# Effects of Chronic Mild Stress in Female Bax Inhibitor-1-Gene Knockout Mice

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**Objective:** The anti-apoptotic protein Bax inhibitor-1 (BI-1) is a regulator of apoptosis linked to endoplasmic reticulum (ER) stress, and BI-1$^{-/-}$ mice exhibit increased sensitivity to tissue damage. The purpose of this study was to investigate the role of BI-1 in the pathogenesis of chronic mild stress (CMS)-induced depression-like behaviors in BI-1$^{-/-}$ mice.
**Methods:** We delivered CMS for 2 or 6 weeks in BI-1-knockout and wild-type mice. Control groups of BI-1-knockout and wild-type mice were left undisturbed. The measured parameters were sucrose consumption at weeks 1, 2, 3, 4, 5, and 6, spontaneous locomotion, and a forced swimming test (FST) at weeks 2 and 6.
**Results:** Significant decreases in sucrose consumption and increases in immobility time in the FST were observed in both stress groups compared with the non-stress groups. Interestingly, at week 2, but not at week 6, BI-1$^{-/-}$-stress mice showed less sucrose intake and greater immobility time than did BI-1$^{+/+}$-stress mice.
**Conclusion:** These results suggest that BI-1 may play role in protecting against the depressogenic effects of CMS in the short term, but not in the long term. Further study is required to deepen understanding of the role of BI-1 in protecting against depression.

KEY WORDS: Bax inhibitor-1; Chronic mild stress; Forced swimming test; Depression; Locomotor activity.

## INTRODUCTION

Bax inhibitor-1 (BI-1) is an evolutionarily conserved, multi-transmembrane protein that resides in the endoplasmic reticulum (ER) and provides cytoprotective functions in both animals and plants.[1,2] BI-1 is expressed in normal cells and tissues including the heart, brain, placenta, liver, skeletal muscle, kidneys, and pancreas.[3] The cytoprotective function of BI-1 was originally discovered in cDNA library screens for human proteins capable of suppressing the death of yeast induced by ectopic expression of mammalian Bax protein.[3] BI-1 overexpression tagged to hemagglutinin or green fluorescent protein in several neuronal cell lines protects neuronal cells from oxygen-glucose deprivation or serum deprivation, a paradigm mimicking ischemia.[4] Cells isolated from BI-1$^{-/-}$ mice

exhibit hypersensitivity to apoptosis induced by ER stress.[5] Targeted ablation of the BI-1 gene in mice results in increased sensitivity to cell death induced by ischemia-reperfusion injury.[6] In contrast, BI-1 is not essential for regulating programmed developmental cell death, as liver tissue of BI-1 deficient mice is histologically normal.[7] Although the exact mechanisms of the BI-1 anti-apoptotic function remain unclear, several lines of evidence suggest that it may be involved in the regulation of intra-ER Ca$^{2+}$ levels[8] and production of reactive oxygen species.[9]

Given the neuroprotective function of BI-1 for ER stress and oxygen-glucose deprivation,[4] BI-1 may be implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and depression. However, up to now, no studies have investigated the role of BI-1 in these neurodegenerative and neuropsychiatric disorders. Chronic mild stress (CMS), an animal model of depression, has been reported to promote caspase-3 dependent neuronal apoptosis in the cerebral cortex[10] and increase serum corticosterone levels in female mice after 1 or 2 weeks of CMS.[11] We hypothesized that BI-1 may be involved in the diverse physio-

logical or behavioral effects induced by CMS. The purpose of the present study was to investigate the role of BI-1 in the pathogenesis of CMS-induced depression-like behaviors using BI-1$^{-/-}$ mice.

## METHODS

### Animals

BI-1$^{-/-}$ (n=18) and BI-1$^{+/+}$ C57BL/6J female mice (n=18, 13 weeks of age) were kindly provided by Dr. John C. Reed of the Stanford-Burnham Institute for Medical Research (La Jolla, CA, USA). Upon arrival, the mice were housed in groups of 3-5 at 21±1°C and 55±5% humidity under a 12-hour/12-hour light/dark cycle (lights on at 07:00). Food and water were available *ad libitum*. Mice were maintained in specific pathogen-free housing and cared for in accordance with the National Institute of Health guidelines for animal care. Permission for this study was given by the animal care and use committee of Chonbuk National University Graduate School of Medicine.

### Study Design

After 10 days of initial acclimation to the laboratory, the mice were trained to drink a sucrose solution. Mice were exposed to two standard drinking bottles on alternating nights during week 1; one bottle contained 2.5% sucrose and the other held tap water. After this preliminary phase, mice were food and water deprived for 8 hours and then exposed to the sucrose solution and water from 18:00 until 09:00 to measure sucrose consumption. Three baseline sucrose consumption tests were performed, separated by 3 days. Each group of BI-1$^{+/+}$ and BI-1$^{-/-}$ mice was divided into two groups, control and stress groups, matched for sucrose consumption and body weight. This resulted in four different groups: BI-1$^{+/+}$ control and stress groups (n=4-6) and BI-1$^{-/-}$ control and stress groups (n=4-6). The stress group was exposed to CMS, and the control group was left unchallenged. We initially conducted one experiment with a 6-week CMS treatment (experiment 1). Significant results were obtained after 2 weeks, so we conducted an additional short experiment with a 2-week CMS treatment (experiment 2). Behavioral tests, spontaneous locomotor activity, and a forced swimming test (FST), were performed between 08:00 and 14:00 after 2 and 6 weeks of exposure to CMS. No stressors were applied 15 hours before the tests (Fig. 1).

### CMS Procedure

The CMS regimen used in this study was based on a protocol described previously,[12,13] with minor modifications. The CMS procedure was delivered to each mice and consisted of a variety of unpredictable mild environmental, social, and physical stressors, including confinement in a small tube (1 hour), an empty cage without sawdust (15 hours), water and food deprivation (15 hours), food restriction (about 50 mg of food pellets, 3 hours), cage tilted at 45° (1-3 hours), damp sawdust (about 200 ml of water per 100 g of sawdust, 3 hours), paired housing in damp sawdust (18 hours), reversal of the light/dark cycle, 4-hour light/dark succession every 30 minutes (09:30-11:30), and light (15-17 hours) dark (4-6 hours) (Table 1). To maximize the unpredictable nature of the stressors, two or three different stressors were applied in any 24 hours period in a pseudo-random and changing sequence throughout the experimental period. Control animals were single-housed during the time of CMS to match the condition but after that, group-housed in a different room and left undisturbed with the exception of general handling (e.g., regular cage cleaning and measuring body weight).

### Sucrose Consumption Test

Sucrose intake and body weight were measured in the control and stress groups once per week on separate days during a 15-hour window (18:00-09:00) following 8 hours of food and water deprivation. The position of the two bottles was varied randomly from trial to trial. Stressors were not applied during the period of sucrose intake measurement, and the stressor of food and water deprivation was never conducted within the 15 hours before the sucrose consumption test or body weight measurements to avoid an effect of acute stress on the measures. Consumption
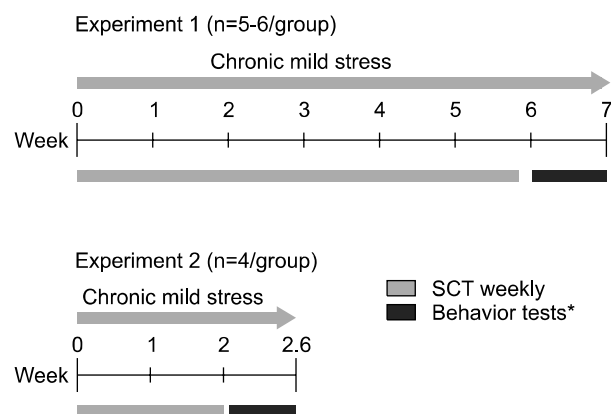


**Fig. 1.** Experiment design of the study. SCT, sucrose consumption test. *Behavior tests include locomotion and forced swimming test.

**Table 1.** Procedure of chronic mild stress

| Week | Start point | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|---|---|
| 1 | 9:00 a.m. | Small tubes (1.5 h) | Small tubes (1 h) | Food restriction (3 h) | Small tubes (1.5 h) | Small tube (1 h) | Reversal of the light/dark cycle (after 9:00 am) | Reversal of the light/dark cycle |
|  | 2:00 p.m. | Cages tilt at 45° (1.5 h) | Damp sawdust (3 h) | Cages tilt at 45° (1.5 h) | Damp sawdust (3 h) | Cages tilt at 45° (1.5 h ) |  |  |
|  | 6:00 p.m. | Empty cage (15 h) | Water and food deprivation (15 h) | Empty cage (15 h) | Cages tilt at 45° (1 h ) | Water and food deprivation (15 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle |
| 2 | 9:00 a.m. | End of reversal cycle (9:00) | Cages tilt at 45° (1.5 h) | Small tube (1.5 h) | Small tube (1.5 h) | Cages tilt at 45° (1.5 h) | 4 light/dark succession every 30 min (9:30-11:30) | 4 light/dark succession every 30 min (9:30-11:30) |
|  | 2:00 p.m. | Small tube (1.5 h) | Small tube (1 h) | Food restriction (3 h) | Cages tilt at 45° (1.5 h) | Small tube (1 h) |  |  |
|  | 6:00 p.m. | Paired housing in Damp sawdust (18 h) | Water and food deprivation (15 h) | Empty cage (15 h) | Water and food deprivation (15 h) | Damp sawdust (3 h) | Light (15-17 h), dark (4-6 h) | Light (15-17 h), dark (4-6 h) |
| 3 | 9:00 a.m. | Body weighing (10:00) | Cages tilt at 45° (3 h) | Small tube (1 h) | Food restriction (3 h) | Damp sawdust (3 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle |
|  | 2:00 p.m. | Small tube (1.5 h) | Damp sawdust (3 h) | Cages tilt at 45° (3h) | Damp sawdust (3 h) | Cages tilt at 45° (1.5 h) |  |  |
|  | 6:00 p.m. | Food restriction (3 h) | Empty cage (15 h) | Small tube (1.5 h) | Water and food deprivation (15 h) | Small tube (1.5 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle |
| 4 | 9:00 a.m. | Body weighing (10:00) | Small tubes (1 h) | Cages tilt at 45° (1 h) | Damp sawdust (3 h) | Small tube (1.5 h) | Reversal of the light/dark cycle (after 9:00 am) | Reversal of the light/dark cycle |
|  | 2:00 p.m. | Small tube (1 h) | Cages tilt at 45° (3 h) | Food restriction (3 h) | Small tube (1.5 h) | Cages tilt at 45° (3 h) |  |  |
|  | 6:00 p.m. | Empty cage (15 h) | Water and food deprivation (15 h) | Cages tilt at 45° (3 h) | Cages tilt at 45° (1.5 h) | Damp sawdust (3 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle |
| 5 | 9:00 a.m. | End of reversal cycle (9:00) | Cages tilt at 45° (1.5 h) | Food restriction (3 h) | Small tube (1 h) | Small tube (1.5 h) | 4 light/dark succession every 30 min (9:30-11:30) | 4 light/dark succession every 30 min (9:30-11:30) |
|  | 2:00 p.m. | Cages tilt at 45° (3 h) | Damp sawdust (3 h) | Small tube (1 h) | Damp sawdust (3 h) | Cages tilt at 45° (1.5 h) |  |  |
|  | 6:00 p.m. | Paired housing in damp sawdust (18 h) | Water and food deprivation (15 h) | Paired housing in damp sawdust (18 h) | Empty cage (15 h) | Damp sawdust (3 h) | Light (15-17 h), dark (4-6 h) | Light (15-17 h), dark (4-6 h) |
| 6 | 9:00 a.m. | Weighing (10 h) | Cages tilt at 45° (3 h) | Cages tilt at 45° (1.5 h) | Small tube (1 h) | Restriction (3 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle |
|  | 2:00 p.m. | Small tube (1 h) | Food restriction (3 h) | Small tube (1 h) | Cages tilt at 45° (3 h) | Cages tilt at 45° (1.5 h) |  |  |
|  | 6:00 p.m. | Water and food deprivation (15 h) | Paired housing in damp sawdust (18 h) | Small tube (1 h) | Damp sawdust (3 h) | Empty cage (15 h) | Reversal of the light/dark cycle | Reversal of the light/dark cycle End of reversal cycle (after 20 h) |

h, hour(s); min, minute(s).

was measured by weighing the pre-weighed bottle at the end of the test. Intake was expressed in relation to the animal's body weight (g/g). Control mice were housed individually during the test.

### Spontaneous Locomotor Activity

Locomotor activity was measured in an open field box (40×50×30 cm) consisting of a white base and black walls, using a Smart video tracking system (Panlab S.L., Barcelona, Spain) in a softly illuminated (40 W), sound-proof room. Each mouse was placed in the center of the open field apparatus, and the distance traveled (cm), locomotor time (seconds), number of times crossing the center (50% of all area), and time spent in the center was re-

corded at 10-minute intervals during a 30-minute period. The open field arena was thoroughly cleaned between tests.

## FST

Each animal was placed in a beaker (18 cm diameter, 24 cm depth) filled with water (temperature 25-26°C) to a height of 16 cm. Immobility (floating with only small movements to keep balance) and swimming were scored throughout the last 4 minutes of the 6-minute test period. Scoring was done by a trained observer who was blind to the treatment paradigm.
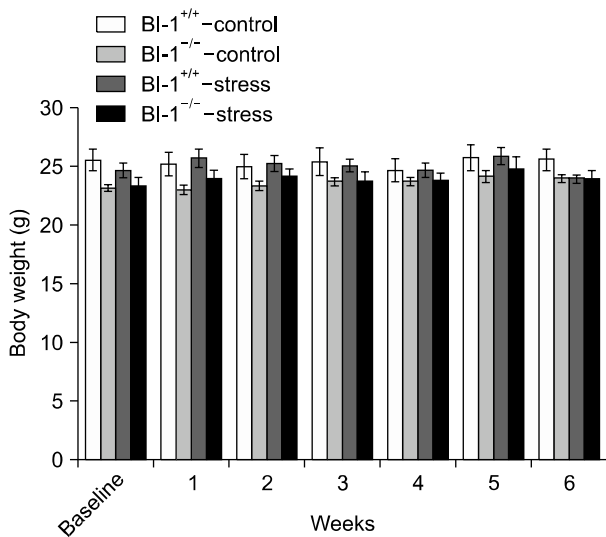


**Fig. 2.** Body weight of four experimental groups during 6-week treatment of chronic mild stress. BI-1, Bax inhibitor-1.

## Statistical Analysis

All data are expressed as the mean±standard error of the mean. Body weight and sucrose consumption were analyzed with a repeated-measures two-way analysis of variance (ANOVA). Individual measures of body weight and sucrose consumption at each time point, locomotor activity, and FST immobility times were compared among the groups using a one-way ANOVA followed by Tukey's *post hoc* test. Differences of $p < 0.05$ were considered significant.

# RESULTS

No significant difference in the main effect of group ($F[3,17]=1.373$, $p=0.285$), but significant differences in the main effect of time ($F[6,102]=6.480$, $p < 0.001$) and a group×time interaction ($F[18,102]=2.652$, $p=0.001$) were observed for body weight (Fig. 2). Further analyses comparing the baseline and 6-week values among the groups revealed no significant differences.

## Sucrose Consumption

The ANOVA yielded a significant main effect of group ($F[3,17]=92.562$, $p < 0.001$) and time ($F[6,102]=44.123$, $p < 0.001$) and a significant group×time interaction ($F[18,102]=4.460$, $p < 0.001$) for sucrose consumption (Fig. 3A, 3B). A *post hoc* group analysis demonstrated significant differences between the BI-1$^{+/+}$-control group vs. the BI-1$^{+/+}$-stress group and between the BI-1$^{-/-}$-stress and BI-1$^{+/+}$-stress groups vs. the BI-1$^{-/-}$-control group as well as between the BI-1$^{-/-}$-stress and the BI-1$^{-/-}$-control group. The BI-1$^{+/+}$-
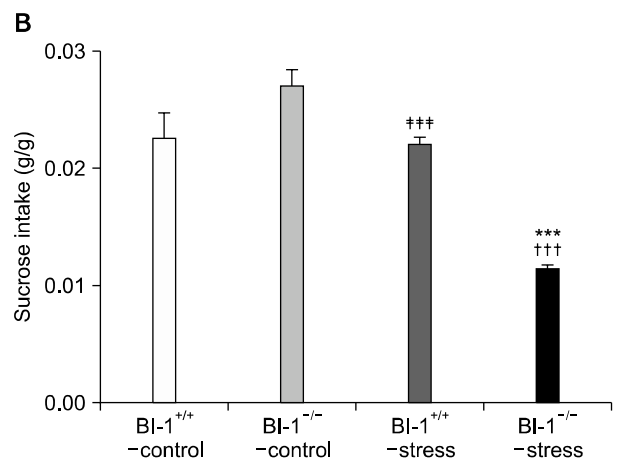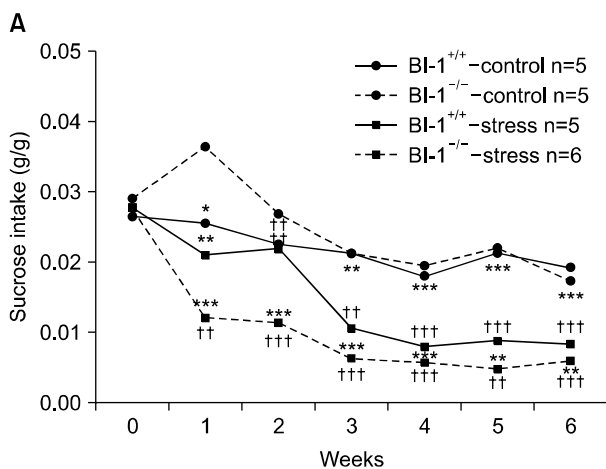


**Fig. 3.** (A) Effect of chronic mild stress on sucrose consumption over 6 weeks and (B) effect of chronic mild stress in sucrose consumption at week 2. *Significant differences from BI-1$^{-/-}$-control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); †significant difference from BI-1$^{+/+}$-control (††$p < 0.01$, †††$p < 0.001$); ‡significant difference from BI-1$^{-/-}$-stress (‡‡$p < 0.01$, ‡‡‡$p < 0.001$). BI-1, Bax inhibitor-1.

**Table 2.** Spontaneous locomotor activities after 2- and 6-week treatment of chronic mild stress

| | Distance traveled (cm) | Locomotor time (seconds) | Number of crossing the center | Time spent in the center (seconds) |
|---|---|---|---|---|
| 2-week CMS (n=4/group) | | | | |
| BI-1$^{+/+}$-control | 14,458.43±1,857.61 | 1,172.70±142.63 | 101.25±16.78 | 343.1±76.39 |
| BI-1$^{-/-}$-control | 8,740.833±183.16 | 655.96±137.03 | 58.00±18.19 | 345.23±182.53 |
| BI-1$^{+/+}$-stress | 17,004.15±3,825.87 | 1,151.80±114.98 | 99.25±31.06 | 583.48±377.12 |
| BI-1$^{-/-}$-stress | 15,707.00±2,645.72 | 1,175.35±126.07 | 103.75±30.86 | 289.78±125.13 |
| 6-week CMS (n=5-6/group) | | | | |
| BI-1$^{+/+}$-control | 7,796.34±1,451.97 | 793.76±78.67 | 18.80±11.34 | 80.52±49.06 |
| BI-1$^{-/-}$-control | 10,212.21±1,084.61 | 970.04±98.53 | 57.40±5.56 | 243.72±70.73 |
| BI-1$^{+/+}$-stress | 12,838.99±1,715.32 | 1,066.32±86.01 | 57.40±13.87 | 277.86±39.92 |
| BI-1$^{-/-}$-stress | 14,966.96±1,289.70$^{\dagger\dagger}$ | 1,144.33±67.77$^{\dagger}$ | 57.00±16.93 | 175.15±77.57 |

$^{\dagger,\dagger\dagger}$Significant differences from BI-1$^{+/+}$-control ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$). BI-1, Bax inhibitor-1.
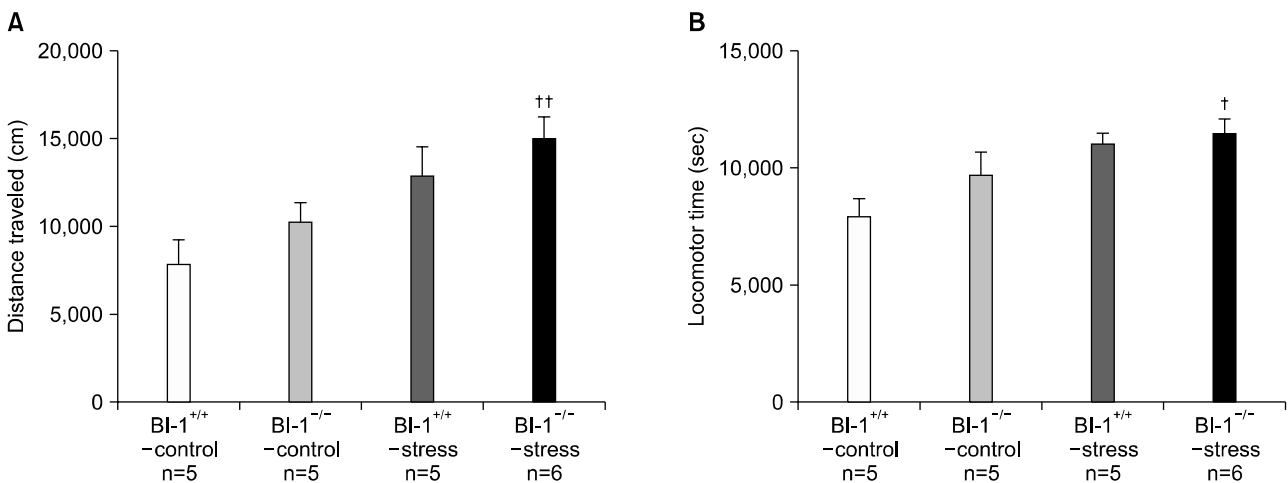


**Fig. 4.** Spontaneous locomotor activities ((A) distance traveled and (B) locomotor time) after 6-week treatment of chronic mild stress. $^{\dagger}$Significant differences from BI-1$^{+/+}$-control ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$). BI-1, Bax inhibitor-1.

stress and BI-1$^{-/-}$-stress groups consumed significantly less sucrose than the BI-1$^{+/+}$-control and BI-1$^{-/-}$-control groups, starting from week 1 and throughout the experiment. No significant differences in sucrose consumption were observed between BI-1$^{+/+}$-control BI-1$^{-/-}$-control groups throughout the experiment, except at week 1, when greater sucrose was consumed in the BI-1$^{-/-}$-control group than in the BI-1$^{+/+}$-control group ($F=20.733$, $p=0.018$). Interestingly, at week 2, less sucrose was consumed in the BI-1$^{-/-}$-stress group than in the BI-1$^{+/+}$-stress group ($F=28.592$, $p<0.001$) (Fig. 3B).

### Spontaneous Locomotor Activity

Significant differences were observed for distance traveled ($F=5.121$, $p=0.009$) and locomotor time ($F=3.418$, $p=0.032$) among the groups in the 6-week CMS treatment, but not in the 2-week CMS treatment. *Post hoc* analyses revealed greater distances traveled and increased locomo-

tor time in the BI-1$^{-/-}$-stress group than in the BI-1$^{+/+}$-control group (Table 2, Fig. 4).

### FST

Significant differences were observed in the immobility and swimming times among the groups for both the 2- and 6-week CMS treatments. *Post hoc* results showed a longer immobility time and a shorter swimming time in the BI-1$^{+/+}$-stress and BI-1$^{-/-}$-stress groups compared with the BI-1$^{+/+}$-control group and BI-1$^{-/-}$-control group, respectively, in the 2-week and 6-week CMS treatments. In experiment 1, with a 2-week CMS treatment, significantly longer immobility time was observed in the BI-1$^{-/-}$-stress group than in the BI-1$^{+/+}$-stress group ($F=42.465$, $p=0.003$) (Fig. 5).

## DISCUSSION

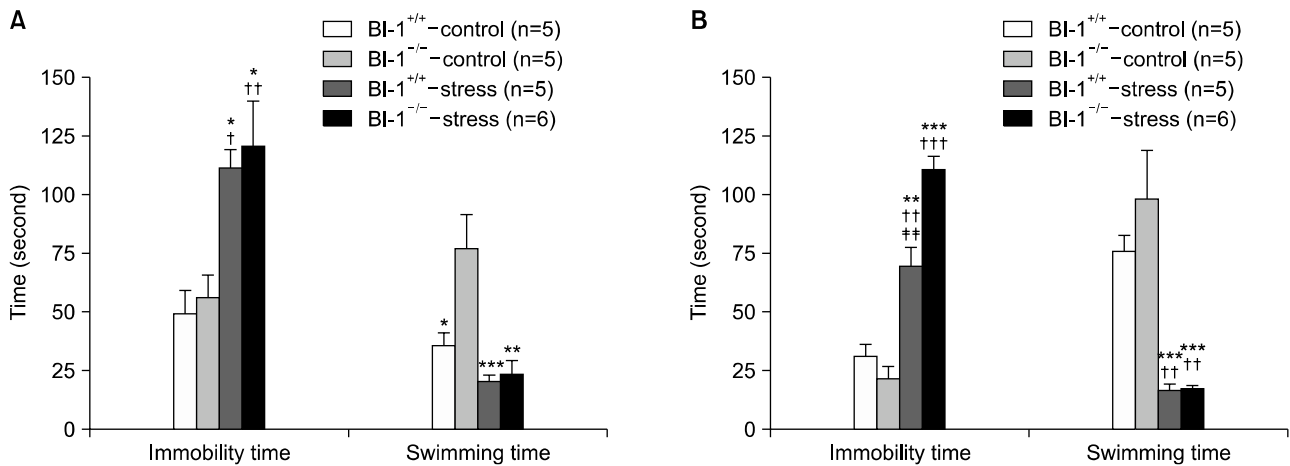The role of BI-1 in producing depression-like behaviors

A



B



**Fig. 5.** Effects of chronic mild stress in the forced swimming test after 2-week (A) and 6-week (B) treatment of chronic mild stress. *Significant differences from BI-1$^{-/-}$-control (*$p<0.05$, **$p<0.01$, ***$p<0.001$); $^{†}$significant difference from BI-1$^{+/+}$-control ($^{†}p<0.05$, $^{††}p<0.01$, $^{†††}p<0.001$); $^{†}$significant difference from BI-1$^{-/-}$-stress ($^{††}p<0.01$). BI-1, Bax inhibitor-1.

induced by CMS was investigated using BI-1-gene knockout mice. We showed that a BI-1 gene deficiency is implicated in producing more severe depression-like behaviors during a short-term CMS treatment (2-week), but not following long-term CMS treatment (6-week).

The CMS procedure decreased sucrose consumption significantly in both the wild-type and knockout mice, which was consistent with the majority of previous reports.[14-16] It should be noted, however, that different strains, such as dilute brown non-agouti (DBA/2) mice, do not show a decrease in sucrose consumption following CMS.[16] Some researchers[17,18] have suggested that a reduction in sucrose intake may not be related directly to CMS *per se*, but secondary to stress-induced weight or appetite loss. However, as we did not observe significant weight changes during the CMS procedure, and because sucrose consumption was expressed as the absolute intake of sucrose per mouse body weight, that hypothesis seems invalid, at least for our study. Mixed findings, such as no change[14,16] or an increase[19] in body weight, have been reported following a CMS procedure in C57BL/6J mice. These discrepancies may be related to the severity of the CMS regimen and other experimental conditions employed in different studies. It is suggested that food and/or water deprivation should be removed from the CMS regimen or only water should be deprived before measuring sucrose consumption. The major finding of the present study was that the BI-1$^{-/-}$-stress group consumed a smaller amount of sucrose than was consumed by the BI-1$^{+/+}$-stress group, suggesting that a BI-1 gene deficiency is involved in producing more severe anhedonic-like behavior following CMS. Thus, this result seems related to a neuroprotective

function of BI-1. However, further studies are required to explore the molecular mechanisms of BI-1 regulation of the pathophysiological pathways induced by CMS. Interestingly, a transient increase in sucrose consumption in the BI-1$^{-/-}$-control group compared with the BI-1$^{+/+}$-control group was observed at week 1. This finding, however, is difficult to interpret because both groups were under the same conditions. Given that food and water deprivation were delivered to both groups as part of the sucrose consumption test and that social isolation, another mild stressor, increases sucrose intake in mice,[20] we speculate that BI-1$^{-/-}$-control mice were more sensitive to the effects of food and water deprivation delivered before measuring sucrose consumption, at least transiently. Recently, Strekalova and Steinbusch (2010)[21] suggested that sucrose preference is a parameter of the sucrose test, which makes absolute sucrose intake suitable for analyzing individual differences in hedonic sensitivity in mice. Despite this suggestion, we measured sucrose intake only because of inconsistent results for sucrose preference in our preliminary experiment (data not shown) due to the very small amount of water taken.

No significant difference in spontaneous locomotor activity was observed between BI-1$^{+/+}$-control and BI-1$^{+/+}$-stress groups, whereas the BI-1$^{-/-}$-stress group showed greater activity than the BI-1$^{+/+}$-control group. These results suggest that CMS had no effect on locomotion in wild-type mice and that a BI-1 deficiency produced enhanced vulnerability to CMS. Anhedonia often accompanies psychomotor retardation in depressed patients.[22] Hence, we expected a decrease in locomotion in BI-1$^{+/+}$-stress mice and a greater reduction in locomotion in BI-1$^{-/-}$-stress

mice, but we obtained the opposite findings. Inconsistent results have been reported regarding the effects of CMS on locomotion, including no effect,[14,19,23] a decrease,[24-27] and an increase.[15,28,29] These inconsistencies may be due to variations in light intensity,[29] temperature, or sound proofness of the experimental room. The exact meaning of hyperlocomotion in BI-1$^{-/-}$-stress mice is unclear because increased locomotion can be interpreted as increased exploratory behavior toward a novel environment, a form of hyperactivity (anxiolytic-like behavior), or agitated behavior (anxiogenic-like behavior). This issue should be addressed in a future study using behavioral tests measuring anxiety such as an elevated plus maze, the dark-light exploration test, or the marble-burying test.

CMS increased immobility time during the FST in the BI-1$^{+/+}$-stress group, which was similar to the results of many previous studies. It was interesting to see a greater increase in immobility time in the BI-1$^{-/-}$-stress group than in the BI-1$^{+/+}$-stress group at week 2, suggesting that BI-1 gene knockout may be associated with producing more severe depression-like behavior following CMS. This finding together with the greater reduction of sucrose consumption in the BI-1$^{-/-}$-stress group than in the BI-1$^{+/+}$-stress group at week 2 suggests that BI-1 has a protective function against the depressogenic effects of CMS in the short term, but not in the long term.

Several limitations of our study should be mentioned. First, it was the lack of baseline locomotor activity data. As interindividual variability in response to the stress of a novel environment often exists, this could be a confounding factor when comparing locomotor activities after CMS. Second, the sample size of each group was relatively small, and therefore there was a possibility of type II error. Additionally, our findings should be considered with related molecular studies to provide more insight into the role of BI-1 in depression. In conclusion, our results suggest the involvement of BI-1 in the production of depression-like behaviors following short-term CMS treatment.

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