Manganese-mediated decrease in levels of c-RET and tyrosine hydroxylase expression *in vitro*

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Abstract

Previous studies showed that overexposure to manganese causes parkinsonism, a disorder of dopaminergic neurons. Previous studies also showed that activity of c-RET kinase controls dopamine production through regulation of tyrosine hydroxylase (TH) expression, suggesting the involvement of c-RET in the development of parkinsonism. To our knowledge, however, there is no report showing a correlation between manganese-mediated parkinsonism and c-RET. In this study, we examined the effect of manganese on the expression and/or activation levels of c-RET and TH in human TH-expressing cells (TGW cells). We first found that treatment with 30 μM and 100 μM manganese resulted in reduction of c-RET transcript level and degradation of c-RET protein through promotion of ubiquitination. We then examined the biological significance of manganese-mediated decrease of c-RET protein expression. Decreased TH expression with decreased c-RET kinase activity was observed in c-RET protein-depleted TGW cells by treatment with manganese (30 μM) as well as by c-RET siRNA transfection. Since TH protein has been shown to be involved in the dopamine-producing pathway in previous studies, our results indicate the possibility that manganese-mediated reduction of TH expression and phosphorylation via decreased expression of c-RET protein in neural cells is involved in parkinsonism induced by manganese.

Key words: Manganese, Metals, Protein expression, RET, tyrosine kinase

Introduction

Excessive exposure to manganese has been reported to cause respiratory, immune and neuronal disorders (Antonini et al. 2012; Han et al. 2009; Olanow 2004). Chronic inhalation of manganese in workplaces such as mining and welding workplaces has been shown to be associated with a neuronal disorder known as manganism, which has symptoms similar to those of idiopathic Parkinson's disease (parkinsonism). Patients with manganism are thought to have neuronal damage in the substantia nigra that leads to deficiency of the neurotransmitter dopamine for the striatum (Huang

et al. 1989; Kwakye et al. 2015; Mena et al. 1970; Mena et al. 1967; Rosenstock et al. 1971).

It has been reported that c-RET encoding a receptor tyrosine kinase (RTK) is the receptor for glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs). To activate c-RET, GFLs bind with high affinity to coreceptors, GFRαs, and then this GFL-GFRα complex binds to c-RET (Baloh et al. 1997; Kato et al. 2002). Previous studies in animals revealed a correlation between c-RET and Parkinson's disease. Decreased expression of c-RET, GFRα1 and GFRα2 was observed in a model for Parkinson's disease (Marco et al. 2002). Deletion of c-RET in the nigrostriatal dopaminergic system in mice caused progressive degeneration of dopaminergic neurons in the substantia nigra *pars compacta* (Kramer et al. 2007). Previous studies in humans showed that GDNF, a ligand for c-RET, is a good tool to decrease symptoms of Parkinson's disease (Barker 2006). Previous biochemical studies also showed that activation of c-RET stimulated by GDNF increases the expression of tyrosine hydroxylase (TH), a rate-limiting enzyme of the dopamine-producing pathway (Xiao et al. 2002). Moreover, c-RET is expressed not only in dopaminergic neurons and motor neurons but also in the brain including the hippocampus and cerebral cortex, which are affected by manganese exposure (Burazin and Gundlach 1999; Gould et al. 2008; Kern et al. 2010).

Despite those previous results, there is no information showing a correlation between manganese-mediated parkinsonism and c-RET. In this study, we hypothesized that manganese causes the development of parkinsonism via modulation of the expression and function of c-RET and subsequently that for TH. Since a human TGW cell line is one of the representative cell lines in which GDNF-mediated activation of c-RET directly affects levels of TH expression and phosphorylation (Tenenbaum and Humbert-Claude 2017; Xiao et al. 2002), we tried to certify the hypothesis using TGW cells in this study.

Materials and Methods

Cell culture and reagents

Human TGW neuroblastoma cells obtained from Human Science Health Research Resources Bank were cultured according to the method described previously (Kato et al. 2011). Briefly, TGW cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (WAKO) at 37ºC in 5% CO2. Manganese (II) chloride (Sigma), epoxomicin (PEPTIDE INSTITUTE, INC.) and GDNF (R&D Systems) were used as manganese, a pharmacological inhibitor for proteasome and a ligand for c-RET, respectively. TGW cells were exposed to manganese after being cultured with 1% FBS for 6 hrs.

Immunoblotting

Immunoblotting was performed according to the method described previously (Kato et al. 2004). Rabbit polyclonal antibodies against RET (IBL), phosphorylation of tyrosine 905 (Y905) in RET (Cell Signaling Technology), phosphorylation of serine (Ser40) in TH (Cell Signaling Technology), and TH (Abcam) and mouse a monoclonal antibody against α-tubulin (Sigma) were used as first antibodies. Since Y905 is a representative tyrosine to regulate c-RET kinase activity (Baloh et al. 1997; Kato et al. 2002), we substituted phosphorylated levels of Y905 in c-RET protein for phosphorylated levels of c-RET protein. Densitometric analysis of immunoblot bands was performed using the software program WinROOF (MITANI Corporation) following a previous report (Ohgami et al. 2010).

Immunoprecipitation

After TGW cells had been extracted with NP40 buffer (50 mM Tris-buffered saline, pH 7.4, 1% Nonidet P-40, 10% glycerol, 200 mM phenylmethylsulfonyl fluoride, 200 mM, 10 μg/L aprotinin), 500 μg of proteins was used for immunoprecipitation with a UbiQapture™-Q kit (Enzo Life Sciences) following the manufacturer's protocol.

Real-time PCR

Real-time PCR analysis was performed by the method previously described (Ohshima et al. 2010). The primers used for TH were designed following a previous report (Lambooy et al. 2003).

Small-interfering RNA transfection

TGW cells were transiently transfected with control siRNA (Invitrogen Life Tech) or c-RET siRNA (Hokkaido System Science) using Lipofectamine RNAiMAX (Invitrogen Life Tech) according to the manufacturer's protocol. The sequence of c-RET siRNA was designed following a previous report (Koga et al. 2010).

Statistical analysis

Statistical analyses using Dunnett's test, Tukey-Kramer's test and Student's *t*-test were performed following the method previously described (Kato et al. 2011). The JMP Pro10 software package (SAS Institute Inc.) was used for statistical analyses. The significance level was set at $p < 0.05$.

Results

Effects of manganese on levels of c-RET protein and transcript.

We examined the effect of manganese on expression levels of c-RET protein in TGW cells. A previous study showed that manganese concentration in human toenails had a distribution ranging from 0.05 to 10.41 μg/g (0.9-189 μM) (Laohaudomchok et al. 2012). Previous studies also showed that manganese concentrations in human hairs had distributions ranging from 0.4 to 49.6 μg/g (7.2-901.8 μM) (Bader et al. 1999) and 0.3 to 20.0 μg/g (5.5-363.6 μM) (Bouchard et al. 2007). TGW cells were treated with 30-100 μM manganese in this study (Figure 1). Treatment with 30, 60 and 100 μM manganese for 18 hours resulted in decreases in c-RET protein expression levels by 46%, 78% and 98%, respectively (Figure 1a and b). Shrinkage was observed for TGW cells treated with 30 or

100 μM manganese (arrowheads in Figure 1c), but there was no significant difference between the viability of untreated cells and that of cells treated with manganese (Figure 1d).

We next examined the effect of treatment with 30 μM manganese for 18 and 42 hours on c-RET expression in TGW cells (Figure 2a-c). Interestingly, treatment with 30 μM manganese for 18 and 42 hours resulted in decreases in expression levels of c-RET protein by 49% and 89%, respectively (Figure 2a and b). The expression levels of c-RET transcript were decreased by only 26% and 58% by treatment with 30 μM manganese for 18 and 42 hours, respectively (Figure 2c). Correspondingly, the expression levels of c-RET protein were decreased by 69% and 99% by treatment with 100 μM manganese for 12 and 18 hours, respectively (Figure 2d and e). On the other hand, the expression levels of c-RET transcript were decreased by 30% and 56% by treatment with 100 μM manganese for 12 and 18 hours, respectively (Figure 2f). Our results showed that manganese-mediated decreases in expression levels of c-RET protein were greater than those of c-RET transcript. These results suggest that posttranslational degradation of c-RET protein as well as decrease in the level of c-RET transcript seems to be correlated with the manganese-mediated decrease in expression level of c-RET protein.

We next examined whether posttranslational degradation of c-RET protein is induced by treatment with manganese. A previous study showed that phosphorylated c-RET protein treated with GDNF was proteasome-dependently degraded after ubiquitination (Pierchala et al. 2006). Therefore, we first examined whether c-RET protein in TGW cells treated with 30 μM manganese is proteasome-dependently degraded (Figure 3a and b). Manganese-mediated decrease in c-RET protein was inhibited by treatment with epoxomicin, a proteasome inhibitor (Figure 3a and b). We next examined whether manganese promotes ubiquitination of c-RET protein, since ubiquitination is the initial step for protein degradation via the ubiquitin/proteasome pathway (Tu et al. 2012). Ubiquitinated c-RET protein was found in TGW cells treated with manganese but not in untreated TGW cells (Figure 3c and d). These results suggest that posttranslational modification of c-RET

protein via the ubiquitin/proteasome pathway in addition to decrease in the level of c-RET transcript are involved in manganese-mediated decrease in c-RET protein.

Biological significance of manganese-mediated decrease in c-RET protein expression level.

We next examined the biological significance of decreased c-RET protein expression level. The expression level of c-RET protein in c-RET siRNA-transfected TGW cells (siRNA-mediated c-RET-depleted cells) was decreased by 53% compared to that in control siRNA-transfected TGW cells (control cells) (lanes 1 and 2 in Figure 4a and b). The phosphorylation level of c-RET protein and the protein and transcript levels of TH expression in the absence of GDNF were undetectably low in both siRNA-mediated c-RET-depleted cells and control cells (lanes 1 and 2 in Figure 4a, c, s and e). Levels of c-RET protein expression, TH protein and transcript expression, and phosphorylated c-RET protein were increased in both siRNA-mediated c-RET-depleted cells and control cells in the presence of GDNF (Figure 4a-e). More importantly, expression levels of TH protein and transcript in siRNA-mediated c-RET-depleted cells were significantly lower than those in control cells in the presence of GDNF (lanes 3 and 4 in Figure 4a, d and e).

We finally examined expression levels of c-RET protein and TH protein and transcript and the phosphorylated levels of c-RET protein and TH protein in 30 μM manganese-treated and untreated TGW cells in the presence or absence of GDNF. As shown in Figures 1-3, the level of c-RET protein expression in cells treated with manganese (manganese-mediated c-RET-depleted cells) was lower than that in untreated cells (control cells) in either the presence or absence of GDNF (Figure 5a and b). The levels of phosphorylated c-RET protein and TH protein and the expression levels of TH protein and transcript in the absence of GDNF were undetectably low in both manganese-mediated c-RET-depleted cells and control cells (lanes 1 and 3 in Figure 5a, c-g). Levels of c-RET protein expression, phosphorylated c-RET, and TH protein and transcript expression were increased by treatment with GDNF in both manganese-mediated c-RET-depleted cells and control cells (Figure 5a-e). More importantly, levels of phosphorylated c-RET protein, phosphorylated

(Ser40) and total TH protein and transcript expression of *TH* in manganese-mediated c-RET-depleted cells were significantly lower than those in control cells in the presence of GDNF (lanes 2 and 4 in Figure 5a, c-g). Thus, our results showed a decreased level of TH with a decreased level of c-RET phosphorylation in manganese-mediated c-RET-depleted cells as well as in siRNA-mediated c-RET-depleted cells.

Discussion

In this study, we demonstrated for the first time that manganese not only decreases the amount of c-RET transcript but also promotes proteasome-dependent degradation of c-RET protein, resulting in a decrease in the level of c-RET protein expression in human TGW cells. Since manganese-mediated reduction of c-Ret protein expression was also found in rat PC-12 cells, which spontaneously express c-Ret (Pelicci et al. 2002) Kumasaka et al. unpublished observation), manganese might decrease c-RET/Ret protein expression in neuronal cells. We then demonstrated that decreases in the levels of c-RET phosphorylation and TH expression and phosphorylation occurred in accordance with a decrease in c-RET protein expression (Figure 6). Since c-RET has been reported to be associated with the dopamine-producing pathway through enhancement of transcription of TH, which encodes the initial and rate-limiting enzyme in dopamine biosynthesis (Mijatovic et al. 2007), TH has been used as one of the indicators to determine the level of dopamine production (Hurley et al. 2003). Moreover, decreased TH expression level has been reported in the rat brain overexposed to manganese (Zhang et al. 1999). There have been various *in vivo* and *in vitro* studies in which TH protein and phosphorylation levels and activity were evaluated (Guilarte et al. 2008; Peres et al. 2016; Posser et al. 2009; Zhang et al. 2011). However, it was difficult to evaluate correlations between the GDNF/c-RET pathway and TH expression/phosphorylation in previous studies because the cell lines or tissues used in previous studies constantly express TH protein. Since TH expression in TGW cells used in this study is initiated by GDNF treatment (see Figure 4), our results demonstrated a correlation between the GDNF/c-RET pathway and TH expression/phosphorylation. Taken together,

the results indicate the possibility that manganese-mediated decrease in c-RET protein expression is involved in parkinsonism via decreased c-RET activity and TH protein expression/phosphorylation. Thus, this study suggested a novel mechanism for manganese-mediated parkinsonism (Figure 6).

Previous studies showed that manganese causes not only parkinsonism (Perl and Olanow 2007) but also various neuropsychological deficiencies with dysfunction of the dopamine system such as hyperactive behaviors (Bouchard et al. 2007), learning deficit (Zhang et al. 1995) and cognitive deficit (Bouchard et al. 2007). Since c-RET has been reported to be associated with the pathway of dopamine production through promotion for TH transcript (Mijatovic et al. 2007), our results showing manganese-mediated decreases in c-RET protein expression and activity and subsequent reduction of TH expression indicate the possibility that manganese is involved not only in parkinsonism but also in various neural disorders derived from dysfunction of the dopamine system through dysfunction of c-RET. In addition, patients with amyotrophic lateral sclerosis (ALS) as well as parkinsonism have been reported in New Guinea and Papua islands, where the well drinking water contains high concentrations of manganese (Nishida 2003). However, it remains unclear whether there is a correlation between manganese and ALS. Since decreased c-RET expression in motor neurons of the lumbar spinal cord has been reported in model mice for ALS (Ryu et al. 2011), our results showing manganese-mediated dysfunction of c-RET indicate the possibility that manganese is also partially correlated with development of ALS via c-RET molecules.

Our results showed manganese-mediated suppression of c-RET mRNA expression. Previous studies showed that manganese affects the activity of metal-responsive transcription factors (Brett et al. 2008; Casalino et al. 2007; Rodionov et al. 2006). Therefore, manganese-mediated repression of c-RET mRNA expression might be caused by modification of the activity of manganese-responsive transcription factors for c-RET. Our results also showed the involvement of manganese-mediated posttranslational modification in reduction of c-RET protein. Previous studies showed that ubiquitination precedes the degradation of many receptors, transporters and channels (Kamsteeg et al. 2006; Miranda and Sorkin 2007; Miranda et al. 2005). An abnormal ubiquitin system causes cancer,

inflammatory and autoimmune diseases, and neurodegenerative disorders (Colland 2006; Diehl et al. 2010; Lee et al. 2008; Lim and Lim 2011; Wang and Maldonado 2006). Previous studies also showed that manganese is involved in activation of the ubiquitin system (Sidoryk-Wegrzynowicz et al.). CBL-B is a representative ubiquitin ligase that is involved in regulation of c-RET protein expression (Pierchala et al. 2006; Scott et al. 2005). However, the expression level of CBL-B protein in TGW cells was decreased by treatment with 30 μM manganese (Kumasaka et al. unpublished observation). Manganese-mediated decrease in c-RET protein expression in TGW cells was also observed in CBL-B depleted TGW cells (Kumasaka et al. unpublished observation). Our results suggest that CBL-B has a limited role in the manganese-mediated decrease in c-RET protein. It is important to clarify the mechanism for manganese-mediated ubiquitination of c-RET in the future.

In conclusion, we proposed the possibility of a linkage between manganese-mediated neural disorders and dysfunction of c-RET and partially clarified the molecular mechanism.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1 Manganese-mediated decrease of c-RET protein expression levels. After incubation in the medium containing 1% FBS for 6 hrs, TGW cells were treated with the indicated concentrations of manganese for 18 hrs. Protein expression levels of c-RET in the cells untreated (lane 1) and treated with 30 μM (lane 2), 60 μM (lane 3) and 100 μM (lane 4) of manganese are presented (**a**). c-RET protein was detected as two protein bands, a band of 175 kDa (mature glycosylated form) and a band of 155 kDa (immature glycosylated form) (**a**) as shown in a previous report (Kato et al. 2000). Expression levels of α-TUBULIN (α-TUB) protein are presented as an internal control (**a**). After calculating c-RET protein expression levels for α-TUBULIN protein expression levels by densitometric analysis, levels of c-RET expression with manganese treatment (lanes 2-4) are presented as relative ratios (means ± SD; n=3) to that in the untreated control (lane 1) (**b**). Morphology of TGW cells not treated with or treated with manganese (30 and 100 μM) (**c**) and viability of TGW cells untreated (lane 1) and treated with 30 μM (lane 2) and 100 μM (lane 3) of manganese for 18 hrs are presented (**d**). Scale bar: 10 μm. * *, significantly different (**, p<0.01) by Dunnett's test.

Fig. 2 Comparison of manganese-mediated decreases in the expression levels of c-RET protein and transcript. After incubation in the medium containing 1% FBS for 6 hrs, TGW cells were treated with 30 μM (**a**-**c**) and 100 μM (**d**-**f**) of manganese for the indicated period (hours) (**a**-**f**). Expression levels of α-TUBULIN (α-TUB) protein are presented as an internal control (**a**). After calculating c-RET protein expression levels for α-TUBULIN protein expression levels by densitometric analysis, levels of c-RET expression (**b** and **e**) with 30 μM manganese treatment for 18 hrs (lane 2 in **b**) and 42 hrs (lane 3 in **b**) and with 100 μM manganese treatment for 12 hrs (lane 2 in **e**) and 18 hrs (lane 3 in **e**) are presented as relative ratios (means ± SD; n=3) to that for 0 hrs (lane 1 in b and e). Levels of c-RET transcript expression (**c** and **f**) with 30 μM manganese treatment for 18 hrs (lane 2 in **c**) and 42 hrs (lane 3 in **c**) and with 100 μM manganese treatment for 12 hrs (lane 2 in **f**) and 18 hrs (lane 3 in **f**) are

presented as relative ratios (means ± SD; n=3) to that for 0 hrs (lane 1 in **c** and **f**). * and **, significantly different (*, $p<0.05$; **, $p<0.01$) by Dunnett's test.

Fig. 3 Mechanism of manganese-mediated decrease of c-RET protein. After incubation in the medium containing 1% FBS for 6 hrs, TGW cells were treated with 30 μM manganese (Mn) for 18 hrs. Then the cells were treated with 3 μM epoxomicin (Epox) for 6 hrs (**a** and **b**). Protein expression levels of c-RET (**a** and **b**) in the cells untreated (lanes 1 and 2) or treated (lanes 3 and 4) with epoxomicin in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of manganese are presented. Expression levels of α-TUBULIN (α-TUB) protein are presented as an internal control (**a**). After calculating c-RET protein expression levels for α-TUBULIN protein expression levels by densitometric analysis, levels of c-RET expression in the cells treated with epoxomicin and/or manganese (lanes 2-4 in **b**) are presented as relative ratios (means ± SD; n=3) to that in the untreated control (lane 1 in **b**). Expression levels of ubiquitinated c-RET protein (top) and constitutive c-RET protein (bottom) in cells in the presence (lane 2) or absence (lane 1) of 30 μM manganese are presented (**c**). After calculating ubiquitinated c-RET protein expression levels for constitutive c-RET protein expression levels by densitometric analysis, levels of ubiquitinated c-RET protein expression in untreated cells (lane 1) are presented as relative ratios (means ± SD; n=3) to that in cells treated with manganese (lane 2) (**d**). **, significantly different (**, $p<0.01$) by Tukey-Kramer's test (**b**) and Student's *t*-test (**d**).

Fig. 4 Depleted c-RET-mediated decrease of TH expression. After transfection of control siRNA or c-RET siRNA (siRET), TGW cells were incubated in D-MEM with 10% FBS for 42 hrs. Then the cells were cultured in the presence or absence of GDNF (25 ng/ml) for 18 hrs after incubation in the medium containing 1% FBS for 6 hrs and were analyzed by immunoblotting (**a**) and real-time PCR (**e**). Expression levels of c-RET (**a** and **b**) and TH (**a** and **d**) proteins and phosphorylated c-RET (p-c-RET; **a** and **c**) in control siRNA (lanes 1 and 3 in **a**-**d**) and c-RET siRNA (lanes 2 and 4 in **a**-**d**)-transfected TGW cells in the presence (lanes 3 and 4 in **a**-**d**) or absence (lanes 1 and 2 in **a**-**d**) of

GDNF are presented. After calculating levels of c-RET protein expression (**b**), phosphorylated c-RET (**c**) and TH protein expression (**d**) for α-TUBULIN (α-TUB) protein expression levels by densitometric analysis, those in the cells treated with c-RET siRNA and/or GDNF (lanes 2-4 in **b**, lanes 1, 2 and 4 in **c** and **d**) are presented as relative ratios (means ± SD; n=3) to that in control siRNA-transfected cells in the absence (lane 1 in **b**) or presence (lane 3 in **c** and **d**) of GDNF. Expression levels of TH transcript in TGW cells treated with siRNA and/or GDNF (lanes 1, 2 and 4 in **e**) are presented as relative ratios (means ± SD; n=3) to that in control siRNA-transfected cells in the presence of GDNF (lane 3 in **e**). * and **, significantly different (*, $p<0.05$; **, $p<0.01$) by Tukey-Kramer's test.

Fig. 5 Decreased TH expression and phosphorylation levels by manganese-mediated depletion of c-RET. After incubation in the medium containing 1% FBS for 6 hrs, TGW cells were treated with 30 μM manganese (Mn) for 24 hrs. Then the cells were cultured in the presence or absence of GDNF (25 ng/ml) for 18 hrs and were analyzed by immunoblotting (**a** and **f**) and real-time PCR (**e**). Expression levels of c-RET (**a** and **b**) and TH (**a** and **d**) proteins and phosphorylated c-RET (p-c-RET; **a** and **c**) in 30 μM manganese-treated (lanes 3 and 4 in **a**-**d**) and untreated (lanes 1 and 2 in **a**-**d**) TGW cells in the presence (lanes 2 and 4 in **a**-**d**) or absence (lanes 1 and 3 in **a**-**d**) of GDNF are presented. After calculating levels of c-RET protein expression (**b**), phosphorylated c-RET (**c**) and TH protein expression (**d**) for α-TUBULIN (α-TUB) protein expression levels by densitometric analysis, those in the cells treated with manganese and/or GDNF (lanes 2-4 in b, lanes 1, 3 and 4 in **c** and **d**) are presented as relative ratios (means ± SD; n=3) to that in untreated control cells (lane 1 in **b**) or in cells treated with GDNF (lane 2 in **c** and **d**). Expression levels of TH transcript in the cells treated with 30 μM manganese and/or GDNF (lanes 1, 3 and 4 in **e**) are presented as relative ratios (means ± SD; n=3) to that in cells treated with GDNF (lane 2 in **e**). Phosphorylation and expression levels of TH (**f**) in 30 μM manganese-treated (lanes 3 and 4 in **f** and **g**) and untreated (lanes 1 and 2 in **f** and **g**) TGW cells in the presence (lanes 2 and 4 in **f** and **g**) or absence (lanes 1 and 3 in **f** and **g**) of GDNF are

presented. After calculating levels of phosphorylated TH (**g**) for α-TUBULIN (α-TUB) protein expression levels by densitometric analysis, those in the cells treated with manganese and/or GDNF (lanes 1, 3 and 4 in **g**) are presented as relative ratios (means ± SD; n=3) to that in untreated control cells (lane 1 in **g**). **, significantly different (**, $p<0.01$) by Tukey-Kramer's test.

Fig. 6 A scheme for a novel mechanism of manganese-mediated parkinsonism via c-RET molecules. Manganese-mediated reduction of c-RET transcript and degradation of c-RET protein through ubiquitination in TGW cells secondarily decreases levels of c-RET kinase activity and TH protein expression and phosphorylation. Since a previous study showed that TH protein is involved in the dopamine-producing pathway (Xiao et al. 2002), manganese-mediated reduction of c-RET protein may cause dopamine-depleted diseases including parkinsonism.

Fig. 1

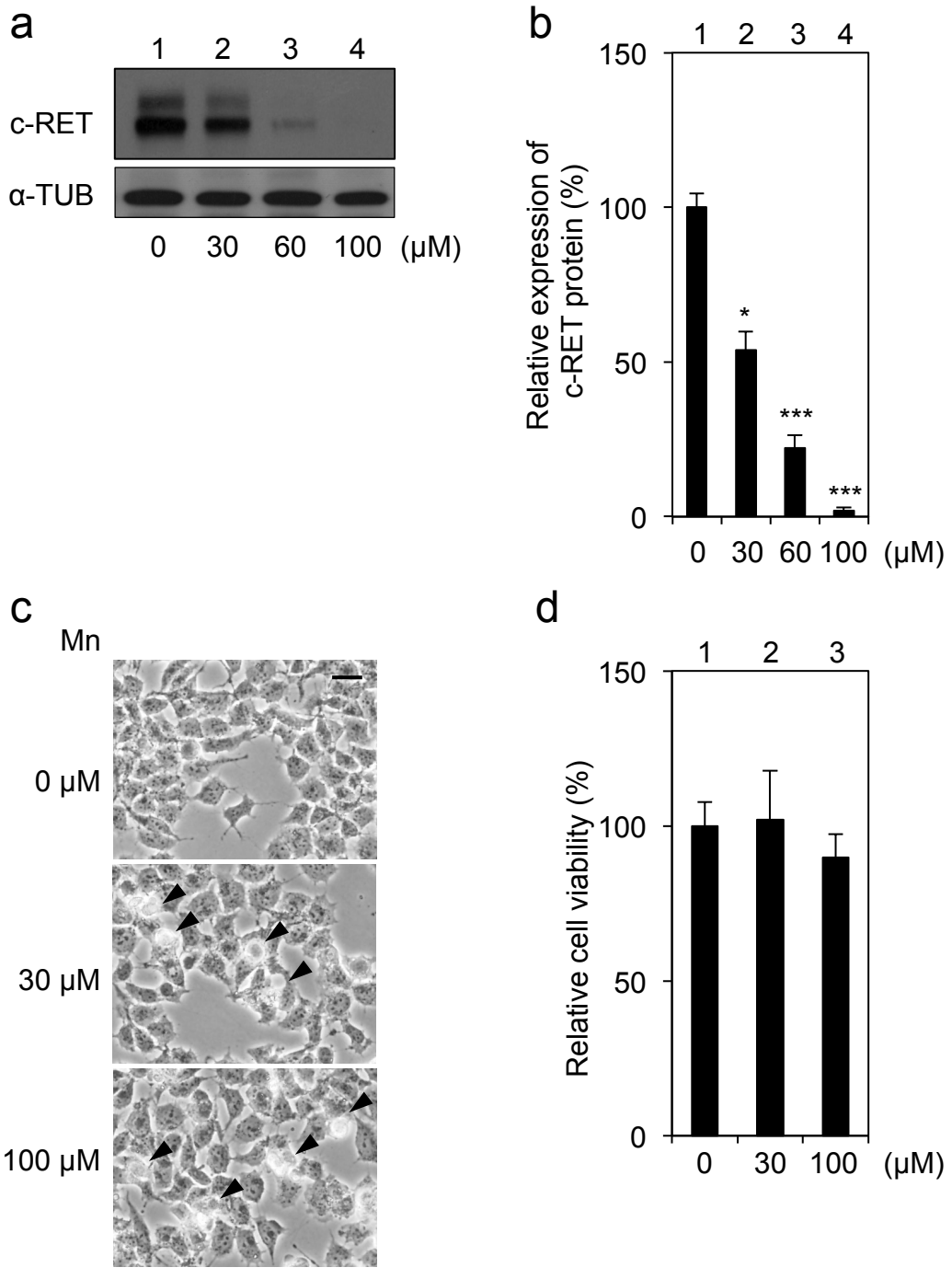

a
1 2 3 4
c-RET
α-TUB
0 30 60 100 (μM)
b
1 2 3 4
150
100
50
0
Relative expression of c-RET protein (%)
*
***
***
0 30 60 100 (μM)
c
Mn
0 μM
30 μM
100 μM
d
1 2 3
150
100
50
0
Relative cell viability (%)
0 30 100 (μM)

Fig. 2

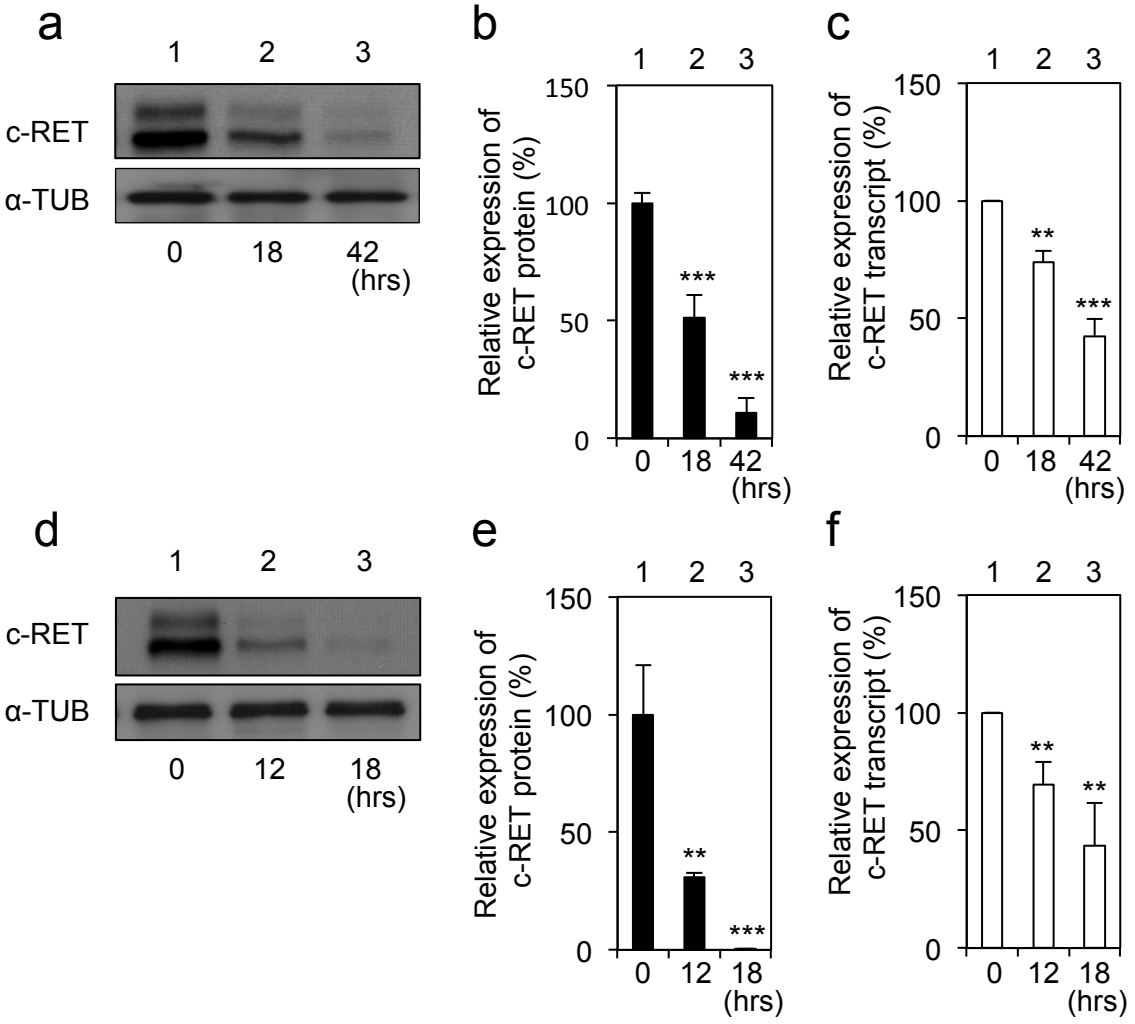

a
1 2 3
c-RET
α-TUB
0 18 42
(hrs)
b
1 2 3
Relative expression of c-RET protein (%)
150
100
50
0
***
***
0 18 42
(hrs)
c
1 2 3
Relative expression of c-RET transcript (%)
150
100
50
0
**
***
0 18 42
(hrs)
d
1 2 3
c-RET
α-TUB
0 12 18
(hrs)
e
1 2 3
Relative expression of c-RET protein (%)
150
100
50
0
**
***
0 12 18
(hrs)
f
1 2 3
Relative expression of c-RET transcript (%)
150
100
50
0
**
**
0 12 18
(hrs)

Fig. 3

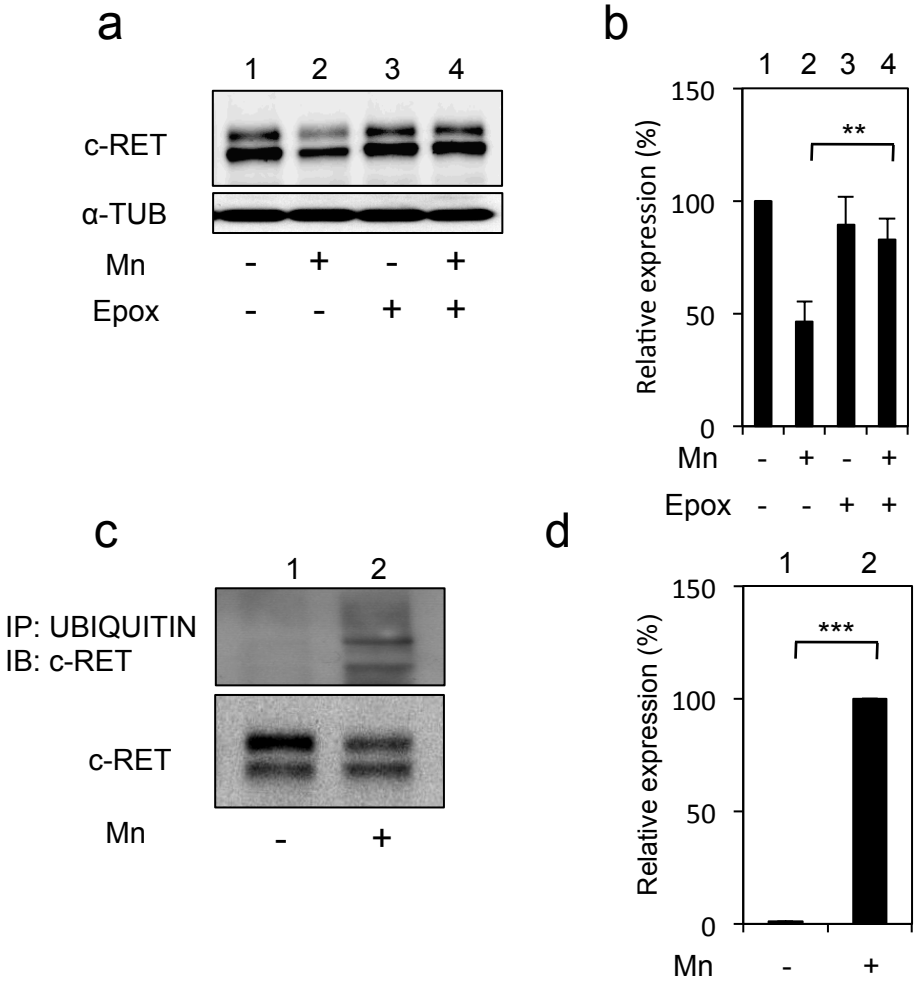

a
1 2 3 4
c-RET
α-TUB
Mn - + - +
Epox - - + +
b
1 2 3 4
150
100
50
0
Relative expression (%)
**
Mn - + - +
Epox - - + +
c
1 2
IP: UBIQUITIN
IB: c-RET
c-RET
Mn - +
d
1 2
150
100
50
0
Relative expression (%)
***
Mn - +

Fig. 4

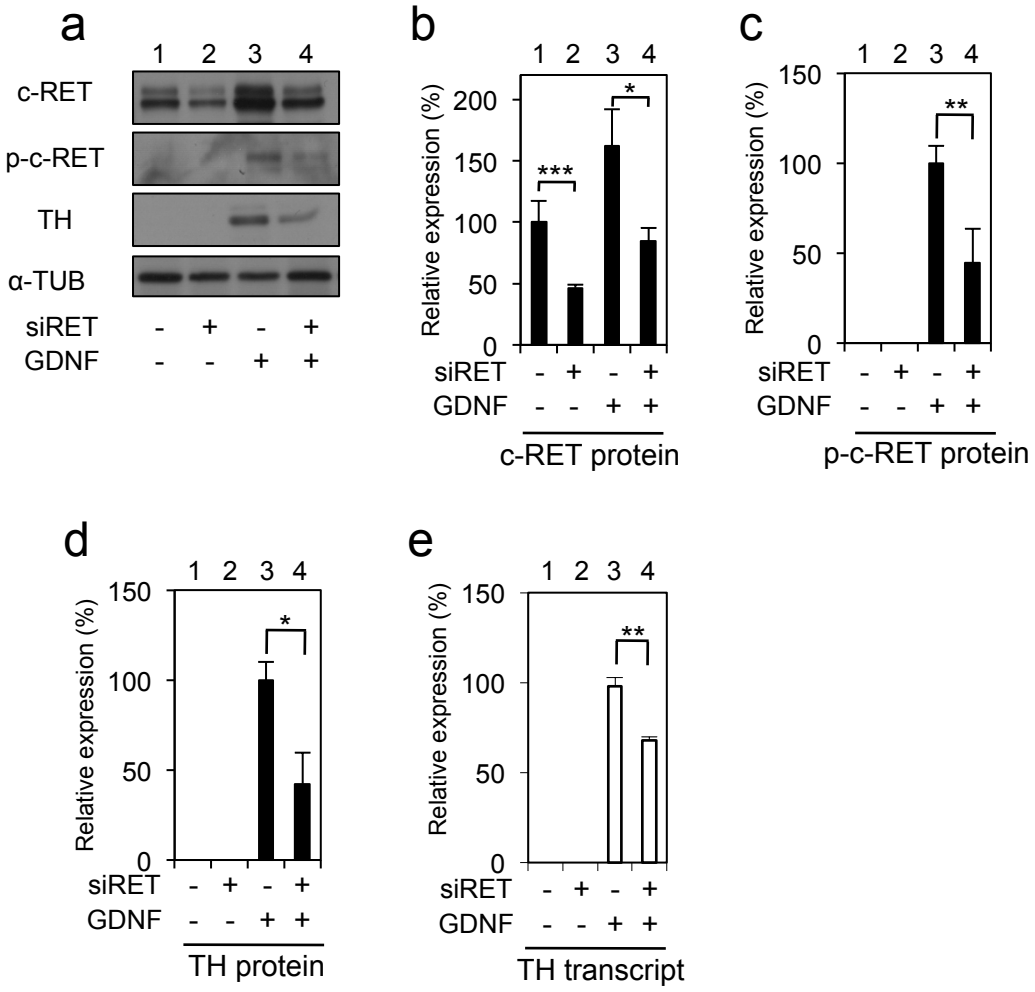
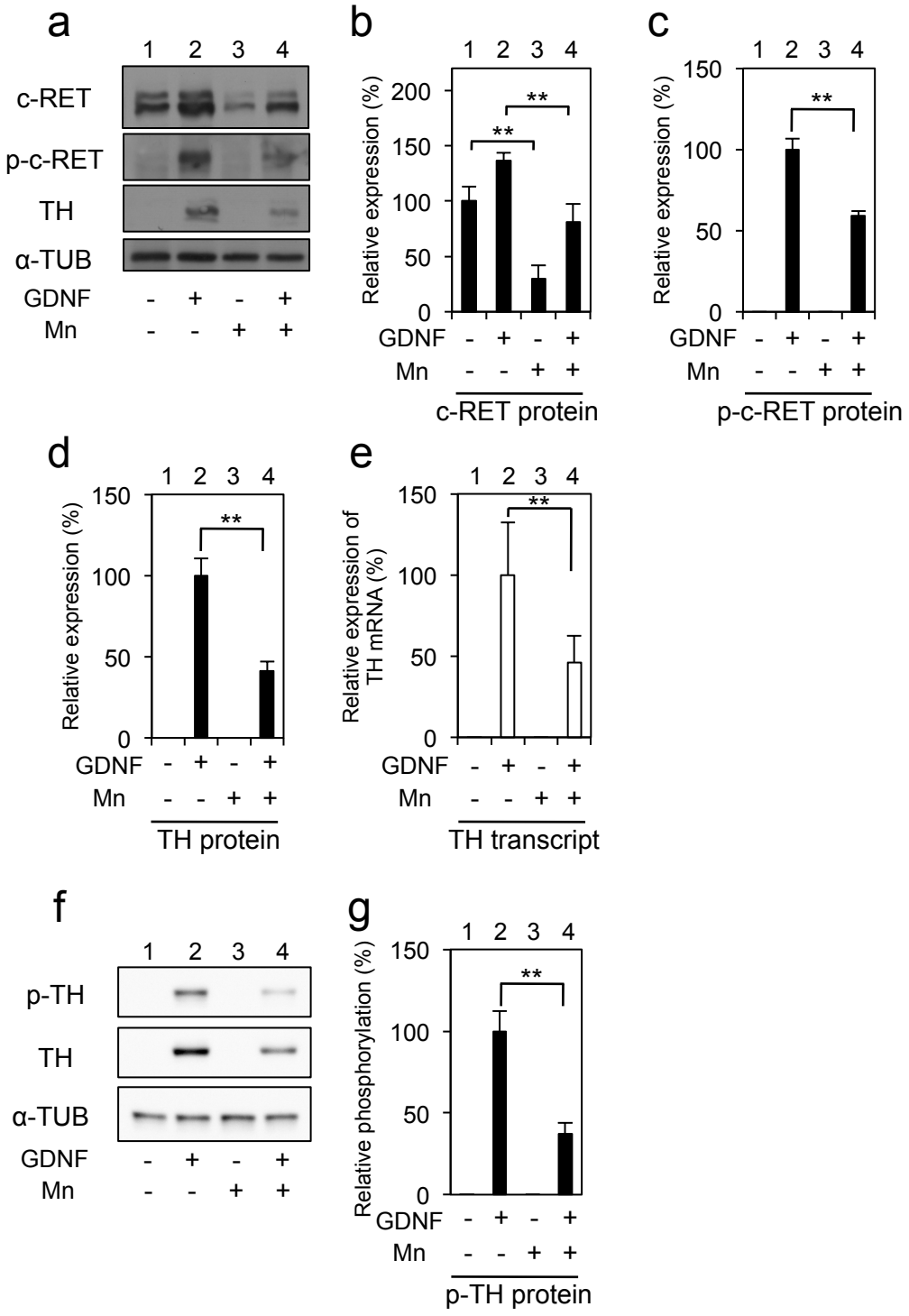

a
1 2 3 4
c-RET
p-c-RET
TH
α-TUB
siRET - + - +
GDNF - - + +
b
1 2 3 4
Relative expression (%)
200
150
100
50
0
***
*
siRET - + - +
GDNF - - + +
c-RET protein
c
1 2 3 4
Relative expression (%)
150
100
50
0
**
siRET - + - +
GDNF - - + +
p-c-RET protein
d
1 2 3 4
Relative expression (%)
150
100
50
0
*
siRET - + - +
GDNF - - + +
TH protein
e
1 2 3 4
Relative expression (%)
150
100
50
0
**
siRET - + - +
GDNF - - + +
TH transcript

Fig. 5


a
1 2 3 4
c-RET
p-c-RET
TH
α-TUB
GDNF - + - +
Mn - - + +
b
Relative expression (%)
200
150
100
50
0
**
**
GDNF - + - +
Mn - - + +
c-RET protein
c
Relative expression (%)
150
100
50
0
**
GDNF - + - +
Mn - - + +
p-c-RET protein
d
Relative expression (%)
150
100
50
0
**
GDNF - + - +
Mn - - + +
TH protein
e
Relative expression of TH mRNA (%)
150
100
50
0
**
GDNF - + - +
Mn - - + +
TH transcript
f
p-TH
TH
α-TUB
GDNF - + - +
Mn - - + +
g
Relative phosphorylation (%)
150
100
50
0
**
GDNF - + - +
Mn - - + +
p-TH protein