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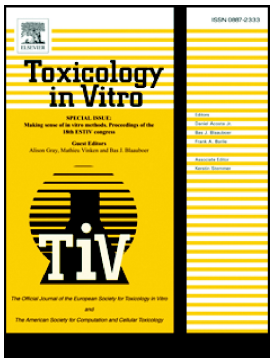

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Manganese exposure: linking down-regulation of miRNA-7 and miRNA-433 with α-synuclein overexpression and risk of idiopathic Parkinson's disease

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Abstract

Manganese is an essential trace element however elevated environmental and occupational exposure to this element has been correlated with neurotoxicity symptoms clinically identical to idiopathic Parkinson's disease. In the present study we chronically exposed human neuroblastoma SH-SY5Y cells to manganese (100μM) and carried out expression profiling of miRNAs known to modulate neuronal differentiation and neurodegeneration. The miRNA PCR array results reveal alterations in expression levels of miRNAs, which have previously been associated with the regulation of synaptic transmission and apoptosis. The expressions of miR-7 and miR-433 significantly reduced upon manganese exposure. By *in silico* homology analysis we identified SNCA and FGF-20as targets of miR-7 and miR-433. We demonstrate an inverse correlation in expression levels where reduction in these two miRNAs causes increases in SNCA and FGF-20. Transient transfection of SH-SY5Y cells with miR-7 and miR-433 mimics resulted in down regulation of SNCA and FGF-20 mRNA levels. Our study is the first to uncover the potential link between manganese exposure, altered miRNA expression and parkinsonism: manganese exposure causes overexpression of SNCA and FGF-20 by diminishing miR-7 and miR-433 levels. These miRNAs may be considered critical for protection from manganese induced neurotoxic mechanism and hence as potential therapeutic targets.

Keywords : Manganese, SH-SY5Y, miRNA, SNCA, Parkinson's disease

Abbreviations

BCL2	-	B-Cell CLL/Lymphoma 2
CCND1	-	Cyclin D1
CCNE1	-	Cyclin E1

CRKL	-	V-Crk Avian Sarcoma Virus CT10 Oncogene Homolog-Like
FGF20	-	Fibroblast Growth Factor 20
FGF7	-	Fibroblast growth factor 7
FOXO1	-	Forkhead Box Protein O1A
GRB2	-	Growth Factor Receptor Bound Protein 2
HMGA1	-	High Mobility Group AT-Hook 1
Mn	-	Manganese
NTRK3	-	Neurotrophic Receptor Tyrosine Kinase 3
PAK1	-	P21 Protein (Cdc42/Rac)-Activated Kinase 1
SLC7A5	-	Solute Carrier Family 7 Member 5
SNCA	-	Synuclein Alpha
WNT3A	-	Wingless-Type MMTV Integration Site Family, Member 3A
α-syn	-	α-synuclein

1. Introduction

Manganese (Mn) is the most prevalent transition metal in the environment but is only required in trace amounts for normal growth and brain development (Sandstead, 1986). Mn is an essential cofactor for various enzymes involved in carbohydrate and protein metabolism and for antioxidant enzymes such as superoxide dismutase (SOD) (Guilarte TR, 2013). Mn levels are tightly regulated in the human body and exposure to elevated concentrations cause manganism, a neurological disorder with clinical symptoms resembling those of idiopathic Parkinson's disease (Racette BA et al., 2012a, 2001b). Mn can be an occupational healthrisk. For example, miners and workers in ferroalloy, smelting and metallurgy industries are often exposed to Mn, primarily

through inhalation (Finkelstein MM, 2007; Lucas E.L et al., 2015). It is possible that rural populations are at greater risk of developing Parkinson's disease considering their frequent exposure to Maneb and Paraquat, pesticides containing Mn (Ritz et al, 2000; Roede JR et al., 2011). Also populations that depend Mn contaminated drinking water at levels exceeding the WHO regulatory standard (400μg/L) have been shown to express increased risk of neurological deficits (WHO, 2006;Ljung K, 2007). Elevated levels of Mn can also be released into the atmosphere as a combustion product since methylcyclopentadienyl manganese tricarbonyl (MMT) is used to improve fuel oil combustion (Crump, 2000; Su C et al., 2015). High dosage consumption of Mn containing psychoactive formulations have been associated with parkinsonism (de Bie R et al., 2007).

The globus pallidus is a primary site for Mn accumulation within the brain while other studies also suggests deposition in the substantia nigra, a brain structure that produces dopamine (Park JD et al., 2007). Progressive loss of dopaminergic neurons in the substantia nigra due to Mn accumulation could be a pathophysiological cause of parkinsonism (Peneder TM et al., 2011). Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons and the presence of Lewy neurites (LNs) and proteinaceous inclusions known as Lewy bodies (LBs) in dopaminergic neurons (Hashimoto, M et al., 2009; Recchia A et al., 2004). A major component of LBs are peptides encoded by α-Synuclein (SNCA) (Spillantini M. G et al., 1997). Altered SNCA expression levels have been found to be associated with several neurodegenerative diseases including Parkinson's disease (Recchia A et al., 2004). Mn exposure can induce overexpression of SNCA, causing neuronal apoptosis (Cai T et al., 2010; Jason P, 2011; Li Y et al., 2010). The upstream regulatory events leading to SNCA over-expression have not been well

established. Our previous work suggests the possible role of Mn induced epigenetic changes in parkinsonism through mitochondrial dysfunction (Tarale et al., 2017, 2016).

MicroRNAs (miRNAs) are 17–24 base small non-coding, single stranded RNAs that regulate gene expression post-transcriptionally via binding to the 3'-untranslated region (UTR) of mRNAs, thereby repressing the translation process (Gartel AL, 2008; He L, 2004). Dysregulation of miRNA expression was recently identified as contributing to a higher risk of neurodegeneration (Johnson R et al., 2012). Altered expression of miRNAs, such as miR-29a/b-1, miR-19, miR-101 and miR-130 has been associated with neurodegenerative disorders (Hébert SS et al., 2008). Additionally, miR-7 and mir-433 have been found to regulate the expression of SNCA in normal and PD brain, as well as in animal models of PD, for example, the worm *C. elegens* (Asikainen S, Rudgalvyte M, 2010; Gillardon F, Mack M, 2008; Junn E et al., 2009; Vartiainen S et al., 2006; Wang G et al., 2008). The potential role of miRNAs in manganese exposed cell models and the implications in the etiology of Parkinson's disease have not been yet explored.

In the present investigation we profiled the expression of 84 different miRNAs known to be differentially expressed during neuronal development or in neurodegeneration. We used a miRNA PCR array and measured expression changes in the dopaminergic human neuroblastoma cell line SH-SY5Y following a chronic manganese exposure. This represents the first study to demonstrate a connection between Mn exposure, miRNA deregulation and Parkinson's disease.

2. Material and Methods

2.1. Cell line

Human neuroblastoma (SH-SY5Y) cell line was obtained from National Center for Cell Science (NCCS), Pune, India. Cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 50 μg/mL streptomycin, and 25 μg/mL Fungizone. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Chronic Mn^{2+} exposure

SH-SY5Y cells were seeded in 25mm^2 flask (5 x10^5 cells/ml) and grown to 70% confluence when they were exposed to 100 μM of Mn^{2+} in the form of $MnCl_2$ (Sigma Aldrich, USA). The Concentration was selected from the dose response relationship (24hrs) for Mn exposed SH-SY5Y cells (Supplementary figure 1). This is also within the range of concentration from previous studies that used human astrocytes and human brain samples. Mn concentrations shown to be toxic are in the range 60.1-158.4 μM (Bowman AB., and Aschner, 2014). Mn exposures ≥50 μM (from 0 to 100μM) have been found to impact the redox processes in human neuroblastoma cell mitochondria corresponding to toxic range of Mn in human brain (Fernandes et al., 2017). Three replicates of controls (culture medium only) and three replicates of Mn-exposed cultures were maintained for a period of 30 days. The medium was changed every 72 hours after each passage throughout the period of exposure. Mn^{2+} was added after each cell passage after cells started to adhere to the surface of the flasks (it took approximately 3-4 hours for cells to adhere). We also evaluated the viability of cells at each cell passage using a trypan blue exclusion assay (Supplementary figure 2). The viability of exposed cells remains at ~85%

throughout the period of chronic exposure. This indicates there is not a development of tolerance for Mn over the period of exposure.

2.3. miRNA profiling

At the end of the exposure period, total miRNA was harvested from the cells using the mirPremier microRNA Isolation Kit (Sigma Aldrich, USA) as per the manufacturer protocol. miRNA was quantified using a NanoDrop 8000 spectrophotometer. miRNA profiling was carried out using the Qiagen human neurological development and disease miScriptmiRNA PCR array, to analyse the differential expression of 84 miRNAs involved in neuronal development or progression of neurological diseases (Supplementary table 1). 12 wells of each miScriptmiRNA PCR array plate were controls (*SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6-6P*). These controls were used for normalization of RT-PCR data for endogenous miRNAs in the samples. 200 ng of miRNA was converted to cDNA using miScript II RT kit (Qiagen, Germany) as per the manufacturer instructions. Real time PCR for mature miRNA expression profiling was performed using diluted cDNA according to the manufacturer's protocol. Applied Biosystems 7300 real time PCR system was used for RT-PCR. Data were collected to perform quantification using the ΔΔCt method of relative quantification and interpretation of control wells (Tarale et al., 2017).

2.4. *In silico* Hybridisation Analysis

In silico hybridisation studies were performed using the mirTarBase and miRANDA software (August 2010 release; http://www.microrna.org/microrna/home.do) to screen and predict possible mRNA targets of differentially expressed miRNAs. miR-433-3p and miR-7-5p were selected for this *in silico* analysis based on their significant differential expression detected

in our study and their established role in Parkinson's disease (Wang G et al., 2008) (Supplementary table 1). Additionally, other miRNAs were screened for their possible mRNA targets. Supplementary file 1 gives the list of these miRNAs along with their target genes and associated biological functions. The list of potential target genes was submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 for functional annotation clustering.

2.5. Real time quantitative PCR

Validation of screened miRNAs (miR-433-3p/miR-7-5p) was carried out using quantitative real time PCR (qRT-PCR). Isolation of miRNAs was carried using the mirPremier microRNA Isolation Kit (Sigma Aldrich, USA) as per the manufacturer protocol. 500 ng of miRNA was converted to cDNA using the NCode VILO miRNAcDNA Synthesis Kit (Invitrogen, USA). Relative quantification of miRNA was performed on Applied Biosystems 7300 real time PCR system using EXPRESS SYBR GreenERqPCRSuperMix containing universal reverse primers (Invitrogen, USA) and forward customized primers of miR-433-3p/miR-7-5p (Table 1). RUN48 was used as an internal control in the real time qRT-PCR (Nurul et al., 2012). ddCT (2^-ddCt) method was used for calculation of fold change.

For expression-validation of possible mRNA targets, total RNA was isolated using the Trizol (Invitrogen, USA) reagent. RNA was quantified using a NanoDrop 8000 spectrophotometer. High Capacity Reverse Transcription kit (Applied Biosystems, USA) was used for converting 2 μg of total RNA to cDNA. qRT-PCR primers for SNCA and FGF-20 were used (Table 1) and validated before relative quantification. RNA polymerase II (RPII) and hypoxanthine phosphoribosyltransferase (HPRT) were used as internal controls (Sain S et al., 2014). Relative

quantification was performed on Applied Biosystems 7300 real time PCR system using EXPRESS SYBR GreenERqPCRSuperMix. Fold change in expression was calculated using the ddCT (2∧-ddCt) method.

2.6. miRNA mimics transfection

mirVana^{TM}miRNA mimics are small, double stranded RNAs that mimic endogenous precursor miRNAs. They increase potency and specificity for enhanced performance in gain of function analysis of miRNAs. hsa-miR-7-5p (mature miRNA sequence-UGGAAGACUAGUGAUUUUGUUGU) and has-miR-433-3p (mature miRNA sequence-AUCAUGAUGGGCUCCUCGGUGU) mimics were purchased from Invitrogen, USA. Transfection efficiency for SH-SY5Y cell line was calculated using Block iTAlexa Fluor Red (Invitrogen, USA). BlockiTAlexa Fluor Red fluorescence (50nM) along with Lipofectamine-2000 (Invitrogen, USA) (1 µl/25µl OptiMEM medium) was transfected to SH-SY5Y cells seeded in 6-well flat bottom plates at 70% confluence level. 4 hrs post incubation after transfection, cells were imaged with fluorescence microscope (Olympus, USA).

SH-SY5Y cells were seeded in 25mm^2 culture flasks at a density of 5x10^5 cells/ml for transfection with miRNA mimics. At 70% confluence cells were transfected with 50nM miRNA mimics (hsa-miR-7-5p ,has-miR-433-3p, mirVanaInvitrogen, USA). Lipofectamine-2000 (Invitrogen, USA) was used as transfection agent at the concentration of 1 µl/25µl in OptiMEM medium. After a 6hr incubation, OptiMEM was replaced with Ham's F-12 medium (2% serum) followed by treatment with Mn^{2+} (100µM) for 24h. After incubation, miRNAs were isolated using mirPremier microRNA isolation kit (Sigma Aldrich, USA) and total RNA was isolated

using Trizol (Invitrogen, USA). qRT- PCR was carried out for miRNAs and RNA as discussed in 2.5.

2.7. Statistical analysis

Student's *t* test (paired, 2-tailed) in Microsoft Excel was used to determine the statistical significance between the values in control and exposed samples.

3. Results

3.1 Deregulation of miRNA expression is implicated in critical neuronal pathways

The miRNAs sampled by the miScriptmiRNA PCR array include a set of miRNAs known to be associated with various neuronal disorders (Supplementary table 1). miRNA expression data were obtained for a total of 84 miRNAs and their differential expression visualized by a heat map (figure 1). Our results indicate significantly altered expression levels of 24 miRNAs ($p<0.05$), six were up-regulated in expression, while the rest were down-regulated (Figure 2A). To identify target genes from the misregulated miRNAs, we screened the online *in-silico* databases mirTarBase and miRANDA. The results of the screen yielded a list of 80 target genes whose mRNAs serve as putative binding sites for the 24 misregulated miRNAs. (Supplementary file 1). The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (https://david.ncifcrf.gov/) was used for functional annotation clustering of putative target genes.

DAVID analysis indicates that these putative target genes are primarily involved in processes of apoptosis (21%), gene expression (21%), phosphorylation (19%) and synaptic transmission

(10%) (Figure 2B). We narrowed our screen of 80 putative miRNA target genes and focused on genes known to be associated with Parkinson's disease (Supplementary table 1). *NTRK3, SNCA, PAK1, SLC7A5 have* been identified as putative target genes for miR-7. *WNT3A, BCL2, CCND1, CCNE1, CRKL, HMGA1, FGF7, FOXO1* and *FGF20, GRB2* have been identified as putative target genes for miR-15A and miR-433, respectively. For the rest of the study, we concentrated on *SNCA* and *FGF-20,* two genes known to play critical roles in the development of Parkinson's disease.

3.2 miR-7 and miR-433 identified as regulators of SNCA expression

An *in-silico* homology analysis identified *SNCA* and *FGF-20* as putative target genes for miR-7 and miR-433, respectively (Junn E et al., 2009) (Figure 3A). miR-7 and miR-433 are predicted to bind the 3'-UTR regions of SNCA and FGF-20 and thereby could interfere with the mRNA translation process. These two miRNAs have been reported to play a role in the pathophysiology of Parkinson's disease (Wang et al., 2008; Doxakis., 2010;).

To validate miR-7 and miR-433 expression levels and those of their potential targets, SNCA and FGF-20, we performed qRT-PCR. The results indicate significant down-regulation ($p<0.05$) of miR-7(-2.13 ± 0.24), and miR-433 (-4.85 ± 0.30) following Mn exposure (Figure 3B). In the same cells, the qRT-PCR results show that Mn exposure resulted in up-regulated expression of both, SNCA (4.02 ± 0.16) and FGF-20 (2.61 ± 0.22) (Figure 3C). SNCA is considered to play a critical role during the onset of Parkinson's disease. It has also been shown that the expression of *SNCA* may be indirectly regulated by FGF-20 (Wang G et al., 2008). The diminished expression of miRNA-433 causing elevated levels of FGF 20 may therefore also influence the regulation of

SNCA expression. The present results imply a role for miR-7 and miR-433 in regulating *SNCA* expression.

3.3 Transfection with miRNA mimics down-regulates SNCA expression

To validate and further explore the roles of miR-7 and miR-433 in regulating the expression of SNCA, we transfected manganese-exposed SH-SY5Y cells with miRNA mimics for mir-7 and miR-433. Transfection efficiency for SH-SY5Y cells was greater than 90% (Figure 4A). qRT-PCR for miR-7 and miR-433 after transfection with miRNA mimics shows an decrease in expression with fold changes of 3.34±0.24 and 4.35±0.30, respectively ($p<0.05$) (Figure 4B). Subsequent qRT-PCR analysis for *SNCA* and *FGF-20* showed that transfection with miRNA mimics correlated with reduced expression of *SNCA* (Figure 4C). Transfection with miR-7 mimics causes significant down-regulation in the expression level of *SNCA* (fold change -2.45±0.18) (Figure 4D). This observation supports the concept that miR-7 has a direct role in regulating the expression of *SNCA*. Transfection with miR-433 mimic down-regulates the expression of *SNCA* and *FGF-20* with fold changes of -1.29 ± 0.18 and -1.98 ± 0.34 respectively ($p<0.05$) (Figure 4C and D). The observed down-regulation in the expression of SNCA upon transfection with mimics for miR-433 strengthens the hypothesis that *FGF-20* indirectly regulates *SNCA* expression.

4. Discussion

Manganese is an crucial trace element however chronic environmental and occupational exposure reported to cause neurological deficits with symptoms clinically resembling to idiopathic Parkinson's disease (Su C et al., 2015). Elevated expression of SNCA considered to

be toxic for dopaminergic neuronal cells and frequent molecular pathological findings in brain tissue samples from Parkinson's disease patients (Recchia A et al., 2010). Various genetic and environmental factors may have the potential to cause elevated SNCA expression (reviewed in Tarale et al., 2016). Mn exposure has been reported to cause over-expression of SNCA in the neuronal SH-SY5Y cell line (Li Yet al., 2009). Our work provides evidence that Mn-exposure leads to deregulation of certain miRNAs. These miRNAs and their potential target mRNAs regulate apoptosis and synaptic transmission, processes which are critically important for neuronal cell health (Figure 2A and B).

Among the list of deregulated miRNAs, we selected miR-7 and miR-433 to further dissect their role in manganese neurotoxicity. Expression of miR-7 and miR-433 was down-regulated following Mn exposure (Figure 3B). We also identified *SNCA* and *FGF-20* as putative targets of miR-7 and miR-433 (figure 3A).

The upstream mechanisms causing down-regulation of miR-7 and miR-433 are unknown. However, Mn exposure is known to cause neuronal cell apoptosis through oxidative stress mechanisms (Stephenson et al., 2013; Fernandes et al., 2017). Alterations in the redox balance of primary human brain cells exposed to iron-plus aluminum-sulfate can cause deregulation of specific miRNAs via ROS generation (Lukiw and Aileen, 2007). We hypothesise that oxidative stress and its effect on redox sensitive transcription factors may lead to altered expression of miRNAs, which in turn influence a gene programme important in PD.

miR-7 is a conserved gene enriched in brain and known to regulate neuronal differential and synaptic plasticity (Doxakis, 2010). Reduction in the expression level of miR-7 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mice (a model of PD) has been reported to cause an elevation in SNCA expression and degeneration of the nigrostriatal system (Shasi et al.,

2000). Mn-containing neurotoxins, including MPTP leads to a demise of dopaminergic neurons in mice (*Musmusculus*) and nematode worms (*C. elegans*) (Cannon JR and Greenamyre, 2010). Our present study suggests a role for miR-7 in Mn-induced neurodegeneration by causing SNCA overexpression.

Fibroblast growth factor 20 (FGF-20) is expressed in the substangia nigra and may preserve the function of neurotrophic activity in dopaminergic neurons (Ohta, 2013). *FGF20* has been suggested as another candidate gene responsible for Parkinson's disease in Japanese and Chinese populations (Pan et al., 2012); though not in Finnish and Greek populations (Satake et al., 2008; Christian et al., 2009).

A sequence polymorphism in the 3' UTR of FGF20 elevates the risk of Parkinson's disease and is most likely caused by disruption of a binding site for microRNA-433 (Wang G et al., 2008). Homozygosity of this *FGF-20* variant is not only associated with an elevation of *FGF-20* expression, but also with an increase in SNCA protein levels (Wang G et al., 2008). In the present work we show that elevated *FGF-20* expression is closely associated with decreased miR-433 levels.

Our transfection study with miRNA mimics strengthens the evidence that manganese exposure may cause elevation of SNCA expression via alteration in miR-7 and miR-433 levels. It will be of interest to replicate and confirm our results using animal models. The present findings indicate that two neurodegenerative disorders - manganism and idiopathic Parkinson's disease may result from misregulation of the same genetic network, which includes altered expression of miRNAs. Our results raise the possibility of using miR-7 and miR-433 as therapeutic targets to prevent or treat manganese induced neurotoxicity.

5. Conclusion

The present study identified a selection of miRNAs which are deregulated upon Mn-exposure in a neuronal cell line. This list of miRNAs provides molecular entry points to further explore their regulatory roles in Mn-induced neurodegeneration. A key finding is that down-regulation of miR-7 and miR-433 is closely associated with elevated SNCA expression. We provide the first indication that manganism and Parkinson's disease share common molecular mechanisms, including deregulation of miRNAs which may be targeted therapeutically.

Figure legends

Figure 1. Heatmap clustering of 84 miRNAs involved in neuronal development or progression of neurological diseases. Red and green colors represent the magnitude of expression.

Figure 2. miScriptmiRNA PCR array profiling of miRNA expression in SH-SY5Y cells chronically exposed to manganese (100 μM) **A)** Expression profile for 24 miRNAs differentially expressed at statistically significant levels ($p<0.05$). **B)** DAVID based function annotation and clustering showing the major biological pathways associated with the target genes of deregulated miRNAs. Predictions are based upon *in-silico* homology using mirTarBase and miRANDA online softwares.

Figure 3. A) *In-silico* hybridization analysis of miR-7 and miR-433 with SNCA and FGF-20.Validation of expression level of miRNA and their target genes by qRT-PCR **B)** Expression levels of miR-7 and miR-433 and **C)** Expression levels of SNCA and FGF-20 in manganese treated samples were normalized with the control.

Figure 4. A) Transfection efficiency of SH-SY5Y cells were determined using Block iTAlexa Fluor Red **B)** Expression profile of miR-7 and miR-433 after transfection with miRNA mimics. **C)** Expression profile of SNCA after transfection with miRNA mimics in manganese exposed cells. **D)** Expression of FGF-20 post-transfection with mimics in manganese exposed cells. Expression of SNCA and FGF-20 were normalized with controls in manganese treated samples.

Table 1.qRT-PCR primers

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

Asikainen S, Rudgalvyte M, H.L. et al., 2010. Global microRNA expression profiling of caenorhabditis elegans Parkinson's disease models. J. Mol. Neurosci. 41, 210–218.

Bowman, A. B., and Aschner, M., 2014. Considerations on manganese (Mn) treatments for in vitro studies. Neurotoxicology 41, 141–142.

Cai T, Yaoa T, Zhenga G, Chena Y, Dua K, Caob Y, Shena X, Chena J, L.W., 2010. Manganese induces the overexpression of α-synuclein in PC12cells via ERK activation. Brain Research. Brain Res. Bull. 1359, 201–210. doi:doi:10.1016/j.brainres.2010.08.055

Cannon JR and Greenamyre, 2010. Neurotoxic in vivo models of Parkinson's disease recent advances. Prog Brain Res 184, 17–33.

Christian Wider, Justus C. Dachsel, Alexandra I. Soto, Michael G.Heckman, Nancy N. Diehl, Mei Yue, Sarah Lincoln, Jan O. Aasly, Kristoffer Haugarvoll, John Q. Trojanowski, Spiridon Papapetropoulos, Deborah Mash, Alex Rajput, Ali H. Rajput, J. Mark Gibson, and O.A.R., 2009. FGF20 and Parkinson's disease: No evidence of association or pathogenicity via α-synuclein expression. Mov Disord 24, 455–459. doi:doi:10.1002/mds.22442

Crump, K.S., 2000. Manganese exposures in Toronto during use of the gasolineadditive, methylcyclopentadienyl manganese tricarbonyl. J.Expo. Anal. Env. Epidemiol 10, 227–239.

de Bie, R. M., Gladstone, R. M., Strafella, A. P., Ko, J. H., and Lang, A.E., 2007. Manganese-Induced Parkinsonism Associated With Methcathinone (Ephedrone) Abuse. Arch. Neurol 64, 886–889.

E, D., 2010. Post-transcriptional regulation of α-synuclein expression by mir-7 and mir-153. J.

Biol. Chem. 285, 12726–12734.

Fernandes Jolyn, Hao Li, Bijli Kaiser M., Chandler Joshua D., O.M., Hu Xin, Jones Dean P., and G.Y.-M., 2017. Manganese Stimulates Mitochondrial H2O2 Production in SH-SY5Y Human Neuroblastoma Cells Over Physiologic as well as Toxicologic Range. Toxicol. Sci. 155, 213–223.

Finkelstein MM, J.M., 2007. A study of the relationships between Parkinson's disease and markers of traffic-derived and environmental manganese air pollution in two Canadian cities. Env. Res 104, 420–432.

Gartel AL, K.E., 2008. miRNAs: Little known mediators of oncogenesis. Semin Cancer Biol 18, 103–110.

Gillardon F, Mack M, R.W. et al., 2008. MicroRNA and proteome expression profiling in early-symptomatic α-synuclein(A30P)-transgenic mice. Proteomics 2, 697–705.

Hashimoto, M., Hsu, L. J., Xia, Y., Takeda, A., Sisk, A., Sundsmo, M., and Masliah, E., 1999. Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. Neuroreport 10, 717–721.

He L, H.G., 2004. MicroRNAs: Small RNAs with a big role in gene regulation. Nat Rev Genet 5, 522–531.

Hébert SS1, Horré K, Nicolaï L, Papadopoulou AS, Mandemakers W, Silahtaroglu AN, Kauppinen S, Delacourte A, D.S.B., 2008. Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/-secretase expression. Proc Natl Acad Sci USA 105, 6415–6420.

Jason P. Covy, B.I.G., 2011. α-Synuclein, leucine-rich repeat kinase-2, and manganese in the pathogenesis of parkinson disease. Neurotoxicology 32, 622–629.

Jing Pan, Hui Lia, Ying Wanga, Jian-Fang Maa, Jin Zhanga, Gang Wanga, Jun Liua, Xi-Jin Wanga, Qin Xiaoa, S.-D.C., 2012. Fibroblast growth factor 20 (FGF20) polymorphism is a risk factor for Parkinson's disease in Chinese population. Park. Relat. Disord 18, 629–631. doi:doi: 10.1016/j.parkreldis.2012.01.017

Johnson R, Noble W, Tartaglia GG, B.N., 2012. Neurodegeneration as an RNA disorder. Prog. Neurobiol. 99. doi:doi:10.1016/j.pneurobio.2012.09.006

Jolyn Fernandes, Li Hao, Kaiser M. Bijli, Joshua D. Chandler, M.O., Xin Hu, Dean P. Jones, and Y.-M.G., 2017. Manganese Stimulates Mitochondrial H2O2 Production in SH-SY5Y Human Neuroblastoma Cells Over Physiologic as well as Toxicologic Range. Toxicol. Sci. 155, 213–223.

Junn E, Lee KW, Byeong SJ, Chan TW, Im JY, M.M., 2009. Repression of α-synuclein expression and toxicity by microRNA-7. Proc. Natl Acad. Sci 106, 13052–13057.

Li Y, Sun L, Cai T, Zhang Y, Lv S, W.Y., 2010. α-Synuclein overexpression during manganese-induced apoptosis in SH-SY5Y neuroblastoma cells. Brain Res Bull 81, 428–433.

Li Y, Sun L, Cai T, Zhang Y, Lv S, W.Y., Li Y , Sun L, Cai T, Zhang Y, Lv S, Wang Y, Y.L., 2009. α-Synuclein overexpression during manganese-induced apoptosis in SH-SY5Y neuroblastoma cells. Brain Res Bull 81, 428–433.

Ljung K, V.M., 2007. Time to re-evaluate the guideline value for manganese in drinking water? Env. Heal. Perspect 115, 1533–1538.

Lucas E.L., Bertrand P., Guazzetti S., Donna F., Peli M., TJursa.P., L.R., D.R., S., 2015. Impact of ferromanganese alloy plants on household dust manganese levels: Implications for childhood exposure. Environ. Res. 138, 279–290.

MR Cookson, 2009. α-Synuclein and neuronal cell death. Mol. Neurodegener. 4. doi:doi:10.1186/1750-1326-4-9

Ohta, N.I. and H., 2013. Roles of FGF20 in dopaminergic neurons and Parkinson's disease. Front Mol Neurosci 6. doi:doi: 10.3389/fnmol.2013.00015

Park JD, Chung YH, Kim CY, Ha CS, Yang SO, K.H. et al, 2007. Comparison of high MRI T1 signals with manganese concentration in brains of cynomolgus monkeys after 8 months of stainless steel welding-fume exposure. Inhal. Toxicol. 19, 965–971.

Peneder TM., Scholze P, Berger ML, Reither H, Heinze G, Bertl, J, Bauer J, Richfield EK, H.O. and P.C., 2011. Chronic exposure to manganese decreases striatal dopamine turnover in humans a-synuclein transgenic mice. Neuroscience 180, 280–292.

Racette B. A., McGee-Minnich L., Moerlein S. M., Mink J. W., Videen T. O., P.J.S., 2001. Welding-related Parkinsonism: clinical features, treatment, and pathophysiology. Neurology 56, 8–13.

Racette BA, Criswella SR, Lundinb JI, Hobsona A, Seixasb N, Kotzbauera PT, Evanoffc BA, Perlmuttera JS, Zhangd J, Sheppardf L, C.H., 2012. Increased risk of parkinsonism associated with welding exposure. Neurotoxicology 33, 1356–61. doi:.doi:10.1016/j.neuro.2012.08.011.

Recchia A., Debetto P., Negro A., Guidolin D., Skaper S. D., G.P., 2004. α-Synuclein and

Parkinson's disease. FASEB J. 18, 617–626.

Ritz, B., and Yu, F., 2000. Parkinson's disease mortality and pesticide exposure in California 1984–1994. Int. J. Epidemiol 29, 323–329.

Roede JR, Hansen JM, Young-Mi Go, J.D., 2011. Maneb and Paraquat-Mediated Neurotoxicity: Involvement of Peroxiredoxin/Thioredoxin System. Toxicol. Sci. 121, 368–375. doi:doi:10.1093/toxsci/kfr058

S. A. Nurul, L. H. Lee, M. S. Shiran, A. R. Sabariah, M. S. Avatarsingh, Y.K.C., 2012. miR-181a regulates multipl pathways in hypopharyngeal squamous cellc arcinoma. African J. Biotechnol. 11, 6129–6137.

Sain S, Naoghare PK, Devi SS, Daiwile A, Krishnamurthi K, Arrigo P, C.T., 2014. Beta caryophyllene and caryophyllene oxide, isolated from Aegle marmelos, as the potent anti-inflammatory agents against lymphoma and neuroblastoma cells. Antiinflamm. Antiallergy. Agents Med. Chem. 13, 45–55.

Sandstead, H.H., 1986. A brief history of the influence of trace elements on brain function. Am. J. Clin. Nutr 43, 293–298.

Satake W, Nakabayashi Y, Mizuta I, Hirota Y, Ito C, Kubo M, Kawaguchi T, Tsunoda T, Watanabe M, Takeda A, Tomiyama H, Nakashima K, Hasegawa K, Obata F, Yoshikawa T, H.Y. and T.T., 2008. Calbindin 1, Fibroblast growth factor 20, and –synuclein in sporadic Parkinson's disease. Hum Genet 124, 89–94. doi:DOI 10.1007/s00439-008-0525-5

Schulz-Schaeffer, W.J., 2010. The synaptic pathology of alpha-synuclein aggregation in dementia with Lewy bodies, Parkinson's disease and Parkinson's disease with dementia.

Acta Neuropathol 120, 131–143.

Shasi V. Kalivendi, Sonya Cunningham, Srigiridhar Kotamraju, Joy Joseph, C.J.H. and B.K., 2004. α-synuclein up-regulation and aggregation during MPP-induced apoptosis in neuroblastoma cells. J Biol Chem 279, 15240–15247. doi:doi: 10.1074/jbc.M312497200

Spillantini M. G., Schmidt M. L., Lee V. M., Trojanowski J. Q., Jakes R., G.M., 1997. Alpha-synuclein in Lewy bodies. Nature 338, 839–840.

Stephenson AP, Schneider JA, Nelson BC, Atha DH, Jain A, Soliman KF, Aschner M, Mazzio E, R.R., 2013. Manganese-Induced Oxidative DNA Damage in Neuronal SHSY5Y Cells: Attenuation of thymine base lesions by glutathione and N-acetylcysteine. Toxicol Lett 218, 299–307.

Su C, Chen K, Zou Y, Shen Y, Xia B1, Liang G, Lv Y, Wang F, Huang D, Y.X., 2015. Chronic exposure to manganese sulfate leads to adverse dose-dependent effects on the neurobehavioral ability of rats. Env. Toxicol. doi:doi: 10.1002/tox.22161

Tarale, P., Chakrabarti, T., Sivanesan, S., Naoghare, P., Bafana, A., Krishnamurthi, K., 2016. Potential Role of Epigenetic Mechanism in Manganese Induced Neurotoxicity. Biomed Res. Int. 2016, 1–18. doi:http://dx.doi.org/10.1155/2016/2548792

Tarale, P., Sivanesan, S., Daiwile, A.P., Stöger, R., 2017. Global DNA methylation profiling of manganese‐exposed human neuroblastoma SH‐SY5Y cells reveals epigenetic alterations in Parkinson ' s disease associated genes. Arch. Toxicol. doi:10.1007/s00204-016-1899-0

TR, G., 2013. Manganese neurotoxicity: New perspectives from behavioral, neuroimaging and neuropathological studies in humans and non-human primates. Front. Aging Neurosci 5, 1–

10.

Vartiainen S, Pehkonen P, Lakso M, Nass R, W.G., 2006. Identification of gene expression changes in transgenic C.elegans overexpressing human α-synuclein. Neurobiol. Dis. 22, 477–486.

Vila M1, Vukosavic S, Jackson-Lewis V, Neystat M, Jakowec M, P.S., 2000. α-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. J Neurochem 76, 721–729.

Walter J. Lukiw and Aileen I., 2007. Induction of specific micro RNA (miRNA) species by ROS-generating metal sulfates in primary human brain cells. J. Inorg. Biochem. 101, 1265–1269. doi:doi:10.1016/j.jinorgbio.2007.06.004

Wang G, van der Walt J.M, Mayhew G, Li Y.J, Zuchner S, Scott W.K, M.E.R. and V.J.M., 2008. Variation in the miRNA binding site of FGF20 confers risk for Parkinson disease by overexpression of a-synuclein. Am. J. Hum. Genet 82, 283–289.

WHO, 2006. Guidelines for Drinking-Water Quality [electronic resource]: Incorporating First Addendum. Available: http://www.who.int/water_sanitation_health/dwq/gdwq0506begin.pdf [accessed 26 January 2007].

Yoontae Lee, Rodney C. Samaco, Jennifer R. Gatchel, Christina Thaller, Harry T. Orr, H.Y.Z., 2008. miR-19, miR-101 and miR-130 co-regulate ATXN1 levels to potentially modulate SCA1 pathogenesis. Nat Neurosci 11, 1137–1139.

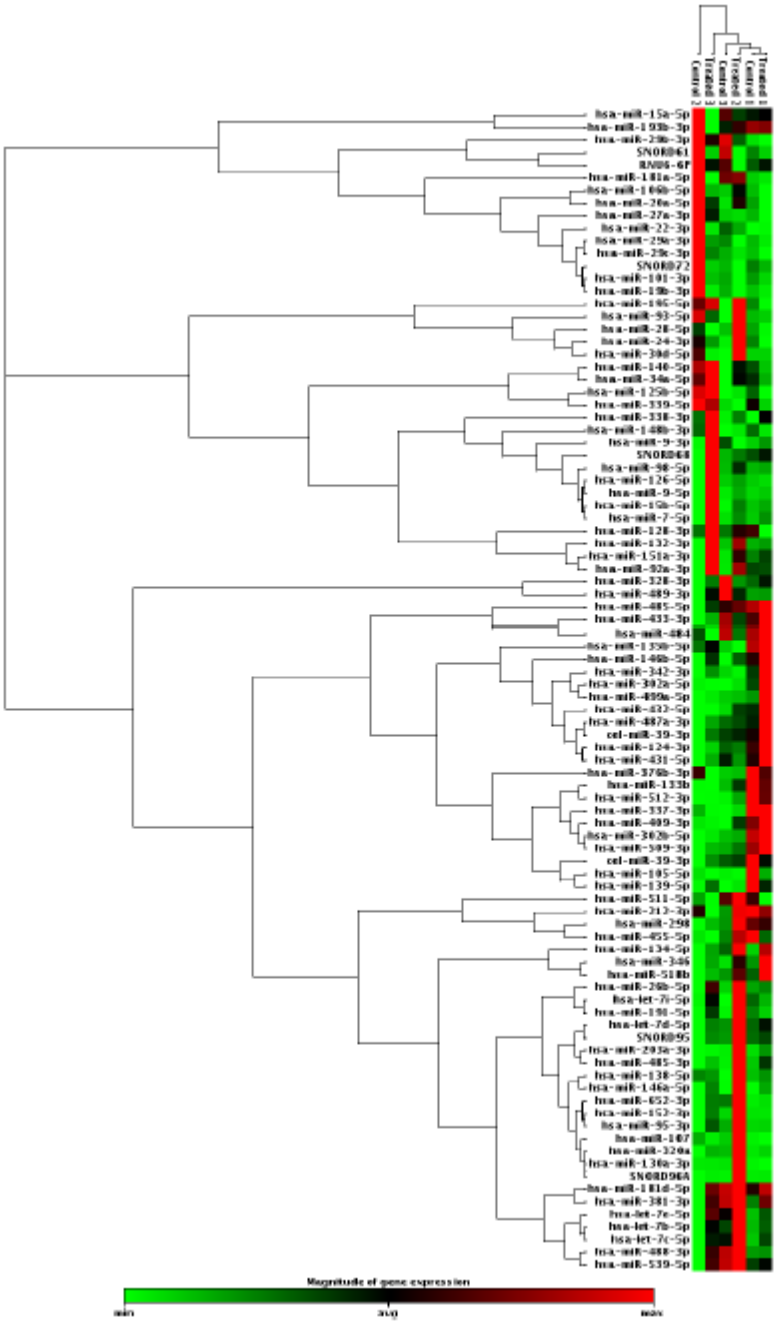


Fig. 1

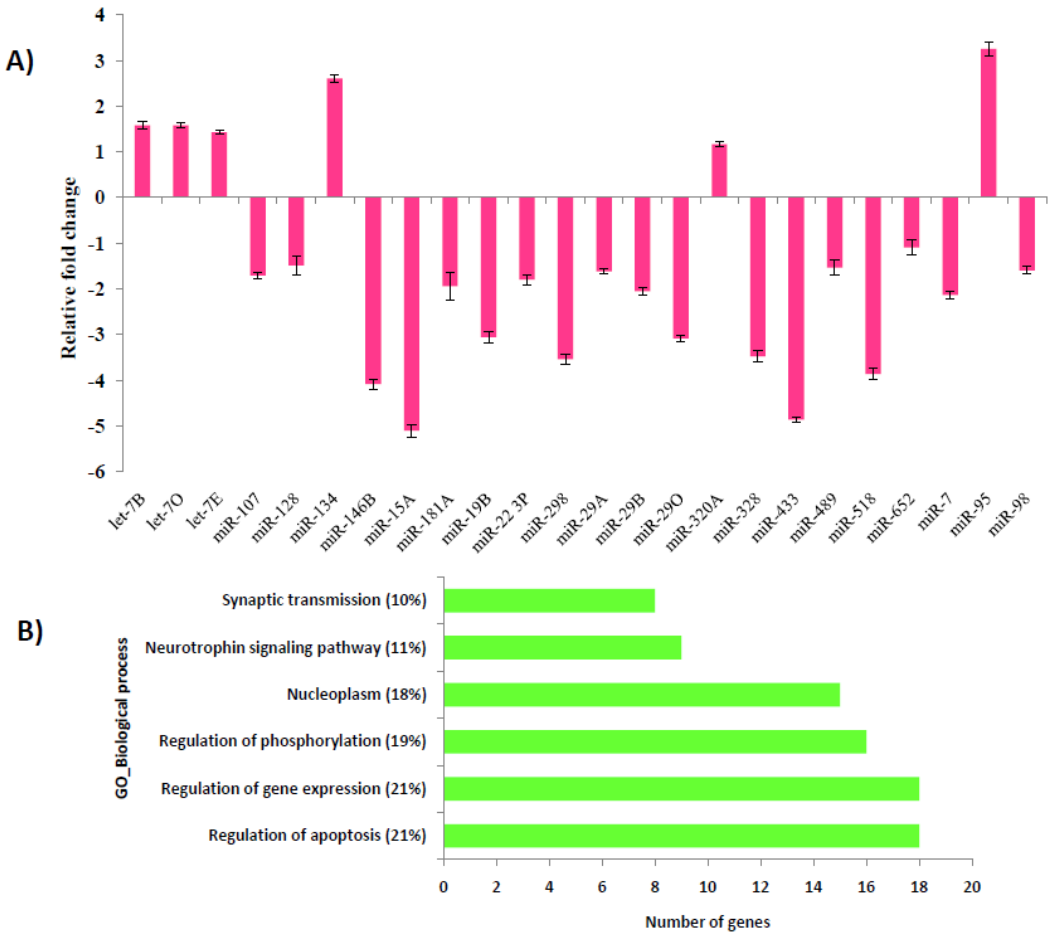


Fig. 2

A)

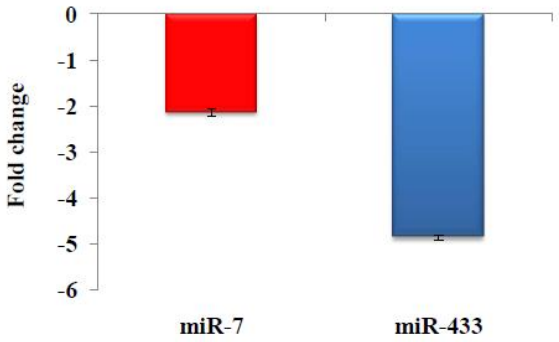
```
      3' uguUGUUUUAGUGA---UCAGAAGGu 5' hsa-miR-7
            |||: :|   ||    ||||||||
102:5' uuuACAGUGUAUCUCGAAGUCUUCCa 3' SNCA
```

Human α-Syn mRNA containing the predicted conserved target site of miR-7

```
      3' ugugGCUCCUCGGGUAGUACUa 5' hsa-miR-433
            | || | :  |||||||
166:5' uugaCUAGAAAUAGAUCAUGAu 3' FGF20
```

Human FGF20 mRNA containing the predicted conserved target site of miR-433

B)



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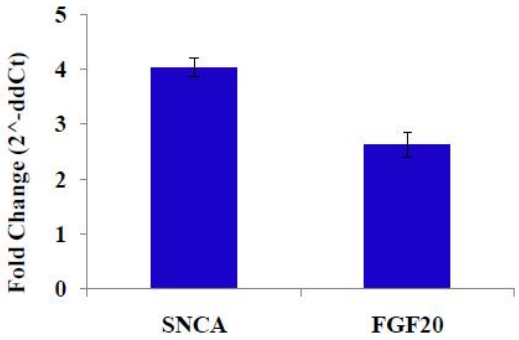

Fig. 3

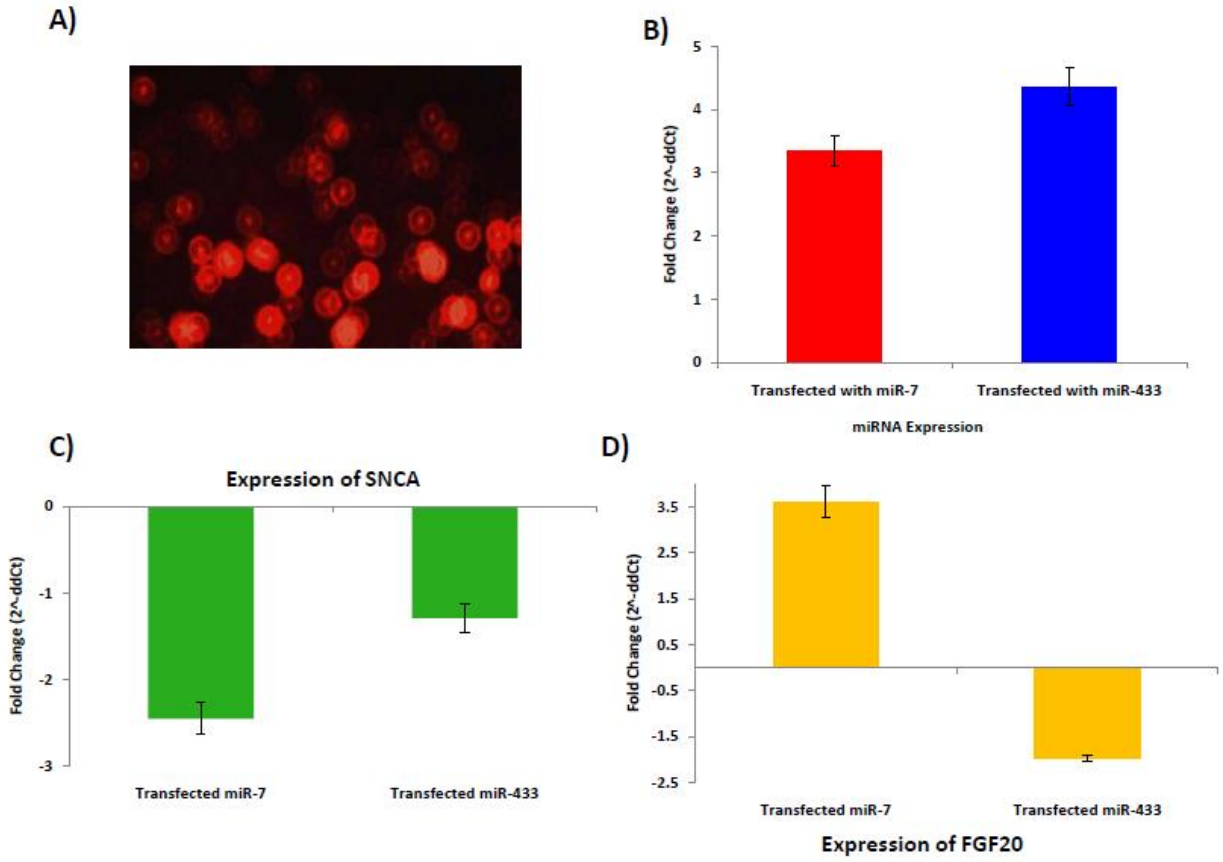

Fig. 4

Table 1: qPCR primers

Sr no	Gene name	Primer	Sequence (5'-3')
		miRNA	
1	miR-433-3p	Forward primer	ATCATGATGGGCTCCTCGGTGT
2	miR-7-5p	Forward primer	TGGAAGACTAGTGATTTTGTTGT
3	RUN48	Forward primer Reverse primer	AGTGATGATGACCCCAGGTAACTC CTGCGGTGATGGCATCAG
		Genes	
4	SNCA	Forward primer	GTGTGGCAACAGTGGCTGAG
		Reverse primer	TGGGGCTCCTTCTTCATTCTTG
5	FGF20	Forward primer	ACAGCCTCTTCGGTATCTTG
		Reverse primer	TCTTGGAGTTCCGTCTTTGT
6	RPII (Internal control)	Forward primer	GCACCACGTCCAATGACAT
		Reverse primer	GTGCGGCTGGTTCCATAA
7	HPRT (Internal control)	Forward primer	ACGAAGTGTTGGATATAAGC
		Reverse primer	ATAATTTTACTGGCGATGTC

Highlights

- Mn causes altered miRNAs expression regulating synaptic transmission and apoptosis
- miR-7 and miR-433 expression is significantly diminished upon chronic Mn exposure
- SNCA and FGF-20 identified as target genes that shows up-regulation in expression
- miRNA mimics transfection confirms the specificity of miRNAs with target genes
- miR-7 and miR-433 are potential therapeutic targets against Mn neurotoxicity