

α -Synuclein Expression in Substantia Nigra and Cortex in Parkinson's Disease

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Summary: Mutations in the human α -synuclein gene have been identified in several families of European descent with early-onset Parkinson's disease (PD). We sequenced the complete α -synuclein cDNA from substantia nigra and cortex from nine patients with PD and eight control subjects. No mutations were found. We then analyzed α -synuclein mRNA levels using a ribonuclease protection assay. Two major protected bands of α -synuclein mRNA, possibly representing two splice variants of the gene, were observed. α -synuclein mRNA was signifi-

cantly diminished in the substantia nigra of patients with PD compared with control subjects but not in the cortex. Our findings suggest that decreased synuclein mRNA may be an early alteration in the SN in PD, and imply that decreased levels of the protein may play a role in the pathogenesis of sporadic cases of the disease. **Key Words:** Parkinson's disease—Substantia nigra— α -Synuclein—Ribonuclease protection assay.

An Ala53Thr substitution in exon 4 of human α -synuclein has been identified in an Italian–American kindred with early-onset Parkinson's disease (PD) and in three unrelated Greek families.¹ A second mutation, Ala30Pro in exon 3, has also been identified in a German family with early-onset parkinsonism, confirming the pathogenic significance of α -synuclein mutations.² Others have failed to find genomic mutations in α -synuclein in sporadic and familial PD, suggesting that α -synuclein mutations are a rare cause of the disease.^{3–6} However, an important new concept in the pathogenesis of sporadic degenerative neurologic diseases is that they may be the result of mutations at the mRNA level⁷ or aberrant mRNA processing.⁸ We therefore considered it important to sequence synuclein cDNA derived from brain tissue mRNA, particularly from the SN. In addition, we considered the possibility that sporadic cases of pathologically proven PD may be associated with an alteration in the levels of expression of α -synuclein mRNA. To

assess this possibility, we have assayed α -synuclein mRNA in the SN and cortex of patients with PD and control subjects by ribonuclease protection assay.

METHODS

We reviewed the postmortem and clinical records of all cases of parkinsonism in the PD Brain Bank at Columbia University. We selected only those patients with: (1) documented Lewy bodies in SN, (2) a clinical history consistent with idiopathic PD, and (3) an available intact, frozen cerebral hemisphere containing the mesencephalon. A total of 11 brains met these criteria. Two cases were excluded because they did not show visible mesencephalic pigment, which we used to guide punch biopsies as described below, leaving nine brains for analysis. We also selected eight control cases who died from causes other than neurodegenerative disease (Table 1). To assess the effects of age and postmortem interval on α -synuclein mRNA levels, we studied the entire control group. For comparison with patients with PD, we used only those control subjects who were 40 years or older at the time of death ($n = 6$). To standardize the site of tissue harvesting, we identified the quadrigeminal plate and the pigmented substantia nigra and took an axial section through the midbrain. We then performed a

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TABLE 1. Clinical and pathologic features of patients with Parkinson's disease and control subjects

Lane	PMT (hrs)	Sex	Age at death	Lewy bodies	SN neuron loss	NFT	Amyl plaq	Duration (yrs)	Dementia	Family history	Other	Medication
Parkinson's disease												
2	6	M	64	SN, DVN, Meynert, hypothalamus, intermediolateral spinal column	moderate	no	no	8	no	no		L-dopa, amantidine, aspirin, lorazepam, nortriptyline
3	15	F	69	SN only	severe			12	no	no		Sinemet, paxiletine, lorazepam, lithium
4	6	M	80	SN, pons, cingulate, entorhinal temporo-occipital, hypothalamus, thalamus	severe	rare	rare	15	no	no		Sinemet
6	14	M	91	SN, locus ceruleus Meynert	severe	rare	moderate	NA		no		NA
7	14.5	M	83	SN	moderate	no	no	>20	yes	yes	Hallucinations	Sinemet
8	4	M	66	SN, locus cereleus, hypothalamus, entorhinal, cingulate, Meynert	moderate	rare	no	12	yes	no	Hearing loss, hallucinations	Sinemet, bromocriptine
9	18	M	79	SN, locus ceruleus, DV, Meynert	severe	no	no	5	no	no		Sinemet, bromocriptine
10	12	F	84	SN, DTN	severe	rare	rare	25	yes	no	Hallucinations	Sinemet
11	14.5	F	86	SN, DTN, Meynert, parahippocampus	severe	rare	moderate	NA	NA	NA		NA
Control subjects												
12	3.5	M	70	no	no	no	no		no	no	CNS vasculitis, hypertension, CVA	Enalapril, aspirin
13	17	M	39	no	no	no	no	no	no	no	Pancreatitis DM, DKA sepsis	Glucotrol, insulin
14	11	M	70	no	no	no	no	no	no	no	Lambert-Eaton	
15	7.5	M	67	no	no	rare	moderate	no	no	no	MI	
16	15	M	44	no	no	no	no	no	no	no	Intersitital lung	
17	7	F	59	no	no	no	no	no	no	no	COPD	
18	12	F	79	no	no	no	no	no	no	NA	"Parkinsonism"* paraparesis	NA
19	12	M	20	no	no	no	no	no	no	no	Embryonal, rhabdomyosarcoma	Dexamethasone, dialysis, neck radiotherapy

PMT, postmortem time; SN, substantia nigra; NFT, neurofibrillary tangle; Amyl plaq, amyloid plaques; N/A, not available; DVN, dorsal vagal nucleus; DTN, dorsal tegmental nucleus; DM, diabetes mellitus; MI, myocardial infarction; DKA, diabetic ketoacidosis; CVA, cerebrovascular accident; COPD, chronic obstructive airway disease; CNS, central nervous system.

* This patient's brain showed no neuronal loss in cortex, SN, striatum or thalamus, or Lewy bodies in cortex or SN, and therefore was included as a control.

3-mm punch biopsy of the frozen pigmented SN. In addition, we biopsied the frontal cortex in all samples.

Total RNA from the biopsies of the SN and cortex was extracted using the ToTALLY RNA extraction kit (Ambion, Austin, TX, U.S.A.). We generated cDNA from the extracted RNA by reverse transcription using a RETROscript kit (Ambion). To facilitate sequencing, the cDNA of α -synuclein (422 base pairs)⁹ was amplified as two overlapping fragments spanning nucleotides 35–389 (SYN5') for 5'-end region using the following primers: forward 5'-AAGGAATTCATTAGCCATGG-3' and reverse 5'-GAATTCCTCCTGTGGGGCT-3'. For 3'-end region (SYN3') spanning nucleotides 370–629, the following primers were used: forward 5'-AGCCCCACA-GGAAGGAATTC-3' and reverse 5'-AGGTACAGAT-ACTTCAATCA-3'. SYN3' was designed to amplify a portion of the cDNA containing an anticipated 84 base pair size difference between two splice variants, SYN140

and SYN112.¹⁰ The amplification profile involved a denaturing step at 95°C for 7 min, followed by 35 cycles at 95°C for 30 sec, 58°C for 1 min, and 72°C for 1.5 min, with the final step at 72°C for 10 min. The amplified products were sequenced using a ABI dRhodamine kit from Perkin-Elmer (Foster City, CA, U.S.A.). For ribonuclease protection assay (RPA), the SYN3' fragment was subcloned into the vector pGEM-T (Promega, Madison, WI, U.S.A.) and sequenced. Reductions in neuronal markers can be attributed to functional decreases or simply to loss of neurons in disease states. To distinguish between these two possibilities for synuclein mRNA in PD, we also measured mRNA for a specific monoaminergic neuron marker, the vesicular monoamine transporter 2 (VMAT2), as an index of the degree of dopamine neuron loss in the PD brains. VMAT2 mRNA is present in all monoaminergic neurons¹¹; in the SNpc, which we sampled, the only monoaminergic neurons

present are dopaminergic. In the analysis of cortex, we used a general neuronal marker, neurofilament-L (NF-L), as an index of the degree of neuron loss. To generate a probe for VMAT2, we performed RT-PCR on cDNA from control SN using the following primers: forward 5'-TGAAGGACCCGTACATCCTC-3' and reverse 5'-GAAGAAGCACAAAGCCACCTC-3'. To generate a probe for NF-L, we performed RT-PCR on cDNA from control cortex using the following primers: forward 5'-ACCGACTCAGTTTCACCAG-3' and reverse 5'-TCAGCCTTAGACGCCTCAAT-3'. All probes were labeled with [α -³²P]UTP using a MAXIScript in vitro transcription kit (Ambion).

RPA, unlike Northern analysis, permits precise quantitation of mRNAs derived from tissues. The principle of the assay is that an extract of total RNA derived from tissue is incubated with an anti-sense cRNA probe for the mRNA to be measured resulting in the formation of an RNA duplex. The mixture is then treated with RNase which digests only single-stranded mRNA and unhybridized, radiolabeled cRNA, leaving the duplex intact. The incubate is then electrophoresed on a gel, the expected size of the duplex (the "protected band") is confirmed,

and the gel is exposed to a phosphorimager cassette for quantitation. The RPA was performed using a RPA II kit from Ambion. The integrated optical density of protected radioactive RNA bands was obtained using the PhosphorImager 445 SI and the ImageQuant image analysis program (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and expressed as phosphorimager counts (PI counts). To subtract background from the phosphorimager counts, the local average option of the ImageQuant program was used and applied to both patient and control autoradiograms. Group comparisons were performed using a *t* test when data was normally distributed; in those instances when data failed a normality test, the nonparametric Mann-Whitney rank sum test was used (SigmaStat; San-del, San Rafael, CA, U.S.A.).

RESULTS

We sequenced the complete cDNA region of α -synuclein generated from SN and cortex mRNA from patients with PD (n = 9) and control subjects (n = 8) and found no mutations in the open reading frame.

RPA analysis, using the SYN3' probe, identified two major α -synuclein mRNA bands in human SN pars com-

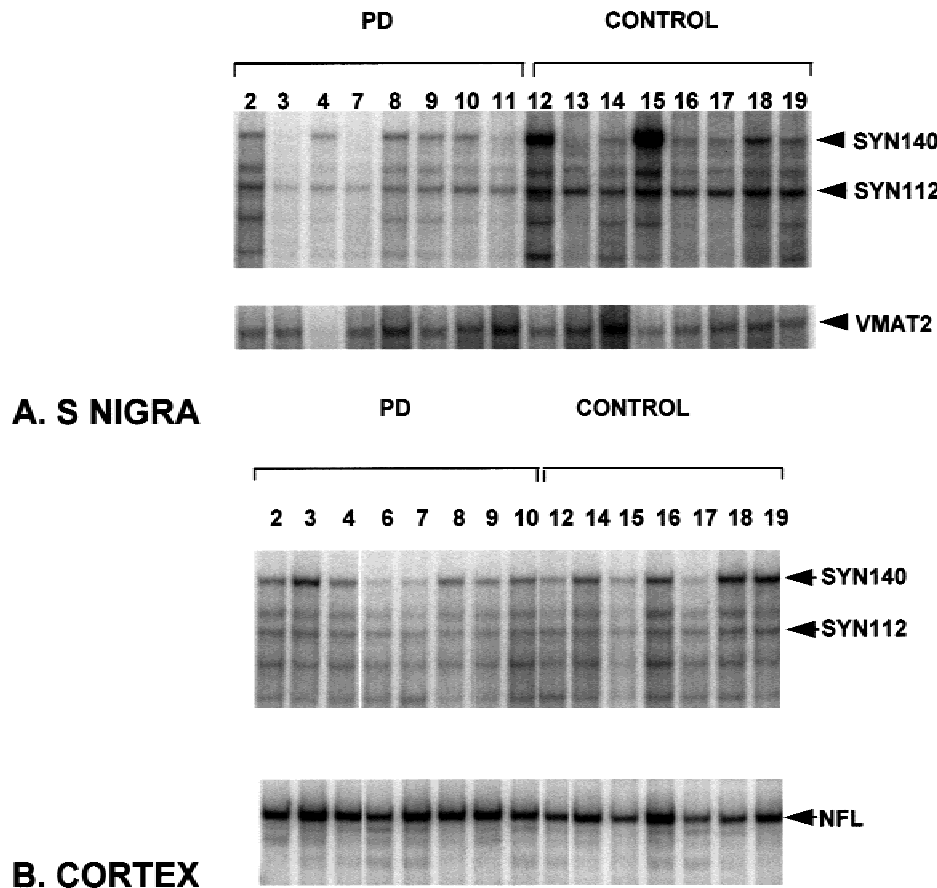


FIG. 1. Ribonuclease protection assay gels. (A) Protected bands for α -synuclein (top panel) and VMAT2 mRNA (bottom panel) obtained from substantia nigra of PD and control brains. (B) Protected bands for α -synuclein (top panel) and NF-L (bottom panel) mRNA obtained from cortex of PD and control brains. The same amount (1 mg) of total RNA was used in all RPA reactions with SYN3', VMAT2, and NF-L probes.

pacta (SNpc), both in PD and control brains (Fig. 1A). Using size standards, the difference between the two bands was approximately 80 base pairs, corresponding to the predicted difference between the SYN112 and SYN140 splice variants.¹⁰ For the control brains ($n = 8$), there was no correlation between age and quantity of either SYN112 ($r = 0.31$, not significant [NS]) or SYN140 ($r = 0.37$, NS). There was also no relationship between postmortem interval and the quantity of either SYN112 ($r = -0.57$, NS) or SYN140 ($r = -0.56$, NS). In the SNpc of control brains, there was greater variability in the level of SYN140 compared with SYN112; however, there was no significant difference in their relative abundance: SYN140, 249 ± 91 PI counts; SYN112, 216 ± 20 PI counts (Fig. 1A). In the control brains, two

splice variants were also identified in the cortex (Fig. 1B). In these brains, SYN112 mRNA was relatively more abundant in the SN than in the cortex: SN, 216 ± 21 ; cortex, 104 ± 14 ($p = 0.001$, t test). A trend for a similar difference was observed for SYN140 but this did not achieve significance: SN, 248 ± 97 ; cortex, 97.4 ± 24 ($p = 0.2$, t test).

For the purpose of comparison of SN mRNA values to those of PD brain, the control patients older than 40 ($n = 6$) were used. There was still a trend for this group to be younger than patients with PD but this did not achieve significance: PD, 76.4 ± 3.1 ; control, 64.8 ± 4.9 ($p = 0.1$). There was no significant difference in the postmortem interval between the two groups: PD, 11.6 ± 1.7 ; control, 9.3 ± 1.7 hours ($p = 0.3$).

We found significantly less α -synuclein mRNA, for both splice variants, in PD substantia nigra (Fig. 2A). VMAT2 mRNA tended to be diminished in PD but this difference failed to achieve significance. When α -synuclein mRNA values in SN were normalized for VMAT2 mRNA values, the difference between PD and control subjects remained significant. There was no difference in the amount of the α -synuclein splice variants mRNA or NF-L mRNA in cortex between PD and control subjects (Fig. 2B).

DISCUSSION

α -Synuclein is a presynaptic nerve terminal protein¹²⁻¹⁵ with no known function. It may be involved in neuronal plasticity, because expression of its avian homolog, synelfin, is increased in the zebra finch during developmental song learning.¹⁶ In the context of human disease, a 35 amino acid internal fragment, the non Ab-component (NAC), has been found in amyloid plaques of patients with Alzheimer's disease,⁹ and α -synuclein has been identified in Lewy bodies of patients with PD.^{17,18} Mutations in α -synuclein are associated with early-onset PD in families of European origin.^{1,2} The biologic mechanism of disease in these familial PD cases is unknown. In our study, we excluded α -synuclein exonic mutations by sequencing cDNA purified from SN and cortex of eight pathologically confirmed sporadic cases of PD. However, we have found a decreased amount of α -synuclein mRNA in the SN, but not in cortex, in these PD brains. It is unlikely that this decrease in expression of α -synuclein simply reflects neuronal loss because a similar difference was observed after normalizing for VMAT2 (a dopaminergic neuronal marker) expression. In fact, levels of VMAT2 mRNA were relatively preserved in our PD group. This was an unexpected finding, and one possible explanation is that by using the pres-

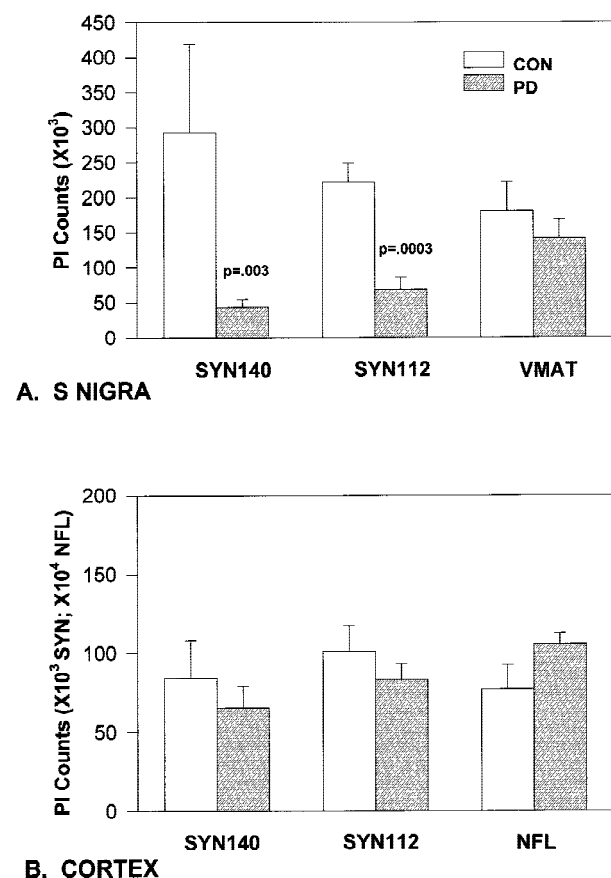


FIG. 2. Quantitative analysis of α -synuclein mRNA expression in patients with PD and control subjects. Radioactivity within protected bands was measured on a phosphorimager and is expressed in arbitrary phosphorimager counts (PI counts). **(A)** In SN, mRNA levels for both the SYN140 and SYN112 splice variants were diminished (Mann-Whitney and t test, respectively). There was a trend for lower VMAT2 levels in PD brain but this did not achieve significance. **(B)** In cortex, mRNA levels for both splice variants and NF-L were not significantly different in patients with PD compared with control subjects.

ence of visible pigment to guide our SNpc punches, we may have selected relatively preserved regions.

There are two principal interpretations of diminished α -synuclein mRNA in the presence of relatively preserved VMAT2. One is that the α -synuclein decrease is a relatively early change in the process leading to neuron degeneration in PD SN preceding changes in dopamine markers. The alternative interpretation of our result is that lower levels of α -synuclein mRNA may be a marker for dopamine neurons which are relatively less vulnerable to the disease. For example, it is known that the ventral tier dopamine neurons are especially vulnerable to degeneration in PD.¹⁹ If synuclein mRNA happens to be more abundant in these neurons, then the apparent decrement in PD brains would be the result of the relative loss of these neurons. At the present time, it is unknown whether the expression of synuclein differs in separate subpopulations of dopamine neurons. Of the two interpretations, the first seems more plausible. In the rodent, synuclein 1 mRNA is of approximately equal abundance in the ventral tegmental area, which is less vulnerable to degeneration in PD, and in the SNpc, which is more vulnerable.²⁰

Although there was a tendency for the control subjects to be younger than the patients with PD, this difference did not achieve significance, and it is unlikely to account for the difference observed in α -synuclein mRNA for the additional reason that we observed no relationship between age and α -synuclein mRNA levels in the control group. The difference in α -synuclein mRNA between patients with PD and control subjects was also unlikely to be the result of differences in the quality of postmortem mRNA preservation, because there was no difference in postmortem interval between the two groups, and there was no difference in values for other mRNAs: VMAT2 in SN, and both α -synuclein and NF-L in cortex.

Thus, the differences we observed are likely to be associated with the disease. However, because all of the patients with PD were under treatment at the time of death, we cannot exclude a medication effect. In addition, because we have examined only patients with PD and not disease-related control subjects, we do not know if the changes observed are specific for PD.

If we accept the possibility that the observed decrease in α -synuclein mRNA may be a manifestation of the disease process, then the fact that changes in synuclein mRNA occur earlier than changes in dopamine markers may suggest that they are a primary mediator of the disease process rather than a secondary change. Such an interpretation might suggest that the mutations of the gene in the familial cases may play a role in pathogenesis

by diminishing the availability or normal function of α -synuclein.

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