α-Synuclein promotes dilation of the exocytotic fusion pore

Todd Logan1,2,4, Jacob Bendor1,4, Chantal Toupin1, Kurt Thorn3 & Robert H Edwards1,2

The protein α-synuclein has a central role in the pathogenesis of Parkinson’s disease. Like that of other proteins that accumulate in neurodegenerative disease, however, the function of α-synuclein remains unknown. Localization to the nerve terminal suggests a role in neurotransmitter release, and overexpression inhibits regulated exocytosis, but previous work has failed to identify a clear physiological defect in mice lacking all three synuclein isoforms. Using adrenal chromaffin cells and neurons, we now find that both overexpressed and endogenous synuclein accelerate the kinetics of individual exocytotic events, promoting cargo discharge and reducing pore closure (‘kiss-and-run’). Thus, synuclein exerts dose-dependent effects on dilation of the exocytotic fusion pore. Remarkably, mutations that cause Parkinson’s disease abrogate this property of α-synuclein without impairing its ability to inhibit exocytosis when overexpressed, indicating a selective defect in normal function.

Despite the established role of multiple proteins in the pathogenesis of neurodegenerative disease, we know remarkably little about their function. In Parkinson’s disease, as well as in the related conditions dementia with Lewy bodies and multiple system atrophy, the peripheral membrane protein α-synuclein accumulates in characteristic inclusions1. Mutations in α-synuclein also produce a dominantly inherited form of Parkinson’s disease2-7, demonstrating that the protein has a causative role. Indeed, α-synuclein gene duplication and particularly triplication produce a severe form of familial Parkinson’s disease8, implicating the wild-type (WT) protein in disease. Synuclein thus has a central role in Parkinson’s disease. However, the normal function of α-synuclein remains poorly understood.

α-Synuclein normally localizes to the nerve terminal, suggesting a role in neurotransmitter release9. Consistent with this, modest overexpression (insufficient to produce inclusions or overt toxicity) inhibits the regulated exocytosis of large dense core vesicles (LDCVs) and synaptic vesicles10-12. 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 the closely related β- and γ- isoforms13,14. Knockout mice lacking α- and γ-synuclein show an increase in evoked dopamine release15, but the physiological change responsible remains unknown. Although overexpression inhibits regulated exocytosis, the role of endogenous α-synuclein has thus remained unknown.

α-Synuclein binds specifically to anionic membranes with high curvature16-18 but can also deform the lipid bilayer. Synuclein aggregates membranes in yeast19,20, tubulates artificial membranes in vitro21 and, when overexpressed in mammalian cells, can produce mitochondrial fragmentation22,23. However, membrane deformation is generally considered to be more important to endocytosis than to exocytosis. The effect of overexpressed synuclein on exocytosis has thus been difficult to explain on the basis of membrane curvature-sensing or curvature-promoting properties. Alternatively, synuclein has been suggested to serve as chaperone for the SNARE complex, but without apparent effect on transmitter release14.

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 reclose during a kiss-and-run event that immediately regenerates the vesicle24. 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 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-run25, and previous work has indeed demonstrated the inhibition of LDCV exocytosis by synuclein overexpression in chromaffin cells10. α-Synuclein-positive Lewy pathology also occurs at high frequency in the adrenal glands of patients with Parkinson’s disease and dementia with Lewy bodies26, with effects on catecholamine release into the circulation27.

RESULTS
Synuclein overexpression accelerates the kinetics of individual exocytotic events in chromaffin cells
To understand how synuclein influences release, we first 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 protein secretogranin II (SgII) confirmed

1Departments of Neurology and Physiology, UCSF School of Medicine, University of California, San Francisco, San Francisco, California, USA. 2Graduate Program in Biomedical Sciences, UCSF School of Medicine, University of California, San Francisco, San Francisco, California, USA. 3Department of Biochemistry & Biophysics, UCSF School of Medicine, University of California, San Francisco, San Francisco, California, USA. 4These authors contributed equally to this work. Correspondence should be addressed to R.H.E. (robert.edwards@ucsf.edu).

Received 4 January; accepted 5 February; published online 13 March 2017; doi:10.1038/nn.4529
expression of the human protein in chromaffin cells (Supplementary Fig. 1a). We also assessed the expression of endogenous α-synuclein using an antibody that recognizes the protein from multiple species. Comparison of chromaffin cells from WT and synuclein TKO mice lacking all synuclein isoforms showed that chromaffin cells express endogenous α-synuclein and the lentivirus confers modest overexpression (Supplementary Fig. 1b,c).

To study individual exocytotic events, we used a fusion of brain-derived neurotrophic factor (BDNF) to the ecliptic pHluorin, a modified form of green fluorescent protein with enhanced pH sensitivity28. Overexpression 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 (F(2,54) = 4.991). n = 19 cells for each group from 3 independent cultures. Bars indicate mean ± s.e.m. (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 diagrams (upper right) illustrate our interpretation of the traces. The proportion of event types differed among all three groups (P < 0.0001 by chi-squared test for pairwise as well as the comparison of all three groups). (d) Synuclein influences the rate of BDNF release. For all full decay events, the time constant of fluorescence decay (τ_decay) was determined by fitting to a single exponential. The histogram represents the distribution of events with different τ_decay (P < 0.0001 for WT versus SYN and TKO versus SYN; P = 0.001 for WT versus TKO by Kolmogorov-Smirnov test). WT, n = 266 events; SYN, n = 167 events; TKO, n = 237 events. (e) For all events with nonzero latency to decay, the time from reaching 90% maximal fluorescence to the onset of decay was determined (WT, n = 134 events; SYN, n = 66 events; TKO, n = 218 events). Boxes represent the middle two quartiles, whiskers 10% and 90% of the events, the line the median and the + the mean. ****P < 0.0001 by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc test; H = 55.22 (d) and 39.45 (e).
However, the fluorescence events fall into at least four distinct classes (Fig. 1c and Supplementary Fig. 4e). Many events decayed immediately to baseline (full decay) whereas others remained at maximum fluorescence for an interval before full decay (plateau–decay). In still others, decay was interrupted, suggesting constriction if not 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. 3c). All events in the process of decay showed quenching by the acidic buffer (Supplementary Fig. 3d), demonstrating exocytosis and either full collapse or persistence of a dilated fusion pore rather than movement away from the plasma membrane. Indeed, the H⁺-ATPase inhibitor bafilomycin did not influence the time course of events in any genotype (Supplementary Fig. 3e), confirming that loss of fluorescence indicates the release of peptide, not reacification. Most of the stable events (plateaus) also showed quenching by low external pH, but a fraction did not (Supplementary Fig. 3d). Thus, complete closure of the fusion pore occurs only in stable events, but incomplete closure can still limit the loss of peptide. Consistent with the acceleration of release, synuclein overexpression increased the proportion of events that undergo full decay (Fig. 1c). Within the group undergoing full decay, however, synuclein overexpression also increased the rate of decay (Fig. 1d and Supplementary Fig. 4f). Thus, synuclein accelerates release independent of effects on pore closure, suggesting a role early in exocytosis in promoting peptide release. Overexpression of synuclein also had no effect on the number of docked vesicles or their luminal pH (Supplementary Fig. 5), arguing against a general disturbance of LDCVs as cause for the change in exocytosis.

**Loss of synuclein prolongs the kinetics of release**

The difficulty detecting clear effects on transmitter release in knock-out mice suggests that α-synuclein overexpression 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 neurons11,13,14, chromaffin cells from synuclein TKO mice showed no clear change in event number relative to WT (Fig. 1a). However, analysis of event distribution revealed an increase in the time to peak fluorescence (Fig. 1b and Supplementary Fig. 4a,b). Loss of the synucleins modestly reduced the proportion of events with full decay (Fig. 1c) and also redistributed the decay time constants to longer values (Fig. 1d). In addition, TKO cells showed greatly increased latency to decay among those events that did not decay immediately (Fig. 1e). (Overexpression did not affect this parameter presumably because it converts those with a short latency to full decay.) Thus, loss of synuclein prolongs release, suggesting a similar role for the endogenous and overexpressed protein in exocytosis. Like overexpression, the synuclein TKO also had no effect on LDCV docking or pH (Supplementary Fig. 5).

Although we used 5 mM external Ca²⁺ for these experiments because it may promote kiss-and-run, we also examined BDNF-pHluorin events at the more physiological 2 mM Ca²⁺. In this condition, as in 5 mM Ca²⁺, overexpression of human α-synuclein both inhibited the exocytosis of chromaffin granules and accelerated the loss of BDNF-pHluorin (Supplementary Fig. 6a,b). The proportion of events again shifted to those with full decay at the expense of those with interrupted release, and the time constant for fluorescence decay shortened (Supplementary Fig. 6c,d). The TKO also showed prolonged decay relative to WT (Supplementary Fig. 6d). Thus, synuclein has similar effects on release at 2 and 5 mM Ca²⁺.

**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. Indeed, the same LDCVs can release different substances at different rates, indicating...
Figure 3  α-synuclein influences fusion pore closure in neurons. (a) Rodent hippocampal neurons were transfected with BDNF-pHluorin and imaged 14–20 d later, stimulating at 50 Hz for 5 s followed immediately by quenching of the cell surface fluorescence at pH 5.5. The arrows indicate an event quenchable at low pH; 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 ± s.e.m.) for BDNF-pHluorin-expressing rat hippocampal neurons cotransfected with α-synuclein (SYN) or empty vector (con) and stimulated as in (a) (P = 0.13 by unpaired, two-tailed t-test; t(16) = 1.582). n = 9 cells from 2 independent cultures. (d) With events classified as either already decayed at the time of acid exposure or, if not, unquenched or quenched by low pH, α-synuclein overexpression reduces the proportion of unquenched events (P < 0.0001 by chi-squared test) (left). n = 281 events (con), 158 events (α-syn). Synuclein overexpression also increases the proportion of quenchable events per coverslip independent of those already decayed (right). *P < 0.05 by unpaired t-test (t(15) = 2.145). Bars indicate mean ± s.e.m. (e) Mouse neurons transfected with BDNF-pHluorin were stimulated at 50 Hz for 5 s and superfused with Tyrode’s solutions oscillating rapidly (1.33 Hz) between pH 7.8 and 6.4. Top trace shows an exocytotic event with oscillation that persisted until fluorescence decay, indicating that the fusion pore remained open until the peptide was released. Middle trace shows an event that did not decay completely but showed oscillation throughout, indicating that the fusion pore did not close. Bottom trace shows an event where the oscillation stopped (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-squared test). n = 100 events from 7 (WT) and 9 (TKO) coverslips. (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 test). Inset plots time to pore closure, with boxes representing the middle two quartiles, whiskers 10% and 90% of the events, the line the median and the + the mean (n.s., not significant). n = 44 events for WT and 63 events for TKO.

that the properties of the aggregated peptide can influence the rate of release. It seems unlikely that a cytoplasmic protein such as synuclein would influence luminal contents, but to distinguish further between effects on the fusion pore and on peptide solubility, we used a construct with the pHluorin inserted into a luminal loop of the vesicular monoamine transporter VMAT2 (ref. 36), a polytopic membrane protein that localizes to LDCVs. Stimulation of endocrine cells expressing the fusion produced discrete exocytotic events consistent with LDCVs, and we used bafilomycin for these experiments to prevent vesicle reacidification. In chromaffin cells, α-synuclein overexpression also inhibited the exocytosis of VMAT2-pHluorin (Fig. 2b). As a membrane protein, VMAT2-pHluorin cannot undergo release, and its decay therefore reflects spread within the plasma membrane and endocytosis, processes limited by the fusion pore37. Indeed, we observed events that spread and others that did not, as well as variation in the time course of fluorescence decay (Fig. 2a). Although the proportion of events with a latency to decay did not differ (46% for synuclein overexpression, 44% for control), the latency to decay shortened with overexpression of α-synuclein (Fig. 2b,c). The long duration of VMAT2-pHluorin events also enabled us to determine how many remain accessible to the external solution. We found that a substantial fraction of the persistent events in control chromaffin cells were protected from quenching by MES-buffered solution at pH 5.5 (Fig. 2d,e), indicating kiss-and-run. In cells overexpressing human α-synuclein, this fraction declined substantially (Fig. 2e), suggesting inhibition of pore closure. However, synuclein overexpression also shortened the time constant of fluorescence decay for VMAT2-pHluorin (Fig. 2f), again supporting an independent effect on the rate of release, and a skew in the distribution of events per cell does not account for the effect on latency to decay or rate of decay (Supplementary Fig. 7). 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 overexpressed synuclein promote fusion pore dilation in neurons

Does synuclein also affect the properties of individual exocytotic events in neurons? To address this, we first expressed human α-synuclein in primary rat hippocampal neurons, imaging cotransfected BDNF-pHluorin (Fig. 3). Quantitative western analysis using an antibody that recognizes both human and rodent α-synuclein showed 4- to 5-fold overexpression (Supplementary Fig. 8). As previously reported38,39, stimulation at 50 Hz evoked discrete exocytotic events primarily in axons (Fig. 3a). The average number of events per coverslip did not significantly change with synuclein overexpression (Fig. 3c), but quenching at low pH revealed a dramatic effect of synuclein on the proportion of events accessible to external solution (P < 0.0001 by chi-squared test) (Fig. 3d). With overexpression, more events decayed before the addition of low-pH solution (Fig. 3a,d), indicating that, as in chromaffin cells overexpressing synuclein, the
events decay more rapidly. Even among those events that remained, however, synuclein overexpression increased the proportion externally quenchable by low pH (Fig. 3b,d), demonstrating that α-synuclein has an effect on the fusion pore 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 had little effect on the proportion of events already decayed, unquenchable or quenchable by low pH (Supplementary Fig. 9). However, BDNF-pHluorin events in neurons persisted for many seconds (Fig. 3b), making it difficult to detect any further prolongation due to the loss of synuclein, and the acid quench limits characterization of the fusion pore to a single time point. We therefore turned to the reporter NPY-pHluorin due to its more rapid release from LDCVs.\(^{34,39}\). To determine how much of the decay in fluorescence reflects peptide release or reacidification, we again used the H\(^+\)-ATPase inhibitor bafilomycin. A small subset of NPY-pHluorin events decayed slowly, with a time constant more than 5 s, and their proportion was reduced by bafilomycin (Supplementary Fig. 10a). At the same time, bafilomycin increased the proportion of events with no decay by a similar amount. Thus, a small fraction of events exhibit pore closure and reacidify slowly after endocytosis. However, the vast majority of events were rapid, with a time constant of decay considerably less than 5 s, and bafilomycin did not significantly change their proportion (Supplementary Fig. 10a). Bafilomycin also did not alter the decay kinetics of most events (Supplementary Fig. 10b). Thus, fast NPY-pHluorin events reflect peptide release and only the longest events undergo pore closure.

Much as in chromaffin cells, overexpression of α-synuclein in neurons shortened both the latency of NPY-pHluorin events to decay and
the time constant for fluorescence decay once this began (Fig. 4a–c). Conversely, the loss of all three synucleins dramatically increased the latency of NPY-pHluorin to decay and the time constant of fluorescence decay (Fig. 4d,e). The TKO thus has 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, and the difference cannot be attributed to a skewed distribution of events per cell (Supplementary Fig. 11). Of note, the effect of synuclein on NPY-pHluorin fluorescence decay appeared to involve only the more slowly decaying exocytotic events (Fig. 4c,e), which the experiment with bafilomycin suggests undergo pore closure (Supplementary Fig. 10). Presumably, it is difficult to detect acceleration of more rapidly decaying events because they are already very rapid. However, we also found that the latency from event appearance to the onset of decay shortened with overexpression of synuclein and lengthened in the TKO, but only for the events that decay rapidly (τ < 1 s), which are more common (Supplementary Fig. 12).

In chromaffin cells and neurons, synuclein overexpression affected 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 fusion pore closure 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 oscillation in fluorescence stops, this indicates pore closure. We used BDNF-pHluorin for this experiment because the events last longer and can thus provide information about pore closure relatively long after exocytosis begins. Figure 3e shows sample traces for events where the pore remained open until peptide release, where closure did not occur during the period imaged, and for a closure event. Inactivation of all three synuclein genes substantially increased the proportion of events with pore closure, at the expense of those without closure (P = 0.01 by chi-squared test) (Fig. 3f). However, loss of synuclein did not affect the time to pore closure (Fig. 3g). Taken together with the NPY-pHluorin data, these results suggest that synuclein prevents interruption of release by pore closure 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 as a presynaptic protein was unexpected. Synuclein localizes to dense core vesicles (Fig. 4c), which the experiment with bafilomycin suggests undergo pore closure (Supplementary Fig. 10). Presumably, it is difficult to detect acceleration of more rapidly decaying events because they are already very rapid. However, we also found that the latency from event appearance to the onset of decay shortened with overexpression of synuclein and lengthened in the TKO, but only for the events that decay rapidly (τ < 1 s), which are more common (Supplementary Fig. 12).

In chromaffin cells and neurons, synuclein overexpression affected 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 fusion pore closure 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 oscillation in fluorescence stops, this indicates pore closure. We used BDNF-pHluorin for this experiment because the events last longer and can thus provide information about pore closure relatively long after exocytosis begins. Figure 3e shows sample traces for events where the pore remained open until peptide release, where closure did not occur during the period imaged, and for a closure event. Inactivation of all three synuclein genes substantially increased the proportion of events with pore closure, at the expense of those without closure (P = 0.01 by chi-squared test) (Fig. 3f). However, loss of synuclein did not affect the time to pore closure (Fig. 3g). Taken together with the NPY-pHluorin data, these results suggest that synuclein prevents interruption of release by pore closure 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, α-synuclein exhibits only weak association with synaptic vesicles by gradient fractionation and photobleaching. We also failed to detect any specific localization of either overexpressed or endogenous synuclein with SgII in chromaffin cells using a number of commercially available antibodies. These antibodies produce diffuse labeling (Supplementary Fig. 1). We were therefore surprised to find that an antibody to the homologous canary protein synelfin specifically labeled LDCVs in these cells. Using this antibody, which recognizes both α- and β-synuclein, overexpressed human α-synuclein colocalized extensively with the LDCV protein SgII by structured illumination microscopy (Fig. 5). The same antibody detected endogenous synuclein on secretory granules in bovine chromaffin cells (M.A. Bittner and R.W. Holz, personal communication). Endogenous synuclein also colocalized with SgII, and the immunoreactivity for synuclein is specific because the TKO showed very low background staining (Fig. 5 and Supplementary Fig. 13). Supporting the specificity for LDCVs, endogenous synuclein showed little colocalization with mitochondria (Fig. 5b and Supplementary Fig. 14). Further, the colocalization persisted on LDCVs away from as well as at the plasma membrane, indicating that synuclein associates with LDCVs before docking. Owing perhaps to conformational specificity for the membrane-bound or multimeric protein, 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.

**Effect on pore dilation is conserved among isoforms but inhibited by mutations associated with Parkinson’s disease**

The N terminus of α-synuclein contains seven 11-amino-acid repeats that form an amphipathic α-helix upon interaction with membranes containing acidic phospholipid headgroups. In addition to membrane association, the repeats may contribute to function, such as on the fusion pore. Since the three synuclein isoforms...
show strong sequence conservation in the repeats and diverge at the more hydrophilic C terminus, we tested the role of the N terminus by examining the effects of β- and γ-synuclein. Transduced into WT mouse chromaffin cells along with BDNF-pHluorin, human β- and γ-synuclein both reduced the number of exocytotic events (Fig. 6a), as in the effect of α-synuclein in chromaffin cells and the effect of multiple synuclein isoforms on synaptic vesicle exocytosis11. In the events that remained, β- and γ-synuclein also accelerated peptide release (Fig. 6b), again like α-synuclein. As the only conserved α-synuclein to inhibit exocytosis, suggests that the Parkinson's disease-associated mutations affected one function of synuclein (pore dilation) but not another (inhibition of exocytosis).

The preserved ability to inhibit exocytosis suggests that the Parkinson's disease-associated mutations localize normally to LDCVs. Indeed, immunofluorescence using the H3C antibody showed unpaired colocalization of both mutants with SgII by TIRF microscopy (Fig. 6c). Since this antibody does not distinguish between endogenous and introduced synuclein, we also examined the mutants in chromaffin cells from synuclein TKO mice and again observed no difference from endogenous synuclein in colocalization with SgII (Fig. 6c and Supplementary Fig. 15).

DISCUSSION
The results show that synuclein influences behavior of the exocytotic fusion pore. Overexpression accelerates the release event, reducing the time to peak fluorescence (pore dilation), 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 overexpressed 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 closure during 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 associate slowly from a luminal matrix, rather than classical transmitters such as glutamate that escape rapidly through even a small pore. Indeed, synuclein overexpression and loss both affect the release of dopamine10,15. In contrast, loss of synuclein has little effect on glutamate release13,14. 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 overexpressed 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 complexes14 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 release47. However, overexpression of synuclein also inhibits the extent of synaptic vesicle exocytosis11, and the results presented here confirm that this effect extends to LDCVs10. It is difficult to reconcile the observed inhibition of release with a role for synuclein as chaperone for the SNARE complex14. Indeed, synuclein inhibits the fusion of membranes in vitro as well as in vivo through direct effects on the lipid bilayer22,48–50.

Alternatively, synuclein may promote SNARE complex accumulation by inhibiting exocytosis, thereby preventing the disassembly of complexes present on vesicles primed for fusion. In either case, synuclein appears to have a dual role. Physiologically, synuclein promotes dilation of the fusion pore, a dose-dependent effect shared by endogenous and overexpressed protein. By contrast, inhibition of exocytosis may be restricted to synuclein overexpression13,15, suggesting a pathological role. Nonetheless, the inhibition of exocytosis appears dose-dependent11, much like the effect on fusion pore kinetics.

The effect of mutations associated with Parkinson’s disease shows that the two activities of synuclein, to promote pore dilation and to inhibit exocytosis, are distinguishable. A30P and A53T mutations do not impair the inhibition of exocytosis by synuclein overexpression. However, they both eliminate the effect of synuclein on fusion pore dilation. Since the loss of synuclein affects fusion pore dilation but not the number of exocytotic events, the Parkinson’s disease mutations appear to produce a selective loss in the normal function of the protein. The preserved ability of overexpressed mutant and WT synuclein to inhibit exocytosis may be required to produce degeneration.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank members of the Edwards laboratory for discussion, D. Jullié for help with the pH oscillation experiment, A. Bertholet (UCSF) for the TOM20 antibody and B. Calaguí and S. Batarni for technical assistance. We also thank K. Bohannan, M. Bittner and R. Holz for sharing data and providing suggestions. This work was supported by grants from NINDS (NS062715), NIDA (DA10154) and the Well Institute for Neurosciences (to R.H.E.), the John and Helen Cahill Family Endowment for Research on Parkinson’s Disease (to R.H.E.), a fellowship from NINDS (to T.L.) and a fellowship from the A.P. Giannini Foundation (to J.B.).

AUThor CONTRIBUTIONS

R.H.E., T.L. and J.B. designed the research and wrote the manuscript. T.L. and J.B. performed the experiments and analyzed the data, with assistance from C.T. K.T. provided essential technical assistance with the chromaffin cell imaging experiments.

COMPEtING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Goedert, M., Spillantini, M.G., Del Tredici, K. & Braak, H. 100 years of Lewy pathology. Nat. Rev. Neurol. 9, 13–24 (2013).
2. Polymeropoulos, M.H. et al. Mutation in the α-synuclein gene identified in families with Parkinson’s disease. Science 276, 2045–2047 (1997).
3. Krüger, R. et al. ALa30Pro mutation in the gene encoding α-synuclein in Parkinson’s disease. Nat. Genet. 18, 106–108 (1998).
4. Zarranz, J.J. et al. The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. Ann. Neurol. 55, 164–173 (2004).
5. Appel-Cresswell, S. et al. Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson’s disease. Mov. Disord. 28, 811–813 (2013).
6. Lesage, S. et al. G51D α-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. Ann. Neurol. 73, 459–471 (2013).
7. Proukakis, C. et al. A novel α-synuclein missense mutation in Parkinson disease. Neurology 80, 1062–1064 (2013).
8. Singleton, A.B. et al. α-Synuclein locus triplication causes Parkinson’s disease. Science 302, 841 (2003).
9. Bendor, J.T., Logan, T.P. & Edwards, R.H. The function of α-synuclein. Neuron 79, 1044–1066 (2013).
10. Larsen, K.E. et al. α-Synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis. J. Neurosci. 26, 11915–11922 (2006).
11. Nemani, V.M. et al. Increased expression of α-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. Neuron 65, 66–79 (2010).
12. Scott, D.A. et al. A pathologic cascade leading to synaptic dysfunction in α-synuclein-induced neurodegeneration. J. Neurosci. 30, 8083–8095 (2010).
13. Gidtren-Harrison, B. et al. α-Synuclein transgenic mice exhibit normal synaptic and motor function. Proc. Natl. Acad. Sci. USA 107, 19573–19578 (2010).
14. Burre, J. et al. α-Synuclein promotes SNARE-complex assembly in vivo and in vitro. Science 329, 1663–1667 (2010).
15. Senior, S.L. et al. Increased striatal dopamine release and hyperdopaminergic-like behaviour in mice lacking both alpha-synuclein and gamma-synuclein. Eur. J. Neurosci. 27, 947–957 (2008).
16. Davidson, W.S., Jonas, A., Clayton, D.F. & George, J.M. Stabilization of α-synuclein secondary structure upon binding to synthetic membranes. J. Biol. Chem. 273, 9443–9449 (1998).
17. Pranke, I.M. et al. α-Synuclein and ALPS motifs are membrane curvature sensors whose contrasting chemistry mediates selective vesicle binding. J. Cell Biol. 194, 89–103 (2011).
18. Jensen, M.B. et al. Membrane curvature sensing by amphiphilic helices: a single liposome study using α-synuclein and annexin B12. J. Biol. Chem. 286, 42603–42614 (2011).
19. Cooper, A.A. et al. α-Synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson’s models. Science 331, 324–328 (2006).
20. Soper, J.H. et al. α-Synuclein-induced aggregation of cytoplasmic vesicles in Saccharomyces cerevisiae. Mol. Biol. Cell 19, 1093–1103 (2008).
21. Varkey, J. et al. Membrane curvature induction and tubulation are common features of synucleins and apolipoproteins. J. Biol. Chem. 285, 32486–32493 (2010).
22. Kamp, F. et al. Inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1, Parkin and DJ-1. EMBO J. 29, 3571–3589 (2010).
23. Nakamura, K. et al. Direct membrane association drives mitochondrial fission by the Parkinson disease-associated protein α-synuclein. J. Biol. Chem. 286, 20710–20726 (2011).
24. Alabi, A.A. & Tsien, R.W. Perspectives on kiss-and-run: role in exocytosis, endocytosis, and neurotransmission. Annu. Rev. Physiol. 75, 393–422 (2013).
25. Taraska, J.W., Perrais, D., Ohara-Imaizumi, M., Nagamatsu, S. & Almers, W. Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. Proc. Natl. Acad. Sci. USA 100, 2070–2075 (2003).
26. Furnimura, Y. et al. Analysis of the adrenal gland is useful for evaluating pathology of the peripheral autonomic nervous system in Lewy body disease. J. Neuropathol. Exp. Neurol. 66, 354–362 (2007).
27. Turkka, J.T., Jujilävi, K.K., Lapinlampi, T.O. & Myllylä, V.V. Serum noradrenaline response to standing up in patients with Parkinson’s disease. Eur. Neurol. 25, 355–361 (1986).
28. Miesenböck, G., DeAngelis, D.A. & Rothman, J.E. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394, 192–195 (1998).
29. Dean, C. et al. Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. Nat. Neurosci. 12, 767–776 (2009).
30. Álés, E. et al. High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. Nat. Cell Biol. 1, 40–44 (1999).
31. Matsuda, N. et al. Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J. Neurosci. 29, 14185–14198 (2009).
32. Holz, R.W. Evidence that catecholamine transport into chromaffin vesicles is coupled to vesicle membrane potential. Proc. Natl. Acad. Sci. USA 75, 5190–5194 (1978).
33. Hu, G. et al. New fluorescent substrate enables quantitative and high-throughput examination of vesicular monoamine transporter 2 (VMAT2). ACS Chem. Biol. 8, 1947–1954 (2013).
34. Perrais, D., Kleppe, I.C., Taraska, J.W. & Almers, W. Recapture after exocytosis causes differential retention of protein in granules of bovine chromaffin cells. J. Physiol. (Lond.) 560, 413–428 (2004).
35. Fulop, T., Radabaugh, S. & Smith, C. Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. J. Neurosci. 25, 7324–7332 (2005).
36. Onoa, B., Li, H., Gagnon-Bartsch, J.A., Elias, L.A. & Edwards, R.H. Vesicular monoamine and glutamate transporters select distinct synaptic vesicle recycling pathways. J. Neurosci. 30, 7917–7927 (2010).
37. Chiang, H.C. et al. Post-fusion structural changes and their roles in exocytosis and endocytosis of dense-core vesicles. Nat. Commun. 5, 3356 (2014).
38. van de Bospoort, R. et al. Munc13 controls the location and efficiency of dense-core vesicle release in neurons. J. Cell Biol. 199, 883–891 (2012).
39. Asensio, C.S. et al. Self-assembly of VPS41 promotes sorting required for biogenesis of the regulated secretory pathway. Dev. Cell 27, 425–437 (2013).
40. Fortin, D.L. et al. Neural activity controls the synaptic accumulation of α-synuclein. J. Neurosci. 25, 10913–10921 (2005).
41. Unni, V.K. et al. In vivo imaging of α-synuclein in mouse cortex demonstrates stable expression and differential subcellular compartment mobility. PLoS One 5, e10589 (2010).
42. George, J.M., Jin, H., Woods, W.S. & Clayton, D.F. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. Neuron 15, 361–372 (1995).
43. Bartels, T., Choi, J.G. & Selkoe, D.J. α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature 477, 107–110 (2011).
44. Burré, J., Sharma, M. & Südhof, T.C. α-Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation. Proc. Natl. Acad. Sci. USA 111, E4274–E4283 (2014).
45. Wang, L. et al. α-synuclein multimers cluster synaptic vesicles and attenuate recycling. Curr. Biol. 24, 2319–2326 (2014).
46. Fortin, D.L. et al. Lipid rafts mediate the synaptic localization of α-synuclein. J. Neurosci. 24, 6715–6723 (2004).
47. Shi, L. et al. SNARE proteins: one to fuse and three to keep the nascent fusion pore open. Science 335, 1355–1359 (2012).
48. Braun, A.R. & Sachs, J.N. α-Synuclein reduces tension and increases undulations in simulations of small unilamellar vesicles. Biophys. J. 108, 1848–1851 (2015).
49. Nuscher, B. et al. α-Synuclein has a high affinity for packing defects in a bilayer membrane: a thermodynamics study. J. Biol. Chem. 279, 21966–21975 (2004).
50. DeWitt, D.C. & Rhoades, E. α-Synuclein can inhibit SNARE-mediated vesicle fusion through direct interactions with lipid bilayers. Biochemistry 52, 2385–2387 (2013).
ONLINE METHODS

Rodent strains. Synuclein triple knockout (TKO) mice were produced by crossing α/β-synuclein double KO mice (Jackson Laboratory, stock no. 006390) to a γ-synuclein KO line13 generously provided by L. Lustig (Columbia University). 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 Biochemicals22, the mouse monoclonal antibody to rodent α-synuclein (Syn-1) from BD Biosciences (610787)23, the goat polyclonal antibody to TOM20 (C-20) from Santa Cruz Biotechnology (sc-11021)24, the guinea pig polyclonal antibody to vesicular glutamate transporter 1 (VGLUT1) from EMD Millipore (AB9055)25 and the rabbit polyclonal antibodies to secretogranin II (K55101R) from Meridian Life Science26 and to actin (A2066) from Sigma27. 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, Iowa. The anti-rat antibody conjugated to Alexa Fluor 488 (A-12088), anti-mouse antibody conjugated to Alexa Fluor 488 (A-11029), anti-goat antibody conjugated to Alexa Fluor 594 (A-11058) and anti-rabbit antibody conjugated to Alexa Fluor 647 (A-11037) were all obtained from Thermo Fisher Scientific. The anti-mouse antibody conjugated to Cy5 (715-175-151) and anti-guinea pig antibody conjugated to Cy3 (706-165-148) were obtained from Jackson ImmunoResearch. The anti-mouse antibody conjugated to IRDye 800CW (925-32210) and anti-rabbit antibody conjugated to IRDye 680LT (925-68021) were obtained from LI-COR Biosciences.

Chromaffin cell culture. Adrenal chromaffin cells were isolated as previously described28. Briefly, whole adrenal glands were collected from 4- to 6-week-old mice and placed in ice-cold Ca2+-free and Mg2+-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 r.p.m. in a thermomixer (Eppendorf). Digested medullae were resuspended completely and the enzymatic reaction quenched with 10 volumes cold CMF-HBSS. Cells were pelleted at 300g 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 dropwise on glass chambers (LabTek) coated with poly-L-lysine and allowed to adhere for 45 min at 37 °C and 5% CO2, followed by addition of prewarmed lentiviral supernatant. Lentiviral transduction was performed overnight and medium replaced the next morning.

Neuronal culture and transfection. Primary neuronal cultures were prepared from P0 Sprague-Dawley rat pups and transfected by electroporation as previously described (Amaxa)31. Briefly, 0.8 µg pCAGGS vectors containing either NPY-pHuorin or BDNF-pHuorin were co-transfected with 0.1 µg synaptophysin-mCherry and either 0.1 µg pCAGGS or 0.1 µg pCAGGS-α-synuclein per 4 × 105 cells. Cultures were maintained in minimum essential medium (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 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 medium 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.

Lentivirus production. Low-passage HEK293T cells were seeded onto six-well plates and transfected overnight with a mixture of the third-generation lentiviral vector pHUMCS encoding the gene of interest, as well as accessory plasmids pREV, pSVSG 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 1,000g. 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 1:13 (H3C), 1:500 (15G7, Syn-1, C-20) or 1:1,000 (K55101R) in blocking buffer and incubated overnight at 4 °C. Fluorescent dye conjugated secondary antibodies were also diluted 1:500 in blocking buffer. For epifluorescence, cell staining was visualized using an upright fluorescence microscope (AxioScope; Zeiss) with a 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).

Cultured hippocampal neurons were fixed in PBS containing 4% formaldehyde and 4% sucrose for 20 min at room temperature, blocked and permeabilized in PBS containing 5% calf serum and 0.05% saponin. The fixed neurons were incubated in the same solution with monoclonal H3C anti-synuclein (1:13) and guinea pig anti-VGLUT1 antibodies (1:5,000), followed by secondary detection using Cy3- or Cy5-conjugated antibodies. Images of fluorescent cell staining were acquired by confocal laser scanning microscopy using a Zeiss LSM510 microscope.

Structured illumination microscopy (SIM). Isolated chromaffin cells were plated onto high-precision coverslips (Zeiss), fixed 3–5 d later and immunostained as described above. Samples were imaged on a Nikon Ti microscope equipped with a 100×, 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 SIM module analysis. Co-localization was quantified with the Coloc2 module in ImageJ.

Total internal reflection fluorescence (TIRF) microscopy. 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 d 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 Xion 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 CaCl2, 1 MgCl2 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 exocytotic events were identified manually in ImageJ software by placing 5 × 5 pixel regions of interest (ROIs) over the center of events and extracting the average intensity profiles using the Time Series Analyzer plug-in. An automated algorithm identified exactly the same events as well as several others that did not rise substantially (>20%) above the background or that reflected the mobility of vesicles with unquenched residual fluorescence and were therefore excluded from the analysis (data not shown). The mean ROI intensity of the 20 preceding frames was used to define event onset, and subtracted as background. The onset of event decay was defined as the first frame dropping below an established higher level. Curve fitting, analysis of event parameters and all subsequent statistical analyses were performed using GraphPad Prism Version 6.05. The representative traces shown in Figures 1c and 2d were normalized to peak ΔF.

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 overlapping events or any latency shorter than the temporal resolution of the experiment (1 frame, 50 ms) were excluded from the analysis. To determine whether reacidification contributes to the decay of BDNF-pHuorin 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 contained 0.6 µM bafilomycin.

© 2017 Nature America, Inc., part of Springer Nature. All rights reserved.
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 5 × 5 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) punctuate fluorescence despite the acid challenge.

For dual-color TIRF imaging experiments, adrenal chromaffin cells transduced with lentivirus encoding BDNF-pHluorin were incubated in basal imaging buffer containing 20 μM FFIN206 (ref. 33) for 1 h in 5% CO2 at 37 °C, washed twice, stimulated and imaged immediately as described above. Dual-color movies were acquired by alternating 30 ms excitation with 405 nm and 488 nm laser lines in triggered acquisition mode (NIS-Elements software, Nikon).

To compare the extent of H3C colocalization with SgII in cells expressing α-synuclein point mutants as well as β and γ isotypes, images were acquired on an inverted TIRF microscope (Ti-E; Nikon) using a 100× Plan Apo 1.49 NA oil objective (Nikon) and an Andor iXon Ultra 897 high speed EMCCD camera (Oxford Instruments). The Colocal2 plugin (ImageJ) was then used to determine the Mander's overlap coefficient for SgII-positive puncta that colabeled with H3C.

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 μM in imaging buffer. WT 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. F0 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 ΔF/ΔF0.

Live cell imaging of neurons. Transfected neurons were imaged at 14–20 DIV at room temperature (24 °C) as previously described11,29. Neurons were imaged in standard Tyrode's solution (in mM, 119 NaCl, 2.5 KCl, 2 MgCl2, 2 CaCl2, 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 exciplic 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 63×, 1.2 N.A. water objective. The representative traces shown in Figures 3b,e and 4a were normalized to peak ΔF.

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. Exocytotic 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 (< 5 s, τ > 5 s or no decay) and compared by the chi-squared test.

Quantitative immunoblotting. Primary rat hippocampal neurons were transfected by electroporation with either pCAGGS or pCAGGS-α-synuclein, plus BDNF-pHluorin and synaptophysin-mCherry as described above. After culturing for 14 d, cells were solubilized in PBS containing 1% Triton X-100, 1 mM EGTA, 1 mM MgCl2 and protease inhibitors (Roche Complete). Lysates were sedimented at 1,300g to remove nuclei, and 6 μg protein was separated by PAGE. After transfer to nitrocellulose, membranes were immunoblotted for α-synuclein (Syn-1; 1:500) and actin as a loading control (A2066; 1:500) followed by detection by IRDye-conjugated secondary antibodies. Immunoreactivity was detected by fluorescence scanner (LI-COR Biosciences) and quantified in ImageJ.

Statistical analysis. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications11,29,31. At least two independent cultures were used for each experiment. Cumulative frequency distributions were compared using the Kolmogorov-Smirnov test and normally distributed data by two-tailed one-way ANOVA with Tukey’s post hoc test for multiple comparisons. In box-and-whisker plots, the boxes represent the middle two quartiles, the whiskers 10% and 90% of the events, the line the median and the + the mean. Event proportions were compared by chi-squared test. The organization of the experiment was not randomized. Data collection and analysis were not performed blind to the conditions of the experiments, except for the NPY-pHluorin data from neurons, of which >80% were analyzed blind to genotypy.

A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

51. Ninkina, N. et al. Neurons expressing the highest levels of gamma-synuclein are unaffected by targeted inactivation of the gene. Mol. Cell. Biol. 23, 8233–8245 (2003).
52. Feller, L. et al. Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia. Glia 61, 349–360 (2013).
53. Baki, S., Tripathi, A.K. & Singh, N. Alpha-synuclein modulates retinal iron homeostasis by facilitating the uptake of transferrin-bound iron: implications for visual manifestations of Parkinson’s disease. Free Radic. Biol. Med. 97, 292–306 (2016).
54. Langore, F. et al. Metformin protects skeletal muscle from cardiotoxic induction of degeneration. PLoS One 9, e114018 (2014).
55. Turner, T.N. et al. Loss of δ-catenin function in severe autism. Nature **520**, 51–56 (2015).
56. Hao, Z. et al. Impaired maturation of large dense-core vesicles in muted-deficient adrenal chromaffin cells. J. Cell Sci. **128**, 1365–1374 (2015).
57. Zhang, S., Wang, P., Ren, L., Hu, C. & Bi, J. Protective effect of melatonin on soluble Aβ1–42-induced memory impairment, astrogliosis, and synaptic dysfunction via the Musashi1/Notch1/Hes1 signaling pathway in the rat hippocampus. Alzheimers Res. Ther. **8**, 40 (2016).
58. Sirkis, D.W., Edwards, R.H. & Asensio, C.S. Widespread dysregulation of peptide hormone release in mice lacking adaptor protein AP-3. PLoS Genet. **9**, e1003812 (2013).
59. Hua, Z. et al. v-SNARE composition distinguishes synaptic vesicle pools. Neuron **71**, 474–487 (2011).
60. Jullié, D., Choquet, D. & Perrais, D. Recycling endosomes undergo rapid closure of a fusion pore on exocytosis in neuronal dendrites. J. Neurosci. **34**, 11106–11118 (2014).