Abstract

Oxidative stress is linked to dopaminergic (DA) neurodegeneration in Parkinson’s disease. Our laboratory reported slowly progressive DA neurodegeneration in the zitter (zi) rat, which is Attractin (Atrn) deficient. However, little is known about the function of Atrn in the central nervous system (CNS). Thus, we investigated whether DA neurodegeneration in the zi rat was induced by oxidative stress, and how Atrn affects oxidative stress. First, we summarize our previous in vivo data, which revealed suppression of DA neurodegeneration using antioxidants (vitamin E and melatonin) in zi rats. Second, our current ex vivo and in vitro studies are introduced. Using primary neuronal cultures of zi mesencephalon as a model of Atrn-deficient neurons or Atrn-GFP-overexpressing HEK293 cells, accumulation of reactive oxygen species (ROS) in mitochondria and cell viability was examined under oxidative stress. Atrn-deficient neurons accumulated excess ROS in mitochondria, resulting in neurodegeneration, whereas Atrn-overexpressing cells showed suppression of ROS accumulation under oxidative stress. These results showed that Atrn plays a suppressive role against ROS and that the loss of Atrn function induced excess ROS accumulation and led to DA neurodegeneration. This is the first report to show that Atrn directly modulates mitochondrial ROS accumulation in the CNS.

Keywords: dopaminergic neurodegeneration, Attractin, reactive oxygen species, Parkinson’s disease, oxidative stress

1. Introduction

Oxidative stress is considered to be the cause of several neurological diseases. In particular, dopaminergic (DA) neurons are vulnerable to oxidative stress, which is normally generated...
by dopamine metabolism [1, 2]. The progressive DA neurodegeneration that occurs with age in Parkinson’s disease (PD) is caused by increased oxidative stress. The neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxydopamine induce free radicals (H$_2$O$_2$, O$_2^-$, and OH) that destroy DA neurons [3, 4] and are used to produce acute animal models of PD. Recently, we reported slowly progressive DA neurodegeneration, which is similar to the symptoms of humans with PD, in a mutant rat, the zitter (zi) rat [5].

The zi rat, which occurred in a colony of Sprague-Dawley (SD) rats, is a spontaneous autosomal recessive mutant rat with body tremors. The gene symbol of the mutation is designated zi (zitter means shake and tremble in German). The rats develop the tremor at around 15 days of age and progressive flaccid paresis of hind limbs at 6 months of age. The behavioral defects are associated with progressive hypomyelination and vacuolation in the central nervous system (CNS) [6]. Zi is homozygous for an 8-bp deletion in intron 12 of Attractin (Atrn) [7]. Atrn is involved in regulating physiological processes such as initial immune cell clustering during an inflammatory response, melanocortin signaling pathways that regulate energy homeostasis, pigmentation, and normal myelination in the CNS. Atrn encodes a protein that is secreted or transmembrane form as a result of alternative splicing of the same mRNA. Both forms of the protein have several domains in common such as epidermal growth factor-related domains, a CUB domain, and a C-type lectin. The transmembrane form, which has a transmembrane domain, has a long N-terminal extracellular domain. Rats and humans express both secreted and transmembrane proteins. However, mice express only the transmembrane protein. The zi rat expresses neither the secreted nor the transmembrane form of Atrn proteins. The abnormalities in the CNS of zi rats are mainly caused by the loss of the transmembrane form [7].

Our laboratory first reported DA neurodegeneration that occurs with age in zi rats [5]. In particular, the nigrostriatal DA pathway shows the most severe progressive neurodegeneration. In the substantia nigra pars compacta (SNc), a significant decrease in the number of DA neurons is detected from 2 months of age in zi rats, whereas no difference is detected at 1 month of age between zi rats and wild-type SD rats [8, 9]. With age, the number of DA neurons decreases significantly [8] (Figure 1). Loss of DA neurons in the SNc results in a decrease in the number of DA fibers and terminals in the dorsolateral caudate putamen (CPu), which receives DA input from the SNc. Consequently, the level of dopamine in the CPu of zi rats at 12 months of age is decreased to one-seventh of that in age-matched SD rats [8].

Figure 1. Progressive dopaminergic neurodegeneration with age in zi rats. Tyrosine hydroxylase-immunoreactive cells in the substantia nigra at 1 month (A), 6 months (B), and 12 months (C) of age. Scale bar: 500 μm.
Previous studies have indicated that zi rats show abnormal activities of some antioxidant enzymes in the brain and tend to accumulate reactive oxygen species (ROS) [10–13]. Examination of the ultrastructure of DA neurons in the SNc of zi rats reveals abnormal mitochondria with disrupted and enlarged cristae [9]. Additionally, Atrn null mice show reduced complex IV activity (cytochrome c oxidase activity) [14]. Complex IV is an enzyme in the respiratory electron transport chain in mitochondria. It was suggested that Atrn regulated ROS production in mitochondria. Therefore, we hypothesized that the neurodegeneration in zi rats is due to oxidative stress, and thus, we investigated whether antioxidants can protect against neurodegeneration in the mutant. Furthermore, to investigate whether Atrn is directly involved in regulation of the ROS-producing system in neurons, we established primary neuronal cultures from zi rats to represent Atrn-deficit neurons, and an Atrn-overexpressing cell line. Using these cells, we examined the viability and ROS accumulation under several oxidative stress conditions.

2. Materials and methods

2.1. Reagents

Materials for cultures were B27 supplement, B27 supplement minus antioxidants (AO (–)), neurobasal medium, Opti-MEM, and penicillin/streptomycin from Gibco (Life Technologies; Carlsbad, CA); MitoTracker Red CM-H2Xros, MitoTracker green FM, and Lipofectamine Plus Reagent from Invitrogen (Life Technologies); cell proliferation reagent WST-1 from Roche Diagnostics (Basel, Switzerland); cytosine-arabinofuranoside, l-glutamine, and poly-l-lysine from Sigma-Aldrich (St. Louis, MO); pAcGFP1-N1 vector from Clontech Laboratories Inc. (Mountain View, CA). Reagents for the immunocytochemistry were purchased from the following sources: biotinylated horse anti-mouse IgG, normal goat serum, and vectastain ABC kit from Vector (Burlingame, CA); anti-tyrosine hydroxylase (TH) antibody from Incstar (Stillwater, MN); Alexa Fluor 488 goat anti-mouse IgG from Molecular Probes (Life Technologies).

2.2. In vivo study

Male zitter rats were housed in-groups of two or three in a cage with food and water ad libitum. The room was maintained at a constant temperature and humidity with a 12 h light/dark cycle in the Laboratory Animal Research Center at Dokkyo University School of Medicine. All procedures in this study were certified by the Animal Welfare Committee at Dokkyo University School of Medicine and were conducted in accordance with NIH guidelines. Between 1 and 10 months of age, we fed zi rats a diet supplied with vitamin E (500 mg d-, l-alpha-tocopherol/kg diet) or administered oral melatonin at dose of 0.5 mg/ml in drinking water, as previously reported [13, 15]. Tissue preparation and immunohistochemical procedure were described previously [5].
2.3. Ex vivo study

Ventral mesencephalic neurons were prepared from fetal zi and SD rats (Charles River Laboratories, Tsukuba, Japan) at gestation day 14. Cells were seeded in 24-well plates at a density of 2 × 10⁵ cells/cm². Wells (for the cell viability assay and ROS assay) or cover glasses (for immunofluorescence, 15-mm diameter) were pre-treated with poly-l-lysine (0.1 mg/ml). After 2 h of initial plating, the medium was changed to neurobasal medium (containing penicillin/streptomycin and l-glutamine) supplemented with B27. Cultures were maintained at 37°C in a humidified 5% CO₂ incubator (Revco Ultima RCO5000T, Thermo Fisher Scientific; Asheville, NC). To obtain neuron-enriched cultures, cytosine-arabinofuranoside (7.5 μM) was added beginning at 2 days in vitro (DIV) to 6 DIV. At 6 DIV, neurons were divided into two groups and cultured in neurobasal medium supplemented with B27 (Cont) or B27 without antioxidants (AO (−)), consisting of B27 supplement without vitamin E, vitamin E acetate, superoxide dismutase (SOD), catalase, or glutathione (GSH). Cultures were used for experiments at 11 DIV. Over 95% of the cells in the cultures were neurons as determined by immunostaining for the neuron-specific marker microtubule-associated protein-2 (data not shown).

The cell viability assay was performed by using the cell proliferation reagent WST-1 according to the manufacturer’s protocol. Cell viability was expressed as a percent of the values of SD-Cont.

ROS accumulation was monitored with a plate reader using MitoTracker Red CM-H₂Xros (MTR). Neurons were co-stained with 0.5 μM MTR and 0.2 μM MitoTracker green (MTG: mitochondria marker) in Opti-MEM for 30 min at 37°C and then assessed (MTR, excitation 535 nm, barrier filter 595 nm; MTG, excitation 485 nm, barrier filter 535 nm). The MTR/MTG ratio of each group was normalized to that of SD-Cont, which was used as an index of ROS content.

Immunofluorescence procedure was described previously [16].

2.4. In vitro study

Atrn cDNA, which encodes full-length membrane-type rat Atrn, was cleaved from pCAGGS-neo-Atrn (kind gift from Dr. Kenzo Sato, Tottori University) and inserted into the pAcGFP1-N1 vector as a ~4.3-kb Eco R1-Bam HI fragment. After cloning, insertion of the fusion sequence into the plasmid was confirmed. HEK293 cells (1.5 × 10⁴ cells/10-mm glass bottom dish) were transfected with 0.1 μg Atrn-GFP or GFP (as a control) plasmid using Lipofectamine Plus Reagent. At 18 h after transfection, cells were incubated with 0.5 μM MTR for 30 min at 37°C. Fluorescence images of living cells were scanned with a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany) using a 60× 1.4-NA oil-immersion objective. Under the microscope, cells were exposed to 1 mM H₂O₂ in Opti-MEM and observed for 60 min.
3. Results

3.1. The effect of antioxidants on DA neurodegeneration in zi rats

We treated zi rats with the antioxidants vitamin E and melatonin. Between 1 and 10 months of age, we fed zi rats a diet supplied with vitamin E, which is a lipid-soluble peroxyl radical scavenger. Chronic vitamin E feeding suppressed DA neuron death in the SNc (Figure 2B). The number of DA neurons in zi rats treated with vitamin E was 1.5 times higher than that in control zi rats [15]. However, vitamin E protected few DA fibers and terminals in the CPu of zi rats (data not shown).

Melatonin, which is a synthetic product of the pineal gland, is a direct free radical scavenger and an indirect antioxidant. Melatonin readily crosses the blood-brain barrier and plays a role in protecting against ROS in the brain. Chronic melatonin administration suppressed DA neuron death in the SNc of zi rats (Figure 2C). The number of DA neurons in zi rats treated with melatonin was two times higher than that in control zi rats [13]. In the CPu, DA fibers and terminals were protected by melatonin, and zi rats administered chronic melatonin showed twice as much dopamine as control zi rats [8, 13]. Melatonin protected DA neuronal somas, fibers, and terminals in both the SNc and the CPu of zi rats.

![Figure 2. Antioxidants suppressed dopaminergic neurodegeneration in zi rats. Tyrosine hydroxylase-immunoreactive cells in the substantia nigra at 10 months of age in zi rats (A) or zi rats treated with vitamin E (B) or melatonin (C) for 9 months. Scale bar: 500 μm.](image)

Antioxidants were effective for preventing DA neurodegeneration in zi rats, indicating that the DA neurodegeneration in the mutant rats is caused by oxidative stress.

3.2. Accumulation of ROS in zi mesencephalic neurons ex vivo

To investigate whether ROS accumulation in mesencephalic neurons is caused by a genetic factor of the Attractin mutation, we established primary mesencephalic neuronal cultures from zi rats and compared them with cultures from wild-type SD rats. Neurons were cultured under an individual condition with (Cont) or without antioxidants (AO (−)), consisting of B27 supplement without vitamin E, vitamin E acetate, SOD, catalase, or GSH. We analyzed the cell viability and ROS content according to two factors: genotype (zi or wild type) and supplement (AO (−) or Cont).
Cell viability was shown in Figure 3A. Two-way analysis of variance (ANOVA) revealed significant effects of the supplement ($P = 1.98 \times 10^{-8}$) and an interaction of genotype × supplement ($P = 8.73 \times 10^{-6}$), but no effect of genotype ($P = 0.493$). Mesencephalic neurons from zi rats showed significantly lower cell viability in AO (−) supplement compared to Cont supplement ($P < 1 \times 10^{-8}$). However, the cell viability of neurons from wild-type SD rats was significantly lower in AO (−) supplement ($P = 0.0085$), the decreasing rate was considerably less than that of zi rats.

Next, ROS accumulation in mitochondria was monitored using MTR, which produces intense fluorescence depending on ROS accumulation in mitochondria (Figure 3B). Two-way ANOVA revealed significant effects of genotype ($P = 6.21 \times 10^{-4}$), supplement ($P = 0.0016$), and an interaction of genotype × supplement ($P = 0.0026$). Mesencephalic neurons from zi rats showed significantly higher ROS content in AO (−) ($P = 0.0011$ compared with Cont supplement in zi rats), whereas that of the wild type showed low ROS content and no difference between AO (−) and Cont supplements ($P = 0.994$). With Cont supplement, the ROS content was not different according to genotype ($P = 0.875$ zi neurons vs. SD neurons).

Because the neurons from zi rats showed excessive ROS accumulation, we examined the morphology of DA neurons using immunofluorescence with anti-TH antibody (Figure 3C). With Cont supplement, TH-immunoreactive (TH-ir) neurons from zi rats had long processes similar to those from wild-type rats (Figure 3C, upper column). However, in AO (−), TH-ir neurons had no long processes and formed cell clusters (Figure 3C, lower column). These culture experiments revealed that mesencephalic neurons from zi rats accumulate excess ROS followed by DA neurodegeneration and death in the absence of exogenous antioxidants.
3.3. Suppression of ROS accumulation in Atrn-overexpressing cells in vitro

To investigate the function of Atrn during oxidative stress, ROS accumulation in mitochondria was observed in HEK293 cells that overexpressed Atrn-GFP.

ROS accumulation in Atrn-GFP-transfected cells was detected 30 min after MTR incubation (Figure 4). Atrn-GFP-transfected cells (Figure 4A) showed lower MTR intensity than the surrounding untransfected cells (Figure 4C and D).

![Figure 4](image_url)

**Figure 4.** ROS accumulation in Atrn-GFP-transfected HEK293 cells. Atrn-GFP (A), the corresponding phase-contrast image (B), and MitoTracker Red CM-H$_2$Xros (C) were merged as shown in (D). White dotted lines (C) show the outline of Atrn-GFP-positive cells (A). Scale bar: 50 μm.

Under the microscope, the time of addition of H$_2$O$_2$ as a form of oxidative stress was set at 0 min, and live images were scanned at 0 and 60 min (Figure 5). Atrn-GFP-transfected cells showed little MTR fluorescent intensity at 0 min (Figure 5C 2–3) and faint intensity at 60 min (Figure 5D 2–3), whereas control transfected cells showed high MTR intensity at 0 min (Figure 5A 2–3) and markedly higher intensity at 60 min (Figure 5B 2–3). Compared with adjacent cells, Atrn-overexpressing cells maintained low MTR intensity under oxidative stress (Figure 5D 2–3).
4. Discussion

This study revealed that the loss of *Atrn* led to ROS accumulation in mitochondria and resulted in DA neurodegeneration in *zi* rats.

*Zi* rats accumulate ROS and are vulnerable to oxidative stress, as observed with fibroblast cultures from the *zi/zi* kidney [11]. Moreover, DA neurons are particularly vulnerable to oxidative stress [1, 2]. Based on these observations, our previous studies revealed that the antioxidants vitamin E and melatonin suppress DA neurodegeneration in *zi* rats [5, 13, 15]. Therefore, we hypothesized that the DA neurodegeneration in *zi* rats was caused by oxidative stress. However, whether oxidative stress is directly caused by the genetic factor, *Atrn*.
gene mutation, was not clear. In the present study, we used ex vivo neuronal cultures and showed that this mutation directly led to ROS accumulation in mesencephalic neurons and caused DA neurodegeneration. B27 without the antioxidants vitamin E, vitamin E acetate, SOD, catalase, and GSH was used in this study. In vivo, SOD and catalase are produced by neurons, whereas vitamin E and vitamin E acetate are not synthesized in animal cells; GSH is mainly produced by astrocytes [17]. Thus, our result showing that wild-type neurons accumulate little ROS in medium without these five antioxidants indicates that the generated ROS is removed by scavenging agents including SOD and/or catalase in neurons. However, zi neurons accumulated significantly excessive ROS in the same medium, indicating that zi neurons have little or no endogenous scavenging agents and produce ROS levels that exceed their scavenging ability. This idea is consistent with previous assays using homogenates of zi brain tissues, which show abnormal \( \text{H}_2\text{O}_2 \) metabolism [10] and mitochondrial function [14]. In addition, morphological analysis using primary neuronal cultures from zi cerebral cortex shows protection from neurodegeneration by the antioxidants, vitamin E and catalase [11]. Additionally, in vitro study using DA neuroblastoma cell line shows that overexpressing Atrn protects against cell death induced by the neurotoxin 1-methyl-4-phenylpyridinium, which enhances ROS production [12]. Thus, Atrn may play a role in the activation of antioxidant enzymes in mitochondria. The observation that overexpression of Atrn suppressed ROS accumulation supports this hypothesis. We suggest that a deficiency in Atrn directly results in an abnormal antioxidant system and excess ROS accumulation in DA neurons. The findings in the present study will contribute to our understanding of the mechanism of not only DA neurodegeneration but also neurodegeneration of other neurons caused by oxidative stress. Further studies are needed to elucidate these possibilities.

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