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REGULAR ARTICLE

Toxic effects of nicotinamide methylation on mouse brain striatum neuronal cells and its relation to manganese

Yayoi Mori · Akiko Sugawara · Masayoshi Tsuji · Takeyasu Kakamu · Satoshi Tsuboi · Hideyuki Kanda · Takehito Hayakawa · Tetsuhito Fukushima

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Abstract
Objective It is well known that manganese (Mn) exposure is involved in parkinsonism. The aim of our study was to test the hypotheses that Mn affects nicotinamide *N*-methyltransferase (NNMT) activity, increases the metabolism of nicotinamide (NA) to 1-methylnicotinamide (MNA), and leads to neurocytotoxicity.
Methods Following demonstration of the effects of Mn concentrations on the survival rate of Mouse CD1 brain striatum neuronal cells (MS cells), the effect of Mn on NNMT activity was investigated by comparing the difference in the amount of MNA produced after various Mn concentrations were added to mouse brain cytosol fractions as an enzyme solution. Toxicity induced by MNA and its precursor NA on MS cells was measured.
Results The survival rate of MS cells decreased significantly with increasing concentrations of Mn in the culture medium. With respect to the influence of Mn on NNMT activity, NNMT activity increased significantly at Mn concentrations of 1 μmol/mg protein. MNA and NA neurotoxicity were compared by comparing cell survival rate. Cell survival rate dropped significantly when the cells were cultivated with 10 mM of MNA. There was also a tendency for the survival rate to fall following the addition of 10 mM NA; however, the difference with the control was not significant.
Conclusions Our study suggests the possibility that Mn causes increased NNMT activity, thereby increasing MNA levels in the brain and bringing about neuron death. Daily absorption of Mn and NA may thus contribute to idiopathic Parkinson's disease.

Keywords Parkinson's disease · Nicotinamide *N*-methyltransferase · Manganese · Mouse CD1 brain striatum neuronal cells · Cytotoxicity

Y. Mori (✉) · A. Sugawara · M. Tsuji · T. Kakamu · S. Tsuboi · H. Kanda · T. Hayakawa · T. Fukushima
Department of Hygiene and Preventive Medicine, Fukushima Medical University School of Medicine, Hikarigaoka 1, Fukushima 960-1295, Japan
e-mail: y-mori@fmu.ac.jp

Introduction

Parkinson's disease (PD) is a neurogenerative disorder with a risk of onset that increases with age [1]. The prevalence rate of PD among the general population of Japan has been reported to be about 150 per 100,000 people [2], while the prevalence rate in Western countries is about 300 per 100,000 people [3]. The search for the cause of PD is a critical social issue as the number of patients have been steadily increasing concurrently with the rapid aging of the Japanese population.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a substance that artificially induces parkinsonism [4]. A number of other exogenous and endogenous substances are also considered to be causative agents of PD, including beta-carboline [5] and or tetrahydroisoquinoline [6, 7]. One substance that has been associated to idiopathic Parkinson's disease (IPD) is 1-methylnicotinamide (MNA), a metabolite of nicotinamide (NA) by methylation in vivo [8–10]. The toxicity of MNA in cultured neuroblastoma cells has also been reported [11], and the ingestion of high quantities of NA, the precursor of MNA, has been suggested to play a role in PD pathogenesis in developed countries [12].

The metabolism of NA to MNA is mediated by nicotinamide *N*-methyltransferase (EC 2.1.1.1) (NNMT).

Differences in in vivo NNMT activity are believed to be negatively associated with gene polymorphism and environmental influences are also considered to be significant [13]. It has been suggested that NNMT activity is affected by the presence of metal ions, such as manganese (Mn; [5, 14]). The link between Mn poisoning and the development of parkinsonism is well known, [15], but it is also suspected that chronic exposure to Mn plays a role in IPD [16, 17]. In an earlier study, we demonstrated the effects of heavy metals such as Mn on NNMT activity and suggested the possibility NNMT activation being involved in the pathogenesis of heavy metal-induced IPD [14]. Although, contrary to our hypothesis, we found no metal-increased NNMT activity, NNMT activity was significantly decreased by copper (Cu^{2+}) and cadmium (Cd^{2+}), while Mn^{2+} and iron (Fe^{2+}) had no effect on NNMT activity. These results indicated that further study was needed to clarify why the effects of Mn^{2+} and Fe^{2+}, which play a major role in PD, on NNMT activity differ from those of other heavy metals. One possibility was that the amount of dithiothreitol (DTT) used in our experiments, 0.125 mM, was insufficient to protect the –SH groups, leading to the observed inhibitory effect of Mn^{2+} on NNMT.

In the study reported here, we performed three experiments with high concentrations of DTT and relatively low concentrations of Mn to verify our hypotheses that Mn brings about a increase in NNMT activity which in turn generates higher concentrations of MNA, subsequently causing neuron death. In the first, the effects of Mn concentrations on the survival rate of mouse CD1 brain striatum neuronal cells (MS cells) were demonstrated. In the second, we observed the effect of different Mn concentrations on NNMT activity, using the mouse brain cytosol fraction as the enzyme solution. In the third we compared differences between the effects of MNA and NA concentrations on the survival rate of MS cells.

Materials and methods

Mn-induced neuron death

Mouse CD1 brain striatum neuronal cells (MS cells) were obtained from Lonza Walkersville (Walkersville, MD). As recommended for the culture of MS cells, we used Neurobasal Medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. B-27 supplement (Invitrogen) which was a commercial culture supplement, including the antioxidant [vitamin E, super oxide dismutase (SOD), catalase, and glutathione] were added to the Neurobasal Medium. MS cells were cultivated in poly-D-lysine-coated 96-well plates, with 8×10^4 cells and 200 μl culture medium in each well. The medium in each well was exchanged 2 h after initiation of the cultivation, and half of the medium in each well was replaced on cultivation day 7. In the experiment designed to observe the influence of Mn, Mn was added to each well on cultivation day 7 to achieve a final Mn concentration of 0 (control), 0.1, 1, 10 and 100 μM, respectively, in a total volume of 200 μl. Six wells at each Mn concentration were cultivated. Mn concentrations were adjusted with $MnCl_2$, and the control (nothing added) was set at 0 Mn. Following the addition of Mn to the wells and ensuring that the concentrations of Mn were maintained, we replaced half of the medium in each well at 3-day intervals. Cell survival rate was compared using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay after 19 days as follows. First, half of the medium in each well was removed, 0.5% MTT reagent (10 μl) in phosphate buffer solution (PBS) was added to each well, and the color reaction was observed for 4 h. Thereafter, acid-isopropyl alcohol (40 mM HCl in isopropyl alcohol) was added to dissolve the dye. The absorbance determination was measured at a wavelength of 570 nm using a microplate reader (model 550; Bio-Rad, Hercules, CA). The control (0 μM Mn) was set at a light absorption of 100%, and survival rates were calculated by comparison of the absorbance at each concentration of Mn.

On cultivation day 19, half of the removed medium was mixed with each Mn concentration, and the concentration of MNA in the medium was measured. As an internal standard, 1-ethylnicotinamide was added, and the media were centrifuged at 4,000 *g* for 10 min. For the fluorometric assay of MNA, an aliquot (400 μl) of the supernatant, 100 μl of 1 M isonicotinamide, and 200 μl of 100 mM acetophenone in ethanol were incubated together for 10 min in a microtube that was placed in an ice-water bath. A 400-μl aliquot of 6 M NaOH was added, and the mixtures were incubated in ice-water for 60 min. Following the addition of 200 μl of formic acid (99% concentration), the mixtures were incubated first for 15 min in ice-water and then for 5 min in a dry-bath. After cooling to room temperature, the samples were filtered through 0.45-μm PTFE membranes, and 50 μl of each filtered sample was injected into a high-performance liquid chromatography (HPLC) system (LC-2000 Plus Series; JASCO Corp, Easton, MD) equipped with an Inertsil ODS-3 V (5 μm) column (4.6 × 250 mm; GL Science, Torrance, CA), and an Inertsil ODS-3 (5 μm) cartridge guard column GL-CART (GL Science) was used for the HPLC system with a fluorescence detector. The mobile phase was acetonitrile–water–formic acid (16:80:2.5), the column temperature was 40°C, and the flow rate was 1.0 ml/min.

Internal standard was added to three concentrations of the MNA standard solution (0.1, 1, 10 μM), and each concentration was measured three times, after which a

calibration curve was plotted. Using the resultant formula, we calculated MNA production from the measured values of the reaction mixture. From there we computed produced MNA (μM) per survival rate (control 1).

The influence of Mn on NNMT activity

An enzyme solution from the mouse brain cytosol fraction was used to investigate the influence of Mn on NNMT activity. Fifteen retired male mice [Crlj:CD1 (ICR)] were purchased from Charles River Laboratories Japan (Yokohama, Japan) and killed by decapitation after being anesthetized with diethylether. Their brains were homogenized in four volumes of 5.0 mM PBS (pH 7.5), and the homogenates were centrifuged at 9,000 *g* for 20 min at 4°C. The protein concentration of the supernatant enzyme extract was measured and the supernatants stored at −40°C prior to assay. The enzyme assay and derivatization of MNA to a fluorescent substance were performed as described by Sugawara et al. [14], with some modifications. The reaction mixture contained the enzyme extract derived from the brain (13.8 mg/ml protein), 50 mM Tris–HCl buffer (pH 8.6), 5 mM DTT, 1 mM NA, and 1 mM *S*-adenosyl-L-methionine. Mn concentrations were adjusted with $MnCl_2$. The control concentration of Mn was the same as the physiological level in mouse brains (0.01 nmol/mg protein), maintained with added $MnCl_2$, which was set at 1; then at tenfold (0.1 nmol/mg protein), 1,000-fold times (10 nmol/mg protein), 10,000-fold (100 nmol/mg protein), and 100,000-fold (1 μmol/mg protein). Five samples of each concentration were measured. After a 40-min incubation at 37°C, the enzyme reaction was stopped with ethanol. As an internal standard, 1-ethylnicotinamide was added and the mixtures treated as described above for the fluorometric assay of MNA. From the value of measured MNA, we calculated the enzyme activity per 1 mg protein concentration and per hour. We compared enzyme activity at each concentration of added Mn.

The experiments presented in this paper were approved by the Animal Research Committee of Fukushima Medical University (No. 22059).

MNA and its precursor NA-induced neuron death

In this experiment, MS cells were cultured using the same method as described above up to day 7, when we replaced half of the medium in each well. To observe the influence of MNA, from day 7 onwards, MNA was added to achieve concentrations of 0 (control), 0.1, 1, and 10 mM in a total volume of 200 μl; six wells of each concentration were cultivated. To gauge the effect of NA, we added NA in the same way as just described to achieve concentrations of 0 (control), 0.1, 1, and 10 mM in a total volume of 200 μl; six wells of each concentration were cultivated. Following the addition of MNA and NA, respectively, to the wells and ensuring that the concentrations of MNA and NA were maintained, we replaced half of the medium in each well at 3-day intervals. Cell survival rate was compared using the MTT assay, as described above, after 19 days. The controls, 0 mM MNA and NA, respectively, were set at a light absorption of 100%, and survival rates were calculated by absorbance at each concentration of MNA and NA.

Statistical analysis

Data analyses were carried out using SPSS software (ver.17; SPSS, Chicago, IL). The results are presented as the mean as ± standard deviation (SD). For comparisons between groups, the Tukey–HSD test was used, with differences considered to be significant at $p < 0.05$ and $p < 0.01$.

Results

Changes in the survival rate of MS cells cultivated at each Mn concentration are shown in Fig. 1. The mean survival rates of MS cells in medium supplemented with 0, 0.1, 1, 10, and 100 μM Mn were 100 ± 16.24, 67.5 ± 8.33, 75.1 ± 8.97, 27.3 ± 8.84, and 7.8 ± 0.63%, respectively. The mean survival rate of MS cells decreased significantly

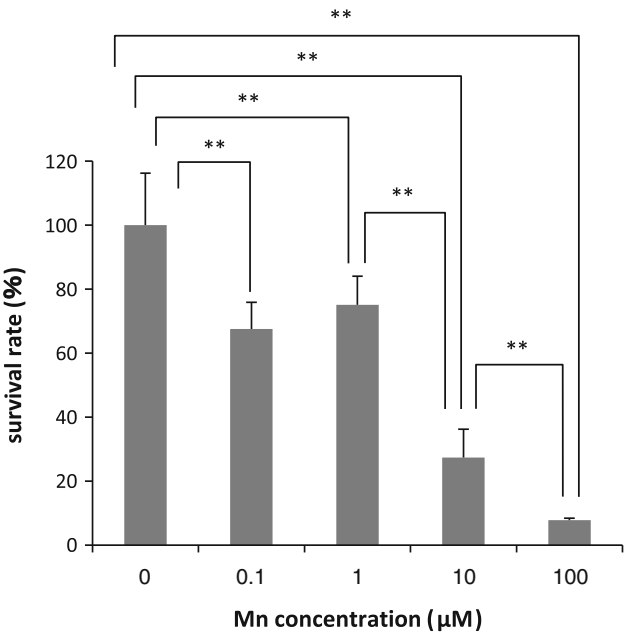


Fig. 1 The effect of manganese (Mn) on mouse CD1 brain striatum neuronal cell (MS cell) survival. Survival rate is shown as a percentage of the control (0 μM Mn). Data are presented as the mean ± standard deviation (SD; $n = 6$). *Double asterisks* Significant difference at $p < 0.01$ between indicated groups

with increasing concentrations of Mn in the culture medium.

The amount of MNA in medium supplemented with 0, 0.1, 1, 10, and 100 μM Mn, respectively on cultivation day 19 was 0.008, 0.030, 0.032, 0.086, and 0.091 μM/survival rate, respectively (Fig. 2). MNA production per survival rate increased with increasing concentrations of Mn in the culture medium.

The influence of Mn on NNMT activity is shown in Fig. 3. When the concentration of Mn was 0.01 (control), 0.1, 10, and 100 nmol/mg protein and 1 μmol/mg protein, the median of NNMT activity was 0.016 ± 0.002, 0.016 ± 0.003, 0.015 ± 0.002, 0.015 ± 0.001, and 0.020 ± 0.003 nmol/mg protein/h, respectively. Assessment of the changes in NNMT activity following the addition of Mn revealed that there was a significant difference among the groups: NNMT activity in culture medium with 1 μmol/mg protein Mn was significantly higher than that in culture media supplemented with 10 and 100 nmol/mg protein Mn, respectively.

The effect of MNA and NA on MS cell survival is shown in Fig. 4. When MNA was added to the culture medium and compared with the control group, no difference in cell survival was observed at 0.1 and 1 mM MNA, but at 10 mM there was a significant decrease to 78% ($p < 0.01$). When NA was added and compared between

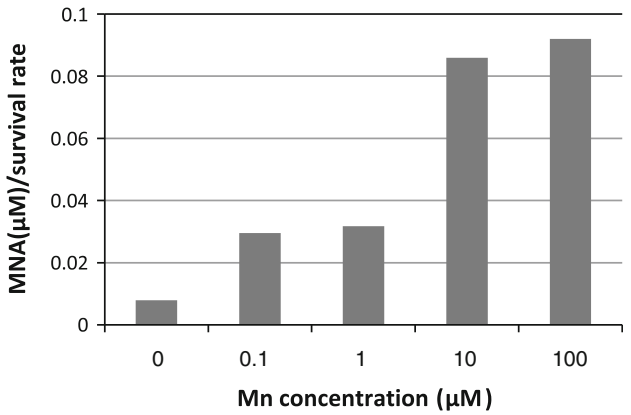


Fig. 2 The amount of 1-methylnicotinamide (*MNA*) in the culture medium on incubation day 19. Each value is expressed as μM/survival rate

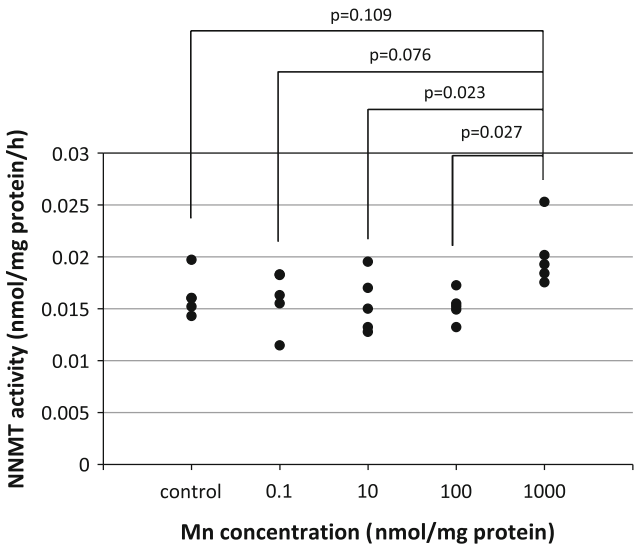
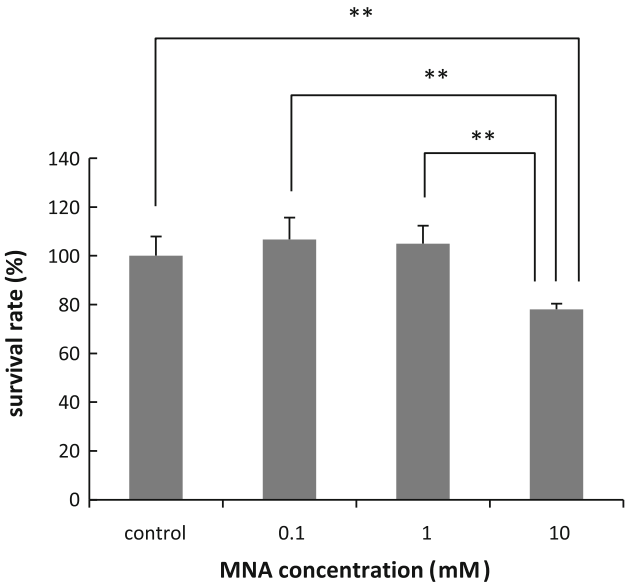


Fig. 3 The effect of different Mn concentrations on nicotinamide *N*-methyltransferase (*NNMT*) activity. NNMT activity is shown as nmol/mg protein/h ($n = 5$). All values for each Mn concentration were plotted. Each p value comparison between groups is shown



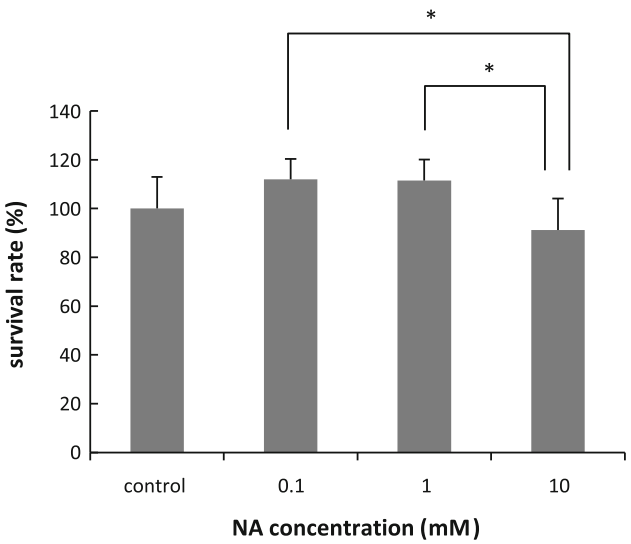


Fig. 4 The effect of MNA and nicotinamide (*NA*) on MS cell survival. Survival rate is shown as the percentage of the control. Data are presented as the mean ± SD ($n = 6$). *Double asterisks* Significant difference at $p < 0.01$ between indicated groups, *single asterisks* significant difference at $p < 0.05$ between indicated groups

multiple groups, there was a significant decrease in survival rate at 10 mM compared to 0.1 and 1 mM ($p < 0.05$).

Discussion

Parkinsonism is known to be induced by chronic exposure to Mn [15, 18], which can occur due to environmental exposure to Mn as well as from the ingestion of Mn, as in the cases of patients on parenteral nutrition who developed parkinsonism due to Mn intoxication from using trace element solutions [19]. It has been thought that the pathogenic mechanism for Mn-induced parkinsonism is the exposure of dopaminergic neurons to oxidative stress caused by the oxidation action of a metal, such as Mn [20, 21]. In our study, we searched for a different mechanism to link Mn with PD and performed three experiments to verify the hypotheses that Mn initiates an increase in NNMT activity, which in turn generates higher concentrations of MNA, subsequently causing neuron death. In the first experiment, we demonstrated the neurotoxicity of Mn on MS cells and observed that the survival rate of MS cells decreased significantly with increasing concentrations of Mn in the culture medium. Since MNA production per survival rate also increased with increasing Mn concentration in the culture medium, we suggest that the Mn-accelerated production of MNA may cause neuronal cell death.

In the second experiment, we observed the effect of different Mn concentrations on NNMT activity using the mouse brain cytosol fraction as the enzyme solution. Although NNMT activity was not increased when the Mn concentration was <0.1 nmol/mg protein (tenfold the normal level found in the mouse brain, it was significantly higher when the Mn concentration was 1 μmol/mg protein (100,000-fold the normal level in the mouse brain) than when it was 10 and 100 nmol/mg protein. These results suggest that NNMT activity may increase with the long-term accumulation of Mn. There is a report linking neurodegeneration and long-term exposure to low levels of Mn [22]. It can be suggested from previous studies and the results of our experiment that excessive accumulation of Mn in daily life plays a possible role in the pathogenesis of IPD.

In the third experiment, we compared differences between the effects of MNA and NA concentrations on the survival rate of MS cells. When MS cells were cultivated with MNA, the cell survival rate dropped at the 10 mM concentration. Based on the results of their study of MNA acute cytotoxicity, Willets et al. [11] reported that at 25 mM concentration MNA, 20% of rat B65 neuroblastoma cells died after 24 h, and 60% died after 3 days. In another study, Ogata et al. [23] demonstrated a 10 mM concentration of MNA for 24 h initiated apoptosis of HL-60 acute myelomonocytic leukemia cells. These studies used tumor cells, but our study used striatal primary cultured cells that were exposed to a lower concentration of MNA than the cells used by Willets et al. [11] and for a longer incubation time—and we were still able to demonstrate neurotoxicity. MNA neurotoxicity has been associated with the destruction of the brain's mitochondrial complex I subunits [9]. Another study showed that injecting rat midbrain substantia nigra with MNA caused dopamine levels of the corpus striatum to drop [10]. Cation ions, such as MNA, cannot pass through blood–brain barriers [24], but NNMT activity exists in the brain, and it has been confirmed that MNA is produced in the brain [25]. Our results suggest that long exposure to MNA influences neuron survival rates.

The addition of MNA's precursor, NA, to the cultivation medium resulted in an increase in cell survival rate at 0.1 and 1 mM NA compared to the control (change was not significant). It is thought that NA acts in a nutritional and neuroprotective manner in the cells. In two previous studies, 5 mM and 25 mM NA added in advance to the culture of primary cultured neurons showed a neuroprotective action against the neurotoxin, homocysteine, after 24 h of incubation [26, 27]; MNA also displayed a neuroprotective action in the same study. NA has an inhibitive effect on oxidative lesions [28]; thus, NA and MNA may play a protective role in neuronal cells during a short-term cultivation. In our long-term cultivation study, NNMT activity increased with increasing NA concentrations, with a resulting decrease in survival rate at 10 mM. The increase in metabolism to MNA resulted in higher MNA concentrations in the cells and, subsequently, cell death. There are also reports linking MNA to oxidative stress-induced neuron death [9, 10]. It is thought that MNA generates reactive oxygen species in the mitochondria, thereby influencing the cell survival rate.

In conclusion, we found that Mn increased NNMT activity. In the presence of Mn, and if the concentrations of NA, the substrate of NNMT, build up, there is the potential of continued high levels of the metabolite, MNA, in the brain. Since MNA causes neuron death, we were able to make a connection between Mn exposure and IPD. Further studies are necessary on the intake levels of Mn from our environment, such as from drinking water, and the intake levels of NA present in supplements and other sources.

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Conflict of interest None.

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