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Alpha-Synuclein Promotes Dilation of the Exocytotic Fusion Pore

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ABSTRACT

A unifying feature of major neurodegenerative diseases is the aggregation and deposition of misfolded proteins. However, our understanding of the normal function of the proteins implicated in the pathology of these disorders is markedly narrow. Specifically, whether the abnormal accumulation of these proteins reflects a cause or a consequence of pathology remains controversial.

The presynaptic peripheral membrane protein α-synuclein has been strongly implicated in both familial and sporadic forms of Parkinson's disease (PD). Point mutations in α-synuclein cause inherited PD, as well as gene duplication and triplication lead to disease, implying a role for the wild-type protein. Recently, it has been demonstrated that overexpression of α-synuclein inhibits exocytosis in primary hippocampal neurons as well as in neuroendocrine cells, implying a role for synuclein in the regulation of transmitter release. However, genetic ablation of α-synuclein and its two family members beta- and gamma-synuclein has yielded very modest changes in exocytosis, raising the question of whether synuclein's effect on exocytosis reflects a normal function of the protein or a toxic gain-of-function.

To understand the role of synuclein in the regulation of exocytosis, we have utilized total internal reflection microscopy (TIRFM) of primary mouse adrenal chromaffin cells to study the dynamics of individual release events. Using BDNF-pHluorin, a fluorescent reporter of dense core vesicle release, we demonstrate that overexpression of α-synuclein accelerates and loss of endogenous synuclein prolongs the kinetics of exocytosis. Experiments with the integral membrane protein VMAT2-pHluorin confirm that this effect is indicative of altered fusion pore dynamics. Further, we demonstrate a similar effect on dense core vesicle exocytosis in primary hippocampal neurons. Using high-resolution structured illumination microscopy, we observe α-synuclein bound directly to secretory granules. Finally, we characterize the behavior of two familial PD-linked synuclein point mutations, A30P and A53T. These mutants exhibit normal binding to secretory granules and inhibition of exocytosis, but fail to accelerate the kinetics of release, suggesting a normal role for the protein in the dilation of the fusion pore that may be dysregulated in disease.

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CHAPTER 1: INTRODUCTION

Synuclein and Parkinson's disease

Nearly twenty years ago, point mutations in α-synuclein were linked to inherited cases of Parkinson's Disease (PD), making it the first causative gene identified [1]. Despite intensive study of the protein in the time since, we still know remarkably little about the normal biology of α-synuclein and whether or not it contributes to the pathogenesis of the disease. Indeed, perhaps the largest unanswered question in the field is whether the underlying pathology stems from a toxic gain of function, due to aggregation or membrane pertubing properties of the protein, or a loss of normal function, which could in part also be triggered by aggregation and sequestration of the functional protein species into Lewy bodies or smaller cytoplasmic inclusions. Regardless of the precise cause, there is an enormous amount of evidence implicating α-synuclein in the pathogenesis of both familial and sporadic forms of the disease. This introduction will attempt to explore the role of α-synuclein in the regulation and maintenance of the endo-lysosomal pathway and how dysregulation of α-synuclein or other genes linked to PD may ultimately lead to pathology.

Clinically, PD is characterized by dramatic loss of dopaminergic neurons in the substantia nigra pars compacta. Post-mortem analysis of subcortical brain tissue shows characteristic proteinacous inclusions known as Lewy bodies. These structures were originally believed to be an artifact of cellular degeneration. It had already been appreciated that α-synuclein was one of the principal immunohistological components of Lewy bodies [2, 3], but once mutations in α-synuclein were linked to familial PD, it suggested a causative role for the Lewy body pathology itself. This remains questionable for several reasons. Cell loss in the substantia nigra precedes the onset

of symptoms and extensive Lewy body pathology, suggesting that protein accumulation and deposition are not primary causes of disease. Along these lines, tissue analysis of many older individuals (up to 30% of centenarians) reveals extensive synucleinopathy (incidental Lewy body disease) with no clear neurological symptoms [4,5]. This has led to the idea that Lewy body pathology may reflect a neuroprotective response, where the cell attempts to sequester away toxic or misfolded proteins. An unwanted side effect of this process could be depletion of functional healthy protein from the intracellular milieu or formation of intermediate structures that confer toxicity.

Lewy pathology was originally considered to involve only α-synuclein, but β- and γ-synuclein can deposit in both PD and Dementia with Lewy Bodies, (DLB) a related synucleinopathy that involves synuclein deposition in cortical areas [6]. Similar to α-synuclein, β-synuclein accumulates pre-synaptically in PD and has been suggested to ameliorate the toxicity of α-synuclein by reducing either its aggregation or its expression [7,8]. However, polymorphisms in β-synuclein predispose to DLB [9] and transgenic mice overexpressing the variant develop degeneration and behavioral abnormalities [10]. Overexpression of β-synuclein appears to be equally as toxic to neurons as overexpression of α-synuclein [11]. There have not been any identified mutations in γ-synuclein that cause familial PD or related disorders, but overexpression of this isoform also produces degeneration in transgenic mice [12], and a polymorphism has been linked to DLB [13]. Similar to α-synuclein, β- and γ-synuclein may thus directly promote degeneration or contribute to disease simply through a decline in their normal protective function [14,15].

In addition to point mutations, duplication and triplication of the chromosomal region surrounding the α-synuclein gene have been found to produce dominantly inherited PD [16,17]. The affected chromosomal region contains several other genes, but the neuropathology reveals deposition of synuclein [18,19], and the phenotype most likely reflects multiplication of the α-synuclein gene. This indicates that simply increasing the dosage of wild-type protein suffices to cause disease, rather than changing the properties of the protein, as could be argued with the mutations. Duplication of α-synuclein leads to a form of PD similar in onset and symptoms to the sporadic disorder, but triplication causes an exceptionally severe phenotype, with much earlier onset and prominent cognitive as well as motor impairment [16,20,21].

α-Synuclein is highly and widely expressed in neurons [22], which may explain the more pronounced neurologic and behavioral deficits observed with gene multiplication as α-synuclein levels are upregulated in larger populations of cells not typically as affected in idiopathic PD. In contrast, the preferential involvement of particular systems such as the nigrostriatal projection in sporadic PD pathology may reflect the upregulation of synuclein within discrete populations of neurons, perhaps in response to oxidative stress. In support of this, genome-wide association studies (GWAS) of genetic risk in idiopathic PD reveal the largest contributions from the synuclein gene itself (as well as the microtubule-associated protein tau) [23]. The polymorphisms implicated in sporadic PD lie outside the exonic regions of α-synuclein and therefore likely affect mRNA transcript expression rather than protein function. It has been demonstrated that a particular polymorphism upstream of the α-synuclein gene (Rep1) can influence expression of a reporter gene in vitro [24].

In addition to increased expression attributable to gene multiplication or polymorphisms, α-synuclein has been repeatedly identified as a gene responsive to toxic insult and growth factors. Injection of quinolinic acid directly into the striatum upregulates α-synuclein in the substantia nigra [25], and oxidative stress induced by insecticide exposure or the loss of antioxidants also increases α-synuclein levels [26,27]. Synuclein may thus upregulate in response to many forms of cellular damage, which could explain this occurrence in the context of many different genetic mutations. It may also explain the selective vulnerability of nigral dopamine neurons, where dopamine itself can be a significant source of oxidative stress. In certain cases, cellular toxicity and cell death cascades appear to work directly through α-synuclein expression. Strikingly, α-synuclein overexpression is capable of protecting cells from the toxic effects of the redox-active herbicide paraquat [28], whereas mice lacking endogenous α-synuclein are resistant to the neurotoxic MPTP [29]. These findings enhance the putative role of α-synuclein as a response hub for cellular insult in disease and perhaps normal aging.

α-Synuclein is merely one in a growing constellation of causal genes linked to inherited PD. It is noteworthy that the presumed mechanism of degeneration for most if not all other genetic risk factors are themselves associated with changes in α-synuclein expression levels, aggregation and related toxicity. The other common theme emerging from studies of these genes is that they all appear to converge on the endo-lysosomal pathway and particularly, vesicular trafficking. With this is mind we will examine briefly some of the background studies of these genes. Here we will not consider Parkin, PINK1 and DJ-1, which have also been linked to autosomal recessive PD and seem to

act to promote mitochondrial function. It is clearly possible, if not likely, that this regulation of mitochondrial function impinges either directly or indirectly on endo-lysosomal trafficking, via changes in energy homeostasis or mitophagy. However, for the sake of brevity, we will consider genes with a clearer relationship to vesicular trafficking.

Endo-lysosomal proteins implicated in inherited PD

VPS35

Mutations in a subunit of the retromer complex, VPS35 (PARK17) are linked to autosomal dominant, late-onset PD [30,31]. This is of particular note, because it is uncommon for a genetic defect to result in a late onset form of the disease that more closely resembles sporadic PD. The retromer is a coat-protein complex that acts to sort protein cargo within the endocytic pathway by shuttling cargo otherwise destined to the lysosome for degradation back to the trans-Golgi network (TGN) [32]. These cargoes include a wide variety of receptors and their ligands, including sortilin and sortilin-related receptor (SORL1), the cation independent mannose-6-phosphate transporter (CIM6PR), glutamate receptors and phagocytic receptors, and the β-amyloid precursor protein (APP) [33,34].

How might mutations in VPS35 cause PD? Studies have suggested that the PD-causing mutations result in a loss of retromer function [35,36]. However, this is not likely due to deficits in retromer assembly, as mutant VPS35 interacts normally with its partners VPS26 and VPS29 to form the cargo-recognition complex. Instead, it appears mutations reduce the interaction of VPS35 with the WASH (WASP and Scar

homologue) complex, which is present on the endosomal surface and serves to induce actin nucleation and fission of tubules that act as transport intermediates during endosomal sorting [35].

The molecular consequences of VPS35 mutation are still being debated. A number of studies suggest mutations interfere with normal retrograde transport of CIM6PR from the endocytic pathway to the TGN [37]. This in turn reduces delivery of Cathepsin D to the endo-lysosomal pathway via constitutively released CIM6PR vesicles [37]. Cathepsin D has been shown to be the major proteolytic enzyme responsible for the lysosomal degradation of α-synuclein [38], raising the possibility that pathology arises from aberrant α-synuclein processing and aggregation. Alternatively, VPS35 may cause mistrafficking of ATG9, resulting in reduced formation and function of autophagosomes [35]. This could act to impair normal autophagy-dependent protein quality control mechanisms, and notably, autophagosomes have been also been linked to intracellular clearance of α-synuclein aggregates. Regardless of the precise mechanism, it is generally agreed that retromer dysfunction results in an increase in α-synuclein aggregates [36].

A more recent study suggests an alternative mechanism to VPS35-mediated neurotoxicity: mitochondrial dysfunction. Expression of PD-associated VPS35 mutants in cultured neurons, mouse substantia nigra dopamine neurons, or patient-derived fibroblasts caused mitochondrial fragmentation and cell death [39]. Interestingly, this cell death could be prevented by inhibition of normal mitochondrial fission via the dynamin-like protein (DLP1) complex. VPS35 mutants displayed an increased interaction with DLP1, which resulted in enhanced turnover of DLP1 complexes and lysosomal targeting

for degradation. Finally, oxidative stress also promoted the VPS35 mutant-DLP1 interaction, which was also enhanced in brains of sporadic PD patients. This provides some novel insight into what may represent a generic source of neurotoxicity in PD.

ATP13A2

Loss-of-function mutations in ATP13A2 (PARK9) underlie an autosomal recessive form of early-onset parkinsonism [40]. It is somewhat clinically atypical of classical PD as it is characterized by pyramidal degeneration and dementia and is also known as Kufor-Rakeb syndrome (KRS). Mutations in this protein also cause a form of neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease [41]. ATP13A2 encodes a lysosomal 5P-type ATPase that is expressed predominantly in neurons [42]. It has been demonstrated that the result of the PD-causing mutations is to redistribute the protein from its normal lysosomal localization to the endoplasmic reticulum (ER) [43]. Interestingly, postmortem analyses of tissue samples from patients with sporadic PD show large reductions in nigral expression of ATP13A2, although the surviving neurons have higher levels relative to controls, suggesting the protein confers a protective phenotype [44].

How does ATP13A2 normally function in neurons? Studies of mutant ATP13A2 performed in patient-derived fibroblasts and dopaminergic neurons have demonstrated that in these cells, lysosomal activity is compromised, leading to decreased degradation of protein and clearance of autophagosomes [44]. This effect seems specific, as it could be completely rescued by expression of functional wild-type ATP13A2 [44]. A relevant consequence of this lysosomal inhibition is that it could serve to prevent proper

clearance of α-synuclein. In both animal and cell culture models of PD, expression of ATP13A2 leads to lower synuclein levels in dopamine neurons [45]. Knockout neurons lacking ATP13A2 also show much higher levels of endogenous α-synuclein than wild-type controls, and ATP13A2 colocalizes with α-synuclein in Lewy bodies [46,47].

Finally, ATP13A2 also has a putative role in mitochondria. Fibroblasts from KRS patients exhibited reduced ATP synthesis rates and lower mitochondrial membrane potential compared with controls [48]. si-RNA knockdown of ATP13A2 in SH-SY5Y cells led to lower autophagy levels and higher amounts of reactive oxygen species and oxidative stress [49]. In neurons, over-expression of ATP13A2 or ablation of ATP13A2 has opposite effects, inhibiting or inducing mitochondrial fragmentation respectively [50]. Although it remains unclear whether the effects of ATP13A2 on mitochondria are due to its direct localization there, or whether it modulates mitochondrial turnover indirectly, the function of ATP13A2 seems to reside at a crossroads of two major disease pathways: mitochondrial health and α-synuclein pathology.

TMEM230

The most recent protein linked to a familial form of PD is TMEM230. Mutations in this protein were discovered in a large North American family of northern European descent, and lead clinically to autosomal dominant PD with confirmed Lewy body pathology [51]. This is particularly worth noting since among the genes linked to inherited PD, SNCA and LRRK2 are the only other causative agents with both dominant inheritance and Lewy body pathology. The TMEM230 locus lies on chromosome 20p and encodes a predicted two transmembrane-pass protein with a short intralumenal loop and cytosolic-facing N- and C- termini. Disease-causing mutations were identified

at a site adjacent to the second transmembrane pass in the lumen (R141L), as well as in both the N-terminal (Y92C) and C-terminal (*184PGext*5/*184Wext*5) cytosolic regions. The C-terminal mutation is in the stop codon and results in the addition of either 7 (*184PGext*5) or 6 (*184Wext*5) extra amino acids. Immunohistochemical analysis confirmed that TMEM230 colocalizes with synaptic vesicle markers, as well as components of recycling endosomes. Interestingly, TMEM230 showed strong colocalization with VPS35, suggesting these two proteins may converge on a common pathogenic pathway that affects vesicle trafficking and recycling. Finally, in transfected mouse neurons, expression of mutant TMEM230 resulted in impaired synaptic vesicle trafficking as evidenced by lower transport speed and displacement.

Synaptojanin and auxilin

Synaptojanin-1 (SYNJ1) is a large (145kDa) protein highly enriched in presynaptic nerve terminals. Similar to dynamin, SYNJ1 interacts with the BAR-domain protein amphiphysin and undergoes dephosphorylation following depolarization [54]. It also interacts with endophilin, and assists in the resolution of clathrin-coated pits during intense stimulation via dephosphorylation of $PI_{4,5}P$ [55]. SYNJ1 has also been shown to act post-synaptically, as it mediates post-stimulus AMPA receptor internalization [56].

A missense Arg258Gln mutation in SYNJ1 has been linked to autosomal recessive early-onset PD [52,53]. This mutation is predicted to result in significant disruption of the protein's Sac1 phosphatase activity, which targets phosphatidylinositol monophosphate. Interestingly, a direct partner of SYNJ1 that aids in its uncoating of synaptic vesicles is auxilin-1, which itself was recently implicated in early-onset atypical PD. Mice lacking either SYNJ1 or auxulin-1 share nearly identical phenotypes of

severely impaired synaptic vesicle recycling and neurological deficits [57,58]. However, these proteins do seem to act on distinct phases of endocytosis, as auxilin-1 is responsible for disassembly of clathrin and clathrin chaperoning (early phase) while SYNJ1 acts later to promote the shedding of the clathrin adaptor from the bilayer surface [58]. Presumably, the severity of the disease phenotype in patients with SYNJ1 or auxilin-1 loss of function mutations reflects the indispensible role of these proteins in the facilitation of endocytosis. Although this may seem unsurprising given the necessity of this process for the normal signaling and functioning of neurons, the severe Parkinsonian phenotype still suggests that the nigral subpopulation is particularly vulnerable to this type of insult. In theory, this could be due to higher endocytic demand in this cell population, or a cell-type specific induction metabolic or oxidative stress of upon failure of endocytosis.

LRRK2

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of inherited PD [59]. LRRK2 is a massive protein (2527 amino acids) that is highly expressed in a many organs and tissues and containing several functional domains, which include a central catalytic domain with both GTPase and kinase activities as well as a number of potential protein-protein interaction domains [59,60]. The disease-causing mutations reside in this central core region, the most notable (and common) of which is the G2019S variant, located in the kinase domain. In vitro studies have conclusively demonstrated that the result of this mutation is an upregulation kinase activity [61]. This

domain is also capable of autophosphorylation of the LRRK2 Ras of complex (Roc) GTPase region, which may therefore allow LRRK2 to self-regulate its function [62].

In neurons, wild-type LRRK2 has been implicated in neurite outgrowth, perhaps by interacting with tubulins [63]. More recently, LRRK2 has been implicated in synaptic vesicle endocytosis by several independent investigators. Both knockdown of LRRK2 with siRNA and overexpression of G2019S mutant LRRK2 result in impairment of endocytosis [64,65]. Additionally, LRRK2 seems to also have effects on exocytosis, as silencing of LRRK2 in primary neurons increases release kinetics [66], while inhibition of LRRK2 kinase activity reduces neurotransmitter release [67].

The potential of LRRK2 function to influence vesicle recycling and release has a remarkable confluence with the emerging roles of α-synuclein at the presynaptic terminal. This may be particularly informative, as there appears to be a close relationship between these two genes in PD pathology as well. LRRK2 mutation generally involves Lewy pathology that may also reflect upregulation of α-synuclein gene expression [68]. Transgenic mice expressing human LRRK2 crossed with mice overexpressing human α-synuclein generate offspring with increased α-synuclein deposition compared with the amount seen in α-synuclein transgenics alone. In contrast, removing endogenous LRRK2 somewhat ameliorated the defects associated with mutant α-synuclein expression [69]. LRRK2 knockout mice also show a dramatic age-dependent accumulation of α-synuclein in the kidney (but not the brain), but there is as yet no explanation for this tissue-specific change in α-synuclein metabolism [70]. At this point though, it seems highly plausible that these two proteins may converge on common functional and pathological pathways, and the fact that they are both highly

implicated as risk factors in sporadic PD suggests that a better understanding of the LRRK2-α-synuclein interaction could prove vital in understanding the early steps in PD pathogenesis.

The Function of Alpha-Synuclein

Identification and initial characterization

α-Synuclein was originally identified using an antibody to purified cholinergic vesicles of the *Torpedo* electric organ [71]. Its expression was found to be predominately restricted to the nervous system, and its synaptic localization, it was found to inhabit a portion of the nuclear envelope, thus its original designation "synuclein". The discovery of α-synuclein also led to the concomitant identification of the closely related β- and γ-isoforms by the same group [72].

Shortly following this, another group discovered changes in α-synuclein mRNA transcripts within specific areas of the zebra finch brain [73]. Relative to other brain regions where synuclein remains at high levels through development and maturity, regions implicated in bird song show large, sustained reductions in synuclein expression during the animal's critical learning period for song acquisition. The tight regulation of synuclein expression in distinct cell populations during a learning period strongly suggests a role in synaptic plasticity, but there has been little progress in understanding this potential function of α-synuclein.

Synuclein was also identified during the biochemical characterization of senile plaques in Alzheimer's disease (AD). Although not as abundant as the β-amyloid, a

proteolytic peptide fragment shown to accumulate in AD, a fragment from the middle of α-synuclein (61–95) now termed the non-Abeta component (NAC) also accumulates at high levels in plaques [74]. Subsequent studies demonstrated that synuclein indeed contributes to the pathology of AD as well as of dementia with Lewy bodies (DLB) [75,76].

Finally, point mutations in α-synuclein were found to cause an autosomal dominant form of Parkinson's disease (PD) [1]. The clinical phenotype resembles idiopathic PD, with typical tremor, rigidity, and bradykinesia, and the pathology shows cytoplasmic Lewy body inclusions characteristic of PD. Mutations in α-synuclein are relatively rare in the PD patient population but the Lewy bodies and dystrophic neurites observed in idiopathic PD label strongly for α-synuclein [2,3,6], strongly suggesting its involvement in sporadic cases of the disease.

Protein structure

The structure of α-synuclein, a small protein of 140 amino acids, can essentially be divided into three regions: The N-terminal membrane binding domain, the hydrophobic core containing the NAC region that is highly abundant in senile plaques found in Alzheimer's Disease, and the C-terminal domain, which is largely unstructured and has been suggested to participate in protein-protein interactions and is a target for phosphorylation [77]. The N-terminal region contains seven 11-residue repeats that are predicted to form an amphipathic α-helix. These repeats are very highly conserved, both across species and among the three different isoforms, suggesting they play a critical role in the function of the synucleins. The motif is also unique, with no similar sequence identified outside the synuclein family. Remarkably, all of the mutations associated with

PD—A53T, A30P, and E46K as well as the more recently described G51D, G51E and H50Q, cluster within this N-terminal domain. It is also somewhat surprising that rodent synuclein normally contains a threonine at position 53, which causes PD in humans. The A53T mutation thus appears pathogenic specifically within the human context. Other model organisms such as *Drosophila*, *S. cerivisiae* and *C. elegans* do not contain any homologues of the synucleins, suggesting that these proteins are not generally essential for normal nervous function.

Purified, recombinant synuclein is an intrinsically disordered protein, as predicted from the sequence, forms an α-helix on binding to artificial membranes [78]. Nuclear magnetic resonance (NMR) studies of synuclein on SDS micelles also reveal an a-helix but bent, presum- ably due to the small size of the micelle [79]. On large unilamellar vesicles (LUVs), which have a larger diameter than micelles, the analysis of spin-labeled protein shows that synuclein adopts the extended 11/3 helix predicted from the sequence [80].

Membrane interactions and the Presynaptic Localization

The presynaptic location of α-synuclein has been recognized since its original identification as a protein associated with synaptic vesicles [71]. In contrast to many proteins involved in neurodegeneration that are distributed throughout the neuron, however, α-synuclein localizes specifically to the nerve terminal, with relatively little in the cell body, dendrites, or extrasynaptic sites along the axon [22,73]. In addition, α-

synuclein is widely expressed by many neuronal populations within both central and peripheral nervous systems, suggesting a general role in neuronal function.

Studies performed in our lab have demonstrated that despite the weak interaction of α-synuclein with cellular membranes, synuclein nonetheless recovers more slowly after photobleaching than GFP, suggesting that it doesn't behave as a purely soluble protein in cells [81]. The N-terminal membrane-binding domain of synuclein seems crucuial to mediate its membrane interaction, as the A30P mutation associated with familial PD in fact disrupts both the association of synuclein with brain membranes and the presynaptic location of synuclein in cultured neurons and accelerates the rate of recovery after photobleaching to that of GFP [81]. The effects of the A30P mutation thus strongly support a role for membrane binding by the N-terminus in pre-synaptic localization.

How then does synuclein localize specifically to presynaptic boutons rather than other cell membranes? The protein seems to prefer negatively charged phospholipids, but acidic headgroups are found on the cytoplasmic leaflet of many intracellular membranes. However, synuclein can also sense membrane curvature and interacts preferentially with high positive curvatures [82,83], making synaptic vesicles, which are the smallest biological organelles, ideal binding targets. Thus, the abundance of synaptic vesicles at nerve terminals may explain the preferential accumulation of α-synuclein in this compartment.

Potential function of α-synuclein at the nerve terminal

The presynaptic location of synuclein and its interaction with membranes have strongly suggested a role in transmitter release. Dopamine release by α-synuclein knockout mice recovers faster from repetitive stimulation than in wild-type animals, and the knockouts show a mild reduction in striatal dopamine stores consistent with increased release [84]. More recently, studies of α-/γ- double and synuclein triple knockouts revealed a substantial increase in striatal dopamine release in vivo not observed with the single knockouts [85,86], further supporting the idea that there is redundancy in function among synuclein isoforms. These animals did not exhibit a change in dopamine transporter activity or tissue dopamine levels, implicating a specific alteration of dopamine release. These effects on dopamine release in vivo are among the most dramatic reported for α-synuclein knockout mice and suggest a major disturbance in the mobilization of synaptic vesicles.

To mimic the increase in expression that causes PD in families with a duplication or triplication of the gene, α-synuclein has also been overexpressed both in culture and in transgenic mice. First studied in chromaffin cells, overexpression of the wild-type human protein was found to inhibit the exocytosis of dense core vesicles as measured by amperometry [87]. Our lab later reported a similar finding in primary hippocampal neurons, where α-synuclein inhibited exocytosis of synaptic vesicles, as measured by live-cell imaging of as well as by electrophysiology [88]. Although there were no overt signs of toxicity and α-synuclein was moderately overexpressed, it remained inconclusive whether this effect reflected a normal function of the protein or a gain-of-toxic function.

These concerns motivated subsequent studies in the synuclein triple-knockout (TKO) animals, which lack all endogenous synuclein. At hippocampal synapses, the effect of the triple knockout has been controversial. According to one report from the Sudhof laboratory, there was no change in baseline transmitter release [89]. However, an independent report by a former member of the same group showed an increase in transmitter release in the triple knockout [90]. The increase was small, possibly accounting for the failure to detect a change by others and raising the possibility that any change in release might be secondary. The loss of all three synuclein genes results in smaller presynaptic boutons [90], suggesting an alternative role for these proteins.

Perhaps the best clue to the normal role for α-synuclein at the presynapse was provided by previous work demonstrating a strong genetic interaction between α-synuclein and the degeneration produced by loss of the presynaptic chaperone cysteine string protein (CSPα) [91]. Knockout of CSPα does not acutely affect synaptic transmission as the animals are healthy shortly after birth, but eventually loss of this protein results in rapidly progressive synaptic degeneration and death within 2 months. CSPα thus appears required for the long-term maintenance of presynaptic function. Remarkably, the overexpression of α-synuclein greatly delays the degeneration due to loss of CSPα, and the loss of synuclein exacerbates the CSPα knockout phenotype [91], suggesting that synuclein may have a role as a chaperone, similar to CSPα. CSPα appears particularly important for the levels of t-SNARE SNAP-25 [92,93]. However, synuclein overexpression does not rescue the decrease in SNAP-25 protein levels, but it does recover SNARE complex assembly to normal levels. The results suggest that whereas CSPa has a specific role with SNAP-25 that secondarily affects SNARE

complex levels, synuclein has a specific role in SNARE complex formation and can bypass the defect in SNAP-25. Uncovering the mechanism behind these effects will undoubtedly aid in understanding the function of α-synuclein at the nerve terminal.

Main findings of dissertation

Despite intensive efforts and many years of study, we still understand remarkably little about the normal function of α-synuclein. Studies from our lab and other groups have implicated a role for α-synuclein in the regulation of neurotransmitter release, both by synaptic vesicles and dense core vesicles [87,88]. However, loss-of-function studies in mice lacking endogenous synuclein isoforms have yielded much more modest results [90], raising the question of whether the effects observed in studies overexpressing α-synuclein may reflect a gain of abnormal function. To date, our lab's studies of α-synuclein has relied mostly on monitoring the electrical stimulation-induced cycling of synaptic vesicles using vGlut1-pHluorin as a pH-sensitive indicator of vesicle exocytosis and subsequent endocytosis and reacidification. A significant limitation of this approach has been the inability to visualize single vesicle release events, which would allow more precise quantitation of release kinetics. In order to overcome these difficulties, we have utilized TIRF microscopy of large dense core vesicles (LDCVs) in chromaffin cells, where we can easily resolve individual secretion events.

In Chapter 2 of this dissertation, we show that in adrenal chromaffin cells, overexpression or genetic ablation of synuclein has opposing effects on the kinetics of individual exocytotic events. We demonstrate that this is caused by synuclein-mediated dilation of the fusion pore, and that synuclein normally acts to resist premature pore

closure after the onset of exocytosis. In complementary studies performed in primary neurons, we are again able to demonstrate opposing effects of loss of synuclein and overexpression on LDCV release kinetics. Further, we show that α-synuclein associates specifically with LDCVs, implying that it can play a primary role in the modulation of the fusion pore. Finally, we report that point mutations in α-synuclein linked to inherited PD fail to promote fusion pore dilation, suggesting for the first time that impairment of the normal function of α-synuclein may be related to disease progression.

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Chapter 2: Synucleins Promote the Dilation of the Exocytotic Fusion Pore

Abstract

The protein α-synuclein has a demonstrated causal role in familial Parkinson's disease as well as strong implications in the etiology of sporadic forms of the disease. Similar to other proteins implicated in neurodegenerative disease, the biological role of α-synuclein in the brain remains poorly understood. Localization of α-synuclein to the nerve terminal suggests a presynaptic role, yet mice lacking all three isoforms of synuclein fail to exhibit any overt changes in synaptic transmission. Using primary adrenal chromaffin cells and hippocampal neurons, we now demonstrate that both over-expressed and endogenous synuclein accelerate the kinetics of individual exocytic events. We find that this promotion of cargo efflux is a consequence of fusion pore dilation as well as a reduced tendency of the pore to reseal ("kiss and run"). Finally, we find that point mutations in α-synuclein implicated in familial forms of Parkinson's disease fail to promote fusion pore dilation, offering the first evidence that loss of α-synuclein normal function may play a causal role in the pathogenesis of the disease.

Introduction

Despite the established role of multiple proteins in the pathogenesis of neurodegenerative disease, we know remarkably little about their function. In Parkinson's disease (PD) as well as in the related conditions Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA), the peripheral membrane protein α-

synuclein accumulates in characteristic inclusions [1-4]. Mutations in α-synuclein also produce a dominantly inherited form of PD [5-10], demonstrating that the protein has a causative role. Indeed, α-synuclein gene duplication and particularly triplication produce a severe form of familial PD [11], implicating the wild type (wt) protein in disease. Synuclein thus has a central role in PD. However, the normal function of α-synuclein remains poorly understood. α-Synuclein normally localizes to the nerve terminal, suggesting a role in neurotransmitter release [12]. Consistent with this, modest over-expression (insufficient to produce inclusions or overt toxicity) inhibits the regulated exocytosis of large dense core vesicles (LDCVs) and synaptic vesicles [13-15]. However, the loss of synuclein has less effect, with minimal or no increase in glutamate release reported in triple knockout (TKO) mice lacking α-synuclein as well as closely related β- and γ- isoforms [16,17]. Knockout mice lacking α- and γ-synuclein show an increase in evoked dopamine release [18,19] but the physiological change responsible remains unknown. Although over-expression inhibits regulated exocytosis, the role of endogenous α-synuclein has thus remained unknown. α-Synuclein binds specifically to anionic membranes with high curvature [20-22], but can also deform the lipid bilayer. Synuclein aggregates membranes in yeast [23,24], tubulates artificial membranes in vitro [25] and when over-expressed in mammalian cells, can produce mitochondrial fragmentation [26,27]. However, membrane deformation is generally considered to have an important role in endocytosis [28] rather than exocytosis. The effect of over-expressed synuclein on exocytosis has thus been difficult to explain on the basis of membrane curvature-sensing or -promoting properties. Alternatively, synuclein has been suggested to serve as chaperone for the SNARE complex, but without apparent effect on transmitter

release [17]. How might membrane deformation by synuclein influence regulated exocytosis? In the course of exocytosis, synaptic vesicles form a fusion pore that dilates before full collapse into the plasma membrane. However, the pore can also close as part of a 'kiss-and-run' mechanism that immediately regenerates the vesicle [29]. Regulation of membrane curvature might thus affect behavior of the fusion pore. Since classical transmitters such as glutamate escape rapidly, postsynaptic recording might not detect a change in fusion pore kinetics. We have therefore used imaging to monitor directly individual exocytotic events. Single synaptic vesicle fusion events are difficult to detect by imaging, so we have focused on peptidergic large dense core vesicles (LDCVs) due to their size (70-200 nm diameter) and relatively slow release. Adrenal chromaffin cells have been used extensively to study the process of regulated exocytosis, including release by kiss-and-run [30], and previous work has indeed demonstrated the inhibition of LDCV exocytosis by synuclein over-expression in chromaffin cells [13].

Results

Synuclein over-expression accelerates the kinetics of individual exocytotic events in chromaffin cells

To understand how synuclein influences dense core vesicle exocytosis, we infected primary cultures from the postnatal mouse adrenal medulla with a lentivirus encoding human α-synuclein. Double staining for human α-synuclein and the LDCV cargo protein secretogranin II (SgII) confirmed expression of the human protein within

chromaffin cells (Supplementary Fig. 1A). We also assessed the relative levels of endogenous and overexpressed α-synuclein using an antibody that recognizes both rodent and human protein isoforms. Comparison of chromaffin cells from wt and synuclein TKO mice lacking all synuclein isoforms shows that chromaffin cells express endogenous α-synuclein at low levels, and the lentivirus confers modest over-expression (Supplementary Fig. 1B,C).

To study individual LDCV exocytotic events, we used a fusion of brain derived neurotrophic factor (BDNF) to the ecliptic pHluorin, a modified form of the green fluorescent protein with enhanced pH sensitivity [31]. Quenched at the acidic pH of LDCVs, BDNF-pHluorin fluorescence increases on exposure to the external medium by exocytosis [32]. Using TIRF microscopy, we first assessed the steady state distribution of "docked" LDCVs by bathing the cells in 50mM NH_4Cl to completely unquench all intravesicular BDNF-pHluorin. There was no change in the relative abundance of docked vesicles among wt, synuclein TKO and α-synuclein-overexpressing cells (Supplementary Fig. 11A). Additionally, we measured the fold change in fluorescence after NH_4Cl unquenching at the sites of individual vesicles as a relative indicator of the intravesicular pH. BDNF-pHluorin punctae showed a similar increase in fluorescence in all conditions, indicating that in our experiments, the relative abundance of synuclein does not affect the pH of LDCVs (Supplementary Fig. 11B).

We next monitored the behavior of BDNF-pHluorin-containing vesicles during depolarization with 45 mM K^+ [33]. Since kiss-and-run may occur more frequently at high external Ca^{++}[34], we used 5 mM external Ca^{++} to sample a wider variety of exocytic events. α-Synuclein over-expression reduces the number of exocytotic events detected

using BDNF-pHluorin (Fig. 1A), as measured previously by amperometry [13]. A change in Ca^{++} entry cannot account for this because Ca^{++} entry does not significantly differ among wt, TKO and SYN (Supplementary Fig. 2).

Individual exocytic events show a series of characteristic changes due to the over-expression of α-synuclein. First, although BDNF-pHluorin rapidly reaches peak fluorescence at the time of exocytosis, the rate increases with synuclein over-expression (Fig. 1B and Supplementary Fig. 3A). Second, after peak fluorescence, the rate of fluorescence decay (due to peptide release) also increases (Supplementary Fig. 3B). These results suggest acceleration of the release event by α-synuclein. However, the fluorescence events fall into at least four distinct classes (Fig. 1C and Supplementary Fig. 3C). Many events decay immediately to baseline (full decay) whereas others remain at maximum fluorescence for an interval before full decay (plateau-decay). In still others, decay is interrupted, suggesting either constriction or complete closure of the fusion pore, with or without a plateau preceding the decay (decay-plateau or plateau-decay-plateau). To determine whether the interruption of fluorescence decay reflects full pore closure, we quenched residual events using external solution adjusted to pH 5.5 with the impermeant buffer MES (Supplementary Fig. 4A). All events in the process of decay show quenching by the acidic buffer (Supplementary Fig. 4A,B), consistent with either full fusion or persistence of a dilated fusion pore rather than movement away from the plasma membrane. In agreement with this, the H^+-ATPase inhibitor bafilomycin does not influence the time course of events in any genotype (Supplementary Fig. 4C), confirming that loss of fluorescence indicates the release of peptide, not reacidification. Interestingly, most of the stable events

(plateau) show quenching by low external pH, but a fraction do not (Supplementary Fig. 4A,B). Thus, complete closure of the fusion pore occurs only in stable events, but incomplete closure (constriction) can still limit the loss of peptide. Consistent with the observed acceleration of release, synuclein over-expression increases the proportion of events that undergo full decay (Fig. 1C). Within the group undergoing full decay, however, synuclein over-expression also increases the rate of decay (Fig. 1D). Thus, synuclein accelerates release independent of effects on pore closure, suggesting a role early in exocytosis that promotes peptide release by dilating the fusion pore.

Loss of synuclein prolongs the kinetics of release

The difficulty detecting clear effects on transmitter release in knockout mice [14, 16, 17] suggests that α-synuclein over-expression may simply produce toxicity that secondarily affects release. It was therefore of great interest to determine how the loss of synuclein influences release kinetics. Consistent with previous work in neurons [14, 16, 17], chromaffin cells from synuclein TKO mice show no clear change in event number relative to wt (Fig. 1A). However, analysis of event distribution reveals an increase in the time to peak fluorescence (Fig. 1B and Supplementary Fig. 3A) and lengthening of BDNF-pHluorin decay time constants (Fig. 1D). In addition, TKO cells show increased latency to decay among those events that do not decay immediately (Fig. 1E) suggesting a defect in fusion pore dilation. Thus, loss of synuclein prolongs release, suggesting a similar role for the endogenous and over-expressed protein in exocytosis.

Although we used 5 mM external Ca^{++} for these experiments because it may promote kiss-and-run, we also examined BDNF-pHluorin events at a more physiological

[Ca^{++}] (2mM). In this condition, as at 5 mM Ca^{++}, synuclein over-expression both inhibits the exocytosis of chromaffin granules and accelerates the loss of BDNF-pHluorin (Supplementary Fig. 5A,B). The proportion of events again shifts to those with full decay, at the expense of those with interrupted release, and the time constant for fluorescence decay shortens (Supplementary Fig. 5C,D). The TKO also shows prolonged decay relative to wt (Supplementary Fig. 5D). Thus, synuclein has similar effects on release at 2 and 5 mM Ca^{++}.

Synuclein inhibits closure of the fusion pore ('kiss-and-run')

The effect of synuclein on release could reflect changes in the fusion pore or in the solubility of dense core vesicle cargo. Previous studies have demonstrated that individual LDCVs can release different substances at different rates [35, 36], indicating that the physical properties of the aggregated peptide can affect the rate of release. It seems unlikely that a cytoplasmic protein such as synuclein would directly influence lumenal cargo, but to distinguish further between effects on the fusion pore and on peptide solubility, we used a construct with the pHluorin inserted into a lumenal loop of the vesicular monoamine transporter VMAT2 [37], a polytopic membrane protein that localizes to LDCVs. Stimulation of endocrine cells expressing the VMAT2-pHluorin fusion produces discrete exocytotic events consistent with LDCVs [38]. To eliminate any contribution of reacidification toward the kinetics of fluorescence decay, we performed these experiments in the presence of bafilomycin. In chromaffin cells, α-synuclein over-expression also inhibits the exocytosis of VMAT2-pHluorin (Fig. 2B). As a membrane protein, VMAT2-pHluorin cannot be released extracellularly, and its decay therefore

reflects either spread within the plasma membrane or endocytosis, both processes potentially limited by the fusion pore [39]. Indeed, we observe events that spread and others that do not, as well as variation in the time course of fluorescence decay (Fig. 2A). Although the proportion of events with a latency to decay does not differ (46% for synuclein over-expression, 44% for control), the latency to decay shortens with over-expression of α-synuclein (Fig. 2B,C). The long duration of VMAT2-pHluorin events also enables us to probe a large number of persistent events with an acute acidic buffer (MES) challenge. We find that a substantial fraction of the persistent events in control chromaffin cells are protected from quenching by MES-buffered solution at pH 5.5 (Fig. 2D,E), indicating kiss-and-run (complete resealing of the fusion pore). In cells over-expressing human α-synuclein, this fraction declines substantially (Fig. 2E), suggesting inhibition of pore closure. However, synuclein over-expression also shortens the time constant of fluorescence decay for VMAT2-pHluorin (Fig. 2F), again supporting an independent effect on the rate of release. The analysis of membrane protein exocytosis thus supports an effect of synuclein on peptide release that cannot be explained by changes in cargo solubility.

Endogenous and over-expressed synuclein promote fusion pore dilation in neurons

Does synuclein also affect the properties of individual exocytotic events in neurons? To address this, we expressed human α-synuclein in primary rat hippocampal neurons [14], imaging cotransfected BDNF-pHluorin. As previously reported, stimulation at 50 Hz evokes discrete exocytotic events primarily in axons [40, 41]

(Fig. 3A). The average number of events per coverslip does not significantly change with synuclein over-expression (Fig. 3C), but quenching at low pH reveals a dramatic effect of synuclein on the proportion of events accessible to external solution ($p < 0.0001$ by Chi-square) (Fig. 3D). With over-expression, more events decay before the addition of low pH solution (Fig. 3A,D), indicating that as in chromaffin cells, the events decay more rapidly. Among those events that remain, synuclein over-expression increases the proportion quenchable by low pH (Fig. 3D), demonstrating that α-synuclein has an effect on the fusion pore (inhibits closure) independent of release rate.

Neurons express high levels of multiple synuclein isoforms, suggesting that the effect of the TKO might be greater than in chromaffin cells, which express modest levels. We were thus surprised that the synuclein TKO showed little effect on the proportion of events already decayed, unquenchable or quenchable by low pH (Supplementary Fig. 6). However, BDNF-pHluorin events in neurons persist tens of seconds (Fig. 3B), making it difficult to detect any further prolongation due to the loss of synuclein. Another limitation of our approach is the acid quench confines characterization of the fusion pore to a single discrete time point. We therefore turned to the reporter NPY-pHluorin due to its more rapid release from LDCVs [35, 41]. To isolate effects on the fluorescence decay attributable to peptide release rather than reacidification, we again used the H^+-ATPase inhibitor bafilomycin. A small subset of NPY-pHluorin events decay slowly, with a time constant more than 5 s, and their proportion is reduced by bafilomycin (Supplementary Fig. 7A). Similarly, bafilomycin increases the proportion of events with no decay by a comparable amount. Thus, a small fraction of events exhibit pore closure and reacidify slowly after endocytosis.

However, the vast majority of events are rapid, with a time constant of decay considerably less than 5 s, and bafilomycin does not significantly change their proportion (Supplementary Fig. 7A). Bafilomycin also fails to alter the decay kinetics of most events (Supplementary Fig. 7B). Thus, fast NPY-pHluorin events reflect peptide release and only the longest events undergo pore closure.

Congruous to the results in chromaffin cells, over-expression of α-synuclein in neurons shortens both the latency of NPY-pHluorin events to decay and the time constant for fluorescence decay once this begins (Fig. 4A-C). Conversely, the loss of all endogenous synucleins dramatically increases the latency of NPY-pHluorin to decay, and the time constant of fluorescence decay (Fig. 4D,E). The TKO thus exhibits a more pronounced effect on the kinetics of LDCV exocytosis in neurons than in chromaffin cells, presumably because neurons express much higher levels of endogenous synuclein. It is interesting to note that the effect of synuclein on NPY-pHluorin fluorescence decay appears to involve only the more slowly decaying exocytic events (Fig. 4C,E), which the experiment with bafilomycin suggests undergo pore closure (Supplementary Fig. 7). Focusing specifically on events with tau > 5 s, we find that synuclein (over-expression or loss) does not affect the latency of these events to decay (Supplementary Fig. 8A,C). In contrast, the latency to decay of more rapid NPY-pHluorin events (tau < 1 s) shortens with over-expression of synuclein and lengthens in the TKO (Supplementary Fig. 8B,D), consistent with a primary role for synuclein in dilation of the fusion pore.

In chromaffin cells and neurons, synuclein over-expression affects the accessibility to quenching by external H^+, as well as the kinetics of peptide release,

indicating changes in behavior of the fusion pore. To monitor the fusion pore status with higher temporal resolution, we oscillated the pH of neuronal cultures between 6.4 and 7.8. If the fusion pore is open, the changes in pH will affect fluorescence of the peptide-pHluorin. When the fusion pore closes, the pH-induced oscillation in fluorescence will cease. We opted for BDNF-pHluorin as a reporter in this experiment because the events have a much longer duration, allowing us to monitor the status of the fusion pore across longer time intervals after exocytosis begins. Figure 3E shows sample traces for the three classes we observe: i) events where the pore remains open until peptide release, ii) events where closure does not occur during the period imaged, iii) events with fusion pore closure. Inactivation of all three synuclein genes substantially increases the proportion of events with pore closure, at the expense of those without closure ($p = 0.01$ by Chi-square) (Fig. 3F). However, loss of synuclein does not affect the time to pore closure (Fig. 3G). Taken together with the NPY-pHluorin data, these results suggest that synuclein prevents interruption of LDCV cargo release in two ways, first by increasing the rate of release and second, by preventing pore closure.

Synuclein localizes to dense core vesicles

Does synuclein affect behavior of the fusion pore directly or indirectly? Despite its presynaptic location, its original identification in a preparation of synaptic vesicles and its preference for artificial membranes with high curvature [21, 42], α-synuclein exhibits only weak association with synaptic vesicles by gradient fractionation and photobleaching [43-45]. We also failed to detect localization of either over-expressed or endogenous synuclein with SgII in chromaffin cells using a number of commercially

available antibodies (Supplementary Fig. 1). We were therefore surprised to find that an antibody to the homologous canary protein synelfin specifically labels LDCVs in these cells. Using this antibody, which recognizes both α- and β-synuclein [46], over-expressed human α-synuclein colocalizes extensively with LDCV protein SgII by confocal and total internal reflection fluorescence (TIRF) (data not shown) as well as structured illumination microscopy (Fig. 5). Similar results were obtained using this antibody to detect human α-synuclein over-expressed in bovine chromaffin cells (personal communication, M.A. Bittner and R.W. Holz.) Endogenous synuclein also colocalizes with SgII, and the immunoreactivity for synuclein is specific because the TKO shows low background staining in both chromaffin cells and primary neurons (Fig. 5, Supplementary Fig. 12). In support of the specificity for LDCVs, endogenous synuclein shows little colocalization with mitochondria (Supplementary Fig. 9). Further, the colocalization persists on LDCVs distributed deeper inside the cell away from the plasma membrane, indicating that synuclein associates with LDCVs in an unbiased manner (not merely to vesicles docked at the cell surface). Due perhaps to conformational specificity for the membrane-bound or multimeric protein [47, 48], this antibody thus provides what may be the first direct histological evidence for the specific localization of synuclein to neurosecretory vesicles, indicating the potential for a direct effect on release.

Fusion pore dilation is a conserved function of the synucleins that is impaired by PD-linked α-synuclein point mutations.

The interaction of synuclein with membranes has been shown to be dependent on its N-terminal region adopting an amphipathic helix conformation (REF). This region of the protein is remarkably well conserved among the synuclein family members implying that it may contribute a common function. Disease-causing point mutations in α-synuclein all cluster around a narrow stretch of this region (between residues 30 and 53), raising the possibility that they may all act to somehow perturb normal protein-membrane interactions. Do other synuclein isoforms and mutant α-synuclein also have the ability to promote fusion pore dilation? To address these questions, we generated lentiviral constructs encoding either human β- or γ-synuclein as well as the first three identified human α-synuclein point mutations (A30P, E46K, and A53T). As in previous experiments, wt chromaffin cells were transduced with BDNF-pHluorin and either empty vector or one of the lentiviral synuclein constructs, cultured for 5 days and imaged by TIRF microscopy.

The behavior of BDNF-pHluorin-containing vesicles was again monitored during a 50s depolarization with 45 mM K^+. Similar to results obtained with WT α-synuclein, over-expression of either β- or γ-synuclein, as well as of mutant A30P or A53T α-synuclein, reduced the frequency of BDNF-pHluorin exocytic events (Fig. 6A). Kinetic analysis of the events revealed that overexpression of β- or γ-synuclein accelerated the release of BDNF-pHluorin (Fig. 6B), consistent with increased dilation of the fusion pore. However, over-expression of mutant α-synuclein had no effect on BDNF-pHluorin release kinetics.

Immunostaining with the H3C antibody revealed that mutant α-synuclein is still capable of associating with secretory granules (Fig. 6C), confirming that the failure of

the mutants to influence release kinetics is not due to aberrant localization or membrane binding capacity of the mutant protein. To ensure that the synuclein immunofluorescence signal we detected is not attributable to endogenous protein, we also over-expressed the point mutants in TKO cells and repeated the immunostaining. Our results demonstrate that mutant α-synuclein maintains its association with secretory granules, even in the absence of any endogenous synuclein (Supplementary Figure 13). Taken together, our findings demonstrate that inhibition of exocytosis and promotion of fusion pore dilation are distinct properties of the synucleins and the latter is selectively disrupted by disease-causing mutations in α-synuclein.

Discussion

The results show that synuclein influences behavior of the exocytotic fusion pore. Over-expression accelerates the release event, reducing the time to peak fluorescence (pore opening), speeding fluorescence decay (peptide release) and preventing pore closure. The loss of synuclein produces opposite effects, increasing the time to peak fluorescence, prolonging decay and increasing the likelihood of pore closure. Thus, both over-expressed and endogenous synuclein promote dilation of the fusion pore. Effects on pore closure are to some extent secondary to changes in the detection of undischarged cargo, but synuclein also appears to prevent the closure of persistent events. Nonetheless, the effect on pore opening suggests that synuclein acts early in exocytosis.

Changes in the fusion pore suggest an explanation for the inconsistent effects of synuclein on transmitter release reported in the literature. Fusion pore dilation would be

expected to limit the release of neuromodulators such as monoamines and peptides that dissociate slowly from a lumenal matrix, rather than classical transmitters such as glutamate that escape rapidly through even a small pore. Indeed, synuclein over-expression and loss both affect the release of dopamine [13, 18] as well as peptide, without major effects on glutamate release monitored postsynaptically [16, 17]. Despite this apparent difference, we anticipate the same effects on fusion of any vesicle to which synuclein binds.

We also provide evidence for the specific association of endogenous as well as over-expressed synuclein with neurosecretory vesicles in cells, indicating the potential for direct effects on the fusion pore. Since synuclein associates with LDCVs before docking at the plasma membrane, it is possible that synuclein acts before fusion to influence its properties. Indeed, previous work showing that synuclein increases the number of SNARE complexes [17] suggests one mechanism for the effects of synuclein reported here. Increased SNARE complex formation might be expected to increase the force that drives fusion pore dilation and hence promote cargo release [49]. However, over-expression of synuclein also inhibits the extent of synaptic vesicle exocytosis [14], and the results presented here confirm that this effect extends to LDCVs [13]. It is difficult to reconcile the observed inhibition of release with a role for synuclein as chaperone for the SNARE complex [17]. Indeed, synuclein inhibits the fusion of membranes *in vitro* as well as *in vivo* through direct effects on the lipid bilayer [26, 50-52]. Alternatively, synuclein may promote SNARE complex accumulation by inhibiting exocytosis, thereby preventing the disassembly of complexes present on vesicles primed for fusion.

We also report what is, to the best of our knowledge, the first confirmation of a specific loss of function in α-synuclein due to disease-causing mutations. The fact that these mutants properly localize to secretory vesicles and can still inhibit exocytosis, yet fail to promote fusion pore dilation suggests that these two effects of the protein are uncoupled. This gives rise to the extraordinary implication that disease pathogenesis could be initiated by a loss of normal function, or that perhaps the mutant protein would be more sensitive to toxic consequences associated with upregulation in expression levels. In further support of this idea, in the CSPα knockout mouse, imminent neurodegeneration can be rescued by crossing the animals with wild-type human α-synuclein transgenics [58]. Crossing with A30P α-synuclein transgenic animals does not rescue the neurodegenerative phenotype, presumably because the mutant protein cannot properly localize to presynaptic terminals [43]. However, crossing CSPα KO mice with A53T α-synuclein mice rescues the neurodegeneration and premature death, but these offspring still develop phenotypes consistent with late-onset parkinsonianism as they age. This suggests the mutant protein retains a function capable of compensating for CSPα-deficiency, but is still deficient in another function (presumably related to fusion pore dilation) that renders it vulnerable to the onset of PD pathology.

How might the familial-PD mutations disrupt α-synuclein's activity toward the fusion pore? Our immunostaining results demonstrate that the mutants remain properly localized to LDCVs (Fig. 6, Supplementary Fig. 13), so their failure to promote pore dilation cannot be attributed to either gross abrogation of or change in the specificity of membrane binding. Previous studies have suggested that the native form of α-synuclein may be tetrameric, implying that the PD-linked mutations could destabilize

this species of the protein, leading to aggregation of unwanted monomer, loss of normal function, or both. Given that our current methods do not distinguish the precise oligomeric state of the protein, we cannot exclude this possibility. Another clue may be afforded us by the clustering of the familial mutations within such a confined region of the protein (between residues 30 and 53), which is predicted to coincide with an area of α-synuclein that could conceivably undergo conformational change from an interrupted helix to an extended helix as curvature of the target membrane diminishes [59,60]. This is precisely the type of curvature change α-synuclein would experience during the fusion of either a synaptic or large dense core vesicle. Perhaps the ability of α-synuclein to undergo this conformational switch during the initiation of exocytosis allows it to remain associated with the membrane surface until the full collapse of the vesicle is completed, and the familial mutations impair this flexibility. In any case, a better understanding of the precise mechanism behind the reported actions of α-synuclein toward the fusion pore should prove invaluable in efforts to understand the early changes in normal cellular function that initiate disease.

Experimental Procedures

Rodent strains

Synuclein triple knockout (TKO) mice were produced by crossing α/β-synuclein double KO mice (Jackson Laboratory, stock # 006390) to a γ-synuclein KO line [53] generously provided by L. Lustig. TKO mice were maintained as homozygotes and C57Bl/6 animals used as wild type (wt) controls since this strain contributed ~90% of the genetic background of the synuclein TKO line (K. Nakamura, personal communication). All rodent procedures were performed according to guidelines established by the UCSF IACUC.

Antibodies

The rat monoclonal antibody to human α-synuclein (15G7) was obtained from Alexis Biochemicals, the mouse monoclonal antibody to rodent α-synuclein (Syn-1) from BD Biosciences, the goat polyclonal antibody to TOM20 (C-20) from Santa Cruz Biotechnology and the rabbit polyclonal antibody to secretogranin II (K55101R) from Meridian Life Science. The H3C antibody to synuclein developed by J. George was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242. The rabbit polyclonal antibody to actin (A2066) was obtained from Sigma-Aldrich. The anti-rat antibody conjugated to Alexa Fluor 488, anti-mouse antibody conjugated to Alexa Fluor 488, anti-goat antibody conjugated to Alexa Fluor 594 and anti-rabbit antibody conjugated to Alexa Fluor 594 were all obtained from Thermo Fisher

Scientific. The anti-mouse antibody conjugated to IRDye800 and anti-rabbit antibody conjugated to IRDye680 were obtained from Rockland Immunochemicals.

Chromaffin cell culture

Adrenal chromaffin cells were isolated as previously described [54]. Briefly, whole adrenal glands were harvested from 4-6 week old mice and placed in ice cold Ca^{++} -, Mg^{++} - free (CMF) Hank's balanced salt solution (HBSS) supplemented with penicillin and streptomycin (pen/strep). Adrenal medullae were isolated and digested in the same solution containing collagenase type I (2.6 mg/ml, Worthington Labs), BSA (3 mg/ml), DNase I (0.15 mg/ml, Sigma) and hyaluronidase I (0.15 mg/ml, Sigma) at 37° C for 30 min, shaking at 800 rpm in a thermomixer (Eppendorf). Digested medullae were resuspended completely and the enzymatic reaction quenched with 10 volumes cold CMF-HBSS. Cells were pelleted at 300 *g* for 10 min at 4□C and resuspended in Dulbecco's Modified Eagle's medium (DME-H21) supplemented with 10% FBS (Hyclone) and pen/strep. Cell suspensions were plated drop-wise on glass chambers (LabTek) coated with poly-L-lysine and allowed to adhere for 45 min at 37° C/5% CO_2, followed by addition of pre-warmed lentiviral supernatant. Lentiviral transduction was performed overnight, and media replaced the next morning.

Lentivirus production

Low passage HEK293T cells were seeded onto 6-well plates and transfected overnight with a mixture of the third generation lentiviral vector pJHUMCS encoding the gene of interest, as well as accessory plasmids pREV, pVSVG and pPRE, using Fugene HD

transfection reagent (Promega) and the manufacturer's instructions. Cells were switched into chromaffin cell culture medium the next morning and 24 h later the culture medium was collected and cell debris sedimented at 1000 *g*. The viral supernatant was either used immediately or aliquoted and frozen at -80° C.

Immunofluorescence

Chromaffin cells were fixed by adding an equal volume of 4% formaldehyde in CMF-PBS to the culture medium and incubating for 20 min at room temperature. Cells were blocked and permeabilized in CMF-PBS containing 2% BSA, 1% fish skin gelatin and 0.02% saponin (blocking buffer). Primary antibodies were either diluted 1:500 (15G7, Syn-1, C-20) or 1:1000 (K55101R) in blocking buffer and incubated overnight at 4° C. Fluorescent dye-conjugated secondary antibodies were also diluted 1:500 in blocking buffer. Cell staining was visualized using an upright fluorescence microscope (Axioskop; Zeiss) with 63× 1.25 N.A. oil objective (Zeiss) and a Coolsnap HQ CCD camera (Photometrics). Images were acquired using Metamorph software and analyzed in ImageJ (NIH).

Structured illumination microscopy (SIM)

Isolated chromaffin cells were plated onto high precision coverslips (Zeiss), fixed 3-5 days later and immunostained as described above. Samples were imaged on a Nikon Ti microscope equipped with a 100x 1.49 NA Apo TIRF objective and an Andor Xyla sCMOS camera. Structured illumination images were acquired as a 15-slice z-stack with 120 nm step size and reconstructed using Nikon Elements software outfitted with

the NIS-A N-SIM analysis module. Colocalization was quantified with the Coloc2 module in ImageJ.

Total internal reflection fluorescence (TIRF) microscopy

Wild type (wt) or synuclein TKO chromaffin cells were plated onto glass chamber slides (Lab-Tek) coated with poly-L-lysine, immediately transduced with lentivirus and imaged live 3–5 days later. Images were acquired at 20 Hz using an inverted TIRF microscope (Ti-E; Nikon) equipped with 50 mW Agilent MLC400B 488 nm laser, quad N-STORM TIRF filter set (405/488/561/647), 525/50 emission filter, 100× Plan Apo 1.49 N.A. oil objective (Nikon) and Andor iXon Ultra 897 high speed EMCCD camera (Oxford Instruments). Cells were imaged in modified Tyrode's solution containing (in mM, 140 NaCl, 10 HEPES-NaOH, pH 7.4, 10 glucose, 4.5 KCl, 5 $CaCl_2$, 1 $MgCl_2$) and exocytosis stimulated by adding an equal volume of high KCl solution (osmotically balanced with NaCl), for a final K^+ concentration of 45 mM. Individual exocytic events were identified manually in ImageJ software by placing 5×5 pixel regions of interest (ROIs) over the center of events and the average intensity profiles extracted using the Time Series Analyzer plugin. The mean ROI intensity during the 20 frames immediately preceding the onset of exocytosis was subtracted as background. Curve fitting, analysis of event parameters and all subsequent statistical analyses were performed using Graphpad Prism Version 6.05.

To quantify the rate of fluorescence decay (full decay events), the decay was fit to a single exponential. To quantify the latency to decay, the traces were fit to a plateau followed by single exponential decay and any latency shorter than the temporal

resolution of the experiment (1 frame, 50 ms) was excluded from the analysis. To determine whether reacidification contributes to the decay of BDNF-pHluorin fluorescence, chromaffin cells were incubated in imaging buffer with Bafilomycin A1 (0.6 μM, EMD Millipore) for 2 min and stimulated with high K^+ in imaging buffer that also contains 0.6 mM bafilomycin.

To assess the state of the fusion pore, imaging buffer at pH 5.5 was applied 30 s after stimulation with high K^+. To determine the fraction of events protected from quenching, all stable exocytotic events visible immediately before the addition of low pH solution were selected using 5x5 pixel ROIs and the background subtracted. Since the spread of VMAT2-pHluorin into the plasma membrane affects local background fluorescence, classification as protected required (i) fluorescence intensity above the local background before event onset and (ii) punctate fluorescence despite the acid challenge.

Fluo-5F Imaging

Fluo-5F AM was reconstituted using anhydrous DMSO to a stock concentration of 3 mM. Immediately before imaging, the stock was diluted to a final concentration of 6 mM in imaging buffer. Wild type and TKO chromaffin cells transduced with lentivirus encoding either empty vector or human α-synuclein were incubated in Fluo-5F for 15 min at room temperature and washed three times with imaging buffer before imaging. Calcium influx was assessed by imaging Fluo-5F fluorescence at an acquisition rate of 20 Hz during depolarization with high K^+ using the same laser and filter settings described above for pHluorin imaging. F_0 values were determined by tracing the outline

of the entire cell footprint and measuring the average pixel intensity during 20 frames immediately before stimulation. The maximum average intensity across the footprint, typically achieved within the first 1-2 s of stimulation, was used to calculate peak DF/F_0.

Neuronal culture and transfection

Primary neuronal cultures were prepared from P0 Sprague-Dawley rat pups and transfected by electroporation as previously described (Amaxa) [14, 55]. Briefly, 0.8 µg pCAGGS vectors containing either NPY-pHluorin or BDNF-pHluorin were cotransfected with 0.1 µg synaptophysin (p38)-mCherry and either 0.1 µg pCAGGS or 0.1 µg pCAGGS-α-synuclein per 4 x 10^5 cells. Cultures were maintained in Minimum Essential Media (MEM) containing 21 mM glucose, 5% FBS, 2% B27 (Gibco), 1% Glutamax (Gibco) and Mito+ serum extender (BD Biosciences). 5-FU and uridine were added on day 3 *in vitro* (DIV3) to inhibit glial growth.

Mouse primary neuronal cultures were prepared from P0 pups, electroporated and plated as described above, except cysteine-activated papain (Worthington) was used to dissociate hippocampi before trituration. On DIV1, 75% of the MEM media was replaced with Neurobasal medium (Gibco) supplemented with 2% B27 and 1.5% Glutamax. 5-FU and uridine were added on DIV8 to inhibit glial growth.

Neuronal α-synuclein overexpression quantitation

Rat hippocampal neurons were isolated, electroporated with BDNF-pHluorin, p38-mCherry and α-synuclein or empty vector and cultured as described above. After 14 days, cells were lysed in PBS containing 1% triton X-100, 1mM EGTA, 1mM $MgCl_2$

and Complete protease inhibitors (Roche). Lysates were sedimented at 1300x g to remove nuclei, and 6 mg protein was separated by electrophoresis through a 15% polyacrylamide gel. Proteins were transferred to nitrocellulose, and membranes were immunoblotted for α-synuclein (using the Syn-1 mAb) and actin (loading control). After detection with fluorescently conjugated secondary antibodies (anti-mouse IRDye800 and anti-rabbit IRDye680, respectively), blots were imaged with an Odyssey system (LI-COR Biosciences) and immunoreactivity was quantified with ImageJ.

Live cell imaging: neurons

Transfected neurons were imaged at 14-20 DIV at room temperature (24° C) as previously described [14, 56]. Neurons were imaged in standard Tyrode's solution (in mM, 119 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 30 glucose and 25 HEPES, pH 7.4). In low pH Tyrode's (pH 5.5 and 6.4), HEPES was replaced with 25 mM MES. All buffers contained the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D,L-2-amino-5-phosphonovaleric acid (APV, 50 μM). To induce LDCV exocytosis, cells were stimulated at 50 Hz for 5 s. The ecliptic pHluorin was imaged at 470/40 nm excitation and 525/50 nm emission, and mCherry at 572/35 nm excitation and 632/60 nm emission. Images were acquired in streaming mode (10 Hz) using a QuantEM:512SC EMCCD camera (Photometrics) and a 63x 1.2 N.A. water objective.

To assess the fusion pore by quenching of BDNF-pHluorin fluorescence at low pH, a 5 s pulse of pH 5.5 Tyrode's solution was applied immediately after the 50 Hz stimulus, followed by washout in standard Tyrode's solution. Exocytic events were

identified manually in the image time series (10 Hz acquisition frequency) recorded from rat (control or +α-synuclein-overexpressing) or mouse (wt or synuclein TKO) neuronal cultures. Stationary events exhibiting rapid and single-step pHluorin unquenching (exocytosis) were scored for sensitivity to ("quenched") or protection from ("unquenched") quenching by acidic Tyrode's solution. Fluorescence events that decayed before application of the low-pH buffer were scored as "already decayed". Data were pooled from 3 independent experiments, with a total of 8-11 coverslips for each condition. Groups were compared by the Chi-square test, and on a per-coverslip basis by the Student's t-test.

To probe further the state of the LDCV fusion pore, mouse neurons expressing BDNF-pHluorin were exposed to rapid pH oscillation as previously described [57]. Neurons were imaged in a chamber containing standard Tyrode's solution (pH 7.4) and stimulated at 50 Hz for 5 s. High pH (7.8) and low pH (6.4) Tyrode's solutions were delivered alternately to the imaging field via a theta pipette (~100 μm tip diameter) every 375 ms and images acquired continuously at 13.3 Hz. To reduce noise, a Kalman filter (Image J) was applied to the image stacks. Exocytotic events were identified manually as for BDNF-pHluorin in the experiment with quenching at pH 5.5, and 4x4 pixel regions of interest (ROIs) used to extract the fluorescence with the Time Series Analyzer plugin of ImageJ. Traces in which the pH-induced oscillation of pHluorin fluorescence stopped abruptly were scored as "pore closure" events, and the time from event onset to termination of oscillation determined. Events that showed oscillation throughout imaging were scored as "no closure". Events that decayed to baseline but exhibited fluorescence oscillation throughout their lifetime were scored as "open until decay".

When required for scoring and quantitation, background fluorescence from areas adjacent to ROIs was subtracted. A total of 100 events each from wild type (7 coverslips) and synuclein TKO (9 coverslips) were scored. Groups were compared by the Chi-squre test, and time-to-closure data displayed as cumulative frequency histograms.

For the analysis of NPY-pHluorin kinetics, exocytotic events imaged at 10 Hz were identified manually as for BDNF-pHluorin, and 4x4 pixel ROIs used to extract the fluorescence. For each event, the kinetics were fit to a plateau (of x seconds) and single exponential decay ($F= IF(x<x_0, F_0, Plateau + F_{span} * e^{-K*(x-x0)})$) by nonlinear regression (GraphPad Prism). Latency to decay (x) and decay time constant (t) values for individual events were pooled across 3 independent experiments (10-11 coverslips total for each condition). Events that failed to decay were scored as “no decay” and included in the analysis. To inhibit reacidification, bafilomycin (0.6 μM) was added to the neurons immediately before imaging. The data, including non-parametric “no decay” events, were displayed as cumulative probability histograms, and groups compared by Kolmogorov-Smirnov. Mean latency and t of decaying events were compared by Student’s t-test. For Bafilomycin A1 experiments, events were grouped by decay kinetics ($t < 5s$, $t > 5s$ or no decay) and compared by the Chi-square test.

Figures

Figure 1. α-Synuclein modulates the kinetics of peptide release.

(a) Chromaffin cells from wt mice were transduced with lentiviruses encoding BDNF-pHluorin and either human α-synuclein (SYN) or empty vector (wt). Over-expression of α-synuclein reduces the number of exocytotic events evoked over 50 s by depolarization with 45 mM K^+. Cells from the synuclein TKO show no difference from wt cells. *, p = 0.01 by one-way ANOVA. n = 19 cells for each group from 3 independent cultures **(b)** Synuclein affects the rise time of exocytotic events. For each exocytotic event, the time to reach 90% maximum fluorescence was determined. Inset shows the average rise time of a single representative cell from each group (wt, n = 46 events; SYN, n = 34 events; TKO, n = 30 events). The histogram represents the frequency of events with rise time in the 50 ms bin indicated ($p < 0.0001$ by Kolmogorov-Smirnov test). wt, n = 473 events; SYN, n = 256 events; TKO, n = 518 events **(c)** Exocytotic events belong to four distinct classes (left). In full decay, the fluorescence immediately decays to baseline. In plateau-decay, the fluorescence decay begins after a variable latency. In decay-closure, the fluorescence decays with no latency but the decay arrests before return to baseline. Plateau-decay-closure involves both a latency before decay and incomplete decay. The diagram (upper right) illustrates our interpretation of the traces. The proportion of event types differed among all three groups ($p < 0.0001$ by Chi-square for pair-wise as well as the comparison of all three groups). **(d)** For all events with non-zero latency to decay, the time from reaching 90% maximal fluorescence to the onset of decay was determined. Bar graph represents the mean ± SEM. ***, $p < 0.0001$ by one-way ANOVA with Tukey's post-hoc test. WT, n = 134

events; SYN, n = 66 events; TKO, n = 218 events **(e)** Synuclein influences the rate of BDNF release. For all full decay events, the time constant of fluorescence decay (τdecay) was determined by fitting to a single exponential. The inset indicates mean ± SEM. **, $p < 0.01$ and ***, $p < 0.001$. The histogram represents the distribution of events with different τdecay ($p < 0.0001$ for WT versus SYN and TKO vs SYN; $p < 0.001$ for WT vs TKO by Kolmogorov-Smirnov test). wt, n = 266 events; SYN, n = 167 events; TKO, n = 237 events

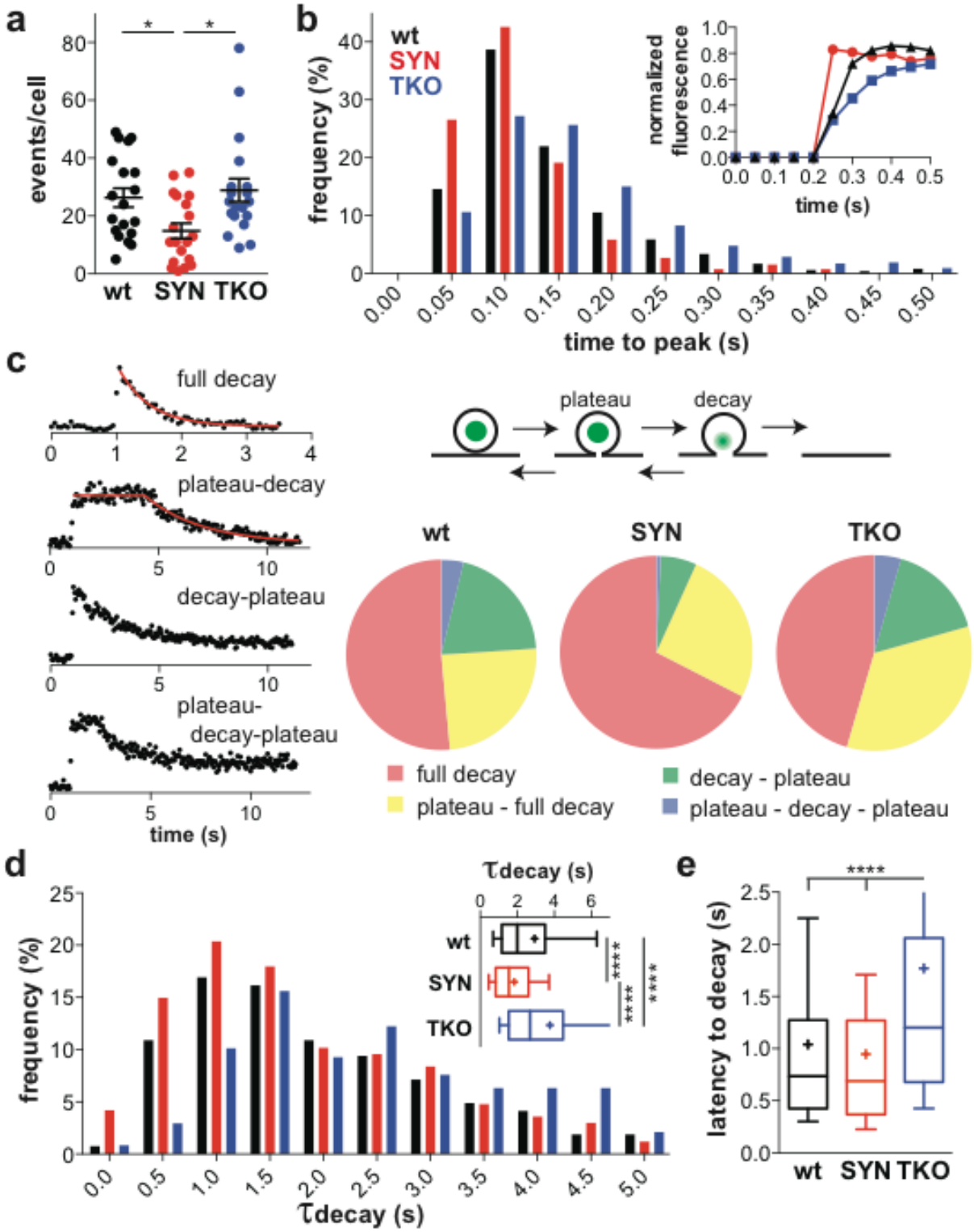

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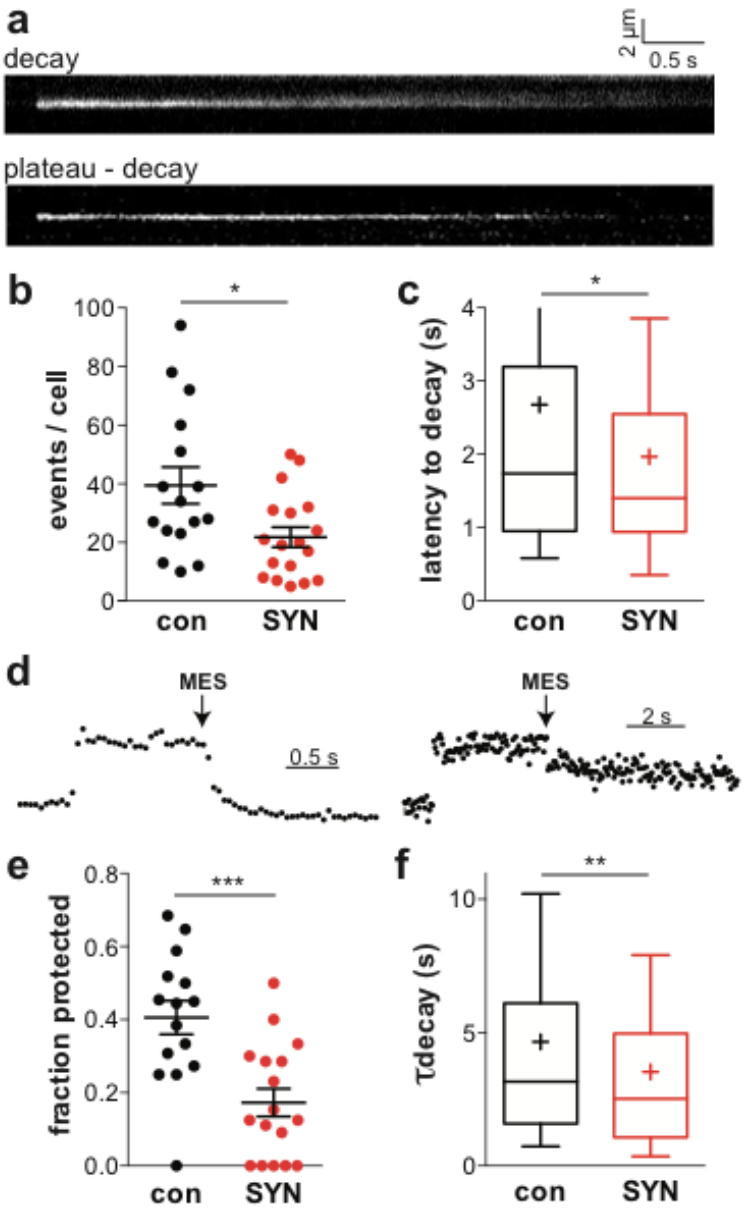
Figure 2. The analysis of VMAT2-pHluorin reveals effects of α-synuclein on the fusion pore.

(a) Wild type chromaffin cells were transduced with lentiviruses encoding VMAT2-pHluorin and either human α-synuclein or empty vector and depolarized 3-5 days later with 45 mM K^+ in the presence of H^+ pump inhibitor bafilomycin to inhibit vesicle reacidification. The kymographs of two exocytotic events illustrate the observed variation in fluorescence time course and spread. after depolarization with 45 mM K^+. Bar indicates 0.5 s. **(b)** α-Synuclein over-expression reduces the number of VMAT2-pHluorin exocytotic events ($p = 0.0154$ by unpaired, two-tailed t test). con, n = 16 cells; SYN, n = 18 cells from 3 independent cultures **(c)** Synuclein over-expression also reduces the latency to fluorescence decay ($p = 0.0244$ by unpaired, two-tailed t test). con, n = 153 events; SYN, n = 128 events **(d)** Representative traces showing a VMAT2-pHluorin event quenched (left) and not quenched (right) by pH 5.5. **(e)** After depolarization for 30 s in 45 mM K^+, the chromaffin cells were challenged at pH 5.5. Over-expression of α-synuclein reduced the proportion of events protected from quenching at low pH ($p = 0.0027$ by two-way ANOVA). con, n = 222 events from 15 cells; SYN, n = 133 events from 16 cells **(f)** The time constant of fluorescence decay shortens with α-synuclein over-expression ($p = 0.042$ by Kolmogorov-Smirnov test). con, n = 348 events; SYN, n = 267 events

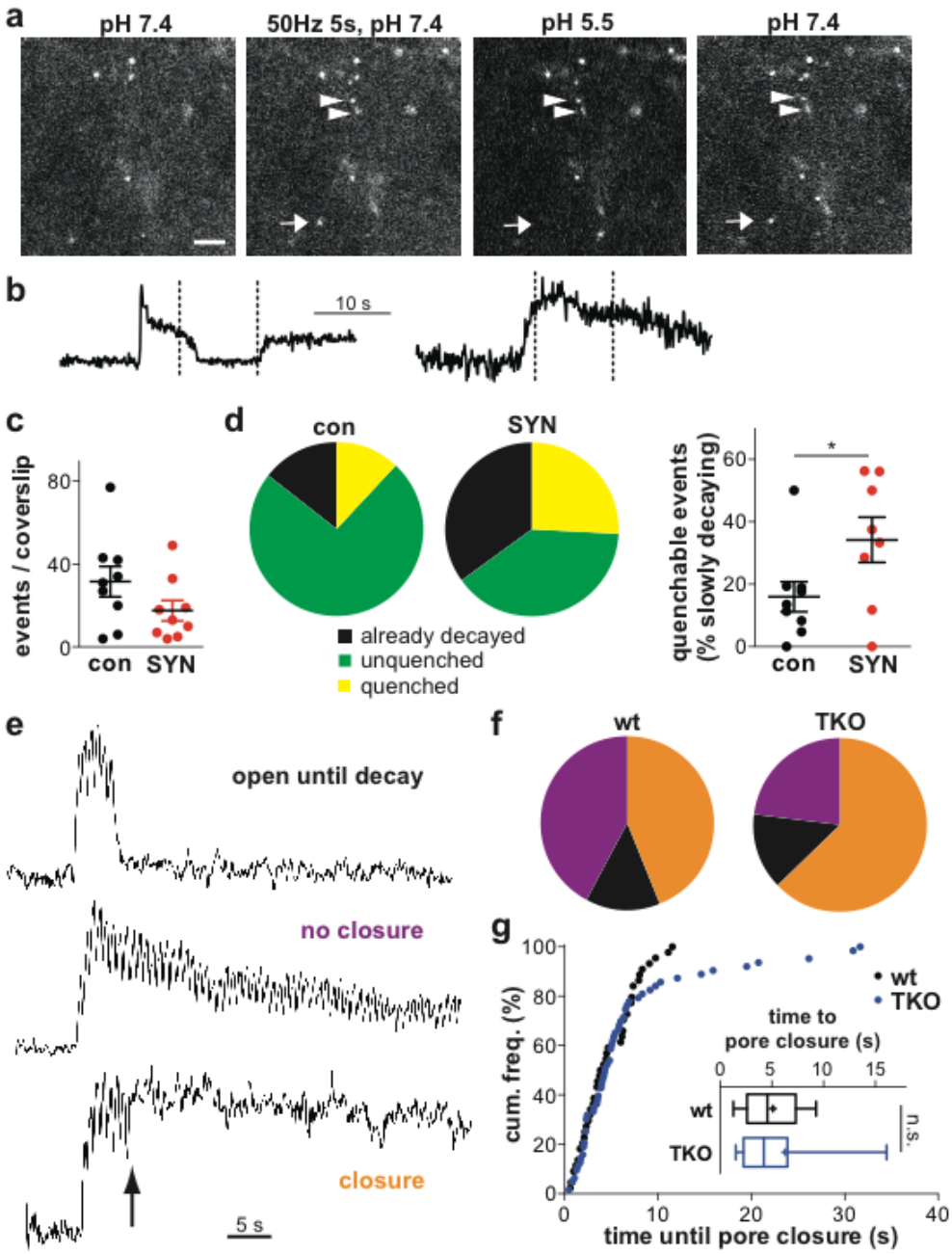


Logan et al., Figure 2

Figure 3. α-Synuclein influences fusion pore closure in neurons.

(a) Rodent hippocampal neurons were transfected with BDNF-pHluorin and imaged 14-20 days later, stimulating at 50 Hz for 5 s followed immediately by quenching of the cell surface fluorescence at pH 5.5. The arrow indicates an event quenchable at low pH, and the arrowheads events resistant to quenching. Scale bar, 5 μm. **(b)** Sample BDNF-pHluorin traces show sensitivity to quenching by pH 5.5 applied between the dashed lines (left) and resistance to quenching (right). **(c)** Average event frequency per coverslip (mean ± SEM) for BDNF-pHluorin expressing rat hippocampal neurons co-transfected with α-synuclein (SYN) or empty vector (con) and stimulated as in (a) (above) ($p = 0.13$). Panel below indicates the event frequency in neurons from wt and synuclein TKO mice. **(d)** Classifying events as either already decayed at the time of acid exposure or if not, unquenched or quenched by low pH, α-synuclein over-expression reduces the proportion of unquenched events ($p < 0.0001$ by Chi-square test) (left panel). n = 281 events (con), 158 events (α-syn) Synuclein over-expression also increases the proportion of quenchable events per coverslip independent of those already decayed (right panel). *, $p < 0.05$ by Student's t-test **(e)** Mouse neurons transfected with BDNF-pHluorin were stimulated at 50 Hz for 5 s and superfused with rapidly oscillating (1.33 Hz) Tyrode's solutions at pH 7.8 and 6.4. Top trace shows an exocytotic event with oscillation that persists until fluorescence decay, indicating that the fusion pore remains open until the peptide is released. Middle trace shows an event that does not decay completely but shows oscillation throughout, indicating that the fusion pore does not close. Bottom trace shows an event where the oscillation stops

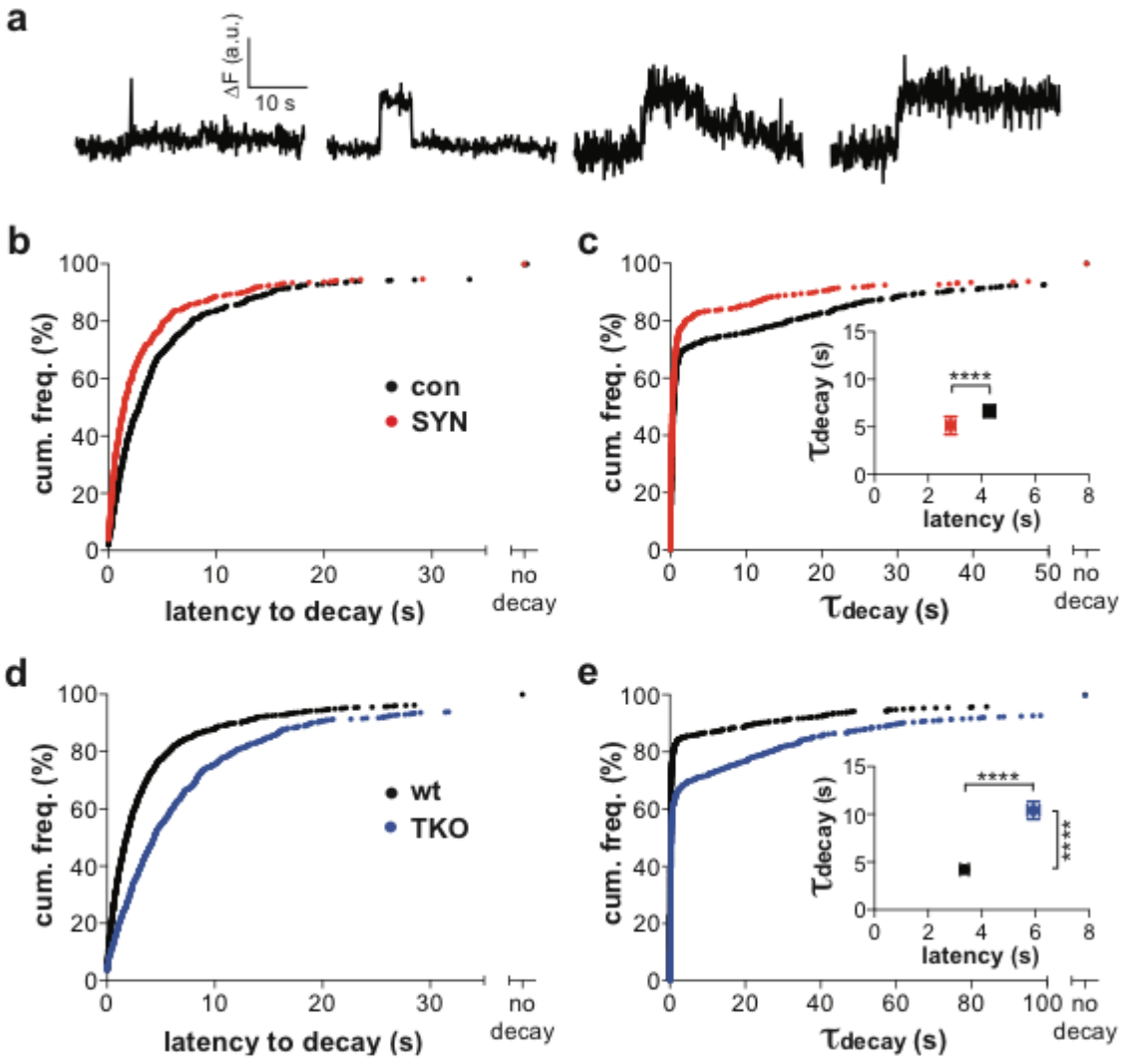
(arrow) before full peptide release, indicating pore closure. **(f)** The proportion of event types differs in wt and synuclein TKO neurons ($p = 0.01$ by Chi-square). **(g)** Among events with pore closure, the cumulative frequency distribution shows no significant difference between wt and synuclein TKO neurons in time to pore closure ($p = 0.63$ by Kolmogorov-Smirnov). Inset shows mean ± SEM (n.s., not significant). n = 44 events for wt and 63 events for TKO



Logan et al., Figure 3

Figure 4. Over-expressed and endogenous synuclein exert similar effects on peptide release.

(a) Sample fluorescence traces of NPY-pHluorin in cultured rodent neurons stimulated at 50 Hz for 5 s. Individual traces were fit to a plateau with single exponential decay. Events that failed to exhibit fluorescence decay (right) were scored as no decay. **(b,d)** The latency to decay was combined with non-parametric "no decay" data and the cumulative frequency distribution plotted. **(b)** Overexpression of α-synuclein (SYN) in rat neurons significantly decreased latency to decay compared to controls transfected with empty vector (con) ($p < 0.0001$ by Kolmogorov-Smirnov). control, n = 652 events / 5 coverslips; α-syn, n = 586 events / 8 coverslips **(d)** Latency to decay of NPY-pHluorin events increased in neurons from synuclein TKO mice relative to wt controls ($p < 0.0001$). wt, n = 928 events / 10 coverslips; TKO, n = 769 events / 11 coverslips **(c,e)** Cumulative frequency histograms for the time constants of fluorescence decay (τ_{decay}) by NPY-pHluorin, including events with no decay. **(c)** Overexpression of α-synuclein in rat neurons significantly reduced τ_{decay} ($p = 0.001$). **(e)** Loss of synuclein in neurons from TKO mice increases NPY-pHluorin τ_{decay} relative to neurons from wild type animals ($p < 0.0001$). Insets in **(c)** and **(e)** indicate mean ± SEM for the latency to decay and τ_{decay} for decaying events in rat **(c)** and mouse **(e)** neurons. ****, $p < 0.0001$ by Student's t-test

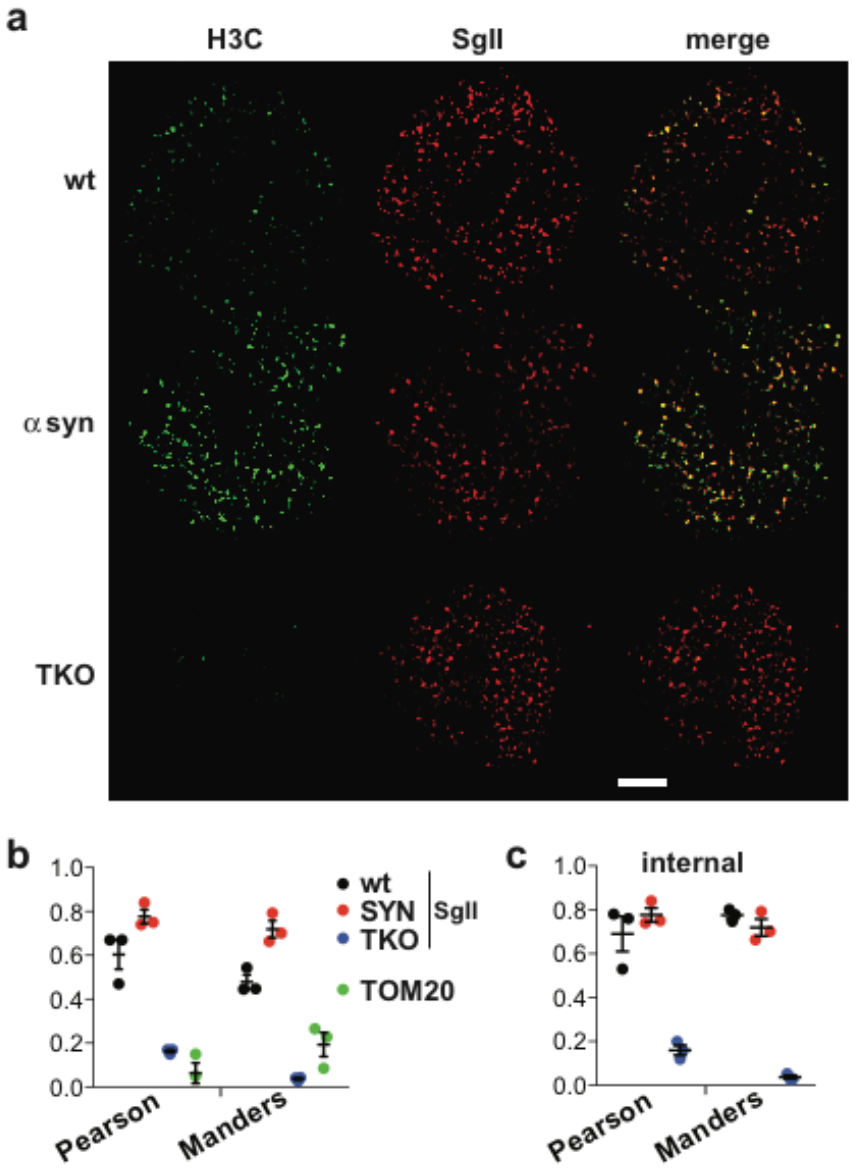


Logan et al., Figure 4

Figure 5. Over-expressed and endogenous synuclein localizes to secretory granules in adrenal chromaffin cells.

(a) Chromaffin cells from wt or synuclein TKO mice were transduced with either lentivirus encoding human α-synuclein (SYN) or empty vector, cultured for 72 h and immunostained for α-synuclein (H3C, green) as well as the dense core vesicle protein secretogranin II (SgII, red) The images were obtained using structured illumination and shown here as reconstructions of a 120 nm-thick slice located within 0.5 μm of the cell-coverglass interface. Size bar, 2.5 μm. **(b)** The extent of synuclein and SgII colocalization was quantified using Pearson's correlation coefficient (R) and Manders overlap coefficient (M1).

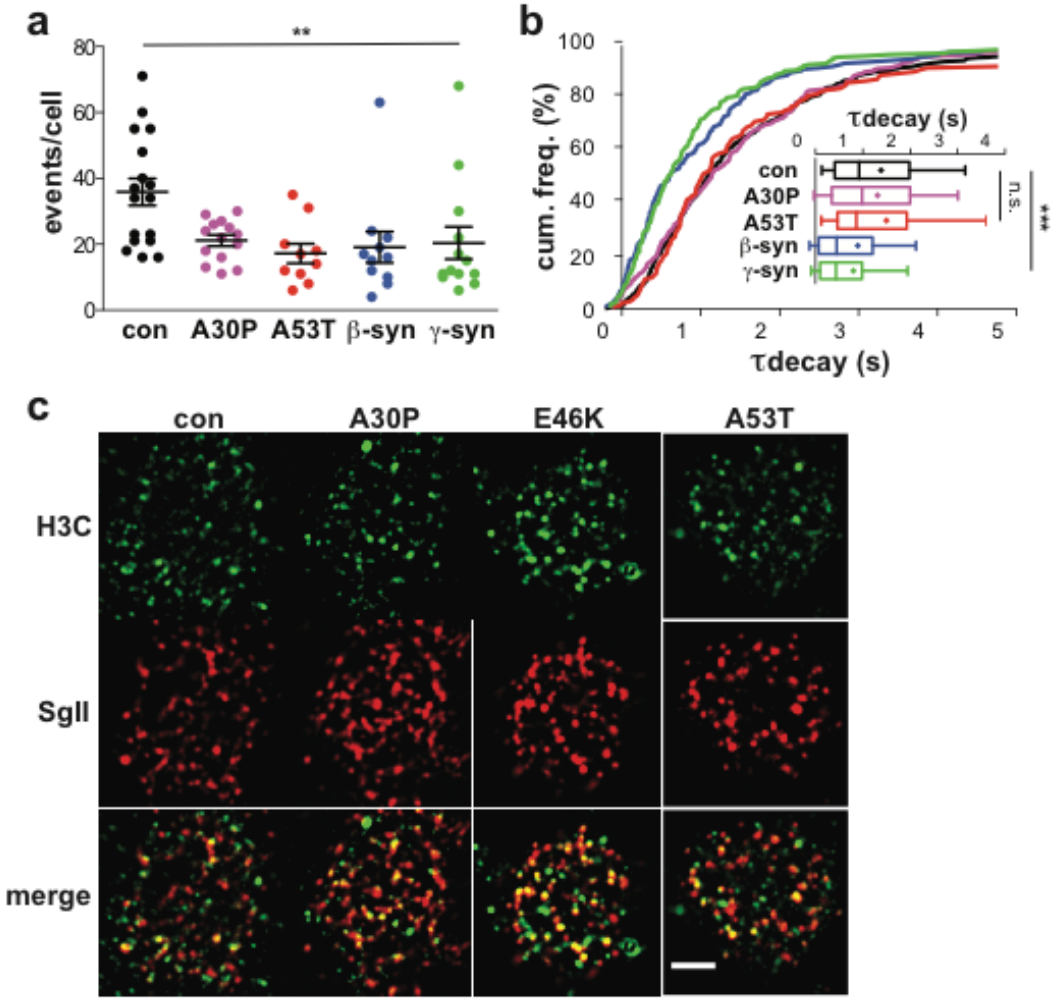
(c) Similar colocalization measures for a slice located 0.5-1.0 μm deeper inside the cell shows that the localization of synuclein to secretory vesicles is not limited to the docked pool. Values indicate mean ± SEM, n = 3 cells for each group.



Logan et al., Figure 5

Figure 6. Fusion pore dilation is a conserved function of the synucleins that is impaired by PD-linked point mutations in α-synuclein.

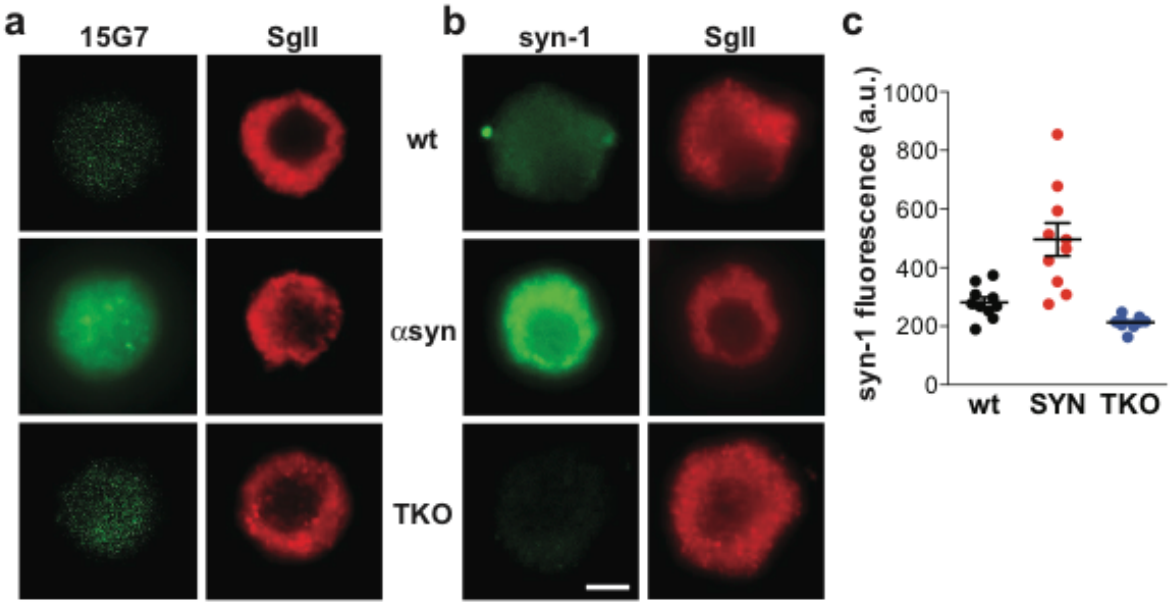
(a) Chromaffin cells from wt mice were transduced with lentiviruses encoding BDNF-pHluorin and either mutant human α-synuclein (A30P, A53T) human β-synuclein (β-syn), human γ-synuclein (γ-syn) or empty vector (ctl). Relative to ctl cells, over-expression of mutant α-synuclein, β-synuclein or γ-synuclein all caused a reduction in the number of exocytic events evoked over 50 s by depolarization with 45 mM K^+. **, p = 0.0046 by one-way ANOVA. ctl, n = 17 cells; A30P, n = 14 cells; A53T, n = 10 cells; β-syn, n = 11 cells; γ-syn, n = 13 cells from 3 independent cultures. **(b)** β-Synuclein and γ-synuclein, but not α-synuclein point mutations, accelerate the kinetics of BDNF-pHluorin release. Cumulative frequency plot displays the distribution of decay constants for all BDNF-pHluorin events that decayed to baseline. Expression of either β-synuclein or γ-synuclein, but not mutant α-synuclein, shifted the decay constants to shorter values relative to ctl cells ($p < 0.0001$ by Kolgomorov-Smirnov test, ctl vs β-syn and ctl vs γ-syn). The inset represents a 10-90 percentile box and whisker plot of the decay constants (mean represented as a "+"). ***, $p < 0.0001$ by one-way ANOVA, ctl vs β-syn and ctl vs γ-syn **(c)** Point mutations do not disrupt the localization of α-synuclein to secretory granules. Cells from wt mice were infected with either empty vector or lentivirus encoding one of three α-synuclein point mutants (A30P, E46K, or A53T), and immunostained for α-synuclein (H3C, green) as well as the dense core vesicle protein secretogranin II (SgII, red). Representative images show extensive colocalization of mutant α-synuclein with secretory granules. Size bar, 2.5um.



Logan et al., Figure 6

Supplementary Figure 1. Endogenous and lentiviral expression of α-synuclein in adrenal chromaffin cells.

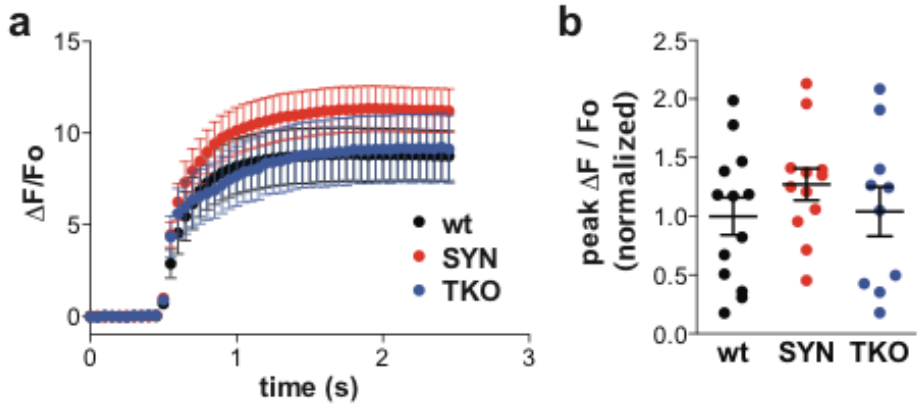
(a) Chromaffin cells from wild type (wt) or synuclein triple knockout (TKO) mice were transduced with either lentivirus encoding human α-synuclein (SYN) or empty vector, cultured for 72 h and immunostained for the over-expressed protein using the human-specific α-synuclein antibody 15G7 (left) as well as the dense core vesicle protein secretogranin II (SgII) to identify chromaffin cells (right). (b) Chromaffin cells from wt and synuclein TKO mice were immunostained using the α-synuclein-specific antibody syn-1, which detects both rodent and human isoforms. Comparison to the TKO shows that wt cells express low levels of endogenous α-synuclein in a diffuse cytosolic distribution. Scale bar, 5 μm. (c) Quantification of whole cell α-synuclein immunofluorescence detected with syn-1 antibody in (b) indicates ~5-fold overexpression after lentiviral infection. Values indicate mean ± SEM, n = 10 cells for each group.



Logan et al., Figure S1

Supplementary Figure 2. Synuclein does not influence calcium influx during stimulation.

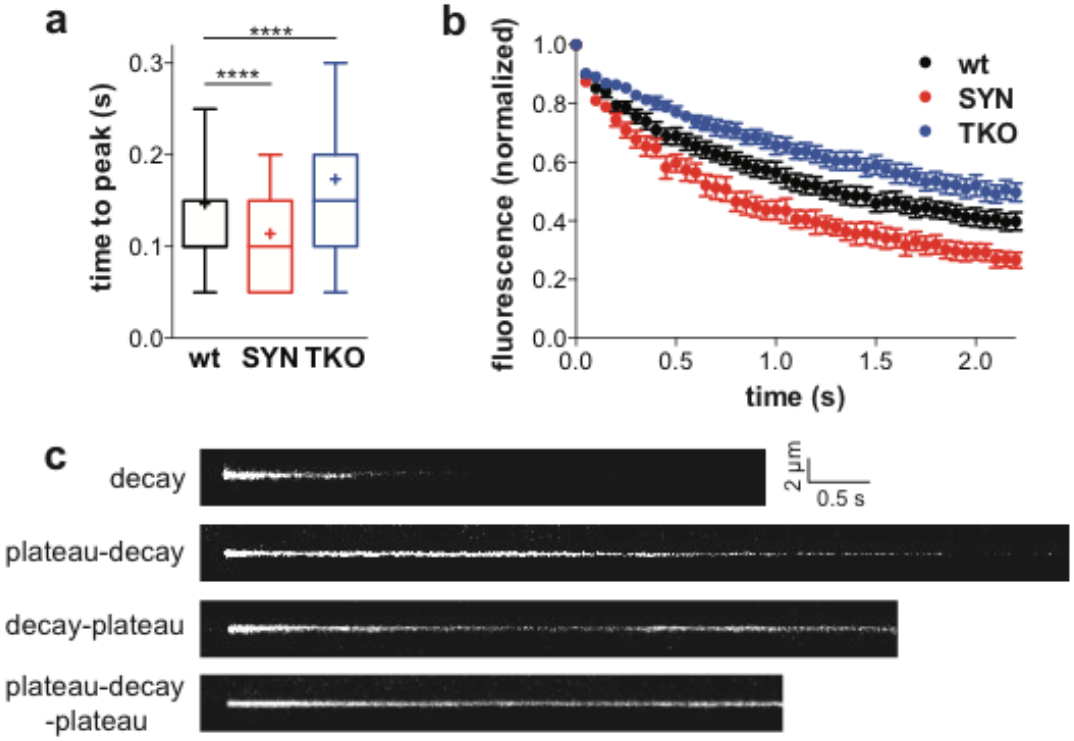
(a) Chromaffin cells from wild type and synuclein TKO mice were transduced with lentivirus encoding wild type human α-synuclein (SYN) or empty vector (wt) 3-5 days before imaging, incubated in Fluo-5F AM (6 μM) for 15 min and imaged by TIRF microscopy during the addition of 45 mM K^+. Values represent mean ± SEM. (b) Peak Fluo-5F fluorescence observed during stimulation is unaffected by synuclein ($p = 0.4571$ by one-way ANOVA). wt, n = 13 cells; SYN, n = 12 cells; TKO, n = 10 cells from two independent cultures.



Logan et al., Figure S2

Supplementary Figure 3. Effect of synuclein on BDNF-pHluorin release.

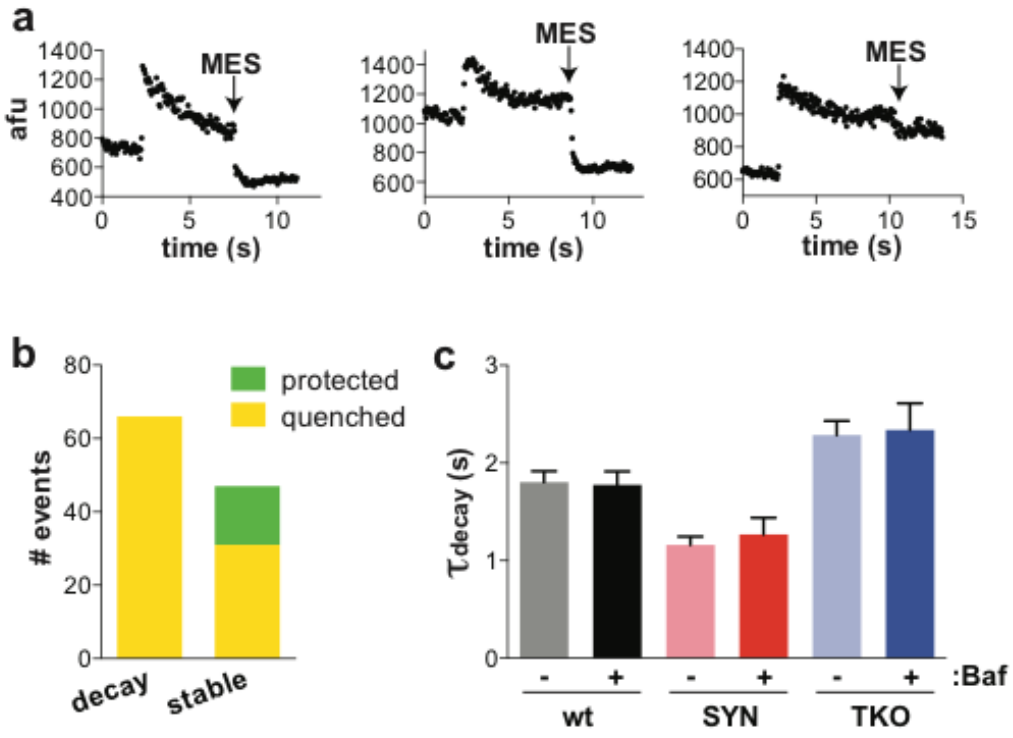
(a) The time to 90% peak BDNF-pHluorin fluorescence was determined as in Figure 1b, and represented here for wild type, α-synuclein overexpression and synuclein TKO as mean ± SEM ****, $p < 0.0001$ by Kruskal-Wallis one-way ANOVA **(b)** The peak fluorescence was normalized and the mean time course of fluorescence decay determined for all exocytotic events on a per cell basis. For all comparisons, $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test. **(c)** Representative kymographs depicting the BDNF-pHluorin event classes presented in Figure 1. n = 19 cells per group **(a,b)**



Logan et al., Figure S3

Supplementary Figure 4. Role of fusion pore closure and reacidification in BDNF-pHluorin fluorescence decay.

(a) Representative traces of a decaying chromaffin cell BDNF-pHluorin event quenched by low pH buffer (MES) (left), a stable event quenched by MES (center), and a stable event protected from quenching by MES (right). (b) All events in the process of decay were quenched by low pH MES buffer, eliminating the possibility that decay of BDNF-pHluorin fluorescence results from vesicle movement out of the TIRF plane. For stable events, MES application did not always result in quenching, indicating that the fusion pore can limit BDNF-pHluorin release but retain continuity with the external solution. In addition, only stable events have the potential for complete pore closure. (c) Experiments performed in the absence and presence of bafilomycin (0.6 μM) showed no difference in the kinetics of BDNF-pHluorin fluorescence decay, excluding a role for vesicle reacidification in the rate of release. wt, n = 213 / 124 events; SYN, n = 117 / 60 events; TKO, n = 178 / 190 events (+ / - bafilomycin)

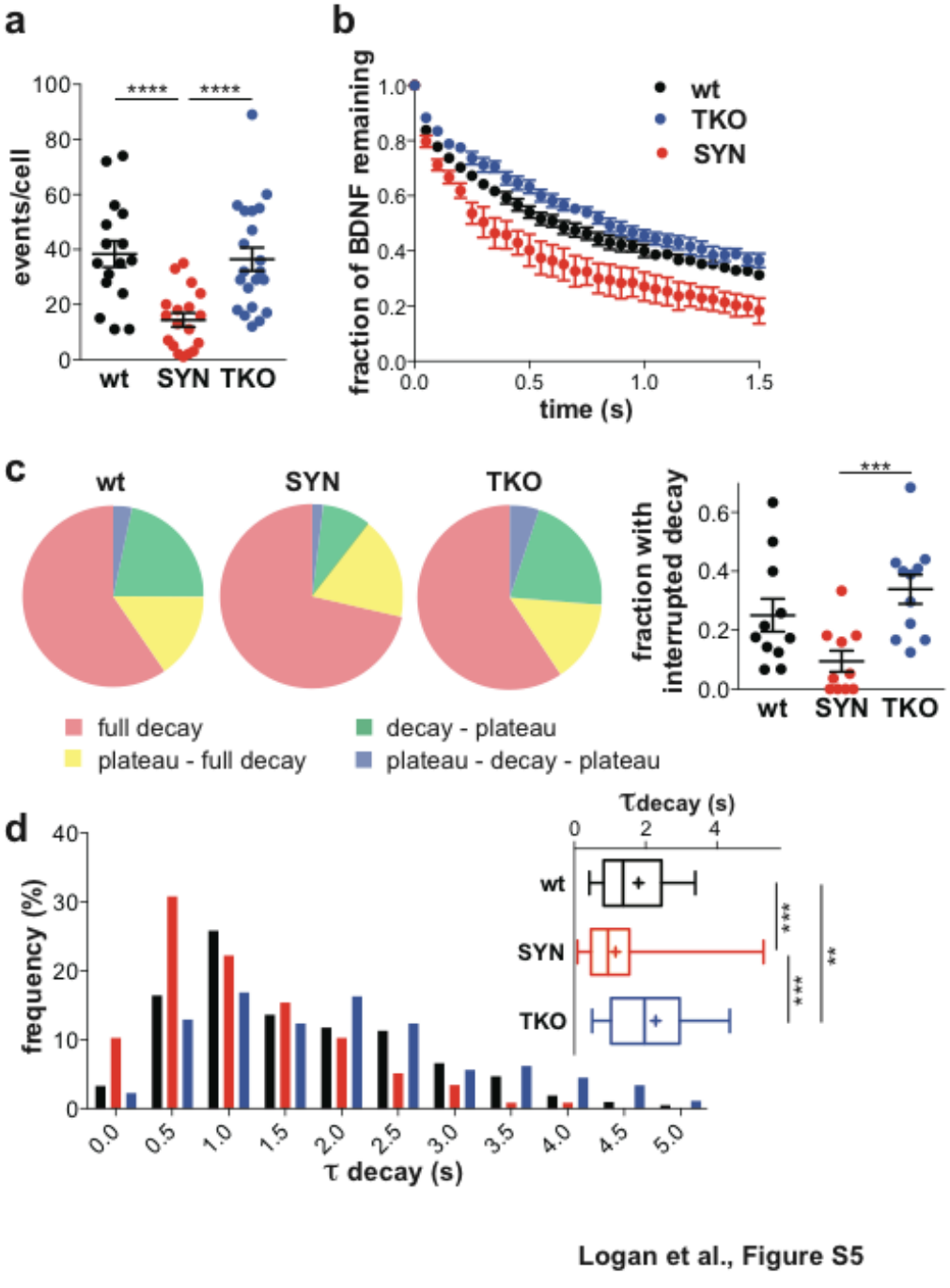


Logan et al., Figure S4

Supplementary Figure 5. Stimulated release of BDNF-pHluorin at 2 mM external Ca^{++}.

(a) Overexpression of α-syn (SYN) reduces the frequency of BDNF-pHluorin exocytotic events (****, $p < 0.0001$ by one-way ANOVA with Tukey's post hoc comparison). wt, n = 16 cells; SYN, n = 18 cells; TKO, n = 21 cells from three independent cultures (b) As described in figure S2, the release of BDNF-pHluorin was determined across all events by normalizing the peak fluorescence and presenting the mean loss of fluorescent cargo per cell. $p < 0.0001$ for all comparisons by one-way ANOVA with Tukey's post hoc comparison. wt, n = 11 cells; SYN, n = 9 cells; TKO, n = 11 cells (c) BDNF-pHluorin event classes (defined in Figure 2C) showed a similar distribution to events recorded at 5 mM Ca^{++}. Synuclein overexpression reduced the fraction of events with pore closure relative to the synuclein TKO (***, $p < 0.001$ by one-way ANOVA with Tukey's post hoc comparison). n = 11 (wt), 9 (SYN) and 11 (TKO)

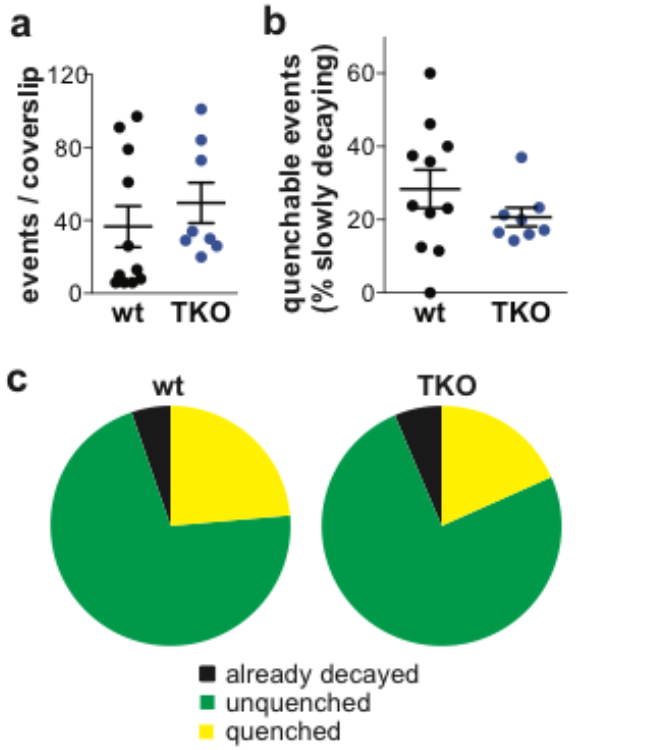
(d) Overexpression of α-synuclein reduces and loss of all synucleins increases the time constant of BDNF-pHluorin fluorescence decay. For all events that decayed completely to baseline fluorescence values, the time constants of decay were determined by fitting single exponentials to the decay component of the trace. The distributions differ with $p < 0.01$ for wt-SYN comparison, $p < 0.05$ for wt-TKO and $p < 0.001$ for SYN-TKO (Kolmogorov-Smirnov). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by Kruskal-Wallis one-way ANOVA with Dunn's post hoc test. wt, n = 213 events; SYN, n = 117 events; TKO, n = 178 events



Logan et al., Figure S5

Supplementary Figure 6. Quenching of BDNF-pHluorin by low pH in wild type and synuclein TKO neurons.

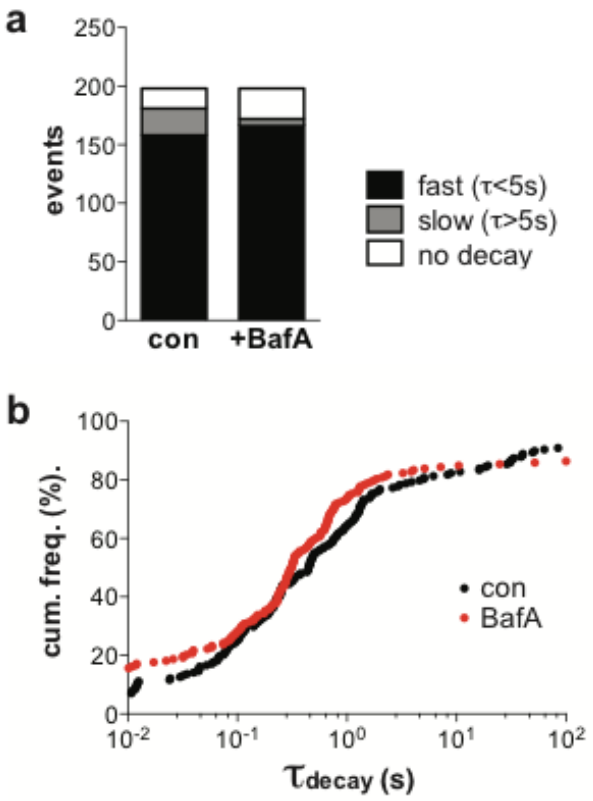
Wild type (wt) and synuclein TKO hippocampal neurons were transfected with BDNF-pHluorin and imaged 14-20 days later, stimulating at 50 Hz for 5 s followed by transient quenching of the cell surface fluorescence at pH 5.5 as in Figure 4. (a,b) Loss of synuclein has no effect on the frequency of exocytotic events per coverslip (a) or the proportion of slowly decaying events in each coverslip quenchable with acidic buffer (b). Data indicate mean ± SEM. (c) Events were classified as already decayed at the time of acid application or for those that were not, either quenched or unquenched by the low pH ($p = 0.16$ by Chi-square test). $n = 397$ events from two cultures for both wt (10 coverslips) and TKO (8 coverslips)



Logan et al., Figure S6

Supplementary Figure 7. Role of reacidification in the decay of NPY-pHluorin fluorescence.

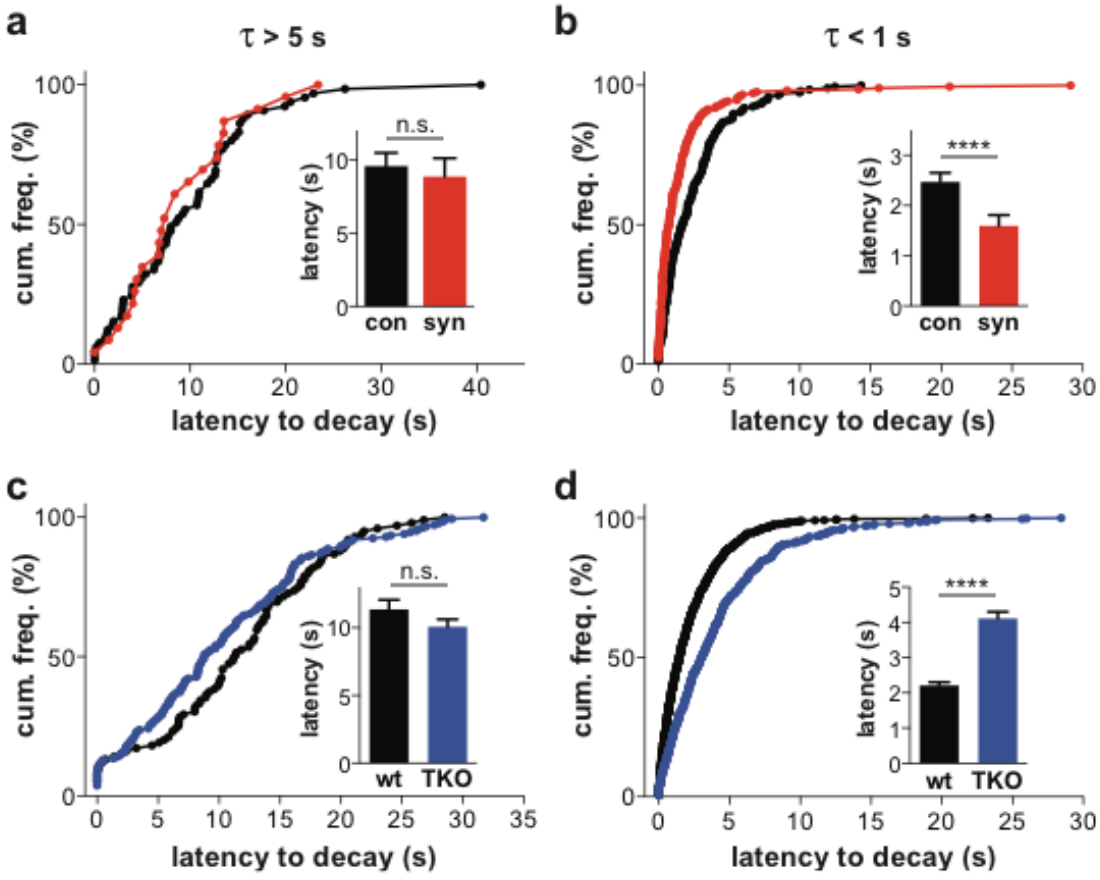
(a) Wild type mouse hippocampal neurons transfected with NPY-pHluorin were stimulated at 50 Hz for 5 s in the absence (con) or presence of bafilomycin (0.6 μM, +BafA). Individual traces were fit to a plateau followed by single exponential decay. Events that failed to show fluorescence decay were scored as no decay, and those that did show decay were classified as fast if $\tau < 5$ s or slow if $\tau > 5$ s ($p < 0.01$ by Chi-square test). n=198 events / 3 coverslips for both conditions. (b) Cumulative frequency distribution of τ_{decay} values, including events that did not decay. $p = 0.25$ by Kolmogorov-Smirnov.



Logan et al., Figure S7

Supplementary Figure 8. Synuclein has a primary effect on fusion pore dilation.

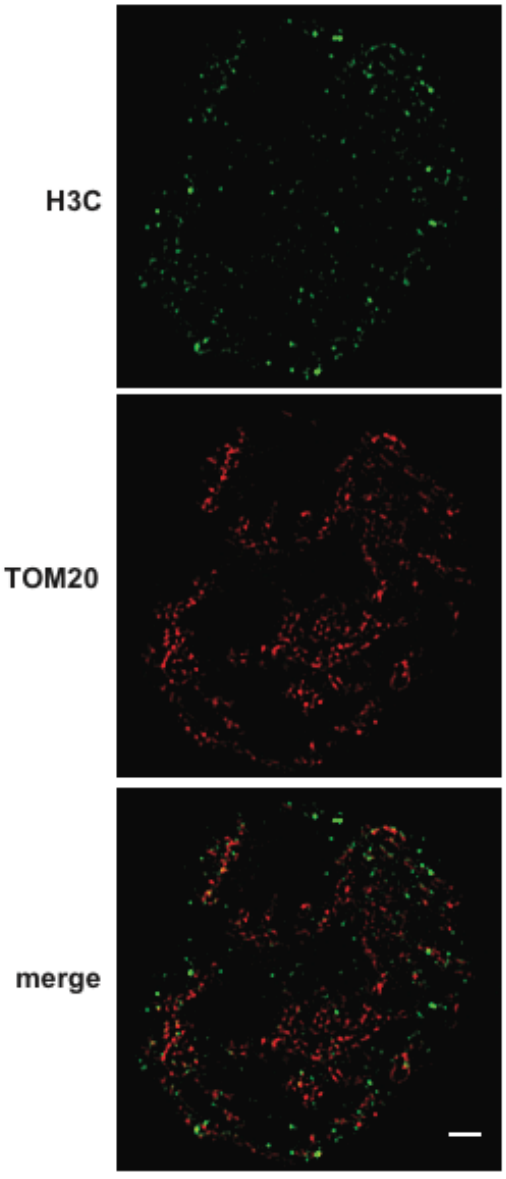
(a,b) NPY-pHluorin events from rat hippocampal neurons over-expressing human α-synuclein (syn) and controls were separated into those with time constants of fluorescent decay more than 5 s (a) and less than 1 s (b), and the latency to decay plotted as either cumulative frequency distribution ($p = 0.675$ for **a** and < 0.0001 by Kolmogorov-Smirnov for b). n(a) = 65 events / 5 control coverslips and 23 events / 8 coverslips for syn; n(b) = 197 events for control and 210 for syn (c,d) NPY-pHluorin events from the hippocampal neurons of wt and TKO mice were separated into similar groups and displayed similarly by cumulative frequency. Synuclein affects the latency to decay only for more rapidly decaying events ($p = 0.160$ for **c** and < 0.0001 by Kolmogorov-Smirnov for d). n(c) = 99 events / 10 cells and 185 events for TKO / 11 cells for TKO; n(d) = 750 events for wt and 474 events for TKO. n.s., not significant; **, $p < 0.01$; ****, $p < 0.0001$ by Mann-Whitney



Logan et al., Figure S8

Supplementary Figure 9. Synuclein does not colocalize with mitochondria in adrenal chromaffin cells.

Chromaffin cells from wt mice transduced with lentivirus encoding human α-synuclein were immunostained for synuclein using the H3C antibody and for mitochondria using an antibody to the outer membrane protein TOM20. Size bar, 1 μm. The images were obtained by structured illumination and are shown here as reconstructions of a 120 nm-thick slice located within 0.5 μm of the cell-coverglass interface. Size bar, 2.5 μm.

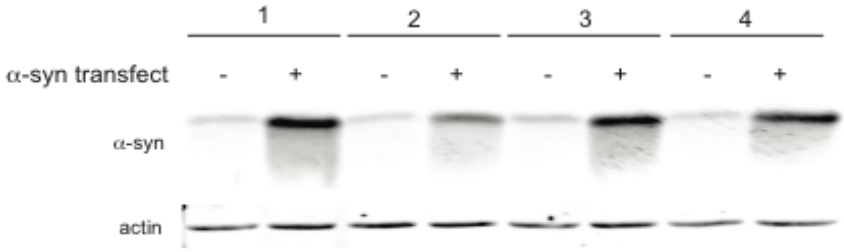


Logan et al., Figure S9

Supplementary Figure 10. Quantification of α-synuclein overexpression in cultured rat neurons.

Primary hippocampal rat neurons were co-electroporated with BDNF-pHluorin, p38-mCherry and either α-synuclein or empty vector exactly as described in figure 3. After culturing for 14 days, neurons were harvested and α-synuclein and actin levels were measured by quantitative fluorescent immunoblotting. **(a)** Representative immunoblot of lysates collected from four independently prepared and transfected cultures (1-4); blots were performed in duplicate. **(b)** α-synuclein immunoreactivity was quantified and normalized to that of actin. Data were expressed as fold-overexpression over empty vector electroporations. Mean ± SEM, 4.6 ± 0.5; n= 4 cultures.

a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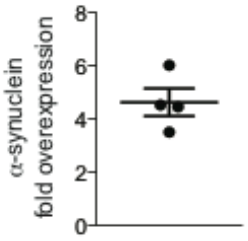
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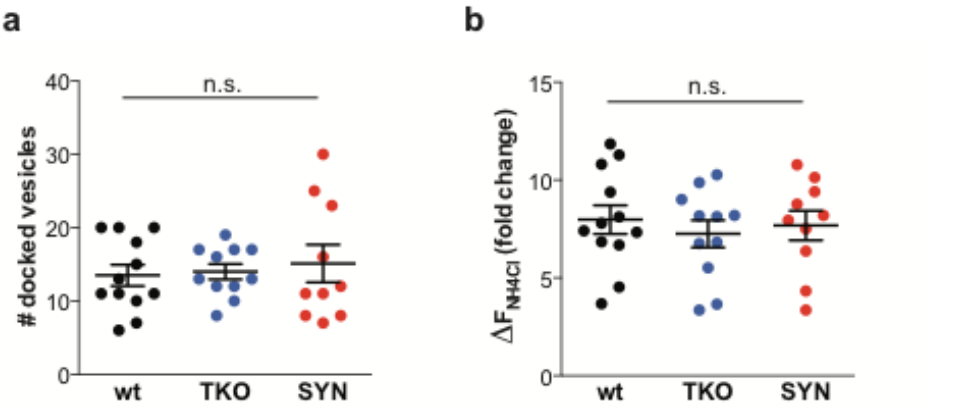
Supplementary Figure 11. Synuclein expression levels do not affect docked vesicle number or intravesicular pH.

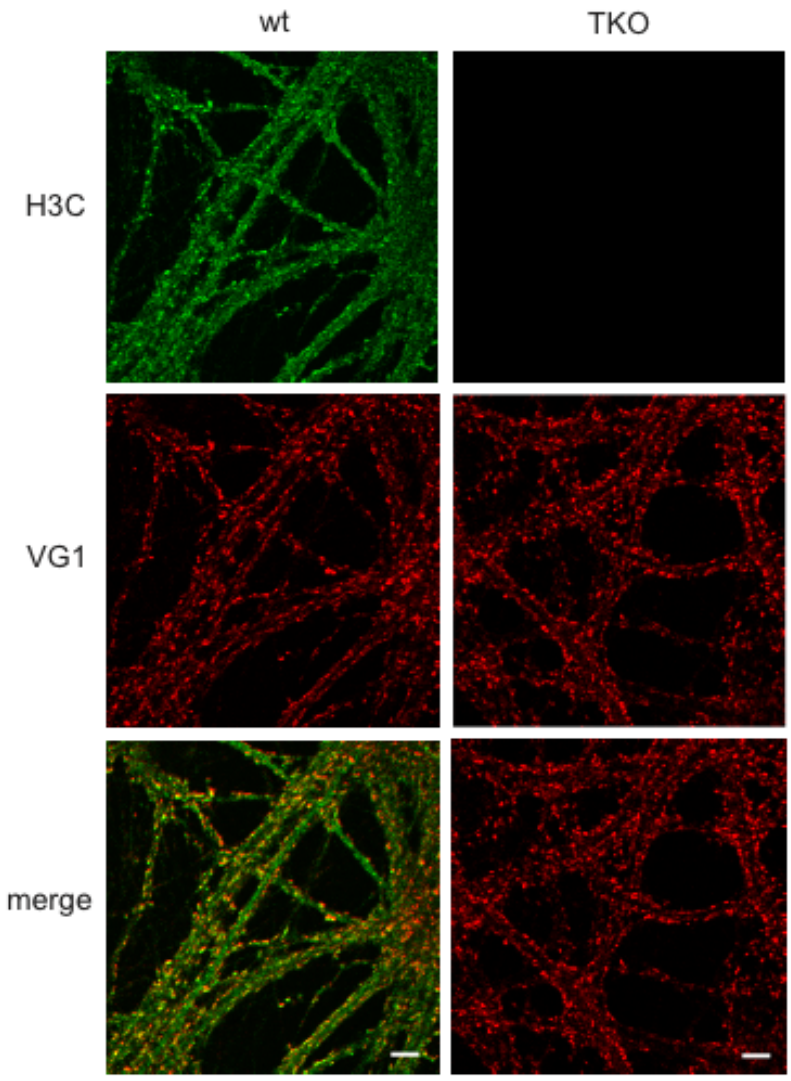
Chromaffin cells from wt or TKO mice were transduced with lentivirus encoding BDNF-pHluorin cultured for 5 days and imaged with TIRF microscopy. BDNF-pHluorin-expressing dense core vesicles were identified by application of 50mM NH_4Cl to neutralize the acidic intragranular pH. Fluorescent punctae were subjected to automated filtering and analysis in ImageJ. **(a)** Synuclein expression levels do not affect the mean number of docked vesicles per cell. BDNF-pHluorin-expressing dense core vesicles were identified by application of 50mM NH_4Cl to neutralize the acidic intragranular pH. Fluorescent punctae were subjected to automated filtering and analysis in ImageJ. **(b)** Synuclein expression levels do not affect intravesicular pH. A binary mask of the identified fluorescent punctae was generated and projected to images taken prior to application of 50mM NH_4Cl to measure the fold change in fluorescence for individual vesicles. Fold change values were averaged per cell. $p = 0.7720$ by one-way ANOVA. wt, n = 12 cells, 162 vesicles; TKO, n = 11 cells, 154 vesicles; SYN, n = 10 cells, 151 vesicles. Data indicate mean ± SEM



Logan et al., Supplementary Figure 11

Supplementary Figure 12. Endogenous synuclein is concentrated at presynaptic terminals in cultured neurons.

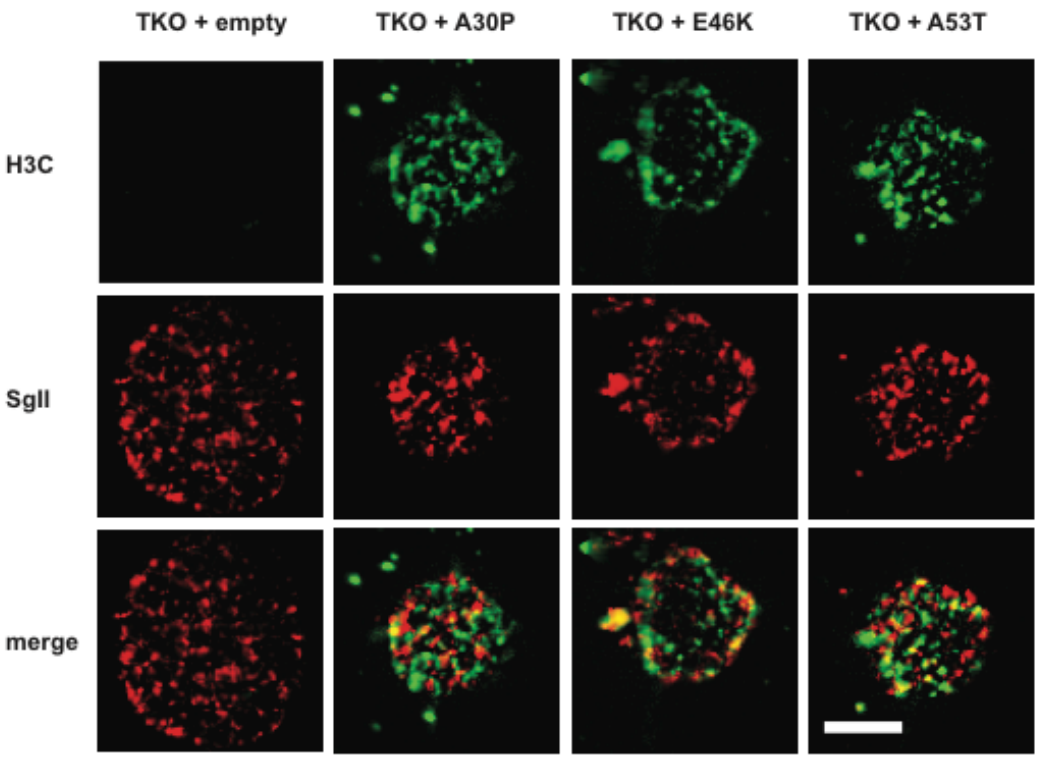
Primary hippocampal neurons from wild type (wt) or synuclein triple knockout (TKO) mice were cultured for 18 days and fixed. Cultures were immunostained for α/β-synuclein using the H3C antibody (green) and for the synaptic vesicle protein vesicular glutamate transporter 1 (VG1, red) and imaged by confocal microscopy. Scale bar, 5 μm.



Logan et al., Supplementary Figure 12

Supplementary Figure 13. Point mutations in α-synuclein do not perturb its localization to secretory vesicles.

Chromaffin cells from TKO mice were isolated and transduced with either empty vector or lentivirus encoding one of three α-synuclein mutants and immunostained 5 days later for α-synuclein (H3C, green) as well as the dense core vesicle protein secretogranin II (SgII, red). Representative TIRF images show that all three mutations still resulted in robust localization to secretory vesicles. Size bar, 2.5 μm.



Logan et al., Supplementary Figure 13

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Chapter 3: Concluding Remarks

Summary of Findings

Although it has been exhaustively studied for over twenty years, the normal function of the presynaptic protein α-synuclein, and how this function may relate to the pathogenesis of PD, remains unclear. The fact that point mutations in α-synuclein [1-6], as well as duplication or triplication of the wild-type gene locus [7] produce inherited forms of PD with extensive deposition of the protein in Lewy bodies [8] suggests a causative role for the protein in disease. Studies from our lab and a number of others have strongly implicated a role for α-synuclein in the regulation of neurotransmitter release [9,10]. However, the surprisingly modest phenotypes documented in the synuclein triple knockout (TKO) animals [11] has raised questions about whether the reported effects on neurotransmitter release reflect the normal function of α-synuclein or a gain-of-toxic function. Our ability to understand the precise mechanism underlying the effects of α-synuclein on exocytosis has been hindered by the inability to measure single synaptic vesicle fusion events by live imaging. In order to overcome these difficulties, we thus utilized TIRF microscopy of large dense core vesicles (LDCVs) in chromaffin cells, allowing us to easily resolve individual exocytotic events.

In Chapter 2 of this dissertation, I show that α-synuclein is expressed in adrenal chromaffin cells, albeit at low levels compared with neurons, and overexpression or genetic ablation of synuclein has opposing effects on the kinetics of individual exocytotic events (Figure 1, Supplementary Figure 1). I demonstrate that this is due to a direct effect of α-synuclein on the fusion pore rather than changes in the solubility of dense core cargo protein, since fluorescence decay of the integral membrane reporter VMAT2-

pHluorin, is also accelerated by α-synuclein overeexpression (Figure 2). I further show α-synuclein disfavors kiss-and-run exocytosis of VMAT2-pHluorin (Figure 2) by testing the fusion pore continuity with external solution during an acidic challenge. The effect of synuclein on LDCV release persisted in primary hippocampal neurons, where we observed even more dramatic effects in the TKO (Figures 3 and 4). By continuously oscillating the pH, we again show that synuclein expression represses closure of the fusion pore (Figure 3). Using an antibody that seems to preferentially recognize a membrane-bound form of α-synuclein, I demonstrate that both endogenous and overexpressed α-synuclein colocalizes extensively with the dense core vesicle cargo secretogranin II, suggesting that α-synuclein's effect on the fusion pore is most likely attributable to a direct physical interaction with the vesicular membrane (Figure 5). The familial PD-linked mutations A30P and A53T did not abolish the interaction of α-synuclein with secretory vesicles or perturb the protein's capacity to inhibit exocytosis (Figure 6, Supplementary Figure 13)). Strikingly though, these mutants were deficient in their ability to accelerate the kinetics of BDNF peptide release (Figure 6). These results suggest that inhibition of exocytosis and fusion pore dilation represent independent functions of α-synuclein, and the latter is selectively perturbed by disease-causing mutations in the protein. This implies that the loss of a normal function of α-synuclein might affect disease susceptibility.

Future Directions

In chapter 2, I present data demonstrating that α-synuclein promotes fusion pore dilation, accelerating release of peptide cargo and preventing pore closure. However, the precise mechanism through which α-synuclein exerts these effects remains unclear. There do exist, however, a number of other known modulators of fusion pore stability that α-synuclein could either act directly through or influence in some form. The cytoskeletal and contractile proteins F-actin and myosin II have been shown to accelerate the kinetics of catecholamine release and promote opening of the fusion pore in chromaffin cells [12,13]. α-Synuclein has been shown to bind actin and modulate its polymerization [14], and so could conceivably influence fusion pore dynamics via effects on the cytoskeleton. A study of fusion pore dynamics using carbon fiber amperometry found that reduction of cellular cholesterol shortened the duration of pre-spike feet, which is an indicator of fusion pore instability prior to rapid dilation of the pore, whereas overload of cholesterol had an opposite effect [15]. The synucleins have been highly implicated in regulation of lipid metabolism [16], and may thus act to alter the composition or distribution of lipids within the vesicular membrane. Alternatively, given the ability of α-synuclein to prevent pore closure, it could exert its effect on pore dynamics by opposing the normal function of the dynamin GTPase scission machinery. It has been recently demonstrated that dynamin plays a role in regulating the transition from hemifusion to full fusion [17]. Given that α-synuclein binds directly to secretory vesicles, it could theoretically sterically occlude the association of dynamin with the nascent fusion pore, resulting in increased pore continuity and content release. Taken together with the identified role for CSPα as a regulator of dynamin [18] and the compelling genetic

interaction between α-synuclein and CSPα, this possibility seems particularly attractive. To this end, ongoing experiments in the lab will seek to determine what effect overexpression of α-synuclein has with either concomitant knockdown of dynamin or expression of a dominant-negative form of dynamin on the kinetics of BDNF-pHluorin release.

I also present data in Chapter 2 revealing that familial mutations in α-synuclein result in a loss of the protein's fusion pore-promoting function. The obvious question to address is whether the other disease-linked point mutations show a similar effect, which would further suggest that disease might be initiated by loss of a normal function of α-synuclein. Ultimately, these experiments hold the potential to uncover a better understanding of the precise molecular events that precede overt cell loss in disease and to potentially elucidate novel biomarkers or therapeutic targets for the prevention or treatment of PD.

Perspectives

The data presented in this dissertation represents a step forward in our understanding of the normal function of α-synuclein and how this function might pertain to Parkinson's disease. Ongoing experiments in our lab will be focused on determining the precise mechanism behind this novel role for α-synuclein, with the hope that in doing so we will gain new insight into the cellular events that precipitate neurodegeneration.

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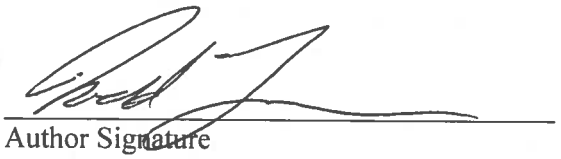

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