The Role of Dopamine Transporter in Selective Toxicity of Manganese and Rotenone

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Abstract: The dopamine transporter has been shown to be the most relevant target site for the specificity of 1-methyl-4-phenylpyridinium ion (MPP^+), a neurotoxin for dopaminergic neurons. In contrast, the mechanisms underlying the selective toxicity of manganese and rotenone, potentially toxic agents implicated in dopaminergic neuronal cell death, remain unknown. The aim of this study was to determine the cellular mechanisms of manganese or rotenone uptake in dopaminergic cells via the dopamine transporter. PC12 cells overexpressing the dopamine transporter, which were exposed to 10 μM MPP^+, showed extensive DNA fragmentation, a biochemical hallmark of apoptosis, whereas wild type PC12 cells or vector-transfected PC12 cells, which were exposed to 5 mM MPP^+, did not show DNA fragmentation. In contrast, manganese and rotenone induced DNA fragmentation at slightly lower concentrations in PC12 cells overexpressing the dopamine transporter compared to control cells. Dopamine transporter inhibitors, such as mazindol, nomifensine, or GBR12909, inhibited MPP^+-induced DNA fragmentation but did not affect manganese- and rotenone-induced DNA fragmentation in PC12 cells overexpressing the dopamine transporter. Finally, manganese accumulated to similar levels in PC12 cells overexpressing the dopamine transporter and control PC12 cells following incubation with manganese chloride. These results suggested that the dopamine transporter dose not confer cytotoxicity to manganese and rotenone.

Key words: dopamine transporter; manganese; rotenone; MPP^+; apoptosis

Abbreviations**:** DAT, dopamine transporter; DMT1, divalent metal transporter-1; ICP-AES, inductively coupled plasma atomic emission spectroscopy; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

1. Introduction

Epidemiological studies have suggested that environmental factors play an important role in the pathogenesis of Parkinson's disease and other related disorders. These include trace metals, pesticides, herbicides, and industrial chemicals (Veldman et al. 1998). Manganese is an essential trace metal for numerous enzymes important in normal cellular function in the central nervous system, including glutamine synthetase, calmodulin-dependent phosphatase, and mitochondrial superoxide dismutase (Prohaska 1987). However, manganese is also neurotoxic at high concentrations. The adverse effects of inorganic manganese dust or vapor among steel manufacturing workers or welders are known, as well as the health risks of exposure to organic manganese compounds including the pesticide Mn ethylenebis (Thiruchelvam et al. 2000; Zhang et al. 2003) and the antiknock agent methylcyclopentadienyl Mn tricarbonyl in unleaded gasoline (Kitazawa et al. 2002). In humans, chronic manganese poisoning is known to produce extrapyramidal symptoms resembling Parkinson's syndrome. (Mena et al. 1967; Cook et al. 1974; Barbeau 1984). Pathological evidence showed that the pallidum, caudate nucleus and putamen are damaged in manganese encephalopathy (Barbeau 1984; Yamada et al. 1986). In experimental animals, manganese is selectively neurotoxic to pathways intrinsic to the basal ganglia. Dopamine levels decreased in the basal ganglia of monkeys intoxicated with manganese (Bird et al. 1984; Eriksson et al. 1992) and in rat striatum directly injected with manganese (Lista et al. 1986; Brouillet et al. 1993). In cultured cells, manganese induces apoptosis, as judged by changes in cell morphology, caspase-3 activation, and DNA fragmentation/TUNEL staining (Desole

et al. 1996; Hirata et al. 1998; Schrantz et al. 1999; Oubrahim et al. 2001). Apoptosis is recognized as a cell death process in several neurodegenerative diseases including Parkinson's disease (Honig and Rosenberg 2000). In addition, the recent finding that manganese may co-operate with a-synuclein in triggering neuronal cell death highlights its relevance as a risk factor for Parkinson's disease (Pifl et al. 2004).

Rotenone, a common pesticide and an inhibitor of complex I of the mitochondrial respiratory chain, induces neuropathological and behavioral symptoms similar to Parkinson's disease in rats (Betarbet et al. 2000). The inhibition of complex I may be implicated in the degeneration of nigrostriatal neurons. This is the proposed mechanism of action of MPP^+, a toxic metabolite to dopaminergic neurons of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an agent widely used as a Parkinson's disease model (Ramsay et al. 1986; Nicklas et al. 1987). The selectivity of MPP^+ for dopaminergic neurons is explained by its selective uptake via dopamine transporter (DAT), which terminates the synaptic actions of dopamine (Javitch et al. 1985; Pifl et al. 1993; Gainetdinov et al. 1997). On the other hand, the selectivity of manganese and rotenone for dopaminergic neurons is not fully understood. Transport of manganese into dopaminergic neurons is controversial. Previous studies have demonstrated the possible role of dopamine uptake mechanisms in manganese transport (Ingersoll et al. 1999; Anderson et al. 2007). In contrast, immunochemical characterization of DAT showed that DAT immunoreactivity was less concentrated in the globus pallidus, a region particularly vulnerable to manganese toxicity, compared to the neostriatum (Ciliax et al. 1995).

On the other hand, as rotenone can slip through any cell membrane, a specific transport system may not be involved in its selectivity for dopaminergic neurons. The present study was done to clarify whether DAT is directly involved in the relative selectivity of manganese and rotenone for dopaminergic neurons using PC12 cells overexpressing DAT.

2. Materials and methods

2.1. Materials

Mazindol, nomifensine, and GBR12909 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rat monoclonal anti-DAT antibody was obtained from Chemicon International (Temecula, CA, USA) and mouse monoclonal anti-actin antibody was obtained from Oncogene Research Products Calbiochem-Novabiochem (La Jolla, CA). Tfx-50 reagent was obtained from Promega Corporation (Madison, WI, USA). MPP^+ was prepared by reacting methyl iodide with 4-phenylpyridine in dichloromethane at room temperature for 24 h (Hirata and Nagatsu 1985).

2.2. Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 7% horse serum (BioWhittaker, Inc., Walkersville, MD, USA) and 4% fetal bovine serum (Biofluids, Inc., Rockville, MD, USA) at 37°C in 5% CO_2. Cells were exposed to various chemicals at the indicated concentrations for 20 h.

2.3. Cell line transfection

The human DAT cDNA (Giros et al. 1992) was subcloned into pRc/CMV (Invitrogen Corporation). PC12 cells were transfected either with pRc/CMV or human DAT subcloned into pRc/CMV using Tfx-50 reagent (Promega) according to the manufacturer's instructions. The day after transfection, cells were selected using 0.8 mg/ml G418 (Invitrogen Corporation, Carlsbad, CA, USA). Surviving cell colonies were screened by Western blotting for DAT immunoreactivity. The cloned cell lines were maintained in PC12 medium supplemented with 0.4 mg/ml G418. Two clones with DAT immunoreactivity (DAT10 and DAT12) were used in the experiments.

2.4. DNA fragmentation

Cells (~ 2 x 10^7 cells) were incubated at 37°C for the indicated periods. After treatment, cells were collected and re-suspended in lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 0.5% Triton X-100). The lysates were centrifuged at 14,000 x g for 10 min and the soluble DNA was isolated and extracted with Tris/EDTA (TE)-saturated phenol and phenol/chloroform (1:1) followed by ethanol precipitation. DNA was dissolved in TE buffer containing RNase A (50 µg/ml) and incubated at 37°C for 1 h. Approximately half of the recovered soluble DNA per condition was separated by electrophoresis in 1.2% agarose gels and visualized with an UV transilluminator.

2.5. Western blotting

Cells were lysed in sodium dodecyl sulfate (SDS)-Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol) and sonicated for ~20 s. Total cell lysates (40 μg protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare UK Ltd., Buckinghamshire, England). Membranes were blocked with phosphate-buffered saline with 0.05% Tween 20 containing 5% non-fat dry milk (Snow Brand, Tokyo, Japan) for 60 min at room temperature. Membranes were probed with the anti-DAT antibody at the dilutions recommended by the manufacturer overnight at 4°C and subsequently incubated with peroxidase-conjugated secondary antibodies for 90 min at room temperature. Immunoblots were developed in enhanced chemiluminescence reagent (GE Healthcare UK Ltd.) and exposed to x-ray film. Blots were re-probed with the anti-actin antibody after stripping in 62.5 mM Tris-HCl (pH 6.7)/100 mM β-mercaptoethanol/2% SDS at 55°C for 30 min.

2.6. Manganese analysis

For the analysis of manganese content, approximately 40 mg of cultured cells were digested in 5 ml of 7 M HNO_3 in a heating block at 90°C for 2 h. The levels of manganese were determined with inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Leeman Labs, Inc., Hudson, NH, USA).

3. Results

To study the uptake of manganese via DAT in neuroendocrine cell types, we generated a PC12 cell line in which the human DAT cDNA was stably transfected

(DAT10- and DAT12-PC12 cells). DAT expression was strictly dependent on transfection with human DAT cDNA and was not detected in either non-transfected or vector-transfected PC12 cells (Fig. 1A). Expressed DAT migrated as diffuse broad bands by SDS-PAGE, indicating that DAT was highly glycosylated as previously suggested (Lew et al. 1991). In fact, a marked decrease in band width and change in migration position were observed after treatment with N-glycosidase F (data not shown). Next, the experiment is designed to verify exogenous DAT function. Thus, we examined the cytotoxic effect of MPP^+ on PC12 cells overexpressing DAT. As shown in Fig. 1B, MPP^+ up to 5 mM did not cause DNA fragmentation, a biochemical hallmark of apoptosis, in PC12 cells after 24 h treatment. On the other hand, MPP^+ caused extensive DNA fragmentation at 50 μM in PC12 cells overexpressing DAT (DAT10). The other clone, DAT12-PC12, showed similar results (data not shown). As MPP^+ is taken up via DAT, these results suggested that stably expressed DAT is functional and confers cytotoxicity to MPP^+ in PC12 cells. In contrast to the effect of MPP^+, staurosporine, a relatively non-selective protein kinase inhibitor, was used as a general inducer of apoptosis, which causes DNA fragmentation to a similar extent in PC 12 cells overexpressing DAT and control PC12 cells (Fig. 1C). This result also demonstrated that the sensitivity difference to MPP^+ can be attributed to the expression of DAT rather than clonal differences.

Next, we examined the effects of manganese and rotenone, which are potential neurotoxins for dopaminergic neurons, in DAT-PC12 cells. Manganese induced DNA fragmentation at lower concentrations in DAT-PC12 cells compared with

control PC12 cells. As shown in Figure 2A, manganese at 50 μM caused DNA fragmentation in DAT-PC12 cells. In contrast, it did not cause DNA fragmentation at all in wild-type PC12 cells. Similarly, rotenone caused DNA fragmentation at slightly lower concentrations in DAT-PC12 cells compared with control PC12 cells. To verify whether DAT was involved in the uptake of manganese and rotenone, we examined the effect of DAT inhibitors on manganese- and rotenone-induced DNA fragmentation. We used mazindol, nomifensine and GBR12909 to block DAT. Mazindol and nomifensine at 10-20 μM did not prevent manganese- and rotenone-induced DNA fragmentation (Fig. 3AB), whereas they did prevent MPP^+-induced DNA fragmentation (Fig. 3B). Furthermore, GBR12909 also did not block, rather it further exacerbated, manganese- and rotenone-induced DNA fragmentation (Fig. 3C). These results demonstrated that manganese and rotenone are not taken up into PC 12 cells via DAT.

Finally, we analyzed the accumulation of manganese in PC12 cells and DAT-PC12 cells using ICP-AES. The endogenous manganese content of control PC12 cells and DAT-PC12 cells was less than 18 pmol per mg wet weight. When the cells were incubated with 0.5 mM manganese for 20 h, cellular contents of manganese increased to 2.76 and 2.46 nmol per mg wet weight in control PC12 cells and DAT-PC12 cells, respectively (Fig. 4). This result confirms that DAT is not involved in selective uptake of manganese.

4. Discussion

The mechanism of the relative specificity of manganese for dopaminergic

neurons is of particular interest since the dopamine rich regions in the basal ganglia, such as the globus pallidus and caudate, are affected during manganese toxicity. The specific cytotoxicity for dopaminergic neurons of MPP^+, a toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an agent widely used in Parkinson's disease models, can be explained by the selective uptake of MPP^+ via DAT (Pifl et al. 1993; Gainetdinov et al. 1997). Immunochemical studies of the localization of DAT in the brain showed that the striatum was densely labeled for DAT and also richly innervated with mesencephalic dopaminergic axon terminals. In contrast, regions with less concentrated labeling (e.g., globus pallidus, entopeduncular nucleus, and medial forebrain bundle) had less dopaminergic innervation (Ciliax et al. 1995). As the globus pallidus is the most affected region in manganese toxicity (Barbeau 1984; Yamada et al. 1986), DAT does not seem to be responsible for the selectivity of manganese. On the other hand, Ingersoll et al. reported that cocaine decreased manganese accumulation in the rat brain after intrathecal administration of manganese, suggesting that the manganese concentration in the brain is related to dopamine reuptake (Ingersoll et al. 1999). Furthermore, it has been recently reported that inhibition of DAT function by GBR12909, a specific DAT inhibitor, significantly decreased manganese accumulation in the globus pallidus *in vivo* and in the striatal synaptosomes *in vitro* (Anderson et al. 2007).

Chronic and low dose delivery of rotenone causes selective degeneration of nigrostriatal dopaminergic neurons, the formation of intraneuronal fibrillar inclusions containing ubiquitin and α-synuclein, and the development of a

behavioral phenotype characterized by hypokinesia, hunched posture, and severe rigidity (Betarbet et al. 2000; Alam and Schmidt 2002). Rotenone is a lipophilic chemical that should diffuse evenly throughout the brain and has been found to cause a uniform inhibition of complex I in the rat brain after systemic administration (Betarbet et al. 2000). Thus, its ability to target dopaminergic neurons cannot be explained by its selective uptake by these cells, as is the case for MPP^+. However, the reason why it specifically damages dopaminergic neurons *in vivo* is not fully understood.

In this paper, we attempted to elucidate the possible cause of the selective toxicity of manganese and rotenone by investigating the direct involvement of DAT in their cytotoxicity. Transfection of human DAT cDNA lead to successful expression of DAT protein that was highly glycosylated, as evidenced by the number of broad bands that appeared on the Western blot. The response of DAT-PC12 cells to MPP^+ was 100 times more sensitive than that of wild-type PC12 cells or vector-transfected PC12 cells. However, DAT expression did not affect the cytotoxicity of staurosporine, a general inducer of apoptosis. These results confirm that DAT is responsible for the uptake of MPP^+ and that PC12 cells expressing the endogenous norepinephrine transporter are relatively resistant to MPP^+ toxicity (Bruss et al. 1997). Manganese and rotenone also induced DNA fragmentation in DAT-PC12 cells at relatively lower concentrations than in parental PC12 cells. However, DAT inhibitors, such as mazindol, nomifensine, or GBR12909, inhibited MPP^+-induced DNA fragmentation but did not affect manganese- and rotenone-induced DNA fragmentation in DAT-PC12 cells. Our data clearly

demonstrate that DAT is not responsible for the cytotoxicity of manganese and rotenone.

Analysis of manganese contents after incubation with PC12 cells showed that the cells accumulated a large amount of manganese. However, DAT expression did not affect the accumulation of manganese in PC12 cells, indicating that DAT is not directly involved in manganese transport. There are several mechanisms that may be responsible for manganese uptake into cells. These include the calcium channel, the divalent metal transporter 1 (DMT1) and the transferrin-dependent mechanism. Our results also showed that manganese transport was not affected by interactions of the DAT protein with these putative manganese transporters. Accumulating evidence indicates that a physiological role for the transport of manganese both by transfferin receptors and DMT-1. Roth et al. have suggested that DMT-1 has a relatively high affinity for manganese and is the major transport proteins responsible for the uptake of manganese into PC12 cells (Roth et al. 2002). However, the reason why the globus pallidus is the target for manganese toxicity cannot be attributed to DMT1 because the expression of DMT1 in the globus pallidus is not high compared to that in the caudate nucleus, putamen, and the substantia nigra pars reticulata in the basal ganglia of monkeys (Huang et al. 2004). Aschner et al. have pointed out that the distribution of transfferin receptors in relationship to brain manganese accumulation is noteworthy. The fact that manganese-accumulating areas in the brain are efferent to areas of high transferrin receptor density suggests that these sites may accumulate manganese through neuronal transport (Aschner et al. 2007). Although a physiological importance of transferrin receptors and DMT-1 in manganese

transport in the brain is not fully understood, DAT does not play a significant role in the selectivity of manganese and rotenone to dopamine-rich cells.

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Legends

Fig.1. Stable human DAT expression in DAT-PC12 cells. (A) Western blot of total cell lysates (40 μg/lane) from PC12 cells or stably transfected with either vector (PC12-V) or human DAT (PC12-DAT clone 10 and 12) cDNAs. The same blot was re-probed with anti-actin antibody and actin protein level served as a loading control. (B) MPP^+ induced extensive DNA fragmentation in PC12 cells overexpressing DAT (DAT10), but not in control PC12 cells. (C) Staurosporine induced internucleosomal cleavage of DNA both in DAT-PC12 cells and control PC12 cells. Control PC12 cells and PC12 cells overexpressing DAT (DAT10) were cultured in the presence of indicated concentrations of MPP^+ or staurosporine for 20 h in regular medium. The soluble DNA was isolated and analyzed as described in Materials and Methods.

Fig. 2. Effects of manganese and rotenone on DNA fragmentation in PC12 cells and DAT-PC12 cells. Control PC12 cells and PC12 cells overexpressing DAT (DAT12) were cultured in the presence of indicated concentrations of manganese or rotenone for 20 h in regular medium. The soluble DNA was isolated and analyzed as described in Materials and Methods.

Fig. 3. DAT inhibitors prevented MPP^+-induced DNA fragmentation but not manganese- and rotenone-induced DNA fragmentation in DAT-PC12 cells. (A) PC12 cells overexpressing DAT (DAT12) were incubated with 50 μM MPP^+, 1 μM rotenone, and 100 μM manganese in the presence or absence of mazindol for 20 h in

regular medium. (B) PC12 cells overexpressing DAT (DAT12) were incubated with 50 μM MPP^+, 100 μM manganese, and 1 μM rotenone in the presence or absence of 10 μM nomifensine for 20 h in regular medium. (C) PC12 cells overexpressing DAT (DAT12) were incubated with 500 μM MPP^+, 100 μM manganese, and 1 μM rotenone in the presence or absence of 5 μM GB12090 for 20 h in regular medium. The soluble DNA was isolated and analyzed as described in Materials and Methods.

Fig. 4. Manganese was accumulated similarly in control PC12 cells and in PC12 cells overexpressing DAT (DAT12). The cells were treated with 0.5 mM $MnCl_2$ in the regular medium for 20 hours. The cells were washed three times with phosphate-buffered saline and digested in HNO_3. Manganese level was determined by ICP-AES as described in Materials and Methods. Data are the mean ± SD (n=3).

Figure
Click here to download Figure: DATMn Fig.1-4.pdf

Fig. 1A

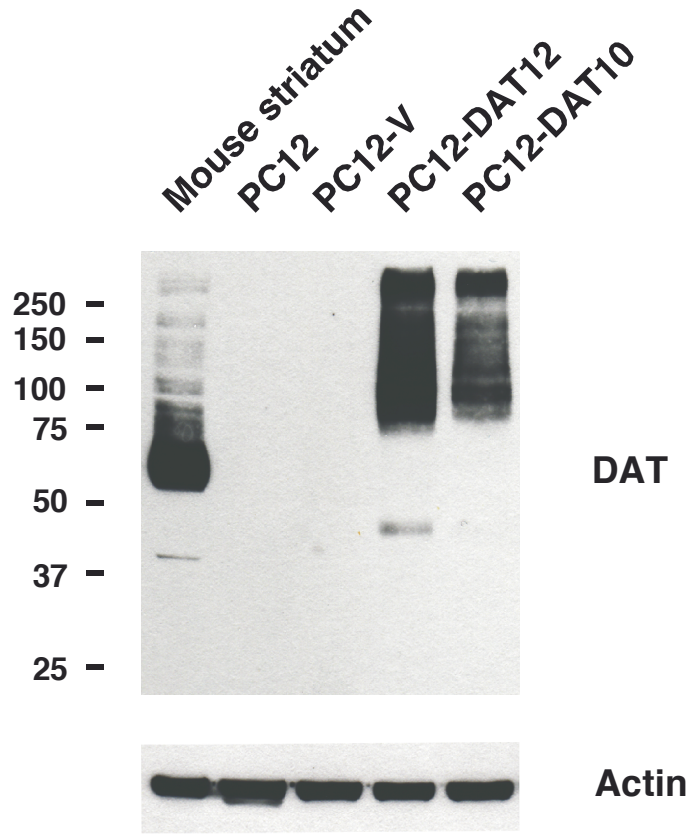


Fig. 1B

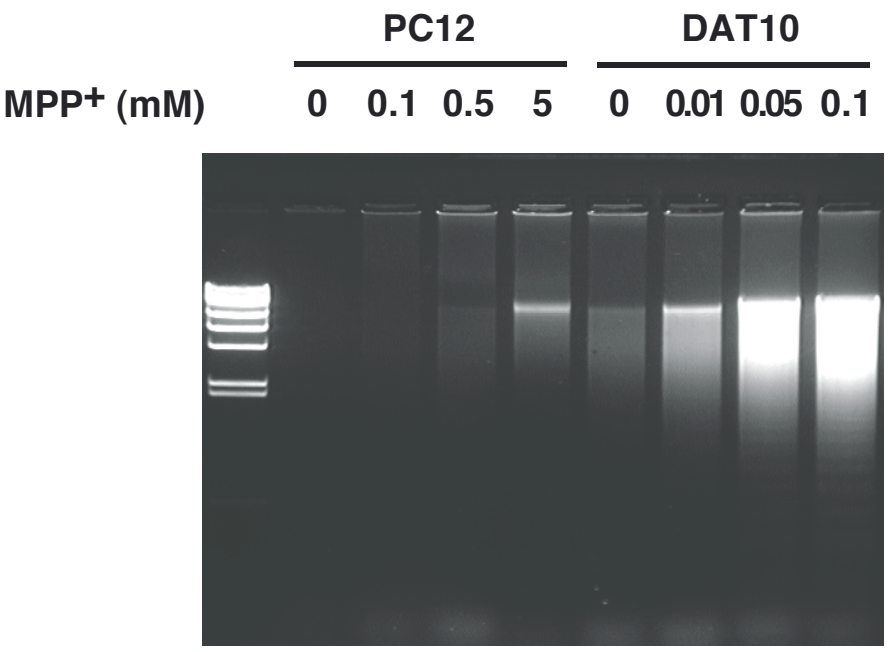


Fig. 1C

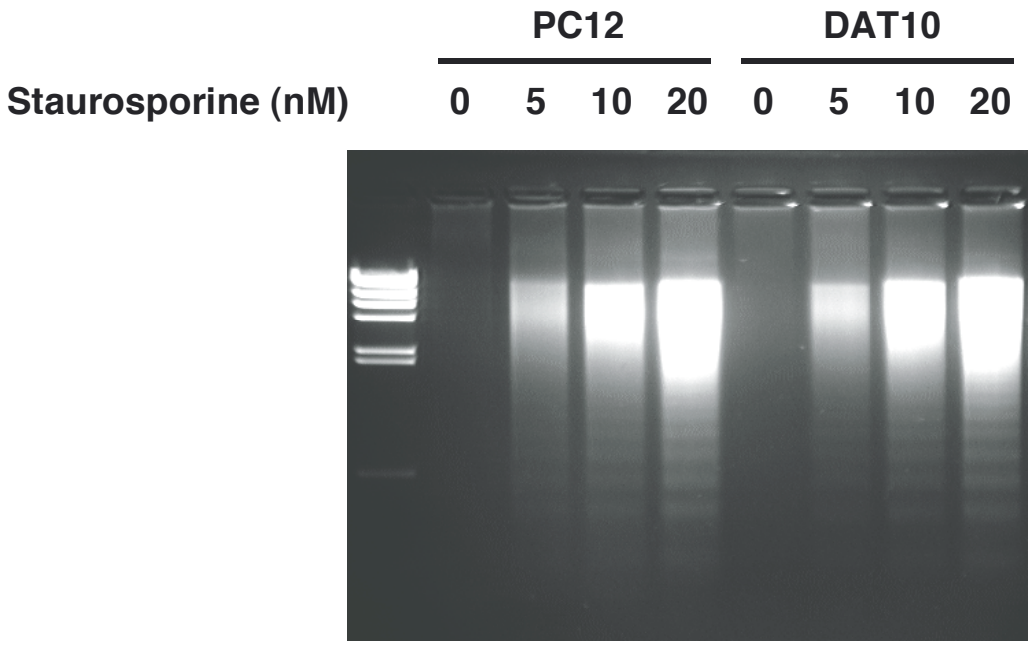
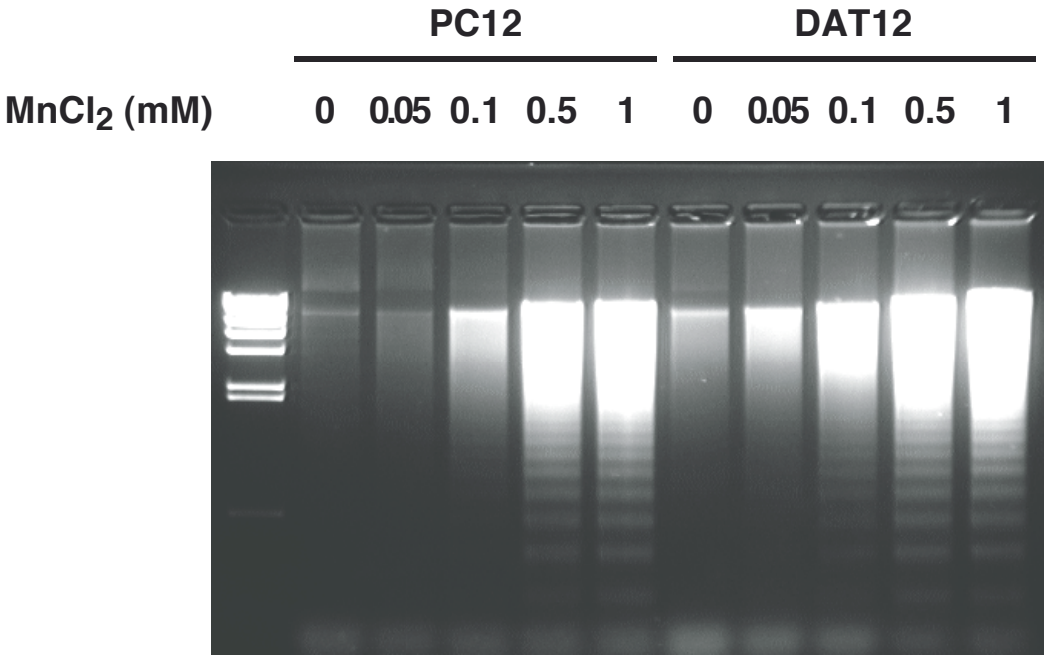


Fig. 2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DAT12
$MnCl_2$ (mM)
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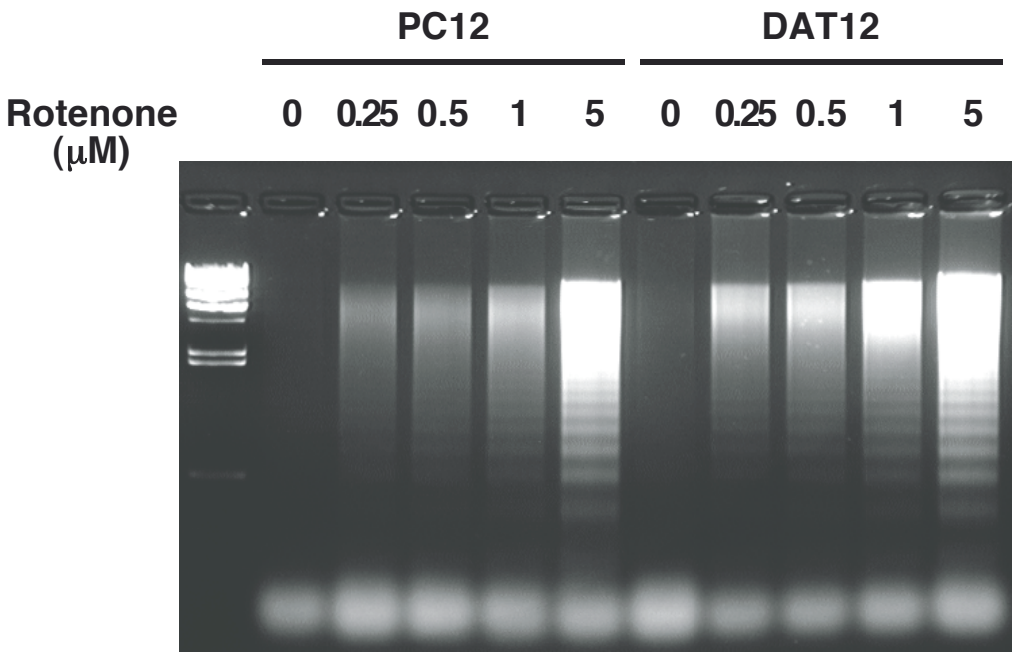
PC12
DAT12
Rotenone (μM)
0 0.25 0.5 1 5 0 0.25 0.5 1 5

Fig. 3A

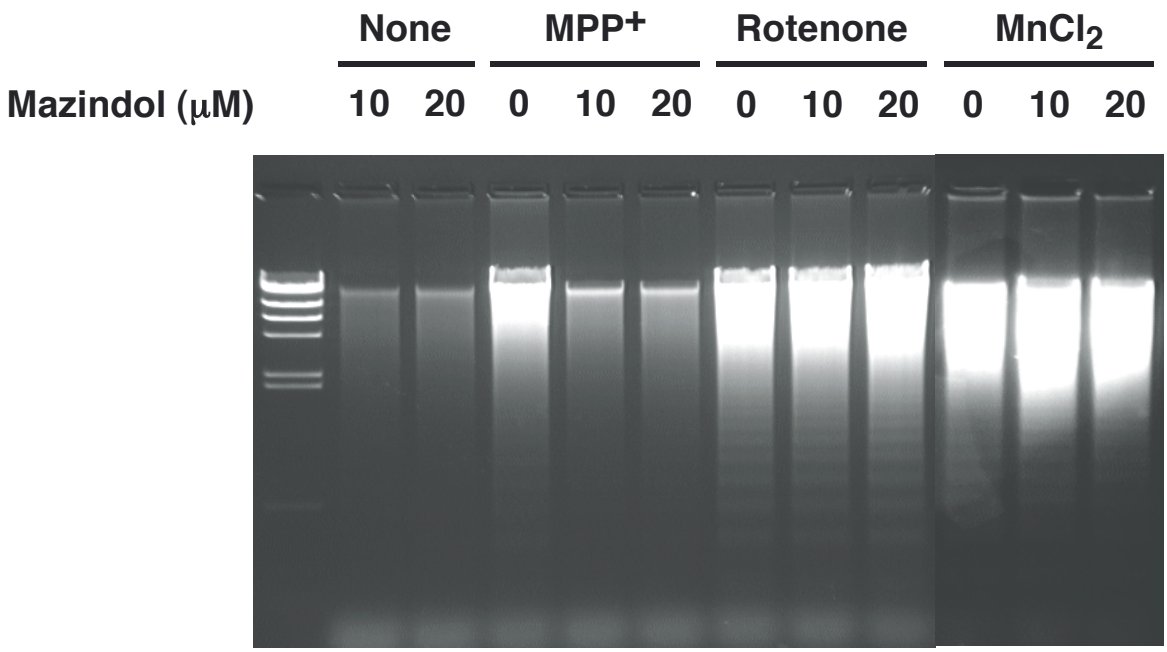

Fig. 3B

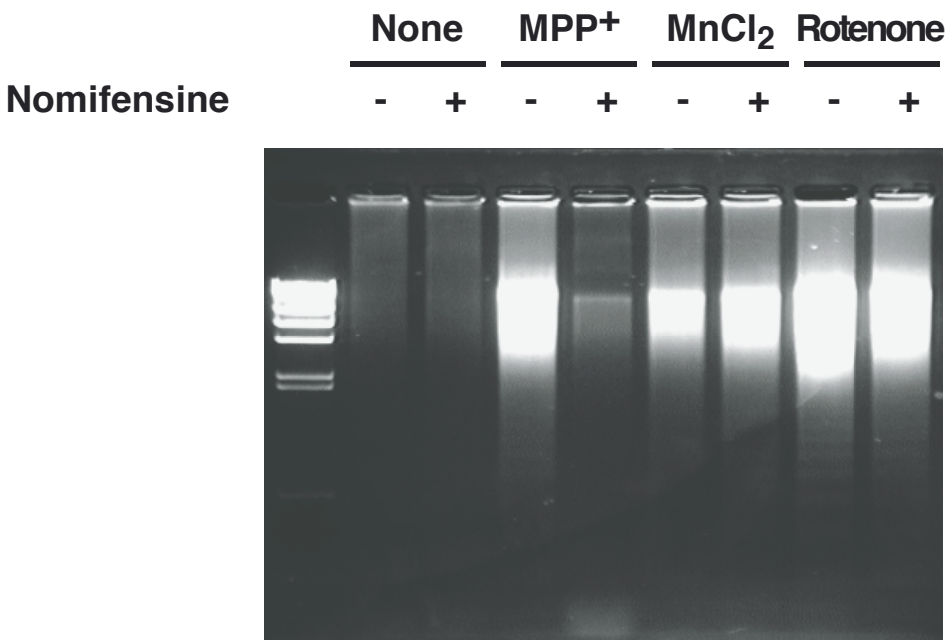

Fig. 3C

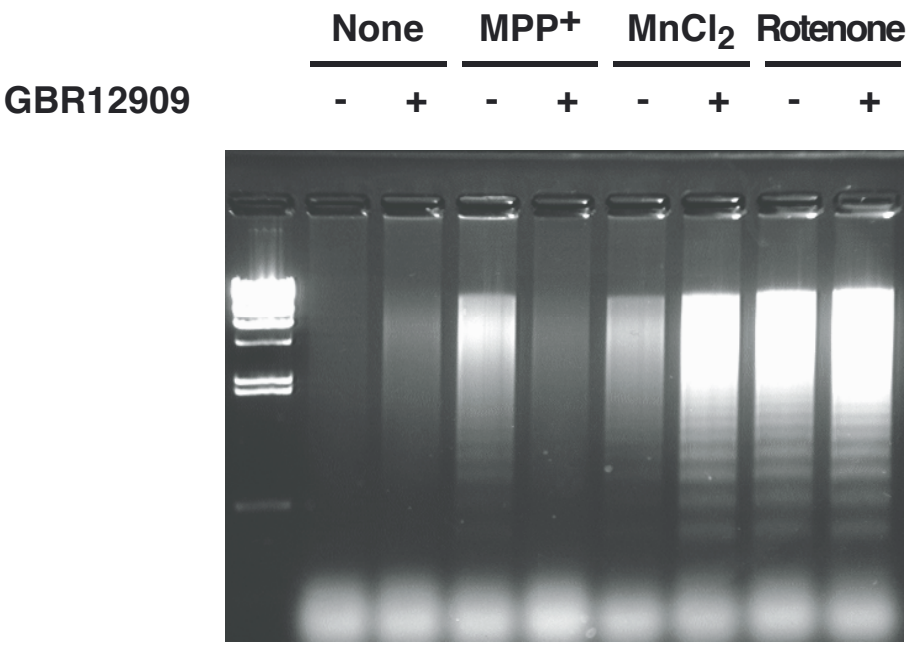

Fig. 4