

Original Article

## Alteration of microRNA expressions in the pons and medulla in rats after 3,3'-iminodipropionitrile administration

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**Abstract:** Although 3,3'-iminodipropionitrile (IDPN) is widely used as a neurotoxicant to cause axonopathy due to accumulation of neurofilaments in several rodent models, its mechanism of neurotoxicity has not been fully understood. In particular, no information regarding microRNA (miRNA) alteration associated with IDPN is available. This study was conducted to reveal miRNA alteration related to IDPN-induced neurotoxicity. Rats were administered IDPN (20, 50, or 125 mg/kg/day) orally for 3, 7, and 14 days. Histopathological features were investigated using immunohistochemistry for neurofilaments and glial cells, and miRNA alterations were analyzed by microarray and reverse transcription polymerase chain reaction. Nervous symptoms such as ataxic gait and head bobbing were observed from Day 9 at 125 mg/kg. Axonal swelling due to accumulation of neurofilaments was observed especially in the pons, medulla, and spinal cord on Day 7 at 125 mg/kg and on Day 14 at 50 and 125 mg/kg. Furthermore, significant upregulation of miR-547\* was observed in the pons and medulla in treated animals only on Day 14 at 125 mg/kg. This is the first report indicating that miR-547\* is associated with IDPN-induced neurotoxicity, especially in an advanced stage of axonopathy. (DOI: 10.1293/tox.2016-0019; J Toxicol Pathol 2016; 29: 229–236)

**Key words:** 3,3'-iminodipropionitrile (IDPN), microRNA, axonal swelling, axonopathy, neurotoxicity

### Introduction

3,3'-iminodipropionitrile (IDPN), an industrial intermediate, is known as a potent neurotoxicant. IDPN induces behavioral abnormalities in humans and experimental animals. The behavioral manifestation referred to as “waltzing syndrome,” which includes circling, repetitive head movements, and hyperactivity, has been reported in IDPN-treated rodents<sup>1–3</sup>. The neurotoxic effects caused by IDPN include proximal axonal neuropathy in the nervous system characterized by accumulation of neurofilaments<sup>4–7</sup>. Although IDPN has been widely used in neurotoxic models, its mechanism for brain impairment has not been fully understood.

microRNAs (miRNAs) are small noncoding RNAs (–25 nucleotides) that play significant roles in regulating a diversity of cellular and biological processes, including

growth, development, differentiation, proliferation, the cell cycle, and cell death<sup>8–10</sup>. Recently, it has been reported that exogenous chemicals can alter miRNA expression profiles in the nervous tissues in association with neurotoxicity<sup>11–17</sup>. However, alteration of miRNA expression caused by IDPN has not been reported. The aim of this work was to investigate miRNA expression alterations that contribute to IDPN-induced neurotoxicity. Using an IDPN-induced rat neurotoxicity model, we observed nervous symptoms and performed histopathological examinations including immunohistochemistry for neurofilaments and glial cells in addition to analysis of miRNA expression alterations in the pons and medulla, which are sites highly susceptible to IDPN, by microarray and RT-PCR (reverse transcription polymerase chain reaction).

### Materials and Methods

All experiments were performed in accordance with the Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd.

#### *Animals and housing condition*

Seventy-seven male Crl:CD(SD) rats (6 weeks old, specific pathogen free) were purchased from Charles River Laboratories Japan, Inc. (Shiga, Japan), and housed individ-

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**Table 1.** Antibody and Staining Information for Immunohistochemistry

Antibody	Marker	Host	Clone	Dilution	Antigen retrieval	Company
NF 68	68 kDa neurofilament	Mouse	NR4	× 500	Microwave in tris-edta (ph9.0), 20 min	Sigma-Aldrich, St. Louis, MO, USA
Olig2	Oligodendrocyte	Rabbit	poly	× 500	Microwave in citrate buffer (ph6.0), 20 min	Immuno-Biological Laboratories, Gunma, Japan
GFAP	Astrocyte	Rabbit	poly	× 2,000	100 Mg/ml proteinase k, 10 min	Dako, Glostrup, Denmark
Iba1	Microglia	Rabbit	poly	× 500	Microwave in citrate buffer (ph6.0), 20 min	Wako Pure Chemical Industries, Osaka, Japan

ually in aluminum cages in a room kept at  $24 \pm 2^\circ\text{C}$  and 40 to 70% relative humidity with a 12-hr light–dark cycle. Tap water via automatic stainless steel nozzles and CRF-1 pellet diet (Oriental Yeast Co., Ltd., Tokyo, Japan) were freely available.

### Experimental design

After a one-week acclimation period, rats were divided into 4 groups (14 or 21 animals per group). Each group was given repeated administrations of distilled water as the vehicle or IDPN (>98% purity, Wako Pure Chemical Industries, Osaka, Japan) at 20, 50, or 125 mg/kg/day via oral gavage. Observation points were set as 3, 7, and 14 days after administration for each dose group (4, 6, or 7 animals per observation point). Clinical symptoms were observed daily. Examination of auditory response by checking animals' responses to auditory stimuli (metal clicker) and measurement of body weight were performed on Days 3, 7, and 14.

### Tissue sampling

After observation periods, animals were anesthetized with isoflurane followed by exsanguination. The nervous tissues (brain, spinal cord, and sciatic nerve) were immediately collected. The brains were weighed and sagittally cut. The left half-brains were fixed in 4% paraformaldehyde phosphate buffer solution for histopathology. The right half-brains were regionally dissected, and the pons and medulla were frozen in liquid nitrogen and preserved at  $-80^\circ\text{C}$  in a freezer for total RNA (including miRNA) extraction. Two rats from each group were perfused *in situ* (4% paraformaldehyde phosphate buffer solution) under deep anesthesia, and their nervous tissues were removed and immersed in the same solution for histopathology and immunohistochemistry.

### Histopathology and immunohistochemistry

Fixed whole brains or half-brains were transversely cut at 4 levels (level 1, forebrain; level 2, center of the cerebrum, including the hippocampus; level 3, cerebellum and pons; level 4, medulla), and spinal cords (cervical, thoracic, and lumbar) and sciatic nerves were embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and microscopically examined. Paraffin-embedded perfused brains from animals sacrificed on Day 14 were subjected to immunohistochemistry using anti-glial fibrillary acidic protein (GFAP), anti-ionized calcium-binding adapter mol-

ecule 1 (Iba1), anti-Olig2, and anti-neurofilament 68 (NF-68) antibodies. The staining conditions are listed in Table 1. After incubation with the primary antibodies, sections were treated with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, Japan). Signals were visualized with a DAB substrate kit (Nichirei).

### miRNA microarray analysis

Total RNA extraction from the pons/medulla (Day 7, control and 125 mg/kg groups; Day 14, control, 50, and 125 mg/kg groups) was carried out in accordance with the protocol for a mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA). Each total RNA sample in the control and 125 mg/kg groups on Day 14 was subjected to miRNA microarray analysis using a Rat miRNA Microarray Kit (miRBase Rel.16.0, Agilent Technologies, Santa Clara, CA, USA). The procedure was conducted basically in accordance with the manufacturer's protocol (Version 2.0), and the Feature Extraction software (Version 10.7.3.1, Agilent Technologies) was used to generate a quantitative signal value and a qualitative detection call for each probe on the microarray. The 75th percentile was used for per-sample normalization. Analysis of miRNA-normalized expression data was performed using Welch's *t*-test (two-tailed) to obtain the miRNAs that were significantly altered in comparison with the expressions in control samples.

### Real-Time RT-PCR

The levels of miRNA expression in the pons/medulla were analyzed by real-time RT-PCR. Reverse transcription and real-time PCR were performed with a TaqMan MicroRNA Reverse Transcription kit (Life Technologies) and TaqMan Universal PCR Master Mix (Life Technologies), and TaqMan MicroRNA Assays (Life Technologies), which include probes and primers, were used for all target miRNAs (miR-547\* and miR-135a) and control miRNAs (U6 ncRNA for the pons/medulla) in accordance with the manufacturer's protocol. Real-time RT-PCR was performed using a PikoReal Real-Time PCR System (Thermo Scientific, Waltham, MA, USA). All reactions were run in duplicate. The data were calculated using the  $\Delta\Delta\text{CT}$  method, and then fold changes compared with the control group were generated.

### Target gene prediction for miR-547\*

For prediction of the target genes for miR-547\*, the TargetScan Human software (<http://www.targetscan.org/>)

**Table 2.** Effects of IDPN Treatment on Body Weights, Brain Weights, Nervous Symptoms, Auditory Response, and Histopathology

Dose (mg/kg)	20			50			125			
	Day	3	7	14	3	7	14	3	7	14
	No.	7	7	7	7	7	7	7	7	7
Body weight	-		↓2%	-	↓1%	↓1%	-	↓3%	▼10%	↓11%
Brain weight (absolute)	-	-	-	-	-	-	-	-	-	-
Nervous symptoms										
Ataxic gait	0	0	0	0	0	0	0	0	0	7 <sup>a</sup>
Spasmodic gait	0	0	0	0	0	0	0	0	0	6 <sup>a</sup>
Decreased locomotor activity	0	0	0	0	0	0	0	0	0	7 <sup>a</sup>
Head bobbing	0	0	0	0	0	0	0	0	0	7 <sup>b</sup>
Circling	0	0	0	0	0	0	0	0	0	6 <sup>b</sup>
Auditory response										
Score 0 (normal response)	4	4	6	7	7	7	7	7	7	3
Score -1 (poor response)	0	0	0	0	0	0	0	0	0	3
Score -2 (no response)	0	0	0	0	0	0	0	0	0	1
Dose (mg/kg)	20			50			125			
Day	3	7	14	3	7	14	3	7	14	
No.	7	7	7	7	7	7	7	7	7	
Cerebellum										
Swelling, axon	±	0	0	0	0	0	0	0	0	4
Pons										
Swelling, axon	±	0	0	0	0	0	1	0	7	0
	+	0	0	0	0	0	0	0	0	7
Medulla oblongata										
Swelling, axon	±	0	0	0	0	0	1	0	7	0
	+	0	0	0	0	0	0	0	0	7
Spinal cord										
Swelling, axon	±	0	0	0	0	0	1	0	7	0
	+	0	0	0	0	0	0	0	0	7

□ Changes related to IDPN administration. <sup>a</sup> Day 9–14. <sup>b</sup> Day 10–14. Percentage values indicate the percentage change compared with the control values (100%). Numbers indicate the numbers of animals showing each finding. Symbols: -, not changed; ▼, significantly lower than the control values; ↓, lower than the control values; ±, grade = slight; +, grade = mild

was used. The search term “Rno-miR-547-5p” was used to obtain target genes.

### Statistical analysis

StatLight (Yukms Co., Ltd., Tokyo, Japan) was used to perform statistical analyses. To test for the homogeneity of variances among the groups, Bartlett’s test was used to analyze data for body weight and brain weight for miRNA expression analysis (miR-547\* expression on Day 14), and the F-test was used for miRNA expression analysis (miR-547\* expression on Day 7 and miR-135a expression on Day 14). Depending on the results of Bartlett’s test, parametric or nonparametric Dunnett’s multiple comparison (two-tailed) was performed for the difference among the groups using the same software. Depending on the results of the F-test, Student’s *t*-test or Aspin-Welch’s *t*-test (two-tailed) was performed. Statistical significance was evaluated at  $p < 0.05$  and  $p < 0.01$ .

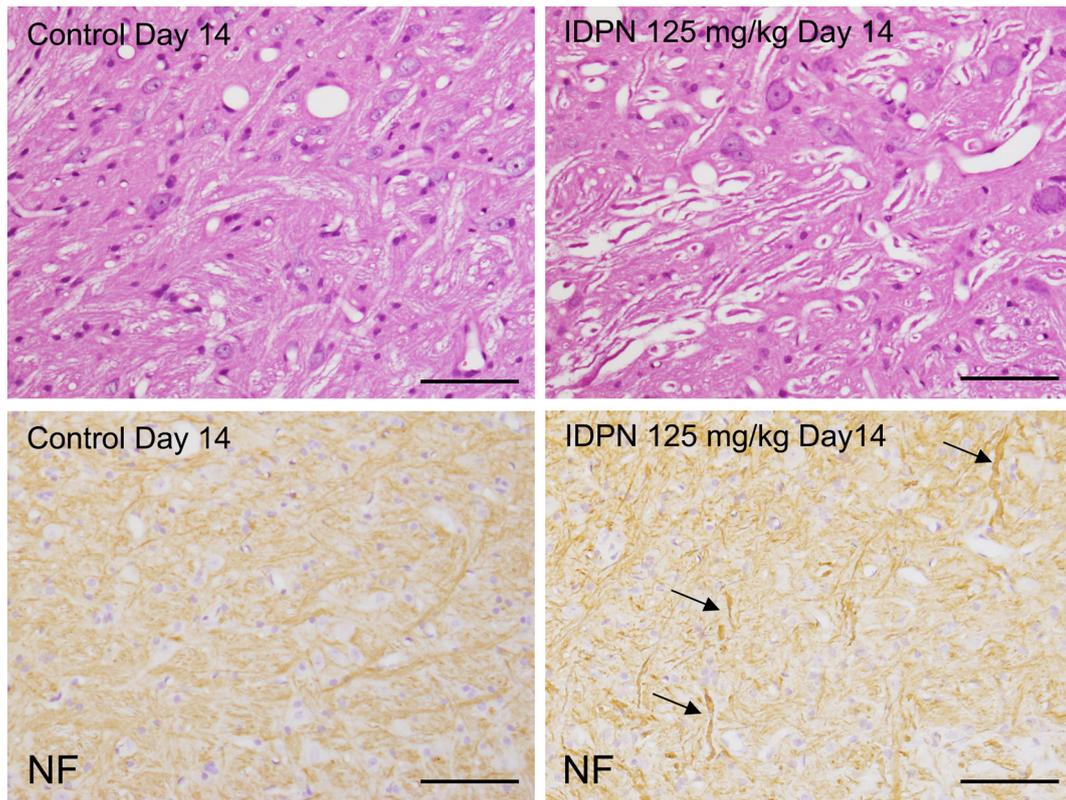
## Results

### General condition, nervous symptoms, and auditory response

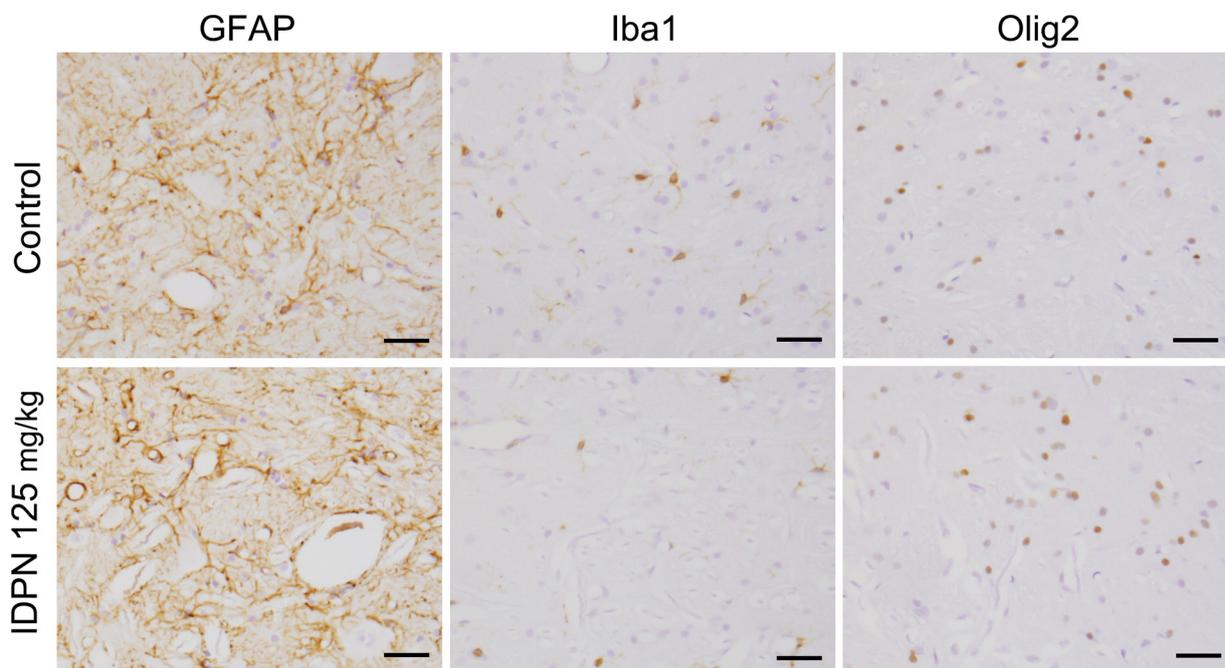
Effects of IDPN treatment on body weight and brain weight, nervous symptoms, and auditory response are shown in Table 2. In the IDPN 125 mg/kg group, body weight was significantly decreased on Day 7 and slightly decreased on Day 14. Absolute brain weight was not changed in any group. Nervous symptoms such as ataxic gait, head bobbing, circling, spasmodic gait, and decreased locomotor activity were observed in most animals at 125 mg/kg from Day 9. Decreased auditory response was observed in animals at 125 mg/kg on Day 14.

### Histopathology and immunohistochemistry for NF-68, GFAP, Iba1, and Olig2

The results of histopathological examination are also shown in Table 2. Photographs of the pons are shown in Fig. 1 (HE and immunohistochemistry for NF-68) and Fig. 2 (immunohistochemistry for GFAP, Iba1, and Olig2). IDPN caused axonal swelling mainly in the pons, medulla, and spinal cord. These changes were slightly observed in



**Fig. 1.** Microphotographs of the pons in control rats and IDPN 125 mg/kg-treated rats on Day 14. Axonal swellings was noted in IDPN-treated rats. Swollen axons noted in IDPN-treated rats showed positive reactions for anti-neurofilament (NF) immunohistochemistry (arrows). HE, upper row; NF, lower row. Scale bars = 50  $\mu$ m.



**Fig. 2.** Microphotographs of immunohistochemistry for GFAP, Iba1, and Olig2 of the pons in control and IDPN 125 mg/kg-treated rats on Day 14. There were no remarkable differences between control and treated rats for any stains. Scale bars = 20  $\mu$ m.

**Table 3.** Up-regulated and Down-regulated miRNAs by Microarray Analysis

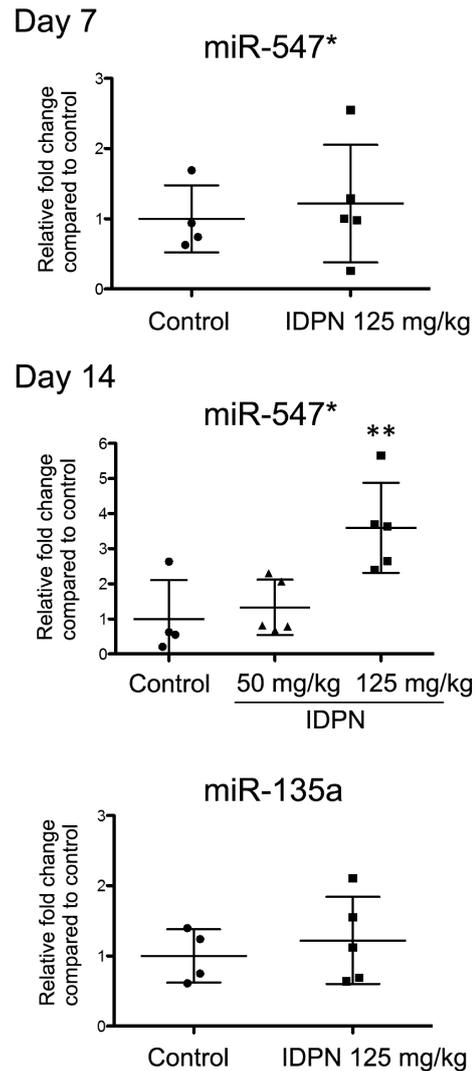
Up-regulated		Down-regulated	
miRNA	fold	miRNA	fold
rno-miR-547*	244.9	rno-let-7c-1*	0.0
rno-miR-3594-3p	135.7	rno-miR-190	0.1
rno-miR-668	117.0	rno-miR-29a*	0.1
rno-miR-487b*	116.7	rno-miR-384-3p	0.2
rno-miR-760-5p	51.3	rno-miR-32	0.3
rno-miR-3550	7.4	rno-let-7a-1*	0.3
rno-miR-540*	5.8	rno-miR-24-2*	0.3
rno-miR-1188-3p	5.4	rno-miR-135a	0.3
rno-let-7i*	5.3	rno-miR-496	0.3
rno-miR-504	5.2	rno-miR-494	0.4
rno-let-7b*	3.3	rno-miR-374	0.4
rno-miR-346	3.1	rno-miR-31*	0.4
rno-miR-207	2.9	rno-miR-126*	0.4
rno-miR-328b-3p	2.8	rno-miR-379*	0.4
rno-let-7d*	2.7	rno-miR-30b-5p	0.4
rno-miR-702-3p	2.7	rno-miR-450a	0.5
rno-miR-485*	2.6	rno-miR-222	0.5
rno-miR-764	2.5	rno-miR-350	0.5
rno-miR-3573-3p	2.5	rno-miR-142-3p	0.5
rno-miR-378*	2.5	rno-miR-365	0.5
rno-miR-466b-2*	2.3	rno-miR-22*	0.5
rno-miR-466c*	2.3		
rno-miR-483	2.3		
rno-miR-347	2.0		

all animals at 125 mg/kg on Day 7, and the severity was increased on Day 14. On Day 14, these findings were also slightly noted in one animal at 50 mg/kg. The same findings were also observed in the cerebellum in animals at 125 mg/kg on Day 14. The swelling of axons was especially noted in vestibular, trigeminal, and reticular formation nuclei of the pons and medulla, and in the ventral horn and funiculus of the spinal cord. No remarkable changes were noted in the other sites in the brain or sciatic nerve. Immunohistochemical stains revealed increased positive reaction for NF-68 in swollen axons, suggesting accumulation of neurofilaments in axons. There were no apparent differences between control and IDPN 125 mg/kg animals in immunoreactivity for glial cells (GFAP, Iba1, and Olig2).

#### Alterations of miRNA expression in the pons/medulla

Table 3 shows the results of microarray analysis of the pons/medulla. Significantly upregulated or downregulated miRNAs with a fold change of 2.0 or more and 0.5 or less, respectively, were observed. Twenty-four miRNAs were up-regulated, and 21 miRNAs were downregulated. miR-547\*, miR-3594-3p, miR-668, miR-487b\*, and miR-760-5p were drastically upregulated, and let-7c-1\*, miR-190, and miR-29a\* were drastically downregulated, though expression levels of these miRNA were extremely low in the control animals. Downregulated miRNAs that ranked highly included miR-190, miR-135a, and miR-496, which have been reported to be altered in the nervous system<sup>14, 18–23</sup>.

We focused on upregulation of miR-547\*, which showed the highest fold change compared with control



**Fig. 3.** Real-time PCR analysis for miR-547\* and miR-135a expressions in the pons/medulla. miRNA levels were expressed as relative fold changes compared with the control mean levels. Individual data were plotted as points, and mean  $\pm$  SEM values were expressed as short and long bars. \*\*Significantly different from the control at  $p < 0.01$  (Dunnett's multiple comparison).

animals (224.9-fold), and downregulation of miR-135a (0.3-fold), alteration of which has been indicated by several reports in association with the nervous system<sup>14, 18–20</sup>.

The results of RT-PCR analysis for miR-547\* and miR-135a are shown in Fig. 3. miR-547\* was significantly increased at IDPN 125 mg/kg on Day 14 (3.6-fold). The fold change in comparison with control animals was lower in RT-PCR than that in microarray analysis. There were no significant differences at 125 mg/kg on Day 7 or at 50 mg/kg on Day 14. Alteration of the miR-135a expression level was not confirmed by RT-PCR at 125 mg/kg on Day 14. The difference in the results between microarray and RT-PCR analysis was considered to be due to the difference in detection sensitivity. In target gene prediction analysis for miR-547\*,

**Table 4.** Target Genes Having Nervous System-related Gene Ontology Predicted for miR-547\* by TargetScan

Human ortholog of target gene	Gene name	Cumulative weighted context++ score	Gene Ontology (GO) associated with nervous system
SNAP23	synaptosomal-associated protein, 23kDa	-0.69	synaptosome, synapse neurotransmitter secretion, nervous system development, brain development, synaptic vesicle, axon, dendrite,
PPT1	palmitoyl-protein thioesterase 1	-0.46	neuronal cell body, negative regulation of neuron apoptosis, synapse, neuron development, regulation of synapse structure and activity
NTNG1	netrin G1	-0.33	nervous system development, axonogenesis

In the top 30 ranking genes ranked by cumulative weighted context++ score, SNAP23 and PPT1 were included. In addition, in the top 100 ranking genes, NTNG1 was included.

genes with nervous system-associated gene ontology, such as SNAP23 (synaptosomal-associated protein, 23 kDa), and PPT1 (palmitoyl-protein thioesterase 1), were found to be involved and to be within the top 30 ranking genes. Within the top 100 ranking genes, NTNG1 (netrin G1) was also included (Table 4).

## Discussion

In this study, we revealed alteration of miRNA expressions in the pons/medulla in IDPN-treated rats. Significantly altered miRNAs in the pons/medulla in microarray analysis included drastically upregulated miR-547\*, miR-3594-3p, miR-668, miR-487b\*, and miR-760-5p and drastically downregulated let-7c-1\*, miR-190, and miR-29a\*. However, these miRNAs showed extremely low expression levels in the control animals. Therefore, the fold changes in these miRNAs need to be considered carefully. In downregulated miRNAs that ranked highly, nervous system-related alterations were previously reported for miR-190, miR-496, and miR-135a. In particular, nervous system-related alteration of miR-135a has been indicated in association with synaptic depression in spine remodeling, RDX-induced neurotoxicity, corticosteroid dependent stress response, cocaine-induced conditioned place preference, and tumorigenesis<sup>14, 18–20</sup>.

In these altered miRNAs, we focused on upregulation of miR-547\* and downregulation of miR-135a and performed RT-PCR analysis. Consequently, only upregulation of miR-547\* was confirmed by RT-PCR. In the time-course and dose-response analysis, the expression level of miR-547\* was only found to be significant at 125 mg/kg on Day 14, at which point nervous symptoms such as ataxic gait, head bobbing, circling, spasmodic gait, and decreased auditory response were most frequently observed, and the severity and incidence of axonal changes, mainly in the medulla, pons, and spinal cord, were increased. Slight axonal changes were also observed at a lower dose (50 mg/kg) on Day 14 and at an earlier time point (on Day 7) at 125 mg/kg; however, they were not accompanied by alteration of miR-547\* expression levels. These results suggested that miR-547\* is related to an advanced stage of IDPN-induced neurotoxicity rather than an onset phase.

Since little is known about the distribution in tissues and cells and function of miR-547\* in any species, the role miR-547\* plays in IDPN-induced neurotoxicity remains unclear. However, SNAP23, PPT1, and NTNG1, which are associated with functions of synapses and axons, were included among the potential target genes for miR-547\* predicted by TargetScan. Of the three genes, SNAP23 was ranked highest. SNAP23 is a member of the SNAP family that is distributed in both non-neuronal tissues and neuronal tissues, and its roles in membrane trafficking have been indicated<sup>24–34</sup>. Regarding neuronal tissues, SNAP23 is distributed in hippocampal and cortical GABAergic neurons, in glutamatergic and GABAergic synapses of the mature cerebellar cortex, and in astrocytes. Its function in the brain is not fully understood, but it is considered an important protein in the exocytotic machinery in neuronal synapses and astrocytes<sup>24–30</sup>. In this study, there was no difference in SNAP23 mRNA levels in the pons/medulla between control and IDPN 125 mg/kg animals on Day 14 (data not shown). However, it remains unclear whether miR-547\* regulates SNAP23 protein expression by translational repression. To clarify this question, analysis of SNAP23 at the protein level will be needed. Moreover, accumulation of neurofilaments was confirmed by immunohistochemistry in our study. Accumulation of neurofilaments has been reported to be caused by a deficit in slow axonal transport of neurofilament proteins<sup>4, 35</sup>. Further study is still needed to determine how miR-547\* is involved in regulating synaptic and axonal function in association with axonal impairment caused by IDPN.

The nervous symptoms and histopathological changes in the present study are in general agreement with some reports on the features of IDPN-induced neurotoxicity<sup>1, 3, 4, 6, 7, 36</sup>. Abnormal behaviors and decreased auditory response might be associated with IDPN-induced vestibular and auditory hair cell injuries, as reported in some studies<sup>2, 37–40</sup>, although the possibility that decreased auditory response might be caused by weakened condition in animals could not be ruled out. In contrast to our result, it has been reported that IDPN induced sciatic nerve degeneration after 28-day (at 100 mg/kg) or 5-week (at 125 mg/kg) oral administration<sup>5, 7</sup>. Since the observation period in our study was shorter than that of the reported studies, no sciatic nerve de-

generation was detected in the present study. Setting a longer administration period may promote progression of the pathological changes and lead to sciatic nerve degeneration. In addition, glial reactions were not apparent in this study. However, it has been reported that an increase in GFAP concentration in the pons/medulla, midbrain, cerebral cortex, and olfactory bulb was induced by IDPN treatment for 3 days at 400 mg/kg/day with a peak on Day 7 after administration<sup>36, 41</sup>. The differences in dose and period of administration and the difference in GFAP detection methods between this study (immunohistochemistry) and the previous studies (measurement of concentration) were suspected as contributing to the differing results for GFAP. The finding that no apparent glial reactions were seen in this study was considered to be supportive data indicating that miR-547\* may regulate genes associated with axonal or synapse function rather than genes associated with other phenomena such as cell death and inflammation.

Regarding the other downregulated miRNAs in microarray in this study, miR-190 and miR-496 have also been reported to be downregulated in relation to the nervous system. *In vivo* and *in vitro* analyses indicates that miR-190 is associated with  $\mu$ -opioid receptor agonists-modulated stability of dendritic spines via regulation of neurogenic differentiation 1 (NeuroD) activity<sup>22</sup>. miR-190 is also downregulated in the contused cortex after traumatic brain injury in mice<sup>23</sup>. Downregulation of miR-496 has been reported in microarray analysis using fetal mouse brains exposed to ethanol<sup>21</sup>. Further investigation for these miRNAs will be the challenge for the future to clarify the roles of miRNAs that contribute to IDPN-induced neurotoxicity.

In conclusion, miR-547\* was considered to be associated with IDPN-induced abnormalities in the pons and medulla. To our knowledge, this is the first report indicating links between upregulation of miR-547\* and neurotoxicity of IDPN in rats. Although the potential mechanisms need to be further clarified, miR-547\* may provide new useful information regarding the mechanism of IDPN neurotoxicity and the pathogenesis of axonopathy.

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**Disclosure of Potential Conflicts of Interest:** The authors declare that they have no conflict of interest.

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