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# Increased thioredoxin-interacting protein in brain of mice exposed to chronic stress

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ABSTRACT

Chronic stress is a key contributor to depression. Previous studies have shown that oxidative stress and inflammation are increased by chronic stress and in subjects with depression. Thioredoxin is a small redox protein that regulates cellular redox balance and signaling. This protein can reverse protein cysteine oxidative modifications such as sulfenylation and nitrosylation, and inhibit stress-regulated apoptosis signal-regulating kinase 1 pathway. Therefore thioredoxin plays an important role in cellular defense against oxidative stress. Thioredoxin-interacting protein is an endogenous thioredoxin inhibitor. In the present study, to understand the role of thioredoxin in chronic stress and depression, we have investigated thioredoxin, thioredoxin-interacting protein, sulfenylation, nitrosylation and apoptosis signal-regulating kinase 1 phosphorylation in brain of mice exposed to chronic unpredictable stress (CUS). We found that mice exposed to CUS displayed decreased exploratory, increased anhedonic and increased despair depressive-like behaviours. We also found that although CUS had no effect on thioredoxin protein levels, it significantly increased levels of thioredoxin-interacting protein in mouse hippocampus and frontal cortex. CUS also increased protein cysteine sulfenylation, protein cysteine nitrosylation and apoptosis signal-regulating kinase 1 phosphorylation in mouse hippocampus and frontal cortex. These findings suggest that chronic stress may upregulate thioredoxin-interacting protein, subsequently inhibiting thioredoxin activity and enhancing oxidative protein cysteine modification and apoptosis signal-regulating kinase 1 pathway. These results also indicate that thioredoxin-interacting protein may have potential for depression treatment.

## 1. Introduction

Depression is a psychiatric disorder, significantly affecting people's mood, thought and well-being. It is among the top 10 causes of disability worldwide (Carney et al., 2002). An early theory of causation points to monoamine dysfunction, based on increased monoamine by antidepressants. However, those drugs increase synaptic monoamines within only minutes, while a clinical response often takes 2–4 weeks to produce. In addition, approximately 40% of patients respond poorly to current antidepressants (Brunello et al., 2002; Hillhouse and Porter, 2015). This suggests that additional factors are also crucial to both its disease mechanism and treatment. However, despite many years of research, the pathophysiological mechanisms of depression are not fully elucidated.

Increased evidence shows that oxidative stress is increased in patients with depression. Oxidative stress is an overproduction of reactive oxygen species (ROS) that overwhelms cellular antioxidant capacity. Overproduced ROS can damage protein, lipid and nucleic acids. We found that RNA oxidation was increased in postmortem hippocampus of depression and bipolar disorder patients; and that antioxidant glutathione was decreased in postmortem frontal cortex of depression patients (Che et al., 2010; Gawryluk et al., 2011). It has also been reported that mitochondrial ROS production, nitric oxide levels and lipid peroxidation were increased, and superoxide dismutase and glutathione peroxidase were decreased in peripheral blood samples of depression patients (Black et al., 2015; Dimopoulos et al., 2008; Herken et al., 2007; Moreno et al., 2013), indicating that oxidative stress contributes significantly to depression development and progression.

Thioredoxin (Trx) is a 12KDa reductase that plays a major role in cellular redox balance and signaling. Trx can reverse protein cysteine oxidative modifications such as sulfenylation and nitrosylation, and also reduce oxidized peroxiredoxin, facilitating peroxiredoxin-induced

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scavenge of peroxides. In addition, Trx can bind to apoptosis signal-regulating kinase 1 (ASK1) and maintain ASK1 in an inactive state, which inhibits ROS and stress-activated apoptosis and inflammatory signaling (Benhar et al., 2008; Netto and Antunes, 2016; Silva-Adaya et al., 2014). Therefore the Trx antioxidant system has an important role in cellular defense against oxidative stress and inflammation. Thioredoxin -interacting protein (Txnip) is an endogenous Trx inhibitor which can inhibit Trx activity (Spindel et al., 2012).

Depression diagnosis is based on clinical symptoms, and there are as yet no biological markers for the disorder. Modeling depression psychopathology in animals is much more difficult than with other somatic diseases. Chronic stress is a major risk for both depression and other psychiatric disorders. Studies show that rodents exposed to stressful situations exhibit depressive-like behaviours (Hollis and Kabbaj, 2014; Yin et al., 2016). In general, the various stress models such as chronic unpredictable stress (CUS), chronic restraint stress, maternal separation and learned helplessness show good face and construct validities. Among these models, CUS is one of most realistic and has been studied extensively. CUS exhibits not only anxiety and despair behaviours, but also anhedonia-like behavior, a major symptom of depression (Mehta et al., 2017; Zhu et al., 2014). Our previous study has shown that oxidative stress was increased but antioxidant capacity was decreased in rodents exposed to CUS (Che et al., 2015). In the current study, we analyzed Trx, Txnip, protein sulfenylation, protein nitrosylation and ASK1 phosphorylation in hippocampus and other brain regions in mice exposed to CUS.

## 2. Materials and methods

### 2.1. Chronic unpredictable stress

Eight-week-old male C57BL/6 mice weighing approximately 20-28 g at the beginning were purchased from Charles River Canada (Montreal, Canada). Upon arrival, animals were maintained at 21 ± 1 °C, with free access to water and food, under a 12:12 h light/dark cycle. Mice were housed at 4–5 in each cage. Mice were divided into stress and control groups. Mice in the control group were kept undisturbed in their home cages, while mice in the CUS group were exposed to two different stressors daily for 28 days. These stressors include restraint, cold swimming, overnight illumination, foot shock, tail clamping, and others as indicated in Table 1. Behavioral tests started at day 29, 24 h after the last stressor. All behavioral tests were performed in the light phase between 09:00 and 17:00 h. After behavioral tests, mice hippocampus, frontal cortex and amygdala were isolated for biochemical analysis. All procedures with mice were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Manitoba.

### 2.2. Open field test

The open-field test was performed in a black Plexiglas square box (50 × 50 × 50 cm). Mice were placed in the center of the testing box and allowed to explore freely for 5 min, and then returned to their home cage. Mice activity was recorded with a video camera connected to the computer. The distance moved, resting time, number of rearings, time spent in the central zone (30 cm diameter) and frequency of center entries were analyzed.

### 2.3. Forced swim test

Mice were individually placed into a glass cylinder (19 cm diameter, 23 cm deep, filled with 23–25 °C water). The depth of water was set to prevent the animals from touching the bottom with their tails, and behavior was recorded using a video camera. The test total lasted 6 min, only the last 4 min were scored for mobility duration. Immobility was defined as remaining motionless or floating, which required the absence of all movement except motions required to maintain balance. Immediately after the test, mice were covered by a dry towel and then placed under a heating lamp until they were dry.

**Table 1**
Daily schedules for the chronic unpredictable stress.

| Day of treatment | Stressor used |
|---|---|
| Day 1 | Wet cage (2 h); Overnight illumination (12*h*) |
| Day 2 | Restraint (4 h); Shaking (4 h) |
| Day 3 | Clamping tail (1 h); Foot shock (20 times, 0.5 mA, 5 s) |
| Day 4 | Cold swimming (5 min, 4°); Water deprivation (24 h) |
| Day 5 | Foot shock (20 times, 0.5 mA, 5 s); Cold swimming (5 min, 4°) |
| Day 6 | Wet cage (2 h); Clamping tail (1 h) |
| Day 7 | Cage tilt (8 h); Overnight illumination (12 h) |
| Day 8 | Restraint (4 h); Cold swimming (5 min, 4°) |
| Day 9 | Foot shock (20 times, 0.5 mA, 5 s); Shaking (4 h) |
| Day 10 | Cold swimming (5 min, 4°); Clamping tail (1 h) |
| Day 11 | Clamping tail (1 h); Water deprivation (24 h) |
| Day 12 | Restraint (4 h); Shaking (4 h) |
| Day 13 | Cage tilt (8 h); Overnight illumination (12 h) |
| Day 14 | Wet cage (2 h); Clamping tail (1 h) |
| Day 15 | Foot shock (20times,0.5 mA,5 s); Cold swimming (5 min 4°) |
| Day 16 | Shaking (4 h); Restraint (4 h) |
| Day 17 | Clamping tail (1 h); Foot shock (20 times, 0.5 mA, 5 s) |
| Day 18 | Cold swimming (5 min, 4°); Water deprivation (24 h) |
| Day 19 | Wet cage (2 h); Overnight illumination (12 h) |
| Day 20 | Restraint (4 h); Shaking (4 h) |
| Day 21 | Foot shock (20 times, 0.5 mA, 5 s), Cold swimming (5 min 4°) |
| Day 22 | Clamping tail (1 h); Overnight illumination (12 h) |
| Day 23 | Restraint (4 h); Foot shock (20 times, 0.5 mA, 5 s) |
| Day 24 | Wet cage (2 h); Water deprivation (24 h) |
| Day 25 | Cold swimming (5 min, 4°); Shaking (4 h) |
| Day 26 | Foot shock (20 times, 0.5 mA, 5 s), Restraint (4 h) |
| Day 27 | Clamping tail (1 h); Wet cage (2 h) |
| Day 28 | Shaking (4 h); Foot shock (20times, 0.5 mA, 5 s) |

Abbreviation: h, hour; min, minute; s, second; mA, milliampere.

### 2.4. Sucrose preference test

Mice were exposed to a 1% sucrose solution for 24 h before test in order to adapt to sucrose solution. During test, mice were deprived of water for 24 h, followed by 4 h of exposure to two identical bottles: one filled with 1% sucrose solution and the other with plain water. The bottle position was switched half way through the test. Sucrose preference will be calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake during 4 h testing period.

### 2.5. Immunoblotting analysis

Mouse hippocampus, frontal cortex and amygdala were dissected on ice and homogenizing in 10:1 (ml/g) ice-cold lysis buffer containing 250 mM NaCl, 30 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 0.5 mM EDTA, 20% glycerol, 1% nonidet P40, 0.1 mM EGTA, and 1× protease inhibitor cocktail (Thermo Scientific, Marietta, OH, USA). The homogenized tissues were kept on ice for 1 h and then centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatants were then collected as protein extract. Bradford protein assay was used to determine protein concentrations (Bradford, 1976). Protein samples were then mixed with a loading buffer containing 100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue and 20% glycerol, separated in 12% SDS polyacrylamide gels for 1 h at 120 V and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) at 200 mA for 1 h. After transfer the membranes were first blocked with 5% milk in Tris-buffered saline with Tween-20 containing 10 mM Tris-HCl (pH 7.5) and 0.1% Tween-20 at room temperature for 1 h, and then incubated with a 1: 1500 dilution of rabbit monoclonal Trx1 antibody (Cell Signaling Technology, Danvers, MA, USA), 1: 2000 dilution of rabbit monoclonal Txnip antibody (Abcam