The Regulation of Thioredoxin System in Chronic Stress-induced Depression

By

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ABSTRACT

Chronic stress is a key contributor to depression. It can cause oxidative stress which is also increased in depression. Thioredoxin (Trx) is a ubiquitous reductase that can reverse protein cystine oxidative modification, including sulfenylation and nitrosylation, and inhibit the apoptosis signal-regulating kinase 1 (ASK1) pathway. Thioredoxin-interacting protein (Txnip) is an endogenous Trx inhibitor.

The chronic unpredictable stress (CUS) animal model is wildly used to mimic human depression. We found that mice exposed to CUS displayed decreased exploratory, increased anhedonic and increased despair depressive-like behaviours. Although Trx protein levels didn't change, Txnip protein levels were significantly increased in hippocampus, frontal cortex and nucleus accumbens of CUS mice. Further investigation demonstrated an increase in protein sulfenylation, nitrosylation and ASK1 phosphorylation in CUS mice hippocampus and frontal cortex.

These findings suggest that chronic stress may upregulate Txnip protein levels, subsequently inhibiting Trx activity and enhancing protein cysteine oxidative modification and the ASK1 pathway.

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me to keep my life in context.

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LIST OF ABBREVIATIONS

$\cdot O_2^-$	superoxide anion
ACTH	adrenocorticotrophic hormone
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
CAT	catalase
CoQ	coenzyme Q
CRH	corticotrophin releasing hormone
CUS	chronic unpredictable stress
Cys	cysteine
Cyt C	cytochrome C
ETC	electron transport chain
fMRI	functional magnetic resonance imaging
GCs	glucocorticoids
GSH	glutathione
Gly	glycine
GSNO	S-nitrosoglutathione
GSSG	oxidized glutathione
GPx	glutathione peroxidase
H_2O_2	hydrogen peroxide
HPA	hypothalamic-pituitary-adrenal
IL-1β	interleukin-1β
IL-18	pro-interleukin -18
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MDD	major depressive disorder
MMTS	methylthiolating agent methyl methanethiosulfonate
NADPH	nicotinamide adenine dinucleotide phosphate
NLRP3	nod-like receptor protein 3
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
OH·	hydroxyl radicals
OXPHOS	oxidative phosphorylation
p38	p38 mitogen-activated kinase
PFC	prefrontal cortex
PVN	paraventricular nucleus
ROS	reactive oxygen species
Prx	peroxiredoxin

Sec	selenocysteine
SOD	superoxide dismutase
Thr	threonine
Trx	thioredoxin
TrxR	thioredoxin reductase
Txnip	thioredoxin interacting protein

CHAPTER 1: INTRODUCTION

1.1 Depression

Depression is one of the most common psychiatric disorders. According to World Health Organization (WHO) reports, depression will be the second most prevalent cause of disability by 2020 and the leading cause of disability worldwide by 2030 [1-3]. Nearly one in six men and one in four women experience depression during their lifetime. In addition, depression is among the most costly medical burdens. It is reported that the total cost of depression in Canada was at least $32.3 billion in 2016 [4-6]. Despite many years of research, the pathophysiology of depression has not yet been fully elucidated. It is postulated that depression is caused by a dysfunction in monoamine function because antidepressants cause an increase in monoamine release. However, increase of synaptic monoamines occurs within only minutes, but a clinical response often takes 2-4 weeks to produce, suggesting that additional factors are also crucial to the therapeutic effect. In addition, current antidepressants also produce adverse effects and approximately 40% of patients respond poorly to them [7-8]. There is a strong need to better understand the disease mechanism and develop more effective treatments for depression.

1.2 Chronic stress and depression

1.2.1 Stress and hypothalamic-pituitary-adrenal axis activation

Stress is an adaptive response to emotional and physiological pressure, which results in a

series of physiological, neurological, and behavioral changes, and mainly mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Acute stress helps the body mobilize energy for various events, while chronic stress is detrimental and is an important modifiable contributor to both physical and mental illnesses. As shown in **Fig.1,** the HPA stress response is initiated by releasing corticotrophin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus. Then CRH stimulates anterior pituitary gland to produce and release adrenocorticotrophic hormone (ACTH). Eventually, ACTH promotes the production of glucocorticoids (GCs) from adrenal cortex (cortisol in humans, corticosterone in rodents). Released GCs then inhibit CRH and ACTH synthesis and secretion, which is known as negative feedback inhibition of the HPA axis. GCs levels are maintained under normal physiological conditions by this negative feedback loop. However, chronic excessive stress impairs this negative feedback, leading to continuous elevation of GC levels that may cause various kinds of cellular damage.

1.2.2 Chronic stress as a major risk factor for depression

Chronic stress has long been recognized as a major risk factor for depression. Studies have found that depressed patients have high levels of GCs in saliva, blood plasma, cerebrospinal fluid and urine [9-10]. Depressed patients who had long term life stress experience showed that CRH secretion was increased and adrenal glands were enlarged, and also showed hypercortisolemia [11-12], indicating that negative feedback of the HPA

axis is impaired.

Exposure to chronic stressful life events has been observed to be associated with the development of depressive symptoms [13]. Stressful conditions such as divorce, job loss, financial problems and assault were found to be strongly and significantly associated with the risk of onset of depressive episodes [14]. Childhood stress such as physical, sexual, emotional or verbal abuse was found to increase the risk of depression in adulthood [15]. Studies on the prospective course of major depressive disorder (MDD) have shown that childhood parental loss, low self-esteem, chronic financial difficulties and ongoing family conflict are strong proximal predictors of MDD onset and are crucial contributors to recurrence [16-19]. Imaging studies on MDD patients have shown that subjects who exposed to chronic stress showed decreased volumes of prefrontal cortex (PFC) and hippocampus [20-21], and decreased serotonin concentrations at the hippocampal synaptic cleft [22]. Chronic stress impairs brain structure and function, leading to changes of learning, memory, decision-making, and emotional responses, which contribute to depression development [23-25]. It has been reported by imaging studies that the size of hippocampus, PFC and striatum was decreased in MDD patients who suffered from stressful life events [26-27]. These changes may lead to deficits in learning and memory in MDD patients [28].

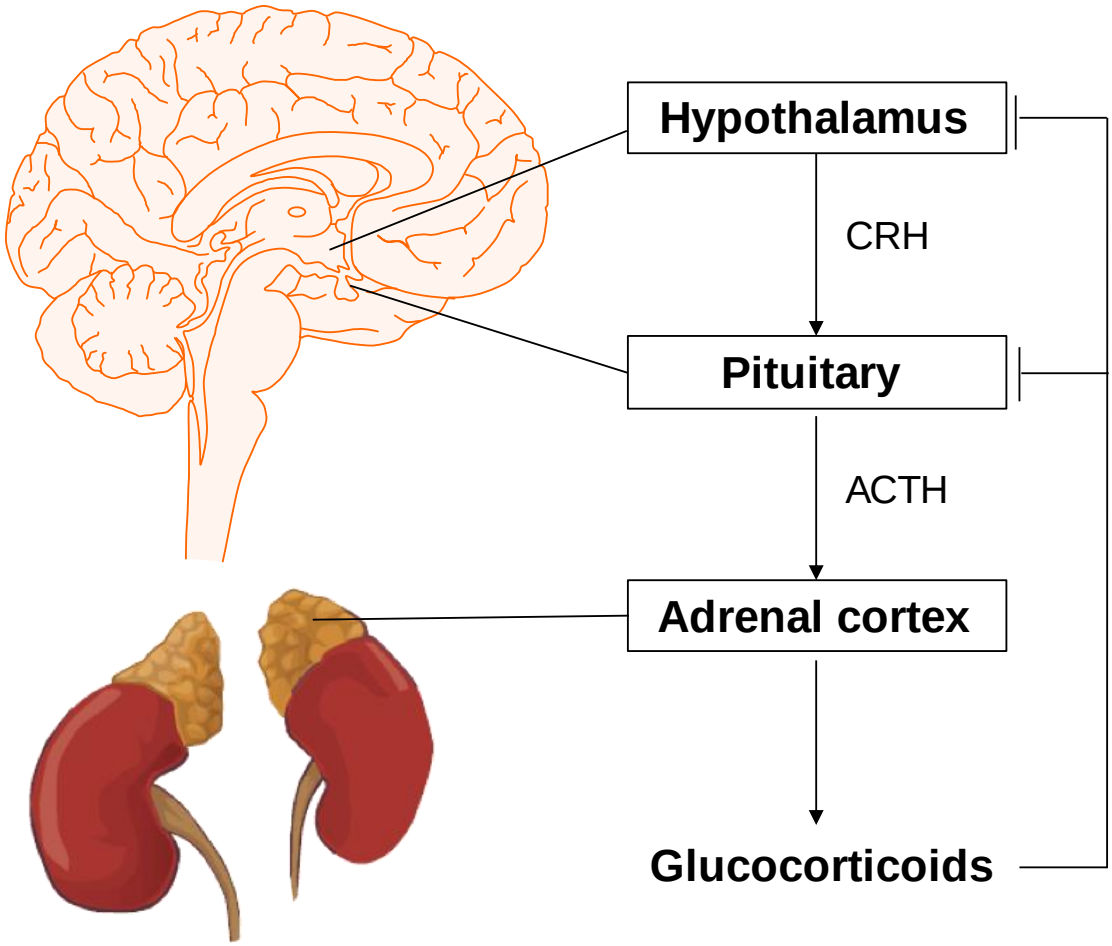

Fig.1 The activation and feedback of hypothalamic-pituitary-adrenal axis activation (HPA) axis. Stress stimulates hypothalamus to release corticotrophin releasing hormone (CRH), which subsequently stimulates pituitary to release adrenocorticotrophic hormone (ACTH). ACTH in turn induces the secretion of glucocorticoids from adrenal cortex. Then glucocorticoids act on hypothalamus and pituitary to inhibit the release of CRH and ACTH as the negative feedback. Under acute stress, this feedback loop maintains glucocorticoids in a normal level. In chronic stress, this feedback is impaired, leading to increased glucocorticoids release. ⊣ ,suppress; → activate

1.2.3 Chronic unpredictable stress animal model in depression research

Animal models are essential tools for studying and understanding human diseases. Much of the current knowledge about pathogenesis of depression has come from studies in animal models. Because chronic stress is a major risk factor for depression, most animal models for depression are stress related. These models include chronic unpredictable stress (CUS), prenatal stress, maternal deprivation, psychosocial stress, and many other stress models.

Face validity, construct validity and predictive validity are three main criteria commonly used to evaluate animal models. Face validity requires that the model has a reasonable degree of symptomatic homology. Construct validity requires that the model has similar causative factors to modeled disease. Predictive validity requires that symptoms of the model can be reversed by pharmacological treatment [29]. Among all the depression models, the CUS paradigm is one of the most commonly used rodent models for depression, which has face, construct and predictive validities [30]. In this model, animals are exposed to a series of different and unpredictable stressors (e.g., foot shock, cold swimming, clamping tail and others) over several weeks to mimic the daily challenges that reportedly provoke the onset of depression in humans.

CUS have been shown to reproduce many of the core behavioral characteristics of

depression, including reduced explorative behavior, impairment of sexual behavior and anhedonia [31]. It was reported that atrophy of the hippocampus occurred in rats exposed to CUS [32-33]. CUS also reduced neurogenesis in the rodent hippocampus [34-35]. CUS was also found to induce significant regression of the apical dendrites of pyramidal cells in medial prefrontal cortex and in CA3 region of the hippocampus in rats [36-37]. These morphological changes are similar to some of the changes seen in post-mortem tissue from patients with MDD. Many studies have shown that multiple classes of antidepressants reverse depressive-like behaviors in CUS animals. For example, tricyclic antidepressants including imipramine, desipramine, and amitriptyline were found to reduce CUS-induced anhedonia in rats [38-41]. Chronic treatment with selective serotonin reuptake inhibitor fluoxetine or serotonin and norepinephrine reuptake inhibitor venlafaxine was found to improve performance in the Morris water maze in CUS rats [42-44], indicating an improvement in spatial learning and memory. In view of all these advantages mentioned above, I have chosen the CUS model for the present study.

1.3 Oxidative stress in chronic stress and depression

1.3.1 Oxidative stress

Oxidative stress is caused by reactive oxygen species (ROS). ROS are ubiquitous, highly reactive, diffusible molecules that are generated within the cell as by-products of aerobic respiration and metabolism. ROS include the superoxide anion ($\cdot O_2^-$), hydrogen peroxide

(H_2O_2), and hydroxyl radicals (OH·). ROS overproduction can cause damage to proteins, lipids, and DNA [45].

The major source of $\cdot O_2^-$ and H_2O_2 is the mitochondrial respiratory system, which is also a target for their damaging effects [46]. The components of mitochondrial respiratory chain include four multimeric complexes (complex I–V). Adenosine triphosphate (ATP) is formed by a process named mitochondrial oxidative phosphorylation (OXPHOS), during which the electrons are transferred from electron donors such as NADH or $FADH_2$ to electron acceptors such as molecular oxygen (O_2) in redox reactions, generating water in complex IV [47]. During the transfer, electrons are leaked as they pass through the chain, mostly at complex I and III, where a small portion of O_2 are converted to $\cdot O_2^-$ radicals. It has been shown that complex I impairment increases ROS production [48-50].

Outside mitochondria, there are also several other pathways involved in the generation of ROS [51-52]. For example, ROS can be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and other oxidases. Redox metals such as $Fe2^+$, $Cu2^+$, and $Mn2^+$ that are essential for electron transfer in many enzymatic reactions, can undergo reactions leading to the production of OH· [53].

Organisms also develop non-enzymatic and enzymatic antioxidant systems to balance

ROS production under physiological conditions. Non-enzymatic antioxidants include ascorbic acid (vitamin C), a-tocopherol (vitamin E) and glutathione (GSH) while enzymatic antioxidant system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and Trx [54]. SOD, the first line of defense against free radicals, catalyzes the dismutation of $\cdot O_2^-$ into H_2O_2. Then H_2O_2 can be transformed into water and O_2 by CAT. Further GPx can catalyze the reaction between GSH and H_2O_2 to generate water and oxidized glutathione (GSSG) [55].

Oxidative stress occurs when production of ROS overwhelms antioxidant defenses. Overproduced ROS may damage proteins, lipids, and DNA, leading to protein cleavage, lipid peroxidation and DNA mutation. Oxidative stress is involved in the pathologies of many diseases such as cancer, diabetes mellitus, cardiovascular, depression, and neurodegenerative diseases. The brain has a high metabolic rate, which means the rate of oxygen consumption is high. On the other hand, the antioxidant levels of brain are relatively low compared to other organs. In addition, neuronal membranes of brain are composed of polyunsaturated fatty acids, which are substrates of ROS. This characteristic makes the brain especially vulnerable to damage caused by oxidative stress [54].

1.3.2 Oxidative stress in chronic stress and depression

Much evidence has shown that oxidative stress is increased in animals exposed to chronic

stress. It has been reported that chronic restraint stress increases ROS production in rat and mouse brain [56-57]. Chronic restraint stress was also found to decrease GSH levels, and activities of SOD, CAT, glutathione transferase and glutathione reductase, but increase oxidative damage to lipids and proteins in rat brain [58-59]. Previously, our laboratory also found that after exposed to CUS for 28 days, rats had significantly lower total antioxidant capacity and lower GPx and CAT activities in hippocampus than the control group [60].

Chronic stress increases secretion of GCs [61-62]. Excessive levels of GCs can lead to oxidative stress [63]. Studies have shown that injections of corticosterone for 3 days decreased activities of SOD and GPx in the rat hippocampus and the cerebral cortex [64]. Injections of corticosterone for 14 days also decreased activities of SOD, CAT and GPx in the rat hippocampus [65]. In our previous study, we also found that after exposed to CUS for 28 days, rats exhibited increased malondialdehyde, which is a marker for lipid peroxidation, in the hippocampus and the frontal cortex [60]. These evidences indicate that oxidative damage can by induced by chronic stress in the brain.

Many studies have shown that oxidative stress is associated with depression. For example, lipid peroxidation products 8-isoprostane and malondialdehyde were found to be significantly increased in the serum of depression patients [66-68]. In addition, studies

also found that oxidative damage to nucleic acids increased in both post-mortem hippocampus and serum of patients with depression [69-71]. Lipid peroxidation and protein oxidation were found to be increased in postmortem brain of patients with manic-depressive bipolar disorder [72-73]. Antioxidant defense system was also found impaired in depression patients. It has been found that plasma vitamin E concentrations in patients with depression were lower than in normal controls [74]. It has also been found that GPx activity in blood was significantly reduced in patients with depression when compared to controls [75]. SOD levels and activity were also found to be decreased in serum of depressed patients [76]. These findings suggest that oxidative stress plays an important role in the development of depression and that the oxidative stress process could be potentially targeted for the treatment of depression.

1.4 Thioredoxin antioxidant system

The thioredoxin (Trx) system is important in defense against oxidative stress. Trx system mainly includes Trx, thioredoxin reductase (TrxR) and thioredoxin interacting protein (Txnip). Dysregulation of this system in humans is found to be involved in metabolic, cardiovascular, malignant, and neurodegenerative disorders.

1.4.1 Introduction to thioredoxin

Trx is a 12 kDa reductase consisting of four α-helices and five β-sheets that is a common

structure among a variety of different organisms. There are three distinct isoforms of Trx, encoded by separate genes: cytosolic Trx (Trx1), mitochondrial Trx (Trx2), and spermatid-specific thioredoxin (Trx3), with catalytic Cys -32 and -35 for Trx1, Cys-90 and -93 for Trx2, and Cys-55 and -58 for Trx3 [77-78]. Among the Trxs, Trx1 was the first identified and is the best characterized. In the present study, we investigated changes in Trx1, observed in an animal model for depression. Beside Cys -32 and -35, mammalian Trx1 has three extra cysteine residues that are cys -62, -69, and -73 [79-80]. These cysteine residues are also responsible for regulating Trx activity by undergoing post-translational modifications including S-nitrosylation, glutathionylation, and dimerization [81-85].

1.4.2 Thioredoxin function

Thioredoxin and protein cysteine oxidative modification: Various crucial cellular functions such as redox homeostasis, signal transduction, gene transcription, enzymatic reaction, metal binding, and structural stabilization are regulated by protein cysteine residues. The cysteine residue is highly nucleophilic and redox sensitive. The thiol (-SH) group in cysteine residue is sensitive to be attacked by H_2O_2 and NO· radical [86-89].

H_2O_2 can oxidize thiols of cysteine to generate reversible sulfenic acid (sulfenylation), subsequently forming a reversible disulfide bond with a nearby thiol (disulfidation).

Cysteine sulfenic acid can also be over oxidized to produce irreversible sulfinic acid (sulfinylation) or sulfonic acid (sulfonylation) (**Fig 2A**). Reversible oxidation of cysteine residues can trigger structural and functional changes in proteins, which is involved in the alteration of signal transduction [90-93]. Trx can transfer electrons from its reactive cysteines thiol to reduce reversible protein sulfenic acid or disulfide to thiol. Therefore, Trx can maintain a protein cysteine thiol group at a reduced state.

Peroxiredoxin (Prx) as an antioxidant enzyme is important in controlling peroxide levels, which also plays an important role in defending the cell against oxidative stress. When Prx reduces peroxides, such as H_2O_2, Prx is oxidized. Trx can reduce oxidized Prx [94-95]. Therefore, Trx can recharge Prx, facilitating Prx-induced scavenging of peroxides.

The NO· radical can induce reversible nitrosylation of cysteine thiol, and cause nitrosative stress (**Fig 2B**). In a cellular environment, Trx can also function as either a denitrosylase or a transnitrosylase in defense against nitrosative stress. Cys 32 of Trx1 is responsible for the denitrosylation while Cys 73 is for transnitrosylation [96-97]. In denitrosylation, Trx reduces cysteine nitrosylated thiol to free thiol through Trx dithiol moiety [98-99]. In transnitrosylation, Trx transfers NO· from nitrosylated thiol on one protein to a reactive cysteine residue on another protein, forming S-nitrosylated and

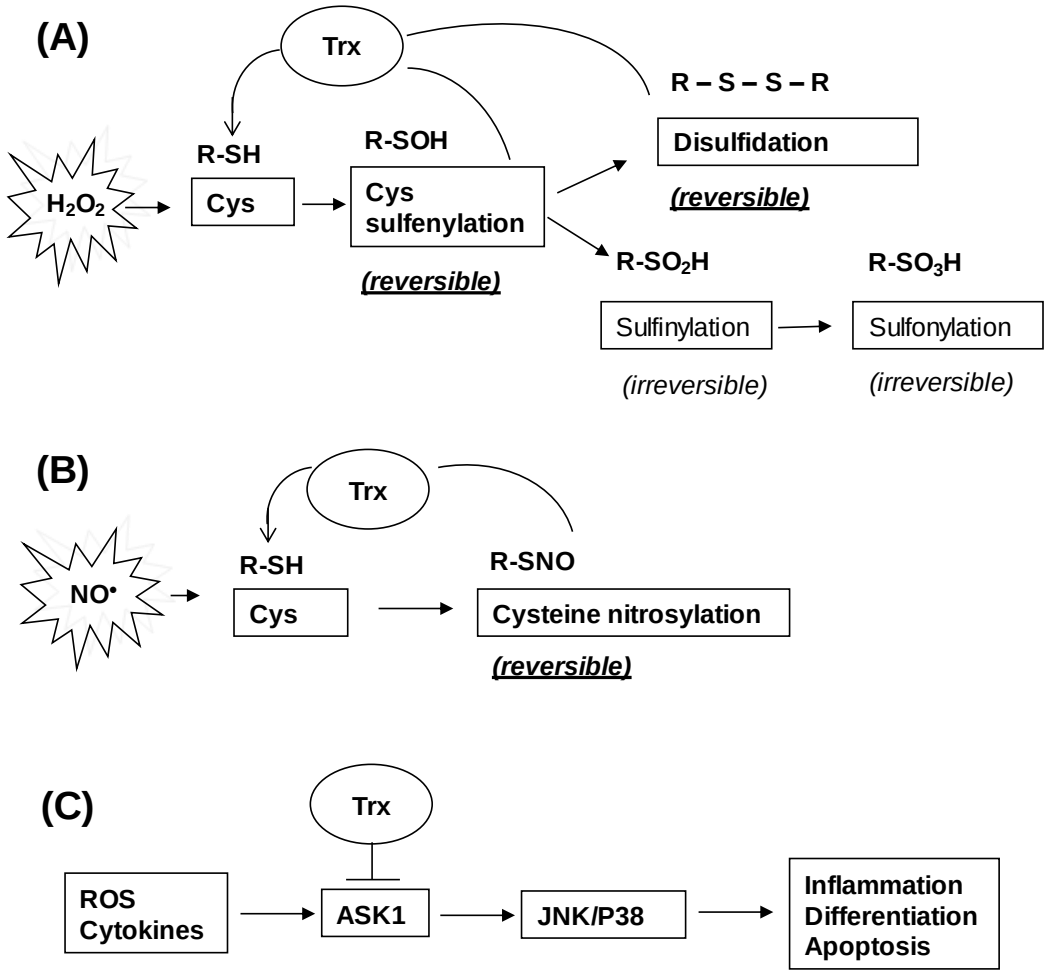

Fig 2. **Function of thioredoxin**. H2O2, hydrogen peroxide; R-SH, thiol group; R-SOH, sulfenic acid; R-S-S-R, disulfide bond; R-SO2H, sulfinic acid; R-SO3H, sulfonic acid; NO• nitric oxide radical; R-SNO, nitrosylated thiol; Trx, thioredoxin; ROS, reactive oxygen species; ASK1, apoptosis signal-regulating kinase 1 ; JNK, c-Jun N-terminal kinase; P38, P38 mitogen-activated kinase

denitrosylated proteins [100-101]. Abnormal S-nitrosylation of specific proteins leads to cell destructive processes and bioenergetic failure, which would trigger neuropathology such as compromised neural function, impaired neurogenesis, and neurodegeneration, which may be involved in illnesses such as schizophrenia, bipolar disorder, and major depression [102-106].

Thioredoxin and apoptosis signal-regulating kinase 1: Stress and inflammatory cytokines activate apoptosis signal-regulating kinase 1 (ASK1), inducing ASK1 Thr845/Thr838 autophosphorylation, subsequently activating c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase (p38) by phosphorylation, which promotes inflammation, differentiation or apoptosis. Under basal conditions, Cys32 or Cys35 in reduced form of Trx can interact with Cys250 of ASK1 to form a complex via an intermolecular disulfide bridge, resulting in inhibiting ASK1 kinase activity and preventing JNK/p38 activation, which inhibits ASK1/JNK/p38-mediated stress and inflammatory signaling [107-110] **(Fig 2C).** ROS, inflammation and other stressors can release ASK1 from Trx and induce ASK1/JNK/p38 activation. Trx1 not only reduces protein cysteine oxidation, but also regulates cell apoptotic pathway by inhibiting ASK-1. In addition, Trx1 can regulate gene expression, signal transduction, and immune function. This multifunctional protein is implicated in many human diseases including cardiovascular diseases, stroke, inflammation, immune diseases, metabolic syndrome,

cancer and neurodegenerative diseases [111-112].

1.4.3 Thioredoxin regulatory proteins

Trx in a reduced state is maintained by TrxR **(Fig 3)**. In the presence of TrxR, the dithiol moieties of Trx are reduced by receiving electrons from NADPH. TrxR is a selenium containing, homodimeric flavoprotein oxidoreductase. Like Trx, mammalian TrxRs also have three isoforms, which are cytosolic TrxR1, mitochondrial TrxR2, and a testis-specific TrxR3 [113-114]. TrxR have a C-terminal extension containing sequence Gly-Cys-Sec-Cys-Gly with the essential Sec residue being essential for catalytic activity, which forms a selenenylsulfide in the oxidized enzyme.

Txnip is an endogenous Trx inhibitor belonging to the α-arrestin protein family. It inhibits Trx reducing activity by interacting with the active center of Trx **(Fig 3)**. Under normal conditions, Txnip is located in the nucleus, whereas in response to oxidative stress, Txnip can shuttle into cytosol or mitochondria to bind with and inhibit Trx1 and Trx2. Cys247 on Txnip can bind to Cys32 on Trx1 and then form a mixed disulfide bond with the Trx catalytic center, which will suppress the activity of Trx, contributing to oxidative stress [115-116].

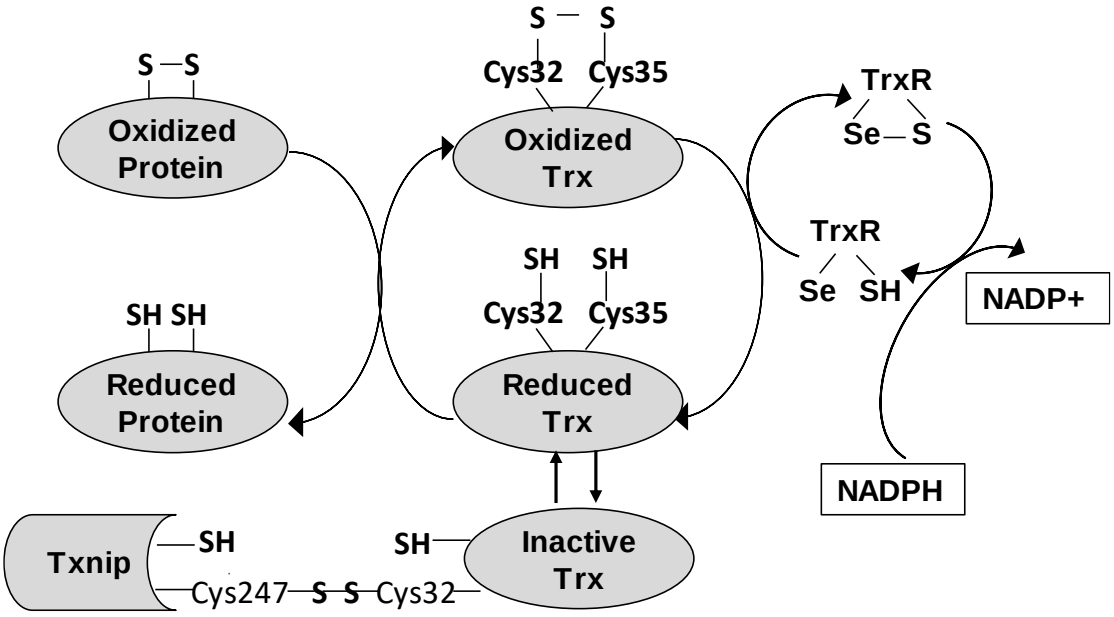

Fig 3. The function of the Trx system. The dithiol moieties of Trx are reduced by receiving electrons from NADPH in the presence of TrxR. Reduced Trx in turn reduces oxidized proteins with disulfide bonds through thiol–disulfide exchange reactions. Trx also binds with ASK1 and inhibits ASK1 activation, thereby inhibits the down stream JNK/p38 stress signaling pathway activation. . Txinp, an endogenous Trx inhibitor, inhibit Trx function by binding to Trx active center. Trx: thioredoxin; TrxR thioredoxin reductase; Txnip: thioredoxin interacting protein.

1.4.4 Trx system in chronic stress and depression

There are several studies focused on Trx in chronic stress and depression. For example, one study reported that Trx protein levels were increased in the hippocampus of rats exposed to chronic intermittent cold stress for 14 days [117]. It has also been found that Trx levels in serum were decreased in patients with bipolar manic episodes as compared to healthy subjects [118]. However, it was reported that plasma Trx levels were not significantly changed in patients with treatment-resistant depression when compared to healthy subjects [119]. Recently our laboratory found that chronic treatment with the stress hormone corticosterone increased Txnip levels in cultured neuronal cells [120]. Although the results are inconclusive, it suggests that Trx, and possibly its regulatory proteins, contribute roles to chronic stress and depression.

1.5 Summary and research questions

Chronic stress is a major risk factor for depression. Many studies have shown that chronic stress increased ROS production, impaired antioxidant defense activity, and increased oxidative damage to proteins, lipids, and DNA in rodent brain. In post-mortem brain and serum of depression patients, studies also found that oxidative damage was increased, suggesting oxidative stress is associated with chronic stress and depression. Trx is an oxidoreductase enzyme and plays an important role in defense against oxidative stress. Trx can reduce protein cysteine oxidative modification such as sulfenylation and

nitrosylation, and also inhibit the ASK1 stress and inflammatory signaling pathway. Trx reducing state is maintained by TrxR, while Trx activity can be inhibited by the Trx endogenous inhibitor Txnip. Our research questions are whether Trx, TrxR, and Txnip are regulated by chronic stress and whether these proteins can be targeted for the treatment of depression.

1.6 Objectives of my project

My project focuses on understanding the role of Trx system in chronic stress. The objectives are:

1) ***To verify if mice exposed to CUS exhibit depressive-like behaviours.***
2) ***To determine the levels of Trx, TrxR, and Txnip in brain of mouse exposed to CUS.***
3) ***To determine if CUS induces protein sufenylation and nitrosylation in mouse brain.***
4) ***To determine if CUS activates ASK1 signaling pathway in mouse brain.***

CHAPTER 2: MATERIALS AND METHODS

2.1 Chronic unpredictable stress model

Eight-week-old male C57BL/6 mice weighing approximately 20-28g 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 12:12h 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 included restraint, cold swimming, overnight illumination, foot shock, tail clamping, and others, as indicated in **Table 1**. Behavioral tests started at day 29, 24h after the last stressor. All behavioral tests were performed in the light phase between 09:00 and 17:00. After behavioral tests, mice hippocampus, frontal cortex, nucleus accumbens, amygdala and cerebellum 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 Evaluation of depressive-like behaviours

Three different behavioural tests including open-field test, forced-swim test and sucrose preference test were used to evaluate the mice depressive-like behaviours. Open-field test

Table 1: Daily Schedules for the Chronic Unpredictable Stress

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

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

was performed at day 29, 24h after final stressor; forced-swim test was performed at day 30; while sucrose preference test was performed on day 31.

2.2.1 Open-field test

The open-field test was performed in a black plexiglas square box (50× 50× 50 cm) **(Fig.4)**. Before testing, all the mice were placed in the behavior room for at least 30 min. Warm red overhead lighting was put inside the room. Mice were placed in the center of the testing box and allowed to explore freely for 5 min, and then returned to their home cages. Mice activity was recorded with a video camera connected to the computer. The distance moved, resting time (a period of inactivity greater than or equal to 1 second), number of rearing (frequency with which the mice stood on their hind legs without support), time spent in the central zone (30 cm diameter) and frequency of center entries were analyzed. The total distance and resting time were analyzed as measures of locomotor ability. The number of rearing, time in the inner zone, and frequency of center entries were analyzed as measures of exploratory behavior.

2.2.2 Forced-swim test

Mice were individually placed into a glass cylinder (19 cm diameter, 23 cm deep, filled with 23–25°C water) **(Fig.4)**. 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 pre-testing handle and room lighting were the same as open-field test. 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. This represents behavioral despair, a typical symptom of depressive-like behavior. Immediately after the test, mice were covered by a dry towel and then placed under a heating lamp until they were dry.

2.2.3 Sucrose preference test

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

2.3 Protein extraction

Mice were first placed in an isoflurane chamber for 20s followed by an immediate decapitation and then their brains were removed. Hippocampus, frontal cortex, nucleus accumbens, amygdala, and cerebellum were dissected using a dissecting

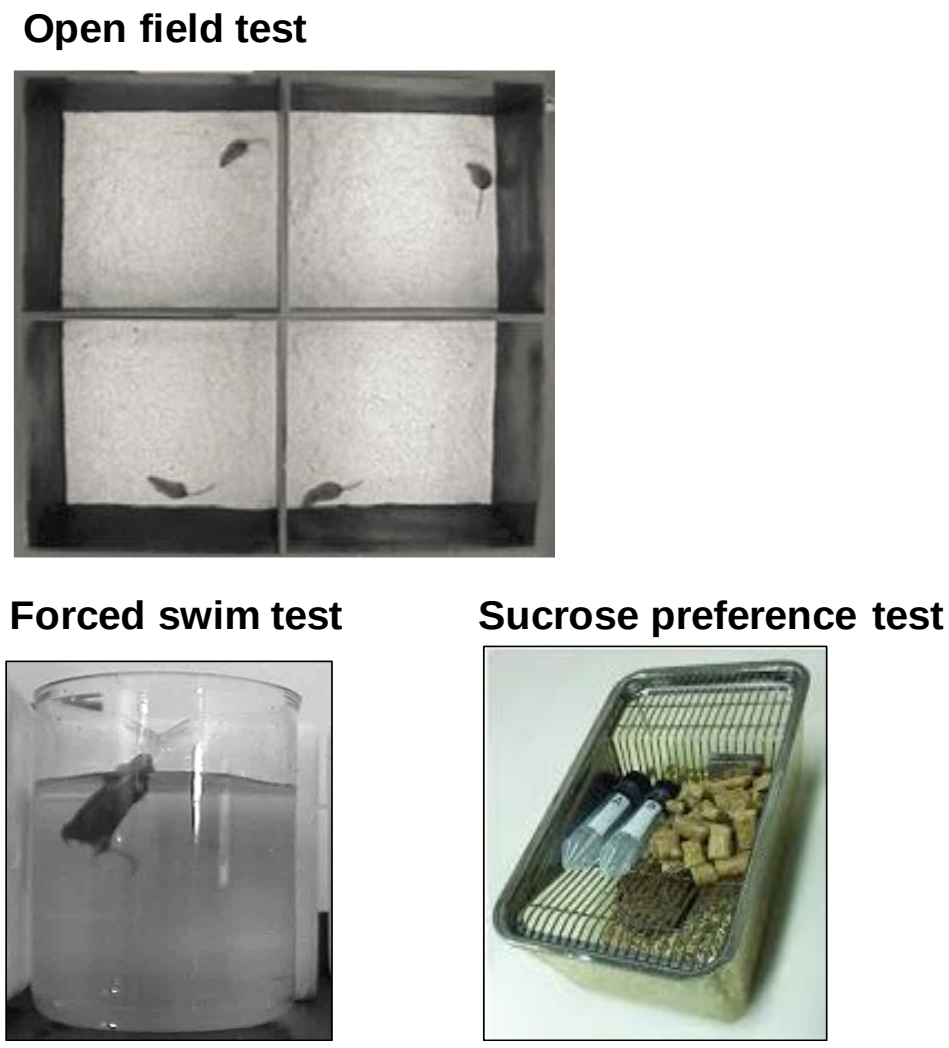

Fig 4. Behavioral apparatuse. Calibration image of the open field apparatus as viewed in EthoVision XT system. The open field apparatus consists of a bare square box (50x50x50cm). Four boxes were put together. A tracking camera was fixed above the boxes. Image forced swim apparatus. The glass cylinder is 19 cm diameter, 23 cm deep, filled with 23–25°C water. Sucrose preference is performed by two identical bottles containing 1% sucrose and plain water respectively. The bottles was switched position half way the test.

microscope according to an anatomical atlas on ice [173]. Brain samples dissected were frozen immediately with dry ice and stored at -80 °C. Decapitation and dissection processes took approximately 2 min for each mouse. The tissue was homogenized in 10:1 (ml/g) ice-cold lysis buffer containing 250mMNaCl, 30mM MgCl2, 20mM HEPES (pH 7.5), 0.5mM EDTA, 20% glycerol, 1% nonidet P40, 0.1mM EGTA, and 1X protease inhibitor cocktail (Thermo Scientific, Marietta, OH, USA). The homogenized tissues were kept on ice for 1 hour and then centrifuged at 10,000x g 4°C for 15 min. The supernatants were then collected as protein extract. The Bradford protein assay was used to determine protein concentrations [121].

2.4 Immunoblotting analysis

Protein samples were then mixed with a loading buffer containing 100mM 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 hour at 120 V and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) at 200 mA for 1 hour. After transfer the membranes were first blocked with 5% milk in Tris-buffered saline with Tween-20 containing 10mM Tris-HCl (pH 7.5) and 0.1% Tween-20 at room temperature for 1hour, 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 Inc., Toronto, ON, Canada),

1:200 dilution of rabbit polyclonal ASK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or 1:100 dilution of rabbit polyclonal phospho-ASK1 antibody (Santa Cruz Biotechnology) overnight at 4°C. Then the membrane blots were further incubated with secondary antibodies conjugated to horseradish peroxidase (Abcam, Eugene, Oregon, USA) for 1 hour at room temperature. Enhanced chemiluminescence reagents (PerkinElmer,Waltham, MA, USA) were used to detect immunoreactive bands and the ChemiDoc MP System (Bio-Rad, Dreieich, Germany) was used to capture imaging. Image Lab software (Bio-Rad) was used to quantify band signal intensity.

2.5 Dimedone conjugation assay for detection of cysteine sulfenylated proteins

Cysteine sulfenylated proteins were detected by dimedone conjugation using anti-cysteine sulfenic acid antibody (Millipore). Fifteen µg of protein was incubated with 2mM dimedone in a buffer containing 0.1% Triton-X100, 12mM Na_2HPO_4, 3mM citric acid, pH 6.0 for 1 hour. Then protein samples were mixed with a loading buffer containing 10mM N-ethylmaleimide, separated in 12% SDS polyacrylamide gels. Sulfenylated proteins were detected by immunoblotting analysis with anti-cysteine sulfenic acid antibody.

2.6 Biotin Switch for detection of cysteine nitrosylated proteins

Cysteine nitrosylated proteins were detected by a biotin-switch assay as previously described [122]. First, a thiol-specific methylthiolating agent methyl methanethiosulfonate (MMTS) was used to block free thiols in unmodified cysteine residues. fifty μg of protein was incubated with a blocking solution containing 40mM MMTS, 2.5% SDS, 250 mM HEPES pH 7.7, 1 mM EDTA and 0.1 mM neocuproine at 50°C for 40 min, then the samples were further incubated with 1ml of acetone at –20°C for 40 min. After that, samples were centrifuged at 13,000x g, at 4°C for 15 min, and then 15μl of HEN buffer (250mM HEPES pH 7.7, 0.1mM neocuproine and 1mM EDTA) with 1% SDS was used to resuspend the pellets. Second, 250 μl of 50 mM ascorbate was used to reduce back the nitrosylated thiols in cysteine to free thiols and then N-[6-(biotinamido) hexyl]-3'- (2'-pyridyldithio) propionamide (biotin-HPDP) was used to label those free thiols. The samples were then incubated with 4mM biotin-HPDP and 50mM ascorbate at room temperature for 1 hour.

2.7 Statistical analysis

IBM SPSS 24.0 software (IBM, Armonk, New York, USA) was used to perform statistical analysis. All results were expressed as mean ± standard error of the mean (SEM). Experimental data were compared using Student's *t*-test and considered statistically significant when $P < 0.05$.

CHAPTER 3: RESULTS

3.1 The effect of CUS on exploratory, despair and anhedonic behaviours

After a 28 days CUS paradigm, open field test, forced swim test, and sucrose preference test were performed on mice to verify whether CUS induces decreased exploratory, despair, and anhedonia depressive-like behaviors.

As shown in **Fig. 5A**, open field test showed that mice exposed to CUS exhibited no changes in the distance moved ($p=0.0640$), but these mice exhibited increased resting time ($p=0.0002$), and decreased number of rearing ($p=0.0002$), frequency of center entry ($p=0.0002$) and time in center zone ($p=0.0005$) when compared to controls. Sucrose preference test showed a reduced sucrose preference ($p=0.0010$) **(Fig. 5B)** and forced swim test showed an increased immobility ($p=0.0205$) **(Fig. 5C)** in mice exposed to CUS when compared to controls. These results suggest that CUS can reduce exploratory, increase anhedonic and despair depressive-like behaviours in mice.

3.2 The effect of CUS on Trx, TrxR and Txnip protein levels in mouse brain

First, the amount of protein used in immunoblotting analysis for Trx, TrxR, and Txnip levels was determined. Protein at amounts of 3.25, 7.5, 15, and 30ug from mice cerebral cortex were loaded in 12% of SDS-PAGE gel. Trx, TrxR, and Txnip levels were measured by immunoblotting analysis. I found that TrxR band can be identified in

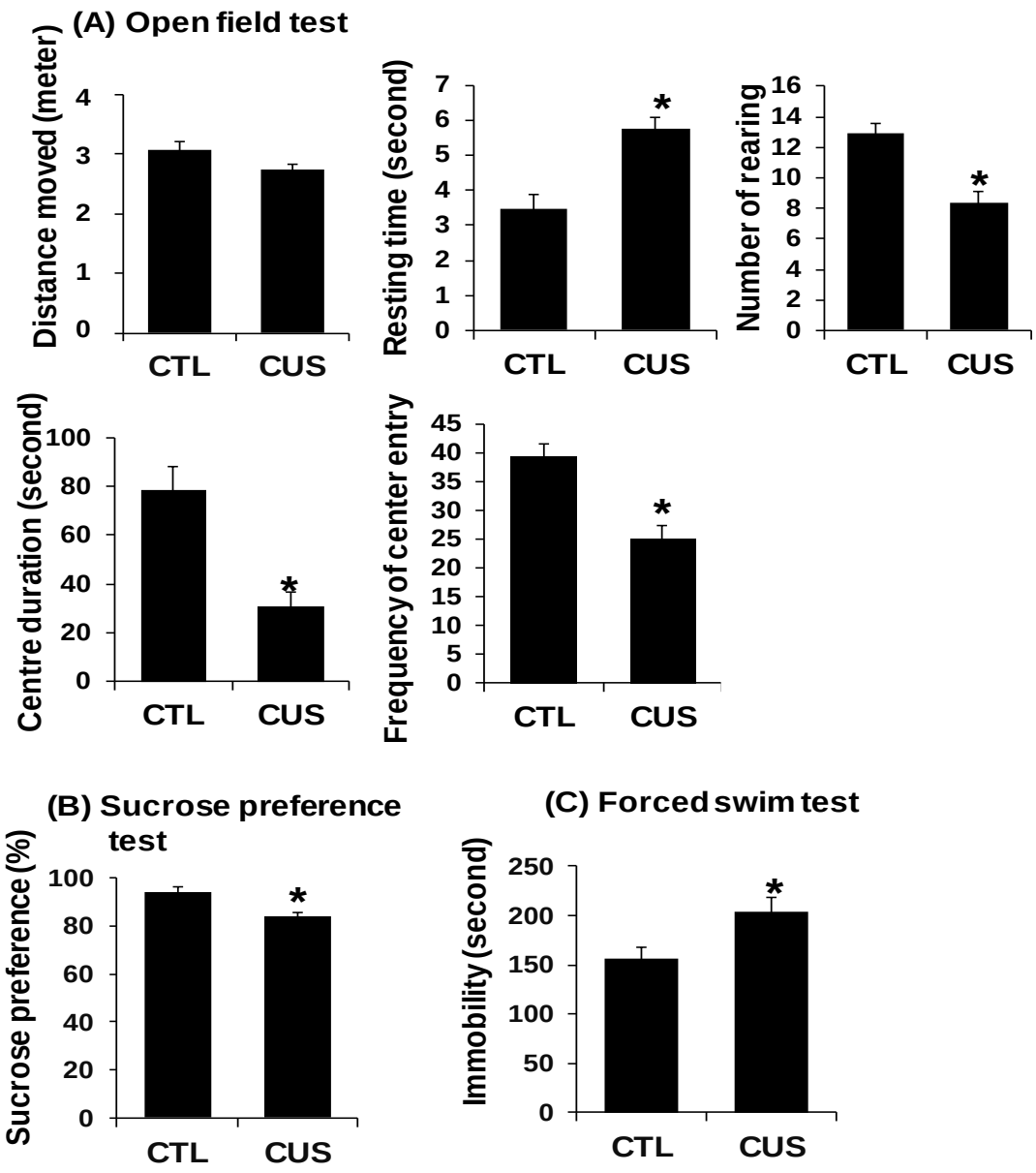

Fig 5. Effects of chronic unpredictable stress (CUS) on mouse behavioral changes. The distance moved, resting time, number of rearings, time spent in the central zone and frequency of center entries were recorded during open field test (A); sucrose preference was recorded during sucrose preference test (B); and immobility was recorded during forced swim test (C). Data are displayed as mean ± SEM (N= 12-13). * indicates $P < 0.05$ by T-test.

cultured cells, but not in mouse brain tissue. Therefore, I only measured Trx and Txnip protein levels. The 12 KD band was identified as Trx **(Fig 6A)**, while 55 KD band was identified as Txnip **(Fig 6B)**. Intensity of Trx and Txnip protein bands was positively correlated with protein amounts. Based on these results, I used 15 μg of protein for Trx and Txnip studies in mouse brain tissue.

The hippocampus is responsible for learning and consolidation of memories. It is a highly stress-sensitive brain region and is associated with cognitive and affective symptoms of depression [123]. Structural imaging studies have shown that hippocampal volumes in MDD patients were approximately 4–6% smaller than matched control subjects [124]. Studies also reported that chronic stress can lead to hippocampal atrophy in rodent [125]. Furthermore, studies reported that the reduced volume of hippocampus is related to the levels of depressive symptoms, such as memory lost and the time and duration of mood episode [126-127]. Frontal cortex is related to mood control, cognition and motor functions. Studies on animal models have reported that chronic stress can cause reduced volumes of frontal cortex [128-129]. Both post-mortem and imaging studies have reported volume reductions of frontal cortex in depressed patients [130-131]. Functional magnetic resonance imaging (fMRI) study has shown that in depressed patients performing cognitive tasks the activity level of frontal cortex was reduced [132]. Trx and Txnip protein levels were measured in hippocampus and frontal cortex of mice exposed

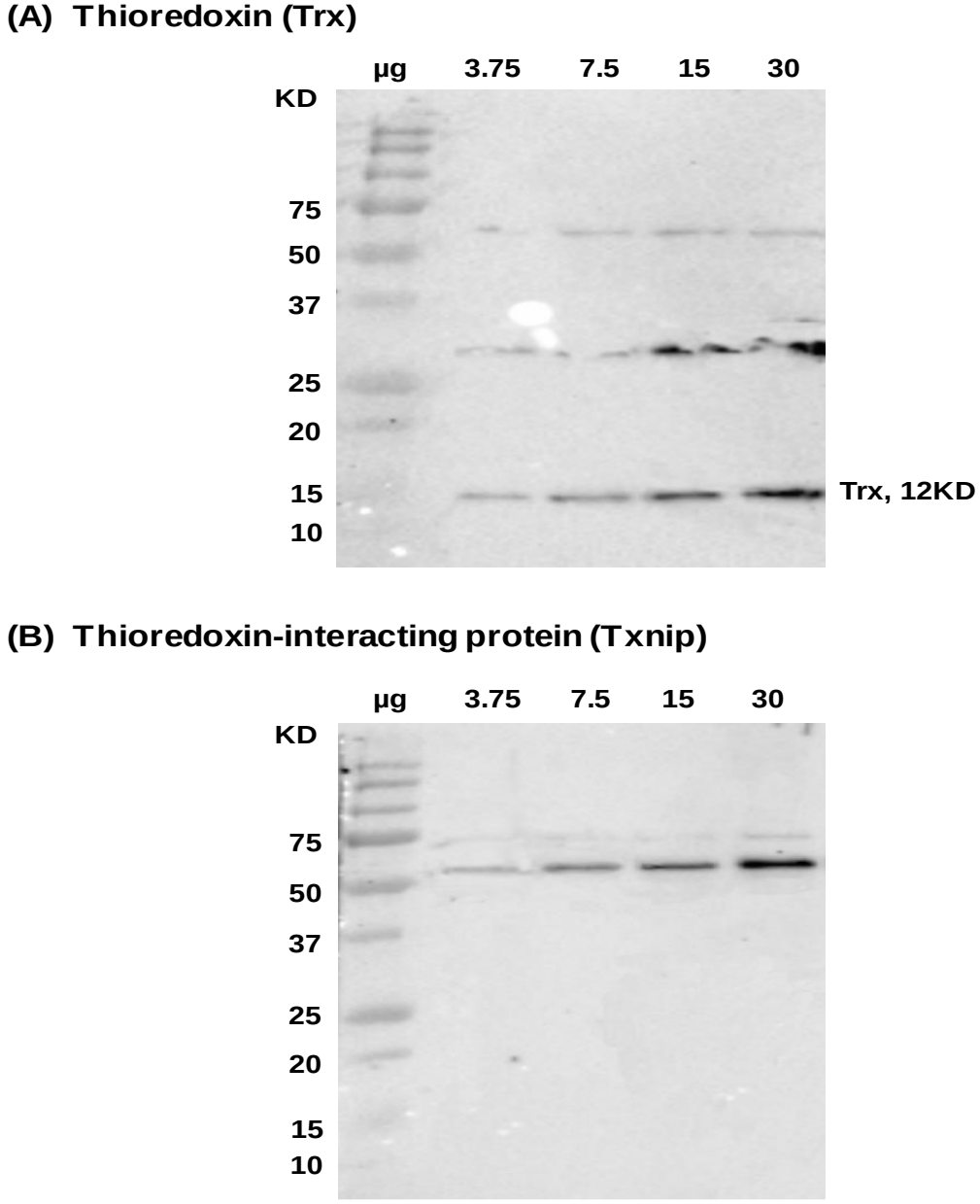

Figure 6. Whole blot of Thioredoxin (A) and Thioredoxin-interacting protein (B). Protein at amount of 3.25, 7.5, 15 and 30µg from mouse cerebral cortex were loaded in 12% of SDS-PAGE gel. Immunoblotting analysis was performed using primary antibody of rabbit monoclonal Trx1 (1:1500 dilution, Cell Signaling Technology, Danvers, MA, USA) (A) or rabbit monoclonal Txnip (1:2000 dilution, Abcam Inc., Toronto, ON, Canada) (B).

to CUS. As shown in **Fig. 7,** in both hippocampus (**Fig. 7A)** and frontal cortex **(Fig. 7B),** there is no significant difference of Trx protein levels between mice exposed to CUS and control group. However, Txnip protein levels were significantly increased in hippocampus (**Fig. 8A**) and frontal cortex (**Fig. 8B**) of mice exposed to CUS when compared to controls. CUS increased Txnip levels by 36.18% ± 0.26 ($p=0.0013$) in hippocampus and by 41.67% ± 0.33 in frontal cortex ($p=0.0079$). These results suggest that CUS can up-regulate Txnip in mouse hippocampus and frontal cortex.

The nucleus accumbens is involved in the mechanisms of natural reward; its dysregulation is thought to relate to anhedonia in depression [133-134]. Animal studies reported that chronic stress causes long-term adaptations of the accumbens reward circuit [135-136] contributing to its dysregulation in MDD [134]. Neuroimaging studies reported depressed patients have attenuated activation of nucleus accumbens in response to positive visual stimuli [137-138]. The function of the amygdala includes producing fear, anxiety, social withdrawal, and sympathetic autonomic arousal. Different from the changes in hippocampus and frontal cortex, chronic stress can increase the size and activity of amygdala [139]. Animal studies have reported that chronic stress can enhance amygdala-dependent fear learning, anxiety and enhance synaptic plasticity, dendritic length, and branching of amygdala neurons [140-142]. Structural imaging studies on MDD patients have found those patients had increased amygdala volume [143-145].

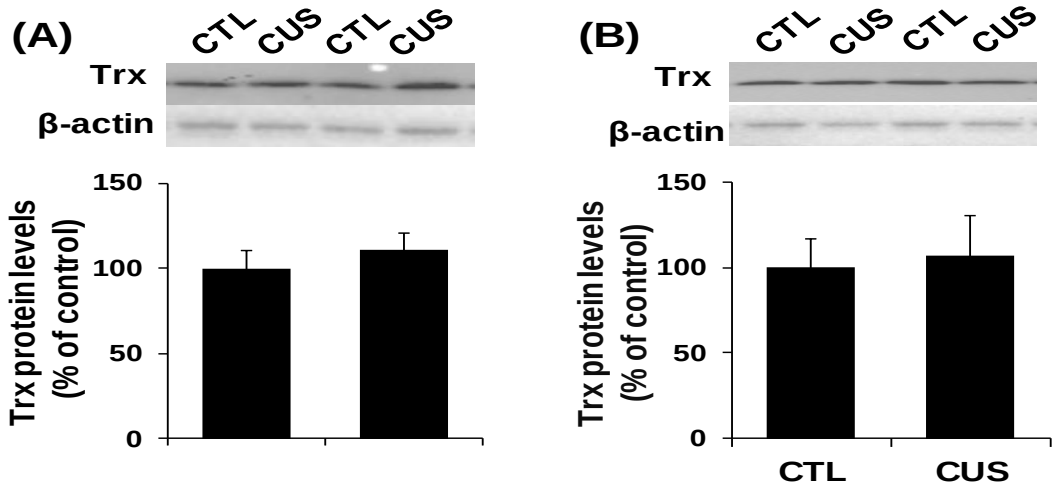

Fig. 7: Effects of chronic unpredictable stress (CUS) on Trx ptotein levels in mouse hippocampus (A) and frontal cortex (B). Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Trx protein levels were measured using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test.

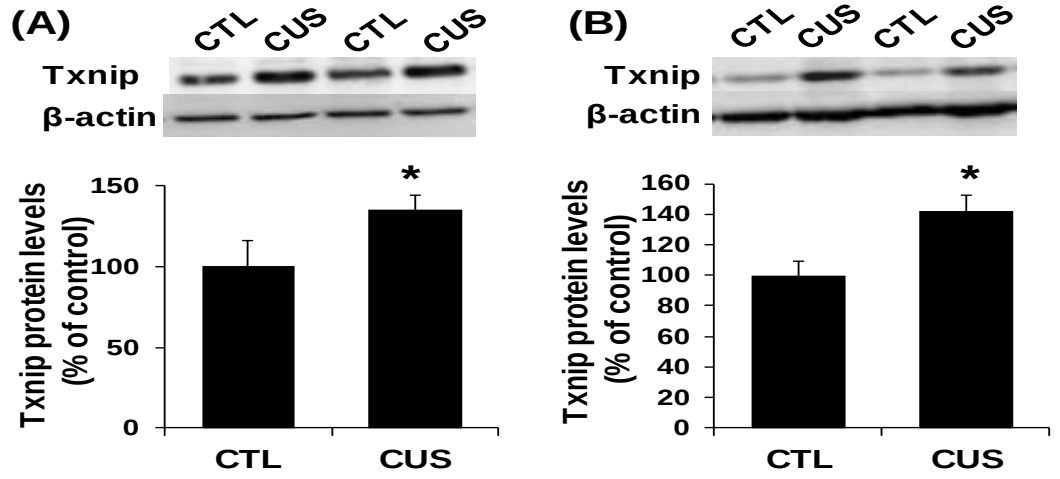

Fig. 8: Effects of chronic unpredictable stress (CUS) on Txnip ptotein levels in mouse hippocampus (A) and frontal cortex (B). Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Txnip protein levels were measured using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test.

Besides, MDD patients are also found to have hyperactive amygdala function which correlates with the intensity of negative affect [146-147]. Txnip protein levels were further measured in nucleus accumbens and amygdala of mice exposed to CUS. The cerebellum was used as a negative control. As shown in **Fig. 9**, Txnip protein levels were significantly increased in nucleus accumbens of mice exposed to CUS when compared to the control group, but Txnip protein levels were not significantly changed in the amygdala and cerebellum. CUS increased Txnip levels by 21.38% ± 0.23 in mouse nucleus accumbens ($p=0.0303$)

3.3 The effect of CUS on protein sulfenylation and nitrosylation in mouse hippocampus and frontal cortex

Because Txnip is a Trx endogenous inhibitor, increased Txnip may further inhibit Trx activity, subsequently exacerbating cysteine oxidative modification such as sulfenylation and nitrosylation. Therefore, protein cysteine sulfenylation and nitrosylation were further measured in hippocampus and frontal cortex of mice exposed to CUS.

As shown in **Fig. 10**, sulfenylated protein levels were significantly increased in hippocampus and frontal cortex of mice exposed to CUS when compared to controls. CUS increased sulfenylated protein levels by 30.66% ± 0.34 ($p=0.0387$) in hippocampus and by 42.71% ± 0.34 ($p=0.0053$) in frontal cortex. Nitrosylated protein levels were also

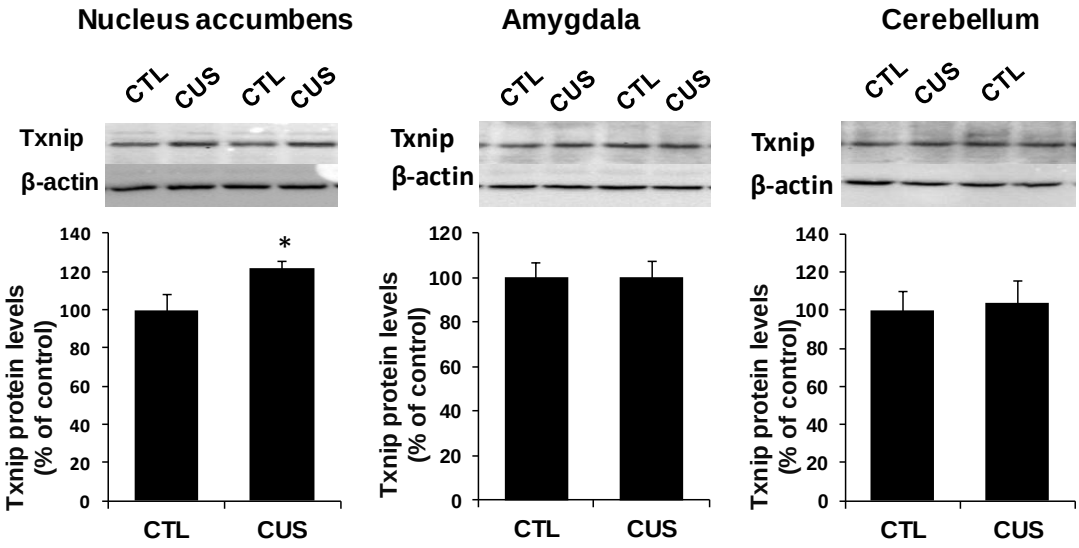

Fig. 9: Effects of chronic unpredictable stress (CUS) on Txnip protein levels in mice nucleus accumbens, amygdala and cerebellum. Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Txnip protein levels were measured using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test.

increased in the same brain regions of CUS mice. CUS increased nitrosylated protein levels by 36.25% ± 0.27 ($p=0.0010$) in hippocampus and by 39.12% ±0.46 ($p=0.0477$) in frontal cortex (**Fig. 11**). These results suggest that CUS increases protein cysteine sulfenylation and nitrosylation in mouse brain.

3.4 The effect of CUS on phosphorylation of ASK1, JNK, and p38 in mouse hippocampus and frontal cortex

Txnip can compete with ASK1 to bind to Trx. Increased Txnip may lead to dissociation of ASK1 from the Trx complex. Dissociated ASK1 will be activated by auto-phosphorylation. Therefore, protein phosphorylation of ASK1 was further measured in hippocampus and frontal cortex of mice exposed to CUS. Phosphorylated ASK1 protein levels were measured by immunoblotting analysis with a phosphor-ASK1 antibody.

As shown in **Fig. 12**, total ASK1 levels in hippocampus and frontal cortex were not different between CUS and control groups; the levels of phosphorylated ASK1 in the same brain region were significantly increased in CUS group when compared to controls. CUS increased phosphorylated ASK1 protein levels by 155.22% ± 0.63 ($p<0.01$) in hippocampus and by 106.61% ± 0.19 ($p<0.01$) in frontal cortex.

ASK1 phosphorylation further triggers phosphorylation of JNK and p38. Previously chronic stress was found to induce JNK and p38 phosphorylation in the mouse brain [148]. Therefore, JNK and p38 phosphorylation was also verified in the brains of mice exposed to CUS. As shown in **Fig 13,** the levels of phosphorylated JNK and p38 in the hippocampus and frontal cortex were significantly increased in CUS group when compared to controls. CUS increased phosphorylated JNK protein levels by 69.48% ± 0.31 ($p<0.01$) in hippocampus and by 59.86% ± 0.36 ($p<0.01$) in frontal cortex. CUS increased phosphorylated p38 protein levels by 15.42% ± 0.18 ($p=0.04$) in hippocampus and by 18.82% ± 0.19 ($p=0.025$) in frontal cortex.

These results suggest that CUS increases phosphorylation of ASK1, JNK and p38 in mouse brain.

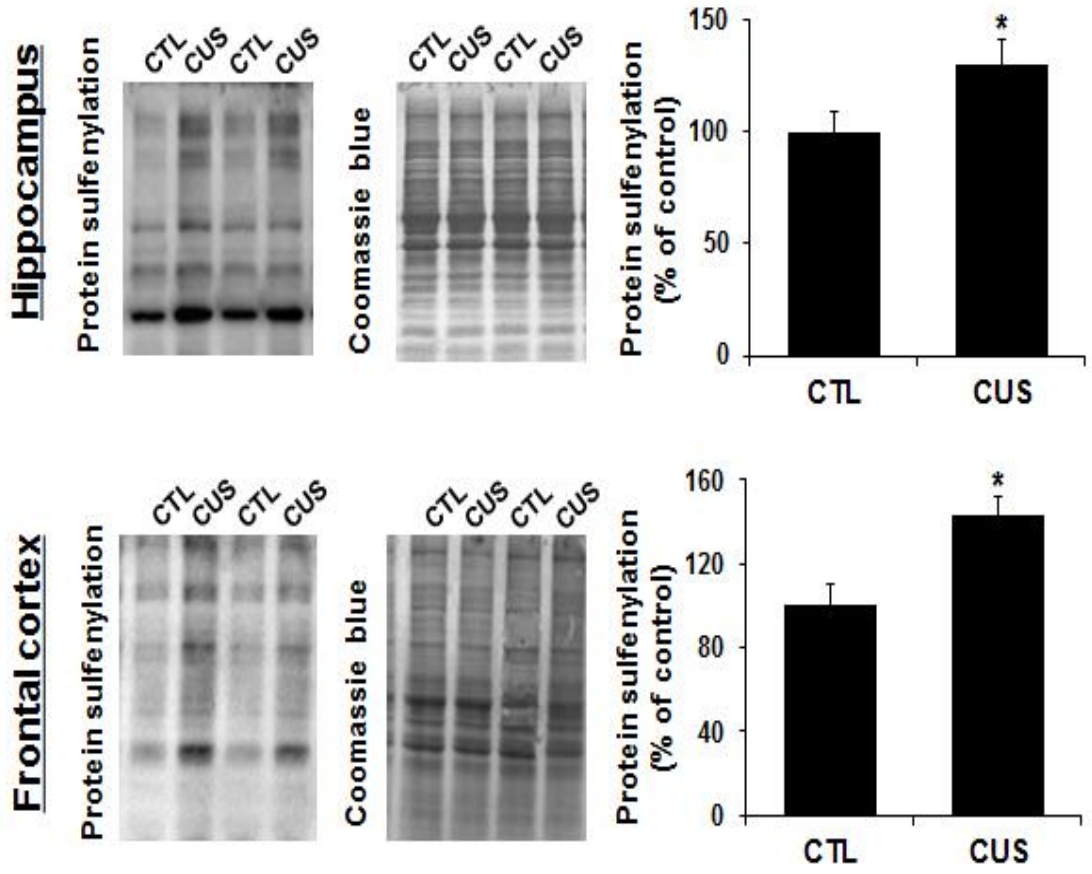

Fig. 10. Effects of chronic unpredictable stress (CUS) on protein sulfenylation in mouse hippocampus and frontal cortex. Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Sulfenylated protein was measured by dimedone conjugation followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test.

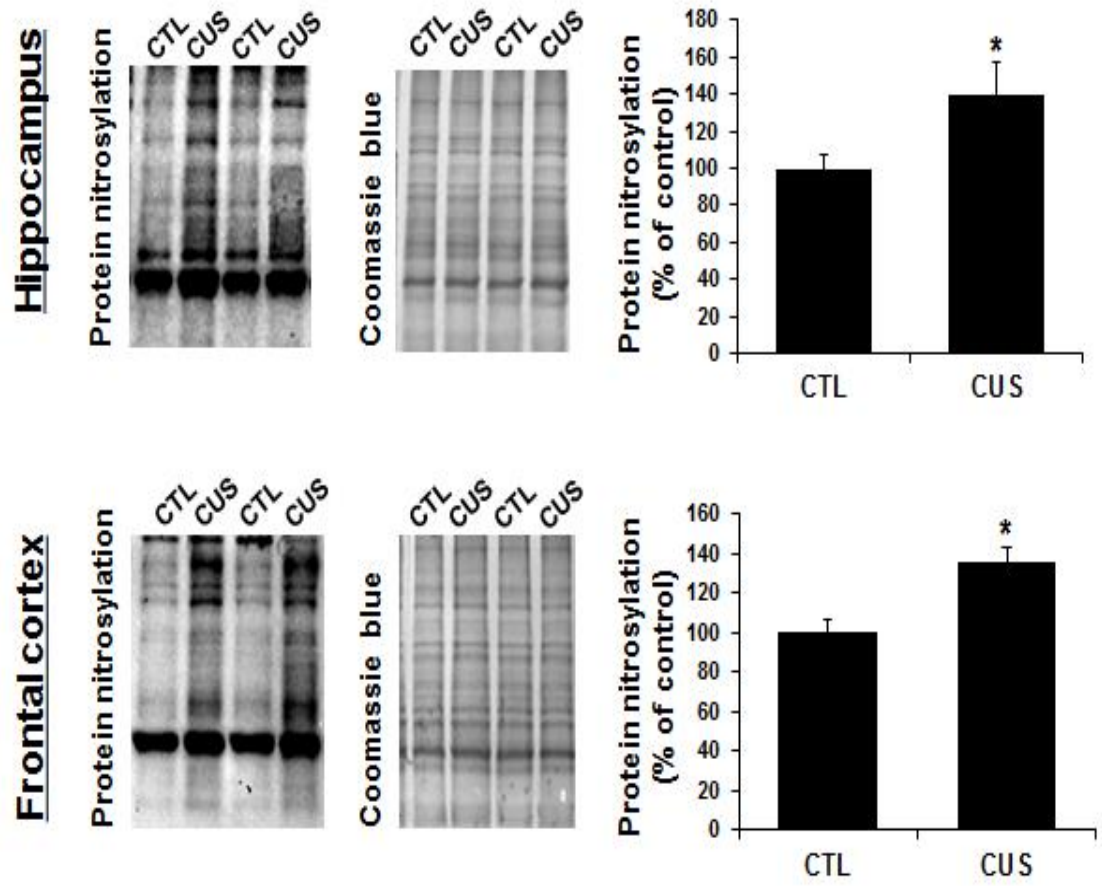

Fig. 11. Effects of chronic unpredictable stress (CUS) on protein nitrosylation in mouse hippocampus and frontal cortex. Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Nitrosylated protein was measured by biotin-switch method, followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 12-13). * indicates $P < 0.05$ by T-test.

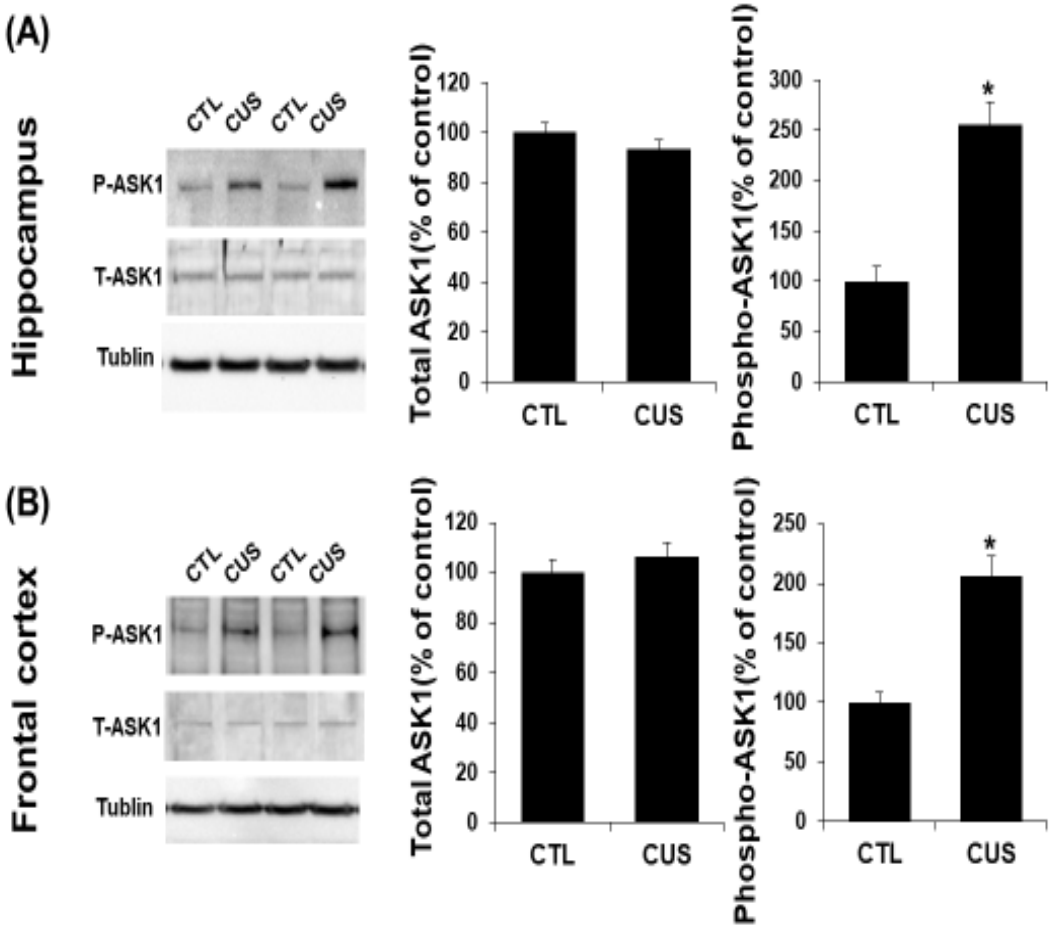

Fig. 12: Effects of chronic unpredictable stress (CUS) on total ASK1 and phosphorylated ASK1 (phospho-ASK1) protein levels in mouse hippocampus (A) and frontal cortex (B). Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. Total ASK1 and phosphor-ASK1 protein levels were measured using immunoblotting analysis. Tublin was used as a normalization standard. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test.

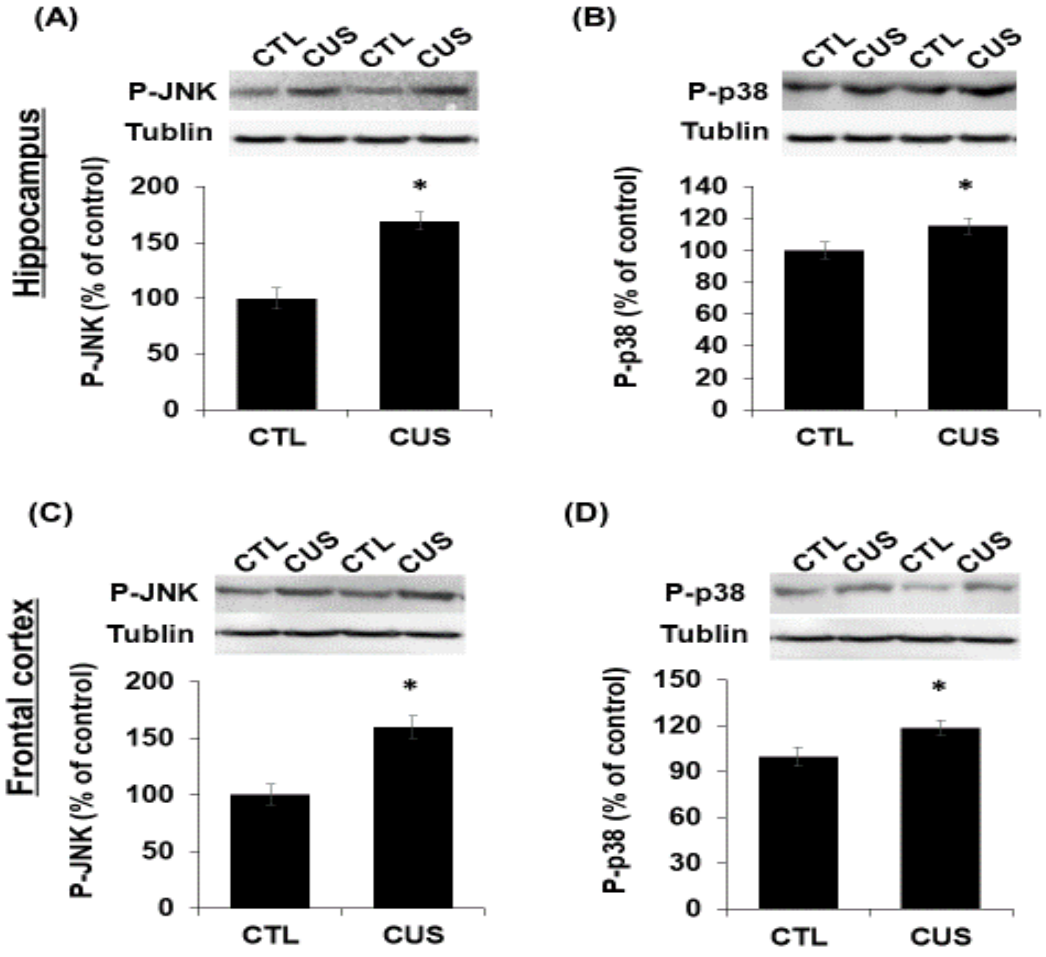

Fig. 13: Effects of chronic unpredictable stress (CUS) on phosphorylated JNK (phospho-JNK) and p-38 (phospho-p38) protein in mouse hippocampus (A-B) and frontal cortex (C-D). Mice in CUS group were exposed to various stressors for 28 days, while mice in control (CTL) group were kept undisturbed in their home cages. phosphor- JNK/p38 protein levels were measured using immunoblotting analysis. Tublin was used as a normalization standard. Data are displayed as mean ± SEM (N=12-13). * indicates $P < 0.05$ by T-test

CHAPTER 4: DISCUSSION

4.1 CUS induces depressive-like behaviour and up-regulates Txnip

In the present study, three behavioral tests were applied to analyze depressive-like behavior in mice exposed to CUS for 28 days. In mice exposed to CUS, open field tests showed an increase in resting time, but a decreased number of rearing, and decreased center duration and frequency of center entry; the sucrose preference test showed a decreased uptake of sucrose; while the forced swim test showed increased immobility. These findings suggest that CUS can induce depressive-like behaviours including decreased exploratory activity, and increased anhedonic and despair conduct, which is consistent with previous studies [149-150].

I also found that although Trx protein levels were not changed, Txnip levels were significantly increased in hippocampus and frontal cortex of mice exposed to CUS. I further observed that Txnip levels were also increased in nucleus accumbens of mice exposed to CUS, suggesting that CUS can up-regulate Txnip in mouse brain. Txnip is a Trx inhibitor. Trx can reduce protein oxidation and inhibit oxidative stress. It has been consistently shown by many studies that oxidative stress can be induced by chronic stress in rodent brain [57][60][62]. CUS-upregulated Txnip may inhibit Trx activity, enhancing the oxidative stress process.

Many studies indicate that hippocampus, frontal cortex, nucleus accumbens and amygdala play an important role in stress response [123][128][129][135][136]. I found that although Txnip levels were increased in hippocampus, frontal cortex, and nucleus accumbens, but not in amygdala of mice exposed to CUS, indicating that Txnip may only contribute a significant role to chronic stress-induced oxidative stress in hippocampus, frontal cortex and nucleus accumbens. Although Txnip levels were not changed in amygdala, previous studies have shown that rodents exposed to stress increased ROS production and lipid peroxidation in amygdala [151-152]. Chronic treatment with the stress hormone corticosterone was also found to increase neuronal nitric oxide synthase (NOS) in the rat amygdala [153]. These studies suggest that Txnip may not have any significant role in stress-induced oxidative and nitrosative damage in amygdala.

4.2 CUS increases protein cysteine oxidative modification

Txnip as a Trx inhibitor can interact with Trx. Txnip cysteine-247 can form a disulfide bond with Trx cysteine-32 active center, thereby breaking the thiol group on Trx active center and inhibiting Trx reducing capability [116]. Because Trx is part of the cellular antioxidant system, impairment in Trx function may exacerbate oxidative damage to cells, promoting protein cysteine oxidative modification such as sulfenylation and nitrosylation [154-155]. Indeed, in the current study I also found that sulfenylated and nitrosylated protein levels were increased in hippocampus and frontal cortex of mice exposed to CUS,

suggesting that CUS-upregulated Txnip may inhibit Trx, facilitating sulfenylation and nitrosylation processes.

Previous studies have shown that chronic stress induced mitochondrial dysfunction, activated inducible neuronal nitric oxide synthase (iNOS) activity, and increased ROS and NO levels in rodent brain [57] [156-157]. Because mitochondrial dysfunction is a major source of ROS, while iNOS is a NO synthesis enzyme, these findings suggest that CUS can increase ROS and NO production. CUS-increased protein cysteine sulfenylation and nitrosylation may also be caused by over produced ROS and NO.

Cysteine residues in proteins are involved in regulation of many protein functions such as redox homeostasis, enzymatic catalysis, signal conduction, metal binding and others [86-87]. Cysteine is highly nucleophilic and redox sensitive when compared with other amino acids. Oxidative modification of cysteine residues may interrupt many cellular processes. Previously, we reported that vesicular monoamine transporter 2 and vesicular glutamate transporters can be nitrosylated, and nitrosylation of these transporters can further reduce vesicular dopamine and glutamate uptake in mouse brain [122]. Nitrosylation of glutamate AMPA receptor was also found to facilitate AMPA receptor phosphorylation, enhancing AMPA receptor sodium channel conductance in primary cultured mouse neocortical neurons [158]. It was also reported that nitrosylation of

calcium channels reduced channel currents [159]. These findings suggest that protein cysteine oxidative modification can regulate neuronal functioning by targeting various proteins. Since Trx can reverse cysteine oxidative modification, these findings also indicate that CUS-increased Txnip may further enhance protein cysteine oxidative modification, subsequently interrupting neuronal function.

4.3 CUS activates ASK1/JNK/p38 stress signaling pathway

ASK1 is a member of the mitogen activated protein kinase kinase kinase (MAPKKK) family that activates the downstream MAPK kinase 4 (MKK4)/MKK7-JNK and MKK3/6-p38 pathways in response to various stress conditions including ROS, endoplasmic reticulum stress, proinflammatory endotoxin lipopolysaccharide, and $Ca2^{+}$ influx. Depending on the cell type and cellular context, the activation of the MAPKs signaling pathway can regulate cell survival, apoptosis, proliferation, differentiation, or inflammation [160]. Under non-stress conditions, the reduced form of Trx binds to the N-terminal region of ASK1 to form a complex via an intermolecular disulfide bridge, which inhibits ASK1 kinase activity. Under stress conditions, Trx is oxidized and dissociates from ASK1, leading to ASK1 activation by autophosphorylation [161]. Over-activation of this kinase is known to cause neuronal degeneration and functional impairment in the central nervous system [108].

Txnip can bind to Trx active center and inhibits its ability to bind ASK1, thereby activating ASK1 [107]. It has been reported that Txnip overexpression by many stimuli, including ROS and stress, increased the binding between Txnip and Trx, whereas the interaction between Trx and ASK1 was significantly decreased, indicating Txnip competes with ASK1 for binding to Trx, thereby releasing ASK1 from Trx inhibition [161]. In the present study, we investigated phosphorylated ASK1 levels in hippocampus and frontal cortex of mice and found that the levels of phosphorylated ASK1 were significantly higher in mice exposed to CUS than in control group while total ASK1 levels were not changed, suggesting that CUS can induce ASK1 phosphorylation and activation. ASK1 phosphorylation can further induce phosphorylation of JNK and p38 kinase and activate JNK/p38 mitogen activated protein kinase pathways [161]. Previous studies have shown that levels of phosphorylated JNK and p38 in the hippocampus and frontal cortex of mice subjected to chronic unpredictable mild stress were higher than control group [148]. In our present study, I also investigated phosphorylated JNK/p38 levels in hippocampus and frontal cortex of mice and verified that the levels of both phosphorylated kinases were significantly higher in mice exposed to CUS than in the control group. It has also been found that antidepressants trazodone and imipramine decreased phosphorylated JNK and p38 levels in hippocampus and frontal cortex of rats subjected to prenatal stress [162]. In addition, studies reported that inhibition of the activity of JNK and p38 with specific blockers produced an antidepressive-like behavior

[163]. Since chronic stress not only increases Txnip levels, but also increases activation of ASK1, JNK and p38, CUS may upregulate Txnip, further inhibit Trx activity, and enhance activation of ASK1 signaling pathway.

4.4 CUS and NLRP3 signaling

The nod-like receptor protein 3 (NLRP3) inflammasome complex is a molecular mechanism that translates stressful stimuli into inflammatory responses. NLRP3 inflammasome promotes the autocatalytic cleavage of pro-caspase-1 to form activated caspase-1. The activated caspase-1 further processes pro-interleukin (IL)-18 and pro-IL-1β into mature IL-18 and IL-1β, and induces pyroptosis, which enhances the inflammatory process [164]. Txnip can bind to NLRP3 that facilitates forming NLRP3 inflammasome complex, resulting in caspase-1 activation, and IL-18 and IL-1β production [165].

Previously it was found that chronic stress increased NLRP3 levels, NLRP3 inflammasome complex formation, IL-1β secretion, and microglial activation in mouse hippocampus and frontal cortex [148] [166-168]. Behavioral dysfunction associated with up-regulation of NLRP3 inflammasome components including the proteins NLPR3, caspase-1, and pro-inflammatory cytokines were also observed in adult rats submitted to prenatal stress [169]. A clinical study has reported that caspase-1, NLRP3 mRNA

expression, and NLRP3 protein levels are increased in the peripheral blood mononuclear cells of patients with MDD compared to non-depressed subjects [170]. On the other hand, antidepressants like fluoxetine were found to reduce NLRP3 activation in chronic unpredictable mild stress rats [167]. Upregulating of Txnip by CUS suggests that Txnip mediates chronic stress-activated NLRP3 inflammasome and proinflammatory processes.

CUS may upregulate Txnip, further increasing Txnip-NLRP3 binding, facilitating formation of NLRP3 inflammasome complex, and activating NLRP3 inflammatory signaling. However, a clear role for Txnip in chronic stress-induced inflammation needs to be further investigated.

4.5 CUS increases production of peroxides

Trx can provide electrons to peroxiredoxin, maintaining peroxiredoxin in a reduced state, facilitating the peroxiredoxin to scavenge peroxides. CUS-upregulated Txnip may further inhibit Trx, increasing accumulation of peroxides. Studies have reported that CUS increased lipid peroxide levels in rat hippocampus, frontal cortex, and striatum [58] [60]. In another study, rats treated with corticosterone for 14 days (10mg/kg/day) also exhibited significantly increased lipid peroxide levels in brain tissue [65]. Clinical studies also found elevated lipid peroxide levels in plasma and brain of patients with depression [66-68]. These studies suggest that Txnip may contribute to CUS-increased peroxides.

CHAPTER 5: CONCLUSIONS, LIMITATION AND FUTURE STUDIES

5.1 Conclusions

In the present studies, we found that:

1. Mice exposed to CUS displayed depressive-like behaviours
2. Txnip protein levels were increased in hippocampus, frontal cortex, and nucleus accumbens of mice exposed to CUS
3. Protein cysteine sulfenylation and nitrosylation levels were increased in hippocampus and frontal cortex of mice exposed to CUS
4. Phosphorylated ASK1/JNK/p38 levels were increased in hippocampus and frontal cortex of mice exposed to CUS

CUS can increase serum corticosterone levels in rodents [171-172]. Recently, we also found that chronic treatment with corticosterone increased Txnip levels and that this effect can be blocked by glucocorticoid receptor inhibitor RU486 [120]. As shown in **Fig 14, o**ur results suggest that CUS may increase Txnip levels by releasing corticosterone and activating glucocorticoid receptors. CUS-increased Txnip may bind to Trx and inhibit Trx reducing activity, subsequently promoting protein cysteine sulfenylation and nitrosylation, thus contributing to the oxidative stress process. CUS-increased Txnip may also inhibit Trx binding to ASK1, facilitating ASK1 phosphorylation that activates

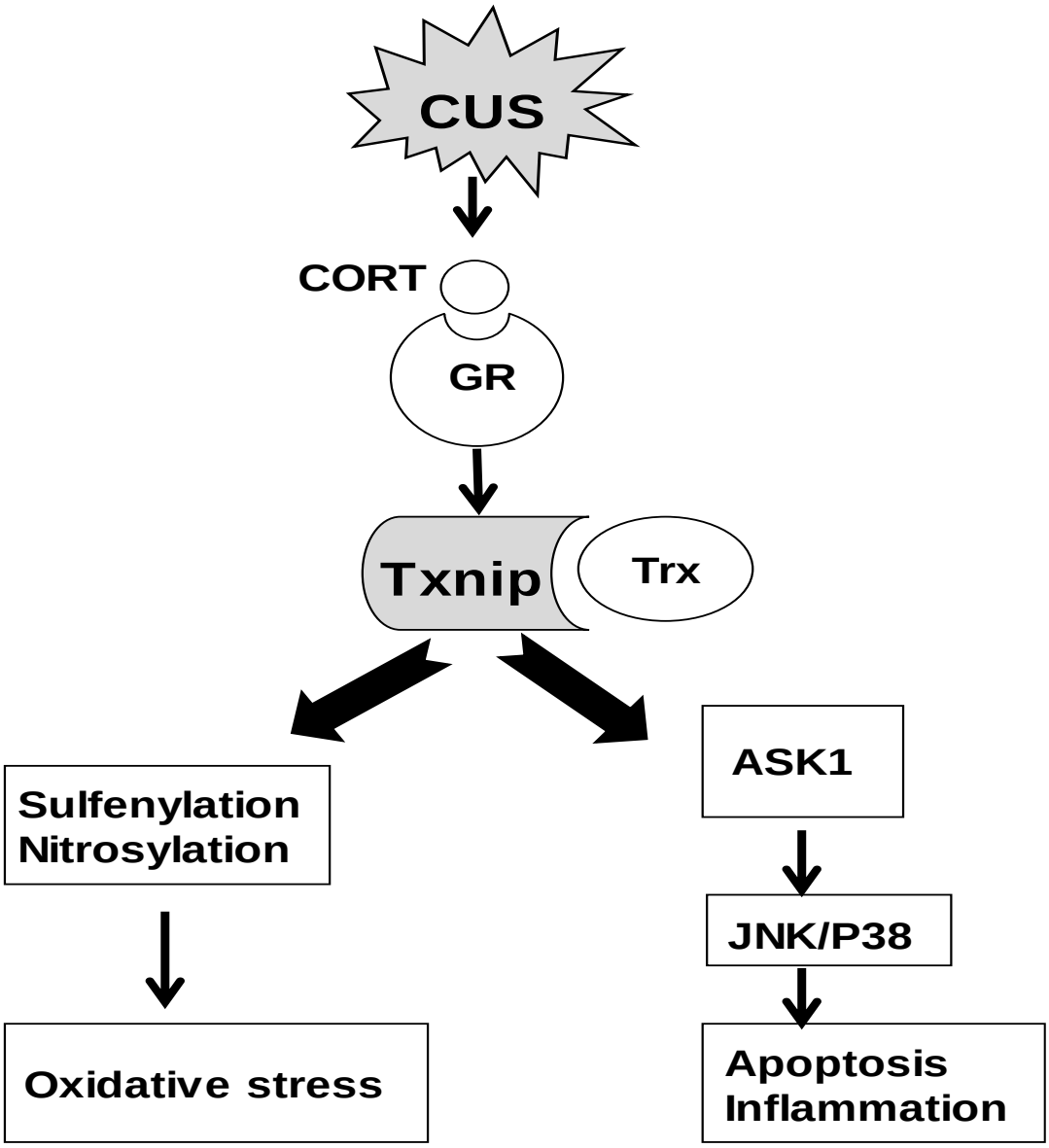

Fig. 14: Possible role of thioredoxin-interaction protein in chronic unpredictable stress-induced oxidative stress and inflammation processes. ASK1, apoptosis signal-regulating kinase 1; CORT, corticosterone; CUS, chronic unpredictable stress; GR, glucocorticoid receptor; JNK, c-Jun N-terminal kinase; Txnip, thioredoxin interacting protein; Trx, thioredoxin

JNK/p38 stress signaling pathway**.** Because Txnip can also enhance NLRP3-mediated inflammatory signaling and peroxiredoxin-dependent peroxide accumulation, upregulated Txnip may also contribute to CUS-increased inflammation and lipid peroxidation. Chronic stress is a major risk for depression and other psychiatric disorders, Txnip may be a potential target for development of new pharmacological treatments for depression.

5.2 Limitations and future studies

In this study, there are several limitations:

First, although our results suggest that CUS-increased Txnip may further bind to Trx and inhibit Trx activity, we only measured Trx protein levels, but did not measure Txnip-Trx binding and Trx enzyme activity. In the future, the effect of CUS on binding and Trx enzyme activity should be analyzed.

Second, although CUS-increased Txnip suggests that CUS may activate inflammatory responses mediated by NLRP3 inflammasome and inhibit scavenging of peroxides medicated by peroxiredoxin, we did not investigate the effect of CUS on NLRP3 and peroxiredoxin systems. In the future, it is necessary to analyze the interaction between Txnip and NLRP3, pro-caspase-1 cleavage, and IL-18/ IL-1β release, and peroxiredoxin expression and activity in the brain of mice exposed to CUS.

Third, although we found that CUS induced depressive-like behaviours, upregulated Txnip, and increased protein cysteine oxidative modification, and ASK1/JNK/p38 phosphorylation in brain of mice exposed to CUS, we do not have direct evidence indicating that Txnip mediates CUS-induced depressive behaviours, increased protein cysteine oxidative modification, and ASK1/JNK/p38 phosphorylation. In the future, Txnip gene can be knocked out by either Txnip shRNA or CRISPR/Cas9 to understand the role of Txnip in CUS-induced behavioural and biochemical changes.

Fourth, we only used male mice to perform behavioural and biochemical tests. However, females have been reported to have increased susceptibility to stress (in both human and rodents). Therefore, in the future, we need to study the regulation of Txnip in female animals.

Fifth, depression is a complex disorder. While no single animal model can capture the entire spectrum of depression, in future we will also study Txnip in other animal models for depression such as prenatal stress, maternal deprivation and chronic restraint stress models.

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