Neurotoxicology 70 (2019) 48–61

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Heterozygous huntingtin promotes cadmium neurotoxicity and neurodegeneration in striatal cells via altered metal transport and protein kinase C delta dependent oxidative stress and apoptosis signaling mechanisms

Gunnar F. Kwakye[a,*], Jessica A. Jiménez[a], Morgan G. Thomas[a], Brett A. Kingsley[a], Matthew McIlvin[b], Mak A. Saito[b], Edmund M. Korley[a]

[a] *Neuroscience Department, Oberlin College, Oberlin, OH, USA*
[b] *Marine Chemistry and Geochemistry Department, Woods Hole Oceanographic Institution, Woods Hole, MA, USA*

ARTICLE INFO

Keywords:
Huntington's disease
Cadmium neurotoxicity
Oxidative stress
Metal transport
Apoptosis
Neurodegeneration
Protein kinase C delta
ERK
Antioxidants

ABSTRACT

Huntington's disease (HD) is functionally linked to environmental factors including cigarette use and dyshomeostasis in the levels of metals. Interestingly, one of the most abundant heavy metals in cigarettes is cadmium (Cd), which also accumulates in the striatum and causes neurotoxicity upon exposure. Thus, we hypothesized that heterozygous huntingtin (HTT), responsible for the majority of cases of HD in patients, in combination with Cd exposure would cause neurotoxicity and neurodegeneration via increased intracellular accumulation of Cd and activation of oxidative stress signaling mechanisms in a mouse striatal cell line model of HD. We report that heterozygous HTT striatal cells are significantly more susceptible to Cd-induced cytotoxicity as compared to wild-type HTT cells upon exposure for 48 h.

The heterozygous HTT and Cd-induced cytotoxicity led to a NADPH oxidase (NOX) mediated oxidative stress that was attenuated by exogenous antioxidants and a NOX inhibitor, apocynin. Heterozygous HTT coupled with Cd exposure caused increased expression of protein kinase C δ (PKCδ) and other key oxidative stress proteins levels, enhanced the activation of caspase-9 and caspase-3 mediated apoptosis, and blocked the overexpression of extracellular signal-regulated kinase (ERK). We observed significantly greater intracellular accumulation of Cd and reduced expression of divalent metal transporter 1 (DMT1) protein in the heterozygous HTT striatal cells upon Cd exposure. Treatment with zinc, manganese, and iron as well as exogenous antioxidants significantly attenuated the Cd-induced cytotoxicity. Collectively, these results demonstrate that heterozygous HTT exhibits greater neurotoxic properties when coupled with Cd exposure to cause cell death via caspase mediated apoptosis, altered metal transport, and modulation of ERK and PKCδ dependent oxidative signaling mechanisms.

1. Introduction

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disease characterized by motor, cognitive, and psychiatric abnormalities (Clabough, 2013). HD is caused by an expansion in the trinucleotide repeat (CAG) in exon 1 of the huntingtin gene (*HTT*) encoding the ubiquitous huntingtin protein (HTT), which results in an abnormally expanded polyglutamine tract in the N-terminus of HTT (Cattaneo et al., 2005). Neurotypical individuals express 6–35 CAGs while most of HD patients express one normal allele and a mutant, disease causing allele, with > 36 CAGs (Snell et al., 1993). Expression of a copy of the normal allele suffices for normal development and neuronal function in adult organisms (Ambrose et al., 1994; Dixon et al., 2004). Wild-type HTT mediates an antiapoptotic role in HD models by interfering with apoptosome formation and consequent caspase activation (Rigamonti et al., 2000, 2001; Zhang et al., 2006).

Mutation in *HTT* results in selective loss of the medium spiny neurons within the striatum prior to the other affected brain regions including the cortex and thalamus, despite widespread expression of the polyglutamine-expanded HTT (Imarisio et al., 2008). Thus, other factors within the striatum may increase the susceptibility of this area to selective degeneration. Environmental factors have been suggested to contribute to variability in age of onset, symptoms, and progression of

* Corresponding author at: Neuroscience Department, Oberlin College, 119 Woodland Street, Oberlin, OH, 44074, USA.
E-mail address: Gunnar.Kwakye@oberlin.edu (G.F. Kwakye).

https://doi.org/10.1016/j.neuro.2018.10.012
Received 23 January 2018; Received in revised form 22 October 2018; Accepted 31 October 2018
Available online 03 November 2018
0161-813X/

HD. For example, the number of CAG repeats contributed to 40% of variation in age of onset in HD patients with the remaining 60% suggested to be caused by other genes and unknown environmental factors (Wexler et al., 2004). Further, identical twin HD individuals exhibit variability in age of onset, symptoms and progression (Sudarsky et al., 1983; Anca et al., 2004; Friedman et al., 2005; Gómez-Esteban et al., 2007).

Heavy metal exposure and cytotoxicity has been implicated in various neurological disorders including HD (Bowman et al., 2011). Metals play important roles in a wide variety of biological processes of living systems, thus their intracellular concentration must be tightly regulated (Jomova and Valko, 2011). Heavy metals are known to cause oxidative stress through interactions with DNA and proteins, and disrupt transcription and signal transduction pathways that play important roles in cell growth, development and apoptosis (Montgomery, 1995). Alteration in homeostasis of redox active metals such as iron (Fe), copper (Cu), and zinc (Zn) may lead to uncontrolled formation of free radicals and enhanced oxidative stress resulting in cell death (Jomova and Valko, 2011).

in vitro and *in vivo* HD studies have revealed pathophysiological interactions mediating neurotoxicity between mutant HTT and heavy metals as well as mutant HTT and pesticides. For example; expression of homozygous mutant HTT (ST*Hdh*$^{Q111/Q111}$) reduced the susceptibility of striatal cell lines to manganese (Mn) neurotoxicity and diminished Mn(II) accumulation as compared to wild-type HTT (ST*Hdh*$^{Q7/Q7}$) in a striatal cell line model of HD (Williams et al., 2010a, 2010b) via selective alterations in the ataxia telangiectasia mutated tumor protein p53 (ATM-p53) signaling pathway (Tidball et al., 2015). A recent study utilized a set of small molecule inhibitors (KU-55933, NU-7441, and LY294002) to demonstrate the role of phosphatidylinositol 3 kinase (PI3K) signaling in modulating Mn-induced signaling and Mn homeostasis in wild-type HTT (ST*Hdh*$^{Q7/Q7}$), Neuro2 A, HEK293, ST14 A, and mouse embryonic fibroblasts (MEF) (Bryan et al., 2018). Impairment in intracellular Fe levels and energy metabolism are both features of HD pathogenesis as HTT has been reported to mediate endocytosed Fe(II) required for oxidative energy production (Lumsden et al., 2007; Agrawal et al., 2018). Further, magnetic resonance imaging studies suggest changes in the metabolism of brain iron during early HD (Rosas et al., 2012; Domínguez et al., 2016; van Bergen et al., 2016). Postmortem studies revealed increased levels of Fe(II), Cu(II), and Zn(II) in the brains of HD individuals as compared to age-matched controls (Dexter et al., 1991, 1992; Fox et al., 2007) suggesting that perturbation in the normal role of HTT in modulating metal homeostasis may contribute to HD neuropathology. *in vivo* and *in vitro* models revealed that exposure to Cu(II) (Fox et al., 2007), pesticides and other mitochondrial inhibitors can accelerate the aggregation of mutant HTT (Deshmukh et al., 2012). We have recently reported that homozygous mutant HTT (ST*Hdh*$^{Q111/Q111}$) striatal cells are significantly more susceptibility to a commonly used organophosphate insecticide, chlorpyrifos, induced neurotoxicity and neurodegeneration compared to wild-type (ST*Hdh*$^{Q7/Q7}$) (Dominah et al., 2017). An explanation for the effect of environmental modulators such as heavy metals and pesticide exposure in the pathogenesis of HD is that both the *HTT* mutation and environmental modulators may be synergistically acting upon shared pathophysiological mechanisms inherent to the medium spiny neurons.

Substance use including alcohol and cigarettes, correlates with the severity of psychiatric symptoms associated with HD. Epidemiological evidence from the National Research Roster on HD suggest that higher levels of alcohol and cigarette use are associated with earlier age of HD symptom onset and higher ratings of psychiatric symptoms (Ehret et al., 2007). Monozygotic twins with identical CAG repeat lengths in the *HTT* gene and discordant for HD by more than seven years revealed increased use of cigarettes and exposure to various industrial toxins as potential explanations for the earlier onset of HD symptoms in one twin (Friedman et al., 2005). Interestingly, Cd(II) is one of the most abundant heavy metals in popular cigarette brands, averaging a concentration of 1.81 $\mu g\,g^{-1}$ (dry weight). Thus, the average quantity of Cd(II) inhaled from smoking one packet of 20 cigarettes is in the range of 0.22–0.78 μg (Ashraf, 2012). These findings are consistent with the possibility of synergistic disease processes of HD and Cd(II) toxicity involving common striatal neurodegeneration.

Cd(II) is a heavy metal found in foods and fertilizers, and dispersed into the environment through phosphate mining and agricultural industries. Cd(II) exposure via oral, dermal, and respiratory routes can accumulate and cause toxicity to organs such as the brain, kidney, liver, pancreas, and lungs. Cd(II) intake ranges from 10 to 40 μg/day to several μg/day in non-polluted and polluted regions, respectively (Jomova and Valko, 2011). A disease-toxicant screen has reported increased susceptibility of homozygous mutant HTT (ST*Hdh*$^{Q111/Q111}$) striatal cells to 50 μM Cd(II)-induced cytotoxicity compared to wild-type upon 30 h exposure (Williams et al., 2010a, 2010b). Understanding Cd(II) toxicity in the context of HD pathophysiology, particularly heterozygous mutant HTT (ST*Hdh*$^{Q7/Q111}$), is of interest because (i) majority of HD patients are heterozygous for the *HTT* mutation (Squitieri et al., 2003); (ii) Cd(II) is abundant in the environment and dispersed between the three main environmental compartments, air, water, and soils (Jomova and Valko, 2011); and (iii) Cd(II) and Zn(II) have similar ionic radius and chemical properties, thus necessitating living organisms to preclude Cd(II) from replacing Zn(II) as a cofactor for proteins. Cd(II) and Zn(II) are group IIB transition elements/metals with similar valence state and electronic configuration and possess similar affinities for ligands. Thus, a coordinate preference of metal ion is required for their ability to interact with enzymes for proper activity and function. Substrate specificity analysis of proteins associated with Zn(II) showed instances of Zn(II) exchanged for Cd(II) in many proteins necessary in living organisms that resulted in altered protein substrate specificity (Zhang et al., 1996). Moreover, the effect of Cd(II) in the HD brain is unknown and of concern to public health due to its long half-life (~15-20 years) in the nervous system and ability to accumulate in the corpus striatum, the brain region most vulnerable in HD, following prolonged exposure (Pal et al., 1993; Miller et al., 2005).

Cd(II) toxicity is known to cause oxidative stress, alter mitochondrial function and activate a caspase cascade resulting in cell death in many cell types including neurons (Yang et al., 2007; Chong et al., 2017). In addition, Cd(II) exposure can activate cellular protein kinases, such as Akt-PI3K (phosphatidylinositol 3 kinase), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC), which results in enhanced phosphorylation of various transcription factors leading to activation of target gene expression (Jomova and Valko, 2011). HD shares similar pathophysiology including oxidative stress, metal accumulation, mitochondrial dysfunction, and protein aggregation with Cd (II) neurotoxicity. Hence, it is likely that Cd(II) toxicity impinges on similar pathogenic pathways known to be affected by mutant *HTT* allele to modify selective striatal neurodegeneration.

Although Cd(II) exposure may not be the primary cause of HD, their pathophysiology in the context of heterozygous *HTT*, found in most HD patients, is critical for our current understanding of selective neuropathology and variability in age of onset, symptoms and progression in HD. Nothing is known about (i) the neurotoxic effects of Cd(II) in heterozygous HTT striatal cells, (ii) heterozygous HTT x Cd(II)-induced caspase activation and apoptosis, (iii) interaction between heterozygous HTT and Cd(II) to modulate ERK, Akt and PKCδ signaling pathways, (iv) intracellular accumulation of Cd(II) in HD striatal cells upon exposure to Cd, (v) modulation of metal transporter systems by heterozygous HTT x Cd(II) interaction, and (vi) potential effect of exogenous antioxidants to protect against Cd(II)-induced neurotoxicity in heterozygous HTT striatal cells. Herein, we investigate these gaps using the striatal neuronal cell line model of HD to better understand the effect of Cd(II)-induced toxicity in medium spiny neurons most vulnerable in HD as well as the association between heterozygous HTT and Cd(II) neurotoxicity and neurodegeneration and its usefulness in the risk assessment of Cd(II) toxicity in humans. We hypothesize that heterozygous

HTT, found in most HD patients, in combination with Cd(II) exposure would cause neurotoxicity and neurodegeneration via increased intracellular accumulation of Cd(II) and activation of oxidative stress signaling mechanisms in a mouse striatal cell line model of HD.

2. Materials and methods

2.1. Chemicals and reagents

Cadmium (II) chloride ($CdCl_2$), manganese (II) chloride ($MnCl_2$), zinc (II) chloride ($ZnCl_2$), and iron (II) chloride ($FeCl_2$) used in the cytotoxicity assays were purchased from Alfa Aesar (Ward Hill, MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt, and CM-H_2DCFDA dye were purchased from Life Technologies (Grand Island, NY). Lactate dehydrogenase (LDH) cytotoxicity assay kit (Item No. 601170) was purchased from Cayman Chemical (Ann Arbor, MI). TBARS lipid peroxidation (MDA) assay kit (ab118970) was purchased from Abcam (Cambridge, MA, USA). Dulbecco's Modified Eagle's Media (DMEM D6546), GlutaMax, G418, penicillin/streptomycin (Pen Strep), dimethyl sulfoxide (DMSO), ascorbic acid, N-acetylcysteine (NAC), apocynin, metaphosphoric acid, nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (SOD) activity assay, radioimmunoprecipitation assay buffer (RIPA) buffer, and reagents for Krebs Ringer buffer (5.6 mM glucose, 1.3 mM EDTA, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4.7 mM KCl, 120 mM NaCl, 16 mM Na_3PO_4) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Caspase-9 substrate (Ac-LEHD-AFC) and caspase-3 substrate (Ac-DEVD-AFC) were obtained from Bachem Biosciences (King of Prussia, PA). Glutathione (GSH) detection kit was purchased from Enzo Life Sciences (catalog # ADI-900-160; Farmingdale, NY). Fetal bovine serum (FBS) was purchased from Biowest (Logan, UT). Annexin V-FITC Apoptosis Detection Kit was purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Cell culture

We used an immortalized striatal derived cell line from a Hdh^{Q111} knock-in transgenic mouse model of HD containing either (i) homozygous HTT loci with a humanized exon 1 containing 7 polyglutamine repeats (ST*Hdh*$^{Q7/Q7}$) or (ii) heterozygous HTT loci with a humanized exon 1 with 111 polyglutamine repeats and the other with a humanized exon 1 with 7 polyglutamine repeats (ST*Hdh*$^{Q7/Q111}$) (Trettel et al., 2000). HD striatal cells (ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q7/Q111}$) were cultured in DMEM with 4.5 g/L glucose supplemented with 10% FBS, 1% P/S, 400 µg/ml G418 and 1% Glutamax at 33 °C with 5% CO_2.

2.3. Cell survival assay

Metal and chemical-induced cytotoxicity were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Chong et al., 2017). Briefly, seeded HTT striatal cells were incubated with the indicated metal and chemical stocks for the indicated time followed by 4 h MTT incubation. Changes in absorbance were measured at 570 nm with a microplate reader (Synergy HT, Biotek Instruments, Winooski, VT). Survival data was normalized by genotype to the vehicle control included in each independent sample set.

2.4. Lactate dehydrogenase activity

We followed the manufacturer's instructions to measure the release of intracellular lactate dehydrogenase (LDH) into the cell-free extracellular supernatant that was used as an index of cell death. Briefly, cells were seeded and exposed to the indicated concentrations of Cd(II) for 48 h. Cultured plates were centrifuged at 400 x g for 5 min and then cell-free extracellular supernatant was added to LDH reaction solution and incubated at 37 °C for 30 min with gentle shaking. Changes in absorbance were measured at 490 nm using a microplate reader and normalized to baseline genotype control in each independent sample set after background subtraction. Percent cytotoxicity was calculated as [(experimental value A490) – (spontaneous release A490) / (maximum release A490) – (spontaneous release A490)] x 100.

2.5. Analysis of intracellular reactive oxygen species production

Reactive oxygen species (ROS) levels in the cells were measured with CM-H_2DCFDA dye that passively diffuses into cells. Cells were seeded in black 96 well plates the evening before treatment and incubated with 10 µM CM-H_2DCFDA for 45 min in Krebs Ringer buffer at 33 °C. Loading buffer was discarded and cells washed with Krebs Ringer buffer followed by 10 minutes de-esterification. The cells were incubated with the indicated concentrations of Cd(II) for the indicated times at 33 °C and fluorescence measurements were made with a microplate reader at excitation 485 ± 20 nm and emission of 528 ± 20 nm wavelengths. Background fluorescence was subtracted from the experimental samples and normalized by genotype to the vehicle control included in each independent sample set.

2.6. Glutathione (GSH) levels

GSH levels in the cells were measured with an enzymatic recycling method that involves the reduction of oxidized glutathione (GSSG) by glutathione reductase to reduced glutathione (GSH). Briefly, cells were exposed to the indicated concentrations of Cd(II) for 48 h, harvested and pellets suspended in 5% metaphosphoric acid. The reagents necessary to catalyze the enzymatic recycling of GSSG to GSH were added as recommended by the manufacturer. Absorbance measurements were made at 405 nm for 10 min at 1 min intervals with a microplate reader. The rate of change of absorbance over time was compared to a generated GSSG standard curve. Intracellular GSH levels were normalized by genotype to the baseline control included in each independent sample set.

2.7. Superoxide dismutase (SOD) activity

Total superoxide dismutase (SOD) activity in the cells was measured by following the manufacturer's instructions (Sigma-Aldrich). Briefly, cells were exposed to the indicated concentrations of Cd(II) for 48 h. Samples and controls were incubated with xanthine oxidase for 30 min and absorbance was measured at 450 nm using a microplate reader. Changes in absorbance were normalized by genotype to the baseline control included in each independent sample set.

2.8. Lipid peroxidation

We followed the manufacturer's instructions for the lipid peroxidation assay kit to measure the levels of malondialdehyde (MDA), a natural bi-product of lipid peroxidation, in the striatal cells. Briefly, cells were exposed to the indicated concentrations of Cd(II) for 48 h and the concentration of MDA was quantified fluorometrically (Ex/Em = 532/553 nm) using a microplate reader.

2.9. NADPH oxidase activity

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity was measured by the lucigenin-enhanced luminescence method as described previously (Dominah et al., 2017). Briefly, cells were exposed to the indicated concentrations of Cd(II) with or without 10 µM apocynin for 48 h at 33 °C. The cell suspensions were homogenized in lysis buffer and homogenates centrifuged. Photon emission in terms of relative light units was measured with a microplate reader after 5 min at 25 °C. There was no measurable activity in the absence of

NADPH. NOX activity expressed as relative luminescence (light) units (RLU) were normalized to protein (μg). Protein content was measured using the Bradford protein assay reagent.

2.10. Caspase-9 and caspase-3 activity

Caspase-9 and caspase-3 activity assay were similar to previously described protocols (Chong et al., 2017). Briefly, cells were exposed to the indicated concentrations of Cd(II) for 48 h, harvested and re-suspended in caspase lysis buffer. Cell-free supernatants were incubated with 50 μM caspase-9 substrate or caspase-3 substrate at 37 °C for 1 h. Formation of 7-amino-4-methylcoumarin (AFC), resulting from caspase-9 or caspase-3 activity, was measured at 400 nm excitation and 508 nm emission wavelengths using a microplate reader. Changes in fluorescence intensity were normalized by genotype to the baseline control included in each independent sample set.

2.11. Flow cytometer analysis

Detection of apoptosis in cultured striatal HD cell lines were performed according to the manufacturer's instruction (Annexin V-FITC Apoptosis Detection Kit, ThermoFisher Scientific). Briefly, cells were washed twice with PBS, 1–5 × 10^5 cells were collected and suspended in binding buffer, followed by the addition of Annexin V-FITC for 10 min and resuspension in binding buffer and propidium iodide (PI). After 5 min of dark incubation at room temperature, the cells were analyzed using flow cytometer (Accuri cytometrics Inc.) within 1 h. After setting the gate around the HD striatal neuron population, the data were analyzed by C6 analysis software (BD). The results were presented as two-color fluorescent diagrams of cells stained with anti-annexin V-FITC and PI. Cell apoptotic rate was calculated as Annexin V-FITC positive staining for early apoptosis, and both Annexin V-FITC and PI positive staining for late apoptosis/death.

2.12. Inductively coupled plasma mass spectrometry (ICP-MS) analysis

Wild-type and heterozygous HTT cell lines were treated with the indicated concentrations of Cd(II) for 48 h. Cells were harvested and flash-frozen for ICP-MS analysis as described previously (Chong et al., 2017). Briefly, samples were dissolved in 1 mL 50% nitric acid (Seastar Chemicals Inc.) with 10 ppb indium (In) and incubated overnight at room temperature; then10 μL each was diluted to 1 mL with 1% nitric acid before running on the ICP-MS (Thermo iCAP-Q isotope ratio mass spectrometer with an ESI SC-4DX autosampler). The ICP-MS was run in KED mode with helium as the collision gas, a dwell time of 0.02 s, and normal resolution. Cd levels were normalized to In. Cd(II) data is expressed as ng/mg protein.

2.13. Western blotting

Equal numbers of wild-type ST*Hdh*$^{Q7/Q7}$ and heterozygous ST*Hdh*$^{Q7/Q111}$ cells were seeded and treated with the indicated concentrations of Cd(II) for 48 h. Whole cell lysates were prepared using RIPA buffer, 1x protease inhibitor cocktail (Sigma), and 1x phosphatase inhibitor cocktails I and II (Sigma) and loaded by equal protein for SDS-PAGE. Protein concentrations in the cell lysates were determined with the Bradford protein assay reagent. Cell lysates containing equal amounts of protein were separated on a 4–25% SDS-polyacrylamide gel. Post separation, proteins were transferred to a nitrocellulose membrane and non-specific binding sites were blocked by treating with 3% BSA or 5% milk. The membranes were incubated overnight with primary antibodies directed against divalent metal transporter 1 (DMT-1) (NRAMP24-A, 1:2000; Alpha Diagnostic International), cytochrome c (catalog # 4272S, 1:1000), pan Akt (catalog # 4685S, 1:1000), phospho Akt (S473) (catalog # 9271S, 1:1000), ERK1/2 (catalog # 4695S, 1:1000), and phosho-ERK1/2 (Thr202/Tyr204) (catalog # 4695S, 1:1000) from Cell Signaling, pan PKCδ (catalog # sc-8402, 1:2000) and phospho-PKCδ (Tyr311) (catalog # sc-18364, 1:1000), nuclear factor erythroid 2-related factor (Nrf2) (catalog # sc-722, 1:500), Heme Oxygenase-1 (HO-1) (catalog # sc-10789, 1:500) from Santa Cruz Biotechnology), and β-actin (catalog #13E5, 1:5000, Cell Signaling). Appropriate secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA) were used at 1:10,000 accordingly. Blots were visualized with Thermo Scientific Pierce Supersignal West Dura Extended Duration Chemiluminescent Substrate (Waltham, MA) on the LI-COR C-DiGit Chemiluminescent Western Blot Scanner (Lincoln, NE). Measurement of integrated density of protein bands were performed using ImageStudioLite software (LI-COR).

2.14. Statistical analysis

Univariate two-way analysis of variance (ANOVA) was performed using SPSS 23.0 software (SPSS, Inc., Chicago, IL). *Post-hoc* analyses were done using Microsoft Excel (Redmond, WA) by Student's t-tests (two-tailed) and error bars are expressed as standard error of the mean (SEM) unless otherwise noted. The alpha level for all analyses was set at $p \leq 0.05$.

3. Results

3.1. Cd(II) exposure increases cytotoxicity in heterozygous HTT striatal cells

Analyses of Cd(II)-induced cytotoxicity in the HTT striatal cell lines revealed that heterozygous HTT increased sensitivity to Cd(II)-induced cytotoxicity when compared to wild-type HTT after 48 h exposure as examined by the MTT metabolic capability assay, an indirect measure of cell survival. Cd(II) concentrations were chosen to generate a survival curve spanning non-toxic to highly toxic concentrations. We observed a 5%, 10%, and 15% decrease in cell survival for both genotypes upon exposure to 5, 10, and 20 μM Cd(II) respectively after 3 and 24 h exposure (Fig. 1A, B). A disease-toxicant screen study has reported that exposure to 50 μM Cd(II) caused significantly greater cytotoxicity in homozygous mutant HTT (ST*Hdh*$^{Q111/Q111}$) striatal cell lines compared to wild-type (ST*Hdh*$^{Q7/Q7}$) upon 26–30 h exposure (Williams et al., 2010a, 2010b). Thus, we reasoned that examining lower concentrations of Cd(II) for longer duration (48 h) using heterozygous HTT (ST*Hdh*$^{Q7/Q111}$) striatal cell line, found in most HD patients, would reveal novel gene-environment interaction. Statistical analysis by two-way univariate ANOVA found a significant effect of genotype ($F_{(1,30)} = 153.5$, $p < 0.001$) and Cd(II) exposure ($F_{(4,30)} = 196.8$, $p < 0.001$) on survival after 48 h exposure. In addition, a two-way interaction between genotype and Cd(II) exposure was detected ($F_{(4,30)} = 20.27$, $p < 0.001$) indicating that each genotype had a unique Cd(II)-response curve after 48 h exposure (Fig. 1C). We used the LDH cytotoxicity assay as an index of cell death after exposure to Cd(II) for 48 h. Statistical analysis by two-way univariate ANOVA confirmed the significant genotype, Cd(II), and genotype x Cd(II) interaction on cell death (Fig. 1D). Further, we observed significant ($p < 0.01$) decrease in survival for both genotypes after Cd(II) exposure when compared to their respective baseline genotype controls as examined by both assays (Fig. 1C, D). This independent cell death analysis confirmed that the Cd(II)-induced cytotoxicity revealed by the MTT assay was due to cell death rather than just differences in mitochondrial reductase activity.

3.2. Heterozygous huntingtin potentiates Cd(II)-induced oxidative stress pathways

Quantitative analysis of ROS levels revealed that the heterozygous HTT line produced significantly ($p < 0.01$) greater intracellular ROS compared to wild-type cells upon 30 min exposure to 10 and 20 μM Cd (II). A two-way interaction between genotype and Cd(II) exposure

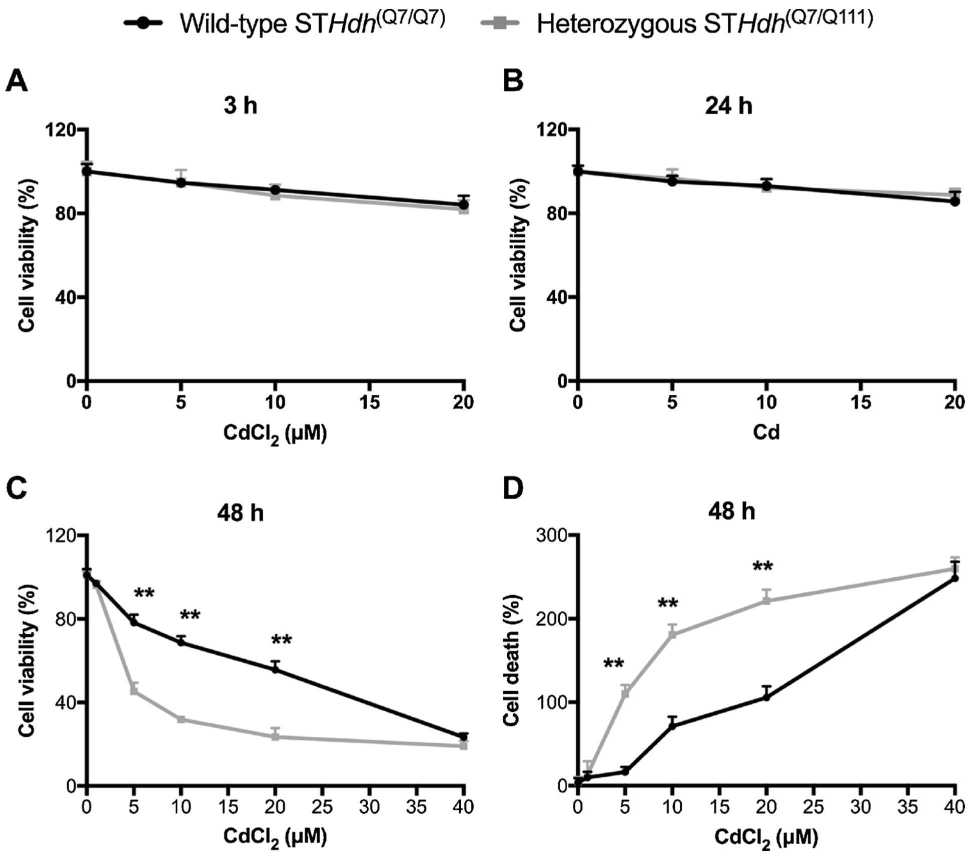


Fig. 1. Heterozygous HTT striatal cell lines are significantly more susceptible to Cd(II) neurotoxicity upon exposure for 48 h. Wild-type (black) and heterozygous HTT (grey) striatal cells were treated with Cd (II) A, 3 h; B, 24 h; C and D, 48 h. Cytotoxicity and cell death were assessed by MTT (A−C) and cell death (D) and LDH release assays respectively. Survival is expressed as percentage of the control groups within each genotype. Results are represented by the mean ± SEM from 4 samples (technical replicates) in each treatment group. N = 4. Significant difference in survival (** $p < 0.01$, Student's *t*-test) between wild-type and heterozygous HTT cells are shown.

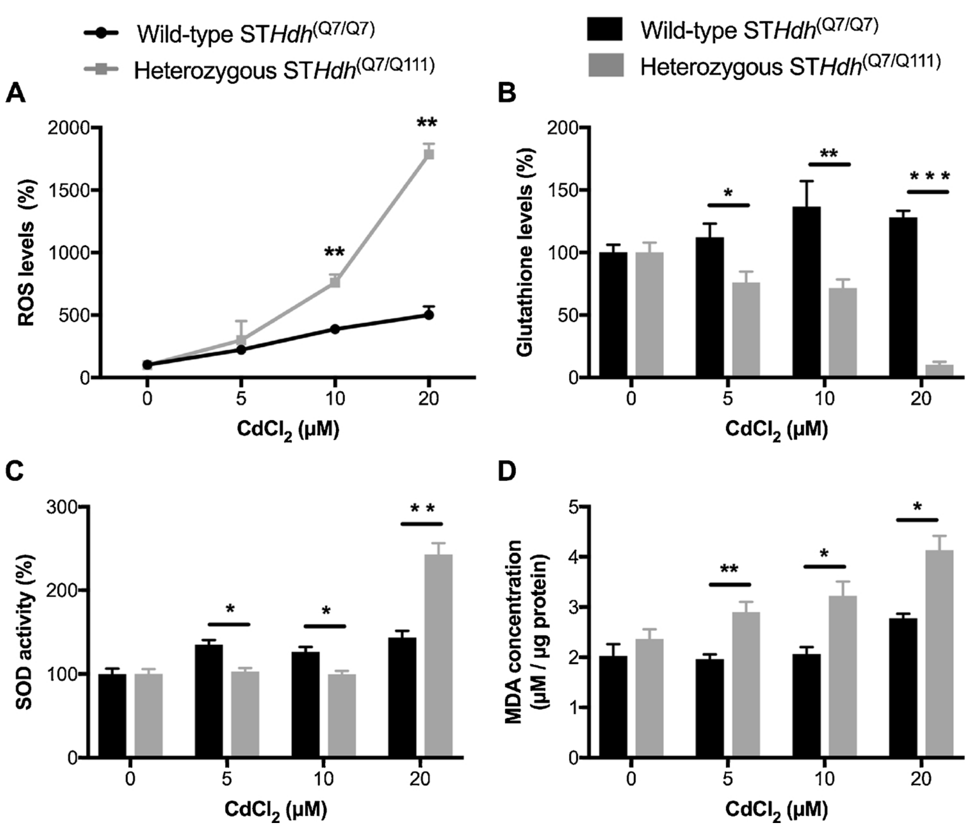


Fig. 2. Heterozygous HTT and Cd(II) co-operatively promote oxidative stress in striatal cells. A, ROS production in wild-type and heterozygous HTT lines after exposure to the indicated concentrations of Cd(II) for 30 min. Fluorescence was normalized to baseline genotype control. Each experiment had 8 independent samples (technical replicates). N = 4 biological replicates. B, Glutathione (GSH) levels and C, superoxide dismutase (SOD) activity in striatal cells after exposure to Cd(II) for 48 h. D, Concentration of MDA levels as an indicator of lipid peroxidation in HTT expressing striatal cells after exposure to Cd(II) for 48 h. Each experiment in B–D had 4 independent samples (technical replicates). N = 4 biological replicates. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test indicates significant differences between wild-type and heterozygous HTT lines.

($F_{(4,30)} = 242.9$, $p < 0.001$) on ROS production was detected indicating that each genotype had a unique ROS production-response curve. *Post-hoc* analysis indicated that Cd(II) exposure significantly increased ROS generation in both HTT lines when compared to their baseline genotype controls after 30 min exposure (Fig. 2A).

To better understand the influence of heterozygous HTT x Cd(II) mediated cytotoxicity on antioxidant defense mechanism we measured the levels of glutathione (GSH) and activity of superoxide dismutase (SOD) after 48 h exposure to Cd(II). Two-way univariate ANOVA found a significant effect of genotype ($F_{(1,30)} = 568.9$, $p < 0.001$), Cd(II) exposure ($F_{(4,30)} = 14.54$, $p < 0.001$), and a two-way interaction between genotype and Cd(II) exposure ($F_{(4,30)} = 38.25$, $p < 0.001$) on the levels of GSH. Wild-type had significantly ($p < 0.05$) greater levels of GSH at 10 and 20 μM Cd(II) when compared to baseline genotype control. In addition, there was a significant ($p < 0.05$) decrease in GSH levels in heterozygous HTT lines at 5, 10, and 20 μM Cd compared to baseline genotype controls. GSH levels in the heterozygous HTT cells were significantly ($p < 0.01$) reduced compared to wild-type at all tested Cd(II) concentrations (Fig. 2B). Further, *post-hoc* analysis indicated that the Cd(II) exposure did not affect the activity of SOD in heterozygous HTT cells at 5 and 10 μM Cd(II) compared to baseline genotype control. However, we observed an unexpected significant ($p < 0.01$) increase in SOD activity in the heterozygous HTT cells at 20 μM Cd(II) exposure when compared to wild-type and baseline genotype control. Cd(II) caused a significant increase in the activity of SOD in wild-type striatal lines at 5, 10, and 20 μM compared to baseline genotype controls (Fig. 2C).

The concentration of MDA was measured to examine Cd(II)-induced lipid peroxidation in the striatal cells. We report significant ($p < 0.05$) increase in the levels of MDA in heterozygous HTT cells upon 10 and 20 μM Cd(II) exposure for 48 h when compared to baseline genotype control. MDA levels were significantly increased in wild-type cells after 20 μM Cd(II) exposure compared to baseline genotype control. *Post-hoc* analysis indicated that the wild-type cells had significantly ($p < 0.05$) lower concentration of MDA at all tested Cd(II) concentrations compared to heterozygous HTT lines (Fig. 2D). This data suggest that heterozygous HTT and Cd(II) potentiate oxidative stress pathways resulting in neurotoxicity.

3.3. NOX activity in HD striatal cells is modulated by heterozygous huntingtin x Cd(II) interaction

To better understand heterozygous HTT and Cd(II) mediated oxidative stress and neurotoxicity, we investigated the role of NOX in Cd (II)-induced ROS production and neurotoxicity in HTT striatal lines following 10 μM Cd(II) exposure for 48 h. Cd(II) exposure induced a significant increase ($p < 0.05$) in NOX activity in both wild-type and heterozygous HTT cells compared to baseline genotype controls. NOX activity was significantly ($p < 0.05$) greater in heterozygous HTT lines compared to wild-type (Fig. 3A). Co-treatment with 10 μM apocynin, a NOX inhibitor, significantly ($p < 0.05$) reduced NOX activity in heterozygous HTT lines as compared to NOX activity in the absence of apocynin treatment. We did not observe a significant effect of apocynin on NOX activity in wild-type cells following 10 μM Cd(II) exposure. In addition, apocynin abolished the genotype difference in NOX activity between wild-type and heterozygous HTT lines following Cd(II) exposure (Fig. 3B). This result suggests that heterozygous HTT x Cd(II)-induced ROS production and neurotoxicity may be mediated, at least in part, through activation of NOX signaling pathway.

3.4. Co-treatment with exogenous antioxidants and apocynin mitigates Cd (II)-induced ROS production in huntingtin striatal cells

Based on the influence of heterozygous HTT x Cd(II) on NOX mediated ROS production, we reasoned that co-treatment with Cd(II) and the exogenous antioxidants, N-acetyl-cysteine (NAC) and ascorbic acid, or NOX inhibitor, apocynin, would attenuate the Cd(II)-induced early ROS production in the HTT striatal cell lines. Wild-type and heterozygous HTT lines were co-treated with the indicated concentrations of Cd(II) and non-toxic concentrations of NAC (250 μM) and ascorbic acid (100 μM), or apocynin (10 μM), and intracellular ROS levels examined after 30 min by DCF-DA assay. The concentration of antioxidants were chosen based on our previous study that used similar concentrations and showed effects in HD striatal cell lines (Dominah et al., 2017). Co-treatment with NAC did not significantly alter Cd(II)-induced ROS production in heterozygous HTT lines but resulted in a subtle and significant decrease in ROS levels in wild-type lines at 10 and 20 μM Cd(II) exposure (Fig. 4A). However, co-treatment with ascorbic acid (Fig. 4B) and apocynin (Fig. 4C) significantly attenuated Cd(II)-induced ROS production in both wild-type and heterozygous HTT cells, and abolished the genotype difference in ROS production at all the tested concentrations of Cd(II) (Fig. 4B, C). There was no significant difference in ROS levels between the two genotypes upon treatment with only NAC, ascorbic acid, and apocynin (Fig. 4D). These results suggest that co-treatment with either 100 μM ascorbic acid or 10 μM apocynin harbors neuroprotective effects against Cd(II)-induced ROS production in HTT striatal cells.

3.5. Treatment with NAC, ascorbic acid, and apocynin greatly attenuates Cd(II)-induced cytotoxicity in heterozygous huntingtin striatal cells compared to no treatment conditions

To better understand the neuroprotective effect of exogenous antioxidants and apocynin on Cd(II)-induced cytotoxicity in HTT striatal lines, we pre and co-treated HTT striatal cell lines with the indicated concentrations of NAC, ascorbic acid, and apocynin. Pre-treatment with NAC, ascorbic acid, and apocynin for 3 h followed by Cd(II) exposure for 48 h significantly ($p < 0.05$) increased cell viability in heterozygous HTT cells as compared to only Cd(II) exposure for each genotype (Fig. 5A–C). While co-treatment with Cd(II) and either NAC, ascorbic acid, and apocynin increased cell viability in the heterozygous HTT cells compared to wild-type lines (Fig. 5C–D), the magnitude of increase in cell viability was greater in the pre-treatment conditions. These results highlight the increased neuroprotective effects of NAC, ascorbic

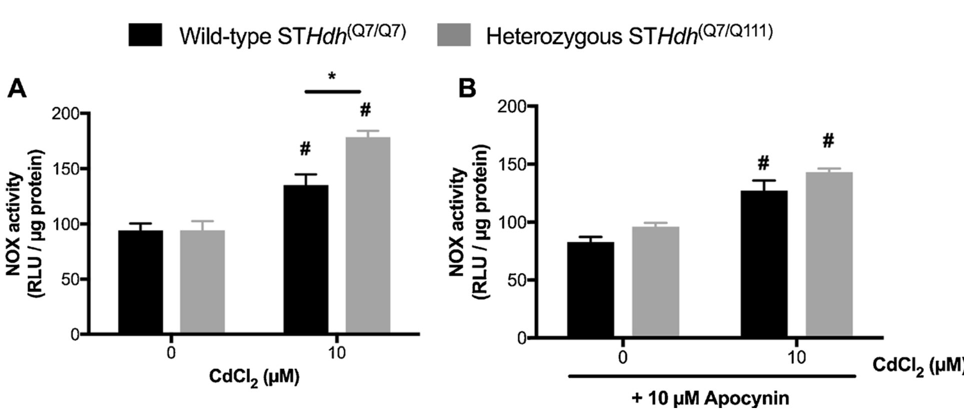


Fig. 3. Examining the role of NOX activity upon Cd(II) exposure. A, Heterozygous HTT lines exhibit greater NOX activity compared to wild-type after exposure to 10 μM Cd(II) for 48 h. B, Co-treatment with apocynin reduced NOX activity in heterozygous HTT lines to wild-type levels after exposure to 10 μM Cd(II) for 48 h. Relative lucigenin-enhanced chemiluminescence (light) units (RLU) was normalized to protein (μg). Each experiment had 3–4 independent samples (technical replicates). N = 4 biological replicates. * $p < 0.05$, Student's *t*-test indicates significant differences in NOX activity between wild-type and heterozygous HTT striatal cells. # $p < 0.05$ denotes significant difference in NOX activity when compared to baseline genotype controls.

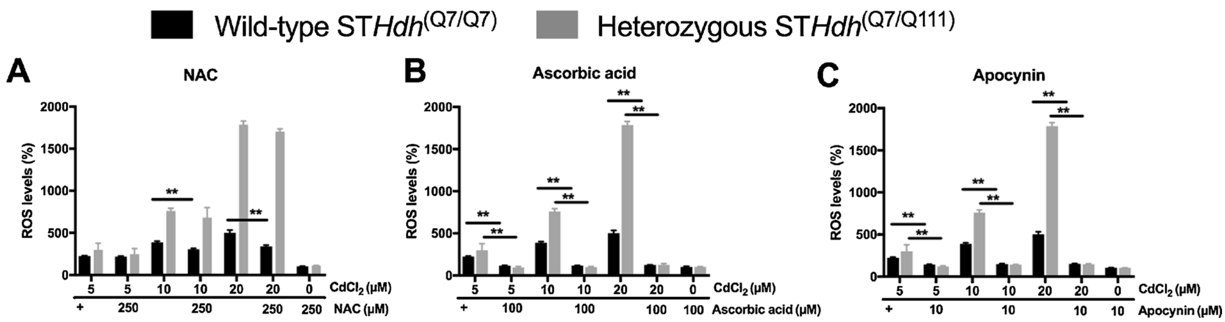


Fig. 4. Co-treatment with ascorbic acid apocynin significantly reduces Cd(II)-induced ROS production in HTT striatal cells. Wild-type and heterozygous HTT cells were co-treated with Cd(II) in the presence of A, 250 μM NAC; B, 100 μM ascorbic acid; and C, 10 μM apocynin for 30 min; and the production of ROS assessed by DCF-DA assay. Results are represented by the mean ± SEM from 8 independent samples (technical replicates). N = 4 biological replicates; ** $p < 0.01$ Student's *t*-test indicates significant differences between Cd(II) only versus co-treatment with antioxidants/apocynin in either wild-type or heterozygous HTT lines.

acid, and apocynin against Cd(II)-induced neurotoxicity in the HTT striatal cells under pre-treatment conditions.

3.6. Heterozygous huntingtin striatal cells exhibit increased apoptosis and Cd(II) exposure enhances caspase-9 and caspase-3 mediated cell death in striatal cells

We examined whether heterozygous HTT modulates the mitochondria mediated activation of caspase 9 and caspase 3 induced by Cd(II) exposure. We report that caspase 9 and caspase 3 are activated in both wild-type and heterozygous HTT cells upon Cd(II) exposure for 48 h. *Post-hoc* analysis indicated that the heterozygous HTT cells had significantly ($p < 0.05$) greater caspase-9 activity at 5, 10, and 20 μM Cd(II) compared to wild-type cells (Fig. 6A). Caspase-3 activation was significantly ($p < 0.05$) greater (~2-fold) in heterozygous HTT cells compared to wild-type at 5, 10, and 20 μM Cd(II) exposure. Notably, we observed greater caspase 3 activity compared to caspase 9 in the HTT lines upon exposure to Cd(II) (Fig. 6A, B). To provide a quantitative assessment of cell death mediated through the caspase-dependent apoptosis pathway, HD striatal cells were exposed to 10 μM Cd(II) and analyzed by a flow cytometry-based apoptosis assay. A quantified summary of the cytogram plots that represent propidium iodide (PI) and Annexin V binding of wild-type and heterozygous HTT striatal cells after 3, 24, and 48 h treatment with 10 μM Cd(II) revealed that the proportion of early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic cells (annexin V-positive, PI-positive) are significantly increased in both baseline and Cd(II) treated heterozygous HTT striatal cell line compared to wild-type cells (Fig. 6C). The number of early and late apoptosis cells was significantly ($p < 0.05$) increased in heterozygous HTT cells compared to wild-type at baseline conditions (control) (Fig. 6C, D). Exposure to 10 μM Cd(II) caused a significant ($p < 0.05$) increase in early and late apoptosis in wild-type striatal cells at 24 and 48 h exposure. Additionally, the heterozygous HTT striatal cells showed a significant ($p < 0.05$) increase in early apoptosis (Fig. 6B) indicating that they may be more primed for apoptosis mediated cell death compared to wild-type cells and may require longer exposure time (48 h) to observe additional increase in early apoptosis upon exposure to Cd(II). We observed a trend towards an increase in late apoptosis upon

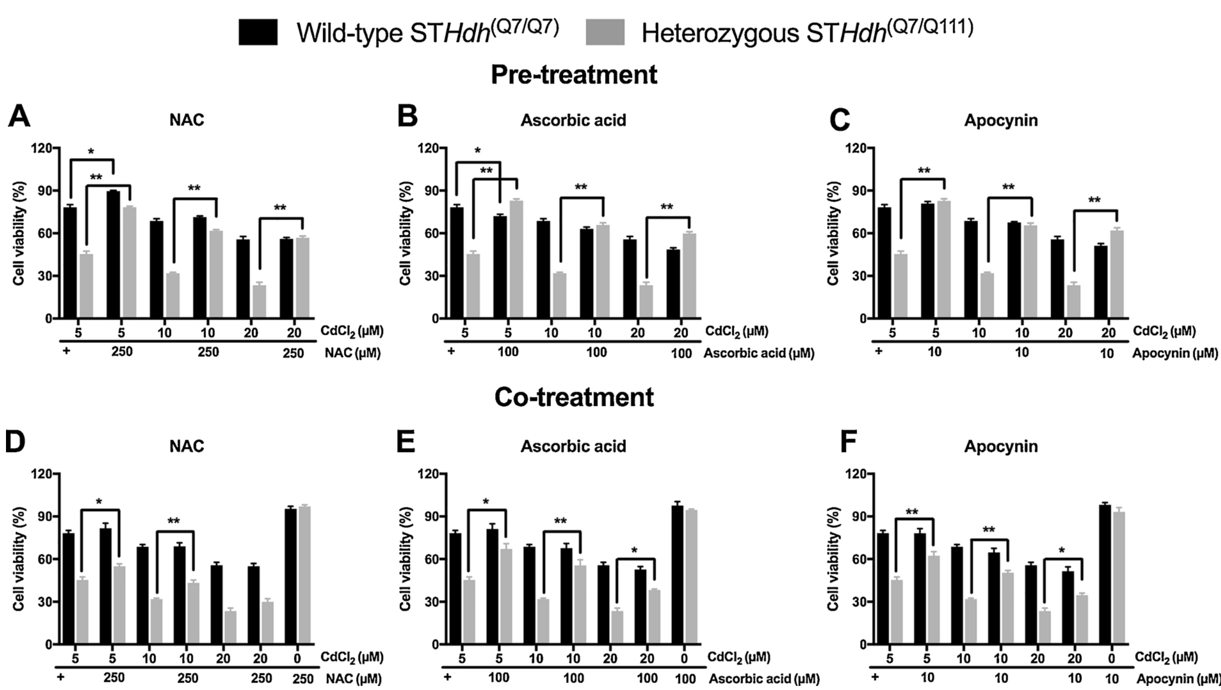


Fig. 5. Pre-treatment with NAC, ascorbic acid, and apocynin significantly attenuates Cd(II) cytotoxicity in heterozygous HTT cells compared to wild-type. Wild-type and heterozygous HTT cells were pre-treated for 3 h with A, 250 μM NAC; B,100 μM ascorbic acid; and C, 10 μM apocynin followed by exposure to Cd(II) for 48 h. Additionally, striatal cell lines were co-treated with the indicted concentrations of Cd(II) containing D, 250 μM NAC; E, 100 μM ascorbic acid; and F, 10 μM apocynin for 48 h and cell survival was examined by MTT assay. Results are represented by the mean ± SEM from 4 independent samples (technical replicates). N = 4 biological replicates. * $p < 0.05$ Student's *t*-test indicates significant difference in survival when compared to baseline genotype controls. concentration of Cd(II).

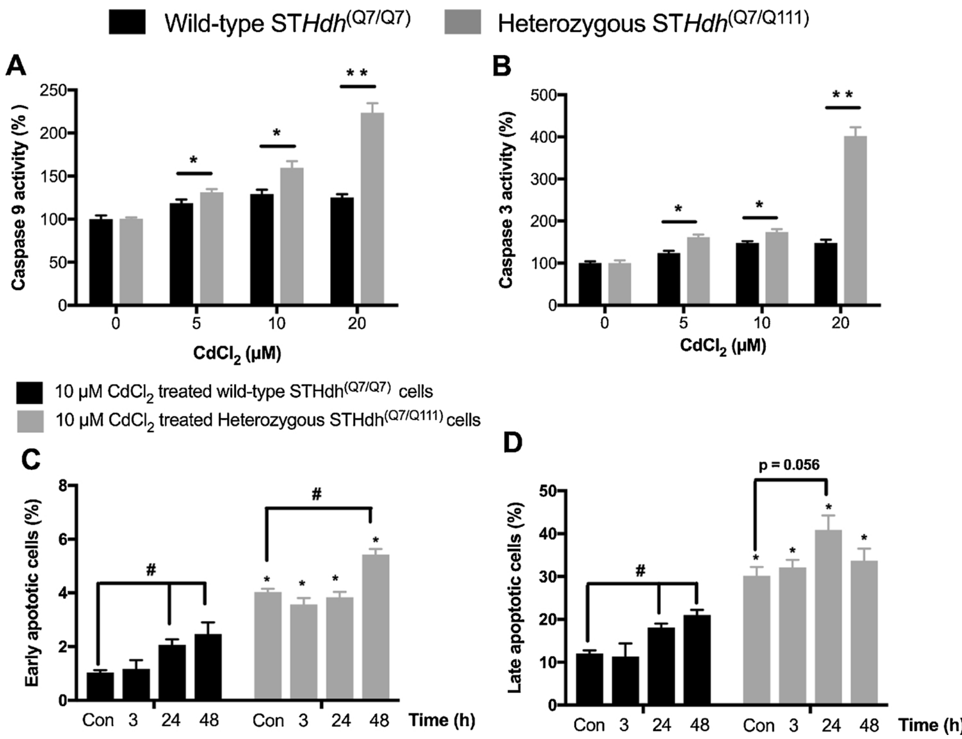


Fig. 6. Cd(II) exposure and heterozygous HTT activate caspase 9 and caspase 3, and heterozygous HTT cell lines exhibit increased apoptosis. Heterozygous HTT cells exhibit greater activation of A, caspase 9; and B, caspase 3 compared to wild-type after exposure to Cd(II) for 48 h. C and D, Analysis of PI and annexin V binding of wild-type and heterozygous HTT striatal cells at 10 μM Cd(II) for 3, 24, and 48 h. Summary data indicate the proportion of (C) early apoptotic cells (annexin V-positive, PI-negative) and (D) late apoptosis/dying cells (annexin V-positive, PI-positive). Results are represented by the mean ± SEM from 3 to 4 samples (technical replicates) in each treatment group. N = 3–4 biological replicates. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test indicates significant differences between wild-type and heterozygous HTT lines at the indicated concentration of Cd(II) or exposure time. # indicates significant ($p < 0.05$) difference between control (Con) and indicated time of exposure of striatal cells to 10 μM Cd (II) within genotype.

exposure to 10 μM Cd(II) for 48 h and 24 h respectively (Fig. 6B). These results suggest that heterozygous HTT enhances Cd(II)-induced sequential activation of caspase 9 and caspase 3. Moreover, heterozygous HTT enhances Cd(II)-induced sequential activation of caspase 9 and caspase 3 to cause cell death.

3.7. Examining Cd(II)-induced neurotoxicity and neurodegeneration on proteins involved in oxidative stress and caspase mediated cell death signaling pathways

Increased generation of intracellular ROS can lead to depolarization of mitochondrial membrane and subsequent release of cytochrome c, resulting in the activation of downstream apoptotic mechanisms (Kaul et al., 2003). We examined whether heterozygous HTT lines in combination with Cd(II) may be more prone to the release of proapoptotic factors including cytochrome c from the mitochondria to the cytosol to initiate the apoptotic cascade and augment cell death in the striatal cells. ANOVA analysis of the densitometry results of Western blot analysis did not detect any significant effect of genotype, Cd(II) exposure and a two-way interaction between genotype and Cd(II) exposure on cytochrome c protein levels (Fig. 7A). This data indicate that Cd(II) does not alter total protein expression of cytochrome c in the HTT striatal cells.

Recognizing the influence of oxidative stress in the heterozygous HTT and Cd(II) mediated neurotoxicity, we investigated the effect of Cd (II) on some key proteins involved in oxidative stress including Nrf2 and HO-1. ANOVA did not detect a significant effect of genotype, Cd(II) exposure and a two-way interaction between genotype and Cd(II) exposure on Nrf2 protein levels. *Post-hoc* analysis showed that the heterozygous HTT cells had significantly ($p < 0.05$) greater Nrf2 protein levels compared to wild-type at 20 μM Cd(II) exposure (Fig. 7B). Furthermore, ANOVA found a significant effect of genotype ($F_{(1,24)} = 9.727$, $p < 0.01$) and Cd(II) exposure ($F_{(3,24)} = 11.81$, $p < 0.001$) on HO-1 protein expression. *Post-hoc* analysis indicated that 10 and 20 μM Cd(II) exposure caused significantly ($p < 0.05$) greater HO-1 protein levels in heterozygous HTT cells as compared to wild-type (Fig. 7C). These data suggest that increased Cd(II)-induced neurotoxicity in heterozygous HTT cells may be mediated, at least in part, by activation of Nrf2 response system to alleviate the oxidative stress and reestablish the redox balance.

3.8. Heterozygous huntingtin increases intracellular accumulation of Cd(II) and exposure to Cd(II) reduces divalent metal transporter 1 (DMT1) protein levels in striatal HTT cells

To explore the basis for the genotype difference in Cd(II)-induced cytotoxicity between heterozygous HTT and wild-type striatal cells, we hypothesized that expression of heterozygous HTT may interfere with net intracellular accumulation of Cd upon Cd(II) exposure resulting in cell death. Thus, we exposed wild-type and heterozygous HTT cells to varying concentrations of Cd(II) for 48 h and measured the net intracellular concentration of Cd(II) by ICP-MS. *Post-hoc* analysis indicated significant ($p < 0.05$) increase in net intracellular concentration of Cd(II) at all tested concentrations of Cd for both genotypes when compared to their baseline controls (Fig. 8A). Additionally, we observed a significant ($p < 0.05$) increase in the net accumulation of Cd (II) in heterozygous HTT cells compared to wild-type after exposure for 48 h to 10 and 20 μM Cd(II).

It is known that DMT1 mediates transport of divalent metals, including Cd(II) into cells (Himeno et al., 2002). In addition, we have previously demonstrated that divalent metal ion transporter (DMT1) protein levels are unchanged in wild-type and mutant HTT expressing striatal cells (Williams et al., 2010a, 2010b). We determined whether heterozygous HTT and Cd(II) exposure differentially modulate the expression of endogenous DMT1 protein levels in HD striatal cells by Western blot analysis. ANOVA found a significant effect of genotype and Cd(II) exposure effects on DMT1 protein levels. *Post-hoc* analysis on the immunoblot results indicated that endogenous DMT1 protein levels in wild-type cells are significantly reduced upon exposure to 5, 10, and 20 μM Cd(II) compared to baseline genotype control. In addition, endogenous DMT1 protein levels were significantly reduced in wild-type compared to heterozygous HTT cells upon 20 μM Cd(II) exposure. Exposure to 5 μM Cd(II) significantly reduced DMT1 protein levels in heterozygous HTT cells compared to baseline genotype control (Fig. 8B). These results suggest that expression of DMT1 protein is modulated in response to Cd(II) exposure and may underlie the

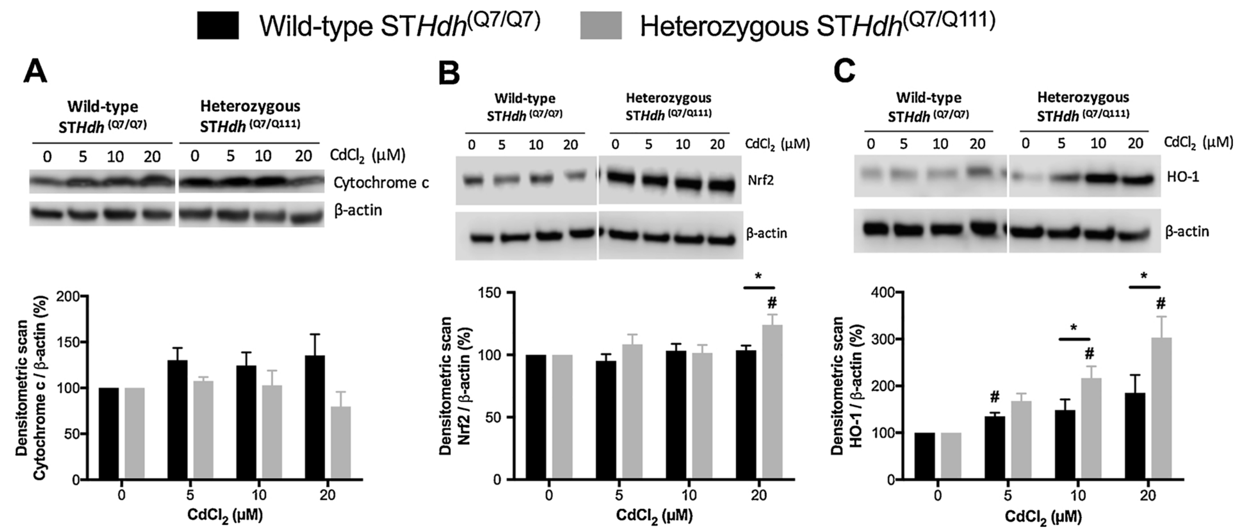
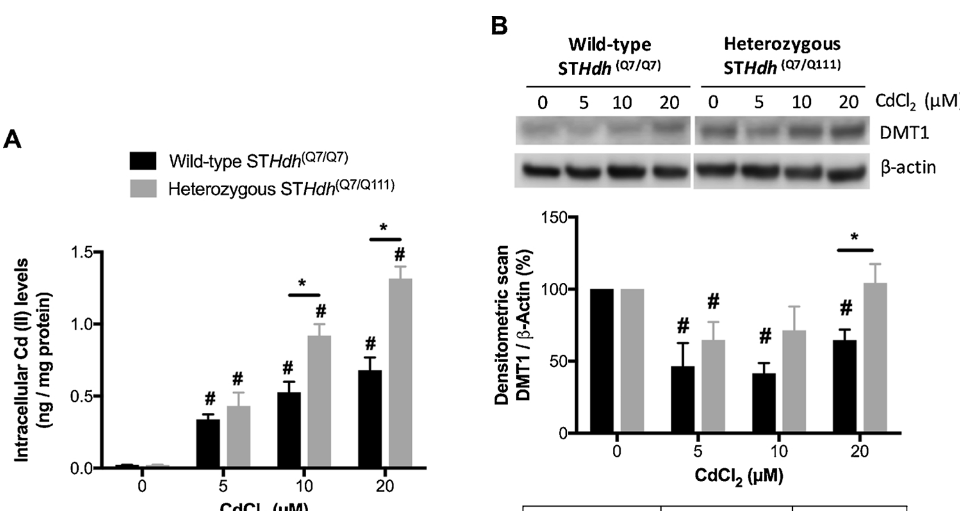


Fig. 7. Exposure to Cd(II) increased the expression of oxidative stress proteins, Nrf2 and HO-1, in HTT striatal cells. Wild-type and heterozygous HTT lines were treated with the indicated dosed of Cd(II) for 48 h and the endogenous protein levels of A, cytochrome c; B, Nrf2; and C, HO-1 were measured by Western blotting. To confirm equal protein-loading in each lane, the membranes were reprobed with β-actin antibody. Quantification of cytochrome c, Nrf2, and HO-1 band intensities are shown below each blot image. Results for each group is represented by the mean ± SEM from 4 independent experiments and separate measurements. * $p < 0.05$, Student's *t*-test indicates significant differences between wild-type and heterozygous HTT lines. # $p < 0.05$ denotes significant difference in the indicated protein expression when compared to baseline genotype controls.

decreased susceptibility of wild-type cells to Cd(II)-induced neurotoxicity.

3.9. Treatment with Zn(II), Mn(II), and Fe(II) partially reduce the susceptibility of heterozygous HTT striatal cells to Cd(II) cytotoxicity as compared to non-treated controls of the same genotype

A disease-toxicant interaction screen has revealed that homozygous mutant HTT (ST*Hdh*$^{Q111/Q111}$) striatal cells did not significantly influence Zn(II) and Fe(II) cytotoxicity but increased susceptibility to Mn(II) cytotoxicity in striatal cells after 26–30 h exposure as compared to wild-type (Williams et al., 2010a, 2010b). We hypothesized that exposure to non-toxic levels of Zn(II), Mn(II), and Fe(II) would activate Zn(II), Mn (II), and Fe(II) transporter systems known to mediate Cd(II) transport, compete, and reduce the influx of Cd(II) into the cytosol and other intracellular organelles, thus diminishing the Cd(II)-induced neurotoxicity in HTT striatal cells. To test this hypothesis, we generated a metal-induced survival curve spanning non-toxic to highly toxic concentrations for 48 h exposure and chose the lowest toxic concentration of Zn(II), Mn(II), and Fe(II) (Fig S1) for the pre- and cotreatment experiments with Cd(II). A comparison between wild-type and heterozygous HTT lines provides an indirect way to assess whether Zn(II), Mn (II), and Fe(II) transporters are involved differently in Cd(II) neurotoxicity between the two genotypes, which may elucidate a mechanism for the increased susceptibility of heterozygous HTT cells to Cd(II) exposure. Pretreatment for 3 h (Fig. 9A–C) and cotreatment for 48 h (Fig. 9D–F) with the indicated concentrations of Cd(II) and either Zn (II), Mn(II), or Fe(II) significantly ($p < 0.05$) increased cell survival in heterozygous HTT striatal cells with no noticeable effect on wild-type lines other than pretreatment with Fe(II) at 5 μM Cd(II) as compared to



ANOVA	F	Sig.
Genotype	$F_{(1,24)}$=7.981	**<0.01**
Cd (II)	$F_{(3,24)}$=7.906	**<0.01**
Genotype × Cd (II)	$F_{(3,24)}$=1.207	0.329

Fig. 8. Substantial increase in net intracellular accumulation of Cd(II) in heterozygous HTT striatal cells. DMT1 protein levels are altered in HTT striatal cells upon exposure to Cd(II). A. Wild-type and heterozygous HTT lines were treated with indicated dosed of Cd(II) for 48 h and net intracellular accumulation of Cd(II) measured by ICP-MS. Heterozygous HTT cells exhibit greater intracellular accumulation of Cd(II) compared to wild-type upon Cd(II) exposure. Each group is represented as mean ± SEM from at least three independent samples in each treatment group. N = 4 biological ICP-MS measurements. B. Total DMT1 protein levels were measured by Western blotting after exposure of HD striatal cells to Cd (II) for 48 h. To confirm equal protein-loading in each lane, the membranes were reprobed with β-actin antibody. Quantification of DMT1 band intensity is shown below the blot image. Results are represented by the mean ± SEM from 4 independent experiments and separate measurements. * $p < 0.05$, Student's *t*-test indicates significant differences between wild-type and heterozygous HTT lines. # $p < 0.05$ denotes significant difference in DMT1 protein levels compared to baseline genotype controls. Two-way univariate ANOVA table of Cd(II) exposure on DMT1 protein levels.

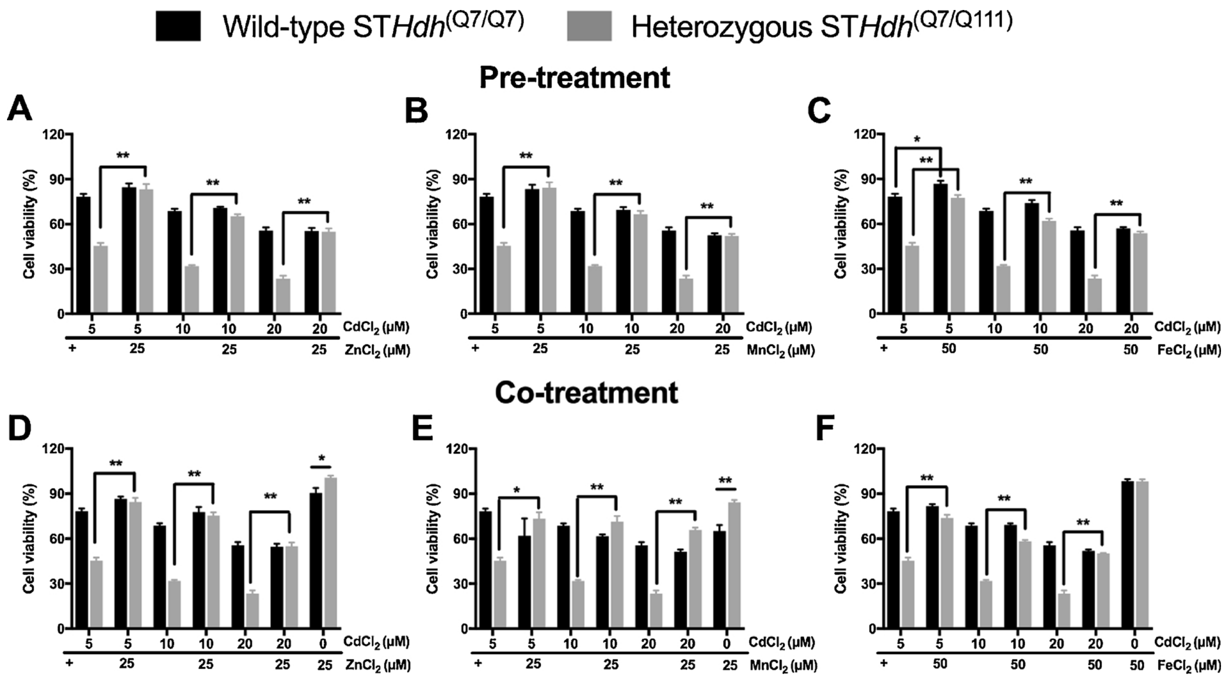


Fig. 9. Pre and cotreatments with Zn(II), Mn(II), and Fe(II) attenuate Cd(II)-induced cytotoxicity in heterozygous HTT striatal cells compared to non-treated controls of the same genotype. Wild-type and heterozygous HTT striatal lines were pre-treated for 3 h with A, 25 μM $ZnCl_2$; B, 25 μM $MnCl_2$; and C, 50 μM $FeCl_2$ followed by exposure to the indicted concentrations of Cd(II) for 48 h. Additionally, cultured striatal lines were co-treated with D, 25 μM $ZnCl_2$; E, 25 μM $MnCl_2$; F, 50 μM $FeCl_2$ and the indicated concentrations of Cd(II) for 48 h. Survival was measured by MTT assay. Results are represented by the mean ± SEM from 4 independent samples (technical replicates). N = 4 biological replicates. * $p < 0.05$ and ** $p < 0.01$, Student's *t*-test indicates significant differences between the Cd(II) exposed striatal cells and their baseline genotype controls.

non-treated controls of the same genotype. These results indicate the contribution of Zn(II), Mn(II), and Fe(II) regulated metal transporters in the Cd(II) neurotoxicity in heterozygous HTT cells.

3.10. Heterozygous HTT blocked the overexpression of ERK and increased PKCδ protein levels upon Cd(II) exposure

Changes in Thr202/Tyr204-phosporylated-ERK (Thr202/Tyr204-P-ERK), S473-phosphorylated-Akt (S473-P-Akt) and Tyr311-phosphorylated-PKCδ (Tyr311-P-PKCδ) protein levels are associated with both HD pathogenesis and Cd(II) toxicity (Gines et al., 2003; Zemskov et al., 2003; Colin et al., 2005; Roze et al., 2008; Thévenod, 2009; Williams et al., 2010a, 2010b; So and Oh, 2016). Thus, we hypothesized that these signaling pathways would show disease-toxicant interactions and reveal the underlying mechanism for the increased susceptibility of heterozygous HTT cells to Cd(II) neurotoxicity. We quantified Thr202/Tyr204-P-ERK levels following 48 h exposure to 5, 10, and 20 μM Cd (II). We observed a Cd(II) dependent increase in Thr202/Tyr204-P-ERK levels ($p < 0.05$) in wild-type, yet heterozygous HTT cells showed no detectable change in Thr202/Tyr204-P-ERK levels when compared to baseline heterozygous HTT cells (Fig. 10A).

The Akt signaling pathway promotes survival in response to extracellular signals (Thévenod, 2009). We quantified S473-P-Akt levels following exposure to Cd(II) for 48 h. This exposure did not lead to Cd (II) or HTT dependent changes in S473-P-Akt levels in both wild-type and heterozygous HTT cells (Fig. 10B). PKCδ is a key oxidative stress-sensitive kinase and considered to be a pro-apoptotic enzyme based on its ability to be activated by caspase 3 (Anantharam et al., 2002). Quantification of Tyr311-P-PKCδ protein levels following Cd(II) exposure revealed that 10 and 20 μM exposure caused a Cd(II)-dependent increase in Tyr311-P-PKCδ protein levels ($p < 0.05$) in heterozygous HTT cells, yet wild-type lines showed no detectable change in Tyr311-P-PKCδ protein levels relative to untreated wild-type cells (Fig. 10C).

4. Discussion

Although the mechanisms underlying striatal degeneration in HD are not completely understood, the influence of environmental factors including exposure to heavy metals and pesticides on the pathophysiology of HD have been recognized and are currently being investigated. Many studies have shown that Cd(II) neurotoxicity is mediated, at least in part, through an oxidative stress mechanism; however, Cd(II) is a non-redox metal and does not directly produce ROS (Flora et al., 2008). Increased oxidative stress induced by Cd(II) disrupts the function of mitochondria, which further enhances intracellular oxidative stress and subsequently triggers apoptosis in neurons and other cell types (Prozialeck and Edwards, 2012). NOX is a multi-protein electron transport system that produces superoxide via the reduction of molecular oxygen (Hernandes and Britto, 2012). Exposure to Cd(II) is known to increase NOX-dependent ROS production that results in cell death in a human hepatocellular carcinoma cell line (Souza et al., 2009). Additionally, NOX activity is enhanced in $HD^{140Q/140Q}$ mouse primary neurons and human HD brains (Valencia et al., 2013) as well as PC12 cells expressing an expanded polyglutamine peptide (Bertoni et al., 2011). Our data showing the involvement of NOX mediated oxidative stress as an early pathophysiological event are noteworthy. Despite the statistically indistinguishable difference in cell survival between wild-type and heterozygous HTT lines at 3 and 24 h Cd(II) exposure, we observed a significant genotype difference in ROS production as early as 30 min indicating that early NOX mediated oxidative stress may trigger the activation of antioxidant defense systems to combat the neurotoxic insults. However, heterozygous HTT may block or diminish the synthesis of antioxidants required to combat the Cd(II) neurotoxicity (Fig. 2B, C).

Lipid peroxidation results from excessive production of ROS that generate oxidized products such as MDA known to oxidize lipids and damage membranes contributing to cell death (Cuypers et al., 2010). Previous studies have demonstrated increased lipid peroxidation in the

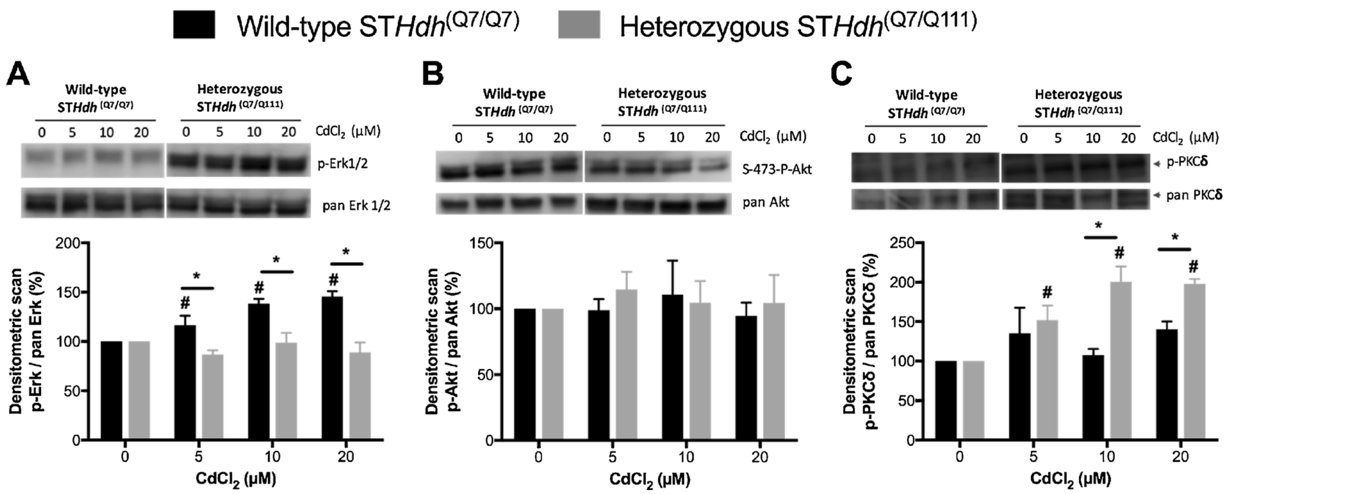


Fig. 10. Cd(II) exposure modulates ERK and PKCδ but not Akt protein levels in HTT expressing striatal cells. Wild-type and heterozygous HTT cells were treated with Cd(II) for 48 h, and then the phosphorylated and pan forms of A, ERK (Thr202/Tyr204-P-ERK and pan ERK1/2); B, Akt (S473-P-Akt and pan Akt); and C, PKCδ (Tyr311-P-PKCδ and pan PKCδ) protein levels were measured by Western blot analysis. Representative images of Western blot analysis are shown. Each group represented by the mean ± SEM from 4 separate measurements. * $p < 0.05$, Student's *t*-test indicates significant differences between wild-type and heterozygous HTT lines. # $p < 0.05$ denotes significant difference in the indicated protein expression when compared to its baseline genotype controls.

caudate and putamen of HD brains (Montine et al., 1999) and striatum of R6/2 HD mice (Lee et al., 2011) as well as increased plasma levels of MDA and reduced activity of GSH and SOD in erythrocytes of human HD patients (Chen et al., 2014). Our data is in agreement with these studies and suggest a response of heterozygous HTT to cause lipid peroxidation and concomitantly decrease the levels of antioxidant to cause striatal neurotoxicity (Fig. 2). Reduction of antioxidant enzymes after Cd(II) exposure has discernible effect on cell survival and suggest a critical role for antioxidant enzymes in maintaining HD bioenergetics and that the specific activation of antioxidant activity in HD striatal cells may be an effective approach to halt neurotoxicity and neurodegeneration as supported by our findings with pre and cotreatment approaches with exogenous antioxidants and apocynin.

Cd(II) and other neurotoxicants can alter mitochondrial membrane integrity and result in the release of cytochrome c from the mitochondria to cytosol triggering sequential activation of caspase 9 and later activation of caspase 3, the executioner caspase responsible for proteolytic cleavage of apoptotic cell death (Kaul et al., 2003). These mitochondrial mediated apoptotic stimuli have been implicated in the pathogenesis of HD. For example, striatal medium spiny neurons are susceptible to sequential caspase cascade in HD patients and the R6/2 transgenic model of HD (Kiechle et al., 2002). Our data indicating no significant change in total cytochrome c protein levels after Cd(II) exposure (Fig. 7A) may be due to a masking effect of mitochondria and cytosolic fractions in our study. A shift in the distribution of cytochrome c from the mitochondria to cytosolic fraction has been associated with greater striatal degeneration in HD patients and R6/2 mouse model of HD (Kiechle et al., 2002). Isolated measurements of mitochondrial and cytosolic cytochrome c levels would be important in future studies. Despite the paucity of evidence *in vivo* connecting HD to apoptosis per se, the processes involved in initiating apoptosis including caspase activation are known to be extremely important for the pathogenesis of HD (Hickey and Chesselet, 2003). *in vitro* studies show that expression of mutant HTT increase the activity of caspase 9 and caspase 3 (Li et al., 2000; Jana et al., 2001), which supports our findings (Fig. 6A, B). The increased apoptosis in heterozygous HTT striatal cells at baseline is consistent with previous HD studies (Li et al., 2000; Jana et al., 2001). Of particular note, a comparison of cell survival, oxidative stress, caspase activity, and apoptosis suggest that Cd(II)-induced oxidative stress and subsequent activation of caspase mediated apoptosis may be the primary and specific pathogenic mechanisms that underlie neurotoxicity and neurodegeneration in heterozygous HTT striatal cells.

Phosphorylation of Akt at S-473 is associated with neuroprotection (Brunet et al., 2001). Yet, we found no statistically distinguishable difference in S473-P-Akt levels between the two genotypes following exposure to Cd(II). Our findings are in agreement with the reported lack of phosphorylation of Akt by Cd(II) exposure in hippocampal slices (Rigon et al., 2008). While these and other observations do exclude a neuroprotective role for Akt signaling in Cd(II) neurotoxicity, they demonstrate that changes in Akt signaling may occur at an earlier time point after Cd(II) exposure as supported by a previous study that showed time-dependent activation of Akt signaling (Huang et al., 2014). In addition to Akt, Cd(II) is known to activate other kinases including ERK, JNK (c-Jun N-terminal kinase) and/or p38 (p38 mitogen-activated protein kinase) in several cell models (Chuang et al., 2000; Rockwell et al., 2004; Kim et al., 2005). Cd(II) was reported to not activate ERK in SH-SY5Y cells (Kim et al., 2005), which is in contrast to previous findings and our results. This may probably be because of the difference in cell types and experimental conditions used. Our findings of increased activation of ERK signaling are in agreement with previous findings that reinforce again that the roles of ERK signaling on either cytotoxic or protective cell responses depend on factors including genotype, Cd(II) or other neurotoxicants, genotype by Cd(II) interaction, and cell-type.

PKCδ is a key oxidative stress-sensitive kinase and plays a pro-apoptotic role following caspase-3 activation (Cross et al., 2000; Anantharam et al., 2002; Kanthasamy et al., 2003). The capacity of Cd(II) to activate PKCδ in cell survival and cell death, and the role of PKCδ in Cd(II)-induced apoptosis have been previously studied with non-coincident results. For example, Cd(II) did not activate PKC in rat mesangial cells (Wang and Templeton, 1998) and Cd(II)-induced apoptosis is independent of PKCδ activation in rat glioma cells (Wätjen et al., 2002). In contrast, Cd(II) activates PKC resulting in apoptosis in lung epithelial cells (Watkin et al., 2003), and PKCδ signaling mediates Cd(II)-induced apoptosis via early membrane translocation and late kinase proteolytic cleavage and phosphorylation on tyrosine residues (Miguel et al., 2005). Our findings are in agreement with the latter findings and suggest that heterozygous HTT may inhibit the degradation of PKCδ as a pro-death mechanism in response to Cd(II) exposure. An increase in the expression of PKCδ in heterozygous HTT cells after Cd(II) exposure may be linked with the increased expression of Nrf2 protein levels. Several reports indicate that the dissociation of Nrf2 from Keap-1, an inhibitory protein that targets Nrf2 for degradation via ubiquitination pathways, is facilitated by members of the PKC family, including PKCδ.

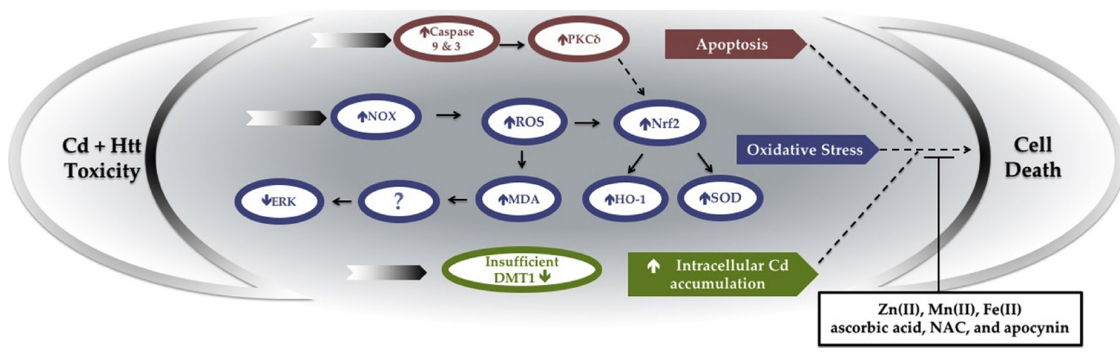


Fig. 11. Schematic representation of mechanisms underlying heterozygous HTT and Cd(II)-induced neurotoxicity and neurodegeneration. Exposure to the environmental neurotoxicant Cd(II) coupled with expression of heterozygous HTT results in elevated intracellular accumulation of Cd(II), which induces NOX mediated oxidative stress as an early response followed by activation of the intrinsic mitochondrial mediated caspase-9 and caspase-3 apoptotic cascade and PKCδ signaling mechanisms. Prolonged treatment with exogenous antioxidants ascorbic acid and NAC, low concentration of metal ions Zn(II), Mn(II), and Fe(II), and NOX inhibitor apocynin significantly rescue the heterozygous HTT striatal cells against Cd(II)-induced neurotoxicity.

Further, a PKC isoform was shown to be involved in mediating the accumulation of Nrf2 in response to Cd(II) exposure in an astrocytoma cell line (Lawal et al., 2015). Thus, our data indicating the increase in PKCδ protein is in line with the increase Nrf2 protein expression observed in heterozygous HTT cells and likely a key contributor to a potential protective mechanism against Cd(II) toxicity.

DMT1 protein expression has been reported to be reduced following metal exposures, which is noteworthy and in agreement with our data (Fig. 8B) due to its role in metal transport necessary for normal brain function (Fan et al., 2016). A previous study demonstrated that neurons exposed to Fe(II) and Cu(II) display increased ubiquitination and degradation of DMT1 resulting in diminished influx of metals and protection against metal toxicity (Howitt et al., 2009). The effect of Cd(II) on ubiquitination and degradation of DMT1 in HTT striatal lines are unknown. Our results suggest that Zn(II), Mn(II), and Fe(II) transporter systems may be involved but are not the major transporters underlying the increased neurotoxic susceptibility of heterozygous HTT cells to Cd (II) neurotoxicity (Fig. 9). The observation that heterozygous HTT cells accumulate greater levels of intracellular Cd(II) compared to wild-type is noteworthy. Of particular note, a comparison of cell survival and net accumulation of Cd(II) reveals that wild-type cells exposed to 5 μM Cd (II) have statistically distinguishable cell survival but indistinguishable total Cd(II) levels relative to heterozygous HTT cells exposed at 5 μM Cd (II) (Fig. 1C versus Fig. 8A). These data strongly suggest that while impairment in Cd(II) accumulation may contribute, at least in part, to the Cd(II) sensitive phenotype of heterozygous HTT cells exposed to 10 and 20 μM for 48 h, the increased susceptibility of heterozygous HTT lines observed at 5 μM Cd(II) is not solely dependent on the net levels of Cd(II) in the cells. Further, Cd(II) levels in heterozygous HTT cells after exposure to 5 μM Cd(II) may dampen or impede the activation of antioxidant defense mechanisms (Fig. 2B, C), and concomitantly potentiate oxidative stress (Fig. 2A, D) resulting in neurotoxicity and cell death (Fig. 6). A comparison between the total levels of Cd(II) accumulation in HD striatal cells upon Cd(II) exposure in our study and the reported average concentration of Cd(II) in humans upon prolonged exposure resulting in its estimated accumulation in urine, blood, and/or tissues of individuals in the United States (Kowal et al., 1979) are comparable. Thus, our observation of heterozygous *HTT* x Cd(II) disease-toxicant interaction is potentially pathologically relevant. Our findings suggest that heterozygous HTT coupled with Cd(II) may regulate other influx, intracellular sequestration/trafficking, and efflux transporter systems involved in Cd(II) cytotoxicity. Based on our current understanding of Mn(II) in HD (Williams et al., 2010a, 2010b; Tidball et al., 2015; Bryan et al., 2018) another possibility consistent with our data would be that Cd(II) exacerbates the limited uptake of Mn (II) by the striatal cells carrying the CAG repeats. Additional work is needed to determine if the specific nature of the Cd(II) accumulation defect is similar between the cellular and animal models (e.g. extracellular versus intracellular accumulation) and the identity of the metal transporter involved in the heterozygous HTT and Cd(II) interaction.

We propose three possible general mechanisms that are not mutually exclusive events for the increased Cd(II) accumulation in the heterozygous ST*Hdh*$^{Q7/Q111}$ cells: (1) an increase in Cd(II) influx, (2) a decrease in Cd(II) efflux, and (3) an increase in the intracellular trafficking and sequestration of Cd(II) into organelles. Future studies will explore the kinetics of the Cd(II) transport defect to elucidate the cellular mechanism and transporters involved. Our findings complement the disease-toxicant screen study that reported increased vulnerability of homozygous HTT (ST*Hdh*$^{Q111/Q111}$) striatal cell line to 50 μM Cd(II) upon 26–30 h exposure as it further supports the dominant toxic gain-of-function of mutant *HTT* and establishes Cd(II) as a known environmental toxicant that may exhibit pathogenic interactions with mutant *HTT* allele to cause striatal neurotoxicity and neurodegeneration. The heterozygous HTT used in the current study is more prevalent in humans and an equally relevant model for these types of studies.

In summary, we present a novel finding that heterozygous HTT in tandem with the environmental neurotoxicant Cd(II) induce NOX dependent oxidative stress as an early event, which is followed by activation of caspase-9/3 cascade and PKCδ, and inhibition of ERK signaling pathways to cause apoptotic striatal neurotoxicity and neurodegeneration. Treatment with NAC, ascorbic acid, and apocynin attenuates Cd(II)-induced oxidative stress and neurotoxicity. Further, Cd(II) exposure reduces DMT1 protein levels in striatal HTT cells and exposure to low concentration of Zn(II), Mn(II), and Fe(II) mitigates the Cd(II)-induced susceptibility of heterozygous HTT cells (Fig. 11). The selective effect of heterozygous HTT suggests that the HD mutation can alter the influence of an environmental toxicant on striatal neurons. In addition, these findings establish Cd(II) as a neurotoxicant that upon exposure can interact and modulate mutant HTT function to cause striatal neurotoxicity and neurodegeneration in HD. Taken together, our findings may enhance our understanding of pathways that mediate Cd(II)-induced neurotoxicity and neurodegeneration in heterozygous HTT striatal cells and reveal neuropathological mechanisms underlying HD.

Funding support

This research was supported in part by Oberlin College Office of Foundation, Government and Corporate Grants (GFK).

Author contributions

Conceived and designed the experiments: GFK. Performed the experiments: GFK, JAJ, MT, MM, BAK, MS, EMK. Analyzed the data: GFK and MT. Contributed reagents/materials/analysis tools: GFK, MS. Wrote the paper: GFK. Provided feedback on paper: GFK, JAJ, MT, BAK, EMK.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

We are deeply grateful to Dr. Monica Mariani, Ari Schaler, and Peter Arden (Oberlin College) for their technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2018.10.012.

References

Agrawal, S., Fox, J., Thyagarajan, B., Fox, J.H., 2018. Brain mitochondrial iron accumulates in Huntington's disease, mediates mitochondrial dysfunction, and can be removed pharmacologically. Free Radic. Biol. Med. 20 (May (120)), 317–329.

Ambrose, C.M., Duyao, M.P., Barnes, G., Bates, G.P., Lin, C.S., Srinidhi, J., et al., 1994. Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. Somat. Cell Mol. Genet. 20 (January (1)), 27–38.

Anantharam, V., Kitazawa, M., Wagner, J., Kaul, S., Kanthasamy, A.G., 2002. Caspase-3-dependent proteolytic cleavage of protein kinase cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. J Neurosci. 22 (March(5)), 1738–1751.

Anca, M.H., Gazit, E., Loewenthal, R., Ostrovsky, O., Frydman, M., Giladi, N., 2004. Different phenotypic expression in monozygotic twins with Huntington disease. Am. J. Med. Genet. A 124A (January (1)), 89–91.

Ashraf, M.W., 2012. Levels of heavy metals in popular cigarette brands and exposure to these metals via smoking. Sci. World J. 12 (March), 729430.

Bertoni, A., Giuliano, P., Galgani, M., Rotoli, D., Ulianich, L., Adornetto, A., et al., 2011. Early and late events induced by polyQ-expanded proteins: identification of a common pathogenic property of polYQ-expanded proteins. J. Biol. Chem. 286 (February(6)), 4727–4741.

Bowman, A.B., Kwakye, G.F., Herrero Hernández, E., Aschner, M., 2011. Role of manganese in neurodegenerative diseases. J. Trace Elem. Med. Biol. 25 (December (4)), 191–203.

Brunet, A., Datta, S.R., Greenberg, M.E., 2001. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. 11 (June (3)), 297–305.

Bryan, M.R., Uhouse, M.A., Nordham, K.D., Joshi, P., Rose, D.I.R., O'Brien, M.T., et al., 2018. Phosphatidylinositol 3 kinase (PI3K) modulates manganese homeostasis and manganese-induced cell signaling in a murine striatal cell line. Neurotoxicology 64 (January), 185–194.

Cattaneo, E., Zuccato, C., Tartari, M., 2005. Normal huntingtin function: an alternative approach to Huntington's disease. Nat. Rev. Neurosci. 6 (December (12)), 919–930.

Chen, S., Ren, Q., Zhang, J., Ye, Y., Zhang, Z., Xu, Y., et al., 2014. N-acetyl-L-cysteine protects against cadmium-induced neuronal apoptosis by inhibiting ROS-dependent activation of Akt/mTOR pathway in mouse brain. Neuropathol. Appl. Neurobiol. 40 (October (6)), 759–777.

Chong, W., Jiménez, J., McIlvin, M., Saito, M.A., Kwakye, G.F., 2017. α-synuclein enhances cadmium uptake and neurotoxicity via oxidative stress and caspase activated cell death mechanisms in a dopaminergic cell model of Parkinson's disease. Neurotox. Res. 32 (August (2)), 231–246.

Chuang, S.M., Wang, I.C., Yang, J.L., 2000. Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. Carcinogenesis.

Clabough, E.B.D., 2013. Huntington's disease: the past, present, and future search for disease modifiers. Yale J. Biol. Med. 86 (June (2)), 217–233.

Colin, E., Régulier, E., Perrin, V., Dürr, A., Brice, A., Aebischer, P., et al., 2005. Akt is altered in an animal model of Huntington's disease and in patients. Eur. J. Neurosci. 21 (March (6)), 1478–1488.

Cross, T., Griffiths, G., Deacon, E., Sallis, R., Gough, M., Watters, D., et al., 2000. PKC-delta is an apoptotic lamin kinase. Oncogene 19 (May (19)), 2331–2337.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., et al., 2010. Cadmium stress: an oxidative challenge. Biometals 23 (October (5)), 927–940.

Deshmukh, R.S., Chaudhary, R.K., Roy, I., 2012. Effect of pesticides on the aggregation of mutant huntingtin protein. Mol. Neurobiol. 45 (June (3)), 405–414.

Dexter, D.T., Carayon, A., Javoy-Agid, F., Agid, Y., Wells, F.R., Daniel, S.E., et al., 1991. Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. Brain 114 (August (Pt 4)), 1953–1975.

Dexter, D.T., Jenner, P., Schapira, A.H., Marsden, C.D., 1992. Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. The Royal Kings and Queens Parkinson's Disease Research Group. Ann. Neurol. 32 (Suppl), S94–S100.

Dixon, K.T., Cearley, J.A., Hunter, J.M., Detloff, P.J., 2004. Mouse Huntington's disease homolog mRNA levels: variation and allele effects. Gene Expr. 11 (5–6), 221–231.

Dominah, G.A., McMinimy, R.A., Kallon, S., Kwakye, G.F., 2017. Acute exposure to chlorpyrifos caused NADPH oxidase mediated oxidative stress and neurotoxicity in a striatal cell model of Huntington's disease. Neurotoxicology 60 (May), 54–69.

Domínguez, J.F.D., Ng, A.C.L., Poudel, G., Stout, J.C., Churchyard, A., Chua, P., et al., 2016. Iron accumulation in the basal ganglia in Huntington's disease: cross-sectional data from the IMAGE-HD study. J. Neurol. Neurosurg. Psychiatry 87 (May (5)), 545–549.

Ehret, J.C., Day, P.S., Wiegand, R., Wojcieszek, J., Chambers, R.A., 2007. Huntington disease as a dual diagnosis disorder: data from the National Research Roster for Huntington disease patients and families. Drug Alcohol Depend. 86 (January (2–3)), 283–286.

Fan, Q., Zhou, Y., Yu, C., Chen, J., Shi, X., Zhang, Y., et al., 2016. Cross-sectional study of expression of divalent metal transporter-1, transferrin, and hepcidin in blood of smelters who are occupationally exposed to manganese. Peer J. 1 (September (4)), e2413.

Flora, S.J.S., Mittal, M., Mehta, A., 2008. Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian J. Med. Res. 128 (October (4)), 501–523.

Fox, J.H., Kama, J.A., Lieberman, G., Chopra, R., Dorsey, K., Chopra, V., et al., 2007. Mechanisms of copper ion mediated Huntington's disease progression. PLoS One 2 (Mar (3)), e334.

Friedman, J.H., Trieschmann, M.E., Myers, R.H., Fernandez, H.H., 2005. Monozygotic twins discordant for Huntington disease after 7 years. Arch. Neurol. 62 (June (6)), 995–997.

Gines, S., Ivanova, E., Seong, I.-S., Saura, C.A., MacDonald, M.E., 2003. Enhanced Akt signaling is an early pro-survival response that reflects N-methyl-D-aspartate receptor activation in Huntington's disease knock-in striatal cells. J. Biol. Chem. 278 (December(50)), 50514–50522.

Gómez-Esteban, J.C., Lezcano, E., Zarranz, J.J., Velasco, F., Garamendi, I., Pérez, T., et al., 2007. Monozygotic twins suffering from Huntington's disease show different cognitive and behavioural symptoms. Eur. Neurol. 57 (1), 26–30.

Hernandes, M.S., Britto, L.R.G., 2012. NADPH oxidase and neurodegeneration. Curr. Neuropharmacol. 10 (December (4)), 321–327.

Hickey, M.A., Chesselet, M.F., 2003. Apoptosis in Huntington's disease. Prog. Neuropsychopharmacol. Biol Psychiatry 27 (April (2)), 255–265.

Himeno, S., Yanagiya, T., Enomoto, S., Kondo, Y., Imura, N., 2002. Cellular cadmium uptake mediated by the transport system for manganese. Tohoku J. Exp. Med. 196 (Jane (1)), 43–50.

Howitt, J., Putz, U., Lackovic, J., Doan, A., Dorstyn, L., Cheng, H., et al., 2009. Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons. Proc. Natl. Acad. Sci. U. S. A. 106 (September(36)), 15489–15494.

Huang, Y.-Y., Xia, M.-Z., Wang, H., Liu, X.-J., Hu, Y.-F., Chen, Y.-H., et al., 2014. Cadmium selectively induces MIP-2 and COX-2 through PTEN-mediated Akt activation in RAW264.7 cells. Toxicol. Sci. 138 (April (2)), 310–321.

Imarisio, S., Carmichael, J., Korolchuk, V., Chen, C.-W., Saiki, S., Rose, C., et al., 2008. Huntington's disease: from pathology and genetics to potential therapies. Biochem. J. 412 (June (2)), 191–209.

Jana, N.R., Zemskov, E.A., Gh, Wang, Nukina, N., 2001. Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum. Mol. Genet. 10 (May (10)), 1049–1059.

Jomova, K., Valko, M., 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283 (May (2–3)), 65–87.

Kanthasamy, A.G., Kitazawa, M., Kanthasamy, A., Anantharam, V., 2003. Role of proteolytic activation of protein kinase cdelta in oxidative stress-induced apoptosis. Antioxid. Redox Signal. 5 (October (5)), 609–620.

Kaul, S., Kanthasamy, A., Kitazawa, M., Anantharam, V., Kanthasamy, A.G., 2003. Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. Eur. J. Neurosci. 18 (September (6)), 1387–1401.

Kiechle, T., Dedeoglu, A., Kubilus, J., Kowall, N.W., Beal, M.F., Friedlander, R.M., et al., 2002. Cytochrome C and caspase-9 expression in Huntington's disease. Neuromol. Med. 1 (3), 183–195.

Kim, S.D., Moon, C.K., Eun, S.-Y., Ryu, P.D., Jo, S.A., 2005. Identification of ASK1, MKK4, JNK, c-jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. Biochem. Biophys. Res. Commun. 328 (March (1)), 326–334.

Kowal, N.E., Johnson, D.E., Kraemer, D.F., Pahren, H.R., 1979. Normal levels of cadmium in diet, urine, blood, and tissues of inhabitants of the United States. J. Toxicol. Environ. Health 5 (November (6)), 995–1014.

Lawal, A.O., Marnewick, J.L., Ellis, E.M., 2015. Heme oxygenase-1 attenuates cadmium-induced mitochondrial-caspase 3- dependent apoptosis in human hepatoma cell line. BMC Pharmacol. Toxicol. 16 (December (1)), 41.

Lee, J., Kosaras, B., Del Signore, S.J., Cormier, K., McKee, A., Ratan, R.R., et al., 2011. Modulation of lipid peroxidation and mitochondrial function improves neuropathology in Huntington's disease mice. Acta Neuropathol. 121 (April (4)), 487–498.

Li, S.H., Lam, S., Cheng, A.L., Li, X.J., 2000. Intranuclear huntingtin increases the expression of caspase-1 and induces apoptosis. Hum. Mol. Genet. 9 (November (19)), 2859–2867.

Lumsden, A.L., Henshall, T.L., Dayan, S., Lardelli, M.T., Richards, R.I., 2007. Huntingtin-

deficient zebrafish exhibit defects in iron utilization and development. Hum. Mol. Genet. 16 (August (16)), 1905–1920.
Miguel, B.G., Rodriguez, M.E., Aller, P., Martinez, A.M., Mata, F., 2005. Regulation of cadmium-induced apoptosis by PKCdelta in U937 human promonocytic cells. Biochim. Biophys. Acta 1743 (April (3)), 215–222.
Miller, D.K., Dopheide, M.M., Smith, S.M., Casteel, S.W., 2005. Dietary cadmium exposure attenuates D-amphetamine-evoked [3H]dopamine release from striatal slices and methamphetamine-induced hyperactivity. Pharmacol. Biochem. Behav. 80 (April (4)), 557–566.
Montgomery, E.B., 1995. Heavy metals and the etiology of Parkinson's disease and other movement disorders. Toxicology 97 (March(1–3)), 3–9.
Montine, T.J., Beal, M.F., Robertson, D., Cudkowicz, M.E., Biaggioni, I., O'Donnell, H., et al., 1999. Cerebrospinal fluid F2-isoprostanes are elevated in Huntington's disease. Neurology 52 (March(5)), 1104–1105.
Pal, R., Nath, R., Gill, K.D., 1993. Influence of ethanol on cadmium accumulation and its impact on lipid peroxidation and membrane bound functional enzymes (Na+, K (+)-ATPase and acetylcholinesterase) in various regions of adult rat brain. Neurochem. Int. 23 (November (5)), 451–458.
Prozialeck, W.C., Edwards, J.R., 2012. Mechanisms of cadmium-induced proximal tubule injury: new insights with implications for biomonitoring and therapeutic interventions. J. Pharmacol. Exp. Ther. 343 (October (1)), 2–12.
Rigamonti, D., Bauer, J.H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., et al., 2000. Wild-type huntingtin protects from apoptosis upstream of caspase-3. J. Neurosci. 20 (May (10)), 3705–3713.
Rigamonti, D., Sipione, S., Goffredo, D., Zuccato, C., Fossale, E., Cattaneo, E., 2001. Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. J Biol Chem. 276 (May (18)), 14545–14548.
Rigon, A.P., Cordova, F.M., Oliveira, C.S., Posser, T., Costa, A.P., Silva, I.G., et al., 2008. Neurotoxicity of cadmium on immature hippocampus and a neuroprotective role for p38 MAPK. Neurotoxicology 29 (July (4)), 727–734.
Rockwell, P., Martinez, J., Papa, L., Gomes, E., 2004. Redox regulates COX-2 upregulation and cell death in the neuronal response to cadmium. Cell Signal 16 (March (3)), 343–353.
Rosas, H.D., Chen, Y.I., Doros, G., Salat, D.H., Chen, N., Kwong, K.K., et al., 2012. Alterations in brain transition metals in Huntington disease: an evolving and intricate story. Arch. Neurol. 69 (July (7)), 887–893.
Roze, E., Betuing, S., Deyts, C., Marcon, E., Brami-Cherrier, K., Pagès, C., et al., 2008. Mitogen- and stress-activated protein kinase-1 deficiency is involved in expanded-huntingtin-induced transcriptional dysregulation and striatal death. FASEB J. 22 (April (4)), 1083–1093.
Snell, R.G., MacMillan, J.C., Cheadle, J.P., Fenton, I., Lazarou, L.P., Davies, P., et al., 1993. Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. Nat. Genet. 4 (August (4)), 393–397.
So, K.-Y., Oh, S.-H., 2016. Cadmium-induced heme-oxygenase-1 expression plays dual roles in autophagy and apoptosis and is regulated by both PKC-δ and PKB/Akt activation in NRK52E kidney cells. Toxicology 31 (August (370)), 49–59.
Souza, V., Escobar, M.D.C., Bucio, L., Hernández, E., Gómez-Quiroz, L.E., 2009. Gutiérrez Ruiz MC. NADPH oxidase and ERK1/2 are involved in cadmium induced-STAT3 activation in HepG2 cells. Toxicol. Lett. 187 (June (3)), 180–186.
Squitieri, F., Gellera, C., Cannella, M., Mariotti, C., Cislaghi, G., Rubinsztein, D.C., et al., 2003. Homozygosity for CAG mutation in Huntington disease is associated with a more severe clinical course. Brain 126 (April (Pt 4)), 946–955.
Sudarsky, L., Myers, R.H., Walshe, T.M., 1983. Huntington's disease in monozygotic twins reared apart. J. Med. Genet. 20 (December (6)), 408–411.
Thévenod, F., 2009. Cadmium and cellular signaling cascades: to be or not to be? Toxicol. Appl. Pharmacol. 238 (August (3)), 221–239.
Tidball, A.M., Bryan, M.R., Uhouse, M.A., Kumar, K.K., Aboud, A.A., Feist, J.E., et al., 2015. A novel manganese-dependent ATM-p53 signaling pathway is selectively impaired in patient-based neuroprogenitor and murine striatal models of Huntington's disease. Hum. Mol. Genet. 24 (April (7)), 1929–1944.
Trettel, F., Rigamonti, D., Hilditch-Maguire, P., Wheeler, V.C., Sharp, A.H., Persichetti, F., et al., 2000. Dominant phenotypes produced by the HD mutation in STHdh(Q111) striatal cells. Hum. Mol. Genet. 9 (November (19)), 2799–2809.
Valencia, A., Sapp, E., Kimm, J.S., McClory, H., Reeves, P.B., Alexander, J., et al., 2013. Elevated NADPH oxidase activity contributes to oxidative stress and cell death in Huntington's disease. Hum. Mol. Genet. 22 (March (6)), 1112–1131.
van Bergen, J.M.G., Hua, J., Unschuld, P.G., Lim, I.A.L., Jones, C.K., Margolis, R.L., et al., 2016. Quantitative susceptibility mapping suggests altered brain iron in premanifest huntington disease. AJNR Am. J. Neuroradiol. 37 (5), 789–796.
Wang, Z., Templeton, D.M., 1998. Induction of c-fos proto-oncogene in mesangial cells by cadmium. J. Biol. Chem. 273 (January (1)), 73–79.
Wätjen, W., Cox, M., Biagioli, M., Beyersmann, D., 2002. Cadmium-induced apoptosis in C6 glioma cells: mediation by caspase 9-activation. Biometals 15 (March (1)), 15–25.
Watkin, R.D., Nawrot, T., Potts, R.J., Hart, B.A., 2003. Mechanisms regulating the cadmium-mediated suppression of Sp1 transcription factor activity in alveolar epithelial cells. Toxicology 184 (March (2–3)), 157–178.
Wexler, N.S., Lorimer, J., Porter, J., Gomez, F., Moskowitz, C., Shackell, E., et al., 2004. Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. Proc. Natl. Acad. Sci. U. S. A. 101 (March (10)), 3498–3503.
Williams, B.B., Kwakye, G.F., Wegrzynowicz, M., Li, D., Aschner, M., Erikson, K.M., et al., 2010a. Altered manganese homeostasis and manganese toxicity in a Huntington's disease striatal cell model are not explained by defects in the iron transport system. Toxicol. Sci. 117 (September (1)), 169–179.
Williams, B.B., Li, D., Wegrzynowicz, M., Vadodaria, B.K., Anderson, J.G., Kwakye, G.F., et al., 2010b. Disease-toxicant screen reveals a neuroprotective interaction between Huntington's disease and manganese exposure. J. Neurochem. 112 (January (1)), 227–237.
Yang, Z., Yang, S., Qian, S.Y., Hong, J.-S., Kadiiska, M.B., Tennant, R.W., et al., 2007. Cadmium-induced toxicity in rat primary mid-brain neuroglia cultures: role of oxidative stress from microglia. Toxicol. Sci. 98 (August (2)), 488–494.
Zemskov, E.A., Jana, N.R., Kurosawa, M., Miyazaki, H., Sakamoto, N., Nekooki, M., et al., 2003. Pro-apoptotic protein kinase C delta is associated with intranuclear inclusions in a transgenic model of Huntington's disease. J. Neurochem. 87 (October (2)), 395–406.
Zhang, F.L., Fu, H.W., Casey, P.J., Bishop, W.R., 1996. Substitution of cadmium for zinc in farnesyl:protein transferase alters its substrate specificity. Biochemistry 35 (June (25)), 8166–8171.
Zhang, Y., Leavitt, B.R., van Raamsdonk, J.M., Dragatsis, I., Goldowitz, D., MacDonald, M.E., et al., 2006. Huntingtin inhibits caspase-3 activation. EMBO J. 25 (December (24)), 5896–5906.