

Oligodendroglial somatic SNCA copy number gains are associated with inclusions and disease onset in multiple system atrophy

Caoimhe Morley

University College London

Ester Kalef-Ezra

University College London

Diego Perez-Rodriguez

University College London

Zane Jaunmuktane

University College London

Christos Proukakis

c.proukakis@ucl.ac.uk

University College London

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Abstract

Multiple system atrophy (MSA) is a rapidly progressive synucleinopathy of unknown aetiology with neuronal and oligodendroglial α -synuclein inclusions. We previously reported somatic copy number variants (CNVs), specifically gains, of *SNCA* (encoding α -synuclein) in MSA brains. Here, we expand on this work by combining fluorescent *in situ* hybridisation for *SNCA* on isolated nuclei with α -synuclein and SOX10 immunofluorescence, to assess oligodendrocyte-specific *SNCA* gains and losses, and their relationship with inclusions across differentially affected regions in two MSA subtypes: striatonigral degeneration (SND) and olivopontocerebellar atrophy (OPCA). Analysis of 13 SND, 12 OPCA, and 15 control brains demonstrated significantly higher somatic *SNCA* CNVs, both gains and losses, in MSA oligodendrocytes compared with controls (gains: 6.3% vs 2.0%; losses: 12.5% vs 7.4%, $p < 0.0001$ for both). Oligodendrocyte *SNCA* gains were high in the preferentially affected regions (putamen in SND, cerebellum in OPCA), where they were associated with a two-fold increased relative risk of α -synuclein inclusions in the same cell ($p < 0.0001$). Higher gain burden correlated with earlier disease onset ($\rho = -0.45$, $p = 0.03$). Oligodendrocyte *SNCA* losses, conversely, showed less regional predilection, limited association with inclusions, and no correlation with onset age. As double strand DNA breaks have been reported in Lewy body diseases, and may cause deletions, we used immunofluorescence for γ H2AX to explore their prevalence in MSA in a subset of experiments. The proportion of γ H2AX-positive cells was significantly higher in MSA than controls, both overall (6.0% vs 2.2%, $p = 0.049$) and in oligodendrocytes (14.4% vs 5.5%, $p = 0.02$), and also in inclusion-bearing cells (22.2% vs 14.9%, $p = 0.02$). These findings define the oligodendrocyte-specific patterns of somatic *SNCA* CNVs in MSA, support a role for gains in MSA pathogenesis, and demonstrate the presence of *SNCA* losses and DNA double strand breaks which require further investigation.

Introduction

Multiple system atrophy (MSA) is a rare synucleinopathy characterised by varying combinations of autonomic failure, parkinsonism, and cerebellar ataxia, and a rapidly progressive disease course [9]. Although neuronal α -synuclein aggregation is a shared hallmark across all synucleinopathies, MSA is uniquely defined by the additional presence of glial cytoplasmic inclusions (GCIs) within oligodendrocytes [13, 59]. Neuronal inclusions, including neuronal cytoplasmic and neuronal nuclear inclusions, are distinct from the Lewy bodies (LBs) and Lewy neurites that characterise Parkinson's disease (PD) and Dementia with Lewy bodies (DLB), which are comparatively rare in MSA [22, 28, 55]. MSA diverges into distinct pathological subtypes, including striatonigral degeneration (SND), olivopontocerebellar atrophy (OPCA), and a mixed subtype. Despite these well-defined pathological patterns, the mechanisms that render oligodendrocytes and specific brain regions selectively vulnerable remain unclear and represent a critical gap to bridge in our understanding of MSA pathogenesis and phenotypic heterogeneity.

In contrast to PD and DLB, where genetic studies have identified multiple Mendelian and risk loci implicating diverse biological pathways, the aetiology of MSA is unknown [27]. MSA shows no clear familial aggregation and very low heritability ($< 7\%$) [10] and to date no major reproducible inherited genetic risk factors have been established [7, 57]. Nevertheless, chronic elevation of α -synuclein is sufficient to drive its aggregation, as demonstrated in PD cases with germline *SNCA* multiplications [53]. These cases highlight the strong dosage sensitivity of α -synuclein: *SNCA* duplications typically lead to later-onset parkinsonism with reduced

penetrance, whereas triplications cause early-onset, rapidly progressive disease [18]. Some individuals with *SNCA* multiplications also exhibit mixed neuronal and glial pathology, suggesting that excess α -synuclein expression can also be detrimental to oligodendrocytes [53, 65]. Experimental models further support this, as oligodendrocyte-specific overexpression of human α -synuclein in mice generates GCI-like pathology, demyelination and neuroinflammation, with higher expression producing earlier and more severe neurodegeneration [52, 63]. Together, these observations suggest that cell- or region-restricted increases in *SNCA* dosage could contribute to the pathogenesis of MSA.

Somatic mutations, arising post-zygotically and resulting in genetic mosaicism within the brain, have emerged as potential contributors to neurodegenerative disease [30, 36, 43, 61]. These alterations encompass a wide range, from single nucleotide variants to large-scale copy number variants (CNVs, gains or losses) and other structural variants. Somatic mutations may result from mitotic errors or diverse types of DNA damage, including highly mutagenic double stranded DNA breaks (DSBs) [46]. DSB repair in post-mitotic cells is primarily by non-homologous end joining (NHEJ) [33] which can lead to deletions [46]. DSBs have been reported in Lewy body diseases [28, 57], but not investigated in MSA to our knowledge. We previously demonstrated through fluorescence *in situ* hybridisation (FISH) studies somatic CNVs, specifically gains, of *SNCA* (encoding α -synuclein) in substantia nigra dopaminergic neurons in PD, with the highest levels reported in two MSA cases [38]. Subsequent work detected higher levels of *SNCA* gains in neurons of the cingulate cortex in both PD and MSA compared to controls, and in non-neurons in MSA only [40]. More recently, we demonstrated increased levels of somatic *SNCA* gains in differentially affected regions in MSA subtypes, the putamen in SND and cerebellum in OPCA, with an increased relative risk for α -synuclein inclusions at the single-cell level [14].

Importantly, these studies did not establish whether *SNCA* gains occur in oligodendrocytes, although their level was higher in oligodendrocytes in the substantia nigra of three SND cases analysed. Moreover, none included region-matched controls from the putamen, cerebellum, and substantia nigra, precluding assessment of whether somatic *SNCA* gains in these regions are truly enriched in MSA. Finally, the potential role of somatic *SNCA* losses has been unexplored and may have distinct functional consequences. Loss of α -synuclein function is not a known driver of any neurodegenerative disease, and experimental models have produced conflicting results regarding its impact [1, 6, 23, 45]. However, aggregated α -synuclein may sequester the protein and diminish its normal physiological functions [51, 62], raising the possibility that dosage reductions could contribute to cellular vulnerability under certain conditions. Together, these observations highlight the need for a systematic, cell-type-resolved and region-matched assessment of somatic *SNCA* CNVs in MSA.

Here, we aim to address these gaps by applying an optimised protocol combining FISH and immunofluorescence on isolated nuclear suspensions, to eliminate sectioning artefacts and reliably call copy number losses in addition to gains. We find that, in MSA, somatic *SNCA* gains are enriched in SOX10⁺ oligodendrocytes relative to controls, in subtype-specific vulnerable regions, and associate with α -synuclein inclusions at both single-cell and regional levels, consistent with a role in pathogenesis. A higher burden of oligodendrocyte *SNCA* gains correlates with earlier disease onset, supporting a model in which pre-existing somatic *SNCA* mosaicism may predispose individuals to α -synuclein aggregation. We now show, for the first time, somatic *SNCA* losses, which are also higher in MSA, though more broadly distributed, with less clear

regional associations with inclusions and no association with age of onset. Our results provide further insights into somatic *SNCA* CNVs in MSA, with oligodendrocyte gains potential drivers of pathogenesis. Furthermore, we perform immunofluorescence staining of tissue sections for phosphorylated H2AX at serine 139 (γ H2AX), an established DSB marker [32], which reveals increased DSBs in MSA compared to controls, notably in oligodendrocytes and in cells with α -synuclein inclusions.

Materials and Methods

Human fresh-frozen post-mortem brain tissue

Fresh-frozen post-mortem brain samples were obtained from the Queen Square Brain Bank for Neurological Disorders in London, United Kingdom. All subjects or their next of kin had given informed consent for brain donation and use in research. Ethics approval was provided by the National Research Ethics Service (07/MRE09/72). Tissue chips and 10 μ m tissue sections were obtained from three brain regions including cerebellar white matter, putamen at the level of the anterior commissure, and the substantia nigra at the level of the red nucleus or decussation of the superior cerebellar peduncle. For clarity, these are hereafter referred to as the *putamen*, *cerebellum*, and *substantia nigra*, respectively. This study included a total of 42 subjects: 27 with pathologically confirmed MSA (15 SND, 12 OPCA) and 15 controls (14 neurologically healthy, 1 with dystonia but normal neuropathology). A summary of experiments performed across each group and brain region are shown in Supplementary Table 1.

Nuclei isolation from fresh-frozen post-mortem brain tissue

Nuclear fractions were isolated from fresh-frozen tissue as previously described [21, 40, 41]. All procedures were performed at 4°C to maintain nuclei integrity. Briefly, 20–50 mg of tissue was added to 500 μ L of Nuclear Isolation Medium (NIM: 25 mM KCl, 5 mM MgCl₂, 10 mM Tris/HCL pH 8.8, 250 mM sucrose, 1X cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, 04693132001) and 1 mM dithiothreitol. Tissue was initially homogenised using a plastic pestle (20 strokes) and supplemented with 0.1% Triton-X 100. Complete homogenisation was performed using Dounce tissue grinders with a loose and tight pestle (5–10 strokes each, Sigma-Aldrich D8938). The homogenate was centrifuged at 1000g for 8 min at 4°C, after which the supernatant was removed. The resulting pellet was resuspended in 700 μ L of 25% Iodixanol prepared using 417 μ L of Optiprep™ Density Gradient Medium (60% w/v Iodixanol, Sigma-Aldrich D1556) in 500 μ L of NIM and 83 μ L of Optiprep Diluent for Nuclei (ODN: 150 mM KCl, 30 mM MgCl₂, 60 mM Tris/HCl pH 8.8 and 250 mM sucrose, 1X cOmplete™ EDTA-free Protease Inhibitor Cocktail). Subsequently, the 25% Iodixanol/homogenate mixture was gently layered using a syringe onto 1 mL of 29% Iodixanol (483 μ L Optiprep™ Density Gradient Medium and 517 μ L ODN) to form a gradient. The tube was ultracentrifuged at 10,300g for 20 min at 4°C. The debris layer and supernatant were discarded, leaving 50 μ L of buffer above the pellet. The pellet was resuspended according to the downstream analysis (see subheading Combined Immuno-Fluorescence *in situ* hybridisation (Immuno-FISH)).

Combined immuno-fluorescence in situ hybridisation (immuno-FISH) on isolated nuclei

We used the previously published FISH protocol on tissue sections [15] with minor modifications for compatibility with isolated nuclei. The same custom-designed red 50kb *SNCA* probe SureFISH (Agilent G110997R-8) was used, and in a subset of experiments a green 767kb Chr 7 chromosome enumeration probe (CEP) probe (G110899G-8) was included for reference. To account for potential variability between experiments, each batch was prepared containing a mixture of MSA subtypes and Controls.

Isolated nuclei were fixed with ice-cold Carnoy's Fixative (3:1 Methanol: Acetic Acid) and incubated on a rotator disk for 1 h at 4°C. The nuclei were dropped onto an Epredia™ SuperFrost Ultra Plus Gold Adhesion slides (Epredia, 11976299) and allowed to fully evaporate. Nuclei were permeabilised using a solution of 0.005% pepsin (Roche, 10108057001) in 10 mM HCl at 37°C for 5 min. Following digestion, slides were transferred to PBS supplemented with 1 mM MgCl₂ for 5 min, then washed twice in PBS. Slides were dehydrated through an ethanol series consisting of 70%, 90%, and 100% molecular-biology grade ethanol, each for 2 min, and then air-dried for 10 min. Next, slides were treated with 70% UltraPure™ formamide (Invitrogen, 15518746) in 2X saline sodium citrate (SSC) buffer at 72°C for 2 min. A second dehydration step was performed using ice-cold ethanol solutions (70%, 90%, and 100%).

FISH probe reactions were prepared according to the manufacturer's guidelines. The FISH probe mixture was denatured at 72°C for 5 min, and 10 µL of the mixture was applied per slide. A 22 × 22 mm coverslip was mounted and sealed using Fixogum rubber cement. Hybridisation was carried out in a humidified chamber at 37°C for 48–72 h. After hybridisation, slides were immersed in 2× SSC buffer at room temperature (RT) for 10 min to allow coverslip removal. They were then washed in preheated Wash Buffer 1 (Agilent, G9401A) at 72°C for 2 min, followed by a 1 min wash in Wash Buffer 2 (Agilent, G9402A) at RT. Finally, slides were washed three times in PBS for 10 min each.

For immunostaining, blocking solution was prepared with 10% goat serum in PBS with 0.2% Triton-X 100 and incubated for 1 h at RT. Primary antibody solutions were prepared in 2% goat serum and 0.2% Triton-X 100 according to the concentrations listed (see subheading *Antibodies used*). All primary antibodies were incubated overnight at 4°C. Following washes, species-specific secondary antibodies were applied and incubated for 1 h at RT. Nuclei were counterstained with 1 µg/mL DAPI (4',6-diamidino-2-phenylindole) solution for 20 min. Slides were cover-slipped with Agilent Mounting Buffer (G9403A).

Slides were imaged using a Leica DM6B epifluorescence microscope coupled to an ORCAII Digital CCD camera (Hamamatsu, Shizuoka, Japan) and controlled by Leica Application Suite X (Leica, Wetzlar, Germany). Z-stacks of 10 images separated by 0.5 µm stacks (5 µm depth total) using appropriate fluorescence filters were obtained with a 40X lens objective. All images were analysed blinded to the donor group and region. FISH signals were visualised using the ImageJ software v1.53t and analysed through manual counting first without the protein markers to obtain unbiased counts and then reviewed with the other colour channels. Exclusion criteria for a cell included (1) autofluorescence, as identified by overlapping signals on more than one channel, obscuring the nucleus, (2) faint, unclear or completely absent FISH signals, and (3) indistinguishable copy number status of overlapping nuclei.

Immunofluorescence on fresh-frozen tissue sections

Fresh-frozen tissue sections were cryosectioned at 10 µm thickness and mounted onto Superfrost Plus slides. Tissue sections were thawed for 20 min at RT and then fixed with 10% neutral-buffered formalin for 20 min at RT followed by permeabilization with 0.3% Triton-X in PBS for 60 min. A blocking solution containing 10% goat serum in 0.2% Triton-X 100 in PBS was applied for 1 hour at RT. Primary antibodies were prepared in 2% goat serum and 0.2% Triton-X 100 according to the concentrations listed (see subheading *Antibodies used*) and incubated overnight at 4°C. Secondary antibodies were prepared in 2% goat serum and 0.2% Triton-X 100 and incubated for 1 h at RT. A 1X solution of TrueBlack® Lipofuscin Autofluorescence Quencher (Biotium, 23007) was prepared in 70% ethanol and was applied to each slide for 1 min. DAPI (4',6-diamidino-2-phenylindole) was applied at a final concentration of 1 µg/mL to each slide for 20 min. Slides were mounted with ProLong™ Gold Antifade (Thermo Fisher Scientific, P36930).

For immunofluorescence markers, image analysis was performed through manual counting using ImageJ software v1.53t. Individual optical Z-stacks (10 stacks at 0.5 µm intervals, corresponding to a total optical depth of 5 µm) were examined to confirm true cellular co-localisation and exclude artefacts arising from overlapping fluorescence in adjacent focal planes. Cell counts and inclusion co-localisation were performed manually within defined regions of interest using merged and single-channel images. Identical exposure and threshold settings were applied across all samples within each staining batch.

Antibodies used

To label oligodendrocytes, a rabbit monoclonal anti-SOX10 antibody (1:50; 0.5 µg/mL; clone SP267, Abcam, ab227680) was used. Alpha-synuclein inclusions were detected using either a mouse monoclonal antibody (1:100; 2 µg/mL; clone 211, Santa Cruz, sc-12767) or a rabbit monoclonal antibody (1:1000; 1 µg/mL; clone MJFR1, Abcam, ab13850), depending on the compatibility with co-stained markers. A mouse monoclonal anti-phospho-Histone H2AX (Ser139) antibody (1:200; 5 µg/mL; clone JBW301) was used to detect DNA DSB repair. Secondary antibodies (anti-mouse/anti-rabbit) were used conjugated to 488 and 647 fluorophores (Thermo Fisher Scientific) at 2 µg/mL (1:500).

Statistics

All statistical analyses were performed in RStudio v2024.12.1 unless otherwise stated. Variables were assessed for normality using the Shapiro-Wilk test, and equality of variance was assessed with Levene's test for homogeneity of variance. Pairwise comparisons of normally distributed data were performed using the Student t-test (with Welch correction for unequal variance) or the nonparametric Mann-Whitney U test accordingly. For comparisons involving more than two groups, a one-way ANOVA for normally distributed data was used with Tukey's HSD post-hoc correction, and the nonparametric Kruskal-Wallis was used with Dunn's post-hoc correction. Where applicable, Bonferroni correction was applied to adjust for multiple comparisons. For normally distributed data, results are presented as the mean ± standard deviation (SD); for non-normal data or non-parametric comparisons, the median and interquartile range (IQR) are reported. Boxplots display the median and IQR, with whiskers extending to 1.5× IQR. Contingency tables were analysed using the Chi-square or Fisher's exact test based on sample size. For correlation assessments, Spearman's rank correlation was performed. Relative risk scores were calculated using MedCalc

(https://www.medcalc.org/en/calc/relative_risk.php). P-values are two-tailed and, unless otherwise stated, are nominal.

Results

Somatic SNCA CNVs are more frequent in MSA than controls and gains are enriched in MSA oligodendrocytes

To explore whether somatic SNCA CNVs are associated with inclusion-bearing cells in MSA, we performed FISH using a custom-designed SureFISH 50kb SNCA probe we previously used [14, 40] combined with immunofluorescence for SOX10, a well-established oligodendrocyte marker [42, 54], which is expressed across oligodendrocyte maturation stages [17] and α -synuclein to visualise inclusions. To characterise somatic losses of SNCA, which cannot be reliably done on tissue sections due to sectioning artefacts, all experiments were done on isolated nuclei from fresh-frozen post-mortem brain tissue (workflow depicted in Fig. 1a). FISH on isolated nuclei allowed us to also significantly reduce the exposure time to pepsin, a protease which is known to variably digest inclusions in MSA [64], therefore allowing for better epitope preservation. We found that, in MSA, α -synuclein inclusions are well-retained in nuclear preparations due to their perinuclear and/or nuclear localisation, with no significant difference observed between levels of inclusions on sections versus nuclei as detected by immunofluorescence for the same case and region ($p = 0.31$, $n = 8$, Supplementary Fig. 1).

The final dataset included at least one brain region from 13 SND cases, 12 OPCA cases, and 15 controls. All three brain regions (putamen, cerebellum, substantia nigra) were analysed in 10 controls, 6 SND, and 7 OPCA cases. There were no statistically significant differences in mean age at death across groups (Table 1) (Controls: 71.3 years; SND: 67.9 years; OPCA: 64.2 years, $p = 0.14$) or in median post-mortem interval (PMI) (Controls: 46.6 hours; SND: 43.8 hours; OPCA: 67.5 hours, $p = 0.12$). Sex distribution was comparable between groups ($p = 0.83$). Among MSA subtypes, there were no significant differences in mean age at disease onset (SND: 61.8 years; OPCA: 57.1 years, $p = 0.14$) or in disease duration (SND: 6.2 years; OPCA: 8.0 years, $p = 0.11$). The overall median total cell count per region from each brain was 88, and the median SOX10⁺ cell count was 46. No significant differences were observed across total cell counts (Kruskal-Wallis $p = 0.46$) or SOX10⁺ cell counts (Kruskal-Wallis $p = 0.15$) analysed across groups within brain regions (Supplementary Fig. 2).

All results divided by disease status, brain region and cell type are shown in Supplementary Table 2. Indicative images of oligodendrocytes with and without CNVs and inclusions are shown in Fig. 1b. We first quantified the percentage of SOX10⁺ cells with SNCA gains in both MSA subtypes and controls by taking the average across the regions analysed. This revealed a significant difference in the percentage of SOX10⁺ gains in MSA, where they were enriched over threefold compared to controls (6.3% vs 2.0%, $p < 0.0001$). SNCA gains in SOX10⁻ cells were also higher (5.7 vs 3.4%) however this was non-significant ($p = 0.07$) (Fig. 1c). We next assessed somatic SNCA losses, which were even higher than gains, and significantly increased in MSA compared to controls in both SOX10⁺ cells (12.5% vs 7.4%, $p < 0.0001$), and SOX10⁻ cells (11.1 vs 8.2%, $p < 0.01$). These results indicate that somatic SNCA gains and losses are both enriched in MSA, preferentially in oligodendrocytes, with less striking enrichment across SOX10⁻ cells.

Table 1
Summary of donor characteristics for the immuno-FISH cohort

Group	n	Age at death (years)		PMI (hrs)		Age at disease onset (years)		Disease duration (years)		Sex (M/F)
		Mean ± SD	Median [IQR]	Mean ± SD	Median [IQR]	Mean ± SD	Median [IQR]	Mean ± SD	Median [IQR]	
Control	15	71.3 ± 11.2	71 [63–75]	64.1 ± 43.4	46.6 [38.6–96.5]	N/A	N/A	N/A	N/A	8 (53.3%) / 7 (46.75%)
SND	13	67.9 ± 8.3	67 [63–72]	49.8 ± 22.1	43.8 [36.8–53]	61.8 ± 8.0	63 [57–67]	6.2 ± 1.3	6 [5–7]	6 (46.2%) / 7 (53.8%)
OPCA	12	64.2 ± 6.8	66.5 [57.8–69.2]	69.3 ± 21.1	67.5 [58.4–86]	57.1 ± 6.5	57 [52–61.5]	8.0 ± 3.3	7 [6–10]	5 (41.7%) / 7 (58.3%)
p-value		ANOVA $p = 0.14$		Kruskal-Wallis $p = 0.12$		Student t-test $p = 0.14$		Student t-test $p = 0.11$		Chi-square $p = 0.83$

Demographic and sample characteristics of control and MSA subgroups. Data are presented as mean ± SD (standard deviation) and median [interquartile range]. PMI, post-mortem interval. SND, striatonigral degeneration. OPCA, olivopontocerebellar atrophy. N/A = not applicable.

Reference probe locus exhibits relative genomic stability in MSA compared to controls and also compared to SNCA

To assess whether the observed *SNCA* CNVs reflect a locus-specific instability rather than a global genomic phenomenon, we omitted the α -synuclein staining and used a reference probe targeting chromosome 7 for most control experiments ($n = 29$), and a subset of MSA ($n = 6$) (representative images in Fig. 2a). SOX10⁺ reference gains were rare and comparable between MSA and controls (median 0% for controls, 0.5% for MSA, adjusted $p = 1$), (Fig. 2b) as were SOX10[−] gains (median 0% for both, adjusted $p = 1$) (Fig. 2c). Reference losses were higher but also showed no significant differences between MSA and controls for SOX10⁺ cells (median 3.4% vs. 3.0%, adjusted $p = 1$) (Fig. 2d) or SOX10[−] cells (median 4.7% vs. 3.3%, adjusted $p = 1$) (Fig. 2e). Within SOX10⁺ cells, gains of both probes were found in only one cell in the MSA case and none in the control group. Among SOX10[−] cells, gains of both probes were identified in a small number of control cells and were not detected in MSA. The median proportions of cells carrying both-probe losses were higher than for gains but were comparable between MSA and controls (SOX10⁺ = 1.2% vs 1.7%, SOX10[−] = 1.3% vs 1.7%).

Next, to directly compare *SNCA* with the reference locus, we performed paired analyses within the same case and region. In controls, *SNCA* gains were significantly more frequent than reference gains in both SOX10⁺ (1.9% vs 0%, adjusted $p = 0.03$) (Fig. 2b) and SOX10[−] populations (2.9% vs 0%, adjusted $p < 0.001$) (Fig. 2c). In

MSA, the same trend was observed in SOX10⁺ gains, though the difference did not reach significance after correction (6.7% vs 0.5%, adjusted $p = 0.13$) (Fig. 2b). There was no difference between SOX10⁻ SNCA and reference gains (1.7 vs 0%, adjusted $p = 1$) (Fig. 2c). A similar pattern was observed for losses. In controls, SNCA losses were significantly more frequent than reference losses in both SOX10⁺ (median = 6.3% vs. 3.0%; adjusted $p < 0.01$) (Fig. 2d) and SOX10⁻ populations (median = 7.5% vs. 3.3%; adjusted $p < 0.01$) (Fig. 2e). In MSA, SNCA losses were likewise higher relative to reference losses in SOX10⁺ cells (median = 12.0% vs. 3.4%) though this did not reach significance (adjusted $p = 0.13$) (Fig. 2d). Similarly, in SOX10⁻ cells there was an increase in SNCA losses compared to the reference though this was non-significant (median = 13.1% vs. 4.7%; adjusted $p = 0.13$) (Fig. 2e). Overall, these results support the relative genomic stability of the reference probe locus, consistent with our previous findings [14, 38, 40] and indicate that CNVs are preferentially enriched at the SNCA locus, including both gains and losses in controls.

Somatic SNCA Gains in Oligodendrocytes Are Most Enriched in Severely Affected MSA Regions and Correlate with α -Synuclein Inclusions

Given the distinct regional patterns of pathology in MSA subtypes, with predominant involvement of the putamen in SND, the cerebellum in OPCA, and the substantia nigra in both, we next assessed whether the frequency of somatic SNCA gains in oligodendrocytes varies across these regions, as we had found in similar work without cell type characterisation [14] (Fig. 3a). In the OPCA subtype, significantly higher proportions of SOX10⁺ SNCA gains were observed compared to controls in all three regions examined: cerebellum (median 8.7% vs. 3.0%, adjusted $p = 0.001$), putamen (9.5% vs. 2.0%, adjusted $p = 0.02$), and substantia nigra (8.4% vs. 2.4%, adjusted $p = 0.001$). In the SND subtype compared to controls, significant increases were observed in the putamen (7.3% vs. 2.0%, adjusted $p = 0.01$) and substantia nigra (6.4% vs. 2.4%, adjusted $p = 0.03$), but not in the cerebellum (adjusted $p = 0.27$). When comparing SNCA oligodendrocyte gain frequencies in a given region between MSA subtypes, we noted a 2.2-fold higher difference in OPCA cerebellum compared to SND cerebellum (8.7% vs 3.9%), however this was non-significant after correction (adjusted $p = 0.09$). We noted no difference between SND and OPCA putamen (7.3% vs 9.5%; adjusted $p = 1$). We next compared differentially affected regions within each MSA type. Within the SND group, we compared the putamen and cerebellum which revealed a 1.9-fold increase in SOX10⁺ gains in the putamen (7.3 vs 3.9%, adjusted $p = 0.09$). Within the OPCA group, there was no difference between the cerebellum and putamen (8.7% vs 9.5%, adjusted $p = 0.40$).

The relative enrichment of gains in the putamen compared to the cerebellum in SND, but lack of regional differences in SOX10⁺ SNCA gains in OPCA, could suggest that our OPCA cohort had a less pronounced cerebellar predominance, with the putamen affected almost to the same extent. To assess this, we compared the proportion of SOX10⁺ α -synuclein⁺ cells in preferentially affected regions (OPCA: cerebellum; SND: putamen) versus lesser affected regions (OPCA: putamen; SND: cerebellum) (Supplementary Fig. 3). In OPCA cases, the median percentage of SOX10⁺ inclusions was 26.1% in the cerebellum and 17.7% in the putamen, reflecting a 1.5-fold difference ($p = 0.12$). In contrast, SND cases exhibited a more pronounced 3.1-fold regional difference, with median values of 35.9% in the putamen and 11.5% in the cerebellum ($p = 0.07$). These findings demonstrate that our OPCA cohort has more regionally extensive pathology than SND, and therefore regional patterns of CNVs, if related to disease, would be expected primarily in SND.

Next, we performed the same analysis for SOX10⁻ cells (Fig. 3b). Significant differences in median percentages of gains were observed in the cerebellum and putamen of the OPCA group compared to controls (cerebellum: 7.9% vs. 3.2%, adjusted $p = 0.03$; putamen: 8.3% vs. 2.3%, $p = 0.02$). In the SND group, the difference between the putamen was not significant (5.7% vs. 2.3%, adjusted $p = 0.09$). A significant difference was observed in the cerebellum between OPCA and SND cases (7.9% vs 0%, adjusted $p = 0.01$), whereas no significant difference was found in the putamen. Interestingly, although the substantia nigra showed significant increases in SOX10⁺ cells, there were no group differences for SOX10⁻ gains. Within each MSA subtype, we noted no differences across any of the regions (OPCA: ANOVA $p = 0.48$; SND: Kruskal-Wallis $p = 0.21$). These results reflect a similar but less pronounced regional trend for SNCA gains in SOX10⁻ cells as there are for SNCA gains in oligodendrocytes.

Next, to determine whether SNCA gains in oligodendrocytes are associated with an increased risk of α -synuclein pathology at the single-cell level, we compared the proportion of SOX10⁺ oligodendrocytes containing inclusions between cells with a SNCA gain and those with copy number 2, stratified by brain region and MSA subtype (Table 2). This revealed a significant relative risk (RR) for presence of an α -synuclein inclusion with an oligodendrocyte SNCA gain in the preferentially affected region of each subtype (OPCA cerebellum, RR: 2.4, $p < 0.0001$, SND putamen, RR: 2.1, $p < 0.0001$) and the substantia nigra (OPCA RR: 2, $p = 0.001$; SND RR 1.8, $p = 0.046$), but not the less affected region in each MSA subtype (OPCA putamen, RR = 1.2, $p = 0.63$; SND cerebellum: RR = 0.3, $p = 0.07$). These findings support the hypothesis that SNCA gains increase the susceptibility of individual oligodendrocytes to inclusion formation in predominantly affected regions. Consistent with this, the regional frequency of oligodendrocyte SNCA gains was significantly correlated with the overall burden of GCIs in the most affected regions of each MSA subtype ($\rho = 0.49$, $p = 0.02$), supporting a role for SNCA gains in determining the regional distribution of pathology.

Recently, evidence suggested that neuronal inclusions may also be a key driver of early MSA pathogenesis [64]. We therefore additionally quantified the RR of SNCA gains in inclusions within SOX10⁻ cells (Table 2), with the limitation that SOX10⁻ cells comprise a heterogeneous mix of cell types, likely neuronal in inclusion-bearing cells, but more diverse among non-inclusion-bearing cells. This analysis revealed a significant RR of 3.6 ($p = 0.002$) for inclusions in SOX10⁻ cells with SNCA gains in the cerebellum of OPCA cases. No other regions in OPCA or any regions in SND showed significant RR. However the low number of inclusion-bearing SOX10⁻ cells may have contributed to the lack of significance elsewhere.

Table 2
Relative risk for α -synuclein inclusions in cells with and without somatic *SNCA* gains.

Region	MSA subtype	Gains			CN2			RR	95% CI	p-value
		Total	aSyn+	%	Total	aSyn+	%			
SOX10 ⁺ cells										
Cerebellum	OPCA	42	26	61.9	276	71	25.7	2.4	1.7–3.3	< 0.0001*
	SND	32	2	6.3	517	114	22.1	0.3	0.07–1.1	0.07
Putamen	OPCA	30	7	23.3	355	70	19.7	1.2	0.6–2.3	0.60
	SND	48	32	66.7	438	136	31.1	2.1	1.7–2.7	< 0.0001*
SN	OPCA	37	16	43.2	371	80	21.6	2.0	1.3–3	0.001*
	SND	30	9	30.0	360	59	16.4	1.8	1–3.3	0.046*
SOX10 ⁻ cells										
Cerebellum	OPCA	47	7	14.9	439	18	4.1	3.6	1.6–8.2	0.002*
	SND	6	0	0	287	15	5.2	1.3	0.1–21	0.84
Putamen	OPCA	41	1	2.4	338	11	3.3	0.7	0.1–5.7	0.78
	SND	24	2	8.3	274	24	8.8	1.0	0.2–3.8	0.94
SN	OPCA	30	2	6.7	375	10	2.7	2.5	0.6–10.9	0.22
	SND	16	0	0	208	7	3.4	0.8	0.05–13.7	0.89

SOX10⁺ and SOX10⁻ cells shown separately. The RR estimates with 95% confidence intervals (CI) and corresponding p-values are indicated. Statistically significant RR p-values (< 0.05) are highlighted with an asterisk (*).

Somatic *SNCA* losses show widespread regional distributions and relative risk of α -synuclein inclusions

Having established overall *SNCA* losses in MSA SOX10⁺ oligodendrocytes and SOX10⁻ cells, we next compared their levels across the cerebellum, putamen, and substantia nigra among OPCA, SND, and control groups (Fig. 4). In SOX10⁺ cells, *SNCA* losses were significantly more frequent in the cerebellum of OPCA cases (median 15.4%) and SND cases (13.0%) compared with controls (6.7%; adjusted $p < 0.0001$ and $p = 0.03$, respectively). In the putamen, both OPCA (15.0%) and SND (13.7%) showed increased *SNCA* losses versus controls (6.4%; adjusted $p = 0.02$ and $p = 0.01$, respectively). In the substantia nigra, we observed a

significant difference between SND and control (11.9% vs 6.5%, adjusted $p = 0.02$) but not OPCA (8.0%, $p = 0.72$). Comparisons between SND and OPCA cases revealed no significant differences across any of the regions. Moreover, region-wise comparisons within each MSA subtype showed no significant differences (Kruskal-Wallis: OPCA $p = 0.06$; SND $p = 0.77$). For SOX10⁻ cells, SNCA losses were significantly elevated in the cerebellum of SND (14.7%) compared to controls (4.3%, adjusted $p = 0.01$), whereas the excess of OPCA losses was not significant (10.9%; adjusted $p = 0.1$). In the putamen, SNCA loss frequencies did not differ significantly between controls and either MSA subtype (controls: 8.8%; OPCA: 11.1%; SND: 15.6%). Similarly, the substantia nigra showed no significant group differences (SND: 10.5%; OPCA: 14.1%; controls: 9.1%). SNCA loss frequencies were also comparable across regions within each MSA subtype (Kruskal-Wallis: OPCA $p = 0.36$; SND $p = 0.81$). These results indicate that SNCA losses in MSA do not show a clear regional predilection based on differential regional involvement in each subtype.

As we had shown a relationship of SNCA gains with α -synuclein inclusions at the single cell level, we next assessed this for SNCA losses. At the single-cell level, a significantly increased RR of SNCA loss in oligodendrocytes with inclusions was observed in the substantia nigra of both MSA subtypes (RR 1.9, $p = 0.003$ SND, RR 2 OPCA $p = 0.0008$) and SND putamen (RR 1.4, $p = 0.03$), however this was not observed in the OPCA cerebellum (RR 1.1, $p = 0.6$) (Table 3). As for non-oligodendrocytes, there was significant RR of SNCA loss in cells with inclusions only in OPCA substantia nigra (RR 3.7, $p = 0.01$), although there was a possible increased risk in all regions (Table 3). Combining all regions together leads to an overall RR for both SOX10⁺ oligodendrocytes (OPCA RR: 1.5, $p = 0.004$; SND RR 1.4, $p = 0.0007$) and SOX10⁻ cells (OPCA RR: 2.7, $p = 0.0006$; SND RR 1.7, $p = 0.07$). These results suggest that, like SNCA gains, losses are also associated with inclusions, but in a more widespread manner, without the clear cell-type and regional predilection seen for SNCA gains.

Table 3
Relative risk for α -synuclein inclusions in cells with and without somatic *SNCA* losses

Region	MSA subtype	Losses			CN2			RR	95% CI	p-value
		Total	aSyn+	%	Total	aSyn+	%			
SOX10 ⁺ cells										
Cerebellum	OPCA	62	18	29.0	276	71	25.7	1.1	0.7–1.7	0.60
	SND	77	21	27.3	517	114	22.1	1.3	0.9–1.9	0.20
Putamen	OPCA	58	14	24.1	355	70	19.7	1.2	0.7–2	0.40
	SND	72	31	43.1	438	136	31.1	1.4	1–1.87	0.03*
SN	OPCA	45	19	42.2	371	80	21.5	2.0	1.3–2.9	0.0008*
	SND	60	19	31.7	360	59	16.4	1.9	1.2–3	0.003*
SOX10 ⁻ cells										
Cerebellum	OPCA	55	5	9.1	439	18	4.1	2.2	0.9–5.7	0.10
	SND	42	5	11.9	287	15	5.2	2.3	0.9–5.9	0.09
Putamen	OPCA	46	4	8.7	338	11	3.3	2.7	0.9–8	0.08
	SND	49	5	10.2	274	24	8.8	1.2	0.5–2.9	0.74
SN	OPCA	61	6	9.8	375	10	2.7	3.7	1.4–9.8	0.009*
	SND	35	3	8.6	208	7	3.4	2.5	0.7–9.4	0.16

The RR estimates with 95% confidence intervals (CI) and corresponding p-values are indicated. Statistically significant RR p-values (< 0.05) are highlighted with an asterisk (*).

***SNCA* gains reflect an intrinsic case-level propensity for gains in other cell types and other brain regions associated with younger age of disease onset**

As we had seen apparent inter-individual variability in our CNV analysis, we explored whether this variability reflects a case-level propensity rather than being restricted to region-specific pathology. We first examined within-individual patterns of oligodendrocyte *SNCA* gains across brain regions by performing pairwise Spearman's rank correlation analyses between the most and lesser affected brain regions within each case, revealing a significant positive correlation ($\rho = 0.56$, $p = 0.02$; Fig. 5a). To determine whether cases with higher mosaicism in oligodendrocytes also had higher mosaicism in other cell types, we compared the

frequency of *SNCA* gains in SOX10⁺ and SOX10⁻ cells across all regions, which also showed a significant positive correlation in MSA ($\rho = 0.5, p = < 0.0001$) (Fig. 5b) but not in controls ($\rho = 0.2, p = 0.2$). Taken together, this supports the idea that *SNCA* gains may represent an intrinsic, case-level tendency in MSA, across regions and cell types. We performed the same analysis for *SNCA* losses. No significant correlations were observed between SOX10⁺ and SOX10⁻ cells ($\rho = -0.002, p = 1$). Losses did, however, show a strong correlation between the most and lesser affected regions ($\rho = 0.7, p = 0.002$).

Given the previous observation in PD of a negative correlation between age of onset and *SNCA* gains in the key affected region, the substantia nigra, and cell type (dopaminergic neurons) [38] we wondered whether such a relationship might exist for MSA. We investigated whether SOX10⁺ oligodendrocyte *SNCA* gains were associated with age of onset by taking the average percentage per case across the regions analysed. This revealed a significant negative correlation ($\rho = -0.45, p = 0.03$) for age of disease onset (Fig. 5c), which was not observed for SOX10⁻ *SNCA* gains ($\rho = -0.12, p = 0.58$) (Fig. 5d). There was no significant correlation with age of death ($\rho = -0.35, p = 0.09$), disease duration ($\rho = 0.10, p = 0.63$), or PMI ($\rho = 0.27, p = 0.19$). We did not detect any significant associations between *SNCA* losses in SOX10⁺ and SOX10⁻ cells and these clinical parameters (Supplementary Table 3). These results suggests that oligodendrocyte *SNCA* gains, but not *SNCA* gains or losses in SOX10⁻ cells, may have a role in determining onset age.

DNA double-stranded breaks are enriched in MSA oligodendrocytes and are associated with α -synuclein pathology

To investigate whether DSBs are associated with MSA pathology, we performed immunofluorescence staining for γ H2AX phosphorylated at Ser139, an established marker of DSBs [32], in at least one region from three controls, three SND cases, and five OPCA cases (demographics summarised in Supplementary Table 4). Subsets of experiments included γ H2AX with either α -synuclein or SOX10 (Supplementary Table 5), as simultaneous detection of all three markers was not possible due to species overlap and secondary antibody compatibility. We observed two γ H2AX staining patterns, diffuse pan-nuclear staining and bright foci (Fig. 6a). Pan-nuclear γ H2AX signalling has been attributed to clustered DNA damage associated with several pathogenic cellular responses such as apoptosis and replication stress [12, 35, 37], as well as more non-specific physiological responses such as increased cellular activity [50]. Accordingly, we focused on analysing the discrete γ H2AX foci, irrespective of pan-nuclear staining, as these are more reliable indicators of an active downstream DNA damage response.

First, to determine if the DSB response is altered in MSA compared to controls, we classified cells with one or more foci as γ H2AX⁺. Across all brain regions analysed, the median percentage of γ H2AX⁺ cells was significantly higher in MSA than in controls (6.0% vs 2.2%, $p = 0.049$) (Fig. 6b), indicating an increase in DNA damage response activity. To identify the affected cell populations, we performed an immunofluorescence experiment with SOX10 on two controls (cerebellum and SN from one, putamen from the other), two OPCA (cerebellum or SN from each) and two SND (cerebellum and SN from both). This revealed a significant enrichment of DSBs in oligodendrocytes in MSA versus controls (14.4% vs 5.5%, $p = 0.02$) (Fig. 6c) indicating that DSBs are present in cell types known to be vulnerable in MSA. We also noted a slightly higher level of

γ H2AX⁺ in SOX10⁻ cells than in controls (3.1% vs 1.6%) (Fig. 6d), however this difference was non-significant ($p = 0.30$). This pattern suggests that the DSB response is enriched within oligodendroglial populations.

We next assessed whether DSB accumulation was linked to α -synuclein pathology. Paired comparisons revealed significantly more γ H2AX foci in α -synuclein⁺ nuclei than α -synuclein⁻ nuclei (22.2% vs 14.9%, $p = 0.02$) (Fig. 6e), suggesting a strong association between α -synuclein aggregation and DSB burden at the single-cell level. This finding is consistent with previous reports in DLB, where γ H2AX accumulation and p53 activation occur in neurons with nuclear α -synuclein [26]. Finally, we examined whether DSB burden varied across brain regions with differential involvement in MSA. Due to the small number of paired samples, statistical comparisons were performed at the group level rather than within-case pairs. Overall, γ H2AX levels were 3.7-fold higher in the more affected regions compared with less affected regions (18.8% vs 5.1%) although this did not reach statistical significance ($p = 0.11$).

Discussion

In this study, we build upon growing evidence implicating somatic *SNCA* copy number gains in MSA by defining their cellular distribution, regional patterns, and relationship to α -synuclein inclusions. We show that *SNCA* gains are increased in MSA relative to controls, particularly in SOX10⁺ oligodendrocytes, the glial cell type predominantly affected by α -synuclein pathology. Within each subtype, oligodendrocyte *SNCA* gains tend to be high in regions that are selectively vulnerable, namely the cerebellum in OPCA and putamen in SND, and the substantia nigra in both. At the single-cell level, oligodendrocyte gains are associated with a 2-fold increased relative risk for α -synuclein inclusions in affected regions, an association that was reflected at the regional level, where the frequency of gains correlated with the overall burden of α -synuclein pathology. These results, taken together with the association between higher oligodendrocyte *SNCA* gain burden and earlier age of disease onset, are supportive of a role in MSA pathogenesis, possibly by locally raising the intracellular α -synuclein to pathogenic thresholds.

The regional distribution of oligodendrocyte *SNCA* gains differ between MSA subtypes. In SND, gains are enriched in the putamen relative to the cerebellum, consistent with the established regional predilection of pathology in this subtype. In contrast, in OPCA, oligodendrocyte *SNCA* gains are present at comparable levels in the cerebellum and putamen. In line with this, we found that the burden of GCIs in our cohort is 3-fold higher in SND in the putamen compared to the cerebellum, but only 1.5-fold higher in OPCA in the cerebellum compared to putamen. This may reflect the broader regional pathology seen in OPCA, where severe cerebellar and putaminal involvement often coexist [20], although, we cannot exclude sampling bias or small sample size effects contributing to the pattern observed in our samples.

We also detect excess gains in SOX10⁻ cells consistent with our prior reports of neuronal *SNCA* mosaicism in MSA cingulate cortex and substantia nigra [38, 40] which may also contribute to disease pathogenesis. Neuronal inclusions are becoming more widely recognised as early events in MSA pathogenesis [64]. However, as we did not include neuronal markers in the present study, we cannot definitively assign SOX10⁻ cells with inclusions to a neuronal lineage. Further experiments using neuronal markers with α -synuclein would be necessary to assess this.

Case-level analyses reveal that the level of *SNCA* gains in MSA is positively correlated across other brain regions and between SOX10⁺ oligodendrocytes and SOX10⁻ cells within the same individual, suggesting that these somatic gains reflect an intrinsic, case-specific propensity that is not observed in controls. These relationships may be consistent with an early clonal origin that produces descendant cells across multiple regions or cell types, supporting a model in which a subset of MSA patients may have pre-existing somatic *SNCA* mosaicism for gains that increases the likelihood of developing α -synuclein aggregation in later life. However, we cannot rule out the other possibility of a general increased susceptibility to *SNCA* copy number variation within MSA, in which case individual cells may carry unique rather than shared CNVs, with different size and distinct breakpoints. While the former could be characterised fully by deep long read whole genome sequencing (WGS), the latter would require single cell WGS. This is presently limited generally to megabase-scale CNVs [21] and could thus miss smaller events, although we did detect one in our pilot MSA single cell WGS study [40]. Long read single cell WGS could detect events of all sizes, and was recently reported in the brain in a small study including MSA [19]. While these findings strengthen the case for a pathogenic role of somatic *SNCA* gains in MSA, our protocol on isolated nuclei also allowed the characterisation of somatic *SNCA* losses. Losses are higher in MSA, and exhibit a more widespread distribution across cell-types and regions, with a variable but overall positive association with α -synuclein pathology, but no correlation with onset age or disease duration.

Collectively, these observations raise important questions regarding the timing and origin of these CNVs, which cannot be resolved with the current data. Gains and losses can arise through distinct mechanisms. Large-scale gains are most likely to reflect mitotic errors such as chromosome mis-segregation or DNA replication defects, and are typically clonal [5, 16]. This raises the likelihood that such gains occur prior to MSA pathogenesis, as the cells known to form α -synuclein inclusions are mostly post-mitotic, although we cannot exclude their emergence during low-level clonal expansion of glial progenitors in disease progression. We also cannot rule out that gains arise secondarily in post-mitotic cells, potentially through rare mechanisms such as cell-cycle re-entry and localised DNA synthesis, which has been reported in other neurodegenerative diseases [39, 48]. Importantly, gains and losses may sometimes occur concomitantly during non-allelic homologous recombination (NAHR), resulting in one daughter cell with a duplication and another with a reciprocal deletion [49], a process that could contribute to the mixed patterns of CNVs we observe. A somatic *SNCA* loss on the other hand could occur post-mitotically secondary to DNA damage in the form of DSBs, rather than a primary event. DSBs can lead to widespread genomic deletions, which is particularly relevant in the brain as the main pathway for DSB repair in post-mitotic cells, NHEJ, is known to be error-prone [34, 44]. To explore this possibility, and to contextualise the *SNCA* CNV landscape within a broader framework of possible genomic instability, we explored DNA DSBs using γ H2AX. We found widespread DNA DSB accumulation in MSA, with γ H2AX foci enriched in SOX10 oligodendrocytes, and in cells with α -synuclein inclusions. This finding suggests an association between α -synuclein aggregation and activation of DNA DSB repair within the oligodendroglial population in MSA. This may also occur in neurons, as shown in Lewy body diseases [26, 56], although we cannot determine this in MSA, as we did not separate neurons from other SOX10⁻ cells. We therefore hypothesise that somatic *SNCA* losses are secondary to disease progression, and may result from mis-repaired DSBs. To conclusively determine, however, whether *SNCA* losses results from DSBs, would require combining FISH with γ H2AX immunofluorescence.

This leads to the question of whether the propensity of the *SNCA* gene to acquire CNVs in MSA reflects specific instability of that gene, which has been suggested to be within a fragile site [47], or whether genome-wide somatic CNVs, known to occur in healthy brain cells [33], are enriched in MSA. The overall higher frequency of *SNCA* CNVs in controls relative to the reference probe suggests that the *SNCA* locus itself may be inherently prone to structural instability, independent of disease status, consistent with previous work showing minimal gains of other reference probes [14, 38]. The reference probe in our study was equally affected by CNVs (predominantly losses) in MSA and controls, arguing against an overall tendency for CNVs in MSA. Our pilot single cell WGS of large CNVs genome-wide did detect them in ~30% of MSA cells, including a putaminal neuron with extensive nuclear inclusions and large-scale genomic losses encompassing *SNCA*, likely to have arisen by NHEJ [40]. Larger scWGS will be required, however, to determine if genome-wide CNVs are enriched in MSA.

Another limitation is that, while we propose sustained overexpression of *SNCA* mRNA as the mechanism by which gains contribute to pathogenesis, we have not demonstrated this. An increase in *SNCA* mRNA in MSA oligodendrocytes has been reported previously through quantitative reverse transcription PCR, though these results were non-significant [2]. More recently, a higher level of *SNCA* transcripts was found in oligodendrocytes with inclusions using RNAScope, though the numbers of cases analysed were limited [25]. It thus remains unclear whether *SNCA* mRNA is increased in MSA, especially across individual cell types. Ideally, methods assessing DNA and RNA in the same cell would clarify these relationships. While *in situ* approaches are limited by DNA-RNA cross-reactivity, sequencing of the DNA and RNA of the same nucleus [60], although challenging, is becoming possible, including in the human brain [8]. Nonetheless, RNA measurements still only represent a snapshot at the point of death of cells surviving in a disease environment, rather than reflect long-term expression patterns, and are influenced by both pre-mortem and post-mortem changes [11, 58].

While our findings strongly implicate somatic *SNCA* gains as contributors to MSA pathology, it is important to acknowledge that these gains may represent only one facet of a multifactorial disease. Somatic mosaicism may serve as a “first hit” or modifier that increases vulnerability to α -synuclein aggregation. Supporting this, we did not find an association of *SNCA* gains with α -synuclein inclusions in the OPCA putamen, despite high levels of *SNCA* gains. Furthermore, both somatic *SNCA* gains and losses have been identified in neurologically healthy individuals from the UK Biobank data, though some of these had reported blood-based cancers, therefore it is unclear if they would be found also in the brain, and others were younger than the typical age of onset for MSA or PD [4]. Similarly, individuals with germline *SNCA* multiplications typically develop a clinical picture resembling PD rather than MSA, and do not always contain GCIs [24]. These observations suggest that *SNCA* copy number variation alone is unlikely to be sufficient to cause MSA, but may act as a contributory or permissive factor whose pathogenicity depends on additional influences, such as cell-type-specific stress, environmental risk factors, coexisting inherited as yet unidentified genetic variants, or indeed other somatic mutations. Such mutations could arise within the brain or originate in the periphery, including in the context of clonal haematopoiesis of indeterminate potential, an age-related process recently reported to be associated with MSA [29].

Nonetheless, our findings reinforce the rationale for therapeutic approaches that lower α -synuclein levels, including antibody-based and antisense strategies already in development [3]. The enrichment of somatic

SNCA gains in affected cells also supports the value of using SNCA overexpression approaches, either alone or alongside other perturbations, to explore how increased α -synuclein dosage interacts with additional cellular processes. More broadly, our results point to somatic variation as a contributor to non-heritable genetic risk in MSA and helps advance our understanding of its aetiology.

Declarations

Competing Interests

Zane Jaunmuktane is a member of the Acta editorial board, but was not involved in the assessment or decision-making process for this manuscript. The other authors report no conflicts of interest.

Author Contribution

C.M. Sample preparation, data collection, statistical analysis, manuscript writing. E.K.E. Experimental supervision and manuscript revision. D.P.R. Experimental supervision and manuscript revision. Z.J. Providing human brain samples and neuropathological characterisation, manuscript revision and supervision of the project. C.P. Conception and design, manuscript revision, and overall supervision of the project.

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Data Availability

All code used to generate the statistical analyses and figures in this study is available on GitHub at <https://github.com/caoimhemorley/somatic-SNCA-CNV-FISH-analysis>. All raw cell counts and summarised datasets supporting the findings of this study are provided in the Supplementary Materials. Representative images are included in the figures, and additional raw image data are available from the corresponding author upon reasonable request.

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Figures

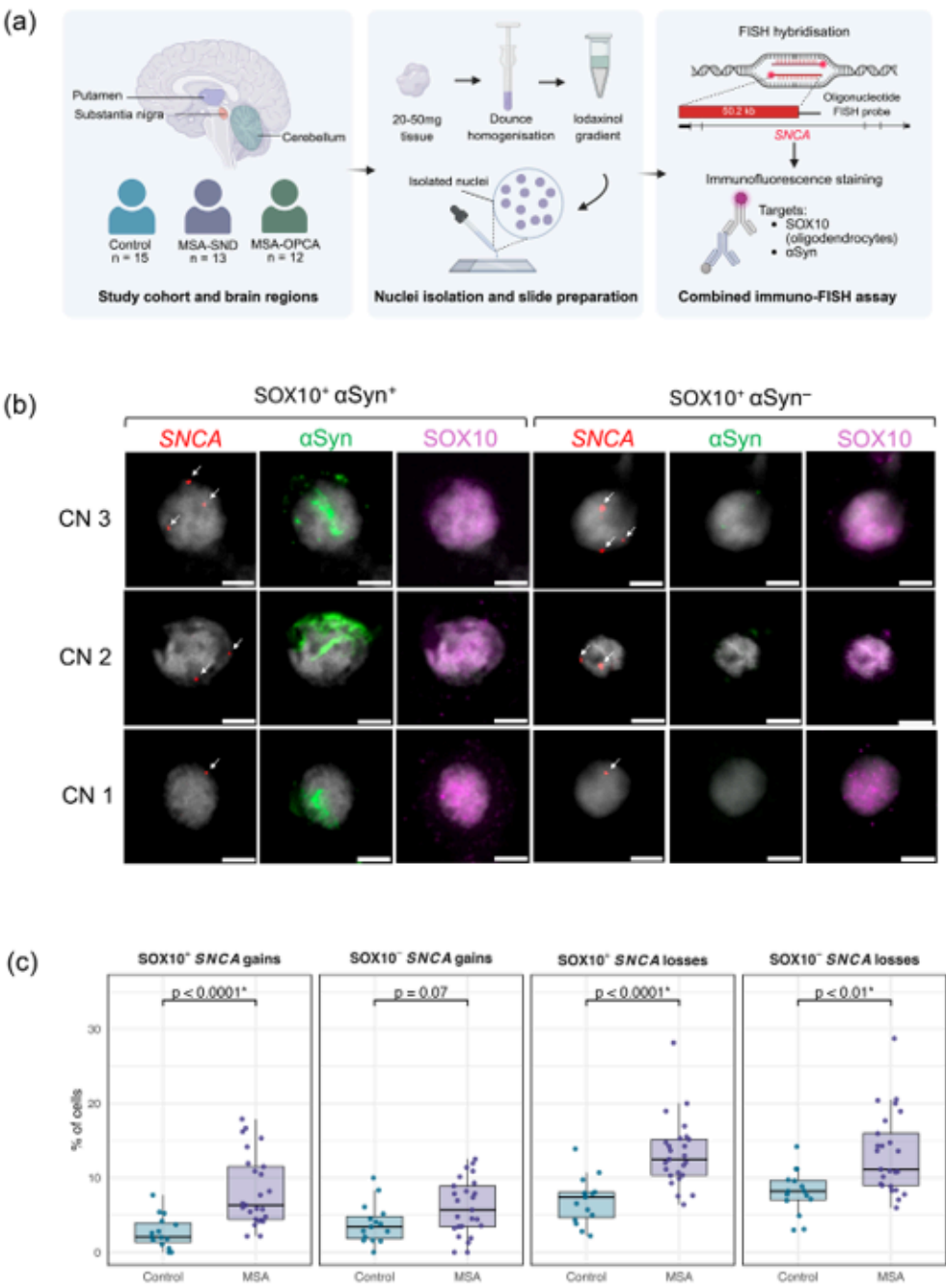


Figure 1

(a) Experimental overview of the immuno-FISH workflow. Fresh-frozen post-mortem tissue from control donors and individuals with SND and OPCA was processed to generate isolated nuclear suspensions. Tissue

was dissociated by Dounce homogenisation and nuclei were separated through an iodixanol gradient before mounting on slides. The combined immuno-FISH assay used an *SNCA* oligonucleotide FISH probe together with immunofluorescent detection of SOX10 to mark oligodendrocyte lineage nuclei and α -synuclein to detect inclusions. Created in BioRender. M, C. (2026) <https://BioRender.com/xtqohau> (b) Representative immuno-FISH images showing *SNCA* FISH signal together with α -synuclein and SOX10 staining. Examples of nuclei with different *SNCA* copy number (CN) states are shown for SOX10⁺ α -synuclein⁺ and SOX10⁺ α -synuclein⁻ nuclei. White arrows indicate *SNCA* FISH signals. Scale bar = 5 μ m. (c) Quantification of somatic *SNCA* CNVs in SOX10⁺ and SOX10⁻ nuclei across MSA (n = 25) and control (n = 15) groups. Box plots display the proportion of nuclei with *SNCA* gain or loss within each group. Group comparisons were performed with the Mann-Whitney U test

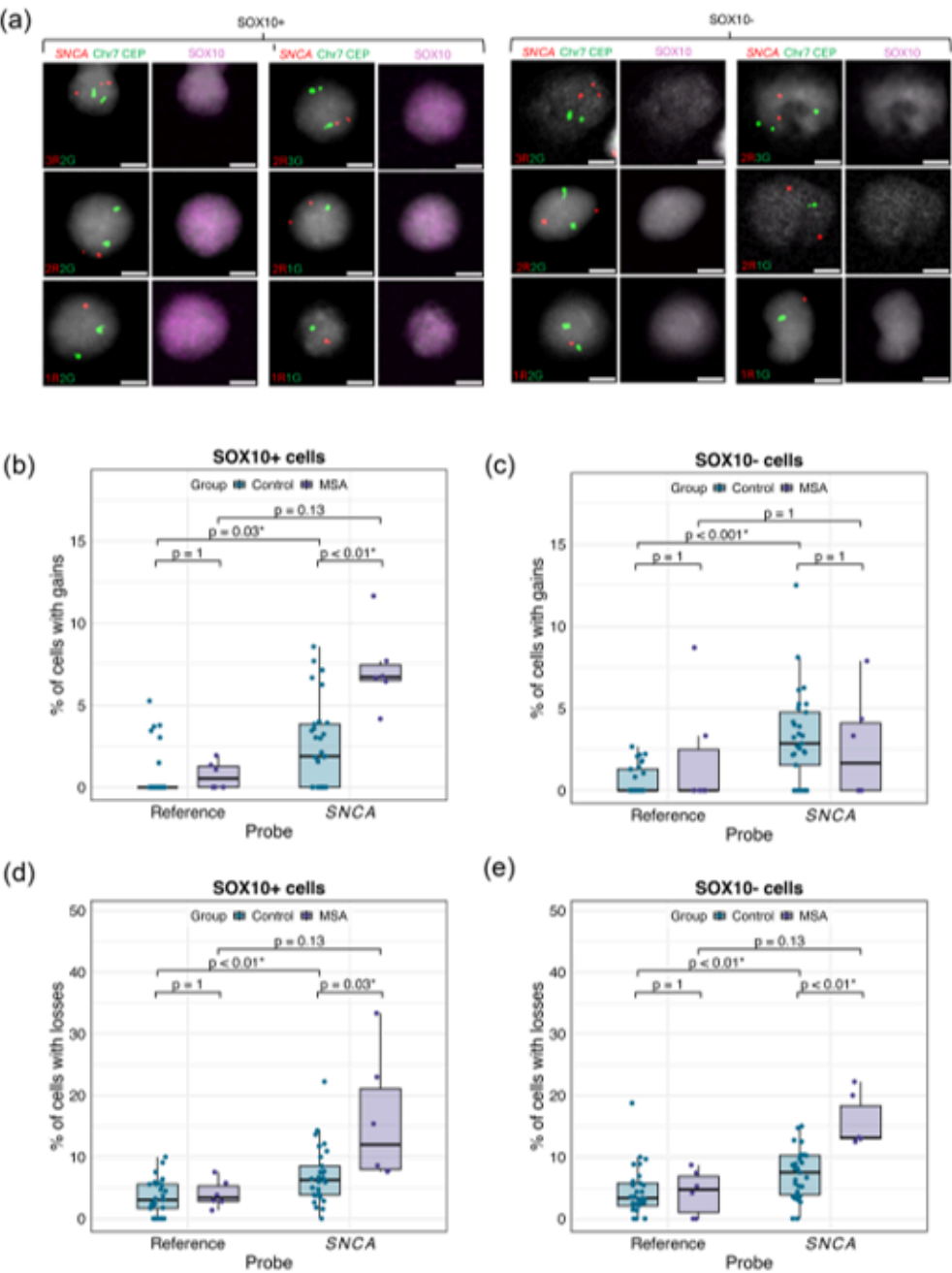


Figure 2

Two-probe FISH analysis across SOX10⁺ oligodendrocytes and SOX10⁻ cells (a) representative immuno-FISH images showing examples of SNCA and reference probe with SOX10 staining and different copy number states. Scale bar = 5 μ m. (b) Reference probe and SNCA gain quantification in SOX10⁺ cells, (c) Reference probe and SNCA gain quantification in SOX10⁻ cells (d) Reference probe and SNCA loss quantification in SOX10⁺ cells (e) Reference probe and SNCA loss quantification in SOX10⁻ cells. Statistical significance was determined using Mann-Whitney U test and paired Wilcoxon signed-rank tests, with Bonferroni correction for multiple comparisons (n = 4)

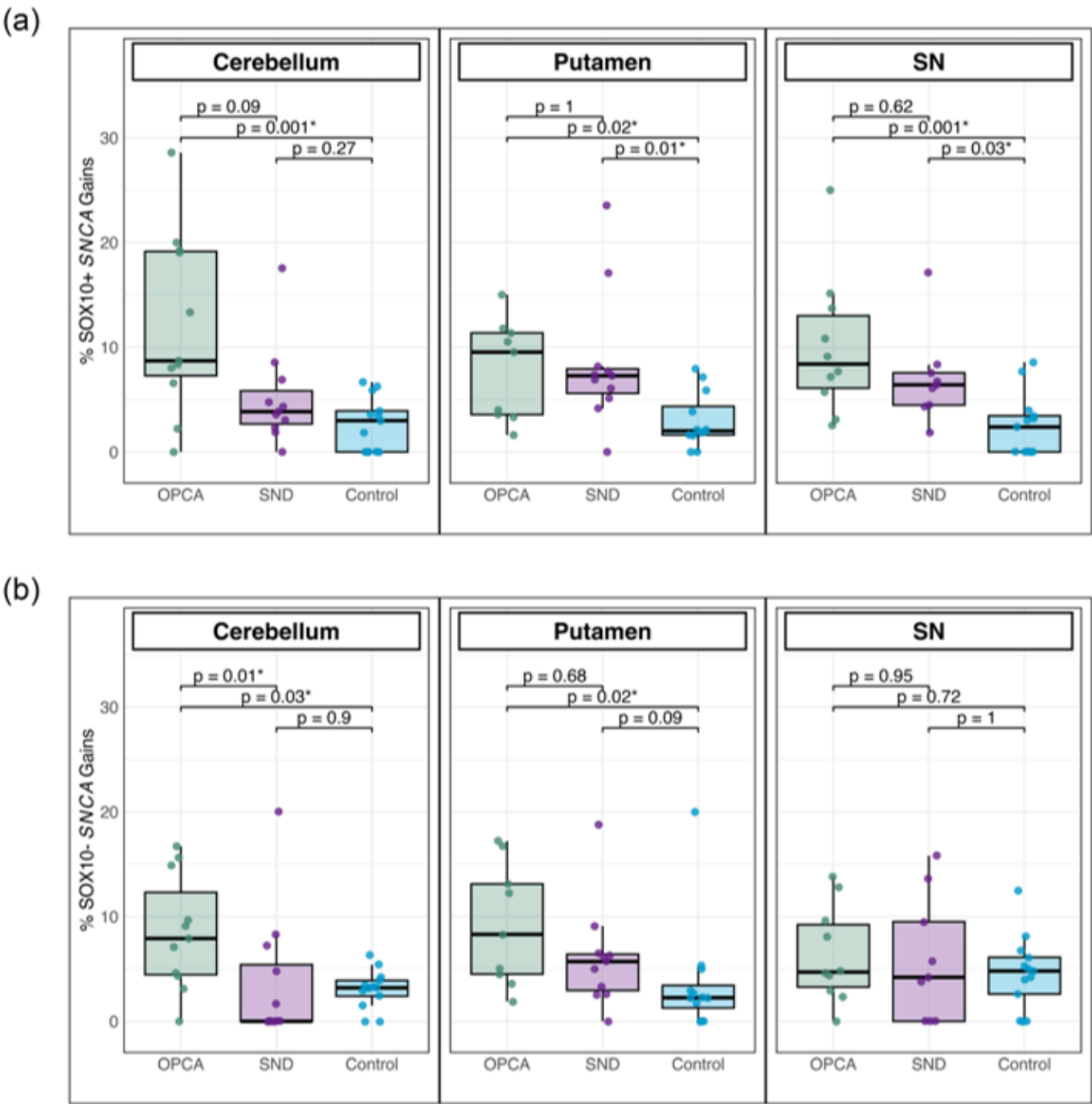


Figure 3

Distribution of percentage *SNCA* gains in (a) SOX10⁺ cells and (b) SOX10⁻ cells across the cerebellum, putamen, and substantia nigra (SN). Regional panels compare three groups: OPCA, SND, and control. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post-hoc tests with Bonferroni correction for multiple comparisons (n = 3). P-values less than 0.05 are indicated by an asterisk (*)

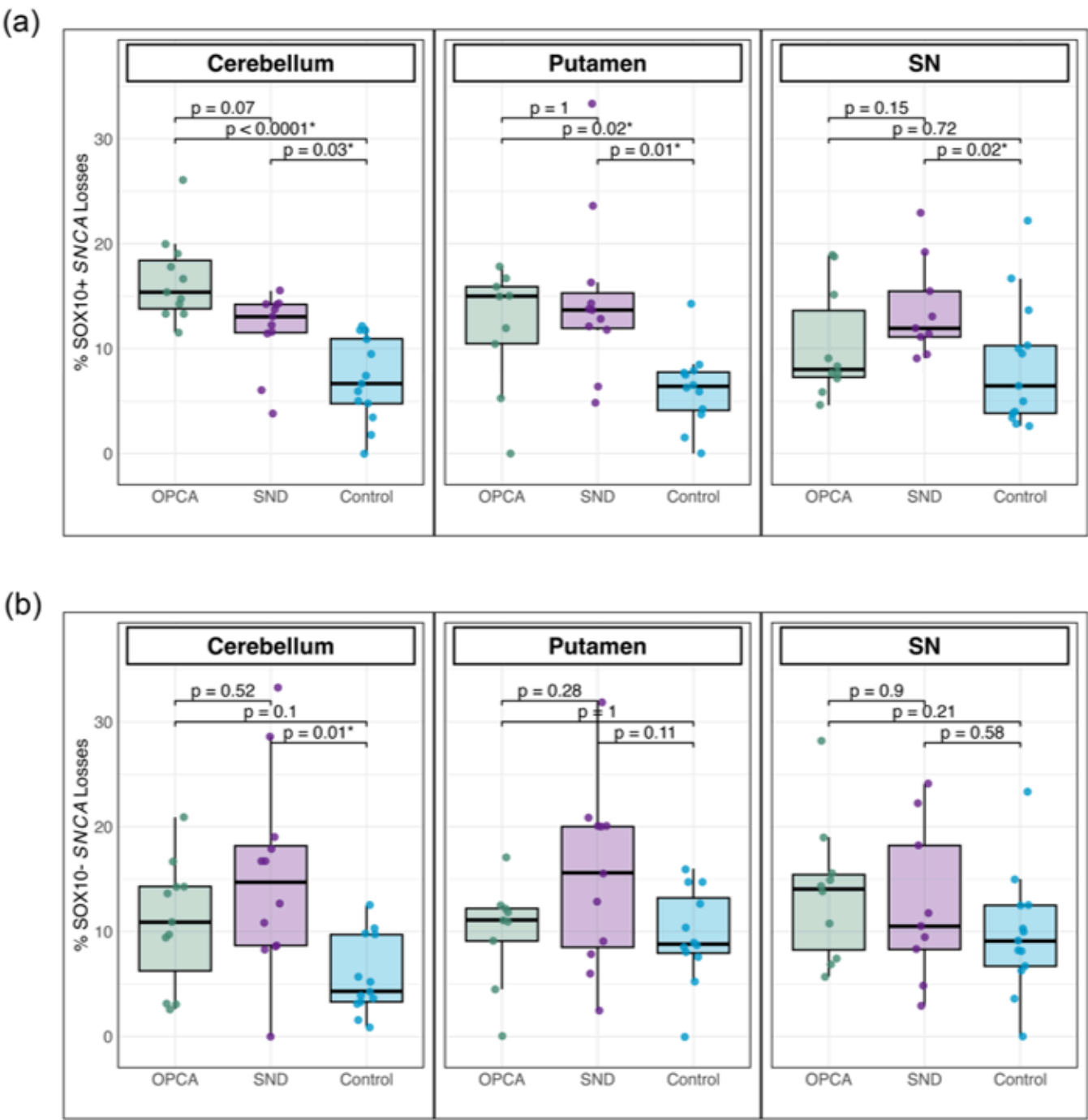


Figure 4

Distribution of percentage *SNCA* losses in (a) SOX10⁺ cells and (b) SOX10⁻ cells across the cerebellum, putamen, and substantia nigra (SN). Regional panels compare three groups: OPCA, SND, and control.

Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post-hoc tests with Bonferroni correction for multiple comparisons ($n = 3$). P-values less than 0.05 are indicated by an asterisk (*)

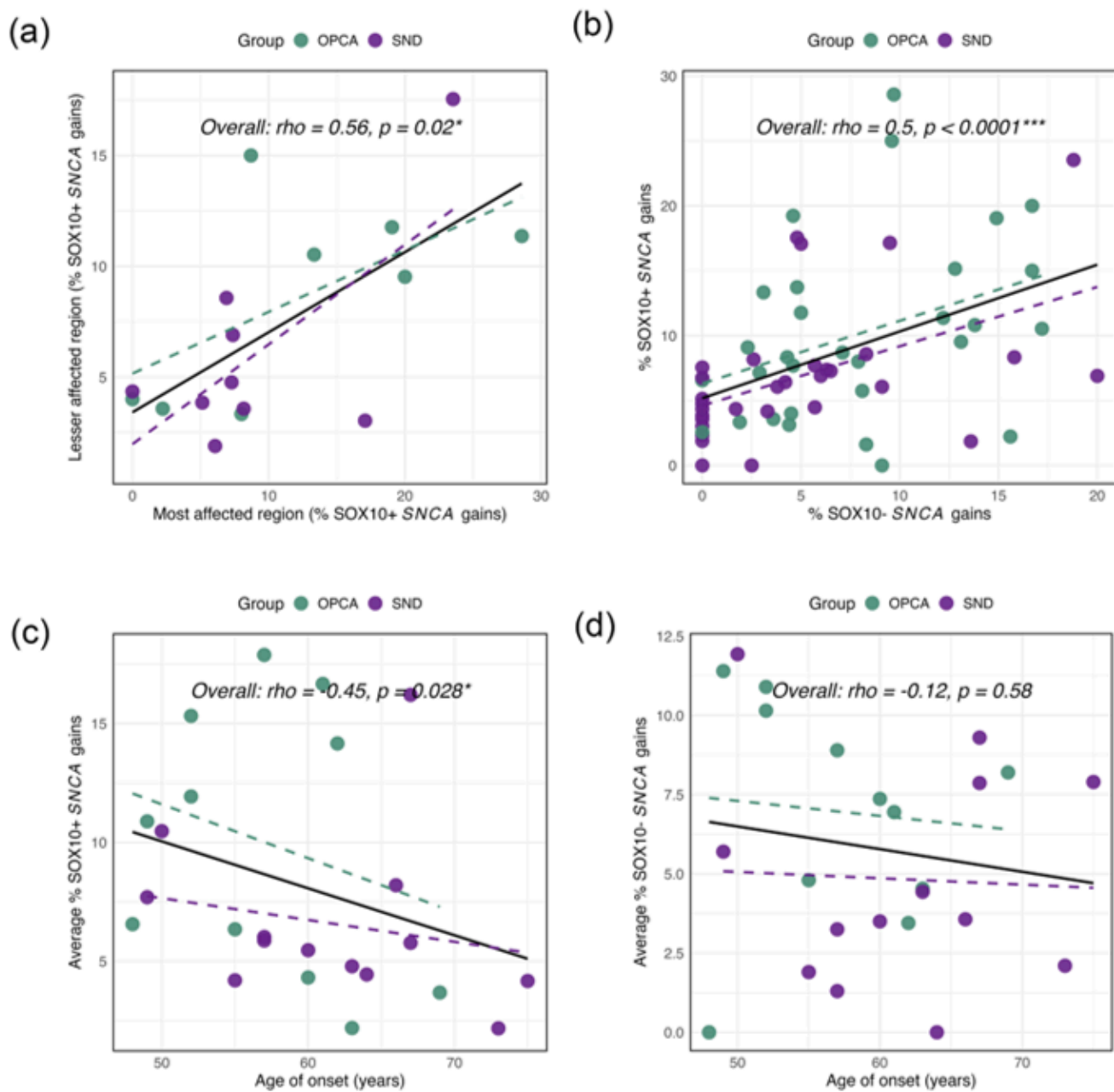


Figure 5

Spearman correlation analyses examining relationships between (a) Percentage of SOX10⁺ SNCA gains in the region most affected in each subtype (OPCA cerebellum; SND putamen) compared with the region lesser affected (OPCA putamen; SND cerebellum). (b) Percentage of SOX10⁺ SNCA gains plotted against SOX10⁻ SNCA gains across all regions analysed. (c) Average percentage of SOX10⁺ SNCA gains plotted against age at disease onset. (d) Average percentage of SOX10⁻ SNCA gains plotted against age at disease onset. Each

panel includes a linear model illustrating the overall trend (black) together with separate trend lines for OPCA and SND

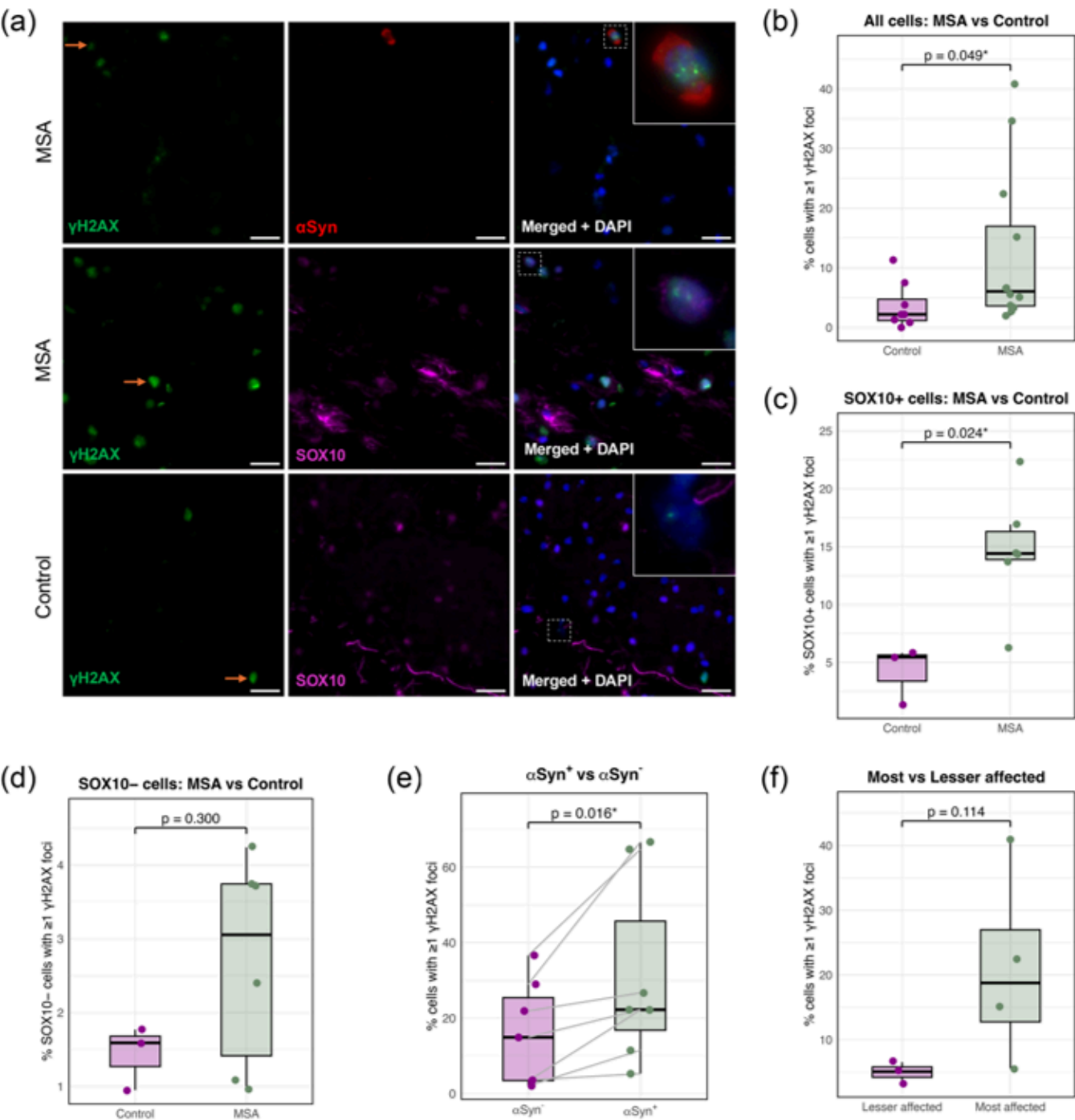


Figure 6

(a) Immunofluorescence images showing representative γH2AX staining. The upper panel of images shows γH2AX staining together with α-synuclein in MSA tissue. The middle set shows γH2AX together with SOX10 in MSA tissue. The lower set shows γH2AX together with SOX10 in control tissue. For each set the individual

markers are shown separately followed by a merged image that includes an inset with representative nuclei containing γ H2AX staining. Orange arrows indicate examples of pan-nuclear γ H2AX signal. Scale bar = 20 μ m. (b) Quantification of cells that contain ≥ 1 γ H2AX foci in MSA compared with control. (c) Proportion of SOX10⁺ cells that contain ≥ 1 γ H2AX labelling foci in MSA compared with control. (d) Proportion of SOX10⁻ cells with ≥ 1 γ H2AX positivity in MSA compared with control. (e) Paired comparison of α -synuclein (aSyn)⁺ versus aSyn⁻ cells with ≥ 1 γ H2AX foci. (f) Percentage of γ H2AX positive cells in the region most affected for each subtype (OPCA cerebellum; SND putamen) compared with the region lesser affected (OPCA putamen; SND cerebellum). Unpaired comparisons were performed using the Mann-Whitney U test and paired comparisons used the Wilcoxon paired test. Significant values <0.05 are indicated with an asterisk (*)

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