Published in final edited form as:

Neurotox Res. 2015 May ; 27(4): 368–383. doi:10.1007/s12640-015-9521-4.

Neurotoxin-induced DNA damage is persistent in SH-SY5Y cells and LC neurons

Yan Wang, Phillip R. Musich, Kui Cui, Yue Zou, and Meng-Yang Zhu

Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA

Abstract

Degeneration of the noradrenergic neurons has been reported in the brain of patients suffering from neurodegenerative diseases. However, their pathologic characteristics during the neurodegenerative course and underlying mechanisms remain to be elucidated. In the present study, we used the neurotoxin camptothecin (CPT) to induce the DNA damage response in neuroblastoma SH-SY5Y cells, normal fibroblast cells, and primarily cultured LC and raphe neurons to examine cellular responses and repair capabilities after neurotoxin exposure. To our knowledge, the present study is the first to show that noradrenergic SH-SY5Y cells are more sensitive to CPT-induced DNA damage and deficient in DNA repair, as compared to fibroblast cells. Furthermore, similar to SH-SY5Y cells, primarily cultured LC neurons are more sensitive to CPT-induced DNA damage and show a deficiency in repairing this damage. Moreover, while N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) exposure also results in DNA damage in cultured LC neurons, neither CPT nor DSP4 induce DNA damage in neuronal cultures from the raphe nuclei. Taken together, noradrenergic SH-SY5Y cells and LC neurons are sensitive to CPT-induced DNA damage and exhibit a repair deficiency, providing a mechanistic explanation for the pathologic characteristics of LC degeneration when facing endogenous and environmental DNA-damaging insults *in vivo*.

Keywords

DNA damage; γ H2AX; p-p53^{ser15}; Noradrenergic neurons; Neurotoxin; Primary cultures; Degeneration

Introduction

The locus coeruleus (LC), a small nucleus located in the pons, is the main source of brain norepinephrine (NE) especially for the hippocampus and forebrain (Maeda 2000). The activity of LC neurons is considered to be involved in numerous important functions including response to stress, attention, emotion, motivation, decision making, learning and memory (Usher *et al.* 1999). It is reported that LC neuronal numbers decrease during normal aging (Mann 1983, Mann *et al.* 1983) and in aging-related diseases (Chan-Palay 1991a,

German *et al.* 1992). Damage and loss of LC noradrenergic neurons are accelerated in certain progressive neurodegenerative diseases such as Alzheimer's Diseases (AD)(Mann *et al.* 1983, Bondareff *et al.* 1987, German *et al.* 1992, Grudzien *et al.* 2007) and Parkinson's Diseases (PD)(Mann & Yates 1983, Arima & Akashi 1990, Chan-Palay 1991b, Forno 1996), which are early pathological indicators of these diseases. The greater neuronal loss was observed in the LC (83% loss in AD; 68% loss in PD) compared with other subcortical nuclei (the nucleus basalis and substantia nigra pars compact) (Lyness *et al.* 2003, Zarow *et al.* 2003), and correlated to a reduced level of NE in the brain (Adolfsson *et al.* 1979, Palmer & DeKosky 1993). However, despite extensive studies of AD and PD, it remains unclear why degeneration of the LC neurons precedes those neurons observed in other subcortical nuclei in these diseases.

It was reported that aging-related diseases are mainly caused by accumulation of nuclear DNA (nDNA) damage in neurons due to insufficient nDNA repair. In the brain there are a large number of non-proliferative neuronal cells, which are vulnerable to defective DNA repair. Deficiencies in repairing DNA damage usually leads to accumulation of DNA lesions; the latter might be considered as the cause of the neuropathology in several neurodegenerative disorders. Certain neurons with a high amount of nDNA damage, like Purkinje cells in the rodent brain, would be removed during physiological aging, while other neurons with less nDNA damage may persist in the brain (Brasnjevic *et al.* 2008). The molecular and cellular mechanisms of the selective neuronal vulnerability during aging/degenerative diseases are currently not clear. Therefore, exploring the pathologic characteristics of LC noradrenergic neuronal loss during the neurodegenerative process is important for elucidating the pathological mechanisms underlying AD and PD.

Camptothecin (CPT) is a cytotoxic quinoline alkaloid and a S-phase-specific anticancer agent which inhibits DNA enzyme topoisomerase I (Liu *et al.* 2000). Generally, administration of CPT produced irreversible DNA double-strand breaks during DNA synthesis, suggesting that this agent should not have toxic effects on non-replicating cells, such as neurons. However, it was reported that CPT can lead to death of post-mitotic rat cortical neurons *in vitro* in a significantly dose-dependent manner (Morris & Geller 1996). Additionally, neurotoxic activity of CPT was found in cultured cerebellar granule neurons, which inhibited both protein synthesis and the neuritic outgrowth (Uday Bhanu & Kondapi 2010). These observations indicate that CPT also exhibits significant toxicity toward neuronal cells *in vitro*.

Effects of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) on NE levels in the peripheral and central noradrenergic system were first reported several decades ago (Ross 1976). *In vivo* DSP4 selectively damages noradrenergic projections originating from the LC by interacting with the NE reuptake system and depleting intracellular NE, finally inducing degeneration of noradrenergic terminals (Winkler 1976, Ransom *et al.* 1985, Dooley *et al.* 1987, Prieto & Giralt 2001). Our previous study showed that DSP4 induces DNA damage response (DDR) in neuroblastoma SH-SY5Y cells in a time- and dose-dependent manner (Wang *et al.* 2014). However, whether DSP4 can induce 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 pathophysiologic characteristics.

DSP4 has been widely used as a supplement neurotoxin to construct AD or PD animal models for the appearance of noradrenergic dysfunction (Srinivasan & Schmidt 2004, Heneka *et al.* 2006, Kalinin *et al.* 2007, Thomas *et al.* 2007). As a DNA-damaging agent CPT is also occasionally used to mimetic cell impairments in the *in vitro* cell model (Malagelada *et al.* 2006, Liu *et al.* 2014). However, the precise pathologic nature of these toxic agents and the response of cells upon their exposure, especially for CPT, have not been fully investigated. We hypothesize that different cells may exhibit alternative responses to neuronal impairments induced by these neurotoxins, and that these differences may reflect their cellular repair rate, which may decide their vulnerability to cellular insults. In the present study, we exposed neuroblastoma SH-SY5Y cells, which are considered as a noradrenergic cell line (Presgraves *et al.* 2004), and primary cultures from the rat LC and raphe nucleus to CPT or DSP4. DDR markers were measured. The results showed that the noradrenergic SH-SY5Y cells and the primary LC neuronal cultures are severely affected by these neurotoxins, reflecting an increased sensitivity to CPT- or DSP4-induced DNA damage, and a deficiency in DNA repair, as compared to fibroblast cell lines or primary cultures from the raphe nucleus.

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 Dulbecco's modified Eagle's medium. Both cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) and grown at 37°C in humidified air containing 5% CO₂. Culture media and supplements were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Cells were seeded into 6-well or 100-mm plates. Drug exposures were started 24 h after 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 & Bjorklund 1992). Mesencephalic tissue pieces containing the raphe nucleus 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°C in a 15ml-centrifuge tube containing 4.5 ml HBSS, 0.5 ml 0.25% trypsin-EDTA and 25 µl RQ1 DNase (0.1 mg/ml deoxyribonuclease). The trypsinization was stopped by addition of 5ml HBSS containing 1mM of pyruvate, 10mM 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 serum free B-27 (Gibco-Invitrogen, Carlsbad, CA, USA), 0.5 mM glutamine, 25 μ M glutamate, penicillin (100 U/ml) and streptomycin (100 μ g/ml). The cells were counted before plating. 1×10^5 /ml cells were transferred into each well of 24-well plate coated with poly-L-lysine (Sigma, St Louis, MO, USA). At 4 day *in vitro* (DIV), the medium was replaced by fresh media without glutamate. Thereafter, 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 diluted with culture media and added to cells to a final concentration of 50 μ M. CPT (Cat. No. C9911, Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide at 10 mM, then diluted with culture media and added to cells to a final concentration of 10 μ M. The selection of the concentration of DSP4 was based on our previous data (Wang et al. 2014). The concentration of CPT was based on published papers (Uday Bhanu & Kondapi 2010). The control group in each experiment was treated with vehicle which is the fresh medium in the case of DSP4, or fresh medium containing the same amount of dimethyl sulfoxide as in the case of CPT.

Western blot 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°C. An equal volume of 2X 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 μ g) 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, respectively, probed with anti- γ H2AX antibody (1:1,000 dilution, Bethyl Laboratories, Inc., Montgomery TX USA), or an antibody specific for p53 tumor-suppressor protein phosphorylated on serine 15 (anti-p-p53^{ser15}) (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 then were subjected to enhanced chemiluminescence (ECL, Amersham Life Sciences, Buckinghamshire, UK) or super 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 μ M, for 2 h) or DSP4 (50 μ M, for 24 h), or vehicle. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2 % Triton X-100 in phosphate buffered saline (PBS) for

10 min. The coverslips then were blocked with 5% goat serum in PBS for 1 h, and incubated overnight with primary antibodies (anti- γ H2AX: 1:200 dilution, GeneTex Inc., Irvine, CA, USA), and anti-p-p53^{ser15} (1:400 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). After 3x10-min washes with PBS, the coverslips were incubated with the secondary antibodies [AlexaFluor[®] 488 Goat Anti-Rabbit IgG (H+L); AlexaFluor[®] 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 60X magnification using an EVOS inverted fluorescent microscope (Advanced Microscopy Group, Washington, USA). We counted cells with at least two γ H2AX foci as γ H2AX-positive cells and increased p-p53^{ser15} expression in nuclei as p-p53^{ser15}-positive cells.

Comet assay

SH-SY5Y cells were treated with 10 μ M CPT for 2 h, and then CPT was washed out. Cells were allowed to recover from the CPT-induced damage for 24, 48 and 72 h. The 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 $\times$ magnification. The overall cell 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 graph as the mean $\pm$ SEM. The number of replicates is enumerated in the figure legends. The statistics were performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA), which was followed by a *post hoc* Newman–Keuls test for planned comparisons.

Results

SH-SY5Y cells are more sensitive to CPT-induced DNA damage as compared to fibroblastic cells

In this study, noradrenergic SH-SY5Y cells, which richly express the noradrenergic hall mark proteins NE transporter (NET) and dopamine β -hydroxylase (DBH), were used. Normal human fibroblast cells which express neither NET nor DBH were used as non-adrenergic cells (Fig. 1). First, to examine whether noradrenergic SH-SY5Y cells were sensitive to CPT-induced DNA damage, SH-SY5Y and fibroblast cells were exposed to 10 μ M CPT for 15, 30, 60, 90 and 120 min. Western blotting and IFA analysis were performed to measure γ H2AX and p-p53^{ser15}, two DDR markers. Figs. 2A, 2B, 3A and 3B show the γ H2AX foci and p-p53^{ser15}-positive cells identified by IFA at different CPT treatment times. As shown in Fig. 2C, the percentage of γ H2AX-positive fibroblast cells was 14.7% after 90 min exposure and 100% after 120 min exposure ($F_{5,12}=377.5$, $p<0.0001$). In contrast,

percentages of γ H2AX-positive SH-SY5Y cells were about 1.3%, 29.3%, 51.2%, 74.0% and 77.7% after CPT exposure for 15, 30, 60, 90 or 120 min ($F_{5,12}=171.3$, $p<0.0001$), respectively (Fig. 2D). The percentage of p-p53^{ser15}-positive fibroblast cells was 37.9% after 120 min exposure (Fig. 3C, $F_{5,12}=45.47$, $p<0.0001$). However, treated SH-SY5Y cells exhibited a significantly higher levels of p-p53^{ser15}-positive cells with the percentage of 36.6%, 47.2% and 49.5% after 60, 90 and 120 min CPT treatment, respectively (Fig. 3D, $F_{5,12}=1816$, $p<0.0001$). Western blot results show that although the effect of CPT on γ H2AX and p-p53^{ser15} levels at 120 min after exposure is similar in both cell lines, the levels of γ H2AX (Figs. 4E and 4G, $F_{5,12}=83.02$, $p<0.0001$) and p-p53^{ser15} (Figs. 4F and 4H, $F_{5,18}=168.5$, $p<0.0001$) were dramatically increased after a 60 min CPT exposure in SH-SY5Y cells (Figs. 4A and 4C, $F_{5,18}=78$, $p<0.0001$; Figs. 4B and 4D, $F_{5,18}=868.9$, $p<0.0001$). These results demonstrated that CPT-induced alterations in γ H2AX and p-p53^{ser15} appeared earlier in SH-SY5Y cells than fibroblast cells, indicating that SH-SY5Y cells are more sensitive to CPT-induced DNA damage.

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 μ M) for 2 h. Cells were then briefly washed with PBS and continued to grow in fresh medium in the absence of CPT for 24, 48, 72 and 96 h. In normal fibroblast cells, numbers of γ H2AX foci and levels of p-p53^{ser15} were significantly reduced within 24 h after removal of CPT (Figs. 5A and 5B). However, in SH-SY5Y cells, the number of γ H2AX-positive cells was gradually reduced over 72 h (Figs. 6A and 6C, $F_{5,12}=453.9$, $p<0.0001$). Unexpectedly, the number of γ H2AX-positive SH-SY5Y cells dramatically decreased from 76.9% to 41.0% within 24 h, but increased from 41.0% to 52.3% from 24 h to 48 h after removal of CPT. The level of p-p53^{ser15} in SH-SY5Y nuclei decreased gradually over 72 h (Figs. 6B and 6D, $F_{5,12}=203.0$, $p<0.0001$). It took almost 96 h for these γ H2AX foci and p-p53^{ser15} positive nuclei to disappear completely after removal of the CPT. Western blot analysis also showed the reduced protein levels of γ H2AX ($F_{4,20}=43.23$, $p<0.0001$) and p-p53^{ser15} ($F_{4,10}=205.3$, $p<0.0001$) at 72 h after washing-out of CPT in SH-SY5Y cells (Fig. 7).

To further explore the repair efficiency, CPT-treated cells were analyzed by neutral and alkaline comet assays. Interestingly, tails in fibroblast cells were detected under both neutral and alkaline conditions, suggesting that CPT induces both double-strand breaks (DSBs) and single-strand breaks (SSBs) in fibroblast cells (Fig. 8A). However, tails were only detected under alkaline condition in SH-SY5Y cells (Fig. 8B), which indicated SSBs. Tails gradually shortened or disappeared over 72 h in SH-SY5Y cells, while they began to short and disappear at 24 h in normal fibroblast cells (Fig. 8A). Thus, by demonstrating the persistence of DNA strand breaks in SH-SY5Y cell, these comet assay data confirm that CPT-induced DDRs are different in both cell lines as indicated by the IFAs and western blotting.

In sum, compared to fibroblast cells, CPT-treated SH-SY5Y cells exhibited a delay in reducing levels of the two DDR markers γ H2AX and p-p53^{ser15}. In addition, comet assays showed that CPT-induced DNA damage was persistent in SH-SY5Y cells. These data indicated that SH-SY5Y cells were deficient in repairing CPT-induced DNA damage.

CPT-induced DDR in cultured LC and raphe neurons—The above observations (Figs. 2, 3, 4, 5, 6, 7 and 8) demonstrate that noradrenergic SH-SY5Y cells were sensitive to CPT-induced DNA damage and exhibit a deficiency in 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 neuronal cultures from the LC and raphe nuclei were similarly treated with neurotoxins. Primary neuronal cultures derived from rodents are widely used to study basic physiological properties of neurons, and represent a useful tool to study the potential neurotoxicity of chemicals. To develop a cell culture system of the LC and raphe nuclei would facilitate investigations of various properties of these noradrenergic and non-noradrenergic neurons under well-controlled conditions (Masuko *et al.* 1986). The LC is the main source of noradrenergic neurons in the brain while the raphe nuclei are the key center for serotonin expressing neurons. Thus, primarily cultured LC and raphe neurons were used to examine whether these neurons respond to DNA damage differently from each other. The LC and raphe tissues were separated from 15- to 18-day rat embryos, then cultured for 12 days. Since we did not get enough neurons to do western blots, therefore in this study only IFA was employed. The primary cultures were treated with CPT (10 μ M) for 2 h. After brief washing, cells were allowed to recover in the media without CPT for another 24, 48 or 72 h. As shown in Fig. 9, significant γ H2AX foci were found in LC neurons after CPT treatment ($F_{4,11}=558.3$, $p<0.0001$). The number of γ H2AX-positive cells was gradually reduced over 72 h after CPT wash-out. However, we could barely detect γ H2AX foci in raphe neurons after CPT treatment (Fig. 10). Therefore, the primarily cultured LC neurons are sensitive to CPT-induced DNA damage and are deficient in repair of this damage.

LC neurons accumulate DSP4-induced DNA damage—The earlier studies showed that effects of DSP4 were initially restricted to the nerve terminals originating from the LC (Fritschy *et al.* 1990). However, the subsequent reports demonstrated that there was loss of LC neuronal cell bodies after DSP4 treatment (Fritschy & Grzanna 1991). Also, the selective effects of DSP4 on noradrenergic neurons are documented (Jonsson *et al.* 1981, Fischer *et al.* 1983, Hallman & Jonsson 1984, Howard *et al.* 1990). Our previous study showed that DSP4 could induce DDR in SH-SY5Y cells (Wang *et al.* 2014). To test the effects of DSP4 on primarily cultured LC and raphe neurons, neuronal cultures were treated with DSP4 (50 μ M) for 24 h, and IFA was performed. As shown in Fig. 11B, γ H2AX foci were detected in DSP4-treated primarily cultured LC neurons after DSP4 treatment. However, no significant γ H2AX foci were found in primarily cultured raphe neurons after DSP4 treatment (Fig. 11A).

Discussion

In the present study, γ H2AX and p-p53^{ser15} were measured as the DDR markers to evaluate the response to DNA damage induced by CPT or DSP4, as well as the repair rate for the DNA damage. Our data showed that SH-SY5Y cells are sensitive to accumulate CPT-induced DNA damage (Figs. 2, 3 and 4) and are deficient in repairing this damage (Figs. 5, 6, 7 and 8), compared to that in fibroblast cells. In order to correlate these *in vitro* findings with those in *in vivo* conditions, we used primary LC and raphe neuronal cultures; such

cultures would facilitate the investigation of various properties of these noradrenergic and non-noradrenergic neurons under well-controlled conditions and mimic the *in vivo* conditions. Our data show that LC neurons are more sensitive to DNA damage induced by CPT or DSP4 than raphe neurons (Figs. 9, 10 and 11). These pathological characteristics are consistent with the *in vivo* observations which demonstrate that degeneration of noradrenergic neurons may occur at an early stage in the brain of neurodegenerative disease patients (Bondareff *et al.* 1982, Mann & Yates 1983, Mann *et al.* 1983, Bondareff *et al.* 1987, German *et al.* 1992, Weinshenker 2008).

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 γ H2AX, and γ H2AX foci can be detected within minutes after the induction of DNA damages (Kang *et al.* 2005). H2AX phosphorylation not only is a DDR, but also 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 & Alt 2004, Thiriet & Hayes 2005). Similarly, p53 tumor suppressor protein, known as a classic “gatekeeper” of cellular fate, is activated in response to genotoxic stress-induced DNA damage (May & May 1999), among which the phosphorylation of serine-15 in p53 is one of the key responses (Hammond *et al.* 2002). P-p53^{ser15} levels may rapidly increase 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 γ H2AX and p-p53^{ser15} can be used to represent a relative time process in CPT-induced DDR and repair.

The LC is important for regulating the amount of NE in the brain (Maeda 2000). A deficiency of the noradrenergic function in the brain, originating largely from loss of cells in the LC, is an early pathological indicator in the progression of multiple neurodegenerative disorders including PD and AD (Marien *et al.* 2004). Also, aging-related cognitive decline is associated with an accumulation of nDNA damage in the neurons (Rutten *et al.* 2003, Rutten *et al.* 2007); this effect may be due to insufficient nDNA repair. Our data show that noradrenergic SH-SY5Y cells and primary LC neuronal cultures exhibit an enhance sensitivity to CPT-treatment, which results in accumulation of DNA damage (Fig. 2, 3 and 4). This sensitivity to CPT could stem from a deficiency of DNA repair in noradrenergic SH-SY5Y cells and LC neurons. Generally, the repair of DNA damage is depended upon functional repair systems (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 & Wang 1988, Chatterjee *et al.* 1989). Therefore, noradrenergic SH-SY5Y cells and LC neurons may be relatively deficient in DNA repair 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 (Figs. 5, 6, 7, 8 and 9).

Additionally, it was reported that oxidative stress-induced DNA damage can lead to cell cycle arrest (Migliore & Coppede 2002). For example, human fibroblasts treated with H₂O₂ undergo either cell cycle arrest or apoptosis. The majority of the apoptotic fibroblasts

are in the S-phase, whereas growth-arrested cells were predominantly accumulated in the G1- or G2/M-phases(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 the S-phase proteins. Hippocampal pyramidal and basal forebrain neurons from AD brains show chromosomal duplication but die before mitosis, which is consistent with cell death in the S-, or G2-phase of the cell cycle(Nagy *et al.* 1997). It was reported that insufficient nDNA repair leads to accumulation of nDNA damage in neurons. So if SH-SY5Y cells were in S- or G2/M-phases when CPT was added, these cells might have an enhanced response with much more γ H2AX foci in damaged nuclei. This hypothesis may also explain our data in Fig. 6A. After a decrease in the number of γ H2AX-positive cells at 24 h after CPT was washed away, they increased again at 48 h after removal of CPT. This effect may be explained as follows: at 24 h after removal of CPT, cells with significant of DNA damage died due to apoptosis, therefore the number of γ H2AX-positive cells dramatically decreased. At 48 h after removal of CPT, the number of γ H2AX-positive cells was less than unrecovered cells but higher than that at 24 h, indicating that cells with a lower amount of DNA damage recruited γ H2AX to repair the damage, but this recruitment was at slower rate than in fibroblast cells. Further studies are needed to measure the viable cell number at each recovery time point and also to determine if cells with higher γ H2AX levels are in any particular cell cycle phase, *i.e.*, the S- or G2/M-phase. The generated data will help to explain why a dramatically decreased expression of γ H2AX occurs at 24 h after wash-out CPT.

In Fig. 8, under neutral conditions, CPT-induced DNA damage was detected as DSBs in fibroblast cells, but not in SH-SY5Y cells. This 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. CPT inhibits topoisomerase I after it has opened one DNA strand; the conversion of such SSBs into DSBs occurs during S-phase when the replication fork collides with the single-strand cleavage complexes formed on DNA by topoisomerase I-CPT complex. These data suggest that the CPT-induced SSBs in SH-SY5Y cells immediately arrest the cell cycle and/or replication in the S-phase cell, thus preventing the conversion of SSB to DSB.

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 & Geller 1996, Uday Bhanu & Kondapi 2010). Taken together, these observations indicate that CPT also exhibits significant toxicity toward neuronal cells *in vitro*. This could explain the results in Fig. 9, in which CPT was observed to induce DDR in LC neurons. In the present study, exposure of the primary cultures from rat raphe nuclei to CPT (Fig. 10) or DSP4 (Fig. 11A) did not cause obvious DDR, indicating a potential neuronal selectivity of these neurotoxin actions. Although to date there is no report about 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 & Elgin 1988). However, it was reported that DSP4 could induce a decrease of 5-HT concentrations in the cerebral cortex, mesencephalon and spinal cord (Fornai *et al.* 1996, Jackisch *et al.* 2008). Further studies are warranted to clarify their selectivity on different neurons, especially *in vivo*.

In summary, in the present study, SH-SY5Y cells and primary cultures from rat LC are more sensitive to neurotoxins CPT- or DSP4-induced DNA damage, and deficient in repairing the damage, compared to fibroblast cells and raphe neurons, respectively. It can be concluded that the more sensitivity to neurotoxins and deficiency in repair capacity in noradrenergic neurons may account for their vulnerability after cellular insults. 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.

Acknowledgements

This work is supported by NIH grants MH080323 (MYZ) and CA86927 (YZ). The authors declare no conflict of interest regarding the work reported here.

References

- Adolfsson R, Gottfries CG, Roos BE, Winblad B. Changes in the brain catecholamines in patients with dementia of Alzheimer type. *Br J Psychiatry*. 1979; 135:216–223. [PubMed: 486847]
- Arima K, Akashi T. Involvement of the locus coeruleus in Pick's disease with or without Pick body formation. *Acta neuropathologica*. 1990; 79:629–633. [PubMed: 2163182]
- Bassing CH, Alt FW. H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle*. 2004; 3:149–153. [PubMed: 14712078]
- Bondareff W, Mountjoy CQ, Roth M. Loss of neurons of origin of the adrenergic projection to cerebral cortex (nucleus locus ceruleus) in senile dementia. *Neurology*. 1982; 32:164–168. [PubMed: 7198741]
- Bondareff W, Mountjoy CQ, Roth M, Rossor MN, Iversen LL, Reynolds GP, Hauser DL. Neuronal degeneration in locus ceruleus and cortical correlates of Alzheimer disease. *Alzheimer Dis Assoc Disord*. 1987; 1:256–262. [PubMed: 3453748]
- Brasnjevic I, Hof PR, Steinbusch HW, Schmitz C. Accumulation of nuclear DNA damage or neuron loss: molecular basis for a new approach to understanding selective neuronal vulnerability in neurodegenerative diseases. *DNA Repair (Amst)*. 2008; 7:1087–1097. [PubMed: 18458001]
- Cassano T, Gaetani S, Morgese MG, *et al.* Monoaminergic changes in locus coeruleus and dorsal raphe nucleus following noradrenaline depletion. *Neurochem Res*. 2009; 34:1417–1426. [PubMed: 19229609]
- Chan-Palay V. Alterations in the locus coeruleus in dementias of Alzheimer's and Parkinson's disease. *Prog Brain Res*. 1991a; 88:625–630. [PubMed: 1726030]
- Chan-Palay V. Locus coeruleus and norepinephrine in Parkinson's disease. *The Japanese journal of psychiatry and neurology*. 1991b; 45:519–521. [PubMed: 1722263]
- Chatterjee S, Cheng MF, Trivedi D, Petzold SJ, Berger NA. Camptothecin hypersensitivity in poly(adenosine diphosphate-ribose) polymerase-deficient cell lines. *Cancer Commun*. 1989; 1:389–394. [PubMed: 2562007]

- Chen QM, Liu J, Merrett JB. Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H₂O₂ response of normal human fibroblasts. *Biochem J.* 2000; 347:543–551. [PubMed: 10749685]
- Chrobak JJ, DeHaven DL, Walsh TJ. Depletion of brain norepinephrine with DSP-4 does not alter acquisition or performance of a radial-arm maze task. *Behav Neural Biol.* 1985; 44:144–150. [PubMed: 3834919]
- Dooley DJ, Heal DJ, Goodwin GM. Repeated electroconvulsive shock prevents increased neocortical beta 1-adrenoceptor binding after DSP-4 treatment in rats. *Eur J Pharmacol.* 1987; 134:333–337. [PubMed: 3032658]
- Downs JA, Lowndes NF, Jackson SP. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature.* 2000; 408:1001–1004. [PubMed: 11140636]
- Dunnett, SB.; Bjorklund, a. Staging and dissection of rat embryos. *Neuroa.* 1992. D. S. B. B. A. ed
- Fischer JB, Waggaman LA, Ransom RW, Cho AK. Xylamine, an irreversible inhibitor of norepinephrine uptake, is transported by this same uptake mechanism in cultured rat superior cervical ganglia. *J Pharmacol Exp Ther.* 1983; 226:650–655. [PubMed: 6887005]
- Ford JM. Regulation of DNA damage recognition and nucleotide excision repair: another role for p53. *Mutat Res.* 2005; 577:195–202. [PubMed: 15927209]
- Fornai F, Bassi L, Torracca MT, Alessandri MG, Scalori V, Corsini GU. Region- and neurotransmitter-dependent species and strain differences in DSP-4-induced monoamine depletion in rodents. *Neurodegeneration : a journal for neurodegenerative disorders, neuroprotection, and neuroregeneration.* 1996; 5:241–249.
- Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol.* 1996; 55:259–272. [PubMed: 8786384]
- Fritschy JM, Geffard M, Grzanna R. The response of noradrenergic axons to systemically administered DSP-4 in the rat: an immunohistochemical study using antibodies to noradrenaline and dopamine-beta-hydroxylase. *J Chem Neuroanat.* 1990; 3:309–321. [PubMed: 2204356]
- Fritschy JM, Grzanna R. Experimentally-induced neuron loss in the locus coeruleus of adult rats. *Exp Neurol.* 1991; 111:123–127. [PubMed: 1984427]
- German DC, Manaye KF, White CL 3rd, Woodward DJ, McIntire DD, Smith WK, Kalaria RN, Mann DM. Disease-specific patterns of locus coeruleus cell loss. *Ann Neurol.* 1992; 32:667–676. [PubMed: 1449247]
- Grudzien A, Shaw P, Weintraub S, Bigio E, Mash DC, Mesulam MM. Locus coeruleus neurofibrillary degeneration in aging, mild cognitive impairment and early Alzheimer's disease. *Neurobiol Aging.* 2007; 28:327–335. [PubMed: 16574280]
- Hallman H, Jonsson G. Pharmacological modifications of the neurotoxic action of the noradrenaline neurotoxin DSP4 on central noradrenaline neurons. *Eur J Pharmacol.* 1984; 103:269–278. [PubMed: 6237924]
- Hammond EM, Denko NC, Dorie MJ, Abraham RT, Giaccia AJ. Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol.* 2002; 22:1834–1843. [PubMed: 11865061]
- Heneka MT, Ramanathan M, Jacobs AH, et al. Locus ceruleus degeneration promotes Alzheimer pathogenesis in amyloid precursor protein 23 transgenic mice. *J Neurosci.* 2006; 26:1343–1354. [PubMed: 16452658]
- Hickson ID, Davies SL, Davies SM, Robson CN. DNA repair in radiation sensitive mutants of mammalian cells: possible involvement of DNA topoisomerases. *Int J Radiat Biol.* 1990; 58:561–568. [PubMed: 1976717]
- Howard BD, Cho AK, Zhang MB, Koide M, Lin S. Covalent labeling of the cocaine-sensitive catecholamine transporter. *J Neurosci Res.* 1990; 26:149–158. [PubMed: 2142222]
- Jackisch R, Gansser S, Cassel JC. Noradrenergic denervation facilitates the release of acetylcholine and serotonin in the hippocampus: towards a mechanism underlying upregulations described in MCI patients? *Exp Neurol.* 2008; 213:345–353. [PubMed: 18625224]
- Jonsson G, Hallman H, Ponzio F, Ross S. DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine)--a useful denervation tool for central and peripheral noradrenaline neurons. *Eur J Pharmacol.* 1981; 72:173–188. [PubMed: 6265244]

- Kalinin S, Gavriluk V, Polak PE, Vasser R, Zhao J, Heneka MT, Feinstein DL. Noradrenaline deficiency in brain increases beta-amyloid plaque burden in an animal model of Alzheimer's disease. *Neurobiol Aging*. 2007; 28:1206–1214. [PubMed: 16837104]
- Kang J, Ferguson D, Song H, Bassing C, Eckersdorff M, Alt FW, Xu Y. Functional interaction of H2AX, NBS1, and p53 in ATM-dependent DNA damage responses and tumor suppression. *Mol Cell Biol*. 2005; 25:661–670. [PubMed: 15632067]
- Liu G, Yu J, Ding J, et al. Aldehyde dehydrogenase 1 defines and protects a nigrostriatal dopaminergic neuron subpopulation. *J Clin Invest*. 2014; 124:3032–3046. [PubMed: 24865427]
- Liu LF, Desai SD, Li TK, Mao Y, Sun M, Sim SP. Mechanism of action of camptothecin. *Ann N Y Acad Sci*. 2000; 922:1–10. [PubMed: 11193884]
- Lyness SA, Zarow C, Chui HC. Neuron loss in key cholinergic and aminergic nuclei in Alzheimer disease: a meta-analysis. *Neurobiol Aging*. 2003; 24:1–23. [PubMed: 12493547]
- Maeda T. The locus coeruleus: history. *J Chem Neuroanat*. 2000; 18:57–64. [PubMed: 10708919]
- Malagelada C, Ryu EJ, Biswas SC, Jackson-Lewis V, Greene LA. RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. *J Neurosci*. 2006; 26:9996–10005. [PubMed: 17005863]
- Mann DM. The locus coeruleus and its possible role in ageing and degenerative disease of the human central nervous system. *Mech Ageing Dev*. 1983; 23:73–94. [PubMed: 6228698]
- Mann DM, Yates PO. Pathological basis for neurotransmitter changes in Parkinson's disease. *Neuropathol Appl Neurobiol*. 1983; 9:3–19. [PubMed: 6133229]
- Mann DM, Yates PO, Hawkes J. The pathology of the human locus ceruleus. *Clin Neuropathol*. 1983; 2:1–7. [PubMed: 6220852]
- Marien MR, Colpaert FC, Rosenquist AC. Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev*. 2004; 45:38–78. [PubMed: 15063099]
- Martin GE, Elgin RJ Jr. Effects of cerebral depletion of norepinephrine on conditioned avoidance responding in Sprague-Dawley and Fischer rats. *Pharmacol Biochem Behav*. 1988; 30:137–142. [PubMed: 3174736]
- Masuko S, Nakajima Y, Nakajima S, Yamaguchi K. Noradrenergic neurons from the locus ceruleus in dissociated cell culture: culture methods, morphology, and electrophysiology. *J Neurosci*. 1986; 6:3229–3241. [PubMed: 2430074]
- May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene*. 1999; 18:7621–7636. [PubMed: 10618702]
- Migliore L, Coppede F. Genetic and environmental factors in cancer and neurodegenerative diseases. *Mutat Res*. 2002; 512:135–153. [PubMed: 12464348]
- Morris EJ, Geller HM. Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J Cell Biol*. 1996; 134:757–770. [PubMed: 8707853]
- Nagy Z, Esiri MM, Smith AD. Expression of cell division markers in the hippocampus in Alzheimer's disease and other neurodegenerative conditions. *Acta neuropathologica*. 1997; 93:294–300. [PubMed: 9083562]
- Nitiss J, Wang JC. DNA topoisomerase-targeting antitumor drugs can be studied in yeast. *Proc Natl Acad Sci U S A*. 1988; 85:7501–7505. [PubMed: 2845409]
- Offer H, Wolkowicz R, Matas D, Blumenstein S, Livneh Z, Rotter V. Direct involvement of p53 in the base excision repair pathway of the DNA repair machinery. *FEBS Lett*. 1999; 450:197–204. [PubMed: 10359074]
- Okorokov AL. p53 in a crosstalk between DNA repair and cell cycle checkpoints. *Cell Cycle*. 2003; 2:233–235. [PubMed: 12734432]
- Palmer AM, DeKosky ST. Monoamine neurons in aging and Alzheimer's disease. *Journal of neural transmission. General section*. 1993; 91:135–159. [PubMed: 8099796]
- Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotoxicity research*. 2004; 5:579–598. [PubMed: 15111235]

- Prieto M, Giralt MT. Effects of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) on alpha2-adrenoceptors which regulate the synthesis and release of noradrenaline in the rat brain. *Pharmacol Toxicol.* 2001; 88:152–158. [PubMed: 11245411]
- Ransom RW, Waggaman LA, Cho AK. Interaction of xylamine with peripheral sympathetic neurons. *Life Sci.* 1985; 37:1177–1182. [PubMed: 3930904]
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
- Ross SB. Long-term effects of N-2-chloroethyl-N-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart. *Br J Pharmacol.* 1976; 58:521–527. [PubMed: 1000130]
- Rutten BP, Korr H, Steinbusch HW, Schmitz C. The aging brain: less neurons could be better. *Mech Ageing Dev.* 2003; 124:349–355. [PubMed: 12663133]
- Rutten BP, Schmitz C, Gerlach OH, Oyen HM, de Mesquita EB, Steinbusch HW, Korr H. The aging brain: accumulation of DNA damage or neuron loss? *Neurobiol Aging.* 2007; 28:91–98. [PubMed: 16338029]
- Srinivasan J, Schmidt WJ. Behavioral and neurochemical effects of noradrenergic depletions with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine in 6-hydroxydopamine-induced rat model of Parkinson's disease. *Behav Brain Res.* 2004; 151:191–199. [PubMed: 15084435]
- Thiriet C, Hayes JJ. Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. *Mol Cell.* 2005; 18:617–622. [PubMed: 15949437]
- Thomas B, von Coelln R, Mandir AS, et al. MPTP and DSP-4 susceptibility of substantia nigra and locus coeruleus catecholaminergic neurons in mice is independent of parkin activity. *Neurobiol Dis.* 2007; 26:312–322. [PubMed: 17336077]
- Uday Bhanu M, Kondapi AK. Neurotoxic activity of a topoisomerase-I inhibitor, camptothecin, in cultured cerebellar granule neurons. *Neurotoxicology.* 2010; 31:730–737. [PubMed: 20600288]
- Usher M, Cohen JD, Servan-Schreiber D, Rajkowski J, Aston-Jones G. The role of locus coeruleus in the regulation of cognitive performance. *Science.* 1999; 283:549–554. [PubMed: 9915705]
- Wang Y, Musich PR, Serrano MA, Zou Y, Zhang J, Zhu MY. Effects of DSP4 on the Noradrenergic Phenotypes and Its Potential Molecular Mechanisms in SH-SY5Y Cells. *Neurotoxicity research.* 2014; 25:193–207. [PubMed: 23996700]
- Weinshenker D. Functional consequences of locus coeruleus degeneration in Alzheimer's disease. *Current Alzheimer research.* 2008; 5:342–345. [PubMed: 18537547]
- Winkler H. The composition of adrenal chromaffin granules: an assessment of controversial results. *Neuroscience.* 1976; 1:65–80. [PubMed: 794758]
- Zarow C, Lyness SA, Mortimer JA, Chui HC. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch Neurol.* 2003; 60:337–341. [PubMed: 12633144]

Figure 1.

Expression levels of DBH and NET proteins in SH-SY5Y and normal fibroblast cells. SH-SY5Y cells are rich in NET and DBH expression. However, normal fibroblast cells express neither NET nor DBH.

Figure 2.

Time course for appearances of γ H2AX foci in normal fibroblast and SH-SY5Y cells after CPT treatment. Cells were treated with CPT (10 μ M) for 15, 30, 60, 90 and 120 min, then IFAs were performed. The top panel shows images of IFA staining of normal fibroblast cells (A, n=3) and SH-SY5Y cells (B, n=3). The bottom panel presents a quantitative analysis of the percentage of γ H2AX-positive cells after CPT exposure in normal fibroblast (C) and SH-SY5Y cells (D), representing averages obtained from 3 separate experiments. Images were taken with 60 $\times$ magnification. At least 200 cells were counted from three random chosen views, from which the positive cell number represented the result from each experiment.

*p<0.01, **p<0.0001, compared to the group of 0 min.

Figure 3.

Time course of increased p-p53^{ser15} levels identified by IFA in nuclei of fibroblast and SH-SY5Y cells after CPT treatment. Cells were treated with CPT (10 μ M) for 15, 30, 60, 90 and 120 min, then IFs were performed. The top panel shows images of IFA staining of normal fibroblast cells (A) and SH-SY5Y cells (B). The bottom panel displays a quantitative analysis for percentage of p-p53^{ser15}-positive cells after CPT exposure in normal fibroblast (C) and SH-SY5Y cells (D), representing averages obtained from 3 separate experiments. Images were taken with 60 $\times$ magnification. At least 200 cells were counted from three random chosen views. * $p < 0.0001$, compared to the group of 0 min.

Figure 4.

Time course of increased protein levels of γ H2AX and p-p53^{ser15} in fibroblast (A, B, C and D) and SH-SY5Y (E, F, G and H) cells after CPT treatment. Cells were treated with CPT (10 μ M) for 15, 30, 60, 90 and 120 min, then western blots were performed. A, B, E and F showed autoradiographs of γ H2AX and p-p53^{ser15} proteins obtained by western blotting. The graphic data, shown in C, D, G and H, represent a quantitative analysis of protein levels of γ H2AX and p-p53^{ser15}, which were normalized to β -actin. These graphic data represent averages obtained from 4 separate experiments. * $p < 0.01$, ** $p < 0.0001$, compared to the group of 0 min; # $p < 0.05$, ## $p < 0.0001$, compared to the group of 120 min.

Figure 5.

The IFA measurement for γ H2AX foci or p-p53^{ser15}-positive fibroblast cells after CPT wash-out. Cells were treated with CPT (10 μ M) for 2 h, then CPT was washed away. Cells were allowed to recover in fresh prepared media without CPT for 24 (R24) or 48 (R48) h. After 2 h CPT treatment, significant γ H2AX foci (A) were found in nuclei, and the level of p-p53^{ser15} (B) was increased in nuclei. The numbers of γ H2AX- or p-p53^{ser15}-positive cells were reduced within 24 h after CPT wash-out. The images were taken with 60 $\times$ magnification. Blue: DAPI; red: γ H2AX; green: p-p53^{ser15}. Con: control group treated with vehicle. R24 h and R48 h: after CPT was washed away, cells continued to grow in media without CPT for 24 or 48 h. DAPI: 4',4-diamidino-2-phenylindole, a fluorescent dye that strongly binds to DNA as a nuclear counterstain.

Figure 6.

The IFA measurement for γ H2AX foci or p-p53^{ser15}-positive cells in SH-SY5Y cells after CPT wash-out. Cells were treated with CPT (10 μ M) for 2 h. After CPT was washed away, cells continued to grow in media without CPT for 24, 48, 72 or 96 h. The numbers of γ H2AX-(A) and p-p53^{ser15}-positive (B) cells were reduced within 72 h after CPT wash-out in SH-SY5Y cells. Analytical data of percentage of γ H2AX- or p-p53^{ser15}-positive cells are shown in C and D, representing averages obtained from 3 separate experiments. At least 200 cells were counted from three random chosen views, from which the positive cell number represented the result from each experiment. * $p < 0.0001$, compared to the control (con); # $p < 0.05$, ## $p < 0.0001$, compared to the CPT group; & $p < 0.05$, && $p < 0.001$, &&& $p < 0.0001$, compared to R24 h group. R72 h and R96 h: after CPT was washed away, cells continued to grow in media without CPT for 72 or 96 h. See Fig. 5 for other abbreviation.

Figure 7.

The protein levels of γ H2AX and p-p53^{ser15} were decreased within 72 h in SH-SY5Y cells after wash-out of CPT. SH-SY5Y cells were treated with CPT (10 μ M) for 2 h, then CPT was washed away, and 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 autoradiographs of γ H2AX and p-p53^{ser15} proteins obtained by western blotting. Quantity analysis data are shown in C and D, representing averages obtained from 6 separate experiments. # $p < 0.001$, ## $p < 0.0001$, compared to the control; * $p < 0.0001$, compared to the CPT group; & $p < 0.05$, && $p < 0.001$, &&& $p < 0.0001$, compared to the R24 h group. See Figs. 5 and 6 for abbreviations.

Figure 8.

CPT-induced DNA damage as determined by the comet assay in fibroblast and SH-SY5Y cells. The cells were exposed to CPT (10 μ M) for 2 h, then CPT was washed away, cells continued to grow in media without CPT for 24 h, 48 h and 72 h. The cells were processed for neutral or alkaline comet assays. Images were taken with 10 $\times$ 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. See Figs. 5 and 6 for abbreviations.

Figure 9.

CPT induces DDR in DBH-positive cultured neurons. Cultured LC neurons were treated with CPT (10 μ M) 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 γ H2AX foci were found in nuclei after CPT treatment. The number of γ H2AX-positive cells was reduced gradually within 72 h. Images were taken with 60 $\times$ magnification. Percentage of γ H2AX positive cells were shown in (B), representing averages obtained from 4 separate experiments. At least 100 cells from three random chosen views were counted. C: Enlarged images from yellow boxes in A show γ H2AX foci in DBH-positive cells, where yellow arrows indicated γ H2AX foci in nuclei. Blue: DAPI; Green: DBH; Red: γ H2AX. * p <0.05, ** p <0.001, *** p <0.0001, compared to the control; # p <0.0001, compared to the CPT group. See Figs. 5 and 6 for abbreviations.

Figure 10.

γ H2AX foci are not detected in serotonin transporter (SERT)-positive cultured neurons. Raphe neurons were treated with CPT (10 μ M) for 2 h, then IF was performed. Only SERT-positive cells were counted. No significant γ H2AX foci were found in nucleus after CPT treatment. Images were taken with 60 $\times$ magnification. Blue: DAPI, Red: SERT, Green: γ H2AX. See Figs. 5 for abbreviations.

Figure 11.

γ H2AX foci were detected in DBH- but not SERT-positive cultured neurons after DSP4 treatment. Cultured neurons were treated with DSP4 (50 μ M) for 24 h, and then IFA was performed. Only DBH- or SERT-positive cells were counted. Images were taken with 60 $\times$ magnification. A: No γ H2AX foci were detected in SERT-positive cultured raphe neurons after DSP4 treatment. B: γ H2AX foci were detected in DBH-positive cultured LC neurons after DSP4 treatment. C: Quantity analysis data for percentage of γ H2AX-positive cells in DBH-positive cultured LC neurons, representing averages obtained from 4 separate experiments. At least 200 cells from three random chosen views were counted, from which the positive cell number represented the result from each experiment. D: Enlarged images from yellow boxes from B show γ H2AX foci in DBH-positive cells, where yellow arrows indicated γ H2AX foci in nuclei. In A, blue: DAPI; red: SERT; green: γ H2AX. In B and D, blue: DAPI; green: DBH; red: γ H2AX. * p <0.0001, compared to the control (con). See Fig. 5 for abbreviations.