**Tau Knockout and α-Synuclein A53T Synergy Modulated Parvalbumin-Positive Neurons Degeneration Staging in Substantia Nigra Pars Reticulata of Parkinson’s Disease-Liked Model**

Meige Zheng\(^{1*}\), Yanchang Liu\(^{1*}\), Zhaoming Xiao\(^{1}\), Luyan Jiao\(^{2}\) and Xian Lin\(^{3,4}\)

\(^{1}\) Department of Orthopaedics, The Second Hospital of Anhui Medical University, Hefei, China; \(^{2}\) Nuwacell Biotechnologies Co., Ltd, Hefei, China; \(^{3}\) Guangdong Province Key Laboratory of Brain Function and Disease, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China; \(^{4}\) Department of Anatomy, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

The loss of parvalbumin-positive (PV\(^{+}\)) neurons in the substantia nigra pars reticulata (SNR) was observed in patients with end-stage Parkinson’s disease (PD) and our previously constructed old-aged Pitx3-A53Tα-Syn \(\times\) Tau\(^{-/-}\) triple transgenic mice model of PD. The aim of this study was to examine the progress of PV\(^{+}\) neurons loss. We demonstrated that, as compared with non-transgenic (nTg) mice, the accumulation of α-synuclein in the SNR of aged Pitx3-A53Tα-Syn \(\times\) Tau\(^{-/-}\) mice was increased obviously, which was accompanied by the considerable degeneration of PV\(^{+}\) neurons and the massive generation of apoptotic NeuN\(^{+}\)TUNEL\(^{+}\) co-staining neurons. Interestingly, PV was not costained with TUNEL, a marker of apoptosis. PV\(^{+}\) neurons in the SNR may undergo a transitional stage from decreased expression of PV to increased expression of NeuN and then to TUNEL expression. In addition, the degeneration of PV\(^{+}\) neurons and the expression of NeuN were rarely observed in the SNR of nTg and the other triple transgenic mice. Hence, we propose that Tau knockout and α-syn A53T synergy modulate PV\(^{+}\) neurons degeneration staging in the SNR of aged PD-liked mice model, and NeuN may be suited for an indicator that suggests degeneration of SNR PV\(^{+}\) neurons. However, the molecular mechanism needs to be further investigated.

**Keywords:** Tau knockout, α-synuclein, parvalbumin, NeuN, degeneration staging

**INTRODUCTION**

The most predominant pathological feature of Parkinson’s disease (PD) is the massive loss of dopaminergic neurons in the substantia nigra pars compacta (SNC) (Poewe et al., 2017). It may be closely related to the massive accumulation of α-synuclein (α-syn) and the aberrant expression of the microtubule-associated protein tau (Bassil et al., 2021; Han et al., 2021), both of which have been identified as the first two
genes of the population attributable risk underlying PD in genome-wide association studies (Simon-Sanchez et al., 2009).

The majority of PD research has focused on the SNC, while substantia nigra pars reticulata (SNR), as the other part of the substantia nigra, has received little attention (Dionisio et al., 2021; Pirooznia et al., 2021). Previous studies showed that the activity of parvalbumin-positive (PV+) neurons in the SNR were affected in PD animal models (Wichmann et al., 1996; Hardman et al., 1996; Jiao et al., 2020). The line of Pitx3-A53T tetO-hTau transgenic mice (pituitary homeobox 3 (Pitx3), in which the expression of human α-syn A53T transgenic mice (tetO-A53T) and human wild-type Tau inducible transgenic mice (tetO-hTau), in which the expression of human α-syn A53T and human wild-type Tau were under the transcriptional control of tetracycline operator (tetO), and the line of Tau−/− mice were used to generate triple transgenic mice and maintained on C57BL/6J background. The mice were reared under specific-pathogen-free (SPF) conditions, with 12-h light/12-h dark cycles and fed a regular diet ad libitum. All experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Immunofluorescence
The mice were perfused transcardially with phosphate-buffered saline (PBS). Brains were separated and soaked in 4% paraformaldehyde for 48 h. Then, 30% sucrose solution was used for dehydration treatment. A cryostat (Leica SM 2010R, Germany) was used to cut the brains into continuous slices with a thickness of 40 µm. The following primary and secondary antibodies were used as recommended by the manufacturers: rabbit anti-human/mouse α-synuclein (α-syn; Santa Cruz Biotechnology, United States, sc-7011-R, 1:1000), mouse anti-PV (Sigma-Aldrich, United States, P3088, 1:500), rabbit anti-PV (Abcam, United States, ab11427, 1:1000), rabbit anti-c-fos (Sigma-Aldrich, United States, F7799, 1:1000), mouse anti-neuronal nucleus (NeuN; Chemicon, United States, MAB 377, 1:500), and Alexa 488 or Alexa 555 conjugated secondary antibody (Invitrogen, United States, 1:500). For antibodies produced in mice, the mouse-on-mouse immunodetection kit (Vector Laboratories, United States, BMK-2202) was used following the manufacturer’s protocol. A laser scanning confocal microscope (LSM 710; Zeiss, Germany) was used for observing and photographing. The paired images in all the figures were acquired under the same conditions and processed uniformly after collection.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Staining
According to the manufacturer’s protocol, apoptotic cells were labeled with an apoptosis detection kit (Roche, United States, 11684795910). The frozen sections were adhered to the slides and air-dried at 50°C for 30 minutes. Slides were rinsed in PBS for 5 min × 4 times, and then immersed in the permeabilization solution (0.1% sodium citrate, containing0.1% TritonX-100) and incubated on ice (2–8°C) for 2 min. Slides were washed in PBS for 5 min × 4 times. Afterward, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture was added to the slides and incubated at 37°C in the dark for 1 h. Slides were then rinsed in PBS for 5 min × 4 times and in ddH2O for 5 min × 3 times. After air-drying for 2 h at 37°C in the dark, the slides were mounted with ProLong® Gold anti-fading reagent (Invitrogen, United States, 1724814) before analysis.

Image Analysis
Cell counting of TUNEL+, NeuN+, and PV+ cells was performed in serial coronal sections across the SNR (every fourth from bregma, −3.28 to −4.04 mm). Fluorescence images of TUNEL, NeuN, and PV on each of the coronal sections in the SNR of 2-, 6-, and 12-month-old Pitx3-A53Tα-Syn × Tau−/− mice were captured with a laser-scanning confocal microscope (LSM 710; Zeiss, Germany) at 20 × magnification. The numbers of TUNEL+, NeuN+, and PV+ cells, and PV and NeuN double-positive cells, and TUNEL and NeuN double-positive cells were counted with NIS-Elements BR Imaging software (Nikon Instruments, Japan). The percentage of PV and NeuN double-positive cells to the total number of PV+ cells and the percentage of TUNEL and NeuN double-positive cells to the total number of TUNEL+ or NeuN+ cells in the SNR were determined.
To quantify the relative immunofluorescence intensity of α-syn, c-fos, and PV in the SNR of mice, images were taken using identical settings and then analyzed using ImageJ software (NIH, United States). To measure c-fos and PV fluorescent intensity, only fluorescence within the PV+/-syn+ co-staining neurons in the SNR was analyzed. All the images were converted to an 8-bit color scale and the background was subtracted. Areas of interest were selected by the freehand selection tools and subjected to measurement by mean gray value to determine the average intensity.

Three mice were used per genotype and at each time point. Counters were blinded to the information of the samples.

Statistics
GraphPad Prism 7 (GraphPad Software, United States) was used for statistical analysis. Data were expressed as mean ± SEM. The one-way analysis of variance was used to compare the means of different groups, followed by Tukey’s honestly significant difference test, and significance was set at P < 0.05.

RESULTS

Tau Knockout Exacerbated the Accumulation of α-Syn in the Substantia Nigra Pars Reticulata of α-Syn A53T Conditional Transgenic Mice

As described in our previous study, we continued to use the Nigra Pars Reticulata of α-Tau A53T for statistical analysis. Data were expressed as mean ± SEM. The one-way analysis of variance was used to compare the means of different groups, followed by Tukey’s honestly significant difference test, and significance was set at P < 0.05.

The expression product of the Fos gene, c-fos, is most prominently related to the accumulation of α-Syn A53T neurons in the Substantia Nigra Pars Reticulata at 18-Month-Old mice had almost the same levels of α-Syn −/− mice as compared to the triple transgenic mice and α−/− mice (Figures 1A,B). These results indicated that the degenerating PV+−/− mice showed practically degeneration of PV+ and c-fos+ neurons in the SNR at old age.

τ Knockout Exacerbated Cell Apoptosis and Parvalbumin-Positive Neuron Loss Asynchronously in the Substantia Nigra Pars Reticulata of α-Syn A53T Conditional Transgenic Mice

To determine whether the decrease of PV+ neurons was due to cell apoptosis, we stained PV and TUNEL in the SNR of 18-month-old mice. The results revealed that the less PV expressed, the more TUNEL signals were detected, as shown in the Pitx3-A53Tα-Syn × Tau−/− mice. However, TUNEL did not costain with PV in the SNR of all the triple transgenic mice (Figure 3). These suggested that the degenerating PV+ neurons could not be labeled by TUNEL, and they might undergo a transitional stage between the expression of PV and TUNEL.

Pitx3-A53Tα-Syn × Tau−/− Mice Presented Massive NeuN+ Neuronal Apoptosis in the Substantia Nigra Pars Reticulata

NeuN is a neuron-specific marker, but its expression in the SNR is species-specific. For example, the neurons in the gerbil SNR do not express NeuN, whereas the neurons in the rats' SNR strongly express NeuN (Mullen et al., 1992; Kumar and Buckmaster, 2007; Gusel'nikova and Korzhhevskiy, 2015). To determine the type of cells which presented massive apoptosis in the SNR but did not co-stain with PV, we examined TUNEL and NeuN staining in the SNR of 18-month-old mice. The results showed that TUNEL was highly costained with NeuN in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice (Figure 4A). The percentage of TUNEL NeuN+ co-staining cells relative to the total number of TUNEL+ or NeuN+ cells reached 94.47 ± 2.12% or 92.72 ± 1.1%, respectively (Figure 4B). However, in the SNR of the other triple transgenic mice and nTG mice, the neurons rarely expressed NeuN, which did not co-stain with TUNEL either (Figure 4A). Considering the previous experimental results (Figure 3), we preliminarily speculated that NeuN may be suited for an indicator of the transitional stage of degenerating neurons in SNR between expression of PV and TUNEL, especially, at the beginning of 6-month-old.
FIGURE 1 | α-syn accumulation was increased in the substantia nigra pars reticulata (SNR) of 18-month-old Pitx3-A53T α-Syn × Tau−/− mice. (A) α-syn immunostaining in the SNR of 18-month-old mice, compared to nTg mice, α-syn was obviously overexpressed in dopaminergic neurons in SNC of the triple transgenic mice. White dotted lines demarcate the boundary between SNC and SNR. The ventrolateral area was considered as SNR. Scale bars: low magnification (10×), 100 µm; higher magnification (40×) in SNR, 25 µm. (B) Quantitative analysis of α-syn fluorescence intensity in SNR of 18-month-old mice. n = 3 per genotype. Values are mean ± SEM. **P < 0.01 (Pitx3-A53Tα-Syn × Tau−/− vs nTg).

NeuN Gradually Replaced Parvalbumin to Mark Apoptotic Neurons in the Substantia Nigra Pars Reticulata of Pitx3-A53Tα-Syn × Tau−/− Mice

To further explore whether NeuN can specifically label the degenerating PV+ neurons, we stained PV and NeuN in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice at 2- and 6-months old. The results showed that PV+ neurons in the SNR did not begin to degenerate at 2-month-old because the expression level of PV was high while the expression level of NeuN was low (Figure 5A). At 6-month-old, NeuN was gradually increased and highly costained with PV (Figures 5A,C). Combining the previous results, while the loss of PV+ neurons (12- and 18-month-old) becoming significantly (Figures 2, 3), NeuN was highly costained with TUNEL (Figures 4, 5B,C). Therefore, we suspected that in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice, the rapidly progressed loss of PV+ neurons may undergo a transitional stage, i.e., from decreasing PV expression, to increasing NeuN expression, finally to TUNEL expression (Figure 6); NeuN may be a compatible marker labeling the degeneration of PV+ neurons at the beginning of 6-month-old.

DISCUSSION

In the present study, we revealed that tau knockout specifically aggravated A53T α-syn-mediated PV+ neurons degeneration staging and α-syn accumulation in the SNR of mice at old age (late-stage). We used the most classic TUNEL assay to stain the apoptotic neurons in the SNR and attempted to demonstrate that the neurons lost in SNR were PV+ neurons. Contrary to our expectations, TUNEL and PV were not co-stained. Indeed, through continuously observing the co-staining of PV and NeuN, NeuN, and TUNEL, we divided three stages by the different active states in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice, as shown in Figure 6. At 2–6 months (initial-stage), PV was expressed normally in the SNR, while NeuN was modestly expressed and costained with PV. At 6–12 months (Middle stage), PV expression was...
FIGURE 2 | Decreased expression of c-fos in the parvalbumin-positive (PV+) neurons in the SNR of α-syn AS3T transgenic mice. (A,B) PV (green) and c-fos (red) costained in the SNR of 6- (A) and 18-month-old (B) mice. Scale bars: 25 µm. (C) Quantification of c-fos and PV fluorescence mean intensity in PV+/c-fos+ co-staining cells in the SNR of 6-, 12- and 18-month-old Ptx3-AS3Tα-Syn × Tau−/− mice (n = 30 cells for each sample, 3 mice for each time point). Values are mean ± SEM. ***P < 0.001 (c-fos, 6-month-old vs 18-month-old Ptx3-AS3Tα-Syn × Tau−/−); #P < 0.05, ##P < 0.01, ###P < 0.001 (PV, 6- and 12-month-old vs 18-month-old Ptx3-AS3Tα-Syn × Tau−/−). (D) Representative dot plots of c-fos fluorescence mean intensity in PV+ neurons in the SNR of 18-month-old mice (n ≥ 30 cells for each sample, 3 mice per genotype). ***P < 0.001 (nTg vs triple transgenic); #P < 0.05, ##P < 0.01, ###P < 0.001 (other triple transgenic vs Ptx3-AS3Tα-Syn × Tau−/−).
FIGURE 3 | Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) (green) and PV (red) double-staining in the SNR of 18-month-old mice. Scale bar: 50 µm.

FIGURE 4 | Pitx3-A53T α-Syn × Tau−/− mice presented massive NeuN+ neuron apoptosis in the SNR at old age. (A) TUNEL (green) and NeuN (red) double-staining in the SNR of 18-month-old mice. Scale bar: 50 µm. Figures in the white boxes represent the high-magnification images of TUNEL and NeuN co-staining in the SNR of 18-month-old Pitx3-A53T α-Syn × Tau−/− mice. (B) The percentage of TUNEL+ NeuN+ co-staining cells relative to the total number of TUNEL+ or NeuN+ cells in the SNR of 18-month-old Pitx3-A53T α-Syn × Tau−/− mice. n = 3, values are mean ± SEM.
FIGURE 5 | NeuN may be suited for an indicator that suggests degeneration of PV+ neurons in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice at the beginning of 6-month-old. (A) PV (green) and NeuN (red) double-staining in the SNR of 2- and 6-month-old Pitx3-A53Tα-Syn × Tau−/− mice. Scale bar: 100 µm. Figures in the white boxes represented the high-magnification images of PV and NeuN co-staining in the SNR of 6-month-old Pitx3-A53Tα-Syn × Tau−/− mice. (B) TUNEL (green) and NeuN (red) co-staining in the SNR of 12-month-old Pitx3-A53Tα-Syn × Tau−/− mice. Figures in the white boxes represented the high-magnification images of TUNEL and NeuN co-staining. Scale bar: 100 µm. (C) Percentage of PV and NeuN co-staining cells to the total number of PV+ cells, and percentage of TUNEL and NeuN co-staining cells to the total number of TUNEL+ cells in the SNR of 2-, 6-, and 12-month-old Pitx3-A53Tα-Syn × Tau−/− mice. n = 3 per genotype per time point. Values are mean ± SEM. ****P < 0.0001 (2- and 12-month-old vs. 6-month-old Pitx3-A53Tα-Syn × Tau−/−). ND, not determined.

decreased and replaced by NeuN, which was increased obviously. At 12–18 months (late-stage), neurons developed apoptosis as TUNEL was expressed and co-stained with NeuN. So, we propose that the loss of PV+ neurons in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice may undergo a transitional stage, i.e., from decreased expression of PV to increased expression of NeuN to TUNEL expression.

Numerous studies have demonstrated that tau plays an important role in maintaining neuronal integrity and axonal transport (Augustinack et al., 2002; Combs et al., 2019). This might be because the main function of tau is responsible for the dynamic assembly of the cytoskeleton in neurons (Venkatramani and Panda, 2019). Tau depletion caused preferential loss of the labile microtubule fraction in the axon (Qiang et al., 2018). Thus, in the case of stalled growth cones of tau-depleted axons, axonal regeneration was retarded extremely (Biswas and Kalil, 2017). In the present study, we have used A53Tα-syn conditionally transgenic mice to construct PD models with different expression levels of tau, all of which had developed selective loss of SNC dopaminergic neurons and severe motor coordination and balance disorders, as described previously (Jiao et al., 2020). Interestingly, we found that different degrees of SNR neuronal death were specifically induced by different tau gene dosages. At 18-month-old of the triple transgenic mice, PV+ neurons degeneration caused by tau knockout was the most significant. It indicates that tau is important for maintaining the activity of PV+ neurons in SNR, and tau knockout could accelerate the progression of PD mediated by A53T α-syn and promote the degeneration of SNR PV+ neurons.

Parvalbumin (PV) is a calcium-binding protein, which accounts for an abundant subpopulation of GABAergic neurons (Hu et al., 2014). GABAergic neurons exhibit fast-spiking patterns and form direct inhibitory synapses with the cell bodies, proximal dendrites, and starting segments of cortical pyramidal neurons (Siemian et al., 2020). Numerous studies have suggested that the number of PV+ GABAergic neurons in the SNR is absolutely dominant (McRitchie et al., 1996; Galaj et al., 2020). Therefore, PV can specifically mark the SNR and reflect the physiological state of this area. In this study, Pitx3-A53Tα-Syn × Tau−/− mice showed specific decreased expression of PV and c-fos in the SNR at old age, which may be
correlated with their increased α-syn aggregates and anxiety-like behavior. However, the mechanism of this specificity in the tau knockout state remains unclear. Uchida et al. (2014) reported that maternal stress and mutations in glutamate decarboxylase (GAD) 67, both risk factors for psychiatric disorders, can cause selective loss of PV+ GABAergic interneurons in the cerebral cortex. This suggests that specific degeneration of PV+ neurons can be associated with dysfunction of tau and GAD67, which needs further study.

Unexpectedly, our study found that tau knockout exacerbated TUNEL+ cell apoptosis and PV+ neuron loss asynchronously in the SNR of α-syn A53T conditional transgenic mice, as TUNEL did not costain with PV. This suggests that the degenerating SNR neurons might undergo a transitional stage between the expression of PV and TUNEL. Although NeuN is usually used as a definitive marker of mature neurons in neurodegenerative diseases, its role has been challenged by recent studies, indicating that NeuN staining is variable and even absent during certain diseases and specific physiological states (Duan et al., 2016). For example, NeuN expression in the SNR is species-specific. While neurons in the rats’ SNR strongly express NeuN, neurons in the gerbil SNR do not express it (Kumar and Buckmaster, 2007). Here we propose that NeuN may be an indicator of the transition of PV+ neurons from normal to degenerating phase in the SNR of mice. The progressive degeneration of PV+ neurons in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice may undergo a transitional process from decreased PV expression to increased NeuN expression and finally to TUNEL expression (Figure 6).

In conclusion, our results suggested that tau knockout can exacerbate α-syn A53T-mediated PV+ neurons degeneration staging, from PV expression reduced to NeuN expression increased to TUNEL expression, in the SNR of mice. The rapid increase of NeuN at the middle stage and co-staining with TUNEL at the late stage made it gradually replace PV as a better

### Figure 6

| Initial stage (Normal physiology, <6M) | Middle stage (Degeneration, 6~12M) | Late stage (Cell death, >12M) |
|---------------------------------------|-----------------------------------|-------------------------------|
| PV+ was expressed normally; NeuN+ was modestly expressed, and costained with PV+; No TUNEL+ expression | PV+ expression reduced, and replaced by NeuN+; NeuN+ expression increased; No TUNEL+ expression | Late-stage: neurons developed apoptosis, PV+ expression decreased rapidly; TUNEL+ was expressed, and costained with NeuN+ |

The character of PV+ — NeuN+ — TUNEL+:

a. PV+: reduced gradually from early-stage to middle-stage; developed a robust loss at late-stage.

b. NeuN+: increased gradually and costained with PV+ from middle-stage to late-stage; costained with TUNEL+ at late-stage.

c. TUNEL+: expressed at late-stage and costained with NeuN+.

We divided the three stages by the different active states in the SNR of Pitx3-A53T α-Syn × Tau−/− mice, which may be occurred simultaneously and overlappingly.

**FIGURE 6** | The loss of PV+ neurons in the SNR of Pitx3-A53Tα-Syn × Tau−/− mice may undergo a transitional stage, i.e., from decreased expression of PV to increased expression of NeuN to TUNEL expression.
indicator of the degeneration of PV+ neurons in the SNR. We hope that this research can provide a reference for long-term observation of the neuron degeneration in the brain of the mouse model of PD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Sun Yat-sen University.

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