

## Original article

**Effect of social isolation stress on saliva BDNF in rat**Yusuke Nakagawa<sup>1)</sup>, Masahiro To<sup>1)</sup>, Juri Saruta<sup>1)</sup>, Yuko Yamamoto<sup>2)</sup>, Toshiharu Yamamoto<sup>1)</sup>, Tomoko Shimizu<sup>3)</sup>, Yohei Kamata<sup>3)</sup>, Masato Matsuo<sup>1)</sup>, and Keiichi Tsukinoki<sup>1)</sup><sup>1)</sup>Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan<sup>2)</sup>Department of Dental Hygiene, Junior College, Kanagawa Dental University, Yokosuka, Japan<sup>3)</sup>Department of Highly Advanced Stomatology, Graduate School of Dentistry, Kanagawa Dental University, Yokohama, Japan

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**Abstract:** Salivary glands produce various compounds, including brain-derived neurotrophic factor (BDNF), which serve as biomarkers of stress-related disorders. Social isolation-induced stress models a form of chronic mild stress that induces neurodegenerative changes in the brain and behavioral alterations. This study employed a rat model to determine whether social isolation stress affects BDNF levels in saliva. Male Sprague-Dawley rats were randomly allocated to social isolation stress (1 animal/cage) or control (3-4 animals/cage) groups and reared for 8 weeks. The concentration of BDNF was quantified in specific brain regions, blood, and saliva using enzyme-linked immunosorbent assay (ELISA). The levels of expression of *Bdnf* and tyrosine kinase B (*TrkB*) mRNA were quantified using reverse transcriptase-polymerase chain reaction. Behavioral alterations were analyzed using the open-field and elevated plus maze assays. The BDNF concentration was lower in the hippocampus, prefrontal cortex, blood, and saliva of the stress group than in those of the controls. *Trkb* expression in the hippocampus and prefrontal cortex was decreased by social isolation stress. Moreover, the social isolation stress group showed behavioral deficits in both tests. In conclusion, these findings indicate that social isolation stress may reduce the expression of BDNF protein in blood and saliva, thus providing a potentially valuable biomarker for diagnosis of stress-related disorders.

Keywords; BDNF, *TrkB*, social isolation stress, hippocampus, saliva, biomarker

**Introduction**

Salivary glands produce various compounds, such as growth and antimicrobial factors, and are involved in oral health maintenance [1]. They work as an endocrine and exocrine organ because some of the secreted products are transferred into the bloodstream via endocrine mechanisms [2]. Neurotrophins, including nerve growth factor and brain-derived neurotrophic factor (BDNF), are secreted by salivary glands and can exert hormone-like effects on organs distant from the salivary glands, such as the brain [3] and adrenal gland [4]. Importantly, the salivary glands are sensitive to stress [5], and saliva may contain biomarkers associated with stress-related disease [6].

Stress is an important risk factor for various neuropsychiatric disorders, including depression and schizophrenia. Several brain areas, such as the hippocampus, amygdala, and prefrontal cortex, are implicated in stress-induced cognitive and affective dysfunction [7,8]. Acute stress is strongly associated with the onset of depression via impairment of neural plasticity [9]. Moreover, chronic stress induces neurochemical alterations and depression-like behavior [10]. Social isolation stress, which models a form of chronic mild stress, induces neurodegenerative changes in the brain [8] and behavioral alterations [11]. Depriving rats of social contact from post-natal day 21 to 42 induces anxiety-like and fear behavior in adulthood [12]. Similarly, social isolation during early life in humans contributes to the occurrence of schizophrenia [13].

Animal models of social isolation stress are frequently used for investigating the mechanisms underlying neuropsychiatric disorders [13]. Socially isolated model rats show behavioral deficits in various tests, including the Morris water maze [11], open-field (OF) [14], and elevated plus maze (EPM) [15]. Additionally, changes in blood metabolites have been observed in response to social isolation stress [8]. A previous study has reported that compounds found in saliva are related to brain function by affecting metabolites in the hippocampus [3]. However, the relationship between social isolation stress and compounds present in saliva remains unclear.

Many studies have focused on BDNF as a biomarker for stress-related disorders [10]. BDNF is produced by the salivary glands, blood, and amygdala [7,16] in young rats in response to acute stress. However, chronic stress can reduce the expression of BDNF in the amygdala, hippocampus, and cingulate cortex [17]. In addition, social isolation-induced stress decreases BDNF in the hippocampus [11,18] and prefrontal cortex [19]. Patients with depression show reduced levels of BDNF in the hippocampus [20]. Similarly, tyrosine kinase B (*TrkB*), which is the BDNF receptor, is also reduced in the hippocampus in schizophrenia [21]. Additionally, model rats with mild chronic stress show a reduction in the concentration of serum BDNF [14], and stressful life events can reduce the serum concentration of BDNF in humans [22]. It is well established that saliva is an extremely useful diagnostic source for several diseases in humans, and could eventually replace blood sampling [23,24]. However, changes in the concentration of BDNF in saliva resulting from chronic mild stress remain to be investigated.

The aim of the present study was to determine whether social isolation stress affects the concentration of BDNF in saliva, employing a rat model. Firstly, to investigate stress-induced brain dysfunction in this model, behavioral changes and alterations of BDNF and *TrkB* expression in the hippocampus, prefrontal cortex, and amygdala were determined. Subsequently, alterations in the concentration of BDNF in blood and saliva were then studied.

**Materials and Methods****Animals**

Male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) were obtained at post-natal day 21. They were kept under controlled conditions (22 ± 3°C; 12-h light/dark cycle, lights on at 7 a.m.) and had free access to water and food.

All experiments were approved by the Ethics Committee for Animal Experiments of Kanagawa Dental University (approval number 18-004) and performed in accordance with the Guidelines for Animal Experimentation of Kanagawa Dental University and the ARRIVE guidelines for reporting animal research.

**Experimental design**

Rats were randomized into social isolation stress or control groups [11]. Rats in the social isolation group were reared in isolation (1 animal/cage) and rats in the control group were reared with littermates (3-4 animals/cage) for 8 weeks (cage dimensions, 260 × 380 × 180 mm) [8]. Body weight was measured at frequent intervals during the study (start of rearing, 0 weeks; reared for 4 weeks; reared for 8 weeks). After 8 weeks, rats were subjected to behavioral tests (day 77; 3-week-old rats were reared for 8 weeks).

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**Table 1** Differences in body weight and corticosterone between each group

|                        | Control group (n = 6) |      | Stress group (n = 6) |       | P value |
|------------------------|-----------------------|------|----------------------|-------|---------|
|                        | Mean                  | SE   | Mean                 | SE    |         |
| Body weight (g)        |                       |      |                      |       |         |
| 0 weeks                | 43.3                  | 1.6  | 44.1                 | 1.7   | >0.99   |
| 4 weeks                | 287.0                 | 3.7  | 281.3                | 6.2   | >0.99   |
| 8 weeks                | 494.1                 | 6.0  | 491.0                | 10.8  | >0.99   |
| Corticosterone (ng/mL) | 38.26                 | 0.07 | 96.74                | 12.13 | 0.015*  |

SE, standard error of the mean. \* $P < 0.05$

Saliva samples were collected under deep anesthesia (sodium pentobarbital, 50 mg/kg, intraperitoneal [i.p.] administration), following injection of pilocarpine-HCl (1 mg/kg, i.p.; Sanpilo 1%; Santen Pharmaceutical Co., Ltd., Osaka, Japan) [3]. Following this, the rats were euthanized. Blood (serum and plasma) and brain (hippocampus, prefrontal cortex, and amygdala) samples were collected, as described previously [25], then stored at  $-80^{\circ}\text{C}$  until further analysis.

### Open-field test

The OF tests were performed as described previously [3]. Briefly, the experimental space was surrounded by a black curtain, and illumination was provided by a fluorescent light (light intensity, 23–25 lux). All behavioral tests were performed between 3 and 7 p.m. The rats were introduced to the experimental room for habituation 1 week before the start of behavioral testing. The performance of the rats during behavioral testing was recorded and analyzed using TopScan (Clever Sys Inc., Reston, VA, USA). After each trial, the instruments were cleaned with 30% (v/v) ethanol solution. First, rats were assessed in the OF, then tested in the EPM 24 h later.

In the OF test, each rat was placed into the center of the OF arena (150 × 45 cm) and behavior was recorded for 10 min. Data were evaluated for each of the following parameters: distance walked, time of movement, and entries into the center.

### Elevated plus maze

The conditions for the EPM test were the same as those for the OP test. The EPM consisted of a plus-shaped maze elevated at a height of 50 cm from the floor with two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 45 cm), spreading out from a central platform (10 × 10 cm). Rats were placed into the center of the EPM, facing one of the open arms, and allowed to explore freely. Their behavior was recorded for 10 min. Data were evaluated for each of the following parameters: total time spent and number of entries in each arm, and total distance walked.

### Quantification of corticosterone and BDNF protein expression

For ELISA, RNA isolation, and immunoblotting, brain samples were processed as described previously [26]. Briefly, the samples were homogenized using a Cryo-press (Microtec Company Limited, Urayasu, Japan) and solubilized in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. After incubation on ice for 30 min, the suspensions were centrifuged (10,000 × g, 15 min, 4°C), and the supernatant was collected.

The concentration of corticosterone in blood serum was determined by ELISA (ADI-900-097, Enzo Life Sciences, Farmingdale, NY, USA), in accordance with the manufacturer's instructions. The serum samples were diluted (1:100) with sample buffer, and data were adjusted based on this dilution ratio after measurement.

The concentrations of BDNF in brain, plasma, and saliva samples were quantified by sandwich ELISA (CYT306, Merck Millipore, Billerica, MA, USA). BDNF standards and samples were incubated at 4°C overnight. Color development after incubation with horseradish peroxidase substrate was measured using an iMark Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

### Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) procedures were performed as described previously [3]. After homogenization, total RNA was isolated using the ISOGEN Kit (Nippon Gene, Tokyo, Japan). cDNA was synthesized using a first-strand cDNA synthesis kit (Sigma-Aldrich, St. Louis, MO, USA). RT-PCR was performed using Takara Ex-Taq (Takara Bio, Kusatsu, Japan). The primer sequences for the Bdnf,

Trkb, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; as a house-keeping gene) genes were designed as described previously [27]. Bdnf/Gapdh and Trkb/Gapdh ratios were quantified using the image analysis application, ImageJ (NIH, Bethesda, MD, USA).

### Statistical analysis

Data for the two groups were compared by Mann-Whitney *U* test using GraphPad Prism (v. 6.05.; GraphPad Software Inc., San Diego, CA, USA). Differences at  $P < 0.05$  were considered to be statistically significant.

## Results

### Body weight and serum corticosterone

There were no differences in mean body weight between the groups at any time point (mean final body weight, control: 494.1 ± 6.0 g; stress: 491.0 ± 10.8 g; Table 1). To confirm the presence of social isolation-induced stress, the concentration of corticosterone in blood serum was measured. The level of corticosterone was significantly elevated in the stress group (96.74 ± 12.13 ng/mL) relative to the control group (38.26 ± 0.07 ng/mL,  $P < 0.05$ ; Table 1), indicating that social isolation stress increased the production of corticosterone without affecting body weight.

### Behavioral changes induced by social isolation stress

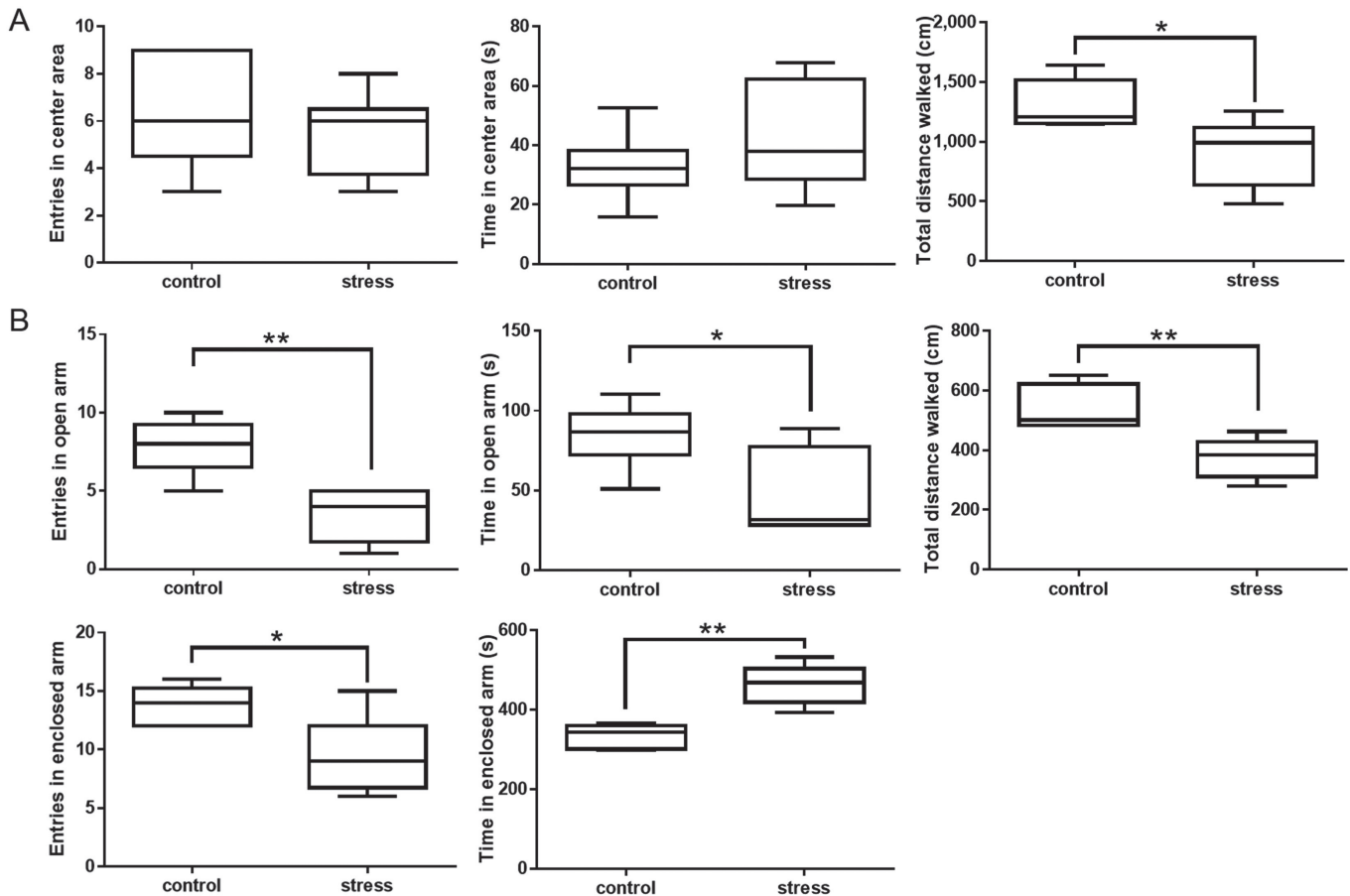
To evaluate the effect of social isolation stress on behavior, rats were assessed using the OF and EPM tests. Total distance walked in both tests was significantly less in the stress group than in the control group ( $P < 0.05$ ; Fig. 1A, B). In the OF test, there was no inter-group difference in the number of entries and time spent in the center of the chamber (Fig. 1A). However, in comparison with the control group, rats in the stress group had significantly fewer entries and spent less time in the open compartments ( $P < 0.05$ ; Fig. 1B). Interestingly, rats in the stress group had significantly fewer entries into the enclosed compartment ( $P < 0.05$ ), but spent significantly longer in this compartment than the rats in the control group ( $P < 0.01$ ). These results indicated that social isolation induced anxiety-like behavior.

### Changes in brain BDNF protein expression induced by social isolation

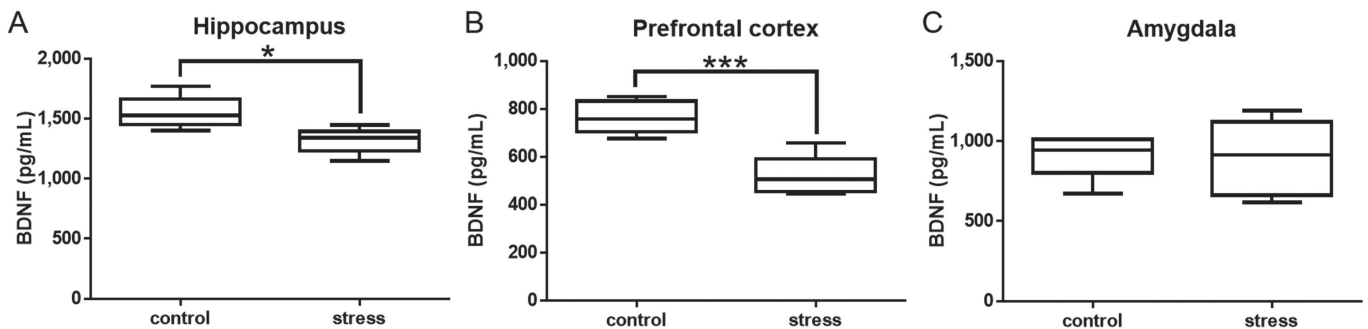
Determination of the levels of BDNF protein in different areas of the brain revealed that the level in the hippocampus was significantly lower in the stress group (1,319.7 ± 48.78 pg/mL) than in the control group (1,551.8 ± 60.45 pg/mL,  $P < 0.05$ ; Fig. 2A). The BDNF concentration in the prefrontal cortex was also significantly lower in the stress group (511.39 ± 33.95 pg/mL) than in the control group (726.6 ± 47.53 pg/mL,  $P < 0.005$ ; Fig. 2B). The concentration of BDNF in the amygdala did not differ significantly between the groups (stress, 897.1 ± 105.87 pg/mL; control, 913.51 ± 62.83 pg/mL,  $P = 0.5$ ; Fig. 2C). These results indicated that social isolation led to a reduction of BDNF expression in the hippocampus and prefrontal cortex.

### Alterations of Bdnf and Trkb mRNA expression in the brain induced by social isolation

The expression of Bdnf mRNA in each brain region was significantly lower in the stress group (hippocampus: 0.39 ± 0.02, prefrontal cortex: 0.28 ± 0.05, amygdala: 0.13 ± 0.04) than in the control group (hippocampus: 0.84 ± 0.08, prefrontal cortex: 1.02 ± 0.07, amygdala: 0.79 ± 0.08,  $P < 0.01$ ; Fig. 3A, B). Moreover, Trkb expression was significantly lower in the stress group (hippocampus: 0.57 ± 0.07, prefrontal cortex: 0.21 ± 0.12, amygdala: 0.14 ± 0.09) than in the control group (hippocampus: 1.31 ± 0.06, prefrontal cortex: 1.18 ± 0.28, amygdala: 1.18 ± 0.21,  $P < 0.05$ ; Fig.



**Fig. 1** Behavioral effects of social isolation in the open field (OF) and elevated plus maze (EPM) tests. (A) Total distance travelled in the OF test was significantly lower in the stress group than in the control group. (B) In the EPM test, all parameters, except for time spent in the enclosed arm, had significantly lower values in the stress group. Data are presented as the box plot of the control group ( $n = 6$ ) or the stress group ( $n = 6$ ); \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 2** Effect of social isolation stress on BDNF protein expression in the hippocampus (A,  $n = 5$ ), prefrontal cortex (B,  $n = 5$ ), and amygdala (C,  $n = 5$ ). BDNF expression was significantly lower in the hippocampus and prefrontal cortex of the stress group than in the control group. Data are presented as the box plot; \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

3A and C).

#### Altered levels of BDNF protein in blood and saliva

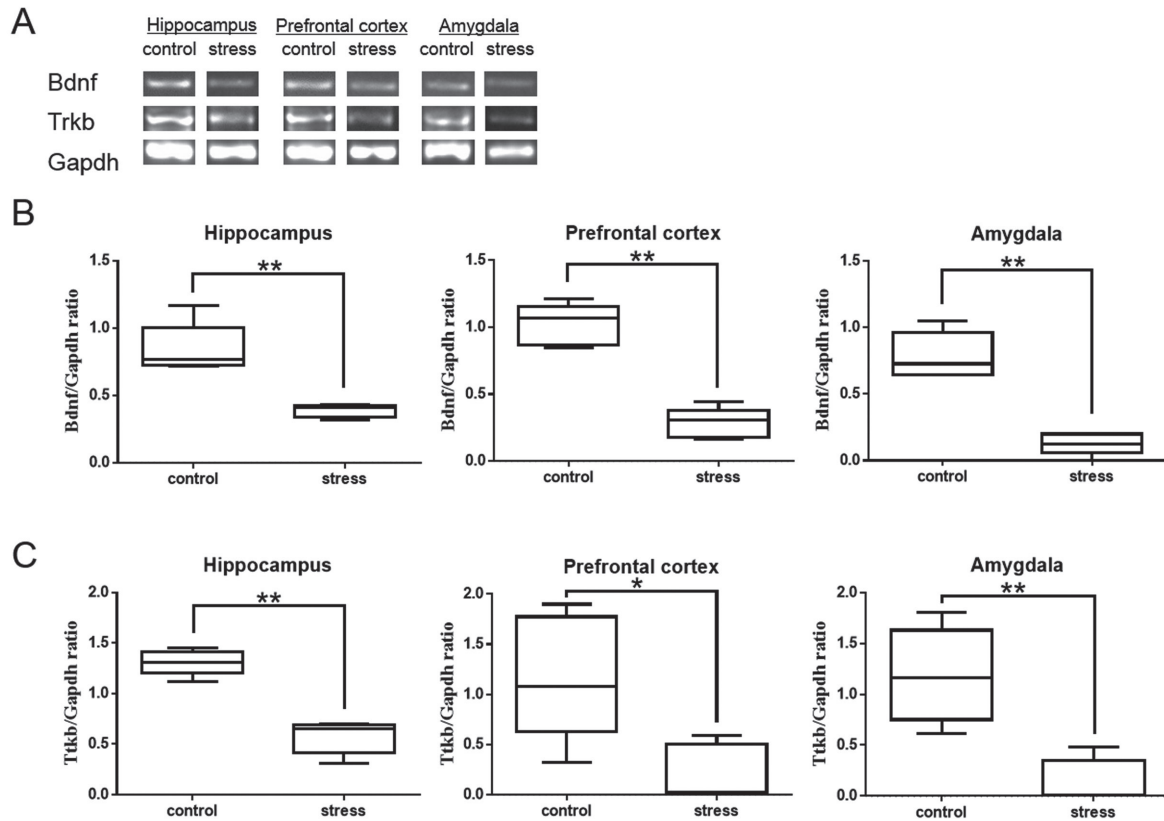
The concentration of BDNF in plasma was significantly lower in the stress group ( $52.64 \pm 8.57$  pg/mL) than in the control group ( $94.21 \pm 13.43$  pg/mL,  $P < 0.05$ ; Fig. 4A). Furthermore, the BDNF concentration in saliva was also significantly lower in the stress group than in the control group ( $38.13 \pm 4.68$  pg/mL; control,  $50.39 \pm 2.86$  pg/mL,  $P < 0.05$ ; Fig. 4B).

#### Discussion

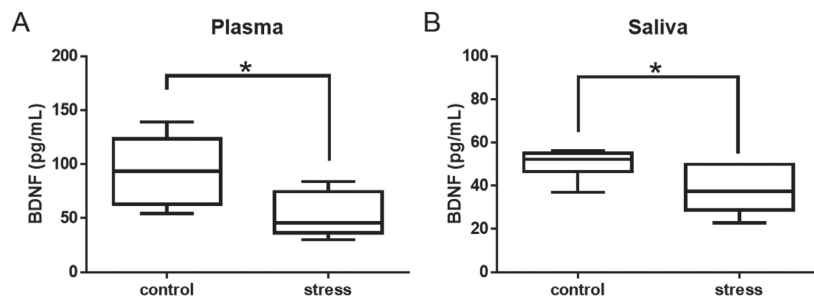
This is the first study to have demonstrated that social isolation stress induces changes in the salivary level of BDNF. Previous studies have indicated that BDNF expression in the salivary glands changes in response to stress [28,29]. Moreover, it is well established that the concentration of BDNF in blood decreases following stress in humans and various animal

species [14,22]. The present findings indicate that social isolation stress also induces a reduction of BDNF in saliva and blood.

In this study, social isolation stress elicited behavioral changes in rats, as assessed using the OF and EPM tests. Rats exposed to chronic stress are known to show reduced locomotor activity in the OF test [14], and in the present study stressed rats showed a reduction in the total distance walked in both tests. In addition, the increased time spent in the enclosed arms was considered due to this lower locomotor activity. Moreover, this study recorded a significantly lower amount of time spent and fewer entries in the open arms of the EPM, in line with a previous study [30]. This suggests that social isolation induces anxiety-like behavior in rats. Several studies have used the Morris water maze to demonstrate the cognitive deficits induced by social isolation stress [11,13]. In addition, the social interaction test has revealed behavioral dysfunction [31]. These changes are accompanied by decreased hippocampal expression of BDNF [14]. In line with these previous findings, the present study found lower hippo-



**Fig. 3** Effect of social isolation stress on expression of Bdnf and Trkb mRNA in the hippocampus, prefrontal cortex, and amygdala. (A) RT-PCR analysis detected expression of mRNA for each gene in each brain area. In the stress group, Bdnf (B) and Trkb (C) expression was significantly lower in the hippocampus ( $n = 5$ ), prefrontal cortex ( $n = 5$ ), and amygdala ( $n = 5$ ) than in the control group. Data are presented as the box plot; \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 4** Social isolation-induced changes in the concentration of BDNF in blood and saliva. The concentration of BDNF in blood (A) and saliva (B) was significantly lower in the stress group than in the control group. Data are presented as the box plot of the control group ( $n = 6$ ) or the stress group ( $n = 6$ ); \* $P < 0.05$ .

campal BDNF expression in rats in the stress group. A previous study has suggested that this difference is partly mediated by increased expression of glucocorticoid [7]. Glucocorticoids are increased by activation of the hypothalamic-pituitary-adrenal (HPA) axis under stress conditions [7]. In the present study, corticosterone release was induced by social isolation stress, suggesting that decreased hippocampal expression of BDNF may be due to HPA axis activation.

The present study demonstrated significantly lower hippocampal BDNF and TrkB gene expression after 56 days in the social isolation stress group. Furthermore, expression of BDNF and TrkB in the prefrontal cortex was also lower in this group. The hippocampus and prefrontal cortex are associated with behavioral changes linked to processing of environmental information, including social environments [32]. Moreover, it has been shown that various neuropsychiatric disorders are associated with downregulation of TrkB in the hippocampus [21]. It is known that TrkB expression is decreased by behavioral dysfunction [33]. Consistent with changes in BDNF in the hippocampus, the concentration of BDNF in the prefrontal cortex is reduced following social interaction-related stress [18,19]. Taken together with these data, the present results indicate that social isolation stress affected brain function through downregulation of

BDNF-TrkB signaling.

Importantly, the present results revealed that social isolation stress reduced the concentration of BDNF in both blood and saliva. Previous human and animal studies have demonstrated reductions in the concentration of blood BDNF in individuals with stress-related disorders [14,34,35]. Furthermore, the concentration of BDNF in blood is significantly correlated with that in the brain [36].

The concentration of glucocorticoids in blood is correlated with that in saliva [32]. A previous study has shown that the expression of BDNF in salivary glands reflects that in the brain [3]. The present study demonstrated a significant difference in the level of salivary BDNF between the groups, reflecting differences in the concentrations of brain and blood BDNF following social isolation stress. Importantly, the present findings indicate that salivary BDNF may be a useful biomarker for detection of social isolation-induced brain dysfunction. Previous studies have shown that the concentration of BDNF in the salivary glands and blood increases following acute and chronic restraint stress [16,29]. In the present study, rats were exposed to mild chronic stress, which may explain the lower concentration of BDNF. Future studies will be required to examine changes in BDNF expression in the salivary glands induced by social isolation stress.



Saliva samples can provide information about environmental factors [37] and the efficacy of therapeutics [38]. In contrast to blood, collection of saliva is non-invasive [23]. As compounds present in saliva are derived from blood, they may reflect physiological conditions [39]. Measurement of stress-related hormones in saliva has been commonly conducted in human biomedical research [32]. The relative abundance of various substances in saliva may reflect their abundance in the brain more precisely than is the case for those measured in blood [40,41]. Blood BDNF passes from the brain through the blood-brain barrier, and therefore alterations in blood the BDNF level may be associated with disease pathology [42]. A previous study has indicated that the level of BDNF in the brain is correlated with its expression in the salivary gland via the blood [3]. Changes in the concentration of BDNF in the blood and salivary glands are identical following stress [16]. The present study also showed a relationship between changes in the concentration of salivary BDNF and social isolation stress. These findings suggest that analysis of BDNF in saliva could be a valuable tool for diagnosis of stress-related disorders. Use of this technique in clinical settings may therefore be warranted.

There were two limitations to the present study. Firstly, it found no difference in the concentration of BDNF protein in the amygdala between the groups. Previous studies have reported that BDNF expression in the amygdala may be increased [7] or decreased [43] following chronic stress. In addition, stress-induced alterations in BDNF expression vary depending on the brain region examined [17,34]. Further examination of such changes in different brain regions may therefore be required. Secondly, behavioral changes induced by social isolation stress were examined using the OF and EPM tests. It would have been preferable to use the social interaction and prepulse inhibition tests to evaluate the behavioral consequences of social isolation-related stress [31]. Further studies employing this model should be performed using the social interaction and prepulse inhibition tests.

In conclusion, the present study has provided evidence that the level of salivary BDNF reflects stress-induced dysfunction in rats. Importantly, social isolation stress was associated with lower levels of salivary BDNF, similar to differences found in the blood and brain. Blood BDNF is a proven biomarker for neuropsychiatric disorders, including depression and schizophrenia; therefore, BDNF in saliva may be a valuable, non-invasive, indicator for diagnosis of these disorders.

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### Conflict of Interest

The authors have no conflict of interest to declare.

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