Sleep-controlling neurons are sensitive and vulnerable to multiple forms of α-synuclein: implications for the early appearance of sleeping disorders in α-synucleinopathies

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Abstract
Parkinson’s disease, Multiple System Atrophy, and Lewy Body Dementia are incurable diseases called α-synucleinopathies as they are mechanistically linked to the protein, α-synuclein (α-syn). α-syn exists in different structural forms which have been linked to clinical disease distinctions. However, sleeping disorders (SDs) are common in the prodromal phase of all three α-synucleinopathies, which suggests that sleep-controlling neurons are affected by multiple forms of α-syn. To determine whether a structure-independent neuronal impact of α-syn exists, we compared and contrasted the cellular effect of three different α-syn forms on neurotransmitter-defined cells of two sleep-controlling nuclei located in the brainstem: the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus. We utilized size exclusion chromatography, fluorescence spectroscopy, circular dichroism spectroscopy and transmission electron microscopy to precisely characterize timepoints in the α-syn aggregation process with three different dominating forms of this protein (monomeric, oligomeric and fibril) and we conducted an in-depth investigation of the underlying neuronal mechanism behind cellular effects of the different forms of the protein using electrophysiology, multiple-cell calcium imaging, single-cell calcium imaging and live-location tracking with fluorescently-tagged α-syn. Interestingly, α-syn altered membrane currents, enhanced firing, increased intracellular calcium and facilitated cell death in a structure-independent manner in sleep-controlling nuclei, and postsynaptic actions involved a G-protein-mediated mechanism. These data are novel as the sleep-controlling nuclei are the first brain regions reported to be affected by α-syn in this structure-independent manner. These regions may represent highly important targets for future neuroprotective therapy to modify or delay disease progression in α-synucleinopathies.

Keywords Neurodegenerative disease · α-synuclein · α-synucleinopathies · Sleep disorders · Laterodorsal tegmental nucleus · Pedunculopontine tegmental nucleus
Abbreviations

α-syn  α-Synuclein
LDT  Laterodorsal tegmental nucleus
PPT  Pedunculopontine tegmental nucleus
PD  Parkinson’s disease
SDs  Sleeping disorders
REM  Rapid eye movement
ThT  Thioflavin T fluorescence spectroscopy
CD  Circular dichroism spectroscopy
TEM  Transmission electron microscope
SEC  Size exclusion chromatography
GPCR  G-protein coupled receptor
bNOS  Brain derived nitric oxide synthase
sEPSCs  Spontaneous excitatory post synaptic currents
α-synF  Fibril form of α-synuclein
α-synO  Oligomeric form of α-synuclein
α-synM  Monomeric form of α-synuclein
Hz  Hertz

Introduction

The neurodegenerative disorders named α-synucleinopathies are a group of incurable diseases, including Parkinson’s disease (PD), Lewy Body Dementia, and Multiple System Atrophy. Characterized by specific, well-defined clinical symptoms, all three diseases exhibit a prodromal interval, that is, an initial period during which symptoms or signs putatively related to neurodegeneration are present, but the full clinical symptom profile upon which a diagnosis is based has not yet developed [1–3]. Thus, the prodromal phase provides a theoretical opportunity for implementation of neuroprotective therapy that could prevent further development of PD, Lewy Body Dementia and Multiple System Atrophy. However, detection of this prodromal period is often in hindsight and after the full clinical disease is recognized. The most notable symptoms indicating the prodromal phase of α-synucleinopathies are sleep disorders (SDs) [6, 7]. Although SDs are not particular for α-synucleinopathies, presence of SDs often precede the cardinal symptoms currently used for clinical diagnosis of these diseases by more than a decade [8]. While several multicenter studies have explored the progression from SDs to diagnosed α-synucleinopathies, the mechanistic basis for the early appearance of SDs in this group of diseases is unknown [9, 10]. Elucidation of this basis will, we believe, have a tremendously powerful impact on the development of specific biomarkers and strategies for earlier treatment or prevention of these diseases.

The most common SDs found in prodromal phases of α-synucleinopathies are: Rapid Eye Movement (REM) Sleep Behavior Disorder, which presents with abnormal motor activity during REM sleep when atonia should be prevalent [11–16] and Excessive Daytime Sleepiness which is characterized by low arousal levels during wakefulness [17, 18]. Both SDs involve altered activity of neurons in two brainstem nuclei that play a role in the reticular activating system and REM sleep control: the laterodorsal tegmental nucleus (LDT) and the pedunculopontine tegmental nucleus (PPT) [19, 20]. Localized at the junction of the midbrain andpons in the brainstem, both of these nuclei are cytologically heterogeneous and are composed of acetylcholine-containing, glutamatergic and GABAergic neurons [20–23] with the cholinergic neurons being the most studied for the role they play in sleep behavior and alert arousal [19, 24]. Interestingly, analyses of post mortem brains of patients with α-synucleinopathies showed extensive degeneration in these two brain nuclei of cholinergic neurons [25–27], which was associated with deposition of aggregated proteins named ‘Lewy bodies’ that are the histological hallmark of the α-synucleinopathies [27–29]. The extensive neuronal loss and presence of Lewy bodies suggest that disease process mechanisms related to α-synucleinopathies affect sleep-controlling nuclei and given the early appearance of SDs, raise the speculation that protein-related neurodegeneration might appear in SD-related nuclei before occurring in other more classically affected motor-controlling neural areas that results in disease-defining symptoms. Thus, following this rationale, our group was the first to investigate the neuronal mechanisms behind the early appearance of SDs in PD. We reported differential cellular effects occurred in the LDT and the PPT in response to α-synucleinopathy-related proteins when compared to effects in the motor-controlling substantia nigra, which suggested mechanistic underpinnings of SDs’ prodromal appearance in PD [30].

Lewy bodies are primarily composed of misfolded aggregates of the protein α-synuclein (α-syn). α-syn, in its native state, is intrinsically disordered in its monomeric form. The physiological function of this protein is not fully understood, but it has been shown to play a role in neurotransmitter vesicle traffic [31]. Prior to Lewy body formation, levels of the monomeric form of α-syn rise and the protein undergoes a series of conformational changes, resulting initially in the formation of intermediate, structured aggregates, known as oligomers and, subsequently, into highly organized and structured amyloid fibrils. In vivo investigations revealed that distinct structures of α-syn display differential seeding capacities, which induce structure-specific pathology and neurotoxic phenotypes, leading to the hypothesis that the different structures of α-syn produce distinct clinicopathological traits within α-synucleinopathies [32–34]. Oligomers are related to the PD phenotype, and fibrils are associated with both PD and MSA phenotypes. Findings from ex vivo and in vitro studies suggest that α-syn induces structure-dependent cellular effects and among the known protein structures, the oligomeric form is toxic to cells [35, 36]. However, a
few studies have suggested that the fibril form is more toxic compared to the oligomeric or monomeric forms [37]. While currently there is disagreement in the literature, and debate about whether oligomeric or fibril forms are more toxic to cells, most studies indicate that the monomeric form of α-syn is not toxic. One caveat of these studies is that they were conducted in brain regions which are only affected late in α-synucleinopathies [26], and we have shown previously that different areas of the brain exhibit distinct sensitivity and vulnerability to different forms of the protein [30]. Additionally, it is highly challenging to characterize the different forms of the protein aggregation given the large size range (nm to μm scales), and the inherent transient nature of the oligomeric species as it progresses along a pathway (on-pathway) to fibril formulation, and thus, the possibility exists that effects attributed to one form may actually have been exerted by the presence of another form. Finally, only few studies have compared and contrasted neuronal and toxicity effects of different forms of the protein within the same study and under identical experimental conditions.

Thus, to further investigate whether α-syn exerts structure-dependent effects on neurons, and to screen for potentially shared neuronal mechanisms of SDs in the prodromal phase of different α-synucleinopathies, we have compared and contrasted the cellular effects in brain slices of three different α-syn samples, each from different stages of the amyloid formation process, on neurotransmitter-defined cells in two sleep-controlling nuclei: the LDT and the PPT. To this end, we adopted a range of methodologies to classify the dominant α-syn species in each of the protein samples. Specifically, the monomeric state in its pure form was isolated from size exclusion chromatography (SEC). The fibrillation process was initiated and intermediate samples with on-pathway α-syn oligomers were extracted from the early elongation phase based on Thioflavin T (ThT) fluorescence spectroscopy and prior knowledge of the volume fraction of the different species [38]. The final fibril form was extracted after significant maturation and confirmed by circular dichroism (CD) spectroscopy, and transmission electron microscope (TEM). For our investigations of α-syn-induced effects, we used mouse brain slices and employed electrophysiology, multiple-cell calcium imaging, single-cell calcium imaging, and live particle tracking of protein interactions with neural structures utilizing fluorescently tagged α-syn. We found that α-syn at all three stages (monomeric, intermediate/oligomeric, and fibril) exerted neuronal and functional effects and induced intracellular calcium rises without any substantive differences between these actions. In addition, we have monitored cell survival by using neurotoxicity assays, and confirmed and extended earlier findings that α-syn heightens cell death. Finally, we show that α-syn in monomeric form interacted with the surface of the membrane resulting in activation of a G-protein coupled receptor (GPCR)-mediated mechanism to induce membrane responses and alterations in intracellular calcium levels.

To our knowledge, this study provides the first report in native mammalian brain tissue of the cellular and toxic effects of α-syn at three different, pre-characterized stages of the fibrillation process. We show that effects were largely protein structure independent. Additionally, we provide novel insights into a new mechanism triggered by α-syn that induces excitation of the neuronal membrane and alters intracellular calcium. As our data suggest neuronal dysfunction in sleep-controlling neurons induced by even simple forms of α-syn, our findings indicate one single mechanistic explanation for the early appearance of SDs in α-synucleinopathies and raise the possibility that these cells represent an important target for neuroprotective therapy to modify or delay the disease process.

Materials and methods

Cellular effects of monomeric, oligomeric and fibril forms of human α-syn were examined on neurons of the LDT and PPT in mouse brain slices. Preparation of the different forms of the protein and the brain slices are presented below, as well as the experimental design.

Preparation of the different forms of α-syn

Monomeric α-syn

Human α-syn was recombinantly expressed and purified as previously described [39] with few modifications. In brief, α-syn was cloned into E. coli BL21DE3 cells using a pET-11a vector construct. Harvested cells were lysed by osmotic shock, and non-heat-stable proteins were removed by boiling and centrifugation. α-syn was isolated by ion-exchange chromatography, and the monomer was isolated by size exclusion chromatography (SEC). Monomer fractions from SEC were pooled, aliquoted to avoid freeze/thaw cycles, and stored in PBS buffer at −80 °C until use, keeping the sample from forming aggregates and/or high-molecular weight species (Supplemental Information, Fig. 1).

Fibrillation assay for fibril and intermediate/oligomeric α-syn samples

α-syn in PBS buffer (0.3–2.2 mg/mL) was fibrillated in a microplate reader (FLUOstar Omega. BMG Labtech) at 37 °C with 3 mm sterile glass beads and orbital shaking (700 rpm for 280 s in each 360 s cycle). Samples were aliquoted (150 μL) in a NUNC 96-well optical polymer-based, clear-bottom black plate (Thermo Fisher scientific 265,301) and sealed with clear polyolefin tape (Thermo
Thioflavin T (ThT; 20 µM) was added to one sample, keeping additional parallel non-ThT containing samples for the electrophysiology experiments. ThT emission was recorded at 480 ± 5 nm upon excitation at 450 ± 5 nm. Fibril samples were produced at 0.9–1.88 mg/mL and collected after 7 days to ensure full fibril maturation (Fig. 1A1, B1). Our previous studies [38] along with measurement of residual concentration measured after fibrillation and centrifugation at 14,000g for 20 min to spin down fibrils confirmed full fibrillation with less than 10% soluble species remaining. Transmission Electron Microscopy (TEM) was used to characterize fibrils. To this end, 10 µL diluted α-syn fibril sample was incubated for 1 min on a carbon coated Cu grid (FCF, 400 MESH) before the excess sample solution was removed. The grid was washed with 10 µL water, subsequently stained with 10 µL of 2% (w/v) uranyl acetate, and finally, excess staining was removed in two washing cycles with water. The grid was dried, and micrographs were subsequently collected on a CM100 TWIN microscope (Fig. 1A2) at the Core Facility for Integrated Microscopy (CFIM), University of Copenhagen. Samples of fibril α-syn were kept at room temperature and used within a maximum of 2 weeks.

Based on initial concentration scans, intermediate samples were extracted after initiation of the fibrillation, approximately when the ThT value reached 20–30% of the final fluorescent value (16 h at 0.3 mg/mL; Fig. 1B2, or 6 h at 1.1 mg/mL), which was the point at which the sample contained a significant fraction of oligomeric species as previously described [38]. However, it should be noted that the intermediate sample is not an isolated form, as this oligomeric species - since it is on-pathway to the fibril form - is only transiently present in an evolving mixture, thus is present alongside approximately equal amounts of monomers and fibrils at this point [38]. However, this approach ensures a minimum perturbation of the on-pathway process and serves as comparison with the purely monomeric and fibrillar samples. Intermediate/oligomeric α-syn samples were kept at room temperature and used within a few hours (maximum 12 h) following extraction.

**LDT and PPT brain slices**

**Animals**

All protocols for preparation of animal tissue used in this study were approved by the European Communities Council Directive (86/609/EEC). Brain slices containing the LDT or the PPT were made from male (13–30 days old) Naval Medical Research Institute mice (Harlan Mice Laboratories, Denmark) and were used for electrophysiological recordings,
calcium imaging studies and neurotoxicity assays. The animals were housed under controlled temperature (22–23 °C) and humidity (45–65%) conditions, were kept on a 12:12 h light–dark cycle, and had a standard rodent diet available ad libitum.

### Brain slice preparation

On the day of the experiments, mice were anesthetized via inhalation of isoflurane (Baxter A/S, Denmark) and decapitation was conducted immediately after determination of sufficient depth of anesthesia. A block of the brain which contained the LDT and the PPT was rapidly removed (Fig. 2A1, A2) and submerged in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF solution (124 NaCl, 5 KCl, 1.2 Na2HPO4·2H2O, 2.7 CaCl2·2H2O, 1.2 MgSO4 (anhydrous), 10 dextrose, 26 NaHCO3; in mM) at pH 7.4 and an osmolality of 298–302 mOsm/kg was saturated with carbogen (95% O2/5% CO2). The brain block was sectioned in 250 µm slices on a vibratome (Leica VT1200S, Leica Biosystems, Germany) following calibration with the vibro-check stabilizer (Leica Biosystems) in order to reduce cellular damage from vertical blade deflections. LDT and PPT-containing brain slices (Fig. 2B1) were placed in a chamber containing oxygenated ACSF and incubated at 37 °C for 15 min. To allow the tissue to equilibrate after the incubation period, the slices were kept at room temperature, and carbogen was continuously supplied for at least 1 h prior to further procedures.

### Compounds applied to brain slices

α-syn samples (in native monomeric, intermediate or fibril states) were diluted in ACSF to a final concentration of 100 nM, unless otherwise noted. Bath application of α-syn was conducted for 3–4 min after a stable baseline had been established for membrane current or, when relevant, for fluorescence sourcing from the calcium indicator.

#### α-syn/AF647 (AF647-MJFR-1)

To investigate the association of α-syn with LDT neurons, which were later immunohistochemically identified, an anti-α-syn monoclonal antibody (MJFR1, Abcam) covalently conjugated to Alexa Fluor 647 NHS Ester (Sigma Aldrich) was used. The conjugate was purified according to published protocols [40], aliquotted, and stored at −20 °C until use. Brain slices containing LDT were incubated for 1 h in 2 mL of ACSF containing the conjugated α-syn. After washing once, the slices were fixated with 200 µL of 4% PFA for at least 8 h before further immunohistochemistry procedures.

### Drugs

Tetrodotoxin (0.5 mM) (TTX; Tocris, Denmark) was used to block voltage-dependent Na+ channels mediating action potentials. Cyclopiazonic acid (10 µM CPA; Sigma) was used to inhibit IP3-mediated, calcium pumps in the sarcoplasmic reticulum, which was prepared in 10 mM stock of DMSO. To block AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (N-methyl-D-aspartate) ionotropic glutamate receptors, the following antagonists were added to the ACSF during the recording: 6,7-dinitroquinoxaline-2,3-dione (DNQX; 15 µM) and (2R)-amino-5-phosphonopentanoate (AP5; 50 µM). To block dopamine receptors, SCH-2339 (Tocris), and 3,5-Dichloro-N- [[(2S)-1-ethyl-2-pyrrolidinyl][methyl]-2-hydroxy-6-methoxybenzamide (Raclopride, Tocris), selective D1-like and D2-like receptor antagonists, respectively, were used at a concentration of 10 µM in the ACSF. All the blockers were bath applied for 5–7 min prior to peptide application. Dopamine hydrochloride (30 µM, Tocris) was bath applied for 7 min. To block voltage operated calcium channels (VOCCs) during the electrophysiological experiments, CdCl2 (200 µM) was added to the ACSF.

### Patch-clamp recordings in LDT and PPT brain slices

Voltage-clamp or current clamp recordings were conducted from neurons in the LDT or PPT. Borosilicate, patch pipette electrodes were fabricated on a horizontal puller (P-97, Sutter Instruments, USA) with a resulting pipette resistance of 6–11 MΩ after filling with an intracellular solution which contained in mM: 144 K-gluconate; 2 KCl; 10 HEPES; 0.2 EGTA; 5 Mg-ATP and 0.3 Na-GTP. Alexa-594 (Thermo Fisher Scientific, USA) was included in the pipette to allow later identification of recorded cells as this dye is photostable following fixation. GTP in the pipette solution was replaced with Guanosine 5′- [β-thio]diphosphate trilithium salt (GDPβS, Sigma-Aldrich) for experiments designed to block G-protein mediated mechanisms. Slices were placed in a chamber on the microscope stage which was constantly perfused with ACSF. Individual neurons within the LDT or the PPT were visualized with a 60× water immersion objective which was coupled to an upright microscope (BX50WI, Olympus; Japan) with an infrared Dodt gradient contrast system (IR-DGC; Luigs & Neumann, Germany) and a CCD camera (CCD-300ETRC; DAGE-MTI, Michigan City, IN). Voltage-clamp recordings were initiated using an EPC9 amplifier (HEKA, Germany) under software control (Patchmaster; HEKA; version 13.0). After membrane breakthrough and a 5–7 min stabilization period, data collection in voltage clamp or current clamp mode commenced with sampling at 10 kHz. In addition to electrophysiological responses being recorded by Patchmaster, voltage and
current responses were monitored in gap free mode using AxoScope 10.2 (Molecular Devices, USA) and an Axon miniDigi 1B digitizer (Molecular Devices Corporation). Amplitudes of membrane currents were measured as the difference between baseline and maximum deflection. To determine current–voltage relationships during α-syn-induced inward currents, a voltage step

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Fig. 2 In sleep-controlling nuclei, α-synF induced similar changes in holding currents regardless of neuronal phenotype or nucleus. A1 Sagittal representation of the mouse brain showing schematically the location of the LDT and PPT. A2 Schematic of a coronal brain slice containing the LDT which shows the boundaries of this nucleus in the blue ovals along with other rostrocaudal, extent-marking anatomical structures, including the DTN shown in white ovals. Detail of the region of the LDT roughly demarcated by the red dots is shown at higher magnification in the photo micrograph to the right. Below are histological images of two representative neurons recorded in this study that were marked with Alexa-594 (left panel) which appears as red fluorescence when viewed under 560 nm. Recorded cells were determined to be cholinergic based on presence or absence of bNOS which appears as green when exposed to 488 nm. As can be seen in the middle panel only one of the two recorded neurons was cholinergic. Superimposition (right panel) of the two images shows that cholinergic recorded neurons appear as yellow, whereas recorded neurons which are bNOS- appear in red. A3 Sample of membrane responses to α-synF, which induced inward currents in A3 cholinergic and A4 non-cholinergic neurons (scale in A3 applies to A4). B Similar to what was seen in the LDT, α-synF induced inward currents in both types of neurotransmitter-defined cells in the PPT. B1 Schematic of, and higher magnification detail from a coronal brain slice containing the PPT, showing the anatomical boundaries of the recorded nuclei in green. Sample membrane responses to α-synF, which also induced excitatory currents in both (cholinergic (B2) and non-cholinergic (B3) PPT neurons (scale in B2 applies to B3). C Histogram showing that the amplitudes of the inward currents induced in cholinergic and non-cholinergic neurons did not differ within the LDT (C1) and the PPT (C2). C3 The proportion of cells responding to, and the amplitude of the current (C4) evoked by, α-synF are shown; neither differed significantly between the two nuclei. α-synF indicates the fibril form of the protein. DTG dorsal tegmental nucleus, SCP superior cerebellar peduncle, bNOS brain Nitric Oxide Synthase.
protocol in which the membrane was stepped from −30 to −110 mV in increments of 10 mV (200 ms step duration) was utilized, and input–output curves were generated. Mini-Analysis (Synaptosoft, USA) was used to detect and analyze spontaneous and miniature synaptic events in voltage clamp data (sEPSCs, mEPSCs) in 30 s epochs selected right before application of α-syn and 30 s around the peak amplitude of α-syn-induced membrane current. The inter-event intervals and the amplitude of events were averaged and analyzed statistically across a population of cells. Firing frequency was monitored in current clamp mode by depolarization of the neuron sufficient to induce a sustained firing of action potentials (−45 mV) by injection of constant current. A 30 s epoch before and a 30 s epoch after application of α-syn was selected, and intervals between action potentials were measured and averaged.

**Perforated patch configuration for recording LDT neurons**

Perforated patch recordings were obtained as previously described [41, 42], with the modification that the perforating agent in the intracellular solution was replaced by 500 nM of α-syn. A pulse of 5 mV, which was 5 ms in duration, was applied in order to monitor potential membrane perforation in voltage-clamp recording mode following establishment of high resistance seals (> 1 MΩ) between the patch pipette and the cell membrane. Thereafter, the recording was conducted in cell-attached configuration to monitor the seal resistance for longer than 40 min. At the end of each experiment, membrane breakthrough was induced.

**Multiple-cell and single-cell calcium imaging**

Calcium imaging was conducted by monitoring fluorescence emitted by the fluorescent calcium indicator dye, Fura-2. To monitor fluorescence across multiple cells in the same slice, we conducted ‘bulk’ dye loading of cells in brain slices following previously published protocols using the acetoxymethyl ester form of Fura-2 (Fura-2 AM) which is cell permeant [43]. For single-cell calcium imaging recordings, the EGTA in the intracellular pipette solution was replaced by 25 µM of Fura 2 (Molecular Probes). The patch-clamp configuration was established and following membrane breakthrough, Fura 2 diffused passively into the cell. Fluorescence intensity emitted was monitored by a CCD fluorescent camera (12-bit Sensicam, PCO Imaging, Germany) on the microscope, which was controlled by the imaging software Live Acquisition (TILL Photonics, Germany). The excitation wavelengths of 340 and 380 nm were rapidly switched allowing recordings for ratiometric analysis (340 nm:380 nm). The analysis of calcium imaging data was conducted using Igor Pro 6 (Wavemetrics, USA). Changes in fluorescence with ascendant deflections were interpreted as rises in calcium, and amplitude of the rises were quantified, and converted to a percentage of baseline fluorescence following subtraction of background fluorescence. A fluorescent change in cells below 4% was considered indiscernible from background noise and those data were excluded. The ratiometric measurement in changes in fluorescence is expressed as %DF/F in the figures.

**Immunohistochemistry**

Immunohistochemistry was used for post hoc identification of the recorded cells within the LDT and PPT as bNOS positive (bNOS+) or bNOS negative (bNOS–), which identifies a LDT or PPT cell as cholinergic or non-cholinergic, respectively. Colocalization of Alexa-594 and bNOS was used to determine a cholinergic phenotype of the recorded cell. Immunohistochemistry was also used to investigate the association of conjugated α-syn/AF647 with bNOS+ cells in LDT. Accordingly, following recordings, brain slices in which cells had been labeled with Alexa-594 or brain slices that had been incubated in the conjugated α-syn/AF567 were submerged in 4% paraformaldehyde for fixation and stored overnight. Slices were then cryoprotected by saturation in a 30% sucrose solution and resectioned on a Leica Cryostat into 40 µM for immunohistochemical processing using the bNOS primary antibody (rabbit anti brain nitric oxide synthase, Sigma) and the fluorescent secondary (Goat, anti-rabbit, Alexa fluor 488 secondary, Molecular probes, USA) following previously reported protocols (Fig. A2) [44].

**Neurotoxicity assay**

Coronal slices containing the LDT were bisected with one half incubated for a period of 7 h in control solution, and the other half in the monomeric or fibril form of α-syn (500 nM) under identical protocols. Another investigator who was blinded to slice treatment conducted the analysis for identification of live and dead cells. For that, propidium iodide (PI; Sigma-Aldrich) and the fluorescent secondary (Goat, anti-rabbit, Alexa fluor 488 secondary, Molecular probes, USA) was used to mark live cells and DAPI to identify dead cells and DAPI. The total cell count was calculated as PI positive plus DAPI positive cells. The slices were incubated for 3 periods of 5 min in a solution which contained 1 μg/mL solution of both PI and DAPI with a pH of 7.4. Data are presented as cell death in α-syn treated hemi-slices as a percentage of control as measured by cell death in the other half of the slice.

**Imaging of immunohistochemistry and neurotoxicity assays**

To detect fluorescent signals in immunohistochemically-treated slices, an upright Zeiss microscope coupled to a monochrome CCD camera (Axiocam MRM, Zeiss, Germany)
controlled by Axioskop 2 software (AxioVision 4.6, Zeiss) and appropriate cube sets (Zeiss 59 fluorescent filter cube sets, wavelengths PI: 472–578 nm; DAPI: 358–463 nm, bNOS+: 380 nm, AF647: 620 nm) were used. To conduct the analysis of images collected, ImageJ (National Institutes of Health, Bethesda, MD) was used, and a macro within the program was utilized to count the number of DAPI and PI-labeled cells. Selected cells were validated by manual confirmation. The same software was also used to identify bNOS + cells, and to analyze their association with the conjugate α-syn/AF647. For this analysis, within 2 brain slices, all of the bNOS + cells were counted and the relative location of the fluorescent signal emitted by α-syn/AF647 was noted and correlated to the position of the bNOS + cells. Data are presented as a calculation of the total percentage of bNOS + cells associated with α-syn/AF647.

Statistics

Statistical treatment of the data was carried out in Graphpad Prism (version 6.0). The results are presented as mean values ± SEM. The Student’s t-test was used to evaluate differences in numerical data. The Fisher’s exact test or a three-way chi-square test were used to evaluate differences in categorical data. Where relevant, to identify which contingency differed significantly from expected outcomes in the categorical data, a post hoc Fisher’s exact test was employed. For all statistical tests, significance was determined when alpha was below 0.05. Figures were prepared using Igor Pro software (Wavemetrics, USA), and Matlab R2018b.

Results

Fibrill α-syn induced membrane currents, affected synaptic signaling and altered cellular excitability of neurotransmitter-defined neurons in sleep-controlling brain nuclei.

We previously reported that fibril α-synuclein (α-synF) induced excitatory membrane responses in LDT and PPT neurons [30], and in accordance with those results, α-synF induced excitatory membrane responses in all of the LDT neurons examined (n = 10/10; Fig. 2A3, C3). The average amplitude of the α-synF-induced inward current in LDT neurons was 7.3 ± 0.8 pA (n = 10; Fig. 2C1). Moreover, we saw no statistical differences between the amplitudes of α-synF-elicited current in populations of neurotransmitter defined LDT cells (bNOS+/cholinergic: 7.6 ± 1.3 pA, n = 5; bNOS-/non-cholinergic: 8.5 ± 1.8 pA, n = 2; p = 0.8; Unpaired t-test; Fig. 2C1). Similarly, α-synF induced excitatory membrane responses in 100% of the recorded PPT neurons (Fig. 2C3). The average amplitude of the α-synF-induced inward current in PPT neurons was 9.3 ± 1.9 pA (n = 8/8; Fig. 2C2). We also found no significant differences between the current amplitudes between cholinergic and non-cholinergic PPT neurons (bNOS+: 9.8 ± 3.0 pA, n = 3; bNOS-: 8.5 ± 3.2 pA, n = 2; Fig. 2C2). When the current amplitude elicited by α-synF was compared between nuclei from all cells irrespective of phenotype, there wasn’t a significant difference (p = 0.4; Unpaired t-test; Fig. 2C4).

Next, we analyzed whether α-synF had effects on synaptic activity in these two nuclei. Relative to baseline, α-synF induced a significant increase (8%) in the amplitude of the spontaneous excitatory postsynaptic currents (sEPSCs) in LDT neurons (control: 10.7 ± 2.1 pA; α-synF: 11.6 ± 2.1 pA; n = 4; p = 0.04; Paired t-test; Fig. 3A1, 2, 3), which was comparable to α-synF-mediated increases in sEPSC amplitudes in PPT neurons (8% increase from baseline; control: 11.3 ± 3.6 pA; α-synF: 12.2 ± 3.7 pA; n = 4; p = 0.02; Paired t-test; Fig. 3B1, 2, 3). No significant changes were induced by α-synF in the frequency of sEPSCs within the LDT (control: 11.0 ± 3.8 Hz; α-synF: 12.8 ± 5.2 Hz, n = 4, p = 0.3; Paired t-test; Fig. 3A4) or PPT (control: 8.4 ± 3.1 Hz; α-synF: 8.2 ± 2.8 Hz, n = 4, p = 0.5; Paired t-test; Fig. 3B4).

The alterations induced by α-synF in membrane currents and synaptic activity of neurons in the LDT and PPT could result in modulation of neuronal excitability. Therefore, we examined directly whether this was the case and found that α-synF significantly increased the firing frequency of LDT neurons by 37.5% (control: 0.43 ± 0.1 Hz; α-synF: 0.55 ± 0.1 Hz; n = 3; p = 0.04; Paired t-test; Fig. 3C1), and the firing frequency of PPT neurons was significantly increased by 39.5% (control: 0.43 ± 0.1 Hz; α-synF: 0.6 ± 0.1 Hz; n = 3; p = 0.03; Paired t-test; Fig. 3C2).

Oligomeric α-syn induced effects on membrane currents, altered synaptic activity and heightened excitability in sleep-controlling nuclei.

The intermediate samples with oligomeric forms of α-syn (α-synO) also induced excitatory currents in the majority of recorded neurons in both the LDT (n = 7/7) and the PPT (n = 4/4) (Fig. 4A3). In contrast to what was seen for the fibril form in which there were no differences between the amplitudes elicited in LDT and PPT neurons, the amplitude of the current in PPT neurons induced by α-synO was significantly smaller than the amplitude induced by the same form of the protein in LDT neurons (LDT: 6.0 ± 1.5 pA; n = 4; PPT: 3.5 ± 1.2 pA; n = 4; p = 0.04; Unpaired t-test; Fig. 4A1, 2, 4). While recovery was low, the population of recorded cells included both cholinergic and non-cholinergic neurons, and there did not appear to be a difference between amplitudes across phenotypes within each nuclei (LDT bNOS+: 6.5 ± 4.9 pA, n = 2; bNOS-: 5.5 ± 2.1 pA, n = 2; p = 0.9;
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Unpaired t-test; PPT bNOS +: 3.7 ± 1.8 pA, n = 2; bNOS−: 5.0 pA, n = 1; Supplemental Information, Fig. 2).

Next, we examined whether α-syn O affected synaptic activity in both nuclei. α-syn O induced a 11.8% increase from baseline in the amplitude of sEPSCs in the LDT which was significant (control: 8.5 ± 1.1 pA; α-syn O: 9.5 ± 1.0 pA; n = 3; p = 0.02; Paired t-test; Fig. 4B1, 3, 5), as well as a 8.4% increase from baseline within the PPT that was significant (control: 10.3 ± 5.0 pA; α-syn O: 11.1 ± 5.0 pA; n = 3; p = 0.01; Paired t-test; Fig. 4B2, 4,

Unpaired t-test; PPT bNOS +: 3.7 ± 1.8 pA, n = 2; bNOS−: 5.0 pA, n = 1; Supplemental Information, Fig. 2).

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6). No significant change in the frequency of sEPSCs was seen in the analyzed neurons within the LDT (control: 13.9 ± 1.7 Hz; α-synO: 13.0 ± 1.2 Hz $p=0.08$; Paired t-test; Fig. 4B7) or the PPT (control: 5.8 ± 1.8 Hz α-synO: 4.4 ± 0.8 Hz; $p=0.6$; Paired t-test; Fig. 4B7).

We also investigated potential effects of α-synO on excitability in neurons within these two nuclei. In the LDT, α-synO induced an increase of 52% from baseline in the firing frequency which was significant (control: 0.63 ± 0.2 Hz; α-synO: 0.96 ± 0.3 Hz; n = 3; $p=0.03$; Paired t-test; Fig. 4C1). Similarly, a significant increase in the firing
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1 3

Fig. 4 α-synO induced inward currents, increased the amplitude of sEPSCs and enhanced the firing frequency in neurons from either sleep-controlling nucleus. A Samples of membrane responses to α-synO, which induced excitatory currents in neurons within the LDT (A1) and the PPT (A2; scale in A2 applies to A1). A3 Histogram from the population of recorded cells showing that the proportion of cells responding to α-synO with inward currents did not differ between the two nuclei. However, the amplitude of the current evoked by α-synO was significantly greater in LDT cells than in PPT neurons (A4). B α-synO induced an increase of the amplitude of sEPSCs in LDT and PPT. B1, 2 Samples of recordings showing synaptic events at baseline (top) and in the presence of α-synO (below) in the LDT and PPT. B3, 4 Detail of sEPSCs at a higher magnification is shown to the right, where it is exemplified that the amplitude was greater following α-synO. B5–7 Paired plots and histograms from the population of recorded cells are shown which reveal a significant increase in the amplitude and no change in the frequency of sEPSCs in both nuclei upon α-synO exposure. C α-synO induced a change in the frequency of firing of APs in both nuclei. C1, 2 Representative examples of the firing frequency of a LDT and a PPT neuron and baseline and in the presence of α-synO. The alterations in firing frequency induced by α-synO were significant in both the LDT and PPT across the recorded population of cells in either nucleus as depicted in the histograms on the right. *p < 0.05. Control reflects measures taken of baseline. Black traces are control and gray traces are following application of α-synO.

frequency of 50% over baseline was induced in PPT neurons (control: 0.82 ± 0.3 Hz; α-synO: 1.2 ± 0.4 Hz; n = 4; p = 0.03; Paired t-test; Fig. 4C2). We found no significant differences between the relative change from baseline in the α-synO-induced firing frequency when comparing this effect between the two nuclei (p > 0.9; Unpaired t-test).

Monomeric α-syn induced membrane currents, altered synaptic activity and heightened excitability in both sleep-controlling nuclei

In our previous report [30], we showed that the monomeric form of α-syn (α-synM) evoked an excitatory membrane response in all of the neurons examined in the LDT irrespective of phenotype (n = 35/35) with an average amplitude of 7.0 ± 2.7 pA (n = 35; Fig. 5A1, 3, 4). In agreement with our earlier report [30], in this study, all tested PPT cells exhibited an excitatory membrane response which was also phenotype independent (Fig. 5A2, 3, 4). While a greater average amplitude of current was elicited in PPT neurons (8.9 ± 2.5 pA, n = 9), this did not constitute a significant difference from the amplitude evoked in the LDT (p = 0.32; Unpaired t-test; Fig. 5A4).

Next, we determined effects of α-synM on sEPSCs and found that this form of the protein induced a significant increase of 18.7% over baseline in the amplitude of the sEPSCs in LDT neurons (control: 8.0 ± 0.8 pA; α-synM: 9.5 ± 0.9 pA; n = 6; p = 0.01; Paired t-test; Fig. 5B1, 3, 4). A significant sEPSCs amplitude increase of 16% was noted in PPT cells (control: 5.1 ± 0.5 pA; α-synM: 6.0 ± 0.5 pA; n = 4; p = 0.008; Paired t-test; Fig. 5B2, 3, 5). In contrast to what was seen for the other forms of the protein, α-synM also induced a significant increase in the frequency of sEPSCs in the LDT (23.3%) (control: 6.0 ± 1.8 pA; α-synM: 7.4 ± 2.2 pA; n = 6; p = 0.04; Paired t-test), which was not an effect we observed in the PPT (control: 5.0 ± 1.8 pA; α-synM: 4.5 ± 1.2 pA; n = 4; p = 0.2; Paired t-test) (Fig. 5B6).

When we investigated excitability changes induced by α-synM, we found an increase of 15% in the firing frequency of LDT neurons, which was a significant change (control: 0.31 ± 0.03 Hz; α-synM: 0.36 ± 0.01 Hz; n = 4; p = 0.03; Paired t-test; Fig. 5C1). In contrast, while the firing rate was relatively higher following α-synM, the increase was not significant in the examined PPT neurons (control: 1.2 ± 0.8 Hz; α-syn: 1.7 ± 1.3 Hz; n = 3; p = 0.35; Paired t-test; Fig. 5C2).

α-syn alters intracellular calcium in sleep-controlling nuclei

The neuronal effects induced by α-syn in its different structural conformations could impact intracellular calcium levels differentially, which could then substantially impact neuronal functioning. To investigate whether calcium is altered by α-syn in a structurally dependent manner, we applied α-synF, α-synO, or α-synM and assessed changes in intracellular calcium levels using a fluorescent calcium indicator.

Fibril α-syn

Similar to findings in our previous study [30], α-synF elicited changes in intracellular calcium dynamics in the LDT and PPT neurons that were comparable for the two nuclei (Fig. 6). Within the LDT, 97% and in PPT, 93% of the cells responded to α-synF with changes in fluorescence indicative of changes in calcium (LDT: n = 60/62; PPT: n = 56/60; Fig. 6C1). Responses elicited were categorized into two types: an increase or a decrease in fluorescence corresponding to increases or decreases in intracellular calcium, respectively. In both nuclei, α-synF elicited an increase in calcium in the majority of cells (LDT: 71.6%, n = 43/60; PPT: 60.7%, n = 34/56; Fig. 6C2). The proportion of cells responding to α-synF was not significantly different in LDT and PPT (p = 0.5, Fisher’s exact test; Fig. 6C1), and the proportion of responding cells exhibiting an increase in intracellular calcium did not differ between these two nuclei (p = 0.6; Fisher’s exact test; Fig. 6C2).

Intermediate/Oligomeric α-syn

α-synO induced a change in fluorescence indicative of an alteration in intracellular calcium in 76.0% (n = 35/46) of cells within the LDT, and in 92.0% of cells within the PPT (n = 23/25) (Fig. 6B2, C1). In both nuclei, the majority of
cells responded with an increase in calcium (LDT: 82.8%, \(n = 29/35\); PPT: 86.9%, \(n = 20/23\); Fig. 6C2). The proportion of examined cells responding to \(\alpha\)-syn\(_{O}\) was not significantly different between the two sleep-related nuclei (\(p = 0.06\); Fisher’s exact test; Fig. 6C2), and there were no nucleus-based differences in the proportion of directionality of the response (\(p = 0.9\); Fisher’s exact test; Fig. 6C2). Taken together, this suggests that \(\alpha\)-syn\(_{O}\) exerts similar effects on intracellular calcium in the LDT and PPT.
Sleep-controlling neurons are sensitive and vulnerable to multiple forms of α-synuclein:…

Fig. 5 α-synM induced an inward current, enhanced synaptic transmission and altered the firing frequency in sleep-controlling nuclei. A Sample of membrane responses to α-synM which induced excitatory currents in neurons from the LDT (A1) and the PPT (A2) (scale in A1 applies to A2). Histograms showing that the proportion of recorded cells responding to α-synM (A3) and the amplitude of the current evoked by α-synM (A4) did not differ significantly between the LDT and the PPT. B α-synM induced an increase in amplitude of sEPSCs in LDT and the PPT neurons (B1-2) which is reflected in representative recordings of the synaptic events from individual cells, and in a higher gain detail of single sEPSCs in an LDT (top) and a PPT (bottom) neuron at baseline and in the presence of α-synM (B3). B4-6 Histograms from the population of LDT and PPT cells recorded are depicted which indicate no change in the frequency of sEPSCs and a significant increase in the amplitude of sEPSCs in the population of cells within the LDT and PPT. C α-synM induces changes in the frequency of action potential firing in LDT neurons. C1, 2 Shown are current clamp recordings from representative LDT and PPT neurons in which APs are elicited by injection of current to depolarize the membrane potential. As can be seen from the single examples, and also from analyses of the firing frequency from the population of cells shown in the histograms, a significant increase in the firing rate was elicited by α-synM in neurons from the LDT. *p < 0.05. Control reflects measures taken of baseline. Black traces are control and gray traces are following application of α-synM.

Monomeric α-syn

Similar to the effects of the other two α-syn conformations, α-synM exposure lead to alterations in intracellular calcium in the majority of cells in the sleep-controlling nuclei (LDT: 88.0%, n = 94/107; PPT: 83.0%, n = 45/54; Fig. 6C1), which we had also observed in our previous study [30]. In both nuclei, the majority of responding cells exhibited increases in calcium (LDT: 64.3%, n = 69/94; PPT: 65.6%, n = 29/45; Fig. 6C2). There were no significant differences in the proportion of cells responding, or the proportion of responding cells which exhibited an increase or decrease in fluorescence between nuclei (p = 0.4; Fisher’s exact test; Fig. 6C2). Taken together, this suggests that the effects of the protein on intracellular calcium are similar in cells within LDT and PPT.

Effects on membrane currents, synaptic events, firing rate, and calcium levels in sleep-controlling nuclei are independent of the structural complexity of α-syn

LDT

All three conformational states of α-syn depolarized the membrane of both cholinergic and non-cholinergic LDT neurons. There were no significant differences between the current amplitudes elicited by the three different conformations of α-synin LDT neurons (Table 1). Similarly, there were no differences between the conformational states when comparing the α-syn effect of increasing the amplitude of sEPSCs; however, only α-synM also enhanced the frequency of sEPSCs within the nucleus (Table 1). An increase in firing frequency was induced by all three forms of α-syn (Table 1). In the majority of LDT cells examined, the three conformational states of α-syn induced changes in fluorescence indicative of alterations in intracellular calcium (Table 1). However, in terms of frequency of response, some variation was found when comparing actions of α-synF, α-synO, and α-synM on alterations in calcium levels. A significantly greater proportion of examined LDT cells responded to α-synF when compared to the proportion responding to α-synO. However, the proportion of responding cells which exhibited an increase in intracellular calcium was not significantly different for these two protein forms, and although this could in part be due to a mixture of species in the intermediate sample, the monomeric and fibrillar state are distinctly different and the amplitude of the rise was not significantly different between the three proteins (structures (Table 1). When taken together, we conclude that overall alterations of membrane currents, synaptic events, excitability and increases in intracellular calcium in neurons of the LDT were not dependent on α-syn conformation as effects of α-synM, α-synO, α-synF on these parameters did not vary greatly. PPT

Similar to actions seen in the LDT, we observed that the 3 conformations of α-syn altered the membrane current in 100% of the PPT neurons tested, and the amplitude of the evoked currents did not vary significantly when comparing α-synM, α-synO, or α-synF (Table 1). These effects were independent of phenotype. The α-syn-elicited increase of the amplitude of the sEPSCs did not vary significantly between the distinct protein samples, and none were found to enhance the frequency of sEPSC within this nucleus (Table 1). The firing rate of PPT neurons was significantly altered by α-synO and α-synF (Table 1). However, there were no significant differences in the proportion of cells responding with changes in intracellular calcium or in the proportion of responding cells exhibiting calcium increases following exposure to the three different protein samples (Table 1).

When taken together, with the exception that α-synM did not induce changes in frequency of firing or EPSCs within the PPT, we conclude that findings in the PPT were similar to those seen in the LDT in that all three forms of α-syn altered membrane currents, synaptic events, excitability and intracellular calcium.
In our earlier work, we were the first to report that α-synM has actions on membrane currents in mammalian neurons in ex vivo studies in the LDT and PPT. Further, we were the first to show that these actions, as well as rises in calcium, were associated with cell death, which we postulated were related. In the present study, we confirmed α-synM-mediated heightened cell death and extended our findings by showing that α-synF also leads to increased cell death by exposing one half of an LDT slice to control solution and the other half to either α-synF or α-synM and comparing cell death. When exposed to α-synF, the proportion of cell death was 13 ± 8.1% over control (n = 5) in the LDT and when exposed to α-synM, 12 ± 3.6% cell death over control (n = 6) was detected (Supplemental Information, Fig. 3). There were no significant
Table 1  Comparison of the membrane current, EPSCs, firing rate and intracellular calcium changes induced by three different structural forms of α-syn in sleep-controlling nuclei

| Structure | ΔIM (µA) | ΔEPSC—Amp | ΔEPSCs—Freq | ΔAP—Freq | ΔDF/F (%) | Increase in DF/F (%) |
|-----------|----------|------------|-------------|-----------|------------|---------------------|
| α-synF    | −7.3 ± 0.8 | ↑ 8.0% | ↑ 11.8% | ↑ 18.7% | 97.0% | 71.6% |
| α-synO    | −6.0 ± 1.5 | ↑ 6.4% | ↑ 23.3% | ↑ 52.0% | #76.0% | 82.8% |
| α-synM    | −7.0 ± 2.7 | ↑ 2.3% | ↑ 16.0% | ↑ 15.0% | 88.0% | 64.3% |

ΔIM, membrane current; α-syn α-synuclein (F: monomers; O: fibrils; M: intermediate/oligomers). Amp, amplitude, Freq, frequency, AP action potential. p value column calculated with either a one-way ANOVA or a Chi-square test.

#Significance in post hoc testing with the Fisher’s exact test. *Proportion of cells showing a change in DF/F

| Structure | ΔIM (µA) | ΔEPSC—Amp | ΔEPSCs—Freq | ΔAP—Freq | ΔDF/F (%) | Increase in DF/F (%) |
|-----------|----------|------------|-------------|-----------|------------|---------------------|
| α-synF    | −9.3 ± 1.9 | ↑ 8.0% | ↑ 8.4% | ↑ 16.0% | 93.0% | 60.7% |
| α-synO    | −3.5 ± 1.2 | ↓ 24.0% | ↓ 10.0% | ↓ 50% | #71.6% | 86.9% |
| α-synM    | −8.9 ± 2.5 | ↓ 16.3% | ↓ 23.3% | ↓ 41.6% | 39.5% | 85.6% |

I<sub>M</sub>, membrane current, α-syn α-synuclein (F: monomers; O: fibrils; M: intermediate/oligomers). Amp, amplitude, Freq, frequency, AP action potential. p value column calculated with either a one-way ANOVA or a Chi-square test.

#Significance in post hoc testing with the Fisher’s exact test. *Proportion of cells showing a change in DF/F

Membrane perforations are not induced by α-syn<sub>M</sub>

The live-cell imaging and fixed tissue fluorescence indicated an association with the membrane and raised the possibility that α-syn<sub>M</sub> could be creating pores which could allow the passage of ions, including calcium across the cell membrane. This mechanism had been suggested previously when it was noted that the presence of 500 nM of α-syn<sub>O</sub> in the pipette induced ruptures of the neuronal membrane following 20 min of exposure in primary cultures of rat hippocampal and dopaminergic neurons from the substantia nigra [42, 45, 46]. To examine this possibility, we conducted perforated patch recordings following well-established protocols [41] to detect the appearance of pores based on large changes in membrane resistance. In a control solution, a stable seal was formed between the membrane and maintained for over 40 min (resistance of the seal at 0 min: 1.7 GOhms; at 40 min: 1.5 Gohms; Fig. 7C1). In a separate population of cells, we added α-syn<sub>M</sub> (500 nM) to the patch pipette solution and monitored whether changes in the seal resistance appeared, putatively due to membrane perforations (Fig. 7C2). Results seen in control conditions were very similar to those obtained when α-syn<sub>M</sub> was present in the pipette solution. A stable seal was obtained for over 40 min (resistance of the seal in 0 min: 1.5 ± 0.4 GOhms; 40 min: 1.2 ± 0.2
GOhms; \( p = 0.1; n = 3; \) Fig. 7C3) suggesting no ruptures of the membrane under the pipette. Therefore, induction of inward current by \( \alpha\text{-syn}_M \) does not appear reliant on pore formation in the membrane of native mouse LDT neurons.
α-synM induced depolarization of the membrane due to direct actions on LDT postsynaptic neurons which was not dependent on IP3-mediated intracellular calcium stores or Voltage-Operated Calcium Channels (VOCCs)

To further investigate α-synM effects on the LDT, we evaluated the actions of α-synM on membrane currents during action potential blockade via inhibition of voltage-dependent Na⁺ channels with TTX. Following determination that α-synM responses could be repeated within cells (p = 0.6; n = 3; Paired t-test), we found that following an initial application of α-synM that induced inward currents, inward currents were still seen with the same frequency and amplitude upon a second application in presence of TTX (Table 2, Fig. 8A1, 2, 3). Current–voltage curves revealed a reversal potential of approximately −55 mV (−55.3 ± 1.9 mV, n = 3) which confirmed findings in our previous report [30]. In order to test a potential role for SERCA pumps which can mediate α-synM-induced calcium changes in cell culture models [47], we blocked IP3-mediated intracellular calcium stores with CPA (10 µM) and found no difference from control applications (Table 2, Fig. 8B1). We also found that Cd²⁺ (200 µM), a non-specific blocker of VOCCs [48] which have been shown to play a role in α-synM-mediated calcium rises [49], did not attenuate the frequency or amplitude but was instead associated with a significantly greater amplitude (Table 2, Fig. 8B2). When taken together with our TTX data and current–voltage curves, we suggest that inward current responses induced by α-synM are not greatly dependent on action potential-mediated release from the presynaptic terminal, and involve a mixed cation conductance at the postsynaptic membrane.

Glutamate and dopamine receptor involvement

To investigate whether a membrane-bound receptor is involved in α-synM-induced inward currents, we next examined the effects of selective antagonists of receptor targets of α-syn previously reported in the literature. While studies have shown that α-synO modulates AMPA/NMDA receptor activation [50], and we saw an increase of the amplitude of eEPSCs, responses were not significantly different in presence of DNQX (15 µM) and AP5 (50 µM), antagonists of AMPA and NMDA receptors, respectively (Table 2, Fig. 8B3). While α-syn has been shown to enhance dopamine (DA)-mediated transmission by modulation of DA type 2 receptors (DR2) [51], we found no differences in α-syn-effects in the presence of the DR2-like and DR1-like receptor antagonists, RAC and SCH-23390, respectively (Table 2, Fig. 8B4).

GPCR-mediated transmission

After exclusion of the most commonly published α-synM membrane targets, we decided to examine whether a GPCR was involved in the mediation of the α-synM membrane effect. Therefore, we replaced GTP in the intracellular solution with GDPβS (250 µM), a membrane impermeable, nonspecific inhibitor of GPCR signaling. To ensure that this manipulation was effective in blocking GPCR-involved signaling, we monitored the effect of DA on the membrane, as all reported actions of DA at DA receptors are mediated by G-proteins [52]. In the presence of TTX, DA (30 µM) induced an outward current in a population of LDT neurons (n = 3; Fig. 8C1). When GDPβS (250 µM) was included in the pipette, DA failed to induce any noticeable effect on the membrane current of LDT neurons (n = 3; Fig. 8C2). Next, we examined the effects of α-synM on LDT neurons in presence of GDPβS, and we observed that α-synM (100 nM) failed to induce an inward current in 76% of the LDT neurons, which was a significant difference from...
Sleep-controlling neurons are sensitive and vulnerable to multiple forms of α-synuclein:... miniature EPSCs (mEPSCs) following exposure to α-synM was not significantly different from baseline (control: 3.8 ± 1.3 Hz; α-synM: 3.5 ± 1.3 Hz; n = 5; p = 0.3; Paired T-test; Supplemental Information, Fig. 5). Further, α-synM did not significantly alter the amplitude of mEPSCs (control: 4.5 ± 0.7 pA; α-synM: 4.6 ± 0.4 pA; n = 5; p = 0.8; Paired T-test; Supplemental Information, Fig. 5). These data suggest that α-synM has actions outside the terminal on excitatory, presynaptic inputs directed to the postsynaptic neuron leading to action potential-dependent alterations in glutamate release from presynaptic terminals. As these glutamatergic terminals could derive from glutamate-containing cells in the LDT, these data support the conclusion that the population of bNOS- cells in which we observed α-synM-mediated membrane effects included local glutamate-containing LDT neurons. However, glutamatergic input also stems from projections directed to the LDT; therefore, the population of glutamate cells affected by α-synM remains to be determined.

**α-synM induces calcium responses directly in postsynaptic neurons which involve G-protein mediated signaling**

We first verified that effects of α-synM on calcium were repeatable. We found that there were no significant differences between the probability of responses between a first and second application of α-synM to the same cells (p = 0.9). However, the amplitude of the change in fluorescence indicative of alteration in calcium (DF/F) elicited by the second application of α-synM was significantly reduced by 18% from that seen with first applications (1st app DF/F: 11.0 ± 1.0%; 2nd app DF/F: 9.0 ± 1.0%; p = 0.0001; Fisher’s exact test; Supplemental Information, Fig. 5). Further, α-synM did not significantly alter the amplitude of mEPSCs (control: 4.5 ± 0.7 pA; α-synM: 4.6 ± 0.4 pA; n = 5; p = 0.8; Paired T-test; Supplemental Information, Fig. 5). These data suggest that α-synM has actions outside the terminal on excitatory, presynaptic inputs directed to the postsynaptic neuron leading to action potential-dependent alterations in glutamate release from presynaptic terminals. As these glutamatergic terminals could derive from glutamate-containing cells in the LDT, these data support the conclusion that the population of bNOS- cells in which we observed α-synM-mediated membrane effects included local glutamate-containing LDT neurons. However, glutamatergic input also stems from projections directed to the LDT; therefore, the population of glutamate cells affected by α-synM remains to be determined.

**α-synM enhances the amplitude and frequency of glutamate release from presynaptic excitatory synapses outside the terminal**

Since we saw effects of α-synM on the membrane current and calcium levels in distinct populations of LDT cells, we wanted to confirm whether these two physiological phenomena occurred in the same cells. To that end, we conducted single-cell calcium imaging by injecting a cell-impermeant form of the calcium indicator dye, Fura 2, via the recording pipette, and concurrently monitored the current of the membrane following application of high concentration of α-synM (500 nM) (Fig. 9B1, 2). In all cases, we observed...
that α-syn M induced an inward current that was greater at
this higher concentration than at 100 nM (− 127.5 ± 12.5 pA,
\( n = 6 \)) [30], and the membrane current was accompanied by
an increase in fluorescence indicative of rises in intracellular
calcium levels in the individual neurons (36.8 ± 10.4%DF/F,
\( n = 6 \)). These findings indicate the likelihood of a common
underlying mechanism mediating calcium and membrane
responses to α-syn M. Therefore, we examined the α-syn M-
mediated rise of calcium in presence of GDPβS. Relative to
baseline, α-syn M (500 nM) induced a significant increase in
intracellular calcium which was 50% lower in amplitude than
under baseline conditions (18.7 ± 6.4%DF/F, \( n = 7, p = 0.04 \),
Unpaired \( T \)-test; Fig. 9B3, 4). These results demonstrate that
α-syn M induces intracellular calcium increases within LDT
neurons that involve GPCR-mediated signaling. As the rise
in calcium was not eliminated, this suggests that additional
mechanisms could be involved, or blockade of GPCR-mediat-
ed mechanisms was not complete.

**Discussion**

To the best of our knowledge, ours is the first study in
native mammalian neural tissue to investigate the under-
lying mechanisms behind cellular and toxic effects of the
most common α-syn forms found in pathological conditions
(monomers, oligomers and fibrils) in sleep-controlling brain
regions. We conducted our examinations by exposing LDT
and PPT mouse neurons to protein samples that were care-
fully characterized before experiments. The monomeric and
fibrillar samples represent the pure start and end states of
the fibrillation process, while intermediate samples contain-
ing oligomeric species were extracted as on-pathway states

| Table 2 | Comparison of the frequency of responses and amplitude of membrane current and intracellular calcium changes induced by α-syn M in
control conditions or in presence of inhibitors |
|-----|-----|-----|
| Control | Inhibitor | \( p \) value |
| \( I_m \) Freq. (%) | TTX | 3/3 | 1.0 |
| | TTX | 1/1 |
| | CPA | 3/3 | 1.0 |
| | CPA | 5/5 |
| | Cd\(^{2+}\) | 4/4 | 1.0 |
| | Cd\(^{2+}\) | 8/8 |
| | DNQX + AP5 | 3/3 | 1.0 |
| | DNQX + AP5 | 5/5 |
| | SCH23390 + RAC | 7/7 | 1.0 |
| | SCH23390 + RAC | 8/8 |
| \( \Delta I_m \) (pA) | TTX | 6.0±1.7 | 3 |
| | CPA | 7.0±1.1 | 3 |
| | Cd\(^{2+}\) | 9.0±0.5 | 4 |
| | DNQX + AP5 | 10.0±4.0 | 3 |
| | SCH23390 + RAC | 6.6±1.1 | 7 |
| \( \Delta DF/F—Amp (%) \) | TTX | 100 | 23 |
| | CPA | 95.6 | 23 |
| | TTX | 9.0±1.0 | 22 |
| | CPA | 7.0±1.0 | 23 |

\( I_m \) membrane current, Freq frequency of responses, \( I_m \) membrane current, Amp amplitude of the response, \( \Delta \) change in the parameter.

A Paired \( t \)-test or Fisher’s exact test was utilized to compare differences and a \( p \) value was calculated when first and second applications of α-syn M were applied to the same cells. Results from individual same-cell applications of α-syn M with or without inhibitor are shown in paired plots in Fig. 8B below single representative examples of membrane responses in inhibitor. In cases where only the inhibitor was applied to de-
termine whether the inhibitors blocked responses, and no responses in those same cells in absence of the inhibitor were recorded, a \( p \) value is not
presented.

\( \Delta I_m \) (pA) | 6.0 ± 1.7 | 6.0 ± 1.7 |
| \( \Delta DF/F—Amp (%) \) | TTX | 3 |
| | CPA | 3 |
| | Cd\(^{2+}\) | 4 |
| | DNQX + AP5 | 3 |
| | SCH23390 + RAC | 7 |
| | TTX | 100 | 23 |
| | CPA | 100 | 23 |
| | TTX | 9.0 ± 1.0 | 22 |
| | CPA | 7.0 ± 1.0 | 23 |

To the best of our knowledge, ours is the first study in
native mammalian neural tissue to investigate the under-
lying mechanisms behind cellular and toxic effects of the
most common α-syn forms found in pathological conditions
(monomers, oligomers and fibrils) in sleep-controlling brain
regions. We conducted our examinations by exposing LDT
and PPT mouse neurons to protein samples that were care-
fully characterized before experiments. The monomeric and
fibrillar samples represent the pure start and end states of
the fibrillation process, while intermediate samples contain-
ing oligomeric species were extracted as on-pathway states
without further manipulation or perturbation. We confirmed
and extended our earlier findings that α-syn induced a post-
synaptic effect on the membrane current in neurotransmit-
ter-defined cells, altered synaptic activity, led to changes in
intracellular calcium, enhanced excitability, and induced cell
death in a structure-independent manner in sleep-controlling
neurons located in the brainstem. Further, using a traceable
α-syn probe, we found that α-synM antibody conjugated to

![Figure 9](image_url)

**Fig. 9** α-synM-induced fluorescence changes in LDT neurons were action potential-independent and reliant on intact G-protein medi-
ation signaling. **A** Sample fluorescent responses to 100 nM α-synM indicative of an increase in intracellular calcium in the presence of
TTX (A1). **A2** The α-synM-induced increase in intracellular calcium
was not dependent on intracellular calcium stores as indicated by
the persistence of calcium responses to α-synM in presence of CPA.
Histograms of the calcium responses from the population of cells
recorded, which show in bar graphs in A3 no significant changes in
the probability of responses in TTX or CPA, and no changes in the
amplitude of calcium increases under TTX conditions. Responses
were not attenuated when SERCA pump-mediated calcium stores
were pharmacologically depleted, and in fact, increases were signifi-
cantly greater in the presence of CPA. **B** In single-cell recordings, in
which the calcium indicator was introduced via the pipette, and the
centration of α-synM was 500 nM, α-synM was shown to induce
calcium rises via a GPCR-mediated mechanism in LDT neurons.
**B1** left panel A sample LDT neuron recorded in patch-clamp con-
figuration for single-cell calcium imaging is shown in bright field,
and in **B2** under fluorescent optics for Fura-2 imaging (380 nm). **B3**
Sample of fluorescence changes of the calcium indicator indicative of
an increase in intracellular calcium following application of α-synM
(top), and in another cell, the α-synM-mediated rise of calcium in
presence of GDPβS is shown, which was of a smaller amplitude when
blockade of G–protein receptor signaling was present. **B4** Histogram
of summary data from the population of recorded cells which shows
that the average amplitude of fluorescence increases in the presence
of GDPβS was significantly smaller than under control conditions.
* * p < 0.05, *** p < 0.0001
a fluorescent molecule associates with the surface of the neuronal membrane of cholinergic neurons, and does not appear to be internalized. Finally, we provide evidence that an unidentified GPCR is involved in the induction of inward currents and intracellular calcium rises.

Every form of protein that was analyzed, α-synF, α-synO, α-synM induced an excitatory inward current in the membrane of cholinergic and non-cholinergic neurons within LDT and PPT in mouse brain slices. While we have not been able to find reports, besides our previous work, that any of these forms of α-syn induces alterations in membrane currents in native mammalian neurons, other studies conducted in cultured cells have shown that α-syn induces a depolarization-evoked calcium rise, that α-synO elicits an increase in membrane conductance as well as permeability [42, 53, 54], and that α-synF alters membrane pump activity and efficiency of Na⁺ extrusion [55]. Although electrical effects on the membrane were not detailed, all of these reported alterations are likely to induce changes in the membrane current. Additionally, our findings complement other studies showing that α-syn can alter both post- and presynaptic conductances. In hippocampal brain slices and cultured cortical cells, 90 min of exposure to α-synO enhanced synaptic transmission by increasing the amount of current elicited by AMPA/NMDA postsynaptic receptors [50]. In cultured neurons, 30 min exposure to α-synM increased calcium influx, which was associated with heightened activity of VOCCs in the presynaptic compartment as documented by calcium imaging targeted to synaptic vesicles [56]. Our EPSC data do suggest activity of α-synM on glutamatergic presynaptic neurons, however, the lack of an α-synM-mediated change of mEPSC frequency does not support a direct action on VOCCs in the presynaptic terminal sufficient to enhance glutamate release, but rather, suggests that alterations in sEPSC frequency were due to excitatory actions upstream of the terminal, and dependent on action potential generation. Further, we failed to see evidence of an enhancement of synaptic currents through AMPA/NMDA receptors as the amplitude of mEPSCs was unchanged by α-synM, suggesting effects on increasing EPSC amplitude were due to actions on the presynaptic cell outside of the terminal. However, as exposure times greatly differed across the studies, this raises the possibility that effects on presynaptic VOCCs and AMPA/NMDA receptor-mediated conductances are sensitive to exposure duration.

Studies focused on the neuronal mechanism of α-syn effects have shown actions of the protein at both intracellular and extracellular locations. α-synM is able to enter into cells without being degraded by cellular proteolytic systems [57, 58] and produce alterations in synaptic transmission and excitability [59]. Alternatively, α-synO can act extracellularly, and lead to formation of 'pore-like structures' in the membrane similar to cation channels which results in increases in intracellular calcium levels [42, 46, 54] as well as interact with AMPA/NMDA receptors [50], potentiate VOCCs, activate SERCA pumps [47, 56], or affect downstream signaling of dopamine D2 receptors [51]. In our studies using the labeled-probe α-syn, internalization of the peptide was not noted. Using the perforated patch-clamp recording method that allowed us to monitor the resistance of the seal formed under the pipette in the presence of α-synM, we did not find evidence of pore formation, as this would have been reflected in large reductions of the membrane seal resistance. Our investigations using blockers of synaptic transmission showed that α-syn induces inward currents likely through a postsynaptic mechanism, and that this membrane excitation was independent of glutamatergic ionotropic receptor activation, VOCC activation, intracellular calcium stores, or enhancement of dopamine receptor signaling. Using a blocker of G-protein coupling, we provide the first evidence that α-synM induces excitatory currents in the membrane of LDT neurons that depend on G-protein signaling, which provides a mechanistic insight into a hitherto unknown process triggered by α-syn. We also showed that the other two forms caused excitation of the membrane as well. While we eliminated a role for the most parsimonious possibilities based on literature, ultimately, we did not identify the specific GPCR responsible for membrane effects induced by the three tested forms of α-syn. Cholinergic and non-cholinergic neurons in the LDT have been shown to contain many GPCRs which when activated lead to cellular depolarization and rises in calcium, including orexin receptors, and corticotropin releasing hormone receptors [60–62]. Stimulation of these receptors by their known canonical agonists at the concentrations used did not appear to cause cell death in those earlier studies, but it is possible that activation by α-syn activates a toxic cascade. While we did test the GPCRs shown in other literature to be affected by α-syn, it was beyond the scope of the present study to test other GPCRs that are present in the LDT, which had not previously been shown to interact with α-syn. Therefore, identification of the relevant GPCR remains a topic for future studies.

Exposure to α-syn (monomeric and oligomeric) leads to alteration in intracellular calcium levels in neurons, and several mechanisms have been implicated in prior reports. α-syn has been shown to directly interact with calcium pumps [35, 47, 63-Preprint] as well as VOCCs [56], and to lead to flux of calcium subsequent to formation of pore-like structures in the plasma membrane [42]. Our data support our previous work showing that α-syn induces rises in intracellular calcium in native mammalian neurons, and we extend that work by showing that rises in calcium occur in a structure-independent manner. We provide evidence that alteration in the calcium rise is induced directly in the postsynaptic cell concomitant with the depolarization of the membrane, and
Sleep-controlling neurons are sensitive and vulnerable to multiple forms of α-synuclein: ... 

while SERCA pumps were not involved in rises as depletion of this calcium store did not reduce calcium increases, this effect on calcium appeared to be linked to G-protein coupled intracellular mechanisms.

We demonstrated that prolonged exposure to α-synM and α-synF heightens cell death. We did not examine whether α-synO induces cell death; however, other studies have reported that cell death in primary cultured neurons is induced by α-synO and this mortality increase is associated with rises in calcium, whereas α-synM, which also leads to rises in calcium, does not appear to induce toxic effects in these cultured cells [34, 58]. It has been well established that sustained overexcitability [64, 65] exposes neurons to excitotoxic levels of glutamate which results in a transient increase in cytosolic calcium concentration that is followed by a profound collapse of the mitochondrial membrane potential leading to neuronal death [66–70]. Furthermore, the sustained rise in calcium induced by α-syn could induce toxicity by damaging mitochondria, triggering lysosomal leakage, disrupting microtubules, and/or increasing levels of ROS leading to the trigger for apoptotic processes. Supporting this hypothesis, blocking intracellular calcium rises reduces cytotoxicity induced by α-synO [35]. Moreover, α-syn-mediated cell death in a yeast model of cultured hippocampal cells was due to the ability of α-synO to increase basal intracellular calcium levels, resulting in an oxidative burst that ultimately lead to apoptotic processes and cell death [71]. Abolishing intracellular calcium elevation prevented cell death. Our data from native LDT neurons are consistent with the interpretation that rises in calcium induced by α-syn contribute to the heightened cell death seen in the LDT following exposure to α-synM and α-synF.

The cell death induced by α-synM which was not seen in primary neuronal cultures from other brain regions could be due to differential sensitivity of distinct neuronal populations to intracellular calcium levels and suggest that as systemic of α-synM increases, damage could be induced primarily in select vulnerable neuronal populations which show this rise in calcium and increase in cellular excitability. While we were focused on the mechanisms of the monomeric form in light of our interest in symptoms present prodromal in α-synucleinopathies, we did examine whether excitatory effects of fibril and intermediate/oligomeric forms involved GPCR mechanisms. Like the monomeric form, excitation of LDT cells by the fibril and the oligomeric forms was dependent on GPCR mechanisms. Therefore, based on our data, we speculate that cell death due to the more aggregated forms of α-syn involves the same unidentified GPCR implicated in cell death seen with the monomeric form of α-syn. However, we do not rule out involvement of other mechanisms by which oligomeric and fibril species could lead to cell death.

Although there are discrepancies in the literature, a tenet has emerged that oligomers and fibrils induce cell death [35, 72], whereas the monomeric form is non-harmful [35]. However, in our previous report, we showed that monomers and fibrils induced a similar neuronal effect on excitatory currents and intracellular calcium levels of sleep-controlling neurons, and that the monomeric form induces cell death putatively through excitotoxic mechanisms, which provided evidence that challenges this tenet [30]. We have extended that work by showing here that native monomeric, intermediate oligomeric and fibril α-syn states induce similar excitatory cellular actions and that the fibril form as well as monomeric form is toxic to sleep-controlling neurons. The lack of concordance between previous studies and ours could derive from multiple factors such as differences in the in vitro model used. Many previous studies have utilized neuronal cultures [42] where it is possible to study single homogeneous cell populations, whereas we have utilized brain slices where the three-dimensional architecture and micro milieu of the cells is closer to that seen in vivo [73]. Another technical difference may be ascribable to the differences in protein preparations in the various studies. Different purification protocols or fibrillation assays could result in various polymorphic structures and in addition not all studies verified the specific structure of the protein applied. Different exposure methods to α-syn were employed across the studies which included intracellular or extracellular exposure, acute applications or incubations for hours or days. In addition, a great variety in the concentrations utilized has been reported with some above those seen under pathological conditions (1 μM, 5 μM). Other differences in findings could result from the disparate brain regions investigated. Many previous studies were conducted recording neurons in the substantia nigra, striatum, cortex and hippocampus [42, 50, 56, 74], while we recorded from cells that regulate behaviors which are first affected in PD. Here, we carefully characterized each sample to ensure identification of the specific form used, which included on-pathway intermediate species. Furthermore, we conducted our recordings in native mammalian neurons of the LDT and PPT that are nuclei located in the brain stem which is a neural region hypothesized to be affected earlier in the disease [8, 15, 75]. Finally, by using nM concentrations, we exposed the neurons to α-syn concentrations similar to what is seen under clinical conditions based on cerebral spinal fluid studies of PD patients [76, 77]. When quantifying the levels of α-syn in cerebral spinal fluid, many studies have not specified protein structural state; however, as patients were included post diagnosis, presumably aggregated forms were predominant in the samples, with the additional caveat that oligomeric species are transitory [78], precluding accurate measuring. Nevertheless, a recent study that used an antibody which
did not recognize the monomeric form reported presence of protofibrils in PD patients at a concentration of 4.4 nM [79]. When taken together, this suggests our use of a concentration of 100 nM was more replicative of clinical physiological exposure than studies using higher concentrations. We believe that our findings suggest that neurons within the LDT and PPT are vulnerable and sensitive to three common forms of α-syn, including one that appears early or prodromal in PD etiology.

Different conformations of α-syn have been linked to distinct symptoms in α-synucleinopathies. Injections of fibrils lead to progressive motor impairment and cell death in a phenotypic PD-like animal model, whereas injections of oligomeric forms result in a distinct phenotype displaying a combination of Multiple System Atrophy traits with PD-like symptoms [34]. While the different α-synucleinopathies are characterized by separate cardinal symptoms, interestingly, sleep disorders can be present during the preclinical phase of many if not all of the α-synucleinopathies, including PD, Lewy Body Dementia and Multiple System Atrophy [10, 11, 80]. The fact that distinct sleep-controlling nuclei are equally affected by the presence of α-syn, regardless of protein structure, provides a mechanistic explanation for the appearance of SDs as a common prodromal symptom in the preclinical phase of this group of diseases. We speculate that neurodegeneration is wrought by rising levels of α-syn present early in the disease interacting with the LDT and PPT, irrespective of protein form. As the LDT and PPT are key regulators of motor control during sleep and arousal during wakefulness, α-synucleinopathies in the prodromal phase would share the common feature of sleeping disorders. Furthermore, in this study, we showed that α-syn induces depolarization and calcium rises in cholinergic and non-cholinergic neurons, and we believe therefore that cell death is not specific to one phenotype in the LDT and PPT. The cell loss and alteration of neuronal transmission induced by α-syn in different neurotransmitter-defined subpopulations within LDT and PPT in the present study suggests that there could be early functional changes in these diseases beyond sleep as the LDT and PPT control behavior elements besides sleep and wakefulness cycles. For example, PD patients develop postural instability and gait disorders which may be due, in part, to loss of cholinergic and non-cholinergic PPT neurons [81–83]. Therefore, our data could shed light on other features and symptoms of α-synucleinopathies that may be altered in the preclinical phase of PD [84]. Several projects are ongoing which based on past results, could confer ability to clinically detect different structural forms of α-syn in individuals [85–87], and if the different structural forms of α-syn can be targeted, this offers hope that those with sleeping disorders, especially RDB and EDS could be screened for changes in α-syn and in the future, rising levels of this protein could be directly targeted. Alternatively, neuroprotective strategies could be implemented at an earlier phase if rising levels of the protein are detected, which might be more successful in slowing or halting neuronal loss. Altogether, our findings provide valuable new insight into the mechanisms associated with α-syn-induced cellular effects. We conjecture cellular effects seen in the LDT and PPT are harmful and would lead to alteration in behaviors controlled by these nuclei.

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Author contributions ABDS, MPK and KAK initiated the study and designed the experimental strategy. LKS, TSP, and AEL expressed and characterized protein structure. ABDS, ST and EM performed and analyzed electrophysiological, and calcium imaging experiments. ABDS and CRRL performed neurodegeneration imaging experiments and data analyzes. ABDS and KAK performed statistical evaluations. LKS, AEL, EM, ST, MPK, ABDS and KAK prepared figures. ABDS, AEL, MPK and KAK wrote the paper, and all authors provided critical feedback on early drafts. All authors read and approved the final manuscript.

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Declarations

Conflict of interest All authors declare that there are no conflicts of interest in regard to this manuscript.

data availability upon acceptance, data are available at a repository at the university of copenhagen and made available upon reasonable request to the authors.

ethics approval Animals were under approval in accordance with European Communities Council Directive (86/609/EEC).

Statement about data used in another study In the data set reporting amplitudes of effects with monomer and fibril, some of the same data were used in a previously published study (2021;11(4):1773–1790. https://doi.org/10.3233/JPD-212554). However, the present report represents a distinctly different study as it examines a hypothesis, and presents conclusions which are different from those in the earlier publication. In addition, we present the results of many new experiments in this report.

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