RESEARCH ARTICLE

Investigating the presence of doubly phosphorylated α-synuclein at tyrosine 125 and serine 129 in idiopathic Lewy body diseases

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INTRODUCTION

Aggregation of the protein α-synuclein (α-syn) into insoluble intracellular assemblies within vulnerable neurons is thought to be a critical event in the pathogenesis of Lewy body diseases (LBD) such as Parkinson’s disease (PD) and dementia with Lewy bodies (DLB) (21,24). Evidence from genetic studies suggested that mutations or duplications in the α-syn gene cause familial PD (4,15).

α-syn is phosphorylated at serine 129 (pS129), suggesting that this is an important disease-related post-translational modification (PTM). However, PTMs do not typically occur in isolation and phosphorylation at the proximal tyrosine 125 (pY125) residue has received considerable attention and has been inconsistently reported to be present in LBs. Furthermore, the proximity of Y125 to S129 means that some pS129 antibodies may have epitopes that include Y125, in which case phosphorylation of Y125 will impede recognition of α-syn. This would potentially lead to underestimating LB pathology burdens if pY125 occurs alongside pS129. To address the apparent controversy in the literature regarding the detection of pY125, we investigated its presence in the LB pathology. We generated pS129 antibodies whose epitope includes or does not include Y125 and compared the extent of α-syn pathology recognized in mouse models of α-synucleinopathies, human brain tissue lysates and fixed post-mortem brain tissues. Our study demonstrated no difference in α-syn pathology recognized between pS129 antibodies, irrespective of whether Y125 was part of the epitope or not. Furthermore, evaluation with pY125 antibodies whose epitope does not include S129 demonstrated no labeling of LB pathology. This study reconciles disparate results in the literature and demonstrates pY125 is not a key component of LB pathology in murine models or human tissues in idiopathic LB diseases.

Despite the apparent importance of α-syn aggregation in LBD, many studies report a poor relationship between the severity of LB pathology and clinical phenotype (22), or neuronal loss (3). Numerous species of α-syn have been described with assumed different relevance to pathological neurodegeneration (21); however, most studies of LBD employ antibodies that recognize both native and pathological species. α-Syn is post-translationally modified (PTM) (19,20) and α-syn phosphorylated at serine 129 (pS129) is

Keywords
alpha-synuclein, dementia with Lewy bodies, Lewy bodies, Parkinson’s disease, phosphorylation.

Abbreviations
α-syn, alpha-synuclein; AD, Alzheimer’s disease; DLBL, dementia with Lewy bodies; ELISA, Enzyme-linked immunosorbent assay; LB, Lewy body; LBD, Lewy body disease; mAb, monoclonal antibody; PD, Parkinson’s disease; PFFs, pre-formed fibrils; pS129-α-syn, phosphorylated α-syn at serine 129; PTM, post-translational modifications; pY125-α-syn, phosphorylated α-syn at tyrosine 125.

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abundant in LBs, suggesting an association with disease states (2,9).

Due to the abundance of pS129-α-syn abundance in LB pathology (2,9), the region around pS129 has received considerable attention and several studies have investigated other C-terminal phosphorylation sites (5-6,10,12,13,16,18). Of these sites, α-syn can be phosphorylated at Y125, Y133 and Y136. While several studies looked into the role of some of these modifications and how they interact to affect the oligomerization of the protein in vitro, only pY125 was investigated in brain tissue (2,5,12). In one immunohistochemical study, pY125 has been found within LBs in a familial PD case with G51D mutation (12); whereas another study reported detecting pY125-α-syn in brain lysates from a *Drosophila* model of PD but failed to detect it in human brain tissues (5). Similarly, a study using proteomic and mass spectrometry analyses of α-syn from patients with DLB reported that pS129 is the dominant pathological modification and failed to detect pY125 (2). Several in vitro studies have also explored the role of Y125 phosphorylation (5,10). One study reported that pY125 inhibits α-syn aggregation implying a neuroprotective role (10); whereas a previous in vitro study using nuclear magnetic resonance spectroscopy reported that pY125 is a priming event for pS129, suggesting that it may be an important precipitating event in the genesis of α-syn aggregation (13). Therefore, there is a pressing need to characterize whether pY125 occurs alongside pS129 in LBs in human brain tissue and animal models of α-synucleinopathy.

PTMs in LBD have typically been investigated in isolation, usually focused on pS129. However, PTMs are unlikely to occur in such an isolated manner under physiological conditions and the same molecule of α-syn may be phosphorylated at numerous sites. Y125 is a phosphorylation site of sufficient proximity to S129 and therefore, can be part of the epitope for many available antibodies against pS129-α-syn. Precise mapping of the epitope for these anti-pS129-α-syn antibodies is needed to provide information on whether they will only recognize pS129-α-synuclein when Y125 is not phosphorylated, but such information is lacking since the precise epitope for most commercial pS129 antibodies is not disclosed. Therefore, previous antibody-based studies of pS129 may have been limited by only detecting aggregates that are singly phosphorylated and not recognizing aggregates that contain doubly phosphorylated α-syn at Y125 and S129. In the present study, we sought to identify whether pY125 is present in α-syn aggregates that characterize LB pathology. Using a panel of four in-house developed and thoroughly characterized antibodies as well as three commercial antibodies, we evaluated the pathological relevance of pY125 and pS129 α-syn in LB pathology.

**MATERIALS AND METHODS**

**Generation and characterization of anti pS129-α-syn antibodies**

Generation of mouse monoclonal antibodies was performed using hybridoma technology as recently described (8). Animal procedures were carried out in accordance with Laboratory Animal Research Center (LARC), Qatar University (QU), Qatar, according to the QU institutional ethical rules and regulations and approved by QU—IACUC & IBC.

**Epitope mapping of antibodies**

To map the epitopes for our mAbs, we performed alanine scanning experiments, a widely used site-directed mutagenesis approach. Synthetic 11 amino acid long peptides spanning residues (124-134) were used (Table S1). The 384-well black MaxiSorb plate (Nunc) was coated with 500 ng/well of the peptides in NaHCO₃, and incubated overnight at 37°C under dry conditions. The following day, the plate was blocked with blocking buffer (PBST containing 2.25% gelatin) for 1 h at RT. The plate was then washed three times with PBST and the mAbs were added at 100 ng/mL for 1 h.

**Tissue culture of HEK 293 human embryonic kidney cells**

HEK cells were grown in Dulbecco’s MEM- high glucose (Gibco BRL, Rockville, MD) supplemented with 15% fetal bovine serum (Gibco BRL, Rockville, MD), 1% penicillin-streptomycin (Gibco BRL, Rockville, MD) and incubated at 37°C in a 5% CO₂/95% air humidified incubator. After plating HEK cells overnight in 6-well plates, cells were transfected with 2 μg of wild type α-syn plasmid DNA by lipofectamine 3000 reagent (Invitrogen, Waltham, MA). One group of α-syn expressing HEK cells was similarly transfected again with 4μg of α-syn seeds (preparation described in Supporting Information) the following day. HEK cells were lysed, 48 hours post-transfection, initially with 1% Triton X-100 in 50 mM Tris, 150 mM NaCl (pH 7.6) containing protease and phosphatase inhibitors to obtain soluble fractions. The pellet was further lysed with 1% SDS in 50 mM Tris, 150 mM NaCl (pH 7.6) with complete inhibitors to attain insoluble fractions. Protein concentration was determined by BCA protein assay (Pierce) prior to analysis on 12% SDS-PAGE and immunoprobing. Primary antibodies used in this study are summarized (Table S2).

**Immunofluorescence analysis of mouse brain sections**

Male wild-type C57Bl/6/C3H mice 2–4 months old (Jackson Laboratory, Bar Harbor, Maine) were used. These have been previously undergone stereotaxic injections with α-syn preformed fibrils phosphorylated at serine 129, as described previously (11). Animals were anesthetized by isoflurane surgical anesthesia and perfused intracardially with ice-cold PBS followed by 4% paraformaldehyde in PBS and post-fixed overnight. Brains were frozen in iso-pentane at −45°C and stored at −80°C. Fluorescent immunohistochemistry was performed in free-floating sections of 35 μm. The sections were treated with antigen retrieval solution (citrate buffer, pH 6) at 80°C for 30 minutes followed by treatment with Proteinase K (PK) (Sigma-Aldrich, USA) 5 μg/
mL in PBS for 10 minutes at 25°C. Immunohistochemical staining with the anti-β Tubulin III antibody (TUJ1; Sigma monoclonal) was performed at a working dilution of 1:1000.

**Preparation of human brain lysates**

Frozen *post-mortem* samples of the frontal cerebral cortex from clinically diagnosed PD (n = 2), DLB cases (n = 2), or age-matched controls (n = 2) were obtained from Newcastle Brain Tissue Resource and processed as previously described (8,25). Briefly, tissues were homogenized on ice in five volumes of TBS buffer (50 mM Tris–HCl, pH 7.4, 175 mM NaCl) containing 5 mM EDTA and cocktail of protease phosphatase inhibitors, and then centrifuged for 30 minutes at 3000 × g at 4°C. The resulting supernatants subsequently referred to as the “aqueous soluble fraction”. Pellets were re-suspended and homogenized in buffer containing the mild detergent cell-lytic and 150 mM NaCl and centrifuged at 3000 × g at 4°C, with the resulting supernatant subsequently referred to as “detergent soluble fraction”. The resulting detergent-insoluble pellets were solubilized in 8 M Urea/5% SDS and will subsequently be referred to as the “insoluble fraction”. Total protein concentration was measured using the Pierce BCA assay.

**Immunoblotting of human brain lysates**

About 10 µg of urea-soluble brain lysates, as well as 50 ng of recombinant human α-syn, or pS129-α-syn (Supporting Information), were mixed with loading buffer (250 mM Tris–HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) and then separated on 12% SDS-PAGE gels. The gels were then transferred to nitrocellulose membranes in transfer buffer (250 mM Tris–HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) and cocktail of protease phosphatase inhibitors, and then centrifuged for 30 minutes at 3000 × g at 4°C. The resulting supernatants subsequently referred to as the “aqueous soluble fraction”. Pellets were re-suspended and homogenized in buffer containing the mild detergent cell-lytic and 150 mM NaCl and centrifuged at 3000 × g at 4°C, with the resulting supernatant subsequently referred to as “detergent soluble fraction”. The resulting detergent-insoluble pellets were solubilized in 8 M Urea/5% SDS and will subsequently be referred to as the “insoluble fraction”. Total protein concentration was measured using the Pierce BCA assay.

**Immunohistochemistry of human post-mortem tissue**

We obtained 6 µm formalin-fixed paraffin-embedded *post-mortem* human brain tissue from the cingulate gyrus and substantia nigra for immunohistochemical and immunofluorescent staining (Table 1). Optimal epitope unmasking was empirically determined, with heat-mediated antigen retrieval in boiling citrate pH 6 followed by 5 minutes in formic acid found to confer a modestly better signal compared to EDTA pH 8 and citrate pH 6 without formic acid for all antibodies. 5B9 (1 µg/mL), 6H5 (1 µg/mL), 9G1 (200 ng/mL), PS129 (in-house; 100 ng/mL), ab51253 (Abcam; 2 µg/mL), pY125 (Abcam ab10789, ab131466; both 2 µg/mL) and pY133 (Abcam ab51104; 5 µg/mL) were incubated for 1 h at room temperature. Immunohistochemical staining used the Menarini MenaPath X-Cell Plus HRP detection kit with Menarini purple chromagen (Menarini Diagnostics, Berkshire, UK).

| Case ID | Diagnosis | Sex | Age | Braak NFT | McKeith LB |
|---------|-----------|-----|-----|-----------|------------|
| Case 1  | PDD       | M   | 76  | 2         | Neocortical|
| Case 2  | DLB       | F   | 78  | 3         | Neocortical|
| Case 3  | DLB       | M   | 74  | 4         | Neocortical|
| Case 4  | DLB       | M   | 81  | 4         | Neocortical|
| Case 5  | DLB       | M   | 80  | 4         | Neocortical|
| Case 6  | Mixed DLB/AD | M  | 83  | 6         | Neocortical|
| Case 7  | Mixed DLB/AD | F  | 91  | 6         | Limbic     |
| Case 8  | Mixed DLB/AD | M  | 77  | 6         | Neocortical|
| Case 9  | Control   | F   | 90  | 2         | No LB      |

For immunofluorescent analysis, antigen retrieval was performed using citrate pH6 and formic acid as before, and sections were blocked in 10% normal goat serum for 1 h at room temperature. Primary antibodies (PS129, 1 µg/mL; ab51253, 4 µg/mL; pY125, 10 µg/mL; pY133, 10 µg/mL) were suspended in 10% normal goat serum and incubated overnight at 4°C. Secondary antibodies for PS129 and ab51253 (goat anti-mouse Alexa Fluor 488, Thermo Fisher #A28175; goat anti-rabbit Alexa Fluor 594 #R37117) or for PS129/pY125 and pS129/pY133 (goat anti-mouse Alexa Fluor 647, Thermo Fisher #A21241 and goat anti-rabbit Alexa Fluor 594, Thermo Fisher #A1101) were diluted 1:100 in 10% normal goat serum for 1 h at room temperature, before being incubated in 1% Sudan Black B and mounted in ProLong Gold Anti-Fade (PS129 and ab51253 used ProLong Gold with added DAPI). Fluorescent images were visualized on a Nikon Eclipse 90i microscope for PS129 and ab51253; and on a Leica SP8 confocal microscope for PS129/pY125 and PS129/pY133.

**RESULTS**

**Epitope mapping of monoclonal antibodies against pS129-α-syn**

Our group has previously generated three mouse monoclonal antibodies (mAbs; 5B9, 6H5, and 9G1) against pS129-α-syn (8). These were thoroughly characterized in combination with our previously generated mAb (PS129) (14) and the commercial mAb (ab51253; abcam). The specificity of all antibodies toward pS129-α-syn was validated. All antibodies showed nanomolar affinity toward pS129-α-syn and did not cross-react with WT-α-syn (8). Epitope mapping of the antibodies showed that 5B9 and 6H5 map to amino acids (128-131) (Figure 1A,B), whereas 9G1 and ab51253 were mapped to amino acids (127-130) (Figure 1C,D). PS129 had a slightly different epitope mapped to residues (124-129) (Figure 1E). Syn-1, which has an epitope between amino acids 91-99 (27) did not react with any of the peptides and was included as a negative control (Figure 1F). We confirmed that all four antibodies can recognize the doubly phosphorylated peptide at Y125 and S129 (Figure 2A–C, E). In contrast, PS129
Figure 1. Epitope mapping of the antibodies. Using indirect ELISA, peptides (Table S1) were coated on 384-well plate and detected with indicated antibodies. Bar chart for each antibody is shown above and the expected epitope is marked in red dash-lined selections (A–E). Syn 1 (F), which has an epitope between amino acids 91–99 was included as a negative control. RLU/10⁻¹ s = relative light units per tenth of a second. Error bars represent the mean of three independent experiments.

Figure 2. Assessing the reactivity of pS129-α-syn mAbs toward doubly phosphorylated-α-syn peptide. Using inhibition ELISA, these antibodies were pre-incubated with the four different peptides: AYEMPpSEEGYQ, ApYEMPSEEGYQ, ApYEMPpSEEGYQ or AYEMPSEEGYQ which are abbreviated, pS129, pY125/pS129, pY125, and S129 respectively. The peptide-antibody mixture was used to detect pre-coated pS129-α-syn. Peptides were used at 50, 100 or 200 µM. Bar graph charts showing the optical density at 450 nm (OD, Y-axis) obtained upon incubation with the corresponding peptide (X-axis) for (A) 5B9, (B) 6H5, (C) 9G1, (D) PS129, or (E) ab51253. No signal indicates strong mAb/peptide binding. Error bars represent the mean of three independent experiments.
only recognized the pS129 peptide and failed to recognize it when it is also phosphorylated at Y125 (Figure 2D). This was expected given that Y125 residue falls within its epitope (Figure 1E) and hence, phosphorylation on this site would disrupt the binding epitope (details in Supporting Information). It is worth noting that Y133 is not part of the epitope for the five mAbs tested, meaning that these mAbs can potentially detect pS129-α-syn if it is also phosphorylated at Y133. Further analysis will be required to validate the sensitivity of these mAbs to multiply phosphorylated pS129-α-syn at Y125 and Y133.

Detection of pY125- and pS129-α-syn in α-syn expressing HEK cells

To verify that our mAbs detect pathologically relevant accumulations in a cell model system, we tested the antibodies using an experimental model for seeded aggregation of α-syn in HEK293 cells. α-Syn phosphorylation and its corresponding seeding-dependent aggregation were induced in α-syn expressing HEK cells, where phosphorylation of α-syn was promoted after a consecutive transfection of WT-α-syn plasmid DNA and α-syn seeds. Immunoblotting of cell lysates showed that pS129-α-syn was detected for all the mAbs tested (Figure 3A). Noticeable increased levels of insoluble pS129-α-syn were seen in the plasmid and seed transfected cells compared to no detection in the non-transfected (negative control) and plasmid-transfected cells, with the latter indicating the seeding-dependent phosphorylation importance. Moreover, soluble fractions showed comparable levels in the two transfected groups using all indicated pS129-α-syn specific antibodies. We have also evaluated two commercially available antibodies specific for pY125-α-syn (ab10789 and ab124955; Abcam). These antibodies can recognize pY125-α-syn when it is also phosphorylated at S129 (S129 is not part of their epitopes; Figure S1). Both antibodies did not detect pY125-α-syn in our cell model (Figure 3B). Total α-syn levels, as detected using Syn-1, was seen in all transfected groups in both soluble and insoluble fractions (Figure 3B).

Investigating the presence of pY125- and pS129-α-syn in brain sections of the PD mouse model

Immunofluorescent staining was performed in the brain sections of C57Bl6/C3H mice who had received stereotaxic injection of pre-formed α-syn fibrils (α-syn PFFs) in the right dorsal striatum, as previously described (11). At 60 dpi, midbrain sections were analyzed by immunofluorescence using the indicated pS129-α-syn specific antibodies. All the five antibodies were able to detect cytoplasmic accumulations of pS129-α-syn surrounded by TUJ1-stained neurons (Figure 4A), with no distinction in staining between antibodies capable of recognizing pY125/pS129-α-syn and an antibody that can only recognize pS129-α-syn. These
accumulations were not observed upon staining with the two antibodies specific for pY125-α-syn (Figure 4B). Our observations indicate the absence of pY125-α-syn in a tissue from the PD mouse model. Although pY125-α-syn was still undetectable in tissues stained without antigen-retrieval methods (data not shown), results need to be interpreted with caution for the known effects antigen-retrieval methods could have on an epitope.

**Immunoblotting of human brain lysates to evaluate the presence of pY125-α-syn**

Next, we used our newly developed antibodies to confirm the detection of pS129-α-syn pathology and explore the presence of pY125-α-syn in post-mortem human brain lysates. Brain lysates were homogenized in a sequential manner to obtain the aqueous-soluble fraction, followed by detergent-soluble fraction and eventually urea-soluble fraction. We have previously reported the accumulation of pS129-α-syn in urea-soluble fractions of human brain lysates using ELISA and immunoblotting (26). It is detected to a lesser extent in detergent-soluble fractions but not in aqueous-soluble fractions (26) (Figure S2). We subjected urea-soluble fractions from the frontal lobes of two PD cases, two DLB cases and two healthy control cases to western blotting analyses. As expected, immunoblotting analyses showed the accumulation of pS129-α-syn in all diseased cases (Figure 5A). These bands were not present in healthy controls, whereas Syn-1 did show immunoreactivity (Figure 5B), presumably representing the native, physiological protein (Figure 5). The above results further confirmed the antibodies’ ability to detect pS129-α-syn pathology in brain samples from patients with synucleinopathies (PD, DLB) and that this immunoreactivity was phosphorylation specific at S129. Notably, we did not observe any significant differences in the signal recognized by the four antibodies that can recognize pY125/pS129-α-syn (5B9, pS129, pY125-α-syn (ab124955), pY125-α-syn (ab10789)), or midbrain (5B9, ab51253, ab10789, ab124955) region of the brain. A. TUJ1 (red) or DAT (red) were used as neuronal markers.
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6H5, 9G1, and ab51253) in comparison to PS129. Moreover, the two antibodies specific for pY125-α-syn did not detect any α-syn pathology in the brain lysates tested (Figures 5B and S2), suggesting that pY125-α-syn does not appear to be present in LB pathology.

**pY125-α-syn is not a component of Lewy bodies in post-mortem human brain tissues**

To further evaluate the pathological relevance of pY125- and pS129-α-syn in LBs from the brains of patients with synucleinopathies, immunohistochemical staining of 5B9, 6H5, and 9G1 in the substantia nigra of an aged control (Case 9) and Parkinson’s disease dementia (PDD; Case 1) case, and in the cingulate gyrus of a control (Case 9) and mixed DLB and Alzheimer’s disease (AD) case (mixed AD/DLB; Case 7). The antibodies 5B9, 6H5, 9G1, as well as PS129, labeled the circumference of intracellular inclusions only in the cases with LB diseases (Figure 6). Antibody 9G1 gave a slightly different pattern of staining, with some labeling of apparent axonal and/or dendritic processes (Figure 6), possibly due to its cross-reactivity with hyperphosphorylated neurofilament protein (8). Importantly, antibodies that can recognize pY125/ pS129-α-syn did not stain distinct or more abundant pathology than the antibody (PS129) that can only recognize pS129, suggesting that pS129-α-syn is the dominant modification and that pY125-α-syn does not exist in LBs (Figure 7).

To further test the hypothesis that pY125 is minimally present in LBs and neurites, immunohistochemical staining was carried out using anti-pY125-α-syn antibodies (ab10789 and ab124955; Abcam). Staining of brain tissue with LBs diseases from cingulate gyrus with both antibodies revealed flame-like profiles reminiscent of neurofibrillary tangles but no LB-type profiles (Figure 8A–C). To determine the specificity of this staining, we pre-incubated both antibodies with the immunizing peptide (ab17030; 10 µg/mL), which eliminated staining of flame-like bodies, indicating specific staining by the antibody (Figure 8D).

Moreover, we performed co-staining of ab10789/ab124955 and PS129 in cingulate gyrus and substantia nigra of one PDD, four DLB and three mixed DLB/AD (Table 1). Ps129 immunoreactivity was observed in a cortical LB in the cingulate gyrus of Case 6, but no co-localization with HP-tau or pY125 (Figure 9Bi–Biv). Similarly, a midbrain Lewy body in the substantia nigra of Case 1 shows a pS129-positive “doughnut” structure, but only weak co-localization with HP-tau and pY125 (Ci–Civ). We observed flame-like bodies recognized by anti-pY125-α-syn antibodies that were morphologically similar to neurofibrillary tangles. As these flame-like bodies were observed in regions with proclivity to developing neurofibrillary tangles, we tested whether these stainings were detecting hyper-phosphorylated tau (HP-tau) in neurofibrillary tangles. Immunofluorescence indicated that flame-like profiles immunoreactive for anti-pY125-α-syn antibodies (ab10789 and ab124955) were co-localized with the HP-tau antibody, AT8 in the cingulate gyrus of Case 6 (Figure 9Ai–Aiv). Similar observations were made in the hippocampus of Case 6, where co-localization between HP-tau and

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**Figure 5. Western blotting analysis of human brain lysates from healthy controls, PD, or DLB.** Samples from the frontal cerebral cortex of healthy controls (n = 2) as well as clinically diagnosed PD (n = 2) or DLB (n = 2) patients have been analyzed on 12% SDS-PAGE, along with 50 ng of the recombinant proteins (WT-α-syn or pS129-α-syn). The gel was transferred into nitrocellulose membrane for western blotting analysis. The blots were then probed using the indicated antibodies against (A) pS129-α-syn, or (B) pY125-α-syn. Syn-1 was included as a positive control (B) and β-Actin as a loading control (B).
Figure 6. Immunohistochemical staining of human brain tissues. Images were obtained in control (Case 9) and PD (Case 1) SN (substantia nigra); and in control (Case 9) and DLB (Case 7) CG (cingulate gyrus). Scale bars = 50 µm.

Figure 7. Co-labeling of PS129 (aa124-129) and ab51253 (aa127-130). Immunofluorescence in PD substantia nigra (Case 1) demonstrates that there is no difference in staining abundance or intensity if an antibody capable of recognizing pY125 and pS129 is used (ab51253) compared to an antibody that only recognizes pS129 (PS129). Scale bar = 50 µm.
pY125-α-syn was observed, but not pS129 (Figure 9Di–Div). Notably, the only instances where anti-pY125-α-syn antibodies showed staining in LBs pathology was only when similar levels of AT8 positivity were observed (Figure 9Ci–Civ). Whilst it is possible that pY125-α-syn is present in neurofibrillary tangles, this seems unlikely as antibodies with epitopes unlikely to be affected by phosphorylation at this reside, such as Syn-1, have not been reported to recognize neurofibrillary tangles. Therefore, as neurofibrillary tangles contain tau phosphorylated at many residues (17), we speculate that this staining may represent cross-reactivity with a phosphorylated tau epitope. However, irrespective of whether pY125-α-syn is or is not in neurofibrillary tangles, it does not appear to be present in LBs based on the present findings. We finally examined whether the other phosphorylation site proximal to S129, Y133, was phosphorylated in α-syn aggregates using a commercially available antibody (ab51104; Table S1). Immunofluorescence showed faint staining only at concentration much higher than that recommended by the manufacturer (Figure S3), whereas immunoblotting of human brain lysates did not show any pathology (Figure S4). Further analyses with more specific immunological tools are needed to evaluate the presence of pY133-α-syn in LBs.

DISCUSSION

α-Syn is known to undergo several post-translational modifications in PD and related disorders, the most abundant being phosphorylation at serine 129 (2,19,20). Over the past decade, immunoreactivity to pS129-α-syn has emerged as a hallmark feature of PD and related synucleinopathies. This raised the question of whether pS129-α-syn plays an important role in initiating misfolding and aggregation of α-syn in the disease state. Various in vitro and in vivo studies that investigated the effect of phosphorylation of α-syn at S129 has failed to reach a consensus as to the role played by this modification (17); nevertheless, it is a consistent marker of LB pathology in PD and other synucleinopathies.

PTMs such as pS129-α-syn might not occur in isolation, and few studies have evaluated the presence of different PTMs and how these may interact in the etiopathology of LBD. Besides its phosphorylation at S129, α-syn has been shown to undergo phosphorylation at several tyrosine residues in the C-terminal region, namely Y125, Y133, and Y136 in vitro. Out of these three residues, phosphorylation at Y125 is thought to be more relevant (1,6,16,18) and is the only phosphorylation site that has been detected in vivo. The few studies that have studied pY125 in LBDs have reported discrepant findings, with one reporting it as present within LBs (10) and another demonstrating a reduction in pY125 in whole tissue lysates from LBD brain tissue compared to aged controls (5). This discrepancy is in part due to the lack of immunological tools needed to address this question. Moreover, the proximity of Y125 to S129 is such that the epitope of pS129-α-syn antibodies may also span Y125, meaning such antibodies would only recognize α-syn when it is not phosphorylated at Y125. Nevertheless, several in vitro studies investigated the effect of pY125 and how it affects the oligomerization of α-syn through interactions with proximal residue pS129, suggesting that pY125 may
act as a priming event for efficient phosphorylation of S129 (13). Given the putative importance of pS129-α-syn in LB pathology, investigating proximal phospho-epitopes is crucial to further understanding of synucleinopathies.

To overcome the discrepant findings regarding the presence of pY125-α-syn in LB pathology, we used a combination of newly developed in-house and commercial antibodies and conducted a comprehensive study of its presence in cellular and rodent models and human post-mortem brain tissue. In this study, we have demonstrated that pY125-α-syn is not a component of α-syn inclusions in cell model systems, rodents injected with α-syn fibrils and post-mortem human brain tissue. In contrast, pS129-α-syn is consistently observed across all systems and tissues. These findings suggest that pY125 is not a major or critical component of LBs.

We performed epitope mapping experiments for the antibodies used herein and showed that three out of four in-house antibodies, as well as ab51253, are specific for pS129-α-syn and their reactivity toward it does not decrease if pS129-α-syn is also phosphorylated at Y125. This is largely attributed to Y125 not being covered in the epitope for these antibodies. It should be noted, however, that these epitopes might not fully represent the exposed epitope in a full-length protein depending on its folding patterns and will, therefore, require further analysis.

Using two commercial pY125-α-syn antibodies we noted immunoreactivity in neurofibrillary tangles and co-localization with hyper-phosphorylated tau. Whilst it is possible that pY125-α-syn is a component of neurofibrillary tangles, previous studies have not reported α-syn-immunoreactive neurofibrillary tangles and it is unlikely this would have been unreported given the importance of such a finding for the interaction of pathologies and the growing interest in multiple pathologies in age-associated neurodegeneration. We speculate that pY125-α-syn antibodies may be cross-reacting with the extensive phosphorylation in tau pathology. Furthermore, we noted that previous reports of pY125-α-syn in LB pathology were observed in the CA1 sector of the hippocampus, a region where the authors also noted some
co-localization of phosphorylated tau and α-syn, in a case carrying a G51D mutation (12). Therefore, one could speculate that a plausible explanation of the discrepant findings is that cases harboring G51D mutations have inclusions consisting of α-syn and tau, and that pY125-α-syn antibodies are detecting tau in these inclusions. However, an equally plausible explanation is that, as G51D mutations are unique amongst disease-causing mutations of α-syn as they inhibit aggregation (7,23) and have atypical neuropathological features (12), the discrepant findings simply underline the differences between idiopathic LB disease and those observed in individuals with G51D mutations.

CONCLUSIONS

In conclusion, we have addressed the discrepancy in the literature regarding the role of pY125-α-syn in LB pathology. Using a panel of in-house developed and commercial antibodies, we have demonstrated that unlike pS129-α-syn, pY125 is not a major component of α-syn inclusions in cell or rodent model systems or post-mortem brain tissue from LBD cases. However, current tools to investigate pY125-α-syn may cross-react with phosphorylated residues in tau pathology, indicating these tools may be sub-optimal for identifying immunopositivity in inclusions in post-mortem brain tissue from individuals with age-associated neurodegeneration given the high prevalence of multiple pathologies in these populations. The present findings have important implications for understanding the genesis and etiology of α-syn pathology and methods for the study of alternative PTMs in LBD.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Newcastle Brain Tissue Resource is a UK Human Tissue Authority approved tissue repository. Brain tissue donors or next of kin provided informed consent to donate tissue and tissue donation was approved by the local UK National Health Service Research Ethics Committee (19-NE-0008).

CONSENT FOR PUBLICATIONS

Not applicable.

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Omar El-Agnaf performed the study design and supervision. Munerah Fayyad, Daniel Erskine Nour Majbour, Nishant Vaikath, Simona Ghanem, Indulekha Sudhakaran and Agaristi Lamprokostopoulou performed the experiments. Christopher Morris and Johannes Attems dissected and neuropathologically characterized human brain tissue. Houari Abdesselem contributed to the data analysis. Omar El-Agnaf, Daniel Erskine, and Kostas Vekrellis contributed to the interpretation of results and critical revision of the draft. Munerah Fayyad wrote the first draft and was responsible for incorporating the revision from other authors. Daniel Erskine equally contributed to the writing and thoroughly revised it. Omar El-Agnaf reviewed the final draft, provided comments and final approval to submit for publication. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Figure S1. Epitope mapping for anti-pY125-α-syn commercial antibodies. Using indirect ELISA, peptides (Table 1) were coated on the plate and detected by different commercial antibodies from abcam (a) ab10789 and (b) ab124955 (clone EPR1719), polyclonal and monoclonal antibodies specific for pY125-α-syn, respectively. (c) ab51253 (clone EP1536Y), monoclonal antibody specific for pS129-α-syn was included as a control. RLU/10-I sec = relative light units per tenth of a second.

Figure S2. Western blotting of the aqueous-soluble and detergent-soluble fractions from human brain lysates. Samples from the frontal cerebral cortex of healthy controls (n = 2) as well as clinically diagnosed PD (n = 2) or DLB (n = 2) patients have been analyzed on 12% SDS-PAGE, along with 50 ng of the recombinant proteins (WT-α-syn or pS129-α-syn). The gel was transferred into nitrocellulose membrane for western blotting analysis. The blots were then probed using the indicated antibodies. pY125-α-syn is detected using ab1078, whereas pS129-α-syn is detected using ab51253. Syn-1 detects total α-syn and β-Actin is included as a loading control.

Figure S3. Immunostaining of post-mortem brain sections with an antibody specific for pY133. Immunostaining in the substantia nigra of Case 1 (Ai), entorhinal cortex of Case 6 (Aii), and cingulate gyrus of Case 6 (Aiii and Aiv) revealed Lewy body-like inclusions. Confocal microscopy of pY133 co-stained with pS129 and AT8 revealed co-localisation of pY133 in cingulate gyrus of Case 6 (Bi-Biv) and also accumulations labelled by pY133 but not pS129 (Ci-Div). Unlike pY125, pY133 did not show co-localisation with HP-tau labelled by AT8, including...
in the hippocampus of Case 6 (Ei-Eiv). Scale bars 200 µm (Ai, Aiii), 50 µm (Aii, Aiv), 10 µm (Bi-Eiv).

**Figure S4. Immunoblotting of human brain lysates.** Samples from the frontal cerebral cortex of healthy controls (n = 2) as well as clinically diagnosed PD (n = 2) or DLB (n = 2) patients have been analyzed on 12% SDS-PAGE, along with 50 ng of the recombinant pS129-α-syn protein. The blots were then probed with antibody against pY133-α-syn (ab51104). Both Urea-soluble fractions (top right panel) and detergent-soluble fractions (bottom right) are shown. Syn-1 was included for total α-syn and β-Actin as a loading control.

**Table S1.** List of peptides designed for epitope mapping of the antibodies.

**Table S2.** List of primary antibodies used in the study.