Published in final edited form as:

Neurotox Res. 2014 February ; 25(2): . doi:10.1007/s12640-013-9421-4.

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, and Meng-Yang Zhu

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

Abstract

Dopamine β -hydroxylase (DBH) and norepinephrine (NE) transporter (NET) are the noradrenergic phenotypes for their functional importance to noradrenergic neurons. It is known that *in vivo* N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) treatment induces degeneration of noradrenergic terminals by interacting with NET and depleting intracellular NE. 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 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 proteins, as well as NET proteins 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.

Keywords

DNA damage; Norepinephrine transporter; Dopamine β -hydroxylase; Neurotoxin; Cell cycle; Degeneration

Introduction

The dopamine β -hydroxylase (DBH, EC 1.14.17.1) and norepinephrine (NE) transporter (NET) are the important proteins of the noradrenergic neurons for their specific functional characteristics in these neurons (Kaufman *et al.* 1965, Chan-Palay *et al.* 1989, Barker *et al.* 1995). DBH catalyzes the oxidation of dopamine to NE and is expressed exclusively in the noradrenergic and adrenergic neurons in the brain. DBH is not the rate-limiting enzyme for NE synthesis. However, it was reported that the amount of DBH available is also a key factor in determining the rate of NE 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 NE into the presynaptic terminals (Axelrod *et al.* 1969). As this reuptake is the main mechanism for inactivation of NE-stimulated transmission, alterations of NET expression remarkably would affect NE 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 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). DSP4 can cross the blood-brain barrier and accumulate intraneuronally. *In vivo* DSP4 selectively damages noradrenergic projections originating from the locus coeruleus (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, Howard *et al.* 1990, Prieto *et al.* 2001). Thus, DSP4 has been widely used as a noradrenergic neurotoxin. However, the precise mechanism of action of DSP4 remains unclear. In addition, little data has 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 (γ H2AX) (Burma *et al.* 2001, Ward *et al.* 2001) and the tumor suppressor protein p53 which is phosphorylated at serine 15 (phospho-p53^{ser15}) (Hammond *et al.* 2002). γ H2AX tags the chromatin sites of DNA damage to initiate the recruitment of DNA repair factors (Zarei *et al.* 2002, Sontag *et al.* 2008) while the phospho-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 has not been addressed in 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°C in humidified air containing 5% CO₂. 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 hours 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 *et al.* 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 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 detected 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 (1 µl 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: 5'-CCTCACTGGCTACTGCACGGACAAG-3' and reverse: 5'-GTGGAGCTGAGAGGCGAAGATGTGG-3'; NET, forward: 5'-CGGTGCCTTCTTGATCCCG-3' and reverse: 5'-CCGTTGTACTGTCCCAGAG-3'; and GAPDH (as a control): forward: 5'-TGCACCACCAACTGCTTAGC-3' and reverse: 5'-GGCATGGACTGTGGTCATGAG-3'. All reactions were performed according to the following protocol: 2 min at 50°C, 2 min at 95°C, followed by 45 cycles of 18 sec at 95°C and 45 sec at annealing temperature (NET 60°C, DBH 56°C, GAPDH 56°C), then continued with the melting curve analysis (55–90°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°C to 66°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 12000 rpm for 10 min at 4°C. An equal volume of 2×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 Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins (40 μ g) 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 (1:1000 dilution; Mab Technology Inc., Stone Mountain, GA, USA, or 1:1000; 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 (1:1000 dilution, R3280, Sigma-Aldrich, St Louis, Mo, USA), anti- γ H2AX antibody (1:1000 dilution, Bethyl Laboratories, Inc., Montgomery, TX USA), or an anti-phospho-p53^{ser15} antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5000 dilution; Amersham Life Sciences, Buckinghamshire, UK) was used as the secondary antibody. The membranes then 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 (1:5000 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 μ M) for 24 hours. After washing with 37°C phosphate buffered saline (PBS), 200 μ l 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 3000 rpm for 10 min at 4°C and fixed by slowly adding 1 ml ice-cold 70% ethanol to re-suspend the cells. The cells were stored at –20°C for 12–24 hours, then collected at 3000 rpm for 10 min at 4°C. The cells were washed once with ice-cold PBSE, then re-centrifuged and re-suspended in 300 μ l of freshly prepared PBSE containing 20 μ g/ml propidium iodide (Sigma, St Louis, MO, USA) and 20 μ g/ml DNase-free RNase A (Invitrogen, Grand Island, NY, USA). After incubation at 37°C for 30 min, the cells were analyzed on an Accuri C6 flow cytometer. The population of G0/G1, S, and G2/M were determined using C6 Flow Cytometer Software. The results are expressed as percentage of the attached cells in each phase.

Immunofluorescence— 2×10^4 cells were grown on coverslips in 24-well plates and treated with or without DSP4 (5, 10 or 50 μ M) for 24 hours. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. 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 an anti-phospho-p53^{ser15} (1:400 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). After three 10-minute 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 100X 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 μ M) or camptothecin (CPT, 10 μ M) for 24 hours. 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

10X 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 $\pm$ 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-depend manner

Effects of DSP4 treatment on mRNA and protein levels of noradrenergic phenotypes in human SH-SY5Y cells were examined. As shown in Fig. 1, exposure of cells to 50 μ M 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 μ M for 24 hours. 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. 2A and 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. 2B&C, and 3B&C). *Post hoc* tests demonstrated that DSP4-induced reduction in mRNA and protein levels of DBH, as well as NET proteins showed in a concentration-dependent manner.

It was reported that the effects of DSP4 on NE levels *in vivo* are reversible (Jaim-Etcheverry *et al.* 1980, Hallman *et al.* 1984b, Wolfman *et al.* 1994, Srinivasan *et al.* 2004, Szot *et al.* 2010). The recovery of DBH/NET protein levels from inhibition by DSP4 was tested further. Cells were exposed to 50 μ M for 24 hours and after brief washing with PBS, cells then were maintained in fresh medium in the absence of DSP4 for 24, 48, 72 and 96 hours. Western blotting analysis showed that washing-out of DSP4 marked affected DBH/NET protein levels ($F_{5,42}=7.56$, $p<0.01$ for DBH; $F_{5,42}=30.48$, $p<0.0001$). While DBH levels were fully recovered within 24 hours, it took 48 hours 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 (Fig. 4).

DSP4 treatment leads to cell cycle arrested

To further study the action mechanisms of DSP4, SH-SY5Y cells were exposed to 5 or 50 μ M DSP4 for 24 hours and the effects of these single-dose treatments on cell growth and viability were studied by trypan blue staining. At 5 μ M, DSP4 inhibited cell proliferation by 25% ($p<0.05$), whereas 50 μ M DSP4 inhibited proliferation by 50% ($p<0.001$), compared to the control group after 24 hours (Fig. 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 *et al.* 2001). Flow cytometry was then used to determine whether cell cycle transit was altered by DSP4. 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. 5B–C). After a 24-hour treatment with 5 or 50 μ M DSP4 the percentage of SH-SY5Y 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.

Next, we examined whether S-phase-arrested cells could resume cell cycle transit after removal of DSP4. Cells were treated with 50 μ M DSP4 for 24 hours, then rinsed with PBS and maintained in fresh medium without DSP4 for another 12 or 24 hours before analysis by flow cytometry. As shown in Fig. 6, arrested cells resumed cycle transit within 12 or 24 hours 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 hours, 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 DNA damage responses

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 μ M DSP4 for different times. As a positive control, one group of cells was treated for 2 hours before cell harvest with camptothecin (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 γ H2AX, phosphor-p53^{ser15} and hyperphosphorylation of RPA. As shown by Fig. 7, γ H2AX and phosphor-p53^{ser15} levels were significantly increased at 2 or 4 hours, respectively, after DSP4 exposure ($F_{6,42}=46.6$, $p<0.001$ for γ H2AX; $F_{6,42}=85.5$, $p<0.001$ for p-p53^{ser15}), indicating DNA damage appeared earlier than reduction of DBH/NET expression.

In the separate experiment, cells were exposed to DSP4 (5, 10 or 50 μ M) for 24 hours. Increased γ H2AX levels were proportional to DSP4 concentrations (Figs. 8A&B), suggesting that DNA strand breaks were induced by DSP4. Also, nuclear γ H2AX foci were observed by immunofluorescence with the number of foci increasing proportionally with DSP4 concentrations or exposure time (Fig. 9A). Tumor suppressor protein p53 also is phosphorylated at serine15 in response to DNA damage or replication stress (Prieto & Giralto 2001). The level of phospho-p53^{ser15} also is increased in response to DSP4 treatment; this increase in phospho-p53^{ser15} is proportional to DSP4 concentration as determined by western blots (Figs. 8C&D) and by immunofluorescence (Fig. 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 *et al.* 2000), which appears as a slower migrating band on SDS-polyacrylamide gels. To determine if RPA becomes hyperphosphorylated in response to DSP4 treatment SH-SY5Y cells were treated with DSP4 (5, 10 or 50 μ M) for 24 hours. As a positive control other cells were treated with CPT for 2 hours before cell harvest. Interestingly, the hyperphosphorylated-RPA32 (hyp-RPA32) band was detected only in the CPT-treated cells (Fig. 8E).

DSP4 induces activation of both ATM and ATR pathways

Cell cycle checkpoints are regulatory pathways that govern the order and timing of cell cycle transitions to ensure completion of one cellular event prior to commencement of the next cell cycle phase (Hatip-Al-Khatib *et al.* 1999). The key regulators of the checkpoint pathways in the mammalian DNA damage response 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 one hour before continuous exposure to 50 μ M DSP4 for 24 hours. Western blotting revealed that ATM inhibition significantly decreased the level of DSP4-induced γ H2AX, compared to that of cells treated with DSP4 alone. In contrast, ATR inhibition increased the level of DSP4-induced γ H2AX over that of cells treated with DSP4 alone. Treatment with both ATM and ATR inhibitors resulted in no net change of γ H2AX levels, compared to that of the group treated with DSP4 alone (Figs. 10A&B). These results indicate that ATM is involved in γ H2AX formation after DSP4 treatment. This is consistent with the significant reduction in DSP4-dependent phosphorylation of 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 (Figs. 10C&D). These results indicate that γ H2AX and phospho-p53^{ser15} formation are downstream product primarily of ATM after DSP4 treatment.

DSP4 induces single-strand DNA breaks (SSBs)

To further explore the type of DNA damage induced by DSP4 treatment, SH-SY5Y cells treated with DSP4 (5, 10 or 50 μ M) for 24 hours were analyzed by neutral or the alkaline comet assays. These assays detect DNA double- vs. single-strand breaks, respectively, by measuring the formation of the nuclear DNA tail (comet) after single-cell gel electrophoresis. As shown in Fig. 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 (Fig. 11, bottom).

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 *et al.* 1986) but neither the serotonin nor dopamine transporters (Lode *et al.* 1995), were used to examine the potential molecular mechanisms underlying DSP4 action on noradrenergic phenotypes. The main findings are: 1) DBH and NET expression in SH-SY5Y 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 manner. 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 was possibly caused by DNA damage as DSP4 treatment significantly increased the DDR markers γ H2AX and phospho-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 NE re-uptake sites on the noradrenergic terminals which were degenerated due to the alkylation of diverse vital neuronal structures (Lee *et al.* 1982, Hallman *et al.* 1984a, Dudley *et al.* 1990). Also, DSP4-

induced reduction of [³H]desipramine binding (Lee et al. 1982) suggested a damaged NE reuptake. 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 & Giralt 2001, Kalinin *et al.* 2006, Waterman *et al.* 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 NE, and finally leads to lower levels of NE in the brain.

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 which 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 *et al.* 2008). In response to DNA damage, checkpoint surveillance mechanisms initiate signaling cascades which coordinate cell cycle arrest and facilitate DNA repair (Shiloh 2003, Bakkenist *et al.* 2004, McGowan *et al.* 2004). If these checkpoint surveillance mechanisms fail in neurons, neurodegeneration eventually occurs (Lavin 1999, Cho *et al.* 2011). There has been a slow but steady accumulation of evidence of DNA damage in various neurodegenerative diseases (Robison *et al.* 1984). For example, an increase of DNA strand breaks in neurons has been reported in Alzheimer disease (AD) (Adamec *et al.* 1999) and Huntington disease (Anne *et al.* 2007). Damage to mitochondrial DNA also has been found in Parkinson's disease (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 μ M DSP4 for 24 hours induced about 50% cell proliferation inhibition. The flow cytometric analysis showed that DSP4 treatment for only 24 hours induced cell cycle arrest in S-phase. DDR measurements confirmed a significant increase in γ H2AX and phospho-p53^{ser15}, as well as increased frequencies of nuclear γ H2AX 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 *et al.* 2000, Rouse *et al.* 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 a major physiological mediators for the phosphorylation of H2AX and p53 (Rogakou *et al.* 1998, Kastan *et al.* 2000, Bakkenist *et al.* 2003). γ H2AX and phospho-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 γ H2AX foci and nuclear phospho-p53^{ser15} were proportional to DSP4 concentrations (Fig. 9). Furthermore, the ATM inhibitor significantly blocked the DSP4-induced enhancement of γ H2AX and phospho-p53^{ser15} levels (Fig. 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 (Fig. 11). Interestingly, earlier studies hypothesized that DSP4 is an alkylating compound and its neurotoxic effects might derive from uptake by NET, then alkylation of NE 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 *et al.* 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 are 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 *et al.* 1991), 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, Jandanhazi-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 *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 & 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 γ H2AX and phospho-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 single-strand DNA breaks, which in turn activated ATM signaling pathways to phosphorylate several DNA damage response 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.

Acknowledgments

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

- Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* 2001; 15:2177–2196. [PubMed: 11544175]
- Adamec E, Vonsattel JP, Nixon RA. DNA strand breaks in Alzheimer's disease. *Brain Res.* 1999; 849:67–77. [PubMed: 10592288]
- Anne SL, Saudou F, Humbert S. Phosphorylation of huntingtin by cyclin-dependent kinase 5 is induced by DNA damage and regulates wild-type and mutant huntingtin toxicity in neurons. *J Neurosci.* 2007; 27:7318–7328. [PubMed: 17611284]
- Axelrod J, Kopin IJ. The uptake, storage, release and metabolism of noradrenaline in sympathetic nerves. *Prog Brain Res.* 1969; 31:21–32. [PubMed: 4900113]
- Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature.* 2003; 421:499–506. [PubMed: 12556884]
- Bakkenist CJ, Kastan MB. Initiating cellular stress responses. *Cell.* 2004; 118:9–17. [PubMed: 15242640]
- Barker, E.; Blakely, R. Norepinephrine and serotonin transporters. Molecular targets of antidepressant drugs. In: Bloom, F.; Kupfer, D., editors. *Psychopharmacology. A fourth generation of progress.* Raven Press; New York: 1995. p. 321–333.
- Bartek J, Lukas J. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol.* 2001; 13:738–747. [PubMed: 11698191]
- Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res.* 1978; 38:3751–3757. [PubMed: 29704]
- Boksa P, Aitken D, Meaney M. Effects of the catecholaminergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) on adrenal chromaffin cells in culture. *Biochem Pharmacol.* 1989; 38:1491–1498. [PubMed: 2719722]
- 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]
- Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem.* 2001; 276:42462–42467. [PubMed: 11571274]
- Caldecott KW. DNA single-strand breaks and neurodegeneration. *DNA Repair (Amst).* 2004; 3:875–882. [PubMed: 15279772]
- Chan-Palay V, Asan E. Quantitation of catecholamine neurons in the locus coeruleus in human brains of normal young and older adults and in depression. *J Comp Neurol.* 1989; 287:357–372. [PubMed: 2570793]
- Cho YJ, Liang P. S-phase-coupled apoptosis in tumor suppression. *Cell Mol Life Sci.* 2011; 68:1883–1896. [PubMed: 21437646]
- Del Bino G, Bruno S, Yi PN, Darzynkiewicz Z. Apoptotic cell death triggered by camptothecin or teniposide. The cell cycle specificity and effects of ionizing radiation. *Cell proliferation.* 1992; 25:537–548. [PubMed: 1333822]
- 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]

- Dudley MW, Howard BD, Cho AK. The interaction of the beta-haloethyl benzylamines, xylamine, and DSP-4 with catecholaminergic neurons. *Annu Rev Pharmacol Toxicol.* 1990; 30:387–403. [PubMed: 2188573]
- Engler H, Doenlen R, Riether C, Engler A, Besedovsky HO, Del Rey A, Pacheco-Lopez G, Schedlowski M. Chemical destruction of brain noradrenergic neurons affects splenic cytokine production. *J Neuroimmunol.* 2010; 219:75–80. [PubMed: 20031236]
- 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.
- 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. Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? *Prog Brain Res.* 1991; 88:257–268. [PubMed: 1726027]
- 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]
- Gesi M, Soldani P, Giorgi FS, Santinami A, Bonaccorsi I, Fornai F. The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci Biobehav Rev.* 2000; 24:655–668. [PubMed: 10940440]
- Gordon JT, Kaminski DM, Rozanov CB, Dratman MB. Evidence that 3,3',5-triiodothyronine is concentrated in and delivered from the locus coeruleus to its noradrenergic targets via anterograde axonal transport. *Neuroscience.* 1999; 93:943–954. [PubMed: 10473259]
- Hallman H, Jonsson G. Pharmacological modifications of the neurotoxic action of the noradrenaline neurotoxin DSP4 on central noradrenaline neurons. *Eur J Pharmacol.* 1984a; 103:269–278. [PubMed: 6237924]
- Hallman H, Sundstrom E, Jonsson G. Effects of the noradrenaline neurotoxin DSP 4 on monoamine neurons and their transmitter turnover in rat CNS. *J Neural Transm.* 1984b; 60:89–102. [PubMed: 6208323]
- 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]
- Hatip-Al-Khatib I, Bolukbasi F. Destruction of the noradrenergic system with DSP4 potentiates the behavioral effects of MK-801 in rats. *Pharmacol Biochem Behav.* 1999; 62:233–237. [PubMed: 9972689]
- Heneka MT, Galea E, Gavrilyuk V, Dumitrescu-Ozimek L, Daeschner J, O'Banion MK, Weinberg G, Klockgether T, Feinstein DL. Noradrenergic depletion potentiates beta -amyloid-induced cortical inflammation: implications for Alzheimer's disease. *J Neurosci.* 2002; 22:2434–2442. [PubMed: 11923407]
- 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]
- 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]
- Iversen LL. Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br J Pharmacol.* 1971; 41:571–591. [PubMed: 4397129]
- Jaim-Etcheverry G, Zieher LM. DSP-4: a novel compound with neurotoxic effects on noradrenergic neurons of adult and developing rats. *Brain Res.* 1980; 188:513–523. [PubMed: 7370771]
- Jardanhazi-Kurutz D, Kummer MP, Terwel D, Vogel K, Dyrks T, Thiele A, Heneka MT. Induced LC degeneration in APP/PS1 transgenic mice accelerates early cerebral amyloidosis and cognitive deficits. *Neurochem Int.* 2010; 57:375–382. [PubMed: 20144675]
- Jeppesen DK, Bohr VA, Stevnsner T. DNA repair deficiency in neurodegeneration. *Prog Neurobiol.* 2011; 94:166–200. [PubMed: 21550379]

- 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, Feinstein DL, Xu HL, Huesa G, Pelligrino DA, Galea E. Degeneration of noradrenergic fibres from the locus coeruleus causes tight-junction disorganisation in the rat brain. *Eur J Neurosci.* 2006; 24:3393–3400. [PubMed: 17229089]
- Kastan MB, Lim DS. The many substrates and functions of ATM. *Nat Rev Mol Cell Biol.* 2000; 1:179–186. [PubMed: 11252893]
- Katyal S, McKinnon PJ. DNA strand breaks, neurodegeneration and aging in the brain. *Mech Ageing Dev.* 2008; 129:483–491. [PubMed: 18455751]
- Kaufman S, Friedman S. Dopamine-Beta-Hydroxylase. *Pharmacol Rev.* 1965; 17:71–100. [PubMed: 14294032]
- Kim CH, Zabetian CP, Cubells JF, Cho S, Biaggioni I, Cohen BM, Robertson D, Kim KS. Mutations in the dopamine beta-hydroxylase gene are associated with human norepinephrine deficiency. *Am J Med Genet.* 2002; 108:140–147. [PubMed: 11857564]
- Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem.* 1999; 274:37538–37543. [PubMed: 10608806]
- Kobayashi K, Morita S, Mizuguchi T, Sawada H, Yamada K, Nagatsu I, Fujita K, Nagatsu T. Functional and high level expression of human dopamine beta-hydroxylase in transgenic mice. *J Biol Chem.* 1994; 269:29725–29731. [PubMed: 7961964]
- Kurose A, Tanaka T, Huang X, Traganos F, Darzynkiewicz Z. Synchronization in the cell cycle by inhibitors of DNA replication induces histone H2AX phosphorylation: an indication of DNA damage. *Cell proliferation.* 2006; 39:231–240. [PubMed: 16672000]
- Lavin MF. ATM: the product of the gene mutated in ataxia-telangiectasia. *Int J Biochem Cell Biol.* 1999; 31:735–740. [PubMed: 10467728]
- Lee CM, Javitch JA, Snyder SH. Characterization of [3H]desipramine binding associated with neuronal norepinephrine uptake sites in rat brain membranes. *J Neurosci.* 1982; 2:1515–1525. [PubMed: 7119871]
- 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]
- Liu Z, Martin LJ. Motor neurons rapidly accumulate DNA single-strand breaks after in vitro exposure to nitric oxide and peroxynitrite and in vivo axotomy. *J Comp Neurol.* 2001; 432:35–60. [PubMed: 11241376]
- Lode HN, Bruchelt G, Seitz G, Gebhardt S, Gekeler V, Niethammer D, Beck J. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of monoamine transporters in neuroblastoma cell lines: correlations to meta-iodobenzylguanidine (MIBG) uptake and tyrosine hydroxylase gene expression. *Eur J Cancer.* 1995; 31A:586–590. [PubMed: 7576974]
- Lyons WE, Fritschy JM, Grzanna R. The noradrenergic neurotoxin DSP-4 eliminates the coeruleospinal projection but spares projections of the A5 and A7 groups to the ventral horn of the rat spinal cord. *J Neurosci.* 1989; 9:1481–1489. [PubMed: 2542474]
- Mann DM, Yates PO, Hawkes J. The noradrenergic system in Alzheimer and multi-infarct dementias. *Journal of neurology, neurosurgery, and psychiatry.* 1982; 45:113–119.
- Matsukawa M, Nakadate K, Ishihara I, Okado N. Synaptic loss following depletion of noradrenaline and/or serotonin in the rat visual cortex: a quantitative electron microscopic study. *Neuroscience.* 2003; 122:627–635. [PubMed: 14622906]
- McGowan CH, Russell P. The DNA damage response: sensing and signaling. *Curr Opin Cell Biol.* 2004; 16:629–633. [PubMed: 15530773]
- Murren JR, Beidler DR, Cheng YC. Camptothecin resistance related to drug-induced down-regulation of topoisomerase I and to steps occurring after the formation of protein-linked DNA breaks. *Ann N Y Acad Sci.* 1996; 803:74–92. [PubMed: 8993502]
- 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]

- Pugh PL, Vidgeon-Hart MP, Ashmeade T, et al. Repeated administration of the noradrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) modulates neuroinflammation and amyloid plaque load in mice bearing amyloid precursor protein and presenilin-1 mutant transgenes. *Journal of neuroinflammation*. 2007; 4:8. [PubMed: 17324270]
- Ransom RW, Waggaman LA, Cho AK. Interaction of xylamine with peripheral sympathetic neurons. *Life Sci*. 1985; 37:1177–1182. [PubMed: 3930904]
- Rass U, Ahel I, West SC. Defective DNA repair and neurodegenerative disease. *Cell*. 2007; 130:991–1004. [PubMed: 17889645]
- Rey NL, Jandanhazi-Kurutz D, Terwel D, Kummer MP, Jourdan F, Didier A, Heneka MT. Locus coeruleus degeneration exacerbates olfactory deficits in APP/PS1 transgenic mice. *Neurobiol Aging*. 2012; 33:426, e421–411. [PubMed: 21109328]
- Richards ML, Sadee W. Human neuroblastoma cell lines as models of catechol uptake. *Brain Res*. 1986; 384:132–137. [PubMed: 3790988]
- Robinson GB, Fluharty SJ, Zigmond MJ, Sciallasi RJ, Berger TW. Recovery of hippocampal dentate granule cell responsiveness to entorhinal cortical input following norepinephrine depletion. *Brain Res*. 1993; 614:21–28. [PubMed: 7688646]
- Robison SH, Bradley WG. DNA damage and chronic neuronal degenerations. *Journal of the neurological sciences*. 1984; 64:11–20. [PubMed: 6234379]
- 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-chlorethyl-N-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart. *Br J Pharmacol*. 1976; 58:521–527. [PubMed: 1000130]
- Rouse J, Jackson SP. Interfaces between the detection, signaling, and repair of DNA damage. *Science*. 2002; 297:547–551. [PubMed: 12142523]
- Serrano MA, Li Z, Dangeti M, Musich PR, Patrick S, Roginskaya M, Cartwright B, Zou Y. DNA-PK, ATM and ATR collaboratively regulate p53-RPA interaction to facilitate homologous recombination DNA repair. *Oncogene*. 2012
- Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev*. 2001; 11:71–77. [PubMed: 11163154]
- Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*. 2003; 3:155–168. [PubMed: 12612651]
- Sontag TA, Hauser J, Kaunzinger I, Gerlach M, Tucha O, Lange KW. Effects of the noradrenergic neurotoxin DSP4 on spatial memory in the rat. *J Neural Transm*. 2008; 115:299–303. [PubMed: 17994182]
- 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]
- Szot P, Miguelez C, White SS, Franklin A, Sikkema C, Wilkinson CW, Ugedo L, Raskind MA. A comprehensive analysis of the effect of DSP4 on the locus coeruleus noradrenergic system in the rat. *Neuroscience*. 2010; 166:279–291. [PubMed: 20045445]
- Tieu K, Zuo DM, Yu PH. Differential effects of staurosporine and retinoic acid on the vulnerability of the SH-SY5Y neuroblastoma cells: involvement of bcl-2 and p53 proteins. *J Neurosci Res*. 1999; 58:426–435. [PubMed: 10518116]
- Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem*. 2001; 276:47759–47762. [PubMed: 11673449]
- Waterman SA, Harding CF. Neurotoxic effects of DSP-4 on the central noradrenergic system in male zebra finches. *Behav Brain Res*. 2008; 188:271–280. [PubMed: 18160108]
- Wenge B, Bonisch H. Interference of the noradrenergic neurotoxin DSP4 with neuronal and nonneuronal monoamine transporters. *Naunyn Schmiedebergs Arch Pharmacol*. 2009; 380:523–529. [PubMed: 19838680]
- Winkler H. The composition of adrenal chromaffin granules: an assessment of controversial results. *Neuroscience*. 1976; 1:65–80. [PubMed: 794758]

- Wolfman C, Abo V, Calvo D, Medina J, Dajas F, Silveira R. Recovery of central noradrenergic neurons one year after the administration of the neurotoxin DSP4. *Neurochem Int.* 1994; 25:395–400. [PubMed: 7820072]
- Yu PH, Davis BA, Fang J, Boulton AA. Neuroprotective effects of some monoamine oxidase-B inhibitors against DSP-4-induced noradrenaline depletion in the mouse hippocampus. *J Neurochem.* 1994; 63:1820–1828. [PubMed: 7931338]
- Zarei M, Stephenson JD. Effects of DSP4 and dizolcipine on connectivity of solid E19 cortical grafts to ablated Sml region of adult rats; an in vivo electrophysiological study. *Brain Res.* 2002; 947:182–190. [PubMed: 12176159]
- 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]
- Zhang J, Perry G, Smith MA, Robertson D, Olson SJ, Graham DG, Montine TJ. Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am J Pathol.* 1999; 154:1423–1429. [PubMed: 10329595]
- Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. *Nature.* 2000; 408:433–439. [PubMed: 11100718]
- Zou Y, Liu Y, Wu X, Shell SM. Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. *J Cell Physiol.* 2006; 208:267–273. [PubMed: 16523492]

Figure 1.

Time-dependent effects of DSP4 (50 μ M) 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 7–9 separate experiments. *** $p < 0.001$, compared to the control group (0h); ### $p < 0.001$, compared to the 6h group; &&& $p < 0.001$, compared to the 12h group; \$\$\$ $p < 0.001$, compared to the 24h group.

Figure 2. Concentration-dependent effects of DSP4 treatment for 24 hours on mRNA (A, measured by qPCR) and protein (B, measured by western blotting; C: the quantitative analysis of band densities in western blotting) levels of DBH in SH-SY5Y cells. 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 8–12 separate experiments. *** $p < 0.001$, compared to the control group (0 μ M); # $p < 0.05$, ### $p < 0.001$, compared to the 5 μ M group; && $p < 0.01$, compared to the 10 μ M group.

Figure 3.

Concentration-dependent effects of DSP4 treatment for 24 hours on mRNA (A, measured by qPCR) and protein (B, measured by western blotting; C: the quantitative analysis of band densities in western blotting) levels of NET in SH-SY5Y cells. 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 9–11 separate experiments. *** p < 0.001, compared to the control group (0 μ M); ### p < 0.001, compared to the 5 μ M group; &&& p < 0.001, compared to the 10 μ M group.

Figure 4.

Recovery of DBH/NET protein levels from inhibition by DSP4. SH-SY5Y cells were exposure to 50 μ M DSP4 for 24 hours (DSP4) and then incubated in fresh media in the absence of DSP4 for 24 (R24h), 48 (R48h), 72 (R72h) and 96 hours (R96h) 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 7 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 R24h group.

Figure 5. Effects of DSP4 treatment on cell proliferation and cell cycle arrest in SH-SY5Y cells. A: The viable number of SH-SY5Y cells decreased significantly after cells were exposed to 5 and 50 μ M 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 Fig. A & C represents data obtained from 6–7 separate experiments. ** $P < 0.01$, compared to 0 μ M group (A) or compared to 0 μ M group in G1-phase. ## $p < 0.01$, compared to 0 μ M group in S-phase. In Fig. B: black: control; red: 5 μ M DSP4; blue: 50 μ M DSP4.

Figure 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 Fig. A, black: control; red: DSP4 for 24 hours; blue: removing DSP4 for 12 hours; green: removing DSP4 for 24 hours. Each bar from Fig. B represents data obtained from 7 separate experiments. ** $P < 0.01$, *** $P < 0.001$, compared to 0 μ M group in G1-phase. ## $p < 0.01$, ### $p < 0.001$, compared to 0 μ M group in S-phase. &&& $p < 0.001$, compared to 0 μ M group in G2/M-phase.

Figure 7.

The time course analysis of DSP4-induced DNA damage as demonstrated by increased phosphorylation of H2AX (γ H2AX) and p53 (p-p53^{ser15}). Cells were exposed to 50 μ M DSP4 for 0.5 – 6 hours. A: autoradiograph obtained by western blotting. B and C: the quantitative analysis of band densities in western blotting of γ H2AX/p-p53^{ser15}. Each bar from Figs. B and C represents data obtained from 6 separate experiments. ***P<0.001, compared to the control (0h).

Figure 8.

DSP4 treatment induced a DNA damage response in SH-SY5Y cells. A & B: western blotting and quantitative analysis revealed an increase in γ H2AX after DSP4 treatment for 24 hours. C & D: western blotting and quantitative analysis showed an increase of phosphorylated p53^{ser15} (p-p53^{ser15}) after DSP4 treatment for 24 hours. E: No phosphorylated RPA32 was observed after exposing cells to 50 μ M DSP4. Each bar from Figs. B & D represents data obtained from 7 separate experiments. CPT: camptothecin, the positive control for DNA damage which results in phosphorylation of H2AX, p53 and RPA32. ** $p < 0.01$, *** $p < 0.001$, compared to 0 μ M group.

Figure 9.

DSP4 treatment induced the of nuclear formation γ H2AX and p-p53^{ser15} foci. A: Immunofluorescence staining showed foci of γ H2AX in the nuclei of cells treated with 50 μ M DSP4 for 24 hours. B: Immunofluorescence staining showed foci of p-p53^{ser15} in the nuclei of cells treated with 50 μ M DSP4 for 24 hours. N=5 for each group. DAPI, 4',4-diamidino-2-phenylindole, a fluorescent dye that strongly binds to DNA as a nuclear counterstain.

Figure 10.

The DSP4-induced DDR is dependent on ATM activation. A & B: western blotting and quantitative analysis show a reduction in DSP4-induced γ H2AX formation in cells treated with ATM or ATR inhibitor. C & D: western blotting and quantitative analysis show a reduction in the DSP4-induced phosphorylated p53^{ser15} (p-p53^{ser15}) formation in cells treated with ATM or ATR inhibitor. The cells were pretreated with the inhibitors for 1 hour and continued for 24 hours with DSP4 treatment. Each bar from both pictures (B & D) represents data obtained from 6 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 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 hours. The cells were processed for comet assays run under neutral and alkaline conditions to identify DNA DSBs *vs.* SSBs, respectively. N=5 for each group.