# Role of the Thioredoxin System in Chronic Corticosterone Treatment-Impaired Neuronal Differentiation and Degeneration

By

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## ABSTRACT

**Background:** Stress response is mediated mainly by the hypothalamic-pituitary-adrenal (HPA) axis. During stress, glucocorticoids are released by the HPA axis into the bloodstream and target various organs including the brain. Chronic stress is a major risk factor for psychiatric diseases such as depression. Previous studies have shown that chronic stress and excessive corticosterone (CORT) treatment can cause oxidative damage to the brain. Thioredoxin (Trx) is an oxidoreductase that can reverse protein cysteine thiol oxidation. Trx can also inhibit the activation of apoptosis signal-regulating kinase 1 (ASK1) and facilitate activation of cAMP-response element binding protein (CREB). Trx is maintained in a reduced state by Trx reductase (TrxR). Trx interacting protein (Txnip) is an endogenous inhibitor of Trx. Studies from our lab showed that chronic stress and chronic CORT treatment upregulated Txnip and induced oxidative damage in cultured mouse neurons and mouse brain, suggesting that Trx system mediates CORT-induced neuronal damage.

**Objective:** The objective is to investigate the role of Trx system in chronic CORT-impaired neuronal differentiation and CORT-enhanced neurodegeneration.

**Methods:** Primary cultured mouse cerebrocortical neurons, human neuroblastoma SH-SY5Y cells and adult mice were used in this study. Trx, TrxR and Txnip protein levels, CREB and ASK1 phosphorylation, and various synaptic protein levels were measured by immunoblotting. Neurite outgrowth was measured by immunocytochemistry. CREB cysteine oxidation was measured by the biotin-switch method followed by immunoprecipitation. Trx and Txnip genes were knocked down using CRISPR/Cas9 technology. Mice were treated with CORT daily for 21

days. CORT-induced depressive-like behavior and cognitive decline were evaluated using behavioral tests.

**Results:** First, we found in primary mouse cerebrocortical neurons that Trx and TrxR protein levels were elevated during the neuronal differentiation period while Txnip was upregulated during neurodegeneration period. Blocking Trx with CRISPR/Cas9/Trx sgRNA or the Trx inhibitor PX12 reduced neurite outgrowth. PX12 treatment also reduced the expression of glutamatergic synaptic markers vGLUT1 and PSD95. Second, we found in primary mouse cerebrocortical neurons that CREB phosphorylation was upregulated during differentiation and that blocking Trx with PX12 reduced CREB phosphorylation. We also found in SH-SY5Y cells that $H_2O_2$ reduced CREB phosphorylation and promoted CREB cysteine oxidation. Treatment with Trx mimetic peptide CB3 reversed $H_2O_2$-reduced CREB phosphorylation and CREB cysteine oxidation. We also found in primary cultured mouse cerebrocortical neurons that ASK1 phosphorylation was increased during the degeneration period and that PX12 increased ASK1 phosphorylation. Third, we found that although CORT treatment had no effect on Trx and TrxR protein levels, it increased Txnip protein levels in primary mouse cerebrocortical neurons. CORT treatment also reduced CREB phosphorylation and increased ASK1 phosphorylation. Moreover, CORT treatment reduced neurite outgrowth, while knocking down Txnip prevented CORT-impaired neurite outgrowth. Finally, we found that chronic CORT injections induced depressive-like behaviors in female and male mice. Knocking down Txnip in the frontal cortex prevented CORT-induced depressive-like behaviors in mice.

**Conclusion:** Our findings suggest that Trx may have neurotrophic and neuroprotective effects. Trx can facilitate neuronal differentiation by preserving the cellular redox balance, inhibiting CREB cysteine oxidation and maintaining CREB activation. Trx can inhibit ASK1 apoptotic signaling and produce a neuroprotective effect. Chronic CORT treatment can upregulate Txnip. CORT-upregulated Txnip may further inhibit Trx activity, suppressing CREB neurotrophic signaling and enhancing ASK1 apoptotic signaling, which inhibits neurite outgrowth and promotes depressive-like behaviors in mice.

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## List of Abbreviations

| | |
|---|---|
| -S-S- | Disulfide |
| -SNO | Nitrosothiols |
| -$SO_2H$ | Sulfinic acid |
| -$SO_3H$ | Sulfonic acid |
| -SOH | Sulfenic acid |
| 11-β HSD | 11-β Hydroxysteroid dehydrogenases |
| 4-HNE | 4-Hydroxy-2,3-trans-nonenal |
| 8-oxo-G | 8-oxoguanine |
| AAV | Adeno-associated virus |
| ACTH | Adrenocorticotropin |
| ALS | Amyotrophic lateral sclerosis |
| AP-1 | Activator protein 1 |
| ARE | Antioxidant responsive element |
| ASK1 | Apoptosis signal kinase 1 |
| ATP | Adenosine triphosphate |
| Bax | Bcl-2-associated X protein |
| Bcl-2 | B-cell lymphoma 2 |
| BDNF | Brain derived neurotrophic factor |
| Biotin-HDPD | N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide |
| CAT | Catalase |

| | |
|---|---|
| CBG | Corticosteroid-binding-globulin |
| ChREBP | Carbohydrate-responsive element-binding protein |
| CNS | Central nervous system |
| CORT | Corticosterone |
| CREB | Cyclic AMP response element binding protein |
| CRH | Corticotropin-releasing hormone |
| CRISPR/Cas9 | Clustered regularly interspaced short palindromic repeats |
| CRMP2 | Collapsin response mediator protein 2 |
| DMEM | Dulbecco's Modified Eagle Media |
| DTT | Dithiothreitol |
| EGTA | Ethylene glycol tetraacetic acid |
| GABA | Gamma-aminobutyric acid |
| GC | Glucocorticoids |
| GPCR | G protein coupled receptors |
| GPx | Glutathione peroxidises |
| GR | Glucocorticoid receptors |
| GRE | Glucocorticoid response elements |
| GSH | Glutathione |
| GSSG | Oxidized glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HPA axis | Hypothalamic-pituitary-adrenal axis |
| IFN- γ | Interferon gamma |
| IL-1β | Interleukin-1β |

| | |
|---|---|
| JNK | c-jun-N-terminal-kinase |
| MAP2 | Microtubule-associated protein 2 |
| MDA | Malondialdehyde |
| MDD | Major depressive disorder |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MR | Mineralocorticoid receptors |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NEM | N-ethylmaleimide |
| NF-κB | Nuclear factor kappa B |
| NGF | Nerve growth factor |
| nGRE | Negative glucocorticoid response element |
| NLRP3 | Nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing 3 |
| NMDA | N-methyl-D-aspartate AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic |
| nNOS | Neuronal nitric oxide synthetase |
| NOR | Novel object recognition test |
| NOS | Nitric oxide synthetase |
| OFT | Open field test |
| P38 | Protein 38 |
| PKA | Protein kinase A |
| Prdx | Peroxiredoxin |
| PX12 | 1-methylpropyl 2-imidazolyl disulfide |

| | |
|---|---|
| RBANS | Repeatable Battery for the Assessment of Neuropsychological Status |
| Ref-1 | Redox-factor 1 |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulphate |
| sgRNA | Single guide RNA |
| SNAP25 | Synaptosomal-associated protein 25 |
| SOD | Superoxide dismutase |
| SP1 | Specificity protein 1 |
| SPT | Sucrose preference test |
| STAR | Steroidogenic Acute Regulatory Protein |
| TNF-α | Tumor necrosis factor alpha |
| Trx | Thioredoxin |
| TrxR | Thioredoxin reductase |
| TST | Tail suspension test |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| Txnip | Thioredoxin interacting protein |
| vGAT | Vesicular gamma-aminobutyric acid transporter |
| vGLUT1 | Vesicular glutamate transporters |
| WHO | World Health Organization |

## COPYRIGHTED MATERIAL

# CHAPTER 1: INTRODUCTION

## 1.1 Chronic stress and corticosterone

### 1.1.1 Physiology of stress

Stress refers to the organism's physiological and psychological response to internal or external stressful stimuli that allows the body to adapt to the situation and maintain homeostasis. While acute stress is beneficial to respond and adapt to different stressors, chronic stress has deleterious effects on the body, producing pathophysiological changes that could lead to the development of psychiatric disorders (Mcewen et al., 2007).

The primary system involved in the stress response is the hypothalamic-pituitary-adrenal (HPA) axis. As shown in figure 1, upon stressful stimuli, the neurons in the paraventricular nucleus of the hypothalamus synthesize corticotropin-releasing hormone (CRH). CRH reaches the anterior lobe of the pituitary gland through the hypophysial portal system and stimulates the secretion of adrenocorticotropin (ACHT). In turn, ACHT prompts the adrenal cortex to release glucocorticoids (GCs) [cortisol in humans and corticosterone (CORT) in rodents) to the systemic circulation (Herman et al., 2016). GCs are molecules derived from cholesterol that exert pleiotropic effects in almost all tissues and organs. GCs serve as the ultimate regulators of the HPA axis. GCs terminate the stress response and control the basal functioning of the HPA axis through negative feedback by acting at the hypothalamus and the pituitary gland to inhibit the synthesis and release of CRH and ACTH respectively (Nicolaides et al., 2014). This negative feedback loop of the HPA axis can be disrupted by chronic stress, resulting in a persistent increase in CRH activity and elevated levels of GCs, which in turn can lead to deleterious effects in different organs (Lucassen et al., 2014).

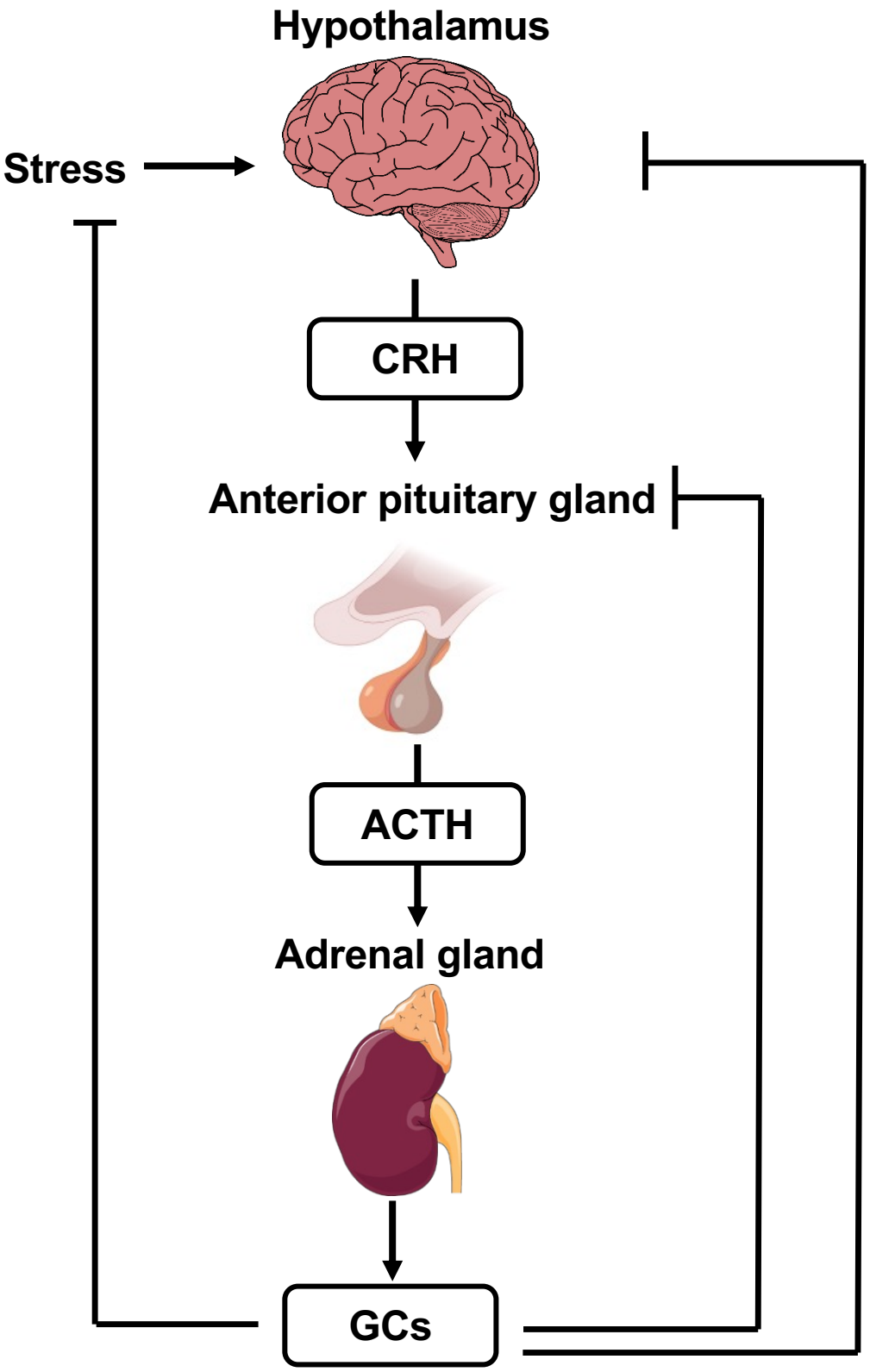


**Figure 1: The activation and regulation of the hypothalamic pituitary adrenal axis.** Stress activates the hypothalamus to release corticotrophin releasing hormone (CRH). CRH then stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH then stimulates the adrenal gland to release glucocorticoids (GCs). GCs act on the hypothalamus and anterior pituitary gland to inhibit the release of CRH and ACTH respectively. Inhibition ⟶ Stimulates ⟙

To exert their pleiotropic function, GCs bind to two types of receptors: mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). MR are located in the nucleus of the target cells and show higher affinity for GCs (Km=0.5 nM). Thus, under physiological conditions, these receptors are fully occupied by GCs (Joëls, 2018). The main function of MR is to regulate electrolytes, fluid balance, and basal circadian and ultradian rhythms in the body (Molina, 2023). On the other hand, GR are located in the cytosol and show lower affinity for GCs (Km=5 nM), thus requiring higher concentrations of GCs for full activation (Joëls, 2018). The GR remain inactive in the cytosol by forming a complex with chaperone heat shock proteins 70 and 90 and immunophilin (Spiers et al., 2014). When GCs cross the cell membrane and bind to the ligand-binding domain of GR, the receptor undergoes a conformational change and dissociates from the chaperone proteins and inhibitory factors. The activated receptor then translocates into the nucleus, where homo- or hetero-dimerizes with another nuclear receptor (Jaszczyk and Juszczak, 2021). The dimer can then interact with a specific DNA sequence, the glucocorticoid response element (GRE) located in the promoter region of target genes, activating their expression. This process is known as transactivation (Spies et al., 2011). The GCs-GR complex can also bind to a negative GRE (nGRE) sequence and inhibit gene expression. This process is known as trans-repression (Juszczak and Stankiewicz, 2018). Moreover, the GCs-GR complex can modulate gene expression in a GRE-independent way. GR can interact with other transcription factors such as the NF-kB, activator protein-1 (AP1), and p53 to enhance or inhibit their transcriptional activity. GCs anti-inflammatory and immunosuppressive activity seems to be mediated by this mechanism (Juszczak and Stankiewicz, 2018; Spies et al., 2011). Although GCs are well known for their genomic actions, they can also induce non-genomic effects. It has been reported that GCs can interact with membrane-bound GR, and their activation leads to kinase signaling

cascades. One example of this non-genomic action of GCs includes the suppression of ACTH secretion from the anterior pituitary gland that leads to the termination of the stress response (Molina, 2023; Persson and Pipkorn, 1990).

Because GR are present in most cells and tissues, GCs have wide-ranging effects on various organ systems. GCs play a crucial role in regulating glucose balance. In the liver, GCs enhance the synthesis of enzymes involved in gluconeogenesis. Second, GCs promote protein catabolism, and fatty acid release while inhibiting muscle protein anabolism. Third, GCs reduce the expression and activity of insulin-like growth factor 1, insulin-like growth factor-binding protein 1, and growth hormone receptors in bone and cartilage. This effect has potential implications for the organism's health since high and sustained circulating GCs levels result in the loss of lean body mass and hinder skeletal growth and bone formation by suppressing osteoblasts and collagen synthesis. Fourth, GCs regulate the immune response by promoting the synthesis of anti-inflammatory cytokines and inhibiting pro-inflammatory cytokines, exerting an overall anti-inflammatory effect. In the vasculature, GCs influence responsiveness to vasoactive substances like angiotensin II and norepinephrine. Deficiency in GCs can lead to hypotension and reduced sensitivity to vasoconstrictor administration. In the central nervous system, GCs modulate perception, emotion, and behavior (Molina, 2023; Spies et al., 2011).

Chronic stress can impair HPA axis negative feedback, which contributes to high levels of circulating GCs. Elevated levels of GCs can then impair GR function and GR transactivation. Previously it has been reported that rats exposed to chronic mild stress showed impaired GR trafficking and GR-mediated transcriptional activity in hippocampus and prefrontal cortex (Guidotti et al., 2013). Elevated GCs are also associated with increased levels of peripheral and CNS pro-inflammatory cytokines, which exacerbate the immune response. A previous report

showed that chronic unpredictable stress for 21 days augmented the levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β in mice hippocampus and prefrontal cortex (Y. Wang et al., 2018). In turn, high cytokine levels alter GR activity by inhibiting GR translocation (Miller et al., 1999; Silverman and Sternberg, 2012); inducing the expression of non-functional GR isoforms (Webster et al., 2001), and activating mitogen-activated protein kinase pathways that inhibit GR activity (Yuan et al., 2016). Moreover, studies have shown that chronic stress can induce epigenetic modifications in the GR such as demethylation of cytosine-guanidine dinucleotides at GREs; histone methylation/acetylation; and chromatin remodeling; which impair GR function and induce alterations in the gene expression profile (Mourtzi et al., 2021). For example, one study showed that early life stress induces demethylation and upregulates the gene encoding FK506 binding protein 5. FKBP5 acts as a negative regulator of the GR signaling by altering GR folding and reducing GR binding affinity (Zannas et al., 2016). Altogether, these findings suggest that chronic stress persistently increases circulating GCs levels and alters GR function, which can lead to deleterious health effects.

### 1.1.2 Corticosterone

#### 1.1.2.1 Synthesis

Corticosterone (CORT) is synthesized in the cells of the zona fasciculata and zona reticularis of the adrenal cortex. CORT synthesis is initiated when the hormone ACTH binds to the melanocortin 2 receptor, a G-protein coupled receptor (GPCR). Upon binding, ACTH activates the G-protein α-stimulatory subunit which increases the intracellular concentration of cyclic AMP. In turn, cAMP binds and activates the protein kinase A (PKA). PKA phosphorylates and activates the cholesterol ester hydrolase. This enzyme makes cholesterol available from the

cytoplasmic membrane and from the cytoplasmic pool of cholesteryl-ester, initiating the cholesterol-derived steroidogenesis pathway. Simultaneously, ACTH increases the synthesis and activity of the steroidogenic acute regulatory protein (STAR), a protein responsible for transferring the released cholesterol to the inner mitochondria membrane for further processing. In the inner mitochondria membrane, cholesterol is converted to pregnenolone by the P450 side-chain cleavage enzyme. This is the rate-limiting step in the steroidogenesis pathway. The enzyme 3β-hydroxysteroid dehydrogenase converts pregnenolone to progesterone, which then is hydroxylated to 11-deoxycorticosterone and further to CORT by the 11 β -hydroxylase enzyme. CORT release depends on the pulsatile release of ACTH by the anterior pituitary gland, and the sensitivity of the adrenal cortex to ACTH throughout the day (Molina, 2023).

#### 1.1.2.2 Corticosterone circadian rhythm

The synthesis and release of CORT follows a circadian rhythm (24-hour period) that depends on environmental and internal factors such as light, sleep, stress, and disease (Mohawk et al., 2007). The CORT release pattern allows the organisms to quickly respond and adapt to environmental changes. CORT circadian rhythm is controlled by the suprachiasmatic nucleus of the hypothalamus (Verhagen et al., 2004). In nocturnal animals such as mice and rats, basal CORT levels usually rise during the resting hours of the sleep/wake cycle after 11:00 am and peak around 5 pm, just before the onset of the active period (Dunn et al., 1972; Verhagen et al., 2004). This circadian pattern prepares the animal for the upcoming period of activity, promoting feeding behavior and mobilization of energy sources (Verhagen et al., 2004). Interestingly, sex-dependent differences are found in plasma CORT levels during the circadian rhythm. A study conducted in female and male Sprague-Dawley rats showed that even though both sexes display

a similar 24-hour pattern in plasma CORT levels, female rats have twice the concentration of plasma CORT levels than male rats (Dunn et al., 1972). The sexual dimorphism observed in the plasma CORT levels is believed to be caused by the activation of HPA axis by ovarian estrogen. Studies indicate that estrogen reduces the HPA axis sensitivity to CORT in the negative feedback loop, causing an increase in ACTH release and thus, higher CORT levels in plasma. Additionally, the plasmatic CORT levels will depend on the estrous cycle stage. During the estrus stage, plasma CORT levels are low but increase drastically during the proestrus period (Atkinson et al., 1997). Lastly, environmental, and internal events can modify the CORT circadian cycle. For instance, chronic alcohol consumption in DBA/2J male mice completely abolishes circadian variations of CORT in plasma (Kakihana and Moore, 1976). In addition, exposing mice to light for 1 hour during the dark period elicits a 2.5 h delay in the onset of the peak of CORT circadian rhythm (Mohawk et al., 2007), suggesting a direct effect of light in the HPA axis (Mohawk et al., 2007).

#### 1.1.2.3 Transport

GCs are lipophilic compounds thus they are usually present in the systemic circulation as sulfates derivates, glucuronide derivates or bound to plasmatic proteins. Around 90% of the released GCs bind in a non-covalent reversible fashion to the glucocorticoid-binding globulin (CBG) or the albumin while less than 10% is present in the unbound form (Molina, 2023). The GCs unbound fraction is biologically active, and it is responsible for the GCs effects. It has been reported that CBG and albumin contribute to the regulation of GC action as transport carrier proteins in two ways. First, CBG and albumin are GCs sensors in the systemic circulation, releasing the GCs into the plasma when the hormone concentration decreases. Second, CBG and

albumin protect the hormone from the peripheral metabolism thus increasing GCs half-life (Jaszczyk and Juszczak, 2021; Persson and Pipkorn, 1990).

#### 1.1.2.4 Metabolism and Excretion

Endogenous CORT is metabolized in tissues by the enzyme 11β -hydroxysteroid dehydrogenase (11β -HSD). Two isoforms of this protein mediate the interconversion of CORT to its inactive metabolite 11-dehydrocorticosterone. The 11β -HSD type 1 is a NADP+ dependent enzyme with low affinity for CORT. This enzyme is expressed in the liver, adipose tissue, lung, skeletal muscle, and CNS. It catalyzes the conversion of 11-dehydrocorticosterone to CORT. On the other hand, 11β -HSD type 2 is a NAD+ dependent enzyme with high affinity for CORT. It is expressed in the distal convoluted tubules and collecting ducts in the kidney. It catalyzes the conversion of CORT to the inactive 11-dehydrocorticosterone for further elimination (Molina, 2023; Vylitová et al., 1998). Studies have reported that CORT exhibits a two-compartment model metabolism, where CORT establishes a quick initial equilibrium with the cell fluid compartments and then the compound degrades more slowly (Birrenkott & Wiggins, 1983). CORT also undergoes extensive metabolism in the liver. Oxidation at C-11; reduction at C-3, C-20 and C-21; and hydroxylation at C-6 are some examples of first metabolic reactions experienced by CORT (Palme et al., 2005). These metabolites are subsequently conjugated to sulfates or glucuronides and excreted via urine and bile.

### 1.1.3 Pathophysiology of stress

#### 1.1.3.1 Chronic stress, corticosterone, and oxidative stress

Oxidative stress is caused by an imbalance between pro-oxidant compounds and the cellular antioxidant defenses in favor of the oxidants. Either overproduction and/or deficiency of the cellular antioxidant defenses can cause oxidative stress. Reactive oxygen species (ROS) are the main contributors to oxidative stress. As shown in figure 2, ROS are oxygen-derived reactive molecules that are produced during cellular metabolism, mainly in the mitochondria, peroxisomes, and endoplasmic reticulum (Melo et al., 2011). Furthermore, oxidases such as xanthine oxidase, NADPH oxidase and monoamine oxidase contribute to the generation of ROS within the cell (Lushchak, 2014). ROS include superoxide radicals, hydroxyl radicals, and hydrogen peroxide ($H_2O_2$). One of the main sources of ROS production within the cell is the mitochondrion electron transport chain. In this process, electrons are transported through four mitochondrial complexes to finally render ATP and $H_2O$. However, the electrons might leak mainly from complex I and complex III and interact with molecular oxygen to produce superoxide radicals (Lushchak, 2014). The superoxide radical is then enzymatically converted to $H_2O_2$. Although $H_2O_2$ is a stable molecule, it can react with transition metals such as $Fe^{2+}$ through the Fenton reaction to render hydroxyl radicals (Spiers et al., 2015). In addition, ROS are produced during hydroxylation reactions catalyzed by the cytochrome P450 family enzymes within the endoplasmic reticulum (Teleanu et al., 2022). Metabolic reactions occurring in the peroxisomes also lead to the production of $H_2O_2$, and superoxide radicals mainly by the action of xanthine oxidase in the lumen, and the NADH/NADPH-dependent electron transport chain in the peroxisomal membrane (Kim, 2020; Sandalio et al., 2023).

In physiological conditions, ROS are scavenged by the cellular antioxidant enzymes, maintaining the cellular redox balance. Cellular antioxidant defenses include the superoxide dismutase (SOD) enzyme, catalase (CAT) enzyme, glutathione (GSH) system, thioredoxin (Trx)

system, and peroxiredoxin (Prdx) enzyme (Valko et al., 2007). SOD converts the highly reactive superoxide radical into $H_2O_2$, which is then reduced to water by the CAT enzyme. $H_2O_2$ can also be converted to water by glutathione peroxidase (GPx) at the expense of GSH. Oxidized GSH is then reduced back by glutathione reductase (GRt) using reduced NADPH. This reaction is especially important in neurons where GPx is expressed in higher concentrations compared to CAT, and in the mitochondria where CAT is not found (Maher, 2006). Trx is an oxidoreductase that reverses protein cysteine oxidative modifications including nitrosylation, sulfenylation and disulfidation, as well as maintains Prdx in a reduced and active state (Nishiyama et al., 2001). Prdx plays a major role in controlling cellular levels of $H_2O_2$ while allowing $H_2O_2$ to function as a signaling molecule (Kamata and Hirata, 1999; Maher, 2006).

Maintaining the redox environment is crucial for the activity of enzymes, transcription factors and normal cellular functioning. However, during oxidative stress, ROS production surpasses the cellular antioxidant capacity and can lead to deleterious effects in the cell. It has been reported that restraint stress for 21 days increases ROS production, and decreases GSH levels and SOD, CAT and GRt activity in the mouse brain (Zafir and Banu, 2009). CORT administration for 14 days also elevated superoxide radical production and decreased the total antioxidant capacity in the hippocampus of rats (Sato et al., 2010a). Treatment with synthetic GCs dexamethasone elevated oxidative status in rat hippocampal slices, an effect that was reversed by GR inhibitor RU486. Moreover, treatment of rat hippocampal slices with dexamethasone reduces GPx and glutathione S-transferase activities and upregulates NADPH oxidase activity (You et al., 2009). Furthermore, chronic social isolation stress for 21 days decreased GSH and SOD levels in the prefrontal cortex of adult male Wistar rats (Zlatković et al., 2014).

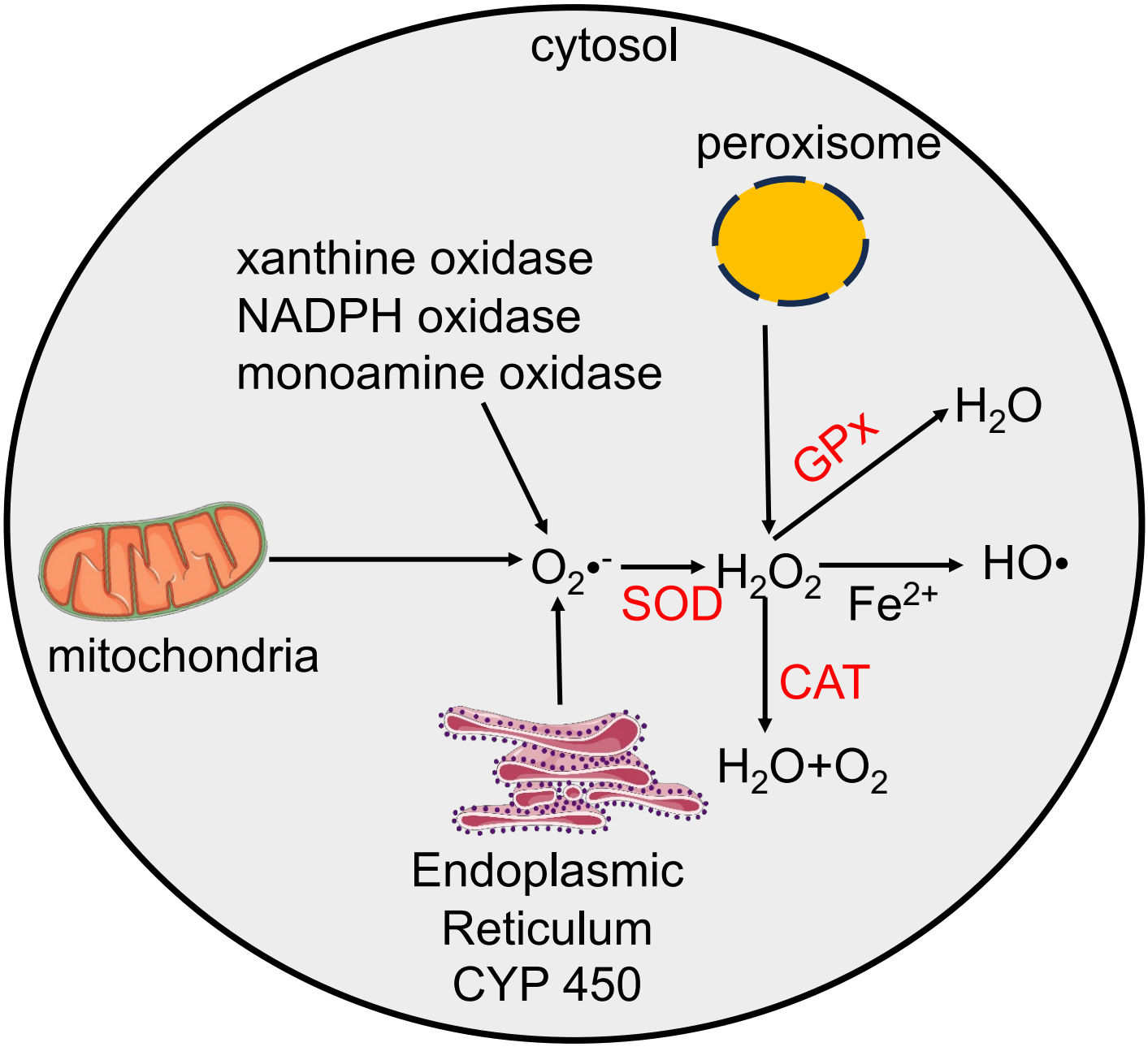


**Figure 2: Reactive oxygen species and antioxidant defense.** Reactive oxygen species (ROS) are produced during cellular metabolism in different cellular compartment including mitochondria, endoplasmic reticulum, and peroxisome. ROS are detoxified by cellular antioxidant systems. Abbreviation: $O_2^-$, superoxide; $H_2O_2$, hydrogen peroxide; HO•, hydroxyl radical; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; CYP450, cytochrome P450

Treatment with 400 μM CORT for 24h also decreased SOD, CAT and GPx activities in PC12 cells (Lew et al., 2020). Our lab has found that chronic CORT treatment for 5 days increased ROS levels in HT22 cells (Bharti et al., 2018). In addition, we found that chronic unpredictable stress decreased GPx and CAT antioxidant activities in rat prefrontal cortex, hippocampus, and striatum (Che et al., 2015). Overall, this evidence suggests that chronic stress and chronic CORT treatment lead to increased ROS production and decreased cellular antioxidant defenses.

The mitochondrion is a major site for ROS production. During aerobic metabolism, electrons leak from complexes I and III in the electron transport chain and then react with oxygen to form superoxide radicals (Spiers et al., 2015). Treatment of primary cortical neurons with 1 μM CORT for 1 day increased mitochondrial oxidation and mitochondrial membrane potential (Du et al., 2009). Previously, it has been reported that chronic unpredictable stress for 28 days reduced the activity of mitochondrial complex I, II, IV and V; and decreased mitochondrial membrane potential in rat hippocampi (Parul et al., 2021). Studies in our lab have found that 24-hour CORT treatment decreased complex I activity in PC12 cells (V. M. Tang et al., 2013). Altogether, this data suggests that chronic stress and CORT treatment compromise mitochondrial membrane integrity and decrease mitochondrial function, leading to the overproduction of ROS and oxidative stress.

The brain is characterized by a high metabolic rate, ample presence of polyunsaturated fatty acids and low levels of antioxidants, which makes it more vulnerable to the action of ROS and oxidative stress. ROS induce oxidative modifications to lipids, proteins, and DNA (Maher, 2006). ROS attack the side chain of polyunsaturated fatty acids to generate harmful by-products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Lipid peroxidation disrupts

the integrity and functionality of the cell membrane. Moreover, ROS can react with protein aminoacidic residues such as proline, arginine, histidine, and lysine to generate carbonyl groups. ROS can also induce cysteine thiol oxidation, sulfenylation and nitrosylation. These modifications can cause protein misfolding, protein aggregation and proteasome-mediated degradation (Melo et al., 2011; Smith et al., 2013). ROS can also induce DNA lesions such as modifications of nucleotides, and DNA single and double-strand breaks. Guanine is the most susceptible to oxidative modification from all the nucleotide bases, leading to the formation of 8-hydroxyguanosine (8-OxoG) (Smith et al., 2013). It has been reported that chronic social isolation stress for 3 weeks increased lipid peroxidation and protein carbonylation in the prefrontal cortex of adult male Wistar rats (Zlatković et al., 2014). Similarly, CORT administration for 28 days and restraint stress for 21 days increased lipid peroxidation and oxidized protein carbonyl groups in the rat brain (Samad et al., 2022; Zafir and Banu, 2009). In another study, chronic mild stress elevated protein carbonyl groups in the striatum, prefrontal cortex and hippocampus (Lucca et al., 2009). Previously, our lab showed that protein carbonylation is increased after 24-hour CORT treatment in rat PC12 cells (V. M. Tang et al., 2013). We also found that chronic CORT treatment for 5 days increases ROS production, protein nitrosylation and protein sulfenylation in HT22 cells (Bharti et al., 2018). Moreover, we found that chronic unpredictable stress increases lipid peroxidation in rat prefrontal cortex, hippocampus, and striatum (Che et al., 2015). Chronic restraint stress for 50 days increased DNA breaks in the hippocampus of male rats (Noschang et al., 2009). Unpredictable chronic mild stress increased the levels of 8-OxoG and DNA breaks in rat hippocampus (Grassi et al., 2017). Restraint stress for 6 hours induced a decrease in DNA repair gene expression in the prefrontal cortex of rats (Forsberg et al., 2015). Similarly, 4-hour restraint stress for 2 weeks led to the

accumulation of DNA damage in the prefrontal cortex of mice (Hara et al., 2013). All these data suggest that chronic stress and CORT treatment induce oxidative modifications in lipids, proteins, and DNA, leading to cellular dysfunction.

#### 1.1.3.2 Chronic stress, corticosterone, and neuronal differentiation

Neuronal differentiation is a complex and highly regulated process. Neural stem cells give rise to neuronal progenitor cells (Vieira, Santos et al. 2018). Then, the neuronal progenitor cells differentiate into immature neurons and start to develop axons and dendrites, and form synaptic connections in order to integrate into the brain (Olguin-Albuerne and Moran et al., 2018). In adulthood, neurons differentiate from neuronal progenitor cells located in the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (Levone et al., 2021). Previously, it has been found that chronic unpredictable stress impairs hippocampal neurogenesis by inducing apoptosis of neural precursor cells in adult rats (Parul et al., 2021). Chronic CORT injections for 21 days also reduced hippocampal neurogenesis in the dentate gyrus in male and female rats (Brummelte and Galea, 2010). In addition, primary hippocampal neuronal progenitor cells exposed to 1μM CORT for 4 hours showed reduced proliferation and differentiation into neurons (Levone et al., 2021). Neurite outgrowth occurs due to dynamic interactions of the cytoskeleton and environmental chemoattractant or chemorepellent cues that will guide the neurites to their final target. Longer neurites with complex arborizations correlate with more diverse synaptic connections and efficient neurotransmission (Kolb and Gibb, 2011). Restraint stress for 21 days reduced dendritic length and branching of pyramidal neurons in rat hippocampal CA3 region and medial prefrontal cortex (Radley et al., 2006). Chronic treatment with CORT 21 days decreased axonal and

dendritic outgrowth in mouse hippocampal pyramidal neurons (Magarinos et al., 1998; Zhang et al., 2021). In another study, exposure of primary rat hippocampal neurons to CORT or GR agonist dexamethasone for 4 and 7 days decreased the length of primary, secondary, and tertiary processes (Levone et al., 2021). The microtubule-associated protein 2 (MAP2) is a neuron-specific cytoskeletal protein highly expressed in dendrites (Chamak, 1987). It has been found that chronic unpredictable stress downregulates MAP2 (Yan et al., 2010). Dendritic spines are small protrusions at the dendrite level that receive and compute excitatory inputs. Dendritic spines play an important role in neurotransmission (Petralia et al., 2014). Rats exposed to chronic unpredictable stress for 28 days displayed reduced spine density of pyramidal neurons and decreased the population of mature mushroom-like spines in the prefrontal cortex indicating a loss of mature spines and synaptic connections (Li et al., 2011). Altogether this evidence suggests that chronic stress and chronic CORT treatment impairs neurogenesis, neurite outgrowth and neurotransmission.

Synapse development starts once the axon growth cone reaches the dendritic target. In this process, specialized cellular machinery recruits presynaptic and postsynaptic domains to their respective compartment (Glantz et al., 2006; Martínez et al., 1998). The presynaptic compartment contains synaptic vesicles, synaptic vesicle-associated transporters such as the vesicular glutamate transporter 1 (vGLUT1) and vesicular gamma-aminobutyric acid (GABA) transporter (vGAT) and SNARE proteins including synapsin I and synaptosomal-associated protein 25 (SNAP25). All these structures and proteins work together to release neurotransmitters into the synaptic cleft (Frank, Wang et al. 2013). In the postsynaptic side, postsynaptic density protein 95 (PSD95) stabilizes and anchors glutamate receptors such as alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartate

(NMDA) receptors at synapses (Dabrowski, Johnson-Venkatesh et al. 2017). Chronic unpredictable stress decreased levels of PSD95 and synapsin I in the hippocampus and prefrontal cortex of adult rats (Li et al., 2011; Ran et al., 2018). Daily restraint stress for 6 hours during a 21-day period also decreased SNAP25 protein levels in the prefrontal cortex compared to non-stressed rats (Müller et al., 2011). Another study showed that chronic treatment with CORT for 4 weeks decreased the expression of AMPA receptor subunit 1 in rat hippocampus, thus impairing glutamatergic synapses and long-term memory consolidation (Kvarta et al., 2015). All these studies suggest that chronic stress and chronic CORT treatment impair synaptogenesis and synaptic transmission.

Neurotrophic factors such as the brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are very important in the regulation of neuronal differentiation (Mcallister, 2001). Neurotrophic factors bind to their tyrosine kinase receptor and activate downstream signaling molecules including the transcription factor cyclic AMP response element binding protein (CREB). Upon phosphorylation, CREB translocates to the nucleus, where it binds to the cyclic AMP response element (CRE) and regulates the expression of various genes that are important in neuronal differentiation and synaptic plasticity (H. Wang et al., 2018). Studies have found that chronic unpredictable stress for 3 to 5 weeks downregulated BDNF mRNA and protein levels, and total and phosphorylated CREB levels in rat hippocampus and basolateral amygdala (Hazra et al., 2017). Chronic stress also decreased BDNF expression in the hippocampus of adult mice, making pyramidal neurons more susceptible to neuronal atrophy and neuronal death (Ran et al., 2018)(Altieri et al., 2004). Moreover, CORT administration for 21 days reduced BDNF mRNA and protein levels in the hippocampus and prefrontal cortex of rats (Dwivedi et al., 2006; Gourley et al., 2008). Primary mouse hippocampal neurons treated with

dexamethasone showed lowered BDNF mRNA levels, an effect that was prevented by pre-treatment with GR antagonist RU-486 (Chen et al., 2017). These findings suggest that chronic stress and chronic CORT exposure may downregulate BDNF and inhibit CREB phosphorylation, thus impairing neuronal differentiation and synapsis formation.

#### 1.1.3.3 Chronic stress, corticosterone, and neurodegeneration

Neuronal degeneration refers to the process where neurons progressively die due to aging or as a result of pathological situations such as neurodegenerative diseases and stroke. Apoptosis is a programmed cell death characterized by the activation of caspases, chromosome condensation and DNA fragmentation (Chen et al., 2018). Apoptosis is initiated by the activation of death receptors or by mitochondrial damage induced by myriad stressors. Stimuli such as DNA damage and oxidative stress impair the activity of mitochondrial pro-survival Bcl-2 family members, resulting in pore formation on the mitochondrial outer membrane and increased mitochondrial permeability (Glantz et al., 2006). Concomitantly, pro-apoptotic Bax proteins translocate to the mitochondria to impair mitochondrial homeostasis. As a result, cytochrome c gets released to the cytosol, then activates caspase 3, induces DNA fragmentation and ultimately causes cell death (Banagozar Mohammadi et al., 2019; Chen et al., 2018). Chronic restraint stress has been shown to induce neuronal loss in rat hippocampus and prefrontal cortex (Carroll et al., 2011). Previously, it has been reported that chronic isolation stress increases cytochrome c release from mitochondria and increases cleaved-caspase 3 levels in rat prefrontal cortex (Filipović et al., 2011). Moreover, mice exposed to chronic restraint stress showed increased levels of Bax and reduced levels of Bcl-2 in the prefrontal cortex and hippocampus (Banagozar Mohammadi et al., 2019). Chronic unpredictable mild stress for 5 weeks increases Bax levels

and caspase-3 positive pyramidal neurons in rat prefrontal cortex and hippocampus (Bachis et al., 2008; Peng et al., 2020). Treatment with CORT for 24 hours also reduces cell viability and augments TUNEL-positive cells in PC12 and SH-SY5Y cell lines (Li et al., 2016; Ma et al., 2020; Yun and Jeong, 2020).

Stressful stimuli can activate apoptosis signaling kinase 1, inducing ASK1 Thr845/Thr838 autophosphorylation. In turn, ASK1 phosphorylates c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase (p38), which leads to apoptosis (Ichijo et al., 1997; Takeda et al., 2000). Our lab has found that chronic unpredictable stress increases phosphorylated ASK1, phosphorylated JNK and phosphorylated p38 levels in mice hippocampi and prefrontal cortex (Zhou et al., 2019). Dysregulated or excessive cell death can lead to pathological conditions including Alzheimer's disease and Parkinson's disease. Alzheimer's disease is a progressive neurological disorder that leads to memory loss and cognitive dysfunction. Some of the hallmarks include deposition of β-amyloid plaques and Tau tangles (Jeong et al., 2006). One study showed that chronic restraint stress for one month increased Tau hyperphosphorylation in mice hippocampi and prefrontal cortex (Carroll et al., 2011). Long-term restraint stress for 8 weeks accelerated the onset and severity of cognitive dysfunction in a mouse model of Alzheimer's disease by elevating β-amyloid plaque deposition and Tau phosphorylation in rat hippocampus and cortex (Jeong et al., 2006). Parkinson's disease is a neurodegenerative disorder that affects movement control. It is characterized by the degeneration of dopaminergic neurons predominantly in the substantia nigra (Smith et al., 2008). Evidence showed that chronic restraint stress and chronic CORT injections induced significant neuronal loss in dopaminergic neurons in ventral tegmentum area and substantia nigra, which exacerbated motor deficit in a rat model of Parkinson's Disease (Smith et al., 2008). Chronic unpredictable stress also exacerbated

lipopolysaccharide-induced loss of dopaminergic neurons in the substantia nigra (De Pablos et al., 2014). Overall, these findings suggest that chronic stress and chronic CORT exposure can lead to neurodegeneration and ultimately to the development of neurodegenerative diseases.

### 1.1.4 Chronic stress as a risk factor for major depressive disorder

Major Depressive Disorder (MDD) is a mental illness characterized by physical, psychological, and behavioral symptoms including psychomotor retardation, feeling of worthlessness, anhedonia, and recurrent thoughts of death among others (American Psychological Association, 1994). It affects around 5% of the adult population, with greater incidence in women than men (Otte et al., 2016). The World Health Organization described MDD as one of the leading causes of disability worldwide (World Health Organization, 2023). In Canada, nearly 4.7% of the population aged 15 years or older has been diagnosed with MDD (Findlay, 2017). Treatments for MDD include antidepressant medications, psychotherapy, and in some cases, electroconvulsive therapy (American Psychological Association, 1994; Kennedy et al., 2001). The estimated costs of these treatments are expected to reach a total of US$147 billion by 2030 (Findlay, 2017).

Chronic stress has been reported to be a risk factor for the development of depression. Clinical studies have demonstrated high levels of GCs in blood, plasma, saliva, urine, and cerebrospinal fluid of patients with depressive disorder. Depressed patients also showed increased CRH levels in plasma and enlarged adrenal glands compared to healthy controls, suggesting that HPA feedback is impaired in MDD (Nemeroff et al., 1984). Moreover, a study comprising over 370,000 individuals revealed that treatment with synthetic GCs increased by two-fold the risk of suicide in patients with MDD (Fardet et al., 2012). Patients diagnosed with

Cushing syndrome, a disease characterized by hypercortisolism, often present depressive symptoms (A. Tang et al., 2013). Stressful life events such as financial distress, divorce, unemployment, and childhood traumas can precipitate the onset of depression (Otte et al., 2016).

Chronic stress impairs the structure, connectivity, and function of the brain, which contributes to the pathophysiology of MDD. For example, imaging studies have reported that MDD patients who suffered from prolonged stress present decreased hippocampal and prefrontal cortex volume (Bittar and Labonté, 2021; Hamilton et al., 2012); thinner orbitofrontal cortex and cingulate cortex (Bittar and Labonté, 2021; Schmaal et al., 2017); and lower levels of serotonin in the hippocampal synaptic cleft (Tafet, 2001). Another study indicated that chronic stress augments the activation of the amygdala and reduces activity in the ventral striatum, which might explain the negative thoughts and lack of reward in MDD patients (Hamilton et al., 2012; Pizzagalli, 2014). Overall, chronic stress induces anatomical and functional changes in the brain that contribute to the development of depression.

## 1.2 Thioredoxin antioxidant system

### 1.2.1 Structure and isoforms of thioredoxin

Thioredoxin (Trx) is a 12 kDa redox protein that can reverse protein cysteine thiol oxidation. This protein has an active conserved site, Cysteine32-Proline-Glycine-Cysteine35, which is essential for its function as an oxidoreductase (Arnér and Holmgren, 2000). Trx is ubiquitously expressed in all organisms ranging from archaea to mammals (Powis and Montfort, 2001), where it plays a key role in maintaining the cellular redox balance. Mammalian cells contain two isoforms of Trx- Trx 1, which is located in the cytosol, and Trx 2, which can be found in the mitochondria (Das, 2005). As shown in figure 3, active Trx reverses protein cysteine

oxidation by transferring electrons from its catalytic cysteine residues to the target protein in a two-step mechanism. First, cysteine residue Cys32, transfers one electron to the substrate protein, forming a transient Trx-substrate protein bond. Then, the cysteine residue Cys35, donates the remaining electron to the substrate protein. At the end of the reaction, the target protein is completely reduced while the catalytic cysteines of Trx are oxidized (Collet and Messens, 2010; Matsuzawa, 2017). Oxidized Trx loses its reducing activity. However, oxidized Trx can be reduced back and reactivated by thioredoxin reductase (TrxR) (Fritz-Wolf et al., 2011). In addition to the catalytic cysteines, mammalian Trx 1 contains three extra cysteine residues: cysteines 62, cysteine 69 and cysteine 73. These cysteines have been reported to be involved in regulating the function of Trx 1 by modulating protein folding and post-translational modifications such as glutathionylation and S-nitrosylation (Casagrande et al., 2002).

### 1.2.2 Thioredoxin Function

***Thioredoxin and oxidative stress***

Trx plays an important role in many physiological processes (Kohárýová and Kollárová, 2015). First, Trx reduces protein cysteine thiol modifications, which contributes to regulating the cellular redox balance and acts as a defense against oxidative stress (Matsuzawa, 2017). Cysteine residues play a crucial role in controlling protein function including catalyzing enzymatic reactions, maintenance of redox balance, and signal transduction (Paul et al., 2018). Cysteines are particularly vulnerable to damage caused by reactive oxygen species (ROS) compared to other amino acids because of their highly nucleophilic character. Under oxidative and nitrosative stress, protein cysteine thiols can undergo oxidative modifications, which can either be reversible or irreversible (Paul et al., 2018).

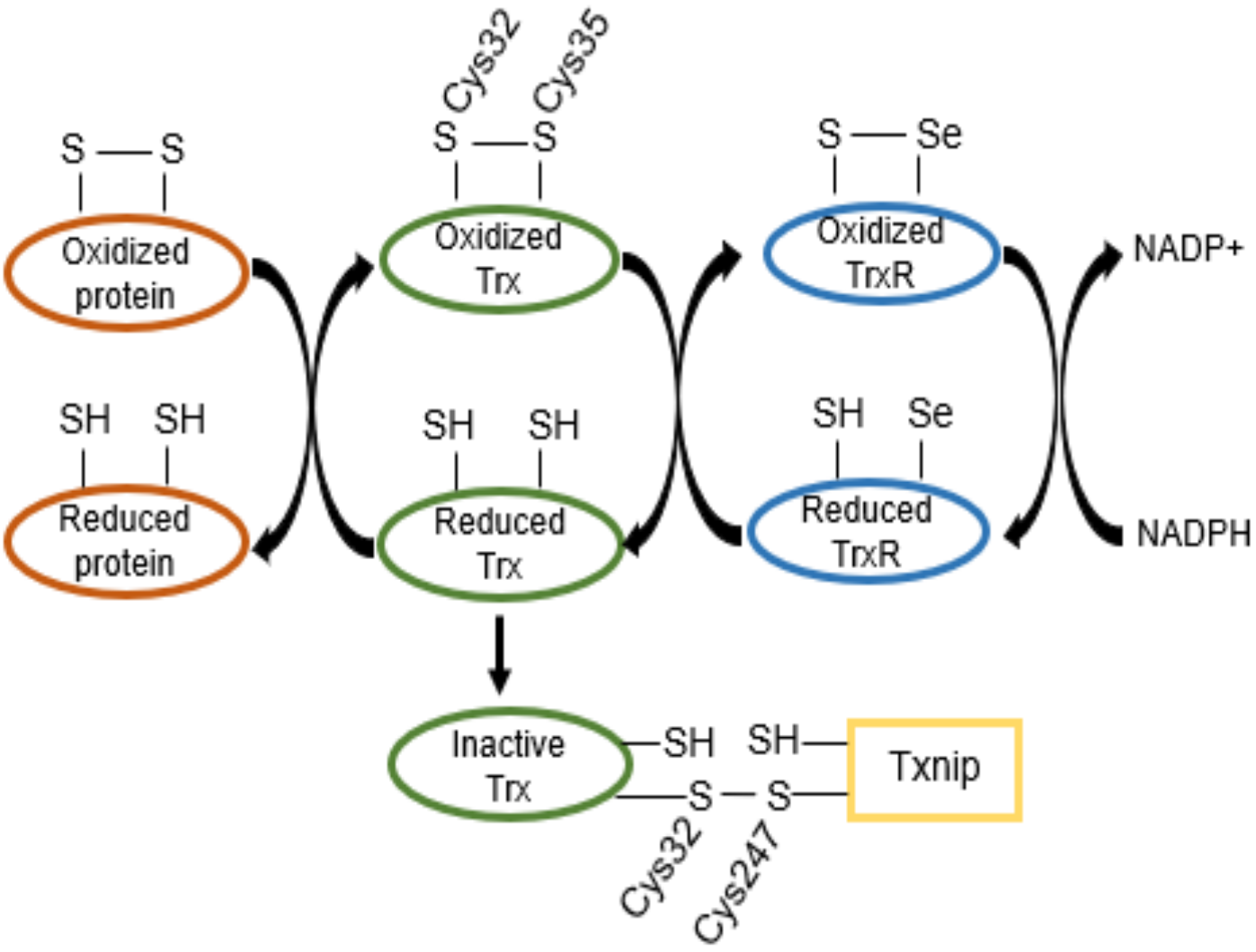


**Figure 3: Thioredoxin mechanism of action:** Protein thiol disulfides are reduced by thioredoxin (Trx). In this process Trx gets oxidized. Thioredoxin reductase (TrxR) reduces back Trx using NADPH. Trx is inactivated by thioredoxin interacting protein (Txnip)

Cysteine oxidative modifications include reversible sulfenylation, disulfidation and nitrosylation, and irreversible sulfinylation and sulfonylation, (Hashemy and Holmgren, 2008).

Under oxidative stress conditions, elevated $H_2O_2$ levels can react with cysteine residues resulting in the formation of sulfenic acid (-SOH) through a reversible process called sulfenylation. Sulfenic acid can further interact with other thiols in the same molecule or thiols from other molecule to form disulfides bonds (-S-S-) in a process known as disulfidation. One of the main functions of Trx is to reduce these disulfide bonds back to reduced cysteine thiols by a thiol-exchange mechanism. Sulfenic acid can undergo further irreversible oxidation reactions including sulfinylation to render sulfinic acid ($-SO_2H$) or sulfonylation to render sulfonic acid ($-SO_3H$). In addition, cysteines can be nitrosylated by nitric oxide (NO) radicals under nitrosative stress conditions, leading to the formation of nitrosothiols (-SNO). This reaction can be reversed by Trx in a process known as denitrosylation. Trx interacts with nitrosothiols through its catalytic site thiols, resulting in the conversion of nitrosothiols back to cysteine thiols, releasing a nitroxyl ion (HNO) in the process (Paul et al., 2018).

In the brain, cysteine oxidative modifications can regulate protein activity in different ways. Previously, it has been reported that GluA1 subunit of AMPA receptors can undergo nitrosylation, which potentiates AMPA receptor phosphorylation, increases receptor conductance and promotes receptor endocytosis (Selvakumar et al., 2013). Similarly, NMDA receptor subunit NR2A can be S-nitrosylated, which reduces the frequency of channel opening and, therefore, channel activity (Choi and Lipton, 2000). Antioxidant protein SOD can also be nitrosylated, which induces protein aggregation. Aggregation of SOD has been associated with degeneration of motor neurons in amyotrophic lateral sclerosis (ALS) (Paul et al., 2018). The monoacylglycol lipase is an enzyme that plays a key role in endocannabinoid signaling by hydrolyzing the

endocannabinoid transmitter endocannabinoid-2-arachidonyl-sn-glycerol. A previous study reported that $H_2O_2$-mediated cysteine sulfenylation of the monoacylglycol lipase prevents the degradation of the endocannabinoid transmitter in primary rat cortical neurons, which increases endocannabinoid signaling (Dotsey et al., 2015). Moreover, cytoskeletal protein tubulin can undergo cysteine thiol oxidation, leading to alterations in tubulin dynamics and polymerization in primary rat cortical neurons (MacKenzie et al., 2011). The cytoskeletal-regulatory protein CRMP2 regulates microtubule function during neuronal development. It has been reported that $H_2O_2$-induced thiol oxidation of CRMP2 stimulates CRMP2 phosphorylation by glycogen synthase kinase-3 and leads to the growth cone collapse of rat dorsal root ganglia neurons (Morinaka et al., 2011). Evidence from our laboratory indicated that nitrosylation levels of vesicular acetylcholine and vesicular glutamate transporter were elevated in the brain of amyloid precursor protein/Presenilin 1 double transgenic mice at 9- and 12-month-old (Wang et al., 2017). Nitrosylation of these transporters decreased vesicular glutamate and acetylcholine uptake in the brain (Wang et al., 2015). All this evidence suggests that protein cysteine oxidative modification can regulate neuronal functioning.

***Thioredoxin and peroxiredoxin***

Trx contributes to regulating the cellular redox balance by keeping peroxiredoxin in a reduced state (Matsuzawa, 2017). Peroxiredoxins are a large and highly conserved family of proteins that scavenge peroxides such as $H_2O_2$, lipid peroxides and peroxynitrite, and prevent lipid peroxidation and protein oxidation. The cysteine residues in the active site of peroxiredoxin react with the peroxide substrate and undergo oxidization. Trx reduces the disulfide bond formed in this step and regenerates the active form of peroxiredoxin (Rhee, 2016).

***Thioredoxin and apoptosis signaling kinase 1***

Trx inhibits programmed cell death by binding to the apoptosis signal-regulating kinase 1 (ASK1) (Saitoh et al., 1998). ASK1 is a ubiquitously expressed Ser/Thr kinase that belongs to the mitogen-activated protein kinase kinase kinase (MAP3K) family. ASK1 consists of three domains: the N-terminal Trx binding domain, the central regulatory domain containing the serine/threonine residues as well as the TRAF-binding domain, and a C-terminal coiled-coil domain. Under basal conditions, ASK1 forms the ASK1 signalosome. The ASK1 signalosome consists of two ASK1 molecules interacting by their C-terminal coiled-coil domain. Trx is bound to the N-terminal domain, blocking the access to the active site of ASK1 kinase domain (Kawarazaki et al., 2014). In addition, the negative regulator 14-3-3 is present (Obsil and Obsilova, 2017). Studies indicate that Trx catalytic cysteine residues might be involved in Trx-ASK1 interaction since the oxidized form of Trx cannot bind ASK1 (Takeda et al., 2003a), and mutagenesis of catalytic Cys32 impairs Trx binding to ASK1 (Shiizaki et al., 2013).

The ASK1 apoptotic pathway is activated by different stressful stimuli including oxidative stress, endoplasmic reticulum stress, cytokines signaling, and activation of TNF receptor and Fas death receptors. Oxidative stress increases the intracellular ROS levels, which oxidizes Trx. The negative regulator 14-3-3 and oxidized Trx dissociate from ASK1. This event leads to the recruitment of TNF-α receptor-associated factors TRAF2 and TRAF6, which bind to the central regulatory domain and induce an open conformation of the regulatory domain. As a result, the ASK1 threonine residue (Thr 845 in mice; Thr 838 in humans) located in the central regulatory region is auto-phosphorylated, leading to ASK1 activation. Activated ASK1 further phosphorylates and activates MKK4/7 and MKK3/6, which in turn phosphorylates c-Jun-N-terminal-kinase (JNK) and protein 38 kinase (p38), leading to apoptosis (Kawarazaki et al.,

2014; Shiizaki et al., 2013). ASK1 apoptotic pathway can also be activated by ER stress generated by the accumulation of unfolded protein in the ER lumen. ER stress induces the oligomerization-dependent autophosphorylation of ER-resident type 1 transmembrane serine-threonine kinases PERK and IRE1. In turn, PERK and IRE1 recruit TRAF2 and TRAF6, which prompts ASK1 autophosphorylation at threonine residue (Takeda et al., 2003b). ASK1 signaling pathway is terminated by dephosphorylation of threonine residue by the protein phosphatase 5. In addition, phosphorylation of serine 966 in the central regulatory domain triggers the association of the 14-3-3 negative regulator with ASK1, hence inducing ASK1 inactivation (Obsil and Obsilova, 2017).

***Thioredoxin and transcription factors***

Trx can translocate to the nucleus and interact with transcription factors to regulate their DNA-binding and transcriptional activity. The redox factor protein 1 (Ref-1) plays a dual role in the cell as a DNA repair enzyme and as a regulator of different transcription factors (Wei et al., 2000). Previously, it has been reported that Trx directly interacts with Ref-1. Co-expression of Ref-1 fusion constructs and wild-type Trx in Cos-7 cell line resulted in a 10-fold increase in the activity of luciferase gene. Moreover, co-expression of a Trx lack-of-function mutant (mutation of catalytic cysteines) and Ref-1 fusion constructs did not induce luciferase activity. These findings suggest that Trx directly interacts with Ref-1 through its catalytic cysteine residues (Hirota et al., 1997). The activator protein 1 (AP-1) is a transcription factor formed by c-Fos and c-Jun nuclear proteins. The DNA binding activity of AP-1 is regulated by Ref-1. Therefore, Trx can indirectly modify AP-1 binding activity by regulating Ref-1 (Hirota et al., 1997). To confirm this, a study conducted in HeLa cells demonstrated that treatment with

increasing concentrations of Trx augmented AP-1 DNA binding activity (Wei et al., 2000). NF-κB is a transcription factor consisting of two DNA-binding subunits p50 and p65. Trx has also been shown to directly associate with NF-κB. In one study, wild-type p50 subunit or a mutant p50 subunit (mutation in Cys 62) was incubated with a recombinant Trx in Jurkat T cells. The DNA binding activity was assessed and compared between the two variants. It was found that the DNA binding activity of wild-type p50 subunit was three times higher than the mutant p50 subunit, suggesting that Trx increases the DNA-binding activity of the NF-kB p50 subunit via Cys 62 (Matthews et al., 1992). Specificity protein 1 (SP1) is a zinc finger transcription factor that can bind to genes containing GCs-rich regions and regulate the expression of housekeeping gene targets. A previous report showed that treating MDA-MB-231 breast cancer cell line with increasing concentrations of human recombinant Trx dose-dependently increased SP1 DNA binding activity (Bloomfield et al., 2003). Cyclic AMP response element-binding protein (CREB) is a gene transcription factor that regulates the expression of various genes that are important in neuronal differentiation and synaptic plasticity (H. Wang et al., 2018). Phosphorylation of CREB is necessary for CREB binding to the cyclic AMP response element (CRE) in the promoter of target genes including c-fos, BDNF and NGF (Bai J. et al., 2003; Zheng et al., 2012). Studies have shown that upregulation of Trx can increase CREB phosphorylation in HEK293 cells, PC12 cells and mouse brain (Hirota et al., 2000; Huang et al., 2018; Lv et al., 2013). In another report, PC12 cells were transfected with an antisense Trx plasmid and pGL3-c-fos-luciferase plasmid containing a CRE sequence upstream. Expression of pGL3-c-fos gene was induced by 4-hour treatment with NGF. Overexpression of antisense Trx contruct reduced NGF-induced expression of luciferase reporter gene by 75%, suggesting that Trx might regulate CREB binding to CRE and CRE-driven gene expression (Bai J. et al., 2003).

Trx can also interact with GR DNA binding domain and restore GR transcriptional activity under oxidative stress conditions (Makino et al., 1999). HeLa cells transfected with GR expression plasmids, and a glucocorticoid-responsive reporter contruct were treated with 1 mM $H_2O_2$, which led to a reduction in the luciferase reporter activity. Interestingly, when the cells were transfected with a Trx antisense plasmid, the repression of the GC response by $H_2O_2$ was enhanced. On the other hand, transfection with a Trx overexpression plasmid reversed this effect, suggesting that GR and Trx have a functional interaction (Makino et al., 1999).

### 1.2.3 Thioredoxin reductase

Trx is maintained in a reduced state by thioredoxin reductase (TrxR) (Collet and Messens, 2010). TrxR is a 55 kDa NADPH-dependent homodimeric oxidoreductase (Arnér and Holmgren, 2000; Mustacich and Powis, 2000). Similar to Trx, this protein contains two isoforms of TrxR, the cytosolic form TrxR1 and the mitochondrial isoenzyme TrxR2 (Fritz-Wolf et al., 2011; Mustacich and Powis, 2000). TrxR contains two cysteine residues -Cys-Xaa-Xaa-Cys- in the catalytic site, which is located in the N-terminal flavin adenine dinucleotide (FAD)-binding site of the enzyme (Collet and Messens, 2010). In addition, TrxR contains a selenocysteine (SeCys) residue -Gly-Cys-SeCys-Gly-COOH, located at the C-terminal segment (Arnér, 2009). At the beginning of the catalytic cycle, the catalytic cysteines, and the C-terminal -Cys-SeCys- are found in the oxidized form. The catalytic reaction starts when NADPH binds to the NADPH-binding site of TrxR. NADPH transfers two electrons to FAD and then FAD reduces the thiol bond between the catalytic cysteines. Through a thiol-disulfide exchange mechanism, the catalytic cysteines reduce the C-terminal -Cys-SeCys- site. Finally, the reduced Cys-SeCys

transfers electrons to the oxidized Trx catalytic cysteines, to render Trx in its reduced form (figure 3) (Arnér, 2009; Arnér and Holmgren, 2000).

### 1.2.4 Thioredoxin-interacting protein.

Thioredoxin-interacting protein (Txnip), also known as vitamin D3 upregulated protein 1 (VDUP1), is a 46 kDa regulatory protein that belongs to the α-arrestin family (Nasoohi et al., 2018). Txnip is ubiquitously expressed in tissues, and it is implicated in various cellular processes (Matsuzawa, 2017). Txnip contains two SH3 binding domains in the NH2-terminal and three SH3 domains in the C-terminal. In addition, Txnip contains two PPxY motifs in the C-terminal domain. The SH3 domains enable Txnip to act as a scaffold in various cellular compartments. On the other hand, the PPxY motif plays a role in regulating ubiquitination and degradation of Txnip (Polekhina et al., 2013). Txnip plays several functions in the cell. Txnip inhibits glucose transporter 1 (GLUT1), thus preventing glucose uptake and glucose metabolism in the pancreas and liver (Shalev, 2014). In addition, Txnip is involved in regulating transcription factor PPAR and insulin secretion (Qayyum et al., 2021; Shalev, 2014). Txnip can also bind to the nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing 3 (NLRP3). Binding of Txnip to NLRP3 induces assembly and activation of the NLRP3 inflammasome, which in turn promotes the cleavage of interleukin-1β precursor by caspase-1 and subsequent inflammatory response (Zhou et al., 2010). Moreover, Txnip regulates cellular redox balance by binding to Trx and inhibiting Trx activity. Txnip binds only to the reduced form of Trx. Txnip interacts with Trx by forming a mixed disulfide bond between the Trx Cys32 catalytic residue and Txnip Cys247 residue (figure 3) (Hwang et al., 2014). Txnip also interacts with α-arrestin to sequester Trx in the nucleus, thus regulating Trx intracellular localization

(Matsuzawa, 2017). Txnip is induced by various types of stress, such as oxidative stress, ultraviolet irradiation, and heat shock. Indeed, binding of Txnip to Trx is augmented during oxidative stress conditions and it is associated with apoptosis signaling (Qayyum et al., 2021). Txnip has been associated to the pathology of many diseases including diabetes, coronary heart disease, and Alzheimer's disease (Matsuzawa, 2017).

### 1.2.5 Regulation of thioredoxin expression

Trx gene expression is upregulated during oxidative stress and contributes to maintaining cellular redox homeostasis. Trx gene promoter region contains four antioxidant-responsive element (ARE) sites (Nakamura, 2004). Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a gene transcription factor. Under oxidative stress conditions, Nrf2 binds to the ARE element in the promoter of Trx gene and promotes Trx gene expression (Kováryová and Kollárová, 2015). Trx can also undergo post-translational modifications that regulate its function and are implicated in signal transduction pathways (Hashemy and Holmgren, 2008). Under nitrosative stress, structural residues Cys-69 and Cys-73 can be S-nitrosylated. This modification has been reported to act as a protective mechanism against irreversible oxidation of catalytic cysteines (Paul et al., 2018). Trx can also be nitrated on Tyr49 by peroxynitrite, which leads to irreversible inhibition of its redox and anti-apoptotic activity (Tao et al., 2006). Recent reports have shown that Trx can undergo glutathionylation at Cys-73 and glycation, which also inhibits Trx-reducing function (Casagrande et al., 2002; Yuan et al., 2010).

### 1.2.6 Thioredoxin system in psychiatric and neurological disorders

Evidence has shown that Trx system is changed in psychiatric and neurological disorders.

Previously, it was reported that male individuals with bipolar disorder had lower plasmatic levels of Trx than healthy subjects (Genc et al., 2015). Conversely, Trx levels were found elevated in the serum of patients with autism spectrum disorder when compared to healthy controls (Zhang et al., 2015). Another report revealed no changes in plasmatic Trx levels in patients with treatment-resistant depression compared to controls (Aydın et al., 2018). A study that compared cognitive scores in 45 schizophrenic patients and 66 healthy subjects using Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) demonstrated a negative correlation between Trx plasma levels and cognitive performance in the patient's group. Conversely, healthy subjects showed a positive correlation between Trx levels and cognitive function. These findings suggest that the cognitive deficit observed in schizophrenic patients may be associated with dysregulation of Trx expression (Zhang et al., 2013). In a similar study, Trx levels were found to be higher in schizophrenic patients after acute psychotic episodes compared to the same patients in remission state after 16 weeks of treatment with atypical antipsychotics. The healthy subjects in this study showed much lower Trx levels in serum than the patient's groups (Bas et al., 2017). These findings suggest that Trx could be used as a marker to help diagnose these disorders. Our lab found that Trx and TrxR protein levels are upregulated in HT22 mouse hippocampal cells after treatment with specific serotonin reuptake inhibitor fluoxetine and specific norepinephrine reuptake inhibitor venlafaxine for 5 days, suggesting that these drugs might exert antidepressant effects by also upregulating Trx system (Bharti et al., 2020). Overall, this evidence suggests that dysregulation of Trx system might contribute to the development of psychiatric disorders.

Evidence also has shown that Trx system is altered in Alzheimer's disease. Previous studies analyzed the Trx levels in different brain areas of post-mortem tissue of patients with Alzheimer's disease. They found that Trx levels were decreased in amygdale, hippocampi, and

frontal cortex of Alzheimer's disease patients compared to healthy subjects (Akterin et al., 2006; Lovell et al., 2000). Furthermore, Trx was found to be reduced in the hippocampus of female 5X FAD mice, a transgenic mouse line featuring the main characteristics of Alzheimer's disease including the development of amyloid β plaque (Pan Q et al., 2020). Findings also showed that TrxR was decreased in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (Liu et al., 2021). However, overexpressing Trx in the brain ameliorated movement disorder and cognitive impairment in MPTP-treated transgenic mice (Zhang et al., 2018). Another study determined the mRNA and protein levels of Trx and TrxR in the plasma of 40 patients with multiple sclerosis. They found that Trx and TrxR levels were significantly elevated in newly diagnosed subjects, patients receiving interferon and patients treated with immunosuppressants compared to healthy individuals (Mahmoudian et al., 2017). These findings suggest that the deficiency of Trx antioxidant system may be involved in these neurological disorders and that Trx and TrxR are potential targets for the treatment of these disorders.

### 1.2.7 Thioredoxin system in chronic stress

Chronic stress contributes significantly to the development of many psychiatric and neurological disorders. Studies indicate that Trx system plays an important role in neuronal damage induced by chronic stress and stress hormones. For example, it has been found that repeated restraint stress for 3 hours daily for 14 days reduced mouse hippocampal Trx protein levels (Kim MH and Leem YH., 2019). Chronic treatment with 20 mg/kg CORT for 21 days was also found to decrease TrxR activity in mice hippocampi (Camargo et al., 2020). On the contrary, TrxR mRNA, protein levels and enzymatic activity were also found elevated in the placenta of

female fetuses but not males after exposure to CORT during pregnancy from E12.5 to E14.5 (Bartho et al., 2019). Moreover, increased Txnip levels were reported in the rat hippocampi after chronic unpredictable stress for 21 days (Song et al., 2018). Similarly, one study found that Txnip protein levels were upregulated in mice hippocampi after chronic unpredictable mild stress for 12 weeks (Su et al., 2017). Txnip levels were also upregulated after 9 days-chronic variate stress in mice hippocampi (Sánchez-Hidalgo et al., 2016). In another study, rats exposed to subcutaneous injections of 40 mg/kg CORT for 21 days displayed elevated Txnip mRNA and protein levels in the prefrontal cortex. In the same study, Txnip was increased in primary rat astrocytes after treatment with 10 nM CORT for 3 days (Pan et al., 2022). Comparable to these findings, studies from our lab showed that Txnip levels were increased in the mouse frontal cortex and hippocampi after chronic unpredictable stress for 28 days. However, Trx and TrxR expression was not changed by chronic stress (Zhou et al., 2019). Moreover, our lab found that chronic CORT treatment for 5 days increased Txnip protein levels but had no effect on Trx and TrxR protein levels in HT22 neuronal cells, primary mouse cortical neurons and N9 microglial cells (Bharti et al., 2019, 2018). Treatment with GR antagonist RU486 or knocking down Txnip prevented CORT-induced oxidative stress and inflammatory response in HT22 and N9 cells respectively (Bharti et al., 2019, 2018). These findings suggest that chronic stress and chronic CORT exposure upregulate Txnip and downregulate Trx, which contributes to chronic stress-induced oxidative damage process.

### 1.3 Summary and hypothesis

***Summary:*** Previous reports have shown that chronic stress and chronic treatment with stress hormone CORT can promote oxidative damage to proteins, DNA, and lipids; decrease

antioxidant capacity; impair neuronal differentiation, plasticity; and induce neurodegeneration, which induces deleterious effects in the brain. Chronic stress is a risk factor for the development of psychiatric disorders such as major depressive disorder.

The thioredoxin system includes Trx, TrxR, and Txnip and plays an important role in cellular defense against oxidative stress. Trx can reverse protein cysteine thiol oxidative modifications and plays a major role in cellular redox balance. Moreover, Trx prevents apoptotic signaling by inhibiting the activation of ASK1. Studies have also shown that Trx can facilitate the activation of CREB neurotrophic signaling. Trx is maintained in a reduced state by TrxR. Txnip is an endogenous inhibitor for Trx. Altogether these findings suggest that Trx system may regulate neuronal differentiation and degeneration and that abnormal Trx system contributes to chronic stress or chronic CORT treatment-induced neuronal damage. Targeting Trx system may have potential for the treatment of depression.

***Hypothesis****:* Chronic CORT treatment may downregulate Trx and upregulate Txnip, thus reducing CREB neurotrophic pathway and activating ASK1 apoptotic pathway, which results in impaired neuronal differentiation and enhanced neurodegeneration. Upregulating Trx antioxidant system may inhibit depressive-like behaviors in rodents.

### 1.4 Objectives

1. To establish the role of Trx system in neuronal differentiation and degeneration.
2. To determine whether CREB neurotrophic pathway and ASK1 apoptotic pathway mediate Trx system-regulated neuronal differentiation and degeneration.
3. To determine whether Trx antioxidant system mediates CORT treatment impaired-neuronal

differentiation and enhanced degeneration.

4. To determine whether Txnip knockdown prevents chronic CORT-induced depressive behaviors and cognitive dysfunction.

# CHAPTER 2: MATERIAL AND METHODS

## 2.1 Reagents

Trx pharmacological inhibitor 2-[(1-methylpropyl) dithio]-1H-imidazole (PX12) was purchased from Sigma Aldrich, Canada (Cat # M5324). PX12 was dissolved in dimethyl sulfoxide and stored at -20$^{o}$C. The Trx mimetic peptide CB3 was synthesized by CanPeptide Inc. (Quebec, Canada). The peptide was dissolved in hexafluoro-2-propanol and aliquoted, then samples were allowed to evaporate and stored at -80$^{o}$C. At the time of use, the peptide was dissolved in water.

## 2.2 Cell culture

### 2.2.1 Primary cultured mouse cerebrocortical neurons

Primary cultured mouse cerebrocortical neurons were cultured as previously reported by our laboratory (Bharti et al., 2018). Pregnant CD1 mouse with 15-18 days of gestation was anesthetized with isoflurane and euthanized via cervical dislocation. Rapidly, embryos were removed and decapitated. The cerebral cortices were dissected, then the meninges and blood vessels were removed, and the cortices were finely chopped. Tissue was digested with 3 mL 0.25% trypsin for 15 min at 37°C. Then, 500 µL DNAse (1 mg/ml) was added for 15 min at 37°C. 3 mL of Minimum Essential Medium Eagle (MEM) supplemented with 20% fetal bovine serum (FBS) was added to stop trypsinization. The supernatant was removed, and cells were dissociated by slowly pipetting 8 times. Cells were resuspended in 1 mL of neurobasal medium (Life Technologies Inc.) containing 1X Glutamax (Life Technologies Inc.), 1X N21 supplement (R&D System, USA) and 1% penicillin/streptomycin. Neurons were seeded in 6-well plates

previously coated with 50 µg/mL poly-D-lysine at a density of 7.50 x $10^5$ cells/well. Neurons were kept in an incubator at 37°C under 5% $CO_2$. Half medium was changed twice a week.

### 2.2.2 Human neuroblastoma SH-SY5Y cell line

SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Life Technologies Inc, Burlington, ON, Canada) with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C under 5% carbon dioxide ($CO_2$).

## 2.3 Protein isolation

Cultured cells were washed twice with cold 1X Phosphate Buffer Saline (PBS). Then, cells were lysed for 1 h with an ice-cold lysis buffer [250 mM NaCl, 30 mM $MgCl_2$, 0.1 mM EGTA, 0.5mM EDTA, 20 mM HEPES, 20% glycerol, 1% NP-40, 0.25 mM DTT and 1× protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA)]. Cell lysates were centrifuged in a refrigerated centrifuge at 4°C for 10 min at 10,000 g and the supernatants containing the protein extracts were collected. Protein concentrations were determined by Bradford protein assay.

## 2.4 Immunoblotting analysis

Immunoblotting analysis was performed as described previously (Bharti et al., 2018). Isolated protein was loaded on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and ran at 120 V for 1 h and 30 min. Proteins were then transferred to polyvinylidene fluoride membranes at 220 mA for 1 hour. Membranes were blocked for 1 hour at room temperature with 5% milk in Tris-buffered saline plus 0.5% Tween-20. Blots were incubated overnight at 4ºC with

the corresponding primary antibodies, followed by incubation at room temperature for 1 hour with their respective secondary antibody conjugated to horseradish peroxidase in blocking buffer. Immunoreactivity was imaged with the ChemiDoc MP System (Bio-Rad, Dreieich, Germany) and quantified using Image Lab software (Bio-Rad, Dreieich, Germany). Primary antibodies: 1: 1500 rabbit monoclonal Trx1 antibody (Cell Signaling Technology, Danvers, MA, USA), 1:2000 rabbit polyclonal TrxR1 antibody (Thermo Fisher Scientific Inc, Canada), 1:3000 rabbit monoclonal Txnip (Abcam, Vancouver, Canada), 1:1000 rabbit monoclonal Txnip (Cell Signaling Technology, Danvers, MA, USA), 1:3000 rabbit polyclonal vesicular GABA transporter (vGAT) antibody (Abcam Inc., Toronto, ON, Canada), 1:1000 rabbit polyclonal vesicular glutamate transporter 1 (vGlut1) antibody (Abcam Inc., Toronto, ON, Canada), 1:3000 rabbit polyclonal postsynaptic density protein 95 (PSD95) (Abcam, Vancouver, Canada), 1:500 mouse monoclonal synaptosomal-associated protein 25 (SNAP25) (kindly provided by Dr. Homer's lab, UBC), 1:500 mouse monoclonal syntaxin 1 (kindly provided by Dr. Homer's lab, UBC), 1:500 mouse monoclonal synaptotagmin (kindly provided by Dr. Homer's lab, UBC), 1: 1500 phosphor-CREB antibody (Thermo Fisher Scientific Inc, Canada), 1:1500 CREB antibody (Thermo Fisher Scientific Inc, Canada), 1:1000 rabbit monoclonal phosphorylated ASK1 (Santa Cruz Biotech Inc.), 1:1000 rabbit monoclonal ASK1 (Abcam, Vancouver, Canada), 1:4000 monoclonal mouse β-actin (Sigma Aldrich, Darmstadt, Germany), and 1:1000 mouse polyclonal α-tubulin (Santa Cruz Biotech Inc).

**2.5 Immunocytochemistry**

Neurons were cultured on coverslips in 24-well cell culture plates at a density of $25x10^3$ cells/well or in 35 mm dishes with glass-like polymer coverslip bottom (Cellvis, California,

USA) at a density of 180 x$10^3$ cells/well. Cells were fixed in 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature. Then, neurons were permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at room temperature, blocked with 10% goat serum for 1 hour at room temperature, and further incubated with 1:500 rabbit monoclonal MAP2 antibody (Abcam Inc., Toronto, ON, Canada) at 4°C overnight. Immunofluorescence was visualized by incubating neurons with either 1:500 Alexa-Fluor 568 or 1:500 Alexa-Fluor 488 (Thermo Fisher Scientific Inc, Canada) in 10% goat serum for 1 hour at room temperature. Images were captured using a Carl Zeiss microscope at 20X magnification. All neurons were imaged with the same magnification and settings. Images were selected randomly for further analysis. The total number of neurons analyzed was N=36-40. The number and length of primary dendrites and dendritic branches (secondary and tertiary dendrites) were analyzed using the NeuronJ plugin (http://www.imagescience.org/meijering/software/neuronj) for ImageJ as reported previously (Turlova et al., 2016). Briefly, neurites of neurons were traced semi-manually and each traced neurite was labeled accordingly into primary, secondary, or tertiary to obtain the number of neurites.

**2.6 Knocking down Trx system in primary cultured mouse cerebrocortical neurons**

To knock down Trx and Txnip in primary cultured mouse cerebrocortical neurons, we used a CRISPR/Cas9 lentivirus transfection approach. Trx single guide RNAs (sgRNA), Txnip sgRNA or scrambled sgRNAs, and CRISPR/Cas9 All-in-One lentivector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro were purchased from ABM Inc (Richmond, BC, Canada). Lentiviral particles were formulated by the University of Manitoba Lentiviral Core Service. Primary cultured neurons were transfected with Trx sgRNAs/CRISPR/Cas9, Txnip

sgRNAs/CRISPR/Cas9 or Scrambled sgRNAs/CRISPR/Cas9 lentivirus at 1 DIV. Cells cultured in 6 well plate was added with 700 µL of neurobasal media containing the lentiviral particles. Neurons were incubated overnight, and the next day neurons were added with neurobasal media to a volume of 1.5 mL. Transfected neurons were selected with 1µg/ml puromycin. The knocking down effect was determined using immunoblotting with Trx and Txnip primary antibodies.

**2.7 Biotin switch assay**

CREB cysteine oxidation was detected using the biotin switch assay followed by streptavidin beads co-precipitation as described previously (Wang et al., 2017). SH-SY5Y cell lysate was diluted to a concentration of 1mg/mL with HEN buffer (250 mM HEPES pH 7.7; 1 mM EDTA and 0.1 mM neocuproine). Then, 50 µg protein was incubated with 150 µM hydrogen peroxide ($H_2O_2$) for 30 minutes at room temperature to induce oxidation. To block free thiols, the samples were first incubated with 200 µL of 40 mM N-ethylmaleimide in HEN buffer with 2.5% SDS for 40 minutes at 50$^{o}$C. Then, 1 mL of ice-cold acetone was added to the samples and incubated at -20$^{o}$C for 20 minutes. Samples were centrifugated at 15,000 g at 4$^{o}$C for 15 minutes and then, the pellet was resuspended in 10 µL HEN buffer with 1% SDS. Second, oxidized thiols were reduced back to free thiols by adding 250 µL of 5mM dithiothreitol and incubating for 20 minutes at room temperature. Then, samples were added with pre-chilled acetone and incubated at -20$^{o}$C for 20 minutes. Samples were centrifuged again at 15,000g at 4$^{o}$C for 15 minutes, and the pellet was resuspended in 50 µL HEN buffer with 1% SDS. The reduced thiols were labeled with 5 µL N- [6-biotinamido hexyl]-3'-(2'-pyridyldithio) propionamide (biotin-HPDP) overnight at 4$^{o}$C. To detect CREB cysteine oxidation, biotinylated proteins were purified with 5 µL of packed streptavidin-agarose beads for 1 hour at room

temperature. The beads were washed 5 times with 10 volumes of Neutralization buffer (20 mM HEPES pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100), and centrifuge at 500 g for 5 seconds at room temperature between each wash. Afterwards, beads were incubated for 10 minutes with 50 μL Elution buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-mercaptoethanol) to recover bound proteins. CREB oxidation was analyzed using immunoblotting analysis with 1:1500 polyclonal rabbit anti-CREB antibody (Thermo Fisher Scientific Inc, Canada). Before the streptavidin co-precipitation, an aliquot of protein mixture was used to determine CREB input for normalization.

**2.8 Animals**

Young male and female CD1 mice (21 days old) were used for all the experiments. Mice were kept on a 12-hour light-dark cycle. All experimental procedures were done during the light phase of the light-dark cycle. Food and water were available *ad libitum* throughout the experiment. All procedures were approved by the Animal Care Committee at the University of Manitoba.

**2.9 Stereotaxic surgery**

Scrambled sgRNA or Txnip sgRNA CRISPR/Cas9 All-in-One adeno-associated virus (AAV) vector pAAV-PGK-saCas9-U6-sgRNAsa-hGH-amp was purchased from ABM Inc (Richmond, BC, Canada) and packaged into AAV virus serotype 1 (SCM: 8 x $10^{12}$ GC/mL; Txnip: 7.3 x $10^{12}$ GC/mL) by the Canadian Neurophotonics Centre- Viral Vector Core (Quebec, Canada). AAV was delivered to the medial frontal cortex of 21-day-old mice using stereotaxic surgery. Mice were put under anesthesia using isoflurane 500-1000 ml/min flowrate and then,

maintained under anesthesia during the surgical procedure with isoflurane 100-200 ml/min flowrate. Mice were injected subcutaneously with Metacam (5 mg/kg). After anesthesia, mice heads were shaved and cleaned with iodine followed by 70% ethanol. Mice were placed in a stereotaxic apparatus and Optixcare eye gel (CLC Medica, ON, Canada) was applied to each eye to keep them hydrated. Body temperature was maintained at 37ºC during surgery. 5% bupivacaine was injected at the incision side. A surgical blade was used to make an incision down the midline of the skull. Bilateral craniotomy was performed using a micro drill over the medial frontal cortex following the coordinates: AP +1.8, ML ± 0.4, DV -3.0, -2.5, -2.0 mm. 1 µL of the virus was delivered bilaterally using a 10 µL gastight syringe (Model 1701, Hamilton Company) at a rate of 10 nL per second using a UltraMicroPump3 (World Precision Instruments, Sarasota, USA). After each injection, the needle remained in place for 5 minutes to allow for diffusion of the virus before being removed from the skull. After completing the AAV injection, the skin was sutured with a monocryl (poliglecaprone 25) violet suture (Ethicon, USA) and saline was injected subcutaneously to facilitate recovery. Mice were placed in a clean cage, injected subcutaneously with 0.1 mg/kg Buprenorphine, and monitored for post-surgical recovery. Metacam (5 mg/kg) was injected subcutaneously 24- and 48 hours post-surgery to minimize discomfort.

**2.10 Chronic corticosterone injection**

CORT (#27840, Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously at a dose of 20 mg/kg daily for 21 days using a 1 mL Hamilton syringe with 26G 3/8" needle. CORT was prepared fresh every day by dissolving 2 mg CORT in 1 mL sesame oil as described previously (Yau et al., 2016). The control group received vehicle alone. Injections were

performed between 8:00 am and 11:00 am when rodent plasma CORT levels were low. In this way, we avoid any contradictory results arising from daily plasma CORT fluctuations due to mice's circadian rhythms. CORT levels in mice increase sharply between 12 pm and 10 pm (Ixart et al., 1977; Ma et al., 2018).

### 2.11 Behavioral tests

Four tests were used to determine depressive-like behavior and cognitive impairment: open field test, novel object recognition test, tail suspension test, and sucrose preference test. The open field test was performed at 1-day post-injection, novel object recognition test at 2 days post-injection, tail suspension test at 4 days post-injection, and sucrose preference test at 5 days post-injection.

#### 2.11.1 Open Field Test

The open field test was used to determine anxiety-like behavior and was performed as described previously (Zhou, 2018). All mice were taken to the behavior room at least 30 minutes before testing. Mice were placed in the center of a black pexiglas square box (50 cm length x 50 cm width x 40 cm height) in a dimly lit room and allowed to explore freely for 5 minutes. Mice activity was recorded with a video camera connected to the EthoVision video tracking system software Noldus XT5.1 (Noldus Information Technology Inc., Leesburg, VA, USA). The open field was sprayed with ethanol, then water, and allowed to dry for 15 minutes between mice. Locomotor activity and exploratory behavior were analyzed by determining the total distance moved, the number of rearing (frequency with which the mice stood on their hind legs without support), time spent in the central zone (30 cm x 30 cm), and frequency of center entries.

### 2.11.2 Novel Object Recognition Test

The novel object recognition test was used to determine cognitive impairment and was performed as described previously (Che et al., 2015). Objects were made of either glass or plastic, and they had different shapes, colors, and textures. Two days after the last injection, mice were placed in a black pexiglas square box (50 x 50 x 40 cm) with two different objects separated by 25 cm and allowed to freely explore them for 10 minutes. The next day, mice were submitted to two test trials within 1-hour interval. In the first trial, mice were placed in the box with the same objects as the day before and allowed to explore them for 5 minutes. In the second trial, one of the familiar objects was replaced by a novel object. The mice were placed back into the box and allowed to explore the objects for 5 minutes. The box and objects were cleaned with ethanol, then water and allowed to completely dry between each trial to remove traces of odor. The total time spent exploring the familiar object (F) and the novel object (N) was recorded with a camera connected to a computer. The recognition index was evaluated using the formula (N-F)/(N+F) x 100.

### 2.11.3 Tail suspension test

The tail suspension test was used to study behavioral despair and coping behavior, which are symptoms of depressive-like behavior. The test was performed as described previously (Botterill et al., 2023). The pre-testing handling and room lighting were the same as previous tests. Mice were suspended by the tail, placing a tape less than 1 cm from the tip of each tail. Mice behavior was recorded for 6 minutes with a camera placed in front of the mice but only the last 4 minutes were used to analyze mobility duration. Mice were considered immobile when hanging passively and motionless with the head facing down.

### 2.11.4 Sucrose Preference Test

The sucrose preference test was used to analyze anhedonia, a recurrent symptom of depressive behavior and was performed as described previously (Zhou, 2018). Before the test, mice were exposed to two bottles with 1% sucrose solution for 24 hours to adapt to the presence of two bottles in the cage and the taste of sucrose solution. During the test, mice were placed in single cages, deprived of water for 24 hours, followed by 24 hours of exposure to one bottle filled with 1% sucrose and one bottle filled with tap water. The bottle position was switched every 4 hours to avoid side preferences. Sucrose preference was calculated as % V (sucrose intake)/ % V (total fluid intake) during a 24-hour testing period.

### 2.12 Blood collection and corticosterone determination.

After the last behavior test, mice were anesthetized with isoflurane and blood was collected directly from the heart's right atrium using a 1 mL Hamilton syringe with a 26 G 3/8" needle. This procedure was performed in the morning to avoid elevated CORT levels due to circadian rhythms. Blood was collected in sterile Eppendorf tubes and allowed to clot at room temperature for 60 minutes. Samples were then centrifugated at 2,000 g for 10 minutes at 4$^{o}$C. Serum was collected and stored at -20$^{o}$C until further processing.

Serum CORT levels were analyzed using a standard corticosterone competitive ELISA kit (Thermo Fisher Scientific Inc, Canada, Cat# EIACORT) following the manufacturer's instruction. Briefly, samples were diluted 1:100 using 1X Assay Buffer. Then, 50 µL of sample or standards were added with 25 µL of corticosterone conjugate and 25 µL corticosterone antibody. The samples were shaken for 1 hour at room temperature and then washed 4 times with 300 µL 1X washing buffer. Next, each well was added with 100 µL of TBM substrate followed

by 30 minutes of incubation at room temperature in the dark. The reaction was stopped by adding 50 µL stop solution. Absorbance was measured at 450 nm. CORT concentration was determined based on a corticosterone standard curve ranging from 10000 pg/mL to 78.125 pg/mL. Each standard was measured in duplicates. Samples were tested in triplicates.

### 2.13 Brain perfusion and collection

After the last behavior test, mice brains were perfused and dissected. Mice were anesthetized with isoflurane and placed in a rack, securing the forelimbs with pins. A cut was made from the sternum and towards the head across the ribs and parallel to the lungs. Then, a cannula connected to a perfusion tube was inserted in the left ventricle of the heart. After, the right atrium was pierced with a needle to allow the outflow of blood from circulation. The brain was perfused with approximately 100 cc of 0.9% saline. After perfusion, the mice were decapitated, and the brain was removed from the skull. The brain was homogenized and lysed on ice for 1 hour for further immunoblotting analysis.

### 2.14 Data Analysis

Data analysis was performed using the IBM SPSS 24.0 software (IBM, Armonk, New York, USA). Results of immunoblotting assays were expressed as means ± standard error. Results of immunocytochemistry are expressed as sums ± standard deviation. Significant differences were analyzed by either Student's-T test or ANOVA followed by Tukey Post-hoc. A p-value of less than 0.05 was accepted as statistically significant.

# CHAPTER 3: RESULTS

## 3.1 To establish the role of Trx system in neuronal differentiation and degeneration.

### 3.1.1 To determine whether Trx, TrxR and Txnip protein levels are changed during neuronal differentiation and neurodegeneration.

To determine whether Trx antioxidant system is regulated during neuronal differentiation and degeneration period, we analyzed Trx1, TrxR1 and Txnip protein levels from 1 to 32 DIV in primary cultured mouse cerebrocortical neurons using immunoblotting analysis. We found that Trx and TrxR protein levels time-dependently increased from 1 to 22 DIV (N=5-6) (Figure 4). After 22 DIV, protein levels plateaued or slightly decreased. On the other hand, Txnip protein levels time-dependently increased with the highest levels at 32 DIV (N=6) (Figure 5). These results suggest that Trx, TrxR and Txnip may play roles in regulating neuronal differentiation, maturation, and degeneration.

### 3.1.2 To determine if Trx inhibition prevents neurite outgrowth.

To determine whether Trx regulates neuronal differentiation, we analyzed the effect of Trx inhibition on dendritic outgrowth in primary cultured mouse cerebrocortical neurons. Microtubule-associated protein 2 (MAP2) is a neuron-specific cytoskeletal protein highly expressed in dendrites. Neurons were stained using immunocytochemistry with MAP2 antibody. Initially, Trx1 gene was knocked down using CRISPR/Cas9/Trx single guide RNA (sgRNA). Trx sgRNA can guide Cas9 to cleave Trx gene. Primary cultured neurons were transfected with lentivirus containing CRISPR/Cas9/Trx sgRNA or CRISPR/Cas9/ scramble sgRNA. Wild-type neurons were used as control.

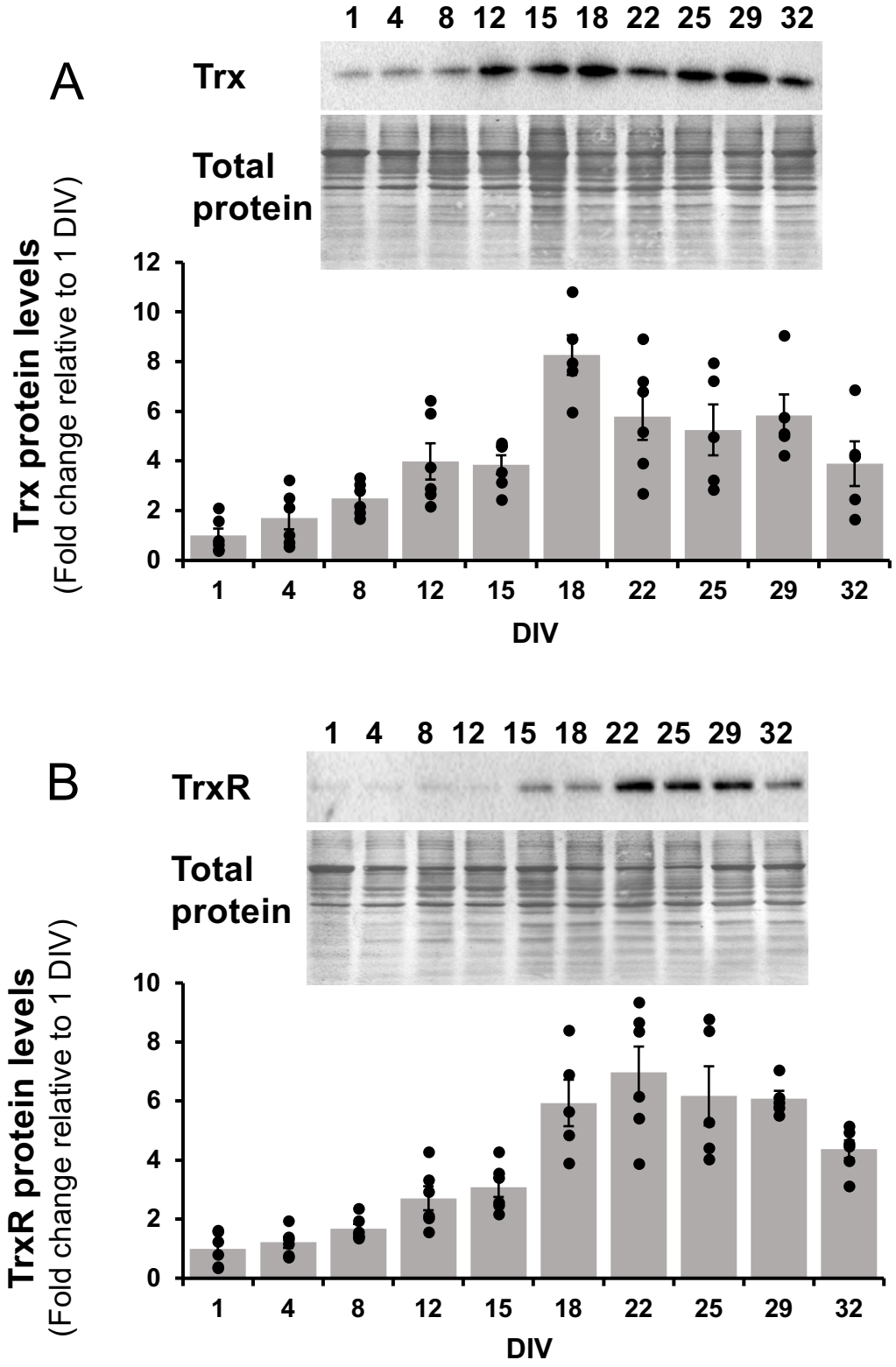


**Figure 4: Protein levels of Trx and TrxR in primary cultured mouse cerebrocortical neurons from 1 to 32 days in vitro (DIV)**. Cells were cultured for 1, 4, 8, 12, 15, 18, 22, 25, 29 and 32 DIV. Protein levels of Trx (A) and TrxR (B) were measured by immunoblotting analysis. Membranes were stained with Coomassie blue to evaluate equal loading. Data is displayed as mean ± SEM (N =5-6).

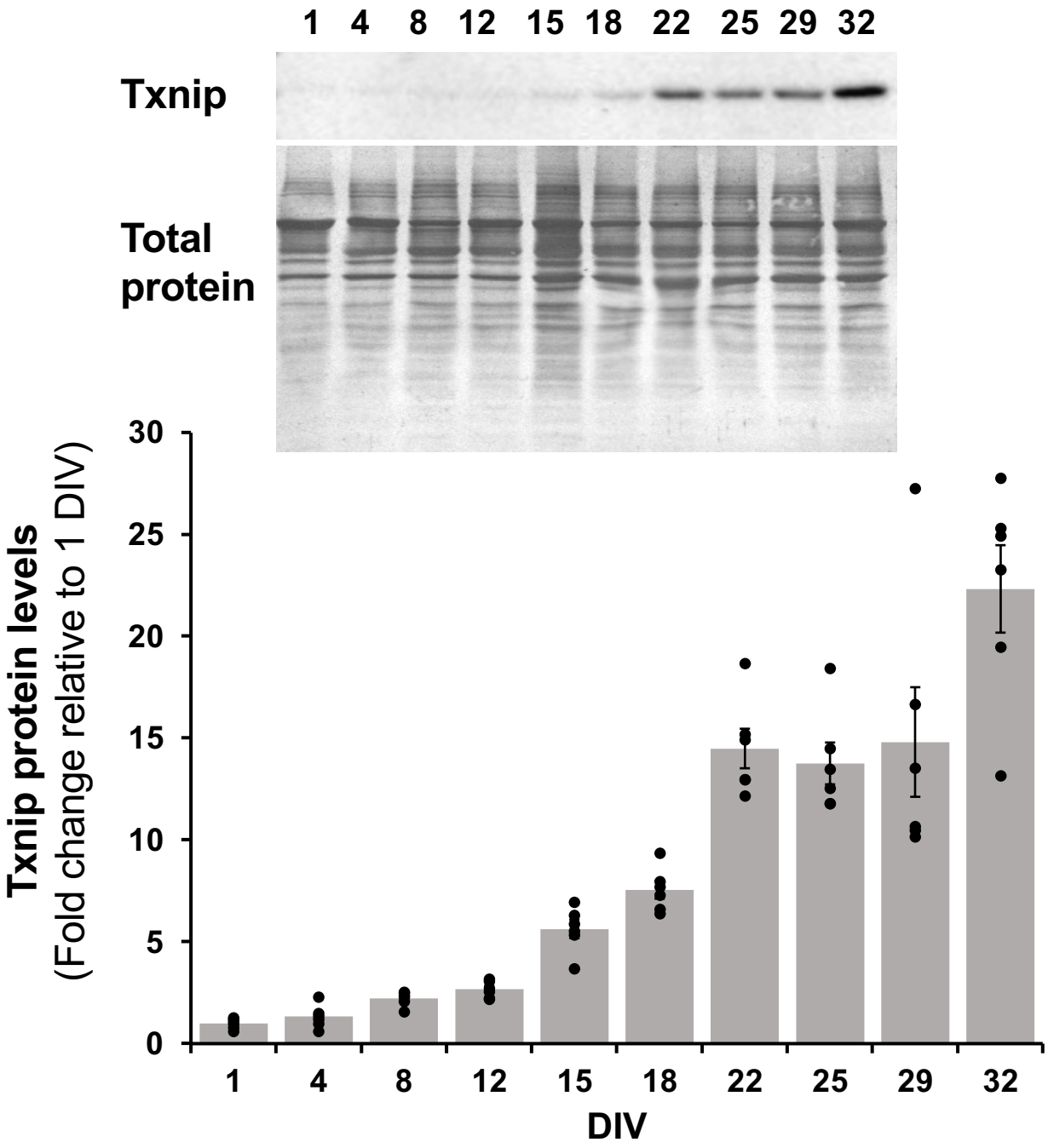


**Figure 5: Protein levels of thioredoxin-interacting protein (Txnip) in primary cultured mouse cerebrocortical neurons from 1 to 32 days in vitro (DIV)**. Cells were cultured for 1, 4, 8, 12, 15, 18, 22, 25, 29 and 32 DIV. Membranes were stained with Coomassie blue to evaluate equal loading. Data is displayed as mean ± SEM (N = 6).

As shown in figure 6, transfection of primary neurons with lentivirus containing CRISPR/Cas9/Trx sgRNA significantly inhibited Trx protein levels at 8 DIV when compared to transfection with CRISPR/Cas9/scramble sgRNA ($p<0.05$, N=5). Our result suggests that Trx sgRNA can knock down Trx gene expression. We found that Trx sgRNA induced a 70% reduction in the length of primary dendrites (Figure 7A) and almost 90% decrease in the total length of dendritic branches compared to the scrambled control (Figure 7B) ($p<0.05$, N=36). In addition, Trx sgRNA also significantly reduced the number of primary dendrites and the total number of dendritic branches by 30% and 70% respectively when compared to the scrambled control ($p<0.05$, N=36) (Figure 7C and 7D). Our result suggests that Trx gene knockdown can inhibit dendritic outgrowth.

Further, we used Trx pharmacological inhibitor PX12 to inhibit Trx activity and analyzed the effect of PX12 on dendritic outgrowth. PX12 induces irreversible thioalkylation of Cys73 of Trx. Cys73 is necessary for post-translational modifications, dimerization, and tertiary structure of Trx. Thus, PX12 inhibits Trx-reducing activity (Islam et al., 2019; May et al., 2018). Primary cortical neurons were treated with 0.5, 1 and 2 μM of PX12 at 2 DIV for 6 days. Neurons were stained using immunocytochemistry with MAP2 antibody. We found that treatment with PX12 for 6 days significantly caused nearly 50% decrease in the length of primary dendrites ($p<0.05$, N=36) (Figure 8A) and around 60% reduction in the total length of dendritic branches compared to control ($p<0.05$, N=36) (Figure 8B). Additionally, this treatment significantly decreased the number of primary dendrites and total number of dendritic branches compared to control ($p<0.05$, N=36) (Figure 8C and 8D). This finding suggests that PX12 can inhibit dendritic outgrowth. Altogether our data suggest that Trx might promote neurite outgrowth in primary cultured mouse cerebrocortical neurons.

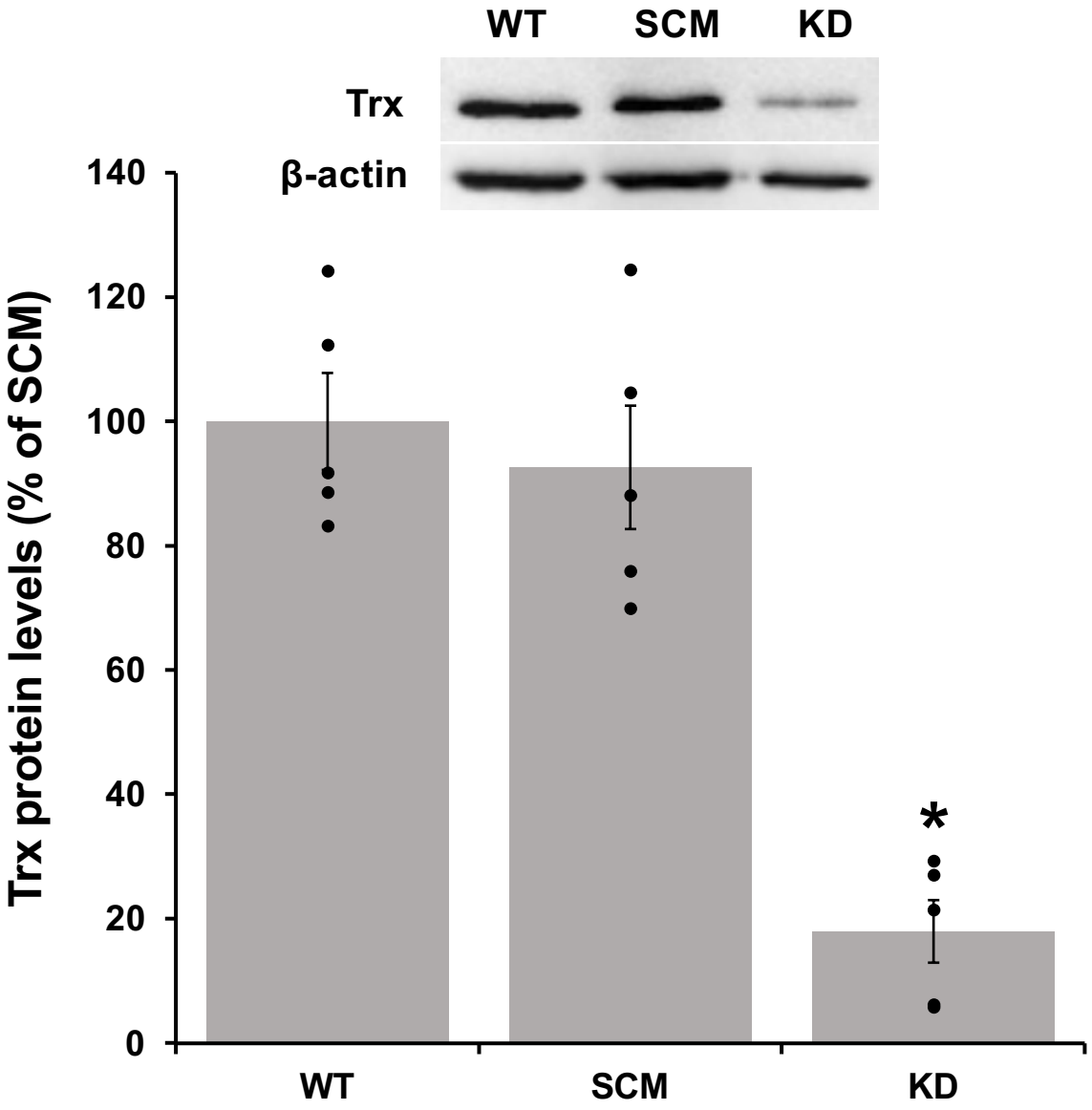


**Figure 6: Knocking down Trx gene in primary cultured mouse cerebrocortical neurons using CRISPR-Cas9 technology**. Cultured neurons were transfected with lentivectors containing Trx sgRNAs/CRISPR/Cas9 (KD) or scrambled sgRNAs/CRISPR/Cas9 (SCM) at 1 DIV. Wild-type cells (WT) were used as control. Trx protein levels were measured by immunoblotting analysis at 8 DIV. Data are displayed as mean ± SEM (N=5). * indicates $p < 0.05$ when KD group was compared to CTL and SCM groups determined by one-way ANOVA followed by Tukey's post-hoc analysis.

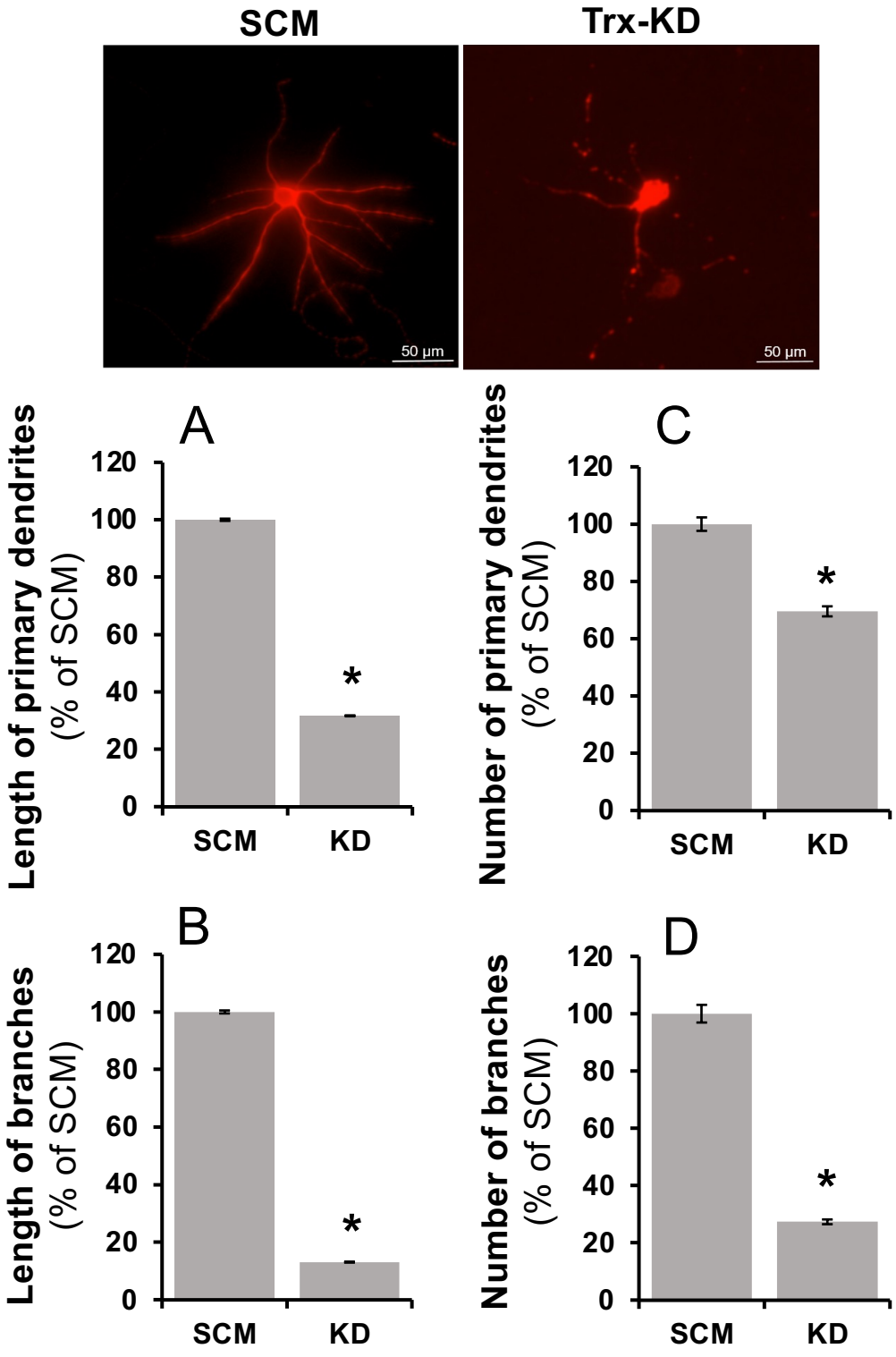


**Figure 7: The effect of knocking down Trx on dendritic outgrowth in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated with scrambled sgRNAs/CRISPR/Cas9 (SCM) or Trx sgRNAs/CRISPR/Cas9 (KD) at 1 DIV. Dendrites were stained with anti-MAP2 antibody at 8 DIV. Pictures of dendrites were taken using a fluorescent microscope. A) Length of primary dendrites, B) length of branches (secondary and tertiary neurites), C) number of primary dendrites, and D) number of dendritic branches were assessed using ImageJ. Data is displayed as sum ± SD (N=36). * indicates $p<0.05$ when compared to the control determined by Student-T test.

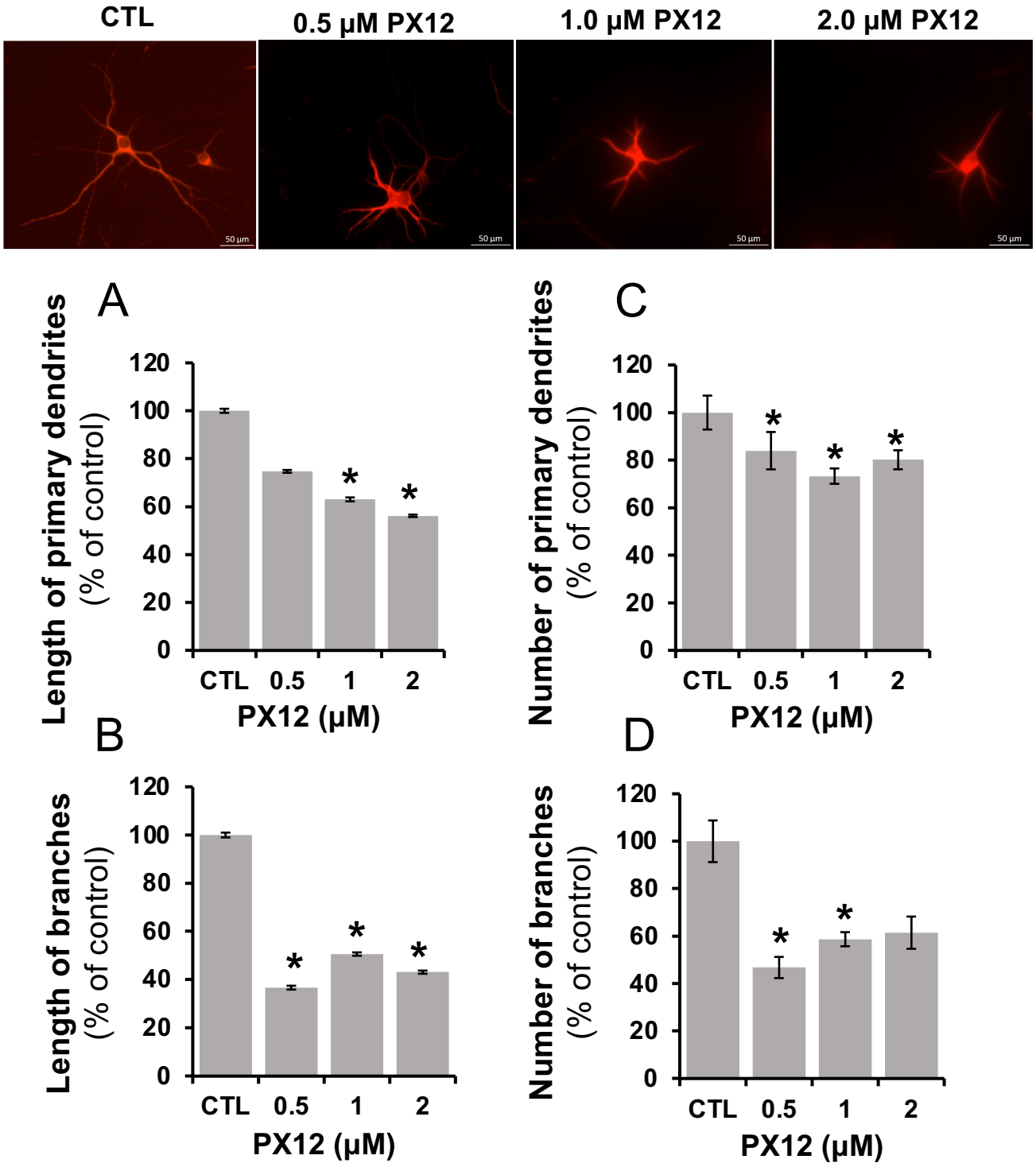


**Figure 8: The effect of Trx inhibitor PX12 on dendritic outgrowth in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with vehicle (CTL) or PX12 at 0.5, 1, and 2 µM for 6 days. Dendrites were stained with anti-MAP2 antibody at 8 DIV. Pictures of dendrites were taken using a fluorescent microscope. A) Length of primary dendrites, B) length of branches (secondary and tertiary neurites), C) number of primary dendrites, and D) number of branches were assessed using ImageJ. Data is displayed as sum ± SD (N=36). * indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis.

In primary cultured mouse cerebrocortical neurons, the majority are excitatory glutamatergic neurons, and some are inhibitory GABAergic neurons. vGlut1 and vGAT are markers of glutamatergic and GABAergic neurons respectively. Therefore, we measured the protein levels of vGlut1 and vGAT by immunoblotting analysis. PSD95 is a scaffold protein in the post-synapse of excitatory neurons. PSD95 protein levels were also measured. In addition, synaptic proteins including SNAP25, syntaxin and synaptotagmin were measured to determine synaptic development. We found that treatment with PX12 for 6 days significantly decreased vGlut1 and PSD95 protein levels, but this treatment had no effect on vGAT ($p<0.05$, N=5-6) (Figure 9). Furthermore, treatment with PX12 also decreased SNAP25 and syntaxin protein levels but had no effect on synaptotagmin protein levels when compared to control ($p<0.05$, N=5-6) (Figure 10). Our results suggest that Trx may promote glutamatergic neuron outgrowth.

**3.2 To determine whether CREB neurotrophic pathway and ASK1 apoptotic pathway mediate Trx system-regulated neuronal differentiation and degeneration.**

**3.2.1 To determine the role of CREB in Trx system-regulated neuronal outgrowth.**

First, we measured phosphorylation of CREB in primary cultured mouse cerebrocortical neurons from 1 to 32 DIV. Phosphorylation of CREB was assessed by measuring the ratio of phospho-CREB/total CREB protein levels using immunoblotting analysis. We found that total CREB protein levels were significantly elevated at 8 and 12 DIV compared to 1 DIV (N=4) (Figure 11A). Phospho-CREB/total CREB levels are also significantly increased from 1 to 12 DIV but decreased after 15 DIV (N=4) (Figure 11B). Our results suggest that CREB phosphorylation is increased during neuronal differentiation period.

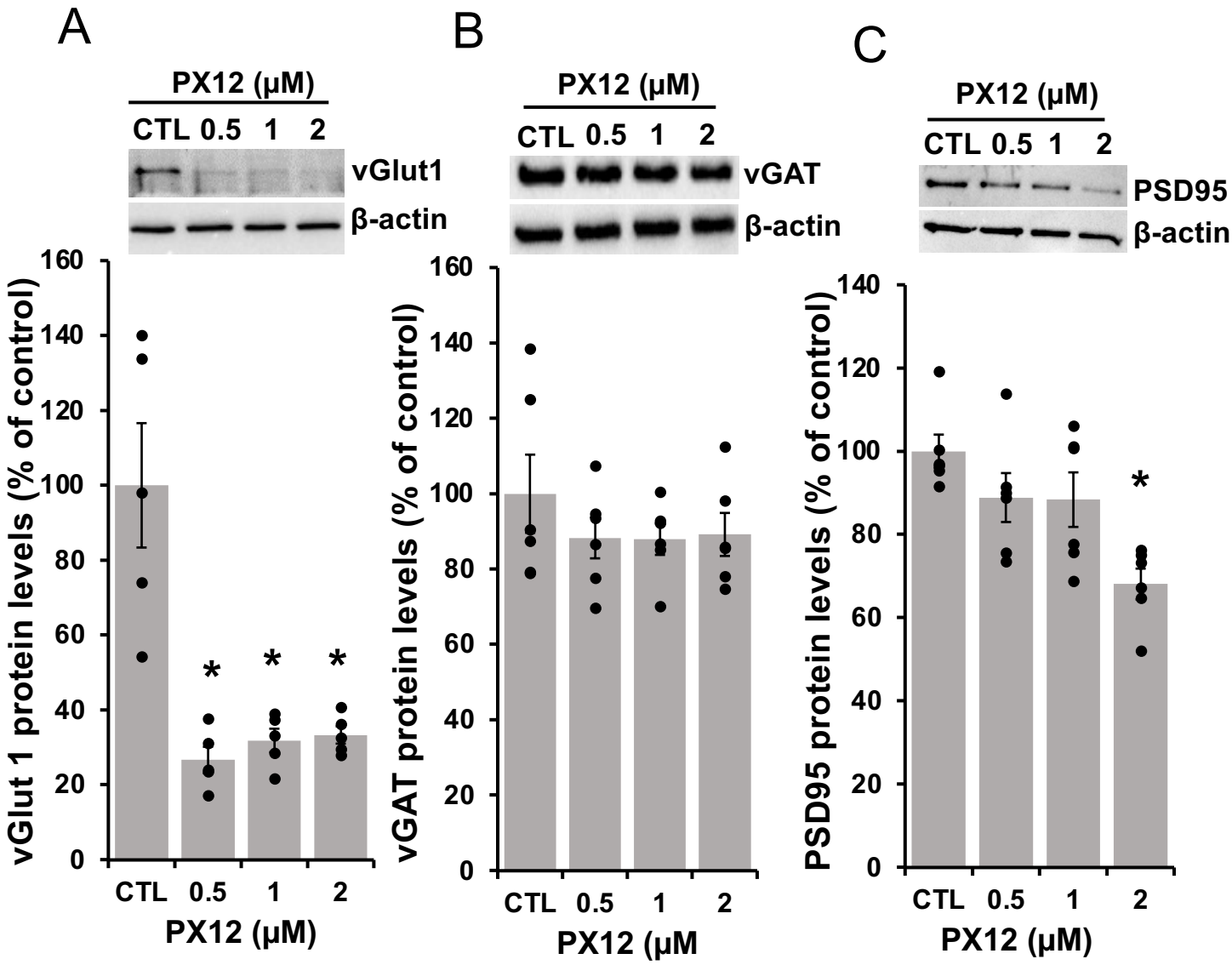


**Figure 9: The effect of Trx inhibitor PX12 on vGlut1, vGAT and PSD95 protein levels in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with vehicle (CTL) or PX12 at 0.5, 1, and 2 μM for 6 days. Protein levels of A) vGlut1, B) vGAT and C) PSD95 were measured at 8 DIV using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N=5-6). * indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis.

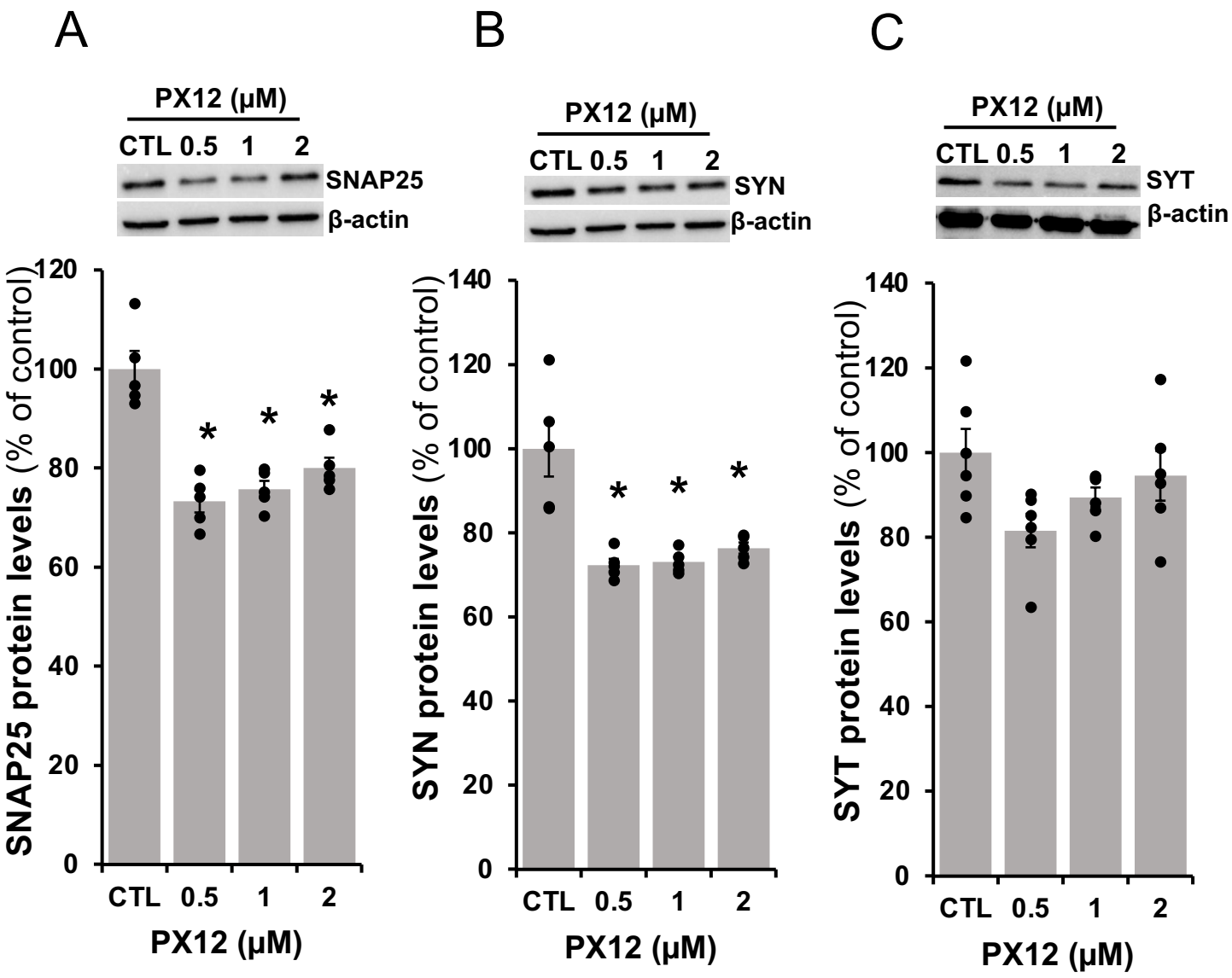


**Figure 10: The effect of PX12 on SNAP25, syntaxin, and synaptotagmin protein levels in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with vehicle (CTL) or PX12 at 0.5, 1, and 2 μM for 6 days. Protein levels of A) SNAP25, B) syntaxin (SYN) and C) synaptotagmin (SYT) were measured at 8 DIV using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =5-6). * indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis.

Second, to determine if Trx mediates CREB phosphorylation, we analyzed the effect of Trx inhibitor PX12 on CREB phosphorylation. Primary cultured mouse cerebrocortical neurons were treated with 0.5, 1 and 2 μM PX12 at 2 DIV for 6 days. We found that although treatment with PX12 had no effect on total CREB protein levels (N=6) (Figure 12A), it significantly reduced the ratio of phosphorylated CREB/total CREB levels ($p<0.05$, N=6) (Figure 12B). This result suggests that blocking Trx activity can inhibit CREB phosphorylation.

### 3.2.2 To determine the role of Trx in redox-regulated CREB phosphorylation in human neuroblastoma SH-SY5Y cells.

To determine whether redox change regulates CREB phosphorylation, we analyzed the effect of $H_2O_2$ on CREB phosphorylation and CREB protein thiol oxidation in SH-SY5Y cells. First, cultured cells were treated with $H_2O_2$ at 75, 150 and 300 μM for 30 minutes. Phosphorylated CREB and total CREB protein levels were evaluated. We found that while treatment with $H_2O_2$ had no effect on total CREB protein levels (N=5) (Figure 13A), treatment with $H_2O_2$ at 150 and 300 μM significantly reduced the ratio of phosphorylated CREB/total CREB levels ($p<0.05$, N=5) (Figure 13B). Second, to investigate whether CREB thiols can be oxidized, we analyzed the effect of $H_2O_2$ on CREB thiol oxidation in SH-SY5Y cells using the biotin-switch method followed by streptavidin-agarose co-precipitation. $H_2O_2$ at 150 μM was incubated with cell extract for 30 min at room temperature. Then, free thiols were blocked with NEM, oxidized thiols were reduced with DTT and labeled with biotin-HPDP. Biotin-labeled proteins were precipitated with streptavidin agarose beads. CREB protein levels were analyzed by immunoblotting analysis.

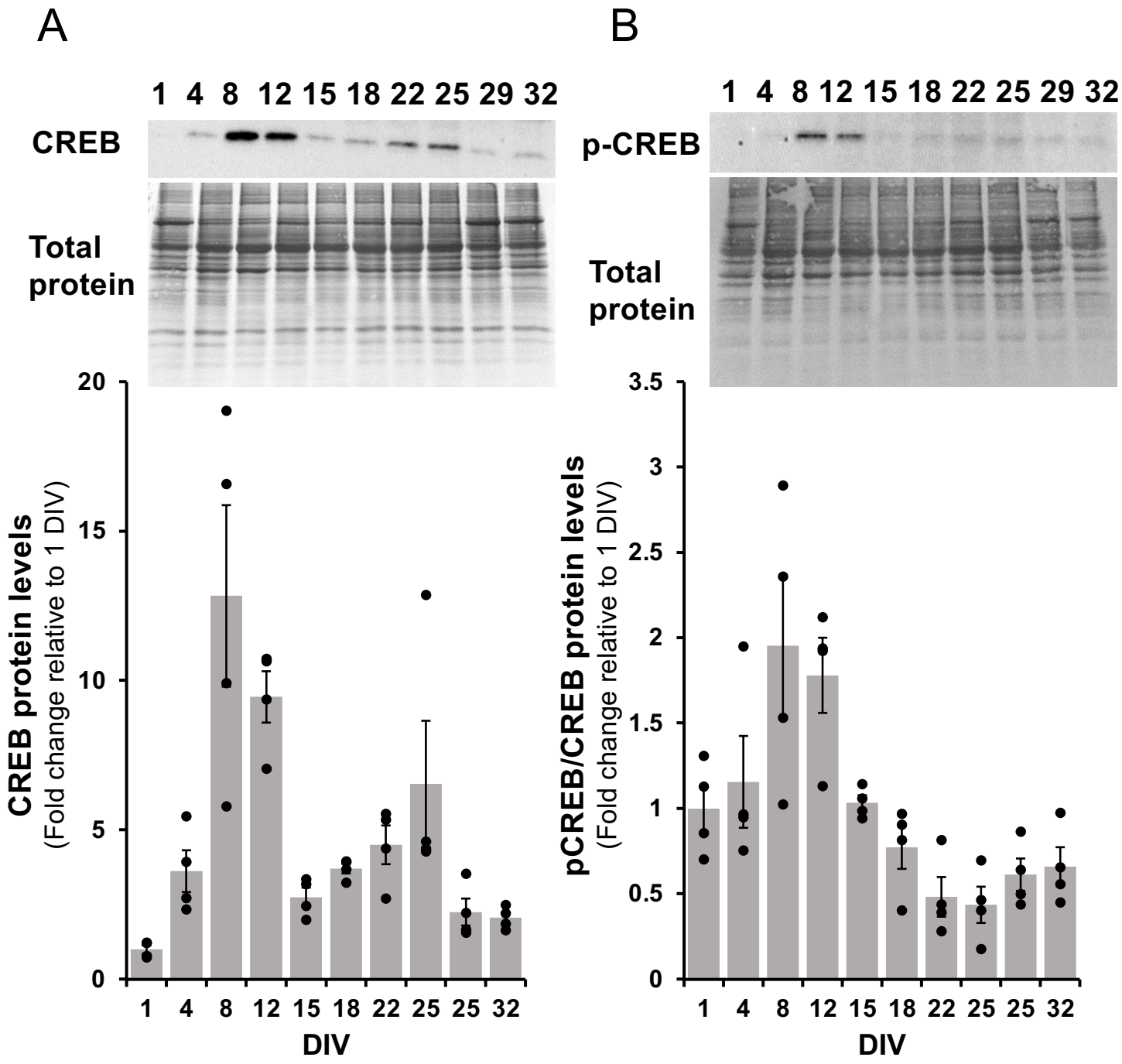


**Figure 11: Protein levels of phosphorylated (P) CREB and total CREB in primary cultured mouse cerebrocortical neurons from 1 to 32 days in vitro (DIV)**. Cells were cultured for 1, 4, 8, 12, 15, 18, 22, 25, 29 and 32 DIV. Total CREB protein levels (A) and the ratio of p-CREB/CREB protein levels (B) were measured by immunoblotting analysis. Membranes were stained with Coomassie blue to evaluate equal loading. Data is displayed as mean ± SEM (N = 4).

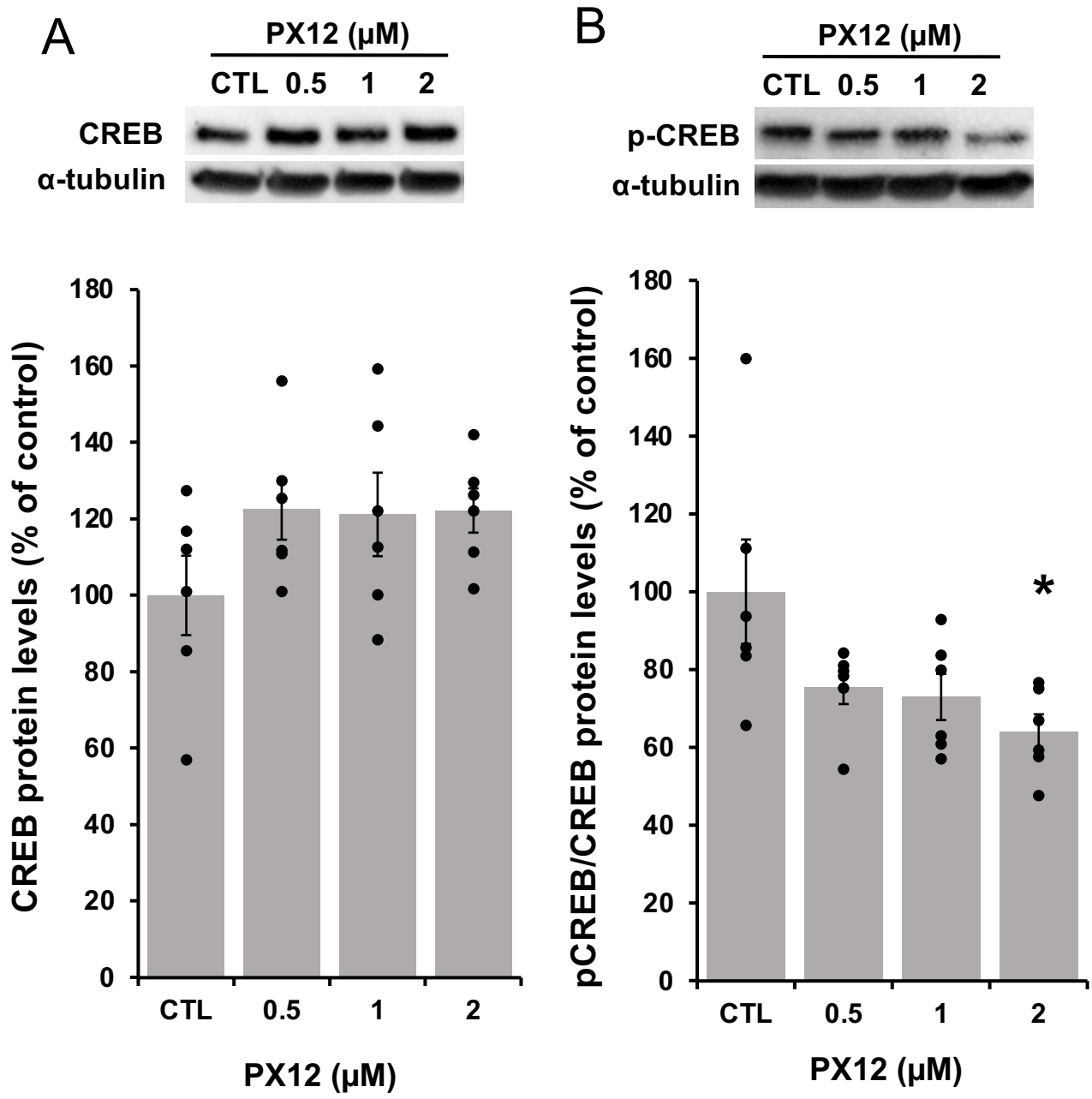


**Figure 12: The effect of Trx inhibitor PX12 on CREB and phosphorylated (p) CREB protein levels in primary cultured mouse cerebrocortical neurons.** Cultured neurons were treated at 2 DIV with vehicle (CTL) or PX12 at 0.5, 1 and 2 μM for 6 days. A) CREB protein levels and B) the ratio of p-CREB /CREB protein levels were measured at 8 DIV using immunoblotting analysis. α-tubulin was used as a normalization standard. Data are displayed as mean ± SEM (N=6). * indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis.

We found that $H_2O_2$ significantly increased oxidized thiol levels of CREB ($p<0.05$, N=6) (Figure 13C). Our results suggest that $H_2O_2$ may inhibit CREB phosphorylation by inducing cysteine thiol oxidation.

To determine whether Trx regulates CREB phosphorylation by maintaining redox homeostasis, we analyzed the effect of a Trx-mimetic tripeptide on $H_2O_2$ -inhibited CREB phosphorylation and $H_2O_2$ -increased CREB oxidation in SH-SY5Y cells. CB3 is a cell-permeable Trx-mimetic tripeptide. We found that treatment with 50 μM CB3 for 2 hours did not change total CREB protein levels (N=7) (Figure 14A), but this treatment significantly prevented $H_2O_2$-inhibited ratio of phosphorylated CREB/total CREB levels ($p<0.05$, N=7) (Figure 14B). In addition, we found that CB3 significantly decreased $H_2O_2$-increased oxidized thiol levels of CREB ($p<0.05$, N=7) (Figure 14C). Our results suggest that Trx can reverse $H_2O_2$-inhibited CREB phosphorylation and $H_2O_2$-increased CREB cysteine thiol oxidation.

### 3.2.3 To determine the role of ASK1 pathway in Trx system-regulated neuronal differentiation.

First, we measured ASK1 phosphorylation in primary cultured mouse cerebrocortical neurons from 1 to 32 DIV. Phosphorylation of ASK1 was assessed by measuring the ratio of phosphorylated ASK1/total ASK1 protein levels using immunoblotting analysis. We found that total ASK1 time-dependently increased during the cell culture period with the highest expression at 18 DIV (N=6) (Figure 15A). On the other hand, phosphorylated ASK1/total ASK1 levels were increased from 22-32 DIV (N=6) (Figure 15B). These results suggest that ASK1 apoptotic pathway is activated during neuronal degeneration.

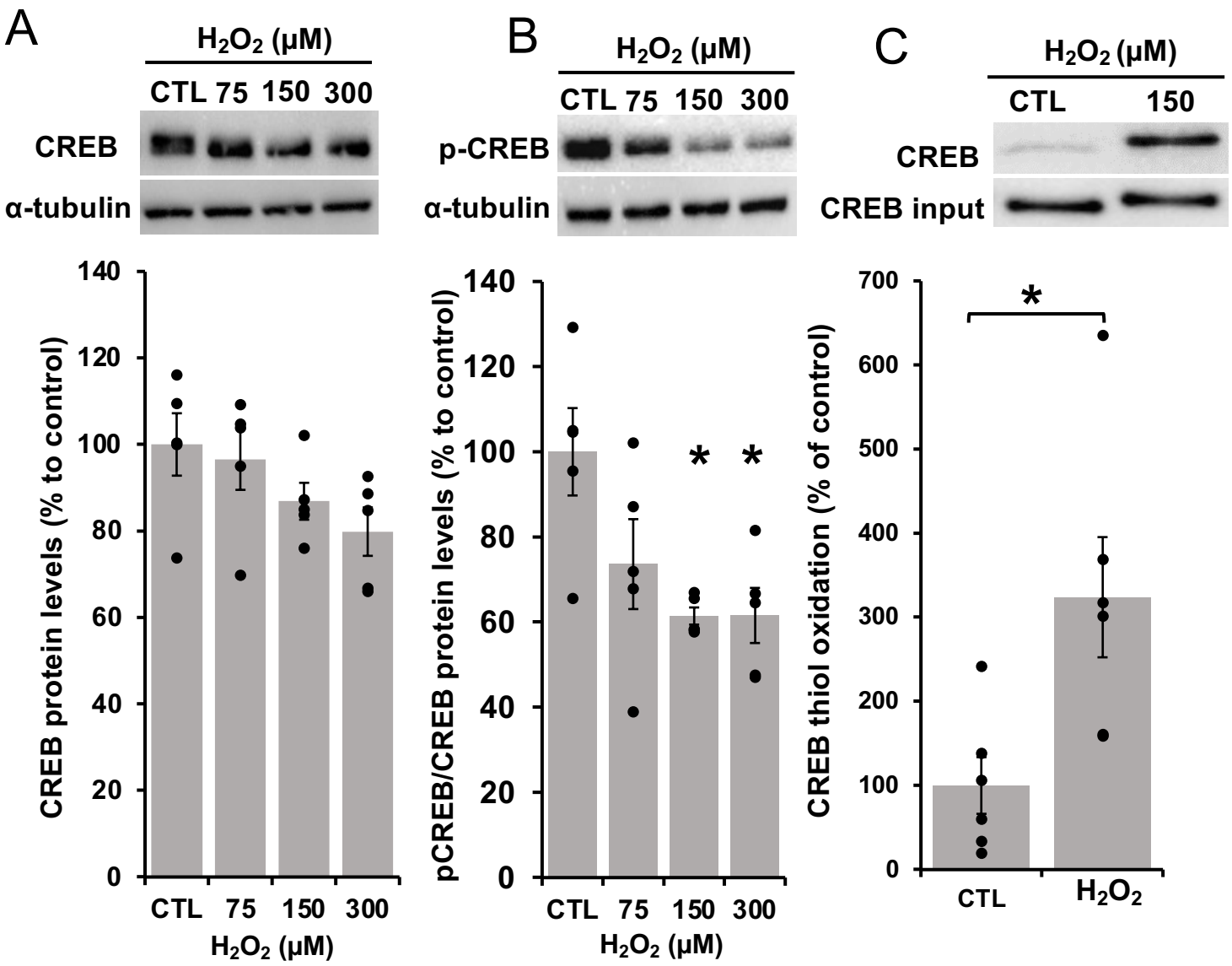


**Figure 13: The effect of $H_2O_2$ on phosphorylated CREB levels and CREB cysteine oxidation in human neuroblastoma SH-SY5Y cell line**. Cells were treated with 75, 150, and 300 μM $H_2O_2$ for 30 min. A) Total CREB and B) Phosphorylated CREB levels were measured using immunoblotting analysis. α-tubulin was used as a normalization standard. Data are displayed as mean ± SEM (N =5). *indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis. C) $H_2O_2$ at 150 μM was incubated with cell extract for 30 min. CREB thiol oxidation was measured with the biotin-switch method followed by streptavidin-agarose co-precipitation. Before the streptavidin co-precipitation, an aliquot of protein mixture was used to determine CREB input for normalization. Data are displayed as mean ± SEM (N =6). * indicates $p < 0.05$ when compared to CTL determined by Student T-test.

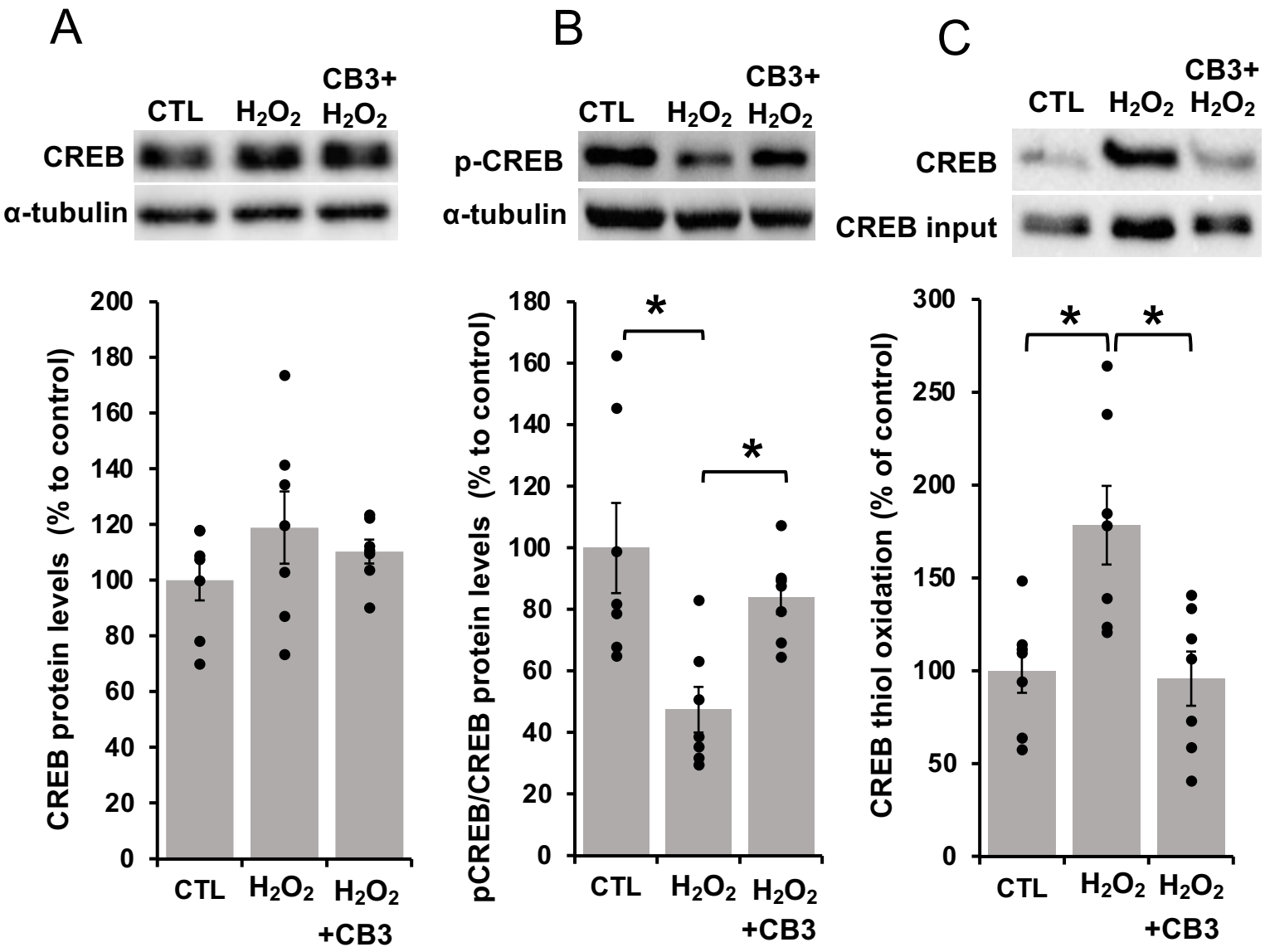


**Figure 14: The effect of CB3 on CREB phosphorylation and CREB cysteine oxidation in human neuroblastoma SH-SY5Y cell line.** Cells were treated with 50 μM CB3 for 2 h and then with 150 μM $H_2O_2$ for 30 min. (A) Total CREB and (B) Phosphorylated CREB levels were measured using immunoblotting analysis. α-tubulin was used as a normalization standard. (C) CREB thiol oxidation was measured with the biotin-switch method followed by streptavidin-agarose co-precipitation. Before the streptavidin co-precipitation, an aliquot of protein mixture was used to determine CREB input for normalization. Data are displayed as mean ± SEM (N =7). * indicates $p < 0.05$ when compared to CTL determined by one-way ANOVA followed by Tukey's post hoc analysis.

Second, we analyzed the effect of Trx inhibitor PX12 on ASK1 phosphorylation. We found that treatment with PX12 at 2 DIV for 6 days did not change ASK1 protein levels ($p<0.05$, N=6) (Figure 16A), but increased protein phosphorylation of ASK1 ($p<0.05$, N=6) (Figure 16B). These findings suggest that Trx is necessary for neuronal survival, and inhibition of Trx activity leads to activation of ASK1 apoptotic pathway and subsequent neurodegeneration.

**3.3 To determine whether Trx antioxidant system mediates CORT impaired-neuronal differentiation and enhanced degeneration.**

**3.3.1 To measure the effect of chronic CORT treatment on Trx, TrxR and Txnip protein levels.**

Primary cultured mouse cerebrocortical neurons were treated with 0.01, 0.1, 1, and 10 μM of CORT at 2 DIV for 5 days. Trx, TrxR and Txnip protein levels were assessed by immunoblotting analysis. We found that although treatment with CORT at 0.01, 0.1, 1, and 10 μM had no effects on Trx and TrxR levels (N=6) (Figure 17A and 17B), these treatments significantly increased Txnip protein levels with the highest increase at 0.1 μM when compared to control ($p<0.05$, N=6) (Figure 17C). This finding suggests that chronic CORT treatment can upregulate Txnip.

**3.3.2 To measure the effect of chronic CORT on phosphorylated CREB and ASK1 levels.**

Cultured neurons were treated with 0.1 μM of CORT at 2 DIV for 5 days. Protein levels of phosphorylated CREB, total CREB, phosphorylated ASK1 and total ASK1 were measured by immunoblotting analysis. We found that treatment with CORT at 0.1 μM significantly increased CREB protein levels when compared to control ($p<0.05$, N=6) (Figure 18A).

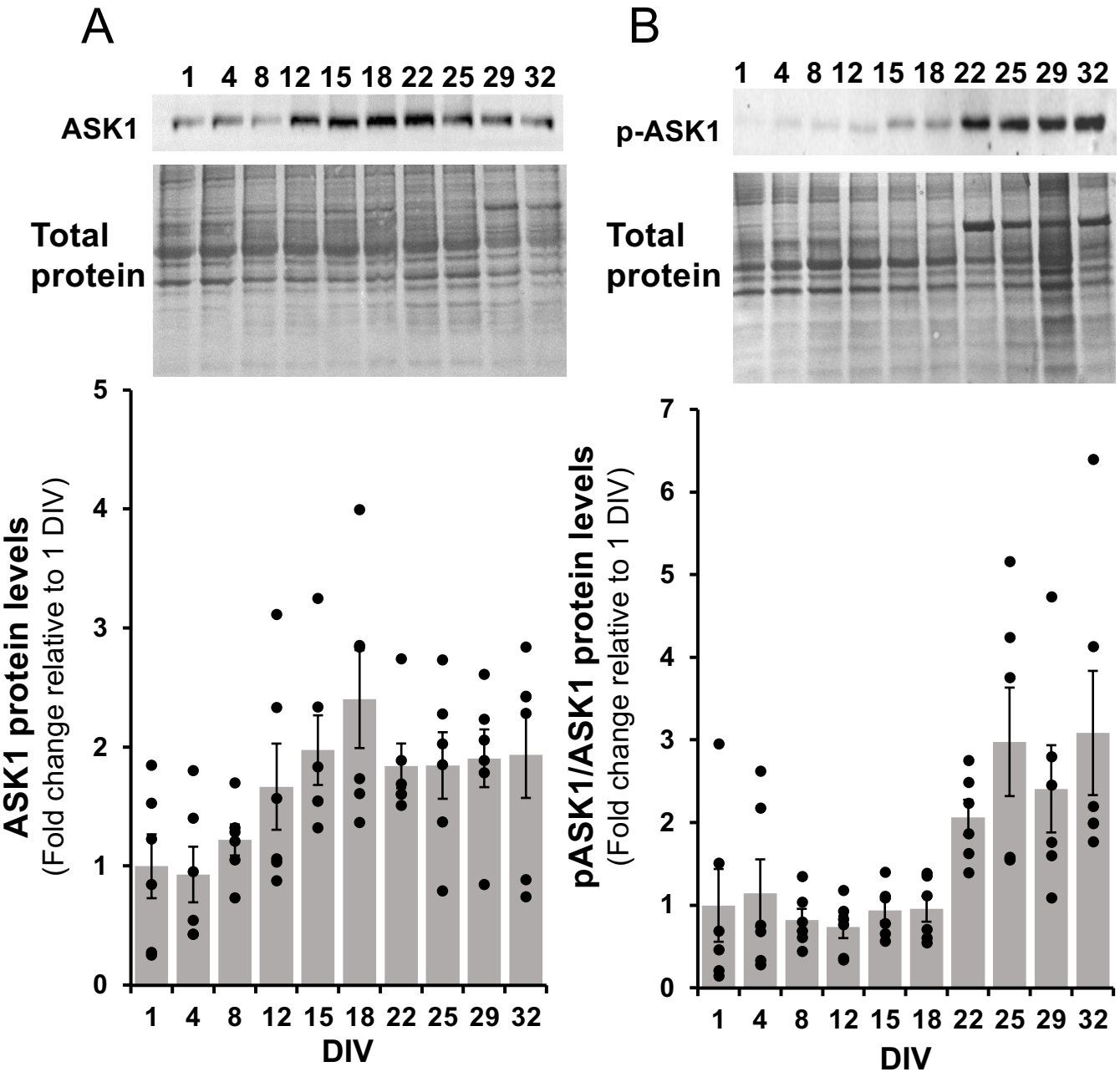


**Figure 15: Protein levels of phosphorylated (p)-ASK1 in primary cultured mouse cerebrocortical neurons from 1 to 32 DIV**. Cells were cultured for 1, 4, 8, 12, 15, 18, 22, 25, 29 and 32 DIV. Total ASK1 protein levels (A) and the ratio of p-ASK1/ASK1 protein levels (B) were measured by immunoblotting analysis. Membranes were stained with Coomassie blue to evaluate equal loading. Data is displayed as mean ± SEM (N =6).

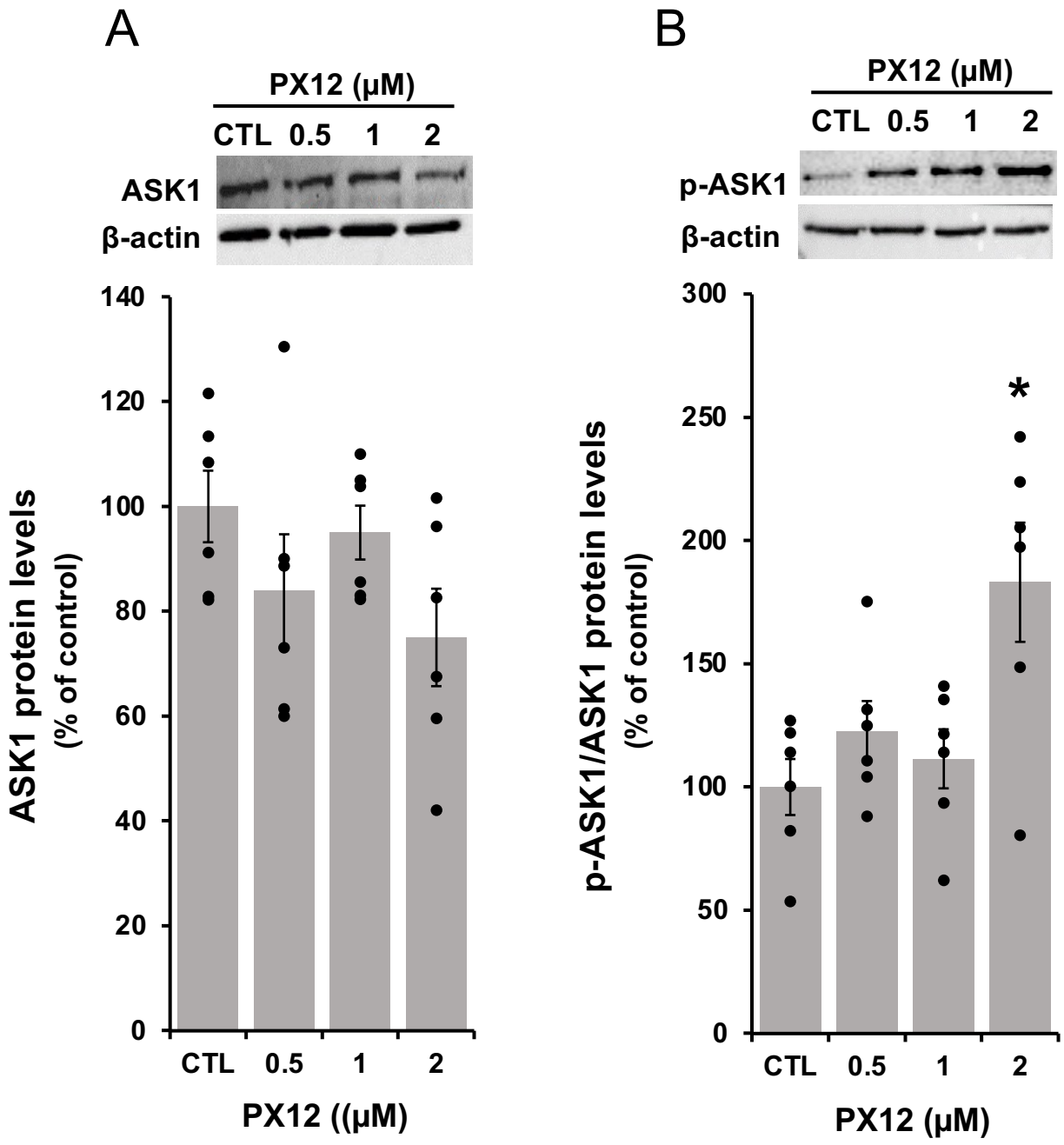


**Figure 16: The effect of Trx inhibitor PX12 on phosphorylated (p)-ASK1 protein levels in primary cultured mouse cerebrocortical neurons**. Primary cultured neurons were treated at 2 DIV with vehicle (CTL) or PX12 at 0.5, 1, and 2 μM for 6 days. Protein levels of A) total ASK1 and B) p-ASK1/ASK1 were measured at 8 DIV using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to control determined by one-way ANOVA followed by Tukey's post hoc analysis.

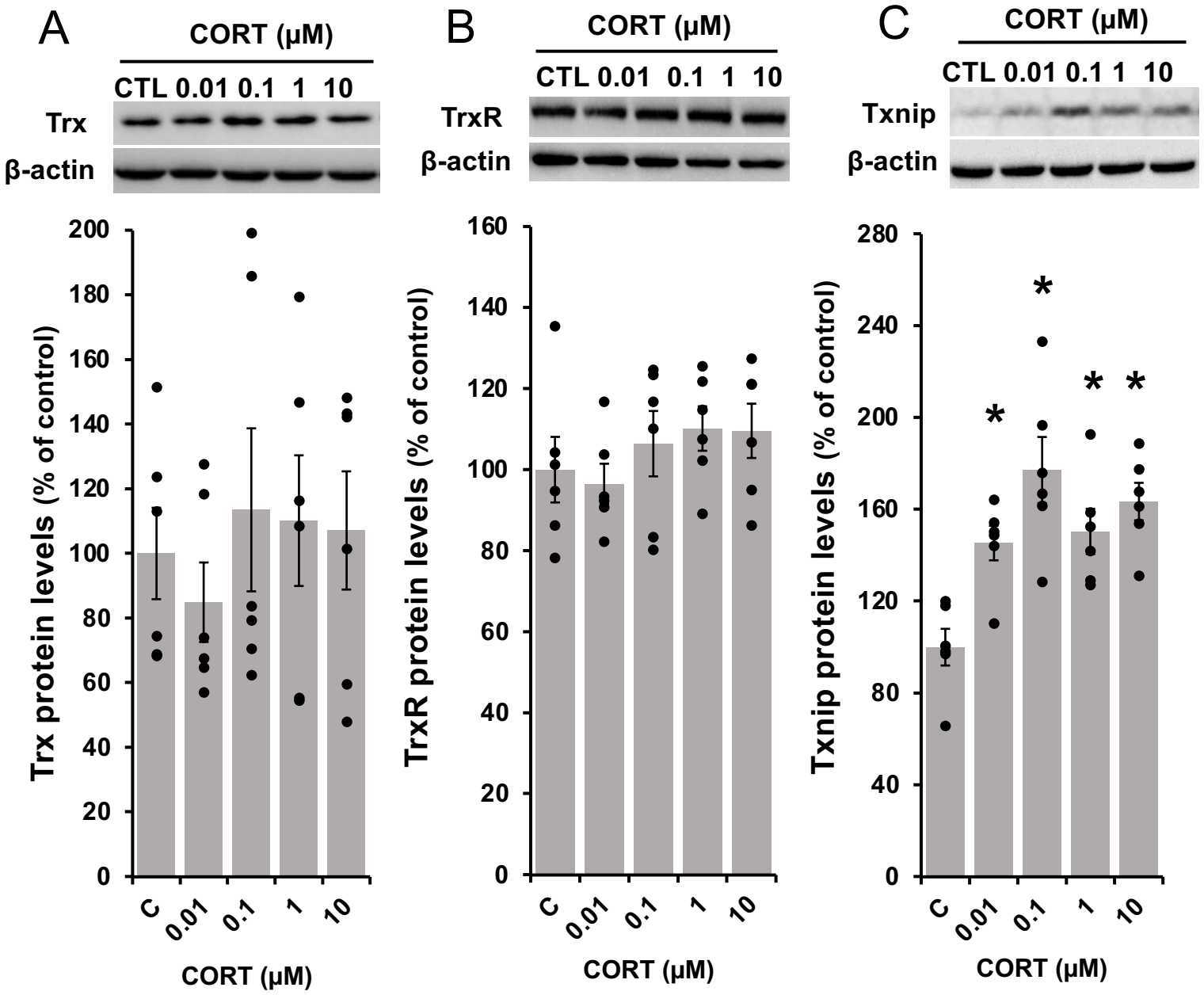


**Figure 17: The effect of corticosterone (CORT) treatment on Trx, TrxR and Txnip system.** Primary cultured mouse cerebrocortical neurons were treated at 2 DIV with vehicle as Control (CTL) or 0.01, 0.1, 1, and 10 μM CORT for 5 days. A) Trx, B)TrxR, and C)Txnip protein levels were measured by immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to CTL determined by one-way ANOVA followed by Tukey's post hoc analysis.

Conversely, treatment with CORT at 0.1μM significantly decreased the ratio of phosphorylated CREB/total CREB levels when compared to control ($p<0.05$, N=6) (Figure 18B). In addition, treatment with CORT at 0.1μM had no effect on total ASK1 protein levels (N=5) (Figure 19A) but this treatment significantly increased the ratio of phosphorylated ASK1/total ASK1 levels ($p<0.05$, N=5) (Figure 19B). Our results suggest that chronic CORT treatment inhibited CREB phosphorylation, but increased ASK1 phosphorylation.

### 3.3.3 To measure the effect of chronic CORT treatment on dendritic outgrowth.

To determine the effect of CORT on neuronal differentiation, we measured the effect of chronic treatment with CORT on dendritic outgrowth in primary cultured mouse cerebrocortical neurons. Cultured neurons were treated with 0.1 μM of CORT at 2 DIV for 5 days. Dendrites were stained with a primary antibody against MAP2 at 7 DIV. We found that treatment with CORT for 5 days significantly reduced the length of primary dendrites by approximately 80% and the total length of dendritic branches by approximately 40% ($p<0.05$, N=40) (Figure 20A and 20B). Moreover, CORT also decreased the number of primary dendrites and the total number of dendritic branches by nearly half ($p<0.05$, N=40) (Figure 20C and 20D). We also examined whether chronic CORT exposure affected the expression of synaptic proteins. After primary neurons were treated with 0.1 μM CORT for 5 days at 2 DIV, protein levels of vGlut1, vGAT and PSD95 were evaluated by immunoblotting analysis. We found that CORT treatment has no effect on vGlut1, vGAT and PSD95 protein levels (N=4) (Figure 21). This evidence suggests that CORT might impair neurite outgrowth but has no effect on vGlut1, vGAT and PSD95 protein levels.

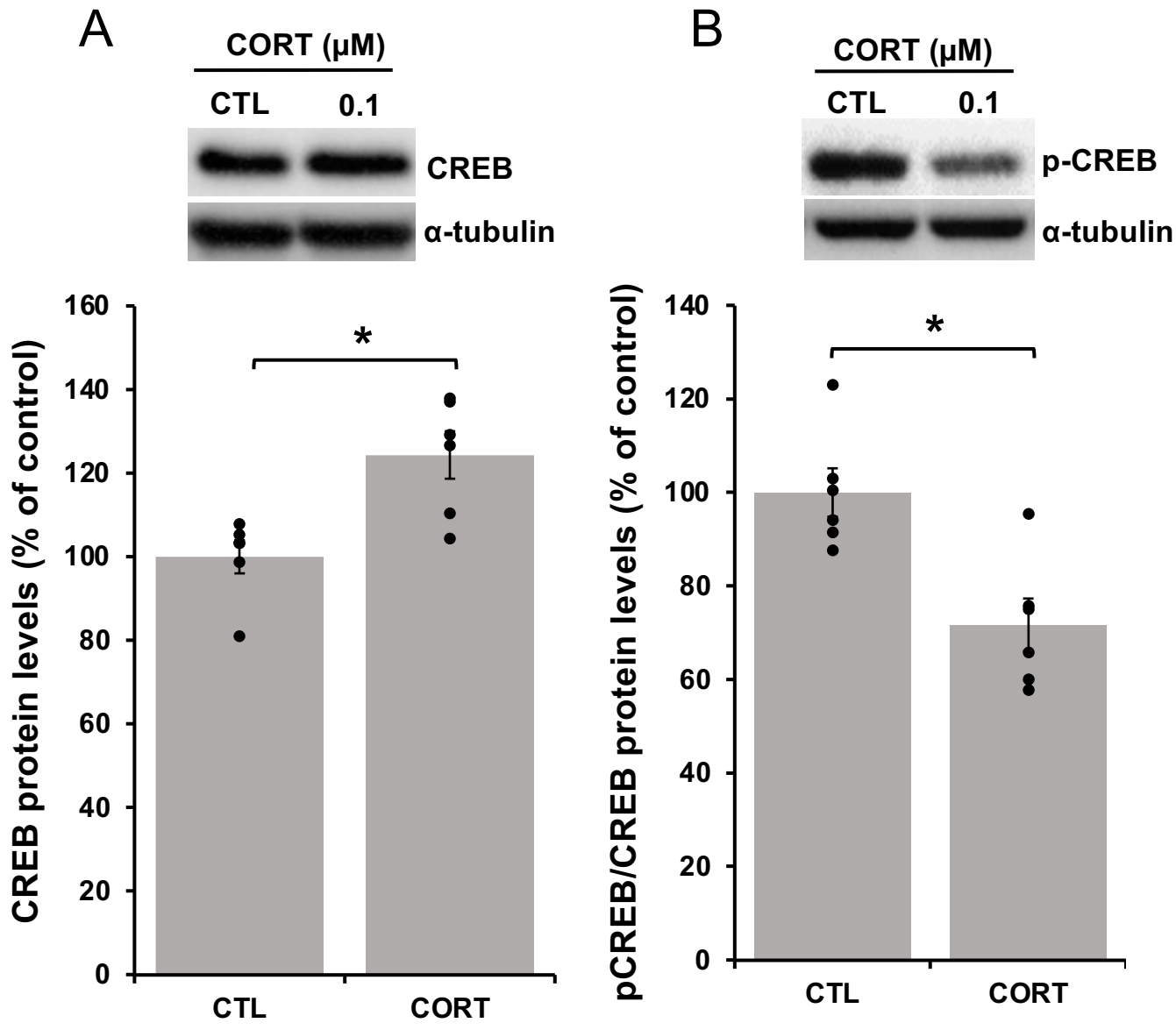


**Figure 18: The effect of corticosterone (CORT) on phosphorylation of CREB in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with the vehicle as Control (CTL) or 0.1 μM CORT for 5 days. The protein levels of A) total CREB and B) the ratio phosphorylated (p)-CREB/ total CREB were measured using immunoblotting analysis. α-tubulin was used as a normalization standard. Data are displayed as mean ± SEM (N =6). * indicates $p<0.05$ when compared to CTL determined by the Student-T test

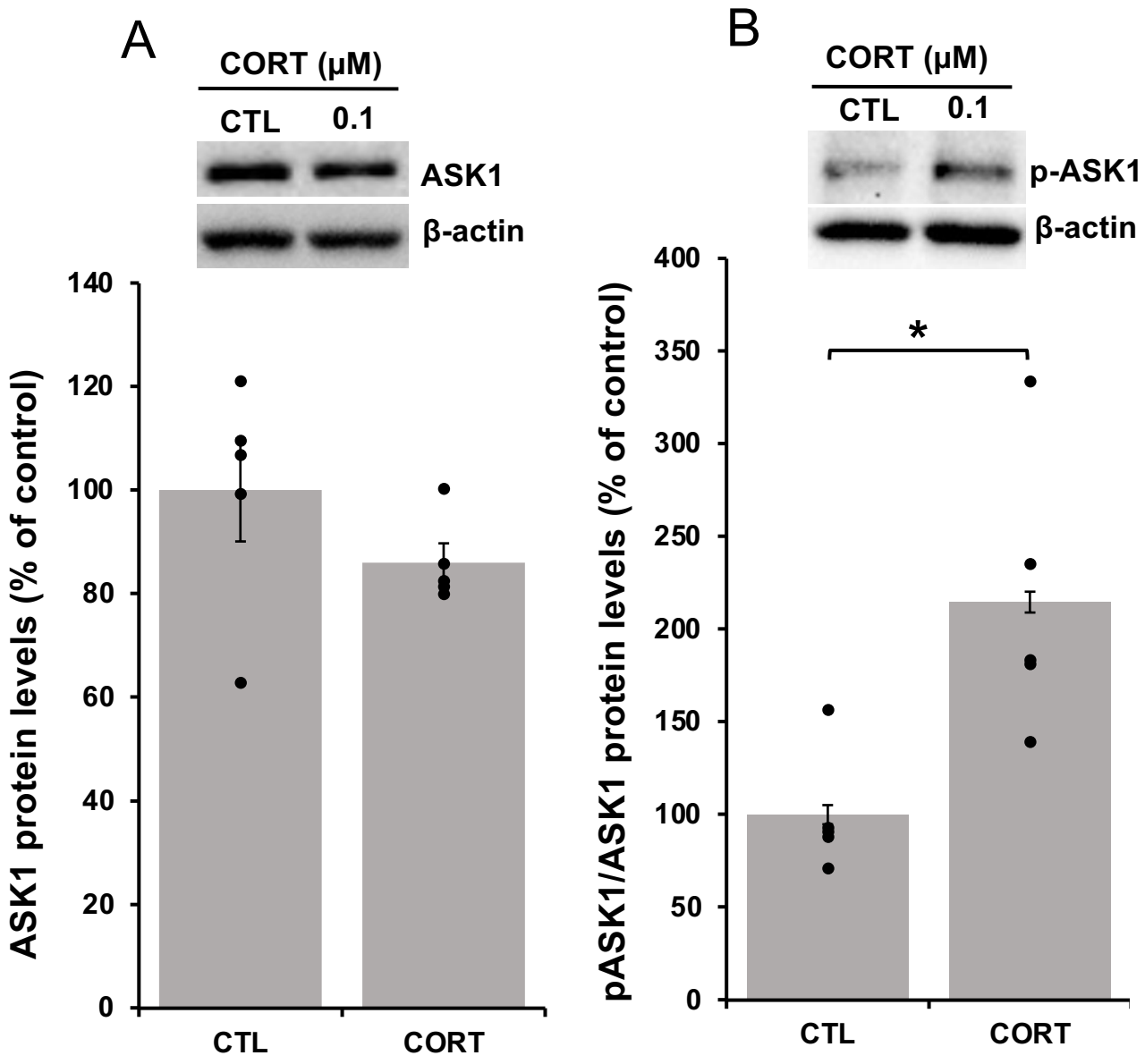


**Figure 19: The effect of corticosterone (CORT) on phosphorylation of ASK1 in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with the vehicle as Control (CTL) or 0.1 μM CORT for 5 days. The protein levels of A) total ASK1, and B) the ratio pASK1/ total ASK1 were measured using immunoblotting analysis. α-tubulin was used as a normalization standard. Data are displayed as mean ± SEM (N =5). * indicates $p<0.05$ when compared to CTL determined by the Student-T test

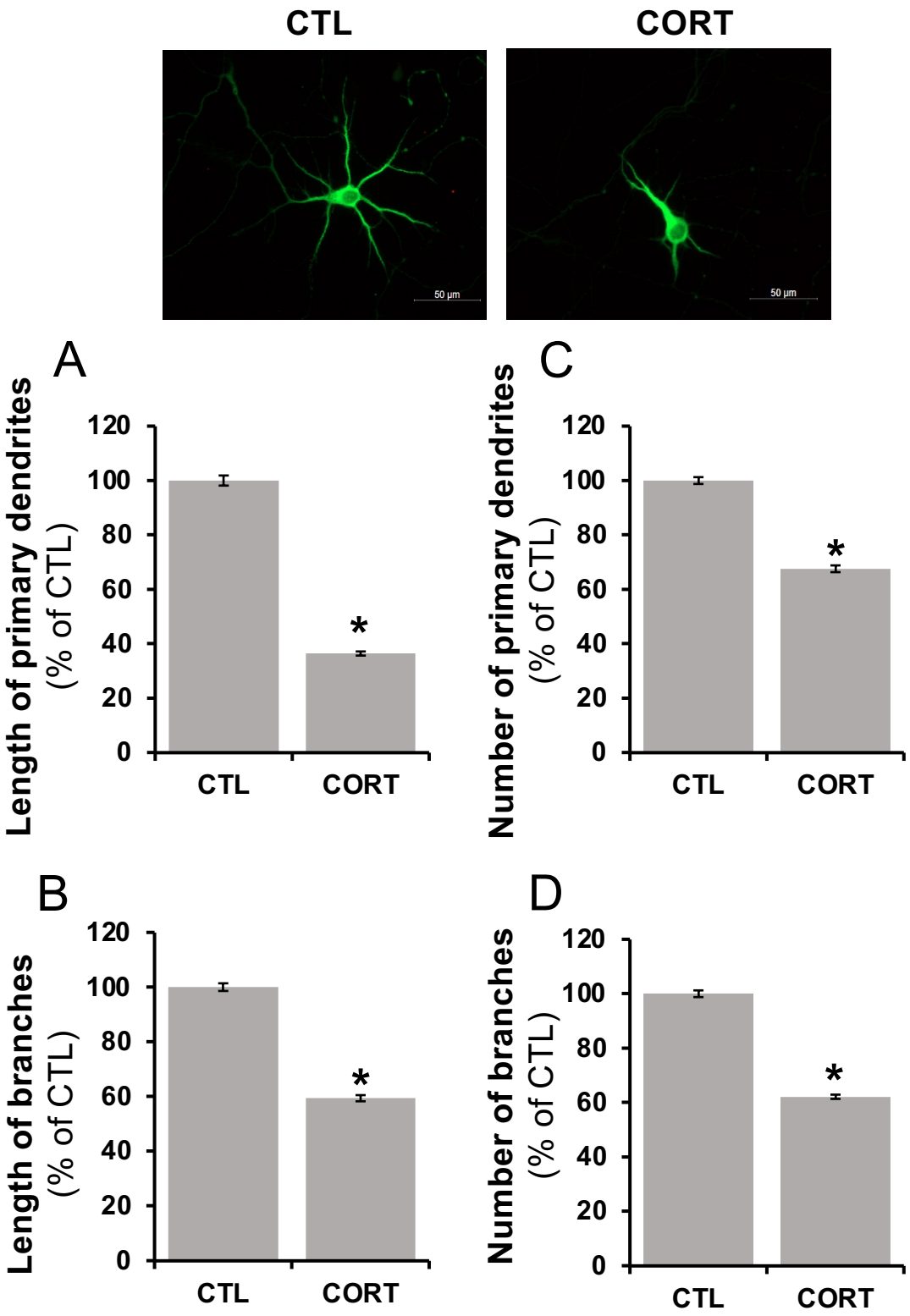


**Figure 20: The effect of corticosterone (CORT) on dendritic outgrowth in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with vehicle as Control (CTL) or 0.1 µM CORT for 5 days. Dendrites were stained with anti-MAP2 antibody at 7 DIV. Pictures of dendrites were taken using a fluorescent microscope. Length and number of primary dendrite and dendritic branches (secondary and tertiary neurites) were assessed using ImageJ. Data is displayed as sum ± SD (N=40). * indicates $p<0.05$ when compared to the control determined by the Student-T test

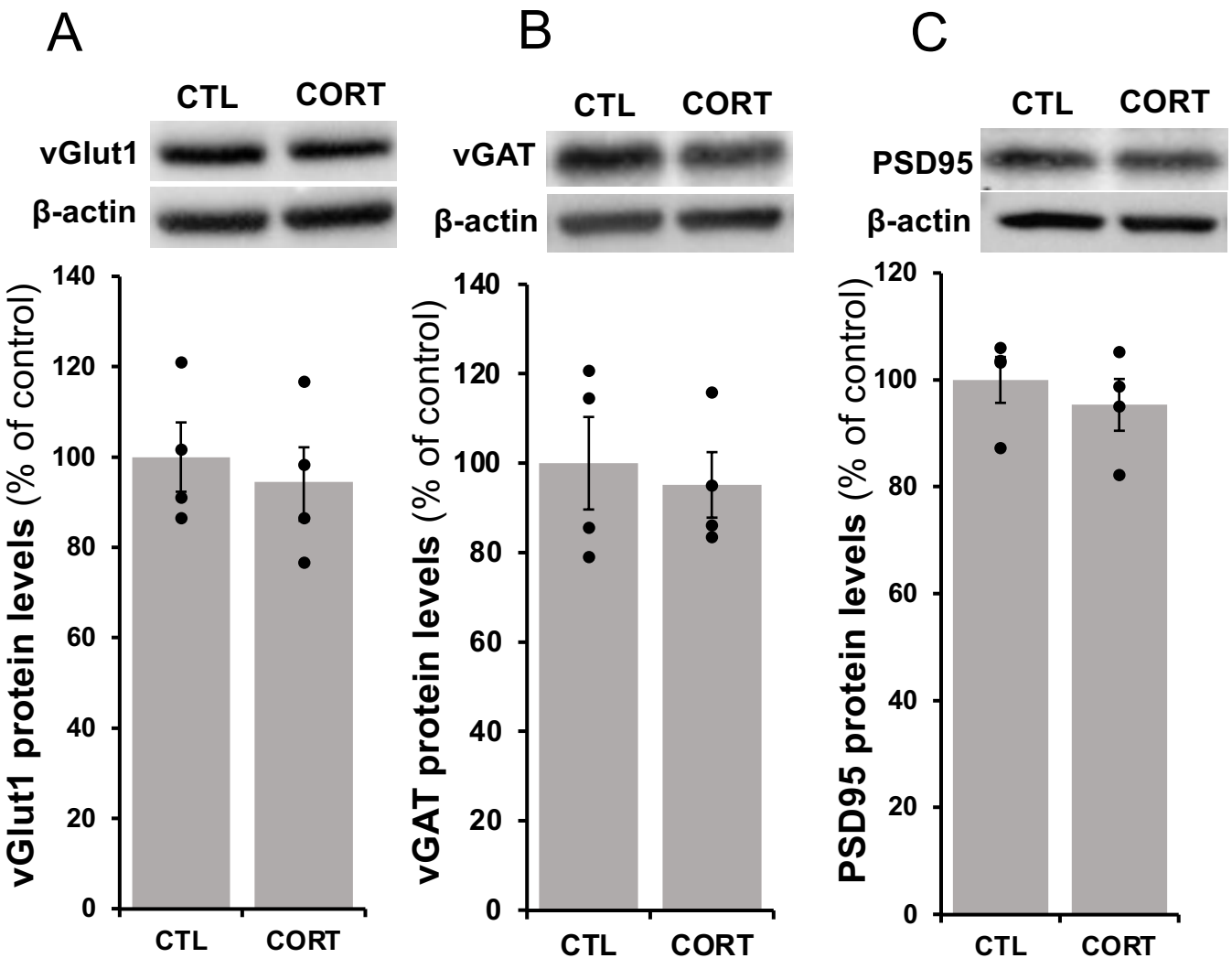


**Figure 21: The effect of corticosterone (CORT) on synaptic protein levels in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated at 2 DIV with vehicle as Control (CTL) or 0.1 μM CORT for 5 days. Protein levels of A) vGlut1, B) vGAT, and C) PSD95 were measured using immunoblotting analysis. β-actin was used as a normalization standard. Data are displayed as mean ± SEM (N =4). * indicates $p<0.05$ when compared to CTL determined by the Student-T test

### 3.3.4 To determine whether Txnip inhibition prevents chronic CORT treatment-impaired neuronal differentiation.

Because CORT upregulates Txnip, we further determined whether knocking down Txnip reverses CORT-impaired dendritic outgrowth. First, primary cultured mouse cerebrocortical neurons were transfected with CRISPR/Cas9/Txnip sgRNA or CRISPR/Cas9/ scrambled sgRNA at 1 DIV. Neurons were stained with MAP2 antibody at 7 DIV using immunocytochemistry analysis. We found that Txnip protein levels were significantly decreased in CRISPR/Cas9/Txnip sgRNA transfected neurons when compared to CRISPR/Cas9/ scrambled sgRNA transfected neurons ($p<0.05$, N=5) (Figure 22), suggesting that Txnip sgRNA can knock down Txnip expression in primary cultured neurons. Second, cultured neurons were transfected with CRISPR/Cas9/Txnip sgRNA or CRISPR/Cas9/ scrambled sgRNA at 1 DIV, and then exposed to 0.1 μM CORT at 2 DIV for 5 days. As shown in figure 23, treatment with CORT at 0.1 μM for 5 days caused a reduction in the length of primary dendrites, and the total length and number of branches ($p<0.05$, N=40) when compared to control in scrambled sgRNA transfected neurons. On the other hand, the length of primary dendrites, and the total length and number of branches were increased in CORT-treated Txnip sgRNA transfected neurons when compared to CORT-treated scrambled sgRNA transfected neurons ($p<0.05$, N=40). Our results suggest that knocking down Txnip gene prevented CORT-impaired dendritic outgrowth. Overall, these results suggest that Txnip might mediate CORT-induced deleterious effects on neuronal differentiation.

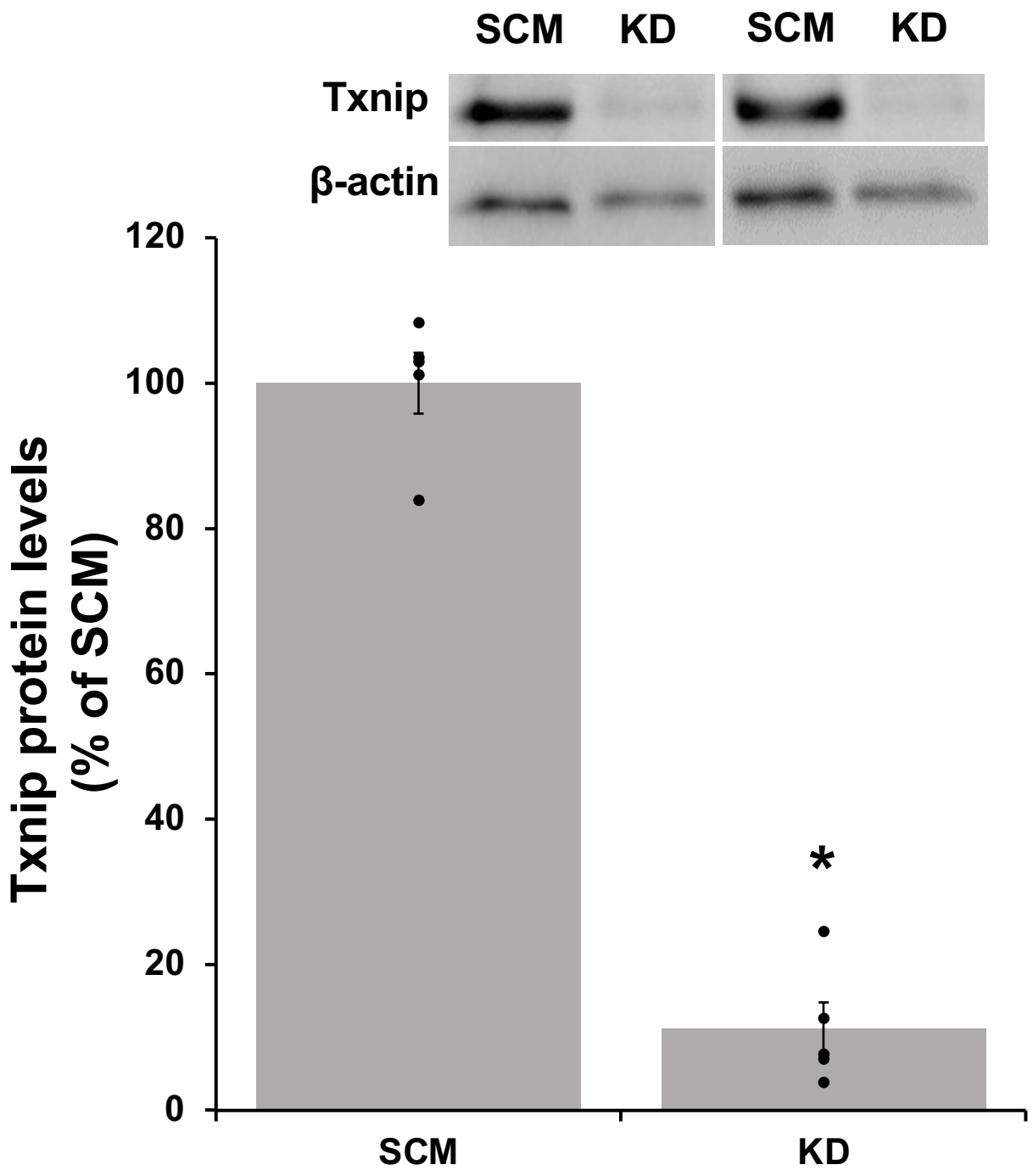


**Figure 22: Knocking down Txnip gene in primary cultured mouse cerebrocortical neurons using CRISPR-Cas9 technology**. Cultured neurons were transfected with lentivectors containing Trx sgRNAs/CRISPR/Cas9 (KD) or scrambled sgRNAs/CRISPR/Cas9 (SCM) at 1 DIV. Txnip protein levels were measured by immunoblotting analysis at 8 DIV. Data are displayed as mean ± SEM (N=5). * indicates $p < 0.05$ when KD group was compared to SCM groups determined by the Student-T test

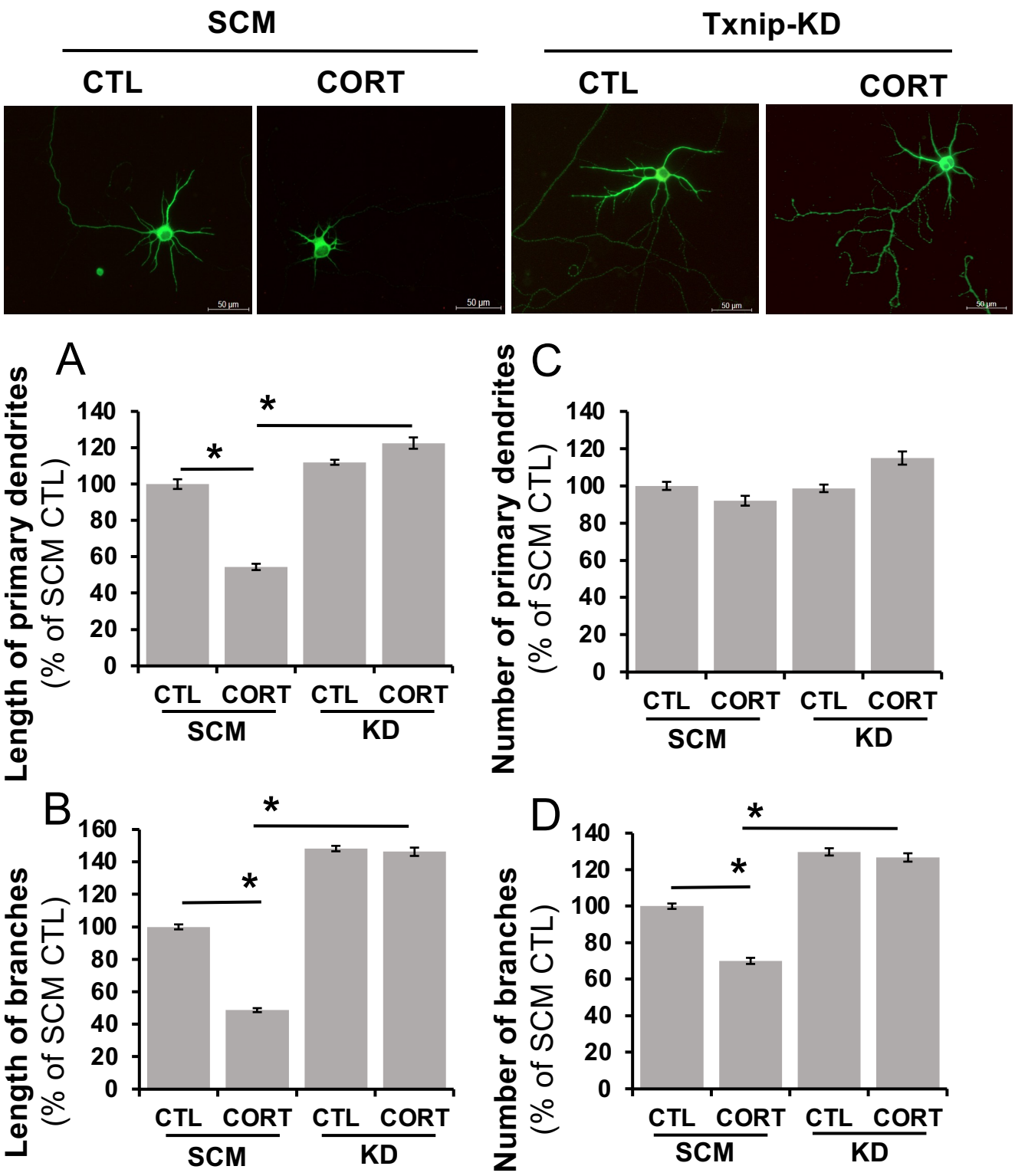


**Figure 23: The effect of knocking down Txnip on corticosterone (CORT)-impaired dendritic outgrowth in primary cultured mouse cerebrocortical neurons**. Cultured neurons were treated with scrambled sgRNAs/CRISPR/Cas9 (SCM) or Txnip sgRNAs/CRISPR/Cas9 (KD) at 1 DIV. Then, neurons were treated with vehicle (CTL) or 0.1 µM CORT at 2 DIV for 5 days. Dendrites were stained with anti-MAP2 antibody. Pictures of dendrites were taken using a fluorescent microscope. The length and number of primary dendrite and dendritic branches (secondary and tertiary neurites) were assessed using ImageJ. Data is displayed as sum ± SD (N=40). * indicates $p<0.05$ when compared to SCM-CTL and KD-CORT determined by one-way ANOVA followed by Tukey's post-hoc analysis.

### 3.4 To determine the role of Txnip in CORT-induced depressive-like behaviors and cognitive dysfunction.

Dysregulation of glucocorticoid signaling due to chronic stress has been associated with the development of depression. Depression diagnosis is based on symptoms. No biological markers are available for the disorder. Modeling depression in animals is much more difficult than other somatic diseases. Anxiety behavior, despair behavior, anhedonia behavior and cognitive dysfunction are major symptoms of depression. In rodents, anxiety, despair, anhedonia, and cognitive dysfunction depressive-like behaviors can be measured by the open field test, tail suspension test, sucrose preference test and novel object recognition test (Acikgoz et al., 2022; Belovicova et al., 2017). In order to understand the role of Txnip in CORT-induced depressive-like behaviors in mice, we first determined if mice injected with CORT exhibit depressive-like behaviors, and then determined if Txnip knockdown prevents CORT-induced depressive-like behaviors.

#### 3.4.1 To determine if mice injected with CORT exhibit depressive-like behaviors.

First, male CD1 mice were injected subcutaneously with vehicle as control (sesame oil) or 20 mg/Kg CORT daily for 14 or 21 days. Then we measured anxiety behavior using the open field test, despair behavior using the tail suspension test, anhedonia behavior using the sucrose preference test and cognitive function using the novel object recognition test.

In the open field test, although chronic CORT exposures for 14 and 21 days had no effect on the total distance travelled in the arena (N=6-8) (Figure 24A), it significantly decreased the time spent in the centre, and the frequency of centre entry ($p<0.05$, N=6-8) (Figure 24B and 24C). CORT treatment for 21 days also significantly reduced the number of rearing compared to

control ($p<0.05$, N=6-8) (Figure 24D). Furthermore, we found that CORT injections for 21 days significantly increased the immobility time in the tail suspension test ($p<0.05$, N=6-8) (Figure 25A) and decreased sucrose consumption in the sucrose preference test compared to the control group ($p<0.05$, N=6-8) (Figure 25B). However, although CORT injections for 14 and 21 days decreased the recognition index in the novel object recognition test (N=6-8) (Figure 25C), this decrease shows statistically no significance. Our results suggest that chronic CORT treatment can induce depressive-like behaviors in mice. Our results also suggest that CORT treatment for 21 days achieved a better depressive phenotype than CORT treatment for 14 days. Thus, for our next experiments, we decided to treat mice with CORT for 3 weeks at a dose of 20 mg/kg.

### 3.4.2 To determine if Txnip knockdown prevents CORT-induced depressive behaviors and cognitive dysfunction.

Next, we evaluated whether knocking down Txnip in the frontal cortex prevents CORT-induced depressive-like behavior. The animal treatment schedule is shown in figure 26. Animals were habituated for 3 days and then scrambled or Txnip sgRNA CRISPR/Cas9 packaged into adeno-associated virus serotype 1/2 (AAV1/2) were bilaterally microinjected into the medial frontal cortex of male and female CD1 mice. Animals were allowed to recover from the surgery for 7 days. Then, mice were subjected to chronic subcutaneous injections with CORT for 21 days at a dose of 20 mg/Kg. Then, depressive-like behaviors and cognitive decline were evaluated in the open field test, novel object recognition test, tail suspension test, and sucrose preference test at 1 day, 2 days, 4 days, and 5 days after the last injection respectively. At 7 days post-injection, blood was collected to detect CORT levels. Moreover, the frontal cortex was isolated to measure Txnip protein levels.

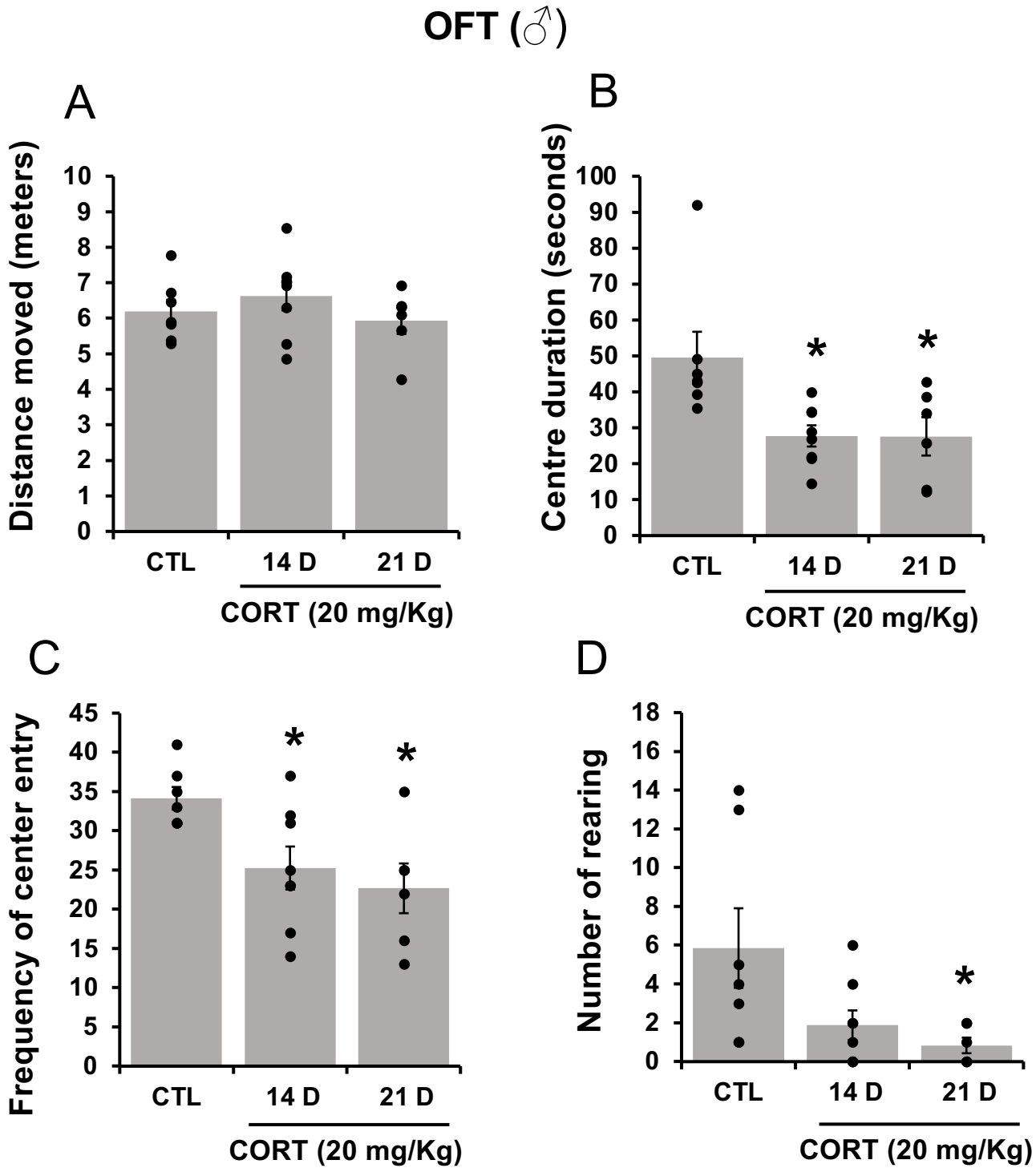


**Figure 24: The effect of chronic corticosterone injection on male mouse behavior in the open field test.** Male mice were exposed to subcutaneous injections of sesame oil (CTL), 20 mg/Kg CORT for 14 days, (14D), or 20 mg/Kg CORT for 21 days (21D). The distance moved (A), time spent in the center (B), frequency of center entries (C), and number of rearing (D) were recorded during the open field test. Data are displayed as mean ± SEM (N = 6–8). * indicates $p < 0.05$ when compared to CTL determined by one-way ANOVA followed by Tukey's post-hoc analysis.

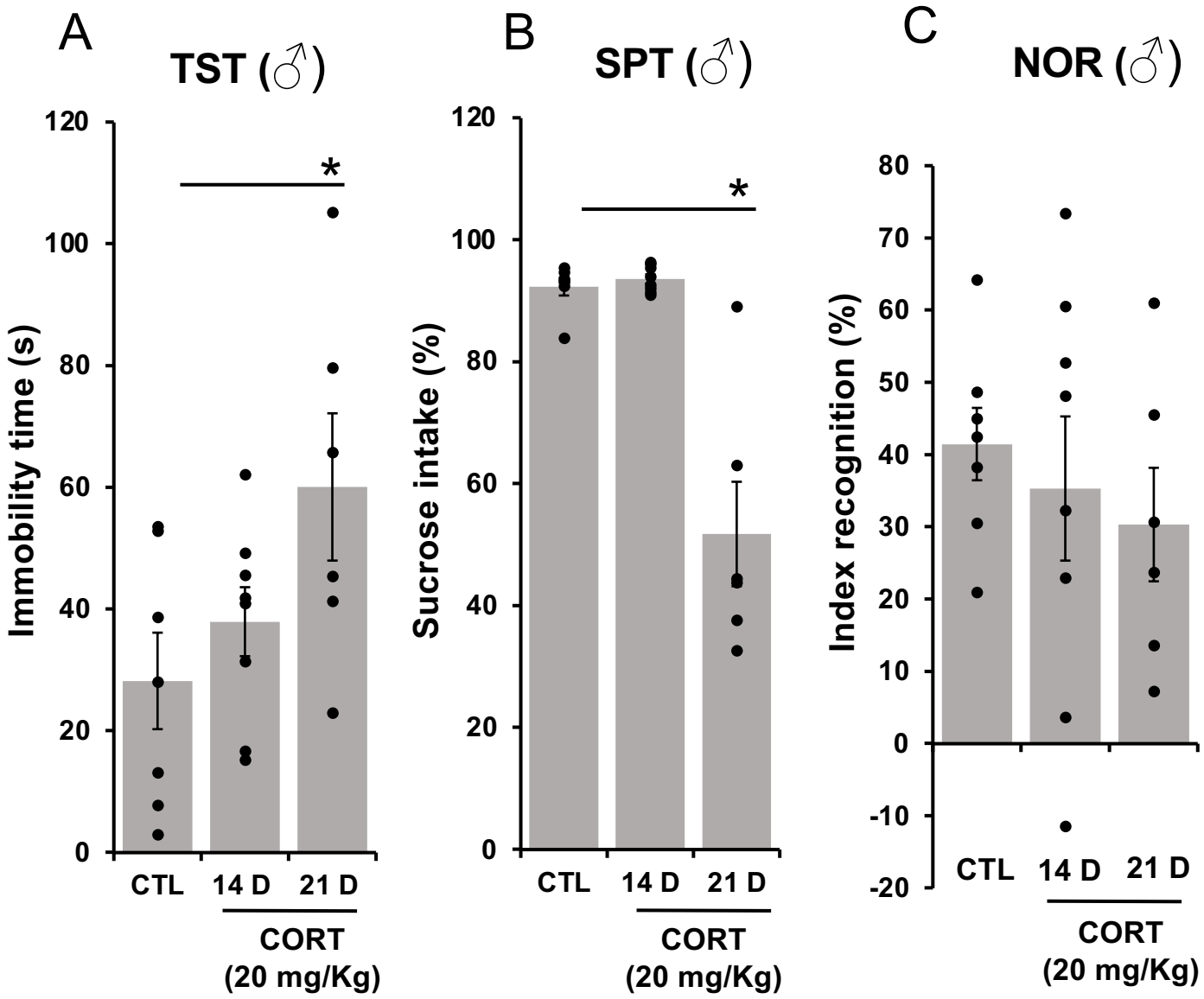


**Figure 25: The effect of chronic corticosterone injection on male mouse behavior.** Male mice were exposed to subcutaneous injections of sesame oil (CTL), 20 mg/Kg CORT for 14 days, (14D), or 20 mg/Kg CORT for 21 days (21D). Immobility was recorded during the tail suspension test (TST) (A), sucrose preference was recorded during the sucrose preference test (SPT) (B), and the recognition index was recorded during the novel object recognition test (NOR) (C). Data are displayed as mean ± SEM (N = 6–8). * indicates $p < 0.05$ when compared to CTL determined by one-way ANOVA followed by Tukey's post-hoc analysis.

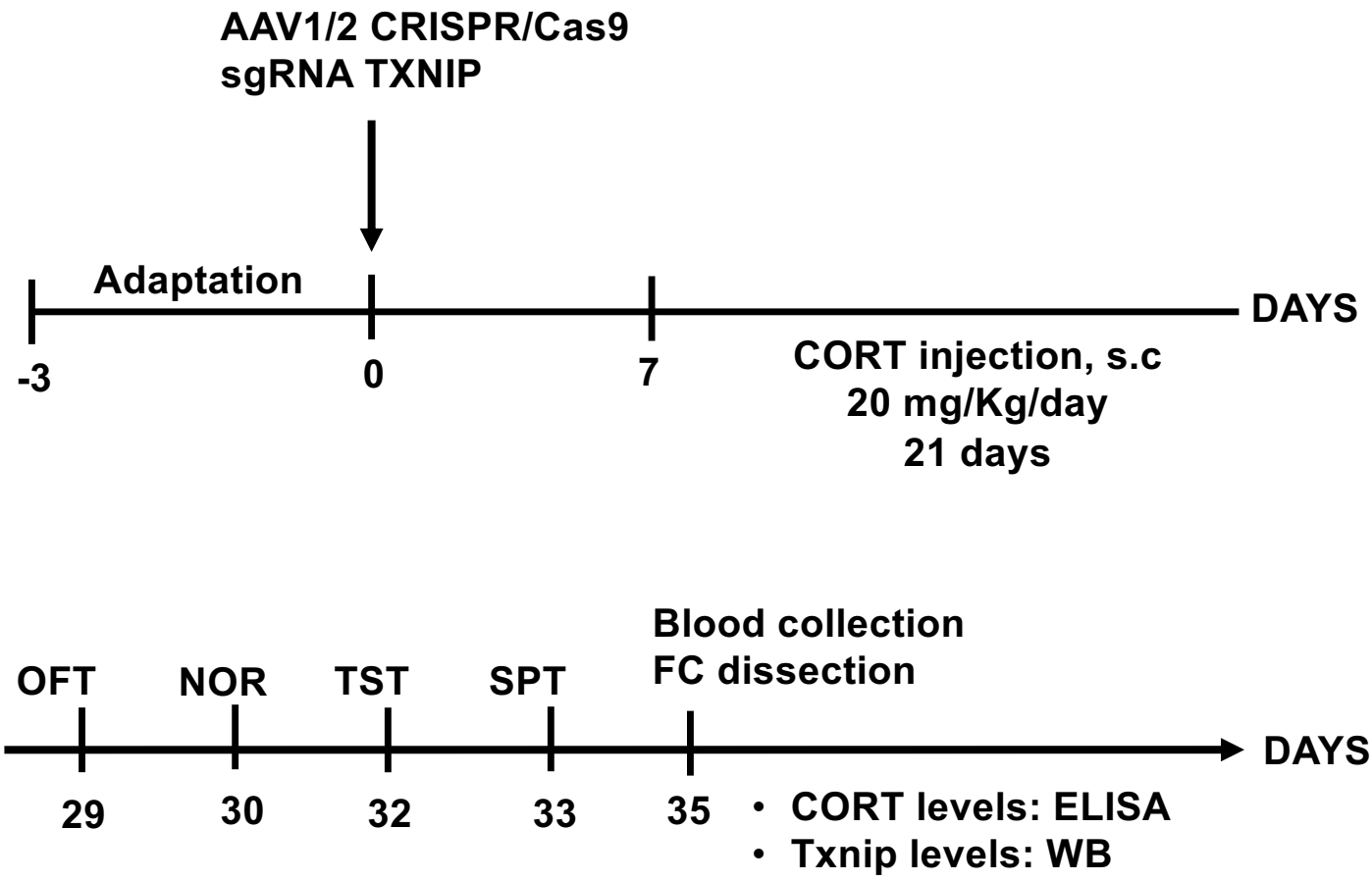


**Figure 26: Animal experimental design.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the frontal cortex (FC) of male and female mice. Mice were then exposed to 20 mg/Kg CORT for 21 days. Behavior was assessed at the end of the experiment in the following order: open field test (OFT), novel object recognition test (NOR), tail suspension test (TST), and sucrose preference test (SPT). After the last behavior test, the frontal cortex and blood were collected. Txnip protein levels were analyzed by immunoblotting analysis (WB). Serum CORT levels were determined by ELISA.

First, we analyzed the effect of injection with Txnip sgRNA CRISPR/Cas9 AAV on CORT-induced depressive-like behaviors in male mice. Txnip sgRNA CRISPR/Cas9 AAV was injected into the medial frontal cortex of male mice. Then mice were injected with CORT at 20 mg/Kg daily for 21 days. As shown in figure 27, chronic CORT injections significantly increased serum CORT levels when compared to controls ($p<0.05$, N=17-18). Injection with Txnip sgRNA CRISPR/Cas9 AAV1/2 also significantly decreased Txnip protein levels in the frontal cortex of male mice by 70% ($p<0.05$, N=9-10) (Figure 28), suggesting that Txnip sgRNA can inhibit Txnip gene expression in male mouse frontal cortex. In the open field test, we found that there is no significant difference in distance moved (N=8-10, Figure 29A) and centre duration (N=8-10, Figure 29B) among vehicle and CORT-treated groups in mice transfected with scrambled sgRNA and mice transfected with Txnip sgRNA. However, we found that chronic CORT treatment significantly decreased the frequency of centre entry in mice transfected with scrambled sgRNA ($p<0.05$), while CORT treatment had no effect on the frequency of centre entry in mice transfected with Txnip sgRNA (N=8-10, $p<0.05$) (Figure 29C). Chronic CORT treatment also decreased the number of rearing in mice transfected with scrambled sgRNA, but there is no statistical significance ($p>0.05$). However, there was a significant increase in the number of rearing in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice ($p<0.05$, N=8-10) (Figure 29D). Further, in the tail suspension test, we found that chronic CORT treatment significantly increased immobility time in mice transfected with scrambled sgRNA ($p<0.05$), while immobility time was significantly decreased in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice (N=8-10, $p<0.05$) (Figure 30A).

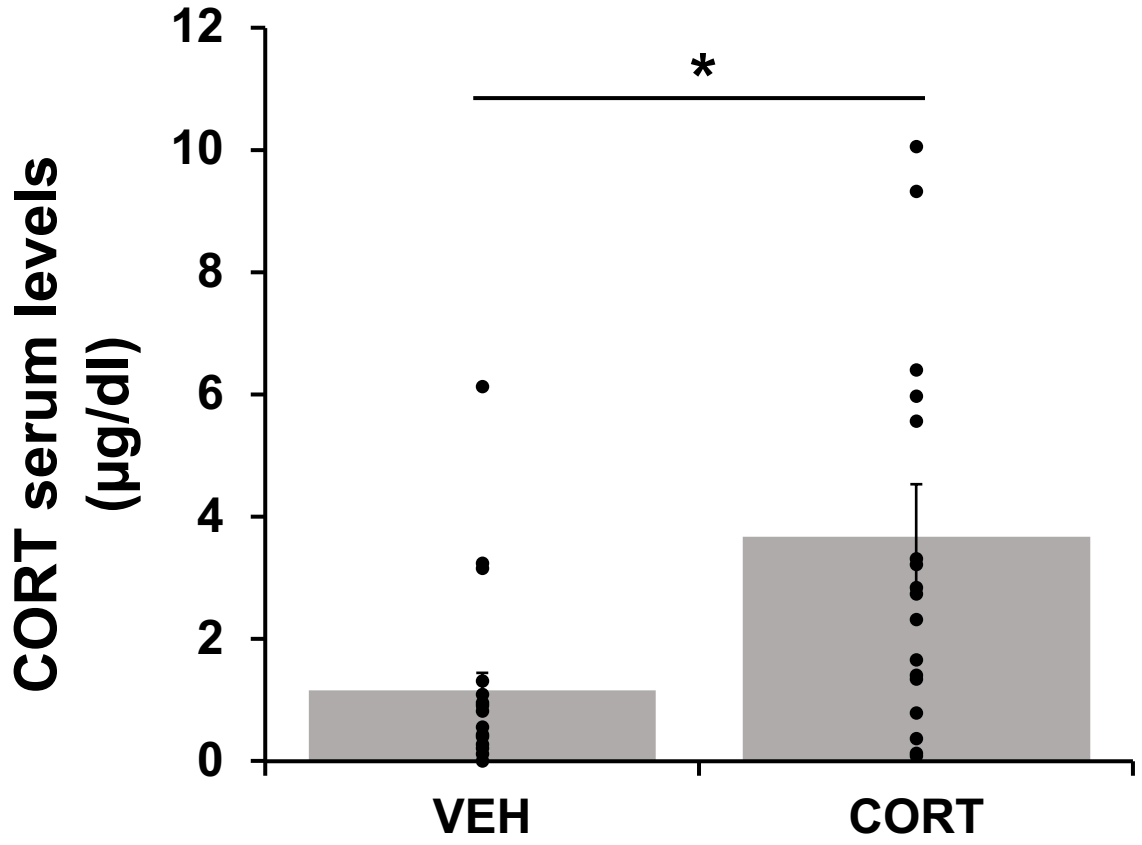


**Figure 27: Serum corticosterone levels in male mice.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of male mice. Mice were then exposed to 20 mg/Kg CORT for 21 days. CORT serum levels were evaluated using ELISA kit. Data are displayed as mean ± SEM (N = 17-18).* indicates $p < 0.05$ when vehicle-injected group was compared to CORT-injected group determined by the Student-T test

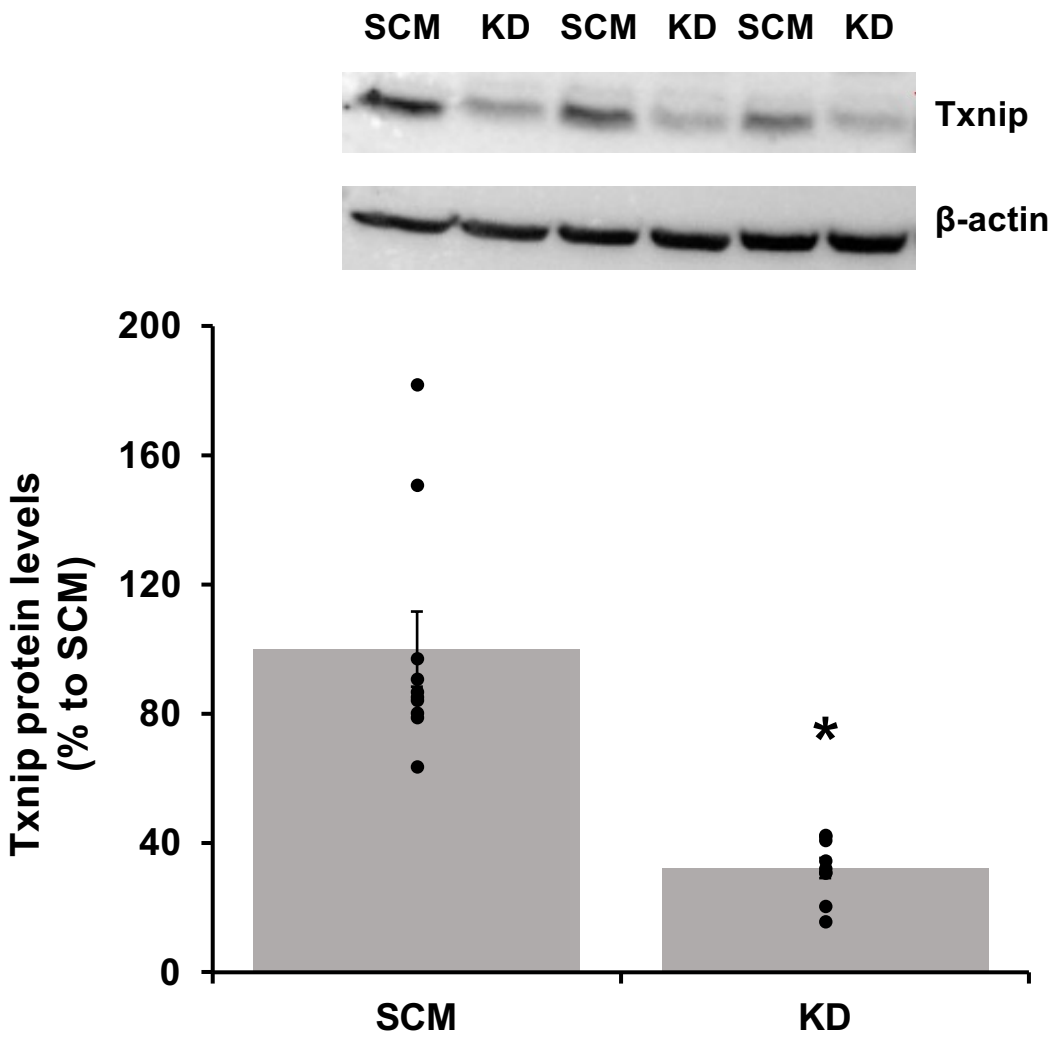


**Figure 28: Txnip protein levels in the frontal cortex of male mice.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of male mice. Mice were then exposed to 20 mg/Kg CORT for 21 days. Txnip protein levels in the frontal cortex were analyzed by immunoblotting. Data are displayed as mean ± SEM (N = 9-10). * indicates $p < 0.05$ when KD group was compared to SCM groups determined by the Student-T test

In the sucrose preference test, we found that chronic CORT treatment significantly decreased sucrose consumption in mice transfected with scrambled sgRNA ($p<0.05$), while sucrose consumption was significantly increased in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice (N=8-10, $p<0.05$) (Figure 30B). In the novel object recognition test, we found that chronic CORT treatment significantly decreased exploring time for the novel object in mice transfected with scrambled sgRNA ($p<0.05$) when compared to control, while CORT treatment had no effect on exploring time for the novel object in mice transfected with Txnip sgRNA (N=8-10, $p<0.05$) (Figure 30C). Our findings suggest that Txnip sgRNA can inhibit CORT-induced depressive-like behaviors in male mice.

Second, to evaluate whether there was sexual dimorphism in the effect of Txnip on CORT response, we also analyzed the effect of injection with Txnip sgRNA CRISPR/Cas9 AAV on CORT-induced depressive-like behaviors in female mice. Txnip sgRNA CRISPR/Cas9 AAV was injected into the medial frontal cortex of female mice. Then mice were injected with CORT at 20 mg/Kg daily for 21 days. As shown in figure 31, chronic CORT injections significantly increased serum CORT levels when compared to controls ($p<0.05$, N=16-18). We also found that Txnip sgRNA CRISPR/Cas9 AAV1/2 significantly decreased Txnip protein levels approximately by 40% in the frontal cortex of female mice when compared to scramble sgRNA group (N=9-10, $p<0.05$) (Figure 32), suggesting that Txnip sgRNA can also inhibit Txnip gene expression in female mouse frontal cortex. In the open field test, we found that there is no significant difference in the distance moved and frequency of center entry among vehicle and CORT-treated groups in mice transfected with scrambled sgRNA and in mice transfected with Txnip sgRNA (N=9-10) (Figure 33A and 33C). However, we found that chronic CORT treatment significantly decreased centre duration in mice transfected with scrambled sgRNA (N=9-10, $p<0.05$).

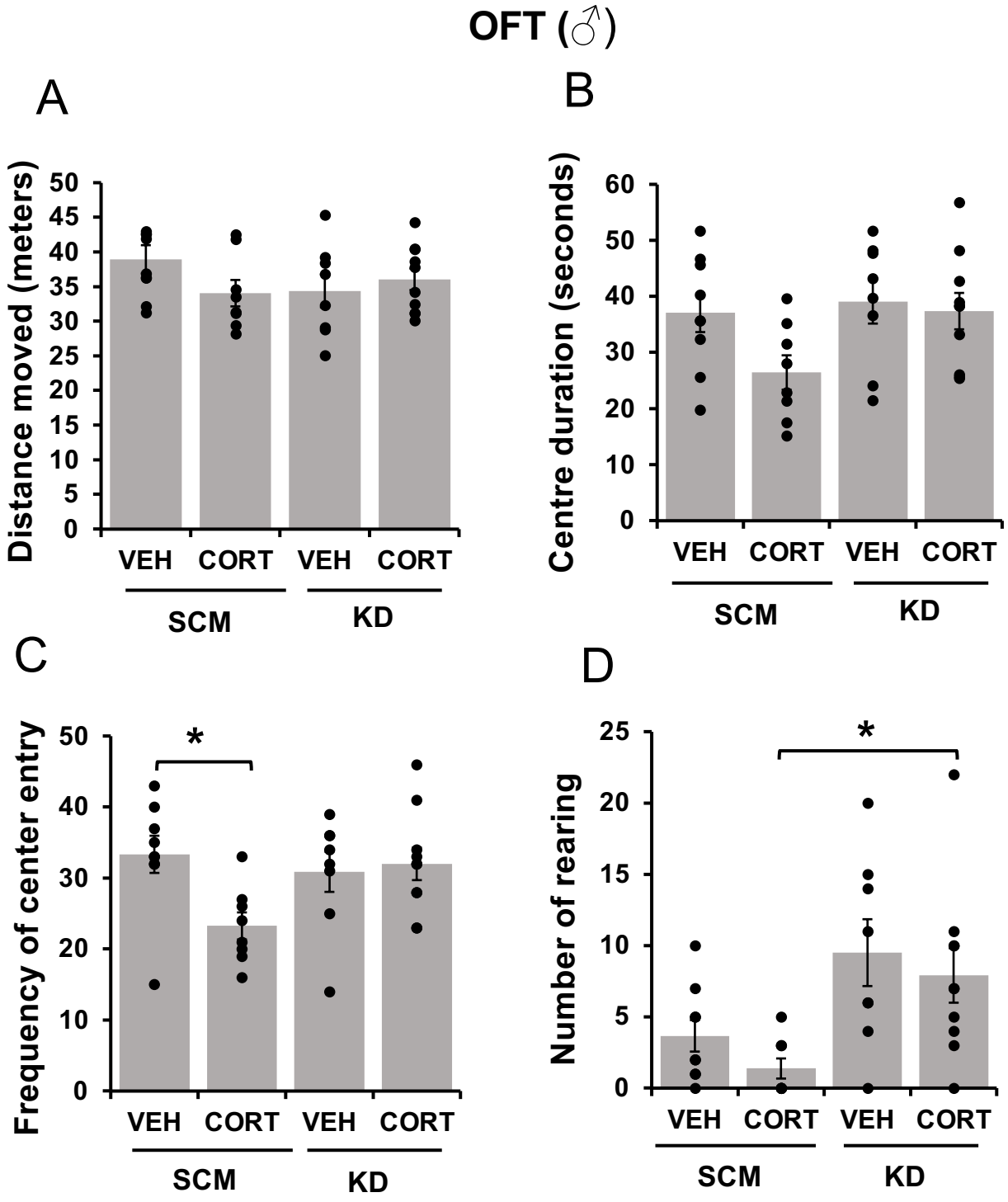


**Figure 29: The effect of knocking down Txnip in male mouse frontal cortex on chronic corticosterone (CORT) injection-induced anxiety behavior.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of male mice. Mice were then subcutaneously injected with 20 mg/Kg CORT dissolved in sesame oil for 21 days. Control mice were injected with sesame oil. The distance moved (A), time spent in the center (B), frequency of center entries (C), and number of rearing (D) were recorded during the open field test. Data are displayed as mean ± SEM (N = 8-10). * indicates $p < 0.05$ when compared to SCM-VEH and KD-CORT determined by one-way ANOVA followed by Tukey's post-hoc analysis.

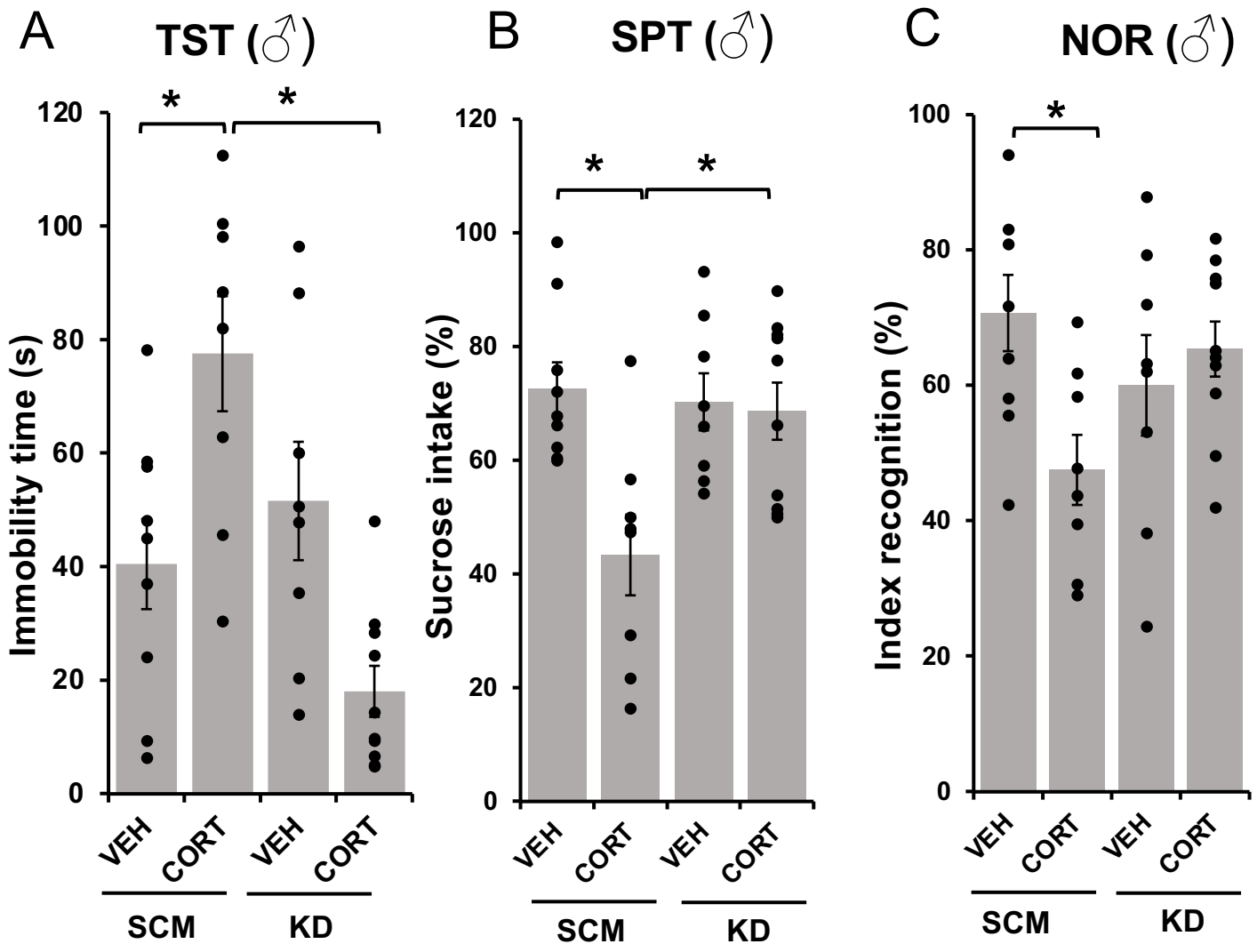


**Figure 30: The effect of knocking down Txnip in male mouse frontal cortex on chronic corticosterone (CORT) injection-induced despair behaviour, anhedonia behaviour and cognitive dysfunction behavior.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of male mice. Mice were then subcutaneously injected with 20 mg/Kg CORT dissolved in sesame oil for 21 days. Control mice were injected with sesame oil. Immobility was recorded during the tail suspension test (TST) (A), sucrose consumption was recorded during the sucrose preference test (SPT) (B), and the recognition index was recorded during the novel object recognition test (NOR) (C). Data are displayed as mean ± SEM (N = 8-10). * indicates $p < 0.05$ when compared to SCM-VEH and KD-CORT determined by one-way ANOVA followed by Tukey's post-hoc analysis.

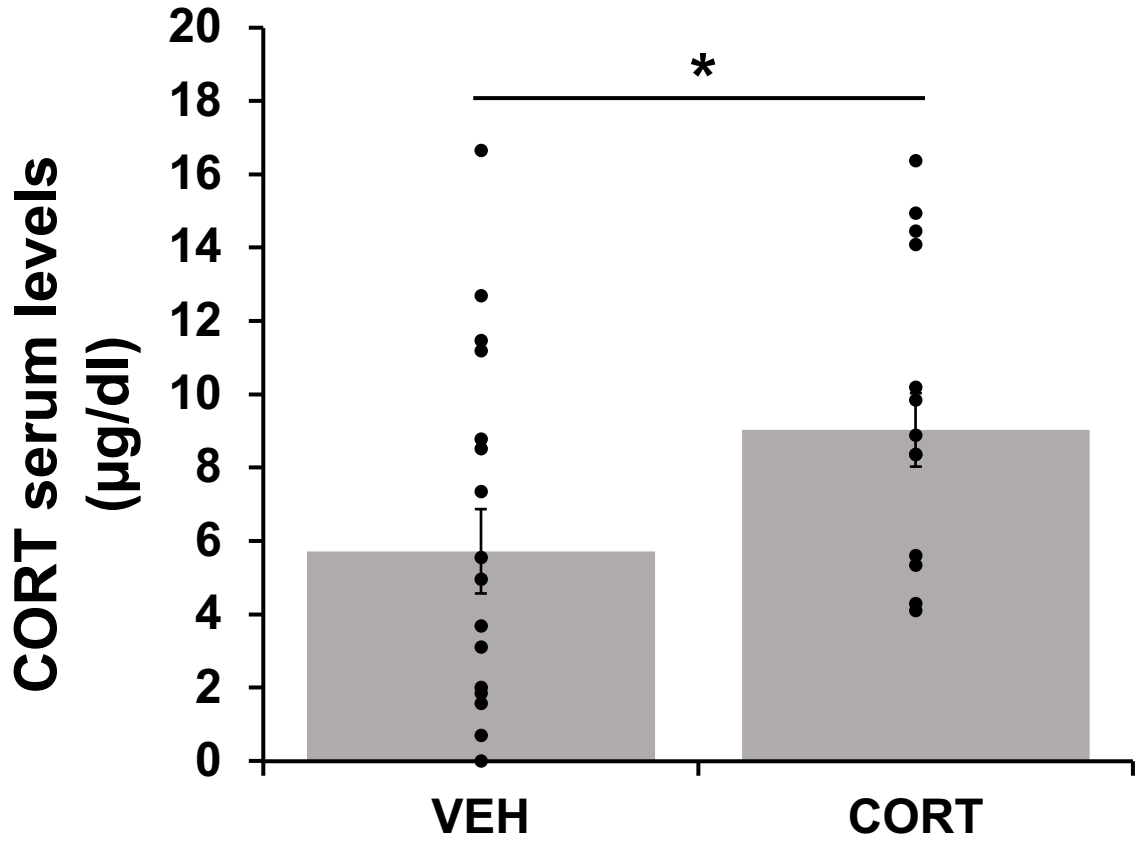


**Figure 31: Serum corticosterone levels in female mice.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of female mice. Mice were then exposed to 20 mg/Kg CORT for 21 days. CORT serum levels were evaluated using ELISA kit. Data are displayed as mean ± SEM (N =16-18).* indicates $p < 0.05$ when vehicle-injected group was compared to CORT-injected group determined by the Student-T test

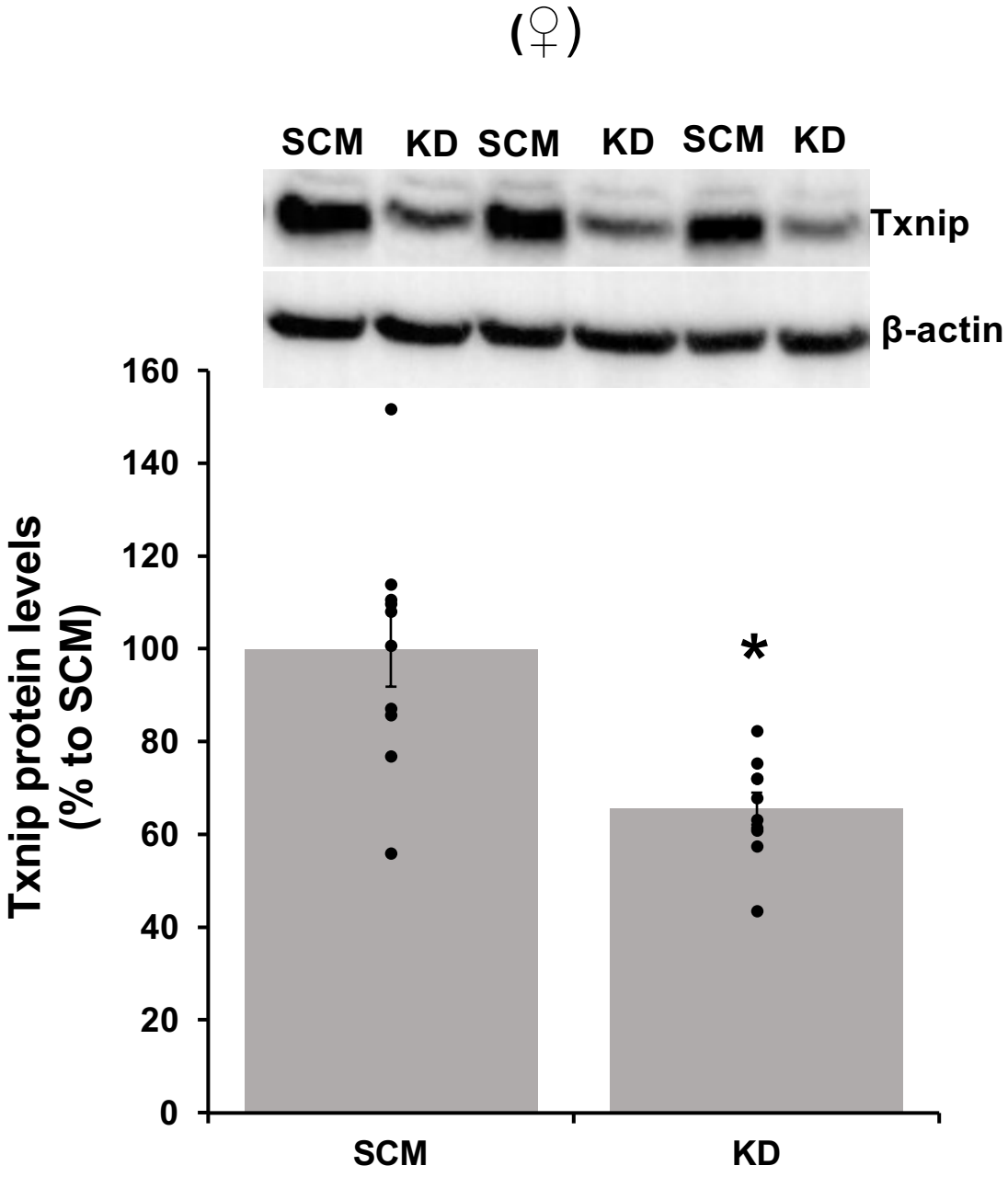


**Figure 32: Txnip protein levels in the frontal cortex of female mice.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of female mice. Mice were then exposed to 20 mg/Kg CORT for 21 days. Txnip protein levels in the frontal cortex were analyzed by immunoblotting. Data are displayed as mean ± SEM (N = 9-10). * indicates $p < 0.05$ when KD group was compared to SCM groups determined by the Student-T test

In addition, centre duration was significantly increased in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice (N=9-10, $p<0.05$) (Figure 33B). We also found that chronic CORT treatment significantly decreased the number of rearing in mice transfected with scrambled sgRNA ($p<0.05$), but CORT treatment had no effect on the number of rearing in mice transfected with Txnip sgRNA (N=9-10, $p<0.05$) (Figure 33D). In the tail suspension test, we found that chronic CORT treatment significantly increased immobility time in mice transfected with scrambled sgRNA ($p<0.05$), while immobility time was significantly decreased in CORT-treated Txnip sgRNA -transfected mice when compared to CORT-treated scrambled-transfected mice (N=9-10, $p<0.05$) (Figure 34A). In the sucrose preference test, we found that chronic CORT treatment significantly decreased sucrose consumption in mice transfected with scrambled sgRNA ($p<0.05$), while sucrose consumption was significantly increased in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice (N=9-10, $p<0.05$) (Figure 34B). In the novel object recognition test, we found that chronic CORT treatment decreased exploring time for the novel object in mice transfected with scrambled sgRNA ($p<0.05$) when compared to control, but there is no statistical significance. However, exploring time for the novel object was significantly increased in CORT-treated Txnip sgRNA-transfected mice when compared to CORT-treated scrambled-transfected mice (N=9-10, $p<0.05$) (Figure 34C). Our findings suggest that Txnip sgRNA can inhibit CORT-induced depressive-like behaviors in female mice.

Overall, our results indicate that injection with Txnip sgRNA can inhibit Txnip gene expression in the frontal cortex of both male and female mice. Chronic CORT exposure induces depressive-like behavior and cognitive dysfunction. Knocking down Txnip can prevent CORT-induced depressive-like behavior and cognitive dysfunction in both female and male mice.

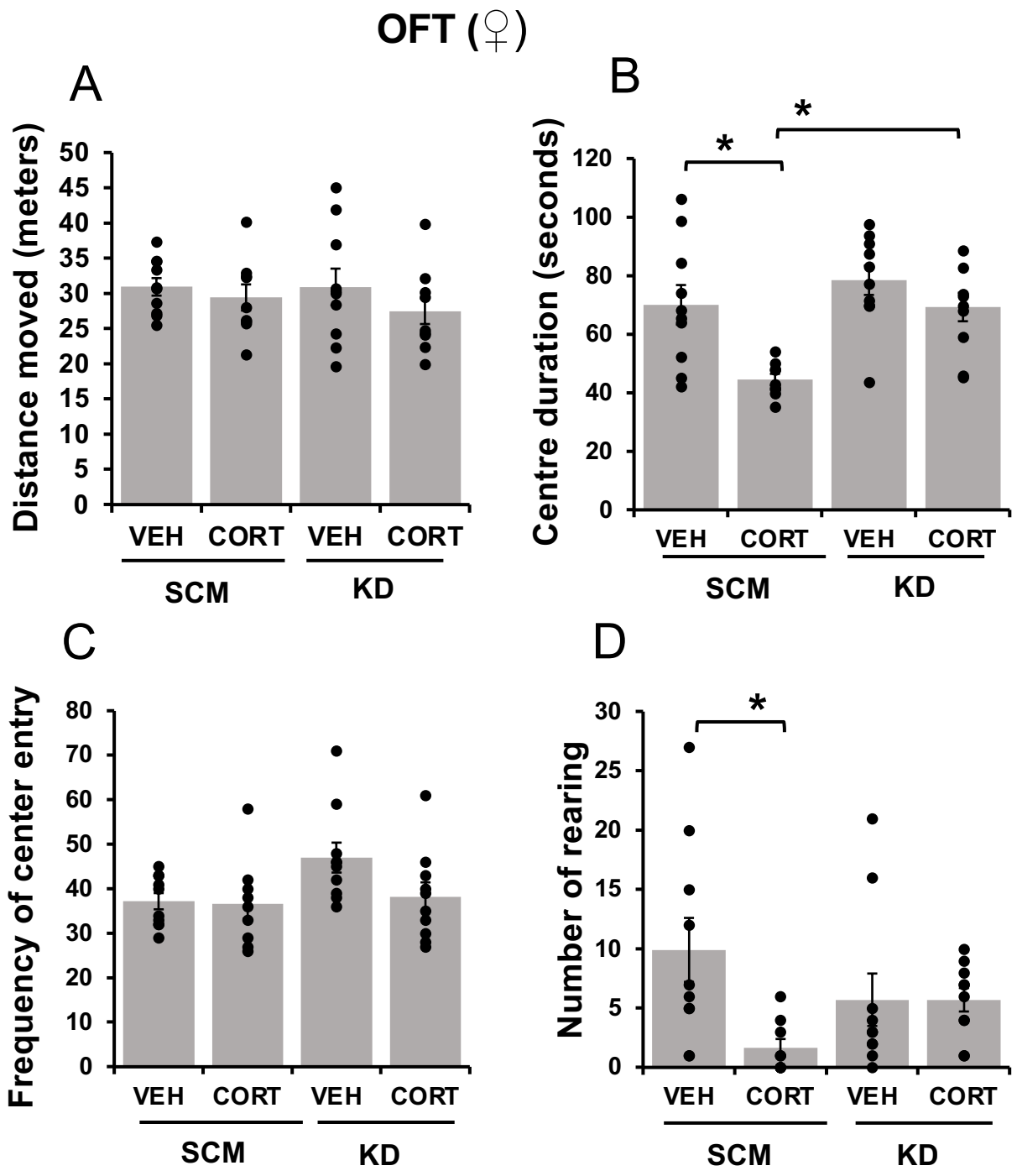


**Figure 33: The effect of knocking down Txnip in female mouse frontal cortex on chronic corticosterone (CORT) injection-induced anxiety behavior.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of female mice. Mice were then subcutaneously injected with 20 mg/Kg CORT dissolved in sesame oil for 21 days. Control mice were injected with sesame oil. The distance moved (A), time spent in the center (B), frequency of center entries (C), and number of rearing (D) were recorded during the open field test. Data are displayed as mean ± SEM (N = 9-10). * indicates $p < 0.05$ when compared to SCM-VEH and KD-CORT determined by one-way ANOVA followed by Tukey's post-hoc analysis.

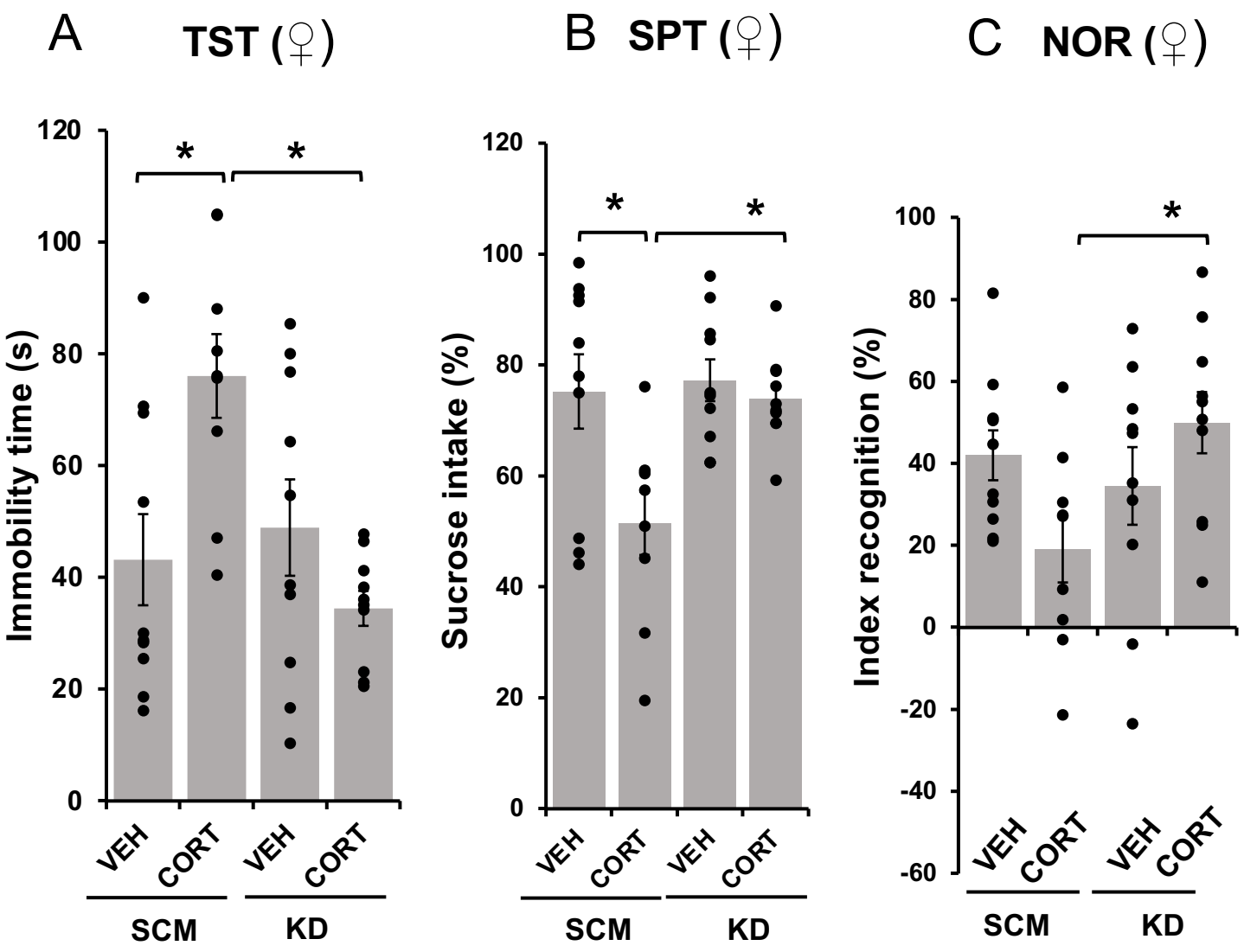


**Figure 34: The effect of knocking down Txnip in female mouse frontal cortex on chronic corticosterone (CORT) injection-induced despair behaviour, anhedonia behaviour and cognitive dysfunction behavior.** Scrambled (SCM) or Txnip (KD) sgRNA CRISPR/Cas9 AAV1/2 was bilaterally injected into the medial frontal cortex of female mice. Mice were then subcutaneously injected with 20 mg/Kg CORT dissolved in sesame oil for 21 days. Control mice were injected with sesame oil. Immobility was recorded during the tail suspension test (TST) (A), sucrose consumption was recorded during the sucrose preference test (SPT) (B), and the recognition index was recorded during the novel object recognition test (NOR) (C). Data are displayed as mean ± SEM (N = 9-10). * indicates $p < 0.05$ when compared to SCM-VEH and KD-CORT determined by one-way ANOVA followed by Tukey's post-hoc analysis.

# CHAPTER 4: DISCUSSION

## 4.1 The role of Trx system in neuronal differentiation and degeneration.

### 4.1.1 Expression pattern of Trx, TrxR and Txnip during neuronal differentiation, maturation and degeneration in primary cultured mouse cerebrocortical neurons.

In the present study, we found that protein levels of Trx and TrxR are time-dependently increased from 1 to 22 days in vitro (DIV) in primary cultured mouse cerebrocortical neurons. In primary mouse neuronal culture, neurons extend processes, differentiate into mature neurons, and start to form synaptic connections in the first 15 DIV. During approximately 15–18 DIV, neurons form an extensive network and synaptic proteins are highly expressed. After 22 DIV, neurons start to degenerate (Lesuisse and Martin, 2002; Aksenova et al., 1999; Porter et al., 1997). Increased Trx and TrxR protein levels during differentiation and maturation suggest that Trx antioxidant system contributes to regulating neuronal differentiation and survival. Conversely, the present study also showed that Txnip protein levels gradually increased from 15 to 32 DIV, with the highest increased Txnip levels at 32 DIV. Because Txnip acts as an endogenous inhibitor of Trx by binding to Trx via cysteine thiol bond, Txnip upregulation may inhibit Trx enzyme activity and lead to neurodegeneration of primary cultured mouse cerebrocortical neurons.

Trx is widely expressed throughout the brain, especially in areas with high metabolic demands that involve redox reactions, where Trx exerts pro-survival and antioxidant functions (Silva-Adaya et al., 2014). Recent studies suggested that Trx may promote cell differentiation. For example, a study conducted in rat adrenal phaeochromocytoma (PC12) cells showed that treatment with nerve growth factor (NGF) at 50 ng/mL for 24 hours and 48 hours induced PC12 cell differentiation, while transfection of PC12 cells with a mutant lack-of-function Trx vector

reduced NGF-induced cell differentiation. In this study, only 8% of mutant lack-of-function Trx vector-transfected cells showed cell differentiation after NGF treatment when compared to control (Bai et al., 2003). Another study also showed that treatment with 1μM rat recombinant Trx for 48 hours induced cell differentiation to the same extent as treatment with 100 ng/mL NGF in PC12 cells (Horstkorte et al., 2010). Because PC12 cells are a sympathetic neuron-like cell line, this evidence suggests that Trx contributes to the neuronal survival and differentiation process. However, the exact role of Trx in these processes and the mechanism involved are not yet fully understood.

### 4.1.2 The role of thioredoxin on neuronal differentiation

To understand the role of Trx in neuronal differentiation, we analyzed the effect of Trx inhibition on dendritic outgrowth. Neurons are highly polarized cells. Their dendrites and axons branch out to establish connections with other neurons, receive signals, compute and store information. In primary cell culture, neurites grow out into dendrites and axons and form nascent synaptic junctions between 5-10 DIV (Björklund et al., 2010; Lesuisse and Martin, 2002). In the present study, we found that Trx gene knockdown using CRISPR/Cas9/Trx sgRNA reduced the length and number of dendritic branches at 8 DIV. Trx inhibitor PX12 can induce irreversible thioalkylation of Cys73 of Trx. Cys73 is necessary for post-translational modifications, dimerization, and tertiary structure of Trx. Thus, PX12 inhibits Trx reducing functioning (Islam et al., 2019; May et al., 2018). We found that PX12 also reduced the length and number of dendritic branches. Our results suggest that inhibition of Trx expression and functioning impairs neuronal outgrowth, supporting that Trx may play an important role in regulating neuronal

differentiation and maturation. Our study provided direct evidence indicating that Trx may be neuroprotective and neurotrophic.

Primary cultured cortical neurons contain both glutamatergic neurons and GABAergic neurons. We also found that PX12 reduced glutamatergic neuronal marker vGlut1 protein levels but had no effect on GABAergic neuronal marker vGAT protein levels. Furthermore, we found that inhibition of Trx activity by PX12 also reduced the expression of presynaptic makers SNAP25, and syntaxin as well as postsynaptic protein PSD95 protein levels. Our results suggest that Trx mainly plays a role in regulating excitatory neurons rather than inhibitory neurons.

***In summary***, we found that Trx and TrxR protein levels are upregulated during neuronal differentiation and maturation while Txnip is increased during neuronal degeneration of primary cultured mouse cerebrocortical neurons. Inhibition of Trx reduces dendritic outgrowth and synaptic protein expression. Our results suggest that Trx plays a key role in promoting neuronal differentiation and preventing neurodegeneration.

## 4.2 The role of CREB and ASK1 in Trx-regulated neuronal differentiation and degeneration.

### 4.2.1 CREB activation is regulated by Trx in primary cultured mouse cerebrocortical neurons.

Cyclic AMP response element-binding protein (CREB) is a gene transcription factor that binds to the cyclic AMP response element (CRE) of target genes and regulates the expression of those target genes. Those genes include NGF, brain-derived neurotrophic factor (BDNF) and others (Jeon et al., 2011; Ryu et al., 2005). CREB regulates the expression of various genes that

are important in neuronal differentiation and synaptic plasticity (Landeira et al., 2018; Ma et al., 2014; Jeon et al., 2011). Phosphorylation is an important step for CREB activation. CREB phosphorylation is necessary for CREB-regulated differentiation. For example, it has been reported that the expression of a mutant recombinant lentivirus containing a CREB protein that cannot be phosphorylated at Ser133 reduced the number and length of dendritic branches, the number of dendritic spines, and the expression of synaptic protein synapsin and spinophilin in mice hippocampi and primary cultured mouse hippocampal neurons (Zhang et al., 2016). Conversely, overexpression of a constitutively active CREB lentivirus promoted dendritic outgrowth and increased the expression of synaptic proteins in mice hippocampi and primary mouse hippocampal neurons (Zhang et al., 2016).

Recent studies suggest that Trx can regulate CREB phosphorylation. It has been found that treatment with recombinant human Trx elevated phosphorylated CREB levels in rat neonatal cardiomyocytes H9C2 cell line (Subramani et al., 2020). Overexpression of Trx in human embryonic kidney HEK293 cells also elevated the levels of phosphorylated CREB (Hirota et al., 2000). Moreover, transgenic mice overexpressing Trx showed increased phosphorylated CREB levels in the ventral tegmentum area, nucleus accumbens (Huang et al., 2018), and hippocampus (Zhang et al., 2018) when compared to wild-type mice. Conversely, studies have found that inhibition of Trx expression using small interfering RNA decreased CREB phosphorylation in cultured human coronary artery endothelial cells in vitro (Subramani et al., 2020). In the present study, we found that both CREB protein levels and the ratio of phosphorylated CREB/total CREB protein levels were upregulated during the neuronal differentiation period in primary cultured mouse cerebrocortical neurons. However, we also found that treatment with Trx inhibitor PX12 reduced the ratio of phosphorylated CREB/total CREB in primary cultured

cerebrocortical neurons. Our findings suggest that Trx can regulate CREB phosphorylation in cultured neurons. These findings together suggest that Trx may regulate neuronal differentiation and maturation by targeting CREB neurotrophic signaling. However, the mechanism of this regulation requires further investigation.

### 4.2.2 CREB activation is regulated by the cellular redox homeostasis.

Trx is an important redox protein and plays a critical role in maintaining redox balance. Recent studies indicate that CREB activation and recruitment of transcriptional coactivators can be regulated by the cellular redox state. It has been reported that treatment with $H_2O_2$ inhibited NGF-increased phosphorylated CREB levels in PC12 cells (Zhang and Jope, 1999) and in rat periodontal ligament stem cells (Fu et al., 2019). In the present study, we found that treatment with $H_2O_2$ directly inhibited phosphorylated CREB levels in cultured human SH-SY5Y neuroblastoma cells. Previously, $H_2O_2$ treatment was also found to decrease the DNA-binding activity of CREB in primary cultured rat mesencephalic neurons (Iwata et al., 1997). These studies suggest that reactive oxygen species can inhibit CREB phosphorylation and CREB-driven gene expression. CREB contains three cysteines: Cys300 and Cys310 reside in the basic region of the DNA-binding domain, while Cys337 is located just outside the DNA-binding domain (Goren et al., 2001). Previously, a study showed that dithiothreitol-induced reduction of the Cys300 and Cys310 residues enhances CREB binding efficiency to DNA and gene expression (Goren et al., 2001). Cysteine thiol groups are vulnerable to attack by $H_2O_2$. In the present study, we found that $H_2O_2$ can directly induce CREB cysteine thiol oxidation. Our findings suggest that CREB cysteine residues can be oxidatively modified and that cysteine oxidation in CREB may contribute to the inhibition of CREB phosphorylation.

### 4.2.3 Trx regulates CREB signaling by maintaining the cellular redox balance.

As described above, thioredoxin regulates the cellular redox balance. CB3 is a cell-permeable Trx-mimetic tripeptide containing the active motif Acetyl-Cys-Pro-Cys-Amide derived from the Trx conserved active site (Atlas, 2021; Bachnoff et al., 2011; Lejnev et al., 2016). Previous studies have reported that treatment with CB3 reduced $H_2O_2$-induced protein carbonylation and lipid peroxidation in NIH3T3 mouse fibroblast cells and primary rat cortical neurons (Bartov et al., 2006). CB3 has also been found to lower TrxR inhibitor aurofin-mediated production of reactive oxygen species and protein oxidation in insulinoma INS 832/13 cell line (Cohen-Kutner et al., 2013). Furthermore, treatment with CB3 significantly decreased nitric oxide donor GSNO-induced protein nitrosylation in human THP-1 monocytes in a dose and time-dependent manner (Kronenfeld et al., 2015). Trx can also reduce oxidized peroxiredoxin, facilitating peroxiredoxin-induced scavenging process of $H_2O_2$ (Matsuzawa, 2017; Netto and Antunes, 2016). Previous reports have indicated that peroxiredoxin can be nitrosylated. Nitrosylation of peroxiredoxin has been shown to inhibit peroxiredoxin enzymatic activity in human leukemia monocytic (THP-1) cell line, and primary cortical neuronal cell culture. Treatment with CB3 reversed peroxiredoxin nitrosylation, thus rescuing peroxiredoxin enzymatic activity (Fang et al., 2007; Kronenfeld et al., 2015). These findings suggest that CB3 acting as Trx produces reducing activity and reduces protein oxidation.

In the current study, we found that treatment with 50 μM CB3 for 2 h can reverse $H_2O_2$-inhibited CREB phosphorylation. We further found that CB3 treatment can inhibit $H_2O_2$-increased CREB cysteine oxidation. Since Trx can reduce $H_2O_2$-induced protein cysteine oxidation, our finding provided direct evidence indicating that CREB oxidation can

downregulate CREB phosphorylation. Our finding also suggests that Trx may regulate CREB neurotrophic signaling by preserving the cellular redox balance.

### 4.2.4 ASK1 activation is regulated by Trx in primary cultured mouse cerebrocortical neurons.

***ASK1 activation induces neurodegeneration:*** ASK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) that can be activated by stressful stimuli such as oxidative stress, endoplasmic reticulum stress, and pathogens, among others. Activation of ASK1 plays an important role in apoptosis and inflammation (Guo et al., 2017; Hayakawa et al., 2012; Obsil and Obsilova, 2017). Phosphorylation of Thr845 is an important step for ASK1 activation. ASK1 activation can further phosphorylate MAP2K, subsequently inducing c-Jun kinase (JNK) and p38 MAP kinase phosphorylation and activation. ASK1 apoptotic pathway is involved in many cellular processes including apoptosis, inflammation, and cell differentiation (Kawarazaki et al., 2014). For example, in a previous report, it was found that immunofluorescence levels of phosphorylated ASK1 were increased in the retina of the Tubby mouse, a model of retinal dystrophy characterized by progressive loss of photoreceptors cells. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) is a widely used method to detect apoptotic cells. Elevated phosphorylated ASK1 levels were found in TUNEL-positive photoreceptor cells. This data suggests that ASK1-apoptotic pathway is upregulated during neurodegeneration of photoreceptors in the Tubby mouse (Kong et al., 2010). In the present study, we found that phosphorylated ASK1 protein levels were upregulated from 25 to 32 DIV in primary cultured mouse cerebrocortical neurons. Previous studies have shown that vacuolated soma, fragmented neurites, and oxidative damage were increased in primary cultured rat neurons after 30 DIV,

suggesting that primary cultured neurons undergo neurodegeneration after 30 days in culture (Aksenova et al., 1999; Lesuisse and Martin, 2002). Increased ASK1 phosphorylation in cultured mouse neurons after 30 DIV indicates that ASK1 activation is involved in neurodegeneration.

ASK1 signaling cascade has been found to be associated with several neurodegenerative processes including Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease. For example, Alzheimer's disease is characterized by the deposition of senile plaques composed of amyloid β peptide derived from the amyloid precursor protein. Immunoprecipitation studies demonstrated that amyloid precursor protein forms a complex with ASK1 in human embryonic kidney (HEK) 293 cells and cortical primary cultured neurons. The same interaction was found in the brain of amyloid precursor protein transgenic mice, which is a common model of Alzheimer's Disease. These observations suggest that amyloid precursor protein-ASK1 downstream signaling might contribute to amyloid β peptide deposition, leading to the development of Alzheimer's disease (Galvan et al., 2007). Phosphorylated JNK and phosphorylated p38 protein levels were also found to be increased in the cerebral cortex of amyloid precursor protein/presenilin 1 (APP/PS1) transgenic mice at 7 and 12 months (Savage et al., 2002). It has also been reported that ASK1 mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity of dopaminergic neurons of MPTP-induced Parkinson's disease mouse model. In this study, phosphorylated ASK1 protein levels were found upregulated in the striatum and ventral midbrain of mice exposed to MPTP. Knocking out ASK1 improved MPTP-induced motor impairment in the rotarod test and reduced MPTP-induced toxicity of dopaminergic neurons (Kang-Woo Lee). A recent paper also showed that ASK1-apoptotic signaling pathway is involved in the development of amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis is a neurodegenerative disease characterized by the presence of a

mutant form of superoxide dismutase (SOD), which leads to motor neuron degeneration. In this study, it was found that ASK1 enzymatic activity was increased in mutant SOD-transfected NSC34 motor neurons. Deleting ASK1 in SOD1 transgenic mice extended motor neuron survival (Nishitoh, 2008). Altogether, these findings suggest that activation of ASK1 leads to neuronal degeneration and the development of neurodegenerative diseases.

***Inhibition of Trx induces ASK1 activation:*** Accumulating evidence has shown that Trx has a protective effect against neurodegeneration. For example, Trx overexpression protected photoreceptors neurodegeneration in the Tubby mouse. The Tubby mouse is a model of retinal dystrophy that causes the loss of photoreceptors in the retinal outer nuclear layers. Double transgenic Tubby mice overexpressing Trx in the retina, displayed a thicker outer nuclear layer and lowered number of TUNEL-positive photoreceptor cells than the control Tubby mice (Kong et al., 2010). Moreover, it has been reported that overexpression of cardiac Trx in transgenic mice decreased infarct lesion size and lowered apoptotic annexin V-positive myocardial cells after myocardial ischemic insults (Jakobs et al., 2017; Subramani et al., 2020), suggesting that Trx upregulation can protect cardiac cells against ischemia. In addition, Trx was found to be downregulated in the brain and cerebrospinal fluid of patients with neurodegenerative disorders such as Alzheimer's disease, and multiple sclerosis (Lovell et al., 2000; Mahmoudian et al., 2017). Overall, this evidence suggests that Trx has cellular protective effect and blocking Trx might lead to degeneration.

Trx can interact with ASK1 and inhibit ASK1 apoptotic signaling. Under pathological conditions, Trx gets oxidized and dissociates from ASK1. In turn, ASK1 is phosphorylated and activates downstream kinases such as JNK and p38 to induce apoptosis. Previously, a study has

shown that treatment with Trx inhibitor PX12 increased ASK1 and JNK phosphorylation when compared to control in mouse neuroblastoma Neuro2a cells (Ren et al., 2015). In addition, immunofluorescent studies showed that PX12 treatment increased ASK1 phosphorylated protein levels in mouse-derived cochlear hair cells. PX12-induced ASK1 phosphorylation was correlated with an increase in the number of dead cochlear hair cells (Ren et al., 2021). In the present study, we found that treatment with PX12 increased ASK1 phosphorylation in primary cultured mouse cerebrocortical neurons. Altogether, these findings suggest that Trx is required for neuronal survival during neuronal differentiation, and that inhibition of Trx activates ASK1 apoptotic signaling, which leads to neurodegeneration.

***In summary***, first, we found that phosphorylated CREB protein levels are upregulated during neuronal differentiation of primary mouse cerebrocortical neurons. Inhibition of Trx activity using the pharmacological inhibitor PX12 decreased CREB phosphorylation. Furthermore, we found that $H_2O_2$ induced CREB cysteine oxidation and reduced CREB phosphorylation in SH-SY5Y cells. CB3, a Trx mimetic peptide, reduced CREB cysteine oxidation and reversed $H_2O_2$-reduced CREB phosphorylation. These results suggest that CREB phosphorylation is regulated by the cellular redox state. Because Trx maintains the cellular redox balance, our results also suggest that Trx regulates neuronal differentiation by upregulating CREB neurotrophic signaling. Second, we found that phosphorylated ASK1 protein levels are greatly increased during neuronal degeneration of primary mouse cerebrocortical cultured neurons. Trx inhibitor PX12 increased phosphorylated ASK1 protein levels. Because Trx can bind and inhibit ASK1 activation, our results suggest that Trx might promote neuronal survival and neuronal differentiation by downregulating ASK1-apoptotic signaling.

### 4.3 Txnip mediates CORT impaired-neuronal differentiation.

#### 4.3.1 CORT upregulates Txnip.

Previously, our laboratory has found that although chronic treatment with CORT had no effect on Trx and TrxR protein levels, this treatment significantly increased Txnip protein levels in mouse HT22 hippocampal cells and mouse N9 microglia cells (Bharti, 2019; Bharti et al., 2019, 2018). Our laboratory also found that treatment with CORT at 10 DIV for 5 days increased Txnip protein levels in primary cultured mouse cerebrocortical cells. In the present study, we found that although chronic treatment with CORT at 2 DIV for 5 days had no effect on Trx and TrxR protein levels, this treatment increased Txnip protein levels in primary cultured mouse cerebrocortical neurons. Because in primary cultured rodent neurons, neurons extend neurite and differentiate to mature neurons in the first 7 days (Gary Banker, et al., 2018), our finding suggests that chronic CORT treatment may upregulate Txnip and then impair neuronal outgrowth.

Txnip establishes a thiol bond between its cysteine residue Cys-247 and Trx cysteine residue Cys-32 and can bind to Trx in cytosolic and mitochondrial compartments. Binding Txnip to Trx can inhibit Trx-reducing activity and cause cellular damage. A study has shown that Txnip overexpression in the substantia nigra of mice caused the loss of dopaminergic neurons (Su et al., 2017). Moreover, Txnip protein levels were upregulated in rat cortex and hippocampus after subarachnoid hemorrhage-induced brain injury compared to control. Txnip was localized in TUNEL-positive cortical and hippocampal cells. Knocking down Txnip using small interfering RNA reduced the number of TUNEL-positive cells in rat brain after subarachnoid hemorrhage insult and prevented subarachnoid hemorrhage-induced neurological deficit (Zhao et al., 2017).

These studies suggest that Txnip can promote neuronal damage, potentially leading to neuronal death.

Chronic stress can induce depressive-like behavior and has been shown to increase serum CORT levels in rodents (He et al., 2022; Parul et al., 2021). Previously, our laboratory found that mice exposed to chronic unpredictable stress for 28 days showed not only increased levels of Txnip in the hippocampus and frontal cortex but also induced anxiety-like behavior in the open field test, anhedonia in the sucrose preference test and behavioral despair in the forced swim test (Zhou, 2018). These results suggest that chronic stress might elevate CORT levels, causing the increase of Txnip expression, which might contribute to stress-induced brain damage and stress-induced depressive-like behaviors.

CORT can bind to MR and GR receptors. Previously, our laboratory found that treatment with GR antagonist RU-486 completely blocked CORT-induced upregulation of Txnip in mouse HT22 hippocampal cells and primary cultured mouse cerebrocortical neurons (Bharti, 2019), suggesting that CORT might increase Txnip protein levels by binding to GR. Previous study has shown that Txnip gene promoter contains a glucocorticoid response element from -940 to -764 bp, and that treatment with GR agonist dexamethasone increased Txnip mRNA levels in mouse thymocytes (Wang et al., 2006). This study suggests that CORT treatment may increase Txnip gene transcription, resulting in increased Txnip protein levels.

### 4.3.2 CORT downregulates CREB signaling and upregulates ASK1 signaling.

CREB is a transcription factor that regulates the expression of genes involved in neuronal differentiation, synaptic formation, and survival. In the present study, we found in primary cultured cerebrocortical neurons that chronic treatment with CORT for 5 days decreased the ratio

of phosphorylated CREB/total CREB protein levels. Our result suggests that chronic CORT treatment can inhibit CREB phosphorylation. Previous studies have also reported that treatment with CORT reduced CREB mRNA and protein levels in mouse HT22 hippocampal cells (Mo et al., 2020) and reduced the ratio of phosphorylated CREB/total CREB protein levels in PC12 cells (Zeng et al., 2016). Another study showed that mice exposed to chronic CORT injections for 21 days not only developed despaired social interactions and reduced food consumption depressive-like behaviors but also reduced the ratio of phosphorylated CREB/total CREB protein levels in the prefrontal cortex (Yang et al., 2022). Similarly, the ratio of phosphorylated CREB/total CREB protein levels was also decreased in mice hippocampi after drinking CORT for 14 days (Gourley et al., 2008). These studies suggest that inhibition of CREB phosphorylation may mediate CORT-induced depressive-like behaviors. As we discussed above, Trx can regulate CREB phosphorylation. Therefore, these findings together suggest that chronic CORT treatment may upregulate Txnip and inhibit Trx activity, resulting in decreased CREB phosphorylation.

ASK1 contributes to cellular apoptosis process. Various extracellular stressors can activate ASK1, which leads to ASK1 phosphorylation. Phosphorylated ASK1 can then phosphorylate and activate c-Jun N-terminal kinase (JNK) and p38 kinase, resulting in the activation of apoptotic signaling (Guo et al., 2017; Ichijo et al., 1997; Takeda et al., 2003). Previously, our laboratory found that mice exposed to chronic unpredictable stress for 28 days showed an increase in the ratio of phosphorylated ASK1/total ASK1 protein levels in the frontal cortex and hippocampus (Zhou, 2018). In the present study, we also found that although chronic treatment with CORT for 5 days had no effect on total ASK1 protein levels, this treatment increased the ratio of phosphorylated ASK1/ total ASK1 protein levels in primary cultured mouse cerebrocortical neurons. Our studies suggest that chronic stress and chronic CORT

treatment can increase ASK1 phosphorylation. A previous study has also shown that prenatal stress increased JNK and p38 phosphorylation in rat prefrontal cortex and hippocampus (B. Budziszewska et al., 2010). CORT was also found to upregulate phosphorylated JNK and phosphorylated p38 protein levels in mice hippocampi (KV et al., 2018; Moretti et al., 2016). A recent study showed that treatment with CORT for 24 hours not only increased JNK and p38 phosphorylation but also increased DNA fragmentation and caspase-3 activity in primary cultured rat hippocampal neurons (Liu et al., 2011). These findings together suggest that CORT treatment can trigger ASK1 activation, resulting in an increase in JNK/p38 phosphorylation and activation of apoptotic process. Since Trx can bind to ASK1 and maintain ASK1 at an inactive state, these results also suggest that chronic CORT treatment may increase Txnip, further inhibiting Trx and activating ASK1 apoptotic signaling.

Altogether, this evidence suggests that CORT might upregulate Txnip, which in turn inhibits Trx activity. Inhibition of Trx activity affects the cellular redox balance thus impairing neurotrophic CREB signaling. On the other hand, inhibition of Trx may induce phosphorylation of ASK1 and upregulate ASK1-apoptotic signaling. Our results suggest that CORT impairs neuronal differentiation by upregulating Txnip expression and further inhibiting Trx neurotrophic and neuroprotective activity.

### 4.3.3 Txnip mediates CORT impaired-neurite outgrowth.

***Chronic CORT treatment impairs neurite outgrowth:*** Neuronal outgrowth is necessary for neurons to differentiate into mature specialized cells and establish synaptic connections (Aksenova et al., 1999; Lesuisse and Martin, 2002). Neurite outgrowth is one of the key indicators for neuronal differentiation. MAP2 is a neuron-specific cytoskeletal protein. MAP2-

immunoreactive neurites are commonly used to analyze neurite outgrowth. In the present study, we found that chronic treatment with CORT for 5 days decreased the number and length of primary dendrites and dendritic branches in primary cultured mouse cerebrocortical neurons. Our result suggests that chronic CORT treatment can impair neurite outgrowth. Retinoic acid can induce cell differentiation in cultured cell lines. Previously treatment with CORT for 24 hours was found to reduce MAP2 levels and neurite length in retinoic acid-treated Neuro2A cells (Ohmoto et al., 2020). It has been also reported that treatment with CORT reduced the total length of neurites and blocking the GR with the GR antagonist RU486 prevented CORT-impaired neurite outgrowth in PC12 cells (Meng, 2019). Furthermore, treatment of hippocampal neural progenitor cells with GR agonist dexamethasone was found to reduce neurite outgrowth (Levone et al., 2021). These results suggest that CORT-impaired neurite outgrowth is mediated by the GR. Studies have shown that chronic stress and chronic CORT exposure decrease neurite outgrowth in rodents' brains in vivo. For example, a previous report indicated that chronic restraint stress reduced apical dendritic length and branching, decreased the total number of terminal spines, and lowered the stubby spine density in the prelimbic cortex of male mice (Moench and Wellman, 2017). Another study indicated that rats undergoing chronic restraint stress for 14 days showed a decreased MAP2 expression in hippocampal and cortical neurons (Yan et al., 2010). Similarly, mice exposed to chronic CORT treatment for 36 days showed lower MAP2 protein levels in the hippocampus compared to the control group (Zhao et al., 2009). These studies together suggest that chronic stress and chronic CORT treatment can impair neurite outgrowth. Since chronic CORT treatment upregulates Txnip, our result also suggests that Txnip may mediate CORT-impaired neuronal differentiation.

***Knocking down Txnip reduces CORT-impaired neurite outgrowth:*** Txnip is induced by various types of stress including oxidative stress. As a result, Txnip binds and inhibits Trx activity, thus promoting oxidative damage and cellular death. In the present study, we found that CORT reduces the length and number of primary dendrites and dendritic branches in primary cultured mouse cerebrocortical neurons. Knocking down Txnip in primary cortical neurons significantly prevented CORT-mediated reduction of dendritic outgrowth and branching. Our study suggests that Txnip might contribute to CORT-impaired neurite outgrowth. Recent studies have also shown that inhibiting Txnip can prevent neuronal damage. Verapamil is a L-type calcium channel blocker. It has been found that verapamil can reduce Txnip mRNA and protein levels in rat insulinoma cell line INS-1 and mouse islets. The same research group found that verapamil may reduce Txnip expression by inhibiting the binding of carbohydrate response element binding protein (ChREBP) to an E-box in the Txnip promoter region (Guanlan Xu, 2012). Therefore, verapamil has been recently used in different studies to reduce Txnip expression (Feng Zhou, 2018; Lingling Xu, 2019, Nasoohi, 2023). For example, treatment of primary neurons with verapamil was found to reduce oxygen-glucose deprivation-increased Txnip expression and also reverse oxygen-glucose deprivation-decreased length of neurites (Nasoohi et al., 2023). In a similar study, rat dorsal root ganglia neurons exposed to the chemotherapeutic oxaliplatin displayed elevated Txnip protein level, and decreased neurite length compared to control. Treatment with verapamil inhibited Txnip expression and reversed oxaliplatin-impaired neurite outgrowth (Junwei Du, 2023). These studies suggest that inhibiting Txnip can prevent impaired neurite outgrowth. Blocking Txnip expression rescues stress-impaired neurite outgrowth. Since inhibition of Trx also showed to impair neuronal outgrowth, our study suggests that chronic CORT treatment may induce upregulation of Txnip and further

inhibit Trx, resulting in impairment of neuronal outgrowth.

***In summary***, we found that chronic CORT treatment upregulates Txnip protein levels but has no effect on Trx and TrxR protein levels in primary cultured mouse cerebrocortical neurons. In addition, we found that chronic CORT treatment decreases CREB phosphorylation and increases ASK1 phosphorylation. Moreover, CORT treatment decreased the length and number of primary dendrites and dendritic branches of primary mouse cerebrocortical neurons. Knocking down Txnip prevented CORT-decreased neurite outgrowth. Since Txnip binds to Trx, and Trx promotes CREB signaling and prevents ASK1 apoptotic signaling, our results suggest that CORT might impair neuronal differentiation by upregulating Txnip and indirectly inhibiting Trx activity, resulting in downregulation of CREB neurotrophic pathway and upregulation of ASK1-apoptotic pathway.

### 4.4 Txnip mediates CORT-induced depressive-like behaviors in mice.

#### 4.4.1 CORT induces depressive-like behavior in mice.

Patients with major depressive disorder display elevated levels of cortisol in serum (Nemeroff et al., 1984; Tafet, 2001). In addition, patients with Cushing syndrome, a hypercortisolism disorder, present depressive symptoms (A. Tang et al., 2013). Chronic stress can increase serum CORT levels and disrupt neuronal differentiation, synaptic plasticity and enhance neurodegeneration. It is considered a major risk factor for major depressive disorder (Juruena, 2014; LeMoult et al., 2020; Pizzagalli, 2014). Elevated levels of glucocorticoids dysregulate the HPA axis and lead to brain damage, which may have an impact on the development of depressive symptoms. Our laboratory previously exposed mice to chronic

unpredictable stress for 28 days, and found that chronic unpredictable stress reduced exploratory behavior, increased anhedonia behavior, and increased despair behaviors in mice, suggesting that chronic stress can cause depressive-like behaviors (Zhou, 2018). In the present study, we also found that subcutaneous injections of 20 mg/kg CORT for 14 days and 21 days significantly decreased exploratory behaviors in the open field test. However, only mice treated with CORT for 21 days displayed significantly increased despair behavior in the tail suspension test, induced anhedonia behavior in the sucrose preference test, and caused cognitive dysfunction in the novel object recognition test. Our result suggests that subcutaneous injections of 20 mg/kg CORT for 21 days can induce depressive-like behaviors in mice.

Chronic CORT administration constitutes a validated preclinical animal model of depressive-like behavior and cognitive dysfunction. This method has shown effective elevation of CORT levels in plasma and serum (Hellsten et al., 2002; Yau et al., 2016). Moreover, chronic CORT treatment has been found to increase the immobility time in the forced swim test (Johnson et al., 2006; Murray et al., 2008), which can be reversed by antidepressant fluoxetine and risperidone (Iijima et al., 2010), indicating that chronic CORT treatment can cause despair depressive-like behavior. Chronic treatment with CORT was also found to decrease hippocampal neurogenesis and reduce hippocampal and cortical volume, which correlates with those observations in depressive patients (Murray et al., 2008). The dose and time of subcutaneous CORT injections may affect the development of depressive-like behavior. It has been reported in the forced swim test that although injection with CORT at 10 mg/Kg once a day for 21 days had no effect on immobility time, injection with CORT at 20 or 40 mg/Kg once a day for 21 days caused an increase in immobility time when compared to control (Johnson et al., 2006). Moreover, subcutaneous injections of 40 mg/Kg CORT for 7-, 14- and 21-days, time-

dependently increased immobility time in the forced swim test with a significant increase in immobility after 21 days of treatment (Lussier et al., 2013). Previously, it was reported that subcutaneous injections of 20 mg/Kg CORT for 10 days (Brotto et al., 2001) or 18 days (Zhao et al., 2009) could not increase mice immobility time in the forced swim test, suggesting that 21 days or more are required to elicit CORT-induced depressive behavior.

Overall, these findings indicate that chronic subcutaneous injection with more than 20 mg/Kg CORT for 21 days can be used as an animal model to study the effect of glucocorticoids on the development of depressive-like behavior in mice.

### 4.4.2 Knocking down Txnip reverses chronic CORT-induced depressive-like behaviors and cognitive dysfunction.

Previously our laboratory has reported that chronic CORT treatment increased Txnip protein levels in cultured neuronal cells (Bharti, 2019; Bharti et al., 2018). CORT-increased Txnip protein levels were also verified in primary cultured mouse cerebrocortical neurons in the present study as discussed above. Further, our laboratory has found that chronic unpredictable stress not only induced depressive-like behaviors in mice but also increased Txnip protein levels in mouse brain (Zhou et al, 2018). Our results suggest that Txnip might mediate CORT-induced depressive-like behavior. To understand the role of Txnip in CORT-induced depressive-like behaviors, we used CRISPR/Cas9 technology to knock down Txnip gene in mouse brain. We found that injection with CRISPR/Cas9/Txnip sgRNA into the medial frontal cortex not only inhibited Txnip gene expression in the frontal cortex but also inhibited chronic CORT-induced depressive-like behaviors and cognitive dysfunction in both male and female mice. Our result suggests that Txnip plays an important role in CORT-induced depressive-like behaviors in both

sexes. Previously, it has been reported that Txnip overexpression in rat frontal cortex astrocytes using a GFAP-driven promoter AAV construct significantly increased immobility time in the forced swim test, decreased sucrose consumption in the sucrose preference test, and reduced time spent in the centre in the open field test, suggesting that upregulating Txnip in astrocytes can also induce depressive-like behaviors. Further, the same group found that Txnip knockdown in rat frontal cortex astrocytes using short hairpin RNA AAV vectors prevented chronic CORT treatment-induced depressive-like symptoms (Pan et al., 2022). These results together suggest that both neuronal and astrocytic Txnip might contribute to the development of chronic CORT treatment-induced depressive-like behaviors.

Txnip is an endogenous inhibitor of Trx. Binding of Txnip to Trx results in the inhibition of Trx-reducing activity, which leads to oxidative stress and protein cysteine oxidative modifications. Recent reports have shown that chronic CORT treatment increased ROS and RNS levels in cultured neuronal cells and animal brains (Sato et al., 2010; Zhou et al., 2011; Zhu et al., 2014). In addition, our laboratory has found that chronic unpredictable stress elevated protein sulfenylation and nitrosylation in mice hippocampi and frontal cortex. Our laboratory has also shown that chronic CORT treatment increased protein sulfenylation and nitrosylation in HT22 cells but knocking down Txnip prevented CORT-induced protein oxidative modifications. These findings suggest that CORT upregulates Txnip, which in turn blocks Trx reducing capacity, leading to the accumulation of protein cysteine oxidative modifications and oxidative stress. CORT-promoted oxidative stress has been associated with the development of cognitive dysfunction and depressive behavior. For example, rats exposed to chronic CORT injections for 14 days showed increased lipid peroxides and protein carbonyl levels together with a decrease in antioxidant protein levels in the hippocampus. Oxidative damage in hippocampus led to an

increase in the number of TUNEL-positive hippocampal neurons and enhanced cognitive dysfunction in the Morris water maze test (Sato et al., 2010). Another study showed that CORT injections for 21 days increased anhedonia and caused behavioral despair in rats. In the same study, CORT upregulated neuronal nitric oxide synthase (nNOS) expression and nitrosative stress in rat hippocampus, suggesting that CORT-induced nitrosative stress might be contributing to the development of a depressive phenotype. Indeed, blocking nNOS activity with nNOS inhibitor 7-Nitroindazole reversed CORT-induced depressive-like symptoms (Zhou et al., 2011). Overall, this evidence suggests that chronic stress may increase CORT levels, subsequently upregulating Txnip. Txnip in turn will inhibit Trx activity, which leads to oxidative damage and neurodegeneration, contributing to the development of depression. Knocking down Txnip reverses CORT-induced depressive-like behaviors, suggesting that Txnip might be a potential therapeutic target for the treatment of depression.

***In summary,*** we found that chronic treatment with CORT for 21 days reduced exploratory behavior, increased anhedonia, promoted behavioral despair, and caused impaired cognitive functioning in both female and male mice. We also found that injection with CRISPR/Cas9/Txnip sgRNA into mouse medial frontal cortex not only inhibited Txnip protein levels in the frontal cortex but also reversed CORT-induced behavioral changes. These results suggest that chronic treatment with CORT can induce depressive-like behaviors and cognitive dysfunction and that knocking down Txnip gene expression can inhibit CORT-induced depressive-like behaviors and cognitive dysfunction. Our results also indicate that Txnip may contribute to the development of depressive-like behaviors.

# CHAPTER 5: SUMMARY, SIGNIFICANCE, LIMITATION AND FUTURE STUDIES

## 5.1 Summary

First, we found in primary cultured mouse cerebrocortical neurons that Trx and TrxR were upregulated during the neuronal differentiation period while Txnip was upregulated during the neurodegeneration period. Blocking Trx with CRISPR/Cas9/Trx sgRNA or the pharmacological Trx inhibitor PX12 impaired dendritic outgrowth. PX12 treatment also reduced the expression of excitatory glutamatergic synaptic markers vGLUT1 and PSD95. These findings suggest that Trx antioxidant system plays an important role in neuronal differentiation.

Second, we found in primary cultured mouse cerebrocortical neurons that CREB phosphorylation was upregulated during the differentiation period and that blocking Trx with PX12 reduced CREB phosphorylation. Furthermore, we found in human neuroblastoma SH-SY5Y cells that $H_2O_2$ treatment not only reduced CREB phosphorylation but also induced CREB cysteine thiol oxidation. Treatment with the Trx mimetic peptide CB3 prevented $H_2O_2$-decreased CREB phosphorylation and $H_2O_2$-increased CREB thiol oxidation. We found in primary cultured mouse cerebrocortical neurons that ASK1 phosphorylation was increased during the degeneration period and that blocking Trx with PX12 increased ASK1 phosphorylation. These findings suggest that CREB phosphorylation is important in regulating neuronal differentiation while ASK1 phosphorylation is important in regulating neurodegeneration. CREB cysteine oxidation may contribute to the inhibition of CREB phosphorylation. Trx may regulate CREB neurotrophic signaling by preventing CREB cysteine oxidation.

Third, we found that chronic CORT treatment increased Txnip protein levels but had no effect on Trx and TrxR protein levels in primary cultured mouse cerebrocortical neurons.

Chronic CORT treatment also decreased CREB phosphorylation and increased ASK1 phosphorylation. Moreover, chronic CORT treatment impaired dendritic outgrowth. Knocking down Txnip using CRISPR/Cas9/Txnip sgRNA prevented chronic CORT-impaired neuronal differentiation. These findings suggest that chronic CORT can upregulate Txnip, which in turn inhibits Trx activity further impairing neuronal differentiation and promoting neurodegeneration.

Fourth, we found that chronic CORT subcutaneous injections for 21 days decreased exploratory behavior, increased despair behavior, increased anhedonia behavior and impaired novel object recognition in both male and female mice, suggesting CORT treatment can induce depressive-like behaviors in mice. Injection with CRISPR/Cas9/Txnip sgRNA into medial frontal cortex reversed chronic CORT treatment-induced depressive-like behaviors in both male and female mice, indicating that Txnip mediated chronic stress-induced depressive behavior.

Overall, our findings suggest that Trx may have neurotrophic and neuroprotective effects. As shown in figure 34, Trx can facilitate neuronal differentiation by preserving the cellular redox balance, inhibiting CREB cysteine oxidation and maintaining CREB activation. Trx can also inhibit ASK1 apoptotic signaling and produce neuroprotective effect. Chronic CORT treatment can upregulate Trx endogenous inhibitor Txnip. CORT-upregulated Txnip may further inhibit Trx activity, suppressing CREB neurotrophic signaling and enhancing ASK1 apoptotic signaling, which inhibits neurite outgrowth and promotes depressive-like behaviors in mice.

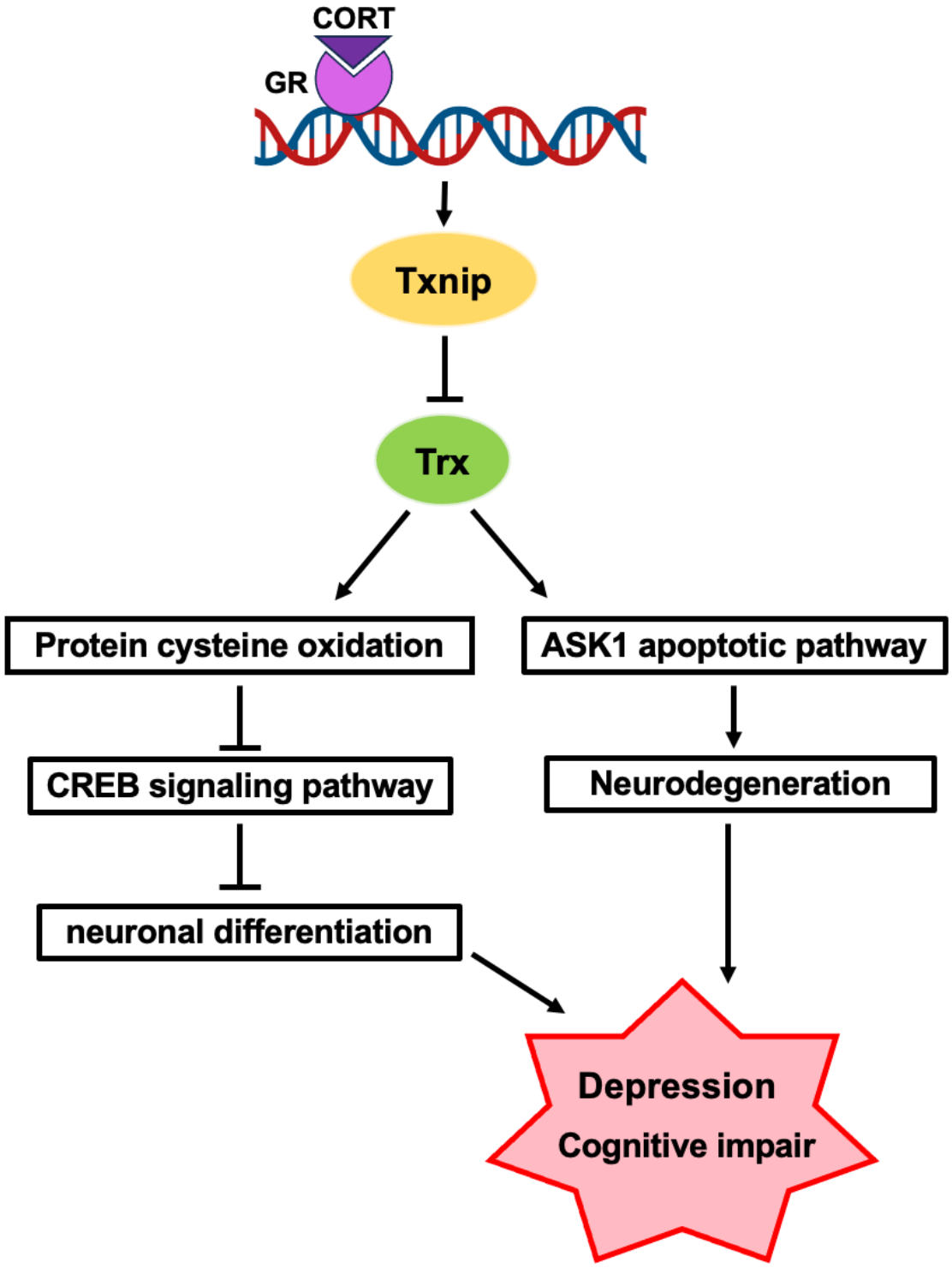


**Figure 35: Summary: Possible role of system in corticosterone-impaired neuronal differentiation and neurodegeneration.** CORT: corticosterone, GR: glucocorticoid receptor; Txnip: thioredoxin interacting protein; Trx: thioredoxin, ASK1: apoptosis signaling kinase 1; CREB: cAMP response element binding protein. Inhibition ⟞ Stimulates ⟶

## 5.2 Significance

Chronic stress plays a major role in the pathophysiology of psychiatric disorders such as anxiety and depression. As discussed in the introduction, many studies have shown that chronic stress can induce oxidative stress, impair neuronal differentiation, and promote neurodegeneration, which in turn contributes to the development and progression of depression. This study helped us to understand the role of Trx and Txnip in chronic CORT treatment-impaired neuronal differentiation and enhanced neurodegeneration. If Trx promotes neuronal differentiation and Txnip promotes neuronal degeneration, new drugs that downregulate Txnip or upregulate Trx might be a new therapeutic approach to treat psychiatric disorders.

## 5.3 Limitations and future studies

In this study, there are some limitations. First, we used CRISPR/Cas9 technology to knockout Trx and Txnip in primary cortical neuronal cell culture. We understand that CRISPR/Cas9/sgRNA may have some off-targets. Our sgRNA CRISPR/Cas9 All-in-One lentivector was purchased from ABM Inc. This company has used CRISPResso Software analysis to determine off-target effects and has predicted no off-target binding sites. However, in the future, GUIDE-seq, CIRCLE-seq, Digenome-seq, DISCOVER-seq, or other methods can be used to identify off-target sites.

Second, we evaluated whether CORT treatment impairs neuronal differentiation in primary cortical neurons. However, my work is merely restricted to primary neurons. Many factors affect the process of neuronal differentiation including glial cells. In the future, we could use a mixed culture of neurons and astrocytes to evaluate the influence of glial cells in these processes. We could also incorporate studies in brain slices.

Third, we found that $H_2O_2$ induced CREB cysteine oxidation in human neuroblastoma SH-SY5Y cell line. Transcription factor CREB contains 3 cysteines. We did not determine which cysteines were affected by oxidation. Furthermore, we did not evaluate whether $H_2O_2$ impairs cyclic AMP response element (CRE)-driven gene expression. In the future, we could use mutational analysis to generate different CREB cysteine mutants to elucidate which cysteines undergo oxidation. In addition, we could evaluate the effect of $H_2O_2$ and Trx on CRE-driven gene expression using a luciferase reporter assay.

Fourth, we explored whether knocking down Txnip in the frontal cortex reverses chronic CORT injections-induced depressive-like behaviors and cognitive dysfunction. We knocked down Txnip levels by injecting sgRNA Txnip CRISPRCas9 AAV into the medial frontal cortex. However, this method does not ensure a complete Txnip knockout. In the future, we could use a brain-specific Txnip KO cre-flox transgenic mice. In addition, we limited our study to the frontal cortex, but various brain areas respond to CORT injections in animal models including the hippocampus and amygdala. Thus, in the future, we should also explore the effects of knocking down Txnip in other brain areas.

**Future studies:**

1. To understand the specific mechanism by which Trx regulates CREB/BDNF neurotrophic pathway to promote neuronal differentiation.
2. To understand whether knocking down Txnip prevents chronic CORT-induced oxidative damage in frontal cortex.

3. To understand whether chronic CORT injections impair hippocampal neuronal differentiation; and whether knocking down Txnip prevents CORT-impaired hippocampal neuronal differentiation.

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