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p73 gene in Dopaminergic Neurons is Highly Susceptible to Manganese Neurotoxicity

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Abstract

Chronic exposure to elevated levels of manganese (Mn) has been linked to a Parkinsonian-like movement disorder, resulting from dysfunction of the extrapyramidal motor system within the basal ganglia. However, the exact cellular and molecular mechanisms of Mn-induced neurotoxicity remain elusive. In this study, we treated C57/BL6J mice with 30 mg/kg Mn via oral gavage for 30 days. Interestingly, in nigral tissues of Mn-exposed mice, we found a significant downregulation of the truncated isoform of p73 protein at the N-terminus (Np73). To further determine the functional role of Mn-induced p73 downregulation in Mn neurotoxicity, we examined the interrelationship between the effect of Mn on p73 gene expression and apoptotic cell death in an N27 dopaminergic neuronal model. Consistent with our animal study, a 300 μ M Mn treatment significantly suppressed p73 mRNA expression in N27 dopaminergic cells. We further determined that protein levels of the Np73 isoform was also reduced in Mn-treated N27 cells and primary striatal cultures. Furthermore, overexpression of Np73 conferred modest cellular protection against Mn-induced neurotoxicity. Taken together, our results demonstrate that Mn exposure downregulates p73 gene expression resulting in enhanced susceptibility to apoptotic cell death. Thus, further characterization of the cellular mechanism underlying p73 gene downregulation will improve our understanding of the molecular underpinnings of Mn neurotoxicity.

Keywords

Manganese; p73; neurotoxicity; Np73

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Conflict of interest

A.G.K. and V.A. are shareholders of PK Biosciences Corporation (Ames, IA), which is interested in translating mechanistic studies into therapies targeting p73 signaling. The other authors have no conflicts of interest.

1. Introduction

Neurodegenerative diseases are becoming more prevalent due to aging populations and exposure to an increasing array of environmental toxicants such as pesticides, neurotoxic metals and industrial byproducts (Caudle et al., 2012; Goldman, 2014; Kanthasamy et al., 2012; White et al., 2015). The transition metal manganese (Mn) is an essential trace nutrient that not only serves as a co-factor for a variety of enzymes including superoxide dismutase 2 and glutamine synthetase, but is also important for various normal physiological functions including bone formation, fat and carbohydrate metabolism, as well as the regulation of blood sugar, cellular energy, the immune system and calcium absorption (Aschner et al., 2009). Mn has been used widely in various industries for more than two centuries. The primary routes of Mn exposure are inhalation in occupational settings and dietary consumption in general. Airborne Mn particles from various chemicals including methylcyclopentadienyl manganese tricarbonyl (MMT) and fungicide are another important source of public exposure (Bowman et al., 2011; Caudle et al., 2012). Mn overexposure causes a neurodegenerative disorder known as manganism. Many clinical symptoms of manganism are also seen in PD patients, including gait dysfunction, postural instability, bradykinesia, rigidity and a mask-like face expression (Guilarte, 2011). Pathologically, both manganism and PD show dopaminergic neuronal cell death in the substantia nigra (SN) and striatum (STR). But unlike PD, manganism is unresponsive to L-dopa therapy and develops psychological abnormalities in its early phase.

The *p73* gene (also known as *Tp73*) belongs to the p53 tumor suppressor gene family, and like other family members, codes for multiple mRNA transcripts (Rosenbluth and Pietsenpol, 2008). Besides playing an important role in regulating differentiation, cell death, and neural stem cells, p73 is also implicated in neurodegenerative diseases such as Alzheimer's disease (Engelmann et al., 2015; Killick et al., 2011; Yang et al., 2002). The *p73* gene has 15 exons and two different transcription start sites. The C-terminal domain of p73 is alternatively spliced generating a variety of isoforms. Transcripts from the upstream promoter generate transactivation domain-containing isoforms of p73 (TAp73), while transcripts from the second promoter produce the N-terminal transactivation domain-truncated isoform subfamily (Np73). Similar to the p53 tumor suppressor, TAp73 induces apoptosis and serves as a tumor suppressor. Interestingly, Np73 inhibits cellular apoptosis by directly inhibiting TAp73 activity (Nakagawa et al., 2002). The p53 family members, including p73, share highly similar protein architectures and cellular targets, but they have different upstream signals (Rosenbluth and Pietsenpol, 2008).

Manganese-induced neurotoxicity has been studied extensively, and several key players, such as protein kinase C delta (PKC δ) (Jin et al., 2011; Latchoumycandane et al., 2005), SLC30A10 (Chen et al., 2015; Leyva-Illades et al., 2014), ATM-p53 (Tidball et al., 2015), and others (Horning et al., 2015) have been identified. Previous evidence also indicates that manganese-induced neurotoxicity involves activation of caspase-3 signaling (Anantharam et al., 2004; Latchoumycandane et al., 2005). Moreover, caspase-3 has been shown to cleave p73 (Sayan et al., 2008). Mn exposure induces mitochondria-mediated apoptosis in neurons, which is partially facilitated by p53 (Wan et al., 2014), and Np73 antagonizes the functional p53 by regulating the expression of anti-apoptotic molecules such as Bcl-xL and

Mcl-1 (Basu and Haldar, 1998; Grob et al., 2001; Huttinger-Kirchhof et al., 2006; Melino et al., 2002). However, the cellular and molecular mechanisms underlying Mn-induced neurotoxicity still have not been delineated. Here, we report that Np73 expression was downregulated during Mn exposure in N27 dopaminergic neuronal cells, mouse primary striatal cells, and in the mouse SN. Through functional studies, we identified that Np73 downregulation makes neuronal cells more susceptible to Mn neurotoxicity. Overexpression of Np73 protected N27 dopaminergic neuronal cells from Mn-induced neurotoxicity. These observations provide new insights into the mechanisms underlying Mn-induced dopaminergic neurotoxicity.

2. Materials and Methods

2.1 Chemicals

Manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 99%) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against Bcl-xL and Mcl-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against Np73 was purchased from Imagnex (San Diego, CA). Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Mouse and rabbit monoclonal antibodies against β -actin were purchased from Sigma-Aldrich. The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRDye800 conjugated anti-rabbit IgG) were purchased from Life Technologies (Grand Island, NY) and Rockland (Gilbertsville, PA), respectively. RPMI 1640, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Life Technologies. We purchased the tetrapeptide caspase-3 inhibitor zDEVD-fmk from ApexBio (Houston, TX).

2.2 Animal studies

Six- to eight-week-old male C57BL/6J mice were housed at RT under a 12-h light cycle with water and food provided *ad libitum*. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (10 or 30mg/kg) was orally administered once daily for 30 days. Animals were cared for in accordance with Institutional Animal Care and Use committee guidelines. After the treatment period, striatal tissues were collected for apoptosis PCR array analysis or for measuring the expression of proteins of interest.

2.3 Cell cultures

A rat-derived dopaminergic neuronal cell line, N27, was kindly gifted from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). N27 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units of penicillin, and 50 $\mu\text{g}/\text{ml}$ of streptomycin in a humidified incubator with 5% CO_2 at 37°C as described previously (Jin et al., 2011).

2.4 Primary striatal cultures and treatment

Primary striatal neuronal cultures were prepared from the ganglionic eminences of gestational 14- to 15-day-old mouse embryos as previously described (Martin et al., 2011). Briefly, after extracting the embryonic brain, the embryonic cerebral cortex was folded away to expose ganglionic eminences followed by dissection of the STR, which were then washed

in Dulbecco's modified eagle media (DMEM) and dissociated via trypsinization. Dissociated cells were seeded into poly-D-lysine-coated plates and maintained in Neurobasal medium supplemented with B-27 supplements, 500 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C. Four- to six-day-old primary striatal neuronal cells were exposed to various concentrations of Mn (100-500 µM) for 24 h and then subjected to Western blot analysis.

2.5 Plasmid constructs

The expression plasmid for the human p73 alpha isoform (HA-p73alpha-pcDNA3) was obtained from Addgene (ID 22102) as described (Jost et al., 1997). To generate the expression vector for the N-terminal transactivation domain-truncated p73 isoform, the appropriate cDNA fragment was PCR-generated from HA-p73alpha-pcDNA3 using a forward primer, 5'-

CAGATTACGCTAGCTGCTGTACGTCGGTGACCCCGCACGGCACCTCGCCACGGCC
CA GTTCAATCTGCTGAGCAGCACC-3', and the SP6 reverse primer. The restriction enzymes of NheI and XhoI were used to generate the 5' overhang cleavage site, which was used for the ligation reaction with pcDNA3 vector. The resulting clone for Np73 was confirmed by sequencing.

2.6 Transfections

N27 cells were transfected with Np73 DNA construct through electroporation. The Amaxa Nucleofector instrument was used according to the manufacturer's protocol. Five million cells were transfected with 10 µg of plasmids using the A-23 program. Transfected cells were seeded in either T-75 flasks or 96-well plates as desired for 24 h before treatment (Zhang et al., 2007b).

2.7 Cell viability assays

Colorimetric MTS assay was performed to measure mitochondrial activity as an indirect measure of cell viability using the Cell Titer 96 Aqueous One Solution Proliferation Assay kit from Promega as described previously (Jin et al., 2014a). Briefly, cells were seeded in 96-well plates before exposure to Mn. At the end of treatment, 20% MTS reagent was added to each well. The MTS tetrazolium compound is bio-reduced in living cells to its colored formazan products. After incubating for up to one hour, absorbance at 490 nm was measured using a SpectraMax spectrophotometric microplate reader (Molecular Devices).

2.8 Quantitative real-time RT-PCR

After treatment, total RNA was extracted from cells using the Absolutely RNA Miniprep kit and converted to cDNA using the High Capacity cDNA Synthesis kit (Life Technologies). SYBR Green quantitative PCR was performed according to the manufacturer's protocol. Forward primer (5'-ACTGCAAGTCCCGTAAGCAG-3') and reverse primer (5'-CATGAGCGACTTCCCTTCAG-3') were used for detecting p73 mRNA. Validated primers for 18S rRNA (Qiagen, #PPR57734E) were used as the housekeeping gene controls. The threshold cycle (C_t) was calculated from the instrument software, and fold change in gene expression was calculated using the C_t method.

2.9 Behavioral measurements

Spontaneous exploratory movements of mice were measured using a computer-controlled open-field apparatus (VersaMax animal activity monitor, model RXYZCM-16, Accuscan, Columbus, OH) as described previously (Zhang et al., 2007a). The transparent activity chamber was $40 \times 40 \times 30.5$ cm and was covered with a transparent ventilated lid. Data were collected and analyzed by a VersaMax analyzer (model CDA-8, AccuScan). Following a 2-min adaptation period, each mouse was given 10 min to explore the open-field monitor while horizontal and vertical movements were recorded automatically *via* an array of infra-red beams.

2.10 Western blotting

Cells were lysed in lysis buffer (1% Triton X-100, 1 mM EDTA, 100 mM NaCl, 1 mM EGTA, 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris-Cl, pH 7.4). Brain homogenates were prepared as described previously (Jin et al., 2011). Western blotting was performed as described previously (Jin et al., 2014b). Briefly, the samples containing equal amounts of proteins were fractionated through a 10-15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Primary antibodies against specific proteins were incubated with the membrane. After thoroughly rinsing in PBS supplemented with 0.1% Tween-20, the membrane was incubated with Alexa Fluor 680 goat anti-mouse, Alexa Fluor 680 donkey anti-goat, or IRDye 800 donkey anti-rabbit secondary antibodies. For the loading control, β -actin antibody was used. Immunoblot imaging was performed with an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). ImageJ software (National Institutes of Health, Bethesda MD, USA) was used to quantify Western blot bands.

2.11 Statistical analysis

Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA). Tukey's multiple comparison test was used after ANOVA for comparing differences between multiple groups. Paired student's *t*-test was used when two groups were being compared. Differences were considered statistically significant for *p*-values <0.05 . All data were determined from at least two biologically independent experiments, each done in triplicate (3 samples from the same culture, flask etc). Data are represented as the mean $\pm$ S.E.M. ($n=6-9$).

3. Results

3.1 Manganese exposure suppresses Np73 expression in mouse substantia nigra

To investigate whether Mn exposure has any effect on the expression of p73 proteins *in vivo*, C57BL mice were orally treated with 30 mg/kg/day of Mn for 30 days and then their nigral tissues were subjected to biochemical analysis. To perform isoform-specific gene expression analyses of *p73* gene products, we used two specific antibodies directed against full-length and truncated p73 proteins, respectively. Interestingly, expression of Np73 in Mn-treated mice was reduced by more than 50% when compared to control mice (Fig. 1C-D). Full-length p73 protein expression was not changed (data not shown). Behavioral performance testing also confirmed that 30 mg/kg Mn treatment caused significant motor deficits (Fig.

1A-B). These findings in an animal model indicate that Mn exposure induced downregulation of Np73 expression.

3.2 Manganese exposure downregulates Np73 expression in N27 dopaminergic cells and mouse primary striatal cultures

We further characterized the Mn-induced downregulation of p73 expression and its functional consequence in the *in vitro* N27 dopaminergic neuronal cell system. N27 cells were exposed to 300 μ M Mn for 6-24 h, and quantitative RT-PCR analysis was performed for *p73* gene expression after 24 h (Fig. 2A). We found ~60% reduction of *p73* gene expression relative to control cells. Furthermore, Western blot analysis revealed that Np73 expression was downregulated upon exposure to 300 μ M in a time-dependent manner with ~60% reduction relative to control cells 24 h after treatment (Fig. 2B-C), which validated the results from our *in vivo* model and also showed that Mn exposure affects dopaminergic neurons through downregulating Np73 expression. Interestingly, N27 cells treated with 300 μ M Mn for 12 h did not trigger cell death (data not shown), indicating that Np73 downregulation takes place prior to cell death. To further validate the Mn-induced downregulation of Np73, we showed that exposing mouse primary striatal cultures for 24 h to increasing concentrations of Mn (100-500 μ M) concentration-dependently repressed Np73 expression (Fig. 3A-B).

3.3 Downregulation of Np73 is not dependent on manganese-induced caspase-3 activation

To investigate the possibility that activated caspase-3 contributes to the reduction of Np73 protein levels by inducing proteolytic cleavage of Np73, we used the cell-permeable, irreversible caspase-3 inhibitor zDEVD-fmk to selectively inhibit caspase-3 activation (Anantharam et al., 2004). N27 dopaminergic cells were pre-exposed to either 20 or 50 μ M of zDEVD-fmk for 1 h before exposure to 300 μ M of Mn. Pre-treatment of N27 cells with zDEVD-fmk did not alter Np73 expression levels (data not shown). Interestingly, the Mn-induced downregulation of Np73 was unaffected by the presence of zDEVD-fmk (Fig. 4A-B), indicating that caspase-dependent proteolytic cleavage is unlikely to be involved in the suppression of Np73.

3.4 Downregulation of Np73 signaling sensitizes neurons to manganese-induced neurotoxicity

We next investigated whether downregulation of Np73 affects Mn-induced dopaminergic neuronal cell death by altering the expression of key anti-apoptotic proteins, such as Bcl-xL and Mcl-1. Interestingly, we found that expression of the anti-apoptotic proteins Bcl-xL (Fig. 5A-B) and Mcl-1 (Fig. 5C-D) was remarkably reduced by ~40-50% in N27 cells treated with 300 μ M Mn. These results indicate that reduced Np73 levels may play an important role in Mn neurotoxicity. To further investigate the functional relevance of Np73 downregulation in N27 cells, we overexpressed Np73 by more than two-fold relative to vector control (Fig. 6A-B), and then exposed the cells to increasing concentrations of Mn for 24 h. The overexpression of Np73 modestly inhibited the Mn-induced loss of cell viability during exposure to 100 and 200 μ M Mn as indicated via MTS assay, but was unable to overcome the toxic effect of 300 μ M Mn (Fig. 6C). Taken together, these data suggest that

Np73 suppression sensitizes dopaminergic cells to Mn-induced cell death and that the suppression of the anti-apoptotic Np73 protein may contribute to Mn-induced neurotoxicity.

4. Discussion

In the present study, we provide evidence that the tumor suppressor gene *p73* is highly susceptible to Mn-induced neurotoxicity in the nigrostriatal system. To our knowledge, this is the first evidence that Mn exerts its neurotoxic effects by suppressing expression of the anti-apoptotic Np73 protein, thereby providing novel mechanistic insights into the molecular events underlying Mn neurotoxicity.

Although several molecules have been identified as key mediators of Mn neurotoxicity leading to apoptosis, including the pro-apoptotic kinase PKC δ (Latchoumycandane et al., 2005; Zhang et al., 2007b), caspase-12, caspase-3 (Chun et al., 2001; Wang et al., 2015), and p53 (Ma et al., 2015; Yang et al., 2007), the exact molecular and cellular mechanisms of how chronic Mn exposure induces dopaminergic neuronal cell death have not been fully characterized. The p73 protein has been shown to participate in a wide variety of cellular events, including cell death, self-renewal and cell cycle regulation (Di et al., 2013; Engelmann et al., 2015; Fatt et al., 2014; Killick et al., 2011). The p73 protein has several isoforms characterized by alternative transcription at the N-terminus and alternative splicing at the C-terminus. Interestingly, TAp73 isoforms having the N-terminal transactivation domain induce apoptosis, whereas Np73 isoforms lacking the transactivation domain antagonize TAp73 functions, thereby favoring cell survival. We examined p73 protein expression in an *in vivo* animal model exposed to 30 mg/kg for 30 days. Mn exposure reduced total distance of movement compared to controls, showing Mn exposure induced SN degeneration (Fig. 1A-B). Importantly, we provide *in vivo* evidence that expression of Np73 (Fig. 1C-D) was downregulated in Mn-treated mice relative to controls, suggesting that Np73 plays an important role in Mn-induced neurotoxicity. Since Mn exposure downregulates Np73 in an isoform-specific manner, this gene has potential value in the development of a biomarker for Mn exposure.

Characterizing the isoform function of p73 may provide a better understanding of how Mn overexposure induces dopaminergic neuronal cell death. Our quantitative RT-PCR results revealed that Mn exposure downregulates *p73* gene expression in N27 dopaminergic neuronal cells (Fig. 2A). The concentration of 300 μ M Mn in N27 cells is lower than the 0.5-1 mM Mn used to induce toxic effects in mouse hippocampal neurons or human neuroblastoma cells (Tamm et al., 2008; Yoon et al., 2011). Furthermore, depending upon the dose, duration and route of exposure, Mn concentrations can reach up to 350 μ M in certain brain regions (Horning et al., 2015; Ingersoll et al., 1999; Roels et al., 1997). Adult mice exposed to 30 mg/kg for 8 weeks showed symptoms of Mn-induced toxicological effects (Moreno et al., 2009). Thus, Mn concentrations used in our study are within the relevant toxicological range and are consistently used in neurotoxicological studies by our group and by many leading laboratories (Aschner et al., 2007; Harischandra et al., 2015; Stredrick et al., 2004). The downregulation induced by 300 μ M Mn occurred over 12 h in N27 cells, indicating that Np73 downregulation occurred during early stages of the cell

death process. Our results from primary striatal neuronal cells exposed to different concentrations of Mn revealed that Mn downregulated the expression of Np73 in a concentration-dependent manner. Caspase-3 was shown to cleave full-length TAp73 under apoptotic conditions, releasing a 45-kDa fragment (Sayan et al., 2008). Our inhibition of caspase-3 failed to recover Np73 suppression. Moreover, we did not observe a ~40-kDa cleaved product of Np73. These data indicate that Np73 suppression involves caspase-3-independent mechanisms. Interestingly, p73 undergoes proteasomal degradation (Asher et al., 2005; Bernassola et al., 2004; Jung et al., 2011), but further research is needed to reveal whether p73 degradation plays a role in Mn-induced Np73 repression.

In addition to caspase-mediated proteolysis and proteasomal degradation, transcriptional suppression of the *p73* gene is another important mechanism to negatively alter p73 activity (Chen et al., 2001; Putzer et al., 2003). Runt-related transcription factor 2 and the transcription factor repressor ZEB bind to p73 promoter and transcriptionally suppress p73 expression (Bui et al., 2009; Fontemaggi et al., 2001; Ozaki et al., 2014). Additionally, epigenetic mechanisms, such as DNA hypermethylation, have been suggested to account for Np73 transcriptional repression (Lai et al., 2014). Mn exposure induces DNA damage via oxidative stress (Oikawa et al., 2006; Stephenson et al., 2013) and upregulates p53 (Wan et al., 2014). Furthermore, genotoxic stress has been shown to selectively degrade Np73 via the antizyme AZ1 (Dulloo et al., 2010). It is plausible that DNA damage induced by Mn exposure via oxidative stress may reduce Np73 and antagonize TAp73 as well as p53. Therefore, altered mRNA stability and protein stability may also contribute to Mn-induced Np73 downregulation. Future studies will focus on providing further evidence and mechanisms for Mn-induced transcriptional repression of the *p73* gene.

Bax is one of the transcriptional targets of the *p73* gene (Graupner et al., 2011; Melino et al., 2004). Consistent with a previous report (Gonzalez et al., 2008), our results indicate that Mn exposure upregulated Bax (data not shown). Bcl-xL and Mcl-1 were reported to suppress activation of Bax and Bak, respectively (Finucane et al., 1999; Sedlak et al., 1995). Downregulation of Bcl-xL and Mcl-1 during Mn exposure (Fig. 5) indicates that downregulation of Np73 may play a role in fine-tuning the activity of p73. When we overexpressed Np73 in N27 dopaminergic neurons, we observed a 2-fold increase in Np73 expression compared to empty vector-transfected N27 cells. After 24 h post-transfection, Np73 overexpression modestly inhibited an Mn-induced loss of viable cells (Fig. 6C) at low concentrations of 100 and 200 μ M Mn, but not against the 300 μ M concentration. Considering that chronic exposure to low doses of Mn leads to neurodegeneration in the long-term, the modest protective effects against low Mn concentrations offered by Np73 overexpression may have important implications for Mn neurotoxicity.

In conclusion, our results demonstrate that Np73 downregulation significantly contributed to Mn neurotoxicity. These observations provide new insights into the mechanisms underlying Mn-induced dopaminergic neurotoxicity. Further characterization of the molecular mechanisms underlying Np73 downregulation will provide a better understanding of Mn-induced neurotoxicity.

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References

- Anantharam V, Kitazawa M, Latchoumycandane C, Kanthasamy A, Kanthasamy AG. Blockade of PKCdelta proteolytic activation by loss of function mutants rescues mesencephalic dopaminergic neurons from methylcyclopentadienyl manganese tricarbonyl (MMT)-induced apoptotic cell death. *Annals of the New York Academy of Sciences*. 2004; 1035:271–289. [PubMed: 15681813]
- Aschner M, Erikson KM, Herrero Hernandez E, Tjalkens R. Manganese and its role in Parkinson's disease: from transport to neuropathology. *Neuromolecular medicine*. 2009; 11(4):252–266. [PubMed: 19657747]
- Aschner M, Guilarte TR, Schneider JS, Zheng W. Manganese: recent advances in understanding its transport and neurotoxicity. *Toxicology and applied pharmacology*. 2007; 221(2):131–147. [PubMed: 17466353]
- Asher G, Tsvetkov P, Kahana C, Shaul Y. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. *Genes Dev*. 2005; 19(3):316–321. [PubMed: 15687255]
- Basu A, Halder S. The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Molecular human reproduction*. 1998; 4(12):1099–1109. [PubMed: 9872359]
- Bernassola F, Salomoni P, Oberst A, Di Como CJ, Pagano M, Melino G, Pandolfi PP. Ubiquitin-dependent degradation of p73 is inhibited by PML. *The Journal of experimental medicine*. 2004; 199(11):1545–1557. [PubMed: 15184504]
- Bowman AB, Kwakye GF, Herrero Hernandez E, Aschner M. Role of manganese in neurodegenerative diseases. *J Trace Elem Med Biol*. 2011; 25(4):191–203. [PubMed: 21963226]
- Bui T, Sequeira J, Wen TC, Sola A, Higashi Y, Kondoh H, Genetta T. ZEB1 links p63 and p73 in a novel neuronal survival pathway rapidly induced in response to cortical ischemia. *miR oxidative stress*. 2009; 4(2):e4373.
- Caudle WM, Guillot TS, Lazo CR, Miller GW. Industrial toxicants and Parkinson's disease. *Neurotoxicology*. 2012; 33(2):178–188. [PubMed: 22309908]
- Chen P, Bowman AB, Mukhopadhyay S, Aschner M. SLC30A10: A novel manganese transporter. *Worm*. 2015; 4(3):e1042648. [PubMed: 26430566]
- Chen X, Zheng Y, Zhu J, Jiang J, Wang J. p73 is transcriptionally regulated by DNA damage, p53, and p73. *Oncogene*. 2001; 20(6):769–774. [PubMed: 11314010]
- Chun HS, Lee H, Son JH. Manganese induces endoplasmic reticulum (ER) stress and activates multiple caspases in nigral dopaminergic neuronal cells, SN4741. *Neurosci Lett*. 2001; 316(1):5–8. [PubMed: 11720765]
- Di C, Yang L, Zhang H, Ma X, Zhang X, Sun C, Li H, Xu S, An L, Li X, Bai Z. Mechanisms, function and clinical applications of DNp73. *Cell Cycle*. 2013; 12(12):1861–1867. [PubMed: 23708520]
- Dulloo I, Gopalan G, Melino G, Sabapathy K. The antiapoptotic DeltaNp73 is degraded in a c-Jun-dependent manner upon genotoxic stress through the antizyme-mediated pathway. *Proc Natl Acad Sci U S A*. 2010; 107(11):4902–4907. [PubMed: 20185758]
- Engelmann D, Meier C, Alla V, Putzer BM. A balancing act: orchestrating amino-truncated and full-length p73 variants as decisive factors in cancer progression. *Oncogene*. 2015; 34(33):4287–4299. [PubMed: 25381823]
- Fatt MP, Cancino GI, Miller FD, Kaplan DR. p63 and p73 coordinate p53 function to determine the balance between survival, cell death, and senescence in adult neural precursor cells. *Cell Death Differ*. 2014; 21(10):1546–1559. [PubMed: 24809925]

- Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem.* 1999; 274(4):2225–2233. [PubMed: 9890985]
- Fontemaggi G, Gurtner A, Strano S, Higashi Y, Sacchi A, Piaggio G, Blandino G. The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation. *Mol Cell Biol.* 2001; 21(24):8461–8470. [PubMed: 11713281]
- Goldman SM. Environmental toxins and Parkinson's disease. *Annual review of pharmacology and toxicology.* 2014; 54:141–164.
- Gonzalez LE, Juknat AA, Venosa AJ, Verrengia N, Kotler ML. Manganese activates the mitochondrial apoptotic pathway in rat astrocytes by modulating the expression of proteins of the Bcl-2 family. *Neurochem Int.* 2008; 53(6-8):408–415. [PubMed: 18930091]
- Graupner V, Alexander E, Overkamp T, Rothfuss O, De Laurenzi V, Gillissen BF, Daniel PT, Schulze-Osthoff K, Essmann F. Differential regulation of the proapoptotic multidomain protein Bak by p53 and p73 at the promoter level. *Cell Death Differ.* 2011; 18(7):1130–1139. [PubMed: 21233848]
- Grob TJ, Novak U, Maise C, Barcaroli D, Luthi AU, Pirnia F, Hugli B, Graber HU, De Laurenzi V, Fey MF, Melino G, Tobler A. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell death and differentiation.* 2001; 8(12):1213–1223. [PubMed: 11753569]
- Guilarte TR. Manganese and Parkinson's disease: a critical review and new findings. *Ciencia & saude coletiva.* 2011; 16(11):4549–4566. [PubMed: 22124833]
- Harischandra DS, Jin H, Anantharam V, Kanthasamy A, Kanthasamy AG. alpha-Synuclein protects against manganese neurotoxic insult during the early stages of exposure in a dopaminergic cell model of Parkinson's disease. *Toxicological sciences : an official journal of the Society of Toxicology.* 2015; 143(2):454–468. [PubMed: 25416158]
- Horning KJ, Caito SW, Tipps KG, Bowman AB, Aschner M. Manganese Is Essential for Neuronal Health. *Annual review of nutrition.* 2015; 35:71–108.
- Huttinger-Kirchhof N, Cam H, Griesmann H, Hofmann L, Beitzinger M, Stiewe T. The p53 family inhibitor DeltaNp73 interferes with multiple developmental programs. *Cell death and differentiation.* 2006; 13(1):174–177. [PubMed: 16341031]
- Ingersoll RT, Montgomery EB Jr. Aposhian HV. Central nervous system toxicity of manganese. II: Cocaine or reserpine inhibit manganese concentration in the rat brain. *Neurotoxicology.* 1999; 20(2-3):467–476. [PubMed: 10385905]
- Jin H, Kanthasamy A, Anantharam V, Rana A, Kanthasamy AG. Transcriptional regulation of pro-apoptotic protein kinase Cdelta: implications for oxidative stress-induced neuronal cell death. *J Biol Chem.* 2011; 286(22):19840–19859. [PubMed: 21467032]
- Jin H, Kanthasamy A, Harischandra DS, Kondru N, Ghosh A, Panicker N, Anantharam V, Rana A, Kanthasamy AG. Histone hyperacetylation up-regulates protein kinase Cdelta in dopaminergic neurons to induce cell death: relevance to epigenetic mechanisms of neurodegeneration in Parkinson disease. *J Biol Chem.* 2014a; 289(50):34743–34767. [PubMed: 25342743]
- Jin H, Kanthasamy A, Harischandra DS, Kondru N, Ghosh A, Panicker N, Anantharam V, Rana A, Kanthasamy AG. Histone Hyperacetylation Upregulates PKCdelta in Dopaminergic Neurons to Induce Cell Death: Relevance to Epigenetic Mechanisms of Neurodegeneration in Parkinson's Disease. *The Journal of biological chemistry.* 2014b
- Jost CA, Marin MC, Kaelin WG Jr. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature.* 1997; 389(6647):191–194. [PubMed: 9296498]
- Jung YS, Qian Y, Chen X. The p73 tumor suppressor is targeted by Pirh2 RING finger E3 ubiquitin ligase for the proteasome-dependent degradation. *J Biol Chem.* 2011; 286(41):35388–35395. [PubMed: 21852228]
- Kanthasamy A, Jin H, Anantharam V, Sondarva G, Rangasamy V, Rana A, Kanthasamy A. Emerging neurotoxic mechanisms in environmental factors-induced neurodegeneration. *Neurotoxicology.* 2012; 33(4):833–837. [PubMed: 22342404]
- Killick R, Niklison-Chirou M, Tomasini R, Bano D, Rufini A, Grespi F, Velletri T, Tucci P, Sayan BS, Conforti F, Gallagher E, Nicotera P, Mak TW, Melino G, Knight RA, Agostini M. p73: a multifunctional protein in neurobiology. *Molecular neurobiology.* 2011; 43(2):139–146. [PubMed: 21380933]

- Lai J, Nie W, Zhang W, Wang Y, Xie R, Wang Y, Gu J, Xu J, Song W, Yang F, Huang G, Cao P, Guan X. Transcriptional regulation of the p73 gene by Nrf-2 and promoter CpG methylation in human breast cancer. *Oncotarget*. 2014; 5(16):6909–6922. [PubMed: 25071010]
- Latchoumycandane C, Anantharam V, Kitazawa M, Yang Y, Kanthasamy A, Kanthasamy AG. Protein kinase Cdelta is a key downstream mediator of manganese-induced apoptosis in dopaminergic neuronal cells. *The Journal of pharmacology and experimental therapeutics*. 2005; 313(1):46–55. [PubMed: 15608081]
- Leyva-Illades D, Chen P, Zogzas CE, Hutchens S, Mercado JM, Swaim CD, Morrisett RA, Bowman AB, Aschner M, Mukhopadhyay S. SLC30A10 is a cell surface-localized manganese efflux transporter, and parkinsonism-causing mutations block its intracellular trafficking and efflux activity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2014; 34(42):14079–14095. [PubMed: 25319704]
- Ma X, Han J, Wu Q, Liu H, Shi S, Wang C, Wang Y, Xiao J, Zhao J, Jiang J, Wan C. Involvement of dysregulated Wip1 in manganese-induced p53 signaling and neuronal apoptosis. *Toxicology letters*. 2015; 235(1):17–27. [PubMed: 25791630]
- Martin DP, Anantharam V, Jin H, Witte T, Houk R, Kanthasamy A, Kanthasamy AG. Infectious prion protein alters manganese transport and neurotoxicity in a cell culture model of prion disease. *Neurotoxicology*. 2011; 32(5):554–562. [PubMed: 21871919]
- Melino G, Bernassola F, Ranalli M, Yee K, Zong WX, Corazzari M, Knight RA, Green DR, Thompson C, Vousden KH. p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation. *J Biol Chem*. 2004; 279(9):8076–8083. [PubMed: 14634023]
- Melino G, De Laurenzi V, Vousden KH. p73: Friend or foe in tumorigenesis. *Nature reviews. Cancer*. 2002; 2(8):605–615. [PubMed: 12154353]
- Moreno JA, Yeomans EC, Streifel KM, Brattin BL, Taylor RJ, Tjalkens RB. Age-dependent susceptibility to manganese-induced neurological dysfunction. *Toxicol Sci*. 2009; 112(2):394–404. [PubMed: 19812362]
- Nakagawa T, Takahashi M, Ozaki T, Watanabe Ki K, Todo S, Mizuguchi H, Hayakawa T, Nakagawara A. Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter. *Mol Cell Biol*. 2002; 22(8):2575–2585. [PubMed: 11909952]
- Oikawa S, Hirokawa I, Tada-Oikawa S, Furukawa A, Nishiura K, Kawanishi S. Mechanism for manganese enhancement of dopamine-induced oxidative DNA damage and neuronal cell death. *Free Radic Biol Med*. 2006; 41(5):748–756. [PubMed: 16895795]
- Ozaki T, Sugimoto H, Nakamura M, Hiraoka K, Yoda H, Sang M, Fujiwara K, Nagase H. Runt-related transcription factor 2 attenuates the transcriptional activity as well as DNA damage-mediated induction of pro-apoptotic TA-p73 to regulate chemosensitivity. *FEBS J*. 2014
- Putzer BM, Tuve S, Tannapfel A, Stiewe T. Increased DeltaN-p73 expression in tumors by upregulation of the E2F1-regulated, TA-promoter-derived DeltaN'-p73 transcript. *Cell Death Differ*. 2003; 10(5):612–614. [PubMed: 12728260]
- Roels H, Meiers G, Delos M, Ortega I, Lauwerys R, Buchet JP, Lison D. Influence of the route of administration and the chemical form (MnCl₂, MnO₂) on the absorption and cerebral distribution of manganese in rats. *Archives of toxicology*. 1997; 71(4):223–230. [PubMed: 9101038]
- Rosenbluth JM, Pietenpol JA. The jury is in: p73 is a tumor suppressor after all. *Genes Dev*. 2008; 22(19):2591–2595. [PubMed: 18832062]
- Sayan AE, Sayan BS, Gogvadze V, Dinsdale D, Nyman U, Hansen TM, Zhivotovsky B, Cohen GM, Knight RA, Melino G. P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis. *Oncogene*. 2008; 27(31):4363–4372. [PubMed: 18362891]
- Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci U S A*. 1995; 92(17):7834–7838. [PubMed: 7644501]
- Stephenson AP, Schneider JA, Nelson BC, Atha DH, Jain A, Soliman KF, Aschner M, Mazzio E, Renee Reams R. Manganese-induced oxidative DNA damage in neuronal SH-SY5Y cells: attenuation of thymine base lesions by glutathione and N-acetylcysteine. *Toxicology letters*. 2013; 218(3):299–307. [PubMed: 23296100]

- Stredrick DL, Stokes AH, Worst TJ, Freeman WM, Johnson EA, Lash LH, Aschner M, Vrana KE. Manganese-induced cytotoxicity in dopamine-producing cells. *Neurotoxicology*. 2004; 25(4):543–553. [PubMed: 15183009]
- Tamm C, Sabri F, Ceccatelli S. Mitochondrial-mediated apoptosis in neural stem cells exposed to manganese. *Toxicol Sci*. 2008; 101(2):310–320. [PubMed: 17977900]
- Tidball AM, Bryan MR, Uhouse MA, Kumar KK, Aboud AA, Feist JE, Ess KC, Neely MD, Aschner M, Bowman AB. A novel manganese-dependent ATM-p53 signaling pathway is selectively impaired in patient-based neuroprogenitor and murine striatal models of Huntington's disease. *Human molecular genetics*. 2015; 24(7):1929–1944. [PubMed: 25489053]
- Wan C, Ma X, Shi S, Zhao J, Nie X, Han J, Xiao J, Wang X, Jiang S, Jiang J. Pivotal roles of p53 transcription-dependent and -independent pathways in manganese-induced mitochondrial dysfunction and neuronal apoptosis. *Toxicology and applied pharmacology*. 2014; 281(3):294–302. [PubMed: 25448048]
- Wang T, Li X, Yang D, Zhang H, Zhao P, Fu J, Yao B, Zhou Z. ER stress and ER stress-mediated apoptosis are involved in manganese-induced neurotoxicity in the rat striatum in vivo. *Neurotoxicology*. 2015; 48:109–119. [PubMed: 25732873]
- White AR, Kanninen KM, Crouch PJ. Editorial: Metals and neurodegeneration: restoring the balance. *Frontiers in aging neuroscience*. 2015; 7:127. [PubMed: 26191002]
- Yang A, Kaghad M, Caput D, McKeon F. On the shoulders of giants: p63, p73 and the rise of p53. *Trends in genetics : TIG*. 2002; 18(2):90–95. [PubMed: 11818141]
- Yang H, Sun Y, Zheng X. Manganese-induced apoptosis in rat myocytes. *Journal of biochemical and molecular toxicology*. 2007; 21(3):94–100. [PubMed: 17623882]
- Yoon H, Kim DS, Lee GH, Kim KW, Kim HR, Chae HJ. Apoptosis Induced by Manganese on Neuronal SK-N-MC Cell Line: Endoplasmic Reticulum (ER) Stress and Mitochondria Dysfunction. *Environmental health and toxicology*. 2011; 26:e2011017. [PubMed: 22232721]
- Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG. Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. *The Journal of pharmacology and experimental therapeutics*. 2007a; 322(3):913–922. [PubMed: 17565007]
- Zhang D, Kanthasamy A, Yang Y, Anantharam V, Kanthasamy A. Protein kinase C delta negatively regulates tyrosine hydroxylase activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons. *J Neurosci*. 2007b; 27(20):5349–5362. [PubMed: 17507557]

Highlights

- Manganese exposure downregulates *p73* gene expression in an isoform-specific manner
- Overexpression of Np73 protects against manganese-induced apoptosis
- Downregulation of Np73 is recapitulated in an animal model of Mn neurotoxicity
- Np73 downregulation sensitizes neuronal cells to manganese-induced apoptosis

Fig 1. Oral Mn administration downregulates Np73 expression in the C57BL mouse model
Male C57BL mice were orally administered 30 mg/kg of Mn for 30 days, and vehicle control animals were administered de-ionized water. After treatments, locomotor activity was measured using an automated VersaMax locomotor activity monitor. Representative activity maps of open-field movements of Mn-treated mice and control mice revealed Mn-induced locomotor deficits (A). Mn treatment also reduced total distance traveled (cm) during a 10-min test of locomotor activity (B). Western blot assays were performed on substantia nigral lysates to measure the expression of Np73 (C). Densitometric analysis was also performed (D). Data are expressed as percent of control and represented as mean $\pm$ S.E.M. from four animals per group. Asterisk (*, $p < 0.05$) indicates significant differences between treatment and control groups.

A **B** **C**

Fig 2. Mn exposure represses Np73 gene and protein expression in N27 dopaminergic cells
 N27 dopaminergic cells were exposed to 300 μM of Mn for 24 h in reduced sera. We examined p73 mRNA expression by qRT-PCR (A). Data are expressed as percent of control and represented as mean ± S.E.M. from six independent experiments. Asterisks (***, $p < 0.001$) indicate significant differences between treatment and control groups. N27 cells were exposed to 300 μM of Mn for 6-24 h. Expression of Np73 protein was measured by Western blot assays (B). Densitometric analysis was performed (C). Data are expressed as percent of control and represented as mean ± S.E.M. from three independent experiments. Asterisks (*, $p < 0.05$) indicate significant differences between treatment and control groups.

A **B**

Fig 3. Mn downregulates Np73 in primary striatal neurons

Primary striatal neurons were exposed to 100-500 μ M of Mn for 24 h in reduced sera. Expression of Np73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Data are expressed as percent of control and represented as mean $\pm$ S.E.M. from three independent experiments. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate significant differences between treatment and control groups.

A **B**

Fig 4. Caspase-3 inhibition does not alter the downregulation of Np73 during Mn-induced neurotoxicity

N27 dopaminergic cells were pre-treated with either 20 or 50 μ M of the caspase-3 inhibitor zDEVD-fmk before exposure to 300 μ M of Mn for 24 h in reduced sera. Expression of

Np73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Data are expressed as percent of control and represented as mean $\pm$ S.E.M. from three independent experiments. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate significant differences between treatment and control groups.

Fig 5. Expression of the downstream effectors of the p73 signaling pathway is also downregulated in Mn-induced neurotoxicity
 N27 dopaminergic cells were exposed to 100-500 μ M of Mn for 24 h in reduced sera. Expression of Bcl-xL (A) and the longer isoform of Mcl-1 (C) was measured by Western blot assays. Densitometric analysis was performed (B and D). Data are expressed as percent of control and represented as mean $\pm$ S.E.M. from three independent experiments for Bcl-xL and four independent experiments for Mcl-1, respectively. Asterisks (*, $p < 0.05$; ***, $p < 0.001$) indicate significant differences between treatment and control groups.

Fig 6. Overexpression of Np73 protects against Mn-induced neurotoxicity

N27 dopaminergic cells were overexpressed with empty vector or Np73. Cells were exposed to 100-300 μM of Mn for 24 h in reduced sera. Expression of Np73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Data are expressed as percent of control and represented as mean ± S.E.M. from two independent experiments. Asterisks (*, $p < 0.05$) indicate significant differences between Np73-expressing and empty vector-expressing groups. Cell survival was measured by MTS assay (C). Data are expressed as percent of control and represented as mean ± S.E.M. from six independent experiments for MTS assay. Asterisks (***, $p < 0.001$) indicate significant differences between Np73-expressing and empty vector-expressing groups.