Title: Leucine-rich repeat kinase 2 impairs the release sites of Parkinson's disease vulnerable dopamine axons

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Abstract

The end-stage pathology of Parkinson's disease (PD) involves the loss of dopamine-producing neurons in the substantia nigra pars compacta (SNc). However, synaptic deregulation of these neurons begins much earlier. Understanding the mechanisms behind synaptic deficits is crucial for early therapeutic intervention, yet these remain largely unknown. In the SNc, different dopamine neuron subtypes show varying susceptibility patterns to PD, complicating our understanding. This study uses intersectional genetic mouse models to uncover synaptic perturbations in vulnerable dopamine neurons, focusing on the LRRK2 kinase, a protein closely linked to PD. Through a combination of immunofluorescence and advanced proximity labeling methods, we found higher LRRK2 expression in the most vulnerable dopamine neuron subclusters. High-resolution imaging revealed that pathogenic LRRK2 disrupts release sites in vulnerable dopamine axons, leading to decreased in vivo evoked striatal dopamine release in mice with LRRK2 mutations. Proteomic and biochemical analyses indicate that mutant LRRK2 increases the phosphorylation of RAB3 proteins, reducing their interactions with RIM1/2 effector proteins and impacting their synaptic functions. Overall, this research highlights the cell-autonomous dysfunctions caused by mutant LRRK2 in the neurons that are primarily affected by the disease. It also provides a framework for therapeutic strategies for early nigrostriatal synaptic deficits in PD.

Introduction

Parkinson's disease (PD) is marked by the progressive degeneration of dopamine neurons in the substantia nigra pars compacta (SNc).[1,2] These neurons are particularly vulnerable due to their unique intrinsic properties, such as large numbers of synaptic sites, massive arborization, and high bioenergetic burden.[3] Notably, in PD, there is a selective loss of dopamine neurons within the SNc, with greater ventral than dorsal loss.[4–6] This suggests subtype-specific dysfunction among SNc dopamine neurons. The difficulty in defining and isolating these subtypes has hindered the direct study of vulnerable neurons. To address this, single-cell profiling studies have identified a ventrally biased *Sox6*+/Aldehyde Dehydrogenase 1A1 (*Aldh1a1*+) dopamine neuron subcluster in humans and mice.[7–9] The location of the cell bodies (ventral SNc) and axonal projections (dorsal striatum) of Aldh1a1+ cells corresponds to the patterns of neuron and projection loss observed in PD.[10–12] Additionally, recent research identified Annexin 1 (*Anxa1*) as a subset of *Aldh1a1*+ dopamine neurons occupying the ventral-most SNc. These *Anxa1*+ neurons project densely to the most dorsal striatum and serve as markers of vulnerability in PD mouse models,[13–15] and in the brains of human PD patients.[16] This molecular classification has enabled the development of new intersectional genetic models [12,13,17] to study these otherwise intermingled SNc dopamine neuron populations, ultimately enhancing our understanding of PD mechanisms.

Deficits in dopamine release and synaptic dysfunction are well-documented hallmarks of early PD. Neuropathological comparison of dopaminergic axon versus cell body loss in PD patients suggests that axons may be the initial site of dysfunction and degeneration.[18–20] Impaired nigrostriatal dopamine transmission often precedes noticeable dopamine neuron loss—a convergent feature in human and numerous preclinical rodent models of PD (comprehensively reviewed in[21]). This suggests that synaptic dysregulation in dopamine neurons is one of the earliest manifestations in the context of diverse PD factors. Additionally, the presynaptic region is a known site of action for gene products associated with PD (e.g., auxilin, synaptojanin, α-synuclein).[22–25] Despite these observations, the field lacks a unified framework for early dopaminergic synaptic impairments and their contribution to subsequent manifestations of PD, in part due to the limited understanding of the precise mechanisms underlying these deficits.

Mutations in the *LRRK2* gene are among the most common genetic causes of PD, reaching up to 30% in specific ethnic groups.[26–28] The most prevalent pathogenic point mutation, G2019S, leads to a hyperactive LRRK2 kinase. Increased kinase activity is also observed in idiopathic PD,[29] suggesting that aberrant LRRK2 kinase activity is a broad pathophysiological mechanism for this disorder. Currently, the clinical development of LRRK2 kinase inhibitors is underway, highlighting their potential as a viable therapeutic option for PD.[30,31] However, despite its clinical relevance, the mechanism by which increased LRRK2 kinase leads to cellular dysfunction and ultimately results in the loss of dopamine neurons remains a critical knowledge gap. Several rodent models were developed to investigate the dysfunctions caused by LRRK2 mutations.[32,33] While these models typically do not show loss of dopamine neurons[34] in the absence of secondary hits, they do exhibit deficits in striatal dopamine transmission,[35–38] suggesting that mutant LRRK2 affects the nigrostriatal synapse. LRRK2 protein is expressed in dopaminergic neurons in the SNc[39], and deficits in striatal dopamine signaling are also observed in mouse models that express the pathogenic LRRK2 variant specifically within dopamine

neurons.[40,41] This indicates a cell-autonomous deregulation of dopamine release in these neurons. However, research on LRRK2 function in dopamine neurons is limited, and to date, no studies have examined the effect of LRRK2 mutations on the dopamine neuron subpopulations that are most vulnerable in disease.

Previous research on non-neuronal cells identified RAB proteins as robust downstream targets of LRRK2 kinase activity. The phosphorylation of RAB proteins by LRRK2 disrupts their normal function in intracellular membrane trafficking, resulting in cellular dysfunction.[42,43] Due to various technical challenges, it remains unclear whether the aberrant phosphorylation of RAB proteins by LRRK2 occurs specifically in the most vulnerable dopamine neurons and how this relates to dopamine release deficits.

Using a combination of advanced intersectional genetic strategies, cell-type-specific proteomics methods, sophisticated biochemical approaches, super-resolution microscopy, and a platform to access in vivo dopamine release, we describe a novel pathophysiological role for LRRK2 in the synapses of the most vulnerable ventral tier SNc neurons. Our study provides high-resolution insights into the underlying mechanisms that lead to functional disruptions in dopamine signaling in a well-established preclinical LRRK2 model for prodromal/early PD. Our findings enhance the understanding of LRRK2 dysfunction and highlight biologically and pathologically relevant cellular changes in the neurons that are primarily affected in the disease.

Results

LRRK2 is expressed in dopamine neurons and is enriched in the vulnerable subpopulation of neurons

Single-cell profiling studies and research on both postmortem PD tissues and preclinical mouse models demonstrated that Sox6/Aldh1a1 markers molecularly define the vulnerable ventral tier dopamine neurons,[11,12] while Calbindin1 (Calb1) marks the resilient dorsal tier neurons (Figure 1A).[1,44] These distinct dopamine neuronal subtypes differ in their molecular profiling; they also exhibit unique projection patterns and functional responses.[10,13,17] Therefore, studying these neurons in a subtype-specific manner can yield important insights into cell-intrinsic mechanisms of vulnerability in PD. We first addressed whether LRRK2 is expressed in dopamine neurons, as this is a prerequisite for any cell-autonomous effects. We performed immunolabeling on mouse brain sections and confirmed LRRK2 expression in SNc dopamine neurons (Figure 1B). No LRRK2 signal was observed by either Western blot (WB) or immunolabeling experiments in *Lrrk2*^{KO} tissue, confirming the specificity of antibodies used (Supplementary Figure 1A, B).

As an orthogonal approach, we developed an independent methodology that focuses on proximity labeling of genetically labeled neuronal cell types in the mouse brain (Supplementary Figure 1C). We achieved specific APEX2 expression in these neurons by crossing the Cre-dependent APEX2 EGFP reporter mouse line[45] with DAT^{cre} mice. APEX2 is a soy peroxidase that rapidly biotinylates proximal proteins. [46]To selectively label proteins in dopamine neurons, acute brain slices of DAT^{Cre}; APEX2 EGFP were prepared, and rapid biotinylation was induced following published protocols optimized for the mouse brain.[45,47,48] Immunostaining with EGFP and tyrosine hydroxylase (TH) confirmed APEX2 expression in dopamine neurons and their axons, as shown in sections of the SNc and striatum, respectively (Supplementary Figure 1D). Following tissue lysis, we purified biotinylated dopamine neuron proteins and subjected them to mass spectrometry analysis (MS). Our analysis, using a publicly available APEX2 proteome of dopamine neurons database as reference, confirmed enrichment of dopamine neuron proteins (Supplementary Figure 1E)[48]. We then conducted streptavidin pulldowns to isolate biotinylated proteins from either dopamine neurons or their axons in the striatum, followed by WB. Control slices from APEX2 EGFP mice (Cre- control) were included to assess non-specific binding. Streptavidin–horseradish peroxidase (HRP) blotting in SNc and striatal lysates showed broad biotinylation in DAT^{Cre}; APEX2 EGFP samples but not in control samples, and TH immunoblotting further indicated labeling specificity (Supplementary Figure 1F, G). Using this refined methodology, we were able to independently confirm the presence of LRRK2 in SNc dopamine neurons, as well as the existence of a faint LRRK2 signal in dopamine axons (Figure 1C, Supplementary Figure 1H and I).

Notably, quantification of our immunolabeling experiments indicated that LRRK2 protein expression was higher in the most vulnerable dopamine neuron populations in the mouse brain, specifically those of the ventral tier SNc, marked by Aldh1a1 (Figure 1D). Additionally, human single-nucleus RNA sequencing datasets show that the *LRRK2* gene is enriched in specific midbrain dopamine neuron subtypes marked by *SOX6* (Figure 1E). These specific SOX6+ populations, including the ALDH1A1+ subcluster, are known to selectively degenerate in human PD.[49]

Altogether, our findings indicate that the LRRK2 protein is enriched in the most vulnerable populations of dopamine neurons in both mice and humans, supporting a cell-autonomous LRRK2 dysfunction in these neurons in PD.

LRRK2 phosphorylates RAB3 isoforms in vivo

We and others previously reported that knock-in mice expressing the pathogenic hyperactive LRRK2^{G2019S} mutation exhibit deficits in dopamine release within the striatum.[35,37] To investigate the underlying molecular mechanisms, we conducted quantitative phosphoproteomic Liquid Chromatography-Mass Spectrometry (LC-MS) studies on striatal synaptosomes from LRRK2^{G2019S} mice. Mice were treated with vehicle control or the potent and specific LRRK2 kinase inhibitor MLi-2.[50] We focused on this comparison, as pharmacological inhibition of the already hyperactive LRRK2^{G2019S} kinase increases the likelihood of identifying in vivo changes in phosphoregulation. First, we confirmed the in vivo effective target engagement in our experimental conditions by assessing the phosphorylation of RAB12 at Ser106, a well-established readout of LRRK2 kinase activity. [51] In extracts from LRRK2^{G2019S} mice, RAB12 phosphorylation was higher compared to wildtype, but it returned to wildtype levels upon MLi-2 treatment (Supplementary Figure 2A, B). We employed our previously described protocols for subcellular fractionation to isolate the striatal synaptosome fractions,[52] which were then subjected to MS for total and phosphoproteomic analyses (Figure 2A). Comparing vehicle to MLi-2 treated LRRK2^{G2019S} mice (unadjusted p value < 0.05 and $|\log2FC|>0.58$) revealed 289 significantly altered phosphopeptides (Figure 2B). We performed pathway enrichment analyses using gene ontology (GO) to annotate the differentially expressed phosphoproteins with at least one altered phosphopeptide. The top 15 enriched pathways highlighted key synaptic biological processes, including signal release from the synapse and neurotransmitter secretion (Figure 2C), as well as presynaptic membrane and axonal localizations (Supplementary Figure 2C). These findings suggest that increased LRRK2 kinase activity causes presynaptic alterations in phosphoproteins, potentially leading to reduced nigrostriatal dopamine release as observed in LRRK2^{G2019S} mice. In contrast, total protein analysis from the same samples revealed only 12 differentially expressed proteins (Supplementary Figure 2D).

One notable finding from our phosphoproteome analysis was the decreased phosphorylation at the T86 site of the RAB3 GTPase in LRRK2^{G2019S} mice treated with MLi-2, compared to the vehicle group (Figure 2B). Given that RAB3 proteins were previously identified as targets of the LRRK2 kinase in cell lines,[42,43] our phosphoproteomic results suggest that LRRK2 may phosphorylate RAB3 proteins in the mouse brain. There are four RAB3 proteins—RAB3A, RAB3B, RAB3C, and RAB3D—that play a role in the synaptic vesicle cycle at the presynaptic site.[53,54] To examine the relative expression of these four proteins in dopamine neurons, we first analyzed a dataset from mouse single-cell RNA sequencing studies,[9] which indicated that RAB3A and RAB3C had the highest expression levels in dopamine neurons (Supplementary Figure 2E). In addition, the analysis of a human dopamine neuron single-nucleus dataset revealed that RAB3B is predominantly present in resilient dopamine neuron subclusters,[49] rather than in the vulnerable ones, which show higher LRRK2 expression (Supplementary Figure 2F). Taken together, these findings suggest that the availability of LRRK2 kinase activity targets differs in vulnerable and resilient dopamine neuron subpopulations.

To evaluate the presence of RAB3A and RAB3C proteins in vulnerable dopamine axons, we labeled Anxa1+ ventral tier dopamine neurons through Cre-dependent viral delivery of APEX2 in the SNc. [47] This proteomics approach confirmed high levels of RAB3A and RAB3C in vulnerable dopamine axons (Supplementary Figures 2G and H). Next, using our APEX2-based biochemical approach, we confirmed the presence of RAB3A and RAB3C in dopamine neurons and their axons. We noted their apparent presence in dopamine axons (striatum), consistent with their presynaptic roles (Supplementary Figure 2I). The higher levels of synaptophysin in pulldowns from striatal as opposed to SNc extracts from the same mouse validated the compartment specificity of our method.

Considering these expression patterns, we focused on RAB3A and RAB3C proteins, examining their phosphorylation in SNc extracts of the mouse brain and how this is influenced by LRKK2 kinase activity. Due to the lack of specific antibodies for phosphorylated RAB3, we used phosTag gels to separate phosphorylated RABs from their non-phosphorylated counterparts by SDS-PAGE[42,55] and we blotted the membranes with RAB3 isoform-specific antibodies (Figure 2D-G). Given the relative homology of RAB3 isoforms, the isoform specificity of the antibodies was determined in extracts from cells transiently transfected with different RAB3 isoforms (Supplementary Figure 2J). The slower migrating RAB3A band (representing the phosphorylated species, closed circle, Figure 2D) was increased in the SNc of LRRK2^{G2019S} mice as compared to littermate controls. This increase returned to wild-type levels when the LRRK2^{G2019S}

mice were treated with MLi-2 (Figure 2D, E). Similarly, an increase in phosphorylated RAB3C protein was observed in the mutant LRRK2 compared to the wild type, which was reversed upon MLi-2 treatment (Figure 2F, G).
Overall, our findings indicate that the phosphorylation of RAB3A and RAB3C is aberrantly increased in the SNc of LRRK2^{G2019S} mice, and this depends on LRRK2 kinase activity.

Phosphorylation of RAB3A decreases its binding to the effector proteins RIM1 and RIM2 from mouse brain

RAB3 proteins play an important role in coordinating presynaptic vesicle trafficking and release.[56] They achieve this by binding to effector proteins such as RIM1/2.[54] Prior studies conducted in vitro and in cells[42,43,57] indicate that LRRK2-induced hyperphosphorylation of RAB proteins disrupts their interaction with regulatory and effector proteins, while promoting interactions with phospho-specific effectors of the RILP family through their conserved RH2 domains.

To investigate the interactome of phosphorylated (p-) versus unphosphorylated RAB3A in the mouse brain, we employed a biochemical strategy combined with MS (Figure 3A). Purified Flag-RAB3A was phosphorylated at T86 by MST3 kinase, which phosphorylates RAB proteins at the same site as LRRK2 and is easier to purify in milligram amounts for biochemical studies.[58,59] Phosphorylated Flag-RAB3A was purified using MonoQ chromatography, and stochiometric phosphorylation was confirmed by PhosTag gel analysis. Additionally, the site of phosphorylation was established as Thr86 through MS experiments (Supplementary Figure 3A-B). Recombinant p-Flag-RAB3A or unmodified Flag-RAB3A were then used as 'bait' for an MS affinity purification experiment using homogenized whole brain extracts from mice administered MLi-2 via subcutaneous injection (30 mg/kg) 2 hours prior to termination (to induce dephosphorylation of endogenous RAB3 phosphorylation). Proteins that interacted with p-RAB3A or RAB3A were detected and quantified using a data-independent acquisition (DIA) approach. This analysis revealed a total of 3,480 proteins, with 69 proteins preferentially interacting with RAB3A compared to p-RAB3A, while another set of 16 proteins exhibited a higher interaction with p-RAB3A as compared to RAB3A (p-value<0.05, |log2FC|=1) (Figure 3D). These data can be further explored using the interactive CURTAIN volcano plot[60] using the weblink provided in the figure legend. The RAB3A effector proteins RIM1 and RIM2 showed a marked preference for binding to unphosphorylated RAB3A (Figure 3D, E). This observation aligns with the finding that LRRK2-mediated phosphorylation of other RAB proteins, which occurs within the effector binding motif, induces steric clashes that impair their interactions with respective effector proteins. RIM1 and RIM2 are essential components of the presynaptic active zone, which serves as the structural and functional platform for dopamine release (Figure 4A).[61]A precise arrangement of active zone proteins is critical for effective neurotransmission. Key active zone proteins, such as ERC1/2 (ELKs) and UNC13 (MUNC13), which are known to bind to RIM1 and RIM2, also exhibited differential binding to unphosphorylated RAB3A (Figure 3D), likely reflecting the decreased interaction between RIM1, RIM2, and p-RAB3A. Conversely, we found that p-RAB3A preferentially associated with RILPL1 and RILPL2 (Figure 3D,F), known phospho-specific effectors that recognize phosphorylated Rabs and have been linked to the regulation of ciliogenesis in neuronal cells.[42,62,63]
These data indicate that increased phosphorylation of RAB3A by LRRK2 disrupts its interaction with the effector RIM1/2 as determined from brain extracts, suggesting a potential regulatory mechanism by which RAB3 phosphorylation may impact neurotransmission.

LRRK2 alters the composition and organization of active zone release sites in dopaminergic striatal synaptosomes

Dopaminergic neuronal release sites have unique characteristics, as only about 25–30% of axonal varicosities contain active zones that support transmitter release.[64–66] RIM1/2 proteins are important scaffolding molecules that organize these release sites (Figure 4B), and a dopamine neuron-specific knockout of RIM1/2 leads to the structural disorganization of active zones and almost completely abolishes evoked dopamine release in the nigrostriatal pathway.[64,67] Our phosphoproteomic analysis revealed that several pathways involved in the organization of presynaptic release sites are altered in response to changes in LRRK2 kinase activity (Figure 2). Furthermore, we observed a decrease in the interaction of phosphorylated RAB3A with various active zone proteins from mouse brain (Figure 3). Therefore, we next examined whether alterations in the number and/or organization of active zones may form the cellular basis for the observed deficits in dopamine release in LRRK2^{G2019S} mice. Striatal synaptosomes were prepared from LRRK2^{WT} and LRRK2^{G2019S} mice (Supplementary Figure 4A, B), stained using antibodies against TH, the vesicular SNARE protein synaptobrevin-2 (VAMP2), and the active zone marker bassoon, and imaged using confocal microscopy (Figure 4C, Supplementary Figure 4C). The LRRK2^{G2019S} mutation did not change the percentage of TH+ synaptosomes that contained VAMP2+ clusters (Supplementary Figure 4D). However, we found a significant decrease in the percentage of

TH+/VAMP2+ synaptosomes that contained the active zone marker bassoon (Figure 4D). In addition, the remaining active zone clusters in LRRK2^{G2019S} TH+/VAMP2+ synaptosomes had an increased average area of bassoon staining when compared to wildtype (Figure 4E), which was not observed in TH-/VAMP2+ synaptosomes (Supplemental Figure 4E).

To determine whether LRRK2 specifically impacts dopaminergic active zone sites rather than synaptic integrity in the striatum, we analyzed the levels of several synaptic markers in striatal synaptosomes. We observed comparable levels of synaptophysin, VAMP2, synaptosomal-associated protein 25 (SNAP25), and the dopamine transporter (DAT) in LRRK2^{G2019S} as compared to wild-type synaptosomal extracts (Figure 4F-H, Supplementary Figure 4F-L). These data are in line with previous reports[34] and indicate that LRRK2 does not affect the number of synaptic vesicles. Similarly, the levels of RAB3 proteins or GDP dissociation inhibitor 1 and 2, which regulate RAB3 function[68] remained unchanged across genotypes (Supplementary Figure 4F-L). However, RIM1 levels were decreased in the LRRK2^{G2019S} synaptosome fraction compared to controls (Figure 4I and Supplementary Figure 4F). In contrast, RIM1 levels in other striatal fractions or SNc lysates did not change (Figure 4J-L), suggesting that LRRK2 specifically affects the synaptic targeting of RIM proteins. Since RIM1 and Bassoon co-cluster to form the active zones,[64] the reduction in synaptic RIM1 aligns with our imaging analysis showing fewer bassoon+ clusters in TH+/VAMP2+ synaptosomes (Figure 4D). Overall, these findings suggest that dopamine axons in LRRK2^{G2019S} mice have fewer varicosities that contain active zones, and that the remaining active zones are disorganized.

LRRK2 disrupts the number and composition of active zone release sites in vulnerable dopamine axons

Since vulnerable dopamine neurons show higher LRRK2 expression (Figure 1), we wondered whether active zone changes may be prominent in vulnerable Aldh1a1+ as compared to resilient Aldh1a1- dopaminergic axons in LRRK2^{G2019S} mice. To accomplish this, we used a previously described intersectional/subtractive approach[13,17] that allows for simultaneous labeling of Aldh1a1+ neurons with EYFP and Aldh1a1- neurons with mCherry, respectively (Figure 5A, Supplementary Figure 5A). Striatal brain sections were then stained with antibodies against bassoon to analyze the number and organization of active zone sites in Aldh1a1+ (EYFP+) or Aldh1a1- (mCherry+) axons from the same mouse (Figure 5B). Due to the high signal of the bassoon in the striatum, we employed structured illumination microscopy (SIM) to achieve improved resolution, followed by 3D reconstruction analysis (Figure 5C). We then used this imaging and analysis pipeline, combined with the intersectional genetic approach, to investigate active zone sites within vulnerable versus resilient dopamine axons. We observed a decrease in the density of bassoon+ clusters in vulnerable Aldh1a1+ but not resilient Aldh1a1- axons of LRRK2^{G2019S}, compared with LRRK2^{WT} mice (Figure 5D). Furthermore, we noted an increase in the volume of the remaining bassoon clusters in Aldh1a1+ but not Aldh1a1- dopamine axons in mutant LRRK2 compared to wild-type control mice (Figure 5E). We confirmed these findings using the Anxa1^{Cre} mice (Figure 5F-H). To further confirm that we are measuring bassoon clusters within the dopamine axons, we conducted the following analysis. First, we assessed the density of bassoon clusters associated with Anxa1+ dopaminergic axons. Next, we rotated the bassoon signal image by 180 degrees, while keeping the GFP channel unchanged and reassessed the density of the bassoon clusters. If we had primarily measured bassoon clusters located outside of the axons, their density would not have changed with rotation. Instead, we observed a decrease in density (Supplementary Figure 5B), which suggests that our analysis mainly captures bassoon clusters within dopaminergic axons.

Since the observed active zone changes may be due to the loss of TH fibers or alterations in dopamine vesicle clustering, we employed confocal imaging and 3D reconstructions to assess TH+ fibers in the dorsolateral striatum. We found no difference in the volume (Figure 5I, J, Supplementary Figure 5C), area, and intensity of TH signal in immunopositive ALDH1A1 fibers (Supplementary Figure 5D, E) in the dorsal striatum. We also observed no change in the area or intensity of vesicular monoamine transporter 2 (VMAT2) vesicle clusters in LRRK2^{G2019S} mice compared with controls (Supplementary Figure 5D, F). These results, along with the unchanged percentage of TH+ striatal synaptosomes and unchanged levels of synaptic markers (Supplementary Figure 4), suggest that there is no significant dopaminergic axonal loss in LRRK2^{G2019S} mice at least at 5-6 months of age. Instead, the LRRK2^{G2019S} mutation disrupts the number and composition of active zones that are essential for dopamine release, particularly in vulnerable dopaminergic axons. This is in line with the reported decrease in dopamine release in the dorsolateral striatum,[35] which is the main projection site of these vulnerable neurons. [13]

To identify the molecular players underlying these structural alterations in mutant LRRK2 dopamine axons, we used a Cre-dependent virus to drive APEX2 proximity labeling within genetically targeted Anxa1 neurons in LRRK2^{WT}

and LRRK2^{G2019S} mouse brains—with or without MLi-2—and coupled this with MS.[48] Cre-negative mice injected with Cre virus controlled for non-specific binding (Supplementary Figure 6A, B). We confirmed that our APEX2 dataset is enriched for dopamine neurons (compared to the publicly available APEX2 DA neuron proteome database).[48] We validated the compartment specificity of our approach by comparing the enrichment of established somatodendritic versus axonal markers between the SNc and the striatum (Supplementary Figure 6C, D). Overall, the number of total proteins altered across different comparisons was limited. 2% and 3% of all identified proteins in our dataset showed significant changes when comparing LRRK2^{G2019S} with LRRK2^{WT} and LRRK2^{G2019S} MLi-2-treated versus vehicle control, respectively (p-value < 0.05, $|Log2FC| > 0.58$). This pattern is consistent with the limited number of proteins that were altered in a similar comparison in our striatal synaptosome-based experiments (Supplementary Figure 2D). Notably, among the significantly altered proteins were known components of active zones and presynaptic function (Supplementary Figure 6E), consistent with the observed structural changes (Figure 5G, H). In either case, the magnitude of effects in proteomic methods highlights that the primary impact of LRRK2 mutations in dopamine axons occurs through phosphoregulation, consistent with LRRK2 function as a kinase.

In vivo evoked dopamine release is reduced in LRRK2^{G2019S} compared to control mice

We next asked whether the changes in synaptic release machinery described here for Anxa1+ dopamine neurons in LRRK2^{G2019S} mice lead to any detectable changes in dopamine release in vivo. In LRRK2^{WT} or LRRK2^{G2019S} mice, we expressed the optogenetic actuator CHRmine[69] in Anxa1+ dopamine neurons in the SNc and expressed the dopamine sensor GRAB-DA3m[70] widely and non-specifically across the dorsal striatum (Figure 6A, Supplementary Figure 7A). We used fiber photometry to record the dorsal striatum dopamine release evoked by optogenetic activation of Anxa1+ dopamine neuron cell bodies in awake mice that were head fixed on a treadmill (Fig. 6B).[13,71] We found that evoked dopamine release was significantly reduced in LRRK2^{G2019S} compared to LRRK2^{WT} mice (Figure 6C, D). Specifically, 4 out of 5 individual LRRK2^{G2019S} mice showed a statistically significant reduction in dopamine release compared to the control group (Figure 6D). This reduction was observed across a range of optogenetic stimulation powers (Supplementary Figure 7B, C). Taken together, the presynaptic anatomical alterations in LRRK2^{G2019S} vulnerable dopamine release sites (Figure 5) functionally manifest as decreased striatal dopamine release. These findings represent the first evidence of in vivo dopamine signaling deficits in the most vulnerable neuronal populations n LRRK2^{G2019S} mice.

Discussion

This study offers novel and important insights into the role of LRRK2 in dopamine neurons in PD. We demonstrate that mutant LRRK2 disrupts the presynaptic architecture (active zone release sites) essential for efficient neurotransmitter release in dopamine axons,[64] leading to reduced in vivo striatal dopamine release. These deficits are specific to defined populations of dopamine neurons vulnerable to PD. Our targeted approach builds on our previous findings of non-subtype-specific ex vivo dopamine release deficits[35] in mutant LRRK2 mice. To our knowledge, this is the first study to date to demonstrate cell-autonomous LRRK2-mediated deficits, specifically within the most vulnerable dopamine neuron subpopulations.

Recent advances in single-cell transcriptomics, combined with studies in postmortem human PD and preclinical mouse models, revealed that the Aldh1a1+/Anxa1+ subpopulation is the most susceptible dopamine neuron subtype in PD.[11–13,16] Our data indicate that this ventral-biased subtype displays elevated expression of LRRK2 protein, further underscoring its unique properties relevant to PD pathophysiology. To further investigate the implications of these findings, we utilized targeted knock-in mice expressing the G2019S pathogenic mutation and implemented intersectional labeling to study vulnerable subsets of dopamine neurons in the SNc, focusing on the Aldh1a1+ and Anxa1+ subpopulation. Super-resolution imaging analysis uncovered a reduction in the number of active zones within the Aldh1a1+ neurons, while such a decrease was not observed in Aldh1a1- neurons. Moreover, the remaining active zones appeared disorganized in Aldh1a1+ neurons. These findings, which were also confirmed in Anxa1+ neurons (a subset of Aldh1a1+ clusters), illustrate a striking example of cell-type-specific effects of LRRK2 mutations in the dorsal striatum, particularly within the most vulnerable populations of dopamine neurons.

Multiple studies highlight notable distinctions in the release sites of dopamine neurons compared to other synapses. Only 25-30% of dopamine neuron varicosities contain active zones, which limits the availability of presynaptic platforms for dopamine release.[64,66] This observation aligns with functional studies that approximately 20% of dopamine varicosities

are capable of releasing dopamine.[65] Consequently, the precise regulation of these sparse release sites becomes increasingly crucial for dopaminergic synapses. Dopamine synapses also show a higher dependence on RIM1/2 proteins as compared to non-dopaminergic synapses,[64] which is likely less functionally redundant with other synaptic proteins, such as those reported in hippocampal synapses.[72,73] Together, these observations suggest that the active zone-like structures in dopamine neurons are molecularly and functionally specialized. Of note, similar, albeit more pronounced, active zone alterations as those observed here for LRRK2^{G2019S} were reported in conditional dopamine neuron-specific RIM1/2 knockout models,[64] suggesting that LRRK2 may directly or indirectly modulate RIM-related roles to influence the structural and functional integrity of vulnerable dopamine neuron release sites.

Given that increased kinase activity is the accepted pathogenic mechanism for mutant LRRK2 effects, our findings in mice and humans showing higher LRRK2 levels in vulnerable dopamine neurons suggest that the aberrant phosphorylation of downstream targets in these neuronal populations may contribute to presynaptic dysfunctions. To investigate the molecular mechanisms by which LRRK2 kinase activity mediates presynaptic defects in dopamine neurons, we performed a phosphoproteomics screen using striatal synaptosomes from LRRK2^{G2019S} mice with or without LRRK2 kinase inhibitor treatment. We identified RAB3 proteins as differentially phosphorylated presynaptic LRRK2 substrates, consistent with previous reports indicating that RAB proteins serve as robust LRRK2 substrates across various cell types.[42,43] RAB3 proteins are known to play a role in presynaptic vesicle trafficking events, and we found RAB3A and RAB3C to be amongst the most highly expressed proteins in our vulnerable dopamine neuron-specific proteome dataset. We demonstrated that LRRK2 phosphorylates these RAB3 proteins in synaptosomes from the SNc and striatum. Our unbiased MS data using mouse brain extracts indicate that increased RAB3 phosphorylation diminishes its interaction with RIM1 and RIM2 proteins. This is only observed with phosphorylated RAB3 but not with overexpressed phosphomimetic RAB3 constructs, used to probe consequences of altered phosphorylation across several studies (Supplementary Figure 8).[74] These findings, together with previous observations[42,63] underscore the importance of using phosphorylated RAB proteins (Figure 3) to elucidate the impact of phosphorylation on RAB-protein interactions.

The diminished interaction between RAB3 proteins and their effectors, RIM1 and RIM2, may influence the synaptic vesicle trafficking function of RAB3[54,56,75–77] and consequently impact the probability of synaptic vesicle release. Previous studies in Drosophila have shown that disruptions in RAB3 function result in a reduced number of active zones, while the remaining active zones exhibit an increased area.[78] These findings align with our observations in the axons of LRRK2^{G2019S}-vulnerable dopamine neurons, highlighting the effect of RAB3 phosphorylation on active zone composition. Recent studies in induced pluripotent stem cells (iPSC)-derived neurons further suggest that LRRK2-mediated RAB3 phosphorylation can impair the axonal trafficking of synaptic vesicle precursors,[74] which may lead to decreased targeting of RIM1 and RIM2 to axonal varicosities. In support of this notion, we observed reduced RIM1 levels in striatal synaptosomes but not in nigral extracts from mutant LRRK2 mice. Altogether, our studies suggest a mechanism whereby LRRK2 phosphorylation impairs RAB3-RIM interactions, leading to a reduction in the number and composition of active zones in vulnerable dopamine axons. Given the dependence of striatal dopamine release on RIM1/2 proteins, [64] these disruptions underlie the in vivo dopamine release deficits we observed specifically in the susceptible populations of the LRRK2^{G2019S} mice (Figure 6).

Dopamine release involves complex presynaptic processes tied to the synaptic vesicle cycle, which are closely associated with the function of various monogenic forms of PD.[79–83] While these presynaptic events are crucial for the function of all neurons, it is the vulnerable dopamine neurons in the SNc that are ultimately lost in PD. This suggests that the unique secretory biology of these neurons[61] may contribute to these presynaptic dysfunctions. Research on mice with mutant LRRK2 expressed specifically in dopamine neurons supports a cell-autonomous mechanism for release deficits. [40] Additionally, non-cell-autonomous factors from cholinergic interneurons or astrocytes in the dorsolateral striatum may also influence striatal dopamine release in the presence of mutant LRRK2. Even LRRK2 mediated functions in the strial projections neurons may affect the associated circuit. [52,84–86] Further investigations focused on specific cell types are necessary to clarify these possibilities. Nonetheless, our results emphasize that dopamine axons are critical sites for understanding disease pathogenesis related to one of the most common genetic risk factors associated with PD, and they establish a foundation for further research into the mechanisms underlying these disruptions.

To date, no therapies can stop or slow PD progression. Treatments often begin too late, after a significant loss of nigrostriatal dopamine function (50–70%) has already occurred. This emphasizes the urgent need for therapies that address early dopaminergic dysfunctions. To effectively implement strategies that could alter the course of the disease, it is crucial

to comprehensively understand the underlying and possibly reversible mechanisms leading to the early dysfunction of dopamine neurons in PD. Although the direct link between dopamine neuron loss and presynaptic deficits is not fully understood, compelling evidence from both preclinical models and human studies suggests that early presynaptic deficits play a significant role in disease progression. These studies reveal that axonal dysfunctions and impaired striatal dopamine transmission occur before the actual loss of dopamine neurons in numerous preclinical models. [21]Brain imaging research in non-manifesting carriers of LRRK2 mutations and individuals at the prodromal stage of sporadic PD has identified presynaptic deficits and dopamine release defects before the appearance of motor symptoms.[87,88] Collectively, these findings suggest that gaining a deeper understanding of synaptic dysfunctions of dopamine neurons may present a window of opportunity for therapeutic interventions.

Our study also has significant translational implications, particularly as several LRRK2 kinase inhibition strategies are currently under clinical development for both familial and sporadic PD. Future studies should focus on investigating the reversibility of these early presynaptic events and dopamine release deficits in vulnerable dopamine axons upon LRRK2 kinase inhibitor treatment in preclinical mouse models.

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Authors contributions

Conceptualization, C.C., L.P.; scientific input, E.G., Y.K., D.A., A.K., D.R.A., D.A.D., S.H., and R.A.; methodology, C.C., E.H.G.T., E.N., M.J., O.R.M., H.S., Y.N., A.S., V.P., K.Q., C.N.; imaging, proximity labeling methods, C.C. Y.K., intersectional genetic strategies development, O.A.M.R, R.A.; in vivo evoked dopamine release, Q.H., K.Q., D.A.D.; biochemical methods, C.C., Y.N., S.H., in vitro phosphorylation of RAB3 protein, E.N., and A.K., mass spectrometry, M.J., and D.A.; data curation, formal analysis, C.C., Q.H., G.T., M.J., H.S., K.Q; writing, review and editing, all authors; supervision, L.P.

Data sharing

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository, with dataset identifiers PXD065003, PXD065006, PXD0651255. Raw tubular data of all experiments, all images, and codes used in this study are available through the open-access option on the Zenodo repository (https://doi.org/10.5281/10.5281/zenodo.15635558) or GitHub. The protocols used in this study were uploaded to protocols.io, and their DOI can be found in the key resources table.

Declaration of interests

The authors declare no competing interests.

Materials and methods

1. Animals

All experiments complied with the guidelines set by the National Institutes of Health and were reviewed by the Northwestern Animal Care and Use Committee. Adult male and female mice, aged 3 to 7 months, were used in all experiments. Structural studies were conducted with mice aged 3-5 months, while analyses involving TH fibers were performed on mice aged 5-6 months. The in vivo dopamine release experiments included mice aged 6-7 months. The mice were group-housed on a standard 12-hour light/dark cycle and provided with standard feed. Littermates were randomly assigned to experimental procedures. Researcher blinding was implemented across experiments.

C57BL/6 mice (JAX:000664), homozygous KI mice expressing the pathogenic mutation LRRK2^{G2019S} (JAX:030961), and homozygous *Lrrk2*^{KO} mice (JAX:016121) were purchased from Jackson Laboratory. The Aldh1a1i^{Cre} and Anxa1i^{Cre} lines were generated by the Transgenic and Targeted Mutagenesis Laboratory at Northwestern University and were characterized in a previous study;[13] these lines were maintained in a heterozygous state. The DAT^{Flpo} line was generated by knocking in Flpo to the 3' end of Dat coding sequences, following a P2A peptide, and will be described in detail in an upcoming study.

The Cre-dependent APEX2 mouse line (DIO-APEX2.NES-P2A-EGFP) was characterized in a prior study. [45] These APEX2-EGFP mice, bred to homozygosity, were crossed with the Dat^{Cre} (JAX:006660) to achieve expression of APEX2 in dopamine neurons of the substantia nigra compacta (SNc). All mice were backcrossed for several generations and maintained on a C57BL/6J background with either the LRRK2^{WT} or LRRK2^{G2019S} allele. Standard genotyping primers are available on the Jackson Laboratory website or in referenced studies. Further details on the mouse strains can be found on the resources table.

2. Immunofluorescence and confocal imaging in brain sections

Mice were perfused with 50 ml of PBS, followed by 50 ml of 4% paraformaldehyde in PBS. The brains were dehydrated using 30% sucrose in PBS for 48 hours and then sectioned coronally at a thickness of 30 µm with a cryostat (Leica Biosystems, CM305). The slices were collected in PBS containing 0.1% sodium azide and stored at 4°C for subsequent immunohistochemical analysis.

Striatal sections were incubated in 5% goat serum with 0.2% Triton X-100/PBS for 2 hours. Antigen retrieval for LRRK2 staining was performed by incubating the sections in antigen-unmasking solution (Vector, H-3300) at 100°C for 30 minutes, followed by cooling to room temperature for an additional 30 minutes. The sections were then washed with PBS for 5 minutes.

Next, the sections were incubated overnight at 4°C in 5% goat serum with 0.2% Triton X-100 in PBS. Then, they were incubated for an additional 48 to 72 hours at 4°C with primary antibodies: anti-TH (1:1000, SYSY), anti-ALDH1A1 (1:300, Abcam), anti-LRRK2/Dardarin clone N241A/34 (1:200, NeuroMab), anti-GFP (1:1000, Invitrogen). After washing with PBS, the sections were incubated in the same buffer used for primary antibodies with secondary antibodies—Alexa Fluor™ 488, Alexa Fluor™ 568, and Alexa Fluor™ 647 (1:300, Invitrogen)—for 3 hours. Subsequently, all slices were washed with PBS and mounted using ProLong™ Diamond Antifade Mountant. Detailed catalog information and identification numbers can be found in the resources table.

Confocal images of the fixed 30 µm-thick striatal and midbrain sections were obtained using the Nikon A1R microscope. Fluorescence images were captured using a 20x objective for LRRK2 expression, TH area, and intensity (Supplementary Figure 6), and a 100x objective for Th fluorescence at a resolution of 1,024 x 1,024 pixels, with a constant laser power maintained across all genotypes for each channel.

3. Stereotactic injections and viruses

For the intersectional labeling of Aldh1a1 and Anxa1 populations, adult mice expressing Aldh1a1i^{Cre};DAT^{Flpo} or Anxa1i^{Cre} were anesthetized with 3% isoflurane, which was maintained at approximately 1.5% during the procedure. Pain management included subcutaneous injections of Rimadyl (3 mg/kg) at the beginning of the surgery, followed by a slow-release injection of Buprenex (0.25 mg/kg) after the surgery, and an additional dose of Rimadyl (3 mg/kg) the following day.

The surgery commenced with the exposure of the skull, using the bregma as the reference point. A 0.5-1 mm hole was drilled into the skull at the stereotactic coordinates of the substantia nigra pars compacta (SNc) (RC: -3.16 mm, ML: -1.50 mm, DV: -4.00 mm). A 0.5 µl Hamilton Neuros syringe was employed for precise delivery, with the needle left in place for 5 minutes after reaching the target depth. A volume of 0.3 µl of the viral vector was slowly injected into the SNc over 5 minutes, followed by another 5-minute wait to allow for proper viral diffusion within the brain parenchyma.

Two viral cocktail options were utilized: (i) a 1:1 mixture of AAV8 hSyn-Con/Fon-YFP (Addgene #55650, titer: $2.4x10^{13}$ GC/ml) and AAV8 Ef1a-Coff/Fon-mCherry (Addgene #137134, titer: $2.2x10^{13}$ GC/ml) delivered unilaterally for imaging experiments in Figure 5, or (ii) a pure injection of the AAV5-CAG-DIO-APEX2-NES (Addgene plasmid #79907, virus packaged by VectorBiolabs; titer: $3.8x10^{12}$ GC/ml), delivered bilaterally for APEX2 proximity labeling. The viral constructs are detailed in the resources table. Following the injection, the cranial wound was closed with staples. The mice were perfused 4 weeks post-injection, and their brains were isolated for subsequent analysis.

4. APEX2 biotinylation in acute brain slices and tissue dissections

Mice were anesthetized with ketamine and underwent transcardial perfusion with 10 ml of ice-cold cutting solution consisting of 110 mM choline, 2.5 mM KCl, 1.25 mM monosodium phosphate, 10 mM glucose, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, and 25 mM sodium bicarbonate, all saturated with 95% O_2 and 5% CO_2. The brains were quickly extracted and placed in ice-cold cutting solution, also saturated with 95% O_2 and 5% CO_2.

Coronal slices of 300 µm were prepared using a Leica VT1200S. After the slices were made in the cold cutting solution, they were transferred to a glass dish containing artificial cerebrospinal fluid (aCSF), which included 2.5 mM KCl, 10 mM glucose, 125.2 mM NaCl, 0.3 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 0.3 mM KH_2PO_4, supplemented with 0.5 mM biotin-phenol. The aCSF was continuously saturated with 95% O_2 and 5% CO_2. The slices were allowed to recover at room temperature for 60 minutes.

Following recovery, APEX2 labeling was initiated by adding 1 mM H_2O_2 to the aCSF at room temperature for 5 minutes. To quench the labeling, the slices were rapidly transferred to a separate glass dish containing quenching aCSF, which consisted of the aforementioned aCSF supplemented with 10 mM Trolox, 20 mM sodium ascorbate, and 10 mM sodium azide, for 5 minutes.

The slices were then rapidly dissected in ice-cold quenching aCSF. The tissues were flash-frozen in liquid nitrogen and stored at -80°C until further use in WB or proteomic experiments.

5. Protein extraction and streptavidin enrichment of APEX2 samples

Frozen APEX2-labeled tissues were homogenized on ice using a glass Dounce homogenizer with 30 strokes of both A and B pestles. The lysis was performed in 0.75 ml of ice-cold tissue lysis buffer, which contained 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 mM Trolox, 10 mM sodium ascorbate, 10 mM sodium azide, and Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After adding 39 µl of 10% SDS to achieve a final concentration of 0.5%, the lysates were rotated for 15 minutes at 4°C. The lysates were then clarified by centrifugation at 21,000 × g for 10 minutes at 4°C. The supernatants were transferred to a new prechilled Eppendorf tube for trichloroacetic acid (TCA) precipitation (for LC-MS) or stored at −80°C (for WB).

To precipitate proteins from the lysates, an equal volume of ice-cold 55% trichloroacetic acid (TCA) was added. The samples were incubated on ice for 30 minutes, followed by centrifugation at 21,000 × g for 10 minutes at 4°C. The protein pellets were then resuspended in 1 ml of acetone prechilled to -20°C and centrifuged again under the same conditions. This resuspension and centrifugation process was repeated three additional times using 1 ml of acetone prechilled to -20°C, for a total of four washes. After removing any residual acetone, the protein pellets were resuspended in Urea Dissolve Buffer, which contained 8 M urea, 1% sodium dodecyl sulfate (SDS), 100 mM sodium phosphate (pH 8), and 100 mM ammonium bicarbonate (NH_4HCO_3). The pellets were dissolved by sonication for 1 minute, followed by gentle agitation on an orbital shaker for 1 hour at room temperature. A small aliquot (5%) of the resuspended protein was flash-frozen and stored at -80°C. The samples were diluted with equal volumes of water to achieve a final concentration of 4 M urea and 0.5% SDS.

Streptavidin magnetic beads (Thermo Fisher #88817) were resuspended and washed three times in Urea Detergent Wash Buffer (4 M urea, 0.5% SDS, 100 mM sodium phosphate, pH 8) for 15 minutes at 4°C. After washing, the streptavidin beads were resuspended in ice-cold Urea Detergent Wash Buffer, and 50 µl containing 0.5 mg of beads was added to each sample. Proteins were incubated overnight with streptavidin beads on a rotary wheel at 4°C. After 14 to 18 hours, the unbound supernatant was discarded, and the beads were resuspended in 1 ml of Urea Detergent Wash Buffer and transferred to a new tube. The beads were washed three times for 10 minutes in 1 ml of Urea Detergent Wash Buffer at room temperature.

After the third wash, the beads were resuspended in 1 ml of Urea Wash Buffer (4 M urea, 100 mM sodium phosphate, pH 8) and transferred to a new tube. This washing step was repeated three times for 10 minutes in 1 ml of Urea Wash Buffer at room temperature. The beads were then resuspended in 200 µl of Urea Wash Buffer and transferred to a new tube. The buffer was removed using a magnetic stand, and the beads were flash-frozen and stored at -80°C.

6. Western blot analysis

***Brain extracts**:* Tissues were extracted and homogenized in 1x cell lysis buffer (Cell Signaling Technologies) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) using pellet pestles for 30 seconds. The protein concentration was determined using BCA Protein Assays (Thermo Scientific). Equal amounts of protein (30 µg of total tissue lysate) were separated by 4–12% NuPAGE Bis-Tris PAGE (Invitrogen) and transferred to membranes using the iBlot2 nitrocellulose membrane blotting system (Invitrogen) according to the manufacturer's protocol.

Synaptosomes: Subcellular fractionation of mouse striatum was performed as previously described.[64] Specifically, mouse striata were dissected and rapidly homogenized in 1ml iced-cold homogenizing buffer (4mM HEPES, 320 mM Sucrose, and Halt protease supplemented with phosphatase inhibitor cocktail (Thermo Fisher Scientific)) using a Teflon homogenizer (15 strokes). The homogenized brain extract was centrifuged at 1,000×g for 10 min at 4°C. The supernatant (S1) was collected and centrifuged at 12,500× g for 15 min at 4 °C. The supernatant (S2) was removed, and the pellet (P2) was re-homogenized in 1 ml homogenizing buffer with 10 strokes in a Teflon homogenizer. After the addition of 1 ml homogenizing buffer, the P2 homogenate was added to the top of a sucrose gradient made of 5ml 1.2M sucrose and 5ml 0.8M sucrose, and was centrifuged at 69,150×g (SW41) for 70 min at 4 °C. The synaptic plasma membrane fraction (SPM) in the interphase between two sucrose fractions was collected using a syringe and transferred to clean ultracentrifuge tubes, and the samples were centrifuged in a swinging bucket rotor (SW55) at 200,000×g for 30 min. The pellet was resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) or in 1x Phosphate-buffered saline (Sigma-Aldrich) for imaging. The protein concentration was determined using the BCA Protein Assay (Thermo Scientific), and 5 µg were separated by 4–12% NuPAGE Bis-Tris PAGE (Invitrogen) and transferred to membranes using the iBlot2 nitrocellulose membrane blotting system (Invitrogen) according to the manufacturer's protocol.

APEX2 based experiments: The protein concentration of frozen tissue lysates was determined using the Bradford assay (Thermo Scientific) and 10 µg APEX2 labeled tissue lysate were loaded into 4–12% NuPAGE Bis-Tris PAGE (Invitrogen) as input. Frozen streptavidin beads were resuspended in ~20 µl of 1× SDS sample buffer supplemented with 20 mM DTT and 2 mM biotin. Samples were boiled for 5 min at 95°C to elute biotinylated proteins. Beads were immediately placed onto a magnetic rack, and the entire sample was loaded into 4–12% NuPAGE Bis-Tris PAGE (Invitrogen) and transferred to membranes using the iBlot2 nitrocellulose membrane blotting system (Invitrogen) according to the manufacturer's protocol.

The membranes were incubated with primary antibodies specific for streptavidin-HRP (1:1000, Abcam), LRRK2/Dardarin clone N137/6 (1:1000, NeuroMab), LRRK2/Dardarin clone N241A/34 (1:1000, NeuroMab), TH (1:1000, Millipore), RAB3A (1:1000, Sigma), RAB3C (1:1000, Proteintech), synaptophysin (1:1000, Cell Signaling), VMAT2 (1:1000, R&D Systems), SNAP25 (1:1000, Proteintech), DAT (1:1000, Millipore), pS1292 LRRK2 (1:100, Abcam), pS935 LRRK2 (1:100, Abcam), RIM1 (1:1000, Proteintech), GDI1 (1:1000, Sigma), GDI2 (1:1000, Novus), phospho-RAB12 (1:1000, Abcam), total Rab12 (1:1000, Proteintech), and β-actin (1:3000, Sigma) overnight at 4°C. Following this, the membranes were incubated with secondary anti-mouse or anti-rabbit antibodies (1:2000, Thermo Scientific) for 1 hour at room temperature. Afterward, they were incubated with Immobilon ECL Ultra Western HRP Substrate (Millipore) for 3 minutes prior to image acquisition.

Chemiluminescent blots were imaged using the iBright imaging CL1500 system (Thermo Fisher Scientific). Further details on the chemicals and antibodies can be found in the resource table.

Phos Tag gels: SNc tissues extracted and homogenized in EDTA-free Lysis buffer (1% Triton X-100, 1% glycerol, 150 mM NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$) using pellet pestles (30 seconds). The lysis buffer was supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The protein concentration was determined using BCA Protein Assays (Thermo Scientific). A total of 10 µg of lysate were separated by 12.5% SuperSep™ Phos-tag™ (Wako). Gels were washed 3 times with 1x TG transfer buffer (Fisher BioReagents) supplemented with 10 % Methanol and 10 mM EDTA, with each wash lasting 10 minutes. After the washes, the gels were incubated with 1x TG transfer buffer with 10% Methanol for an additional 10 minutes and then transferred to Nitrocellulose membranes (Thermo Scientific) using the Mini Gel Tank and Blot Module (ThermoFisher) at 15V for 3 hours.

The membranes were incubated in 3% BSA in 1x Tris buffered saline (Sigma) supplemented with 0.1% Tween 20 for 1 hour, followed by incubation with primary antibodies specific for RAB3A (1:1000, Sigma) and RAB3C (1:1000, Proteintech) at 4 °C overnight, followed by probing with secondary anti-rabbit antibodies (1:2000, Thermo Scientific), as described above. Chemicals and antibodies are detailed in the resource table.

7. Striatal synaptosome preparation for imaging

Synaptosomes were diluted 5,000 times in 1x PBS, spun down at 4,000 rpm for 10 min at 4°C on #1.5 poly-d-lysine coated coverslips and fixed for 10 minutes in 4% PFA in PBS. Synaptosomes were blocked in 5% donkey serum in PBS for 1 hour and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes. Incubation with primary antibodies (VAMP2 1:200, R&D; TH 1:500, SYSY; bassoon 1:300, Enzo) was carried out in 0.1% Triton X-100, 1% donkey serum PBS overnight at 4°C. After three 5-min washes in 1X PBS, synaptosomes were incubated with the secondary antibodies (Donkey anti-Rabbit Alexa Fluor™ 488; Donkey anti-goat Alexa Fluor™ 647; Cy™3 AffiniPure™ Donkey Anti-Guinea Pig) in 0.1% Triton X-100, 1% donkey serum PBS for 90 min and mounted (ProLong™ Diamond Antifade Mountant) onto Superfrost™ slides. A single optical section for each area of synaptosomes was acquired with the confocal Nikon A1 laser scanning microscope system using a 100X 1.49NA objective.

8. Imaging analysis

LRRK2 signal in SNc sections: The protein signals for TH, ALDH1A1, and LRRK2 were measured using Imaris 10.1 software (Bitplane, Concord, USA). The surface rendering function was used to segment TH/ALDH1A1 cells. Background subtraction was enabled; the diameter for the largest sphere was set to 10 µm and automatically thresholded, with a smoothing surface set to 3 µm. Mean intensity of ALDH1A1 protein within the TH surface above 1.5 times the average TH protein channel mean intensity was considered positive. The LRRK2 intensity within TH-positive cells was measured automatically by the software.

Synaptosome analysis: Synaptosome imaging analysis was performed using FIJI software.[89] To define synaptosomes that are positive for a specific protein, background was subtracted from all channels using the "rolling ball" FIJI plugin with a radius of 33.0 pixels. Automatic segmentation was applied with the following parameters: default threshold, particle size ranging from 0.04 to 0.40, and circularity between 0.30 and 1.00. This process allowed the definition of regions of interest (ROIs) first in the VAMP2 channel. The average intensity of the signal was measured within each ROI, and only those ROIs that were positive for VAMP2 were retained. An ROI is classified as containing the target protein if the average fluorescence intensity of that protein within the ROI is more than twice the average intensity of all pixels in the image. Conversely, an ROI was considered negative for the target protein if the intensity within the ROI fell below this threshold. To identify synaptosomes that contained both VAMP2 and TH, the intensity of the TH signal within the VAMP2-positive ROIs was measured. The intensity of the bassoon signal was measured in the double-positive ROIs, retaining only those that were triple-positive.

To assess the bassoon area, automatic segmentation was used to define the ROIs set both for bassoon and TH (threshold: default; analyze particles: size 0.04-0.40 for bassoon and size 0.06-0.60 for TH; circularity: 0.30-1.00). These bassoon and TH ROI sets were then combined to define the overlapped ROIs, using the “AND” function in the ROI Manager. The intensity of the VAMP2 signal helped define the bassoon/TH overlapped ROIs that were positive for VAMP2. Finally, the average area value of bassoon localized within TH and VAMP2 double-positive synaptosomes was calculated using the “Set Measurement” and “Measure” FIJI functions. The percentage of bassoon positive clusters within VAMP2 or double

703 TH/VAMP2-positive was calculated based on the intensity signal within ROIs as described above.
704
705 ***Striatal TH expressing fibers analysis:*** After performing perfusion and immunofluorescence as described above, dorsal
706 striatal sections were stained using the primary antibodies: anti-TH (1:1,000, SYSY), anti-ALDH1A1 (1:300, Abcam),
707 and anti-VMAT2 (1:200, Immunostar). The resource table provides detailed catalog and identification numbers for these
708 antibodies.
709
710 Analysis was performed in fluorescence images acquired using different microscopes and settings as follows: a.Volume
711 measurements of TH fibers. Nikon CSU-W1 SoRa with a 60x objective at 0.1 mm intervals at 1,024 x 1,024 pixel
712 resolution. TH protein signal was measured using Imaris 10.1 software (Bitplane, Concord, USA). Surface rendering
713 function was used to segment the TH fibers. Background subtraction was enabled, the diameter of the largest sphere was
714 set at 0.805 μm and threshold set at 2,000. Segments were filtered with "Number of voxels Img=1" set above 50. TH
715 volume was measured automatically by software.
716
717 b. Intensity and area of TH expressing axons. To eliminate any confounding factors related to magnification settings, we
718 measured TH axons using both high and low magnification. For the high magnification analysis (Figure 5), we utilized a
719 Nikon A1R microscope with a 100x oil immersion objective (Numerical Aperture = 1.45) and a resolution of 1,024 x
720 1,024 pixels to assess the intensity of TH axons in the striatum across different genotypes. Rolling ball background
721 subtraction was performed using Nikon Elements software. The protein signals were analyzed using Imaris 10.1 software
722 (Bitplane, Concord, USA), utilizing the surface rendering function to segment TH, ALDH1A1, and VMAT2 proteins.
723 Background subtraction was disabled and automated thresholds were applied with manual adjustment made as needed.
724 Segments were filtered with "Number of voxels Img=1" set above 50. For the ALDH1A1 positive TH axon, filtering was
725 applied based on the "overlapped area ratio to surface surface=TH-ALDH1A1" above 0.4. Similarly, VMAT2 within TH
726 axonal terminals was filtered with "overlapped area ratio to surface surface= TH-VMAT2" above 0.4. Area and intensity
727 were measured automatically by software. For the low magnification analysis (Supplementary Figure 6), images were
728 captured using the same Nikon A1R microscope with a low magnification (20x) objective (NA=0.75), with a 1,024 x 1,024
729 pixel resolution. Stitched images of the whole striatum were automatically generated with Nikon Element. The TH protein
730 signal was analyzed with Imaris 10.1 software (Bitplane, Concord, USA) employing the surface rendering function to
731 segment TH fibers. Background subtraction was disabled and the threshold was set to 1,500 with manual adjustments made
732 if needed. Segments were filtered with "Number of voxels Img=1" set above 50. TH area and intensity were measured
733 automatically by software.
734
735 **9. LC-MS sample preparation**
736 ***Synaptosomes***
737 Mouse striata were dissected two hours after administering a dose of MLi-2 (10 mg/kg) or control vehicle via oral gavage.
738 The tissues were flash frozen and stored at −80°C until use. Subcellular fractionation of the mouse striatum was performed
739 as previously described.[84, 90]
740
741 In this process, three mouse striata were pooled and rapidly homogenized in four volumes of ice-cold Buffer A (0.32 M
742 sucrose, 5 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$) supplemented with a Halt protease and phosphatase inhibitor
743 cocktail (Thermo) using a Teflon homogenizer with 12 strokes. The homogenized brain extract was then centrifuged at
744 1,400 g for 10 minutes. The supernatant (S1) was saved, and the pellet (P1) was homogenized in Buffer A with a Teflon
745 homogenizer (five strokes). After centrifugation at 700 g for 10 minutes, the supernatant (S1') was pooled with S1.
746
747 Next, the pooled S1 and P1 were centrifuged at 13,800×g for 10 minutes, resulting in a crude synaptosomal pellet (P2)
748 and its corresponding supernatant (S2). The P2 pellet was resuspended in Buffer B (0.32 M sucrose, 6 mM Tris, pH 8.0),
749 also supplemented with the protease and phosphatase inhibitor cocktail, using a Teflon homogenizer (five strokes). It was
750 then carefully loaded onto a discontinuous sucrose gradient (0.8 M/1 M/1.2 M sucrose solution in 6 mM Tris, pH 8.0) with
751 a Pasteur pipette, followed by centrifugation in a swinging bucket rotor at 82,500×g for 2 hours.
752
753 The synaptic plasma membrane fraction (SPM) located at the interphase between the 1 M and 1.2 M sucrose fractions was
754 collected using a syringe and transferred to clean ultracentrifuge tubes. Samples were then centrifuged in a swinging bucket
755 rotor at 200,000×g for 30 minutes. The supernatant was removed and discarded, while the SPM pellet was flash frozen
756 and stored at −80°C.

The SPM pellet samples were processed by Tymora Analytical Operations in West Lafayette, Indianapolis. For the lysis process, 200 µL of phase-transfer surfactant lysis buffer (PTS), which contains 12 mM sodium deoxycholate, 12 mM sodium lauroyl sarcosinate, 10 mM TCEP, and 40 mM CAA, was added to each tissue sample. A phosphatase inhibitor cocktail 3 (Millipore-Sigma) was also included.

After the initial lysis, the samples were incubated for 10 minutes at 95°C, pulse-sonicated several times, and then incubated for an additional 5 minutes at 95°C. The lysed samples were centrifuged at 16,000 × g for 10 minutes to remove debris, and the supernatant was collected. The samples were diluted fivefold with 50 mM triethylammonium bicarbonate, and a BCA assay was performed to determine protein concentration, normalizing all samples by protein amount.

The samples were then normalized to 300 mg protein in each and digested with 6 mg Lys-C (Wako) for 3 h at 37°C. 6 mg trypsin was added for overnight digestion at 37°C. The supernatants were collected and acidified with trifluoroacetic acid (TFA) to a final concentration of 1% TFA. Ethyl acetate solution was added at a 1:1 ratio to the samples. The mixture was vortexed for 2 min and then centrifuged at 16,000 × g for 2 min to obtain aqueous and organic phases. The organic phase (top layer) was removed, and the aqueous phase was collected, dried completely in a vacuum centrifuge, and desalted using Top-Tip C18 tips (Glygen) according to manufacturer's instructions. The samples were dried completely in a vacuum centrifuge and subjected to phosphopeptide enrichment using PolyMAC Phosphopeptide Enrichment kit (Tymora Analytical) according to manufacturer's instructions, and the eluted phosphopeptides dried completely in a vacuum centrifuge.

The full phosphopeptide sample was dissolved in 10.5 µl of 0.05% trifluoroacetic acid with 3% (vol/vol) acetonitrile and 10 µl of each sample was injected into an Ultimate 3000 nano UHPLC system (Thermo Fisher Scientific). Peptides were captured on a 2-cm Acclaim PepMap trap column and separated on a 50-cm column packed with ReproSil Saphir 1.8 µm C18 beads (Dr. Maisch GmbH). The mobile phase buffer consisted of 0.1% formic acid in ultrapure water (buffer A) with an eluting buffer of 0.1% formic acid in 80% (vol/vol) acetonitrile (buffer B) run with a linear 90-min gradient of 6–30% buffer B at flow rate of 300 nL/min. The UHPLC was coupled online with a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode, in which a full-scan MS (from m/z 375 to 1,500 with the resolution of 60,000) was followed by MS/MS of the 15 most intense ions (30,000 resolution; normalized collision energy - 28%; automatic gain control target (AGC) - 2E4, maximum injection time - 200 ms; 60sec exclusion).

APEX2

This analysis was performed in Biognosys AG (Schlieren, Switzerland). Samples (beads with proteins attached via streptavidin pulldown as described in section 5) were solubilized and digested overnight with sequencing grade trypsin (Promega) in a urea-containing denaturation buffer. Each sample is the eluted proteins from one mouse: 4-8 mice/genotype, treatment combination as shown in Supplementary Figures 1 and 6 except Cre- control mice n=3 mice/condition. Beads were collected on a magnetic rack, supernatant was transferred to a new tube and used for the clean-up. Purification for mass spectrometry was carried out using Oasis HLB µElution Plate 30µm plate (WATERS) according to the manufacturer's instructions. Peptides were dried down to complete dryness using a SpeedVac system and dissolved in LC solvent A (1 % acetonitrile in water with 0.1 % formic acid) containing Biognosys' iRT-peptide mix for retention time calibration. Peptide concentrations in mass spectrometry ready samples were measured using the mBCA assay (Thermo Scientific Pierce). For DIA LC-MS/MS measurements, peptides were injected on an in-house packed reversed phase column on a Thermo Scientific EASY-nLC 1200 nano-liquid chromatography system connected to a Thermo Scientific Orbitrap Exploris 480 mass spectrometer equipped with a Nanospray Flex ion source and a FAIMS Pro ion mobility device (Thermo Scientific). LC solvents were A: water with 0.1 % FA; B: 80 % acetonitrile, 0.1 % FA in water. The nonlinear LC gradient was 1 – 50 % solvent B in 171 minutes followed by a column washing step in 90 % B for 7 minutes, and a final equilibration step of 1 % B for 0.5 column volumes at 64 °C with a flow rate set to a ramp between 450 to 271 nl/min (min 0: 450 nl/min, min 172: 271 nl/min, washing at 400 nl/min). The FAIMS-DIA method consisted per applied compensation voltage of one full range MS1 scan and 34 DIA segments, as adapted from previous work.[91,92]

10. MS Data analysis

S*ynaptosomes:* The raw files were searched directly against the mouse database with no redundant entries, using Byonic (Protein Metrics) and Sequest search engines loaded into Proteome Discoverer 2.3 software (Thermo Fisher Scientific). The data from the two search engines were combined. In most cases, the same peptides were identified by both. The final

812 data is from both search engines, including identifications reported by only one search engine. MS1 precursor mass
813 tolerance was set at 10 ppm, and MS2 tolerance was set at 20 ppm. Search criteria included a static carbamidomethylation
814 of cysteines (+57.0214 Da), and variable modifications of phosphorylation of S, T and Y residues (+79.996 Da), oxidation
815 (+15.9949 Da) on methionine residues and acetylation (+42.011 Da) at N terminus of proteins. Search was performed with
816 full trypsin/P digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence
817 database. The false-discovery rates of proteins and peptides were set at 0.01. All protein and peptide identifications were
818 grouped, and any redundant entries were removed. Only unique peptides and unique master proteins were reported. All
819 data were quantified using the label-free quantitation node of Precursor Ions Quantifier through the Proteome Discoverer
820 v2.3 (Thermo Fisher Scientific). For the quantification of phosphoproteomic data, the intensities of phosphopeptides were
821 extracted with initial precursor mass tolerance set at 10 ppm, minimum number of isotope peaks as 2, maximum ΔRT of
822 isotope pattern multiplets – 0.2 min, PSM confidence FDR of 0.01, with hypothesis test of ANOVA, maximum RT shift
823 of 5 min, pairwise ratio-based ratio calculation, and 100 as the maximum allowed fold change. For calculations of fold-
824 change between the groups of proteins, total phosphoprotein abundance values were added together, and the ratios of these
825 sums were used to compare proteins within different samples. This analysis identified 2,155 unique phosphoproteins, 8,008
826 unique phosphopeptides that map to 2,155 unique phosphoproteins, 24,238 unique peptides that map to 3,130 unique
827 proteins. The threshold for significance was set to $p<0.05$ (unadjusted p-value) & FC (difference on the log2 intensity
828 values) ≥ 0.58. To capture a greater phosphorylation for GO-term and kinase-substrate analysis, the statistical cut-off for
829 inclusion was set at uncorrected $p > 0.05$.
830
831 ***APEX2 study:*** The DIA mass spectrometric data were analyzed using Spectronaut software (Biognosys, version 19.0).[93]
832 The false discovery rate on peptide and protein level was set to 1 %. A mouse UniProt .fasta database (Mus musculus,
833 2024-07-01) was used for the search engine, allowing for 2 missed cleavages, carbamidomethylation of cysteine as fixed
834 modification and up to 5 variable modifications (N-terminal acetylation, methionine oxidation, deamidation of asparagine
835 or glutamine and phosphorylation at serine or threonine or tyrosine).
836
837 HRM mass spectrometric data were analyzed using Spectronaut software (Biognosys, version 19.0). The false discovery
838 rate on peptide and protein level was set to 1 %, and data were filtered using row-based extraction. The directDIA+ library
839 generated in this project was used for the analysis. The HRM measurements analyzed with Spectronaut were normalized
840 using global median normalization.
841
842 Proteins that were not enriched (enriched: q-value < 0.05, |log2FC| > 0) in $Anxa1^{Cre}$; $LRRK2^{G2019S}$ vehicle vs. non-
843 transgenic control were removed from the dataset of samples from SNc and striatum, respectively. The filtered dataset was
844 used for further analysis. For testing of differential protein abundance, protein intensities for each protein were analyzed
845 using a two-sample Student's t-test. The following thresholds were applied for candidate identification: p-value < 0.05;
846 absolute average log2 ratio > 0.58 (fold-change > 1.5). Distance in heat maps was calculated using the "manhattan"
847 method, and clustering was performed using "ward.D" for both axes. Principal component analysis was conducted in R
848 using prcomp and a modified ggbiplot function for plotting.
849
850 **11. SIM imaging and analysis**
851 $Aldh1a1^{Cre}$-DAT^{flop} or $Anxa1^{Cre}$ mice crossed with $LRRK2^{G2019S}$ mice or control littermates were labeled with viruses as
852 described in sterotactic injections and viruses sections and processed as in Immunofluorescence and confocal imaging in
853 brain sections. They were probed with the primary antibodies anti-EGFP (1:1000, Invitrogen), anti-mCherry (1:1000,
854 Invitrogen), and anti-bassoon (1:300, Enzo). Detailed catalog and identification numbers are shown in the resource table.
855
856 Multichannel SIM images were obtained with a Nikon N-SIM Structured Illumination super-resolution microscope in the
857 Center for Advanced Microscopy at the Nikon Imaging Center at Northwestern University. The imaging was conducted
858 with a 100x objective lens with a numerical aperture (NA) of 1.4. [62] The acquisition settings were set to 10 MHz, and 16
859 bit depth with electron multiplication (EM) gain and no binning. Exposure times ranged between 200 and 900 ms, with
860 the EM gain multiplier kept below 1,500. The conversion gain was maintained at 1x. The laser power was adjusted to keep
861 LUTs within the first quarter of the scale. LUTS were kept similar across comparisons.
862
863 Single images were processed and analyzed using Nikon Elements software. 3D reconstructions in a single plane used
864 nine images captured with 2D SIM and reconstructed with 3D SIM to increase xy and z resolution, were generated by
865 Nikon Element, and the illumination modulation contrast was set automatically by the software. Images were cropped

using 3D crop function to remove the edges, and a resolution of 1,950x1,950 was applied for further analysis with Imaris 10.1. The segmentation of bassoon particles and axons was conducted using the surface creation tool. In the surface wizard, the source channel for axons was selected, corresponding to either Alexa488 (EGFP) or Alexa568 (mCherry), with object-object statistics activated.

To create a clean surface of axon surface, the smoothing surface detail option was enabled (Surface Grain Size = 0.0643 µm). Background subtraction was performed using local contrast with a diameter of Largest Sphere set to 0.241 µm for better separation of the background. The auto-threshold value was applied, with user adjustments made as required. The segments were then filtered by setting the "Number of Voxels Img=1," above the lower automatic threshold. The settings were saved and manually checked and validated with 10 different images before they were applied to all the images in the dataset. The fluorescence signal outside the surface was removed by using the "Mask channel" function, selecting the corresponding channel for either the Alexa488 (EGFP) or Alexa568 (mCherry), and voxel intensity outside the surface was set to 0.

Axon terminal segments' length was measured with the filaments tool. In the tool wizard, the source channel for axonal terminal segments was again selected, corresponding to the masked Alexa488 (EGFP) or masked Alexa568 (mCherry), with the seed points threshold set at 6,000. Machine learning pixel classification was performed by training Imaris to discard unwanted segments (not overlapping with the surface) while keeping segments that overlapped with the surface until they covered over 99% of the visible surface. The settings were again saved and verified using 10 randomly chosen images before being applied to all images without further user adjustments.

The source channel for bassoon particles was selected based on Alexa647, with object-object statistics on. To create a clean surface of bassoon particles, the smoothing surface detail was activated (Surface Grain Size = 0.06 µm). Background subtraction (local contrast) (Diameter of Largest Sphere =0.15 µm) was employed to separate the background. An auto-threshold value was applied with user adjustments as required. Additionally, the Region Growing Estimated Diameter was set to 0.100 µm based on intensity to help split touching objects. The segments were then filtered by "quality" between lower and upper automatic thresholds, followed by a further "volume" filter between 0.003 and 0.04 μm^3. Bassoon clusters within axonal terminals were filtered based on "overlapped volume ratio to surface," where the ratio of EGFP/mCherry was set above 1.0. These settings were saved and, as before, checked with 10 different randomly chosen images before being applied to all the images.

The number of bassoon objects, their volume, and the length of axon terminal segments were automatically measured and calculated. Density calculations were performed in Excel using the number of bassoon objects relative to the total length of axonal terminal segments, with bassoon object number and axonal terminal segment length determined in Excel.

12. p-RAB3A vs. RAB3A interactome experiment:

Preparation of recombinant p-RAB3A (pT86): The cDNA for His-FLAG-RAB3A (residues 18-190, Q81L) and MST3 kinase (residues 1-431), codon optimized for *E.coli* expression, was ordered from Genscript and cloned into the pET28a vector at the NdeI/BamH1 sites. FLAG-RAB3A and MST3 were expressed in 2xYT and Lysogeny Broth (LB) respectively, supplemented with 30 mg/mL kanamycin at 37 °C. At an OD600 of 0.6, FLAG-RAB3A was induced with 0.5 mM IPTG, and MST3 was induced with 0.1 mM IPTG. The cells were then grown overnight at 18°C. Cells were harvested by centrifugation and lysed by sonication in extraction buffer (20 mM Tris-Cl, pH 8, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$). Following centrifugation at 20,000 x g for 40 minutes at 4°C to remove cellular debris, the supernatants were applied to a gravity flow column containing Ni^{2+}-agarose resin. The resin was washed with an excess of extraction buffer and wash buffer (extraction buffer supplemented with 40 mM imidazole) and then eluted in extraction buffer supplemented with 200 mM imidazole. The His-tag was removed from FLAG-RAB3A by overnight dialysis at 4°C in gel filtration buffer (20mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$) with thrombin protease, followed by a second Ni^{2+}-column. FLAG-RAB3A, not intended for phosphorylation, was further purified by gel filtration chromatography using a Superdex 75 column (10/300 GL) equilibrated in gel filtration buffer. To generate phospho-FLAG-RAB3A, purified FLAG-RAB3A was incubated with MST3 (8:1 wt/wt ratio) in phosphorylation buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, pH 7.5), similar to previous protocols for other RABs at room temperature for 40 minutes.[58,59] The reaction generated more than 50% phosphorylated RAB3A, and the mixture was subsequently exchanged into a low-salt buffer (10 mM Tris-HCl, pH 7, 10 mM NaCl, 1 mM DTT, 5 mM MgCl2) for separation by ion-exchange chromatography on a Mono Q 5/50 GL column (GE Healthcare). Phospho-FLAG-

920 RAB3A was separated from the non-phosphorylated species using a 0-35% gradient from low-to-high salt buffer (low salt
921 buffer supplemented with 1M NaCl) and confirmed by PhosTag gel electrophoresis. Finally, phospho-FLAG-RAB3A was
922 further purified by gel filtration chromatography as previously described.
923
924 ***Immunoprecipitation of p-RAB3A and RAB3A interactors:*** p-FLAG RAB3A and FLAG-RAB3A were used as 'bait' for
925 the immunoprecipitation of proteins from mouse brain lysate to identify binders that interact differentially. Brains from
926 mice administered a subcutaneous injection of MLi-2 (30 mg/kg, 2 hours). Whole brain was isolated snap frozen in liquid
927 nitrogen and stored at -80º C. The brains were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10%
928 [w/v] glycerol, 10 mM sodium-glycerophosphate, 10 mM sodium pyrophosphate, 0.5% [v/v] NP40 - supplemented with
929 Complete Mini [EDTA-free] protease inhibitor (Merck), PhosSTOP phosphatase inhibitor (Merck), 5 mM $MgCl_2$). The
930 lysate was clarified by centrifugation at 17,000×g for 15 minutes at 4°C and quantified using the Bradford assay. Phospho-
931 FLAG-RAB3A and FLAG-RAB3A were bound to PierceTM Anti-DYKDDDDK Magnetic Agarose at 4°C on a rotary
932 wheel for 1 hour. The supernatant was removed, and the beads were washed twice with PBS-T (PBS supplemented with
933 0.01% Tween-20, 5 mM $MgCl_2$). Brain tissue lysate (750 mg) was added to the beads in a 500 ml volume and incubated
934 on a rotary wheel for 1 hour at 4°C. The supernatant was removed, and the beads were washed twice with high-salt lysis
935 buffer (lysis buffer supplemented with 500 mM NaCl), followed by regular lysis buffer, and then frozen at -80°C for mass
936 spectrometry analysis, as described below.
937
938 ***Animals:*** For affinity MS pulldown experiments of pRAB3A/RAB3A interactors, 12 C57BL/6j male mice at 10 weeks of
939 age were used. Mice were maintained under specific pathogen-free conditions at the University of Dundee (UK). All
940 animal studies were ethically reviewed and carried out by the Animals (Scientific Procedures) Act 1986 and regulations
941 set by the University of Dundee and the U.K. Home Office. Animal studies were approved by the University of Dundee
942 ethical committee and performed under a U.K. Home Office project license. Mice were housed at an ambient temperature
943 (20–24°C) and humidity (45–55%) and were maintained on a 12 hr light/12 hr dark cycle, with free access to food and
944 water. Mice were injected subcutaneously with vehicle (40% [w/v] 2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich
945 #332607) or MLi-2 dissolved in the vehicle at a 30 mg/kg final dose. Mice were sacrificed by cervical dislocation 2 hours
946 following treatment, and the collected tissues were rapidly snap frozen in liquid nitrogen for downstream analysis.
947
948 ***Sample preparation for quantitative proteomics:*** Immunoprecipitation samples were eluted from magnetic beads in 40 µL
949 of lysis buffer containing 2% SDS (w/v), 20 mM HEPES (pH 8), with a complete EDTA-free protease inhibitor cocktail
950 (Roche) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Samples were reduced with 10 mM TCEP for 30
951 minutes at 60°C, followed by alkylation of cysteine residues with 40 mM IAA for 30 minutes at 25°C in the dark. 20% SDS
952 was then added to achieve a final concentration of 5% SDS in the samples, which were subsequently acidified by the addition
953 of Trifluoroacetic acid to a final concentration of 1%. Samples were subsequently processed for on-column tryptic digestion
954 using a micro S-trap (Protifi, USA). Briefly, samples were diluted 6-fold with wash buffer (90% methanol, 10% 100 mM
955 TEABC) and loaded onto microcolumns, with centrifugation at 1000g for 1 minute, and the flow-through discarded. After
956 sample loading, the S-Trap columns were washed four times with 150 µL wash buffer, followed by centrifugation at 1000g
957 for 1 minute. On-column digestion was performed by incubating 40 µl (1 µg) of Trypsin/Lys-C mix (MS grade, Promega,
958 UK) in 50 mM TEABC solution (pH 8) at 47°C for 1 hour and 20 minutes, followed by incubation at 22°C overnight. The
959 samples were then eluted with the addition of 40 µl 50mM TEABC (pH 8), 40 µl 0.15% (v/v) formic acid (FA), and 3x 40 µl
960 80% acetonitrile (ACN), 0.15% FA. Peptides were then dried using a vacuum centrifuge at room temperature and stored at -
961 20°C until mass spectrometry analysis.
962
963 ***Quantitative Proteomic Analysis:*** Peptides were resuspended in 0.1% formic acid supplemented with 0.015% N-Dodecyl-
964 β-D-Maltoside (DDM). Approximately 200 ng of peptides were analyzed with a Vanquish Neo nano-liquid chromatography
965 system in-line with an Orbitrap Astral mass spectrometer (ThermoScientific). Peptides were trapped and eluted using an
966 Acclaim™ PepMap™ 100 C18 HPLC column (3 µm particle size, 75 µm diameter, 150 mm length) and separated using an
967 EASY-Spray™ PepMap™ Neo UHPLC column (2 µm C18 particle, 75 µm diameter, 150 mm length) with buffer A: 0.1%
968 formic acid and buffer B: 80%ACN, 0.1% formic acid. Peptides were separated across a 13-minute gradient as follows: 0-
969 0.7 min, 1% buffer B, 1.8 µl/min; 0.7-1.0 min, 4% buffer B, 1.8 µl/min; 1.0-7.7 min, 8% buffer B, 1.8 µl/min; 7.7-11.4 min,
970 22.5% buffer B, 1.8 µl/min; 11.4-11.8 min, 35% buffer B, 1.8 µl/min; 11.8-12.3 min, 55% buffer B, 2.5 µl/min; 12.3-13 min,
971 99% buffer B, 2.5 µl/min.
972
973 Samples were analyzed in DIA mode, with MS1 scans performed at a resolution of 240,000 across an m/z range of 380-980,

using a normalized AGC target of 500% and a maximum injection time of 3 milliseconds. DIA scans were performed on the precursor mass range with an isolation window of 4 m/z, a scan range of 150-2,000 m/z, and a normalized collision energy of 25%.

Raw Mass Spectrometry Data Analysis: Raw data were searched using DIA-NN (version 1.9.2) against a predicted spectral library generated from the reviewed mouse UniProt database (downloaded January 2023; 17,124 entries with isoforms). Spectra were searched with strict Trypsin specificity (cleavage at K or R residues), allowing a maximum of one missed cleavage. Peptides with an amino acid length of 7-30 were considered using default settings, with cysteine carbamidomethylation enabled as a fixed modification.

Statistical Analysis and Data Visualization: Statistical analysis and visualization of DIA-NN output files were performed with Python (version 3.11.5, Jupyter Notebook; Project Jupyter) using packages pandas (2.0.3), matplotlib (3.7.2), seaborn (0.12.2), numpy (1.26.1), and then uploaded to the CURTAIN web tool (https://curtain.proteo.info/#/).[60] Proteins identified by a single peptide were excluded, with log2-transformed protein intensities then filtered by 100% detection in at least one sample condition. Missing values were imputed by random draws from a Gaussian distribution centered at the 1% quantile of global protein group intensities per sample. The Limma package (version 3.56.2; R, version 4.3.1) was used to fit the data to a linear model using the lmfit function, with eBayes correction to compute moderated t-statistics and false-discovery rate controlled by Benjamini-Hochberg correction.

Confirmation of in vitro p-RAB3A phosphorylation site with MS. 1 µg of purified phosphorylated FLAG-Rab3a protein was diluted 1:39 in 50 mM triethylammonium bicarbonate (TEABC, pH 8) in triplicate. Triplicate samples of the non-phosphorylated FLAG-RAB3A protein were also prepared as controls. Protein was reduced by adding Tris(2-carboxyethyl) phosphine (TCEP) to a final concentration of 10 mM at 60°C with mixing at 1,100 rpm (ThermoMixer C, Eppendorf) for 30 minutes. Samples were then alkylated by the addition of Iodoacetamide (IAM) to a final concentration of 40 mM at 25°C with mixing at 1,100 rpm for 30 minutes in the dark. 100 ng of Trypsin/Lys-C MS grade protease (Pierce, ThermoScientific) was then added to samples for tryptic digest at 37°C overnight (approximately16 hours), followed by acidification with 1% formic acid to a final concentration of 0.1%. Samples were then dried at room temperature and resuspended in 0.1% formic acid with 0.015% n-dodecyl-β-d-maltoside (DDM) in LC-grade water, with agitation at 1,800 rpm for 30 minutes at room temperature. Peptides were desalted using C18 EvoTips (EvoSep) and eluted in 40% acetonitrile. Samples were again dried using a vacuum centrifuge and stored at -20°C. For mass spectrometry analysis, samples were resuspended in 0.1% formic acid with 0.015% n-dodecyl-β-d-maltoside (DDM) in LC-grade water. 5 ng of peptides from each sample were injected into a Vanquish Neo nano-liquid chromatography system, in-line with an Orbitrap Astral mass spectrometer (Thermo Scientific), as previously described.

Raw data were searched against a fasta file generated from the Flag-RAB3A protein sequence using DIA-NN (version 1.9.2), with phosphorylation set as a variable modification (UniMod:21 with mass delta 79.9663 at STY, maximum number allowed=1) and precursor FDR set at 0.01. DIA-NN matched the expected phosphorylation site of peptide: YRT(UniMod:21)ITTAYYR, in the 2+ charge state. This was confirmed by manual inspection of the precursor and fragment peptide traces in Skyline (idotp=0.99, Skyline version 24.1). The DIA-NN spectral database search also identified another phosphorylation site in the peptide TSFLFRY(UniMod:21)ADDSFTPAFVSTVGIDFK, in the 4+ charge state. However, manual inspection of this peptide trace in Skyline confirmed this to be a false positive, as no precursor ion was found within the retention time window to match the observed fragmentation spectra traces (idotp=0).

13. Experiments in HEK-293 cells

***Cell culture*:** HEK-293 cells were cultured in low glucose DMEM (Gibco, #11885-084) supplemented with 10% fetal bovine serum (Gibco, #A5256801), 1% MEM non-essential amino acids (Gibco, #11140050), and 1% penicillin/streptomycin (Gibco, #15140122). At 90% confluency, cells were trypsinized with 0.25% trypsin/EDTA (Gibco, #25200072) and plated at a 1:4 dilution into a 100 mm tissue culture dish.

RAB3, RIMS1, and pCMV plasmids were transformed into competent bacteria (One Shot TOP10 chemically competent E. coli, Invitrogen, #C404003). Single colonies were selected and cultured in 3 mL of LB medium supplemented with the corresponding antibiotics for 8 h at 37 °C. After that, 1 mL of bacterial culture was grown in 200 mL of LB medium plus antibiotics overnight at 37°C. Plasmids were purified using ZymoPURE II Plasmid MidiPrep kits (Zymo Research, #D4201) according to the manufacturer's instructions.

Transfection: For cotransfection of RAB3 plasmids and RIMS1, HEK-293 cells at 80% confluency were plated into 6-wells at a 1:20 split ratio. The following day, cells were co-transfected with HA-RAB3 constructs and myc-flag-RIMS1, or with myc-flag-RIM1 alone. For each 6-well, 200 ng of HA-RAB3 plasmids and 2 µg of myc-flag-RIM1 plasmid were mixed in a 1.5 ml centrifuge tube in 50 µl high glucose DMEM (Gibco, #11965092), and 6 µl of LipoD293 (Signagen Laboratories, #SL100668) was mixed in another 1.5 ml centrifuge tube in 50 µl high glucose DMEM. Three minutes later, the contents were mixed, briefly vortexed, and incubated at room temperature for 15 minutes. The media was exchanged to fresh complete medium, and the plasmid/LipoD293 mix was added dropwise to the wells. 24 hours after transfection, cells were transferred into 100 mm dishes and grown for an additional 24 hours.

For the determination of isoform-specificity of the RAB3 antibodies, HEK-293 cells at 80% confluency were plated into 6-well plates. The following day, they were co-transfected with HA-RAB3 constructs (200 ng) and pCMV (2 µg), or with pCMV alone (2 µg) as described above. After 24 hours, the transfected cells were transferred into 100 mm dishes and grown for an additional 24 hours.

Immunoprecipitation: For HA-RAB3 and myc-flag-RIM1 co-transfections, HEK293 cells were collected 48 h after transfection. For each 100 mm dish, cells were washed with ice-cold 1 x PBS, and lysed with 1 ml of IP lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.3% Triton-X100, 10% glycerol, and 1X cOmplete, Mini, EDTA-free protease inhibitor cocktail) in a 1.5 ml low protein binding tube for 30 min on a rotary wheel at 4 °C. Lysates were centrifuged at 10,000 rpm for 10 minutes at 4 °C, and 50 µl of the supernatant set aside as immunoprecipitation input. Magnetic anti-HA beads (25 µl) (Pierce) were washed for 30 min with IP wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 10% glycerol) on a rotary wheel at 4°C. Beads were pulled down with magnet, followed by equilibration in IP lysis buffer for 3 min on ice. Beads were pulled down again and incubated with 1 ml of lysed supernatant for 1h at 4 °C on a rotary wheel. Beads were washed three times for 5 min with 1 mL of IP wash buffer, and proteins were eluted with 40 µL of 2x Laemmli sample buffer and boiled at 95°C for 5 min to release bound proteins.

For the determination of RAB3 antibody specificity, cells were washed and collected 48 h after transfection in 1 ml of IP lysis buffer. Lysates were incubated for 30 minutes on a rotary wheel at 4°C, and then centrifuged for 10 minutes at 10,000 rpm at 4 °C. For each transfection, five µl of supernatant was resolved on SDS-PAGE gels and analyzed by WB as described above.

14. In vivo evoked dopamine release

Surgery: Anxa1iCre; LRRK2^{WT} and Anxa1iCre; LRRK2^{G2019S} mice were used for virus injection and fiber implantation at 6 months of age. Mice were anesthetized with isoflurane (5% at induction and 1–2% for maintenance). Two craniotomies, 0.5–1mm in diameter, were made over the right substantia nigra (−3.20 mm caudal, +1.6 mm lateral from bregma) and the right rostral striatum (+0.5 mm caudal, +1.8 mm lateral from bregma). A small volume (0.4 µl total) of virus (pAAV-Ef1a-DIO-ChRmine-mScarlet-WPRE, Addgene, 130998-AAV5, titer 2.20E+13 vg/mL), diluted 1:1 in PBS, was pressure injected through a pulled glass micropipette into the SNc at four depths (−3.8, −4.1, −4.4, and −4.7 mm ventral from dura surface, 0.1 µl per depth). Similarly, 0.1 µl of virus (pAAV-hSyn-GRAB-gDA3m, Addgene, 208698-AAV1, titer ≥ 7×10^{12} vg/mL), diluted 1:2 in PBS, was injected into the striatum at -1.9 mm ventral from the dura surface. Following injections, a 4mm long, 400 um diameter optic fiber (MFC_400/430-0.66_4.0mm_TS3.0_C60, Doric Lenses) for opto-stimulation was implanted through the same craniotomy just above the SNc, and a 2mm long, 200-diameter optic fiber (MFC_200/250-0.66_2.0mm_ZF1.25(G)_FLT, Doric Lenses) for fiber photometry was implanted in the dorsal striatum. The fibers and a custom-made metal headplate were secured on the skull with metabond (Parkell).
For details, see the online protocol at dx.doi.org/10.17504/protocols.io.e6nvwd6qdlmk/v1.

Fiber photometry and optogenetics setup: A custom-made photometry setup, based on a previously published design[13] was used for recording. Blue excitation light (470-nm LED, Thorlabs, M70F3) and purple excitation light (for the isosbestic control) (405-nm LED, Thorlabs, M405FP1) were coupled into the optic fiber such that a power of 0.5 mW emanated from the fiber cannula tip. Then, 470-nm and 405-nm excitation were alternated at 100 Hz using a waveform generator, each filtered with a corresponding filter (Semrock, FF01-406/15-25 and Semrock, FF02-472/30-25) and combined with a dichroic mirror (Chroma Technology, T425lpxr). The excitation light was collimated and coupled to a

fiber-optics patch cord (MBP_400/430/3000-0.57_1m_FCM-2xZF1.25(F), Doric Lenses), which connects to the fiber cannula implant. Green fluorescence was separated from the excitation light by a dichroic mirror (Chroma Technology, T505lpxr) and further filtered (Semrock, FF01-540/50-25) before collection using a GaAsP PMT (H10770PA-40, Hamamatsu; signal amplified using Stanford Research Systems SR570 preamplifier). A red LED light for opto-stimulation (LEDFRJ_635, Doric Lenses) was similarly coupled to a fiber-optic patch cord and connected to implanted fiber-optic cannulas. The frequency, power, and duration were constructed as stimulation trains and controlled by Doric Studio software (Doric Lenses) using complex waveforms. The stimulation trains were triggered by a custom LabVIEW script, which introduces random time delays between every train within a time interval. LED powers at the tip of the cannula were measured with a digital power meter (PM100D, ThorLabs). The rotational velocity of the treadmill during animal locomotion was sampled at 1 kHz by a rotary encoder (E2-5000, US Digital) attached to the treadmill's axle, using a custom Arduino script. A DigiData data acquisition system (Axon Digidata 1550B, Molecular Devices) was used to record and synchronize fluorescence and optogenetic light triggers at a sampling rate of 2 kHz.

In vivo optogenetic activation of Anxa1+ DA neurons: 4 weeks after surgery, mice were head-fixed with their limbs resting on a one-dimensional cylindrical Styrofoam treadmill ~20 cm in diameter by 13 cm wide. A separate red LED light was placed in front of their face to mask the red light delivery to the brain. After 2 days of habituation, GRAB-DA3m signals were recorded for 20 minutes. During recordings, TTL-triggered red LED stimulation was delivered in pseudorandom order at 0.1, 0.5, 1, and 4 mW. Each pulse train consisted of 8-ms on, 8-ms off cycles for 500 ms. Stimuli were spaced at least 20 seconds apart and repeated 8 times per power level.

Fiber photometry signal processing: Simultaneous traces of optogenetic stimulation triggers, treadmill velocity, green fluorescence (520 nm), excitation light function generator, and camera triggers were collected as times series at 2kHz from DigiData as ABF files. The files are then loaded into MATLAB and analyzed using custom scripts similar to previously described. [13] The 470-nm and 405-nm excited fluorescence was first separated based on function generator signals and then all traces were re-binned at 100 Hz for analysis. The fluorescence generated from 470 nm excitation was used for functional measurements, while the non-functional fluorescence generated from 405 nm excitation (isosbestic point) was used as a control for movement artifacts. Raw fluorescence traces first underwent correction procedures to eliminate background fluorescence and slow drifts. Background fluorescence was estimated from cortex recordings where there is no GRAB-DA labeling and calculated as a percentage of the baseline (defined as the 8th percentile of total fluorescence using a 20-s sliding window). Background fluorescence was subtracted from 470 nm and 405 nm independently. The background-subtracted traces were normalized by baseline (newly defined as the 8th percentile of the background-subtracted trace using a 20-s window) division. To calculate the $\Delta F/F$, the normalized traces were then converted to % $\Delta F/F$ by subtracting the baseline (defined as the 8th percentile of the whole trace) and dividing by it. For triggered averages of $\Delta F/F$ on opto-stimulation light-on periods, integral values were normalized to a range of -0.25 to 1 for plotting.

Quantification and statistical analysis: Data were analyzed using custom code in MATLAB deposited in Github. Link can be found in the resource table.

For tests of statistical significance, all *P* values reported were calculated using a 2-sample t-test (ttest2 function in MATLAB). For individual-to-group comparisons, the Welch t-test was used because of the assumption of unequal variance. The specific test used is stated in the main text and figure legends where *P* values are reported. All tests were corrected for multiple comparisons using the Bonferroni correction: multiplying the *P* values by the number of comparisons made.

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications. Surgeries, data collection, and signal processing were performed in a blinded manner, concerning the mouse type ($LRRK2^{WT}$ or $LRRK2^{G2019S}$). Post-hoc histological verification of virus expression and fiber placement were used as exclusion criteria, which led to the exclusion of one G2019S mouse due to the abnormality of GRAB-DA3m and ChRmine expression.

15. Pipelines for proteome analysis and data visualization

Gene Ontology (GO) analysis, related to Biological Process, Cellular Components, and Molecular Function, was evaluated using the clusterProfiler 4.16.6 package and the org.Mm.eg.db 3.20.0 database in R 4.4.3. Other packages such as readxl/1.4.5, openxlsx/4.2.8, dplyr/1.1.4, and ggplot2/3.5.1 were used for filtering and visualization. Pathways with an

adjusted p-value <0.05, based on a hypergeometric test and a Benjamini-Hochberg correction for multiple testing, are considered significantly enriched for the specified terms.

The functional analysis of significantly altered phosphopeptides was performed in proteins with at least one peptide showing significant differences in its phosphorylation status between synaptosomes of vehicle-treated and MLi2-treated LRRK2^{G2019S} mice. Significance was defined as $|\log_2 FC| > 0.58$ and $p \leq 0.05$ as determined by Student's t-tests. The top 15 pathways are specified in Figures 2C and Supplementary Figure 2C. The functional analysis of differentially expressed proteins was performed on proteins with a significant difference in the APEX2 proteomes of vehicle-treated vs MLi2-treated LRRK2^{G2019S} mice. Significance was defined as $|\mathrm{Log}_2 FC| > 0.58$ and $p \leq 0.05$ as determined by two-sample Student's t-tests. Selected pathways relevant to presynaptic functions are shown in Supplementary Figure 6E.

The complete list of analyses will be uploaded to Zenodo and in Supplementary File 1 and Supplementary File 2 for synaptosome experiment (Figure 2) and APEX2 experiment (Supplementary Figure 6), respectively.

Volcano plots: All quantified peptides in the phosphopeptidome analysis comparing vehicle-treated and MLi2-treated LRRK2^{G2019S} mice were graphed according to their Log2FC and -log(p-value), as determined by multiple unpaired t-tests. All proteins identified in the proteomics analysis of vehicle- and MLi2-treated LRRK2^{G2019S} mice were plotted according to their Log2FC and log (p-value), as determined by multiple unpaired t-tests. Visualization was performed using the ggplot2 R package in R version 4.4.3. Phosphopeptides and proteins with $|\mathrm{Log}_2 FC| > 0.58$ and the $p \leq 0.05$ were considered significantly altered.

Heatmaps were generated using the ComplexHeatmap R package.

Plotting human genes in dopamine neurons: Feature plots were generated using R (RRID: SCR_001905) ShinyCell package[93] (RRID: SCR_022756) to display the expression of LRRK2 and RAB3 genes in molecularly defined subclusters of dopamine neurons from a single-nuclei RNA dataset from Kamath et al.[49] (GEO Accession # GSE178265)

16. Statistics

Group statistical analyses were performed using GraphPad Prism 10.1 software (GraphPad, La Jolla, CA). Sample size (n value) is defined by the number of observations (i.e., neurons, sections, or mice) and is indicated in the legends. All data are expressed as mean ± SEM or individual plots. Unless stated otherwise, statistical significance was determined by two-tailed Student's t-tests for two-group comparisons. For multiple group comparisons, one-way or two-way analysis of variance (ANOVA) tests were used for normally distributed data, followed by post-hoc analyses. All statistical tests are specified in the figure legends.

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1381 **Resources table**

RESOURCE TYPE	*RESOURCE NAME*	*SOURCE*	*IDENTIFIER*
Antibody	Bassoon (SAP7F407)	Enzo, ADI-VAM-PS003	RRID:AB_10618753
	Tyrosine hydroxylase	SYSY, 213-104	RRID:AB_2619897
	Tyrosine hydroxylase	Millipore, MAB318	RRID:AB_2313764
	ALDH1A1	Abcam, ab52492	RRID:AB_867566
	ALDH1A1	Proteintech, 60171-1-Ig	RRID:AB_10693634
	anti-GFP	Invitrogen, A10262	RRID:AB_2534023
	anti-GFP	Abcam, ab6556	RRID:AB_305564
	anti-mCherry	Invitrogen, A11217	RRID:AB_2536611
	phospho-RAB12 (S106)	Abcam, ab256487	RRID:AB_2884880
	RAB12	Proteintech, 18843-1-AP	RRID:AB_10603469
	β-Actin	Sigma-Aldrich, A5441	RRID:AB_476744
	LRRK2/Dardarin cloneN241A/34	NeuroMab, 75-253	RRID:AB_10675136
	LRRK2/Dardarin cloneN137/6	NeuroMab, 75-188	RRID:AB_2234791
	LRRK2 (phospho S935)	Abcam, ab133450	RRID:AB_2732035
	LRRK2 (phospho S1292)	Abcam, ab203181	RRID:AB_2921223
	Streptavidin-HRP	Abcam, ab7403	
	RAB3A	Sigma-Aldrich, WH0005864M1	RRID:AB_1843230
	RAB3C	Proteintech, 10788-1-AP	RRID:AB_2177217
	Synaptophysin	Cell signaling, 4329	RRID:AB_1904154
	VAMP2	R&D systems, AF5136	RRID:AB_10571702
	SNAP25	Proteintech, 14903-1-AP	RRID:AB_2192051
	Dopamine Transporter	Sigma-Aldrich, MAB369	RRID:AB_2190413
	RIM1	Proteintech, 24576-1-AP	RRID:AB_2879618
	GDI1	Sigma-Aldrich, WH0002664M8	RRID:AB_1841744
	GDI2	Novus, NBP3-03650	RRID:AB_3532284
	VMAT2	Immunostar, 20042	RRID:AB_10013884
	GAPDH (6C5)	Santa Cruz, Sc-32233	RRID:AB_627679
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	anti-c-myc	Sigma, M4439	RRID:AB_439694
	IRDye® 680RD Goat anti-rat IgG	LiCor, 926-68076	RRID:AB_10956590
	IRDye 800CW Goat anti-mouse IgG	LiCor, 926-32210	RRID:AB_3096013
	IRDye 800CW Goat anti-Rabbit IgG	LiCor, 926-32211	RRID:AB_621843
	IRDye® 680RD Goat anti- rabbit IgG	LiCor, 926-68071	RRID:AB_10956166
	Goat anti-Mouse IgG (H+L), HRP	Thermo Fischer Scientific, #626520	RRID:AB_2533947
	Goat anti-Rabbit IgG (H+L), HRP	Thermo Fischer Scientific, #656120	RRID:AB_2533967
	Goat anti-Chicken IgY (H+L), Alexa Fluor™ 488	Invitrogen, A11039	RRID:AB_2534096
	Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 488	Invitrogen, A21206	RRID:AB_2535792
	Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 568	Invitrogen, A11075	RRID:AB_141954

	Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 647	Invitrogen, A31573	RRID:AB_2536183
	Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 647	Invitrogen, A31571	RRID:AB_162542
	Cy™3 AffiniPure® Donkey Anti-Guinea Pig IgG (H+L)	Jackson Immuno Research, 706-165-148	RRID:AB_2340460
Virus strain	AAV8 hSyn-Con/Fon-YFP	Addgene #55650	RRID:Addgene_55650
	AAV8 Ef1a-Coff/Fon-mCherry	Addgene #137134	RRID:Addgene_137134
	AAV-CAG-DIO-APEX2NES	Addgene plasmid #79907	RRID:Addgene_79907
	pAAV-Ef1a-DIO-ChRmine-mScarlet-WPRE	Addgene #130998-AAV5	RRID:Addgene_130998
	pAAV-hSyn-GRAB-gDA3m	Addgene #208698-AAV1	RRID:Addgene_208698
Plasmids	pcDNA5D-frt/to-HA-RAB3A	mrcppureagents.dundee.ac.uk	DU36574
	pcDNA5D-frt/to-HA-RAB3A T86A	mrcppureagents.dundee.ac.uk	DU26352
	pcDNA5D-frt/to-HA-RAB3A T86E	mrcppureagents.dundee.ac.uk	DU26353
	pcDNA5D-frt/to-HA-RAB3A Q81L	mrcppureagents.dundee.ac.uk	DU36588
	pcDNA5D-frt/to-HA-RAB3A T36N	mrcppureagents.dundee.ac.uk	DU36587
	pCMV-HA-RAB3B	mrcppureagents.dundee.ac.uk	DU55007
	pCMV-HA-RAB3C	mrcppureagents.dundee.ac.uk	DU55048
	Myc-flag-RIMS1	OriGene	RC213013
	pET15b 6HIS MST3 TV2	mrcppureagents.dundee.ac.uk	DU62878
	FLAG-RAB3A	customized gene synthesis and generated by Genscript	
Chemical, peptide, or recombinant protein	MLi-2	Abcam, ab254528	
	Hydroxypropyl-β-Cyclodextrin	Cayman, 16169	
	Sodium chloride solution	Sigma-Aldrich, S5150	
	ProLong™ Diamond Antifade Mountant	Invitrogen, P36965	
	OCT embedding medium	Fisher Scientific #4585	
	Phosphate buffered saline	Sigma-Aldrich, P5493	
	Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	Thermo Scientific, 78441	
	Tris Buffered Saline	Sigma-Aldrich, T5912	
	TWEEN® 20	Sigma-Aldrich, P1379	
	Sodium chloride	Sigma-Aldrich, S3014	
	Potassium chloride	Sigma-Aldrich, P9541	
	Sodium bicarbonate	Sigma-Aldrich S5761	
	Monosodium phosphate	Sigma-Aldrich S3139	
	Calcium chloride	Sigma-Aldrich C5670	
	D-(+)-Glucose	Sigma-Aldrich G7021	
	Sodium dodecyl sulfate solution	Sigma-Aldrich 71736-100ML	
	Ammonium bicarbonate	Fisher Chemical A643-500	
	MgCl2	Sigma-Aldrich M8266	
	HEPES	Sigma-Aldrich 54457	
	Antigen Unmasking Solution, Citrate-Based	Vector, H-3300	
	Triton X-100	Sigma-Aldrich, T8989	
	Glycerol	Sigma-Aldrich, G5516	
	Ethylenediaminetetraacetic acid disodium salt solution	Sigma-Aldrich, E7889	
	Methanol	Fisher Scientific #412	

Sucrose	Sigma-Aldrich, RDD023
Paraformaldehyde	Sigma-Aldrich, P6148
Choline chloride	Sigma-Aldrich, C7527
(+)-Sodium L-ascorbate	Sigma-Aldrich, A4034
Sodium azide	Sigma-Aldrich, S2002
Potassium phosphate monobasic	Sigma-Aldrich, 795488
Tris (1 M), pH 8.0	Invitrogen, AM9856
Trolox	Sigma-Aldrich, 238813
Sodium dodecyl sulfate solution	Sigma-Aldrich, 71736
Trichloroacetic acid	Sigma-Aldrich, T6399
urea	Thermo Scientific, 29700
Streptavidin magnetic beads	Thermo Scientific, 88817
Sodium phosphate, 0.5M buffer soln., pH 8.0	Thermo Scientific, J60825.AP
Bovine Serum Albumin	Sigma-Aldrich, A9647
Biotinyl tyramide	Sigma-Aldrich, SML2135
DL-Dithiothreitol	Sigma-Aldrich, D9779
2x YT Broth	FORMEDIUM, YDB0102
LB broth	Sigma-Aldrich, 102123542
IPTG	Fluorochem, M02726
Kanamycin Sulfate	Thermo Fisher Scientific, 11815032
Thrombin protease	Cytiva, 27-0846-01
Imidazole	Sigma-Aldrich, 102117349
Trizma base	Sigma-Aldrich, 0000349338
Magnesium chloride hexahydrate	Sigma-Aldrich, 102790662
2-Mercaptoethanol	Sigma-Aldrich, M3148
Adenosine 5'-triphosphate Disodium Salt Trihydrate	Thermo Fisher Scientific, BP413
Amicon® Ultra Centrifugal Filter, 10 kDa MWCO	Millipore, UFC9010
Phos-tag (TM) Acrylamide	FIJIFILM Wako, AAL-107
HisPur™ Ni-NTA Resin	Thermo Fisher Scientific, 88222
Pierce™ Anti-DYKDDDDK Magnetic Agarose	Thermo Scientific, A36798
Superdex 75 10/300 GL	Cytiva, 17517401
Mono Q 5/50 GL column	Cytiva, 17516601
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche, 11836170001
PhosSTOP Phosphatase Inhibitor	Roche, 4906845001
12.5% SuperSep™ Phos-tag™	FUJIFLIM Wako, 199-18011
NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%	Invitrogen™, NP0336BOX
iBlot™ 2 Transfer Stacks, nitrocellulose	Invitrogen™, IB23001
NuPAGE™ MES SDS Running Buffer (20X)	Invitrogen™, NP0002
NuPAGE™ Antioxidant	Invitrogen™, NP0005
NuPAGE™ Transfer Buffer (20X)	Invitrogen™, NP0006
NuPAGE™ LDS Sample Buffer (4X)	Invitrogen™, NP0008
NuPAGE™ Sample Reducing Agent (10X)	Invitrogen™, NP0009
10x TG transfer buffer	Fisher BioReagents, BP1306-4

	Pierce™ BCA Protein Assay Kits	Thermo Scientific™, 23225	
	Pierce™ Detergent Compatible Bradford Assay Kit	Thermo Scientific™, 23246	
	Restore™ Western Blot Stripping Buffer	Thermo Scientific™, 21059	
	BLUeye Prestained Protein Ladder	Sigma-Aldrich, 94964	
	Immobilon ECL Ultra Western HRP Substrate	Millipore, WBULS0500	
	Cell Lysis Buffer (10X)	Cell Signaling, 9803	
	OCT embedding medium	Fisher Scientific, 4585	
	Superfrost™ Clipped Corner Slides	Fisher Scientific, 12-550-016	
	Nitrocellulose Membranes, 0.45 µm	Fisher Scientific, 88018	
	low glucose DMEM	Gibco, 11885-084	
	high glucose DMEM	Gibco, 11965092	
	Fetal bovine serum	Gibco, A5256801	
	MEM non-essential amino acids	Gibco, 11140050	
	trypsin/EDTA	Gibco, 25200072	
	One Shot TOP10 chemically competent E. coli	Invitrogen, C404003	
	ZymoPURE II Plasmid MidiPrep kits	Zymo Research, D4201	
	LipoD293	Signagen Laboratories, SL100668	
	Complete, Mini, EDTA-free protease inhibitor cocktail	Roche,11836170001	
	Magnetic anti-HA beads	Pierce, 88836	
	Acetonitrile ≥99.9%	VWR, 1.00030.2500	
	Formic Acid, 99.0+%, Optima™ LC/MS Grade	Thermo Fisher Scientific, A117-50	
	Water, for HPLC-MS	Fisher Scientific, 10777404	
	EPA Screw Vial Assembled Kit, 20mL amber glass EPA	Thermo Scientific, 11543750	
	Sodium Dodecyl Sulfate (SDS)Micropellets	Thermo Fisher Scientific, 15450685	
	Hepes 99.6%	Formedium, HEPES10	
	Bond-Breaker™ TCEP Solution, Neutral pH	Thermo Scientific, 77720	
	Iodoacetamide, BioUltra	Sigma-Aldrich, I1149-25G	
	Trifluoroacetic acid (TFA)	Sigma-Aldrich, 302031-100ML	
	Triethylammonium bicarbonate buffer (TEABC)	Sigma-Aldrich, T7408-500ML	
	Trypsin/Lys-C Mix	Thermo Fisher Scientific, #15956915	
	n-Dodecyl-beta-Maltoside Detergent (DDM)	Thermo Scientific, 89902	
	S-Trap micro columns	Protifi, C02-micro-80	
	Complete EDTA-free protease inhibitor cocktail	Roche, 11873580001	
	PhosSTOP phosphatase inhibitor cocktail tablets	Roche, 4906837001	
	Micro Tube 2ml Low Binding	Sarstedt, 72.695.600	
	Eppendorf twin.tec PCR Plates LoBind (96-well)	Thermo Fisher Scientific, 15280735.	
	Mounting Medium with DAPI	VECTASHIELD, H-2000	
	C&B-Metabond Dental Cement	Fisher Scientific NC0877382	
Mouse model	*LRRK2 G2019S*	Jackson Laboratory 030961	RRID:IMSR_JAX:030961
	C57BL/6	Jackson Laboratory 000664	RRID:IMSR_JAX:000664
	LRRK2 KO	Jackson Laboratory 016121	RRID:IMSR_JAX:016121

	Aldh1a1-cre	Transgenic and Targeted Mutagenesis Laboratory, Northwestern University, Awatramani, [13]	
	DAT-cre	Jackson Laboratory 006660	RRID:IMSR_JAX:006660
	Anxa1-icre	Transgenic and Targeted Mutagenesis Laboratory, Northwestern University Awatramani, [13]	
	DATflpo	Transgenic and Targeted Mutagenesis Laboratory, Northwestern University Awatramani	
	Gt(ROSA)26Sorem1(EEF1A1-APX1*)Ykozo	MGI:7529474[45]	RRID:MGI:7529479
Dataset	The Parkinson's disease kinase LRRK2 impairs release sites of vulnerable dopamine axons-APEX2 proteome	ProteomeXchange	PXD065003
	Mass spectrometry-synaptosome	ProteomeXchange	PXD065006
	LRRK2 alters the composition and organization of active zone release sites in dopaminergic striatal synaptosomes	BioImage Archive	S-BIAD2074
	LRRK2 is expressed in dopamine neurons and is enriched in the vulnerable subpopulation	BioImage Archive	S-BIAD2070
	TH fibers and dopamine vesicle clustering alterations	BioImage Archive	S-BIAD2072
	LRRK2 disrupts the number and composition of active zone release sites in vulnerable dopamine axons	BioImage Archive	S-BIAD2071
	Primary data	Zenodo	10.5281/zenodo.15635558
	The Parkinson's disease kinase LRRK2 impairs release sites of vulnerable dopamine axons	ProteomeXchange	PXD065125
Software/code	Imaris 10.1	Bitplane Inc	RRID:SCR_007370
	GraphPad Prism 10	GraphPad Software Inc	RRID:SCR_002798
	BioRender	Biorender	RRID:SCR_018361
	ImageJ	doi:10.1038/nmeth.2089	RRID:SCR_003070
	iBright CL1500 Imaging System	Invitrogen	RRID:SCR_026565
	analysis code-phosphopeptidesGSMLi2vsGSvehpublication	Zenodo	10.5281/zenodo.15635558
	analysis code-volcanoPlots	Zenodo	10.5281/zenodo.15635558
	analysis code-WTvsLRRK2MLI2publication	Zenodo	10.5281/zenodo.15635558
	DIA-NN 1.9.2	https://github.com/vdemichev/DiaNN	RRID:SCR_022865
	CURTAIN	https://curtain.proteo.info	RRID:SCR_024465
	Limma	R software package	RRID:SCR_010943
	Jupyter Notebook	https://jupyter.org/	RRID:SCR_018315
	Axon Digidata 1550B	Molecular Devices	
	MATLAB	The MathWorks, Inc.	RRID:SCR_001622
	Doric Neuroscience Studio 5.4.1	Doric Lenses	RRID:SCR_018569
	LabVIEW	National Instruments	RRID:SCR_014325
	lrrk2_photometry_analysis	Github	https://github.com/DombeckLab/lrrk2_photometry_analysis

Protocol	Immunofluorescence and confocal imaging in brain sections and image analysis	protocols.io	dx.doi.org/10.17504/protocols.io.yxmvmm6q6v3p/v1
	Axonal proteome analysis and Western Blot via APEX2 labeling	protocols.io	dx.doi.org/10.17504/protocols.io.n2bvjb79bgk5/v1
	Liquid Chromatography-Mass Spectrometry (LC-MS) with striatal synaptosomes	protocols.io	dx.doi.org/10.17504/protocols.io.eq2lyqk6mvx9/v1
	SIM imaging and analysis for active zone release sites in vulnerable dopamine axons	protocols.io	dx.doi.org/10.17504/protocols.io.ewov1mpdpvr2/v1
	Western blot and Phostag gels	protocols.io	dx.doi.org/10.17504/protocols.io.j8nlkrxd5v5r/v1
	Striatal synaptosome preparation for imaging	protocols.io	dx.doi.org/10.17504/protocols.io.dm6gpqpo1lzp/v1
	Experiments in HEK-293 cells	protocols.io	dx.doi.org/10.17504/protocols.io.6qpvrqjm2lmk/v1
	Expression and Purification of FLAG-RAB3A Phosphorylated at pThr86	protocols.io	dx.doi.org/10.17504/protocols.io.dm6gpq4ejlzp/v1
	FLAG-tag mediated co-immunoprecipitation of phosphorylated Rab GTPases with cellular interacting proteins V.2	protocols.io	dx.doi.org/10.17504/protocols.io.5qpvowjn9l4o/v2
	Proteomics Sample Preparation for Affinity-Purification Mass Spectrometry	protocols.io	dx.doi.org/10.17504/protocols.io.8epv5owj6g1b/v1
	Surgical procedures for simultaneous optogenetic manipulation in the SNc and acute fiber photometry in the dorsal striatum	protocols.io	dx.doi.org/10.17504/protocols.io.e6nvwd6qdlmk/v1.

1382

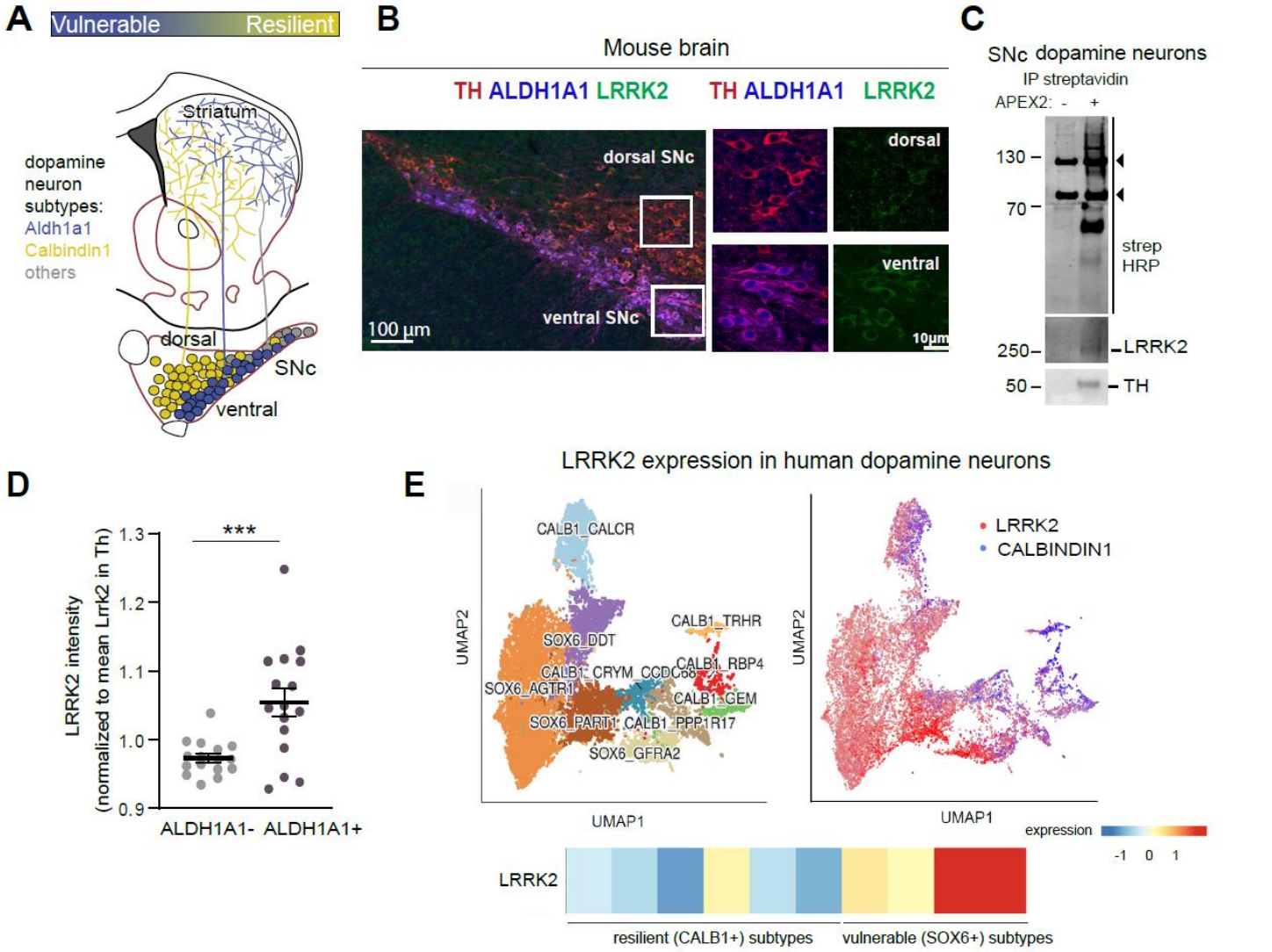


Figure 1: LRRK2 is expressed in SNc dopamine neurons and enriched in vulnerable subpopulations. **A.** Schematic illustrating the localization of selective dopamine neuron subtypes in the mouse SNc and their projection patterns in the striatum. Aldh1a1 defines the vulnerable dopamine neuron populations in PD that are located more ventrally. The resilient dopamine neuron subtypes are characterized by calbindin 1 (*Calb1*+) and are located more dorsally.[11–13] **B.** *Left,* Representative image showing TH, ALDH1A1, and LRRK2 immunostaining in the SNc of wildtype mice. *Right,* High magnification images of the boxed regions in the dorsal (ALDH1A1-) and ventral (ALDH1A1+) SNc areas. **C**. WB analysis of streptavidin pulldowns from SNc dissections from DAT^{cre} mice crossed with APEX2 EGFP mice (detailed in Supplementary Figure 1) probed for streptavidin, LRRK2, and TH. Arrowheads to the right for the strong bands at ~75 and ~150 kDa indicate endogenous biotinylated carboxylase proteins. Each lane contains an equal amount of proteins eluted from the streptavidin beads. **D.** Quantification of LRRK2 intensity from B in Aldh1a1- (less vulnerable) and Aldh1a1+ (vulnerable) dopamine neurons. Each dot represents the average LRRK2 intensity of 60-170 cells/section. n=16 sections/3 mice. Data are represented as mean ± SEM. Asterisks show statistical significance for the unpaired t-test. ***$p < 0.001$. **E.** *Left,* Clusters of dopamine neuron subtypes based on the human snRNA sequencing dataset from[49] colored by subtype and plotted using Shinny Cell.[93] *Right,* Feature plots from the same dataset showing limited coexpression between *LRRK2* and *CALB1*+. *Bottom,* Heatmap showing expression of *LRRK2* with apparent enrichment in vulnerable (*SOX6*+) but not resilient (*CALB1*+) subclusters.

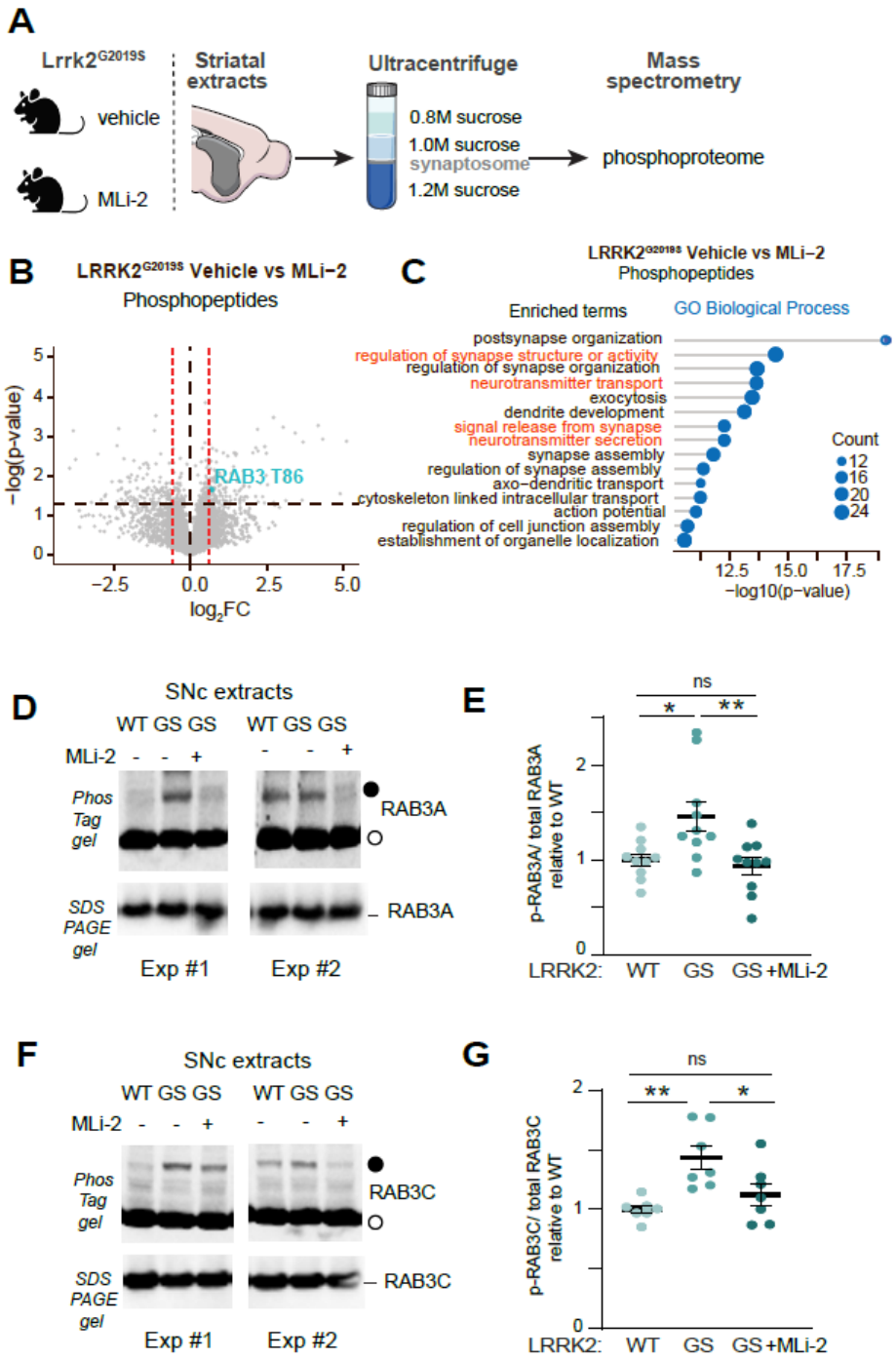


Figure 2: LRRK2 phosphorylates RAB3 proteins in vivo.
A. Experimental workflow of LC/MS-MS analysis in striatal synaptosomes isolated by subcellular fractionation from 3 technical replicates, with each technical replicate performed using 3 LRRK2^{G2019S} mice treated with either MLi-2 or the corresponding vehicle for 2 hours. Treatment conditions: Vehicle for MLi-2, 40% 2-hydroxypropyl-β-cyclodextrin; MLi-2, 10 mg/kg. This treatment leads to a decrease in LRRK2 kinase activity in vivo (see Supplementary Figure 2). **B.** Volcano plot comparing the significantly altered phosphopeptides between vehicle and MLi-2-treated LRRK2^{G2019S} mice ($p \leq 0.05$ by multiple unpaired t-tests, $|Log_2FC| > 0.58$). **C.** Gene Ontology analysis of proteins with at least one differentially regulated phosphopeptide in striatal synaptosomes from vehicle vs MLi2-treated LRRK2^{G2019S} mice. The top 15 pathways are significantly enriched in the biological process term analysis. All enriched pathways have been uploaded to Zenodo (link in the key resources table) and presented in Supplementary File 1. **D.** Representative PhosTag and SDS PAGE gels (upper and lower panels, respectively) from two independent experiments of LRRK2^{WT} and LRRK2^{G2019S} mice (indicated WT, GS,

respectively) with or without MLi-2 treatment (10 mg/kg, 2 hours). SNc extracts from these mice were probed for RAB3A (closed/open circles = phosphorylated (p)- RAB3A/unphosphorylated RAB3A species, respectively). **E.** p-RAB3A/total (phosphorylated +unphosphorylated) RAB3A quantification. n=10 mice/treatment. All samples were normalized to the average signal of all WT samples in the same blot. **F.** Same as D, but probing for RAB3C. **G.** Same as E, but for RAB3C. n=7 mice/treatment. In **E** and **G,** data represent mean±SEM. Asterisks indicate statistical significance, as determined by Šídák's multiple comparisons test following a two-way ANOVA. E: Treatment factor $F(1,27)=11.33$ $p=0.0023$, Genotype factor $F(1,27)=8.593$ $p=0.0068$. G, Treatment factor $F(1,18)=7.398$ $p=0.0140$, Genotype factor $F(1,18)=14.76$ $p=0.0012$. *$p<0.05$, **$p<0.01$.

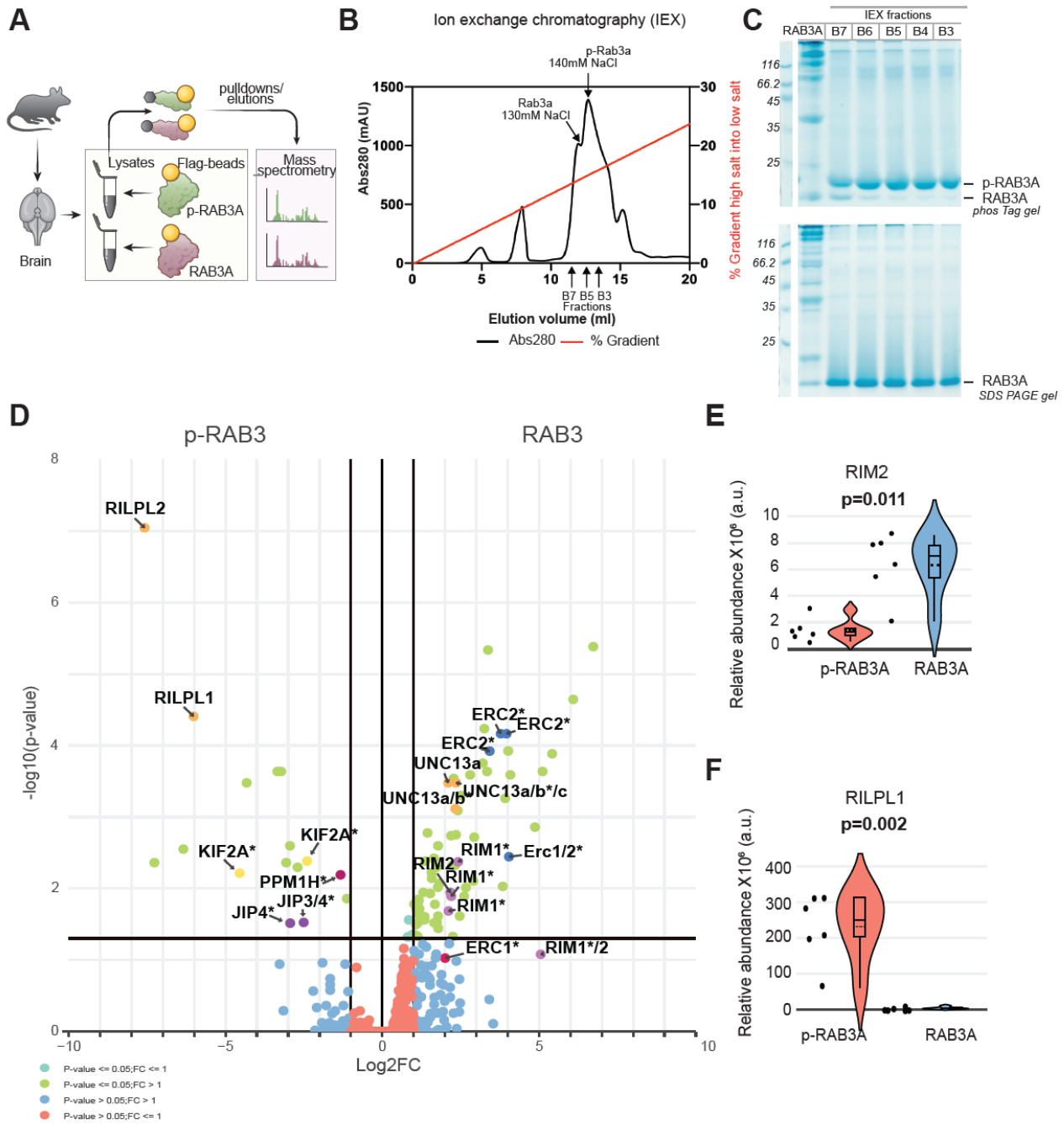


Figure 3: Differential interactome between p-RAB3A and unphosphorylated RAB3A in extracts from mouse brain. **A**. Experimental workflow. Purified Flag-tagged RAB3A or p-RAB3A were incubated with mouse brain lysates. The pulldown samples containing proteins that bound to either RAB3A or p-RAB3A were subjected to MS analysis (Orbitrap Astral). **B.** For purification, p-Flag-RAB3A was subjected to ion exchange chromatography on a MonoQ column and eluted with a 35% 1M NaCl gradient followed by gel filtration. **C.** Ion exchange chromatography fractions from p-RAB3A and RAB3A were resolved on either phos-Tag (upper panel) or SDS-PAGE gels (lower panel). **D**. Volcano plot of proteins with differential affinity for p-RAB3A as compared to RAB3A. The RAB3A effectors RIM1 and RIM2, along with other active zone proteins such as MUNC13 (UNC13) and ELKs (ERC1/2), preferentially interact with unphosphorylated RAB3A. In contrast, other proteins, such as RILPL1 and RILPL2, preferentially bind to p-RAB3A. In the volcano plot, proteins quantified by peptides matching the canonical sequence and a combination of their known isoforms are denoted by *, while a slash (/) indicates protein groups quantified through peptides shared between proteins.

Different color codes indicate proteins of interest. For RAB3A: purple, RIM1, RIM2; dark blue, ERC; orange, UNC isoforms. For p-RAB3A, orange: RILPL1, RILPL2. Data were visualized using the interactive Curtain tool and can be accessed through the CURTAIN link: https://curtain.proteo.info/#/7eb667fc-a390-4484-8894-31e8ba78cbb2 **E, F.** Violin plots showing the relative protein abundances of RIM2 and RILPL1 (DIA-NN 1.9.2) through interactions between RAB3A (RIM2; p-value 0.011) and p-RAB3A (RILPL1; p-value 0.002). Univariate statistical testing conducted via paired Student's t-test (Scipy version 1.11.1). N=6 replicates per condition.

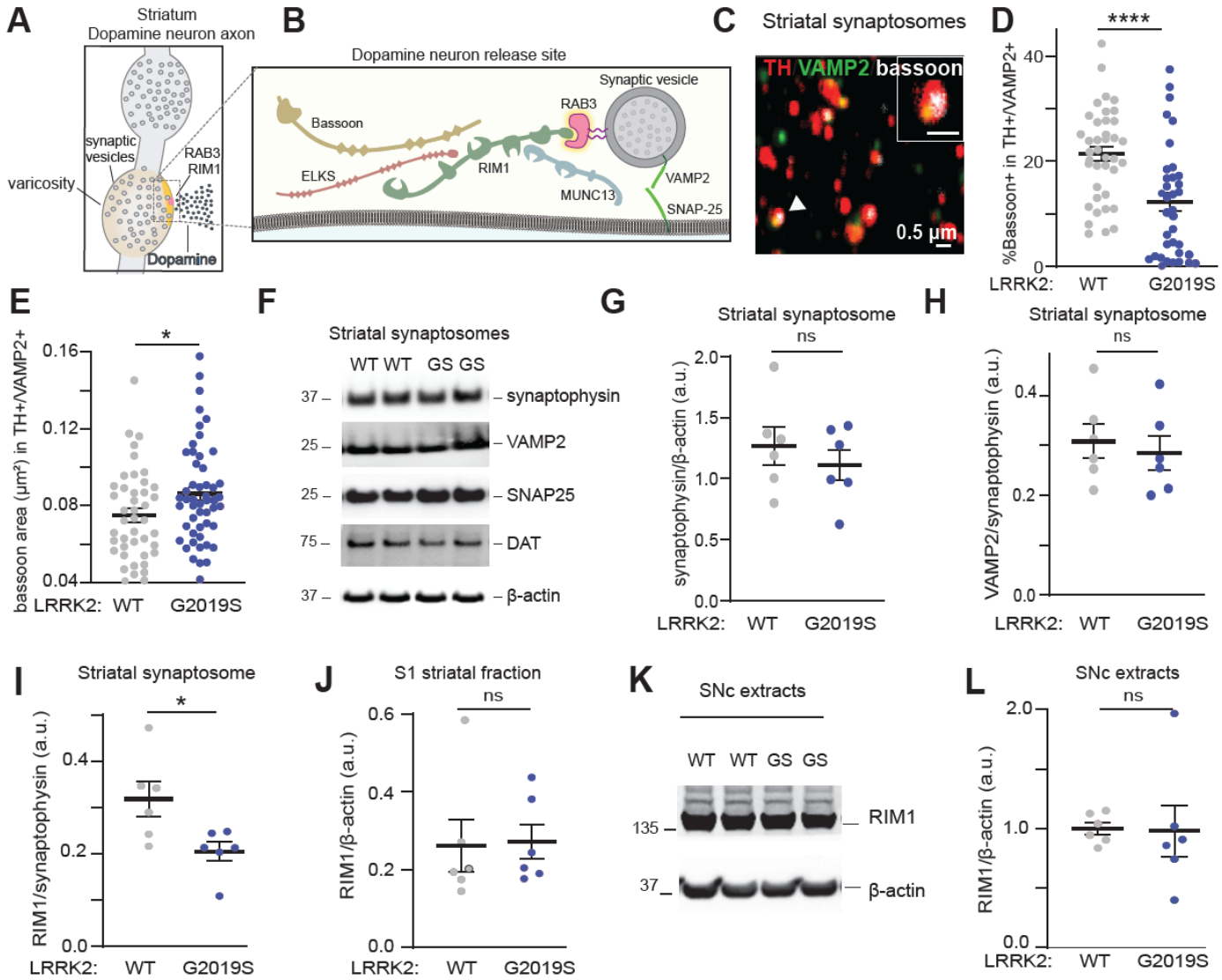


Figure 4: LRRK2 affects the composition of active zone sites in dopaminergic striatal synaptosomes. A. Schematic illustrating varicosities in dopamine axons, with active zone-like release site in orange. **B.** Dopaminergic axonal release site, with key molecular components as indicated**. C.** Confocal image of purified striatal synaptosomes stained with bassoon, VAMP2, and TH. The arrowhead indicates a TH+ striatal synaptosome (also shown in the inset), positive for both bassoon and VAMP2. Scale bar, 0.5µm **D.** Quantification of the percentage of TH+/VAMP2+ synaptosomes that contain bassoon from LRRK2^{WT} and LRRK2^{G2019S} mice. **E**. Quantification of area of bassoon staining in TH+/VAMP2+ synaptosomes. For data in D and E, each circle represents the average result of ~1,000 synaptosomes scored per imaging area. N=10-14 areas/3 mice per genotype. Data are mean± SEM. Asterisks indicate statistical significance after unpaired t-tests. *p <0.01, **** p<0.0001. **F.** Purified striatal synaptosome fractions from LRRK2^{WT} and LRRK2^{G2019S} mice (indicated WT, GS, respectively) were subjected to WB analysis and probed for the antibodies shown on the right. **G**. Quantification of synaptophysin/β-actin ratio in the striatal synaptosome preparations shows no difference between LRRK2^{WT} and LRRK2^{G2019S} mice. Synaptophysin was used as a loading control for further comparisons across genotypes. **H-I** Quantification of indicated proteins in striatal synaptosome fractions from LRRK2^{WT} and LRRK2^{G2019S} mice (RIM1 representative blot is shown in Supplementary Figure 5F). **J.** Quantification of RIM1 in the S1 striatal fraction. **K.** WB analysis of SNc extracts from LRRK2^{WT} and LRRK2^{G2019S} mice using RIM1 and β-actin antibodies. **L.** Quantification of RIM1 signal in SNc extracts across different genotypes. Equal amounts of proteins were loaded in each lane, and duplicate lanes represent biological replicates. Data represent mean±SEM. Asterisk shows significance after the unpaired t-test. *p <0.01. Ns = no significant difference. N=6 mice/genotype.

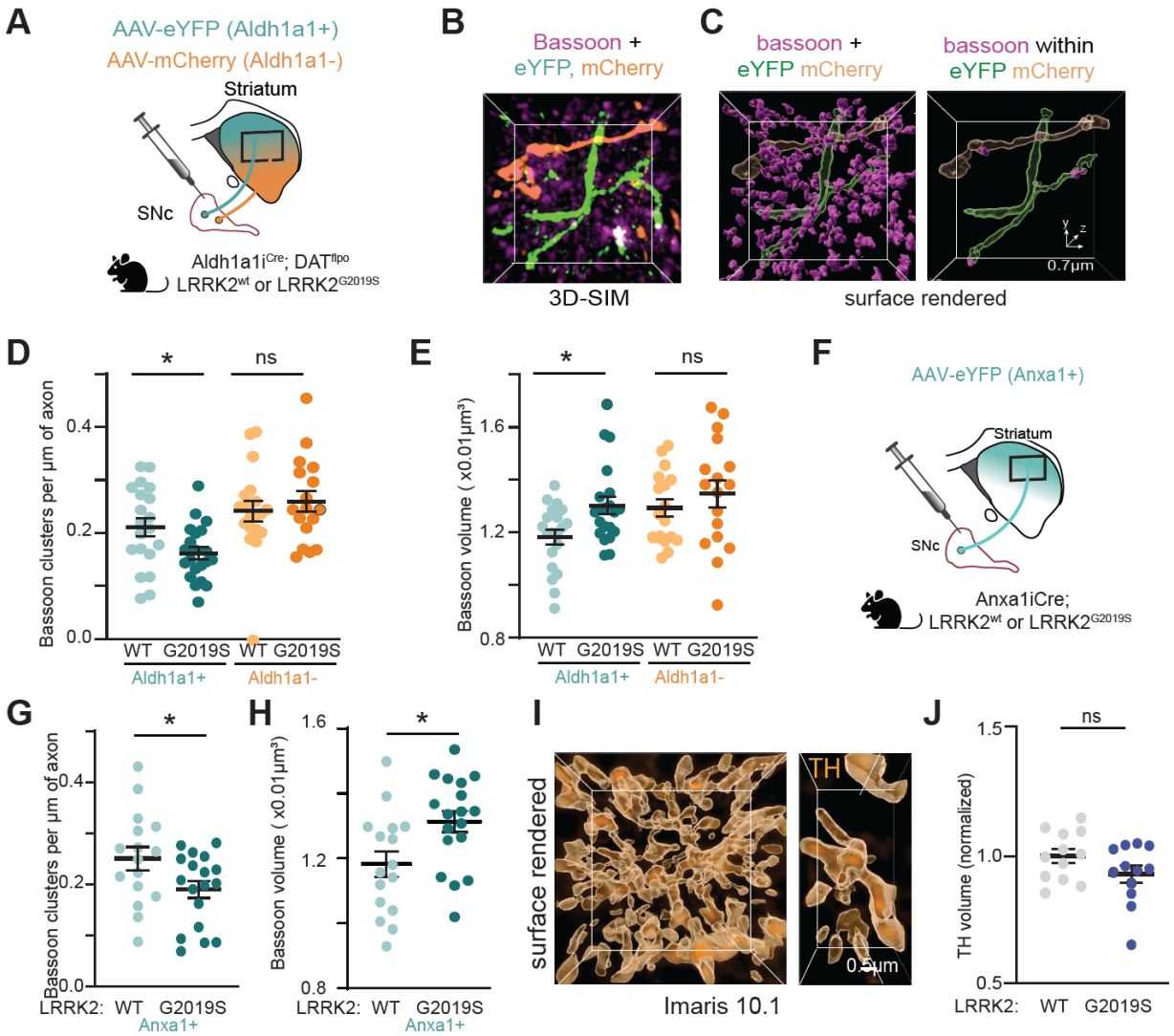


Figure 5: LRRK2 preferentially impacts active zone release sites in vulnerable dopamine neurons. A. Simultaneous intersectional/subtractive viral strategy to label SNc Aldh1a1+ and Aldh1a1- dopamine neuron subtypes and their projections in the striatum of Aldh1a^{Cre}; DAT^{flpo} LRRK2^{WT} and LRRK2^{G2019S} mice. **B, C.** Representative 3D-SIM image and its surface rendering in Imaris 10 showing distribution of bassoon clusters (magenta) in the whole field and within Aldh1a1+ (green) and Aldh1a1- (orange) axons in the dorsal striatal slices. **D.** Quantification of bassoon cluster density in Aldh1a1+ and Aldh1a1- dopamine axons. **E.** Quantification of bassoon cluster volume in Aldh1a1+ and Aldh1a1- dopamine axons. In D and E, each circle represents the average value from a section containing 25,000-35000 bassoon clusters; n=17-21 (4-5 sections/mouse, 4-5 mice/group). Data are represented as mean±SEM. Asterisk indicates statistical significance with Šídák's multiple comparisons test following a two-way ANOVA test. *p<0.05. (D: Cell type factor F(1, 71)=12.75 p=0.0006, Genotype factor F(1,71)=1.277, p=0.2622, interaction F(1,71)=4.838 p=0.0311). E: Cell type factor F(1, 69)=4.607, p=0.0354, Genotype factor F(1,69)=1.277, p=0.0252, interaction F(1,69)=0.8688, p=0.3545 **F.** Strategy used to label SNc Anxa1i^{Cre} dopamine neuron subtypes and their projections in the striatum of Anxa1i^{Cre} LRRK2^{WT} and LRRK2^{G2019S} mice. **G.** Quantification of bassoon cluster density in Anxa1i^{Cre} dopamine axons. **H.** Quantification of bassoon cluster volume in Anxa1i^{Cre} dopamine axons. Each circle represents the average value from a section containing 15,000-40000 bassoon clusters; n=16-18 (4-5 sections/mouse, 4-5 mice/group). Data are represented as mean±SEM. Asterisk shows unpaired t-test. *p<0.05 **I.** Surface-rendered 3D image (Imaris 10.1) acquired with Nikon SoRa confocal instrument in dorsal striatum showing TH immunostaining. **J.** Quantification of the total volume occupied by TH staining (axon fibers) in the dorsal striatum. Data represent mean±SEM. Each circle is the average of a section; n=12 (8 images/section, 3-4sections/mouse, 3-4 mice/group). No statistical significance after the unpaired t-test.

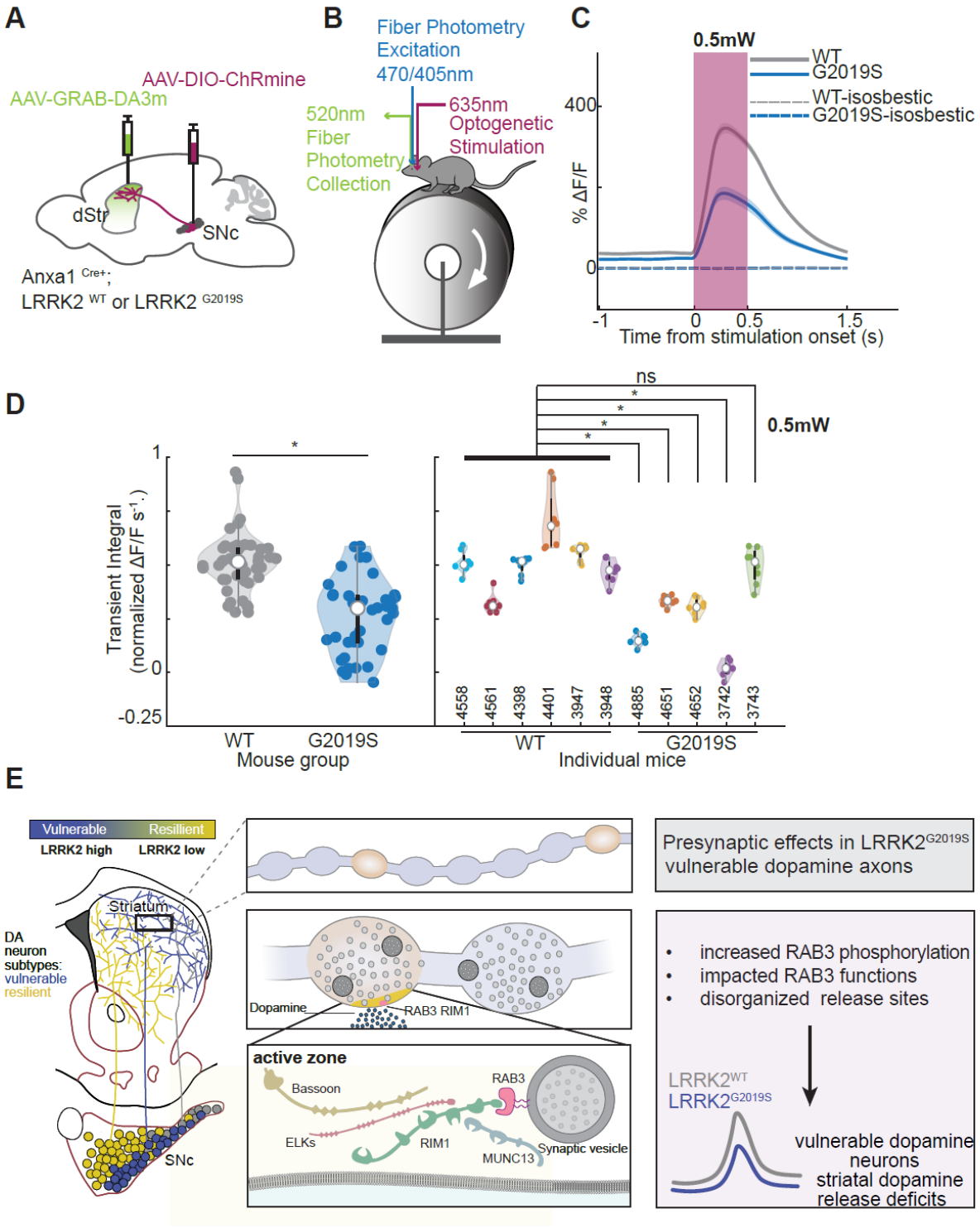
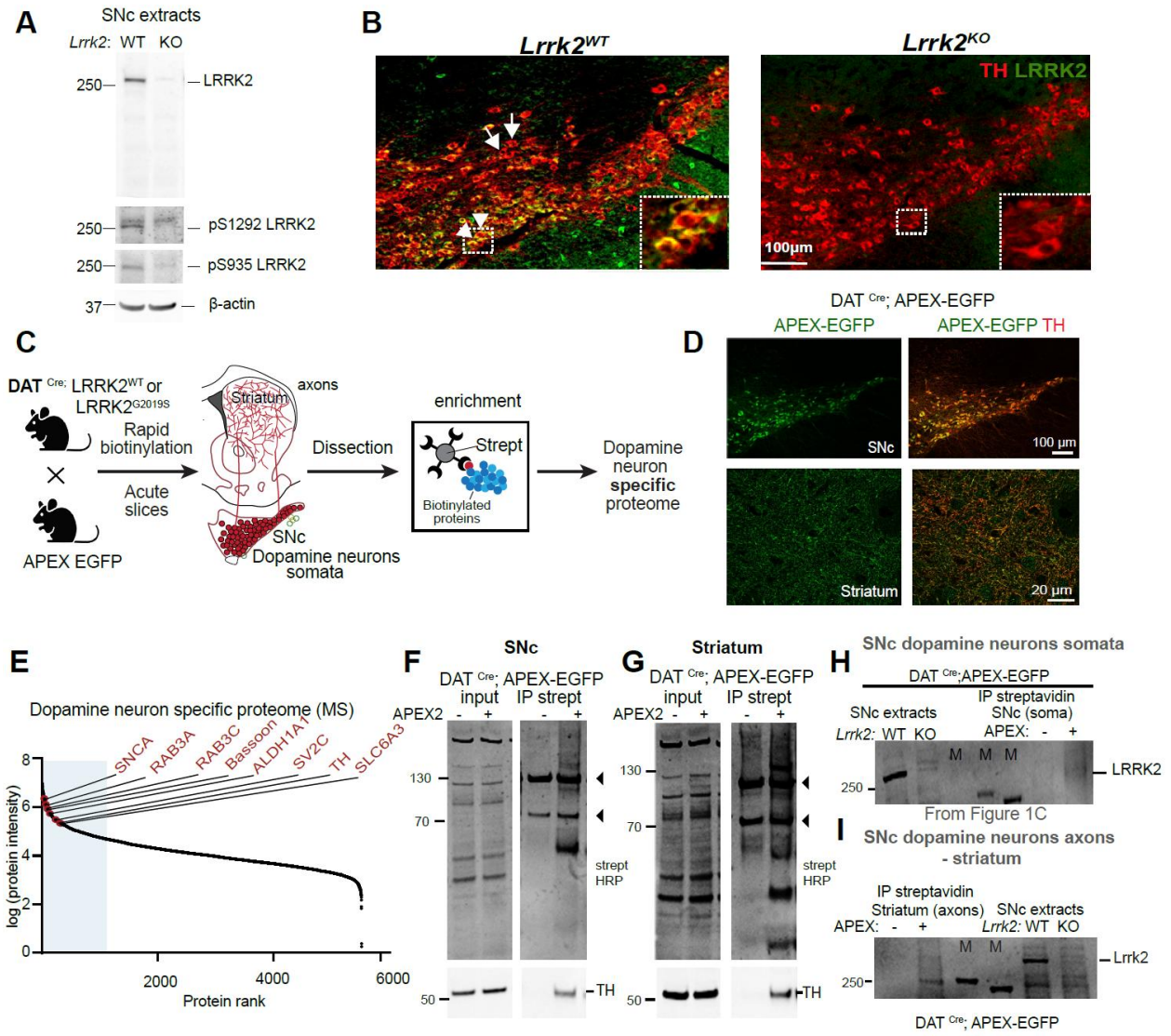


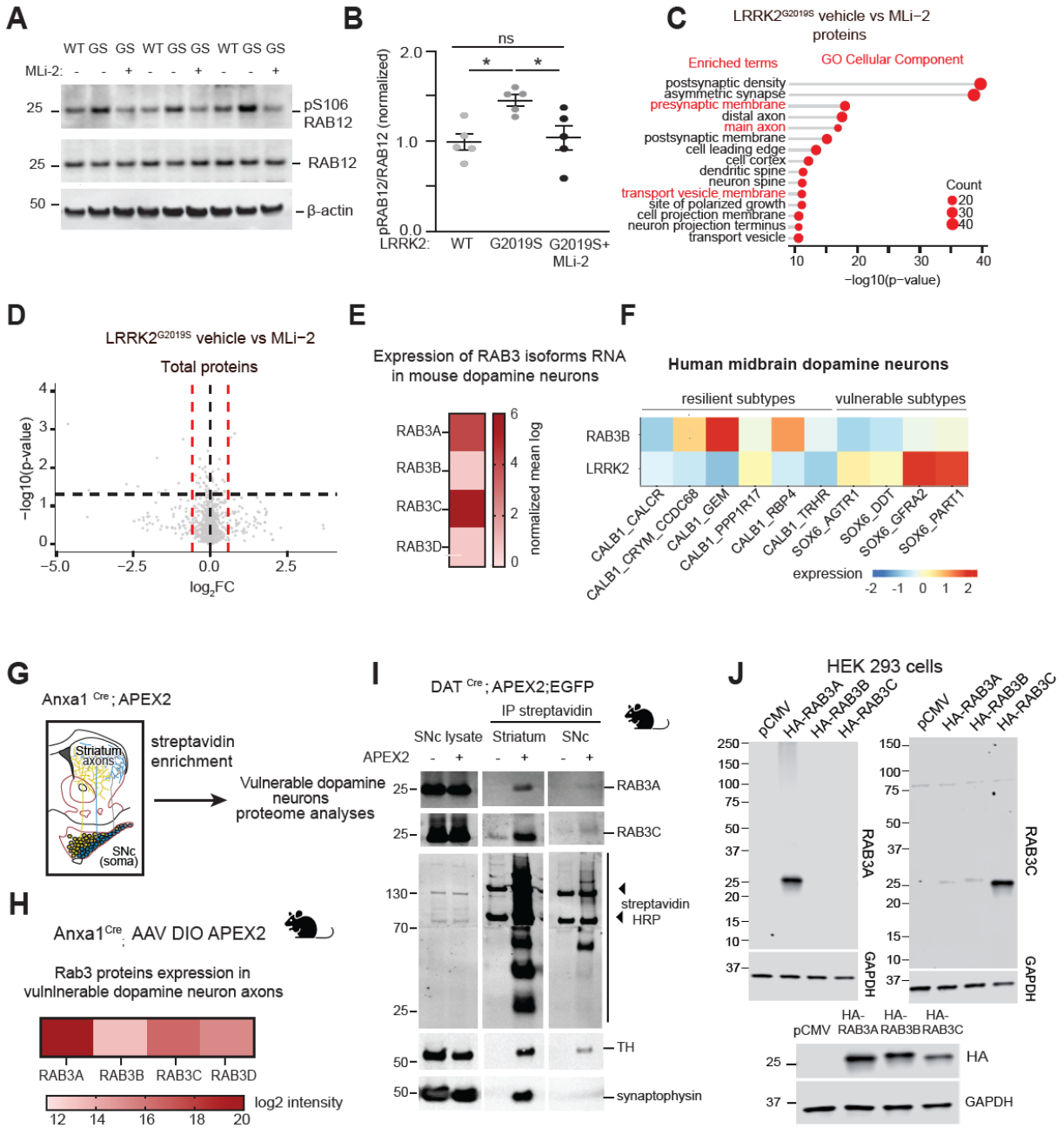
Figure 6. In vivo evoked dopamine release is reduced in LRRK2^{G2019S} compared to control mice.
A. Strategy used to label Anxa1+ dopamine neurons in the SNc of LRRK2^{WT} or LRRK2^{G2019S} mice with a red-shifted channelrhodopsin, ChRmine, and to express the fluorescent dopamine sensor, GRAB-DA3m, in the dorsal striatum (dStr) for measuring dopamine release (ΔF/F). **B.** Schematics of optogenetic stimulation (in SNc) and fiber photometry recording (dStr) setup. **C.** Evoked dopamine release triggered on optogenetic simulation: Average GRAB-DA3m ΔF/F triggered on optogenetic stimulation light onset with 0.5mW light power (blue trace, LRRK2^{G2019S} mice = 5, n = 40 stimulations; grey trace, WT mice = 6, n = 48 stimulations; red, light stimulation time). Dashed lines, isosbestic control. Shaded regions denote mean ± SEM across stimulations. **D.** Violin plots of optogenetically evoked (integrated) dopamine release response with

0.5mW stimulation light power (normalized integral of GRAB-DA3m ΔF/F during the optogenetic-stimulation light-on period). White dots denote group means and black bars denote 25th to 75th percentile. Left, group-level comparisons ($LRRK2^{G2019S}$ n = 40 stimulations, WT n = 48 stimulations; p-value = 2.01E-11, unpaired t-test). Right, Violin plots of optogenetically evoked (integrated) dopamine release response in individual mice. Numbers denote mouse ID (n = 8 stimulations/mouse; p-value (4885) = 5.32E-10, p-value (4651) = 0.0004, p-value (4652) = 8.02E-5, p-value (3742) = 2.32E-14, p-value (3743) = 0.75). Asterisks (*) on the right denote p-value < 0.01 from Welch's t-test with Bonferroni correction ($\alpha = 0.05/5 = 0.01$) comparing individual $LRRK2^{G2019S}$ mice to the WT group. **E.** Summary graphical abstract of this study.



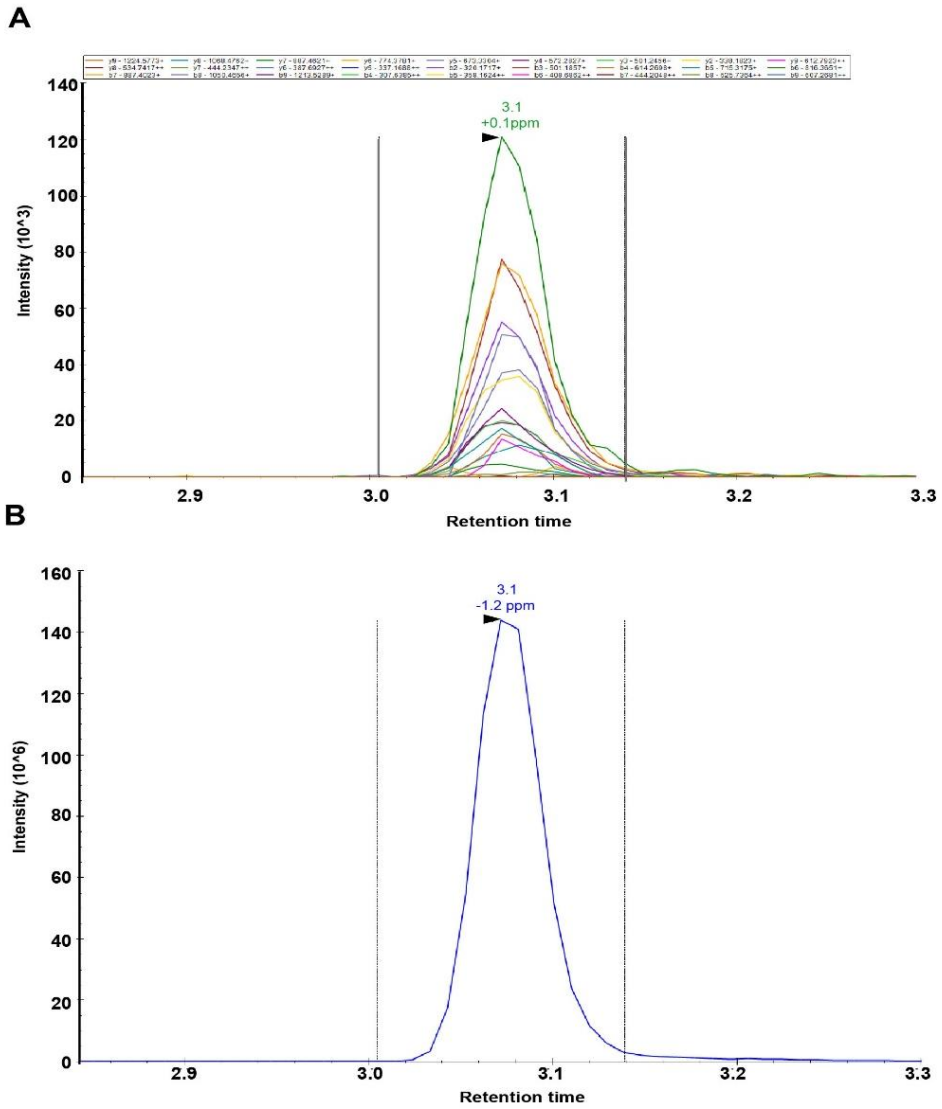
Supplementary Figure 1. LRRK2 protein is expressed in SNc dopamine neurons (linked to Figure 1). A. WB analysis of SNc brain extracts from *Lrrk2*^{WT} and *Lrrk2*^{KO} mice probed with the antibodies indicated on the right. Note the absence of the LRRK2 signal in the *Lrrk2*^{KO} tissues. **B.** Brain sections from *Lrrk2*^{WT} and *Lrrk2*^{KO} mice were stained with the LRRK2 antibody from A, along with TH. **C.** Workflow of APEX2-based proximity labeling within genetically labeled dopamine neurons in the mouse brain. Acute brain slices from DAT^{Cre}; LRRK2^{WT} and DAT^{Cre}; LRRK2^{G2019S} mice crossed with the APEX2 EGFP reporter mouse line[45] were rapidly biotinylated, quenched, and dissected. Tissues were lysed and precipitated to remove free biotin. Tissue proteins were subjected to streptavidin bead purification to enrich biotinylated proteins. On-bead digestion yielded peptides enriched from dopamine neurons, which were analyzed using mass spectrometry. **D.** Representative images showing the cre-dependent expression of EGFP reporter along with Th immunostaining in the SNc and striatum of a DAT^{Cre}; LRRK2^{WT}; APEX2 EGFP mouse. **E.** 85% of the top 55 mDA neuron marker genes (e.g., TH and DAT; SLC6A3) from a publicly available APEX2 proteome dataset (dataset identifier PXD026229 ProteomeXchange Consortium) were detected in all our mass spectrometry samples from DAT^{Cre}; APEX2 EGFP mice. 40% of these markers were among the top 20% (1105/5525) (shaded light blue) of the most highly expressed proteins by intensity. **F.** WB analysis of biotinylated protein inputs and their streptavidin pulldowns from dissections of the indicated brain regions probed with antibodies as indicated on the right. Input, 10 µg of total protein (~1% of striatal lysate, and ~5% of SNc); equal volume of eluted proteins was loaded in each lane. **G**. Full gel from experiment presented in Figure 1C showing SNc extracts from *Lrrk2*^{WT} and *Lrrk2*^{KO} mice for reference of LRRK2 protein size. **H.** WB showing streptavidin pulldowns from equal volumes of proteins from the striatum of DAT^{Cre}; APEX2 EGFP mice or Cre- controls; SNc lysates from *Lrrk2*^{WT} and *Lrrk2*^{KO} are used for reference for LRRK2 protein size. M, lanes loaded with molecular size markers.

[44]s.



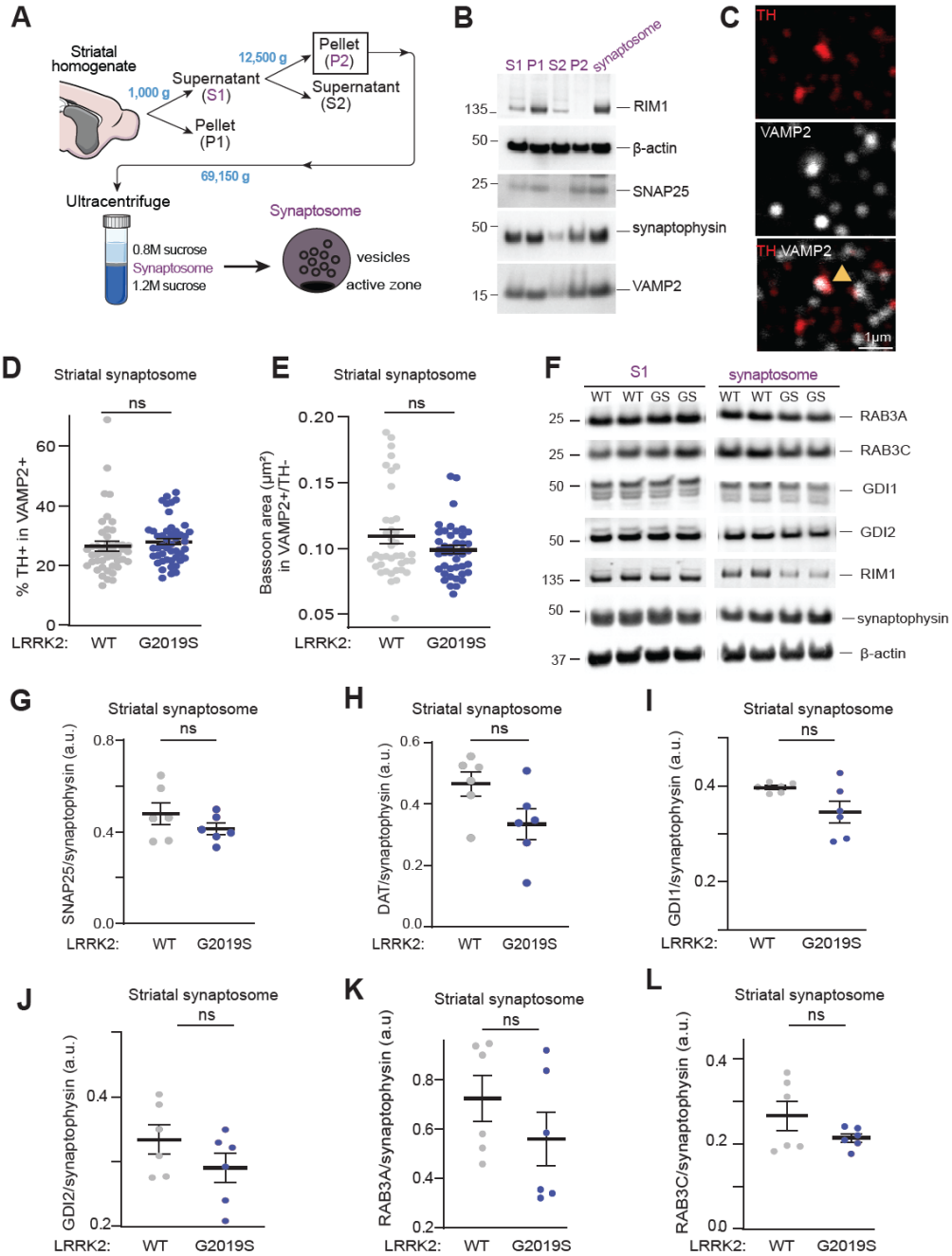
Supplementary Figure 2. Heterogeneous RAB3 isoform expression across dopamine neuron subtypes (linked to Figure 2). A. WB analysis of SNc extracts from mice previously treated with MLi-2 (10 mg/kg) or vehicle for 2 h and probed for pS106 RAB12 (LRRK2 kinase target), total RAB12, and β-actin. **B.** Quantification of p-RAB12 band intensities normalized to total RAB12. n=5 mice/condition. Data are represented as mean±SEM (error bars). Asterisks denote statistical significance for Šídák's multiple comparison tests after two-way ANOVA. *p< 0.05. (Treatment factor F(1,12)=8.614 p=0.0125, Genotype factor F(1, 12)=10.64, p=0.0068) **C.** Gene Ontology analysis of proteins with at least one differentially regulated phosphopeptide in striatal synaptosomes from vehicle vs MLi2-treated LRRK2^{G2019S} mice. The top 15 pathways significantly enriched in the cellular component term analysis (adjusted *p*-value ≤ 0.05 by hypergeometric test with HB correction). All enriched pathways have been uploaded to Zenodo. Link in the resources table.**D.** Volcano plot comparing the significantly altered total proteins (|Log2FC| > 0.58 and unadjusted *p*-value ≤ 0.05 by multiple unpaired t-tests) between vehicle and MLi-2-treated LRRK2^{G2019S} striatal synaptosomes. **E**. Heat map of the expression of RAB3 isoforms in a dataset of single cell RNA sequencing.[9] The differential expression of RAB3 isoforms in TH+ SNc neurons compared to the remaining non-TH SNc neurons is expressed as normalized mean log values

(RAB3A = 4.11, RAB3B = 0.693, RAB3C = 5.59, and RAB3D = 0.693). **F.** Heat map illustrating the expression of LRRK2 and RAB3B isoform in human dopamine neuron subpopulations from a single nucleus RNA sequencing dataset,[49] plotted with Shinny Cell.[931] **G.** Workflow of APEX2 experiment for dopamine neuron subpopulation-specific analysis with subcellular compartment resolution. Cre-dependent APEX2 expressing AAV (AAV5-CAG-DIO-APEX2-NES) was injected into the SNc of Anxa1^{Cre} LRRK2^{WT} mice for Anxa1+ dopamine neuron-specific APEX2 labeling. **H.** Relative expression of RAB3 protein isoforms in our Anxa1+ dopamine axon subcluster proteomic dataset (for further details see Supplementary Figure 6)**. I.** Equal amounts of eluted proteins from streptavidin pulldowns from the striatum and SNc from either a LRRK2^{WT} or a LRRK2^{G2019S} DAT^{Cre} mouse injected with APEX2 AAV**. J.** HEK293T cells were transiently transfected with either pCMV or HA-tagged RAB3 isoforms and blotted with isoform-specific antibodies or anti-HA, and with GAPDH for loading control. Equal expression of all isoforms is shown in the panel below.

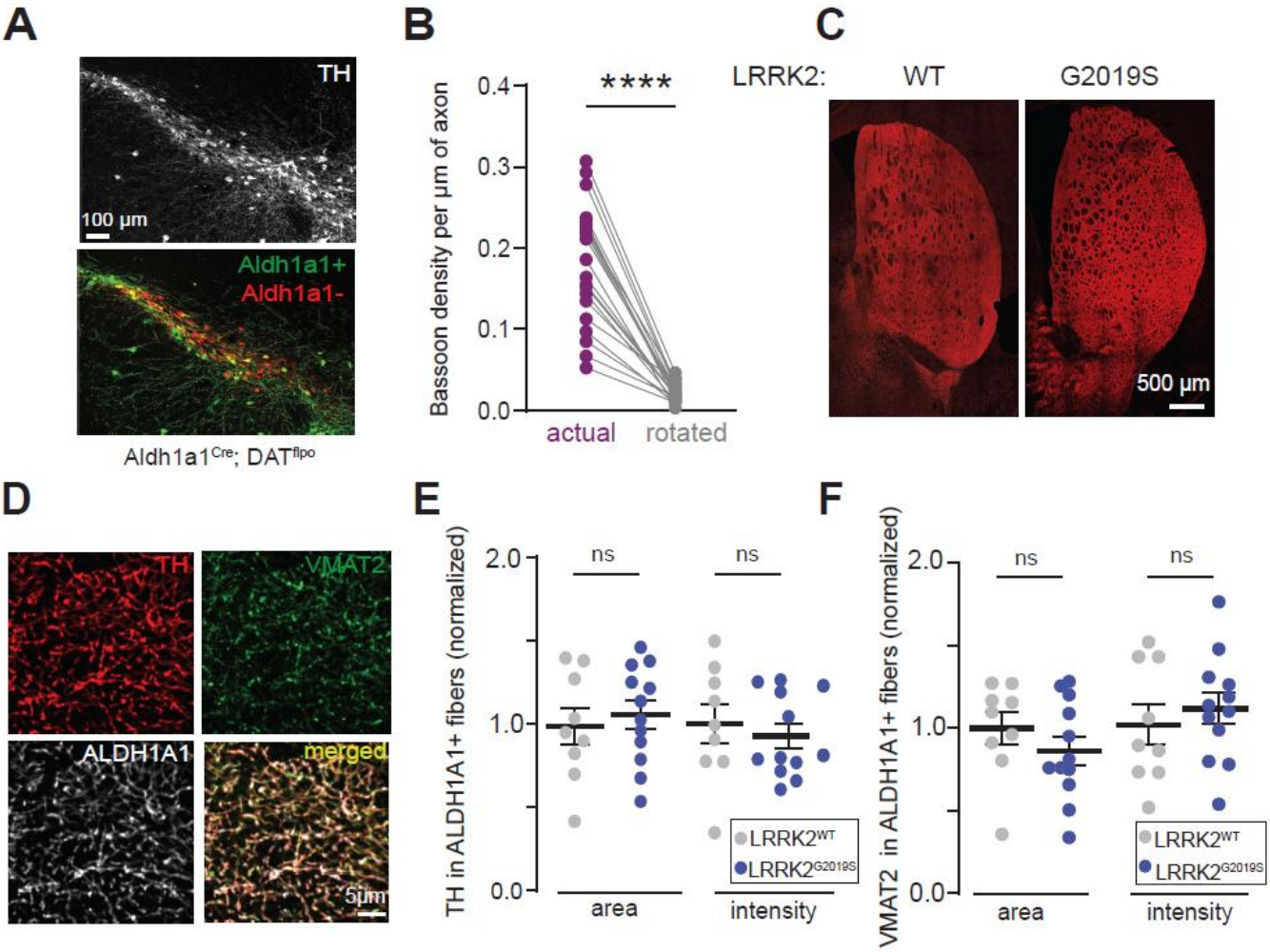


Supplementary Figure 3. Differential interactome of p-RAB3A and unphosphorylated RAB3A in extracts from mouse brain (linked to Figure 3).

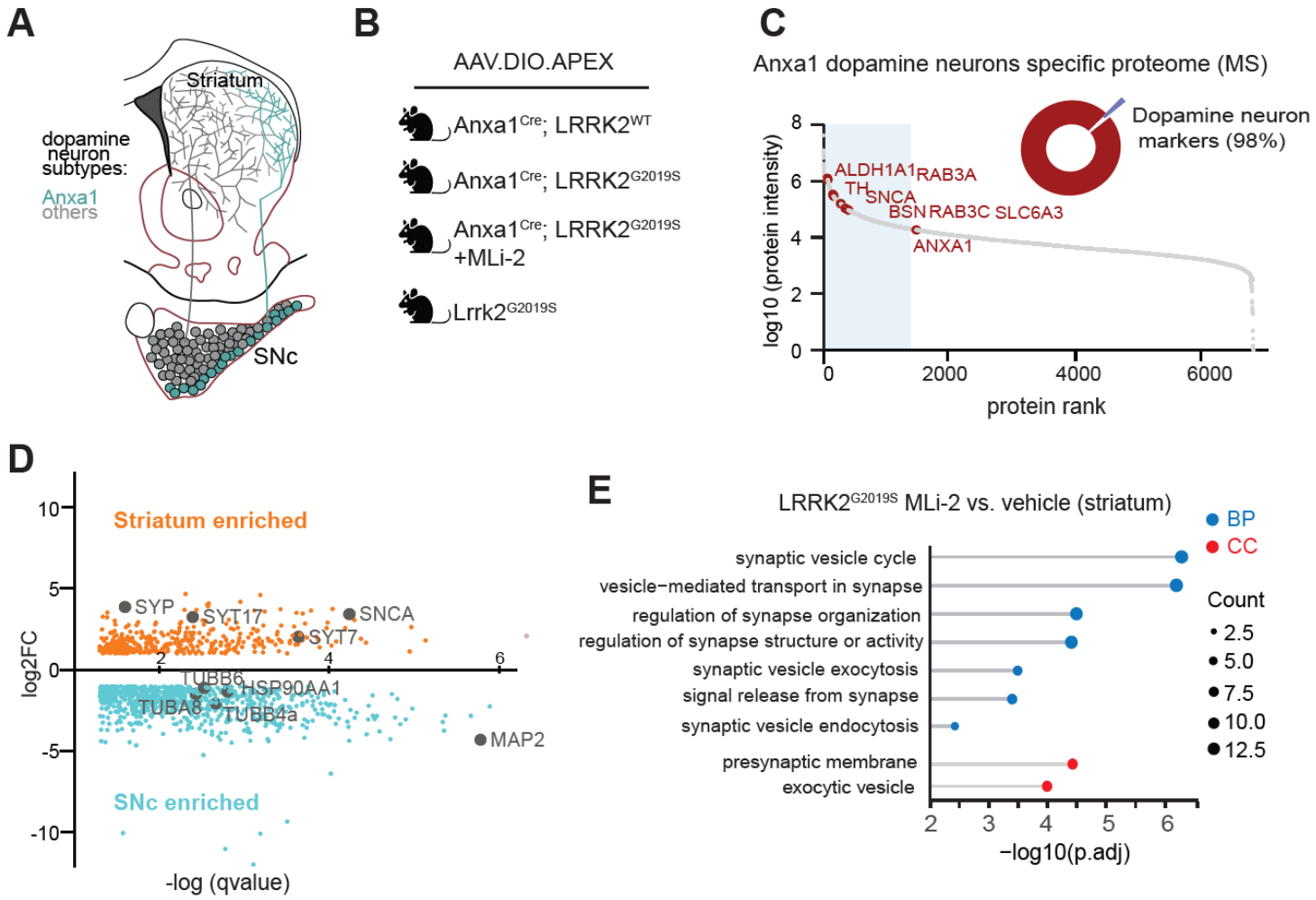
A. Peptide fragment ions of the phosphopeptide; YRTITTAYR, of the 2+ precursor ion **(B).** which spans phosphorylation site T86 in recombinant p-Flag-RAB3A protein. All chromatograms elute at the same retention time, with peptide identification at a false-discovery rate of <1% (DIA-NN 1.9.2).



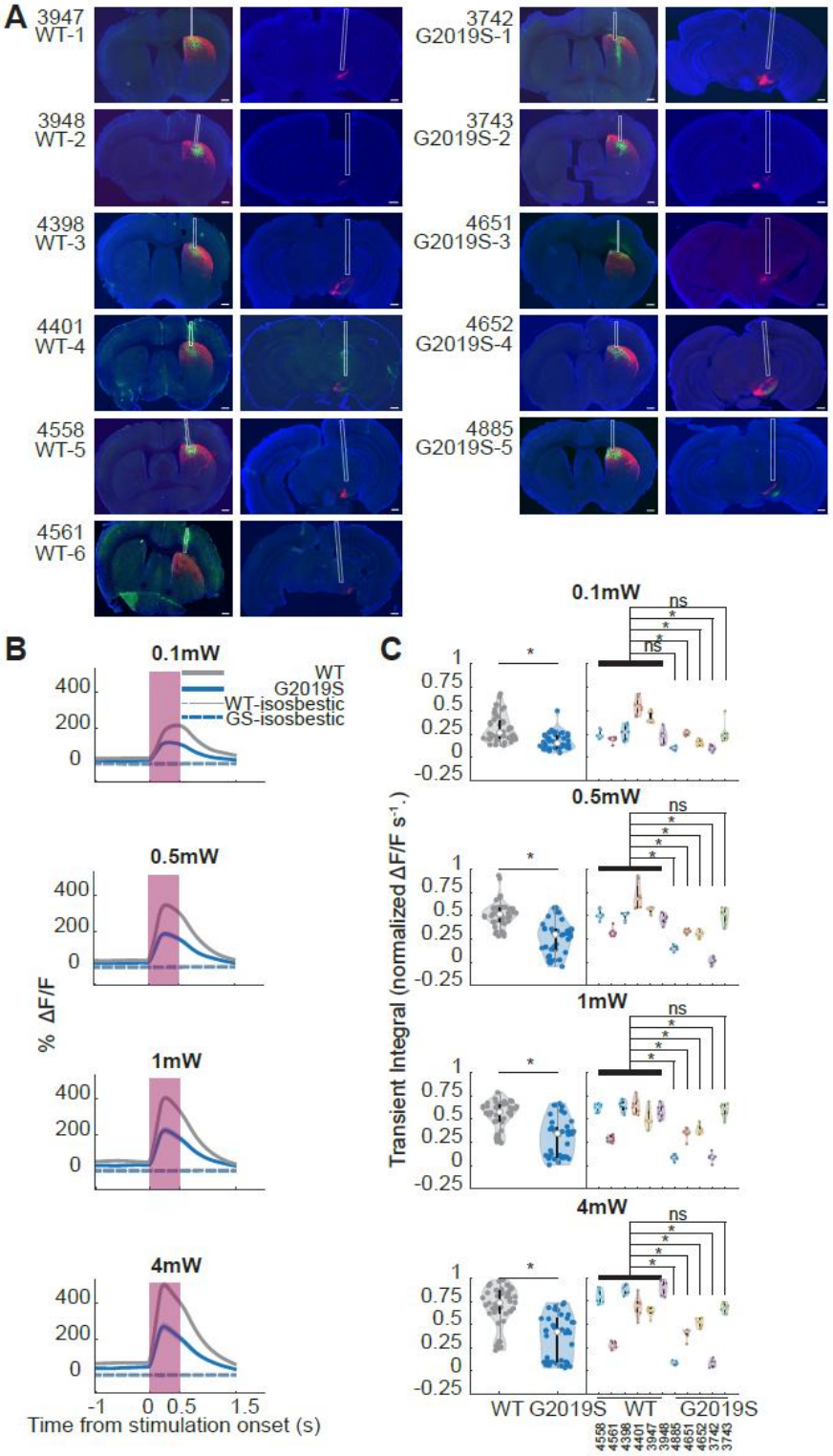
Supplementary Figure 4. LRRK2 alters the distribution and organization of active zones in striatal synaptosomes (linked to Figure 4). A. Schematic representation of the steps for striatal synaptosome fraction preparation**. B.** WB analysis of selective fractions from striatal subcellular fractionation, probed with antibodies as indicated on the right. This analysis shows enrichment of synaptic vesicle and active zone proteins. **C.** Confocal image of striatal synaptosomes stained with TH and VAMP2. A double positive synaptosome is indicated with the arrowhead. **D.** Quantification of the percentage of TH+/VAMP2+ striatal synaptosomes in LRRK2^{WT} and LRRK2^{G2019S} mice (denoted by WT and GS, respectively). Each circle represents the average result of an area with around 1,000 synaptosomes. n = 10-14 areas/4 mice per genotype. **E**. Quantification of bassoon area in VAMP2+ but TH- synaptosomes. Each circle represents the average of an image with ~1,000 synaptosomes. n=10-14 areas/4 mice per genotype. Data in D and E represent mean± SEM. Ns denotes no statistical significance after unpaired t-tests. **F.** WB analysis of S1 and synaptosome fraction from LRRK2^{WT} and LRRK2^{G2019S} mice using the antibodies shown on the right. **G-L**. Quantification of the indicated protein signals in striatal synaptosomes from LRRK2^{WT} and LRRK2^{G2019S}. Data are mean± SEM. Ns =non-significant differences in comparisons after unpaired t-tests. n= 6 mice/genotype. Equal amounts of proteins were loaded in all experiments, and duplicates represent biological replicates.



Supplementary Figure 5. LRRK2^{G2019S} changes the number and composition of active zones without affecting overall integrity of TH fibers (linked to Figure 5). A. SNc sections from Aldh1a1^{Cre}; DAT^{flpo} mice injected with viral plasmids stained with EGFP and mCherry to label Aldh1a1+ and Aldh1a1- dopamine neurons, respectively. **B**. Image with bassoon signal in control mice was rotated by 180°, whereas the eGFP channel remained unaltered. Quantification of bassoon density within dopamine axons before and after image rotation. Each circle represents the average result of a region containing 25,000-35,000 bassoon clusters. Data represent mean ±SEM. n=18 (3-6 sections/mouse, 4 mice). Significant differences in comparisons after unpaired t-tests. **** $p<0.0001$. **C.** Representative low magnification striatal sections from 6-month-old LRRK2^{WT} and LRRK2^{G2019S} mice stained with TH. **D**. Representative high magnification dorsal striatal sections from adult (6 months) LRRK2^{WT} and LRRK2^{G2019S} mice stained with TH, ALDH1A1, and VMAT2 antibodies. **E**. Quantification of TH area and fluorescence intensity in ALDH1A1 immunostained dopamine axons across genotypes in dorsal striatum. **F.** Average VMAT2 area and intensity in TH+/ALDH1A1+ dopamine axons across genotypes in dorsal striatum. For E and F, each dot represents the mean area or intensity of a striatal section, and data show mean±SEM; n=9-12 (2-3sections/mouse, 4-5mice/group). Ns, not significant with unpaired t test**.**



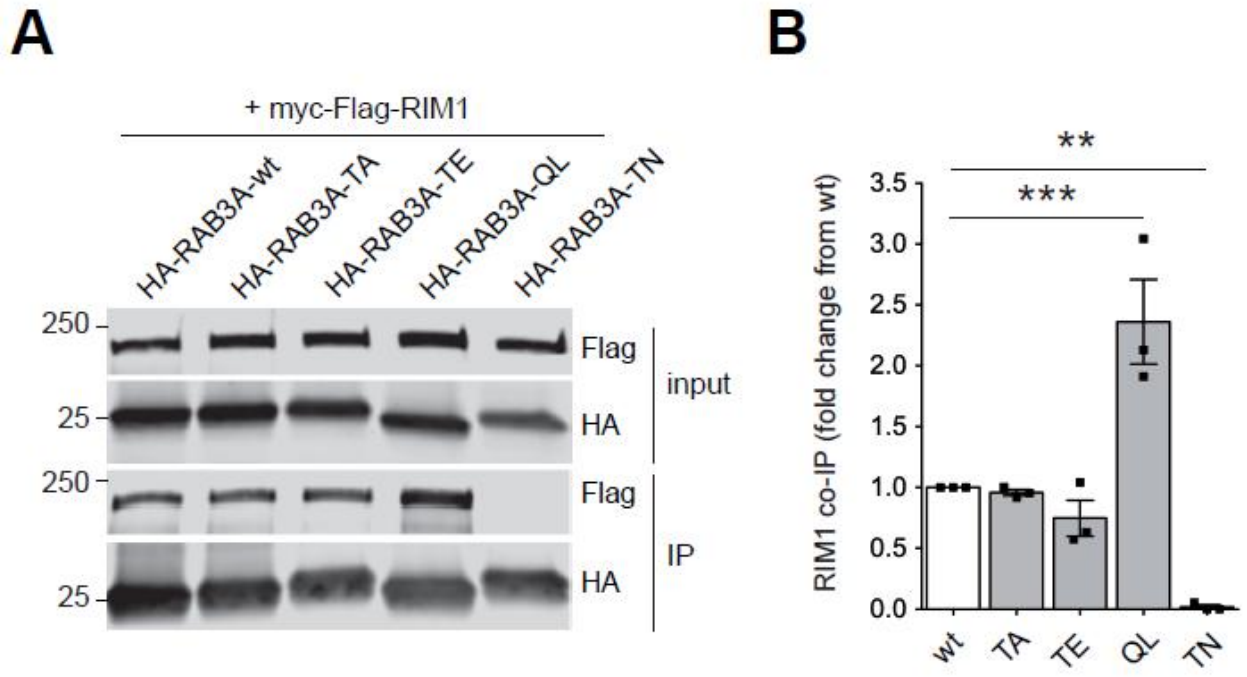
Supplementary Figure 6. Axonal and somatic proteomics of Anxa1+ neurons (linked to Figure 5). A. Schematic illustrating the localization of Anxa1 dopamine neuron subtype in the mouse SNc and their projection patterns in the striatum. **B.** Experiment groups of APEX2-based proximity labeling within genetically labeled dopamine neurons in the mouse brain. **C**. 98% of the top 55 mDA neuron marker genes (e.g., TH and DAT (SLC6A3)) from a publicly available APEX2 proteome dataset (dataset identifier PXD026229 ProteomeXchange Consortium) were detected in all our mass spectrometry samples from DAT^{Cre}; APEX2 EGFP mice. **D.** Differential expression comparison of SNc and Striatum APEX+ streptavidin pulldown samples. Proteins colored orange or blue indicated significantly enriched in Striatum vs. SNc, respectively. ($|Log2FC| > 0.58$ and unadjusted p-value ≤ 0.05 by multiple unpaired t-tests) **E.** Gene Ontology analysis of proteins with at least one differentially regulated phosphopeptide in striatum from vehicle vs. MLi2-treated Anxa1iCre LRRK2^{G2019S} mice. BP, Biological Process; CC, Cellular Component refer to categories in GO. All enriched pathways have been uploaded to Zenodo; the link can be found in the key resources table.



Supplementary Figure 7. Histology and power dependence for in vivo evoked dopamine measurements (linked to Figure 6).

A. Histological verification of viral expression and fiber placement in each animal. Left two columns, coronal sections of the dorsal striatum region (dStr) and the Substantia Nigra pars compacta (SNc) of each LRRK2^{WT} mouse used in the analysis. Red fluorescence indicates the expression of ChRmine in the somas (SNc) and the axons (dStr) of Anxa1+ dopaminergic neurons. Green fluorescence indicates the expression of GRAB-DA3m in the dorsal striatum. White rectangles indicate where the photometry (dStr) and optogenetics (SNc) fibers were placed. Right two columns, same as

left, but in LRRK2^{G2019S} mice. Scale bar = 0.5 mm. **B.** Same as Figure 6C but with 0.1 mW, 0.5 mW, 1 mW, and 4 mW optogenetic light powers from top row to bottom row (blue trace, LRRK2^{G2019S} mice = 5, n = 40 stimulations; grey trace, LRRK2^{WT} mice = 6, n = 48 stimulations). Shaded regions denote mean ± SEM across stimulations. **C.** Same as Figure 6D but with 0.1 mW, 0.5 mW, 1 mW, and 4 mW optogenetic light powers from top row to bottom row. White dots denote group means and black bars denote 25th to 75th percentile. Left, group comparisons at each power (LRRK2^{G2019S} n = 40 stimulations per power, LRRK2^{WT} n = 48 stimulations per power; p-value (0.1 mW) = 4.17E-7, p-value (0.5 mW) = 2.01E-11, p-value (1 mW) = 1.97E-9, p-value (4 mW) = 7.02E-10, unpaired t-test). Right, dopamine release response in individual mice. Numbers denote mouse ID. Asterisks (*) on the right denote p-value < 0.01 from Welch's t-test with Bonferroni correction ($\alpha = 0.05/5 = 0.01$) comparing individual LRRK2^{G2019S} mouse to the LRRK2^{WT} group.



Supplementary Figure 8. Phosphomimetic RAB3 constructs fail to reproduce the effects of phosphorylated RAB3 proteins (linked to Figure 3).
A. HEK293 cells were transiently co-transfected with HA-tagged RAB3A constructs and myc-flag-RIM1. Extracts were subjected to pulldown with anti-HA beads, and input and immunoprecipitated material (IP) subjected to blotting with the indicated antibodies. WT, phospho-deficient (RAB3A-T86A) and phospho-mimic (RAB3A-T86E) RAB3A show similar binding to tagged RIM1. In contrast, and as expected, the RAB3A mutant mimicking the GTP-bound state (Q81L) shows increased RIM1 binding, whilst the RAB3A mutant mimicking the GDP-bound state (T36N) shows loss of RIM1 binding. **B**. Quantification of experiments of the type shown in A. Co-immunoprecipitation of myc-flag-RIM1 with the indicated HA-tagged RAB3A constructs was normalized to that obtained with wild-type (wt) RAB3A. n=3 independent experiments (mean±SEM). Asterisk indicates statistical significance as determined by one-way ANOVA with Dunnett's multiple comparisons. ***$p<0.001$; **$p<0.0$.