into the center of an open square arena (60 cm × 60 cm, enclosed by walls of 48 cm). Each mouse was allowed 10 minutes in the arena, during which time its activity was recorded and analyzed by TopScan (Clever Sys Inc, Vienna, VA, USA). Specifically, the parameters measured include distances traveled in the periphery and in the center of the arena (36 cm × 36 cm), the total distance traveled, and the time spent in the center of the arena. The total

distance traveled provides a measure of exploratory activity while the time and distance ratio of arena center exploration provide an indication of anxiety level. In addition, the number of fecal boli was counted as an additional measure of physiological response to anxiety. The arena was cleaned with 30% ethanol between trials to remove any odor cues.

Elevated T-maze test (ETM)

The elevated T-maze is an ethologically based approach-avoidance conflict test targeting the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit open area. The T-maze consists of two open arms (30.5 cm × 15.5 cm) and an enclosed arm (46 cm × 10 cm) positioned in the shape of a 'T', with the enclosed arm as the stem of the T. The whole apparatus was elevated 88cm above the floor. The open and enclosed arms of the T-maze generate exploratory behavior and the avoidance of elevated open arms is considered a reflection of anxiety state. Each mouse was placed at the end of the closed arm facing toward the open arms and was allowed to explore the maze for 5 minutes. The behavior and movement of each mouse was recorded by a video camera and subsequently scored by an experimenter blinded to the experimental groups. Anxiety was indicated by the time spent on the open arms as well as the number of open arm entries. After each test, the mouse was returned to its home cage and the apparatus was cleaned with 30% ethanol.

Forced swim test (FST)

Forced swim test is one of the most commonly used rodent behavioral tests for screening antidepressant drugs (Cryan and Mombereau, 2004). Mice were placed individually in a transparent cylinder (21 cm diameter, 24 cm height) containing water (25 ± 2°C) to a depth of 15 cm for 6 minutes. At the end of each trial, mice were dried and returned to their home cage. Trials were video-recorded and a blinded experimenter scored the amount of time mice remained immobile as a measure of depressive-like behavior.

Novelty suppressed feeding test (NSF)

NSF assesses hyponeophagia, in which exposure to a novel environment suppresses feeding behavior (Samuels and Hen, 2011). NSF has been used to study anxiety- and depression-related behaviors since it is sensitive to anxiolytic and chronic antidepressant treatments. Mice were fasted overnight, with food removed at 1700 hr. The testing phase was conducted the next morning at 1000 hr. Mouse was individually placed into a brightly lit, novel open cage (40 cm × 28 cm × 20 cm). A piece of white filter paper (7 cm diameter) was placed in the center of the cage with a single pre-weighed food pellet. The latency to consumption (first bite of the food pellet) was measured. The cut-off time was 10 min. To assess if there was any difference in consummatory drive, each mouse was placed in a standard cage with the pre-weighed food pellet after its first bite or at cut-off time if it failed to eat within 10 min. The amount of food consumed in 5 min was calculated.

Metabolic chamber analysis

Following eight weeks of social crowding (or control housing), a subset of mice (n = 8 per group) was individually placed in the monitoring chambers of the Oxymax Lab Animal

Monitoring system (Columbus Instruments, OH) for 3 days. Following at least 24 hours of acclimatization in the chambers, measurements of food intake, activity, heat generated and respiratory exchange ratio (RER) were recorded from day 2 to day 3.

Euthanization and tissue collection

Nine weeks after the start of SC housing, mice were culled by decapitation under isoflurane anesthesia. Trunk blood was collected and serum was isolated by centrifugation and stored at −20°C until assayed. Brown adipose tissue (BAT), inguinal (WATi), retroperitoneal (WATr), and epididymal (WATe) white adipose tissue were dissected and weighed to determine body composition and adiposity. WAT depots were collected from one side only. In addition, the hypothalamus was dissected out using a mouse brain matrix and stored at −80°C until RNA extraction. For a subset of mice, WAT was stored fresh frozen at −80°C until RNA extraction, while the rest was immersed in 4% paraformaldehyde and paraffin-embedded for subsequent morphological analysis.

WAT staining and adipocyte size analysis

Paraffin-embedded WAT was cut into 20μm sections using a cryostat and mounted on slides. Slides were stained with hematoxylin and eosin (H&E) to examine the degree of adipocyte hypertrophy. Adipocyte diameter was measured using the Quick Measure Circle Command in Stereo Investigator 7 (MBF Bioscience, Willeston, VT) using a 10x objective. Software generated 150μm grids were drawn over the entire section; the cells within every other grid were circled to estimate diameter of the circle, excluding any incomplete cells. At least 10 cells per section were measured and then averaged.

Serum biomarker analysis by ELISA

Serum was analyzed for leptin, adiponectin and IGF-1 using DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction. Serum corticosterone level was determined using Enzyme Immunoassay Kit at 1:200 dilution according to the manufacturer's instruction (Assay Designs, Inc., Ann Arbor, MI, USA).

In a separate experiment (n = 8 mice per group), blood was collected by submandibular bleeding and serum was isolated by centrifugation for analysis of corticosterone at the early time-points of social crowding. Blood sample was taken at 1900 h (1 hour after light off) one week after social crowding began. Another blood sample was taken at 1000 h (during the light phase) following a 2 week recovery period. Serum corticosterone level was determined as described above.

Quantitative RT-PCR

Total RNA from hypothalamus and WATr tissue was isolated using RNeasy Mini Kit or RNeasy Lipid Mini Kit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturer's instruction. First-strand cDNA was generated using TaqMan Reverse Transcription Reagent (Applied Biosystems, Roche, Branchburg, NJ, USA) and quantitative PCR was carried out using a LightCycler Sequence Detection System (Roche, Indianapolis, IN, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems). Specific

primers that have been previously validated (Cao et al., 2009; Cao et al., 2010b; Cao et al., 2011; Lin et al., 2011; Liu et al., 2014; McMurphy et al., 2014) were used to detect the following mouse mRNA: *Actb* (beta-actin), *Crh* (corticotropin-releasing hormone), *Crh1r* (corticotropin-releasing hormone receptor 1), *Crh2r* (corticotropin-releasing hormone receptor 2), *Npy* (neuropeptide Y), *Npy1r* (neuropeptide Y receptor Y1), *Npy2r* (neuropeptide Y receptor Y2), *Vgf* (nerve growth factor inducible), *Avp* (arginine vasopressin), *Avpr1a* (arginine vasopressin receptor 1A), *Sgk1* (serum/glucocorticoid regulated kinase 1), *Nr3c1* (nuclear receptor subfamily 3, group C, member 1, also known as glucocorticoid receptor), *Nr3c2* (nuclear receptor subfamily 3, group C, member 2, also known as mineralocorticoid receptor), *Ucp1* (uncoupling protein 1), *Ucp2* (uncoupling protein 2), *Ucp3* (uncoupling protein 3), *Adrb1* (adrenoceptor beta 1), *Adrb2* (adrenoceptor beta 2), *Adrb3* (adrenoceptor beta 3), *Lep* (leptin), *Pparg* (peroxisome proliferator-activated receptor gamma), *Fasn* (fatty acid synthase), *Gpat* (glycerol-3-phosphate acyltransferase), *Pomc* (proopiomelanocortin), *Agrp* (agouti-related peptide), *Mc4r* (melanocortin 4 receptor). Specific primers were checked against the NCBI nucleotide database for specificity. All primer pairs generated a single melt curve product. Primer sequences are provided in Table 1. PCR data analysis was performed using the comparative $2^{-\Delta\Delta C}$T method with *Actb* as endogenous reference (Schmittgen and Livak, 2008).

Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC, USA). Student's *t*-test was used to compare the two experimental groups - control and social crowding. For activity, heat generated and RER over time, we determined the overall significance by repeated measure ANOVA followed by comparison of individual time-points using Student's *t*-test. Statistical significance was set at $P < 0.05$. All data are presented as means ± standard error of the mean (S.E.M).

Results

Effects of social crowding on anxiety-related behaviors

In the OF, chronic SC did not affect locomotion or time traveled in the center of OF but significantly reduced the center/total distance ratio ($P < 0.05$; Fig. 1), suggesting an increase in anxiety-like behavior. In addition, a significant increase in defecation was observed in the SC group in the OF test ($P < 0.05$), which can be considered a physiological reaction reflecting the increase in anxiety. Defecation was also increased in the SC group in the ETM test (1.25 ± 0.54 vs. 0.09 ± 0.09 in control group), although the difference did not reach statistical significance ($P = 0.09$). Other parameters were unchanged in the ETM test (Fig. 1).

In the NSF, latency to eat was significantly increased in the SC group ($P < 0.05$, Fig. 2), suggesting an increased anxiety. Interestingly, the increase in latency might in part be due to a reduced appetitive drive, since the SC group showed a trend for reduced total amount of food consumed in 5 min ($P = 0.055$).

Effects of social crowding on depressive-like behaviors

Immobility behavior in the FST is commonly used as an indicator of depressive-like behavior (Cryan and Mombereau, 2004). We did not observe any significant difference in the time mice remained immobile between the control and SC groups (Fig. 2).

Effects of social crowding on body weight and adiposity

Two months of SC significantly increased adiposity without increase body weight (Fig. 3). Basal body weight was comparable between the groups (control, 26.5 ± 0.29 g vs. SC, 26.4 ± 0.35 g, $P = 0.88$). Body weights were comparable between the groups throughout the experiment (Fig. 3A). Brown adipose tissue (BAT) weight was increased by 1.44 fold ($P < 0.01$). WATi, WATe, and WATr weights showed increases of 1.74 fold ($P < 0.01$), 1.62 fold ($P < 0.05$), and 2.30 fold ($P < 0.05$), respectively.

Using Stereo Investigator 7 software to measure the diameters of the adipocytes, we found that the adipocytes in the WATi and WATr were larger in the SC compared to the control group (Fig. 3 and 4, $P < 0.001$ for both WATi and WATr). In contrast, WATe adipocyte sizes were comparable between the two groups.

Effects of social crowding on metabolic profile

Activity level as determined when mice were individually placed in the metabolic chamber were comparable between SC and control mice (Fig. 5B, Group effect, $P = 0.251$). Repeated measure ANOVA revealed a significant effect over time ($P < 0.0001$), but the interaction effect between time and housing condition was not significant ($P = 0.292$). Comparing the two groups over time, SC mice exhibited slightly higher activity 4 hours after the onset of dark phase ($P < 0.05$). Similarly, energy expenditure as determined by heat generated was comparable between groups (Fig. 5A, Group effect, $P = 0.399$) and there was no significant interaction between time and housing conditions ($P = 0.159$). A significant effect over time was observed ($P < 0.0001$), and SC mice exhibited increased energy expenditure at 4 hours after lights off ($P < 0.05$), concomitant with the slight increase in activity observed. An interaction effect between time and housing condition was detected for RER ($P < 0.01$), suggesting that SC exerted differential effects depending on the time of day. This was likely due to a lower RER in the SC group during the dark but not the light phase (Fig. 5C). However, the effect was subtle since differences at individual time points did not reach statistical significance. Mean RER did not differ between the two groups either (control, 0.90 ± 0.02 vs. SC, 0.92 ± 0.02; $P = 0.516$). Daily food intake was not significantly different between the SC and control mice (Fig. 5D, $P = 0.591$).

Effects of social crowding on serum biomarkers

Consistent with the increase in adipose tissues, SC group showed marked increase in serum leptin level ($P < 0.01$, Fig. 6A). There were no changes in serum insulin, adiponectin, IGF-1 or corticosterone following 2 months of social crowding. To determine if the lack of change in the corticosterone level in the social crowding paradigm might be due to habituation to a chronic stressor, we analyzed serum from the SC mice at 1 and 3 weeks in the dark and light

phase respectively. At both time points, serum corticosterone levels were increased by 2-fold over control animals whether in the light or dark phase (Fig. 6B).

Effects of social crowding on hypothalamic gene expression

Using quantitative real-time PCR, we compared selected gene expression in microdissected hypothalamus from control and SC mice (Fig. 6C). Expression of *Crh* mRNA was increased more than twofold in the SC mice ($P < 0.01$), but its receptors *Crh1r* and *Crh2r* were unchanged. SC increased hypothalamic expression of *Npy* and its receptor *Npy1r* ($P < 0.01$, $P < 0.05$ respectively), but not *Npy2r*. Concomitantly, *Agrp* expression was also increased by SC ($P < 0.05$). In contrast, a twofold reduction in *Sgk1* expression was observed in the SC group ($P < 0.05$). SC did not change expression of *Avp*, *Avpr1a*, *Vgf*, *Nr3c1* (glucocorticoid receptor), *Nr3c2* (mineralocorticoid receptor), *Pomc* and *Mc4r*.

Effects of social crowding on WAT gene expression

We also found changes in WATr gene expression induced by chronic SC (Fig. 6D). These include marked increases in *Ucp2* (2.2 fold, $P < 0.001$) and *Lep* (2.5 fold, $P = 0.01$). *Adrb1* and *Fasn* were reduced in the SC group ($P < 0.05$) and there was a strong trend for reduced *Ucp3* ($P = 0.06$). Expression of *Adrb2*, *Adrb3*, *Pparg* and *Gpat* were not statistically significantly different between the control and SC mice.

Discussion

In this study, we report a chronic stress model that increased anxiety-like behaviors and adiposity prior to any significant body weight gain. Animal models of chronic stress are important tools for the study of the pathophysiology and molecular mediators of stress-related disorders such as anxiety and metabolic syndromes. These animal models are also needed for the verification of potential treatments. The most often used chronic stress models include chronic mild stress, social defeat, chronic subordinate colony housing and social isolation (Toth and Neumann, 2013). Although rodents in overcrowding conditions were studied since the 1970s (Gartner et al., 1973; Pasley et al., 1978; vom Saal and Howard, 1982), they were mostly focused on the reproductive and immunological consequences of overcrowding. There were only a handful of studies that examined the behavioral and metabolic effects of social crowding in mice. In a study by Reiss and colleagues (2007), mice were housed in groups of seven (crowded condition) from 3 weeks of age for 13 weeks (Reiss et al., 2007). Compared to individually housed controls, these mice displayed increased signs of anxiety-like behaviors in the open field and the elevated plus maze tests, and exaggerated acoustic startle response. The reduced center to total distance ratio in the OF exhibited by the SC mice is a parameter widely used as an indication for increased anxiety (Swanson et al., 1998; Bontekoe et al., 2002; Salas et al., 2003; Walz et al., 2006). Moreover, stress induces colonic transit and an increase in stress-induced defecation in the open field was observed in rodents that exhibited increased anxiety behaviors (O'Malley et al., 2010). While the increased latency to feed in the NSF might not be entirely due to hyponeophagia and could be partly due to altered consummatory drive, the change was consistent with an increase in anxiety. Together, these results suggest an anxiogenic effects of social crowding. It is interesting to note that we used adult male mice

in our study. While the potential of fighting may result in the loss of animals if adult mice are used, we did not observe any increase in aggressive behavior or fight wounds in the SC mice compared to controls in our study. The SC mice appeared physically healthy with similar fur coat condition as the control mice. Our study thus expands previous findings (Reiss et al., 2007; Tramullas et al., 2012) and shows that social crowding of adult mice for a month was sufficient to produce a mild anxiety-like phenotype.

More recently, overcrowding has been used as a stressor in the mixed social defeat and overcrowding (SD/OC) chronic psychosocial stress model (Reber et al., 2006; Finger et al., 2012; Slattery et al., 2012). The SD/OC model consisted of 19 days of repeated exposure to social defeat with intermittent 24 hour overcrowding. This paradigm is thus distinct from the overcrowding stress model presented here, with additional stressor, increased stress intensity and the unpredictability factor of the different residents and scheduling of the stressors. Nevertheless, the SD/OC model produced a similar behavioral phenotype to the SC model with an increase in anxiety-like behaviors but no effect on depressive-like behaviors (Slattery et al., 2012).

In the SC mice, serum corticosterone was increased in both the dark and light phases following 1 and 3 weeks of social crowding, respectively, but returned to comparable level to the control group by 2 months. This was likely due to an adaptation to the chronic stress. A return to comparable baseline corticosterone following chronic psychosocial stress has been reported by others using the chronic subordinate colony housing paradigm in mice (Reber et al., 2007; Uschold-Schmidt et al., 2012). The increase in corticosterone levels along with the higher *Crh* expression in the hypothalamus indicate enhanced stress in the SC mice. In addition, the increase in anxiety-like behaviors and physiological responses that are known to be induced by stress (Nishiyama et al., 2004; Julio-Pieper et al., 2012; Campos et al., 2013; McCormick and Green, 2013), are also consistent with increased stress in the SC mice.

Our study shows that chronic social crowding lead to a marked increase in adiposity prior to any change in body weight, indicating a change in body composition with a greater ratio of fat mass. All fat depots collected, including WATi, WATr, WATe and BAT, were significantly larger in the SC mice compared to control mice. Histological examination of the fat depots showed that the adipocytes of WATi and WATr were enlarged. In contrast, WATe adipocyte sizes were comparable between the SC and control mice, suggesting that the enlargement of this particular fat depot might be due to an increase in adipocyte proliferation rather than hypertrophy. The SC model may therefore be useful to study the distinct mechanisms underlying fat mass regulation. The dramatic increase in serum leptin is concomitant with the increase in fat mass. The metabolic phenotype of SC model is thus distinct from other chronic social stress models, such as SD/OC or chronic subordinate colony housing (CSC) paradigms. In both paradigms, body weight was reduced during the presence of stressor (Finger et al., 2012; Slattery et al., 2012). Some studies reported increased body weight gain upon termination of the stressors (Melhorn et al., 2010; Slattery et al., 2012). Closely resembling to the SC model described in this study is the group housed (six per cage) female hamsters, which also exhibited significantly increased adiposity (as determined by percentage of body fat), body weight and body length (Borer et al., 1988;

Meisel et al., 1990). However, it should be noted that in these studies, as for the SD/OC studies mentioned above, individual housed animals were used as controls. Considering that social isolation is also often used as a psychosocial stressor (Yamada et al., 2000; Siegfried et al., 2003; Matsumoto et al., 2005), interpretation of data using these animals as controls could be confounded and thus remain to be validated using group housed animals as controls.

In addition to *Crh*, SC also increased hypothalamic expression of *Agrp, Npy* and *Npy1r*, and a reduction in *Sgk1*. Hypothalamic NPY stimulates appetite and feeding, partly through the Y1 receptors (Kanatani et al., 1996; Lopez-Valpuesta et al., 1996). Both chronic stress and glucocorticoid administration have been shown to upregulate hypothalamic *Npy* and *Npy1r* expression (Larsen et al., 1994; Sergeyev et al., 2005). The orexigenic AgRP colocalizes with NPY in the arcuate nucleus and promotes feeding by blocking the melanocortin receptors (Morton and Schwartz, 2001; Koch and Horvath, 2014). The increases in hypothalamic AgRP and NPY signaling likely counteract with the appetite suppressive effect of CRH (Drescher et al., 1994; Pelleymounter et al., 2000) under chronic SC condition, result in the lack of change in food intake in SC mice. The increase in basal expression of *Npy* and *Agrp* despite dramatically elevated circulating leptin suggests an induction of leptin resistance in the SC paradigm. The robust reduction in *Sgk1* expression is intriguing. *Sgk1* is transcriptionally regulated by a number of hormones, mediators and stressors (Lang and Stournaras, 2013). *Sgk1* gene expressions in the hippocampus and cortex have been shown to be induced by various stressors such as restraint stress, water-immersion and transient ischemia (Nishida et al., 2004; Murata et al., 2005). However, the role of Sgk1 in the hypothalamus is poorly understood. One study showed an increase in hypothalamic *Sgk1* mRNA level following fasting (Nonogaki et al., 2006), although the physiological consequence of this increase was not explored. A follow-up study showed that hypothalamic *Sgk1* mRNA is induced by acute isolation stress and likely contributes to the isolation-induced body weight reduction in response to fasting (Kaji and Nonogaki, 2010). The role of hypothalamic Sgk1 in energy homeostasis and metabolism, particularly in response to stress, is an interesting direction for further study.

In the retroperitoneal white adipose tissue, chronic SC led to increases in *Ucp2* and *Lep*, and decreases in *Adrb1* and *Fasn* mRNA expression. Increases in *Lep* mRNA expression have been reported in animal models of obesity (Lopez et al., 2003; Sato et al., 2010). Our study shows that adipocyte leptin expression is transcriptionally modulated by social crowding stress and contributes to the elevated circulating leptin. Higher leptin level might account for the increase in *Ucp2* expression. Unlike Ucp1, Ucp2 is widely expressed in many tissues, including WAT (Lang and Stournaras, 2013). *Ucp2* gene expression was highly increased in the epididymal, retroperitoneal, and subcutaneous fat tissue of hyperleptinemic Zucker rats (Zhou et al., 1997). In another study, exogenous leptin administration for 1 week led to an increase in *Ucp2* expression in the WATe but not in WATr (Scarpace et al., 1998). Ucp2 is upregulated by free fatty acids and is suggested to be involved in fatty-acid metabolism and may be an adaptive response to protect against oxidative damage caused by excessive fatty acid peroxidation (Reilly and Thompson, 2000; Nedergaard and Cannon, 2003; Brand and Esteves, 2005; Souza et al., 2011). The reduced expressions of both *Adrb1* and *Fasn* in

WATr suggest that the enlarged adipose mass was due to the reduced lipolysis through β1 adrenergic receptor suppression rather than an increase in lipogenesis mediated by the lipogenic enzyme fatty acid synthase. β1-adrenergic receptors have been shown to play an important role in catecholamine-induced lipolysis as specific Adrb1 antagonists markedly inhibit this response (Germack et al., 1997; Louis et al., 2000). Moreover, transgenic mice overexpressing *Adrb1* in adipose tissue exhibit increased lipolytic rate and are partially resistant to diet-induced obesity (Soloveva et al., 1997). On the other hand, increased expression of *Adrb1* mRNA and protein were detected in the WAT of patients with cancer cachexia, a condition exhibiting enhanced lipolysis (Cao et al., 2010a).

The lack of changes in metabolic parameters measured by the metabolic chamber, including food intake, energy expenditure, RER and activity, together with the specific gene expression changes in WAT suggest that the increase in adiposity may due to a local rather than systemic change in metabolism. This could also account for the increase in adiposity without observable increase in body weight since a significant change in energy intake or expenditure would more likely lead to a more significant change in body weight. The increase in adiposity without body weight gain following 2 months of social crowding stress indicates a body composition change. Future studies using MRI or DEXA can determine if there is a decrease in lean or skeletal mass that accompanies the gain in adipose tissue mass.

In summary, our study described a simple mild chronic stress model that induces a moderate anxiety phenotype and a robust change in body composition with marked increase in adiposity. The social crowding model offers several advantages over other stress models. Compared to social isolation, it is more cost-effective due to reduced housing cost. It is also less labor intensive as no daily application of stressors is required as for the social defeat or chronic mild stress models. Although the social defeat model has been shown to elicit changes within days (Lutter et al., 2008; Chuang et al., 2010; Patterson et al., 2013), the SC paradigm may be a better mild ‘chronic’ stress model since the durations of the other stress models were more subchronic compared to the SC paradigm. The SC model thus represents a good alternative for the study of metabolic process and stress-induced metabolic dysfunctions.

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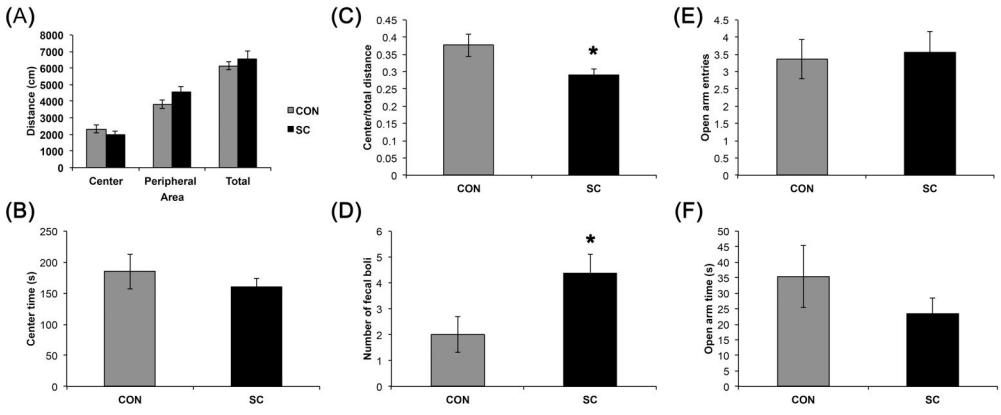

Figure 1.

SC mice exhibited a modest increase in anxiety-like behaviors in the open field test but not in the elevated T-maze. (A) Exploration as assessed by total distance traveled in the open field was comparable between the two groups. Distances traveled in the center or the periphery were not significantly different between the groups. (B) The time spent in the center of the open field was similar between SC and control groups. (C) SC mice had a reduced center/total distance ratio and (D) an increased defecation compared to the control group. No significant differences in open arm entries (E) or open arm times (F) were observed in the SC mice. Data shown are mean ± S. E. M. (n = 11 for control, n = 16 for SC). * $P < 0.05$.

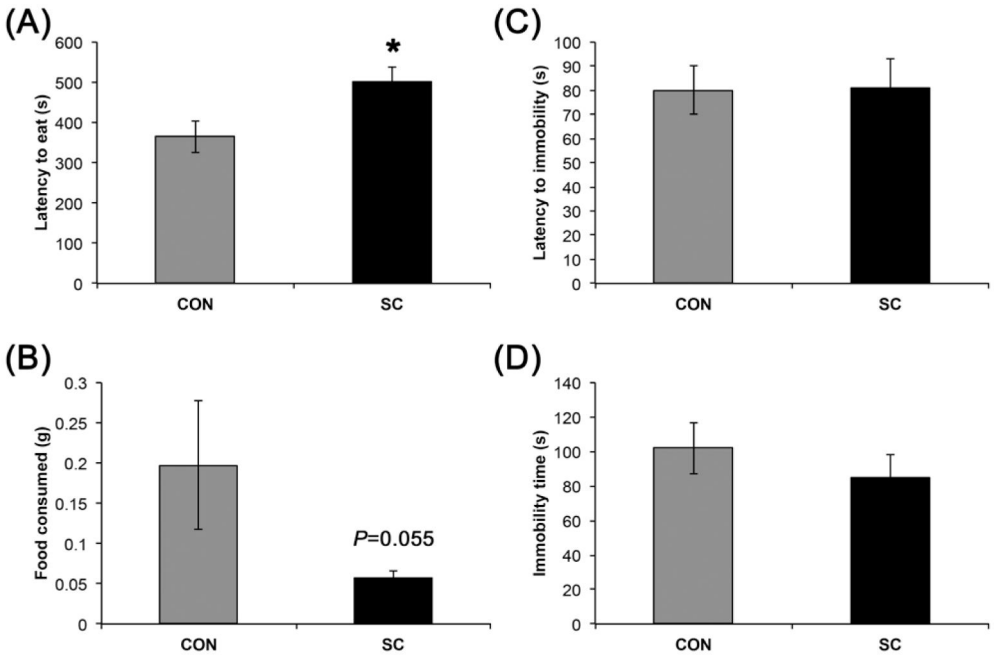

Figure 2.

SC mice exhibited anxiety-like behavior in the novelty suppressed feeding test but not depressive-like behavior in the forced swim test. (A) SC mice had increased latency to eat in a novel environment after overnight fasting. (B) SC mice ate less in 5 minutes after the start of food consumption in the NSF. (C and D) Depressive-like behavior as indicated by immobility in the FST was not affected by SC. Data shown are mean ± S. E. M. (n = 12 for control, n = 16 for SC). * $P < 0.05$.

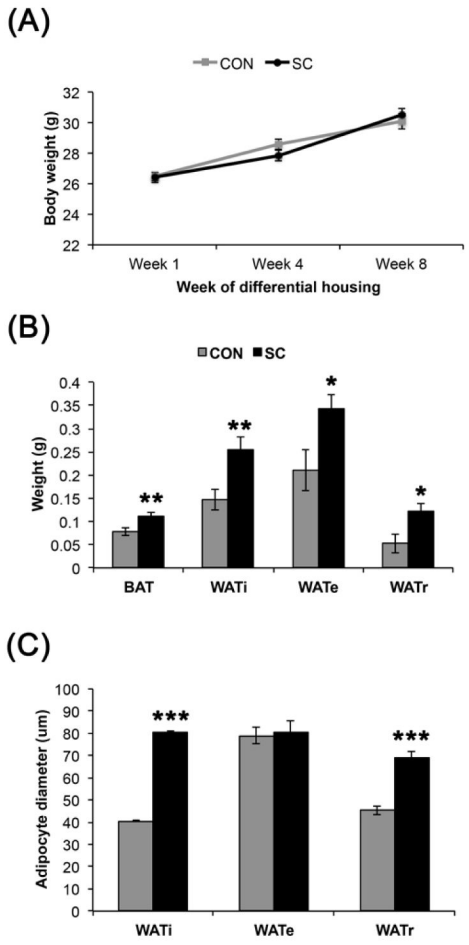

Figure 3.

SC increased adiposity without significant impact on body weight. (A) Body weight was comparable between the SC and control groups. (B) All fat depots assessed were markedly larger in the SC mice compared to the controls. (C) The WATi and WATr adipocytes were significantly larger in SC as compared to control group. Data shown are mean ± S. E. M. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. For (A), n = 12 for control, n = 16 for SC. For (B), n = 10 per group. For (C), n =3–4 per group, 2 sections for each WAT depot.

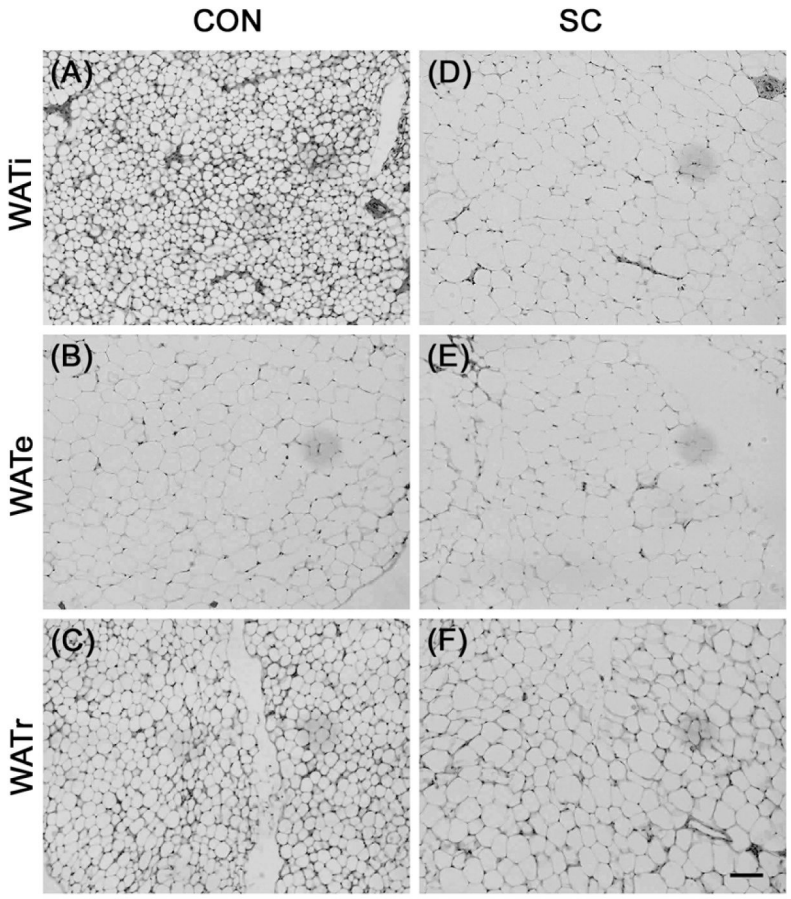

Figure 4.

Representative sections of H&E stained adipose tissue showing WATi and WATr adipocytes were enlarged in SC mice (D and F, respectively) compared to control C57BL/6 mice (A and C, respectively). In contrast, WATe adipocytes were comparable in size between the two groups (B and E). Scale bar = 100 μm.

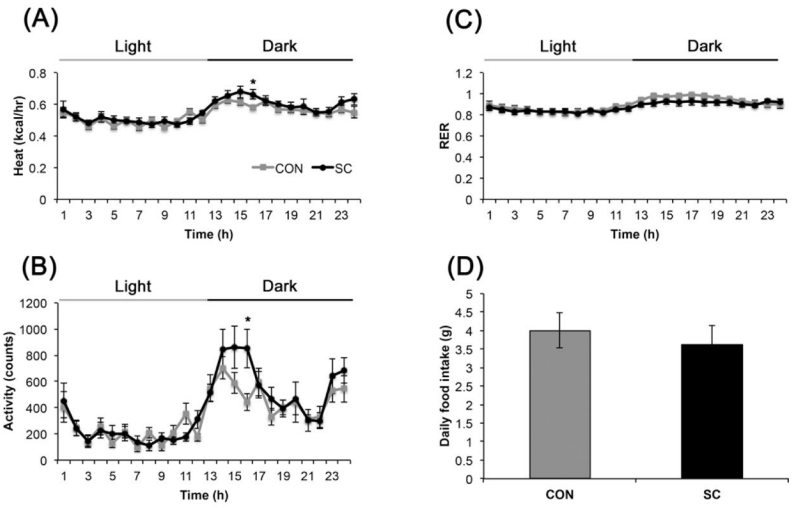

Figure 5.

Effect of eight weeks of SC on (**A**) energy expenditure, (**B**) physical activity (**C**) respiratory exchange ratio and (**D**) food intake (n = 8 per group). Data shown are mean ± S. E. M. * $P < 0.05$.

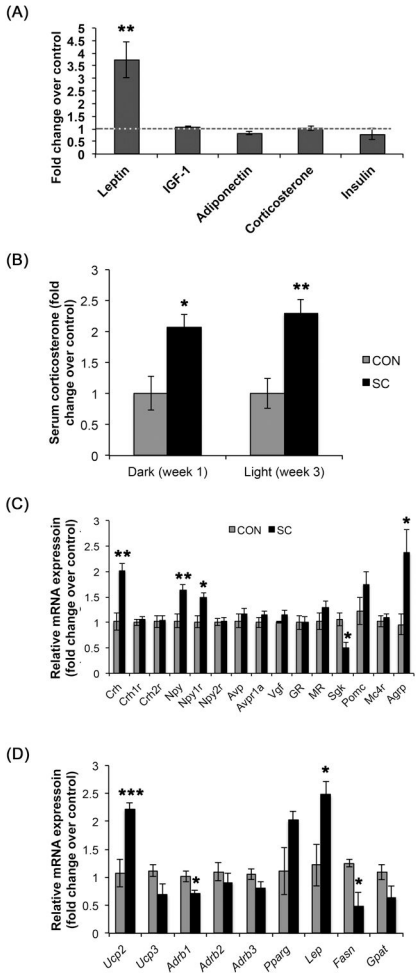

Figure 6.

Nine weeks of SC altered (A) serum profile, (C) hypothalamic and (D) WAT gene expression profile. (B) Serum corticosterone was elevated in the SC mice at 1 and 3 weeks post SC initiation during the dark and light phase, respectively. Data shown are mean ± S. E. M. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. For (A), n = 12 for control, n = 16 for SC. For (B), n = 8 per group. For (C), n = 4 – 6 per group, data were derived from averages of duplicate or triplicate reactions. For (D), n = 11 for control and n = 12 for SC.

Table 1

Primer sequences.

Gene	Primer	Primer sequence
Actb	Forward	ACCCGCGAGCACAGCTT
	Reverse	ATATCGTCATCCATGGCGAACT
Crh	Forward	TGGCCCCAAGGAGGAAA
	Reverse	CCACTGCAGCTCCAAATAAAAA
Crh1r	Forward	TCCGCTACAACACCACAAACA
	Reverse	TCCTGGCACTCAGAATAATTCACA
Crh2r	Forward	CCGAGTACTTCAATGGCATCAA
	Reverse	CCCGTTCTCCAGGCACTCT
Npy	Forward	CTCCGCTCTGCGACACTACA
	Reverse	AGTGTCTCAGGGCTGGATCTCT
Npy1r	Forward	GCATATGACAAAGAGTTTTACATTGTGTT
	Reverse	GGTGGTGACTGCTTTTGAAATGA
Npy2r	Forward	TTGCTTGAAATTCCTGGATTCC
	Reverse	CCAGTTCACTCTCACTTGGCTGTA
Vgf	Forward	GGGCGCCCCGATGT
	Reverse	TCAGCTACCTGCCCATTATGC
Avp	Forward	CGCTCTCCGCTTGTTTCCT
	Reverse	TGGGCAGTTCTGGAAGTAGCA
Avpr1a	Forward	GTTTGGACCGATTCCGAAAA
	Reverse	CAGCTGTTCAAGGAAGCCAGTA
Sgk1	Forward	CCCTCTCCTCCGCCAAGT
	Reverse	TTGGCGTGAGGGTTGGA
Nr3c1	Forward	CAGCATGCCGCTATCGAAA
	Reverse	CGCGGCAGGAACTATTGTTTT
Nr3c2	Forward	TGTCTCAGACCTTGGAGCGTTC
	Reverse	TTGTTCGGAGTAGCACCGGAA
Ucp1	Forward	CGATGTCCATGTACACCAAGGA
	Reverse	CCCGAGTCGCAGAAAAGAAG
Ucp2	Forward	TCATCACTTTCCCTCTGGATACC
	Reverse	GCGCACTAGCCCTTGACTCT
Ucp3	Forward	AACGCTCCCCTAGGCAGGTA
	Reverse	GTCCCTCCTGAGCCACCAT
Adrb1	Forward	GGACTTCGGTAGATGTGCTGTGT
	Reverse	CGGTCCAGGGCGATGAC
Adrb2	Forward	CCTTCGCAGGTCTTCTTCGA
	Reverse	GTCCGTTCTGCCGTTGCTA
Adrb3	Forward	GGACGCTGTTCCTTTAAAAGCA

Gene	Primer	Primer sequence
	Reverse	TCCATCTCACCCCCCATGT
Lep	Forward	ATTTCACACACGCAGTCGGTAT
	Reverse	AGCCCAGGAATGAAGTCCAA
Pparg	Forward	GTGCCAGTTTCGATCCGTAGA
	Reverse	GGCCAGCATCGTGTAGATGA
Fasn	Forward	GATCCTGGAACGAGAACACGAT
	Reverse	TGTCAGTAGCCGAGTCAGTCTTG
Gpat	Forward	CAACACCATCCCCGACATC
	Reverse	TGACCTTCGATTATGCGATCAT
Pomc	Forward	GGCCTTTCCCCTAGAGTTCAA
	Reverse	GGACCTGCTCCAAGCCTAATG
Agrp	Forward	GCGGAGGTGCTAGATCCA
	Reverse	AGGACTCGTGCAGCCTTA
Mc4r	Forward	CACTGTGTCAGGCGTCCTCTT
	Reverse	ATGGAAATGAGGCAGATGATGA