

Comparison of Gut Tight Junction Gene Expression in C57BL/6J and BALB/c Mice After Chronic Social Defeat Stress

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Abstract

Chronic social defeat stress (CSDS), which subjects a male mouse to repeated social subordination by an aggressor male mouse, results in the onset of depression-like behaviors. CSDS in rodents is a useful model for studying the mechanisms that underlie anxiety and depression. We investigated the effect of CSDS on expression of tight junction (TJ) components in colon in C57BL/6J and BALB/c mice to address the correlation of CSDS and the development of inflammatory intestinal disease associated with epithelial barrier dysfunction. After 10 consecutive days of CSDS, BALB/c mice displayed highly social aversive behavior compared to C57BL/6J mice, which was accompanied by the suppression of intestinal Claudin-1 expression. These observations suggest that increased susceptibility to CSDS in BALB/c mice was caused by the downregulation of Claudin-1. The CSDS model with BALB/c mice is a potentially useful system for evaluating food components for psychological stress control.

Discipline: Food

Additional key words: claudin, elevated plus-maze test, social interaction test

Introduction

Psychological stress is associated with the initiation of an intestinal inflammatory response, which can lead to abnormal gastrointestinal function and increased risk of chronic intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)

(Camilleri et al. 2012a, Camilleri et al. 2012b). Several studies have demonstrated that the exposure of animals to various stressors can affect the abnormal gastrointestinal function. For example, acute restraint stress (Ait-Belgnaoui et al. 2005), early life stress (Moussaoui et al. 2014) and water avoidance stress (Santos et al. 2000) can cause the breakdown of intestinal barrier function.

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Impaired colonic epithelial barrier integrity often results in the onset of inflammatory intestinal disorders. To address the correlation of psychological stress and the onset of inflammatory intestinal disease, we have investigated the effect of psychological stress on expression of tight junction (TJ) components in colon.

During intestinal inflammation, intestinal barrier function is controlled mainly by interactions between TJ proteins that connect adjacent epithelial cells (Camilleri et al. 2012a). The TJ consists of several families of proteins, including the transmembrane proteins Claudins and occludin and the cytoplasmic scaffolding protein zonula occludens (ZO) that links Claudins and occludin to the actin cytoskeleton. A decrease in Claudins expression was reported in IBS patients that displayed an increase in intestinal permeability (Ivanov et al. 2004).

We have utilized a rodent chronic social defeat stress (CSDS) model to investigate the effects of psychological stress on gut function and the metabolites (Goto et al. 2015, Aoki-Yoshida et al. 2016). CSDS, which subjects a male mouse to repeated social subordination by an aggressor male mouse, results in the onset of depression-like behaviors such as social avoidance and a decreased preference for sucrose. The majority of these behaviors can be reversed by chronic antidepressant treatment (Berton et al. 2006). Thus, CSDS in rodents is an effective animal model of depression that provides valuable insights into the biology of chronic stress in humans.

Strain-specific characters which provide a basis for behavior might affect the expression of TJ proteins when subjected to CSDS. Krishnan et al. (2007) investigated that all BALB/c mice exposed to CSDS showed a susceptible phenotype displaying social avoidance, whereas C57BL/6J mice exposed to CSDS were divided into two phenotypes, susceptible and unsusceptible. Unsusceptible/resilient mice display social interaction (SI) behavior similar to unstressed mice regardless of exposure to CSDS. Brinks et al. (2007) observed naïve BALB/c and C57BL/6J mice spent more time in the closed arm compared to BALB/c mice in the elevated plus-maze test, suggesting their strain-specific characters.

To address the correlation of psychological stress and onset of inflammatory intestinal disease, we used two inbred mouse strains, BALB/c and C57BL/6J to investigate the effect of psychological stress on the expression of TJ components in the colon.

Materials and Methods

1. Social defeat stress

Intruder male BALB/c and C57BL/6J mice, both at 8 weeks old, and resident male ICR mice at 5 months old

were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were singly housed with ad libitum access to AIN93G standard chow and fresh water. All the animal procedures were carried out in accordance with the animal experimentation guidelines of the National Agriculture and Food Research Organization (NARO Ibaraki, Japan). A previously reported method was used to subject the mice to CSDS (Golden et al. 2011). In brief, a test mouse was allowed to physically interact with a different aggressor ICR mouse for 10 min. After the interaction, the mice were housed in the same cage, separated by a plastic perforated divider that enabled the transmission of visual and olfactory cues for 24 h (Fig. 1A). If the stressed mouse was excessively wounded, the duration of the physical interaction was reduced to 5 min. The mice were subjected to CSDS for 10 consecutive days. The control mice were housed two per cage, one on each side of the divider. All the mice were individually housed after the

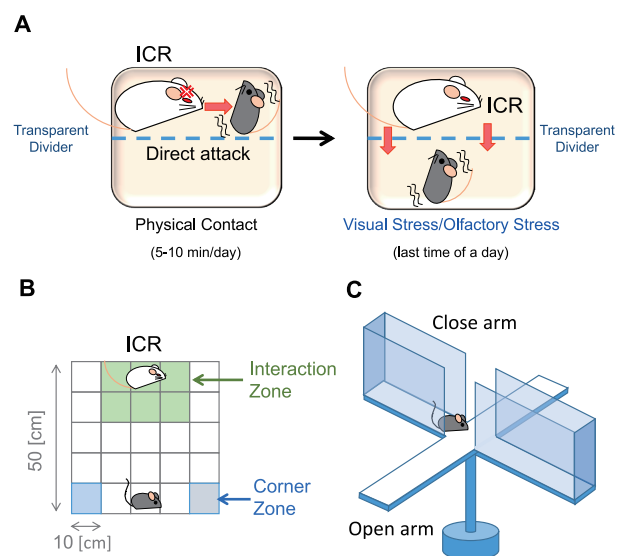


Fig. 1. Experimental design of chronic social defeat stress (CSDS) using the resident intruder paradigm.

(A) A test mouse (intruder) was allowed to physically interact with an ICR mouse (resident) for 10 min. After the interaction, the mice were housed in the same cage, separated by a plastic perforated divider that enabled the transmission of visual and olfactory cues for 24 h. (B) A schematic diagram of open field for social interaction test. The social target was represented as a white mouse (ICR). The experimental animal (C57BL/6J or BALB/c mouse) was defined as a black mouse. (C) An elevated plus maze apparatus consists of two opposite open arms without wall, crossed with two closed arms surrounded by walls.

last course of CSDS.

2. Behavior analysis

(1) Social interaction (SI) test

An SI test was performed 11 days after the initiation of CSDS. A mouse ($n = 6$ per group) was placed in an open field ($50 \text{ cm} \times 50 \text{ cm}$) in a small animal cage and their movements were tracked for 2.5 min in the absence of an aggressor ICR mouse, followed by 2.5 min in the presence of an aggressor ICR mouse (Fig. 1B). The time spent in the interaction zone was measured by video tracking software (O'Hara & Co., Ltd., Tokyo, Japan). The SI scores (%) were estimated as $100 \times (\text{duration of time spent in interaction zone with the ICR mouse present} / \text{duration of time spent in interaction zone with the ICR mouse absent})$.

(2) Elevated plus maze test

The mice were placed at the center of an elevated plus-maze (25 cm [arm length] \times 5 cm [arm width] \times 20 cm [wall height]) under dim lighting and their behavior was videotaped for 10 min (Fig. 1C). The time spent in the closed and open arm, as well as the number of explorations of the open and closed arms, was determined by video tracking software (O'Hara & Co., Ltd.). Student's *t*-test was used to determine the statistical difference between the two groups. The significance level was set at $p < 0.05$. All the data are expressed as the mean \pm SD.

(3) Quantitative real-time PCR

The mRNA levels of Claudins (*Cldn1*, *Cldn3*, *Cldn4*, and *Cldn7*) and zonula occludens-1 (*Tjp1*) were measured by quantitative real-time polymerase chain reaction (qPCR). After the mice were euthanized, a 1-cm segment of the proximal colon was dissected and stored in RNAlater™ (QIAGEN, Valencia, CA, USA). The total RNA was extracted with an RNeasy® Mini Kit (QIAGEN) following the manufacturer's instructions. The ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan) was used to synthesize cDNA from the total RNA, and the THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) and the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) were used to perform the qPCR. The primer sequences were as follows: 5'-cgggcagatacagtcaaaag-3' (forward) and 5'-acttcacgcaatggtggac-3' (reverse) for *Cldn1*, 5'-aagccgaatggacaaagaa-3' (forward) and 5'-ctggcaagtactgcagtg-3' (reverse) for *Cldn3*, 5'-cgctactcttgccattacg-3' (forward) and 5'-actcagcacaccatgacttg-3' (reverse) for *Cldn4*, 5'-agggtctgctctggtcctt-3' (forward) and 5'-gtacgcagctttgcttca-3' (reverse) for *Cldn7*, 5'-tggaacagcacacagtgc-3' (forward) and 5'-gctggcctcctttaaacac-3' (reverse) for *Tjp1* and

5'-gatgctccccgggctgtatt-3' (forward) and 5'-ggggctacttcagggtcagga-3' (reverse) for β -actin (*Actb*). The relative amount of each transcript was normalized to the relative amount of *Actb* in the same cDNA.

(4) Western blotting analysis

Western blotting analysis was performed to measure the expression levels of tight junction proteins in BALB/c mice that experienced CSDS for 10 days. A segment of proximal colon was lysed with RIPA buffer (Nacalai Tesque, Kyoto, Japan), and was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and was electrically transferred to a PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK). Subsequently, the membrane was treated with blocking reagent (Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk) for 2 h at room temperature. Polyclonal antibodies against Claudin-1, -3 (Thermo Fisher Scientific, Waltham, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz, Dallas TX, USA) were used for primary antibody and further reacted with a secondary antibody conjugated with horseradish peroxidase. The membrane was treated with an ECL Prime Western blotting detection reagents (GE Healthcare Life Sciences) and the chemiluminescent signals were detected by exposure to X-ray film (Kodak/Carestream Health, Rochester, NY, USA).

Results

Two mouse strains, BALB/c and C57BL/6J were used in this study. C57BL/6J mice were selected because they are widely used in studies of CSDS, whereas BALB/c mice were used because they exhibit increased social avoidance behavior compared to C57BL/6 mice (Golden et al. 2011, Razzoli et al. 2011). The exposure of BALB/c mice to CSDS resulted in a reduction of the SI score, indicating an aversion to SI (Fig. 2A). However, several C57BL/6J mice were resilient to CSDS and did not develop obvious social avoidance (Fig. 2A)

To examine the effects of CSDS on anxiety-like behavior in mice, an elevated plus-maze test was conducted 12 days after the initiation of CSDS (Fig. 2B). Compared with control mice, stressed C57BL/6J mice spent more time in the closed arms of the elevated plus-maze (Fig. 2B). By contrast, there was no significant difference between control and stressed BALB/c mice in the time spent in open and closed arms (Fig. 2B).

In the stressed BALB/c mice, the mRNA levels of *Cldn1*, *Cldn3*, and *Cldn7* decreased by 40%, 68% and 70%, respectively, compared with the control (Fig. 3A). The *Cldn4* mRNA level increased by CSDS. By contrast, CSDS did not affect the expression levels of Claudins and

ZO-1 in the proximal colon of C57BL/6J mice (Fig. 3B). CSDS decreased Claudin-1 protein level in BALB/c mice (Fig. 3C), consistent with mRNA expression. However, Claudin-3 protein level was not affected by CSDS.

Discussion

Our results show that the exposure of two mouse strains to CSDS resulted in strain-specific behavioral responses (Fig. 2). The results of SI test (Fig. 2A) and elevated plus maze test (Fig. 2B) agree with the previous

results (Krishnan et al. 2007, Brinks et al. 2007). Compared with the C57BL/6J mice, the BALB/c mice showed more movement in the open arms even in unstressed condition, suggesting that an anxious phenotype of C57BL/6J mice might be due to their nature of decreased exploration in a new environment.

Stressed BALB/c mice displayed higher social aversive behavior than the C57BL/6J mice did, which was accompanied by the suppression of intestinal *Cldn1*, *Cldn3*, and *Cldn7* mRNA expression. The contribution of Claudin-1 on permeability was well-defined. Claudin-1 knockout mice die due to a loss of fluid and electrolytes through the impaired epidermal barrier (Fukase et al. 2002). An increased intestinal permeability with a reduced expression of Claudin-1 was observed in IBS patients (Bertiaux-Vandaële et al. 2011). Although the Claudin-1 protein level decreased in the small bowel of IBS patients, the results are conflicting for Claudin-3 and -4 (Ivanov et al. 2004, Lu et al. 2013), suggesting that the increased susceptibility to CSDS in BALB/c mice was caused by the downregulation of Claudin-1.

Regarding the mechanism responsible for the stress-induced alteration of TJ components, several studies have reported differences in the composition of intestinal microbiota between IBS patients and healthy individuals (Codling et al. 2010, Carroll et al. 2011, Durbán et al. 2012). Different microbes can directly regulate TJ protein expression and/or localization in *in vitro* and *in vivo* models (Camilleri et al. 2012a). For example, the abundance of *Oscillibacter* moderately increases in the feces of stressed mice compared with the control (Aoki-Yoshida et al. 2016). *Oscillibacter* is an intestinal commensal bacterium, and its abundance correlates with increased intestinal permeability and decreased TJ protein levels (Lam et al. 2012). Despite the fact that these observations were obtained from C57BL/6J mice, we speculate that probiotics or food components that could affect the microbiota lead to an increase in TJ proteins in BALB/c mice as well as C57BL/6J mice. Beneficial effects of probiotics on gut integrity has been reported in BALB/c mice (Moratalla et al. 2014, Yang et al. 2009). Therefore, the composition of the gut microbiota may underlie the changes in intestinal epithelial TJ expression by CSDS. Additional studies are required to determine the role of microbiota and their metabolites on the susceptibility of mice to CSDS.

There is growing evidence that the bidirectional relationships between the gastrointestinal tract and the brain, called brain-gut-microbiota interaction, plays an important role in both host physiological function and behavior. The efficacy of food components and gut microbiota on intestinal physiological functions is

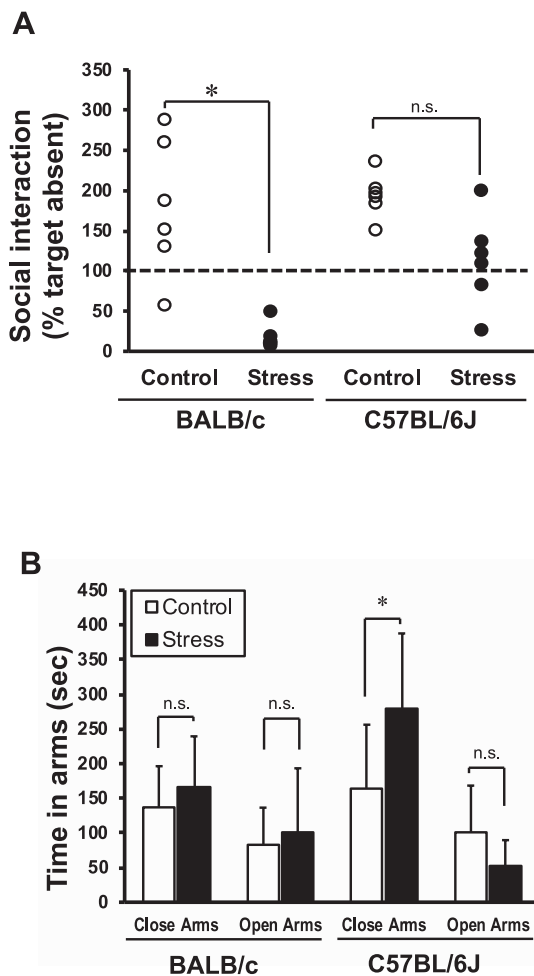


Fig. 2. Effects of chronic social defeat stress (CSDS) on social interaction and anxiety-like behaviors.

(A) Social interaction score of BALB/c and C57BL/6J mice that underwent CSDS (n = 6 per group). Social interaction scores (%) were estimated as 100 × (duration of time spent in interaction zone with the ICR mouse present / duration of time spent in interaction zone with the ICR mouse absent). (B) Effects of CSDS on anxiety-like behavior in the elevated plus-maze in C57BL/6J and BALB/c mice (n = 6 per group). All values are means ± SD (n = 6 per group), *p < 0.05 vs. the control, n.s.: not significant.

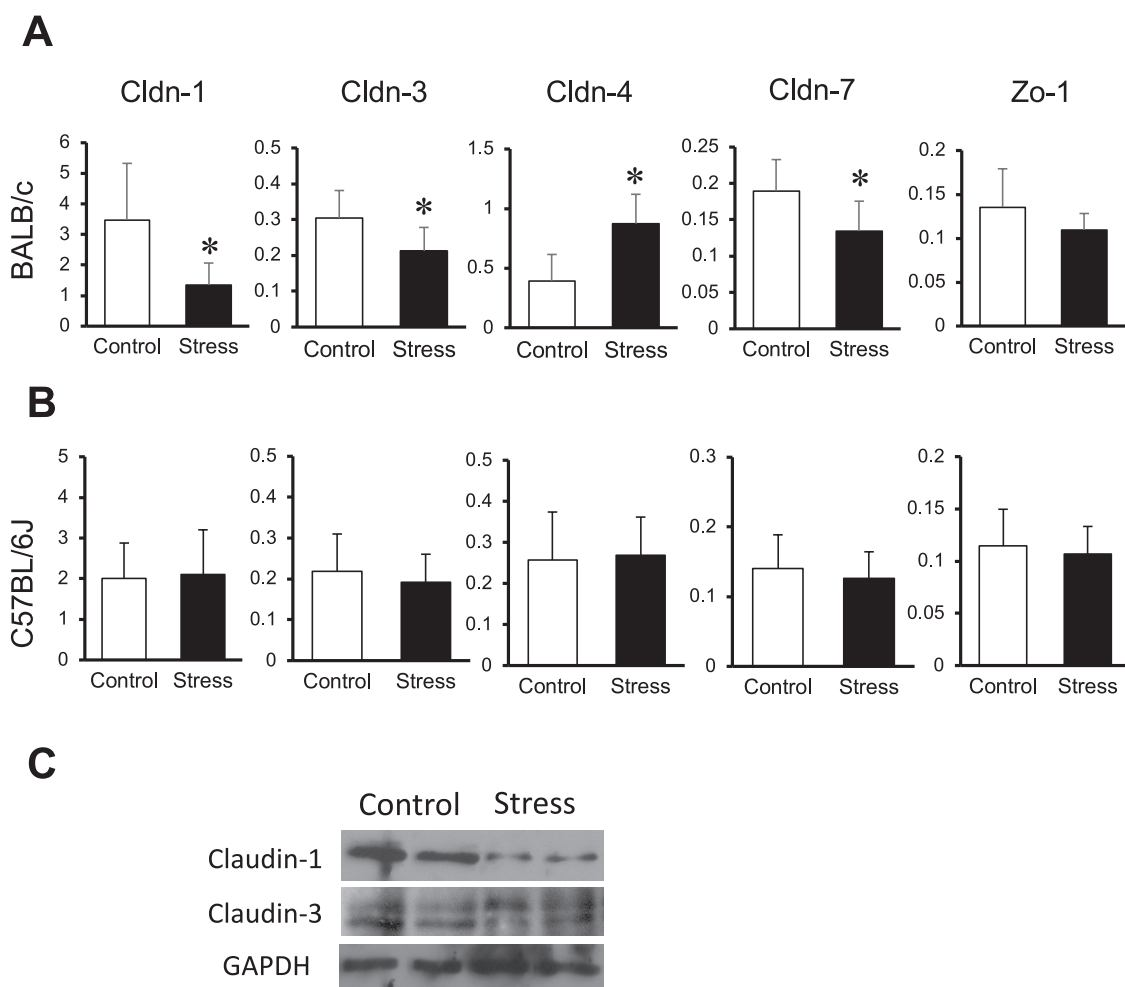


Fig. 3. Effects of CSDS on the expression of intestinal tight junction components in the colon.

The relative mRNA levels of Claudins (*Cldn1*, *Cldn3*, *Cldn4*, and *Cldn7*) and zonula occludens-1 (*Tjp1*) were determined by qPCR after normalization to the β -actin mRNA level in the colon of BALB/c (A) and C57BL/6J mice (B). All values are means \pm SD ($n = 6$ per group), * $p < 0.05$ vs. the control. (C) The expression levels of Claudin-1 and Claudin-3 were measured by Western blotting in BALB/c mice.

well-known (Ulluwishewa et al. 2011, Suzuki 2013). Therefore, there is considerable potential for food components to ameliorate the biological response to psychological stress by using brain-gut-microbiota interaction. Despite the fact that the strain-specific differences of microbiota profiles could be affected to psychological stress response, the CSDS model with BALB/c mice is a potentially useful system for evaluating functional foods for psychological stress control.

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