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A Comprehensive Study of the Effects of Neurotoxins on Noradrenergic Phenotypes, Neuronal Responses and Potential Intervention by Antidepressants in Noradrenergic Cells

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by

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ABSTRACT

A Comprehensive Study of the Effects of Neurotoxins on Noradrenergic Phenotypes, Neuronal Responses and Potential Intervention by Antidepressants in Noradrenergic Cells

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It has been reported that *locus coeruleus* (LC) degeneration precedes the degeneration of other neurons in the brain in some neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD). However, the precise mechanisms of neurodegeneration remain to be elucidated. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) has been widely used as a noradrenergic neurotoxin in the development of AD and PD animal models for specific LC degeneration. However, the precise mechanism of action of DSP4 remains unclear. An increased systemic DNA damage caused by neurotoxin or oxidative stress has been found to be related to the pathogenic development of neurodegeneration. The process of neurodegeneration is not well understood, so current therapeutic approaches are limited to disease management and symptoms relief, such as using antidepressants for depression symptoms, which often accompany neurodegenerative disorders. To date, few studies have explained why different groups of antidepressants have similar clinical effects on relieving depression. Our data demonstrate that DSP4 induces the DNA damage response (DDR) and results in down-regulation of dopamine β-hydroxylase (DBH) and the norepinephrine transporter (NET), which are 2 noradrenergic phenotypes. DSP4 results in cell cycle arrest in S and G2/M phases, which is reversible. The comet assays verify that DSP4 induces single-strand DNA breaks (SSBs).

Furthermore, the neurotoxins camptothecin (CPT) and DSP4 were used to induce the DDR in SH-SY5Y cells, fibroblast cells, and primary cultured neurons. Data show that both CPT and DSP4 induce the DDR in SH-SY5Y cells and primary cultured LC neurons. Compared to fibroblast cells, SH-SY5Y cells and LC neurons are more sensitive to the accumulation of DNA damage when treated with CPT or DSP4. Persistent phosphorylated H2AX (γH2AX) and p53 (p-p53^{ser15}) levels indicate a deficient repair in noradrenergic SH-SY5Y cells and LC neurons. In addition, the current study demonstrates that some antidepressants reduce the DDR induced by DSP4 or CPT in SH-SY5Y cells. Flow cytometry data show that selective antidepressants protect cells from being arrested in S-phase. Together, these effects suggest that blocking DNA damage is one important pharmacologic characteristic of antidepressants, which may explain why different antidepressants could alleviate depression symptoms in neurodegenerative patients.

DEDICATION

I dedicate this manuscript to my families whose support and solace has made this achievement possible. Without the love, encouragement, and understanding of my lovely husband Meijian, my adorable son Greyson, my parents Zhe and Sheying, my brother Weiguo, and my parents-in-law Wanming and Changfeng, I would not have been able to achieve this goal.

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CHAPTER 1

INTRODUCTION

Neurodegenerative Diseases

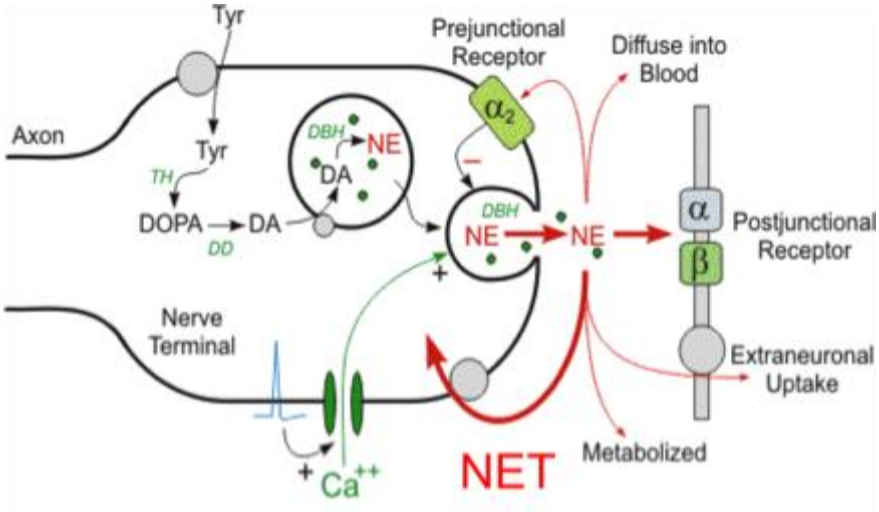
Neurodegenerative diseases are a group of incurable and debilitating diseases that result in progressive loss of neuronal structure and function, eventually neuronal death. Population statistical data show that there are currently about 5 million Alzheimer's disease (AD), 1 million Parkinson's disease (PD), and 30,000 Huntington's disease (HD) patients in the United State of America. Because neurodegenerative diseases primarily affect in middle to late stage of life, the incidence increases as the population ages. It is estimated that more than 12 million Americans will suffer from neurodegenerative diseases by the year 2030. However, there are no known cures or treatments for neurodegenerative diseases, so current therapeutic approaches are limited to disease management and symptoms relief. Therefore, it is urgent to find potential treatments and cures for neurodegenerative diseases.

The nervous system is built with neurons, including the brain, spinal cord and nerves. The body cannot replace damaged or dead neurons because they are known not to reproduce or replace themselves. These particular conditions lead to progressive brain damage and neurodegeneration. Although neurodegenerative disorders manifest with different clinical features, the disease processes appear to be similar at the cellular level, such as the risk of oxidative stress-induced DNA damage or DNA mutation increases with aging (Uttara et al. 2009). It may benefit patients if research explorations focus on these similarities in

neurodegeneration that occur in the neurodegenerative diseases. Therefore, identification of new drug targets and therapeutic approaches are now reaching an important turning point.

Norepinephrine System

Dopamine β-hydroxylase (DBH, EC 1.14.17.1) and the norepinephrine transporter (NET) are 2 important proteins of the noradrenergic neurons for their specific functional characteristics in these neurons (Kaufman et al. 1965, Chan-Palay et al. 1989). DBH catalyzes dopamine to norepinephrine and is expressed exclusively in the noradrenergic neurons in the brain (Figure 1-1). Although, DBH is not the rate-limiting enzyme for norepinephrine synthesis, it was reported that the amount of DBH available is a key factor in determining the rate of norepinephrine synthesis (Kobayashi et al. 1994, Kim et al. 2002). The NET is located on presynaptic terminals of noradrenergic neurons in the central and peripheral nervous system (Iversen 1971), and functions to reuptake more than 90 % of released norepinephrine back into the presynaptic terminals (Axelrod et al. 1969) (Figure 1-1). As this reuptake is the main mechanism for inactivation of norepinephrine transmission, alterations of NET expression would affect norepinephrine levels in the synapses or synapse clefts to influence its transmission. As such, changes in the expression of DBH and NET not only affect norepinephrine levels *in vitro* and *in vivo* but also reflect alterations in activity and function of these neurons in the brain. Therefore, measurement of their expression can yield important information regarding the functional status of neuronal cells and underlying mechanisms of neurotransmission (Zigmond et al. 1989).



Tyr=tyrosin TH=tyrosine hydroxylase DD=DOPA decarboxylase

DA=dopamine DBH=dopamine β-hydroxylase

NE=norepinephrine NET=norepinephrine transporter

Figure 1-1. Norepinephrine synthesis and release. Norepinephrine is the primary neurotransmitter for postganglionic sympathetic adrenergic nerves. It is synthesized inside the nerve axon, stored within vesicles, and then released by the nerve when an action potential travels down the nerve. Figure 1-1 is cited and modified from http://www.cvpharmacology.com/norepinephrine.htm, and used with the permission from Dr. Richard Klabunde (Klabunde 2012).

LC-norepinephrine System in Neurodegenerative Diseases

The *locus coeruleus* (LC) is a small nucleus located in the pons. It is the main source of brain norepinephrine, especially for the hippocampus and forebrain (Maeda 2000). The activity of LC neurons has been considered to be involved in numerous important functions, for example, response to stress (Usher et al. 1999). It is reported that LC cell numbers are reduced during normal aging and in aging-related diseases, as are brain norepinephrine levels (Marien et al. 2004). Damage and loss of LC noradrenergic neurons is accelerated in certain progressive neurodegenerative diseases including AD (Mann et al. 1983, Bondareff et al. 1987, German et al. 1992, Weinshenker 2008) and PD (Mann et al. 1983, Rommelfanger et al. 2007),

representing an early pathological indicator of disease progress. The greatest neuronal loss was observed in the LC (83% loss in AD; 68% loss in PD) compared with other subcortical nuclei (Lyness et al. 2003, Zarow et al. 2003). Patients with AD have reduced levels of norepinephrine compared with controls (Adolfsson et al. 1979, Palmer et al. 1993). However, it remains unclear how and why LC cell death influences the pathogenesis of AD or PD. Therefore, exploring the pathologic characteristics of LC noradrenergic neuronal loss during neurodegeneration is important for elucidating the mechanisms underlying AD and PD.

DSP4

The effects of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) on norepinephrine levels in the peripheral and central noradrenergic system were first reported several decades ago (Ross 1976). DSP4 can cross the blood–brain barrier and accumulate intraneuronally. It was hypothesized that DSP4 selectively damages noradrenergic projections originating from the LC by interacting with the norepinephrine reuptake system and depleting intracellular norepinephrine, finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom et al. 1985, Dooley et al. 1987, Howard et al. 1990, Prieto et al. 2001). In addition, little data have been reported from *in vitro* studies on the mechanism of DSP4-induced neuronal degeneration. Although DSP4 has been widely used as a noradrenergic neurotoxin as a tool to construct AD or PD animal models with LC degeneration (Heneka et al. 2006, Rey et al. 2012), the precise mechanism of action of DSP4 remains unclear. Thus, elucidating the molecular mechanism by which DSP4 induces its neurodegenerative effect may facilitate finding novel therapeutic strategies for treatment of degenerative diseases.

Mechanism of CPT

DNA topoisomerase I (topo I) relaxes supercoils by creating a SSB (nick) in the DNA duplex, which allows the broken strand to rotate and remove local supercoils during transcription and DNA replication (Wang 1996). CPT is a cytotoxic quinoline alkaloid and a S-phase-specific anticancer agent, which binds to the topo I-DNA complex and inhibits topo I (Liu et al. 2000). During replication, the replication fork meets the topo I-DNA complex, which results in late S and early G2 phases arrest with conversion of SSBs to double-strand breaks (DSBs) and ultimately cell death (Hsiang et al. 1985, Li et al. 2001, Pommier et al. 2003) (Figure 1-2).

Generally, administration of CPT produced irreversible DNA DSBs during DNA replication, suggesting that this agent should not have toxic effects on nondividing cells such as neurons. However, it was reported that CPT could lead to death of postmitotic rat cortical neurons *in vitro* in a significant dose-dependent manner. And this effect was not mediated by topo I but dependent upon DNA transcription (Morris et al. 1996). Additionally, neurotoxic activity of CPT also was found in cultured cerebellar granule neurons, which inhibited both protein synthesis and the neuritic outgrowth of primary cultured cerebellar granule neurons (Uday Bhanu et al. 2010). Taken together, these observations indicate that CPT exhibits significant toxicity toward neuronal cells *in vitro,* which are not dependent on topo I.

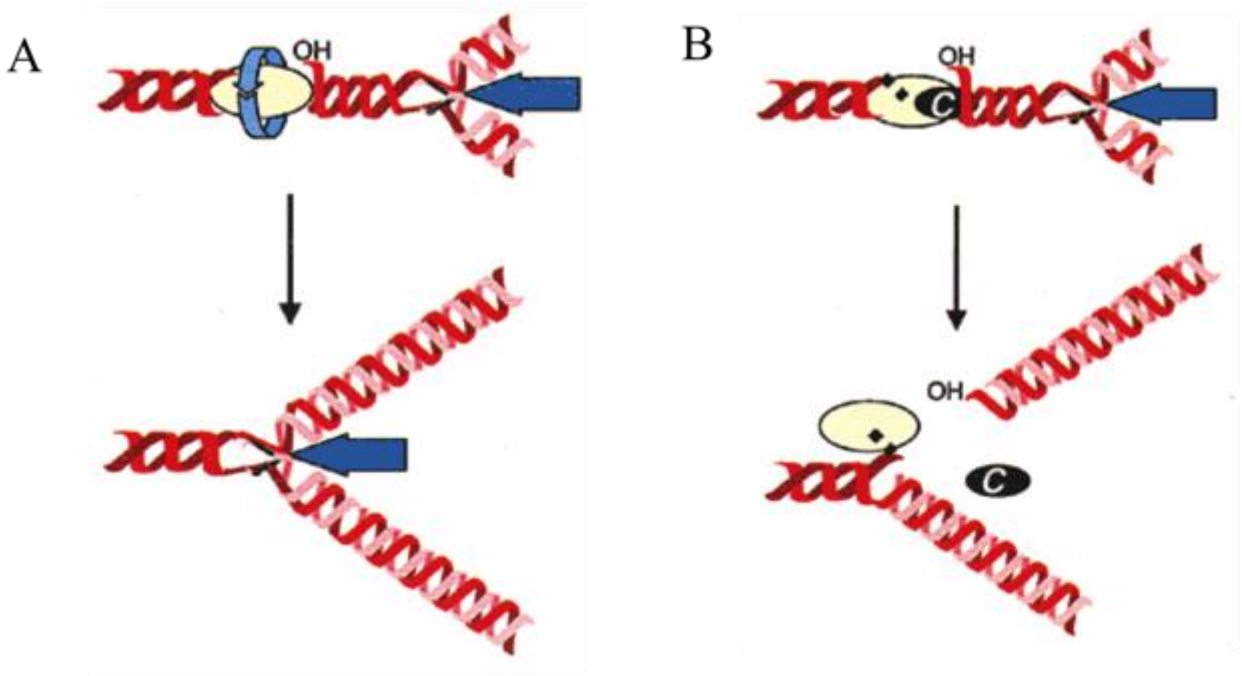


Figure 1-2. Mechanism of action of topoisomerase I poisons. (A) Normally, topoisomerase I introduces a nick in the DNA backbone allowing the rotation of one strand around the other. This releases the torsional strain that otherwise accumulates in front of the advancing replication fork (large arrow). The DNA break is extremely transient and is religated almost immediately at the same time that the topoisomerase I releases the other strand. (B) When a drug such as irinotecan is present (black oval with C), it binds to the topoisomerase I-nicked DNA complex. This prevents the religation of the nicked strand and the release of the enzyme. Eventually, the replication fork collides with the complex, causing the formation of a double-strand break. Figure 1-2 is used with permission from (Rivory 2002).

DNA Damage and Repair in Neurodegenerative Disease

Brain volume and function decline with aging, which in neurodegenerative diseases might be caused by the permanent loss of neurons (Brazel et al. 2004). The "free radical theory of aging hypothesis" indicates that oxidative damage accumulation leads to the cellular decline and aging-associated deterioration (Harman 1981). Imbalanced metabolism and excess reactive oxygen species (ROS) generation lead to various disorders such as AD, PD, aging, and many other neural disorders. Oxidative stress may cause DNA damage because DNA is perhaps one

of the major targets for oxyradicals. It was reported that DNA DSBs in neuronal cells occur during normal brain functions such as learning (Suberbielle et al. 2013). Many researchers have established that there is accumulated oxidative DNA damage in the cells of patients with AD (Kadioglu et al. 2004) and PD (Zhang et al. 1999). Many other studies have shown that there is both increased DNA damage and decreased DNA repair in patients with AD (Fishel et al. 2007). Oxidative stress and DNA damage also are studied in PD (Dias et al. 2013, Hwang 2013). Dopaminergic neurons in the substantia nigra are severely affected by the neurodegenerative process that occurs in PD. Increased levels of oxidative stress have been detected in the substantia nigra region of the brain in PD patients (Fukae et al. 2005).

Evidence shows that oxidative damage plays critical roles in the aging process (Golden et al. 2002, Bokov et al. 2004, Balaban et al. 2005), as well as neurons have very high rates of oxygen metabolism. It has been suggested that deficiencies in the repair of oxidative DNA damage with aging (Weissman et al. 2009). It is known that increased exposure to damaging agents and/or deficiency of DNA repair lead to higher levels of DNA damage (Subba Rao 2007). It was reported that aging-related diseases are mainly caused by accumulation of nuclear DNA (nDNA) damage in neurons due to insufficient nDNA damage repair. The brain consists of large number of nonproliferative neuronal cells that are vulnerable to defective DNA repair. Deficiency of repairing DNA usually leads to “accumulation” of unrepaired DNA lesions that might be considered as the cause of the neuropathology in several neurodegenerative disorders.

DNA Damage Response Markers

As a very early step in the cellular response to DNA damage, histone H2AX is phosphorylated at the C-terminal serine residues Ser136 and Ser139 (Rogakou et al. 1998). Phosphorylated H2AX, called γH2AX, and γH2AX-enriched foci can be detected within minutes post-DNA damage (Kang et al. 2005). H2AX phosphorylation has an important role in the initiation of DNA repair (Downs et al. 2000) including the recruitment of DNA repair or damage-signaling factors, maintenance of the integrity of the DNA damage response, and bringing the broken DNA ends closer together (Bassing et al. 2004, Thiriet et al. 2005).

Known as a classic "gatekeeper" of cellular fate, p53 tumor suppressor protein is activated in response to genotoxic stress-induced DNA damage (May et al. 1999). Phosphorylation of serine15 (p-p53^{ser15}) is one of the major responses (Hammond et al. 2002). P-p53^{ser15} levels can be rapidly increased several folds after DNA damage is detected. Also, phosphorylated p53 has been linked to DNA repair processes such as activation of DNA repair pathways and stalling the cell cycles (Offer et al. 1999, Okorokov 2003, Ford 2005). Therefore, γH2AX and p-p53^{ser15} were measured as the DDR markers to evaluate the appearance as well as the repair rate for CPT- or DSP4-induece DNA damage.

Cell Cycle and Checkpoints

The cell cycle is a well-ordered event, which contains 4 phases. The G1 phase is required for cell growth and preparation of the chromosomes for replication. DNA is synthesized in S phase. The G2 phase is needed for cell growth and preparation for mitosis. The last phase is M (Mitosis). Cells divide into 2 daughter cells in this phase.

Flow cytometry is a method in cell biology to distinguish cells in different phases of the cell cycle (Figure 1-2A). Cells are constantly under the stress of intrinsic and extrinsic agents that cause DNA damage or interference with DNA replication. Cell cycle checkpoints are set at various stages of the cell cycle to arrest cell cycle transit and facilitate DNA repair pathways. When cells have DNA damage that has to be repaired, cells activate DNA damage checkpoints, which arrest cell cycle transit to repair the damage. Based on the cell cycle stages, DNA damage checkpoints are classified into at least 3 checkpoints: G1/S checkpoint, intra-S phase checkpoint, and G2/M checkpoint (Figure 1-2B). G1/S checkpoint ensures that everything is ready for DNA synthesis. Intra-S phase checkpoint stops or slows DNA replication. G2/M checkpoint is to determine if the cells are ready to enter M phase and divide into daughter cells. If the damage is so severe that it cannot be repaired, the cell self-destructs by apoptosis (Figure 1-2C).

Aberrant Cell Cycle Activity in Neurons

As in other cell types, the cell cycle in the central nerve system is tightly regulated. However, aberrant cell cycle activity has been detected during the progression of neurodegenerative conditions. Specifically, key components of the cell cycle proteins, like cyclins and cyclin-dependent kinases (CDKs), have been found to be up-regulated after exposure to severe conditions such as oxidative stress (Busser et al. 1998, Kruman et al. 2004, Murray 2004, Currais et al. 2009). Oxidative stress can lead to both DNA mutation and the formation of damaged proteins; therefore, it is considered as an important risk factor for neurodegenerative diseases. Cell cycle activity in neurons can also be induced by neurotoxic

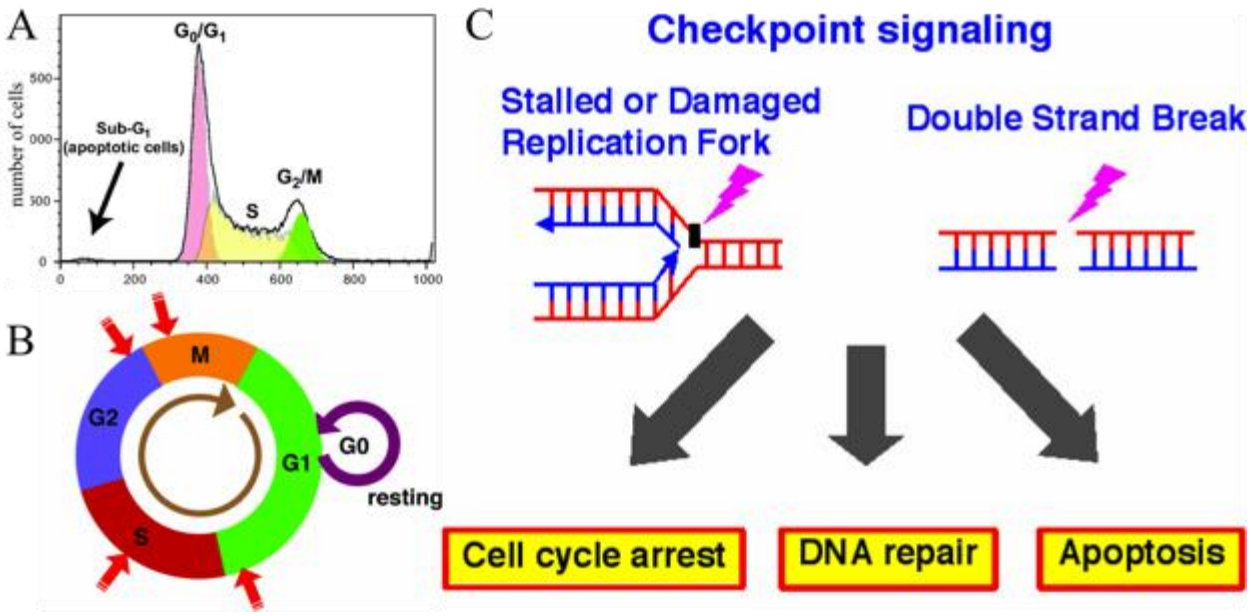


Figure 1-3. Cell cycle and the checkpoints. (A) Cytometry-based DNA content analyzed by flow cytometry. Populations in the G_0/G_1, S, and G_2/M phases are shaded in pink, yellow, and green, respectively. Figure 1-3A is used with permission from Dr. Alex Zambon from Department of Pharmacology at University of California at San Diego (Henderson et al. 2013). (B) The checkpoints are indicated by red arrows. C. Genome maintenance mechanisms. Figure 1-3B and 1-3C are cited from http://eishinoguchi.com/checkpoint.htm, and used with the permission of Dr. Eishi Noguchi from Department of Biochemistry and Molecular Biology at Drexel University (Noguchi 2004-2006).

insults (Klein et al. 2003). For example, up-regulated cell cycle proteins CDK2, cyclin E, and E2F-1 were found in kainic acid–treated cerebellar granule cells (Verdaguer et al. 2002). Similarly, when rat embryonic cortical neurons were cultured with toxic concentrations of Aβ peptides, aberrant cell cycle activity and neuron death were found (Copani et al. 1999). These neurons showed abnormal increased cell cycle proteins expression, like cyclin D1, cyclin E, cyclin A, and phosphorylated retinoblastoma protein. In addition, oxidative DNA damage is correlated with cell cycle arrest (Migliore et al. 2002). For example, human H_2O_2-treated fibroblasts undergo either cell cycle arrest or apoptosis (Chen et al. 2000). The majority of the apoptotic fibroblasts were found in the S phase, whereas growth-arrested cells were

predominantly accumulated in the G1 or the G2/M phase (Chen et al. 2000). This apoptotic death of fibroblasts in the S phase is consistent with the death of neurons that have aberrant cell cycle activity and express S-phase proteins. Dorsal root ganglion neurons go to apoptosis in the S phase (ElShamy et al. 1998), and the apoptotic neurons express S-phase proteins (Folch et al. 2012). Hippocampal pyramidal and basal forebrain neurons from AD brains show chromosomal duplication and die before mitosis. These are consistent with cell death in the S or G2 phase of the cell cycle (Nagy et al. 1997b). In addition, DNA damage in apoptotic neurons is dependent on ATM activation, which suggests that neurons are affected by the same cell cycle checkpoints that regulate apoptosis in other cell types (Kruman 2004). Taken together, all these findings indicate that differentiated neurons may have aberrant cell cycle activity, which is a critical element of the DDR of postmitotic neurons leading to cell death.

Antidepressants in Neurodegenerative Diseases

The earliest main biochemical theory of depression is the "monoamine hypothesis" (Schildkraut 1965), which states that depression is caused by dysregulation of monoaminergic neurotransmitters at certain sites in the brain. Serotonin and norepinephrine are 2 neurotransmitters primarily to regulate mood and emotions (Butler et al. 2008). This theory is based on early clinical observations that monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) were able to ameliorate depression syptoms by increasing levels of serotonin or norepinephrine (Crane 1956, Kuhn 1958). Although the "monoamine hypothesis" of depression has been proposed for a long time, the pathologies and mechanisms for depression disorders are still partially understood. A number of new proposed mechanisms for depression are brought into light such as diminishing neurotrophic factors (Czeh et al. 2007)

and neuroinflammation (Muller et al. 2007). There is also evidence shown that oxidative and nitrosative stress are involved in the pathophysiology of depression (Maes et al. 2009, Maes et al. 2011). Therefore, it is important to elucidate potential mechanisms of depression for finding new antidepressants targets and candidates to treat depression.

The process of neurodegeneration is not well understood, so there are no known cures and treatments for this group of diseases. Current therapeutic approaches are limited to disease management and symptoms relief. Depression symptoms often accompany neurodegenerative disorders that can be relieved by using antidepressants (Table. 1-1) (Briley et al. 1993, Martin 2008). For example, depression in patients with PD can be alleviated by the selective norepinephrine reuptake inhibitor (NRI) reboxetine (McNamara et al. 2006). The most important classes of antidepressants are the selective serotonin reuptake inhibitors (SSRIs) (Geddes et al. 2004), NRIs, TCAs and MAOIs. SSRIs and NRIs are considered to increase the exracellular levels of serotonin and norepinephrine by blocking the serotonin transporter (SERT) and NET. Most TCAs primarily act by inhibiting serotonin and norepinephrine reuptake into the cell; this results in an elevation of the synaptic concentrations of these neurotransmitters (Tatsumi et al. 1997, Gillman 2007).

Table 1-1. Four Classes of Antidepressants and Their Principal Actions

NAME	PRINCIPAL ACTION
SSRIs	Increase the synaptic concentration of serotonin by inhibiting serotonin transporter.
Fluoxetine	
Paroxetine	
NRIs	Increase the synaptic concentration of norepinephrine by inhibiting norepinephrine transporter.
Reboxetine	
Viloxazine	
TCAs	1.Increase the synaptic concentration of norepinephrine and serotonin by inhibiting norepinephrine and serotonin transporter respectively. 2. Act as antagonists at 5-HT, adrenergic, NMDA receptors. 3. Most TCAs potently inhibit sodium channel.
Amitriptyline	
Imipramine	
Desipramine	
MAOIs	Inhibit the activity of monoamine oxidase, thus preventing the breakdown of monoamine neurotransmitters.
Deprenyl	
Pargyline	

Questions to be Answered in These Studies

First, in the study published in Neurotoxicity Research (2014, 25(2): p193-207) and presented here in Chapter 2 (Wang et al. 2014), we reveal a detailed neurotoxic function of DSP4. It is known that *in vivo* DSP4 treatment induces degeneration of noradrenergic terminals by interacting with NET and depleting intracellular norepinephrine. It has been reported that DSP4 induced LC axon lesions in LC cell bodies (Fritschy et al. 1991a). However, DSP4's precise mechanism of action remains unclear. We hypothesize that DSP4 down-regulates the noradrenergic phenotypes, which may be mediated by its actions on DNA replication, leading to replication stress and cell cycle arrest. We used SH-SY5Y, an immortal neuroblastoma cell line that expresses the noradrenergic markers DBH and NET, to test the hypothesis that DSP4 down-regulates their expression. Further efforts have been focused on the exploration of possible mechanisms underlying DSP4-induced down-regulation of these noradrenergic phenotypes and for DSP4 toxicity associated with DDR marker proteins.

Second, it has been reported that degeneration of the noradrenergic neurons proceeds to other neurons in the brain in some neurodegenerative diseases like AD and PD. However, their pathologic characteristics during degenerative course and certain mechanisms remain to be elucidated. DSP4 is considered as a useful tool in studies of the mechanisms of LC neuron degeneration. CPT also exhibits significant toxicity toward neuronal cells *in vitro* (Morris et al. 1996, Uday Bhanu et al. 2010). We hypothesize that noradrenergic SH-SY5Y cells and LC neurons are sensitive to CPT- or DSP4-induced DNA damage and they are deficient to repair the damage. This may be part of the mechanism for LC degeneration. In Chapter 3, we treated noradrenergic SH-SY5Y cells and primary LC cultures and nonnoradrenergic fibroblast cells and raphe neurons with CPT or DSP4. Western blots and immunofluorescence assays (IFAs)

were used to test the appearance of 2 DDR markers. Comet assays were employed to test the DNA damage repair.

Finally, depression symptoms that could be relieved by using antidepressants often accompany neurodegenerative disorders. To date, few studies have elucidated why different groups of antidepressants have the similar effects on relieving depression. There is evidence showing that DNA damage by oxidative stress is involved in the pathophysiology of depression (Maes et al. 2009, Maes et al. 2011). Therefore, we hypothesize that certain antidepressants can reduce the DDR in noradrenergic SH-SY5Y cells induced by DSP4 or CPT. In Chapter 4, four groups of antidepressants, TCAs, SSRIs, NRI, and MAOIs, were used to treat SH-SY5% cells. We demonstrate that several antidepressants reduce the DDR induced by neurotoxins DSP4 or CPT in SH-SY5Y cells. Flow cytometry data show that selective antidepressants protect cells from being arrested in S phase.

CHAPTER 2

EFFECTS OF DSP4 ON THE NORADRENERGIC PHENOTYPES AND ITS POTENTIAL MOLECULAR MECHANISMS IN SH-SY5Y CELLS

Yan Wang, Phillip R. Musich, Moises A. Serrano, Yue Zou, Jia Zhang, Meng-Yang Zhu

Abstract

DBH and NET are the noradrenergic phenotypes for their functional importance to noradrenergic neurons. It is known that *in vivo* DSP4 treatment induces degeneration of noradrenergic terminals by interacting with NET and depleting intracellular norepinephrine. However, DSP4's precise mechanism of action remains unclear. In this study various biochemical approaches were employed to test the hypothesis that DSP4 down-regulates the expression of DBH and NET, and to determine the molecular mechanisms that may be involved. The results showed that treatment of SH-SY5Y neuroblastoma cells with DSP4 significantly decreased mRNA and protein levels of DBH and NET. DSP4-induced reduction of DBH mRNA and protein levels, as well as NET protein levels showed a time- and concentration-dependent manner. Flow cytometric analysis demonstrated that DSP4-treated cells were arrested predominantly in the S-phase, which was reversible. The arrest was confirmed by several DNA damage response markers (phosphorylation of H2AX and p53),

suggesting that DSP4 causes replication stress which triggers cell cycle arrest via the S-phase checkpoints. Moreover, the comet assay verified that DSP4 induced single-strand DNA breaks. In summary, the present study demonstrated that DSP4 down-regulates the noradrenergic phenotypes, which may be mediated by its actions on DNA replication, leading to replication stress and cell cycle arrest. These action mechanisms of DSP4 may account for its degenerative consequence after systematic administration for animal models.

Introduction

DBH and NET are the important proteins of the noradrenergic neurons for their specific functional characteristics in these neurons (Kaufman and Friedman 1965, Chan-Palay and Asan 1989, Barker E 1995). DBH catalyzes the oxidation of dopamine to norepinephrine and is expressed exclusively in the noradrenergic and adrenergic neurons in the brain. DBH is not the rate-limiting enzyme for norepinephrine synthesis. However, it was reported that the amount of DBH available is also a key factor in determining the rate of norepinephrine synthesis (Kobayashi et al. 1994, Kim et al. 2002). The NET is located on presynaptic terminals of noradrenergic neurons in the central and peripheral nervous system (Iversen 1971), and functions to reuptake more than 90 % of released norepinephrine into the presynaptic terminals (Axelrod and Kopin 1969). As this reuptake is the main mechanism for inactivation of norepinephrine transmission, alterations of NET expression remarkably would affect norepinephrine levels in the synapses and, in turn, highly influence noradrenergic transmission. As such, changes in the expression of these proteins not only affect NE levels *in vitro* and *in*

vivo, but also reflect alteration in activity and function of these neurons in the brain. Therefore, measurement of their expression can yield important information regarding the functional status of neuronal cells and underlying mechanisms of neurotransmission (Zigmond et al. 1989).

The effects of DSP4 on norepinephrine levels in the peripheral and central noradrenergic system were first reported several decades ago (Ross 1976). DSP4 can cross the blood–brain barrier and accumulate intraneuronally. *In vivo* DSP4 selectively damages noradrenergic projections originating from the LC by interacting with the NE reuptake system and depleting intracellular norepinephrine, finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom et al. 1985, Dooley et al. 1987, Howard et al. 1990, Prieto and Giralt 2001). Thus, DSP4 has widely been used as a noradrenergic neurotoxin. However, the precise mechanism of action of DSP4 remains unclear. In addition, little data have been reported from *in vitro* studies on the mechanism of DSP4-induced neuronal degeneration. Thus, elucidating the molecular mechanism by which DSP4 evokes its neurodegenerative effect may promote the effort to find novel therapeutic strategies for treatment of degenerative diseases.

Aberrant cell cycle activity and DNA damage have been observed during the progression of neurodegenerative conditions. Many cytotoxic and genotoxic agents including neurotoxins arrest the cell cycle at the different phases (Sontag et al. 2008). Also, neurons are continuously exposed to endogenous and environmental DNA-damaging insults, inducing DNA strand breaks and base adducts, eventually leading to neurodegeneration. Whether these events are involved in DSP4's toxicity to the noradrenergic neurons is an important but unresolved issue. Genotoxic damage can occur in any of the four phases of the cell cycle, G1,

S, G2, or M. Neurons are terminally differentiated cells and no longer progress through the cell cycle. However, neurons require continuous gene expression to maintain their high metabolism and machinery for neurotransmission and genome integrity is essential for such an expression program. Thus, like cycling cells the LC and other neurons remain susceptible to DNA damage and would be expected to have active DNA damage response (DDR) mechanisms and cell cycle checkpoints to remedy such damage. Ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinases are early damage-sensing components of DDR pathways, especially in response to double- and single-strand DNA breaks (Abraham 2001). Protein substrates of the activated ATM and ATR kinases include histone H2AX, which is phosphorylated at serine 139 (γH2AX) (Burma et al. 2001, Ward and Chen 2001) and the tumor suppressor protein p53 is phosphorylated at serine 15 (p-p53^{ser15}) (Hammond et al. 2002). γH2AX tags the chromatin sites of DNA damage to initiate the recruitment of DNA repair factors (Zarei and Stephenson 2002, Sontag et al. 2008) while the p-p53^{ser15} enhances transcription of DDR genes and modifies the interaction of DNA metabolism proteins (Serrano et al. 2012). In cycling cells responses to DNA damage arrest cell cycle progression to allow DNA repair; however, the sequence of events for the DDR in highly differentiated, non-dividing cells have not been addressed in this part because of the experimental limitations in performing such studies.

In this study, we used SH-SY5Y, an immortal neuroblastoma cell line which expresses the noradrenergic markers DBH and NET, to test the hypothesis that DSP4 down-regulates their expression. Further efforts have been focused on the exploration of possible mechanisms underlying DSP4-induced down-regulation of these noradrenergic phenotypes and for DSP4 toxicity associated with DDR marker proteins.

Materials and Methods

Cell Culture and Drug Exposure

The human neuroblastoma cell line SH-SY5Y was used in these experiments (Biedler et al. 1978). SH-SY5Y cells were maintained in a 1:1 mix of RPMI 1640 and F12 media, which was supplemented with 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 ^{o}C in humidified air containing 5 % CO_2. Culture medium and supplements were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Cells were seeded into 6-well or 100-mm plates. Drug exposures were started after 24 h of each subculture. DSP4 (Sigma, St Louis, MO, USA) dissolved in distilled water at 50 mM was diluted with culture media and added to cells to a final concentration of 5, 10, or 50 μM, alone or in combination with the ATM inhibitor KU55933 (10 μM, Selleckchem, USA), and/or the ATR inhibitor Nu6027 (10 μM, Santa Cruz, CA, USA) for the times as indicated in the text. The selection of the concentration of DSP4 was based on the reports about its IC50 in the literature (Boksa et al. 1989, Tieu et al. 1999, Wenge and Bonisch 2009) and our preliminary experiments. Only SH-SY5Y cells prior to passage 15 were used. Cell viability was determined by exclusion of trypan blue dye; cell viability was 90–95 % in the untreated cells.

RNA Isolation, RT-PCR, and Relative Quantitative qPCR Analysis

SH-SY5Y cells with or without DSP4 treatment were collected from 6-well plates (Sigma, St Louis, MO, USA) and isolation of total RNA was carried out using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Quality and

quantity of total RNAs were measured at 260 and 280 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies LLC, Wilmington, Delaware, CA, USA). Equal amounts of total RNA (1 μg) from each sample were primed with random primers and reverse transcribed to cDNAs using the Superscript First-strand Synthesis Kit (Invitrogen, Grand Island, NY, USA) following the manufacturer's recommendation. Aliquots of first strand cDNA (1μl for DBH, NET, or GAPDH) were amplified by PCR in a 25 μL reaction mix containing Platinum PCR Supermix (Invitrogen, Grand Island, NY, USA) and primers at appropriate concentrations in an Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany). Primers were synthesized by Invitrogen or Integrated DNA Technologies (Coralville, IA, USA). qPCR was run on the Mx 3000P QPCR system (Agilent Technologies, La Jolla, CA, USA) using the SYBR green Platinum Quantitative PCR supermix (Invitrogen, Grand Island, NY, USA). The primers were designed as follows: DBH, forward: 50-CCTCACTGGCTACTGCACGGACAAG-30 and reverse: 50-GTGGAGCTGAGAGGCGAAGATGTGG-30; NET, forward: 50-CGGTGCCTTCTTGATCCCG-30 and reverse: 50-CCGGTTGTACTGTCCCAGAG-30; and GAPDH (as a control): forward: 50-TGCACCACCAACTGCTTAGC-30 and reverse: 50-GGCATGGACTGTGGTCATGAG-30. All reactions were performed according to the following protocol: 2 minutes (min) at 50 ^{o}C, 2 min at 95 ^{o}C, followed by 45 cycles of 18 s at 95 ^{o}C and 45 s at annealing temperature (NET 60 ^{o}C, DBH 56 ^{o}C, GAPDH 56 ^{o}C), then continued with the melting curve analysis (55–90 ^{o}C) to verify the product specificity. Annealing temperature of each gene was determined by running gradient qPCR with a range of annealing temperatures starting from 55 to 66 ^{o}C. Comparative cross threshold (Ct) method was used to measure gene expression in response to DSP4 treatments.

Western Blotting Analysis

Whole cell extracts for western blot analysis were prepared by lysing cells in ice-cold Nonidet P-40 (NP-40; Sigma, St Louis, MO, USA) buffer (0.5 % NP-40, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) for 30 min, after which nuclei and cell debris were removed by centrifugation at 12,000 rpm for 10 min at 4 ^{o}C. An equal volume of 29 sodium dodecyl sulfate (SDS) gel-loading buffer then was added to the supernatant and the samples were denatured at 70 ^{o}C for 5 min. Protein concentrations in cell extracts were quantified prior to addition of the loading buffer with the Micro BCA Protein Assay Kit (Thermo Science, Rockford, IL USA). Proteins (40 μg) were electrophoretically separated on a 10 % or a 15 % SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK). For protein detection, the blots were, respectively, probed with a mouse monoclonal anti-NET antibody (1:1,000 dilution; Mab Technology Inc., Stone Mountain, GA, USA, or 1:1,000; Alpha Diagnostic Intl. Inc., San Antonio, Texas USA), anti-DBH antibody (1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-replication protein A (RPA) antibody (1:1,000 dilution, R3280, Sigma-Aldrich, St Louis, Mo, USA), anti-γH2AX antibody (1:1,000 dilution, Bethyl Laboratories, Inc., Montgomery, TX USA), or an anti-p-p53^{ser15} antibody (1:1,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5,000 dilution; Amersham Life Sciences, Buckinghamshire, UK) was used as the secondary antibody. The membranes were subjected to enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) or super enhanced ECL (Sigma Chemical Co., St Louis, MO, USA) and autoradiography. To check for equal loading and transfer, the membranes were reprobed with a mouse IgG monoclonal anti-b-actin antibody (1:5,000 dilution, Amersham

Life Sciences, Buckinghamshire, UK).

Flow Cytometry

Cells, sub-cultured in a 6-well plate at 2×10^4 cells/well, were exposed to DSP4 (5 and 50 μM) for 24 h. After washing with 37 ^{o}C phosphate buffered saline (PBS), 200 μL of 0.25 % trypsin–EDTA (Gibco, Carlsbad, CA, USA) was added per well and the plate was incubated at 37 ^{o}C for 1 min. The trypsin was aspirated off and the cells were suspended with 1 mL ice-cold PBS containing 0.5 mM EDTA (PBSE). The cells were collected by centrifugation at 3,000 rpm for 10 min at 4 ^{o}C and fixed by slowly adding 1 mL ice-cold 70 % ethanol to resuspend the cells. The cells were stored at -20 ^{o}C for 12–24 h, and then collected at 3,000 rpm for 10 min at 4 ^{o}C. The cells were washed once with ice-cold PBSE, then recentrifuged and resuspended in 300 μL of freshly prepared PBSE containing 20 μg /mL propidium iodide (Sigma, St Louis, MO, USA) and 20 μg /mL DNase-free RNase A (Invitrogen, Grand Island, NY, USA). After incubation at 37 ^{o}C for 30 min, the cells were analyzed on an Accuri C6 flow cytometer. The population of G0/G1, S, and G2/M was determined using C6 Flow Cytometer Software. The results are expressed as percentage of the attached cells in each phase.

Immunofluorescence Assay (IFA)

2×10^4 cells were grown on coverslips in 24-well plates (Sigma, St Louis, MO, USA) and treated with or without DSP4 (5, 10, or 50 μM) for 24 h. The cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized with 0.2 % Triton X-100 in PBS for 10 min. Coverslips were then blocked with 5 % goat serum in PBS for 1 h, and incubated overnight with primary antibodies (anti-γH2AX: 1:200 dilution, GeneTex Inc., Irvine, CA, USA), and an anti-p-p53ser15 (1:400 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). After

three 10-min washes with PBS, the coverslips were incubated with the secondary antibodies [Alexa Fluor®488 Goat Anti-Rabbit IgG (H+L); Alexa Fluor®568 Goat Anti-Mouse IgG (H+L), EMD Millipore Corporation, Billerica, MA, USA] diluted in PBS with 5 % goat serum. Coverslips were mounted onto microscope slides using Fluoromount-G mounting medium (Invitrogen, Grand Island, NY, USA). Slides were viewed and photographed at 100× magnification using an EVOS inverted fluorescent microscope (Advanced Microscopy Group) with attached CCD camera.

Comet Assay

SH-SY5Y cells were treated with different concentrations of DSP4 (5, 10, or 50 μM) or camptothecin (CPT, 10 μM) for 24 h. Then, neutral or alkaline comet assays were carried out using the Comet Assay System (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Fluorescence images were captured at 10× magnification. At least 50 cells were assessed per treatment. In parallel with the comet assay, cell cultures with the same treatments were harvested for the protein analysis by western blotting.

Statistics

All experimental data are presented in the text and graph as the mean ± SEM. The number of replicates is enumerated in the figure legends. Data were analyzed using one-way analysis of variance (ANOVA), which was followed by a post hoc Newman–Keuls test for planned comparisons.

Results

DSP4 Treatment Down-Regulates the Expression of DBH and NET in a Time- and Concentration-Dependent Manner

Effects of DSP4 treatment on mRNA and protein levels of noradrenergic phenotypes in human SH-SY5Y cells were examined. As shown in Figure 2-1, exposure of cells to 50 μM DSP4 (this concentration was selected based on preliminary experiments and the literature) for different times resulted in a significant reduction of DBH ($F_{4,45}=34$, $p<0.0001$) and NET ($F_{4,34}=30.8$, $p<0.0001$) proteins. Post hoc tests revealed that compared to the control (0 time), DSP4-induced reduction of DBH/NET protein levels was time dependent. For the concentration course, cells were exposed to DSP4 at concentrations of 5, 10, and 50 μM for 24 h. qPCR analyses showed that DSP4 significantly decreased mRNA levels of DBH ($F_{3,48}=85.2$, $p<0.0001$) and NET ($F_{3,44}=17.97$, $p<0.001$) (Figs. 2-2A, 3A). Consistently, protein levels of DBH ($F_{3,27} = 56.2$, $p<0.0001$) and NET ($F_{3,36} = 69.3$, $p<0.0001$) also were significantly reduced as analyzed by western blotting (Figs. 2-2B,2-2C,2-3B,2-3C). Post hoc tests demonstrated that DSP4-induced reduction in mRNA and protein levels of DBH, as well as NET protein levels showed in a concentration-dependent manner. It was reported that the effects of DSP4 on NE levels *in vivo* are reversible (Jaim-Etcheverry and Zieher 1980, Hallman et al. 1984, Wolfman et al. 1994, Srinivasan and Schmidt 2004, Szot et al. 2010). The recovery of DBH/NET protein levels from inhibition by DSP4 was tested further. Cells were exposed to 50 μM for 24 h, then after brief washing with PBS, cells were then maintained in fresh medium in the absence of DSP4 for 24, 48, 72, and 96 h. Western blotting analysis

showed that washing-out of DSP4 significantly affected DBH and NET protein levels ($F_{5,42}=7.56$, $p<0.01$ for DBH; $F_{5,42}=30.48$, $p<0.0001$ for NET). While DBH levels were fully recovered within 24 h, it took 48 h for full recovery of NET protein levels ($p<0.01$). Nevertheless, these results indicated that DSP4-induced inhibition of DBH and NET expression in SH-SY5Y cells is reversible (Figure 2-4).

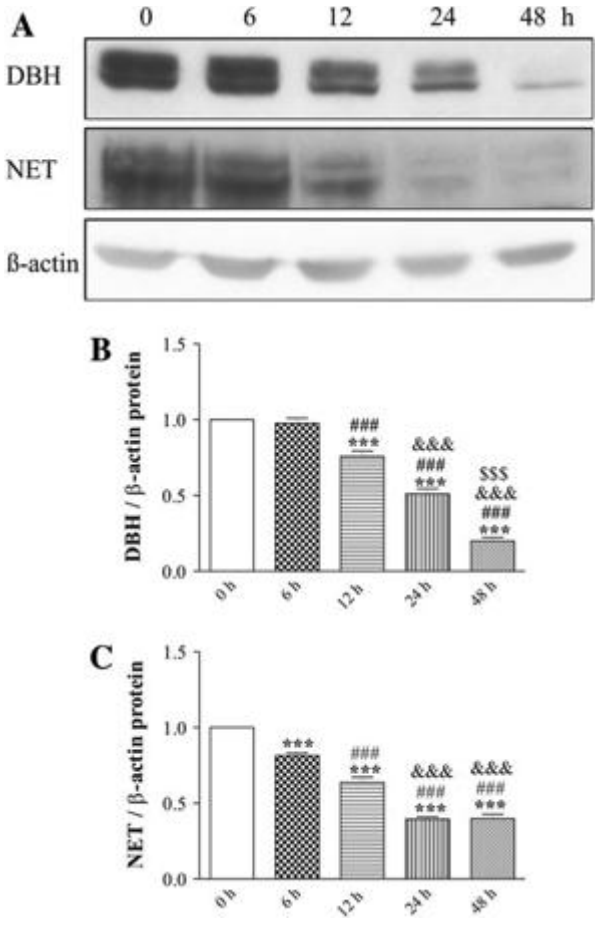


Figure 2-1. Time-dependent effects of DSP4 (50 μM) treatment on protein levels of DBH and NET in SH-SY5Y cells. (A) Autoradiograph obtained by western blotting. (B) and (C) The quantitative analysis of band densities in western blotting of DBH/NET. Values of protein levels of DBH/NET were normalized to those of β-actin in the same measurement. The graphic data represent averages obtained from 7 to 9 separate experiments. $^{***}p<0.001$, compared to the control group (0 h); $^{###}p<0.001$, compared to the 6 h group; $^{\&\&\&}p<0.001$, compared to the 12 h group; $^{\$\$\$}p<0.001$, compared to the 24 h group.

DSP4 Treatment Leads to Cell Cycle Arrest

To further study the action mechanisms of DSP4, SHSY5Y cells were exposed to 5 or 50 μM DSP4 for 24 h and the effects of these single-dose treatments on cell growth and viability were studied by trypan blue staining. At 5 μM, DSP4 inhibited cell proliferation by 25 % ($p<0.05$), whereas 50 μM DSP4 inhibited proliferation by 50 % ($p<0.001$), compared to the control group after 24 h (Figure 2-5A). This result suggests that the cell cycle was arrested. S-phase checkpoint monitors the integrity of the genome and halts DNA synthesis, arresting cells in S phase, following DNA damage (Bartek and Lukas 2001). Flow cytometry was used to determine whether cell cycle transit was altered by DSP4 (Figure 1-2). The DNA profile clearly demonstrates that DSP4 profoundly affects the SH-SY5Y cell cycle. In an untreated cell population 52.63, 18.73, and 27.17% of the cells were distributed among the G1-, S-, and G2/M-phases, respectively (Fig 2-5B, 5C). After a 24-h treatment with 5 or 50 μM DSP4 the percentage of SHSY5Y cells in S-phase increased to 28.77 and 32.83%, respectively, compared to 18.73% in untreated control cells. These cytometric results demonstrate that DSP4-treated SH-SY5Y cells accumulated predominantly in S-phase due to cell cycle arrest.

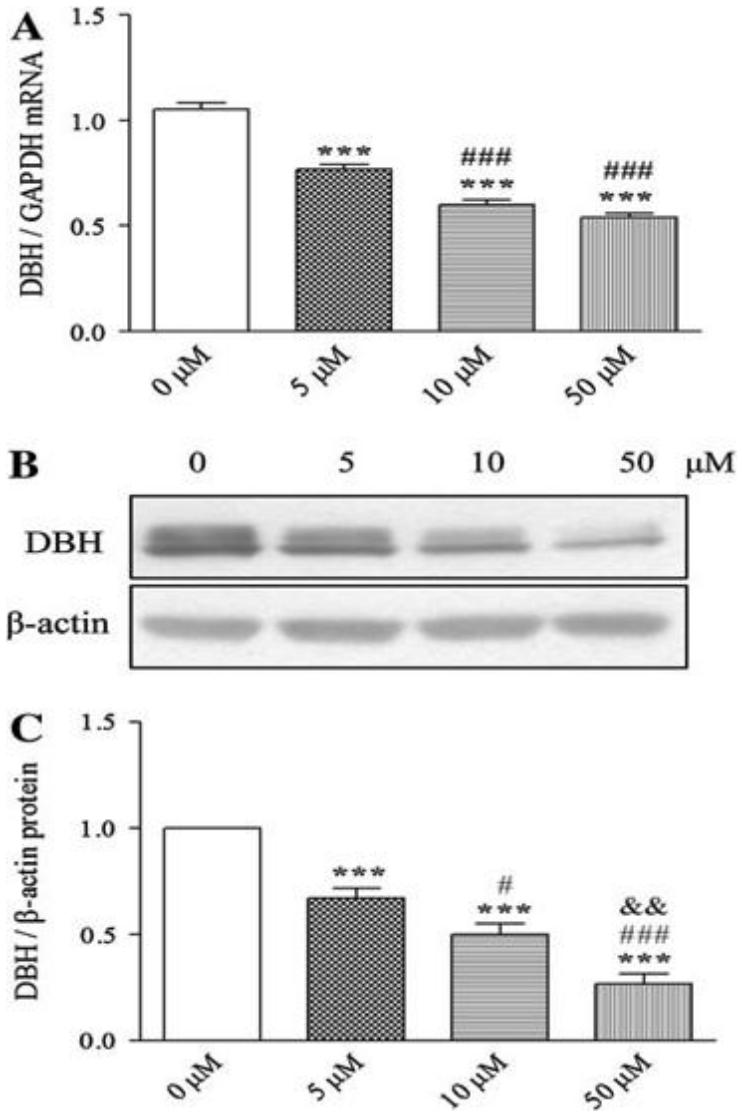


Figure 2-2. Concentration-dependent effects of DSP4 h on mRNA and protein levels of DBH in SH-SY5Y cells. (A) measured by qPCR, (B) measured by western blotting and (C) showed the quantitative analysis of band densities in western blotting. Values of mRNA and protein levels of DBH were normalized to those of GAPDH or β-actin in the same measurement. The graphic data represent averages obtained from 8 to 12 separate experiments. $^{***}p<0.001$, compared to the control group (0 μM); $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, compared to the 5 μM group; $^{\&\&}p<0.01$, compared to the 10 μM group.

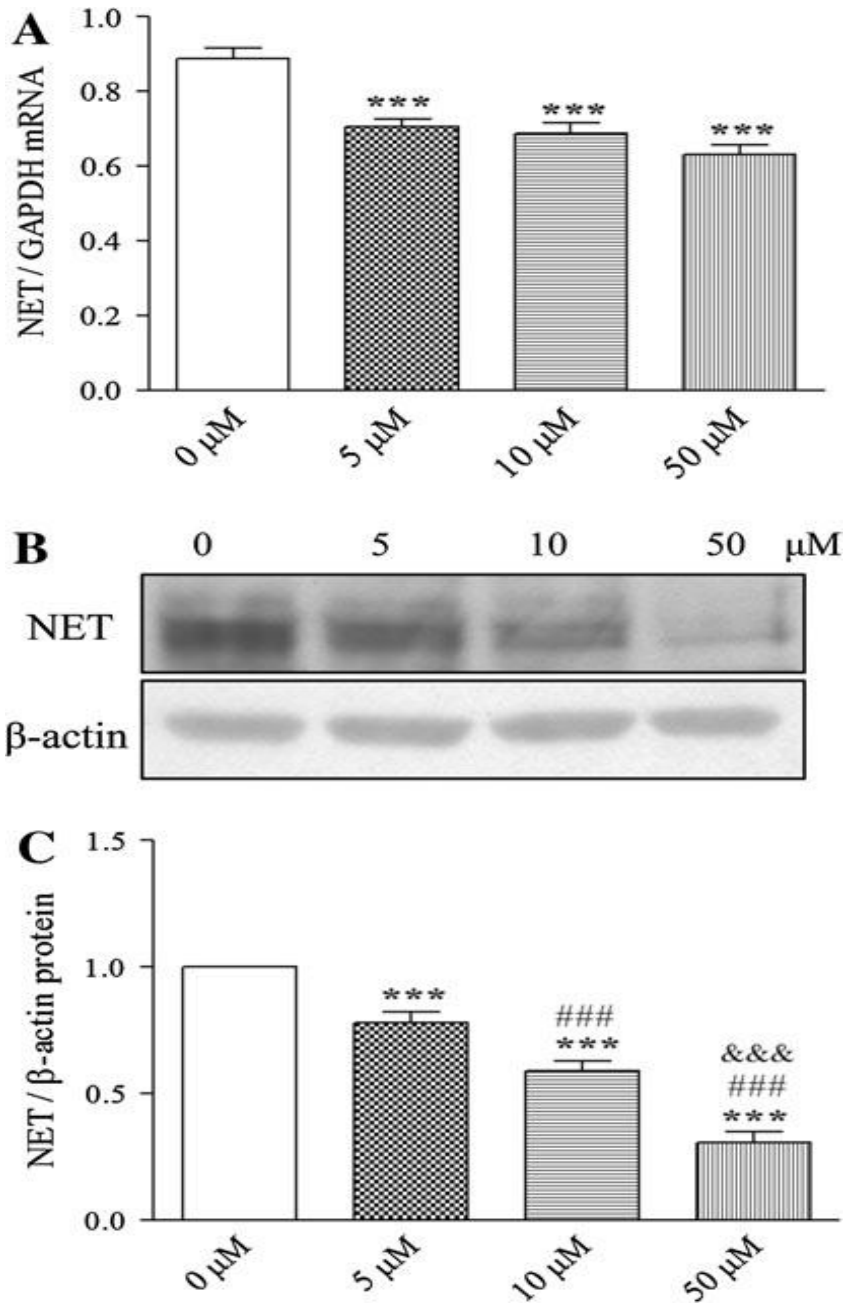


Figure 2-3. Concentration-dependent effects of DSP4 on mRNA and protein levels of NET in SH-SY5Y cells. (A) measured by qPCR, (B) measured by western blotting and (C) showed the quantitative analysis of band densities in western blotting. Values of mRNA and protein levels of NET were normalized to those of GAPDH or β-actin in the same measurement. The graphic data represent averages obtained from 9 to 11 separate experiments. $^{***}p<0.001$, compared to the control group (0 μM); $^{###}p<0.001$, compared to the 5 μM group; $^{&&&}p<0.001$, compared to the 10 μM group.

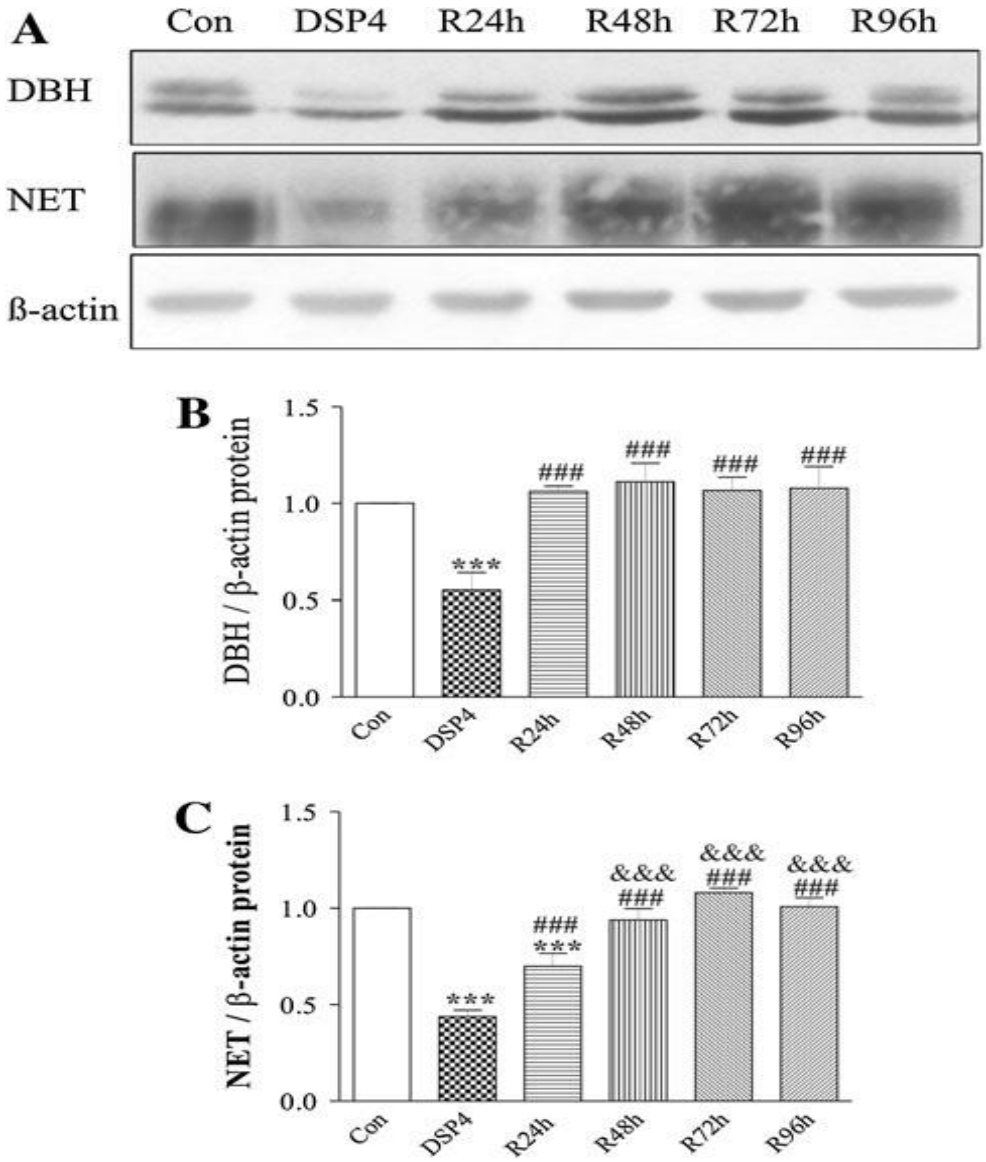


Figure 2-4. Up-regulation of DBH/NET protein levels from inhibition by DSP4. SH-SY5Y cells were exposed to 50 μM DSP4 for 24 h (DSP4) and then incubated in fresh media in the absence of DSP4 for 24 h (R24 h), 48 h (R48 h), 72 h (R72 h), and 96 h (R96 h) after brief washing by PBS. The control cells were exposed to the vehicle (Con). (A) Autoradiograph obtained by western blotting. (B) and (C) The quantitative analysis of band densities in western blotting of DBH/NET. Values of protein levels of DBH/NET were normalized to those of β-actin in the same measurement. The graphic data represent averages obtained from seven separate experiments. $^{***}p<0.001$, compared to the control group (Con); $^{###}p<0.001$, compared to the DSP4 group; $^{&&&}p<0.001$, compared to the R24 h group.

Next, we examined whether S-phase-arrested cells could resume cell cycle transit after removal of DSP4. Cells were treated with 50 μM DSP4 for 24 h, then rinsed with PBS and maintained in fresh medium without DSP4 for another 12 or 24 h before analysis by flow cytometry. As shown in Fig 2-6, arrested cells resumed cycle transit within 12 or 24 h after DSP4 removal. Although more cells were in S-phase compared with the control, the proportion of cells in G1-phase returned to normal. Interestingly, after removal of DSP4 for 24 h, fewer cells were in G2-phase compared to the control group ($p<0.05$). These data indicate that DSP4-arrested cells were able to resume cell cycle transit after removal of DSP4.

DSP4 Treatment Induces DDRs

How does DSP4 treatment lead to cell cycle arrest with an accumulation of cells in S phase? To address this question, SH-SY5Y cells were treated with 50 μM DSP4 for different times. As a positive control, one group of cells was treated for 2 h before cell harvest with CPT alone, a cytotoxic drug which inhibits topoisomerase I, thus inducing DNA double-strand breaks (DSBs) in the subsequent S-phase (Del Bino et al. 1992, Kurose et al. 2006). The harvested cell samples were analyzed by western blotting for the DDR markers γH2AX, p-p53^{ser15}, and hyperphosphorylation of RPA. As shown by Figure 2-7, γH2AX and p-p53^{ser15} levels were significantly increased at 2 or 4 h, respectively, after DSP4 exposure ($F_{6,42} = 46.6$, $p<0.001$ for γH2AX; $F_{6,42} = 85.5$, $p<0.001$ for p-p53^{ser15}), indicating that DNA damage appeared earlier than reduction of DBH/NET expression.

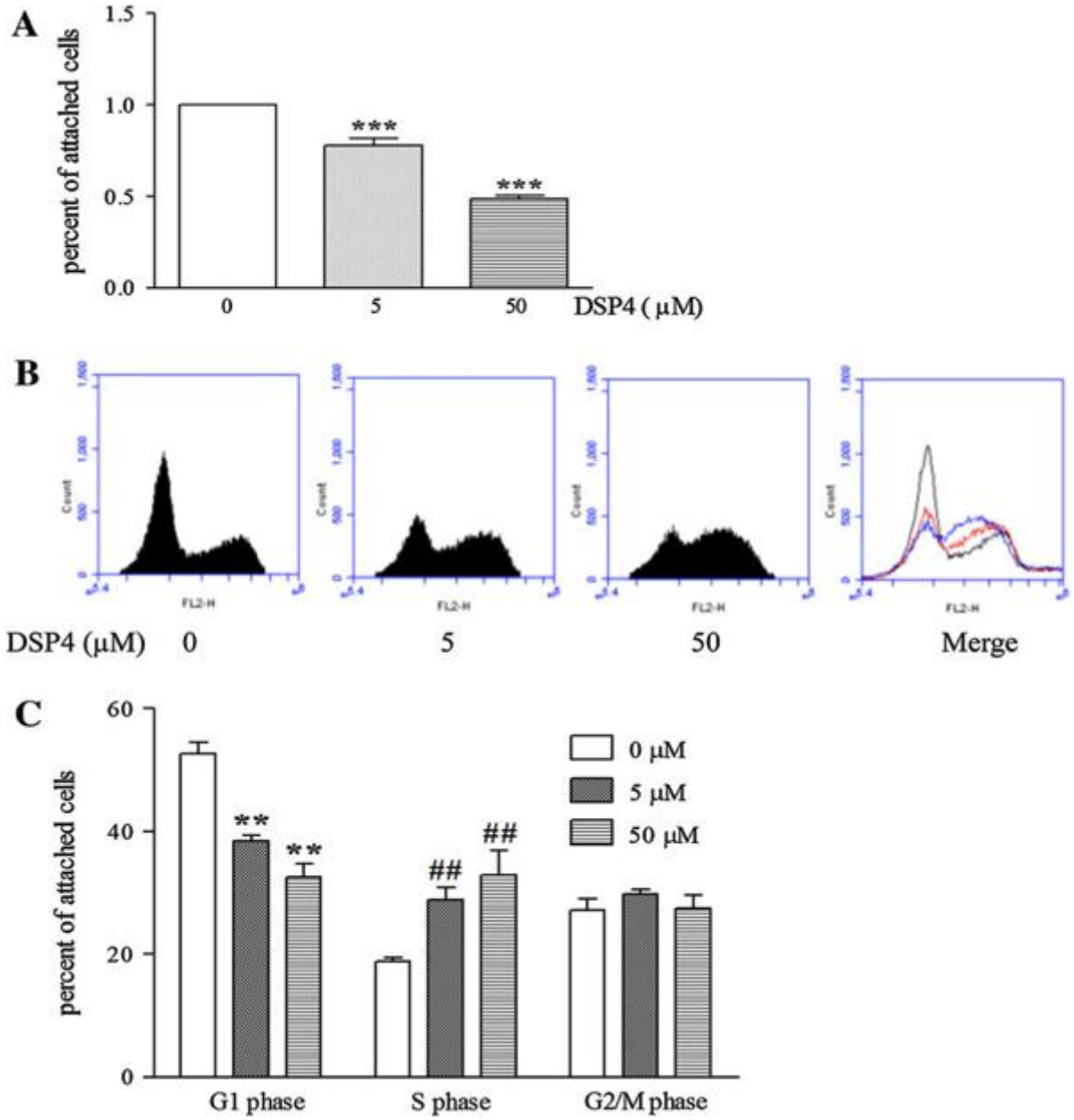


Figure 2-5. Effects of DSP4 treatment on cell proliferation and cell cycle arrest in SHSY5Y cells. (A) The viable number of SH-SY5Y cells decreased significantly after cells were exposed to 5 and 50 μM DSP4 for 24 h. Representative flow-cytometric histograms (B) and quantitative evaluation of DSP4 effects (C) showed DSP4-induced cell cycle arrest. Each bar from a and c represents data obtained from 6 to 7 separate experiments. $^{**}p<0.01$, compared to 0 μM group (A) or compared to 0 μM group in G1-phase. $^{\#\#}p<0.01$, compared to 0 μM group in S-phase. In figure B, black-control, red-5 μM DSP4; blue-50 μM DSP4.

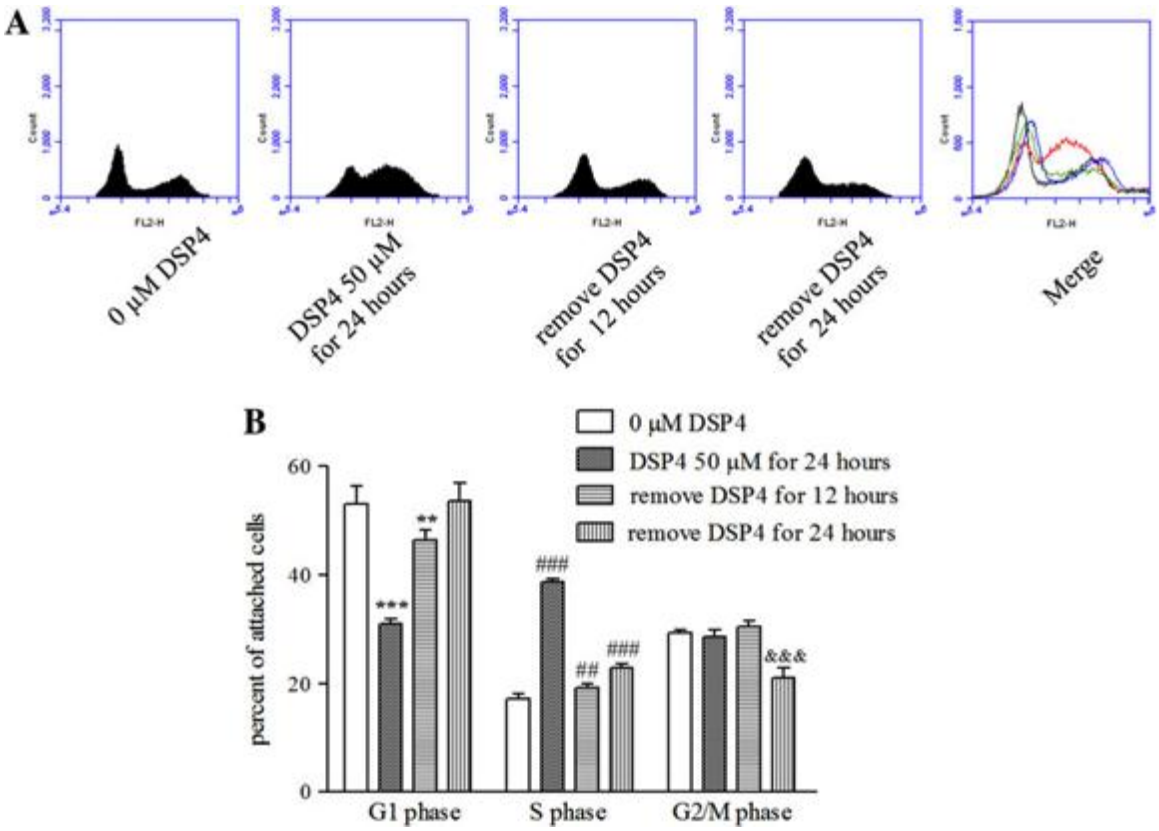


Figure 2-6. The DSP4-induced cell cycle arrest is reversible. Representative flow-cytometric histograms (A) and quantitative evaluation of DSP4 effects (B) showed that the DSP4-induced cell cycle arrest is reversible. In figure (A), black-control, red-DSP4 for 24 h, blue-removing DSP4 for 12 h, green-removing DSP4 for 24 h. Each bar from b represents data obtained from seven separate experiments. $^{**}p<0.01$, $^{***}p<0.001$, compared to 0 μM group in G1-phase. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, compared to 0 μM group in S phase. $^{\&\&\&}p<0.001$, compared to 0 μM group in G2/M-phase.

In a separate experiment, cells were exposed to DSP4 (5, 10, or 50 μM) for 24 h. Increased γH2AX levels were proportional to DSP4 concentrations (Figure 2-8A, 2-8B), suggesting that DNA strand breaks were induced by DSP4. Also, nuclear γH2AX foci were observed by immunofluorescence with the number of foci increasing proportionally with DSP4 concentrations or exposure time (Figure 2-9A). Tumor suppressor protein p53 also is phosphorylated at serine 15 in response to DNA damage or replication stress (Prieto and Giralt

2001). The level of p-p53^{ser15} also is increased in response to DSP4 treatment; this increase in p-p53^{ser15} is proportional to DSP4 concentration as determined by western blots (Figure 2-8C, 2-8D) and by immunofluorescence (Figure 2-9B).

Human RPA is a single-strand DNA-binding protein that is involved in many aspects of DNA metabolism (Zou et al. 2006). The 32 kDa subunit of RPA (RPA32) is hyperphosphorylated in response to DNA DSBs and to some other types of DNA damage (Zou et al. 2006). Also, CPT induces DNA DSBs and RPA hyperphosphorylation (Murren et al. 1996, Liu and Martin 2001), which appears as a slower migrating band on SDS-polyacrylamide gels. To determine if RPA becomes hyperphosphorylated in response to DSP4 treatment in SH-SY5Y cells were treated with DSP4 (5, 10, or 50 µM) for 24 h. As a positive control other cells were treated with CPT for 2 h before cell harvest. Interestingly, the hyperphosphorylated-RPA32 (hyp-RPA32) band was detected only in the CPT-treated cells (Figure 2-8E).

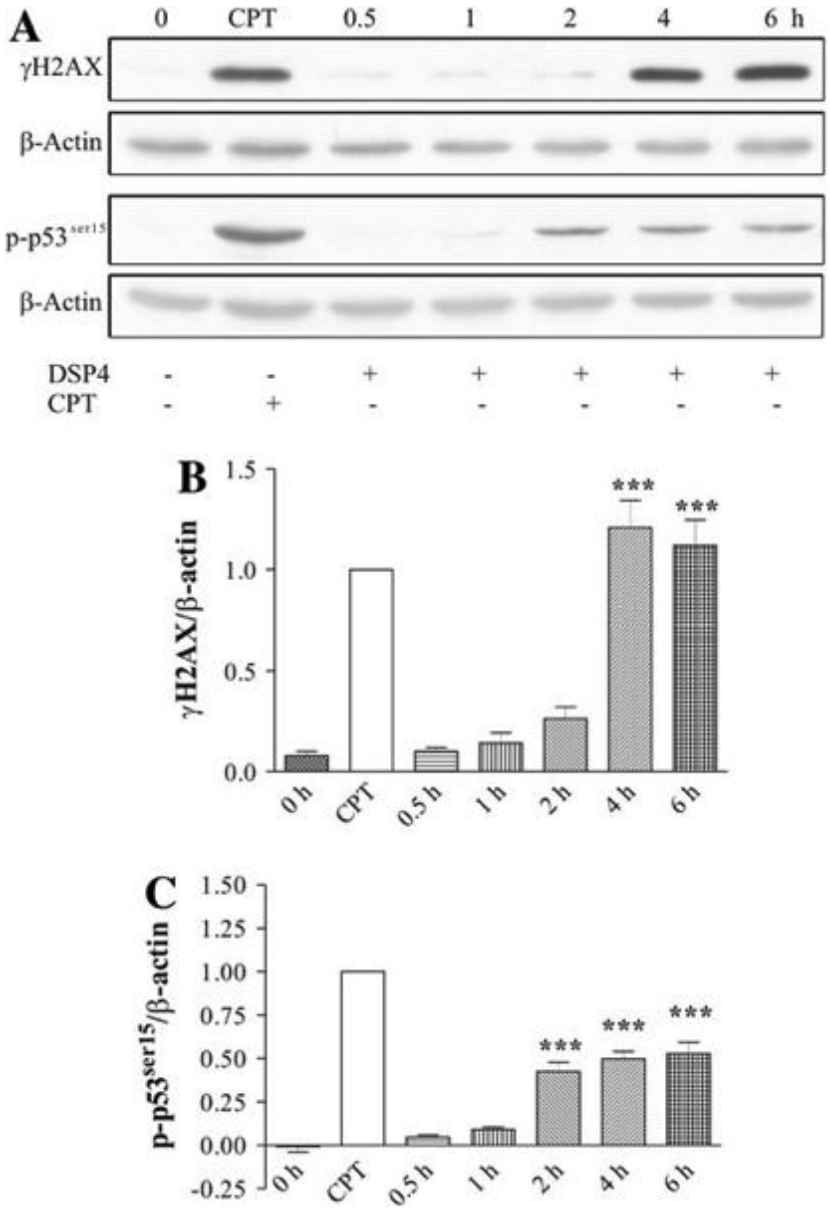


Figure 2-7. The time-course analysis of DSP4-induced DNA damage as demonstrated by increased γH2AX and p-p53^{ser15}. Cells were exposed to 50 μM DSP4 for 0.5–6 h. (A) Autoradiograph obtained by western blotting. (B) and (C) showed the quantitative analysis of band densities in western blotting of γH2AX or p-p53^{ser15}. Each bar from (B) and (C) represents data obtained from six separate experiments. $^{***}p<0.001$, compared to the control (0 h).

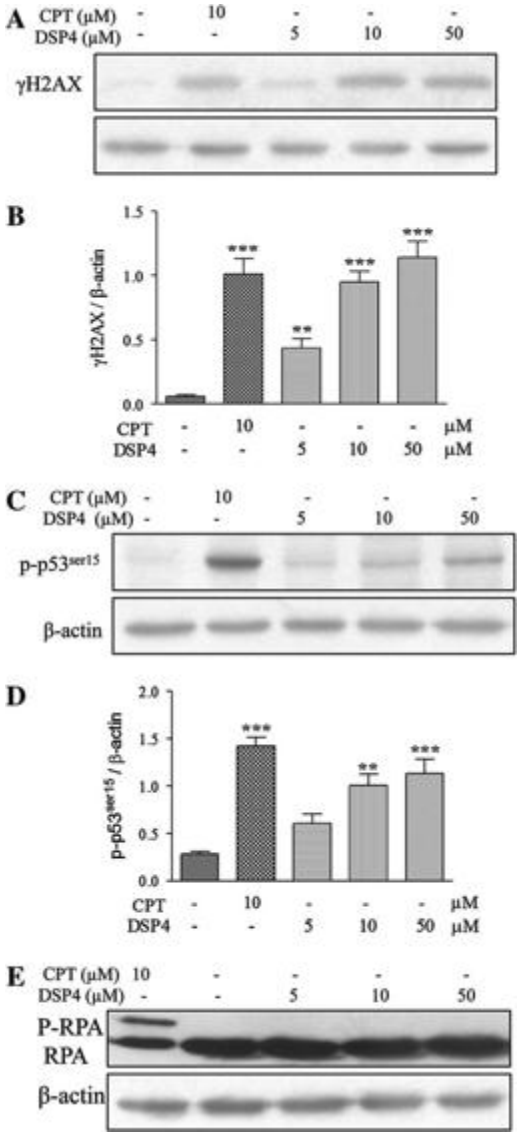


Figure 2-8. DSP4 treatment induced a DNA damage response in SHSY5Y cells. (A) and (B) Western blotting and quantitative analysis revealed an increase in γH2AX after DSP4 treatment for 24 h. (C) and (D) Western blotting and quantitative analysis showed an increase of p-p53^{ser15} after DSP4 treatment for 24 h. (E) No phosphorylated RPA32 was observed after exposing cells to 50 μM DSP4. Each bar from (B) and (D) represents data obtained from seven separate experiments. CPT results in phosphorylation of H2AX, p53, and RPA32. $^{**}p<0.01$, $^{***}p<0.001$, compared to 0 μM group.

DSP4 Induces Activation of ATM Pathway

Cell cycle checkpoints are regulatory pathways that govern the order and timing of cell cycle transitions to insure completion of one cellular event prior to commencement of the next cell cycle phase (Hatip-Al-Khatib and Bolukbasi 1999). The key regulators of the checkpoint pathways in the mammalian DDR are the ATM and ATR protein kinases, members of the serine–threonine PIKK kinases family (Abraham 2001, Shiloh 2001). Although ATM and ATR appear to phosphorylate many of the same cellular substrates (Kim et al. 1999), they generally respond to distinct types of DNA damage. ATM and ATR inhibitors were used to elucidate the role of these kinases in the DSP4-induced DDR. SH-SY5Y cells were treated with ATM or ATR inhibitors, alone or in combination, for 1 h before continuous exposure to 50 μM DSP4 for 24 h. Western blotting revealed that ATM inhibition significantly decreased the level of DSP4-induced γH2AX, compared to that of cells treated with DSP4 alone. In contrast, ATR inhibition increased the level of DSP4-induced γH2AX over that of cells treated with DSP4 alone. Treatment with both ATM and ATR inhibitors resulted in no net change of γH2AX levels compared to that of the group treated with DSP4 alone (Figure 2-10A, 10B).

These results indicate that ATM is involved in γH2AX formation after DSP4 treatment. This is consistent with the significant reduction in DSP4-dependent p-p53^{ser15} by ATM inhibition and increased levels by ATR inhibition. Similarly, treatment with both kinase inhibitors did not show a net reduction in phospho-p53^{ser15} levels below compared to DSP4 treatment alone (Figure 2-10C, 2-10D). These results indicate that γH2AX and p-p53^{ser15} formation are downstream product primarily of ATM after DSP4 treatment.

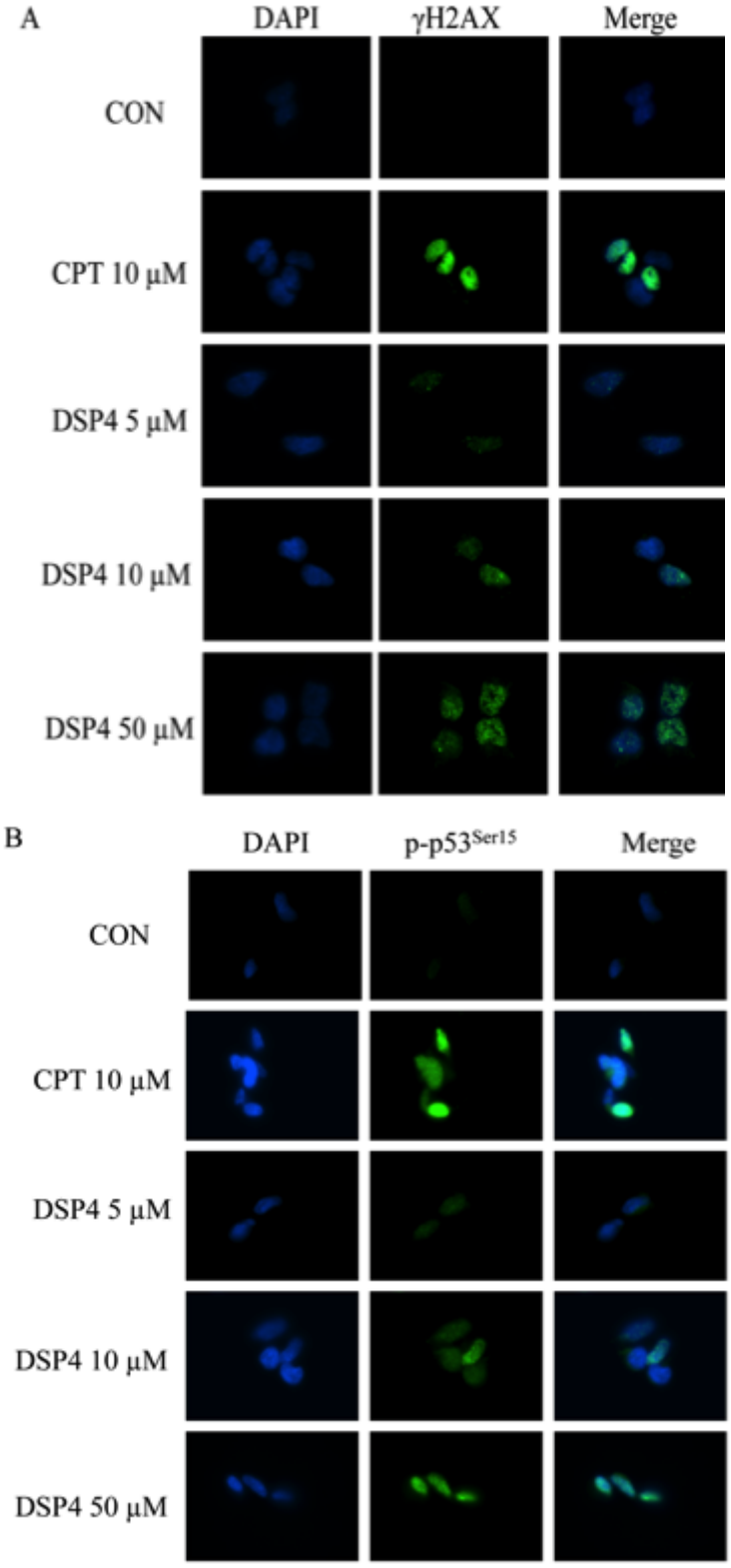

A
DAPI
γH2AX
Merge
CON
CPT 10 μM
DSP4 5 μM
DSP4 10 μM
DSP4 50 μM
B
DAPI
p-p53Ser15
Merge
CON
CPT 10 μM
DSP4 5 μM
DSP4 10 μM
DSP4 50 μM

Figure 2-9. DSP4 treatment induced the formation of nuclear γH2AX foci and p-p53^{ser15}. (A) Immunofluorescence staining showed foci of γH2AX in the nuclei of cells treated with 50 μM DSP4 for 24 h. (B) Immunofluorescence staining showed p-p53^{ser15} in the nuclei of cells treated with 50 μM DSP4 for 24 h. N = 5 for each group. DAPI, 4',6-diamidino-2-phenylindole, a fluorescent dye that strongly binds to DNA as a nuclear counterstain.

DSP4 Induces Single-Strand DNA Breaks

To further explore the type of DNA damage induced by DSP4 treatment, SH-SY5Y cells treated with DSP4 (5, 10, or 50 μM) for 24 h were analyzed by neutral or alkaline comet assays. These assays detect DNA double- versus single- strand breaks, respectively, by measuring the formation of the nuclear DNA tail (comet) after single-cell gel electrophoresis. As shown in Figure 2-11, no obvious nuclear tails were observed in the neutral comet assay after DSP4 treatment, indicating that DSP4 did not lead to detectable DNA DSBs. However, exposure of SH-SY5Y cells to DSP4 resulted in extensive SSBs as reflected in the significant tail lengths observed in the alkaline comet assay (Figure 2-11, bottom).

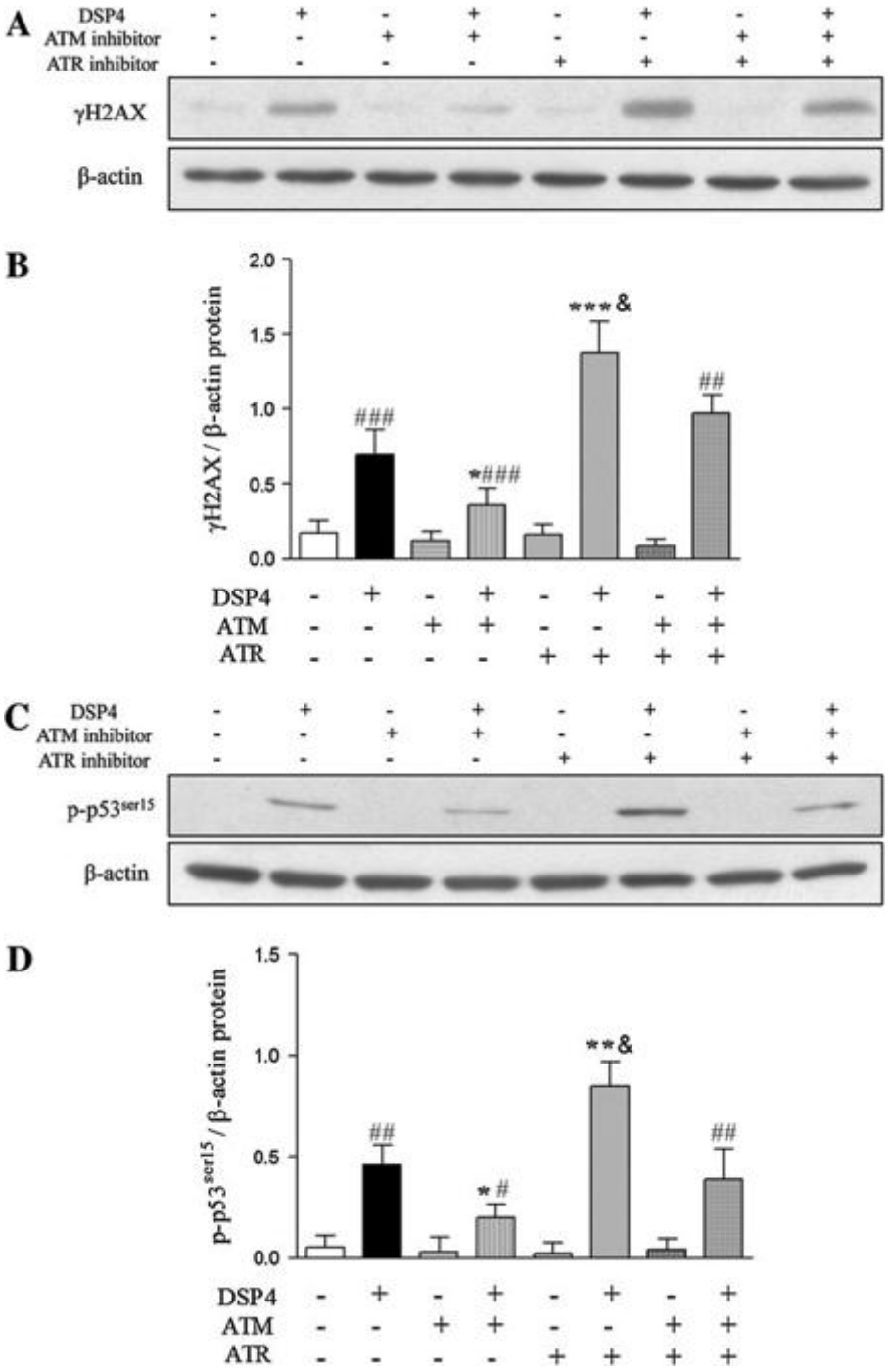


Figure 2-10. The DSP4-induced DDR is dependent on ATM activation. (A) and (B) Western blotting and quantitative analysis show reduction in DSP4-induced γH2AX formation in cells treated with ATM or ATR inhibitor. (C) and (D) Western blotting and quantitative analysis show a reduction in the DSP4-induced p-p53^{ser15} formation in cells treated with ATM or ATR inhibitor. The cells were pretreated with the inhibitors for 1 h and continued for 24 h with DSP4 treatment. Each bar from both pictures (B) and (D) represents data obtained from

six separate experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared to the DSP4-only group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, compared to the DSP4 plus ATR inhibitor group; $^{\&}p<0.05$, compared to the DSP4 plus both inhibitors group.

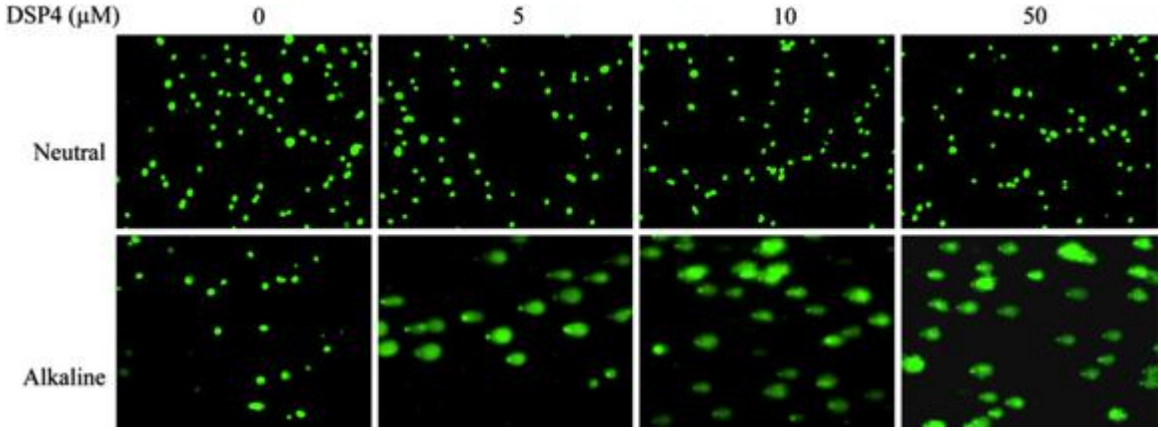


Figure 2-11. DSP4 induces single-strand DNA breaks as determined by the comet assay. SH-SY5Y cells were exposed to DSP4 in a dose dependent manner for 24 h. The cells were processed for comet assays run under neutral and alkaline conditions to identify DNA DSBs versus SSBs, respectively. N = 5 for each group.

Discussion

DSP4 is a well-known neurotoxin that selectively damages the noradrenergic projection originating from the LC (Jonsson et al. 1981, Fritschy et al. 1990). In the present study, SH-SY5Y neuroblastoma cell line, which naturally expressing DBH and NET (Richards and Sadee 1986) but neither the serotonin nor dopamine transporters (Lode et al. 1995), was used to examine the potential molecular mechanisms underlying DSP4 action on noradrenergic phenotypes. The main findings are: (1) DBH and NET expression in SHSY5Y cell line was down-regulated by DSP4 and DSP4-induced reduction of their protein levels and DBH mRNA

level exhibited a concentration and time-dependent course. (2) DSP4 treatment resulted in cell cycle arrest predominantly in S phase. (3) Removal of DSP4 allowed the arrested cells to resume the cell cycle and to replenish the reduced DBH and NET protein. (4) DSP4-induced cell cycle arrest possibly was caused by DNA damage as DSP4 treatment significantly increased the DDR markers γH2AX and p-p53^{ser15}. (5) DSP4 treatment activated the ATM pathway as part of the DDR. (6) DSP4 treatment of SH-SY5Y cells induced DNA SSBs but not DSBs. Thus, these results suggest that down-regulation of the noradrenergic phenotypes caused by DSP4 may stem from the DSP4-induced DNA damage, which activated the ATM pathway and eventually resulted in cell cycle disruption.

Previously, most studies of DSP4 neurotoxicity were carried out under *in vivo* conditions. DSP4 has been considered to interact at high affinity with the norepinephrine reuptake sites on the noradrenergic terminals, which were degenerated due to the alkylation of diverse vital neuronal structures (Lee et al. 1982, Hallman et al. 1984, Dudley et al. 1990). Furthermore, a reduced immunoreactivity of DBH and tyrosine hydroxylase in the LC and other brain regions caused by administration of DSP4 revealed an inhibition of noradrenergic phenotypes (Ross 1976, Gordon et al. 1999, Prieto and Giralt 2001, Kalinin et al. 2006, Waterman and Harding 2008, Engler et al. 2010). In the present study DSP4 treatment was found to drastically down-regulate expression of the DBH and NET in SH-SY5Y cells, which is consistent with those observations *in vivo*. These results confirm that the neurotoxicity of DSP4 is primarily mediated through inhibition or reduced expression of the noradrenergic phenotypes: reduced expression of DBH/NET diminishes the synthesis/reuptake of norepinephrine, finally leads to lower levels of norepinephrine in the brain (Figure 1-1).

Reduced expression of DBH/NET could be the end result rather than the cause of the

neurodegeneration. DNA is vulnerable to damage throughout the cell cycle due to diverse types of pathological insults including oxidative stress and cytotoxins that upset DNA metabolism. Though neurons are non-cycling cells neurotoxicity may be mediated by similar metabolic upsets. One consequence is DNA damage induced by active metabolites which cause DNA base adducts or strand breaks (Katyal and McKinnon 2008). In response to DNA damage, checkpoint surveillance mechanisms initiate signaling cascades, which coordinate cell cycle arrest and facilitate DNA repair (Shiloh 2003, Bakkenist and Kastan 2004, McGowan and Russell 2004). If these checkpoint surveillance mechanisms fail in neurons, neurodegeneration eventually occurs (Lavin 1999, Cho and Liang 2011). There has been a slow but steady accumulation of evidence of DNA damage in various neurodegenerative diseases (Robison and Bradley 1984). For example, an increase of DNA strand breaks in neurons has been reported in AD (Adamec et al. 1999) and HD (Anne et al. 2007). Damage to mitochondrial DNA also has been found in PD (Zhang et al. 1999). Therefore, DNA damage with alteration of cell cycle is likely involved in DSP4-induced degeneration of noradrenergic neurons. The present study demonstrated that exposure of cells to 50 μM DSP4 for 24 h induced about 50 % cell proliferation inhibition. The flow cytometric analysis showed that DSP4 treatment for only 24 h induced cell cycle arrest in S phase. DDR measurements confirmed a significant increase in γH2AX and p-p53^{ser15}, as well as increased frequencies of nuclear γH2AX foci; such foci normally represent formation of protein complexes at sites of DNA damage (Sontag et al. 2008). All these observations indicated that DSP4-induced cell cycle arrest occurred primarily in S-phase and was mediated through DNA damage signaling.

DNA damage and replication stress responses are a cascade signal transduction process. The DDR consists of multiple interconnected pathways, which impact the cell cycle, DNA

replication and repair, transcriptional regulation, chromatin remodeling, and other cellular metabolic processes (Zhou and Elledge 2000, Rouse and Jackson 2002). Actually, the activation of DNA damage-induced signaling pathways serves to arrest the cell cycle while DNA repair occurs. In response to DNA strand breaks and during replication stress, ATM and ATR kinases are considered as major physiological mediators for the phosphorylation of H2AX and p53 (Rogakou et al. 1998, Kastan and Lim 2000, Bakkenist and Kastan 2003). γH2AX and p-p-p53^{ser15} function as downstream mediators in this signaling pathway. The present study demonstrated that these signal pathways are involved in the DSP4-induced down-regulation of the noradrenergic phenotypes. After exposure of cells to DSP4, increased levels of γH2AX foci and nuclear p-p53^{ser15} were proportional to DSP4 concentrations (Figure 2-9). Furthermore, the ATM inhibitor significantly blocked the DSP4-induced enhancement of γH2AX and p-p53^{ser15} levels (Figure 2-10). Thus, ATM signaling pathways play a significant role in the DDR to DSP4's toxic effects.

DNA strand breaks can occur as either SSBs or DSBs. Compared to DSBs, SSBs are the more common lesion induced by exogenous genotoxins such as ionizing radiation and alkylating agents (Jeppesen et al. 2011). Also, SSBs can collapse a replication fork and be converted into DSBs, a threat to genetic stability if not dealt with properly (Caldecott 2004). DNA SSBs were reported to be the source of much of the DNA damage in the brain and are associated with neurodegenerative diseases (Rass et al. 2007). The comet assay in the present study demonstrates that DSP4 exposure induced SSBs, rather than DSBs (Figure 11). Interestingly, earlier studies hypothesized that DSP4 is an alkylating compound and its neurotoxic effects might derive from uptake by NET, then alkylation of norepinephrine uptake sites by covalently binding to these sites in neurons (Ross 1976). It was reported that the

nervous system has a lower tolerance for DNA strand breaks than other tissues. For example, exposure of adult motor neurons in suspension to oxidizing agents induced SSBs followed by neuronal degeneration (Liu and Martin 2001). Also, SSBs in neuronal DNA can physically block transcription, thereby triggering loss of cell function due to the absence of one or more essential gene products; such damage eventually leads to apoptosis. Thus, the current observations are consistent with the hypothesis that DSP4-induced SSBs trigger the down-regulation of noradrenergic phenotypes and lead to the neuronal degeneration.

Noradrenergic neuronal loss is an important pathogenic characteristic of neurodegenerative diseases. For example, in PD the greatest loss of neurons was found in the LC (83.2 %) rather than in the substantia nigra (77.8 %) (German et al. 1992). In AD, LC neurons were reduced by 67.9 %, compared to 41.1 % in the nucleus basalis (Zarow et al. 2003). Furthermore, the loss of LC neurons in PD and AD is best correlated with the duration and severity of illness (Bondareff et al. 1982, Gesi et al. 2000). These data not only indicate that LC neuron loss is possibly due to a primary involvement of the LC itself in the early pathogenesis of PD and AD (Mann et al. 1982, Gesi et al. 2000), but also that it might influence the onset and progression of PD and AD. Although the original hypothesis was that DSP4 affected only LC terminals (Fritschy and Grzanna 1991b), studies in recent years revealed that DSP4 also caused neuronal degeneration in the LC regions (Yu et al. 1994). However, this action may depend on the dose and treatment period, as well as the species of animals. For example, a single dose (50 mg/kg) of DSP4 did not significantly affect neuronal number in the LC of rats (Lyons et al. 1989, Matsukawa et al. 2003, Szot et al. 2010) or rabbit (Robinson et al. 1993), but treatment with two or more 50 mg/kg doses resulted in LC cell loss as revealed by tyrosine hydroxylase staining in rats (Heneka et al. 2002) and mice (Heneka et

al. 2006, Pugh et al. 2007, Jardanhazi-Kurutz et al. 2010, Rey et al. 2012). It has been suggested that mice may exhibit more sensitivity to DSP4 (Fornai et al. 1996). Accordingly, now DSP4 is used mostly *in vivo* studies as a selective neurotoxin to mimic in animal models the pathology of human PD and AD in terms of degeneration of LC neurons and subsequent NE depletion in the brain (Heneka et al. 2002, Srinivasan and Schmidt 2004). Since little is known about how DSP4 induces neurodegeneration *in vivo*, it remains uncertain whether systematic administration of DSP4 would induce DNA damage in the brain similar to that observed in the present study. Nevertheless, this study provides fundamental information regarding the potential action mechanisms of DSP4 *in vivo*. That is, administration of DSP4 in these animal models can result in DNA SSBs which in turn activate ATM signaling pathways including formation of γH2AX and p-p53^{ser15}, as well as other checkpoint factors and DDR processes. Therefore, therapies to minimize DNA damage may be beneficial in the future treatments of neurodegenerative diseases.

Collectively, the present studies demonstrate that DSP4 treatment significantly decreased expression of DBH/NET in a concentration-dependent manner. These alterations may be mediated through DSP4-induced SSBs, which in turn activated ATM signaling pathways to phosphorylate several DDR markers, resulting in cell cycle arrest in S phase. These action mechanisms of DSP4 may account for its degenerative consequence after systematic administration for animal models. Further elucidation of the molecular mechanisms underlying the DSP4-induced DDR process and the genetic interactions between different DDR pathways are underway since they are of critical importance in the development of new therapeutic strategies for the treatment of many degenerative diseases.

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CHAPTER 3

NEUROTOXIN-INDUCED DNA DAMAGE IS PERSISTENT IN SH-SY5Y CELLS AND LC NEURONS

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Abstract

Degeneration of the noradrenergic neurons has been reported in the brain of neurodegenerative disease patients. However, their pathological characteristics in neurodegenerative course and certain mechanisms remain to be elucidated. In the present study, we used neurotoxins CPT and DSP4 to induce the DDR in neuroblastoma SH-SY5Y cells, normal fibroblast cells, and primary cultured LC and raphe neurons. Our studies first show that noradrenergic SH-SY5Y cells are more sensitive to CPT-induced DNA damage and they are deficient to repair it, as compared to fibroblast cells. In order to confirm the findings of SH-SY5Y cells in *in vivo* studies, primary LC and raphe neuron cultures were used. Similar to SH-SY5Y cells, LC neurons are more sensitive to CPT- or DSP4-induced DNA damage and show a deficiency in

repairing CPT-induced DNA damage. Moreover, neurotoxins CPT and DSP4 do not induce DNA damage in neuronal cultures from raphe. Taken together, noradrenergic SH-SY5Y cells and LC neurons are sensitive to CPT-/DSP4-induced DNA damage and they show a repair deficiency. Therefore, these data might be used to explain the pathological characteristics of LC degeneration.

Introduction

The LC is a small nucleus located in the pons. It is the main source of brain norepinephrine, especially for the hippocampus and forebrain (Maeda 2000). The activity of LC neurons has been considered to be involved in numerous important functions, for example, response to stress (Usher et al. 1999). It is reported that the number of LC cells is reduced during normal aging and in aging-related diseases, as are brain norepinephrine levels (Marien et al. 2004). Patients with AD have reduced levels of norepinephrine compared with controls (Adolfsson et al. 1979, Palmer and DeKosky 1993). Damage and loss of LC noradrenergic neurons are accelerated in certain progressive neurodegenerative diseases (Mann and Yates 1983, Mann et al. 1983, Bondareff et al. 1987, German et al. 1992, Rommelfanger and Weinshenker 2007, Weinshenker 2008), which are early pathological indicators of AD and PD. The greatest neuronal loss was observed in the LC (83% loss in AD; 68% loss in PD) compared with other subcortical nuclei (Lyness et al. 2003, Zarow et al. 2003).

It was reported that aging-related diseases are mainly caused by accumulation of nuclear DNA (nDNA) damage in neurons due to insufficient nDNA damage repair. The brain

consists large number of non-proliferative neuronal cells, which are vulnerable to defective DNA repair. Deficiency of repairing DNA damage usually leads to "accumulation" of unrepaired DNA lesions, which might be considered as the cause of the neuropathology in several neurodegenerative disorders. Certain neurons with a high amount of nDNA damage, like Purkije cells in the rodent brain, would be removed during physiological aging, while those with lower amount of nDNA damage will remain there in the brain (Brasnjevic et al. 2008). The molecular and cellular mechanisms of the selective neuronal death during aging are currently not clarified. Therefore, exploring the pathological characteristics of LC noradrenergic neuronal loss in neurodegenerative process is important for elucidating the pathological mechanisms underlying AD and PD.

CPT is a cytotoxic quinoline alkaloid and a S-phase-specific anticancer agent which inhibits DNA topo I (Figure 1-2) (Liu et al. 2000). Generally, administration of CPT produces irreversible DNA DSBs during DNA replication, suggesting that this agent should not have toxic effects on non-dividing cells, such as neurons. However, it was reported that CPT could lead to death of post-mitotic rat cortical neurons *in vitro* in a significant dose-dependent manner. Additionally, neurotoxic activity of CPT also was found in cultured cerebellar granule neurons, which inhibited both protein synthesis and the neuritic outgrowth of primary cerebellar granule neurons (Uday Bhanu and Kondapi 2010). These observations indicate that CPT also exhibits significant toxicity toward neuronal cells *in vitro*.

The effects of DSP4 on norepinephrine levels in the peripheral and central noradrenergic system were first reported several decades ago (Ross 1976). It was reported that *in vivo* DSP4 selectively damages noradrenergic projections originating from the LC by interacting with the norepinephrine reuptake system and depleting intracellular norepinephrine,

finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom et al. 1985, Dooley et al. 1987, Prieto and Giralt 2001). DSP4 has been used as a noradrenergic neurotoxin to construct AD or PD animal models. Our previous study showed that DSP4 induces the DDR in neuroblastoma SH-SY5Y cells in a time- and dose-dependent manner (Wang et al. 2014). However, whether DSP4 can induce the DDR in primary cultured neurons remains unclear. To date, there are limited studies about the effects of neurotoxins on primary cultured neurons; therefore, it is essential to conduct this experiment to elucidate their pathophysiological characteristics.

In the present study, we exposed SH-SY5Y cells, which are considered as a noradrenergic cell line, and primary-cultured LC and raphe neurons to CPT or DSP4. Two DDR markers were measured. The results show that noradrenergic SH-SY5Y cells or primary LC neurons are severely affected by these neurotoxins. They are sensitive to CPT- or DSP4-induced DNA damage and they are deficient in repairing it, as compared to fibroblast cells or raphe neurons.

Materials and Methods

Cell Culture and Drug Exposure

Cell lines: The human neuroblastoma cell line SH-SY5Y and human normal fibroblast cells (AG08498) were used in these experiments. SH-SY5Y cells were maintained in a 1:1 mix of RPMI 1640 and F12 media. Normal fibroblast cells were maintained in DMEM. Both cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100

U/mL), and streptomycin (100 µg/mL) at 37 ^{o}C in humidified air containing 5 % CO_2. Culture media and supplements were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Cells were seeded into 6-well or 100-mm plates. Drugs exposures were started after 24 h of each subculture. Only SH-SY5Y cells prior to passage 10 were used. Cell viability was determined by exclusion of trypan blue dye; cell viability was 90–95 % in the untreated cells.

Primary tissue cultures: Timed pregnant Sprague Dawley rats at day 12-15 of gestation (ED 12-15; the day following nocturnal mating being considered as ED 1) were anaesthetized with ketamine/xylazine (100mg/10mg/kg. i.p.). After laparotomy and hysterectomy, the embryos were removed and their brains dissected under a stereomicroscope based on the published paper (Dunnett and Bjorklund 1992). Mesencephalic tissue pieces containing the raphe or LC were collected in ice-cold Hank's balanced salt solution (HBSS) (Gibco-Invitrogen, Carlsbad, CA, USA) and incubated for 15 min at 37^{o}C in a 15 mL-centrifuge tube containing 4.5 mL HBSS, 0.5 mL 0.25% trypsin-EDTA (Gibco-Invitrogen, Carlsbad, CA, USA) and 25 µL RQ1 DNase (0.1 mg/mL deoxyribonuclease). The trypsinization was stopped by addition of 5 mL HBSS containing 1 mM of pyruvate, 10 mM HEPES and 1 mL of FBS. Subsequently, the cells were dissociated by gentle trituration using a fire-polished Pasteur pipette. The suspension was centrifuged at 3000 rpm for 5 min and the pellet was suspended in culture medium, which contains neurobasal medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with B-27 (Gibco-Invitrogen, Carlsbad, CA, USA), 0.5 mM glutamine, 25 µM glutamate and penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were counted before plating. 1×10^{5} cells were transferred into 24-well plate (Sigma, St Louis, MO, USA) coated with poly-L-lysine (Sigma, St Louis, MO, USA). At 4 day *in vitro* (DIV), the medium was replaced by new medium, which is similar to the original one but without glutamate. For

maintaining cell cultures, half of the medium was changed every 3 days. Cells were used for drug treatment at 12 DIV.

DSP4 (Sigma, St Louis, MO, USA) was dissolved in distilled water at 50 mM, then was diluted with culture media and added to cells to a final concentration of 50 μM. CPT (Cat. No. C9911, Sigma, St Louis, MO, USA) was dissolved in DMSO at 10 mM, then was diluted with culture media and added to cells to a final concentration of 10 μM. The selection of the concentration of DSP4 was based on our previous data (Wang et al. 2014). The concentration of CPT was based on a published paper (Uday Bhanu and Kondapi 2010).

Western blotting analysis

Whole cell extracts for western blot analysis were prepared by lysing cells in ice-cold Nonidet P-40 (NP-40; Sigma, St Louis, MO, USA) buffer (0.5 % NP-40, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) for 30 min, after which nuclei and cell debris were removed by centrifugation at 12,000 rpm for 10 min at 4 ^{o}C. 5× SDS gel-loading buffer then was added to the supernatant and the samples were denatured at 70 ^{o}C for 5 min. Protein concentrations in cell extracts were quantified prior to addition of the loading buffer with the Micro BCA Protein Assay Kit (Thermo Science, Rockford, IL USA). Proteins (40 μg) were electrophoretically separated on a 10 % or 15 % SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK). For protein detection, the blots were probed with anti-γH2AX antibody (1:1,000 dilution, Bethyl Laboratories, Inc., Montgomery TX USA), or an anti-p-p53^{ser15} antibody (1:1,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5,000 dilution; Amersham Life Sciences, Buckinghamshire, UK) was

used as the secondary antibody. The membranes were subjected to enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) or super enhanced ECL (Sigma Chemical Co., St Louis, MO, USA) and autoradiography. To check for equal loading and transfer, the membranes were reprobed with a mouse IgG monoclonal anti-β-actin antibody (1:5,000 dilution, Amersham Life Sciences, Buckinghamshire, UK).

Immunofluorescence Assay (IFA)

2×10^4 cells were grown on coverslips in 24-well plates and treated with CPT (10 μM, for 15, 30, 60, 90 or 120 min) or DSP4 (50 μM, for 24 h), or vehicle. The cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized with 0.2 % TritonX-100 in PBS for 10 min. Coverslips were blocked with 5 % goat serum in PBS for 1 h, and incubated overnight with anti-γH2AX (1:200 dilution, GeneTex Inc., Irvine, CA, USA), anti-p-p53^{ser15} (1:400 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-DBH (1:200 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-SERT (1:200, Calbiochem, EMD Millipore Corporation, Billerica, MA, USA). After 3×5-min washes with PBS, the coverslips were incubated with the secondary antibodies [Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L); Alexa Fluor®568 Goat Anti-Mouse IgG (H+L), EMD Millipore Corporation, Billerica, MA, USA] diluted in PBS with 5 % goat serum. Coverslips were mounted onto microscope slides using Fluoromount-G mounting medium (Invitrogen, Grand Island, NY, USA). Slides were viewed and photographed at 20 or 60× magnification using an EVOS inverted fluorescent microscope (Advanced Microscopy Group, Washington, USA). We counted cells with at least two γH2AX foci for γH2AX-positive cells and increased p-p53^{ser15} expression in nuclei as p-p53^{ser15}-postitive cells.

Comet assay

SH-SY5Y cells were treated with CPT (10 μM) for 2 h, then CPT was washed out, cells continued to grow for 24, 48 and 72 h. Then neutral and alkaline comet assays were carried out using the Comet Assay System (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Fluorescence images were captured at 10× magnification. The overall shape resembles a comet with a circular head corresponding to the undamaged DNA and a tail of damaged DNA. The level of damage can be measured by length of the tail. At least 50 cells were assessed per treatment. In parallel with the comet assay, cell cultures with the same treatments were harvested for the protein analysis by western blotting.

Statistics

All experimental data are presented in the text and graphs as the mean ± SEM. The number of replicates is enumerated in the figure legends. Data were analyzed by using one-way analysis of variance (ANOVA) in GraphPad Prism.

Results

SH-SY5Y cells are sensitive to CPT-induced DNA damage

In this study, noradrenergic SH-SY5Y cells, which richly express the noradrenergic hall markers DBH and NET, were used. Normal human fibroblast cells with neither DBH nor NET expression were used as non-noradrenergic control cells (Figure 3-1). First, we used CPT to induce the DDR in SH-SY5Y cells, which was determined by two DDR makers γH2AX and p-

p53^{ser15}. We wanted to examine whether noradrenergic SH-SY5Y cells were sensitive to CPT-induced DNA damage. SH-SY5Y or fibroblast cells were exposed to 10 μM CPT for 15, 30, 60, 90 or 120 min, followed by western blot analysis and IFAs. As shown in Figure 3-2A and 3-2C, the percent of γH2AX-positive cells was 14.7% after 90 min exposure and 100% after 120 min exposure ($F_{5,12}=377.5$, $p<0.0001$). In contrast, γH2AX foci were found in about 1.3%, 29.3%, 51.2%, 74.0% and 77.7% in SH-SY5Y cells after CPT exposure for 15, 30, 60, 90 or 120 min ($F_{5,12}=171.3$, $p<0.0001$), respectively (Figure 3-2B and 3-2D). The percent of p-p53^{ser15}-positive fibroblast cells was 37.9% after 120 min exposure (Figure 3-3A and 3-3C, $F_{5,12}=45.47$, $p<0.0001$). However, a significant amount of p-p53^{ser15}-positive cells was 36.6%, 47.2% and 49.5% in SH-SY5Y cells after 60, 90 and 120 min CPT treatment, respectively (Figure 3-3B and 3-3D, $F_{5,12}=1816$, $p<0.0001$). Western blot results showed that the levels of γH2AX (Figure 3-4E and 3-4G, $F_{5,12}=83.02$, $p<0.0001$) and p-p53^{ser15} (Figure 3-4F and 3-4H, $F_{5,18}=168.5$, $p<0.0001$) were dramatically increased after 60 min CPT exposure in SH-SY5Y cells, but after 120 min in fibroblast cells (Figure 3-4A and 3-4C, $F_{5,18}=78$, $p<0.0001$; Figure 3-4B and 3-4D, $F_{5,18}=868.9$, $p<0.0001$). These results indicate that SH-SY5Y cells are sensitive to CPT-induced DNA damage, compared to that in fibroblast cells.

Figure 3-1. Expression levels of DBH and NET in SH-SY5Y and normal fibroblast cells. SH-SY5Ycells are rich in NET and DBH expression; however, normal fibroblast cells express neither NET nor DBH.

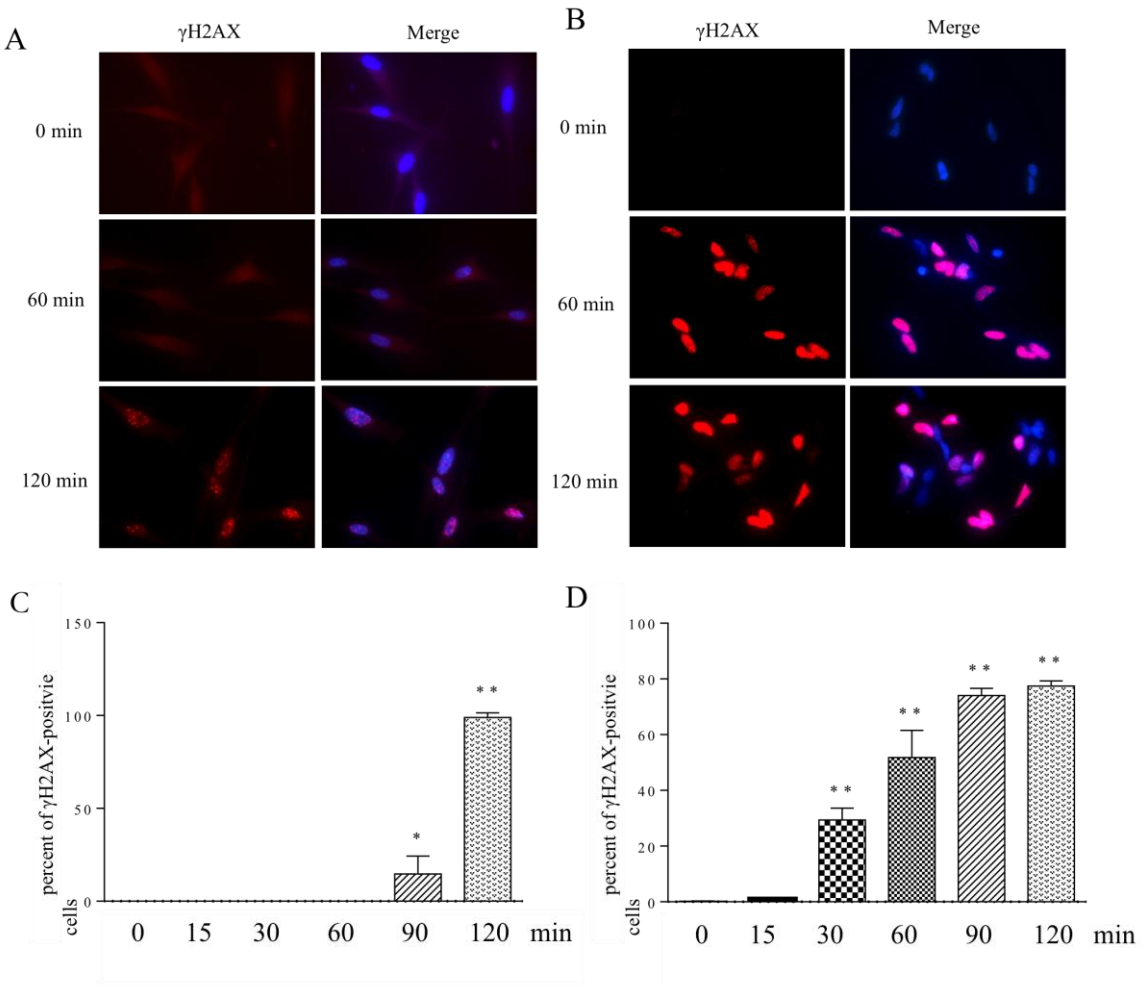


Figure 3-2. γH2AX foci are detected in normal fibroblast and SH-SY5Y cells after CPT treatment. Cells were treated with CPT (10 μM) for 15, 30, 60, 90 and 120 min, then IFAs were performed. (A) 100% of fibroblast cells are found with more than two γH2AX foci in nuclei within 120 min CPT treatment. (B) Significant γH2AX foci are found in nuclei after CPT treatment in SH-SY5Y cells. Images were taken with 60× magnification. Data of percent of γH2AX-positive cells are shown in (C) and (D). At least 200 cells were counted from three random chosen views. *$p<0.01$, **$p<0.0001$, compared to the 0 min.

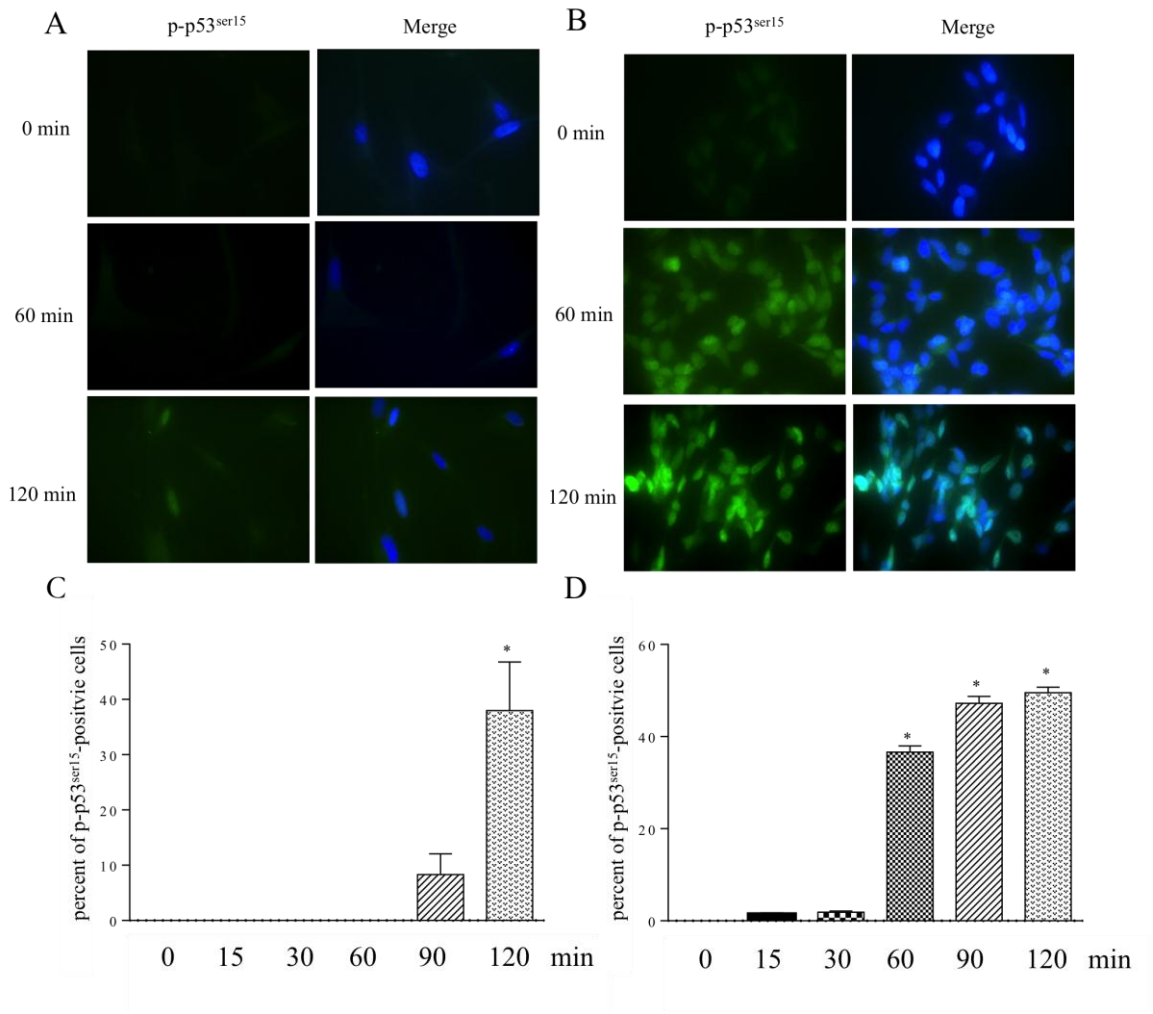


Figure 3-3. P-p53^{ser15} levels increase in nuclei of fibroblast and SH-SY5Y cells after CPT treatment. Cells were treated with CPT (10 μM) for 15, 30, 60, 90 and 120 min, then IFAs were performed. (A) Significant increased number of p-p53^{ser15}-positive cells is detected within 120 min CPT treatment in fibroblast cells. (B) Number of p-p53^{ser15}-positive cells is dramatically increased after 60 min CPT treatment in SH-SY5Y cells. Images were taken with 60× magnification. Percent of p-p53^{ser15} positive cells is shown in (C) and (D). At least 200 cells were counted from three random chosen views. *$p<0.0001$, compared to 0 min.

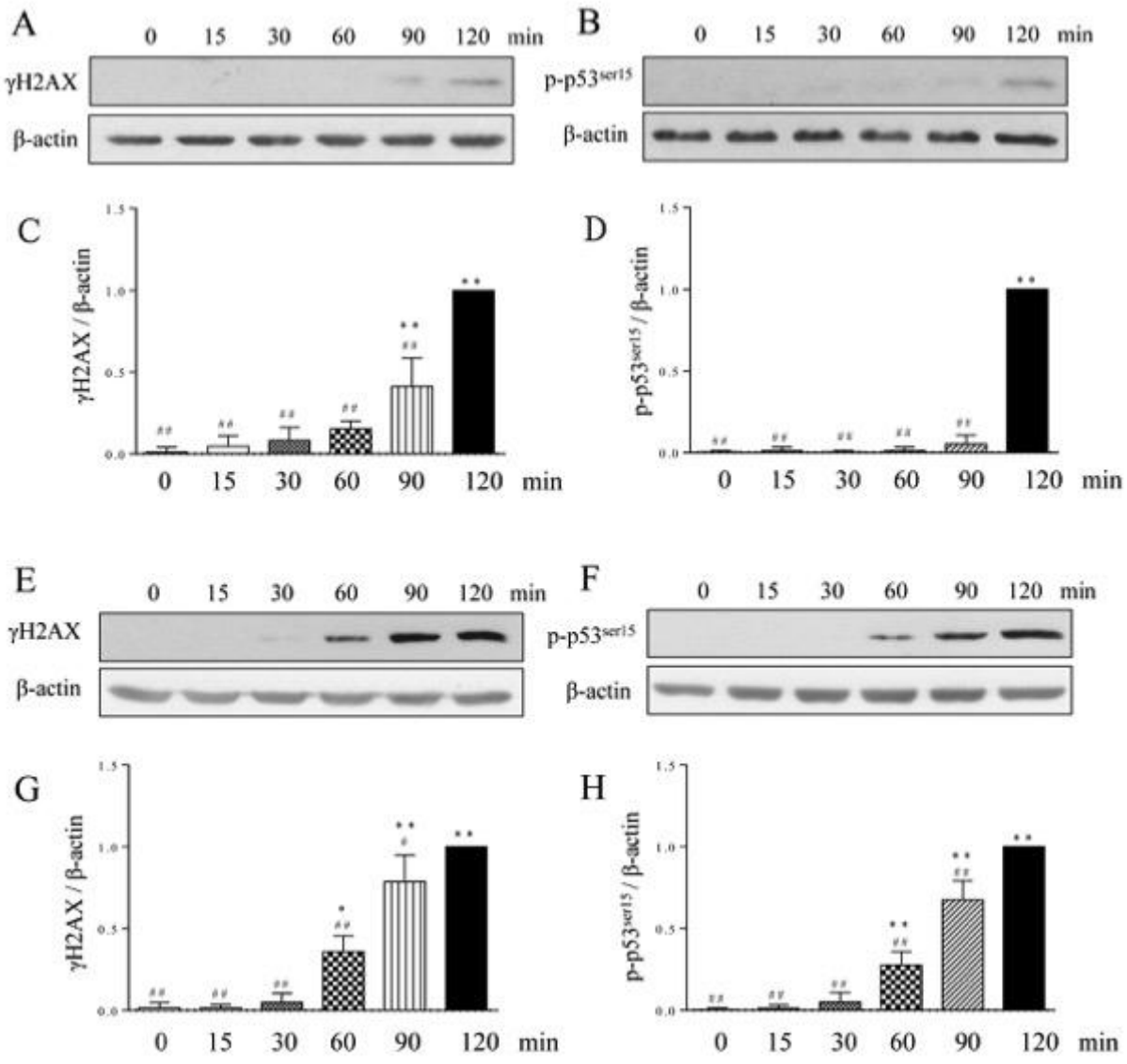


Figure 3-4. The levels of γH2AX and p-p53^{ser15} increase after CPT treatment. Fibroblast and SH-SY5Y cells were treated with CPT (10 μM) for 15, 30, 60, 90 and 120 min, then western blots were performed. (A) γH2AX and (B) p-p53^{ser15} levels were dramatically increased after 120 min CPT treatment in fibroblast cells. (E) γH2AX and (F) p-p53^{ser15} levels were dramatically increased after 60 min CPT treatment in SH-SY5Y cells. The graphic data, shown in (C), (D), (G) and H, represent quantitative analysis of protein levels of γH2AX and p-p53^{ser15}, which were normalized to β-actin. Compared to 0 min, $^{*}p<0.01$, $^{**}p<0.0001$; compared to 120 min, $^{\#}p<0.05$, $^{\#\#}p<0.0001$.

SH-SY5Y Cells are deficient in repairing CPT-induced DNA Damage

In order to examine whether DNA damage caused by CPT in SH-SY5Y cells was resistant to repair, we treated SH-SY5Y and fibroblast cells with CPT (10 μM) for 2 hours, then cells were briefly washed with PBS. Cells continued to grow in fresh medium in the absence of CPT for 24, 48, 72 and 96 h. In normal fibroblast cells, number of γH2AX foci and the level of p-p53^{ser15} significantly reduced within 24 h after removal of CPT (Figure 3-5). However, in SH-SY5Y cells, the number of γH2AX-positive cells was gradually reduced over 72 h (Figure 3-6A, $F_{5,12}=453.9$, $p<0.0001$). Unexpectedly, the number of γH2AX-positive SH-SY5Y cells dramatically decreased from 76.9% to 41.0% within 24 h, but increased from 41.0% to 52.3% from R24 h to R 48 h. The level of p-p53^{ser15} in SH-SY5Y nuclei decreased gradually over 72 h (Figure 3-6B, $F_{5,12}=203.0$, $p<0.0001$). Western blotting analysis also showed in SH-SY5Y cells, reduced γH2AX ($F_{4,20}=43.23$, $p<0.0001$) and p-p53^{ser15} ($F_{4,10}=205.3$, $p<0.0001$) protein levels over 72 h after washing-out of CPT (Figure 3-7).

To further explore the repair efficiency, CPT-treated cells were analyzed by comet assays. We treated both fibroblast and SH-SY5Y cells with CPT, and then neutral and alkaline comet assays were performed. Interestingly, tails in fibroblast cells were detected under both neutral and alkaline conditions, which suggest that CPT induces both DSBs and SSBs in fibroblast cells. However, tails were only detected under alkaline condition in SH-SY5Y cells, which indicated SSBs. Tails gradually shorted or disappeared over 72 h in SH-SY5Y cells, while began to short and disappeared at 24 h in normal fibroblast cells (Figure 3-10).

In sum, compared to fibroblast cells, CPT-treated SH-SY5Y cells exhibited a delay in reducing levels of two DDR markers γH2AX and p-p53^{ser15}. In addition, comet assays showed

that CPT-induced DNA damage were persistent in SH-SY5Y cells. These data indicated that SH-SY5Y cells were deficient to repair CPT-induced DNA damage.

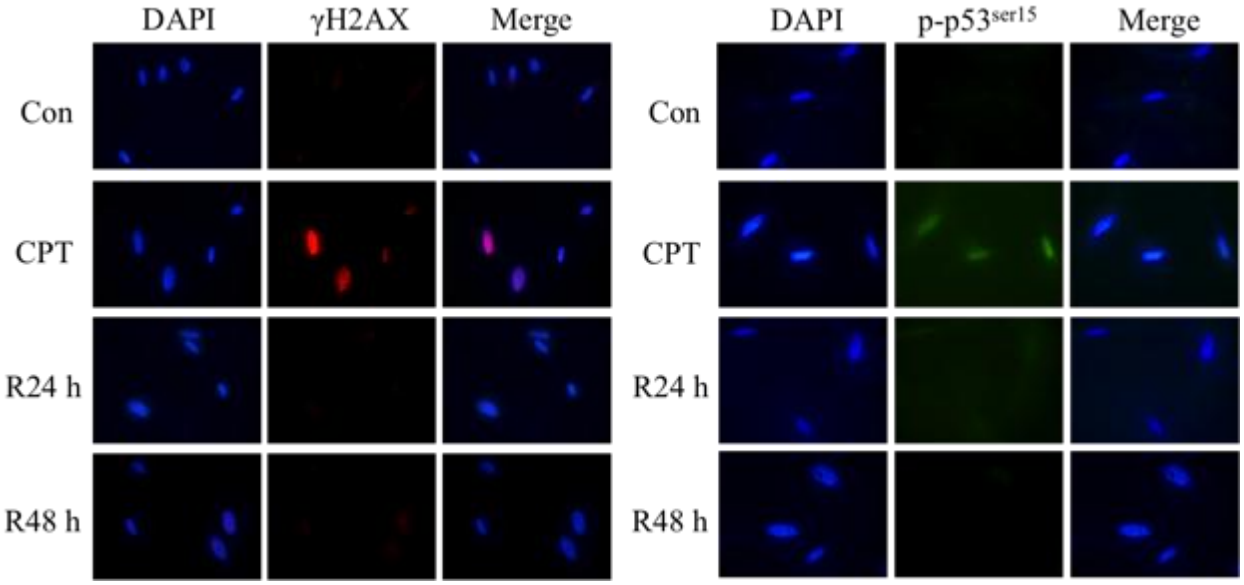


Figure 3-5. The number of γH2AX- or p-p53^{ser15}-positive cells is reduced within 24 h in fibroblast cells. Cells were treated with CPT (10 μM) for 2 h, then CPT was washed away. Cells continued to grow in fresh prepared media without CPT for 24 or 48 h. After 2 h CPT treatment, significant γH2AX foci were found in nuclei (A), and the level of p-p53^{ser15} (B) was increased in nuclei. The numbers of γH2AX- or p-p53^{ser15}-positive fibroblast cells were reduced within 24 h. The Images were taken with 60× magnification. Blue: DAPI, Red: γH2AX.

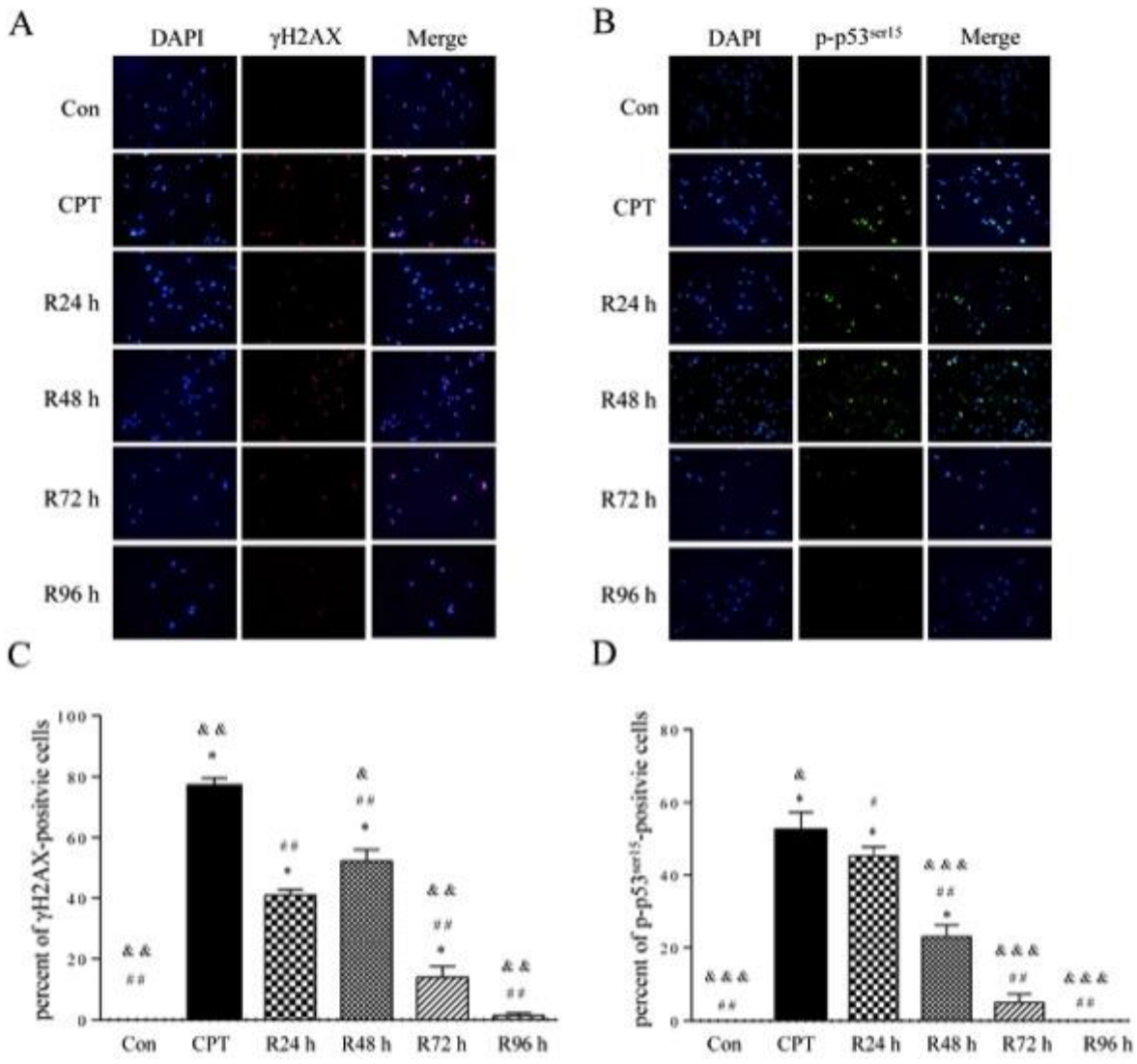


Figure 3-6. The number of γH2AX- and p-p53^{ser15}-positive cells is reduced within 72 h in SH-SY5Y cells. Cells were treated with CPT (10 μM) for 2 h. After CPT was washed away, cells continued to grow in media without CPT for 24, 48, 72 or 96 h. (A) The number of γH2AX-positive cells decreased within 72 h. (B) The level of p-p53^{ser15} was gradually decreased within 72 h. Analytical data of percent of γH2AX- or p-p53^{ser15}-positive cells are shown in (C) and (D). At least 200 cells were counted from three random chosen views. $^{*}p<0.0001$, compared to the control; $^{\#}p<0.05$, $^{\#\#}p<0.0001$, compared to CPT, $^{\&}p<0.05$, $^{\&\&}p<0.001$, $^{\&\&\&}p<0.0001$, compared to R24 h.

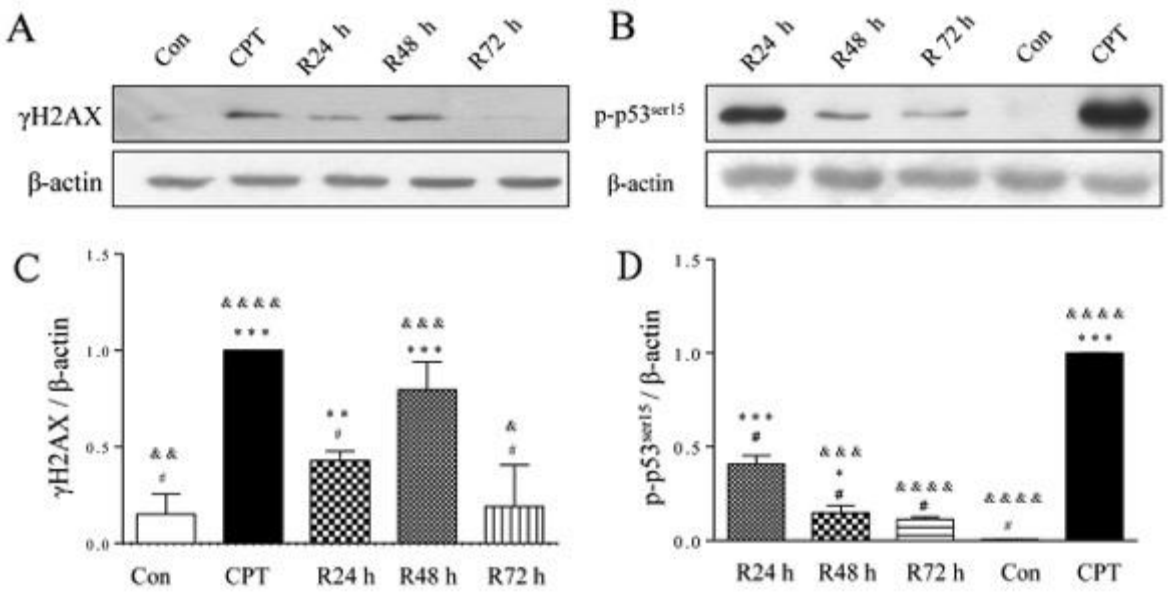


Figure 3-7. The levels of γH2AX and p-p53^{ser15} are decreased within 72 h in SH-SY5Y cells after washing out of CPT. SH-SY5Y cells were treated with CPT (10 μM) for 2 h, then CPT was washed away, cells continued to grow in media without CPT for 24 h , 48 h and 72 h. Then western blot analyses were performed. (A) and (B) show western blots results. Quantity analysis data are shown in (C) and (D) (N=5). $^{\#}p<0.001$, $^{\#\#}p<0.0001$, compared to the control; $^{*}p<0.0001$, compared to CPT group; $^{\&}p<0.05$, $^{\&\&}p<0.001$, $^{\&\&\&}p<0.0001$, compared to R24 h group.

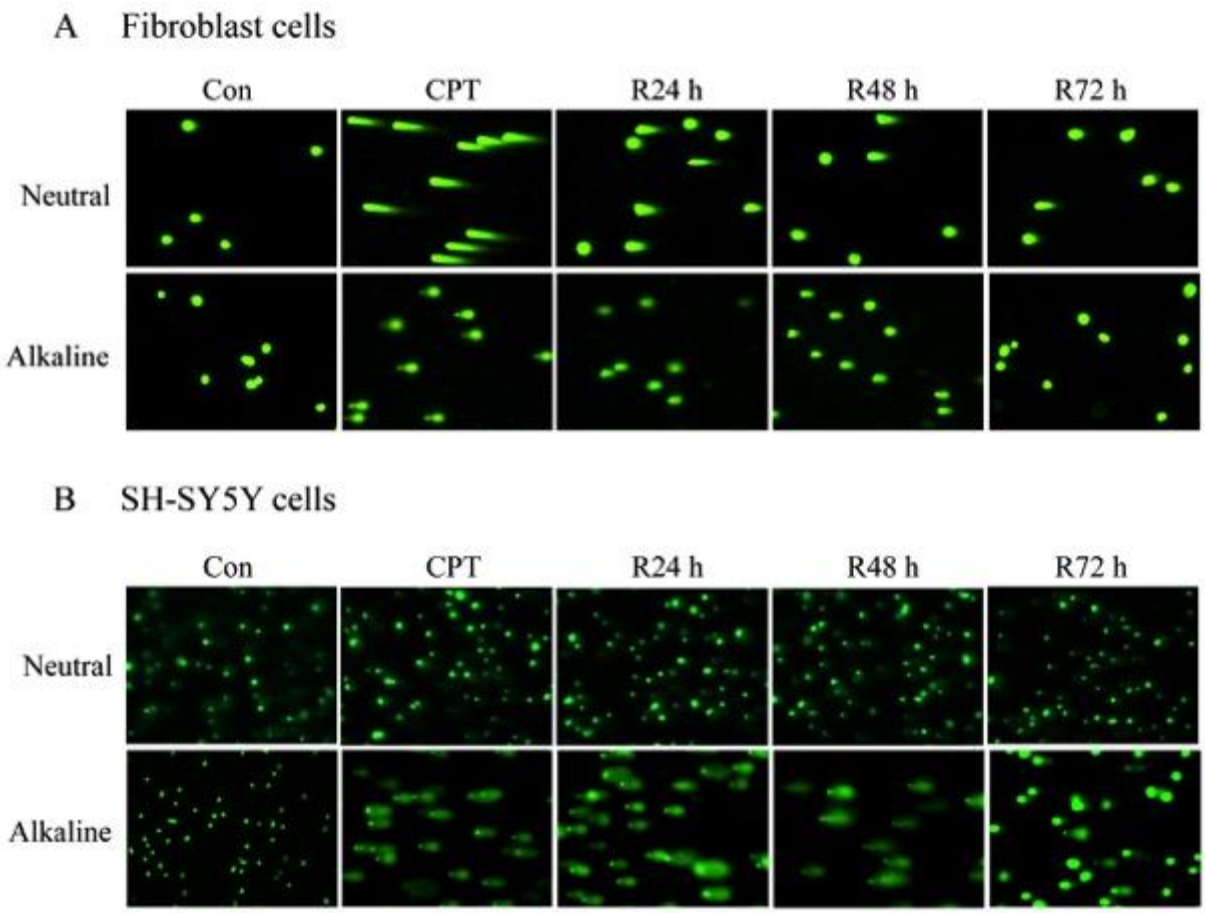
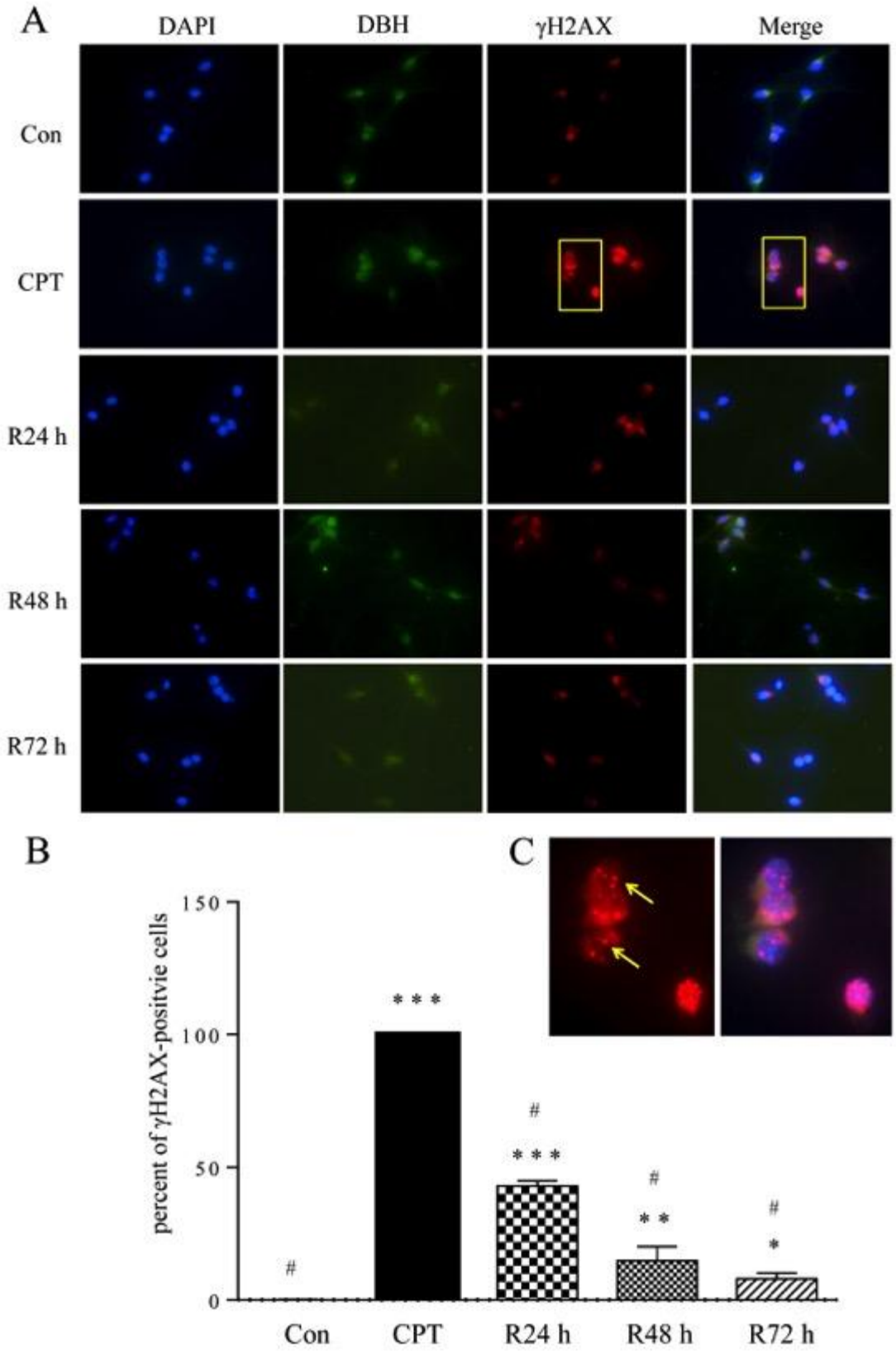


Figure 3-8. CPT-induced DNA damage can be reduced. Fibroblast and SH-SY5Ycells were exposed to CPT (10 μM) for 2 h, then CPT was washed away, cells continued to grow in media without CPT for 24 h (R24 h), 48 h (R48 h) and 72 h (R72 h). The cells were processed for neutral or alkaline comet assays. Images were taken with 10× magnification. (A) After CPT treatment, both neutral and alkaline comet assay detected comet tails in fibroblast cells. Comet tails were shorted or disappeared at 24 h. (B) After CPT treatment, only alkaline comet assay detected comet tails in SH-SY5Y cells. Comet tails were shorted or disappeared within 72 h.

CPT-induced the DDR in cultured LC and raphe neurons

Previous data (Figure 3-2, 3-3, 3-4, 3-5, 3-6, 3-7 and 3-8) showed that noradrenergic SH-SY5Y cells were sensitive to CPT-induced DNA damage and showed deficiency of repairing the damage, compared to fibroblast cells. In order to confirm that SH-SY5Y cells can be used as an appropriate *in vitro* noradrenergic cell model for *in vivo* studies, primary LC and raphe neuronal cultures were used. Primary neuronal cultures derived from rodents are very useful tool to study basic physiological properties of neurons and the potential neurotoxicins effects *in vitro*. To develop a cell culture system of the LC and raphe, which would facilitate the investigation of various properties of these noradrenergic and non-noradrenergic neurons under well-controlled conditions (Masuko et al. 1986). LC is the main source of noradrenergic neurons while raphe is a key center for serotonin expressing neurons. Thus, primary cultured LC and raphe neurons were used to examine whether these neurons respond to DNA damage differently from each other. LC and raphe tissues were separated from 15 to 18 days rat embryos, then cultured for 12 days. Since we did not get enough neurons to do western blots, therefore in this part we mainly focused on IFAs. LC neurons are DBH positive and raphe neurons are SERT positive. These primary cultures were treated with CPT (10 μM) for 2 h, and then after brief wash, cells continued to grow in the media without CPT for another 24, 48 or 72 h. As shown in Figure 3-9, significant γH2AX foci were found in LC neurons after CPT treatment ($F_{4,11}=558.3$, $p<0.0001$). The number of γH2AX-positive cells were gradually reduced over 72 h after CPT wash-out. However, we could barely detect γH2AX foci in raphe neurons after CPT treatment (Figure 3-10). Together, primary cultured LC neurons are sensitive to CPT-induced DNA damage and deficient to repair the damage.


A
DAPI
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R24 h
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Figure 3-9. CPT induces the DDR in DBH-positive cultured neurons. Cultured LC neurons were treated with CPT (10 μM) for 2 h, then continued to grow in the media without CPT for 24 h, 48 h and 72 h. Only DBH-positive cells were counted. (A) Significant γH2AX foci were found in nuclei after CPT treatment. The number of γH2AX-positive cells was reduced gradually within 72 h. Images were taken with 60× magnification. Percent of γH2AX positive cells were shown in (B). At least 100 cells from three random chosen views were counted. (C) γH2AX foci in DBH-positive cells. Enlarged images from yellow boxes in (A), yellow arrows indicated γH2AX foci in nuclei. Blue: DAPI, Green: DBH, Red: γH2AX. $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$, compared to the control. $^{\#}p<0.0001$, compared to CPT.

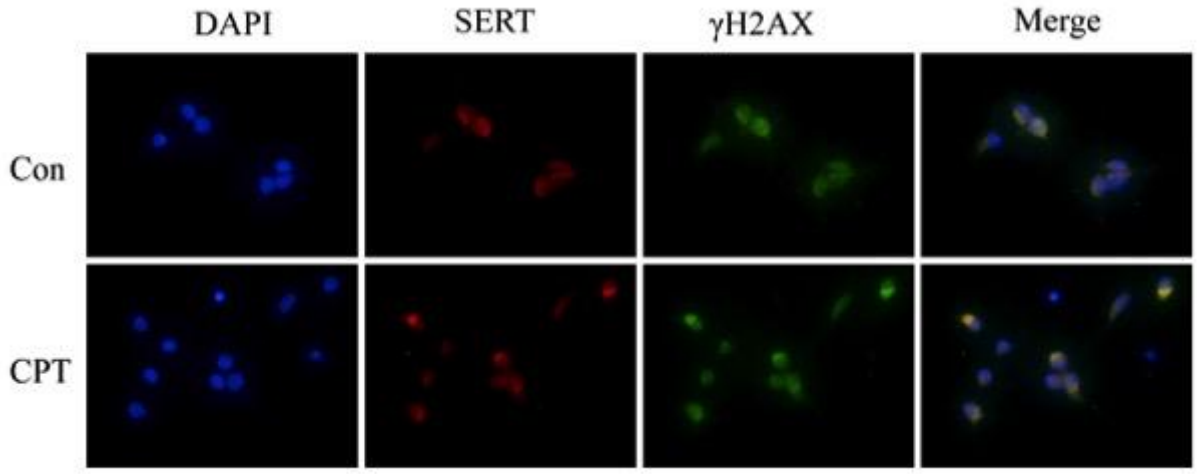

Figure 3-10. γH2AX foci are not detected in SERT-positive cultured neurons. Raphe neurons were treated with CPT (10 μM) for 2 h, then IFAs were performed. Only SERT-positive cells were counted. No significant γH2AX foci were found in nucleus after CPT treatment. Images were taken with 60× magnification. Blue: DAPI, Red: SERT, Green: γH2AX.

LC Neurons Accumulate DSP4-induced DNA Damage

DSP4 has been thought to damage the nerve terminals originating from the LC. Most of the cell bodies of the LC neurons were intact for weeks despite substantial loss of NE nerve terminals in the projection field. The reason for such different effects is unknown. Our previous study showed that DSP4 could induce the DDR in SY5Y cells (Wang et al. 2014). To

test the effect of DSP4 on primary cultured LC and raphe neurons, cells were treated with DSP4 (50 μM) for 24 h, and IFAs were performed. As shown in Figure 3-11B, γH2AX foci were detected in DSP4-treated primary cultured LC neurons after DSP4 treatment. However, no significant γH2AX foci were found in primary cultured raphe neurons after DSP4 treatment (Figure 3-11A).

Discussion

Our data showed that SH-SY5Y cells are sensitive to accumulate CPT-induced DNA damage (Figure 3-2, 3-3, 3-4, 3-5, 3-6) and deficient to repair the damage (Figure 3-7, 3-8, 3-9, 3-10), compared to that in fibroblast cells. γH2AX and p-p53^{ser15} were measured as the DDR markers to evaluate CPT-induced DNA damage and repair rate. In order to conform these *in vitro* findings in *in vivo* conditions, we used primary LC and raphe neuronal cultures, which would facilitate the investigation of various properties of these noradrenergic and non-noradrenergic neurons under well-controlled conditions and mimicked the *in vivo* conditions. Our data show that LC neurons are more sensitive to DNA damage induced by CPT or DSP4 than raphe neurons (Figure 3-11, 3-12, 3-13). These pathological characteristics are consistent with the *in vivo* observation, which demonstrate that degeneration of noradrenergic neurons occurs in an early stage in the brain of neurodegenerative disease patients (Bondareff et al. 1982, Mann and Yates 1983, Mann et al. 1983, Bondareff et al. 1987, German et al. 1992, Weinshenker 2008).

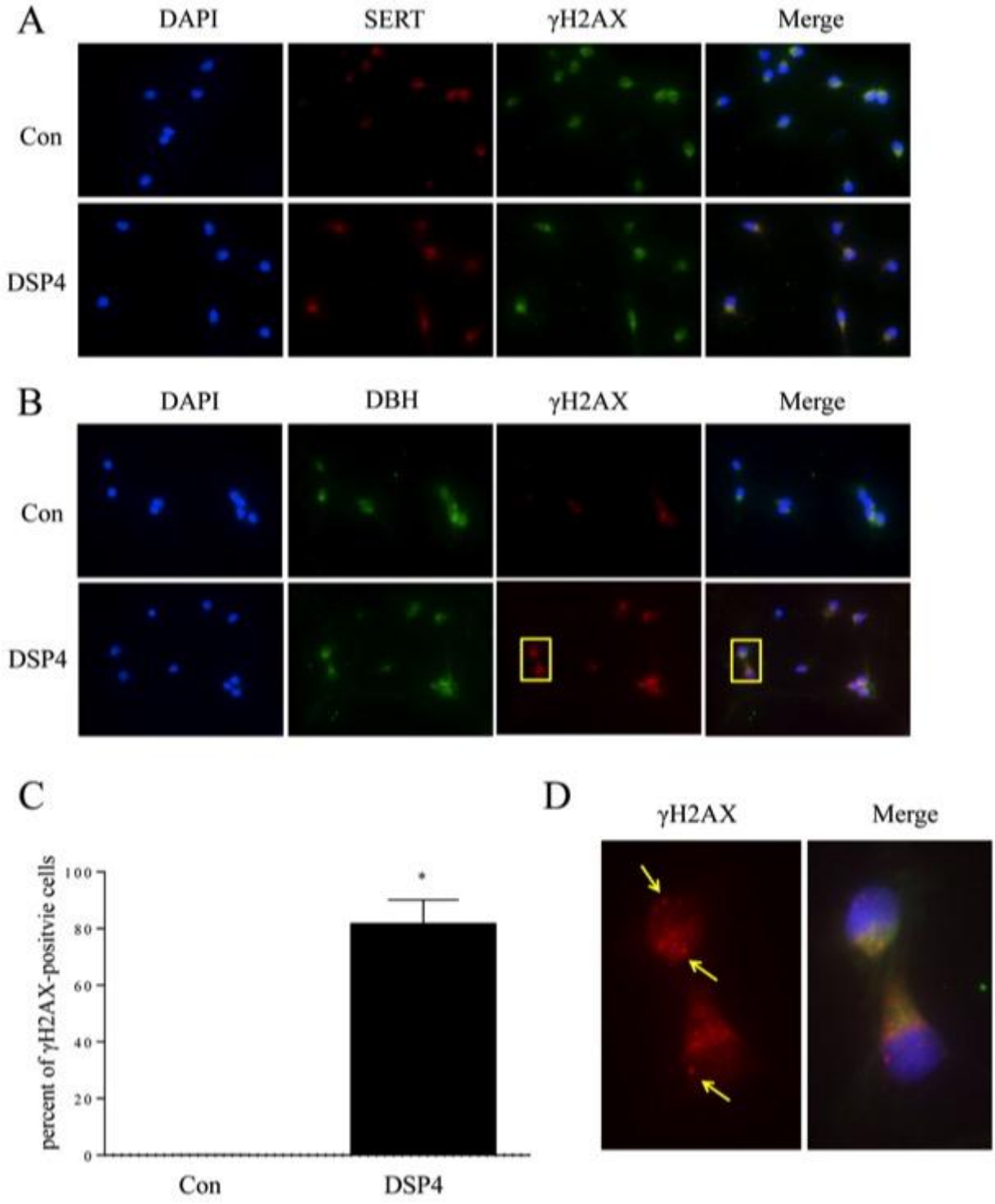
A
DAPI
SERT
γH2AX
Merge
Con
DSP4
B
DAPI
DBH
γH2AX
Merge
Con
DSP4
C
percent of γH2AX-positvie cells
100
80
60
40
20
0
*
Con
DSP4
D
γH2AX
Merge

Figure 3-11. γH2AX foci were detected in DBH- but not SERT-positive cultured neurons after DSP4 treatment. Cultured neurons were treated with DSP4 (50 μM) for 24 h, and then IFAs were performed. Only DBH- or SERT-positive cells were counted. Images were taken with 60× magnification. (A) No γH2AX foci were detected in SERT-positive cultured raphe neurons after DSP4 treatment. (B) γH2AX foci were detected in DBH-positive cultured LC neurons after DSP4 treatment. (C) Quantity analysis data for percent of in γH2AX-positive cells in (B). At least 100 cells from three random chosen views were counted. (D) γH2AX foci in DBH-positive cells. Enlarged images from yellow boxes from B, yellow arrows indicated γH2AX foci in nuclei. In Figure 3-11A, Blue: DAPI, Red: SERT, Green: γH2AX. In Figure 3-11B and 3-11D, Blue: DAPI, Green: DBH, Red: γH2AX. $^{*}p<0.0001$, compared to control.

γH2AX and p-p53^{ser15} were measured as DDR markers to evaluate the response to DNA damage, as well as the repair rate for CPT- or DSP4-induced DNA damage. As a very earlier step in the cellular response to DNA damage, histone H2AX is phosphorylated at C-terminal serine residues (Ser136 and Ser139) (Rogakou et al. 1998). This phosphorylated H2AX, called γH2AX, and γH2AX foci can be detected within minutes after the introduction of DNA damages (Kang et al. 2005). H2AX phosphorylation has an important role in the initiation of DNA repair (Downs et al. 2000), including the recruitment of DNA repair or damage-signaling factors to the damage site, maintenance of the integrity of the DDR, and bringing the broken DNA ends closer (Bassing and Alt 2004, Thiriet and Hayes 2005). Known as a classic "gatekeeper" of cellular fate, p53 tumor suppressor protein is activated in response to genotoxic stress-induced DNA damage (May and May 1999), among which phosphorylation of serine-15 is one of the key responses (Hammond et al. 2002). P-p53^{ser15} levels can be rapidly increased several folds after DNA damage. Phosphorylated p53 has been linked to DNA repair processes, such as activation of DNA repair and stalling the cell cycle (Offer et al. 1999, Okorokov 2003, Ford 2005). Therefore, the formation and disappearance of γH2AX and p-p53^{ser15} can be used to represent a relative time process in CPT-induced DDR and repair rate.

LC is important to regulate the amount of norepinephrine in the brain. A deficiency in the noradrenergic system of the brain originating from LC cell loss is an early pathological indicator in the progression of several neurodegenerative diseases, including PD and AD (Marien et al. 2004). Aging-related cognitive decline is associated with accumulation of nDNA damage in neurons (Rutten et al. 2003, Rutten et al. 2007), and this effect is due to insufficient nDNA repair. Our data shows that noradrenergic SH-SY5Y cells and LC neurons are sensitive to CPT-treatment, which results in accumulation of DNA damage (Figure 3-2, 3-3, 3-4). Deficiency in DNA repair could be one possible explanation of SH-SY5Y cell and LC neuron CPT sensitivity. The repair of DNA damage is depend on functional repair system (Hickson et al. 1990). For example, it has been reported that cells without some DNA repair genes or DNA repair enzymes are hypersensitive to CPT and cannot repair CPT-induced DSBs (Nitiss and Wang 1988, Chatterjee et al. 1989). Therefore, noradrenergic SH-SY5Y cells and LC neurons may be relatively deficient in DNA repair system and consequently sensitive to DNA damage produced by CPT. This explanation is consistent with our data that SH-SY5Y cells and LC neurons are deficient in repairing CPT-induced DNA damage (Figure 3-5, 3-6, 3-7, 3-8, 3-9).

Additionally, the literature on oxidative stress induced DNA damage lead to cell cycle arrest (Migliore and Coppede 2002). For example, human H_2O_2-treated fibroblasts undergo either cell cycle arrest or apoptosis (Chen et al. 2000). The majority of the apoptotic fibroblasts were found in the S phase, whereas growth-arrested cells were predominantly accumulated in the G1 or the G2/M phase (Chen et al. 2000). This apoptotic death of fibroblasts in the S phase is consistent with the death of neurons that have aberrant cell cycle activity and express S-phase proteins. Dorsal root ganglion neurons go to apoptosis in the S phase (ElShamy et al. 1998), and the apoptotic neurons express S-phase proteins (Folch et al. 2012). Hippocampal

pyramidal and basal forebrain neurons from AD brains show chromosomal duplication and die before mitosis, these are consistent with cell death in the S or G2 phase of the cell cycle (Nagy et al. 1997b). It was reported that insufficient nDNA repair system leads to accumulation of nDNA in neurons. So if SH-SY5Y cells were in S or G2/M phase when CPT was added, these cells might respond severely with much more γH2AX foci in nuclei. This hypothesis also can be used to explain our data in Figure 3-6A. The number of γH2AX-positive cells decreased at R24 h then increased at R48 h compared to R24 h. This effect might be explained as at R24 h, the number of γH2AX-positive cells dramatically decreased due to cells with high amounts of DNA damage went to apoptosis. At R48 h, the number of γH2AX-positive cells still decreased but higher than that at R24 h, this indicated that cells with lower amount of DNA damage recruited γH2AX to repair the damage. To measure the number of survived cells at each time point and test if cells with more γH2AX are in S or G2/M phase, these further experiments will help to explain why a dramatically decreased expression of γH2AX after wash-out CPT at 24 h.

In Figure 3-10, under neutral condition, CPT-induced DNA damage was detected in fibroblast cells not in SH-SY5Y cells, which indicates that CPT does not induce DSBs in SH-SY5Y cells. However, CPT induced-DNA damage could be detected in both SH-SY5Y and fibroblast cells under alkaline condition, which suggests that CPT induces SSBs in both cell lines. Toxicity of CPT is primarily a result of conversion of SSBs into DSBs during the S phase when the replication fork collides with the cleavage complexes formed by DNA and Topo I-CPT complex. Therefore, our data suggest that SH-SY5Y probably has special repair system, which blocks CPT-induced DSB formation.

CPT induces DNA DSBs during DNA synthesis (S phase), suggesting that this agent

should not be toxic to non-dividing cells, such as neurons. However, CPT induces significant and dose-dependent cell death of post-mitotic rat cortical neurons and its neurotoxic activity also was found in cultured cerebellar granule neurons (Morris and Geller 1996, Uday Bhanu and Kondapi 2010). Taken together, these observations indicate that CPT also exhibits significant toxicity toward neuronal cells *in vitro*. This could explain the results in Figure 3-9 and 3-11B, which CPT induces DDR in LC neurons. In the present study, exposure of the primary cultures from rat raphe to CPT (Figure 3-10) and DSP4 (Figure 3-11A) did not cause obvious DDR. Although to date there is no report about the effects of CPT on serotonergic neurons *in vitro* or *in vivo*, the result of DSP4 is in agreement with previous studies in that DSP4 did not change the amount of 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid in the hippocampus (Jackisch et al. 2008) and dorsal raphe nucleus (Cassano et al. 2009). Also, previous studies have demonstrated that DSP4 treatment of Fischer 344 rats affects only noradrenergic neurons, leaving serotonergic and dopaminergic neurons intact (Chrobak et al. 1985, Martin and Elgin 1988).

In summary, in the present study, SH-SY5Y cells and primary cultures from rat LC are sensitive to neurotoxins CPT- or DSP4-induced DNA damage, and deficient in repairing the damage, compared to fibroblast cells and raphe neurons, respectively. These pathological characteristics may be consistent with the *in vivo* observation that degeneration of noradrenergic neurons occurs earlier than other neuronal systems in the brain of neurodegenerative diseases. The present study may serve as an initial effort to explore the molecular mechanisms underlying pathophysiological alterations of LC neurons in PD and AD.

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CHAPTER 4

THE EFFECTS OF ANTIDEPRESSANTS ON DSP4-/CPT-INDUCED DNA DAMAGE RESPONSE IN NEUROBLASTOMA SH-SY5Y CELLS

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Abstract

DNA damage is a form of cell stress and injury. An increased systemic DNA damage is related to the pathogenic development of neurodegenerative diseases. Also, depression occurs in a relatively high percentage of patients suffered from degenerative diseases, for whom antidepressants are often used to relieve depression symptoms. To date, however, few studies have elucidated why different groups of antidepressants have the similar effects on relieving depression. Previously, we demonstrate that neurotoxins DSP4 and CPT induce the DDR in SH-SY5Y cells. SH-SY5Y cells are predominately arrested in S and G2/M phases with DSP4 treatment. The current study shows that some antidepressants reduce the DDR, which is induced by DSP4 or CPT in SH-SY5Y cells. Flow cytometry data demonstrate that selective

antidepressants protect cells from being arrested in S phase. These effects suggest that blocking DNA damage may be the common pharmacologic characteristic of antidepressants, which may explain why different antidepressants could alleviate depression symptoms in neurodegenerative patients.

Introduction

DNA damage is a form of cell stress and injury. An increased systemic DNA damage caused by neurotoxins, psychological and oxidative stress has been found to be related to the pathogenic development of neurodegenerative and psychiatric diseases (Martin 2008). Progressive neuronal DNA damage in aging brains has been closely linked with the onset of neurodegenerative disorders (Lindahl 1993). Brain is one of the most important organs, but studies of DNA transactions were neglected for a long time. This is because adult brain cells are considered in low levels of DNA synthesis and repair (Subba Rao 2007). However, increased evidence shows that oxidative stress in the brain affects the brain's DNA repair pathways and genomic stability. Deficiency in DNA repair system has been linked to cognitive decline with aging-related diseases, but the mechanisms that protect neurons from genotoxic stress remain unclear (Dobbin et al. 2013).

DSP4 has widely been used as a noradrenergic neurotoxin to construct AD and PD animal models. Ross first reported the effects of DSP4 on norepinephrine levels in the peripheral and central noradrenergic system several decades ago (Ross 1976). It was hypothesized that DSP4 selectively damages noradrenergic projections originating from the LC

by interacting with the norepinephrine reuptake system and depleting intracellular norepinephrine, finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom et al. 1985, Dooley et al. 1987, Howard et al. 1990, Prieto and Giralt 2001).

Aberrant cell cycle activity also has been detected during the progression of neurodegenerative conditions. Oxidative DNA damage is correlated with cell cycle arrest (Migliore and Coppede 2002). For example, human H_2O_2-treated fibroblasts undergo either cell cycle arrest or apoptosis (Chen et al. 2000). The majority of the apoptotic fibroblasts were found in the S phase, whereas growth-arrested cells were predominantly accumulated in the G1 or the G2/M phase (Chen et al. 2000). This apoptotic death of fibroblasts in the S phase is consistent with the death of neurons that have aberrant cell cycle activity and express S-phase proteins. Dorsal root ganglion neurons go to apoptosis in the S phase (ElShamy et al. 1998), and the apoptotic neurons express S-phase proteins (Folch et al. 2012). Hippocampal pyramidal and basal forebrain neurons from AD brains show chromosomal duplication and die before mitosis, these are consistent with cell death in the S or the G2 phase of the cell cycle (Nagy et al. 1997b). Some neurotoxins can arrest the cell cycle in different phases (Klein and Ackerman 2003). We demonstrate that DSP4 induces the DDR in SH-SY5Y cells and DSP4 treatment results in cell cycle arrest predominantly in the S (Wang et al. 2014) and the G2/M phase. CPT is found to induce cell death of post-mitotic rat cortical neurons *in vitro* (Morris and Geller 1996) and neurotoxic activity of CPT also was found in cultured cerebellar granule neurons (Uday Bhanu and Kondapi 2010). We demonstrated in Chapter 3 that CPT-induced DDR occurred in primary cultured LC and raphe neurons *in vitro* (Wang et al. 2014).

The common behavioral symptoms of neurodegenerative disorders include depression, mood swings, and social withdrawal. The process of neurodegeneration is not well understood,

so there is no known cure for this group of diseases. Current therapeutic approaches are limited to disease managements and symptomatic relief. Depression symptoms often accompany neurodegenerative disorders, which could be relieved by using antidepressants. Antidepressants are drugs used for the treatment of major depression disorder and other conditions (Briley and Moret 1993, Martin 2008). They can be used alone or in combination with other medications. For example, depression in patients with PD can be alleviated by the NRI reboxetine (McNamara and Durso 2006).

The most important classes of antidepressants are the SSRIs (Geddes and Cipriani 2004), SNRIs, TCAs and MAOIs (Table 1-1). Antidepressants are often used to treat patients suffering from depression, however, few studies have shown why these drugs or combination of drugs help to alleviate depression symptoms. Furthermore, neurodegenerative diseases always accompany depression symptoms for which antidepressants are often prescribed. On the other side, DNA damage is associated with the pathophysiological process of neurodegenerative diseases and some psychiatric diseases. Therefore, in the present study, we tried to examine whether antidepressants influence neurotoxins DSP4- and CPT-induced DNA damage. The present results demonstrated that most tested antidepressants could reduce the DDR induced by DSP4 or CPT.

Materials and Methods

Cell Culture and Drug Exposure

The human neuroblastoma SH-SY5Y cells were used in these experiments. Cells were maintained in a 1:1 mix of RPMI 1640 and F12 media, which was supplemented with 10 %

heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in humidified air containing 5% CO_2. Culture medium and supplements were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Cells were seeded into 6-well or 100-mm plates. Drug exposures were started after 24 h of each subculture. DSP4 (Sigma, St Louis, MO, USA) was dissolved in distilled water at 50 mM, then diluted with culture media and added to cells to a final concentration of 50 µM, alone or in combination with antidepressants for the times as indicated in the text. CPT was dissolved in 10 mM dimethyl sulfoxide , then diluted with culture media and added to cells to a final concentration of 10 µM, alone or in combination with antidepressants for the times as indicated in the text. Different antidepressants were used in this study: fluoxetine (1 and 5 µM), reboxetine (1 and 5 µM), desipramine (1 and 5 µM), paroxetine (1 and 5 µM), imipramine (50 and 100 µM), amitricyclin (10 and 50 µM), deprenyl (50 and 100 µM), and pargyline (1, 5, 10, 50 µM). Antidepressants were dissolved in water. The selection of the concentration of DSP4 was based on our previous data (Wang et al. 2014). The concentration of CPT and antidepressants were based on published papers (Lai and Yu 1997, Leskiewicz et al. 2013, Serrano et al. 2013). Only SH-SY5Y cells prior to passage 15 were used. Cell viability was determined by exclusion of trypan blue dye; cell viability was 90–95% in the untreated cells.

Western Blotting Analysis

Whole cell extracts for western blot analysis were prepared by lysing cells in ice-cold Nonidet P-40 (NP-40; Sigma, St Louis, MO, USA) buffer (0.5% NP-40, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) for 30 min, after which nuclei and cell debris were removed by centrifugation at 12,000 rpm for 10 min at 4^{o}C. An equal volume of sodium dodecyl sulfate (SDS) gel-loading buffer then was added to the supernatant and the samples were denatured at

70°C for 5 min. Protein concentrations in cell extracts were quantified prior to addition of the loading buffer with the Micro BCA Protein Assay Kit (Thermo Science, Rockford, IL USA). Proteins (40 µg) were electrophoretically separated on a 10% or a 15% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK). For protein detection, the blots were probed with anti-γH2AX antibody (1:1,000 dilution, Bethyl Laboratories, Inc., Montgomery, TX, USA), or an anti-p-p53^{ser15} antibody (1:1,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5,000 dilution; Amersham Life Sciences, Buckinghamshire, UK) was used as the secondary antibody. The membranes were subjected to enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) or super enhanced ECL (Sigma Chemical Co., St Louis, MO, USA) and autoradiography. To check for equal loading and transfer, the membranes were reprobed with a mouse IgG monoclonal anti-ß-actin antibody (1:5,000 dilution, Amersham Life Sciences, Buckinghamshire, UK).

Flow Cytometry

SH-SY5Y cells were sub-cultured in a 6-well plate at 2×10^4 cells/well, then cells were pretreated with fluoxetine (1 and 5 µM), reboxetine (1 and 5 µM), desiprimine (DMI, 1 and 5 µM), paroxetine (1 and 5 µM), imipramine (50 and 100 µM), amitricyclin (10 and 50 µM), and deprenyl (50 and 100 µM) for 1 h, and then DSP4 (5 µM) was added for another 24 h. Cells were washed with 37°C warm phosphate buffered saline (PBS), 200 µl of 0.25 % trypsin–EDTA (Gibco, Carlsbad, CA, USA) was added per well, and the plate was incubated at 37°C for 1 min. The trypsin was aspirated off and the cells were suspended with 1 ml ice-cold PBS containing 0.5 mM EDTA (PBSE). The cells were collected by centrifugation at 3,000

rpm for 10 min at 4°C and fixed by slowly adding 1 ml ice-cold 70% ethanol to resuspend the cells. The cells were stored at -20°C overnight, and then collected at 3,000 rpm for 10 min at 4°C. The cells were washed once with ice-cold PBSE, then recentrifuged and resuspended in 300 µl of freshly prepared PBSE containing 20 µg/ml propidium iodide (Sigma, St Louis, MO, USA) and 20 µg/ml DNase-free RNase A (Invitrogen, Grand Island, NY, USA). After incubation at 37°C for 30 min, the cells were analyzed on the BD Accuri C6 flow cytometer. The population of G0/G1, S, and G2/M was determined using C6 Flow Cytometer Software. The results are expressed as percentage of the attached cells in each phase.

Statistics

All experimental data are presented in the text and graphs as the mean ± SEM. The number of replicates is enumerated in the figure legends. Data were analyzed by using one-way analysis of variance (ANOVA) in GraphPad Prism.

Results

DSP4-induced DNA Damage Response Can be Reduced by Some Antidepressants

Our previous study demonstrated that DSP4 as a neurotoxin induced DDR in SH-SY5Y cells (Wang et al. 2014). To test the effects of antidepressants on DDR induced by DSP4, in this study, SH-SY5Y cells were pretreated with different antidepressants for 1 h before 4 h DSP4 (50 µM) treatment. Antidepressants include SSRIs (fluoxetine and paroxetine), NRI (reboxetine), TCAs (imipramine, amitriptyline and desipramine) and MAOIs (deprenyl and pargyline). As shown in figures 4-1, 4-2, 4-3 and 4-4, SSRIs, NRI and TCAs when given alone

to SH-SY5Y cells in the absence of DSP4 did not induce any significant changes in protein levels of γH2AX and p-p53^{ser15}, as measured by western blottings. However, most tested antidepressants significantly attenuated DSP4-induced DDR levels, as compared to DSP4 alone groups (for effects on γH2AX: desipramine: $F_{5,12}$=379.6, p<0.0001; imipramine: $F_{5,12}$=46.9, p<0.0001; amitriptyline: $F_{5,18}$=36.2, p<0.0001; fluoxetine: $F_{5,24}$=249.6, p<0.0001; paroxetine: $F_{5,15}$=387.2, p<0.0001; reboxetine: $F_{5,16}$=203.8, p<0.0001; deprenyl: $F_{5,18}$=200.0, p<0.0001. For effects on p-p53^{ser15}: desipramine: $F_{5,16}$=111.3, p<0.0001; imipramine: $F_{5,16}$=285.2, p<0.0001; amitriptyline: $F_{5,12}$=369.4, p<0.0001; fluoxetine: $F_{5,18}$=81.5, p<0.0001; paroxetine: $F_{5,12}$=159.1, p<0.0001; reboxetine: $F_{5,12}$=119.3, p<0.0001; deprenyl: $F_{5,12}$=212.9, p<0.0001). Further analyses revealed some specific outcomes: 1) The alleviatory effects of some antidepressants on DSP4-induced DDR seem to be concentration-dependent. For example, while both concentrations of desipramine (1 and 5 μM), imipramine (50 and 100 μM), amitriptyline (10 and 50 μM), fluoxetine (1 and 5 μM), paroxetine (1 and 5 μM), reboxetin (1 and 5 μM), and deprenyl (50 and 100 μM) significantly inhibited DSP4-induced increases of γH2AX and p-p53^{ser15}, and lower concentrations of desipramine (1 μM, Figures. 4-1A and 4-1B), fluoxetine (1 μM, Figs. 2A and 2B) and paroxetine (50 μM, Figures. 4-2E and 4-2F) showed more significant effects than their higher concentrations on reducing γH2AX and p-p53^{ser15} levels. Furthermore, the effect of amitriptyline (Figure 4-1J), reboxetine (Figure 4-3B) and deprenyl (Figure 4-4B) also reduced the p-p53^{ser15} levels in a concentration-dependent manner. 2) Both deprenyl and pargyline are MAOIs to inhibit the activity of monoamine oxidase, thus preventing the breakdown of monoamine neurotransmitters and thereby increasing their availability. While pargyline (1, 5, 10 and 50 μM) did not have any effects on DSP4-induced DDR in SH-SY5Y cells (Figure 4-4E), deprenyl (50 and100 μM) suppressed

DDR (Figs. 4-4A and 4-4B).

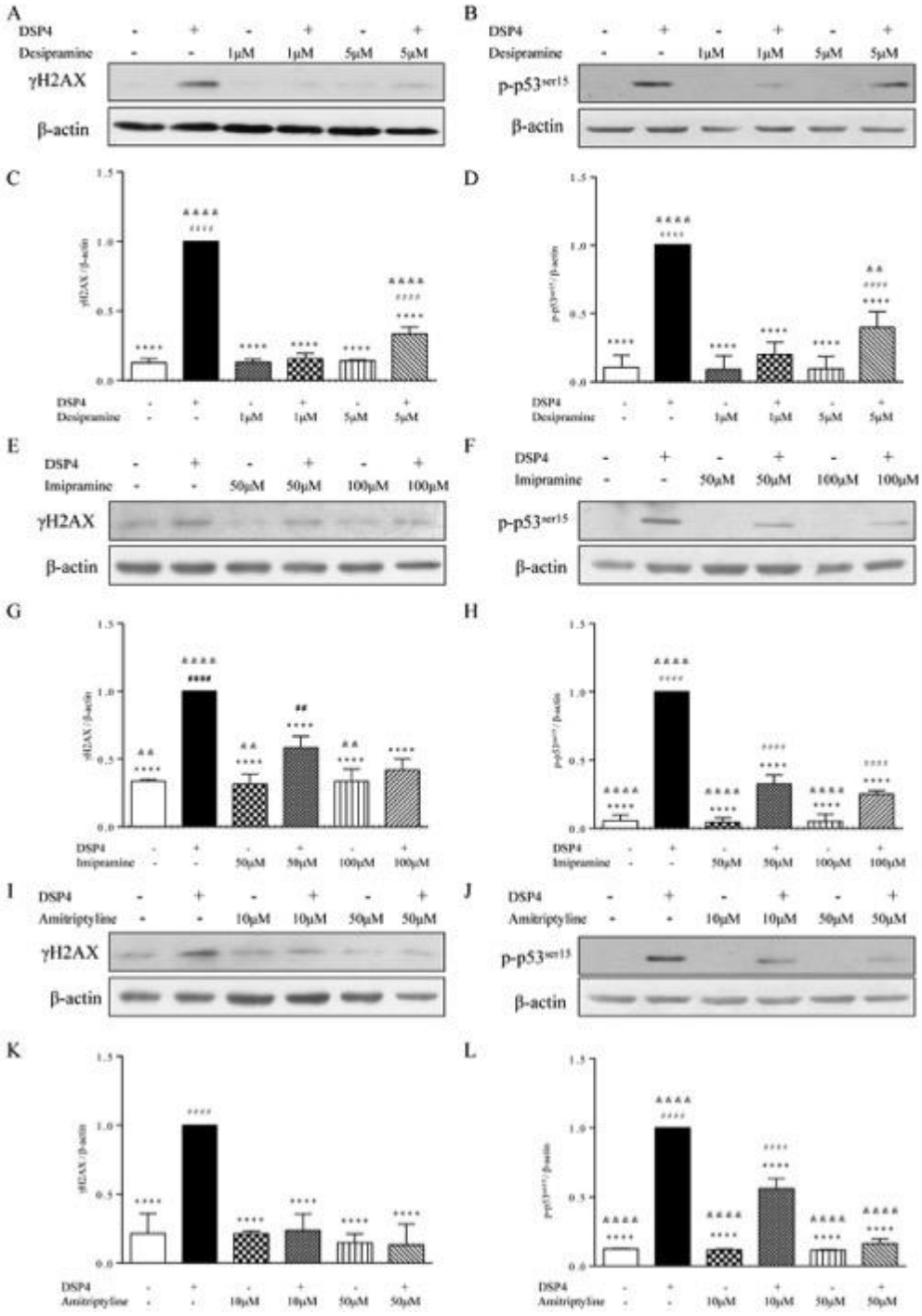

Figure 4-1. TCAs desipramine, imipramine and amitriptyline reduce DSP4-induced DDR in SH-SY5Y cells. Cells were pretreated with desipramine (1 and 5 μM), imipramine (50 and 100 μM), and amitriptyline (10 and 50 μM) for 1 h, then DSP4 (50 μM) was added for another 4 h. γH2AX and p-p53^{ser15} were used as DDR markers. Western blots data were shown

in (A), (B), (E), (F), (I) and (J). Quantified data analysis were shown in (C), (D), (G), (H), (K) and (L). $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{****}p<0.0001$, compared to the DSP4 group; $^{\&\&}P<0.01$, $^{\&\&\&\&}p<0.0001$, compared to desipramine (1 μM), imipramine (50 μM), or amitriptyline (10 μM).

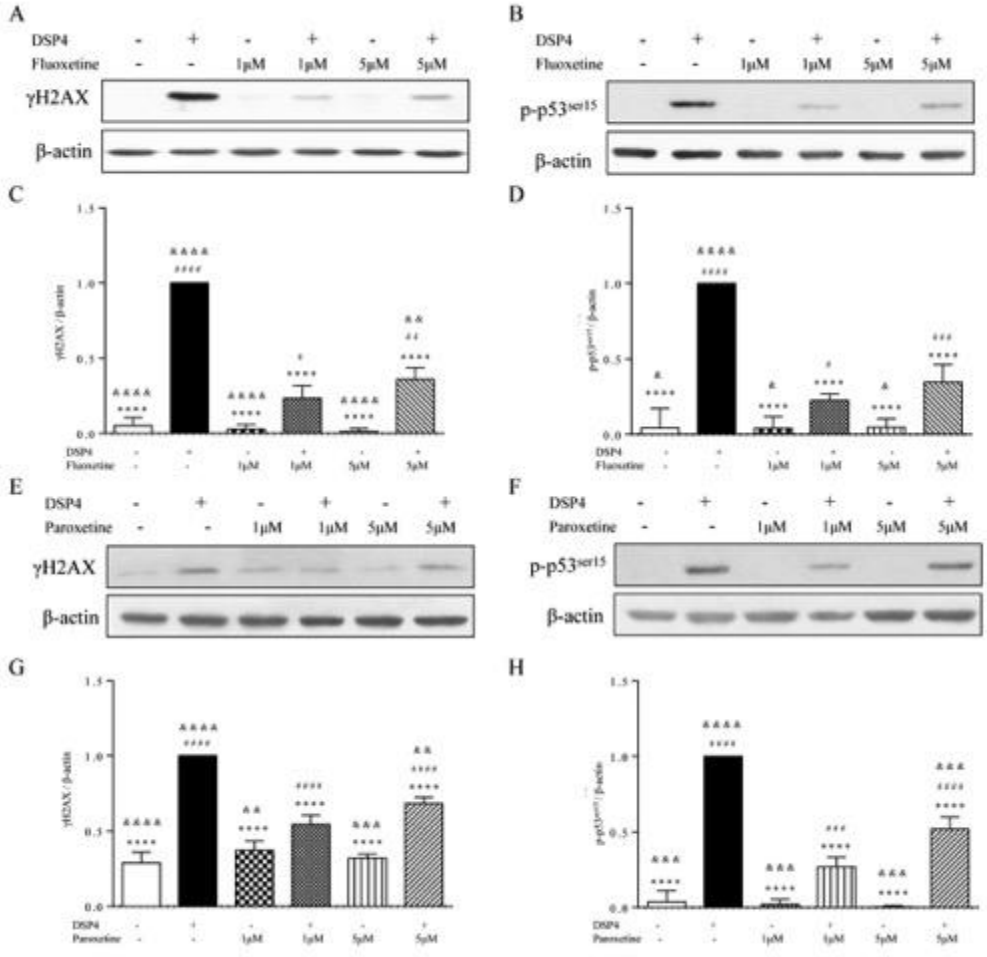

Figure 4-2. SSRIs antidepressants fluoxetine and paroxetine reduce DSP4-induced DDR in SH-SY5Y cells. Cells were pretreated with fluoxetine (1 and 5 μM) and paroxetine (1 and 5 μM) for 1 h, and then DSP4 (50 μM) was added for another 4 h. γH2AX and p-p53^{ser15} were used as DDR markers. Western blots data were shown in (A), (B), (E) and (F). Quantified analysis data were shown in (C), (D), (G) and (H). The graphic data represent averages obtained from 3-5 separate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{****}p<0.0001$, compared to the DSP4; $^{\&}p<0.05$, $^{\&\&}P<0.01$, $^{\&\&\&}p<0.001$, $^{\&\&\&\&}p<0.0001$, compared to groups of fluoxetine (1 μM), or paroxetine (1 μM).

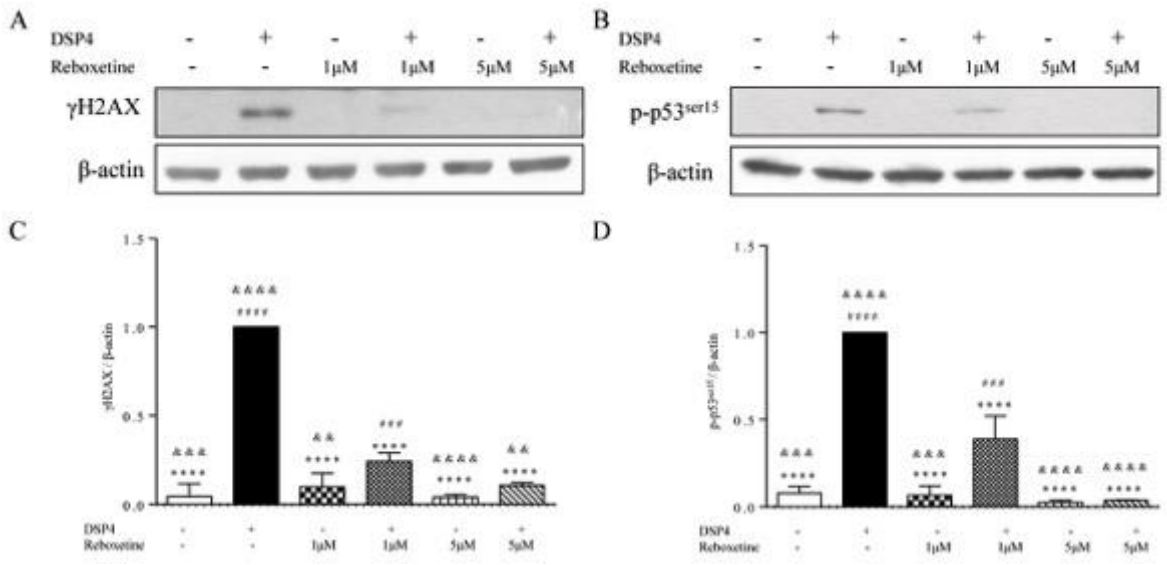

Figure 4-3. NRI antidepressant reboxetine reduces DSP4-induced DDR in SH-SY5Y cells. Cells were pretreated with reboxetine (1 and 5 μM) for 1 h, and then DSP4 (50 μM) was added for another 4 h. γH2AX and p-p53^{ser15} were used as DDR markers. Western blots data were shown in (A) and (B). Quantified analysis data were shown in (C) and (D). The graphic data represent averages obtained from 3-5 separate experiments. $^{###}p<0.001$, $^{####}p<0.0001$, compared to the control; $^{****}p<0.0001$, compared to the DSP4; $^{\&\&}P<0.01$, $^{\&\&\&}p<0.001$, $^{\&\&\&\&}p<0.0001$, compared to the group of reboxetine (1 μM).

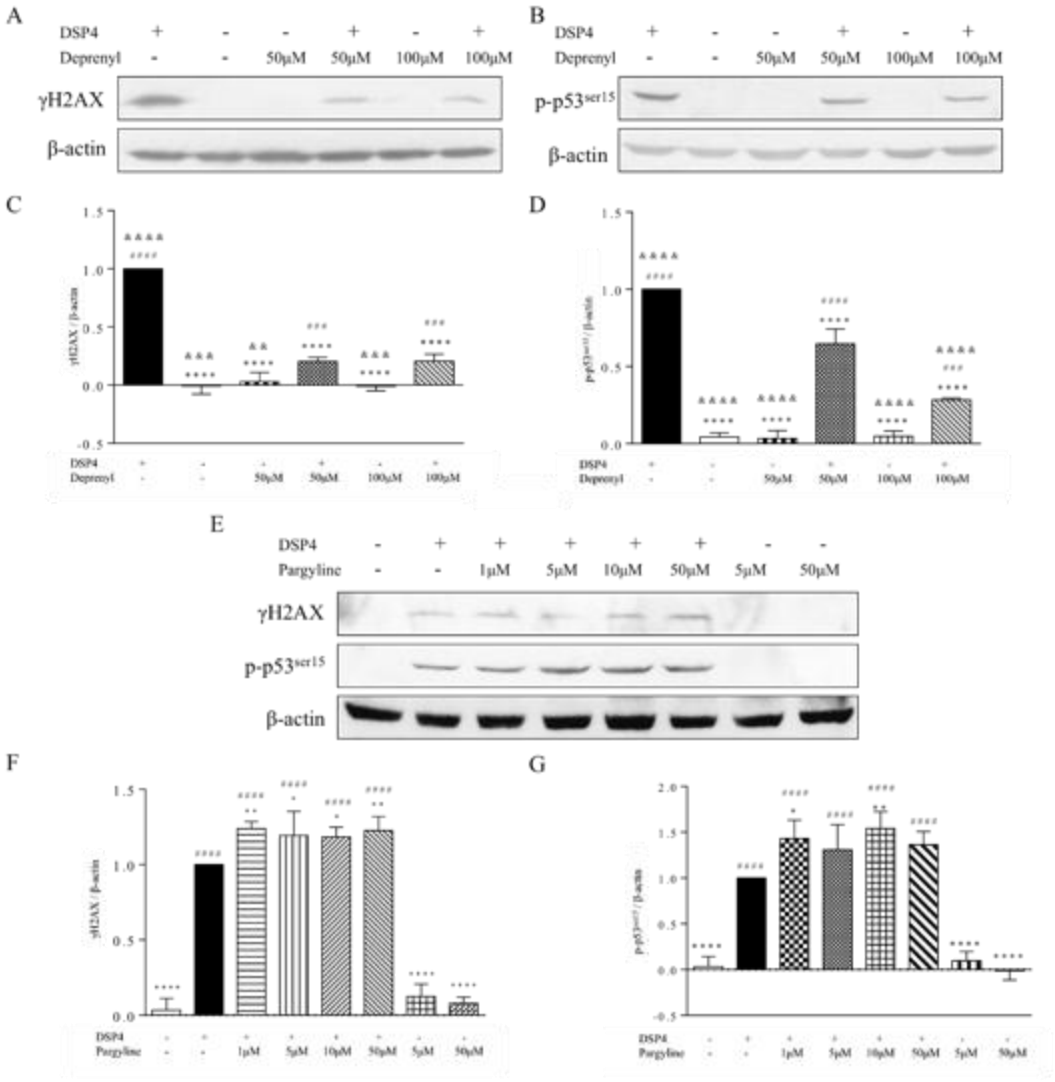

Figure 4-4. MAOI antidepressant deprenyl reduces DSP4-induced (DDR) in SH-SY5Y cells while pargyline does not. SH-SY5Y cells were pretreated with deprenyl (50 and 100 μM) or pargyline (1, 5, 10 and 50 μM) for 1 h, then DSP4 (50 μM) was added for another 4 h. γH2AX and p-p53^{ser15} were used as DDR markers. Western blots data were shown in (A), (B) and (E). Quantified analysis data were shown in (C), (D), (F) and (G). The graphic data represent averages obtained from 3-5 separate experiments. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$, compared to the DSP4.

Table 4-1. Summary of the effects of antidepressants on reducing γH2AX (Green) and p-p53^{ser15} (Blue) levels. This table shows two concentrations for each antidepressants. The effects of DSP4 on levels of γH2AX and p-p53^{ser15} are considered 100%, which is shown as +++. The effects of antidepressants are normalized by DSP4. -: 0-25%; +: 25-50%; ++: 50-75%; +++: 75-100%; ++++>100%.

A

Name	Lower Concentration		Higher Concentration	
	Unit (μM)			
DSP4	50	+++		
TCAs				
Desipramine	1	-	5	+
Imipramine	50	++	100	+
Amitriptyline	10	-	50	-
SSRIs				
Fluoxetine	1	+	5	+
Paroxetine	1	++	5	++
SNRIs				
Reboxetine	1	-	5	-
MAOIs				
Deprenyl	50	-	100	-
Parygyline	5	++++	50	++++

B

Name	Lower Concentration		Higher Concentration	
	Unit (μM)			
DSP4	50	+++		
TCAs				
Desipramine	1	-	5	+
Imipramine	50	+	100	-
Amitriptyline	10	++	50	-
SSRIs				
Fluoxetine	1	-	5	+
Paroxetine	1	+	5	++
SNRIs				
Reboxetine	1	+	5	-
MAOIs				
Deprenyl	50	++	100	+
Parygyline	5	++++	50	++++

Selected Antidepressants Reduce CPT-induced DNA Damage Response

CPT is commonly used as a DNA topo I inhibitor to induce DNA DSBs (Liu et al. 2000). CPT induces significant DDR in SH-SY5Y cells as early as 1 h (Figure 3-4, 3-6). We pretreated SH-SY5Y with antidepressants used above for 1 h then CPT (10 μM) was added for another 1 h. Interestingly, we only found that paroxetine (1 and 5 μM), imipramine (10 and 50 μM) and amitrycycline (50 and 100 μM) could attenuate CPT-induced DDR in SH-SY5Y cells, as shown by reduced levels of γH2AX and p-p53^{ser15} (Figure 4-5).

Effects of Selected Antidepressants on Protecting Cells from Arresting in S phase

Previously, our study showed that DSP4 could arrest SH-SY5Y cells in S (Wang et al. 2014) and G2 phases. Since G2/M phase was not significantly affected by antidepressants and DSP4, therefore we focused on discussing S phase arrest in Chapter 4. To test the effect of antidepressants on S phase arrest caused by DSP4, two parallel experiments were carried out. In the first experiment, cells were treated with different antidepressants alone for 25 h. In the second experiment, cells were pretreated with antidepressants for 1 h, and then DSP4 (5 μM) was added for another 24 h. Cells were collected and flow cytometric analyses were performed.

As shown in Figure 4-6, control cells with neither antidepressants nor DSP4 treatment, distributed 51.7/52.1 in G1 phase and 17.6/17.8 % in S phase (See Con in Figures 4-6 and 4-7). For the groups treated with 5 μM DSP4 only, cells were distributed 40.1/41.0 in G1 phase and 30.6/30.4% in S phase (See DSP4 in Figures 4-6 and 4-7). Compared to control cells, DSP4 significantly decreased G1- and increased S-phase cell populations. In contrast, compared to the magnitude of changes in G1 and S phases caused by DSP4, effects of most tested antidepressants did not affect G1 and S phases (Figure 4-6).

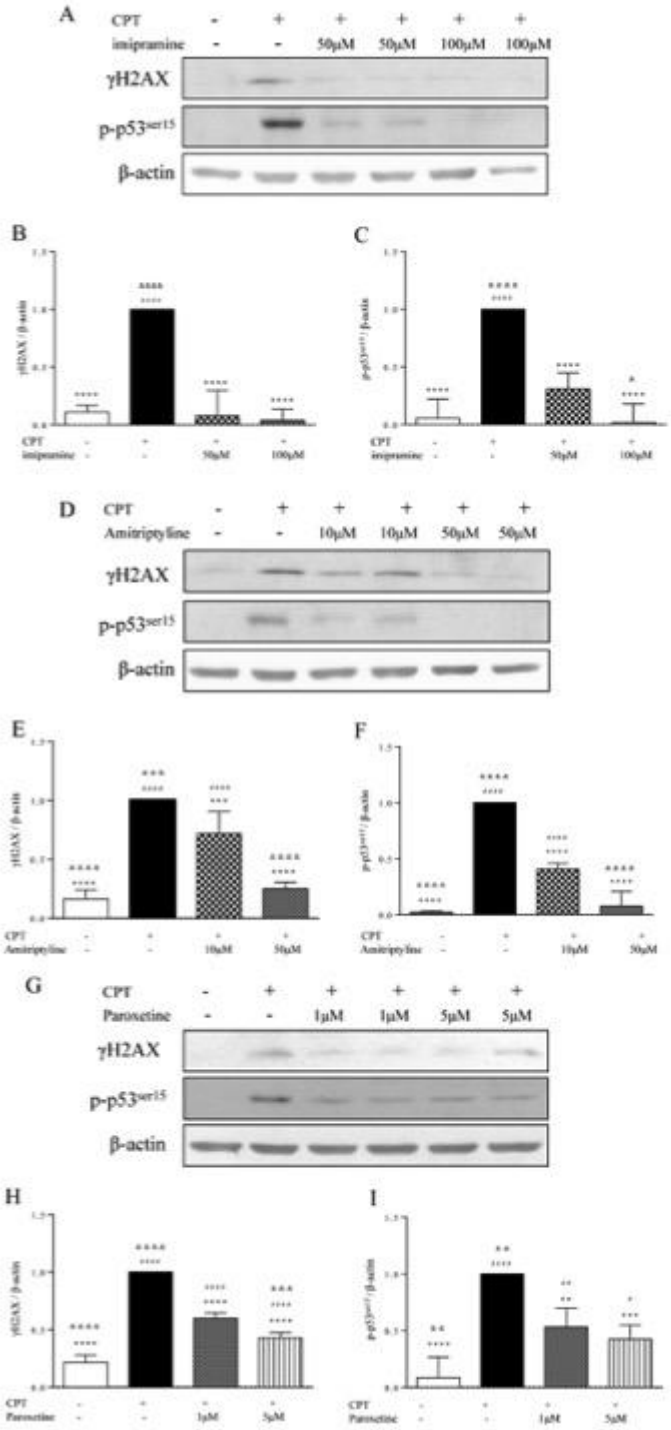

Figure 4-5. CPT-induced DNA damage response is reduced by imipramine, amitriptyline and paroxetine. SH-SY5Y cells were pretreated with imipramine (50 and 100 μM), amitriptyline (10 and 50 μM) or paroxetine (1 and 5 μM) for 1 h, then CPT (10 μM) was added for another 1 h. γH2AX and p-p53^{ser15} were used as DDR markers. Western blots data

were shown in (A), (D) and (G). Quantified analysis data were shown in (B), (C), (E), (F), (H) and (I). The graphic data represent averages obtained from 3-5 separate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, compared to the DSP4; $^{\&}p<0.05$, $^{\&\&\&}p<0.001$, $^{\&\&\&\&}p<0.0001$, compared to of imipramine (50 μM), amitriptyline (10 μM), or paroxetine (1 μM).

DSP4 actives intra-S and G2/M checkpoints, therefore cells go through G1 phase and accumulate in S (Wang et al. 2014) and G2 phases. Flow cytometric data show significantly decreased G1 phase and increased S phase population after DSP4 treatment in SH-SY5Y cells. Interestingly, as shown in Figure 4-7 and flow-cytometric histograms in Figure S-1, effects of DSP4 on changing of cells distribution partially were decreased when co-treated with some antidepressants. After SH-SY5Y cells co-treated with some antidepressants, the effects of increased G1 and decreased S phases population were detected (Figure 4-7). However, 1 μM fluoxetine, 50 and 100 μM deprenyl, 5 and 10 μM pargyline, they did not show significant effects on G1 population. In addition, co-treatment of antidepressants still showed increased S phase population except desipramine (1 and 5 μM) and reboxetine (1 and 5 μM), compared to control. However, compared to DSP4, significantly decreased S phase population except 50 μM deprenyl was detected with antidepressants co-treatment. A summary of antidepressants' effects was shown in Table 4-2. These data suggested that some antidepressants could protect cells from arresting in S phase and increasing cell population in G1 phase.

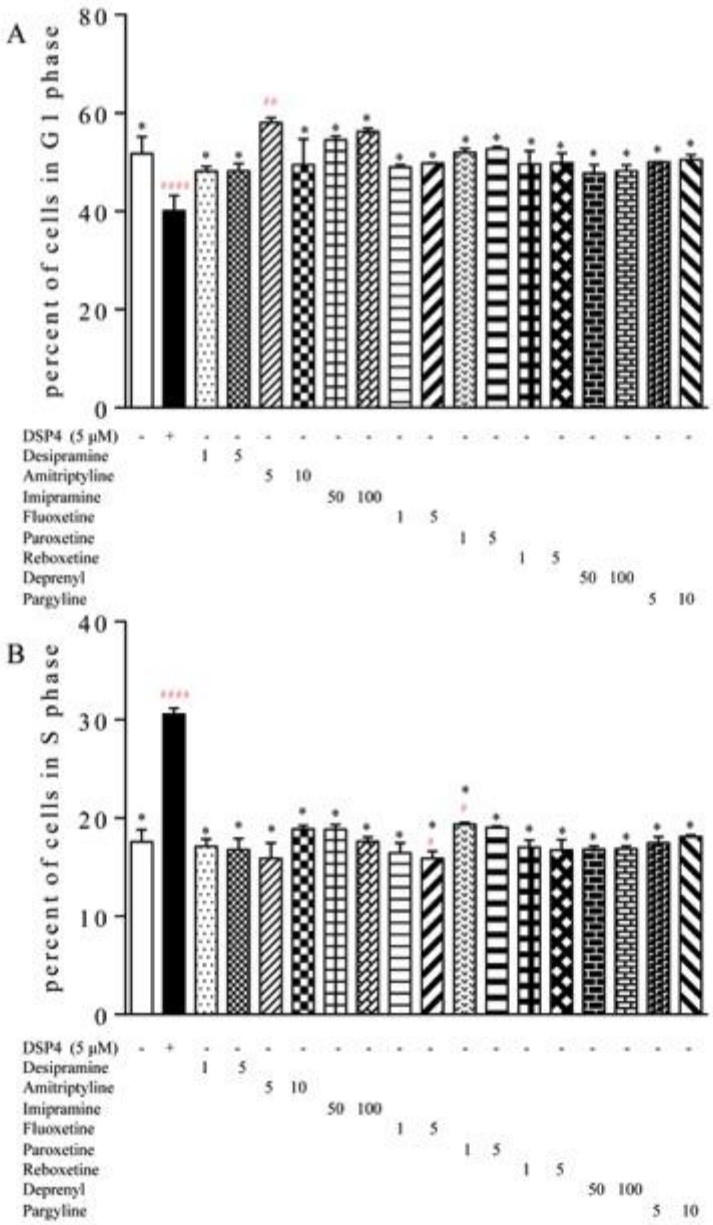

Figure 4-6. Effects of some antidepressants on cell cycle in SH-SY5Y cells. Cells were pretreated with imipramine (50 and 100 μM), amitriptyline (5 and 10 μM), desipramine (1 and 5 μM), fluoxetine (1 and 5 μM), paroxetine (1 and 5 μM), reboxetine (1 and 5μM) and deprenyl (50 and 100 μM) for 25 h. The percents of cells in G1 and S phases were shown in (A) and (B), respectively. Each bar represents data obtained from 3 to 6 separate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{*}p<0.0001$, compared to the DSP4.

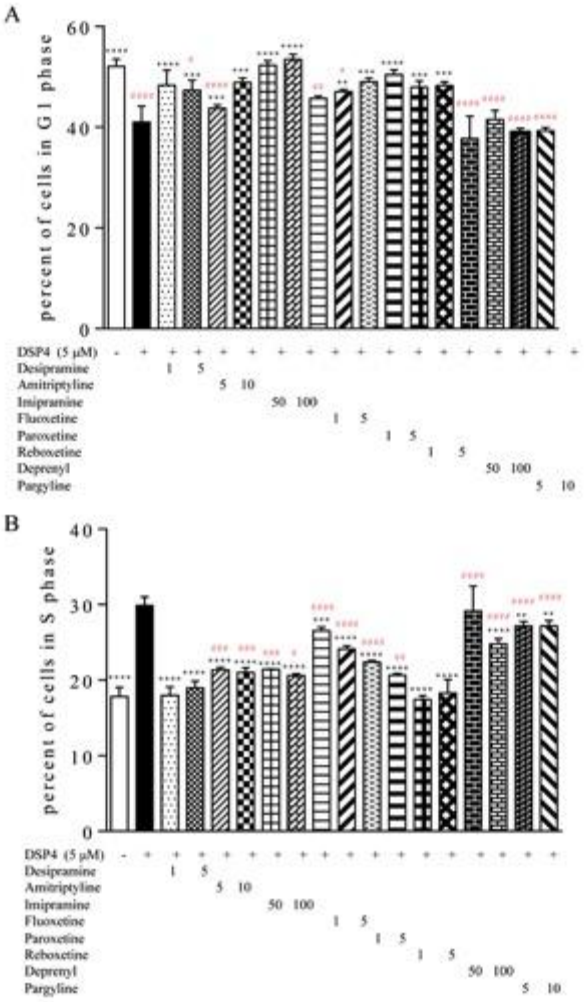

Figure 4-7. Effects of some antidepressants on cell cycle transitions in SH-SY5Y cells with DSP4 co-treatment. SH-SY5Y cells were pretreated with imipramine (50 and 100 μM), amitriptyline (10 and 50 μM), desipramine (1 and 5 μM), fluoxetine (1 and 5 μM), paroxetine (1 and 5 μM), reboxetine (1 and 5μM) and deprenyl (50 and 100 μM) for 1 h, and then DSP4 (5 μM) was added for 24 h. The percents of cells in G1 and S phase were shown in (A) and (B), respectively. Each bar represents data obtained from 3 to 7 separate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$, compared to the control; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, compared to the DSP4.

Table 4-2. Summary of the effects of some antidepressants on cell cycle transitions in SH-SY5Y cells with (B) or without (A) DSP4 co-treatment. ↑/↓$p<0.05$, ↑↑/↓↓$p<0.01$, ↑↑↑/↓↓↓$p<0.001$, ↑↑↑↑/↓↓↓↓$p<0.0001$, compared to the control; ↑/↓$p<0.05$, ↑↑/↓↓$p<0.01$, ↑↑↑/↓↓↓$p<0.001$, ↑↑↑↑/↓↓↓↓$p<0.0001$, compared to the DSP4. Blank boxes show no significant difference.

A Antidepressants

Name	G1 phase	S phase	G2/M phase
DSP4 (5 μM)	↓↓↓↓	↑↑↑↑	
Desipramine (1 μM)			
Desipramine (5 μM)			
Amitriptyline (5μM)	↑↑		
Amitriptyline (10 μM)			
Imipramine (50 μM)			↓↓
Imipramine (100 μM)			↓↓
Fluoxetine (1 μM)			
Fluoxetine (5 μM)		↓	
Paroxetine (1 μM)		↑	
Paroxetine (5 μM)			
Reboxetine (1 μM)			
Reboxetien (5 μM)			
Deprenyl (50 μM)			
Depreenyl (100 μM)			↑
Pargyline (5 μM)			
Pargyline (10 μM)			

B Antidepressants + DSP4

Name	G1 phase		S phase		G2/M phase	
Con		↑↑↑↑		↓↓↓↓		
DSP4 (5 μM)	↓↓↓↓		↑↑↑↑			
Desipramine (1 μM)		↑↑↑↑		↓↓↓↓		
Desipramine (5 μM)	↓	↑↑↑		↓↓↓↓		
Amitriptyline (5μM)	↓↓↓↓	↑↑↑	↑↑↑	↓↓↓↓	↑↑↑↑	↑↑↑↑
Amitriptyline (10 μM)		↑↑↑	↑↑↑	↓↓↓↓	↑↑↑↑	↑↑↑↑
Imipramine (50 μM)		↑↑↑↑	↑↑↑	↓↓↓↓		
Imipramine (100 μM)		↑↑↑↑	↑	↓↓↓↓		
Fluoxetine (1 μM)	↓↓		↑↑↑↑	↓↓↓		
Fluoxetine (5 μM)	↓	↑↑	↑↑↑↑	↓↓↓↓		
Paroxetine (1 μM)		↑↑↑	↑↑↑↑	↓↓↓↓		
Paroxetine (5 μM)		↑↑↑↑	↑↑	↓↓↓↓		
Reboxetine (1 μM)		↑↑↑		↓↓↓↓		
Reboxetien (5 μM)		↑↑↑		↓↓↓↓		
Deprenyl (50 μM)	↓↓↓↓		↑↑↑↑			
Deprenyl (100 μM)	↓↓↓↓		↑↑↑↑	↓↓↓↓	↑↑	↑
Pargyline (5 μM)	↓↓↓↓		↑↑↑↑	↓↓	↑↑	↑
Pargyline (10 μM)	↓↓↓↓		↑↑↑↑	↓↓	↑↑	↑

Discussion

In the present study, we attempt to elucidate potential new mechanisms of antidepressants, SH-SY5Y cells co-treated with SSRIs, NRI and TCAs and MAOIs antidepressants and neurotoxins DSP4 and CPT. Our results showed that pretreatment of SH-SY5Y cells with antidepressants resulted in protection effects on reducing DSP4-induced DDR. For example, exposure of cells to TCAs SSRIs, NRI and deprenyl blocked DSP4-induced elevation of γH2AX and p-p53^{ser15} (Table 4-1). Furthermore, pretreatment of cells with imipramine, amitriptyline and paroxetine also showed a similar protective effect on CPT-induced DNA damage. Moreover, flow cytometric data showed that selective antidepressants could reduce the effects of DSP4-induced S-phase arrest in SH-SY5Y cells. These results reveal that although these tested antidepressants have different pharmacologic mechanisms regarding their clinical use, they may have a common feature to protect cells from DNA damage, specifically by protecting cells from S-phase arrest. This is because the majority of dead neurons are in S phase (Yang et al. 2001).

The common behavioral symptoms of neurodegenerative disorders include depression, mood swings, and social withdrawal. About 30-50% of AD patients have depression symptoms (Brown and Jahanshahi 1995, Cummings and Masterman 1999, Lee and Lyketsos 2003). Several pathological events have been explained that the coborbility may be due to depletion of the LC neurons (Zubenko and Moossy 1988). In Chapter 2, DSP4 has been found to reduce expression of DBH in SH-SY5Y cells, mediated by its action of DDR (Wang et al. 2014). Further study in Chapter 3 shows that primary-cultured LC neurons are sensitive to DSP4- and CPT-induced DNA damage. Recent post-mortem study demonstrated that major depression

disorder was associated with oxidative stress (Shelton et al. 2011) and accumulated oxidative DNA damage was detected in brain cells of AD (Kadioglu et al. 2004) and PD (Zhang et al. 1999) patients.

Antidepressants are used for the treatment of major depression disorder and depression symptoms in other diseases (Briley and Moret 1993, Martin 2008). They can be used alone or in combination with other medications in other diseases. Although antidepressant drugs have been clinically used in the treatment of depression for decades, the precise mechanism of their therapeutic action is still unclear. Currently, the pharmacological mechanisms of the most clinically used antidepressants are related to the "monoamine hypothesis" (Schildkraut 1965), which states that antidepressants, such as SSRIs, NRIs, TCAs and MAOIs, increase neurotransmitter levels, especially serotonin and norepinephrine, in the synapses. This action in turn restore the neurotransmission and functions of brains caused by deficiency of these neurotransmitters. However, this hypothesis has been challenged due to the conflict between rapid increases in serotonin and norepinephrine levels induced by antidepressants and the delayed appearance of therapeutic efficacy. Therefore, new molecular mechanisms are needed for antidepressant actions. In the present study, almost all tested antidepressants effectively ameliorate the DDR caused by neurotoxins, indicating that blocking neuronal damage, such as DNA damage, may be the common pharmacologic action of antidepressants. This notion is supported by some new observations related to the etiology of depression. For example, oxidative and nitrosative stress are involved in the pathophysiology of depression (Maes et al. 2009, Maes et al. 2011). DNA is perhaps one of the major targets for oxyradicals, therefore, oxidative stress may cause DNA damage. Furthermore, antidepressants may protect cells against neurotoxicity caused by several toxic compounds. For example, fluoxetine suppresses

kainic acid-induced neuronal loss in the rat hippocampus, which might be associated with its anti-inflammatory effects. It was reported that both R and S isomers of fluoxetine attenuated chronic neurodegeneration induced by a commonly used inflammogen lipopolysaccharide (Zhang et al. 2012). Moreover, some studies suggest that antidepressants and mood stabilizers may act as antioxidant mechanisms (Berk et al. 2011, Maes et al. 2011), and antioxidants have antidepressant properties (Berk et al. 2008, Scapagnini et al. 2012). Therefore, it is important to elucidate potential mechanisms of antidepressants for new drug target discovery in the treatment of depression.

It is important to note that in the present study, pargyline is the only antidepressant that did not inhibit the formation of DSP4- or CPT-induced DNA damage and cell cycle arrest. In contrast, the MAOI deprenyl exhibited the similar effects on DNA damage as other antidepressants. Currently, we do not have a satisfactory explanation for this difference. One potential explanation is that at least monoamine inhibition activity of these MAOIs does not account for the effect of blocking DDR. Rather, it depends upon the other specific pharmacologic activity of these compounds. It was reported that pretreatment with deprenyl prevented the effect of specific neurotoxins like DSP4. Deprenyl pretreatment prevented the depletion of norepinephrine induced by DSP4 in the rat hippocampus (Magyar and Haberle 1999). This could be due to the uptake inhibitory effect of deprenyl and mainly to its metabolite methylamphetamine, which is a more potent inhibitor of the re-uptake than the parent compound. Moreover, pretreatments of SH-SY5Y cells with imipramine, amitriptyline, desipramine, reboxetine, paroxetine, fluoxetine, and deprenyl showed protective effects on DSP4-induced DNA damage. However, only imipramine, amitriptyline and paroxetine showed protective effects on CPT-induced DNA damage. It is difficult to explain why DSP4 and CPT

have different responses to these antidepressants, since the mechanisms of DSP4 and CPT to induce DNA damage are not totally understood.

In Chapter 2, we showed that DSP4 decreased G1 and increased S phase cell population in SH-SY5Y cells. In this Chapter, our data showed that co-treatment some antidepressants with DSP4 increased G1 and decreased S phase cell population. Damage and loss of LC noradrenergic neurons is accelerated in certain progressive neurodegenerative diseases including AD (Mann and Yates 1983, Bondareff et al. 1987, German et al. 1992, Weinshenker 2008) and PD (Mann et al. 1983, Rommelfanger and Weinshenker 2007), representing an early pathological indicator of AD and PD. It is believed that a neuron loses its capacity to divide and differentiate once it is born. Differentiated neurons were considered to be irreversibly post-mitotic, however, some cell cycle proteins were found in neuronal-programmed apoptotic cells, such as cyclins and CDKs have been found to be up-regulated after exposure to severe conditions, such as oxidative stress (Kruman et al. 2004, Murray 2004 Currais et al. 2009). Cyclins, CDKs, and other cell cycle proteins can be expressed in the AD brain after exposuring to stress (Nagy et al. 1997a, Vincent et al. 1997, Smith et al. 1999). Flow cytometric data revealed that a significant increased S-phase neuron population after exposure to different genotoxic insults (Kruman et al. 2004). Also a significant percent of apoptotic neurons with incorporate BrdU indicated that neurons underwent apoptosis during S phase (Kruman et al. 2004). Our data indicated that antidepressants might play a role in preventing cell cycle activity in noradrenergic LC neurons, therefore to decrease LC neurons loss in AD and PD. LC dysfunction plays an important role in the development of neurodegenerative diseases, so the present data may provide experimental evidence for reasonable use of antidepressants in the neurodegenerative diseases to eliminate depression symptoms and DNA damage in the LC

region.

In summary, our data showed that selective antidepressants protected SH-SY5Y cells from DSP4- or CPT-induced DNA damage and cell cycle arrest, indicating a new potential mechanism of antidepressants. The effects of antidepressants against DNA damage can be used to explain their clinical uses to relieve depression symptoms in psychiatric and neurodegenerative diseases. Further exploration of underlying mechanism may shed light on the efforts to improve therapeutic strategies for treatment of these diseases.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Neurodegenerative diseases primarily affect in middle to late life period; therefore, the incidence increases as the population ages. It is estimated that approximately 1 in 5 Americans will be over the age of 65, and more than 12 million will suffer from age-related neurodegenerative diseases including AD and PD by the year 2030. Neurodegenerative diseases are incurable and debilitating conditions that result in progressive loss of neuronal structure and function and neuronal death. It is reported that LC cell numbers are reduced during normal aging, as are brain norepinephrine levels (Marien et al. 2004). Accumulated oxidative DNA damage was found in brain cells of patients with AD (Kadioglu et al. 2004) and PD (Zhang et al. 1999). Damage and loss of LC noradrenergic neurons is accelerated in certain progressive neurodegenerative diseases including AD (Mann et al. 1983, Bondareff et al. 1987, German et al. 1992, Weinshenker 2008) and PD (Mann et al. 1983, Rommelfanger et al. 2007), representing an early pathological indicator of these diseases. Both increased DNA damage and decreased DNA repair were detected in AD patients (Fishel et al. 2007), and oxidative stress and DNA damage also are implicated in PD (Fukae et al. 2005). The number of LC neurons during aging and some neurodegenerative disorders might be reduced because of a high amount of nDNA damage and their deficiency in repairing the damage.

In Chapter 3, our data show that noradrenergic SH-SY5Y cells and LC neurons are sensitive to CPT-induced DNA damage. γH2AX and p-p53^{ser15} were measured as DDR

markers that are persistent in noradrenergic SH-SY5Y cells and LC neurons, indicating a deficiency in repairing the DNA damage caused by CPT. These pathological characteristics may be consistent with the *in vivo* observation that degeneration of noradrenergic neurons occurs earlier in the brains of patients with neurodegenerative diseases. The present study may serve as an initial trial to explore the molecular mechanisms underlying pathophysiological alterations of LC neurons in PD and AD. Also, SH-SY5Y cells should be considered as an ideal noradrenergic *in vitro* model. Further studies are preferred to elucidate whether CPT or DSP4 have similar effects on LC *in vivo*, which will provide strong supportive evidence for the current hypothesis.

DSP4 has been used as a noradrenergic neurotoxin in the development of AD or PD animal models with LC degeneration (Heneka et al. 2006, Rey et al. 2012). It is hypothesized that neurotoxin DSP4 selectively damages noradrenergic projections originating from the LC by interacting with the norepinephrine reuptake system and depleting intracellular norepinephrine, finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom et al. 1985, Dooley et al. 1987, Howard et al. 1990, Prieto et al. 2001). Our data in Chapter 2 support this hypothesis. The expression levels of DBH and NET were down-regulated by DSP4 in SH-SY5Y cells. However, limited data have been reported from *in vitro* studies on the mechanism of DSP4-induced neuronal degeneration. Thus, elucidating the molecular mechanism by which DSP4 evokes its neurodegenerative effect may promote the effort to find novel therapeutic strategies for treatment of degenerative diseases. The study in Chapter 2 of this dissertation also shows that DSP4 induces DNA SSBs and arrested cells in the S and the G2/M phases. According to Figure 2-5C, we primarily focused on discussing the S-phase arrest caused by DSP4 when we published the paper. In addition, the proportion of

cells remaining in the G2/M phase was relatively constant after DSP4 treatment. These cytometric results demonstrated that the S-phase and the G2/M-phase checkpoints were activated by DSP4 treatment of SH-SY5Y cells. This arrest resulted in cells transiting G1 phase or already in S phase to be accumulated in S phase, while those cells in G2/M phases remained there. In addition, as shown in Fig 2-6, arrested cells resumed cycle transit within 12 or 24 h after DSP4 removal. Although there still are more cells in S phase compared to the control, the proportion of cells in G1 phase returned to normal. Interestingly, after removal of DSP4 for 24 h, fewer cells were in G2 phase compared to the control group. These data indicate that DSP4-arrested cells were able to resume cell cycle transit after removal of DSP4. It is important for cells to delay mitotic entry, which allows cells to repair any DNA damage that may have accumulated after S phase. Our data are consistent with the death of neurons that have aberrant cell cycle activity. Dorsal root ganglion neurons go to apoptosis in the S phase (ElShamy et al. 1998), and the apoptotic neurons express S-phase proteins (Folch et al. 2012). Hippocampal pyramidal and basal forebrain neurons from AD brains show chromosomal duplication and die before mitosis. These are consistent with cell death in the S or the G2 phase of the cell cycle (Nagy et al. 1997b). In addition, DNA damage in apoptotic neurons is dependent on ATM activation, which suggests that neurons are affected by the same cell cycle checkpoints that regulate apoptosis in other cell types (Kruman 2004). Our data showed that DSP4 treatment activated the ATM pathway as part of the DDR (Wang et al. 2014). Taken together, these results suggest that down-regulation of the noradrenergic phenotypes caused by DSP4 stems from the DSP4-induced DDR and replication stress, which affected the transcriptional rate of the DBH and NET.

Based on our findings in Chapters 2, we demonstrate that DSP4 induces the DDR in

SH-SY5Y cells. How does DSP4 cause DDR? Oxygen radicals are involved in many biochemical activities of cells such as signaling transduction and gene transcription (Uttara et al. 2009). Although oxygen is imperative for life, imbalanced oxidative metabolism and excess production of ROS lead to several disorders such as AD and PD. Toxicity of these free radicals contributes to damage of proteins and DNA, inflammation, and subsequent cellular apoptosis. The most common cellular free radicals are hydroxyl (OH·), superoxide (O_2–·), and nitric monoxide (NO·). One of the hypotheses indicates that DSP4 depletes intracellular norepinephrine to induce LC degeneration. Norepinephrine is synthesized inside the nerve axon and stored in vesicles (Figure 1-1). Many enzymes are involved in the process of norepinephrine synthesis, such as tyrosine hydroxylase, DOPA decarboxylase, and DBH. These processes lead to formation of some ROS. ROS are a product of processes taking place during the oxygen metabolism. Therefore, we might explain that DSP4 induces oxidative stress, which damages DNA because of excessive ROS formation due to excessive intracellular norepinephrine synthesis. To elucidate this explanation, we need to test if DSP4 induces oxidative DNA damage in SH-SY5Y cells. We could treat SH-SY5Y and fibroblast cells with DSP4 and measure 8-hydroxyguanosine, which is a classical marker of oxidative damage to DNA. We expect to see a higher level of 8-hydroxyguanosine in SH-SY5Y cells than in fibroblast cells, because fibroblast cells do not express noradrenergic phenotypes and no norepinephrine is synthesized. Moreover, it has been reported that stress hormones such as norepinephrine can increase DNA damage (Flint et al. 2007). It has been proposed that the mechanism why norepinephrine induces DNA damage is by creation of ROS (Djelic et al. 2003). Both DSP4 hypotheses support that excessive norepinephrine is released extracellularly. So cells are exposed to a higher concentration of norepinephrine, which induces oxidative

DNA damage.

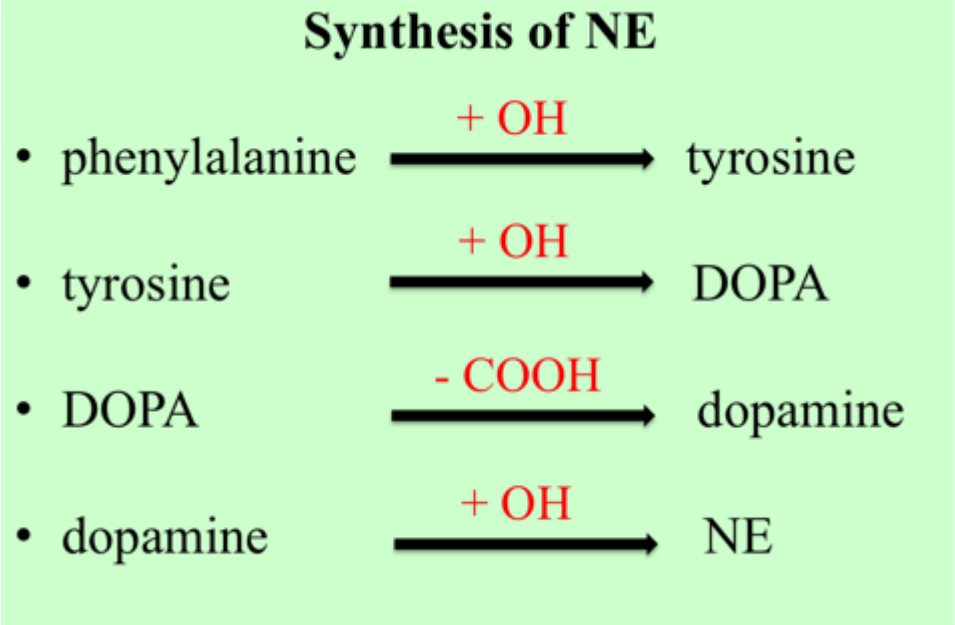

Figure 5-1. Enzymes involved in norepinephrine synthesis

The exact mechanisms of neurodegeneration are still unknown, so there is no cure for neurodegenerative diseases. Therefore, it is urgent to find treatments and cures for neurodegenerative diseases. Depression symptoms often accompany neurodegenerative disorders. Antidepressants are used to treat major depression disorder (Briley et al. 1993, Martin 2008) and are clinically used to relieve depression symptoms in neurodegenerative patients. The "monoamine theory" of depression has been proposed for a long time, but the pathologies and mechanisms for depression disorders remain unclear. Within the last decade, increasing evidence showed oxidative/antioxidant effects of antidepressants and discussed the relevance of intracellular oxidative pathways in the pathophysiology of depression (Michel et al. 2007, Maes et al. 2009, Maes et al. 2011, Behr et al. 2012, Michel et al. 2012). It has been reported that some antidepressants could protect cells form oxidative stress. For example,

fluoxetine reduces oxidative stress in brain (Omar M.E. Abdel-Salam 2011), and desipramine's protective effect against ischemia/reperfusion-induced oxidative stress was found in mice (Gaur et al. 2010). Also, it has been reported that venlafaxine protects against stress-induced oxidative neuronal DNA damage (Abdel-Wahab et al. 2011), and deprenyl was found to protect neurons in the substantia nigra from oxidative stress (Wu et al. 1993). Considering that the pathophysiology of depression is not fully clarified, the present findings suggest that one important action of antidepressants that may contribute to therapeutic efficacy in the treatment of depression is protection from DNA damage. In Chapter 4, the experiments demonstrate that some antidepressants reduce DSP4-induced DDR in SH-SY5Y cells. These effects might be ascribed to the abilities of some antidepressants in scavenging hydroxyl radicals or up-regulating the expression of antioxidant defense enzymes. In all, the present findings that some antidepressants could protect cells from DSP4-induced DNA damage may add a new feature to the neuroprotective potency of these antidepressants. To test this hypothesis, we could pretreat SH-SY5Y cells with these antidepressants before DSP4 treatment. 8-hydroxyguanosine also can be used to measure oxidative stress level. We expect to see a lower level of 8-hydroxyguanosine after cotreatment with antidepressants.

In addition, it is shown that DSP4 irreversibly inhibits the human NET, SERT, and dopamine transporter (DAT) (Wenge et al. 2009). However, this inhibition includes a reversible component at the DAT and SERT but not at the NET. Thus, DSP4's high-affinity uptake through the NET and its interaction with NET may support it to be a noradrenergic neurotoxin. Moreover, although SSRIs and NRIs antidepressants are clinical important, key aspects of their molecular mechanisms such as the binding sites of these antidepressants are still unclear. Recently, it has been reported many antidepressants bind to key residues in S1

pocket; Sorensen et al. mutated 6 S1 residues in SERT and NET to determine the potency of some SSRIs and NRIs antidepressants (Sorensen et al. 2012). This finding can serve as a future model for studying the molecular mechanisms of antidepressants at SERT and NET. Another explanation for the protective effects of some antidepressants on reducing DSP4-induced DDR in SH-SY5Y cells is that these antidepressants compete with DSP4 for binding to the transporters. Further experiments are needed to demonstrate this explanation. For example, we need to test if antidepressants bind to DSP4 or antidepressants bind to the transporters to block DSP4 uptake.

In this ease, deprenyl and pargyline belong to the type B MAOIs. The enzyme in SH-SY5Y cells is only type A (Maruyama et al. 1997), so deprenyl and pargyline did not function as type B MAOIs. This is probably why deprenyl and pargyline have less effect on reducing DSP4-induced DDR in SH-SY5Y cells.

In summary (Figure 5-2), our data indicate that the neurotoxin DSP4 can be used to cause LC degeneration, which is because of its effects on inducing the DDR. Noradrenergic SH-SY5Y cells and LC neuron cultures are sensitive to DNA damage and deficient in repairing the damage, which might be an explanation of why LC degeneration is an early indicator of AD and PD. The DNA damage caused by DSP4 activates the ATM pathway and arrests cells in S and G2/M phases. Some antidepressants partially protect cells from DDR and cell cycle arrested caused by DSP4, which suggests a common mechanism of antidepressants to explain their clinical use to ameliorate depression symptom in AD or PD.

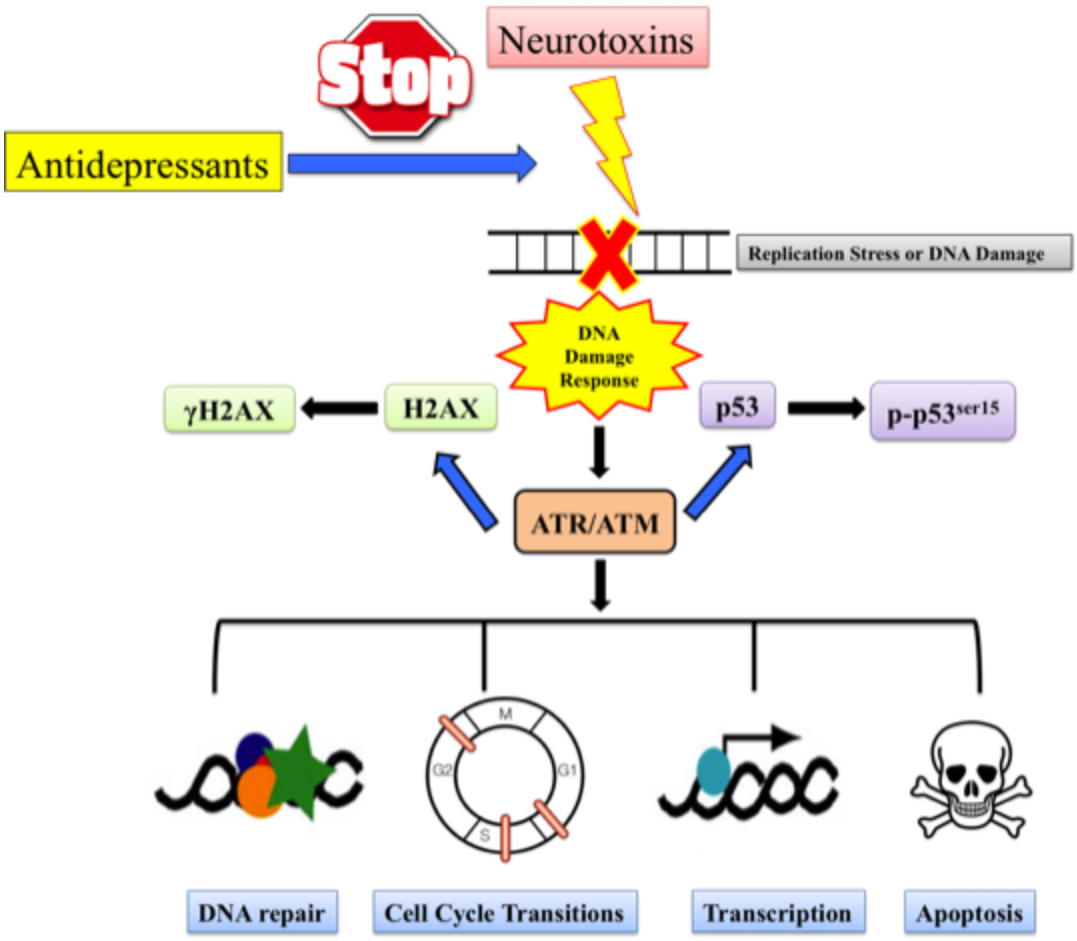

Figure 5-2. Proposed mechanisms of neurotoxins-induced DNA damage response. The presence of neurotoxin leads to replicative stress or DNA damage, which further results in activation of the ataxia-telangiectasia-mutated (ATM) protein kinase or ataxia telangiectasia and Rad3-related (ATR) protein kinase. ATM or ATR phosphorylate downstream targets; including p53 and the histone H2AX. In this way, ATM/ATR can influence cell cycle transitiitions and DNA damage response, transcription, in addition to cell death through apoptosis. The orange bars in cell cycle transitions indicate the three main cell-cycle checkpoints. Selective antidepressants reduce neurotoxins-induced DNA damage response.

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APPENDICES

APPENDIX A

SUPPLEMENTAL FIGURES

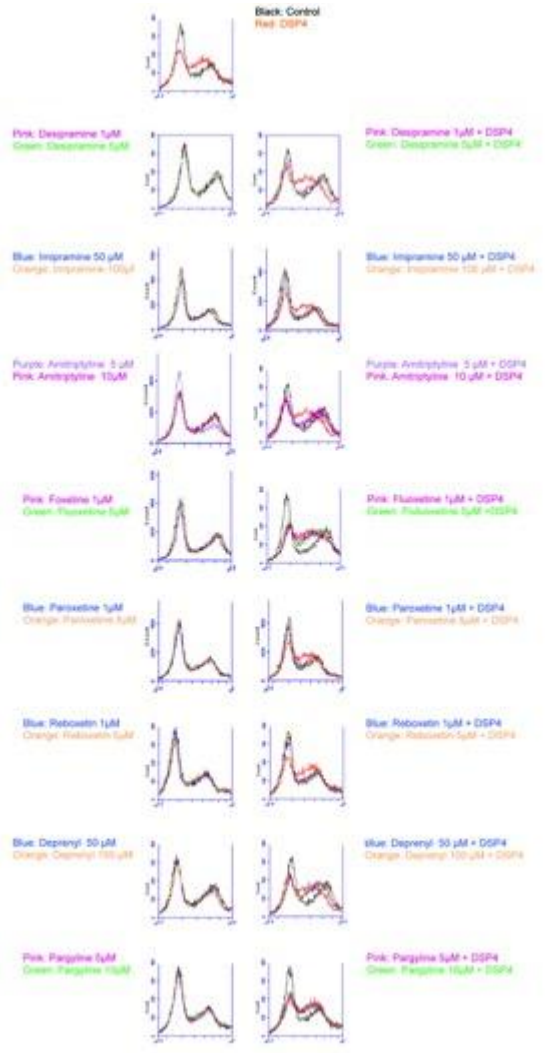

Figure S-1. Representative flow-cytometric histograms show effects of antidepressants on cell cycle with or without DSP4 co-treatment. Different colors show different drugs treatments.

APPENDIX B

ABBREVIATIONS

AD, Alzheimer's Disease

ATM, Ataxia telangiectasia mutated

ATR, Ataxia telangiectasia mutated and RAD 3-related

CDKs, cyclin-dependent kinases

CPT, camptothecin 132

DAPI, 4',6-diamidino-2-phenylindole

DAT, dopamine transporter

DBH, dopamine β-hydroxylase

DDR, DNA damage response

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DIV, day *in vitro*

DSBs, double-strand breaks

DSP4, N-(2-chloroethyl)-Nethyl-2-bromobenzylamine

EDTA, ethylenediaminetetraacetic acid

FBS, fetal bovine serum

HBSS, Hank's balanced salt solution

HD, Huntington's Disease

IFA, immunofluoresce assay

LC, locus coeruleus

MAOIs, monoamine oxidase inhibitors

nDNA, nuclear DNA

NE, norepinephrine

NET, norepinephrine transporter

NO·, nitric monoxide

NRIs, norepinephrine reuptake inhibitors

O_2–·, superoxide

OH·, hydroxyl

PBS, phosphate buffered saline

PD, Parkinson's Disease

ROS, reactive oxygen species

RPA, replication protein A

RPMI, Roswell Park Memorial Institute

RNA, ribonucleic acid

SERT, serotonin transporter

SSRIs, selective serotonin reuptake inhibitors

SDS-PAGE, sodium docecyl sulfate-polyacrylamide gel electrophoresis

topo I: topoisomerase I

APPENDIX C

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	Zha Q, Wang Y, Fan Y, Zhu MY (2011) Dexamethasone-induced up-regulation of the human norepinephrine transporter involves the glucocorticoid receptor and increased binding of C/EBP-beta to the proximal promoter of norepinephrine transporter. J Neurochem 119:654-663.
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Wang Y, Hilton BA., Cui K, Zhu MY, The effects of antidepressants on DSP4/CPT-induced DNA damage response in neuroblastoma SH-SY5Y cells (submitted to Journal of Neurobiochemistry).

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