Loss of nuclear REST/NRSF in aged-dopaminergic neurons in Parkinson’s disease patients

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A B S T R A C T

Parkinson’s disease (PD) is the second most common neurodegenerative disease. Lewy bodies and pale bodies in dopaminergic neurons in the substantia nigra are pathological hallmarks of PD. A number of neurodegenerative diseases demonstrate aggregate formation, but how these aggregates are associated with their pathogenesis remains unknown. It has been reported that repressor element-1 silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) is induced in the nuclei of aged neurons, preserves neuronal function, and protects against neurodegeneration during aging through the repression of cell death-inducing genes. The loss of REST is associated with Alzheimer’s disease pathology. However, its function in dopaminergic neurons remains unknown. Here we demonstrated that REST enters the nucleus of aged dopaminergic neurons. On the other hand, REST is partially sequestered in Lewy bodies and is mostly absent from the nucleus of neurons in brains with PD and dementia with Lewy bodies (DLB). Dopaminergic neuron-specific autophagy-deficient mice exhibit REST accumulation in aggregates. Defects in the protein quality control system induce REST mRNA expression; its gene product mainly appears in aggregates. Our results suggest that Lewy pathology disturbs normal aging processes in dopaminergic neurons by sequestering REST and the loss of REST may associate with the PD pathology.

1. Introduction

Aging is a primary risk factor in several neurodegenerative disorders, including Parkinson’s disease (PD). PD is the second most common neurodegenerative disease. It is characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN) [14]. It is predicted that PD affects over 1% of the population at age 60, but the rate of PD is five times higher by age 80, suggesting that senescence of the brain or neurons is related to the onset of PD [2,9,16]. Although it remains unclear how aging causes the malfunctioning of protein quality control system in neurons, the appearance of cytoplasmic inclusions such as Lewy bodies and pale bodies, hallmarks of late-onset sporadic and familial PD and dementia with Lewy bodies (DLB) [4], suggests there is a close relationship between protein inclusions and neuronal death.

Lu et al. [6] have demonstrated that the repressor element-1 silencing transcription factor (REST), also known as neuron-restrictive silencer factor (NRSF), is transcriptionally induced in the aging human brain, and induction is decreased in patients with Alzheimer’s disease (AD). They also showed that induced REST is localized in the nucleus, where it represses the transcription of pro-apoptotic and AD-related proteins in normal aging brain, suggesting that REST induction and nuclear accumulation is neuroprotective. On the other hand, REST is a primary factor in the transcriptional and epigenetic regulatory circuitry that modulates the expression of genes containing the neuron-restrictive silencer element (NRSE) in their promoter region [1,13]. Since NRSE is found in most neuron-specific genes, REST is believed to act exclusively in non-neuronal cells where it represses the expression of NRSF-controlled neuron-specific genes [8]. If REST only represses neuronal genes, REST induction and nuclear accumulation in aging neurons would be injurious. Thus, it is important to clarify which situation occurs and identify the neuroprotective functions of REST in...
Table 1

| Disease | Age, years | Sex |
|---------|------------|-----|
| Control | 47         | F   |
| Control | 61         | M   |
| Control | 72         | M   |
| Control | 79         | M   |
| Control | 81         | F   |
| PD      | 76         | M   |
| PD      | 73         | M   |
| PD      | 79         | M   |
| PD      | 80         | F   |
| PD      | 77         | M   |
| DLB     | 82         | M   |
| DLB     | 78         | M   |

Table 1: Characteristics of human brain samples (n = 14).

PD, Parkinson’s disease; DLB, dementia with Lewy bodies.

2. Material and methods

2.1. Antibodies

Anti–REST antibodies (Bethyl Lab IHC-00141, Millipore 07-579), anti-TH antibody (Sigma T2928), anti-p62 antibody (Progen p62C), anti-ubiquitin antibody (FK2 04-263), anti-LDH antibody (Santa Cruz H-160 sc-33781), anti-XRCC1 antibody (Cell Signaling Technology #2735S) were purchased.

2.2. Human brain sections

Postmortem human brain tissue (n = 14) was obtained from Juntendo University Hospital and the Jikei University Hospital (Table 1). Human brain samples included the SN and cortex.

2.3. Animals

All animals were kept in a pathogen-free and odor-free environment, which was maintained under a 12 h light/dark cycle at ambient temperature. Procedures were approved by the Animal Experimental Committee of the Juntendo University Graduate School of Medicine and were performed in accordance with the guidelines of the U.S. National Institutes of Health (NIH) and the Juntendo University Graduate School of Medicine. Previously characterized floxed Atg7 mice [3] and were crossed with TH-Cre mice carrying the knock-in construct with TH fused to Cre on the 3’ end (gift from Dr. Ted M. Dawson, Johns Hopkins University, Baltimore, MD, USA) to generate dopaminergic neuron specific Atg7 conditional knockout mice (Atg7fl/oX/fl/oX:TH-Cre mice).

Fig. 1. Age-dependent REST expression and localization in dopaminergic neurons Histological analysis of the human substantia nigra (SN) in middle-aged controls (A: age, 47 years and B: age, 61 years) and in elderly controls (C: age, 79 years, D: age, 72 years and E: age, 81 years). Paraffin sections were immunostained for REST. In middle age, there was slight REST accumulation in the cytosol, but accumulation was not observed in the nucleus. In old age, REST accumulated not only in the cytosol but also in the nucleus. Scale bar: (A), (C), (E): 20 μm. (B), (D): 50 μm.
2.4. Immunohistochemical analysis

After fixation with 10% neutral phosphate-buffered formalin, 6-μm-thick paraffin embedded tissue sections were cut and stained with hematoxylin and eosin. For immunohistochemistry (IHC), we used antibodies against REST (IHC-00141 from Bethyl Laboratories, Inc., Montgomery, TX, USA, for human samples and Millipore 07-579 from Merck, Darmstadt, Germany, for mouse samples). Paraffin sections were deparaffinized, rehydrated, and treated with 0.3% H2O2 in methanol for 30 min at room temperature (RT). Antigen retrieval was performed in 0.05 M Tris buffer (pH 7.6) for 10 min at 121 °C. Sections were incubated with anti-REST antibodies diluted 1:200 with 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA), 0.1% Tween 20 (Sigma), 5% bovine serum albumin (Sigma), and 0.01 M phosphate-buffered saline (PBS) for 30 min at RT, and then incubated with primary antibodies diluted with each blocking solution overnight at 4 °C. Biotinylated secondary antibodies (Vector Laboratories) and 3,3-diaminobenzidine (DAB) as a chromogen. For signal amplification, Tyramide Signal Amplification (PerkinElmer Inc., Waltham, MA, USA) was used. Counterstaining for cell nuclei was performed with Mayer’s hematoxylin. In negative controls, the primary antibody was omitted.

For immunofluorescence, secondary antibodies diluted in 2% bovine serum albumin (BSA) and 0.1% Triton in PBS were coupled to Alexa fluorophores (1:200, Invitrogen) for 1 h. Sections were incubated with 0.1% Sudan Black in 70% ethanol for 10 min to suppress lipofuscin autofluorescence.

3. Results

To determine whether the REST is located in the nucleus in neurons of a normal, aging human brain, different areas of the postmortem human brain, including the cortex and SN, were immunohistochemically analyzed (Table 1). A total of six control cases (age range, 47–81 years) underwent immunostaining with DAB. We first performed antibody validation using commercially available antibodies (Millipore 07-579, Abcam 28018, 21635, Bethyl IHC-00041, and Santa Cruz H-290, F-3). Although all of these antibodies recognized both endogenous and recombinant REST proteins by immunoblotting against the cell lysate of cultured human neuroblastoma cell line, SHSY-5Y (Supplementary Fig. 1A), we found that only the Bethyl antibody could detect REST in postmortem human samples. In previous studies, this antibody has shown reactivity against the full-length REST protein in neurons [6,12].

In cortical neurons of normal aging human brain, REST immunoreactivity was distributed throughout the cell, including the nucleus in most cases, suggesting that REST enters the nucleus of senescent neurons and could be a marker for neuronal senescence.

Next, we investigated whether REST could be detected in dopaminergic SN neurons. In middle-aged cases (age 47 and 61 years), REST was not observed in the nucleus and there was a small amount of cytosolic accumulation (Fig. 1A). In elderly cases (age 72, 79 and 81 years), REST was observed in both the nucleus and cytosol (Fig. 1B), suggesting that nuclear entry of REST is senescence-dependent. This observation was consistent with previous results, in which a cytoplasmic distribution of REST was observed without nuclear staining in...
neurons in the cortex, caudate nucleus, cerebellum, and hippocampus in cases aged 44–60 years [12]. These results indicate that nuclear accumulation of REST is a universal feature of senescent neurons. In contrast to control cases, REST immunoreactivity in patients with PD and DLB was decreased in dopaminergic SN neurons (Fig. 2A–E) and in cortical neurons (Fig. 2F). Importantly, the REST signal was strongly detected in Lewy bodies and pale bodies (Fig. 2C–F). Since a previous report showed that REST is incorporated into cytoplasmic protein aggregates in the cortical neurons of patients with AD, DLB, and FTD [6], it is consistent with our result that REST was detected in Lewy bodies and pale bodies. These results suggest that protein inclusions have a propensity to sequester the REST protein and may prevent senescence-dependent nuclear entry.

Disease-related aggregation-prone proteins such as alpha-synuclein in PD, Aβ or Tau in AD, huntingtin in Huntington’s disease (HD), and TDP-43 in frontotemporal dementia (FTD) have a tendency to form amyloid-like structures and protein aggregates because of their stable structure in the misfolded state. These aggregates generated in a cell contain many kinds of ubiquitinated proteins and p62/SQSTM1, which is known as the major autophagy receptor for selective autophagy [5,15]. To determine whether REST has a propensity to interact with ubiquitinated protein aggregates that are not aggregation-prone, we

![Fig. 3. REST expression is preceded by deficiencies in proteolysis and the REST gene product is localized to aggregates (A) Immunofluorescence labeling of TH (a, f), DAPI (b, g), p62 (c, h), and REST (e, j) in the substantia nigra (SN) of a 12-month-old Atg7fl/fl:TH-Cre (a–e) and Atg7fl/fl (f–j) mice. REST and p62 colocalized in the aggregates in TH-positive neurons. Scale bar: 10 μm. (B) Schematic representation of REST including REST4. Primer 1: (Ex3–Ex4, 892-1054), Primer 2: (Int3–Ex4, -1072), Primer 3 (Ex4, 3007-3100) (C) Quantification of REST mRNA in SH-SYSY cells treated with 5 μM of MG132 for 12 h. qRT-PCR primer: primer 1, second half of exon 2 to exon 3 (REST4); primer 2, first half of exon 4; primer 3, second half of exon 4. REST4 mRNA levels were increased, but there were no remarkable changes in the levels of full-length REST mRNA. (D) REST degradation inhibition. Immunoblotting using SH-SYSY cells lysate treated with MG132 (10 μM), rotenone (1 mM) and lactacystin (1 or 2 μM) for 7 h (lane 1-6) and 14 h (lane 7-12) as indicated.
evaluated REST localization in the brain of dopaminergic neuron-specific Atg7 conditional knockout mice (Atg7flox/−ox:TH-Cre) [11]. In the SN of control mice aged 12 months, REST and p62 immunoreactivity was under the detection limit in tyrosine hydroxylase (TH)-positive neurons (Fig. 3A–j). Of note, we used the Millipore anti-REST antibody to detect the mouse REST protein immunohistochemically because the Bethyl anti-REST antibody is poorly reactive in mouse brain tissue (Supplementary Fig. 1B). In contrast, REST immunoreactivity was detected in p62-positive punctate structures in TH-positive neurons of Atg7flox/−ox:TH-Cre mice, suggesting that induced REST is incorporated into cytoplasmic aggregates that accumulate via autophagy dysfunction (Fig. 3Aa–e). Furthermore, REST co-localized with p62 bodies (Fig. 3Ac–e), suggesting that it is part of a lesion associated with Lewy body [11,7] and the neuronal REST may be constitutively degraded through p62-mediated selective autophagy pathway under normal conditions.

Because the autophagy deficiency results in the REST accumulation in TH-positive neurons, the level of REST in TH-positive neurons may be altered by cellular stress response. To test whether the accumulation of ubiquitinated proteins induces REST gene expression, we measured the level of REST in human neuroblastoma cell line, SH-SYSY, by quantitative RT-PCR analysis using three different primer sets under the proteasome inhibitor, MG132, treated condition. The REST mRNA was amplified more than 2.5 times only by primer set 1 in MG132 treated SH-SYSY cells (Fig. 3B). In neuronal cells, the transcribed REST RNA is processed by neuron specific splicing complex and converted into neuron specific variant, REST4 [10]. Because the primer set 1 amplifies both mRNAs of full-length REST and neuron specific splicing variant, REST4, but primer set 2 and 3 do only the full-length REST mRNA, increased amounts of REST transcripts by MG132 treatment may be spliced into the REST4 mRNA. The immunoblot analysis revealed that 10 μM MG132 treatment resulted in the accumulation of REST protein, but not the more proteasome specific inhibitor, lactacystin, treatment, supporting the idea that REST is degraded through autophagy-lysosome pathway (Fig. 3D), because high concentration of MG132 inhibits both proteasomal- and lysosomal-protein degradation. Intriguingly, interfering with the electron transport chain in mitochondria by Rotenone treatment reduced the REST accumulation. As rotenone is a possible Parkinson-causing agent, loss of neuronal REST accumulation in aged-neurons may relate to the PD pathology.

4. Discussion

In this study, we demonstrated nuclear accumulation of REST in cortical and SN neurons in the normal aging human brain using immunohistochemistry. On the other hand, REST immunoreactivity was observed in Lewy bodies and pale bodies in the postmortem human brain of patients with DLB and PD. We recently reported that Atg7flox/−ox:TH-Cre mice demonstrate neuronal loss and typical Lewy body disease pathology, including p62, ubiquitin, and synuclein, a pattern which resembles PD [11]. In addition, as these mice age, they develop neuronal loss and motor dysfunction. Interestingly, REST is sequestered in protein aggregates in the brain of Atg7flox/−ox:TH-Cre mice. As the accumulation of both p62 and ubiquitin in protein aggregates is a common pathological hallmark of several neurodegenerative diseases including PD [17], the accumulation of aggregates may prevent neuroprotective signaling through the mechanism of REST sequestration.

These results suggest that malfunctioning cellular homeostasis alters neuronal REST accumulation to protect cells and the protective function might be regulated by REST nuclear entry in aged-neurons. Indeed, it is unclear what kinds of neuronal stress induce REST expression. In our in vitro studies, MPTP treatment did not alter the amount and the localization of REST in SH-SYSY cells (Supplementary Fig. 2), but malfunction of protein quality control systems induced REST/REST4 mRNA expression that might preserve neuronal function and prevent neuronal degeneration. Regarding neuronal inclusions, it remains unclear whether these inclusions are protective of neurons or not. We propose that REST sequestration in Lewy bodies or pale bodies disturbs neuronal survival responses against environmental stress and induces disease progression in PD. The loss of nuclear REST in dopaminergic neurons may be related to PD onset. Senescence-induced REST expression may result in its nuclear accumulation and repression of target genes, which may differ from conventional neuron-specific genes. This needs to be clarified if REST plays critical roles of neuronal survival in aged-human brain and relates to the PD pathology.

In conclusion, nuclear accumulation of REST occurs as a normal aging process and Lewy pathology disturbs the process in dopaminergic neurons by sequestering REST. The alteration of neuronal aging processes including the loss of REST in neurons may associate with the PD pathogenesis.

Declaration of conflicts

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.01.042.

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