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Phosphorylation of α -synuclein directs trafficking of the v-SNARE synaptobrevin-2

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Title: Phosphorylation of α -synuclein directs trafficking of the v-SNARE synaptobrevin-2

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Keywords

α -synuclein, synaptobrevin-2, endocytosis, protein trafficking, exocytosis, neurotransmission.

Short title: Phospho- α -syn directs synaptobrevin-2 trafficking

ABSTRACT

α -Synuclein has been extensively studied for its role in Parkinson's disease, however its native function remains unclear. Several lines of evidence suggest it modulates presynaptic function. As many presynaptic proteins have their functions regulated by phosphorylation, we sought to determine whether α -synuclein, a known phosphoprotein, might similarly have phospho-dependent roles. We identified two residues in α -synuclein, Ser42 and Thr81, that undergo activity-dependent phosphorylation in response to neuronal depolarisation. We employed a molecular replacement strategy, introducing α -synuclein phosphovariants into neuronal cultures from α -synuclein knockout (KO) mice (of either sex), and revealed that ablating phosphorylation at Thr81 altered the subcellular distribution of synaptobrevin-2/VAMP2, a crucial fusogenic vesicular SNARE protein and known binding partner of α -synuclein. Ablating phosphorylation of α -synuclein at Thr81 specifically accelerated endocytic retrieval of synaptobrevin-2 during

stimulation without impacting global endocytosis, evoked exocytosis, or the size of the recycling pool. Conversely, mimicking phosphorylation at Thr81 augmented post-stimulation retrieval of synaptobrevin-2. This suggests that activity-dependent phosphorylation of Thr81 modulates the trafficking of synaptobrevin-2 to direct its retrieval via different endocytic pathways, thereby impacting presynaptic functionality and plasticity.

SIGNIFICANCE STATEMENT

The presynaptic proteins α -synuclein has been extensively studied for its role in Parkinson's disease, however its native function remains unclear. As many presynaptic proteins have their functions regulated by phosphorylation, we sought to determine whether α -synuclein, a known phosphoprotein, might similarly have phospho-dependent roles. We identified two residues in α -synuclein, Ser42 and Thr81, that undergo activity-dependent phosphorylation. Mimicking or ablating phosphorylation of α -synuclein at Thr81 specifically and differentially altered retrieval of synaptobrevin-2 without impacting global endocytosis, evoked exocytosis, or the size of the recycling pool. This suggests that activity-dependent phosphorylation of Thr81 modulates the trafficking of synaptobrevin-2 to direct its retrieval via different endocytic pathways, thereby impacting presynaptic functionality and plasticity.

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DECLARATIONS OF INTEREST

The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS

Conceptualisation: S.L.G and E.H.A.; Data Curation: E.H.A.; Formal Analysis: E.H.A., J.P.C., H.M., M.E.G.; Funding Acquisition: S.L.G.; Investigation: E.H.A., J.P.C., H.M., K.E-K., M.E.G.; Visualisation: E.H.A.; Supervision: S.L.G, M.E.G., D.H. Writing-original draft: E.H.A. (lead), S.L.G., M.E.G.; Writing-review and editing: E.H.A., S.L.G., J.P.C, M.E.G. All other authors reviewed and approved this manuscript.

INTRODUCTION

α -Synuclein is a small, 140 amino acid protein that has been implicated in the pathogenesis of a group of neurodegenerative diseases including Parkinson's disease, Lewy body dementia, and multiple system atrophy, collectively known as synucleinopathies (Goedert et al., 2017). These disorders are characterised histopathologically by intracellular inclusions containing high levels of aggregated α -synuclein (Spillantini et al., 1998). α -Synuclein has thus been extensively characterised in a pathological context; however, the physiological function of α -synuclein remains poorly understood.

α -Synuclein is highly and widely expressed in the brain, accounting for up to 1% of total cytosolic protein (Iwai et al., 1995), and is strongly enriched at the presynaptic terminals of neurons (Maroteaux et al., 1988). Accordingly, α -synuclein has a range of proposed roles in the synaptic vesicle cycle - the fusion (exocytosis), recycling (endocytosis) and clustering of synaptic vesicles in nerve terminals (Chanaday et al., 2019). Tight regulation of this process is crucial for the maintenance of high-fidelity neurotransmission and precise dissemination of signals throughout the neural networks of the nervous system.

α -Synuclein is a cytosolic protein with an N-terminal membrane-associated region that reversibly binds to membranes, preferably of high curvature such as synaptic vesicles (Davidson et al., 1998; Bussell and Eliezer, 2003). The acidic C-terminal tail can bind to synaptobrevin-2 (also known as VAMP2; vesicle-associated membrane protein 2), the vesicular SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein that is essential for fusion of vesicles with the plasma membrane (Burré et al., 2010). These structural features give rise to an array of diverse suggested roles for α -synuclein such as enhancing SNARE complex formation (Burré et al., 2010), modulating fusion pore dilation (Logan et al., 2017), inducing membrane curvature during endocytosis (Westphal and Chandra, 2013), and facilitating the clustering of synaptic vesicles (Atias et al., 2019). The modulation of and relationship between each of these functions of α -synuclein remains unclear.

Various key proteins involved in the synaptic vesicle cycle, such as synapsin-1 (Ceccaldi et al., 1995) and dynamin-1 (Clayton et al., 2009), have functions that are regulated via phosphorylation. α -Synuclein is also a presynaptic phosphoprotein, raising the appealing possibility that it may operate in a phosphorylation-dependant manner. Phosphorylation of all 18 serine, threonine and tyrosine residues of human α -synuclein has been detected. (Okochi et al., 2000; Ellis et al., 2001; Negro et al., 2002; Paleologou et al.,

2010; Mahul-Mellier et al., 2014; Engholm-Keller et al., 2019; Schweighauser et al., 2020; Sano et al., 2021; Reimer et al., 2022; Ramalingam et al., 2023; Zhang et al., 2023), and their contribution to protein fibrilisation and aggregation, and cellular toxicity, have been highly studied (Yoo et al., 2022). However, recently there has been heightened focus on how these sites are modulated in physiological contexts (Engholm-Keller et al., 2019; Schweighauser et al., 2020; Sano et al., 2021; Reimer et al., 2022). For example, phosphorylated Ser129 (pSer129) has long been associated with aggregated α -synuclein in Lewy Bodies (Fujiwara et al., 2002), but has only recently been shown to occur in response to neuronal depolarisation over minutes to hours (Ramalingam et al., 2023). Soluble pSer129 α -synuclein is present throughout the mouse brain under physiological conditions and in the absence of disease (Arlinghaus et al., 2023). Phosphorylation at Ser129 modulates the association of α -synuclein with membranes, synaptobrevin-2 and synapsin, influences synaptic plasticity and is important for the ability of over-expressed α -synuclein to attenuate vesicle recycling and cluster vesicles (Parra-Rivas et al., 2023; Ramalingam et al., 2023).

Even with these recent advancements, the role of α -synuclein phosphorylation on neuronal physiology remains unclear. As such, we sought to identify, in an unbiased manner, residues of α -synuclein at which phosphorylation is dynamically altered within seconds of neuronal depolarisation. We identified two sites, Ser42 and Thr81, that underwent an activity-dependent increase in phosphorylation. We then sought to investigate how phosphorylation at these sites impacts α -synuclein function, identifying a novel phosphorylation-dependent function of α -synuclein as a modulator of synaptobrevin-2 trafficking.

MATERIALS & METHODS

Materials

Synaptobrevin-2-pHluorin (syb2-pHluorin) was supplied by Prof. G. Miesenböck (Oxford University, Oxford, England), synaptotagmin-1-pHluorin (syt1-pHluorin) was from Prof. V. Haucke (Leibniz Institute of Molecular Pharmacology, Berlin, Germany), vesicular glutamate transporter 1-pHluorin (VGLUT1-pHluorin) was from Prof. R. Edwards (University of California), and pcDNA3.1/Zeo(+), FLAG-synaptobrevin-2 (FLAG-syb2), mCerulean (mCer) and mCherry constructs were supplied by Prof. M. Cousin (University of Edinburgh, Edinburgh, Scotland). pHAHA vector was from Addgene (Plasmid #12517). QuikChange II site-directed mutagenesis kit was from Agilent Technologies (Santa Clara, CA, USA). Neurobasal (NB) media, penicillin-streptomycin, L-glutamine, B-27 supplement, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-

12), Lipofectamine 2000, Minimum Essential Medium (MEM), TMT11plex reagent, goat anti-chicken Alexa Fluor 488, goat anti-chicken DyLight 550, donkey anti-mouse DyLight 550, donkey anti-mouse Alexa Fluor 647, 40 kDa tetramethylrhodamine (TMR) Dextran, and Pierce anti-HA magnetic beads were obtained from ThermoFisher Scientific (Scoresby, VIC, Australia). The mouse anti- α -synuclein antibody was from BD Biosciences (Franklin Lakes, NJ, USA), and the chicken anti-GFP antibody was obtained from Abcam (Cambridge, UK). Dako Fluorescence Mounting Medium was obtained from Agilent Technologies (Santa Clara, CA, USA). 13 mm and 25 mm #1.5 coverslips were from VWR (Radnor, PA, USA) and papain was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from AdooQ Bioscience (Irvine, CA, USA). DL-2-Amino-5-phosphonopentanoic acid (AP5) and Bafilomycin A1 was from Cayman Chemical (Ann Arbor, MI, USA). Lys-C was from FUJIFILM Wako Pure Chemical Corporation (Japan), Sep-Pak tC18 3cc Vac cartridges were from Waters Corporation (Rydalme, Australia), and ReproSil Pur C18 AQ columns were obtained from Dr. Maisch (Germany). All other reagents were obtained from Sigma-Aldrich (Castle Hill, Australia).

Experimental model details

B6;129X1-*Snc*^{tm1Rosl}/J α -synuclein knockout (KO) mice (Abeliovich et al., 2000) were obtained from The Jackson Laboratory (Bar Harbour, ME, USA) and backcrossed onto C57BL6/J background for 10 generations. All procedures were approved by the Florey Animal Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Mouse colonies were group housed in individually ventilated cages in a temperature controlled (approximately 21°C) room on a 12-hour light-dark cycle with food and water freely available. Animals were time-mated overnight and visual confirmation of a vaginal plug the following morning was considered embryonic day (E) 0.5.

Cortical and hippocampal primary neuronal cultures

As previously described, (Baker et al., 2018; Melland et al., 2023; Park et al., 2024) dissociated primary cortical and hippocampal neuronal cultures were prepared from E16.5-18.5 wild-type or α -synuclein KO mouse embryos of both sexes by incubation of isolated cortical hemispheres or hippocampi, respectively, in papain followed by trituration in DMEM/F-12 supplemented with 10% v/v FBS and 1% v/v

penicillin/streptomycin to obtain a single cell suspension. After centrifugation at 363 g for 5 minutes, DMEM/F-12 was replaced with neurobasal media supplemented with B-27, 0.5 mM L-glutamine and 1% v/v penicillin/streptomycin. Primary cortical cultures were plated at a density of 1.2×10^6 cells/coverslip on 25 mm coverslips in 6-well plates. Hippocampal cultures were plated at a density of 3.5×10^4 cells/coverslip on 13 mm coverslips in 24-well plates for immunolabelling or 6×10^4 cells/coverslip on 25 mm coverslips in 6-well plates. All coverslips were coated with poly-D-lysine and laminin prior to plating. Cultures were kept in supplemented neurobasal media at 37°C and 5% CO₂ in a humidified incubator. After 2-3 days 1 μ M cytosine β -d-arabinofuranoside was added to cultures to inhibit glial cell proliferation. Hippocampal cultures were transfected after 6-8 days in culture with Lipofectamine 2000 as previously described. (Gordon et al., 2011; Baker et al., 2018) For 24-well plates, each coverslip was co-transfected using 1 μ L of Lipofectamine 2000 with 0.5 μ g of either an empty vector (pcDNA3.1/Zeo(+); Addgene plasmid # V86020) or α -synuclein variant plasmid, and 0.5 μ g of either syb2-pHluorin or syt1-pHluorin. For 6-well plates, each coverslip was co-transfected using 2 μ L Lipofectamine 2000 with 1 μ g of an α -synuclein variant, 1 μ g of either syb2-pHluorin, syt1-pHluorin, or VGLUT1-pHluorin, and 1 μ g of mCherry as a transfection marker.

Method details

Plasmid Preparation

Human α -synuclein was *de novo* synthesised and cloned into pcDNA3.1(+) or pHAHA vector by GenScript. Site-directed mutagenesis was used to introduce the following mutations into human α -synuclein in pcDNA3.1(+) or pHAHA vector: S42E, S42A, T81E, T81A. Mutagenesis was performed using QuikChange II site-directed mutagenesis kit as per manufacturer's instructions. All mutations were confirmed by Sanger sequencing (Australian Genome Research Facility, Melbourne, Australia).

Mass spectrometry detection for quantification of synuclein phosphopeptides

Washing, stimulation and lysis were all performed at 37°C. On Day *In Vitro* (DIV) 12, WT and α -synuclein KO primary cortical neuronal cultures were washed with standard saline imaging buffer (136 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) and then either mock-stimulated with standard saline imaging buffer (control treatment) or depolarised with 60 mM KCl buffer (78.5 mM NaCl, 60 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, 10 mM HEPES,) (KCl treatment). Buffer aspiration was initiated after 8 seconds and cells were then lysed by the addition

of lysis buffer at 10 seconds (2% SDS, 50 mM HEPES, 2 mM EDTA, 2 mM EGTA, pH 7.4, supplemented with one Roche PhosSTOP phosphatase inhibitor tablet per 10 mL of buffer) and cell lysates scraped from coverslips and collected and immediately frozen on dry ice. Lysates from three individual coverslips were combined to constitute a single sample.

Each frozen lysate was thawed and a 500 μ L aliquot was reduced with 10 mM tris(2-carboxyethyl)phosphine. The samples were incubated at 85°C for 10 min. Iodoacetamide was added to a final concentration of 20 mM and the samples were incubated at 23°C for 30 min in the dark. Each sample was precipitated using the chloroform-methanol method (Wessel and Flügge, 1984) and dissolved in 20 μ L solution containing 7.8 M urea, 50 mM triethylammonium bicarbonate (TEAB) and 5 μ g of Lys-C. Samples were incubated at 25 °C for 8 h. The samples diluted 8-fold with 50 mM TEAB to 160 μ L, then 4 μ g of Glu-C was added, and the samples incubated at 25 °C for 8 h. An additional 1.4 μ g of Glu-C was added, and the incubation was repeated. The protein content of each sample was estimated using an Implen Nanophotometer and absorption of 280 nm light. An aliquot of each sample containing 200 μ g was made up to 100 μ L, mixed with 41 μ L of anhydrous acetonitrile containing a TMT11plex reagent and incubated at 23 °C for 2 h. The labelling scheme was as follows: KO control bio-replicate 1, 126; KO KCl bio-replicate 1, 127C; KO Control bio-replicate 2, 128C; KO KCl bio-replicate 2, 129C; KO control bio-replicate 3, 130C; KO KCl bio-replicate 3, 131C; WT control bio-replicate 1, 126; WT KCl bio-replicate 1, 127N; WT control bio-replicate 2, 128N; WT KCl bio-replicate 2, 129N; WT control bio-replicate 3, 131N; WT KCl bio-replicate 3, 130N (Lot # UA275089 and TA265136). A 0.3 μ L aliquot was tested for derivatisation efficiency, which was at 100% for the 100 most readily detected proteins. The reagents were quenched with 8 μ L of 5% hydroxylamine. All WT samples were combined, and all KO samples were combined. The two combined samples were acidified to pH < 3 with formic acid and then dried. Each sample was made up to 1 mL in water with 0.1% trifluoroacetic acid and desalted using a Sep-Pak tC18 3cc Vac cartridge (200 mg sorbent). The eluates were applied to the "TiSH" method of phosphopeptide enrichment and hydrophilic interaction chromatography (HILIC) fractionation as described previously (Engholm-Keller and Larsen, 2016). The mainly non-phosphorylated peptides that did not bind to the first titanium dioxide step in the TiSH method were collected. An aliquot equal to 0.5% of the total was desalted and applied to HILIC. Thus, both phosphopeptide and non-phosphopeptides were fractionated prior to LC-MS/MS.

HILIC was performed on a Dionex UltiMate 3000 HPLC system using a Tosoh Bioscience TSKgel Amide-80 1 mm inside diameter x 250 mm long column. The HILIC buffer A was 90% acetonitrile, 9.9% water and 0.1% trifluoroacetic acid. Buffer B was 99.9% water and 0.1% trifluoroacetic acid. The four samples, two mono-phosphopeptide enriched samples and two non-phosphopeptide samples, were made up to 150 μ L in buffer A, separately injected into a 250 μ L sample loop and loaded onto the column at a flow rate of 60 μ L/min with buffer A at 100% for 10 min. The gradient was from 100% A to 60% A for 35 min at a flow rate of 50 μ L/min. Fractions were collected every 1 min in a 96-well plate using a LC Packings Probot Micro Fraction Collector. Chromeleon 6.8 and μ Carrier 2.0 were used to control the HPLC and Probot, respectively. Selected HILIC fractions were combined based on the intensity of the ultraviolet signal at 214 nm to get similar peptide amounts in each fraction, then dried and dissolved in 0.1% formic acid for LC-MS/MS analysis.

The LC-MS/MS was performed using a Dionex UltiMate 3000 RSLC nano system and Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer. The content of HILIC fractions was loaded directly onto an in-house 300 $\times$ 0.075 mm column packed with ReproSil Pur C18 AQ 1.9 μ m resin. The column was heated to 50 $^{\circ}$ C using a PRSCOV1 Sonation Lab Solutions column oven integrated with the Nanospray-Flex ion source with an electrospray operating at 2.3 kV. The S lens radio frequency level was 60 and capillary temperature was 250 $^{\circ}$ C. The 5 μ L sample was injected into a 20 μ L loop and loaded onto the column in 99% reversed phase buffer A and 1% buffer B for 25 min at 300 nL/min. The gradient, at 250 nL/min, was from 99% buffer A to 95% buffer A in 1 min, to 75% buffer A in 74 min, to 65% buffer A in 8 min, to 1% buffer A in 3 min, held at 1% buffer A for 2 min, to 99% buffer A in 1 min and held for 10 min. MS acquisition was performed for the entire 120 min.

All samples and fractions were analysed using data-dependent acquisition LC-MS/MS. The mono-phosphopeptide enriched HILIC combined fractions 24-27 were analysed by targeted acquisition LC-MS/MS using the remaining 33% of the sample. For data-dependent acquisition, the MS scans were at a resolution of 70,000 with an automatic gain control target of 1,000,000 for a maximum ion time of 100 ms from m/z 375 to 1500. The MS/MS scans were at a resolution of 35,000 with an automatic gain control target of 200,000 and maximum ion time of 115 ms. The loop count was 12, the isolation window was 1.2 m/z, the first mass was fixed at m/z 120 and the normalised collision energy was 34. Singly charged ions and those with charge >8 were excluded from MS/MS and dynamic exclusion was for 25 s. For targeted acquisition, using parallel reaction monitoring (Peterson et al., 2012), the MS scans were at a resolution of 70,000

with an automatic gain control target of 3,000,000 counts for a maximum ion time of 150 ms from m/z 375 to 1500. The MS/MS scans were at a resolution of 35,000 with an automatic gain control target of 200,000 and maximum ion time of 150 ms. The isolation window was 1 m/z, the first mass was fixed at m/z 120 and the normalised collision energy was 34. An inclusion list was used to target the α -synuclein phosphopeptides, controls and standards in specified time windows determined from the data-dependent LC-MS/MS. See Figure S1 for inclusion list.

Fixation and immunocytochemistry of neurons

On DIV13-14 primary hippocampal neuronal cultures were washed with standard saline imaging buffer (136 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 1.3 mM MgCl_2 , 10 mM glucose, 10 mM HEPES, pH 7.4) and then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), quenched with 50 mM NH_4Cl in PBS, permeabilised in 0.1% TritonX100 in 1% bovine serum albumin (BSA) in PBS, and blocked in 1% BSA in PBS as previously described (Baker et al., 2018). The cells were incubated with mouse anti- α -synuclein (1:500, BD Biosciences, 610787) and chicken anti-GFP (which also recognises pHluorin, a modified GFP; 1:2000, Abcam, ab13970) primary antibodies diluted in 1% BSA in PBS for 1.5-2 hours in a dark humidification chamber at room temperature. Cells were then incubated with either goat anti-chicken Alexa Fluor 488 (1:500, Invitrogen, A11039) or goat anti-chicken DyLight 550 (1:500 Invitrogen, SA5-10071), and either donkey anti-mouse DyLight 550 (1:200, Invitrogen, SA5-10167) or donkey anti-mouse Alexa Fluor 647 (1:500, Invitrogen, A31571) secondary antibodies diluted in 1% BSA in PBS for 1 hour (with three PBS washes after each incubation). Immunolabelled coverslips were washed in Milli-Q® Type 1 ultrapure H_2O , dried, and mounted on microscope slides with Dako Fluorescence Mounting Medium.

Fluorescence imaging of neurons

Fixed immunolabelled cells were imaged using a Zeiss Axio Observer.Z1 epifluorescence microscope and HXP 120 V mercury arc lamp. A Zeiss EC Plan Neon-Fluar 40x air objective (NA 0.75) and Zeiss Axiocam 506 mono microscope camera were used to capture 14-bit images with 2x2 binning resulting in 0.227 μm pixels. Alexa Fluor 488 was visualised through a single bandpass GFP filter (excitation 450-490 nm; emission 500-550 nm; beam splitter 495 nm). DyLight 550 was visualised through a single bandpass 43 HE DsRed filter (excitation 538-562 nm; emission 570-640 nm; beam

splitter 570 nm). Alexa Fluor 647 was visualised through a single bandpass Cy5 filter (excitation 590-650 nm; emission 662-737 nm; beam splitter 660 nm).

Live hippocampal cultures mounted in a Warner imaging chamber with embedded parallel platinum wires (RC-21BRFS) were imaged with a Zeiss Axio Observer 7 epifluorescence microscope on DIV13-15. A Zeiss Plan-Neofluar 40x oil-immersion objective (NA 1.3) and Zeiss Axiocam 506 mono microscope camera were used to capture 14-bit images with 2x2 binning resulting in 0.227 μ m pixels. pHluorin-tagged syb2/syt1/VGLUT1 was visualised using a 475 nm LED and a single bandpass 38 GFP filter (excitation 450-490 nm; emission 500-550 nm; beam splitter 495 nm) and mCherry was visualised using a 567 nm LED and a single bandpass 43 HE DsRed filter (excitation 538-562 nm; emission 570-640 nm; beam splitter 570 nm). TMR-dextran was visualised using a 567 nm LED and single bandpass Cy3 filter (excitation 545-565 nm; emission 575-604 nm; beam splitter 575 nm).

Determination of the vesicular fraction of syb2-/syt1-pHluorin was performed using a modified version of a previously described protocol (Gordon et al., 2011; Luo et al., 2021). Standard saline imaging buffer was perfused over cultures (surface fluorescence), followed by acidic imaging buffer (136 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, 20 mM MES, pH 5.5) to quench surface syb2-/syt1-pHluorin (background fluorescence), followed by a wash with standard saline imaging buffer. Alkalisising NH₄Cl imaging buffer (86 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 50 mM NH₄Cl, pH 7.4) was then perfused over cultures to reveal the total syb2-/syt1-pHluorin pool (total fluorescence).

To monitor the trafficking of syb2-/syt1-/VGLUT1-pHluorin, cultures were continuously perfused with standard saline imaging buffer (supplemented with 10 μ M CNQX and 50 μ M AP5) and stimulated with a train of either 300 action potentials at 10 Hz (30 s, 35 mA), or 400 action potentials at 40 Hz (10 s, 35 mA). Images were captured at 0.5 or 1 Hz intervals. This was followed by perfusion with alkalisising imaging buffer to reveal total syb2-/syt1-/VGLUT1-pHluorin fluorescence. Assessment of the recycling pool of synaptic vesicles and exocytosis was performed by continuous perfusion with standard saline imaging buffer (supplemented with 10 μ M CNQX, 50 μ M AP5 and 1 μ M bafilomycin A1) and stimulating with a train of 1,200 action potentials (10 Hz, 35 mA). Images were captured at 2 Hz for the first 50 s of stimulation and then at 0.5 Hz intervals for the remaining frames. This was followed by perfusion with alkalisising NH₄Cl imaging buffer to reveal total pHluorin fluorescence.

The tetramethylrhodamine-dextran (40 kDa; TMR-dextran) uptake assay was performed by stimulating mCerulean-transfected neuronal cultures with a train of 400 action potentials (40 Hz, 10 s) in the presence of TMR-dextran (50 μ M) and standard saline imaging buffer (supplemented with 10 μ M CNQX, 50 μ M AP5). Neurons were perfused with standard saline buffer for at least 3 minutes before imaging, and continuously throughout image acquisition.

Co-immunoprecipitation

HEK293 cells in a 10 cm dish were co-transfected with syb2-FLAG and WT, T81E, or T81A α -synuclein-HA plasmids using Lipofectamine3000, according to the manufacturer's instructions. 24 hours after transfection, cells were washed twice with PBS and lysed in 0.15% Triton X-100 in PBS supplemented with cComplete Mini Protease Inhibitor Cocktail (Roche). Lysates were incubated on ice for 20 minutes and clarified by centrifugation at 16,000 $\times$ g for 10 minutes at 4°C.

Equal amounts of protein (1-2 μ g) in 300 μ l lysis buffer (0.15% Triton in PBS) were incubated with either 25 μ l Pierce anti-HA magnetic beads or 25 μ l anti-FLAG M2 Magnetic Beads for 2 hours at 4°C with end-over-end rotation. Beads were washed 3 times with lysis buffer and bound proteins were eluted by boiling in Laemmli sample buffer containing 50 mM DTT for 10 min at 95°C.

Input lysates and immunoprecipitated samples were separated by SDS-PAGE using a TGX Stain-Free FastCast Acrilamide kit (Bio-Rad) and transferred to 0.22 μ m PVDF membranes using iBlot2 Transfer System (ThermoFisher Scientific). Membranes were blocked in 3% (w/v) BSA in PBST and incubated with either mouse anti- α -synuclein (1:2000 BD Biosciences, Cat # 610787) or rabbit anti-synaptobrevin-2 (1:2000 Abcam, Cat # ab3347). Following incubation with either Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (1:2000 ThermoFisher Scientific, Cat # 65-6120) or Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (1:2000 ThermoFisher Scientific, Cat # 31430), immunoreactive bands were visualized with Clarity Western ECL substrate (Bio-Rad) chemiluminescence.

Quantification and statistical analysis

Mass spectrometry quantification of synuclein phosphopeptides

The raw LC-MS/MS data was processed with MaxQuant v1.5.8.3 (Tyanova et al., 2016) using the following settings. Variable modifications were oxidation (M), acetyl (protein N-

terminus), deamidation (NQ) and phosphorylation (STY). Carbamidomethyl was a fixed modification. Digestion was set to specific for LysC and GluC with a maximum of 3 missed cleavages. The *Mus musculus* reference proteome with canonical and isoform sequences downloaded Aug 6, 2019, was used as well as the inbuilt contaminants file. Minimum peptide length was 5 and maximum peptide mass was 7000 Da. Second peptides and dependent peptides searches were enabled. Peptide spectrum matching and protein false discovery rates were set at 1%. Minimum score for modified peptides was 40. Modified peptides and counterpart non-modified peptides were excluded from protein quantification. All other parameters were default. The TMT reporter ion intensities from the MaxQuant output file "Phospho (STY)Sites.txt" were used to determine relative phosphorylation levels at sites within the phosphopeptides. All phosphorylation sites were localised with a certainty >75%. Since there was variation in the protein content (Figure S1), each phosphopeptide intensity was corrected. The phosphopeptide intensity was normalised using the summed peptide intensity for the corresponding protein. The KCI-treated intensity was divided by the control, log2 transformed and comparisons were made via multiple unpaired t-tests and then adjusted for multiple hypotheses using the false discovery rate approach (5%, two-stage linear step-up procedure (Benjamini et al., 2006)) using GraphPad Prism 10.2.0. Note that WT and KO experiments were independent experiments since they were separate TMT6plex experiments (Figure 1A). However, the same approximate amount of protein was processed in parallel on the same days and analysed sequentially by HILIC and LC-MS/MS using the same columns and mass spectrometer, within a matter of days. The KO samples were always analysed first to avoid carry over of α -synuclein peptides.

Mass spectrometry data availability

The raw data files and search output for the targeted LC-MS/MS for WT and KO monophosphopeptide fractions 24-27 have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD049095.

The raw data for the remaining fractions are not supplied since they are intended to be explored and reported in a separate publication. The signal for α -synuclein phosphorylated at Thr81 (pThr81), T(ph)VEGAGNIAAATGFVK, was not detected by targeted acquisition. The quantitative data for pThr81 was from the data-dependent LC-MS/MS experiment. The data-dependent LC-MS/MS data was processed by Proteome Discover software (version 2.0.0.802) in conjunction with Mascot 2.6.2 by Matrix Science to obtain the annotated MS/MS spectrum for T(ph)VEGAGNIAAATGFVK that is provided

in Figure S2. Mascot settings were: LysC/GluC proteolysis; 1 missed cleavage allowed; fixed modifications were carbamidomethyl (C), TMT6plex (N-terminal) and TMT6plex (K); variable modifications were deamidation (NQ), oxidation (M) and phosphorylation (STY); peptide mass tolerance was 10 ppm; fragment mass tolerance was 0.01 Da; instrument type was electrospray ionisation-Fourier transform ion cyclotron resonance and the data was searched against the *Mus musculus* proteome from the May 2019 version of SwissProt with canonical isoforms only.

Fluorescence data quantification

Images were processed offline using ImageJ 1.52g software. To correct for slight XY-plane drift over the time series, stack registration was performed by rigid body alignment using the MBGReg ImageJ plugin (Donal Stewart, Edinburgh, UK), a custom variant of the FIJI StackReg plugin. Quantification of presynaptic protein expression in immunolabelled neurons was performed as previously described (Baker et al., 2018) and was calculated as a fluorescence ratio of transfected/non-transfected protein expression levels (with background fluorescence subtracted). Transfected nerve terminals were identified by visualisation of syt1-pHluorin enhanced by anti-GFP immunolabelling. The diffuseness of fluorescence along axons was determined by calculating the coefficient of variation (CV) as previously described (Gordon and Cousin, 2013; Baker et al., 2018). For CV analyses, 'n' refers to an individual field of view.

For fluorescence imaging, circular regions of interest (ROIs) with a diameter of 10 pixels were placed over presynaptic terminals using the Time Series Analyzer V3 ImageJ plugin, and the total fluorescence intensity was measured for each frame. The vesicular fraction of syb2-/syt1-pHluorin as a percentage of total pHluorin was calculated using the equation: $[\text{maximum} - \text{baseline fluorescence}] / [\text{maximum} - \text{background fluorescence}] \times 100$. For the protein/vesicle trafficking and exocytic assays, background fluorescence decay was corrected with the ImageJ Bleach Correction plugin (v2.0.3-SNAPSHOT) (Schindelin et al., 2016), using ≥ 20 ROIs overlaying areas in the field of view containing no cells, the "exponential decay" setting and all image frames prior to NH_4Cl imaging buffer perfusion. An in-house screening tool (Holly Melland) was used to manually screen the fluorescence time trace of each ROI for genuine responses. ROIs were only included if fluorescence increased from baseline upon electrical stimulation and reached maximum fluorescence during perfusion with alkylising NH_4Cl buffer. Only coverslips with greater than 20 responsive ROIs were included in the final data. The pHluorin fluorescence change over time was calculated as $\Delta F/F_0$ (change in fluorescence from

baseline) and for each frame of the time series, fluorescence values were averaged for all ROIs in a field of view. Time courses of mean $\Delta F/F_0 \pm$ SEM of pHluorin fluorescence were then normalised to either maximal fluorescence (in presence of alkylating NH_4Cl buffer) or peak fluorescence induced by stimulation, as indicated. The mean initial rate of fluorescence change was calculated from linear fit of $\Delta F/F_0$ per second over the first 10 s (for 10Hz) or 4 s (for 40Hz) of stimulation. The time constant (τ) values were determined by fitting one-phase exponential curves to each time course, either over the entire time course of stimulation (for exocytosis) or after cessation of stimulation (for decay). The size of the recycling pool was calculated as the maximal $\Delta F/F_0$ evoked by 1200 action potentials (in the presence of bafilomycin), as a percentage of total fluorescence in alkylating NH_4Cl buffer.

For the dextran uptake assay, dextran puncta within a single view were manually counted along multiple lengths of axon and the average number of puncta per 100 μm of axon was calculated. Axons were identified by visualisation with mCerulean and axon length was measured in ImageJ using the Measure function on freehand line ROIs manually drawn along axons.

Statistical Analyses of fluorescence imaging

Statistical analyses were performed using GraphPad Prism Version 10.2.0 and Microsoft® Excel software. Each data set was tested for normality using the Shapiro-Wilk test and statistical analyses were performed accordingly. For data conforming to normal distribution, the following analyses were used: one-way ANOVA with Sidak's multiple comparison test was used to compare all data to empty vector (EV), and phosphovariants to WT; one-way ANOVA with Dunnett's multiple comparison test was used to compare phosphovariants to WT; and one-way ANOVA with Tukey's multiple comparison test was used to compare between WT, T81A and T81E (i.e. to compare between all variants). For data that did not conform to normal distribution (exocytic τ), non-parametric Kruskal-Wallis test with Dunn's multiple comparisons was used to compare between WT, T81E and T81A (i.e. to compare between all variants). Time courses of change in fluorescence were analysed via two-way repeated measures ANOVA or repeated measures mixed-effects model with Tukey's multiple comparisons post-hoc test, to compare between all variants (i.e. WT, T81E and T81A) at every individual time point. For all analyses, $P < 0.05$ was considered statistically significant where the degree of significance is represented by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All data are displayed as mean $\pm$ SEM, and individual data points are

shown as diamonds and indicated in figure legends. For fixed experiments 'n' refers to an individual field of view and 1-2 fields of view were analysed for each independent coverslip. For live experiments (vesicular protein, trafficking, exocytosis and dextran uptake assays) 'n' refers to an individual field of view from an independent coverslip unless otherwise stated. No data that met inclusion criterion has been excluded from this study. Experimenters were blinded to the variant for image acquisition and analysis for fixed assays, and at the image analysis stage for live imaging. No strategy for randomisation of samples has been followed, and sample sizes were not chosen based on pre-specified effect sizes. Instead, several independent experiments were performed across multiple independent cultures, with all experiments repeated across at least three independent cultures (and each culture comprising at least three embryos), with the exception of the α -synuclein expression and localisation experiment which was performed across two independent cultures. To minimise confounds, controls (WT and empty vector as appropriate) were performed in each batch of experiments alongside phosphovariants.

RESULTS

Identification of activity-dependent phosphorylation sites in α -synuclein

To determine the phosphorylation sites of α -synuclein that are altered upon neuronal depolarisation, cultured cortical neurons from wild-type (WT) and α -synuclein knockout (KO) mice were either mock-stimulated or depolarised with 60 mM KCl for 10 s, a stimulus duration previously shown to induce activity-dependent phosphorylation of presynaptic proteins (Engholm-Keller et al., 2019). Lysates from these samples were sequentially digested with LysC and GluC to produce peptides that favoured the detection of synuclein phosphorylation sites (Figure S1) (Schmid et al., 2013). The peptides were analysed using a quantitative phosphoproteomics approach involving phosphopeptide enrichment and fractionation by hydrophilic interaction chromatography (Engholm-Keller and Larsen, 2016). Protein phosphorylation site levels from mock (control) and KCl-depolarised neurons were compared using the reporter ions from tandem mass tag (TMT) 10plex derivatisation of samples originating from WT and α -synuclein KO mice (Figure 1A). Using information from preliminary analysis of the samples by standard data-dependent acquisition LC-MS/MS (Figure S1), all detectable α -synuclein phosphopeptides in both the WT and KO samples were then targeted in a subsequent LC-MS/MS analysis that exclusively monitored a selection of precursors ions. This included two positive controls for KCl-depolarisation: strongly down-regulated

microtubule affinity-regulating kinase 2 (MARK2) Ser453 and strongly up-regulated protocadherin-1 Ser880 (Engholm-Keller et al., 2019). Both positive controls had a similar response in the WT and KO samples (Figure 1B).

Four α -synuclein phosphorylation sites were identified: Ser129, Ser122, Thr81 and Ser42. Phosphorylation of the Ser129 site of α -synuclein was not significantly altered upon depolarisation (Figure 1B). The Ser122 phosphorylation site has not previously been detected in mice and is not conserved in human α -synuclein (Wu et al., 2008), and was comparable in depolarised and control samples (Figure 1B). However, Thr81, a recently identified phosphorylation site (Schweighauser et al., 2020), was up-regulated in response to depolarisation (Figure 1B). None of these three α -synuclein-specific sites were detected in the KO samples (Figure 1B). The Ser42 peptide is present in both α - and β -synuclein; phosphorylation of Ser42 increased in both the depolarised WT and KO samples in comparison to their respective control samples. However, the signal for the precursor ion of the phosphorylated Ser42 (pSer42) peptide was five-fold lower in the KO sample (Figure S1). The same protein amount was used in both the WT and KO workflows, which indicates that the bulk of the pSer42 originated from α -synuclein. The depolarisation-induced up-regulation of pSer42 was lower in magnitude in the KO sample (Figure 1B), but this difference was not significant when adjusted for multiple hypotheses ($P = 0.04$, $Q = 0.13$, 5% false discovery rate). Overall, we identified Ser42 and Thr81 as α -synuclein phosphorylation sites sensitive to depolarisation, and phosphorylation of Ser42 on β -synuclein also occurs at a lower abundance.

Phosphorylation of α -synuclein does not influence expression or targeting of the protein

To determine the impact of the activity-dependent phosphorylation at Ser42 and Thr81 on the localisation and function of α -synuclein, amino acid substitutions that mimic phosphorylation (to glutamate; S42E and T81E) or abolish phosphorylation (to alanine; S42A and T81A) were introduced into the coding sequence of human α -synuclein at these residues (Figure 1C). We first assessed whether the phosphomutations affected protein synaptic protein levels and trafficking. Cultured wild-type hippocampal neurons co-transfected with synaptotagmin-1 (synt1)-pHluorin (as a marker for transfected nerve terminals) and an α -synuclein phosphomutant or empty vector (EV) were immunolabelled for GFP (which also recognises pHluorin, thus enhancing pHluorin fluorescence) and α -synuclein. The level of α -synuclein in presynaptic terminals transfected with the empty vector construct was approximately equal to endogenous α -

synuclein in non-transfected terminals (Figure 2B, EV 0.942 ± 0.057). Neurons transfected with phosphomutants exhibited α -synuclein levels approximately 2.3- to 2.5-fold higher than non-transfected cells, and were comparable to those transfected with wild-type (WT) α -synuclein (Figure 2B; WT 2.276 ± 0.064 ; S42E 2.334 ± 0.104 ; S42A 2.548 ± 0.110 ; T81E 2.433 ± 0.161 ; T81A 2.400 ± 0.113 ; all $P < 0.0001$ compared to EV, $P > 0.05$ for phosphovariants compared to WT). Therefore, introduction of α -synuclein variants into α -synuclein KO cells would be expected to result in α -synuclein protein levels approximately 1.3 to 1.5-fold that of endogenous levels (in non-transfected wild-type neurons).

We next established whether phosphomutations in α -synuclein influence the targeting of the protein to presynaptic terminals. α -Synuclein knockout (KO) hippocampal neurons were transfected with α -synuclein variants and immunolabelled for α -synuclein. WT α -synuclein displayed a punctate distribution along axons, indicative of enrichment at nerve terminals, and all α -synuclein phosphomutants displayed a similar profile to the WT protein (Figure 2C). To confirm this, the coefficient of variation (CV) of α -synuclein immunofluorescence along axons was calculated to quantify the relative enrichment of α -synuclein phosphomutants at presynaptic terminals. Comparable CV values were observed across the cohort (Figure 2D; WT 60.13 ± 2.81 ; S42E 58.88 ± 3.52 ; S42A 59.83 ± 2.79 ; T81E 55.68 ± 1.97 ; T81A 54.28 ± 2.61 ; $P > 0.05$ compared to WT). Therefore, phosphomutations in α -synuclein do not impact the localisation of the protein at the presynaptic terminal.

Ablating phosphorylation of Thr81 in α -synuclein specifically impacts the distribution of synaptobrevin-2, but not synaptotagmin-1, between membrane compartments.

α -Synuclein directly binds to synaptobrevin-2 (Burré et al., 2010; Sun et al., 2019), and the copy number of vesicle-associated α -synuclein matches that of vesicular synaptobrevin-2 (Takamori et al., 2006; Fakhree et al., 2016), suggesting a one-to-one relationship between these molecules. There is a reduction in vesicular synaptobrevin-2 in $\alpha\beta\gamma$ -synuclein triple knockout (KO) synapses (Vargas et al., 2014), supporting that α -synuclein is important for the trafficking of synaptobrevin-2. Therefore, we next examined whether the subcellular localisation of synaptobrevin-2 is controlled by phosphorylation of α -synuclein. Integral synaptic vesicle cargo proteins such as synaptobrevin-2 are retrieved and recycled at synapses from the plasma membrane back to synaptic vesicles and recycling endosomes. The trafficking of these proteins to and from the cell surface

can be tracked by tagging their luminal domains with the pH-sensitive green fluorescent protein pHluorin. pHluorin fluorescence is quenched by the acidic environment inside vesicles, and unquenched when exposed to the neutral pH of the extracellular environment (Figure 3A). Perfusion with NH_4Cl deacidifies vesicles and reveals total pHluorin fluorescence. Thus, sequential perfusion of neuronal cultures with neutral, acidic, and alkaline buffers can be used to reveal the fraction of vesicular cargo proteins localised intracellularly on vesicles versus on the plasma membrane, relative to the total protein pool (Figure 3B). We combined this approach with a molecular replacement strategy whereby endogenous α -synuclein was replaced with phosphomutant α -synuclein via transfection of α -synuclein KO hippocampal neurons with α -synuclein variants.

The proportion of intracellular synaptobrevin-2 (syb2)-pHluorin was similar in the presence of most α -synuclein phosphomutants compared to WT α -synuclein. (Figure 3C; WT $48.79\% \pm 1.68$; S42E $51.22\% \pm 1.93$; S42A $51.62\% \pm 1.98$; T81E $49.15\% \pm 1.72$; all $P > 0.05$ compared to WT). However, in the presence of T81A α -synuclein there was a significant enrichment in internal synaptobrevin-2 (Figure 3C; T81A $59.69\% \pm 1.65$; $P = 0.0002$). To ascertain whether this change in distribution across membrane compartments was affecting other synaptic vesicle proteins, we repeated the assay using pHluorin tagged to the vesicular calcium sensor synaptotagmin-1. However, no significant differences were observed between neurons expressing WT, T81E or T81A α -synuclein (Figure 3D; WT $52.94\% \pm 3.03$; T81E $50.04\% \pm 2.90$; T81A $44.97\% \pm 1.74$; all $P > 0.05$ compared to WT). As such, these changes are not occurring at the level of the synaptic vesicle; ablating phosphorylation of α -synuclein at Thr81 specifically increases the proportion of syb2-pHluorin on internal membranes (i.e. synaptic vesicles and/or endosomes) but not other synaptic vesicle proteins such as synaptotagmin-1 (syt1)-pHluorin.

Trafficking of synaptobrevin-2 during neuronal activity is perturbed by ablating phosphorylation at Thr81 in α -synuclein

Increased vesicular syb2-pHluorin may result from changes to the activity-dependent trafficking of the protein (Gordon et al., 2011). To investigate this possibility, we co-transfected α -synuclein KO hippocampal neurons with syb2-pHluorin and either WT, T81E or T81A α -synuclein, stimulated them with a train of 300 action potentials (10 Hz, 35 mA) and measured changes in syb2-pHluorin fluorescence over time (Figure 4A). pHluorin fluorescence increases as vesicles undergo fusion and the pHluorin moiety gets exposed to the neutral extracellular environment, and then decreases with endocytic retrieval of vesicles and protein cargo and the reacidification of vesicles (Figure 3A).

We observed that the relative change in syb2-pHluorin fluorescence over time was significantly reduced in the presence of T81A α -synuclein compared to both WT and T81E α -synuclein (Figure 4A; $P < 0.05$ T81A vs. WT at 12-62 and 66-82 seconds, $P < 0.05$ T81A vs. T81E at 10-62 seconds). Correspondingly, the peak amplitude of syb2-pHluorin fluorescence induced by neuronal stimulation was significantly decreased in the presence of T81A α -synuclein compared to T81E α -synuclein, and T81A α -synuclein also trended towards a lower peak height than WT (Figure 4B; WT 0.3453 ± 0.0168 ; T81E 0.3759 ± 0.0235 ; T81A 0.2782 ± 0.0138 ; $P = 0.0503$ for T81A vs. WT, $P = 0.002$ for T81A vs. T81E). Furthermore, the initial rate of fluorescence increase within the first 10 s of stimulation was significantly slower in the presence of T81A α -synuclein compared to both WT and T81E α -synuclein (Figure 4C; WT $0.0164 \Delta F/F_0 \text{ s}^{-1} \pm 0.0014$; T81E $0.0192 \Delta F/F_0 \text{ s}^{-1} \pm 0.0012$; T81A $0.0118 \Delta F/F_0 \text{ s}^{-1} \pm 0.0007$; $P = 0.0179$ for T81A vs. WT, $P < 0.0001$ for T81A vs. T81E). Thus, ablating phosphorylation of α -synuclein at Thr81 alters the trafficking of syb2-pHluorin during stimulation.

Exocytosis is not impacted by ablating phosphorylation of α -synuclein at Thr81

The altered activity-dependent trafficking of syb2-pHluorin (slower rise time and lower peak amplitude, Figure 4) could be driven by several factors, including: 1) reduced availability of vesicles, 2) impaired fusion of vesicles, 3) increased rate of synaptic vesicle endocytosis, or 4) increased retrieval of synaptobrevin-2 to endocytosing vesicles during stimulation. We thus first investigated whether T81A α -synuclein slowed and/or reduced synaptic vesicle exocytosis. α -Synuclein KO hippocampal neurons co-transfected with α -synuclein variants and syb2-pHluorin were stimulated with a train of 1200 action potentials (10 Hz, 35 mA) in the presence of vATPase-inhibitor bafilomycin and changes in syb2-pHluorin fluorescence were observed over time (Figure 4D). Bafilomycin blocks vesicle reacidification and therefore the increasing pHluorin fluorescence specifically reports on cumulative exocytosis.

To examine whether α -synuclein phosphomutants reduce vesicle availability, we measured the size of the entire recycling pool of vesicles (i.e. those mobilised by the end of the stimulus train) as a proportion of total vesicles. The recycling pool of vesicles was similar in the presence of all α -synuclein variants (Figure 4E; WT $38.15\% \pm 2.50$; T81E $43.61\% \pm 1.00$; T81A $39.65\% \pm 1.27$; $P > 0.05$ for all comparisons).

We then ascertained whether there were changes in the rate at which recycling pool vesicles are mobilised. There was no difference in fluorescence change across the entire time course of stimulation (Figure 4D; $P > 0.05$ across all time points). Moreover, there

was no difference in either the initial rate of exocytosis during the first 10 s of stimulation (Figure 4F; WT $0.0086 \Delta F/F_0 s^{-1} \pm 0.0017$; T81E $0.0098 \Delta F/F_0 s^{-1} \pm 0.0011$; T81A $0.0080 \Delta F/F_0 s^{-1} \pm 0.0010$; $P > 0.05$ for all comparisons) or the overall exocytic τ time constant (Figure 4G; WT $43.04 s \pm 6.38$; T81E $35.84 s \pm 3.40$; T81A $48.57 s \pm 9.71$; $P > 0.05$ for all comparisons) between any of the α -synuclein variants. These data demonstrate that the ablation of α -synuclein phosphorylation at Thr81 does not reduce vesicle availability or impair exocytosis but must instead modulate endocytosis or protein trafficking during stimulation.

Synaptotagmin-1 trafficking is unaffected by α -synuclein phosphomutants

To determine whether α -synuclein phosphomutants alter global endocytosis, we co-transfected α -synuclein KO hippocampal neurons with α -synuclein variants and synaptotagmin-1 (synt1)-pHluorin. Synaptotagmin-1 is another integral synaptic vesicle protein and thus acts as an independent marker of synaptic vesicle dynamics. Neurons were stimulated with a train of 300 action potentials (10 Hz, 35 mA) and changes in synt1-pHluorin fluorescence over time were measured. Unlike what was observed with synt2-pHluorin, no differences in the change in fluorescence for synt1-pHluorin were observed between conditions across any time points (Figure 5A; $P > 0.05$ for all comparisons). Moreover, there were no differences in either peak amplitude of synt1-pHluorin fluorescence induced by stimulation (Figure 5B; WT 0.2515 ± 0.0173 ; T81E 0.2664 ± 0.0221 ; T81A 0.2396 ± 0.0188 ; $P > 0.05$ for all comparisons), or the initial rate of fluorescence increase within the first 10 s of stimulation (Figure 5C; WT $0.0164 \Delta F/F_0 s^{-1} \pm 0.0022$; T81E $0.0189 \Delta F/F_0 s^{-1} \pm 0.0022$; T81A $0.0174 \Delta F/F_0 s^{-1} \pm 0.0019$; $P > 0.05$ for all comparisons) between any of the variants. Furthermore, the τ time constant of post-stimulation endocytic retrieval of synt1-pHluorin was similar between α -synuclein variants (Figure 5D; WT $33.84 s \pm 4.15$; T81E $24.60 s \pm 2.79$; T81A 28.46 ± 5.60 ; $P > 0.05$ for all comparisons). Similar results were observed when pHluorin was tagged to another synaptic vesicle protein, VGLUT1 (Figure S3). Therefore, phosphorylation of α -synuclein at Thr81 specifically impacts the trafficking of synaptobrevin-2 but not the retrieval of other synaptic vesicle proteins, suggesting that endocytosis *per se* is not impacted.

Phosphorylation of α -synuclein at Thr81 augments post-stimulation retrieval of synaptobrevin-2

Neurons expressing T81A α -synuclein have a faster rate of endocytic retrieval of synaptobrevin-2 during stimulation, causing a decreased peak syb2-pHluorin fluorescence (Figure 4B) and slower initial rate of fluorescence increase (Figure 4C). To better visualise whether these changes in synaptobrevin-2 trafficking continue following the cessation of stimulation, syb2-pHluorin fluorescence time course traces were normalised to peak amplitude during stimulation (Figure 6A, same source data as Figure 4A). One-phase exponential decay curves were fit to the fluorescence traces following the end point of 300 action potentials of stimulation (10 Hz, 35 mA; Figure 6B). Intriguingly, the syb2-pHluorin post-stimulation endocytic retrieval time constant τ was lower in the presence of T81E α -synuclein in comparison to T81A α -synuclein (Figure 6B; WT $58.05 \text{ s} \pm 2.41$; T81E $46.33 \text{ s} \pm 5.53$; T81A $64.43 \text{ s} \pm 5.97$; $P = 0.0416$ for T81E vs. T81A). This indicates that while endocytic retrieval of synaptobrevin-2 is faster in the presence of T81A α -synuclein during stimulation, following the cessation of stimulation the endocytic retrieval of synaptobrevin-2 is faster in the presence of T81E α -synuclein.

To further tease apart how synaptobrevin-2 retrieval is impacted by α -synuclein phospho-variants, we stimulated neurons with a short, high frequency train of action potentials (40 Hz, 400 action potentials, 35 mA) known to upregulate activity-dependent bulk endocytosis, a form of endocytosis which is strongly temporally linked to stimulation (Clayton et al., 2008). Under these conditions, the recycling of synaptobrevin-2 was similar in the presence of WT and T81A α -synuclein (Figure 6C; $P > 0.05$ across all time points), displaying similar stimulus-induced peak amplitudes of syb2-pHluorin fluorescence (Figure 6D; WT 0.3242 ± 0.033 ; T81A 0.2864 ± 0.034 ; $P > 0.05$) and initial rate of fluorescence change (Figure 6E; WT 0.0388 ± 0.0044 ; T81A 0.0317 ± 0.0041 ; $P > 0.05$). In contrast, synaptobrevin-2 trafficking was altered in the presence of T81E α -synuclein (Figure 6C; $P < 0.05$ T81E vs. WT at 10-20 seconds, $P < 0.05$ T81E vs. T81A at 5 and 10-61 seconds), with synaptobrevin-2 retrieval during stimulation being delayed, as evidenced by a larger peak amplitude in syb2-pHluorin fluorescence (Figure 6D; T81E 0.4549 ± 0.036 ; $P = 0.0326$ vs. WT, $P = 0.0075$ vs T81A) and higher rate of fluorescence change (Fig 6E; T81E $0.0571 \Delta F/F_0 \text{ s}^{-1} \pm 0.0054$; $P = 0.0306$ vs. WT, $P = 0.0036$ vs. T81A). This large increase in syb2-pHluorin peak amplitude was not due to an increase in evoked exocytosis upon 40 Hz stimulation (Figure S4). To better visualise the impacts to synaptobrevin-2 trafficking continue following the cessation of stimulation, syb2-pHluorin fluorescence time course traces were normalised to peak amplitude evoked by stimulation (Figure 6F). Synaptobrevin-2 retrieval following stimulation was clearly faster in the presence of T81E α -synuclein (Figure 6F; $P < 0.05$ T81E vs. WT and T81A over time points indicated), and post-stimulation syb2-pHluorin retrieval time constant τ was significantly lower in the presence of T81E α -synuclein compared to T81A α -synuclein

(Figure 6G; WT $84.34 \text{ s} \pm 5.68$; T81A $124.6 \text{ s} \pm 28.52$; T81E $57.50 \text{ s} \pm 8.89$; $P = 0.0184$ T81E vs. T81A). The change in synaptobrevin-2 trafficking was not due to a change in global bulk endocytosis, as there was a similar uptake of 40 kDa TMR-dextran, which labels bulk endosomes, in the presence of all α -synuclein variants (Figure 6H-I; WT 2.54 dextran puncta per $100 \mu\text{m}$ of axon ± 0.25 ; T81A 3.42 ± 0.54 ; T81E 3.34 ± 0.31 ; $P > 0.05$ for all comparisons).

Finally, we explored whether phosphorylation of α -synuclein at T81 impacts the binding of the protein to synaptobrevin-2. HEK293 cells were transfected with HA-tagged α -synuclein variants and syb2-FLAG. Syb2-FLAG was co-immunoprecipitated alongside all α -synuclein variants when incubated with anti-HA beads (Figure 6J, S5 and S6), and all α -synuclein variants were co-immunoprecipitated when syb2-FLAG was pulled down (Figure S6), suggesting that phosphovariants are all able to bind to synaptobrevin-2.

DISCUSSION

Here, we provide the first evidence of a new function for α -synuclein modulated by the activity-dependent phosphorylation of Thr81. We identified two phosphorylation sites on α -synuclein - Ser42 and Thr81 - that become more highly phosphorylated upon brief neuronal depolarisation. Ser42 was first reported as an activity-dependent phosphorylation site in a phosphoproteomic screen of neurons (Engholm-Keller et al., 2019). Both Ser42 and Thr81 were recently identified as physiological phosphorylation sites of protein kinase R (Reimer et al., 2022), and have been detected in human post-mortem brain tissue (Schweighauser et al., 2020; Zhang et al., 2023). However, the functional and mechanistic relevance of phosphorylation of α -synuclein at these sites has never been explored. We establish here that α -synuclein modulates the trafficking of synaptobrevin-2 in a phosphorylation-dependent manner.

Interestingly, we observed no change in pSer129 within 10 seconds of KCl-induced depolarization in cortical cultures. Ser129 can undergo phosphorylation in response to neuronal activity, albeit over a longer time course than in this study; pSer129 levels were elevated in neuronal cultures following 15-minute incubation with picrotoxin, 3-hour incubation with 4-aminopyridine, or 60 seconds of 10 Hz electrical stimulation (Parra-Rivas et al., 2023; Ramalingam et al., 2023). Future work should establish the time course and level of neuronal activity required to potentiate Ser129 phosphorylation.

Syb2-pHluorin allows us to monitor synaptobrevin-2 trafficking in dynamic flux as it moves between the plasma membrane and vesicles. Ablating Thr81 phosphorylation reduced both the maximum amplitude and rate of increase of syb2-pHluorin fluorescence

during moderate (10 Hz) stimulation. This was not associated with impacts to recycling pool size or exocytosis (at 10 Hz or 40 Hz), or changes to global endocytosis *per se*. Instead, these changes must be driven by a faster rate of retrieval/trafficking of synaptobrevin-2 to endocytosing vesicles. The enhanced retrieval of synaptobrevin-2 by phosphonull T81A α -synuclein specifically occurred during the stimulus train, while the phosphomimetic variant T81E accelerated the post-stimulus retrieval of synaptobrevin-2. Together, this suggests that phosphorylation of α -synuclein acts as a modulatory switch to direct trafficking of synaptobrevin-2 to newly endocytosing vesicles/endosomes in an activity-dependent manner.

Multiple modes of endocytosis contribute to the retrieval of proteins and lipid membrane for the renewal of synaptic vesicle pools, occurring on varied timescales and requiring different facilitatory proteins (Soykan et al., 2017; Chanaday and Kavalali, 2018; Chanaday et al., 2019). Ultrafast endocytosis (UFE) is initiated within 50-100 ms of an action potential, scales with the extent of exocytosis and terminates soon after stimulation stops or once UFE capacity is reached within a synapse (Watanabe et al., 2013; Delvendahl et al., 2016). Activity-dependent bulk endocytosis (ADBE), involving the internalisation of large areas of plasma membrane from which new synaptic vesicles can then bud, is activated during stimulation, upregulated with increasing stimulus intensity, and terminates when stimulation stops (Clayton et al., 2008; Watanabe et al., 2014). In contrast, single-vesicle endocytosis including clathrin-mediated and clathrin-independent endocytosis occurs on a timescale of multiple seconds and persists even after the cessation of stimulation (Kim and Ryan, 2009; Kononenko et al., 2014; Kononenko and Haucke, 2015; Soykan et al., 2017; López-Hernández et al., 2022).

We propose that phosphorylation of α -synuclein modulates the trafficking of synaptobrevin-2 to these different endocytic pathways. However, global endocytosis itself is not impacted, as syt1- and VGLUT1-pHluorin trafficking and dextran uptake are similar in the presence of phosphomimetic and phosphonull α -synuclein (Figures 5, 6 and S3). T81A α -synuclein accelerates synaptobrevin-2 endocytic retrieval specifically during stimulation, suggesting that Thr81-dephosphorylated α -synuclein supports synaptobrevin-2 being retrieved via UFE and/or ADBE. Correspondingly, since T81E α -synuclein accelerates synaptobrevin-2 endocytic retrieval only after stimulation, Thr81-phosphorylated α -synuclein may promote synaptobrevin-2 retrieval via single-vesicle endocytosis (Figure 7). In support of this, under conditions that potentiate ADBE, synaptobrevin-2 retrieval is similar in the presence of both T81A and WT α -synuclein; in contrast, T81E α -synuclein delays synaptobrevin-2 endocytic retrieval during high frequency stimulation and increases retrieval after stimulation.

Interestingly, during moderate 10 Hz stimulation, synaptobrevin-2 trafficking in the presence of WT α -synuclein (which is able to undergo dynamic phosphorylation/dephosphorylation) more closely resembles that of phosphomimetic rather than phosphonull α -synuclein (Figure 4A), suggesting that under these conditions a greater proportion of α -synuclein is phosphorylated than dephosphorylated at T81. In contrast, under high frequency 40Hz stimulation synaptobrevin-2 retrieval in the presence of WT α -synuclein is more similar to phosphonull α -synuclein (Figure 6C). This supports that phosphorylation of α -synuclein at T81 is indeed highly dynamic and activity-dependent, which likely helps ensure an appropriate copy number of synaptobrevin-2 is being retrieved via different modes of endocytosis. The specific mechanism by which this occurs remains unclear; we hypothesise that Thr81 phosphorylation modulates the biochemical properties of α -synuclein to confer this regulatory function.

One possibility is that phosphorylation may impact membrane affinity of α -synuclein. Thr81 is within the membrane-associated region (Figure 1C) on the lipid-binding face of α -synuclein (Jao et al., 2008), and phosphomimetic mutations to this region can weaken binding to synaptic vesicles (Reimer et al., 2022). Phosphorylation of α -synuclein may also impact its binding to synaptobrevin-2 or other third-party proteins. α -Synuclein binds directly to synaptobrevin-2 (Burré et al., 2010); we demonstrate here that all Thr81 phosphomutants are able to co-immunoprecipitate synaptobrevin-2 (Figures 6, S5 and S6), suggesting that these proteins can maintain their interaction under different phosphorylation states.

Intrinsic synaptic vesicle proteins such as synaptobrevin-2 have distinct trafficking partners to support their retrieval (Gordon and Cousin, 2016). Synaptobrevin-2 lacks classical recognition motifs for AP-2; instead, several protein binding partners have been shown to support the recycling and retrieval of synaptobrevin-2 to endocytosing vesicles, including synaptophysin (Gordon et al., 2011; Gordon and Cousin, 2014; Kokotos et al., 2019) and the monomeric adaptor protein AP180/CALM (Koo et al., 2011; Koo et al., 2015). α -Synuclein also co-immunoprecipitates with AP180/CALM, and this interaction is upregulated following stimulation (Vargas et al., 2014). Phosphorylation of α -synuclein may alter the relative binding affinity between α -synuclein, synaptobrevin-2, and these other binding partners to thereby direct trafficking of the protein. α -synuclein has been observed to co-localise with AP180 and clathrin in concentric rings on the plasma membrane (Vargas et al., 2023). Related to this, α -synuclein may also promote nanoclustering of synaptobrevin-2 on the cell surface. For example, SV2A regulates the retrieval of synaptotagmin-1 in a phosphorylation-dependent manner (Zhang et al., 2015; Harper et al., 2020) by forming cell-surface nanoclusters that control its availability to the

endocytic machinery (Small et al., 2024). Future studies will investigate how phosphorylation of α -synuclein impacts these interactions. Identification of the molecular mediators of activity-dependent phosphorylation of α -synuclein is also needed. Both Ser42 and Thr81 can be phosphorylated by protein kinase R and dephosphorylation is inhibited by the protein phosphatase 1/2A inhibitor okadaic acid (Reimer et al., 2022), but whether these are the native enzymes for these sites in neurons is unclear.

α -Synuclein has been suggested to regulate synaptic plasticity (Sharma and Burré, 2023), and the modulatory mechanism described in this study may have knock-on consequences for presynaptic physiology and plasticity over time that will be explored in future work. Distinct endocytic pathways repopulate different pools of vesicles (Cheung et al., 2010). Since phosphorylation of α -synuclein at Thr81 seemingly modulates the pathway by which synaptobrevin-2 is retrieved (Figure 7), this represents a mechanism that could define the distribution of this crucial vesicular SNARE protein across different pools and thus define the fusion properties of these respective pools. Interestingly, there was an increase in the vesicular fraction of synaptobrevin-2 observed in the presence of T81A α -synuclein. This suggests that alterations to synaptobrevin-2 trafficking may indeed result in changes to the molecular physiology of vesicles, representing a form of presynaptic plasticity. We note that we use relatively strong trains of stimuli (10 or 40 Hz) to evoke synaptic vesicle cycling and thus synaptobrevin-2 trafficking; it will be interesting and important to establish the impact of α -synuclein phosphorylation under a broader range of stimuli (i.e. in response to single action potentials, or under conditions where UFE predominates). Similarly, establishing the stoichiometry of α -synuclein phosphorylation under different conditions will be of interest.

These findings likely have implications for the pathogenicity underlying the synucleinopathies. There is a decrease in soluble α -synuclein in Parkinson's disease as the protein becomes sequestered into aggregates (Zhou et al., 2011). Depleting soluble, functional forms of α -synuclein, which undergoes dynamic changes in phosphorylation, may disrupt synaptobrevin-2 trafficking and vesicular distribution and contribute to synaptic dysfunction and pathogenesis in these disorders.

Limitations of the study

Here, we have used phosphonull and phosphomimetic variants to probe the physiological impact of T81 phosphorylation. Such approaches have long been used to investigate the impacts of protein phosphorylation, including recent work demonstrating that Ser129 phosphorylation modulates α -synuclein function (Ramalingam et al., 2023).

There was good alignment of the N-terminal α -helical domains of WT, T81A and T81E α -synuclein structures generated using AlphaFold3 (Figure S7), suggesting that there is little impact of either substitution on the structure or helicity of the proteins. Additionally, mutations do not impact protein levels or targeting to synapses (Figure 2). Nevertheless, results should be interpreted with caution. Additionally, modelling phosphorylation with a glutamic acid substitution does not introduce the same overall charge difference to the protein as addition of a phosphate group.

SUPPLEMENTAL MATERIAL

Figure S1 shows the alignment and peptide coverage for α -synuclein, inclusion list for MS/MS, the protein intensity values for α -synuclein, β -synuclein, MARK2 and protocadherin-1 for each condition and replicate for the LC-MS/MS data, and the intensity of the pS42 peptide precursor ion in the data-dependent acquisition and targeted LC-MS/MS analyses. Figure S2 shows the annotated spectrum and associated Mascot identification report for the peptide identifying α -synuclein pT81 for the LC-MS/MS data. Figure S3 shows the trafficking time course of VGLUT1-pHluorin during and after 300 action potentials (10 Hz, 35 mA). Figure S4 shows the trafficking time course of syt1-pHluorin during 400 action potentials (40 Hz, 35 mA). Figures S5 and S6 show uncropped immunoblots for the co-immunoprecipitation assay. Figure S7 shows the aligned 3D structures of α -synuclein variants predicted by AlphaFold3.

DATA AVAILABILITY

Data are available upon request to the corresponding author. The raw data files and search output for the targeted LC-MS/MS for WT and KO mono-phosphopeptide fractions 24-27 have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD049095. Plasmids generated in this study are available from the lead contact upon request and their distribution is regulated by an institutional MTA.

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FIGURE TITLES AND LEGENDS

Figure 1. Ser42 and Thr81 are α -synuclein activity-dependent phosphorylation sites. (A) Independent cultures from wild-type (WT) or α -synuclein knockout (KO) mice were mock-treated (control) or treated with 60 mM KCl for 10 s and lysed. Lysates were reduced, alkylated, precipitated, digested with LysC followed by GluC and labelled with TMT11plex reagents to make a WT and KO sample combined from two treatments and three replicates. The WT and KO samples were separately enriched for phosphopeptides and analysed by LC-MS/MS. (B) Plot of the $\log_2(\text{KCl}/\text{Control intensity})$ for α/β -synuclein phosphorylation sites and positive controls (KCl-responsive MARK2 Ser453 and protocadherin-1 Ser880) from the KO and WT experiments. Plot shows individual n and mean $\pm$ SD, $n = 3$ individual experiments. Significance was determined by multiple t-tests followed by a false discovery rate approach (see Methods). Thus, a Q value is reported for each comparison, indicating two α -synuclein activity-dependent phosphorylation sites. * $Q < 0.05$, ** $Q < 0.01$, *** $Q < 0.001$. The KO and WT experiments were analysed by LC-MS/MS independently. However, the samples were processed and analysed under very similar conditions and the ratio of KCl to control was calculated, thereby removing the dependence on peptide amount and magnitude of intensity. Thus, WT and KO data is shown in the same plot and the α -synuclein pSer42 response was compared. The peptides for the α -synuclein-specific sites Thr81, Ser122 and Ser129 were undetectable in the KO samples. (C) Protein diagram of α -synuclein showing the membrane-associated region, including the non-amyloid β component and acidic C-terminal domain. Phosphorylation sites in mouse α -synuclein identified by our phosphoproteomic screen are labelled according to whether they are upregulated by brief neuronal depolarisation (Ser42 and Thr81, orange) or not (Ser122 and Ser129, black). * S122 is not conserved in human α -synuclein, which instead has asparagine at this residue.

Figure 2. α -Synuclein phosphomutants do not impact synaptic protein levels or trafficking to presynaptic terminals. (A) Representative images of wild-type hippocampal neurons co-transfected with syt1-pHluorin and either empty vector (EV) or WT, S42E, S42A, T81E or T81A α -synuclein; green panels (left) show transfected neurites (syt1-pHluorin, enhanced with anti-GFP immunolabelling) and false-colour panels (right) show α -synuclein immunofluorescence staining. Filled arrows indicate transfected nerve terminals and empty arrows indicate non-transfected nerve terminals. (B) Synaptic protein levels of α -synuclein variants, $n = 6$ (EV, T81E, T81A) or 9 (WT, S42E, S42A) individual fields of view. **** $P < 0.0001$ for EV vs. WT, S42E, S42A, T81E and T81A, $P > 0.9999$ for WT vs. S42E, $P = 0.3727$ for WT vs. S42A, $P = 0.9619$ for WT vs. T81E, $P = 0.9921$ for WT vs. T81A, one-way ANOVA with Sidak's multiple comparisons post-hoc test comparing conditions against EV and WT. (C) Representative images of α -synuclein KO hippocampal neurons co-transfected with syb2-pHluorin and either WT, S42E, S42A, T81E or T81A α -synuclein. False-colour panels show α -synuclein immunofluorescence staining. (D) Coefficient of variation (CV) analysis was used to determine the distribution of α -synuclein variants along axonal segments, $n = 10$ (S42E, S42A, T81E) or 12 (WT, T81A) individual fields of view. $P = 0.9936$ for S42E, $P > 0.9999$ for S42A, $P = 0.6289$ for T81E, $P = 0.3493$ for T81A, one-way ANOVA with Dunnett's multiple comparisons post-hoc test against neurons transfected with WT α -synuclein. Scale bars are 5 μm . Plots show individual n and mean $\pm$ SEM.

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Figure 3. Ablation of phosphorylation at Thr81 of α -synuclein specifically alters the distribution of synaptobrevin-2 between membrane compartments. (A) pHluorins (diamonds) are tagged to the luminal domain of synaptic vesicle proteins (dark grey) such as synaptobrevin-2 (syb2) or synaptotagmin-1 (syt1). pHluorin fluorescence is quenched in acidic environments (yellow). After exocytosis pHluorins are exposed to the neutral extracellular environment (white) and can fluoresce. Upon reacidification of a newly endocytosed vesicle pHluorin fluorescence is once again quenched. (B) Top: Schematic of the protein distribution assay protocol showing raw pHluorin fluorescence upon exposure to neutral saline buffer (white), acidic buffer (yellow) or alkaline NH_4Cl buffer (green) to distinguish the internal (vesicular) and plasma membrane (PM) fractions of synaptic vesicle proteins. Bottom: Representative images of relative syb2-pHluorin fluorescence intensity of neurons in the presence of different buffers. Scale bar is 10 μm . (C,D) Vesicular (internal) fraction of syb2-pHluorin (C) or syt1-pHluorin (D) in α -synuclein KO hippocampal neurons co-transfected with syb2/syt1-pHluorin and either WT, S42E, S42A, T81E or T81A α -synuclein (C) $n = 10$ (S42A), 11 (S42E, T81A) or 12 (WT, T81E) individual fields of view from independent coverslips. *** $P = 0.0002$ for WT vs. T81A, $P = 0.7359$ for WT vs. S42E, $P = 0.6472$ for WT vs. S42A, $P = 0.9997$ for WT vs. T81E, one-way ANOVA with Dunnett's multiple comparisons post-hoc test comparing against WT. (D) $n = 8$ (WT, T81A) or 9 (T81E) individual fields of view. $P = 0.6542$ for WT vs. T81E, $P = 0.0875$ for WT vs. T81A, one-way ANOVA with Dunnett's multiple comparisons post-hoc test comparing against WT. Plots show individual n and mean $\pm$ SEM.

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Figure 4. Synaptobrevin-2 trafficking during stimulation is perturbed by phosphonull (T81A) α -synuclein, while exocytosis is unaffected. α -Synuclein KO hippocampal neurons co-transfected with syb2-pHluorin and either WT, T81E or T81A α -synuclein were stimulated at 10 Hz and 35 mA with either a train of 300 action potentials without bafilomycin (A-C), or 1200 action potentials in the presence of 1 μM bafilomycin to block vesicle reacidification (D-G), and the change in syb2-pHluorin fluorescence over time was measured. Neurons were then perfused with NH_4Cl to reveal the total syb2-pHluorin fluorescence. Stimulation is indicated by labelled black bar. (A, D) Graphs display the time course of mean $\Delta F/F_0 \pm \text{SEM}$ of syb2-pHluorin fluorescence normalised to total fluorescence (A) or peak fluorescence (D). Striped bars indicate timepoints when $P < 0.05$ between specified conditions, two-way repeated measures ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (B) Peak amplitude of syb2-pHluorin $\Delta F/F_0$ during stimulation, normalised to total fluorescence, ** $P = 0.0020$ for T81E vs. T81A, $P = 0.5050$ for WT vs. T81E, $P = 0.0503$ for WT vs. T81A, one-way ANOVA with Tukey's multiple comparisons comparing all conditions. (C, F) Mean initial rate of fluorescence change. (C) **** $P < 0.0001$ for T81E vs. T81A, * $P = 0.0179$ for WT vs. T81A, $P = 0.1850$ for WT vs. T81E, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (F) $P = 0.7718$ for WT vs. T81E, $P = 0.9449$ for WT vs. T81A, $P = 0.5626$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (E) Recycling pool (i.e. % vesicles mobilised by stimulation train), $P = 0.0544$ for WT vs. T81E, $P = 0.8055$ for WT vs. T81A, $P = 0.1976$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (G) Time constant

of exocytosis (τ , seconds); $P > 0.9999$ for all comparisons, Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test comparing all conditions. (A-C) $n = 9$ (WT) or 11 (T81E, T81A), and (D-G) $n = 8$ (WT, T81A) or 11 (T81E) individual fields of view from independent coverslips. Bar graphs show individual n and mean $\pm$ SEM.

Figure 5. Synaptotagmin-1 trafficking is unaffected by α -synuclein phosphomutants. α -Synuclein KO hippocampal neurons co-transfected with syt1-pHluorin and either WT, T81E or T81A α -synuclein were stimulated with a train of 300 action potentials at (10 Hz, 35 mA, indicated by labelled black bar) and the change in syt1-pHluorin fluorescence over time was measured. Neurons were then perfused with NH_4Cl to reveal the total syt1-pHluorin fluorescence. (A) Graph displays the time course of mean $\Delta F/F_0 \pm$ SEM of syt1-pHluorin fluorescence normalised to total fluorescence. $P > 0.05$ at all timepoints, two-way repeated measures ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (B) Peak amplitude of syt1-pHluorin $\Delta F/F_0$ during stimulation, normalised to total fluorescence, $P = 0.8622$ for WT vs. T81E, $P = 0.9139$ for WT vs. T81A, $P = 0.6026$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (C) Mean initial rate of fluorescence change, $P = 0.6838$ for WT vs. T81E, $P = 0.9450$ for WT vs. T81A, $P = 0.8585$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (D) Decay time constant (τ , seconds), $P = 0.3004$ for WT vs. T81E, $P = 0.6686$ for WT vs. T81A, $P = 0.7885$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (A-D) $n = 7$ (WT), 8 (T81A) or 9 (T81E) individual fields of view from independent coverslips. Bar graphs show individual n and mean $\pm$ SEM.

Figure 6. T81E α -synuclein increases post-stimulus retrieval of synaptobrevin-2 compared to T81A. (A-G) α -Synuclein KO hippocampal neurons co-transfected with syb2-pHluorin and either WT, T81E or T81A α -synuclein were stimulated with a train of 300 action potentials (AP) at 10 Hz (A-B) or 400 AP at 40 Hz (C-G) and the change in syb2-pHluorin fluorescence over time was measured. Stimulation is indicated by labelled black bar. (A) Graph displays the time course of mean $\Delta F/F_0 \pm$ SEM of syb2-pHluorin fluorescence, normalised to peak amplitude during 10 Hz stimulation (same source data as Figure 4A). Striped bars indicate timepoints when $P < 0.05$, two-way repeated measures ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (B) Decay time constant (τ , seconds), * $P = 0.0416$ for T81E vs. T81A, $P = 0.2746$ for WT vs. T81E, $P = 0.6719$ for WT vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (A-B) $n = 9$ (WT) or 11 (T81E, T81A) individual fields of view from independent coverslips. (C, F) Graph displays the time course of mean $\Delta F/F_0 \pm$ SEM of syb2-pHluorin fluorescence normalised to total fluorescence revealed by NH_4Cl perfusion (C) or peak amplitude evoked by 40 Hz stimulation (F). Striped bars indicate timepoints when $P < 0.05$ between specified conditions, repeated measures mixed-effects model with Tukey's multiple comparisons post-hoc test comparing all conditions. (D) Peak amplitude of syb2-pHluorin $\Delta F/F_0$ evoked by 40Hz stimulation, normalised to total fluorescence, ** $P = 0.0075$ for T81E vs. T81A, * $P = 0.0326$ for WT vs. T81E, $P = 0.7429$ for WT vs. T81A, one-way ANOVA with Tukey's multiple comparisons comparing all conditions. (E) Mean initial rate of

fluorescence change, ** $P < 0.0036$ for T81E vs. T81A, * $P = 0.0306$ for WT vs. T81E, $P = 0.5749$ for WT vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (G) Decay time constant (τ , seconds), * $P = 0.0184$ for T81E vs. T81A, $P = 0.4435$ for WT vs. T81E, $P = 0.2124$ for WT vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. (C-G) $n = 8$ (WT), 9 (T81E) or 7 (T81A) individual fields of view from independent coverslips. (H-I) α -Synuclein KO hippocampal neurons co-transfected with mCerulean (transfection marker) and WT, T81E or T81A α -synuclein were stimulated with 400 AP at 40 Hz in the presence of 40 kDa TMR-dextran. (H) Representative images of dextran labelling. Scale bar = 10 μ m. (I) Number of dextran puncta per 100 μ m of axon, $P = 0.3737$ for WT vs. T81E, $P = 0.2864$ for WT vs. T81A, $P = 0.9886$ for T81E vs. T81A, one-way ANOVA with Tukey's multiple comparisons post-hoc test comparing all conditions. $n = 8$ (WT, T81E) or 9 (T81A) individual fields of view from independent coverslips. (J) HEK293 cells were untransfected (UT) or co-transfected with syb2-FLAG and empty vector ($-\alpha$ -syn) or WT, T81E, or T81A α -synuclein-HA. α -Synuclein-HA was immunoprecipitated using anti-HA agarose beads, and co-immunoprecipitated syb2-FLAG was detected by immunoblotting with an anti-syb2 antibody. Immunoprecipitated α -synuclein-HA was detected using an anti- α -synuclein antibody. Representative immunoblot showing input lysates and anti-HA immunoprecipitates (IP:HA). See Figure S5 for full blots, and S6 for independent biological replicate. (A-I) Bar graphs show individual n and mean $\pm$ SEM.

Figure 7. Model for functional impact of phosphorylation of α -synuclein at Thr81.

(A) Synaptic vesicles are docked/primed at the plasma membrane by the partial assembly of the *trans*-SNARE complex composed of synaptobrevin-2, syntaxin and SNAP-25. Upon presynaptic depolarisation, the full zippering of the SNARE complex drives membrane fusion, resulting in neurotransmitter release. NSF and α SNAP then facilitate the disassembly of the *cis*-SNARE complex on the plasma membrane. (B) Following fusion and SNARE complex disassembly, synaptobrevin-2 needs to be retrieved to synaptic vesicles or endosomes, which are newly formed via one of multiple modes of endocytosis. α -Synuclein undergoes activity-dependent phosphorylation at Thr81 upon neuronal depolarisation. Phospho-Thr81 α -Synuclein directs synaptobrevin-2 to be retrieved via clathrin-mediated endocytosis and other endocytic modes that can be active beyond presynaptic depolarisation. α -Synuclein that is dephosphorylated at Thr81 directs synaptobrevin-2 to be retrieved via endocytic modes that are only active during presynaptic depolarisation, including ultrafast endocytosis and activity-dependent bulk endocytosis.













