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Alpha-Synuclein mRNA Expression in Oligodendrocytes in MSA

Yasmine T. Asi,¹ Julie E. Simpson,² Paul R. Heath,² Stephen B. Wharton,²
Andrew J. Lees,^{1,3} Tamas Revesz,¹ Henry Houlden,^{3,4} and Janice L. Holton¹

Multiple system atrophy (MSA) is a progressive neurodegenerative disease presenting clinically with parkinsonian, cerebellar, and autonomic features. α -Synuclein (α syn), encoded by the gene *SNCA*, is the main constituent of glial cytoplasmic inclusion (GCI) found in oligodendrocytes in MSA, but the methods of its accumulation have not been established. The aim of this study is to investigate alterations in regional and cellular *SNCA* mRNA expression in MSA as a possible substrate for GCI formation. Quantitative reverse transcription polymerase chain reaction (qPCR) was performed on postmortem brain samples from 15 MSA, 5 IPD, and 5 control cases to investigate regional expression in the frontal and occipital regions, dorsal putamen, pontine base, and cerebellum. For cellular expression analysis, neurons and oligodendrocytes were isolated by laser-capture microdissection from five MSA and five control cases. *SNCA* mRNA expression was not significantly different between the MSA, IPD and control cases in all regions (multilevel model, $P = 0.14$). After adjusting for group effect, the highest expression was found in the occipital cortex while the lowest was in the putamen (multilevel model, $P < 0.0001$). At the cellular level, MSA oligodendrocytes expressed more *SNCA* than control oligodendrocytes and expression in MSA neurons was slightly lower than that in controls, however, these results did not reach statistical significance. We have demonstrated regional variations in *SNCA* expression, which is higher in cortical than subcortical regions. This study is the first to demonstrate *SNCA* mRNA expression by oligodendrocytes in human postmortem tissue using qPCR and, although not statistically significant, could suggest that this may be increased in MSA compared to controls.

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Key words: α -synuclein, multiple system atrophy, oligodendrocytes, glial cytoplasmic inclusions, laser-capture microdissection

Introduction

Multiple system atrophy (MSA) is a progressive neurodegenerative disease that clinically presents with parkinsonian, cerebellar and autonomic features and pathologically with glial cytoplasmic inclusions (GCIs) found in oligodendrocytes, myelin damage, neuronal loss, and gliosis (Ahmed et al. 2012; Ubhi et al. 2011). The main constituent of GCIs is α -synuclein (α syn), as such, classifying MSA as part of the α -synucleinopathy group of diseases, which also includes Parkinson's disease (PD) and dementia with Lewy bodies (DLB). MSA is pathologically subtyped into striatonigral (SND), olivopontocerebellar (OPC) or mixed type reflecting the predominance of pathological burden in the respective anatomical structures. However, the basis for this regional vulnerability is poorly understood (Jellinger et al. 2005;

Ozawa et al. 2004). The mechanism by which α syn accumulates in oligodendrocytes as GCIs is as yet unknown but is hypothesized to result from the uptake of α syn, released from neurons, by oligodendrocytes or by overexpression of *SNCA* mRNA in the disease condition (Reyes et al. 2014; Ubhi et al. 2011). Mutations and duplications in *SNCA*, the gene encoding α syn, have been identified in some cases of familial PD and these have occasionally been found to have oligodendroglial inclusions resembling GCIs (Gwinn-Hardy et al. 2000; Kiely et al. 2013; Markopoulou et al. 2008; Obi et al. 2008). Despite this, no mutations in *SNCA* have been associated with MSA (Gwinn-Hardy et al. 2000; Kiely et al. 2013; Markopoulou et al. 2008; Obi et al. 2008). There are limited studies in the literature of *SNCA* expression in MSA. In a small case study, *SNCA* mRNA levels in cortical regions

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Address correspondence to J.L. Holton, Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom.
E-mail: janice.holton@ucl.ac.uk

From the ¹Queen Square Brain Bank, Department of Molecular Neuroscience, UCL Institute of Neurology, London, United Kingdom; ²Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield, United Kingdom; ³Reta Lila Weston Institute of Neurological Studies, UCL Institute of Neurology, London, United Kingdom; ⁴Department of Molecular Neuroscience, UCL Institute of Neurology, London, United Kingdom.

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(frontal, temporal, or occipital) of MSA brains did not differ from those in controls using quantitative PCR (qPCR) (Ozawa et al. 2001). Cortical regions are less severely affected by GCI pathology and neuronal loss in MSA and this may explain these results. Based on findings of our study, it may be plausible to suggest that expression differences are more likely to arise in areas more severely affected in MSA such as the pons and cerebellum. However, there are conflicting data with regards to *SNCA* expression in the pons. Jin et al. (2008) observed no difference in *SNCA* copy number and mRNA expression in the pons of MSA and control cases, while down-regulation was reported by Langerveld et al. (2007). Different isoforms of *SNCA* resulting from alternative mRNA splicing were investigated in PD, DLB, and MSA and isoform *SNCA* 98 was found to be up-regulated in the frontal cortex of MSA, while there was no significant difference in the level of *SNCA* 126 (Beyer et al. 2008).

Previous studies focused on understanding *SNCA* mRNA expression in MSA at the regional level and there is little information relating to cellular expression. *In situ* hybridization studies of *SNCA* mRNA expression in PD and control cases have successfully identified expression in neurons but not in oligodendrocytes (Jin et al. 2008; Kingsbury et al. 2004; Miller et al. 2005; Solano et al. 2000). Unravelling the cellular expression profile of *SNCA* in MSA will help to shed light on the possible contribution of overexpression in oligodendrocytes as a mechanism for GCI formation. Therefore, the aim of this study was to investigate the regional and cellular expression profile of *SNCA* in MSA subtypes and controls using qPCR.

Materials and Methods

Tissue Collection

Samples were obtained from brains donated to the Queen Square Brain Bank (QSBB) for Neurological Disorders, Department of Molecular Neuroscience, UCL Institute of Neurology, London. The donations were made according to ethically approved protocols, and tissue is stored under a license issued by the Human Tissue Authority (No. 12198). One half of the brain is sliced in the coronal plane, flash frozen, and stored at -80°C on arrival to the QSBB, while the other half is fixed in 10% buffered formalin. Tissue was sampled from the frozen half brain.

Case Selection

MSA cases were pathologically typed based on published criteria into mixed, SND and OPCA subtypes (Ozawa et al. 2004). Five MSA-SND, five MSA-OPCA, five MSA-mixed subtypes were selected and sex and age matched to five IPD and four normal control cases to study regional α syn mRNA expression (Table 1). For cellular mRNA expression, five MSA-mixed subtype and six neurologically normal controls were used to isolate neurons and oligodendrocytes (Table 2).

Regional Sampling, RNA Extraction, and Reverse Transcription

Frozen tissue (~ 100 mg) from the posterior frontal region (cortex and subcortical white matter), occipital region (cortex and subcortical white matter), dorsal putamen, pontine base, and cerebellum (white matter) was collected and homogenised using TissueRuptor (Qiagen, Germany). RNA extraction was carried out using RNeasy Mini Kit (Qiagen, Germany) and cDNA synthesized using SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen, UK) according to the manufacturer's instructions.

Cellular Sampling, RNA Extraction, and Reverse Transcription

Neurons from the pontine base ($\sim 1,000$ cells) and oligodendrocytes from the cerebellar white matter ($\sim 1,000$ cells) were isolated in five MSA and six control cases using the PixCell II laser-capture microdissection (LCM) system (Arcturus Engineering, Mountain View, CA). Neurons were identified using toluidine blue stain and oligodendrocytes using a rapid immunohistochemistry (IHC) protocol as previously described (Waller et al. 2012). Briefly, 7- μm frozen section were collected on uncharged, sterile glass slides and warmed to RT for 30 sec. The sections were then fixed in ice-cold acetone for 3 min and immunostained using oligodendrocyte-specific protein (OSP) primary antibody (Abcam, UK, ab7474) and VECTASTAIN Elite ABC kit (Vector labs, Burlingame, CA, PK-6101). Sections were blocked with normal goat serum (2%) for 3 min then incubated in OSP (1:10) for 3 min at RT. After a brief rinse with tris-buffered saline (TBS), sections were incubated in 5% biotinylated secondary antibody for 3 min at RT, then rinsed again with TBS. The avidin-biotin complex solution was then added to the section for 3 min at RT then rinsed off. DAB peroxidase substrate kit (Vector labs, Burlingame, CA, SK-4100) was used to visualize the reaction by incubating section for 3 min at RT. The sections were rinsed with TBS, dehydrated in graded alcohol (70, 95, 100, 100% for 1 min each), cleared in two changes of xylene and allowed to air dry in an air-flow hood for an hour prior to LCM. This rapid IHC protocol was carried out under sterile conditions and using diethylpyrocarbonate (DEPC)-treated water. RNA extraction of LCM samples was carried out using the Arcturus PicoPure RNA isolation kit (Applied Biosystems, UK, KIT0204) and cDNA synthesized using SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen, UK) according to manufacturer's instructions. RNA quality and concentration was then assessed using the Nanodrop and Agilent Bioanalyser 2100. Maintenance of RNA quality was adequate as assessed by the RIN of samples before and after LCM was carried out. Prior to LCM, samples had a mean RIN of 4.7 (range 4.2–5.8) declining to 2.6 (range 2.0–3.1) after LCM and this is in keeping with previous findings (Waller et al. 2012).

Quantitative PCR

qPCR was performed on Stratagene MX3000p (Agilent technologies, CA) using 50-ng cDNA and Power SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. All primers were run at the following thermal profile: one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 min and

TABLE 1: Demographics of Regional Expression Cohort

Case	Age	Sex	Diagnosis	MSA pathological type	PMI (hr)
1	56	F	MSA	Mixed	28
2	62	M	MSA	Mixed	20
3	64	F	MSA	Mixed	52
4	66	M	MSA	Mixed	105
5	70	F	MSA	Mixed	65
6	50	F	MSA	SND	4
7	54	F	MSA	SND	47
8	58	F	MSA	SND	11
9	63	M	MSA	SND	27
10	72	M	MSA	SND	50
11	56	M	MSA	OPCA	37
12	60	F	MSA	OPCA	62
13	61	M	MSA	OPCA	ND
14	64	M	MSA	OPCA	35
15	66	F	MSA	OPCA	74
16	61	F	IPD	NA	26
17	63	M	IPD	NA	37
18	65	M	IPD	NA	43
19	67	F	IPD	NA	108
20	70	M	IPD	NA	75
21	57	M	Control	NA	79
22	69	M	Control	NA	ND
23	71	M	Control	NA	39
24	73	F	Control	NA	24

NA: not applicable; ND: not documented; PMI: post-mortem interval.

60°C for 1 min. Three reference genes, UCB, TBP, and GAPDH, were determined as appropriate to normalize regional expression data using qBase plus software (Biogazelle, Belgium). Due to limited sample volume, one reference gene (UBC) was used to normalize LCM expression data. Primers for the gene of interest (GOI), *SNCA*, were designed in house while the reference genes were obtained from RTprimerDB (Table 3).

Expression Analysis and Statistics

Standard curves were used to extrapolate expression value of each gene in each region or cell. The GOI was then normalized to the geometric mean of the reference genes as recommended by Vandesompele et al. (2002). A multilevel statistical model was used for regional expression analysis while the Mann–Whitney U test was used to analyze cellular expression results with the significance levels set at $P < 0.05$.

Results

Regional *SNCA* mRNA expression was examined in the posterior frontal region, occipital region, dorsal putamen, pontine base, and cerebellar white matter of three MSA subtypes (mixed, SND, and OPCA), IPD, and control cases (Fig. 1). *SNCA* expression level was highest in IPD and lowest in MSA-SND, however, data analysis using a multilevel statistical model showed that there was no statistically significant difference between the different groups (multilevel statistical model, $P = 0.14$). After adjusting for group effect, the highest expression was found in the occipital cortex while the lowest was in the putamen. The differences between regions were found to be statistically significant (multilevel statistical model, $P < 0.0001$).

TABLE 2: Demographics of Cellular Expression Cohort

Case	Age	Sex	Diagnosis	MSA pathological type	PMI (hr)	RIN before LCM	RIN after LCM
1	50	M	MSA	Mixed	30	4.8	2.6
2	70	F	MSA	Mixed	65	5.8	ND
3	56	M	MSA	Mixed	75	4.7	ND
4	64	F	MSA	Mixed	99	4.4	2.8
5	64	M	MSA	Mixed	100	5.1	3.1
6	69	M	Control	NA	ND	4.4	ND
7	82	F	Control	NA	ND	4.8	3.6
8	73	F	Control	NA	24	5.3	2.0
9	85	M	Control	NA	78	4.2	2.2
10	80	F	Control	NA	49	3.9	2.5
11	83	M	Control	NA	63	4.2	2.3

NA: not applicable; ND: not documented; PMI: post-mortem interval.

Next *SNCA* expression in neurons and oligodendrocytes isolated by laser capture was explored in the pontine base and cerebellar white matter, respectively, as these regions are affected in MSA. *SNCA* expression was detected in neurons and oligodendrocytes of both MSA and control cases, with the highest level of expression being found in MSA oligodendrocytes (Fig. 2A). The fold changes in mRNA expression between the different cell types and groups were also calculated (Fig. 2B). Although none of the differences identified reached statistical significance, there was a slight increase in expression in MSA oligodendrocytes as compared to control oligodendrocytes (fold change = 0.7, Mann–Whitney U test, $P = 0.18$). Expression in MSA neurons was slightly decreased as compared to control neurons (fold change = -0.4 , Mann–Whitney U test, $P = 0.46$). Comparing the different cell types within the same groups revealed that control oligodendrocytes express 0.3 more *SNCA* than control neurons (Mann–Whitney U test, $P = 0.92$). The greatest difference was seen between MSA oligodendrocytes and neurons where expression in MSA oligodendrocytes was 3.1 times higher than MSA neurons (Mann–Whitney U test, $P = 0.16$).

Discussion

α Syn has been localized in the brain to neuronal presynaptic terminals and may play a role in neuronal plasticity, vesicular transport, and membrane interaction (Iwai et al. 1995; Kahle et al. 2000; Reynolds et al. 2011). There has been great interest in the possible mechanisms by which α syn forms primarily oligodendroglial inclusions in MSA since its identification as the main constituent of the GCIs. Understanding the

expression pattern of *SNCA* in MSA at both regional and cellular levels is essential to determine if changes in expression play a role in MSA pathogenesis and whether this influences regional vulnerability to disease. The regional expression analysis in this study now demonstrates that *SNCA* expression level varies across different brain regions in control, MSA, and IPD groups. The greatest expression is found in the occipital grey matter and the lowest level in the putamen. However, *SNCA* mRNA expression is not significantly different between the MSA subgroups, IPD and control cases in all regions. The expression trend found in MSA and control cases used in this study is similar to that documented in expression databases such as UKBEC and Allen Brain Atlas, which analyze gene expression in control human brains (Hawrylycz et al. 2012; Trabzuni et al. 2011). The similarity in trend, where *SNCA* is generally expressed more in cortical regions than subcortical and cerebellar regions, has at least two main implications. The first is that the vulnerability of StrN and OPC regions is not associated with a higher baseline expression of *SNCA*. It could be hypothesized that the concentration of GCIs in specific regions is due to higher expression levels of *SNCA* in normal conditions in those regions that is exacerbated by disease. Our data do not support this hypothesis as *SNCA* mRNA levels are higher in cortical than subcortical regions in both normal and disease cases, while GCI pathology is greater in subcortical and hindbrain structures such as the StrN and OPC regions in MSA. This also indicates that the disease does not cause an alteration in expression levels in areas of greater vulnerability. The absence of a difference in *SNCA* expression levels between the different MSA groups, IPD, and controls may imply that factors

TABLE 3: Primers Information

Gene	SNCA	TBP	UBC	GAPDH
Gene name	synuclein, alpha (non A4 component of amyloid precursor)	TATA box binding protein	Ubiquitin C	Glyceraldehyde-3-phosphate dehydrogenase
RefSeq no.	NM_001146055.1	NM_003194	NM_021009	NM_002046
Primer sequence	F: CAACAGTGGCT GAGAAGACCA R: GCTCCTTCTTCA TTCTTGCCCA	F: TGCACAGGAGC CAAGAGTGAA R: CACATCACAGCTCCCCACCA	F: R: ATTTGGGTGCGGGTCTTG	F: GAAATCCCATCACC ATCTCCAGG R:
Alignment	F: base 228 to 249 R: base 369 to 390	F: base 892 to 913 R: base 1004 to 1024	R: TGCCCTTGACATTCTCGATGGT F: base 399 to 418 R: base 511 to 532	F: base 313 to 337 R: base 413 to 433
Amplicon length (bp)	163	132	133	120
Organism	Homo sapiens	Homo sapiens	Homo sapiens	Homo sapiens
Source	In house design	RTPrimerDB (ID: 2630)	RTPrimerDB (ID: 8)	RTPrimerDB (ID: 1108)

contributing to pathogenic mechanisms of MSA may be further downstream and not at the transcriptional level and could suggest that impaired α syn degradation may contribute to the disease. Another possibility is that any differences at the transcriptional level are masked by the cellular heterogeneity of the samples. To overcome this problem, we used LCM to obtain samples highly enriched in neurons and oligodendrocytes.

It has long been suggested that mature oligodendrocytes lack α syn (Jin et al. 2008; Miller et al. 2005; Solano et al. 2000). However, cell-culture evidence indicates that rat and mouse oligodendrocytes express α syn protein and mRNA, and in some cases this expression is transient, appearing in precursor cells and declining as they mature (Culvenor et al. 2002; Nielsen et al. 2006; Richter-Landsberg et al. 2000). To date, there is one published study addressing the question of *SNCA* mRNA expression in MSA oligodendrocytes (Miller et al. 2005). This study used double-labeling *in situ* hybridization of *SNCA* and proteolipid protein (PLP), an oligodendrocyte marker, and showed that *SNCA* is not expressed in oligodendrocytes in MSA or control brains. As ISH has limited sensitivity, the findings of this study do not exclude the possibility of *SNCA* mRNA expression by oligodendrocytes (Deglincerti and Jaffrey 2012; Miller et al. 2005).

Analyzing *SNCA* expression in LCM-isolated neurons and oligodendrocytes, our data indicate that this highly conserved protein is expressed in human oligodendrocytes and that expression in MSA oligodendrocytes is greater than control oligodendrocytes. In our hands, LCM isolation of OSP-positive oligodendrocytes provides an oligodendrocyte-enriched sample (Waller et al. 2012). This suggests that *SNCA* overexpression in MSA oligodendrocytes may be a possible mechanism contributing to GCI formation. The cellular expression of α syn in MSA in this study may also explain the pathological profile of the disease. GCIs are found in greater abundance than NCIs in MSA, and this greater susceptibility of oligodendrocytes to inclusion formation may be a reflection of overexpression of α syn by oligodendrocytes in MSA.

The novel findings of this LCM study demonstrating expression of *SNCA* mRNA in oligodendrocytes open the door to further exploration of the molecular pathogenesis of MSA. Expanding the current study to examine cellular *SNCA* mRNA expression in other affected and unaffected brain regions in MSA may help to elucidate the selective vulnerability of StrN and OPC regions. In addition to affected and unaffected brain regions, examination of *SNCA* expression in MSA cases with short disease duration and long disease duration may provide further insight into the role of *SNCA* mRNA expression on clinical outcome. A further step would be to characterize *SNCA* mRNA expression

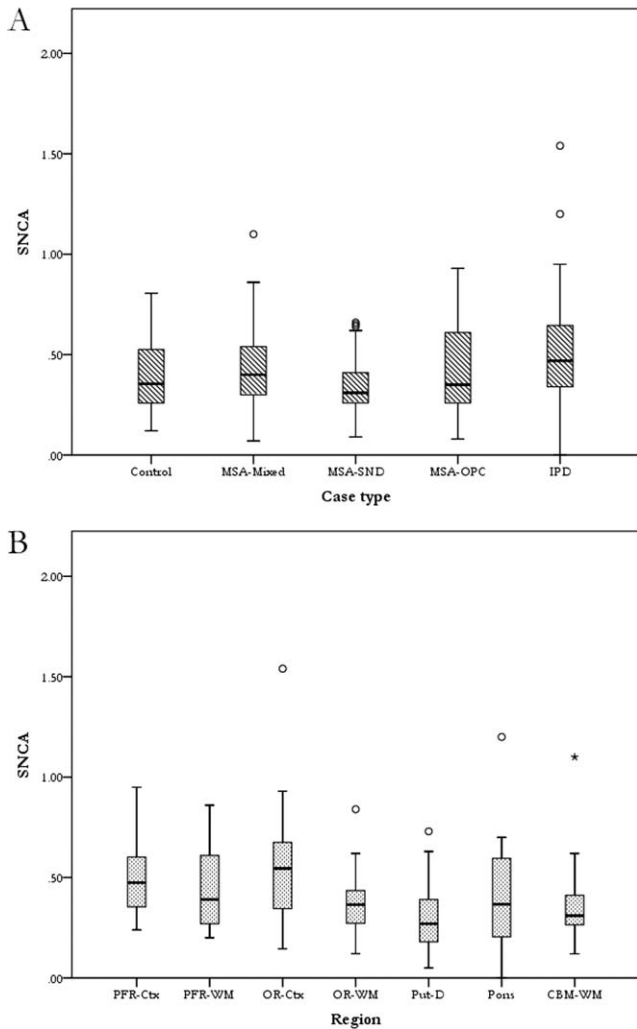


FIGURE 1: SNCA mRNA regional expression (A) Expression between the different cases (adjusted for regions) shows no statistically significant differences between the groups although the lowest level of expression was found in MSA-SND and the highest in IPD cases ($P = 0.14$). **(B)** After adjusting for case type, a statistically significant difference in SNCA expression between regions was found ($P < 0.0001$), with the lowest expression in the dorsal putamen and the highest in the occipital cortex. Multi-level model test with significance level set at $P=0.05$. The box-plot show median values as the line within the box, the box reflects the interquartile range and the whiskers the range of the values. PFR: posterior frontal region; OR: occipital region; Put-D: dorsal putamen; CBM: cerebellum; Ctx: cortex; WM: white matter; MSA: multiple system atrophy; SND: striatonigral degeneration; OPC: olivopontocerebellar; IPD: idiopathic Parkinson's disease; SNCA: α -synuclein.

in oligodendrocytes with and without GCIs to determine the contribution of SNCA expression to inclusion formation in oligodendrocytes. In addition to GCI+/- oligodendrocytes, SNCA mRNA expression may also be explored at different stages of oligodendrocyte maturation. The oligodendrocyte lineage comprises precursor, immature, mature nonmyelinating, and mature myelinating cells and α syn protein in GCIs has been found in mature but not pre-

cursor oligodendrocytes in MSA (Ahmed et al. 2013; Papp et al. 1989). Therefore, determining SNCA mRNA expression in the different maturational stages may provide further insight into the vulnerability of mature oligodendrocytes to α syn aggregation.

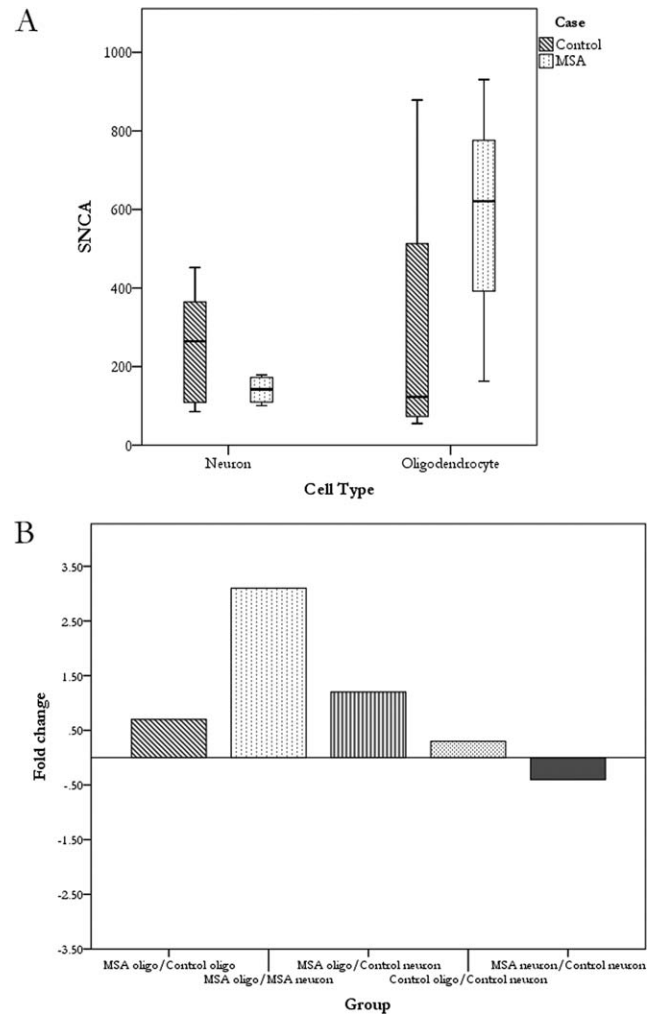


FIGURE 2: SNCA mRNA cellular expression(A) SNCA is expressed in neurons and oligodendrocytes of both MSA and control cases. Expression in neurons is greater in controls as compared to MSA ($P = 0.47$), in contrast to oligodendrocytes where expression is greater in MSA ($P = 0.18$) but these results did not reach statistical significance. **(B)** Bar graph representing fold change of expression values between the different cases and cell types. No statistically significant differences were found although there was a trend toward increased expression in MSA oligodendrocytes when compared to control oligodendrocytes ($P = 0.18$), MSA neurons ($P = 0.16$) and control neurons ($P = 0.18$). In contrast, there was a slight decrease in α syn expression in MSA neurons as compared to control neurons ($P = 0.46$). The greatest difference seems to be the increase in expression in MSA oligodendrocytes compared with MSA neurons. Mann-Whitney U test with significance level set at $P=0.05$. The box-plot shows median values as the line within the box, the box reflects the interquartile range and the whiskers the range of the values. Oligo: oligodendrocytes; MSA: multiple system atrophy; SNCA: α -synuclein.

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