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Absence of α-synuclein mRNA expression in normal and multiple system atrophy oligodendroglia

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Summary. α-Synuclein is a major constituent of glial cytoplasmic inclusions (GCIs), which are pathognomic for multiple system atrophy (MSA). We have previously demonstrated that in normal human brain, α-synuclein mRNA has a restricted pattern of neuronal expression and no apparent glial expression. The current study used double-label *in situ* hybridization to determine if α-synuclein mRNA is expressed by oligodendroglia of MSA cases. Analysis of MSA brain tissue revealed depletion of regional signal for this transcript in many brain areas due to extensive neurodegeneration. Cellular analysis of oligodendroglia in crus cerebri, a GCI-rich region ventral to substantia nigra, revealed an absence of α-synuclein mRNA signal in control and MSA cases. However, an abundance of this transcript was detected in melanin-containing neurons of substantia nigra. Therefore, oligodendroglia do not express α-synuclein mRNA in control and MSA cases suggesting that involvement of α-synuclein in GCI pathology of MSA is due to its ectopic presence in oligodendroglia.

Keywords: Glial cytoplasmic inclusion, *in situ* hybridization, synucleinopathy.

Introduction

Multiple system atrophy (MSA) is a neurodegenerative disease with variable clinical presentations, including parkinsonism, cerebellar ataxia and autonomic dysfunction (Wenning et al., 1997). Compared to Parkinson's disease (PD), MSA is not as responsive to dopamine replacement therapy, which suggests that the parkinsonism in MSA is due to more widespread degeneration than merely dopaminergic neurons of substantia nigra and is associated with significant pathology in the basal ganglia. MSA is associated with neuronal loss in caudate, putamen, substantia nigra, pontine nuclei, inferior olives, and cerebellum. The distribution of pathology in brain is variable and is reflected by the predominant clinical phenotype, with the major clinical forms being associated

with predominant pathology in the nigrostriatal system (MSA-P) or the olivopontocerebellar system (MSA-C) (Gilman et al., 1999).

The unifying histopathologic hallmark of MSA is the glial cytoplasmic inclusion (GCI), which is a round or crescent-shaped inclusion in the cytoplasm of oligodendroglia in white matter of affected brain regions (Papp et al., 1989; Lantos, 1998). The most sensitive and specific method for detecting GCIs is immunostaining for α-synuclein (Arima et al., 1998; Spillantini et al., 1998; Wakabayashi et al., 1998), a small soluble protein that is more abundant in neurons than glia (Irizarry et al., 1996; Iwai et al., 1995; Maroteaux et al., 1988; Solano et al., 2000). While α-synuclein is an abundant component of GCIs, it is unknown whether this protein actually originates in oligodendroglia.

Overexpression of α-synuclein is a common theory for disease pathogenesis in synucleinopathies. Genetic studies have indicated that *SNCA* gene multiplication (α-synuclein overexpression) can cause PD (Singleton et al., 2003; Farrer et al., 2004), but such a direct relationship in MSA has not been established. Multiplication of the *SNCA* gene results in an overexpression of α-synuclein transcript in brain and α-synuclein protein in blood, while in brain the overabundance of α-synuclein protein drives its aggregation (Miller et al., 2004b). GCI-like pathology in addition to Lewy body pathology has been reported in *SNCA*-triplication cases (Gwinn-Hardy et al., 2000; Miller et al., 2004a). Moreover, glial inclusions can be caused by α-synuclein overexpression via oligodendroglial-specific promoters in transgenic mice (Kahle et al., 2002; Yazawa et al., 2005). Thus, an overabundance of α-synuclein in oligodendroglia can cause GCI formation. Therefore, we examined the possibility that overexpression of α-synuclein mRNA may occur in oligodendroglia of MSA cases.

Methods

Human brain tissue

Fresh-frozen blocks of human brain tissue were obtained from Harvard Brain Tissue Resource Center, the Massachusetts General Hospital Alzheimer's Disease Resource Center (MGH-ADRC), and the Michigan-ADRC. MSA cases were characterized as either MSA-P ($n = 5$, age range 62–72) or MSA-C ($n = 5$, age range 46–77) based on clinical symptoms, the pathologic profile of neuronal cell loss, and the distribution of GCIs, which was confirmed by immunohistochemical staining for α-synuclein-containing GCIs (see below). Control cases ($n = 10$, age range 60–82) had no history of neurological disease and all tissue had a post-mortem interval less than 24 hours. Frozen sections (12 μm) from multiple regions were cut at −20°C, mounted on slides, and stored at −70°C.

Immunohistochemistry

Frozen, slide-mounted sections were thawed at room temperature and fixed in 4% paraformaldehyde for 10 min. After rinsing in 0.1 M phosphate-buffered saline (PBS), sections were treated for 60 min with blocking solution (3% normal goat serum, 0.3% Triton X-100, and 3% H_2O_2 in PBS). After rinsing with PBS (3×5 min), the sections were incubated with primary antibody (H3C, 1:5000) overnight at 4°C. H3C is a mouse monoclonal antibody that recognizes the C-terminus of human α-synuclein (generously provided by D. Clayton and J. George, Univ. of Illinois). Excess primary antibody was rinsed from the sections with PBS (3×5 min) after which they were incubated in biotinylated goat-anti-mouse secondary antibody (1:500; Jackson ImmunoResearch Inc.) for 60 min. Slides were rinsed with PBS (3×5 min) and an amplification step was conducted by incubating slides in ABC mixture (ABC Vectastain Elite, Vector Laboratories

Inc., Burlingame, CA) for 60 min followed by thorough rinsing with PBS (3×10 min). Immunoreactivity was visualized by exposing slides to PBS containing 0.5 mg/ml diaminobenzidene and 0.03% H_2O_2 for 4–8 min. Slides were rinsed, counterstained with hematoxylin, and then examined via light microscopy to assess α-synuclein immunoreactive GCIs. Negative controls showed a negligible level of background stain. Photographs were taken at 40× magnification.

In situ hybridization

α-Synuclein mRNA expression was analyzed by using *in situ* hybridization with a radiolabeled cRNA probe for human α-synuclein as previously described (Solano et al., 2000). The transcript for proteolipid protein (PLP), an abundant protein in myelin, was also examined using a radiolabeled cRNA probe generated against the open reading frame of PLP. *In situ* hybridization was carried out using a standard protocol of this laboratory (Kerner et al., 1998). Double-label *in situ* hybridization (DL-ISH) was also conducted using digoxigenin-labeled cRNA probe for PLP (dig-PLP) along with radiolabeled riboprobe for α-synuclein. cRNA hybridization to tissue sections was conducted at 50°C for 4 hours. Sections were processed through stringent post-hybridization washes that included 0.1 M sodium saline citrate (SSC, 70°C) and RNAse A (37°C). The sections were then dehydrated in ethanol and air-dried prior to film exposure.

Film autoradiograms were prepared by apposing the radiolabeled slides to Hyperfilm β-Max (Amersham Co., Sweden) for 3 days (PLP) or 18 days (α-synuclein). Following film exposure, slides were dipped in Ilford K5 autoradiographic emulsion (Polysciences Inc., Warrington, PA; diluted 1:1 with distilled-deionized water), dried overnight, stored at 4°C, and developed after 1 week (PLP) and 6 weeks (α-synuclein).

Previous DL-ISH studies in our laboratory have demonstrated that co-hybridization of digoxigenin-labeled cRNA probes along with radiolabeled cRNA probes does not interfere with the signal obtained from the latter (Counihan et al., 1998; Kerner et al., 1998). Such was the case in the current study. Hybridization signal for α-synuclein mRNA was quantified in oligodendroglia that were identified by the presence of dig-PLP, which appears as purple cytoplasmic stain following alkaline-phosphatase-mediated detection of digoxigenin-tagged residues (Kerner et al., 1998). A total of 20 dig-PLP-positive cells in the interfascicular tracks of crus cerebri were analyzed in each of five cases for each disease condition (control, MSA-P, MSA-C). Additionally, α-synuclein mRNA hybridization signal was quantified in neuromelanin-containing neurons of the substantia nigra for each case.

Emulsion autoradiographic analysis was performed under bright and dark field optics. Labeled cells were visualized using bright field optics under a 100× water immersion lens (Leitz). Quantitative microscopic analysis of hybridization intensity and distribution was performed using the computer-assisted M1 image analysis system (Imaging Research, St. Catharine's, Ontario, CA). This system was used to outline the soma of each labeled cell and then quantify the overlying silver grains. The number of silver grains and the size of the soma (μm^2) were recorded in order to calculate the number of grains per 1000 μm^2. Background signal was evaluated by encircling an equal number of similarly sized area of proximal neuropil and quantifying grains within that area as a comparison to cell body grain clusters.

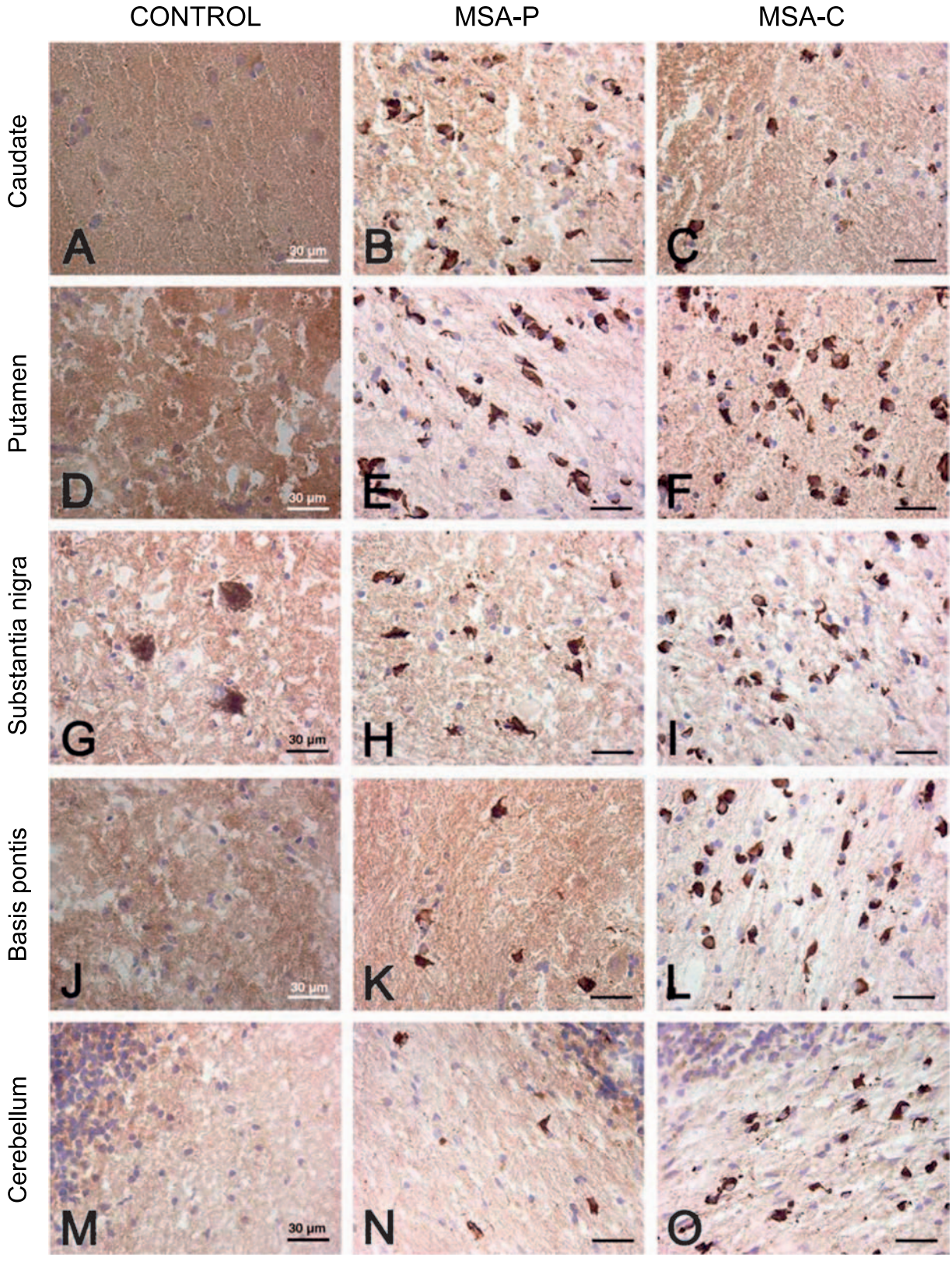
Statistical analysis

Statistical analysis was performed on a commercial statistics software package (SuperANOVA; Abacus Concepts, Inc., Berkeley, CA). Cellular mRNA hybridization signal was quantified from emulsion autoradiograms and analyzed using an ANOVA with repeated measures design followed by Fisher's PLSD post-hoc comparisons (significance $P < 0.05$).

Results

GCI distribution in MSA brains

Immunohistochemical staining for α-synuclein in MSA brain tissue revealed GCIs in numerous brain regions including caudate, putamen, midbrain, pons,


CONTROL
MSA-P
MSA-C
Caudate
Putamen
Substantia nigra
Basis pontis
Cerebellum
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
30 µm

and cerebellum (Fig. 1). Differences in GCI distribution were seen between MSA-P and MSA-C. In MSA-P, GCIs predominated in basal ganglia while in MSA-C, GCIs were not only abundant in basal ganglia, but also in pons and cerebellum. This pattern of GCI distribution was similar to that outlined by Jellinger and colleagues (Jellinger et al., 2005) and therefore validates the grouping of cases into the two disease phenotypes MSA-P and MSA-C. Interestingly, the general neuropil staining of α-synuclein protein seen in controls was dramatically decreased in GCI-rich regions of both types of MSA cases. This loss of staining may reflect either severe neurodegeneration or endocytosis of α-synuclein by oligodendroglia. GCI density in crus cerebri was similar for both MSA subtypes and therefore provided a fortuitous brain region for further analysis at the cellular level.

Regional α-synuclein mRNA expression

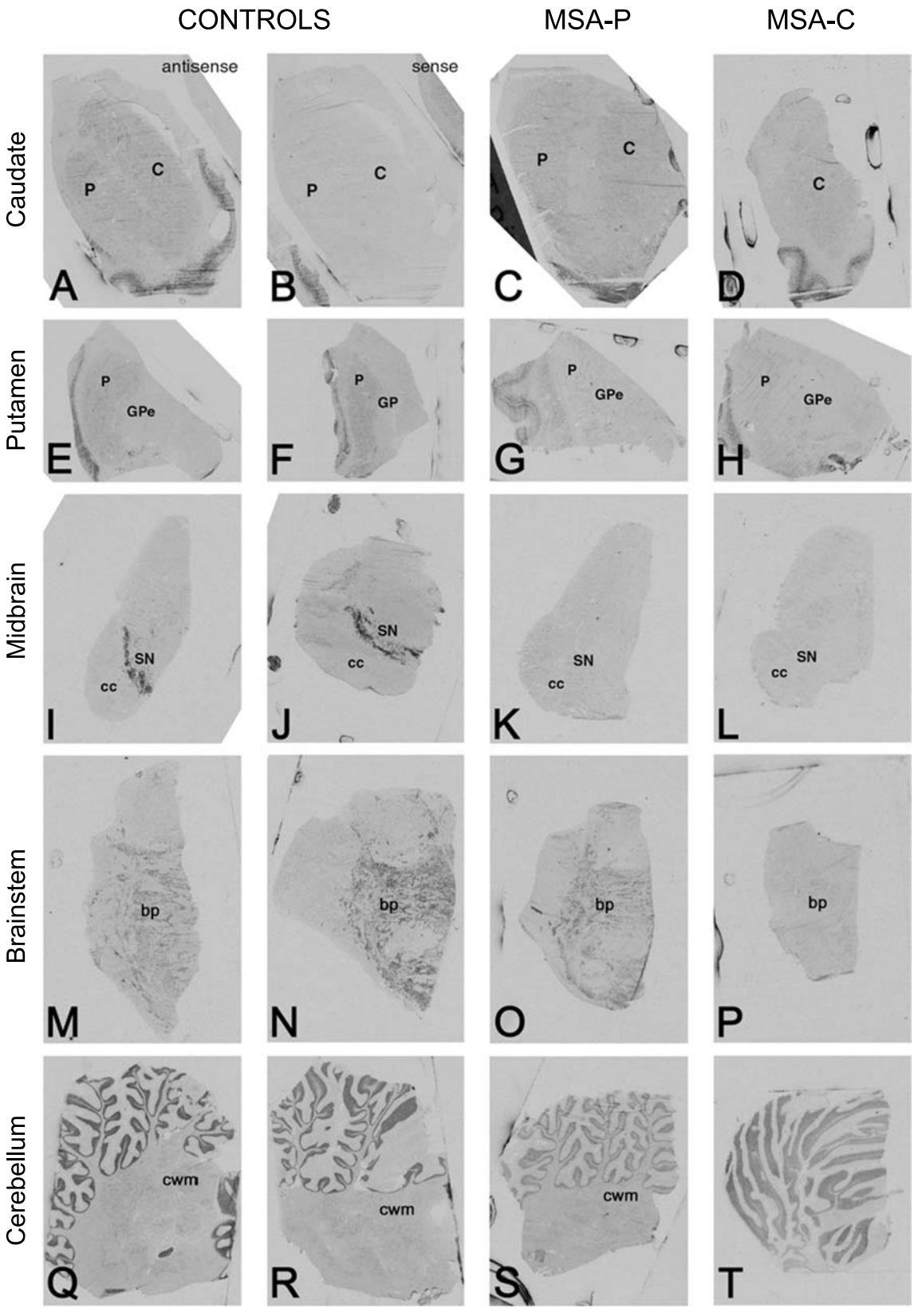
Hybridization of a radiolabeled cRNA probe for α-synuclein confirmed the restricted, neuronal expression pattern of this transcript in control human brain tissue (Fig. 2). As described previously (Solano et al., 2000), there was no apparent expression of α-synuclein mRNA in white matter areas such as crus cerebri and white matter of cerebellum. In MSA brain tissue there was a clear decrease in regional signal for α-synuclein mRNA, which is likely to be due to severe neuronal loss (e.g. substantia nigra).

We next compared the expression pattern of α-synuclein mRNA to that of a transcript enriched in oligodendroglia, proteolipid protein (PLP). Based on our hypothesis that α-synuclein mRNA expression would be elevated in GCI-rich regions of MSA cases, we predicted that expression of this transcript would resemble that of PLP. However, this comparison in the midbrain revealed that these two transcripts have opposite patterns of expression suggesting that oligodendroglia do not overexpress α-synuclein mRNA in MSA (Fig. 3).

Cellular α-synuclein mRNA expression

To test the hypothesis that α-synuclein mRNA expression is elevated in MSA oligodendroglia, we quantified the signal for α-synuclein mRNA hybridization in individual oligodendroglia of MSA brain tissue relative to controls. Crus cerebri is a GCI-rich brain region that is affected in both forms of MSA as verified via α-synuclein immunostaining in adjacent sections (Fig. 4B). In our cellular analysis, oligodendroglia of crus cerebri were identified via co-hybridization of digoxigenin-labeled cRNA probe for PLP (dig-PLP), which yields

Fig. 1. α-Synuclein immunoreactivity in control and MSA brain tissue. The strong neuropil staining for α-synuclein in control tissue was greatly diminished in both MSA subtypes, which reflects massive neurodegeneration. GCIs were detected in many brain regions of MSA cases with subtype-specific distributions. In caudate (**A**–**C**), GCIs were more abundant in MSA-P than in MSA-C. In putamen (**D**–**F**) and substantia nigra (**G**–**I**), GCIs were similarly abundant in both MSA subtypes. Note the significant loss of neuromelanin-containing neurons in substantia nigra of MSA. In pons (**J**–**L**) and cerebellar white matter (**M**–**O**), GCIs were more abundant in MSA-C than in MSA-P. Such GCI distributions are indicative of MSA-P and MSA-C. Scale-bars = 30 μm


CONTROLS
MSA-P
MSA-C
Caudate
Putamen
Midbrain
Brainstem
Cerebellum
antisense
sense
A
B
C
D
E
F
G
H
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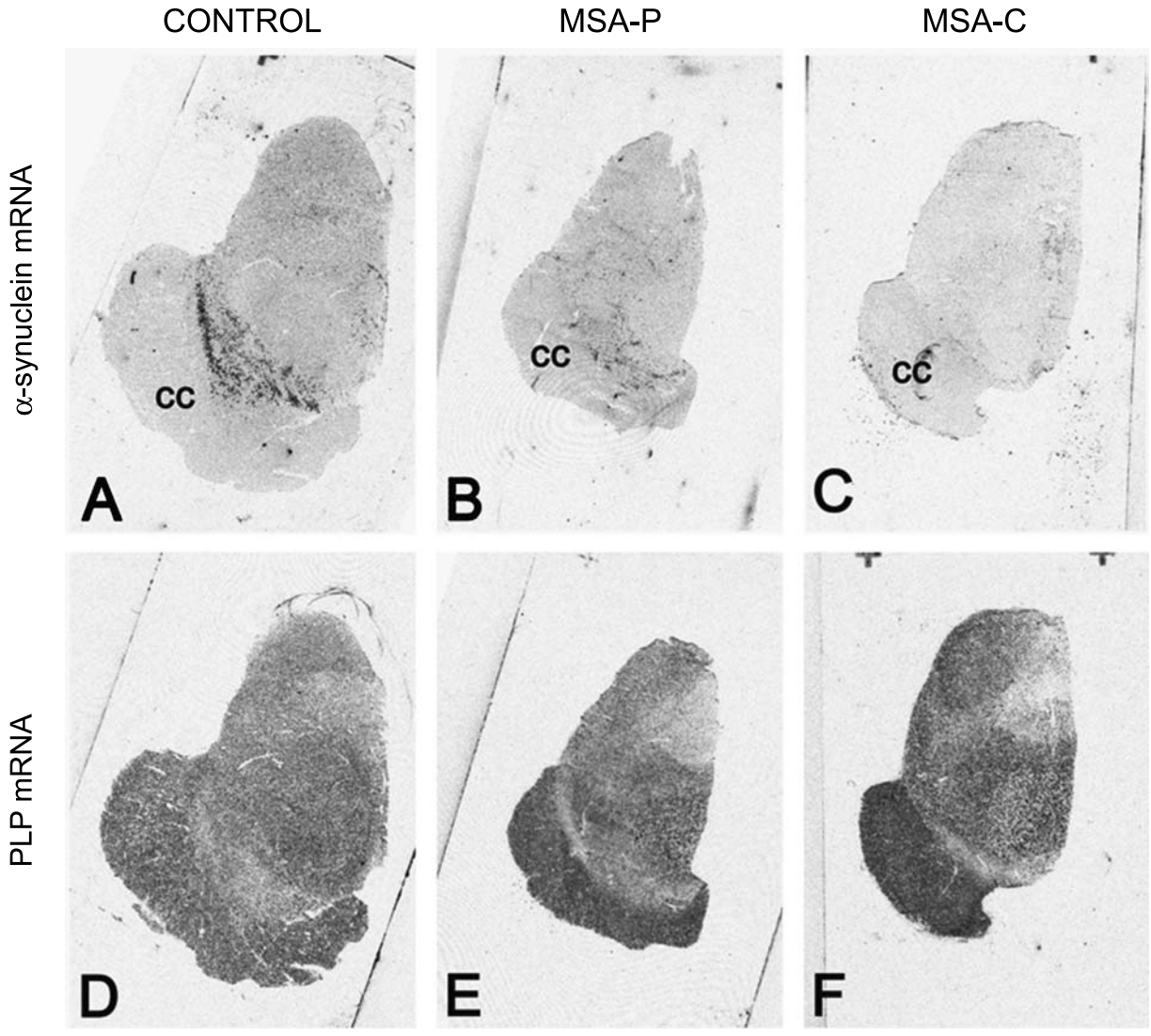

Fig. 3. Relative mRNA expression for α-synuclein (**A**–**C**) and proteolipid protein (PLP; **D**–**F**) in human midbrain. Oligodendroglia contain an abundance of PLP and therefore the transcript for this protein serves as a useful marker of these cells. Opposite patterns of α-synuclein and PLP mRNA expression are apparent. Note the robust signal for PLP mRNA and the negligible signal for α-synuclein mRNA throughout white matter, particularly in crus cerebri (cc)

Fig. 2. Film autoradiograms of α-synuclein mRNA distribution in control and MSA brain tissue. In sections of caudate the specificity of the antisense cRNA probe (**A**) is revealed by comparison with the negligible signal for the sense version of this probe (**B**). The hybridization signal for α-synuclein mRNA occurs in a restricted, neuronal pattern in control cases. However, much of this signal is lost in both subtypes of MSA due to extensive neurodegeneration in areas such as putamen (**G**, **H**) and substantia nigra (**K**, **L**). Cell loss in basis pontis also underlies decreased α-synuclein mRNA signal in MSA-C (**P**), but not in MSA-P (**O**). The expression of α-synuclein mRNA is relatively unaltered in the caudate of either subtype of MSA compared to control (**C**, **D**). Abbreviations: basis pontis (bp), caudate (C), crus cerebri (cc), cerebellar white matter (cwm), external globus pallidus (GPe), putamen (P), substantia nigra (SN)

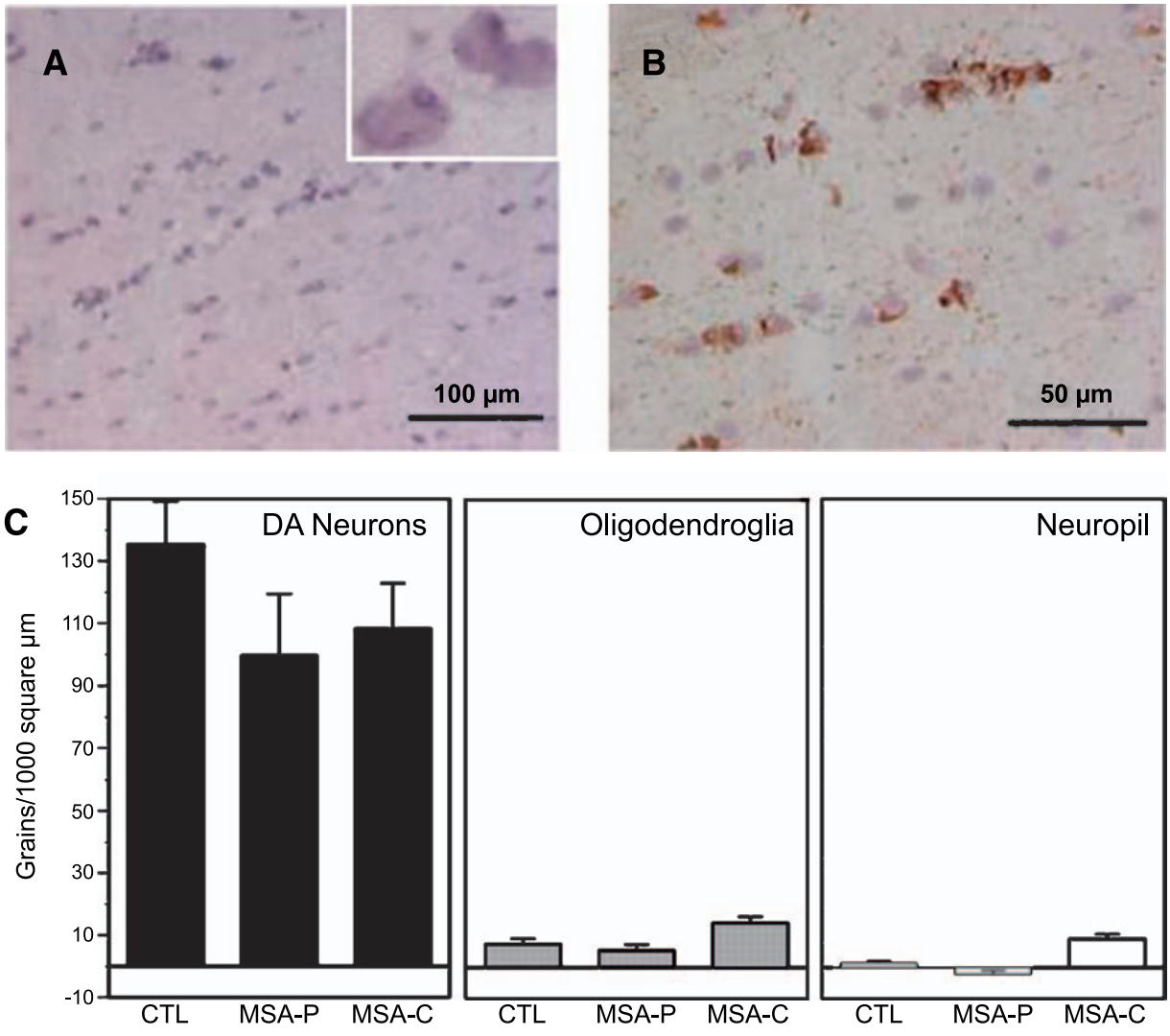

Fig. 4. Quantitative analysis of α-synuclein mRNA hybridization signal in oligodendroglia of MSA and control cases. **A** Oligodendroglia of crus cerebri were identified via dig-PLP labeling (purple chromagen; inset, 100×). Neuropil was sampled from proximal areas vacant of cells. Scale-bar = 100 µm. **B** Crus cerebri is a GCI-rich brain region in MSA cases as revealed by α-synuclein immunostaining in an adjacent section. Scale-bar = 50 µm. **C** Quantification of overlying silver grains, reflecting hybridization of isotopic cRNA probe for α-synuclein, revealed no significant differences between α-synuclein mRNA expression in dig-PLP-labeled oligodendroglia and neuropil of MSA-P, MSA-C, and control cases. This demonstrates that oligodendroglia do not express α-synuclein mRNA in either MSA or normal adult human brain. The robust expression of α-synuclein mRNA in nigral dopamine neurons serves as comparative reference. Bars represent the mean density of autoradiographic grains overlaying each cell ± SEM

a purple chromagen (Fig. 4A). The pattern of α-synuclein hybridization signal observed via single-label *in situ* hybridization (Solano et al., 2000) was unaltered by co-hybridization of the dig-PLP riboprobe. The cellular signal for α-synuclein mRNA hybridization in dig-PLP-stained oligodendroglia was not

significantly different from background signal quantified in proximal neuropil, which suggests that oligodendroglia express a negligible, if any, level of α-synuclein mRNA. This result was the same in controls and both subtypes of MSA (Fig. 4C). Moreover, the level of α-synuclein mRNA signal was nearly 10-fold greater in neuromelanin-containing dopamine neurons of substantia nigra than in oligodendroglia and neuropil.

Discussion

Our analysis of α-synuclein mRNA in MSA brain tissue revealed that regional signal for this transcript was always depleted in GCI-rich brain areas. Similarly, α-synuclein protein staining in neuropil of these brain areas was greatly decreased. These two observations are likely to be attributed to massive neurodegeneration. Immunostaining for α-synuclein revealed GCIs distributed throughout the brain in a pattern typical of MSA pathology in which basal ganglia regions were primarily affected in MSA-P, and cerebellum and pons were primarily affected in MSA-C. Each MSA case also had an abundance of GCIs present in crus cerebri, regardless of disease subtype. Indeed, a grading scale for MSA pathology indicates that crus cerebri is typically affected in both MSA-P and MSA-C (Jellinger et al., 2005). Cellular analysis of α-synuclein mRNA expression in oligodendroglia of crus cerebri, a GCI-rich brain region, revealed that these cells do not express α-synuclein mRNA in either control or MSA brain tissue.

Our study in human brain indicates an absence of α-synuclein mRNA expression in oligodendroglia. This result concurs with the lack of α-synuclein mRNA expression found in oligodendroglia of normal mice (Yazawa et al., 2005). However, α-synuclein has been reported to be transiently expressed by cultured rat oligodendrocytes (Richter-Landsberg et al., 2000) and low levels of the protein have been detected in glia of proteinase K-treated sections of normal human brain (Mori et al., 2002). It is possible that a very low level of α-synuclein mRNA expression may be beyond the detection limits of *in situ* hybridization. Moreover, subsets of oligodendroglia may differentially express α-synuclein in MSA, even if the overall average expression is undetectable. Nonetheless, the contrast between high levels of expression in nigral dopamine neurons relative to an absence of expression in oligodendroglia demonstrates that an overexpression of α-synuclein mRNA does not occur in this disease.

The lack of elevated α-synuclein mRNA in MSA reported here and by others (Ozawa et al., 2001) suggests that MSA pathogenesis is not mediated by α-synuclein overexpression. An alternative mechanism may involve altered interplay between neurons and oligodendroglia in which neuronal α-synuclein may be translocated to oligodendroglia. Such a phenomenon may not be unique to α-synuclein since other predominantly neuronal proteins have also been found in GCIs (Honjyo et al., 2001; Nakamura et al., 1998). α-Synuclein is present in human cerebrospinal fluid suggesting that it may be released from neurons (Borghi et al., 2000; El-Agnaf et al., 2003). Indeed, some cell lines are capable of Rab5-dependent endocytosis of α-synuclein from media (Sung et al., 2001). Whether mature oligodendroglia have this capability *in vivo* is unknown,

but pathologic oligodendroglia of MSA do aberrantly express the endocytosis regulatory proteins Rab5 and Rabaptin-5 (Nakamura et al., 2000). Once in oligodendroglia, α-synuclein may be improperly catabolized due to an absence of cellular machinery found in neurons. Hyperphosphorylated α-synuclein is present in GCIs (Piao et al., 2001; Fujiwara et al., 2002) and in GCI-like inclusions of transgenic mice that overexpress this protein specifically in oligodendroglia (Kahle et al., 2002). Therefore, protein kinase hyperactivity may contribute to the pathogenesis of MSA.

In summary, our results indicate that oligodendroglia do not express α-synuclein mRNA in either control or MSA cases. This suggests that MSA pathogenesis is not mediated by an overexpression of α-synuclein mRNA in oligodendroglia. The involvement of α-synuclein in MSA is likely to involve misprocessing of α-synuclein protein rather than aberrant mRNA overexpression in oligodendrolglia. α-Synuclein found ectopically in oligodendroglia of MSA may arise from neurons. Future studies will examine the likely role of neuronal α-synuclein in MSA pathogenesis.

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