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# Preferentially increased nitration of α-synuclein at tyrosine-39 in a cellular oxidative model of Parkinson's disease

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## Abstract

α-Synuclein is a major component of Lewy bodies, proteinacious inclusions which are a major hallmark of Parkinson's disease (PD). Lewy bodies contain high levels of nitrated tyrosine residues as determined by antibodies specific for 3-nitrotyrosine (3NT) and via mass spectrometry (MS). We have developed a multiple reaction monitoring (MRM) mass spectrometry method to sensitively quantitate the 3NT levels of specific α-synuclein tyrosine residues. We found a nine-fold increase (relative to controls) in levels of 3NT at Tyr-39 of α-synuclein in an inducible transgenic cellular model of Parkinson's disease in which monoamine oxidase B (MAO-B) is overexpressed and which emulates several features of PD. Increased nitration of Tyr-39 on endogenous α-synuclein via elevations in MAO-B levels could be abrogated by the addition of deprenyl, a specific MAO-B inhibitor. The increased levels of 3NT was selective for Tyr-39 as no significant increases in 3NT levels were detected at other tyrosine residues present in the protein (Tyr-125, Tyr-133, and Tyr-136). This is the first report of increased 3NT levels of a specific tyrosine in a PD model and the first use of MRM mass spectrometry to quantify changes in 3NT modifications at specific sites within a target protein.

## Introduction

The nature and function of α-synuclein and its role in PD have been heavily studied since the discovery of a dominant mutation in the α-synuclein gene in a number of Greek and Sicilian families with a familial form of the disorder[1]. α-Synuclein was subsequently identified to be a major component of Lewy bodies in sporadic cases of the disease[2]. The exact function of α-synuclein, the mechanism by which it aggregates and becomes a major component of Lewy bodies is a matter of intense investigation.

Qualitatively, Lewy bodies appear to contain high levels of nitrated tyrosine residues as indicated by antibodies specific for 3NT[3]. Use of these antibodies has shown that α-synuclein is a particularly sensitive target for nitrating agents and that nitrated α-synuclein is present in the Lewy bodies of postmortem tissues not only from PD patients but also other neurodegenerative synucleinopathies[4]. Nitrated α-synuclein has also been detected in the substantia nigra (SN) and ventral midbrain of mice treated with the PD-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[5]. Recombinant α-synuclein treated with nitrating agents (peroxynitrite/$CO_2$ myeloperoxidase/ $H_2O_2$/nitrite) generates highly stable nitrated α-synuclein oligos and aggregates as a consequence of oxidation[6-9] and suggests that nitration is sufficient for α-synuclein aggregation.

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Levels of the neurotransmitter dopamine were found to be selectively decreased in the corpus striatum and SN of PD patients[10], and subsequently dopamine deficiency was found to be associated with the loss of dopaminergic cells within the SN[11]. Of the catacholamine neurotransmitters, dopamine is the most chemically predisposed towards oxidation[12]. Oxidative stress produced by dopamine metabolism may be exacerbated in PD patients in part due to increases in MAO-B activity which has been shown to increase with age and is associated with the disease itself[13, 14]. MAO-B is primarily expressed within astrocytes which themselves have high levels of protective antioxidants to detoxify reactive oxygen species; however $H_2O_2$ produced via MAO-B has a high membrane permeability and may diffuse to adjacent dopaminergic neurons[15, 16] which are more vulnerable to oxidative stress[17-19] leading to dopamine specific degeneration, a hallmark of PD. Our laboratory has established a doxycycline (dox)-inducible dopaminergic PC12 cell line which stably expresses human MAO-B at elevated levels comparable to those observed during normal aging and in PD. The elevated expression of MAO-B in PC12 cells was found to recapitulate other aspects of PD including mitochondrial complex I (CI) deficiency[20] which is also associated with PD[21-23]. Administration of the CI inhibitors rotenone and MPTP result in dopaminergic neuronal loss similar to what is observed in PD patients[24, 25]. It is of interest to note that MAO-B is the enzyme responsible for the conversion of MPTP to its protoxic form 1-methyl-4-phenylpyridinium (MPP+) which is subsequently transported into dopaminergic neurons resulting in CI inhibition and ensuing dopaminergic toxicity[26, 27].

The low abundance of certain protein post-translation modifications (PTMs) such as 3NT combined with the small but consequential changes contribute to the difficulty of identifying and quantifying PTMs present at a specific position of a target protein. To conclusively identify and quantify α-synuclein PTMs in this study, we have first analyzed in vitro nitrated human recombinant α-synuclein by MS/MS to identify 3NT modified α-synuclein peptides and to determine the optimal targets for Multiple Reaction Monitoring (MRM) MS based quantitation. MRM is the most sensitive and quantitative mass spectrometry approach available[28, 29] and has the advantage that it can independently measure each tyrosine in α-synuclein. This sensitive MS based technique allowed us to examine the affects of increased MAO-B expression in a PC12 cell model on 3NT modification of α-synuclein, emulating what occurs during normal aging and PD and which may contribute to α-synuclein aggregation.

## Experimental Section

### Cell Lines and Culture Conditions

Creation and maintenance of dox-inducible MAO-B PC12 cells was previously described by our laboratory[20]. MAO-B was induced by treating cells with 40 $\mu$g/mL of dox for 24 hours; PC12 cells were neuronally differentiated by using 50 ng/mL nerve growth factor (NGF) for 2 days prior to addition of dox. As a positive control, MAO-B PC12 cells (no dox treatment) were treated with 150 $\mu$M $H_2O_2$ for 24 hours. To inhibit MAO-B activity, 10 $\mu$M of deprenyl was added to the medium just prior to dox treatment.

### α-Synuclein Immunoprecipitation

Treated or control cells were lysed with RIPA lysis buffer (Sigma) containing complete mini protease inhibitor tablets (Roche). Cell lysate protein concentrations were adjusted to 5 $\mu$g/$\mu$L, then pre-cleared with EZview protein A affinity gel (Sigma) for 4 hours at 4° C. The lysate was then incubated with anti-α-synuclein antibody (Syn 202, Santa Cruz Biotechnology) overnight at 4° C with continuous gentle mixing followed by incubation of the lysate with EZview protein A affinity gel (Sigma) overnight at 4° C with continuous mixing. The resin was then washed 3 times with 1 mL of RIPA buffer and eluted by boiling in SDS-PAGE (reducing) loading buffer, then separated by SDS-PAGE on a 10% gel (1 mm, Bis-Tris

NuPAGE Invitrogen). The gel was stained with Imperial protein stain (Thermo Fisher Scientific) and the band corresponding to the α-synuclein protein excised, washed, and digested with 40 ng of Asp-N (Roche) or trypsin (Porcine Sequencing grade, Promega) for 16 hours at 37° C. No reduction/alkylation step was necessary as α-synuclein contains no cysteine residues.

### α-Synuclein Nitration

Recombinant human α-synuclein (Sigma) was nitrated using a modified procedure described in Cassina *et. al*.[30]. Briefly, 250 ng of recombinant α-synuclein was treated with 40 mM tetranitromethane in 0.1 M Tris-HCl (pH 8.0) and 0.1 M KCl at room temperature for 30 minutes. The sample was then run on a SDS-PAGE gel (as described above), excised and digested as described above for immunoprecipitated α-synuclein.

### Mass Spectrometry

To identify sites of PTMs, tetranitromethane-treated human recombinant α-synuclein and α-synuclein immunprecipited from rat PC12 cells were subjected to in-gel protease digestion with Asp-N and trypsin and the resulting peptides were analyzed by electrospray HPLC-MS/MS using a hybrid quadrupole time-of-flight mass spectrometer (QSTAR, Applied Biosystems MDS SCIEX, Concorde, CAN) as described previously[31, 32]. Detailed protocols are listed in the legend of Supporting Information Table S-1, titled QSTAR Elite parameters. All peptide assignments from the resulting mass spectrometric data were made using the bioinformatics search engine Mascot version 2.2.04 (Matrix Sciences, London, United Kingdom)[33]. A complete listing of the parameters used for Mascot database searches are listed in the figure legend of Supporting Information Table S-1A, Mascot Database Searches. To provide a quantitative comparison of the levels of tyrosine nitration, MRM analysis targeted the levels of several 3NT modified peptides and their unmodified counterparts. For these experiments, a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems) was used as described previously[34]. Further details of the MRM methods can be found in the legend of Supporting Information Table S-2 under MRM parameters.

## Results

### Identification of 3-nitrotyrosine residues in tetranitromethane treated human recombinant α-synuclein

Due to the relatively low expression of α-synuclein in rodent PC12 cell cultures, nitrated recombinant human α-synuclein was first analyzed by mass spectrometry to provide a tandem mass spectral data set to use as a positive control to aid in the identification of nitrated tyrosine in α-synuclein immunoprecipitated from MAO-B PC12 cell lysates. The analytical strategy for identifying and quantifying PTMs present on peptides expressed at low levels is demonstrated on Figure 1. Although human α-synuclein is not identical to the homologues expressed in rodents, it is highly conserved; both rat and mouse sequences are 95% identical (133 out of 140 amino acids) to the human sequence. The human form also contains 4 of the 5 tyrosine residues (Tyr-39, Tyr-125, Tyr-133, and Tyr-136) found in the mouse and rat homologs, although both mouse and rat α-synuclein have an additional tyrosine at position 107 not present in humans. Given this similarity, nitrated human α-synuclein samples were digested with trypsin and Asp-N and the resulting peptides analyzed by HPLC MS/MS. Analysis of the spectral data showed that a near complete sequence map of α-synuclein had been obtained, covering 99% or 89% of the protein, respectively (Supporting Information Tables S-1A and S-1B). Included in these MS/MS data were a set of high quality spectra that encompassed one or more nitrated tyrosine residues; and these were examined in detail to develop unique and chemically specific criteria for identifying α-synuclein peptides containing 3-nitrotyrosine as described below (Figure 2A, 2B and Supporting Information).

Three of the four tyrosines of interest are located in the C-terminal domain of α-synuclein at positions 125, 133 and 136. Trypsin digestion produced a large, highly-charged 38 amino acid peptide spanning residues 103-140 (m/z 1076.4+4, M = 4301.5 Da) that contained all three of these C-terminal tyrosines. While we were able to unambiguously identify this large C-terminal peptide by MS/MS, its relatively large size precluded an unequivocal determination of the precise site(s) of 3NT modification. In contrast, digestion with Asp-N resulted in a set of peptides that were more amenable to PTM identification, as they generated peptides where each of these 3 tyrosines can be separately examined. Although Asp-N digestion can be prone to producing peptides with missed cleavages, these missed cleavage products appeared consistently from digest to digest. A complete list of peptide ions, their sequence assignments and corresponding Mascot scores obtained by searching against the Swiss-Prot database are listed in the Supporting Information section (Table S-1).

## Criteria for 3NT Identification by MS

To better identify peptides containing 3-nitrotyrosine, we used a set of stringent criteria to avoid incorrect assignments. This criteria consisted of: (1) observation of both unmodified and 3NT modified peptides that showed similar fragmentation patterns, (2) peptides containing a 3NT modification showed a chromatographic shift to a later eluted time (~2-4 minutes) than the corresponding unmodified peptide due to the increased hydrophobicity, (3) mass measurements of the peptide molecular weight showed good mass accuracy (< 0.15 Da, Table S-1A in Supporting Information) for both the unmodified and 3NT modified peptide, and where the latter 3NT containing peptide showed a 45 Da mass shift ($+NO_2$, -H; Δ45 Da), (4) MS/MS fragmentation spectra of the 3NT containing peptides required the presence of 3NT "flanking" y- or b- fragment ions, and (5) peptides containing a 3NT must show 3NT immonium ions at m/z 181.1, while peptides containing an unmodified tyrosine residue showed immonium ions at m/z 136.1. Using these criteria, four 3NT modified peptides (Tyr-39, Tyr-125, Tyr-133, and Tyr-136) were identified in human α-synuclein from their corresponding MS/MS spectra (Figure 2B, and Supporting Information data Figures S-1B, S-2B, and S-3B).

The employment of these assignment criteria for 3NT-containing peptides is illustrated in Figure 2 for the α-synuclein peptide E-35-GVLYVGSKTK-45 (containing Tyr-39). The chromatographic shift in retention time is depicted in the total ion chromatogram in Figure 3 where the nitrated peptide eluted ~2 minutes after the corresponding unmodified peptide. The MS/MS spectra for the unmodified peptide and the peptide containing an 3NT modifcation at Tyr-39 are shown in Figure 2A and 2B, respectively, and clearly demonstrate the presence of either a tyrosine (m/z 136.1) or nitrotyrosine immonium ion (m/z 181.1) in the low mass region. In regards to sequence ions, the $y_7$ ion containing the tyrosine residue was observed to be 163 Da larger than the $y_6$ ion in the unmodified peptide, while the 3NT residue results in a $y_7$ ion that is 208 Da larger than the $y_6$ ion in the MS/MS spectra of the 3NT containing peptide (note that fragment ions that do not contain the tyrosine residue, such as the $y_6$ ion at 619.4 m/z, have identical masses). The masses of the unmodified and modified peptides deduced from their respective triply charged ions at m/z 394.2 and 409.2, also show the expected 45 Da shift. In all, this and the other MS/MS spectra of nitrated and unmodified tyrosine-containing peptides (see Supporting Information), were particularly valuable for validating the nitrated peptides obtained in the rat PD cell model as described below.

## Identification of alterations in 3NT α-synuclein in a dopaminergic cell line with inducibly elevated MAO-B levels

α-Synuclein was immunopurified from PC12 cells treated with dox to induce MAO-B overexpression, dox + deprenyl (an MAO-B inhibitor), or $H_2O_2$ versus control cells (no dox). The immunopreciptated fractions were then subjected to SDS-PAGE separation, and the band (s) corresponding to α-synuclein were digested by trypsin or Asp-N, and analyzed by HPLC

MS/MS. Sequence coverage obtained from PC12 cell lysates after immunoprecipitation and mass spectrometric analysis of the gel-separated proteins was reasonable, i.e., 51% and 57% for Asp-N and trypsin, respectively, and well within our expectations given the significantly lower amount of material available (see Supporting Information Table S-1C and S-1D). Nonetheless, this coverage was considerably lower than what we obtained from our in vitro experiments using recombinant human α-synuclein and not sufficient to accurately quantify sites of nitration. Therefore, to both identify and quantify these potential sites of nitration in the PC12 cell model, we developed a highly targeted and sensitive multiple reaction monitoring (MRM) approach.

These MRM experiments were developed and validated first using the human recombinant proteins (tetranitromethane-treated and untreated) and then used to assess relative changes in tyrosine nitration status amongst the four conditions used in the PC12 model. The tyrosine-containing peptides used for MRM quantitation were E-35-GVLYVGSKTK-45, E-126-MPSEEGYQ-134, D-135-YEPEA-140 for human and rat samples, since their sequence were identical in these regions. However, as the rat and human peptides contained Tyr-125 varied at two residues, D-115-MPVDPDNEAY-125 was used for human and D-115-MPVDPSSEAY-125 was used for rat samples. For Tyr-107 (present only in rat α-synuclein) the peptide D-98-QMGKGEEGYPQEGILE-114 was used. A partial list of MRM transitions and other MRM parameters used is presented in Table 1, details of all MRM transitions are listed in Supporting Information Table S-2.

As determined by MRM analysis, approximately 1% of Tyr-39 residues on α-synuclein obtained from PC12 cells contain a 3NT modification under control conditions. A nine-fold increase (as compared to controls) in levels of 3NT at Tyr-39 was detected by MRM in the inducible transgenic cellular (PC12) model of Parkinson's disease in which MAO-B was overexpressed (Figure 4). The increased nitration of Tyr-39 could be abrogated by the addition of deprenyl, a specific MAO-B inhibitor, reversing 3NT levels to approximately control levels (1.3 fold change). This suggests that MAO-B activity is a critical component for the observed increase in 3NT levels, presumably due to increased hydrogen peroxide and indirect nitrative stress created as a by-product of MAO-B metabolism of dopamine[35]. This conclusion is corroborated by a similar increase in 3NT levels (~6 fold increase) observed following treatment of control PC12 cells with 150 $\mu$M $H_2O_2$ for 24 hours.

The increase in levels of 3NT was selective for Tyr-39; no significant increases in 3NT levels were detected at other tyrosine residues present in α-synuclein (Tyr-125, Tyr-133, and Tyr-136). While our data also suggested 3NT levels of Tyr-136 increased after MAO-B over-expression the signal was near the limit of quantitation of the MRM assay and led to significant variability between biological replicates and did not reach statistical significance. No detectable levels of 3NT modification were observed on peptides containing either Tyr-125 or Tyr-133 from any of the treatments however the unmodified peptides were detected by MRM from α-synuclein immunopurified from MAO-B PC12 cells (data not shown). Neither the unmodified or the 3NT-modified peptide containing Tyr-107 was detected from immunopurified samples by MRM despite the unmodified peptide being detected by MS/MS analysis; further MRM transition optimization may be required.

## Discussion

We have used an MS-based strategy to identify low abundant peptides with PTMs that involved the following: (1) identifying a set of well-validated peptide spectra using recombinant protein; (2), using these peptides to design very selective and specific MRM transitions; and (3), using well-vetted MRM transitions to exploit the sensitivity of MRM-MS to identify and quantify 3NT PTMs present on α-synuclein expressed at relatively low levels in PC12 cells prepared

under four separate conditions. Here we report for the first time a collection of MS/MS spectra of α-synuclein peptides containing 3NT modifications. More importantly, utilizing the sensitivity and selectivity of MRM-MS, we demonstrated a selective 9-fold increase in 3NT levels at Tyr-39 of α-synuclein in an oxidative cellular model of Parkinson's disease.

While mass spectrometry has been previously utilized to discover PTMs such as 3NT with some success[36, 37], these studies were unable to accurately quantitate differences in PTM levels between samples. The importance of rigorously scrutinizing MS data was illustrated by a recent investigation by Laszlo Prokai and associates[38] in which 3NT modified peptides reported by other groups were proven to be misassigned. Our criteria for identifying PTMs was particularly stringent due to this concern. MRM-MS has been previously utilized to measure the levels of free tyrosine and 3NT levels[39, 40] however to our knowledge there are no published reports to date of the use of MRM-MS to quantify 3NT levels in a targeted fashion on a specific protein.

Aggregated α-synuclein present in Lewy bodies has been reported to be highly modified post-transcriptionally including both phosphorylation at Ser-129[41] and nitration of tyrosine residues[3, 4]. However, neither the degree of nitration nor the identification of the specific tyrosines modified has been previously elucidated. α-Synuclein appears to be a particularly sensitive target for nitration by peroxynitrite[5] and nitrated α-synuclein monomers and dimers have been shown to accelerate protein fibril formation[42]. Tyrosine residues within α-synuclein are also required for aggregation caused by oxidation[43] and proteasomal degradation of α-synuclein has been shown to require nitration[42]. Nitration of Tyr-39 in particular has been shown to decrease binding of α-synuclein to synthetic vesicles[42] which may lead to increased oligomerization; mutation of Tyr-39 to a cysteine residue results in increased formation of α-synuclein fibrils and neurotoxicity[44]. Protein conformation can impact on its accessibility to peroxynitrite and subsequent 3NT modification; when α-synuclein is in a "collapsed" formation, Tyr-39 is required for aggregation but not if denaturants are present[45]. It is therefore quite significant that we have found this same tyrosine residue to be selectively nitrated under conditions of oxidative MAO-B overexpression and that the observed nine-fold increase could be reversed with deprenyl, a selective MAO-B antagonist. While the exact source of the nitration is difficult to identify, it has been demonstrated previously that neuronal nitric oxide synthase (NOS) levels are increased in PC12 cells following inhibition of proteasomal activity[46] and $H_2O_2$ treatment of human cardiac microvascular endothelial cells increases NOS levels[47]. Thus increased NOS levels via increased MAO-B and subsequent $H_20_2$ production could result in elevated NO and subsequent α-synuclein nitration.

PTMs on α-synuclein resulting from increases in oxidative and/or nitrosative stress may enhance formation of aggregates through stabilization of α-synuclein complex structures and/or by proteasomal inhibition[48]. Under physiological conditions α-synuclein is a natively unfolded protein, this state of intrinsic disorder may predispose α-synuclein to oxidative and nitrative modifications[49, 50]. This sensitivity coupled with the environment of the substantia nigra, where increases in oxidative stress including age-related increases in MAO-B levels may result in oxidative/nitrative modifications to α-synuclein. This in turn may lead to aggregation of α-synuclein and decreased proteasomal activity which may contribute to dopaminergic cell death in this brain region. We hope that by unraveling the mysteries surrounding the specific mechanisms contributing to α-synuclein aggregation we will better understand the neurodegeneration observed in PD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgment

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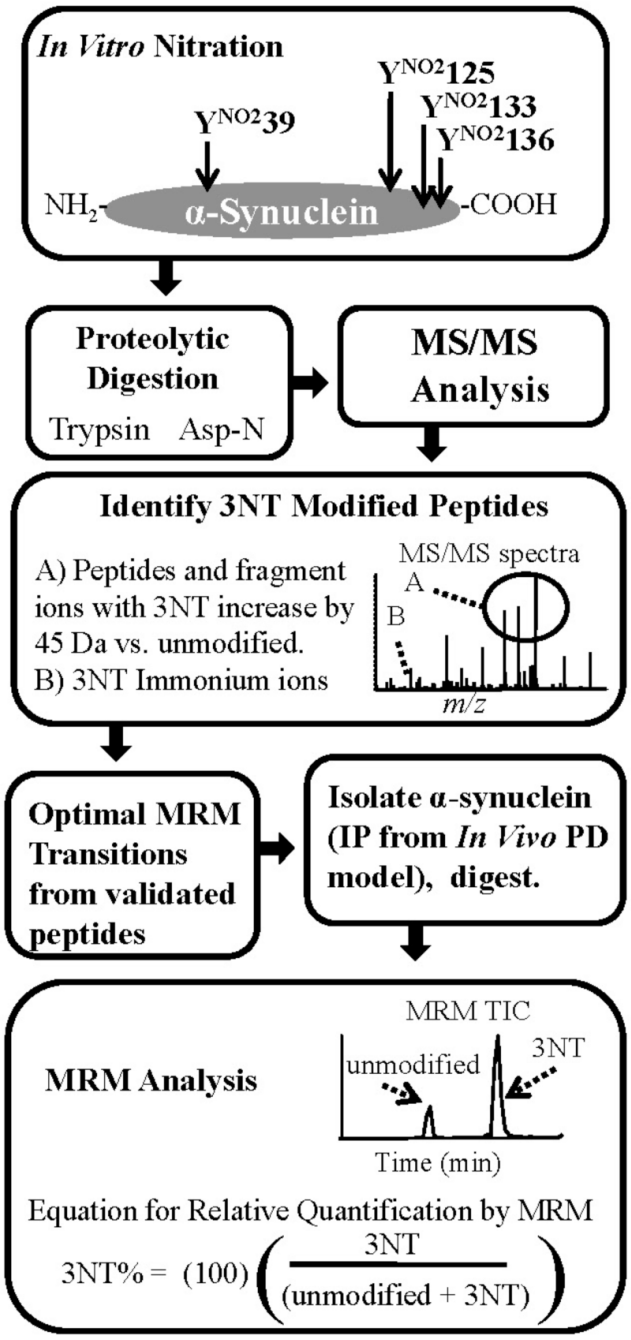


**Figure 1.**

Diagram depicting summarized strategy to identify and quantify PTMs (3NT) on low abundant peptides, starting with *in vitro* nitrated (tetranitromethane) recombinant α-synuclein which was subsequently digested and analyzed by MS/MS to identify 3NT modified peptides using rigorous criteria described in the Results section. Optimized MRM transitions were then used to quantify 3NT modified and unmodified peptides present on endogenous α-synuclein immunoprecipitated (IP) from a cellular model of PD using the equation listed in the final step titled MRM analysis (TIC = Total Ion Chromatogram).

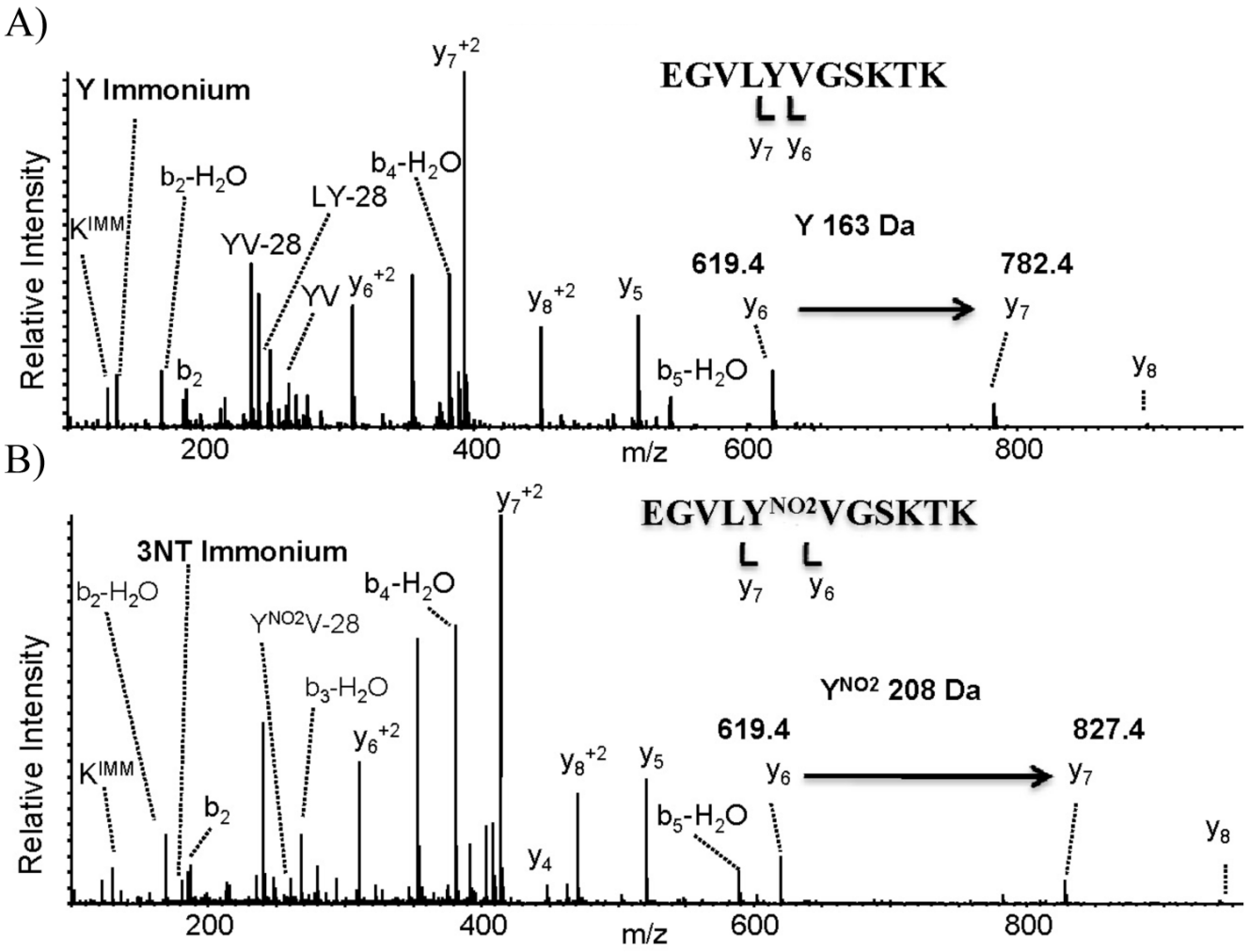


**Figure 2.**

Identification of α-synuclein peptide E-35-GVLY$^{NO2}$VGSKTK-45 containing 3NT modification at Tyr-39. Human α-synuclein was *in vitro* nitrated, proteolytically digested with Asp-N and then analyzed by MS/MS. A) MS/MS spectra of unmodified human α-synuclein peptide (Asp-N digested) containing Tyr-39, E-35-GVLYVGSKTK-45 at *m/z* 394.2, (M=1179.6 Da). B) Corresponding MS/MS spectra of the peptide containing 3NT, E-35-GVLY$^{NO2}$VGSKTK-45 (Y$^{NO2}$ = 3NT) at *m/z* 409.2 (M=1224.5 Da). Note the low mass immonium (IMM) ions (+$NH_2$=CH-R), for Tyr and 3NT at *m/z* 136.1 and 181.1, respectively.

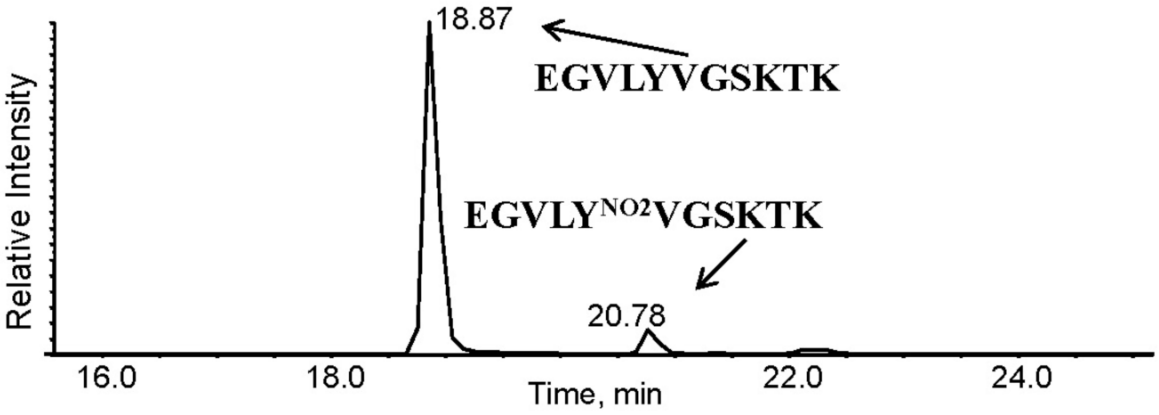


**Figure 3.**

Chromatographic retention time shift of 3NT modified peptide. MRM total ion chromatogram of unmodified and 3NT modified peptide (Asp-N digest) containing Tyr-39 showing retention time delay of ~2 min for the nitrated peptide relative to the unmodified form. Four MRM transitions were used each for the unmodified and 3NT modified peptides, all of which are listed in Table S-2 (Supporting Information).

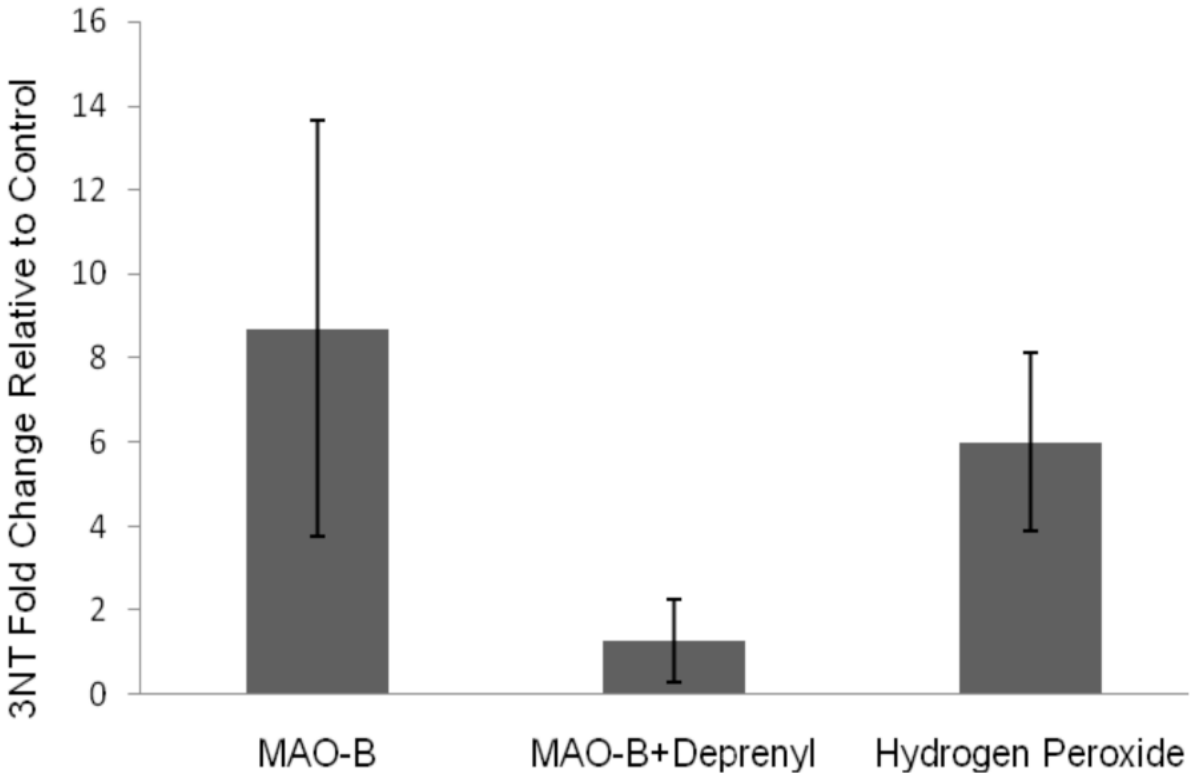


**Figure 4.**

Increased 3NT levels at Tyr-39 of endogenous α-synuclein due to MAO-B overexpression in dopaminergic PC12 cells as determined by MRM (4000 QTRAP). A 9-fold increase of 3NT levels at Tyr-39 was observed in PC12 cells overexpressing MAO-B relative to control levels. This increase was abrogated by the addition of the MAO-B inhibitor deprenyl. As a positive control, cells were treated with 150 $\mu$M of $H_2O_2$ for 24 hours which also exhibited a large increase (6-fold) in 3NT levels as compared to control cells. Quantitation was done using the MRM transitions (Q1/Q3): unmodified 394.2/520.3 and 3NT modified 409.2/520.3. Error bars are the standard deviation of 3 biological replicates analyzed in singlicate using MRM transitions for unmodified and 3NT modified peptides.

**Table 1**

Partial list of α-Synuclein MRM Transitions

| AA Start - End | Peptide Sequence | Q1 | Q3 | z (+) | Ion | Dwell Time (ms) | Collision Energy (CE) | Species |
|---|---|---|---|---|---|---|---|---|
| 35-45 | EGVLYVGSKTK | 394.2 | 619.4 | 3 | $y_6$ | 65 | 16 | Human, Rat |
| | | 394.2 | 391.7 | 3 | $y_7^{(2+)}$ | 65 | 16 | Human, Rat |
| 35-45 | EGVLY$^{NO2}$VGSKTK | 409.2 | 619.4 | 3 | $y_6$ | 195 | 17 | Human, Rat |
| | | 409.2 | 414.2 | 3 | $y_7^{(2+)}$ | 195 | 17 | Human, Rat |
| 135-140 | DYEPEA | 362.2 | 634.2 | 2 | $b_5$ | 70 | 18 | Human, Rat |
| | | 362.2 | 505.2 | 2 | $b_4$ | 70 | 18 | Human, Rat |
| 135-140 | DY$^{NO2}$EPEA | 384.6 | 679.2 | 2 | $b_5$ | 200 | 19 | Human, Rat |
| | | 384.6 | 550.2 | 2 | $b_4$ | 200 | 19 | Human, Rat |
| 126-134 | EMPSEEGYQ | 535.2 | 809.3 | 2 | $y_7$ | 70 | 28 | Human, Rat |
| | | 535.2 | 443.2 | 2 | PSEE | 70 | 28 | Human, Rat |
| 126-134 | EMPSEEGY$^{NO2}$Q | 557.7 | 854.3 | 2 | $y_7$ | 195 | 30 | Human, Rat |
| | | 557.7 | 443.2 | 2 | PSEE | 195 | 30 | Human, Rat |
| 115-125 | DMPVDPDNEAY | 633.3 | 1013.4 | 2 | $b_9$ | 70 | 35 | Human |
| | | 633.3 | 884.4 | 2 | $b_8$ | 70 | 35 | Human |
| 115-125 | DMPVDPDNEAY$^{NO2}$ | 655.8 | 1013.4 | 2 | $b_9$ | 190 | 36 | Human |
| | | 655.8 | 884.4 | 2 | $b_8$ | 190 | 36 | Human |
| 115-125 | DMPVDPSSEAY | 605.8 | 958.4 | 2 | $b_9$ | 70 | 32 | Rat |
| | | 605.8 | 829.3 | 2 | $b_8$ | 70 | 32 | Rat |
| 115-125 | DMPVDPSSEAY$^{NO2}$ | 628.2 | 958.4 | 2 | $b_9$ | 200 | 33 | Rat |
| | | 628.2 | 829.3 | 2 | $b_8$ | 200 | 33 | Rat |
| 98-114 | DQMGKGEEGYPQE GILE | 940.4 | 1095.4 | 2 | $b_{10}$ | 60 | 46 | Rat |
| | | 940.4 | 785.4 | 2 | $y_7$ | 60 | 46 | Rat |
| 98-114 | DQMGKGEEGY$^{NO2}$P QEGILE | 962.9 | 1140.4 | 2 | $b_{10}$ | 190 | 47 | Rat |
| | | 962.9 | 785.4 | 2 | $y_7$ | 190 | 47 | Rat |

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