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Effects of Manganese on Monoamine Neurotransmitter Metabolism and Dopaminergic Cell Viability

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Abstract

Exposure to high levels of manganese through mining, welding, ground/well water or use of manganese-containing pesticides can cause accumulation of this essential trace metal in the dopaminergic cells of the basal ganglia resulting in an idiopathic Parkinsonism-like disease. This accumulation causes mitochondrial dysfunction, oxidative stress, protein aggregation and other signs of neurotoxicity. The catecholamine/catecholaldehyde hypothesis suggests disruption of normal dopamine metabolism often triggered by mitochondrial dysfunction or oxidative stress, leads to an accumulation of toxic 3, 4-dihydroxyphenylacetaldehyde (DOPAL) and cell death. The objective of this study is to determine whether manganese exposure affects viability of dopaminergic cells and alters extracellular levels of dopamine and its metabolites. SH-SY5Y and differentiated PC12 dopaminergic cell lines were exposed to increasing concentrations of manganese, ranging from 200 μM to 1000 μM, for 36, 48 or 60 hours. Cell viability was determined using the trypan blue exclusion assay. SH-SY5Y cells displayed a decrease in viability with increasing concentrations of manganese, but the effect was dependent on time whereas PC12 cells responded to increasing concentrations of manganese with a decrease in viability regardless of time. Samples of media surrounding the exposed cells were collected prior to measuring cell viability and used to perform high performance liquid chromatography (HPLC) with electrochemical detection. HPLC was used to measure the presence and relative levels of specific extracellular monoamine neurotransmitters including dopamine, epinephrine, norepinephrine and serotonin as well as their major metabolites. Preliminary results suggest a decrease in the levels of extracellular dopamine and serotonin in the surrounding media of SH-SY5Y cells exposed to manganese; complete analysis may reveal additional changes in the levels of these neurotransmitters and their metabolites. Future studies will 1) study the mechanism through which manganese exposure may alter extracellular levels of dopamine and 2) determine whether or not alterations in the levels of these neurotransmitters contribute to the observed manganese-induced changes in dopaminergic cell viability.

Keywords: manganese, neurodegeneration, catecholamines

1. Introduction

Manganese is a natural mineral and an essential trace element for all living organisms[1, 2]. It is involved in normal cellular and physiological processes including metabolism, reproduction, and normal brain function[2-4]. As an essential trace element, the body tightly regulates the homeostatic levels of manganese through the processes of ingestion and excretion[5]. However, this regulation can be lost when challenged by chronic exposure to high levels of manganese particularly through inhalation or ingestion from sources such as occupational exposure, well water contamination, and atmospheric exposure[1-3, 6, 7]. The effects of this exposure can involve multiple systems within

the body, with the most notable associated with a loss of neurological function[1-3, 6, 7]. Most severe cases of chronic exposure are associated with occupation and can result in a disorder referred to as manganism[1-3, 5-8].

Manganism is a progressive disorder that like Parkinson's disease is associated with altered neurological function of the basal ganglia[1-3, 5-8]. Although distinct medical conditions, both display an overlap of symptoms stemming from a loss of neurological function in part through the disruption of normal neurochemical signaling[5, 6, 9]. The basal ganglia represent a complex region of the brain associated with overlapping excitatory and inhibitory pathways tightly regulated to maintain functionality of the whole system. Disruption in one area or neurochemical pathway can disrupt the regulation of associated pathways[2, 5, 9]. Individuals with Parkinson's disease display a characteristic loss of dopaminergic cells of the substantia nigra pars compacta, which contributes to the symptoms and progression of this neurodegenerative disease[10]. Exposure to high levels of manganese results in its accumulation in a distinct set of cells with GABAminergic activity within the globus pallidus of the basal ganglia[5, 7]. This accumulation results in the neurotoxicity of these cells and deregulation of glutamate homeostasis[5, 7, 11]. Regardless of the initial site of injury in these two diseases, the overlap of symptoms and overall clinical manifestation has led to a continued debate regarding the role of manganese as a risk factor for idiopathic Parkinson's disease[1-3, 5-7, 9, 12].

Understanding how manganese affects the tightly controlled balance of neurotransmitter production, metabolism and release could contribute to the understanding of the development and progression of manganism and/or idiopathic Parkinson's disease. This study investigated the effect(s) of manganese on the levels of two important monoamine neurotransmitters, dopamine and serotonin. Dopamine is produced in cells found in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc)[13]. The VTA projects primarily to the nucleus accumbens and the prefrontal cortex to regulate the experiences of reward, pleasure and euphoria as well as some cognitive function and memory, while the SN projects primarily to the striatum to regulate motor control[13]. The monoamine neurotransmitter serotonin is produced primarily within the pair of raphe nuclei in the central nervous system and function by modulating neurotransmitter responses throughout the brain. This control by serotonin can regulate mood, cognitive function and memory processing[14].

The objectives in this study were to elucidate the effects of manganese on monoamine neurotransmitter metabolism and cell viability in dopaminergic cells. Specifically, we hypothesized that manganese may disrupt the metabolism of monoamine neurotransmitters in dopaminergic cells, which in turn could result in decreased cell viability. The presence of manganese was found to elicit a significant decrease in the extracellular concentration of dopamine, serotonin and 5-hydroxyindoleacetic acid secreted from SH-SY5Y dopaminergic cells. The effect of manganese on dopaminergic cell viability was more complicated. SH-SY5Y cell viability responded to the interaction between time and manganese treatment. However, differentiated PC12 cell viability was predicted by both time and manganese treatment, independently of one another. Although the presence of manganese was able to nearly abrogate the extracellular levels of monoamine neurotransmitters surrounding SH-SY5Y cells, higher concentrations of manganese were required to elicit decreases in dopaminergic cell viability. Thus, the decreased extracellular concentrations of monoamine neurotransmitters were not sufficient to induce detectable decreases in dopaminergic cell viability. Future studies are needed to investigate the mechanism(s) through which manganese elicits decreased extracellular concentrations of monoamine neurotransmitters and cell viability in different dopaminergic cell lines.

2. Methodology

2.1 Cell culture

SH-SY5Y cells were grown in DMEM high glucose media supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. PC12 cells were grown in RPMI 1640 media with 10% heat inactivated horse serum and 5% fetal bovine serum. Both cell lines were grown in tissue culture dishes coated with rat collagen I. SH-SY5Y cells were cultured for 48 hours prior to treatment with manganese. PC12 cells were induced to differentiate with 100 ng/mL nerve growth factor (NGF) in complete media for 10-14 days prior to manganese treatment. All experiments were conducted in 12-well plates.

2.2 Treatment with manganese

SH-SY5Y and differentiated PC12 dopaminergic cell lines were exposed to increasing concentrations of manganese chloride, ranging from 200 μM to 1000 μM, for 36, 48 or 60 hours in complete media. Cells exposed to sterile, tissue culture grade water (vehicle) in complete media were used as a control. Each treatment at each time was conducted in quadruplicate.

2.3 HPLC analysis

Samples of extracellular media surrounding control or treated cells were analyzed using high performance liquid chromatography (HPLC) to determine the levels of catecholamines and indolamines tested according to a method described previously[15]. For each 100 μL of sample, 400 μL of a perchloric acid solution was added. The solution contained 0.05M $HClO_4$ with 0.4 mM $Na_2S_2O_5$, 0.8 mM EDTA, and 450 nM 3, 4-dihydroxybenzylamime (DHBA). HPLC measurements were done using a Suppleco Discovery C18 column, 5 mm resin size, 15 cm long by 4.6 mm internal diameter with a 2 cm guard column. The column pressure was kept between 2000 and 2200 psi, achieved by using a flow rate between 0.75 and 1.0 mL/min. The HPLC mobile phase was a pre-made phosphate based solution with acetonitrile called MD-TM Mobile Phase purchased premixed from ESA. The analysis was done using electrochemical detection on a BSA LC-4C detector set at +0.75V. The internal standard was DHBA. Retention times of chromatogram peaks representing known standards were used to identify chromatogram peaks of metabolites in experimental samples. The known standards were prepared as a mixture of neurotransmitters, each at a known concentration of 1 μM. The peak area of each identified metabolite of interest was used to estimate the metabolite's concentration. This was done by normalizing the peak area of the metabolite to the peak area of the internal standard, DHBA, followed by normalization to the relative area of the standard at a concentration of 1 μM. For each metabolite, the average estimated concentration was compared relative to the same metabolite's average estimated concentration in the control sample after 36 hours to determine the relative concentration of the metabolite. A two-factor analysis of variance (ANOVA) was conducted to determine the effect of time, treatment and the interaction on the relative concentration of each metabolite studied. With a significant p-value of either main effect, post-hoc tests using a Bonferroni adjustment were used to establish significant differences between treatments.

2.4 Cell viability analysis

Cells were photographed at a total magnification of 200X. Cells were collected by centrifugation, resuspended in complete media and mixed 1:1 with trypan blue. Cell viability was determined using the trypan blue exclusion assay with a TC10 automated cell counter. Each sample was counted at least twice and averaged. Two-factor ANOVA was conducted to determine the effect of time, treatment and the interaction on the average percent cell viability. With a significant interaction, the main effects were considered separately. With a significant p-value of either main effect, post-hoc tests using a Bonferroni adjustment were used to establish significant differences between treatments.

3. Results and Discussion

In order to elucidate the effects of manganese on monoamine neurotransmitter metabolism in dopaminergic cells, SH-SY5Y cells were used. These cells are dopaminergic cells derived from a human neuroblastoma and are commonly used as a cell model to study dopamine metabolism, particularly as it relates to neurodegenerative diseases[16, 17]. In control- or manganese-treated cells, the extracellular levels of the monoamine neurotransmitters dopamine (DA), serotonin (5-HT) and the serotonin metabolite, 5-hydroxyindoleacetic acid (5HIAA), as well as the neurotransmitters epinephrine (E) and norepinephrine (NE) were measured using high performance liquid chromatography (HPLC) with electrochemical detection. A mixture of these neurotransmitters and others were prepared at known concentrations (1 μM) and analyzed using HPLC with electrochemical detection to identify the chromatogram peaks of each metabolite of interest. The retention time of each neurotransmitter within the standard mixture was used to identify the metabolites of interest in experimental samples. The metabolites of interest were

identified when the retention time of their chromatogram peak matched the retention time of a known standard chromatogram peak. Representative chromatograms used to identify metabolites in experimental samples and measure peak areas to elucidate metabolite concentrations are shown in Figure 1.

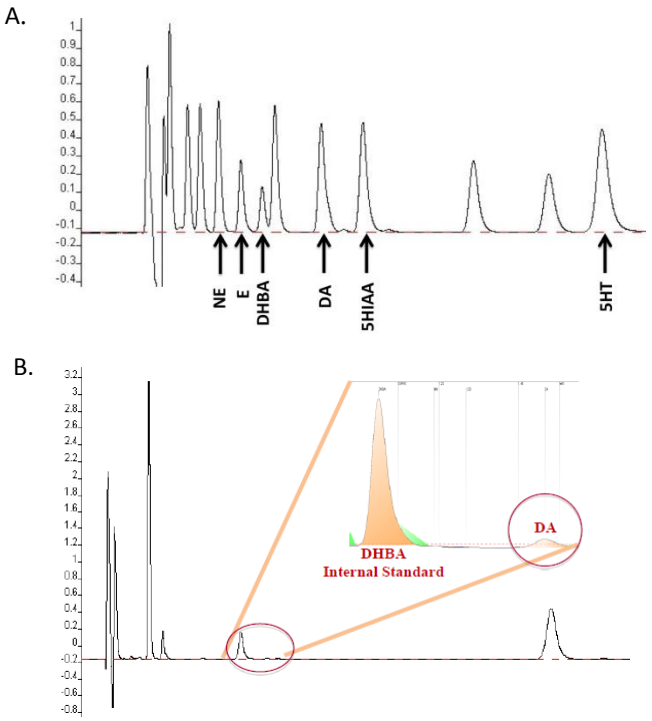

Figure 1. Representative chromatograms following HPLC analysis with electrochemical detection of A. a standard mixture of neurotransmitters at known concentrations and B. an experimental sample. The potential on the y-axis represents the relative concentration of each metabolite. The x-axis represents retention time off the column. Chromatogram peaks for metabolites of interest were labeled.

Dopaminergic cells are characterized by their ability to produce and secrete DA. The methodology used in this study should be able to detect increased extracellular concentrations of DA in the extracellular media surrounding SH-SY5Y dopaminergic cells in response to time. Prior studies have shown that acute manganese (Mn) exposure, typically 24 hours or less, results in decreased extracellular levels of DA surrounding some cell types. It is unknown whether Mn-induced decreases in the extracellular concentration of DA persist when treated for longer lengths of time. In this experiment, SH-SY5Y cells were treated with different amounts of Mn (0, 200, 400, 600, 800 or 1000 μM) for 36, 48 or 60 hours. The average concentration of DA in the extracellular media surrounding control- or Mn-treated SH-SY5Y cells was determined (data not shown). The relative concentration of DA was determined by normalizing the average concentration of DA for each experimental group to the average concentration of DA in the control group after 36 hours, which was set to one. A two-factor analysis of variance (ANOVA) was conducted to analyze the effect of time, Mn treatment and the interaction on the relative extracellular concentration of DA. There was no interaction between time and Mn treatment on the relative concentration of extracellular DA ($F_{10, 50} = 1.6$, $p = 0.146$), allowing the main effects to be interpreted separately.

There was a significant increase in the extracellular concentration of DA in response to time ($F_{2, 50} = 4.1$, $p < 0.05$, Figure 2A). The relative concentration of extracellular DA was significantly higher after cells were grown in culture for 60 hours following treatment compared to cells grown in culture for 36 hours (Figure 2A). These results demonstrate that more DA is produced in or secreted from SH-SY5Y dopaminergic cells with more time. Although extracellular DA levels increased over time, it appeared to do so at a decreasing rate. The relative concentration of extracellular DA increased approximately 60% when cells were grown for 48 hours compared to 36 hours, whereas the observed increase in the relative concentration of extracellular DA after 60 hours was only 80% compared to the concentration at 36 hours (Figure 2A). Despite the 2-fold increase in time, the relative concentration of extracellular DA did not increase linearly.

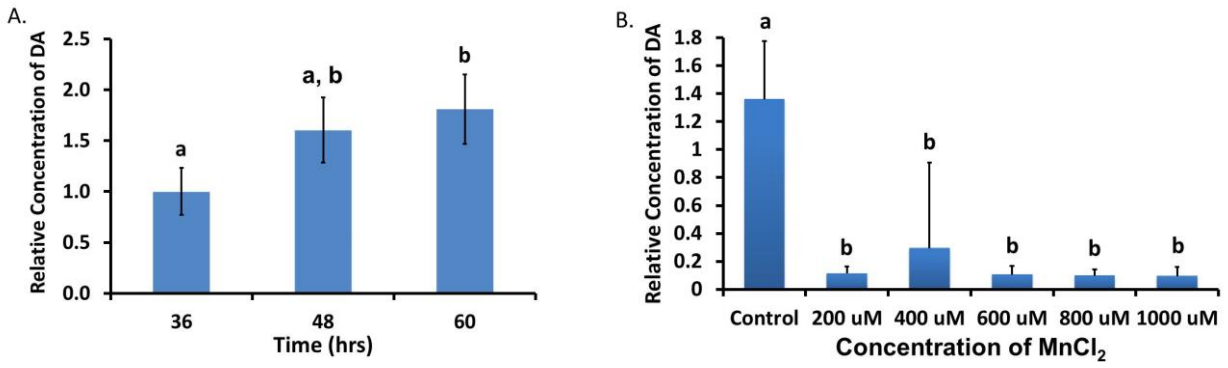

Figure 2. Relative concentration of extracellular DA surrounding SH-SY5Y cells.

Data were normalized to the average relative concentration of extracellular DA surrounding control-treated cells grown in culture for 36 hours. A. relative concentration of extracellular DA in response to time B. relative concentration of extracellular DA in response to Mn treatment. Error bars represent standard deviation of the mean. Where lower case letters are the same, relative DA levels do not differ significantly.

Extracellular DA concentration continued to increase after 60 hours, even following Mn treatment, but Mn treatment significantly suppressed the extracellular concentration of DA overall compared to control-treated cells ($F_{5, 50} = 34.4$, $p < 0.001$, Figure 2B). Treatment of SH-SY5Y cells with any amount of Mn used in this study resulted in a significant drop in the extracellular levels of DA surrounding Mn-treated cells compared to control-treated cells. These results indicate that the presence of low doses of Mn (200 μM) are sufficient to significantly reduce the extracellular concentration of DA.

Significant decreases in the relative concentrations of extracellular serotonin (5HT; $F_{5, 50} = 44.8$, $p < 0.001$) and its major metabolite, 5-hydroxyindoleacetic acid (5HIAA; $F_{5, 50} = 26.6$, $p < 0.001$) were also detected when SH-SY5Y cells were treated with Mn (Figure 3). The extracellular concentrations of these monoamine neurotransmitters were not affected by time (5HT; $F_{2, 50} = 1.8$, $p = 0.180$ and 5HIAA; $F2_{, 50} = 1.4$, $p = 0.257$; data not shown). Similar to the effect of Mn on DA, the presence of at least 200 μM of Mn was sufficient to reduce the levels of these metabolites across all time points. The only significant difference observed between treatments was between control-treated and Mn-treated SH-SY5Y cells. The concentration of Mn used to treat cells did not alter the effect on extracellular levels of 5HT or 5HIAA. These results suggest that Mn can affect the extracellular levels of more than one monoamine neurotransmitter. To determine if metabolites of DA may also be affected by Mn treatment, we tried to measure the relative extracellular concentrations of epinephrine and norepinephrine following treatment of SH-SY5Y cells with Mn. However, the levels of these neurotransmitters were not detectable (data not shown). Further studies are necessary to explore the effects of Mn on other DA metabolites.

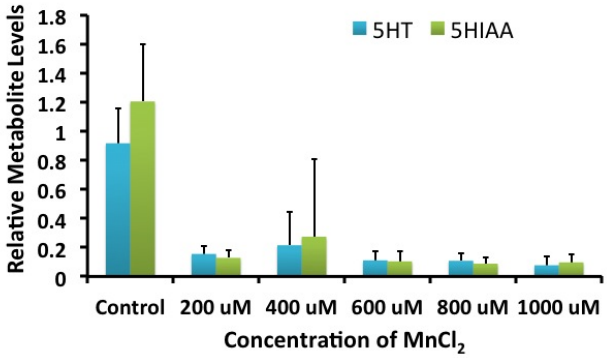

Figure 3. Relative concentrations of extracellular 5HT and 5HIAA surrounding SH-SY5Y cells in response to Mn treatment.

Data were normalized to the average relative concentration of extracellular DA surrounding control-treated cells grown in culture for 36 hours. Error bars represent standard deviation of the mean. The relative metabolite levels were significantly decreased across all treatments with Mn compared to the control. There were no differences observed between groups treated with Mn.

Dopaminergic cells normally adhere to tissue culture plates and appear relatively flat with extensions. Cells treated with higher concentrations of Mn appeared rounder, and they began to clump together and float in the media suspension (data not shown). These qualitative characteristics are consistent with a loss of cell viability. Thus, studies were designed to quantify these observations and determine the effect of Mn treatment on dopaminergic cell viability. Two widely used dopaminergic cell lines, SH-SY5Y and PC12, were used. PC12 cells are derived from a rat pheochromacytoma and upon differentiation with nerve growth factor (NGF) take on a neuronal-like phenotype[18, 19]. PC12 cells are another commonly used cell line to model dopamine metabolism and neurodegenerative diseases[20-22].

In order to elucidate the effect of Mn on dopaminergic cell viability, SH-SY5Y or differentiated PC12 cells were treated with different concentrations of Mn (0, 200, 400, 600, 800 or 1000 μM) for 36, 48 or 60 hours. Cells were collected, and a trypan blue exclusion assay was conducted on the cell suspension using an automated cell counter. The viability of SH-SY5Y cells was dependent on both Mn treatment and time ($F_{10, 53} = 8.7$, $p < 0.001$) making interpretation of the significant main effects, Mn treatment ($F_{5, 53} = 218.2$, $p < 0.001$) and time ($F_{2, 53} = 44.3$, $p < 0.001$) difficult. Therefore, the effect of Mn treatment was considered separately for each time (Figure 4). For each time, 36-, 48- and 60 hours, cell viability declined with increasing exposure ($F_{5, 17} = 30.2$, $p < 0.001$; $F_{5, 17} = 111.5$, $p < 0.001$; $F_{5, 17} = 175.4$, $p < 0.001$, respectively). SH-SY5Y cell viability was high, nearly 90%, in control-treated cells at each time and decreased more than 50% after treatment with 1000 μM of Mn for 60 hours. Significant decreases in SH-SY5Y cell viability were observed following treatment with 800 μM of Mn after 36 hours or 600 μM of Mn after 48 or 60 hours when compared to control-treated cells. Treatment of SH-SY5Y cells with 200 or 400 μM of Mn did not result in a significant decrease in cell viability, regardless of time.

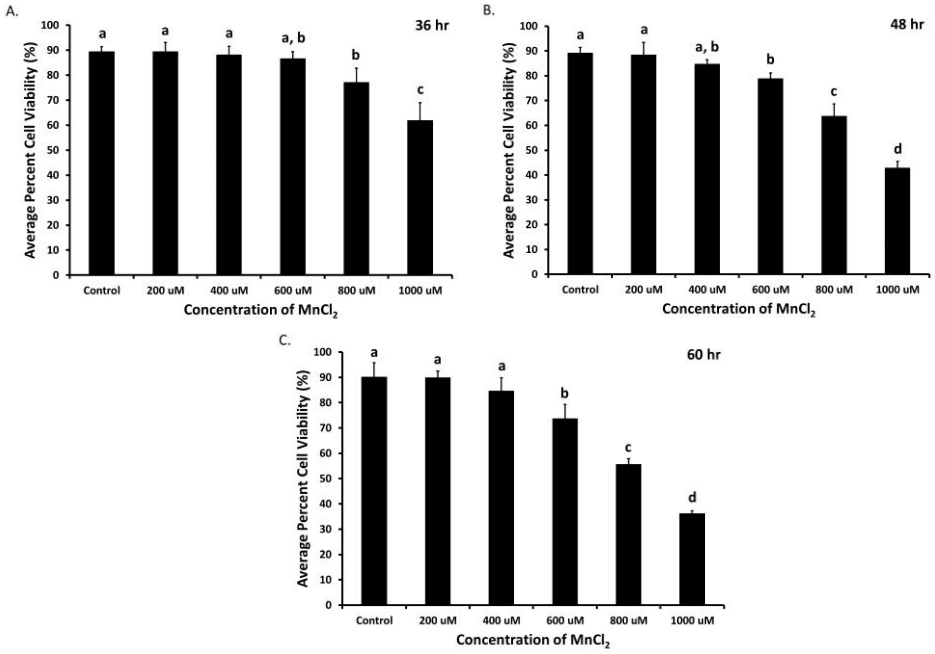

Figure 4. Effects of Mn treatment and time on SH-SY5Y cell viability. SH-SY5Y cell viability was dependent on both Mn treatment and time.

The main effect of Mn treatment is considered separately for each time. Data are shown as the average percent of cell viability after Mn treatment for A. 36-, B. 48- or C. 60 hours. Error bars represent standard deviation of the mean. Where lower case letters are the same, the average percent cell viability (%) does not differ significantly.

Differentiated PC12 cells responded differently than SH-SY5Y cells. There was no interaction between Mn treatment and time on the average percent cell viability of differentiated PC12 cells ($F_{10, 53} = 1.8$, $p = 0.08$) allowing the main effects to be interpreted separately. There was a significant decrease in differentiated PC12 cell viability in response to time ($F_{2, 53} = 72.6$, $p < 0.001$; Figure 5A), and Mn treatment ($F_{5, 53} = 99.3$, $p < 0.001$; Figure 5B). Overall differentiated PC12 cell viability is considerably lower (~45%) than SH-SY5Y cell viability (~90%). In order to differentiate PC12 cells, they were maintained in culture for 10-14 days prior to Mn treatment, whereas SH-SY5Y cells were maintained for only 48 hours before Mn treatment. The methodology used to prepare each cell line for Mn treatment may, in part, explain the differences in overall cell viability. Differentiated PC12 cell viability decreased approximately 10% after cells were grown in culture for 48 hours following treatment compared to 36 hours, but did not appear to decrease further over time. In response to Mn treatment, 600 μM of Mn was sufficient to elicit a significant decrease in differentiated PC12 cell viability compared to control-treated cells.

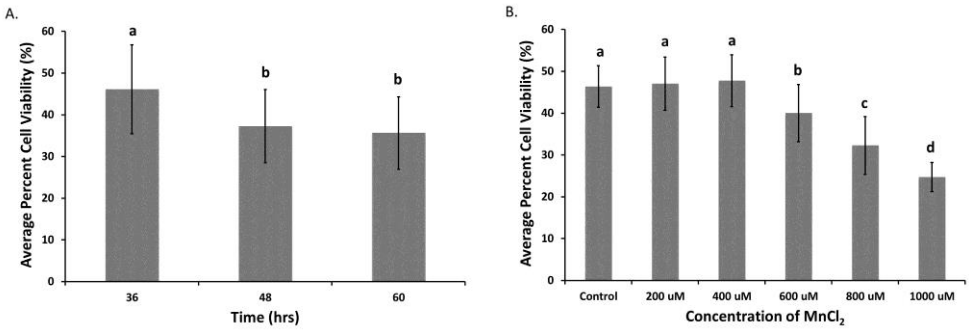

Figure 5. Effect of time or Mn treatment on differentiated PC12 cell viability.

A. average percent cell viability of differentiated PC12 cells in response to time and B. average percent cell viability of differentiated PC12 cells in response to Mn treatment. Error bars represent standard deviation of the mean. Where lower case letters are the same, the average percent cell viability (%) does not differ significantly.

4. Conclusions

Mn treatment of SH-SY5Y dopaminergic cells leads to significant decreases in the concentration of extracellular DA, 5HT and 5HIAA. Extracellular concentrations of monoamine neurotransmitters did not appear to decline in a dose responsive manner to Mn. The presence or absence of Mn was sufficient to describe the response. The extracellular concentration of DA around SH-SY5Y cells was also found to increase over time, but at a decreasing rate. The extracellular DA concentration's response to time might be explained by a decrease in the number of viable SH-SY5Y cells, a decrease in the rate of DA produced in or secreted from SH-SY5Y cells or a combination of these factors. SH-SY5Y and differentiated PC12 cell viability both decreased in response to Mn. SH-SY5Y cell viability is affected by an interaction between time and Mn treatment. Thus, time is important to consider when interpreting the effect of Mn concentration on SH-SY5Y cell viability. For differentiated PC12 cells, both time and Mn treatment predict viability, independently of each other. Differentiated PC12 cell viability declines in response to time, but the magnitude of the effect differs depending on amount of Mn. Together, these results suggest that the effect of Mn treatment on dopaminergic cell viability may be more complicated than the effect of Mn treatment on extracellular concentrations of monoamine neurotransmitters. Future studies are necessary to explain the different responses to Mn treatment observed between these two dopaminergic cell lines.

Treatment of SH-SY5Y cells with at least 200 μM of Mn was sufficient to elicit decreases in the relative concentrations of extracellular DA, 5HT and 5HIAA, but this same concentration of Mn was not sufficient to decrease SH-SY5Y cell viability. These findings suggest that a decrease in the concentration of extracellular monoamine neurotransmitters surrounding SH-SY5Y cells is not sufficient to elicit a decrease in cell viability.

In conclusion, these studies demonstrated that 1) Mn treatment of SH-SY5Y dopaminergic cells results in decreased extracellular concentrations of DA, 5HT and 5HIAA and 2) Mn treatment results in decreased dopaminergic cell viability, though decreases in the extracellular concentration of monoamine neurotransmitters are not sufficient to elicit decreases in the viability of SH-SY5Y cells. Future studies will explore the mechanism(s)

through which Mn treatment alters the extracellular concentrations of DA, 5HT and 5HIAA and dopaminergic cell viability. Understanding these mechanisms may provide insight into how Mn exposure may lead to the loss of normal neurochemical signaling, which can result in manganism or idiopathic Parkinson's disease.

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