

Cell type-specific synucleinopathy reveals differential vulnerability and dysfunction across catecholaminergic circuits

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Abstract

Synucleinopathies are defined by pathological α -synuclein aggregation, yet how this pathology produces selective neuronal vulnerability and distinct symptom dimensions remains unclear. Here, we used a Cre-dependent viral strategy to express A53T- α -synuclein in two vulnerable catecholaminergic populations, dopaminergic neurons of the substantia nigra pars compacta and noradrenergic neurons of the locus coeruleus, and combined this approach with circuit-sensitive behavioral analysis. Although the same pathogenic trigger induced aggregation and neurodegeneration in both nuclei, the resulting trajectories diverged. Nigral synucleinopathy produced earlier and more severe degeneration together with progressive motor impairment, whereas locus coeruleus synucleinopathy followed a slower degenerative course and revealed behavioral consequences only in a linear track paradigm, where mice showed mild anxiety-like avoidance without overt motor impairment. These findings indicate that α -synuclein pathology is translated through neuronal and circuit context, supporting synucleinopathies as multisystem circuitopathies resolvable at cell-type resolution.

Introduction

The pathological accumulation of alpha-synuclein (α Syn) is a defining feature of synucleinopathies and is thought to play a central role in neuronal dysfunction and degeneration that characterize this group of neurodegenerative disorders^{1–3}. Yet, despite extensive work on the molecular mechanisms of α Syn misfolding, aggregation, and propagation, it remains unclear how this pathology translates into selective patterns of neuronal vulnerability and circuit dysfunction^{4–6}. This question is particularly important because α Syn is widely expressed and supports normal synaptic function throughout the nervous system, whereas neurodegeneration and clinical symptoms emerge in a strikingly non-uniform manner across cell populations, neuromodulatory systems, and functional domains^{7–9}. Understanding synucleinopathies, therefore, requires going beyond molecular pathology itself to examine how α Syn accumulation differentially affects defined cell types within specific circuits.

Parkinson's disease (PD), the most prevalent synucleinopathy, provides a paradigmatic example of this dissociation between the broad distribution of α Syn pathology and the selective nature of disease progression^{9,10}. Historically viewed as a motor disorder driven by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and dysfunction of the nigrostriatal dopaminergic system, PD has long been defined by its cardinal motor signs—bradykinesia, rigidity, resting tremor, and postural instability. It is now clear, however, that PD encompasses a much broader clinical spectrum, including non-motor symptoms such as anxiety, depression sleep disturbances, cognitive impairment, and autonomic dysfunction^{11–14}. Many of these symptoms precede motor onset by years, or even decades, indicating that disease progression cannot be explained solely by motor circuit dysfunction²⁰. This broader clinical picture has progressively shifted the classical view of PD from a disorder primarily driven by nigrostriatal degeneration toward a framework in which it is understood as a multisystem neurodegenerative disease causing circuit dysfunction¹⁵.

Yet, much of the experimental and conceptual framework of PD remains organized around a dopaminergic, motor-centered axis. Although this perspective has been instrumental in linking synucleinopathy, nigrostriatal degeneration, and motor symptoms, it has also narrowed the disease concept to its most clinically visible manifestations¹⁶. As a result, the early involvement of other vulnerable nuclei has received comparatively less attention, limiting our ability to explain mechanistically how non-motor dimensions of the clinical syndrome emerge and evolve. This limitation is particularly evident given neuropathological evidence that α Syn pathology in PD affects multiple neuronal populations beyond the nigrostriatal dopaminergic system¹⁷.

Among these, the locus coeruleus (LC), the principal noradrenergic nucleus of the brainstem, shows early pathology and degeneration during prodromal stages of the disease, in some cases before substantial nigral cell loss has occurred^{18–21}. This is especially relevant because the LC exerts widespread modulatory control over circuits involved in arousal, affective regulation, stress responses, autonomic control, and sensory processing through its diffuse projections to limbic, cortical, autonomic, and brainstem regions. Accordingly, LC dysfunction has been linked to several non-motor dimensions of PD, including anxiety, sleep disturbances, and mood dysregulation. Thus, LC involvement does not merely add another affected structure, but suggests that disease progression reflects the engagement of distinct neuromodulatory systems with diverse functional consequences^{22–31}.

Understanding how α Syn pathology affects different, albeit interconnected, neuronal populations requires experimental approaches with sufficient anatomical and cellular resolution to selectively target defined cell types. Although current preclinical models have greatly advanced our understanding of PD, many still lack the ability to compare how the same pathogenic process unfolds across distinct vulnerable populations^{32–37}. Neurotoxin-based paradigms have been instrumental in establishing the relationship between nigrostriatal degeneration and the motor syndrome, but their acute, lesion-based nature captures only limited aspects of the slow, staged progression of human pathology and the involvement of systems beyond the dopaminergic axis^{38–42}. Genetic and transgenic α Syn-based models address some of these limitations by incorporating disease-relevant mechanisms such as α Syn misfolding, aggregation and toxicity. Nevertheless, many induce pathology broadly, lack cell-type specificity, or are limited by restricted temporal control and variable phenotypic penetrance^{43,44}.

In parallel, the association of SNCA variation with PD risk, together with the identification of pathogenic mutations such as A53T, has strengthened the view that altered α Syn biology does not merely accompany disease, but can play a causal role in its progression^{54–56}. The A53T mutation increases the propensity of α Syn to misfold and aggregate, making it useful for modeling aggressive forms of synucleinopathy. Even so, it remains difficult to determine with causal precision how α Syn accumulation in specific neuronal populations shapes disease progression and functional impairment^{45–47}.

In this context, experimental strategies are needed that not only reproduce α Syn pathology but also allow it to be induced selectively in specific cell types. To address this limitation, we developed a conditional adeno-associated viral (AAV) strategy enabling Cre-dependent expression of human A53T- α Syn in

defined neuronal populations. By stereotactically delivering this vector into DAT-Cre and NET-Cre mice, we selectively induced synucleinopathy in dopaminergic neurons of the SNpc and noradrenergic neurons of the LC, respectively. We then monitored neurodegeneration and associated behavioral consequences using classical behavioral assays and a novel linear track paradigm designed to assess how graded spatial openness modulates anxiety-related locomotor behavior. This approach allowed us to compare, at cell-type resolution, how α Syn pathology evolves in two neuromodulatory systems that differ in physiology and connectivity, and to evaluate their differential relevance for motor and non-motor dimensions of disease.

Results

AAV-A53T- α Syn induces cell-type-specific aggregation, differential neurodegeneration and distinct apoptotic burden in the SNpc and LC

To induce synucleinopathy in defined neuronal populations, we generated a Cre-dependent AAV vector expressing human mutant A53T- α Syn fused to a Myc tag (AAV2/9-CreON-p α Syn; Fig. 1a, top). The transgene cassette was arranged in a double-floxed inverted orientation (DIO) configuration⁴⁸, thereby restricting expression to Cre-expressing cells and enabling spatially and genetically defined induction of pathology. This strategy allowed us to target two catecholaminergic populations susceptible to PD-related synucleinopathy, but with potentially distinct contributions to disease progression^{13,49}: dopaminergic neurons in the SNpc of DAT-Cre mice and noradrenergic neurons in the LC of NET-Cre mice (Fig. 1a, left and right, respectively). By imposing the same pathogenic process across populations, this approach provided a controlled framework for comparing their relative susceptibility to α Syn toxicity.

We first asked whether this system reproduced two defining pathological features of synucleinopathy: α Syn aggregation and degeneration of vulnerable neurons. To assess aggregate formation, we performed proteinase K (PK) digestion, which removes soluble protein and unmask PK-resistant α Syn species. By 6 weeks post-injection, PK-resistant α Syn immunoreactivity was readily detected in both the SNpc and the LC, consistent with the formation of pathological α Syn aggregates (Fig. 1b,c). As a specificity control, tyrosine hydroxylase (TH) immunoreactivity was abolished by PK treatment, indicating that the residual signal reflected aggregated α Syn rather than soluble protein. Thus, selective A53T- α Syn expression was sufficient to drive pathological aggregation in both catecholaminergic systems within a relatively short experimental time window.

We next examined whether aggregate formation was accompanied by degeneration of the targeted neuronal populations. Viral transgene expression and injection spread were verified by Myc immunoreactivity within the intended target regions, the SNpc and LC. Neuronal loss in the corresponding catecholaminergic populations was quantified as TH⁺ cell density using an in-house automated image-analysis pipeline (findmycells; see Methods and Supplementary Fig. 1). Supporting

analyses of individual post-injection time points suggested different temporal profiles of detectable neuronal loss in the two nuclei: SNpc TH⁺ dopaminergic cell density was already significantly reduced in DAT-paSyn mice at 10 weeks, whereas LC TH⁺ noradrenergic cell density showed non-significant reductions in NET-paSyn mice at 8, 10 and 12 weeks post-injection (Supplementary Fig. 2).

Fourteen weeks after bilateral injection of AAV2/9-CreON-paSyn or the control vector AAV-hSyn-DIO-mCherry, both DAT-paSyn and NET-paSyn mice showed significant loss of TH⁺ neurons relative to their respective controls. However, degeneration was markedly more pronounced in the SNpc than in the LC, with DAT-paSyn mice showing severe dopaminergic cell loss and NET-paSyn mice showing a more moderate reduction in noradrenergic neuron survival (Fig. 1i,p, respectively). These findings indicate that the same α Syn-induced insult was sufficient to induce neuronal loss in both nuclei, but that the magnitude and apparent onset of detectable degeneration differed between them. We therefore asked whether this differential vulnerability might be associated, at least in part, with distinct degrees of apoptotic activation.

This question is particularly relevant because, in synucleinopathies, pathological α Syn accumulation is closely linked to proteostatic stress, mitochondrial dysfunction, oxidative damage and inflammatory processes, all of which can converge on apoptotic pathways⁵⁰. To address this, we quantified cleaved caspase-3 (CC3), a widely used marker of execution-phase apoptosis, reasoning that it would provide a readout of ongoing synucleinopathy-induced cellular stress beyond aggregate burden or cumulative neuronal loss alone⁵¹. We measured CC3 14 weeks after bilateral AAV2/9-CreON-paSyn injection into either the SNpc or LC, using region-matched AAV-mCherry-injected animals as controls.

We first quantified relative CC3 density as an index of tissue-level apoptotic activation. In NET-paSyn mice, CC3 signal was significantly elevated relative to controls, indicating robust activation of apoptotic pathways at the population level (Fig. 1q, left). By contrast, although CC3 density tended to increase in DAT-paSyn mice, this effect did not reach significance (Fig. 1j, left). Thus, at the level of bulk tissue signal, apoptotic activation appeared more pronounced in the LC than in the SNpc at this late stage of the model.

However, because both nuclei also exhibited substantial neuronal loss by this time point, tissue-level CC3 alone could not resolve whether the surviving neuronal populations were exposed to comparable apoptotic pressure. We therefore calculated an apoptotic load index by normalizing CC3 signal to neuronal survival on a per-animal basis. This analysis revealed a significant increase in relative apoptotic burden in both nuclei compared with their respective controls (Fig. 1j,q, right). Importantly, the apoptotic load index was higher in DAT-paSyn than in NET-paSyn mice, indicating greater death-related stress in surviving dopaminergic neurons than in their noradrenergic counterparts.

Together, these data show that our Cre-dependent viral strategy recapitulates key pathological features of synucleinopathy—aggregate formation, neurodegeneration and apoptotic engagement—in a cell-type-specific manner. At the same time, they reveal a clear dissociation in vulnerability across the two

catecholaminergic populations: although both are susceptible to α Syn pathology, degeneration is more severe in the SNpc, whereas tissue-level apoptotic activation is more prominent in the LC. When normalized to neuronal survival, however, apoptotic burden is greater in the SNpc, indicating that dopaminergic and noradrenergic neurons differ not only in the extent of pathology they develop, but also in how α Syn accumulation is translated into overt cell death.

Supplementary Fig. 2. Earlier detectable loss of TH⁺ neurons in the SNpc than in the LC after A53T- α -synuclein expression. Normalized TH⁺ cell density in control and paSyn mice at intermediate time points before the 14-week endpoint. paSyn mice were injected with AAV2/9-CreON-paSyn and control mice with the Cre-dependent vector AAV-hSyn-DIO-mCherry. (a) Normalized TH⁺ cell density in the SNpc of DAT-Cre mice 8 weeks after virus injection. (b) Normalized TH⁺ cell density in the SNpc of DAT-Cre mice 10 weeks after virus injection. (c–e) Normalized TH⁺ cell density in the LC of NET-Cre mice 8, 10 and 12 weeks after virus injection, respectively. Dots correspond to mean values per animal. Box plots show mean $\pm$ min/max values. Statistical comparisons were performed using two-tailed unpaired t-tests. DAT week 8: $p = 0.2903$; DAT week 10: $p = 0.0103$; NET week 8: $p = 0.1917$; NET week 10: $p = 0.1167$; NET week 12: $p = 0.3859$. ns, not significant; * $p < 0.05$.

Specific α -synuclein aggregation and cell loss selectively drive motor, but not anxiety-like, phenotypes in classical behavioral assays

Having established cell-type-restricted synucleinopathy in dopaminergic and noradrenergic neurons, we next asked whether A53T- α Syn expression in these two neuromodulatory systems translated into detectable behavioral dysfunction. To this end, DAT-Cre and NET-Cre mice received bilateral injections of AAV2/9-CreON-paSyn into the SNpc or LC, respectively, whereas control mice received AAV-hSyn-DIO-mCherry. Behavioral performance was assessed longitudinally at 1, 4, 8, 12 and 14 weeks post-injection using classical assays for motor coordination, spontaneous locomotion and anxiety-like behavior (Fig. 2a).

We first assessed motor coordination using the accelerating rotarod. A53T- α Syn expression in SNpc dopaminergic neurons produced a progressive motor impairment in DAT-paSyn mice, with performance beginning to diverge from controls after the first post-injection time point and becoming significant at later stages of the experiment (Fig. 2c). This temporal profile was consistent with the emergence of α Syn aggregation and subsequent dopaminergic cell loss in the SNpc. In contrast, NET-paSyn mice showed no detectable impairment on the rotarod across the same longitudinal interval (Fig. 2d), indicating that noradrenergic synucleinopathy alone was insufficient to disrupt motor coordination in this classical assay.

We next examined spontaneous locomotion and anxiety-related behavior in the open field test (OF). Consistent with the rotarod phenotype, DAT-paSyn mice developed a progressive hypolocomotor profile, characterized by reduced distance travelled together with increased immobility (Fig. 2f) at week 14. By contrast, anxiety-related measures derived from the same assay, including center entries and center

occupancy, habituated similarly across groups and did not reveal sustained differences between DAT-control and DAT-paSyn mice (Fig. 2g).

In NET-Cre mice, LC-targeted A53T- α Syn expression was not associated with detectable alterations in either locomotor or anxiety-related open-field measures. NET-paSyn mice remained indistinguishable from NET-control mice across OF parameters examined, including distance travelled, immobility, center entries and center occupancy (Fig. 2i,j, respectively). Thus, although LC neurons developed α Syn aggregation, neuronal loss and apoptotic activation, these pathological changes did not translate into measurable deficits in the classical behavioral assays used here.

Together, these findings show that selective α Syn pathology in dopaminergic neurons is sufficient to drive progressive motor dysfunction in standard behavioral paradigms, whereas pathology in noradrenergic LC neurons does not elicit a comparable phenotype under the same conditions. This difference may be explained in part by the lower extent of LC neurodegeneration relative to SNpc degeneration. Nevertheless, because NET-Cre mice showed α -synuclein aggregation and LC neuronal loss, the absence of a detectable phenotype in classical assays suggests that paradigms such as the OF may not be optimally suited to reveal subtle or early behavioral consequences of noradrenergic dysfunction. This motivated us to develop more selective readouts for prodromal behavioral phenotypes.

In the Opening Track maze, anxiety-like behavior emerges selectively after LC synucleinopathy

Classical behavioral assays failed to detect anxiety-like alterations in either DAT-Cre or NET-Cre mice, despite α -synuclein aggregation and neuronal loss in the targeted populations. This mismatch between histopathology and behavioral readouts prompted us to develop the Opening Track (OT), a spatial-openness paradigm designed to probe exposure-dependent defensive behavior and its interaction with locomotion. Rather than measuring locomotor activity or exploration alone, the OT quantifies how animals distribute immobility and movement along a controlled gradient of perceived spatial exposure. This distinction is important because anxiety-like behavior in this task is inferred not simply from reduced movement, but from a spatially organized avoidance of open, exposed regions together with preferential immobility near enclosed or protected zones.

Mice have evolved under threat from aerial predators and thus inherently avoid open spaces⁵². The OT exploits this behavior using a narrow, elevated linear track equipped with interchangeable red acrylic walls—rectangular (OTR), triangular (OTT) or exponential (OTE)—that generate distinct profiles of enclosure and openness along the same locomotor axis. This design allows defensive immobility to be dissociated from general locomotor suppression, motivational deficits or reduced exploratory drive by asking where immobility occurs relative to the openness gradient. Conceptually, this approach aligns with human assays, which use visual threat cues in a virtual plank test to study the effects of anxiety on locomotion in the context of PD⁵³.

To determine whether the OT captures behavioral responses to graded spatial threat, we first characterized performance in wild-type mice (Fig. 3a,b). Immobility was not distributed uniformly along the track, but varied systematically with wall geometry. In the rectangular condition, where enclosure remained constant, immobility was shifted toward the distal end. Under the triangular configuration, immobility was distributed across both proximal and distal portions of the track, consistent with an intermediate and spatially shifting threat gradient. Under the exponential configuration, immobility was concentrated preferentially in the proximal, more enclosed segment, corresponding to the region of greatest perceived protection. Thus, immobility distribution depended on geometry-mediated openness, indicating that the OT is sensitive to perceived exposure and the defensive strategies it elicits.

We next used this assay to test whether cell-type-specific synucleinopathy could reveal anxiety-related phenotypes that remained undetected in standard paradigms. OT behavior was assessed longitudinally at the same post-injection time points: weeks 1, 4, 8, 12 and 14. In DAT-Cre mice expressing A53T- α Syn, immobility patterns were indistinguishable from controls at early time points. At week 14, however, DAT-paSyn mice showed a shift in immobility position toward more distal track segments across wall configurations (Fig. 3c). Because this redistribution was not characterized by immobility near the proximal, enclosed segment, it did not match the spatial profile expected for an anxiety-like avoidance phenotype. Instead, given the progressive motor impairment observed in these mice in the rotarod and OF, the distal redistribution of immobility is more consistent with altered locomotor capacity, impaired task progression or disrupted movement organization. Thus, the DAT-Cre phenotype illustrates the importance of interpreting OT behavior in relation to independent measures of motor function.

In contrast, NET-Cre mice expressing A53T- α Syn exhibited an openness-avoidant phenotype in the OT that remained hidden in classical assays (Fig. 3d). By week 14, NET-paSyn mice displayed sustained immobility near the proximal, enclosed end of the track, together with reduced progression toward more open regions across wall configurations. Importantly, this spatially biased immobility occurred in the absence of detectable motor impairment in rotarod or open-field measures, arguing against a generalized locomotor deficit as the primary explanation. Instead, the clustering of immobility within the protected proximal segment indicates persistent avoidance of exposed space despite preserved motor performance, consistent with a mild anxiety-like defensive state.

Together, these findings show that the OT reveals a behavioral dimension of noradrenergic synucleinopathy that is missed by conventional assays. More broadly, they indicate that LC dysfunction gives rise to a spatially biased anxiety-like phenotype in the absence of overt motor impairment, establishing the OT as a sensitive tool for detecting non-motor consequences of synucleinopathy.

Motor dysfunction re-emerges in the Opening Track Maze and dissociates from LC-driven anxiety

Because the OT constrains exploration along a single spatial axis while preserving a direct relationship between locomotor execution, pausing and progression, it also provides a sensitive framework in which

motor dysfunction can be resolved alongside anxiety-related behavior. We therefore asked whether the task would recapitulate the motor phenotype detected in classical assays while dissociating it from the anxiety-like profile.

In DAT-Cre mice expressing A53T- α Syn, the OT robustly recovered a progressive motor phenotype. By week 14, DAT-paSyn mice travelled shorter distances, spent a lower proportion of time moving, moved at lower speeds during active movement and showed longer intervals between movement bouts relative to controls (Fig. 4a–c). Thus, the OT did not merely reveal altered spatial positioning of immobility in DAT-Cre mice, but also reproduced the broader locomotor impairment associated with nigral synucleinopathy.

This impairment extended beyond gross locomotor output and was also evident in the fine structure of movement. DAT-paSyn mice developed less straight, more tortuous and more meandering trajectories over time, indicating reduced directional stability and impaired movement efficiency along the track (Fig. 4g–i). Together, these findings indicate that dopaminergic synucleinopathy disrupts not only how much animals move, but also how effectively they organize movement in space.

By contrast, NET-Cre mice expressing A53T- α Syn did not develop the same broad motor phenotype observed in DAT-paSyn mice. At week 14, NET-paSyn mice showed largely preserved locomotor capacity, with distance travelled, movement speed and interbout interval remaining comparable to controls, despite a reduction in the proportion of time spent moving (Fig. 4d–f). This pattern suggests reduced movement engagement rather than a generalized inability to traverse the track. Consistent with this interpretation, trajectory-structure analyses showed only a minor alteration in meander, whereas straightness and tortuosity remained largely preserved (Fig. 4j,k). Thus, unlike DAT-paSyn mice, which developed less straight, more tortuous and more meandering trajectories, NET-paSyn mice did not show a broad disruption of trajectory efficiency. Together, these findings indicate that LC-targeted synucleinopathy affects selected aspects of OT behavior without producing the global locomotor deterioration characteristic of dopaminergic synucleinopathy.

This distinction is critical for interpreting the anxiety-related phenotype detected in the same task. In DAT-Cre mice, the redistribution of immobility toward more distal track positions occurred in the context of progressive motor deterioration, suggesting that altered task progression and disrupted movement organization contributed to the spatial phenotype. In NET-Cre mice, however, the proximal immobility bias emerged in the absence of the broad locomotor deterioration observed after SNpc targeting. Thus, sustained immobility near the enclosed segment and reduced progression toward exposed regions are most consistent with an anxiety-like avoidance phenotype induced by LC dysfunction rather than a generalized motor deficit. Together, these data show that the OT resolves two dissociable behavioral consequences of cell-type-specific synucleinopathy within a single assay: progressive motor dysfunction following SNpc targeting, and anxiety-like avoidance without major locomotor compromise following LC targeting.

Discussion

The present study establishes a cell-type-resolved synucleinopathy model that enables direct comparison of how the same α -synuclein-based pathological process differentially affects two catecholaminergic systems: dopaminergic neurons of the SNpc and noradrenergic neurons of the LC. By driving Cre-dependent overexpression of human A53T- α -synuclein in these two populations, we show that a common pathogenic trigger does not elicit a uniform degenerative or behavioral response. Instead, SNpc dopaminergic neurons showed higher degenerative vulnerability associated with progressive motor dysfunction, whereas LC noradrenergic neurons followed a slower and less severe degenerative trajectory accompanied by anxiety-related avoidance of exposed/open regions. In this sense, our data support a view of synucleinopathies as multisystem circuitopathies in which α -synuclein toxicity is shaped not simply by the presence of aggregates, but by the intrinsic properties and circuit context of vulnerable neuronal populations.

A major strength of this model is that it induces pathological α -synuclein aggregation and neurodegeneration within an experimentally tractable time window, yet one that remains sufficiently extended to resolve disease progression. Compared with many germline transgenic models, this approach provides a more progressive and spatially controlled induction of pathology, while, unlike neurotoxin-based paradigms, it does not rely on an acute nigrostriatal lesion^{42–44,54}. Instead, degeneration is driven by the expression of a pathogenic factor directly relevant to synucleinopathy. This intermediate timescale proved particularly informative, as it allowed us not only to detect pathology in both nuclei but also to compare their temporal trajectories. In the SNpc, A53T- α -synuclein expression led to early aggregate formation by 6 weeks post-injection (Fig. 1, b), followed by marked neuronal loss that became evident by 10 weeks post-injection (Supplementary Fig. 2), indicating a relatively rapid transition from pathological accumulation to overt degeneration. In the LC, by contrast, pathogenic α -synuclein also accumulated by 6 weeks post-injection (Fig. 1. c) and ultimately induced neuronal loss, but in this case only by 14 weeks post-injection (Fig. 1, p), indicating that the degenerative process unfolded more slowly and with lower overall severity. Thus, even under the same pathogenic driver, SNpc and LC neurons differed not simply in the extent of pathology they developed, but in the tempo with which pathology progressed toward degeneration.

Importantly, this temporal pattern should be interpreted in the appropriate context. Although the Braak framework is often invoked to support early involvement of the LC in PD, it primarily describes the topographical progression of Lewy pathology across anatomically connected regions rather than the relative speed with which different neuronal populations progress to overt degeneration once the pathogenic insult is established^{18,19,55,56}. More recent body-first and brain-first models further reinforce this distinction by proposing that the anatomical sequence of involvement depends on the site of origin and route of pathological spread^{57–59}. Within this framework, the LC may become involved early, particularly in body-first trajectories, without this necessarily implying that LC neurons degenerate more rapidly once pathology is established locally^{4,21,32,37,60}. Our model addresses a different question: how an equivalent α -synuclein insult, restricted by cell type and initiated directly within the brain, is translated

into neuronal loss in two vulnerable catecholaminergic populations. In this context, degeneration in the LC was slower and less severe than in the SNpc, indicating that early anatomical involvement and intrinsic degenerative vulnerability are not synonymous.

One possible explanation for this dissociation is the greater intrinsic susceptibility of nigral dopaminergic neurons. As proposed by Surmeier and others, these neurons operate under a particularly demanding physiological regime characterized by sustained autonomous activity, calcium entry, and high metabolic and oxidative burden, features that may place them closer to a threshold for failure under additional proteostatic stress⁹. From this perspective, an equivalent α -synuclein burden may be more readily converted into overt degeneration in the SNpc than in the LC. Evidence from other PD-relevant stressors is consistent with this interpretation. In particular, Yee et al. directly compared the effects of rotenone on LC neurons and dopaminergic neurons of the SNpc and found that, although rotenone suppressed activity in both populations, LC neurons were less sensitive to its acute actions. Because rotenone disrupted mitochondrial membrane potential, promoted reactive oxygen species generation and engaged K-ATP-dependent mechanisms, these findings provide convergent support for the idea that LC and SNpc neurons differ in their intrinsic resilience to disease-relevant cellular stress⁶¹. Although this paradigm is mechanistically distinct from A53T- α -synuclein-driven synucleinopathy, it nevertheless supports the broader notion that the spatial order of pathological involvement and the intrinsic vulnerability to degeneration are related but dissociable dimensions of PD pathogenesis.

We next quantified CC3, a marker of execution-phase apoptosis, to assess whether SNpc and LC vulnerability involved distinct engagement of cell-death pathways. LC tissue showed a larger overall CC3 increase at the symptomatic stage, whereas normalization to neuronal survival revealed a greater apoptotic burden per remaining cell in the SNpc. This dissociation between tissue-level apoptotic activation and survival-normalized burden indicates that α -synuclein pathology is not converted into cell death uniformly across catecholaminergic populations^{62–64}.

At the behavioral level, our results highlight an important limitation of standard assays. Classical paradigms such as the accelerating rotarod and the OF robustly captured the progressive consequences of dopaminergic synucleinopathy. DAT-Cre mice expressing A53T- α -synuclein developed clear and temporally coherent deficits in motor coordination, locomotion, and immobility, concomitant to SNpc degeneration. This convergence strongly supports a causal relationship between selective nigral pathology and impaired motor control. However, those same assays failed to detect an equally robust phenotype in animals with LC involvement, despite the clear α -synuclein aggregation and neuronal loss observed histologically. This negative result is not trivial. Rather, it highlights that the sensitivity of a given task depends on the relationship between the structure of the assay and the function of the circuit being disrupted.

This aspect is particularly relevant for the LC. Noradrenergic neurons are centrally involved in arousal, threat appraisal, stress responsiveness, vigilance, and the regulation of exploration under uncertainty^{22,23}. Many of these functions are difficult to capture with coarse OF readouts such as center

time or center entries, especially in assays in which locomotion and anxiety are only imperfectly coupled and center-related measures provide only an indirect index of defensive state⁶⁵. The absence of a robust LC phenotype in the OF should therefore not be interpreted as evidence that noradrenergic synucleinopathy is behaviorally silent. Rather, our data suggest that conventional readouts are poorly matched to the computational and behavioral domain in which LC dysfunction is expressed. This interpretation is reinforced by recent work combining behavior with autonomic readouts in classical anxiety assays. Signoret-Genest et al. showed that integrated cardio-behavioral measures discriminate contextual threat levels robustly in the elevated plus maze and light–dark box, but much less clearly in the OF, where transitions between putatively low- and high-threat subareas were not accompanied by comparably distinct heart-rate signatures⁶⁶. These findings suggest that the OF provides relatively poor physiological resolution of defensive state, reinforcing the idea that its conventional readouts may be insufficiently sensitive to detect subtle anxiety-related phenotypes linked to LC dysfunction⁶⁶.

It was precisely this mismatch between conventional behavioral readouts and the functional domain of the LC that motivated the development of the OT, one of the central methodological and conceptual advances of the present study. Unlike the OF, the OT parametrically manipulates environmental openness along a single spatial axis, preserving a direct relationship between exploratory progression, defensive pausing, and exposure to uncertainty. In wild-type animals, immobility was spatially redistributed as a function of wall geometry, indicating that the task selectively modifies openness and allows to assess the applied defensive strategies. This validation is important because it shows that the OT does not simply measure locomotion in a novel apparatus, but rather how animals structure their exploration in response to changes in perceived environmental safety. Within this framework, dopaminergic and noradrenergic synucleinopathy again diverged clearly. In DAT-Cre animals, synucleinopathy-induced changes in immobility position emerged, but these effects were modest and are most parsimoniously interpreted as a consequence of progressive locomotor impairment rather than a genuine anxiolytic-like phenotype. By contrast, NET-Cre animals developed a clear proximal immobility bias together with reduced exploration of open segments on the OT, despite preserved motor performance. This pattern is consistent with persistent avoidance of exposed space and, therefore, with an enhanced anxiety-like phenotype that becomes visible only under conditions of graded spatial exposure. That this phenotype is evident in the OT but not in the OF shows that the non-motor consequences of noradrenergic synucleinopathy may remain hidden unless animals are challenged with a task whose structure is aligned with the function of the affected circuit.

More broadly, these findings reinforce the idea that distinct symptomatic dimensions of PD may arise from partially dissociable trajectories across vulnerable neuromodulatory systems. In our model, dopaminergic pathology preferentially generated a progressive motor syndrome, whereas noradrenergic pathology altered exploratory control under conditions of graded openness. Although this dissociation necessarily simplifies the multisystem complexity of human disease, it captures an important organizational principle of PD: the same molecular pathology can give rise to different behavioral outcomes depending on the neuronal population and circuit in which it unfolds. From this perspective,

combining circuit-specific pathology with circuit-sensitive behavioral assays may be essential for understanding the prodromal and non-motor dimensions of disease progression^{13,30,49}.

Several limitations should nevertheless be acknowledged. Viral overexpression generates relatively high and spatially restricted levels of α -synuclein compared with endogenous disease progression. Although this is advantageous for controlled manipulation of defined circuits and neuronal populations, it does not fully recapitulate the anatomical spread, stochastic seeding, or multisystem onset observed in patients. Moreover, our analyses focused on selected post-injection time points, leaving open the possibility that LC pathology may produce more pronounced degeneration or additional behavioral deficits at later stages. The OT also remains a newly developed assay and will require further validation across additional models, pharmacological manipulations, and physiological readouts to fully establish the behavioral dimension it captures. Finally, because TH⁺ cell density was used as a primary readout of neuronal integrity, future work combining TH quantification with additional neuronal and functional markers could further distinguish neuronal loss from phenotypic downregulation. By design, our viral synucleinopathy model isolates specific neuronal populations and therefore cannot encompass all dimensions of human PD, similar to most other rodent models. Yet, by enabling selective cellular targeting, our model allows for characterization of the specific susceptibility of neuronal populations to synucleinopathy and precise attribution of resulting dysfunctions to identified circuit elements.

Overall, we present a rapid, cell-type-resolved synucleinopathy model that allows for dissection of molecular, cellular, and behavioral features relevant to PD. It reveals nucleus-specific patterns of synucleinopathy-induced apoptotic burden, degeneration, and symptom expression. Consequently, our findings show that SNpc dopaminergic neurons sustain enhanced apoptotic burden per surviving cell and give rise to a progressive motor phenotype, whereas LC noradrenergic neurons follow a slower degenerative trajectory and produce anxiety-like alterations that become detectable only with a circuit-sensitive behavioral assay. More broadly, these results support a framework in which α -synuclein pathology interacts with intrinsic neuronal and circuit properties to determine not only where degeneration occurs, but also when and how symptoms emerge. This approach provides a strong experimental foundation for studying PD as a multisystem circuitopathy as the basis for developing strategies aimed at detecting and modifying early disease states.

Material & Methods

Animals

This study was conducted using transgenic DAT-Cre (B6.SJL-Slc6a3^{tm1.1(cre)1}/J, Stock No. 020080) mice obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and NET-Cre (C57BL/6J-Slc6a2^{tm1.1(cre)1}/J) mice, generously provided by Dr. Andreas Lüthi (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) with permission from their original creator, Susumo Tonegawa⁶⁷. Breeding colonies were maintained by crossing transgenic animals with wild-type C57BL/6J mice in the institutional animal facility.

For all experimental procedures, male and female mice aged between 4 to 6 months at the time of intervention were used. A total of 38 female and male DAT-Cre mice and 48 female and male NET-Cre mice were included in the study. In addition, 13 wild-type C57BL/6J mice, bred in-house, were used for the validation of the behavioral paradigm.

One week prior to the onset of experimental procedures, animals were individually housed under controlled environmental conditions ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$ relative humidity) on a 12-hour light/dark cycle, with food and water provided ad libitum. All experiments were conducted during the light phase to minimize circadian variability.

All procedures were approved by the local government of Lower Franconia and veterinary authorities (Regierung von Unterfranken: licenses 2532-2-1356 and 2532-2-1067), and were performed under the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines⁶⁸.

Viral Constructs

AAV vectors were used to achieve cell-type-specific expression of the constructs of interest in DAT-Cre and NET-Cre mice. To induce pathological overexpression of A53T, we employed a Cre-dependent vector pAAV2/9-pCAG-FLEX-alpha-synuclein(A53T)-3×Myc-WPRE (titer: 3×10^{12} gc/ml).

Control animals were injected with a Cre-dependent reporter virus, pAAV-hSyn-DIO-mCherry (titer: 4.56×10^{14} gc/ml), which enables visualization of Cre-expressing neurons and serves as a control for viral delivery and recombination. This virus was produced in an in-house viral production facility using a plasmid obtained from Addgene (a gift from Bryan Roth; Addgene plasmid #50459, <http://n2t.net/addgene:50459>, RRID: Addgene_50459).

Stereotaxic Injections

To ensure continuous analgesia throughout the surgical procedure, mice received a subcutaneous injection of buprenorphine (0.05–0.1 mg/kg; Buprenovet®, Bayer, Leverkusen, Germany) 30 minutes before surgery. Anesthesia was induced and maintained with isoflurane (Iso-Vet®, Chanelle Pharma, Loughrea, Ireland; distributed by Harvard Apparatus, Holliston, MA, USA) in oxygen-enriched air (4% for induction, 1.5–2% for maintenance). Body temperature was maintained using an infrared heating pad (RightTemp Jr., Kent Scientific, Torrington, CT, USA), and respiratory and heart rates were continuously monitored.

After confirming a stable anesthetic plane through the absence of pedal reflexes and a consistent respiratory rate of ~ 1 Hz, mice were positioned in a stereotaxic frame (Model 1900, Kopf Instruments, Tujunga, CA, USA). Local analgesia was provided by subcutaneous injection of ropivacaine (8 mg/kg; Naropin®, AstraZeneca, Cambridge, UK) beneath the scalp. Following a midline scalp incision, bilateral craniotomies (0.4 mm diameter) were drilled at stereotaxic coordinates relative to bregma to target the

SNpc in DAT-Cre mice (anteroposterior, -3.3 ± 0.2 mm; mediolateral, $\pm 1.1 \pm 0.2$ mm) or the LC in NET-Cre mice (anteroposterior, -5.4 ± 0.2 mm; mediolateral, $\pm 0.8 \pm 0.2$ mm).

A calibrated glass micropipette (1–5 μ L; Drummond Scientific, Broomall, PA, USA) filled with the appropriate viral solution was slowly lowered to the target depth (dorsoventral, -4.0 ± 0.2 mm for SNpc and -3.8 ± 0.2 mm for LC). Viral suspensions were infused at a rate of 50 nl/min, with a total volume of 100–150 nl per site. After infusion, the pipette was left in place for 10 min to minimize backflow and then withdrawn slowly over 10 min. To assess pathological progression, unilateral injections were used in histological cohorts collected at 4, 8, 10, and 12 weeks post-injection, allowing intra-individual comparisons between injected and non-injected hemispheres. By contrast, bilateral injections were performed in mice assigned to behavioral testing to ensure broader transduction, as well as in animals collected at 14 weeks post-injection for the final histological endpoint.

Following injection, the incision was sutured and disinfected with antiseptic solution. Postoperative analgesia was provided by subcutaneous administration of meloxicam (5 mg/kg; Metacam®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) immediately after surgery and every 12 hours for three consecutive days. Mice were housed individually in recovery cages under a heating lamp and monitored daily for one week to ensure complete recovery.

Histology and Microscopy

Viral expression and neurodegeneration. To assess viral expression, cell loss, and apoptosis, mice were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with ice-cold phosphate-buffered saline (PBS; 10010023, Thermo Fisher Scientific, Waltham, MA, USA), followed by 4% paraformaldehyde (4% PFA; P6148, Sigma Aldrich, St. Louis, MO, USA) in PBS. Brains were post-fixed overnight at 4°C in 4% PFA, rinsed in PBS, embedded in 6% agarose (6%; A9539, Sigma Aldrich, St. Louis, MO, USA), and coronally sectioned at 60 μ m using a vibratome (VT1200; Leica Microsystems, Wetzlar, Germany).

Immunofluorescence was performed on free-floating sections. Sections were blocked for 2 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBS-T; Tris-buffered saline + 0.1% Tween-20; TBS: BP337-500, Thermo Fisher Scientific, Waltham, MA, USA; Tween-20: T8787, Sigma-Aldrich, St. Louis, MO, USA; prepared in-house), 5% donkey serum (017-000-121, Jackson ImmunoResearch, West Grove, PA, USA), and 0.3% Triton X-100 (T8787, Sigma-Aldrich, St. Louis, MO, USA), and then incubated for 72 h at 4°C with primary antibodies diluted in fresh blocking solution.

For viral expression and cell loss, primary antibodies were chicken anti-tyrosine hydroxylase (TH; 1:500; ab76442, Abcam, Cambridge, UK), and either goat anti-Myc (1:500; ab32, Abcam, Cambridge, UK) or rabbit anti-RFP (1:5000; 600-401-379, Rockland Immunochemicals, Limerick, PA, USA), depending on the injected viral construct. Corresponding secondary antibodies were donkey anti-mouse Cy5 (1:1000; 715-175-150, Jackson ImmunoResearch, West Grove, PA, USA), donkey anti-chicken Alexa Fluor 488

(1:800; 703-545-155, Jackson ImmunoResearch, West Grove, PA, USA), and donkey anti-rabbit Cy3 (1:1000; 711-165-152, Jackson ImmunoResearch, West Grove, PA, USA).

For apoptosis analysis, sections were incubated with sheep anti-TH (1:500; PA1-4679, Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-cleaved caspase-3 (1:1000; 9664, Cell Signaling Technology, Danvers, MA, USA), and either goat anti-Myc (1:1000; ab9132, Abcam, Cambridge, UK) or goat anti-tdTomato (1:1000; ABIN6254170, Antibodies-Online, Aachen, Germany), depending on the injected viral construct. Corresponding secondary antibodies were donkey anti-sheep Alexa Fluor 488 (1:800; 713-545-147, Jackson ImmunoResearch, West Grove, PA, USA), donkey anti-goat Cy3 (1:800; 705-165-147, Jackson ImmunoResearch, West Grove, PA, USA), and donkey anti-rabbit Cy5 (1:1000; 711-175-152, Jackson ImmunoResearch, West Grove, PA, USA).

Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 5 µg/mL; D8417, Sigma-Aldrich, St. Louis, MO, USA), and NeuroTrace 435/455 Blue Fluorescent Nissl Stain (1:200; N21479, Invitrogen, Waltham, MA, USA) was used as a counterstain for apoptosis experiments. Sections were mounted on SuperFrost Plus glass slides (631-0108, VWR, Radnor, PA, USA) and coverslipped with Fluorostab (custom-made glycerol-based mounting medium) using 24 × 50 mm coverslips (Epremedia, Portsmouth, NH, USA).

α-Synuclein aggregates To detect PK-resistant α-synuclein aggregates, a separate cohort was perfused with ice-cold PBS containing 5 U/mL heparin (H3393, Sigma-Aldrich, St. Louis, MO, USA), without PFA fixation. Brains were embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), snap-frozen in pre-cooled isopentane (I9516, Sigma-Aldrich, St. Louis, MO, USA), and stored at – 80°C. Coronal sections (10 µm) were cut on a cryostat (CM1950; Leica Microsystems, Wetzlar, Germany) and mounted onto SuperFrost Plus slides (631-0108, Thermo Fisher Scientific, Waltham, MA, USA).

Sections were equilibrated to room temperature, preincubated in PBS (BP337-500, Thermo Fisher Scientific, Waltham, MA, USA) at 43°C for 5 min, and then incubated with PK (1:30 dilution from a 10 mg/mL stock; P2308, Sigma-Aldrich, St. Louis, MO, USA) for 19 min at 43°C in a humidified chamber. After two washes in ice-cold PBS, sections were blocked for 1 h in PBS containing 2% bovine serum albumin (BSA; A7030, Sigma-Aldrich, St. Louis, MO, USA) and 10% normal goat serum (NGS; X0907, Dako, Glostrup, Denmark), followed by overnight incubation at 4°C with rabbit anti-α-synuclein (1:1000; S3062, Sigma-Aldrich, St. Louis, MO, USA) and chicken anti-TH (1:500; ab76442, Abcam, Cambridge, UK).

After washing, sections were incubated for 1 h at room temperature with goat anti-rabbit Cy3 (1:300; 111-165-144, Jackson ImmunoResearch, West Grove, PA, USA) and goat anti-chicken Alexa Fluor 488 (1:300; A11039, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were counterstained with DAPI (D8417, Sigma-Aldrich, St. Louis, MO, USA), and sections were mounted with Aqua-Poly/Mount (18606, Polysciences Inc., Warrington, PA, USA).

Imaging. Low- and high-magnification images used for quantification were acquired on a Zeiss AxioImager 2 fluorescence microscope equipped with an Axiocam 506 mono camera and ZEN software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Brain overviews were acquired using a Zeiss AxioZoom V16 macroscope. Confocal images of α -synuclein aggregates were acquired on a Zeiss LSM 980 using 20 $\times$ and 63 $\times$ oil-immersion objectives. Acquisition settings were kept constant within each experiment, and image analysis was performed blind to the experimental group.

Behavior

Behavioral experiments were conducted in two different sound-attenuated chambers (external dimensions: 100 cm length $\times$ 80 cm width $\times$ 116 cm height), each illuminated from above with a circular LED lamp (LED240; Proxistar GmbH, Göttingen, Germany) and maintained at a constant temperature of $22.5 \pm 1^\circ\text{C}$. The chamber used for the OF and OT paradigms was lined with black, low-reflective acoustic foam, whereas the chamber used for the Rotarod test was lined with white foam of the same material. Prior to each session, the corresponding apparatus was cleaned with 1% acetic acid (OF and OT) or ethanol (Rotarod). A Petri dish containing the same cleaning solution was placed in one corner of the chamber to provide a consistent olfactory context.

Behavioral testing was performed at weeks 1, 4, 8, 12, and 14 post-viral injection to monitor longitudinal changes. Each testing period spanned five consecutive days, with the order of behavioral paradigms randomized across animals and testing weeks to minimize order and habituation effects. The Rotarod and OF tests were conducted on single days, while the OT was assessed across three consecutive days per period. The three wall configurations of the OT (see below) were tested in randomized order across animals and weeks. Only animals with post hoc histological confirmation of bilateral viral expression in the targeted structure were included in behavioral analyses.

Accelerating Rotarod Test Motor coordination was assessed using an accelerating Rotarod device (Ugo Basile, Gemonio, Italy), following the protocol described by Esposito et al.⁶⁹. The apparatus consisted of a rotating rod (external diameter: 3 cm; length: 30 cm) elevated 16 cm above a padded surface and divided into five individual lanes. Each trial lasted 5 minutes, during which the speed increased linearly from 5 to 50 rpm. Mice were pretrained before surgery with five trials per day for five days. During the experimental phase, habituation trials were conducted on days – 10 and – 5. On the testing day, each mouse performed four consecutive trials (5-minute inter-trial intervals); the first trial was excluded from analysis due to its typically high variability.

Open Field Test Spontaneous locomotor activity and innate anxiety-like behavior were assessed in a white acrylic OF arena (50 cm $\times$ 50 cm $\times$ 50 cm). Each mouse was placed in the center of the arena and allowed to explore freely for 15 minutes. Movement tracking and zone occupancy analysis were used to evaluate locomotor activity and anxiety-related avoidance of the center.

Opening Track Maze The OT was designed to assess exploratory behavior and anxiety-related responses across a spatial gradient of environmental openness. The apparatus consisted of a transparent

polyacrylic baseplate (50 cm length × 4 cm width) mounted on four 30 cm-high posts, with a 35 cm × 5 cm backplate providing reinforcement. Two triangular stabilizing feet were symmetrically positioned at 5 cm and 25 cm along the baseplate for structural stability. Transparent red sidewalls of varying geometries were mounted bilaterally to systematically manipulate perceived enclosure.

The rectangular wall configuration was a flat panel (35 cm length × 30 cm height × 0.3 cm thickness), providing full enclosure along the entire track. The triangular wall formed a right triangle with a base and height of 35 cm and 30 cm, respectively, and an inner angle $\alpha = 40.6^\circ$, producing a linear decrease in wall height toward the open end. The exponential wall followed the function:

$$y = 30 \times e^{\{-xb\}}$$

where:

$$b = \frac{\log(30)}{35}$$

This geometry generated a continuous height gradient from 30 cm to 1 cm along the 35 cm length, simulating a smooth transition from enclosed to nearly open space. This parametric design enabled fine-grained modulation of spatial constraints, allowing for systematic analysis of behavioral responses to varying degrees of environmental exposure.

During each session, mice were individually placed at the enclosed end of the track and allowed to explore freely for 10 minutes. Each animal completed one session per wall configuration over three consecutive days. The order of wall types was randomized across animals and testing weeks to avoid systematic biases and account for novelty or prior exposure effects.

Acquisition System Behavior was recorded with an overhead, hardware-synchronized video setup comprising an RGB camera (Pike F-032C, Allied Vision, Stadtroda, Germany; distributed by Campden Instruments, Loughborough, UK) and a thermal camera (A655sc, FLIR Systems, Wilsonville, OR, USA). The thermal stream served exclusively for quality assurance (e.g., detecting occlusions or illumination/contrast shifts and verifying timing integrity) and was not analyzed quantitatively. Both streams were acquired in CinePlex Studio (Plexon, Dallas, TX, USA).

Session onset/offset and frame timing were locked to hardware by transistor–transistor logic (TTL) signals—an initial burst followed by a continuous 1 Hz clock—generated via an OmniPlex controller and Radiant/PlexBright outputs (Plexon), ensuring a shared timestamp for every frame and event. A custom MATLAB interface (R2022b; MathWorks, Natick, MA, USA) integrated through RPDvsEx/ActiveX (Tucker-Davis Technologies, Alachua, FL, USA) provided pre-recording calibration (scale and focus), real-time preview, and standardized session control, yielding identical recording durations across animals and weeks.

Analysis

Quantification with findmycells. The methods implemented in findmycells are detailed in Fig. S2. In the post-processing step, identified features that were smaller than the experts' labels, as well as those with no contact to a predefined region of interest (ROI) covering the SNc were excluded from the quantification.

Open Field Analysis. Behavioral analysis in the OF was performed as previously described⁶⁶. Top-view RGB videos were processed using a custom MATLAB pipeline based on the SignDCL framework. A reference background image was generated from the 80th percentile intensity projection of a subset of frames and subtracted from each frame. The mouse was segmented by thresholding and refined by sequential morphological operations. From the resulting binary mask, the contour of the animal and its center of mass were extracted frame by frame.

A spatial calibration factor was obtained for each recording by manually defining a reference segment of known length. Total distance traveled and instantaneous speed were calculated from the center-of-mass coordinates and lightly smoothed using a median filter. A motion index was additionally computed as the proportion of non-overlapping pixels between consecutive mouse masks relative to total mask area. Immobility was quantified as the percentage of time spent immobile for at least 2 s.

For spatial analysis, the arena was divided into a 5×5 grid, with the center defined as the middle 2×2 squares. Center occupancy was calculated as the cumulative time spent in the center zone (s), and center entries were defined as transitions from the outer area into the center. Occupancy heat maps were generated using the FMAToolbox with 250 bins and no initial smoothing, followed by post hoc two-dimensional smoothing while excluding zero-occupancy bins to minimize edge artifacts.

Rotarod Analysis. Motor performance on the accelerating Rotarod was quantified as latency to fall, averaged across the last three trials of each testing session and compared across time points. To assess gait-related parameters during rod running, mice were recorded from behind and analyzed using DeepLabCut for markerless pose estimation^{70,71}. The resulting tracking data were processed using custom-written Python scripts. The base of the tail was used as a proxy for mouse position on the rod, defined relative to the lower-left corner of the video frame.

Tracking data were segmented by session and averaged separately for a slow rotation phase, including time points during which the rod rotated between 6 and 10 rpm, and a fast rotation phase, including time points between 10 and 50 rpm. In addition, heart rate and mouse position were analyzed around fall events using peristimulus time histograms. For heart rate analysis, values were normalized to the mean heart rate during the baseline period from 20 to 11 s before the fall, and mean heart rate was then compared between the intervals 11–6 s and 5–0 s before falling. Mouse position on the rod was analyzed analogously by comparing mean position during 11–6 s and 5–1 s before the fall.

Opening Track Analysis. Behavior in the OT was analyzed using DeepLabCut for automatic tracking of the snout, both ears, and tail base, without the need for physical markers attached to the animal^{70,71}. Videos were processed using a custom Python pipeline, tracking quality was visually inspected, and

missing detections were not corrected by interpolation. To enable comparisons across animals and sessions, coordinates were aligned to a common reference frame of the apparatus.

Spatial position was defined along the longitudinal axis of the track using the tail base as reference. From these coordinates, total distance traveled, average speed, proportion of movement, and the interval between movement episodes were calculated. Immobility was defined as periods during which the animal remained immobile for at least 2 s, and the spatial position associated with these episodes was extracted for further analysis. Maximum distance from the starting point and longitudinal exploration range were also calculated.

Trajectory geometry was characterized using straightness, tortuosity, and meander. Straightness and tortuosity were calculated in 1-s sliding windows, considering only windows in which movement was detected. Straightness was defined as the ratio between the straight-line distance from the beginning to the end of each window and the total path length traveled within that window, whereas tortuosity was defined as the inverse of this relationship. Meander was calculated as the accumulated angular change normalized by distance traveled. All variables were quantified separately for each wall configuration and each testing time point. To minimize technical bias, only valid OT sessions were included in the behavioral analyses. Sessions in which animals fell from the apparatus or failed to complete a given wall configuration were excluded from the corresponding analysis. For immobility-position analyses, sessions without detectable immobility bouts were additionally excluded, as no spatial immobility-position value could be computed.

Statistics

Normality was assessed using the Shapiro–Wilk test, the Kolmogorov–Smirnov test with Lilliefors correction, or the D’Agostino–Pearson omnibus normality test, where appropriate. Homogeneity of variances was assessed using the Brown–Forsythe test. For comparisons between two independent groups, two-tailed unpaired Student’s t-tests were used when assumptions of normality and equal variance were met. Welch’s t-test was used when variances were unequal. For paired comparisons, two-tailed paired Student’s t-tests were used when appropriate. When datasets did not meet normality criteria, non-parametric tests were applied: the Mann–Whitney U test for unpaired comparisons and the Wilcoxon signed-rank test for paired data.

For comparisons involving more than two groups or conditions, one-way or two-way ANOVA was used when assumptions of normality and homoscedasticity were satisfied, followed by Šídák- or Bonferroni-corrected post hoc tests as appropriate. When assumptions for parametric testing were not met, the Kruskal–Wallis test was used followed by appropriate multiple-comparison procedures. For longitudinal datasets, repeated measurements or datasets with missing values, mixed-effects models were used when appropriate.

Opening Track immobility-position data were analyzed using linear mixed-effects models fitted to all detected immobility bouts, with fixed effects defined according to the experimental comparison and

animal identity included as a random intercept to account for within-animal non-independence. For OT locomotor and trajectory-structure metrics averaged across wall configurations, linear mixed-effects models were fitted to animal-level values with group, week and their interaction as fixed effects and animal identity as a random intercept. Direct comparisons of DAT-paSyn and NET-paSyn Z-scored immobility-position values were performed using two-sided Mann–Whitney U tests.

All statistical analyses were performed in GraphPad Prism 10.2.1 (GraphPad Software) and custom Python scripts. Data are reported as mean $\pm$ s.d. unless otherwise stated. Exact sample sizes, statistical tests and p values are provided in the figure legends or source data. A threshold of $p < 0.05$ was considered statistically significant.

Declarations

Ethics

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

Data and Code Availability

Data supporting this study are available from the corresponding author upon reasonable request. The findmycells tool is available at <https://github.com/DefenseCircuitsLab/findmycells>, with documentation at https://defense-circuits-lab.github.io/findmycells/tutorials/gui_tutorial.html. Custom behavioral preprocessing and contour-based tracking code is available at <https://github.com/DefenseCircuitsLab/>. Additional scripts are available from the corresponding author upon request.

Clinical trial number:

Not applicable

Competing interests

The authors declare no competing interests.

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Author Contribution

H.V.M., A.L., M.S.E and P.T. conceived the project and designed the experiments. H.V.M., A.L. and P.T. wrote the manuscript with contributions from all other authors. H.V.M., A.L., N.S., M.S.E., A.I.T., K.K. and S.R.R. performed histochemical experiments. H.V.M., A.L., M.S., A.I.T. and K.K. ran behavioral experiments. H.V.M., D.D., J.S.G, K.K., and M.S. set up and designed the analysis pipeline. H.V.M., A.L., D.D., J.S.G., K.K., M.S., N.S. and S.R.R. analyzed the data. M.S.E. provided the viruses and supported the experiment designing. C.W.I. provided the empty vector virus, as well as his expertise regarding the viral model approach and analyses.

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Figures

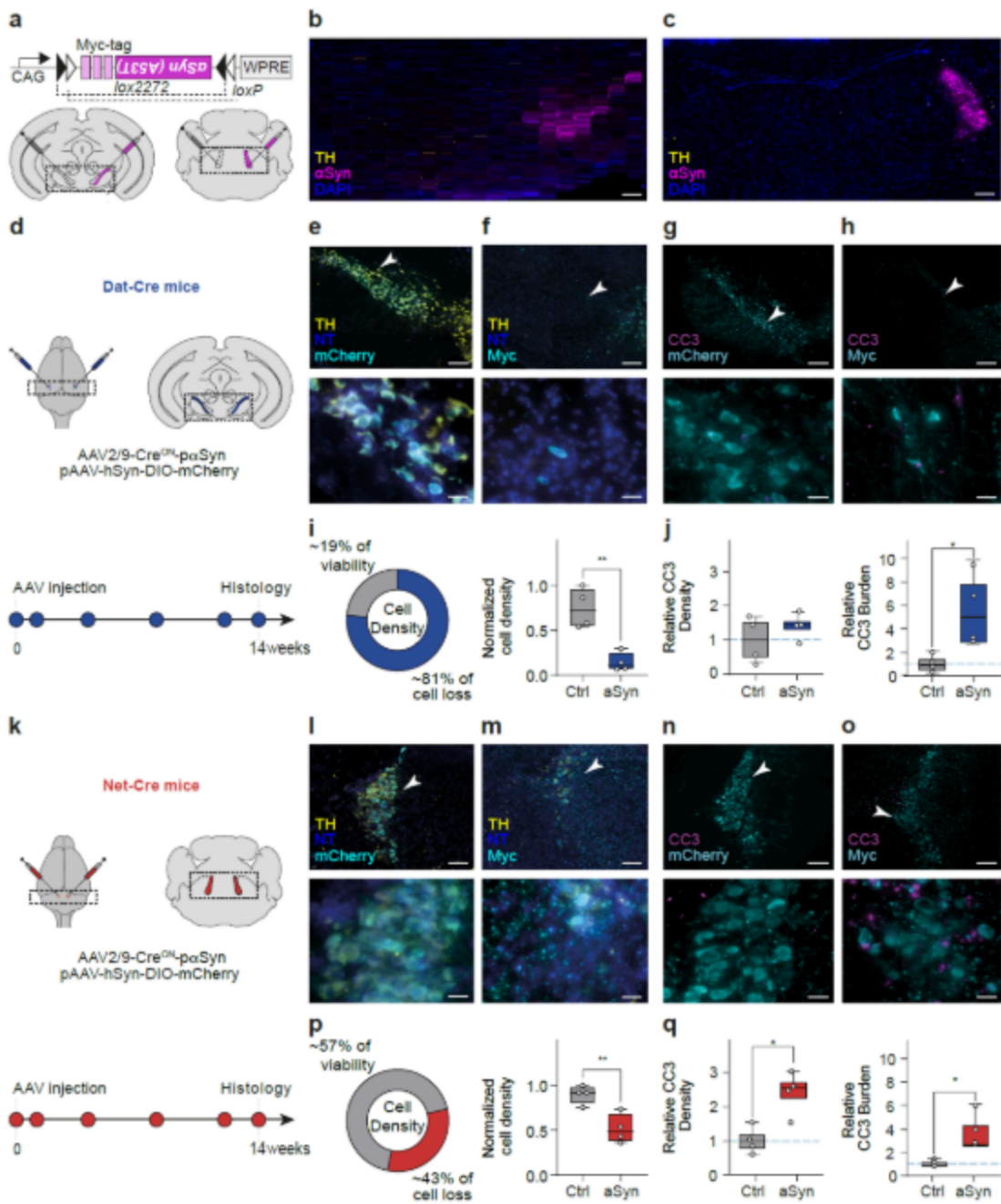


Figure 1

Cell-type-specific A53T-α-synuclein expression induces pathological aggregation, neuronal degeneration and apoptotic activation in SNpc and LC catecholaminergic neurons. **a**, Viral strategy for cell-type-specific induction of synucleinopathy. The Cre-dependent AAV2/9-CreON-paSyn construct drives expression of human mutant A53T-αSyn fused to a Myc tag. Schematics illustrate the unilateral

injection strategy used for aggregate detection in DAT-Cre or NET-Cre mice, in which AAV-hSyn-DIO-mCherry was injected into one hemisphere and AAV2/9-CreON-paSyn into the contralateral hemisphere, allowing intra-animal comparison. **b,c**, Representative images of proteinase K-resistant α -synuclein immunoreactivity in the SNpc (b) and LC (c) 6 weeks after viral injection. TH immunoreactivity was abolished by proteinase K treatment, indicating that the remaining signal reflects aggregated α Syn rather than soluble protein. **d**, Experimental design for analysis of neuronal loss and apoptotic activation in DAT-Cre mice following bilateral injection of AAV2/9-CreON-paSyn or the control vector AAV-hSyn-DIO-mCherry, with histological analysis performed 14 weeks post-injection. **e,f**, Representative SNpc images from DAT-control (e) and DAT-paSyn (f) mice showing TH immunoreactivity together with mCherry in control animals or Myc signal in α Syn-expressing animals. Bottom panels show higher-magnification views. **g,h**, Representative SNpc images showing CC3 immunoreactivity together with mCherry in DAT-control animals (g) or Myc in DAT-paSyn animals (h). Bottom panels show higher-magnification views. **i**, Quantification of normalized TH⁺ cell density in the SNpc 14 weeks post-injection. DAT-paSyn mice showed reduced dopaminergic neuron survival relative to DAT-controls, with approximately 19% residual viability and 81% cell loss (n = 4 mice per group; unpaired two-tailed t-test, p = 0.0027). **j**, Quantification of apoptotic activation in the SNpc. Relative CC3 density did not differ significantly between DAT-control and DAT-paSyn mice (n = 4 mice per group; Welch t-test, p = 0.340), whereas relative apoptotic burden was significantly increased after normalization to neuronal survival (n = 4 mice per group; Welch t-test on log-transformed values, p = 0.024). **k**, Experimental design for analysis of neuronal loss and apoptotic activation in NET-Cre mice following bilateral injection of AAV2/9-CreON-paSyn or AAV-hSyn-DIO-mCherry, with histological analysis performed 14 weeks post-injection. **l,m**, Representative LC images from NET-control (l) and NET-paSyn (m) mice showing TH immunoreactivity together with mCherry in control animals or Myc signal in α Syn-expressing animals. Bottom panels show higher-magnification views. **n,o**, Representative LC images showing CC3 immunoreactivity together with mCherry in NET-control animals (n) or Myc in NET-paSyn animals (o). Bottom panels show higher-magnification views. **p**, Quantification of normalized TH⁺ cell density in the LC 14 weeks post-injection. NET-paSyn mice showed reduced noradrenergic neuron survival relative to NET-controls, with approximately 57% residual viability and 43% cell loss (n = 4 mice per group; unpaired two-tailed t-test, p = 0.0078). **q**, Quantification of apoptotic activation in the LC. Relative CC3 density was significantly increased in NET-paSyn mice relative to NET-controls (n = 4 mice per group; Welch t-test, p = 0.012), and relative apoptotic burden was also significantly increased after normalization to neuronal survival (n = 4 mice per group; Welch t-test on log-transformed values, p = 0.0036). Box-and-whisker plots show median, interquartile range and minimum-to-maximum whiskers. Individual points represent individual animals. Statistical annotations indicate significant comparisons as shown (*p < 0.05, **p < 0.01, ***p < 0.001).

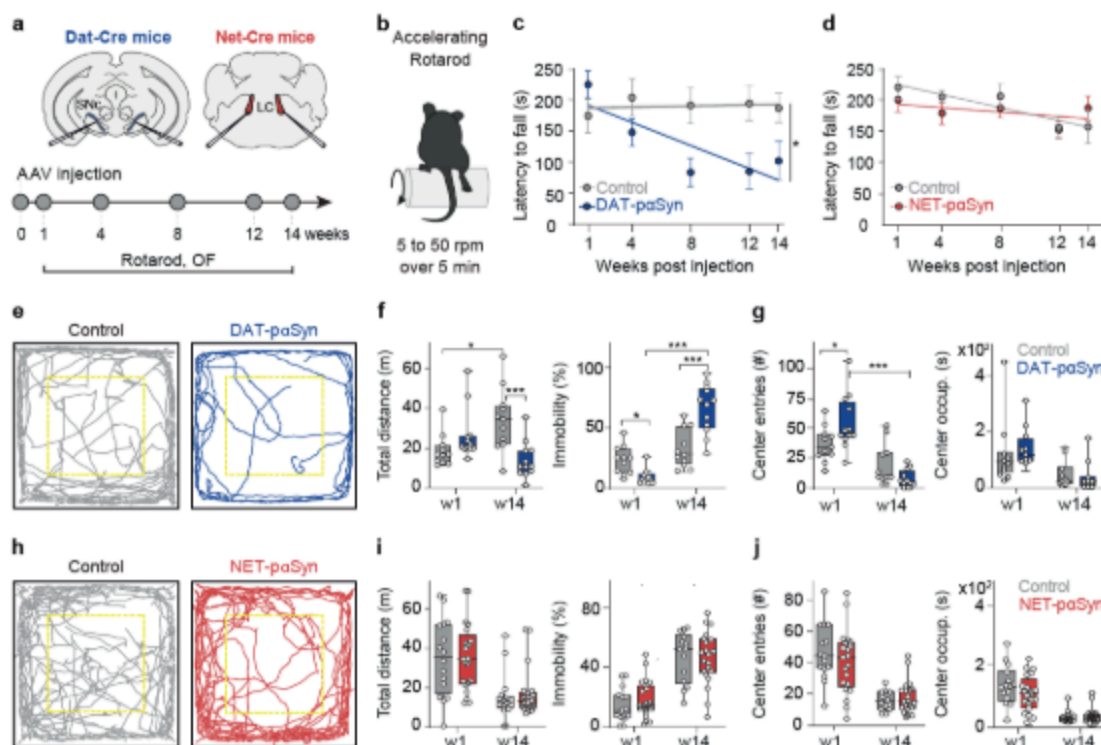


Figure 2

Progressive motor impairment in DAT-Cre, but not NET-Cre mice in classical behavioral assays. **a**, Experimental design and behavioral timeline. DAT-Cre and NET-Cre mice received bilateral injections of AAV2/9-CreON-paSyn targeting the SNpc or LC, respectively, whereas control mice received AAV-hSyn-DIO-mCherry. Rotarod and open-field behavior were assessed at 1, 4, 8, 12 and 14 weeks post-injection. **b**, Accelerating rotarod paradigm, in which mice were tested on an accelerating rod from 5 to 50 rpm over 5 min. **c,d**, Rotarod performance in DAT-Cre (**c**) and NET-Cre (**d**) mice across successive weeks post-injection. Data are shown as mean $\pm$ SEM, and lines indicate linear regression fits. A53T- α Syn expression induced a progressive decline in latency to fall in DAT-Cre mice (DAT-control, $n = 7$; DAT-paSyn, $n = 9$; linear regression slope comparison and mixed-effects model with Šídák multiple comparisons, $p = 0.0324$), but not in NET-Cre mice (NET-control, $n = 9$; NET-paSyn, $n = 14$; linear regression slope comparison and mixed-effects model with Šídák multiple comparisons, $p = 0.521$). **e,h**, Representative OF trajectories from DAT-Cre (**e**) and NET-Cre (**h**) mice at week 14. The yellow square indicates the center area of the arena. **f,g**, Locomotor (**f**) and anxiety-like (**g**) OF measures in DAT-Cre mice at weeks 1 and 14. DAT-paSyn mice displayed reduced locomotor activity (DAT-control, $n = 11$; DAT-paSyn, $n = 12$; $p = 0.004$) and increased immobility ($p = 0.0001$), whereas center entries and center occupancy were not significantly altered. **i,j**, Locomotor (**i**) and anxiety-like (**j**) OF measures in NET-Cre mice. NET-paSyn mice showed no detectable differences from NET-control mice across locomotor or anxiety-like OF measures (NET-control, $n = 15$; NET-paSyn, $n = 21$). For line plots, data are shown as mean $\pm$ SEM. For box-and-whisker plots, boxes show median and interquartile range, and whiskers indicate minimum-to-maximum values. Individual points represent individual animals. OF statistical analysis was performed using

ANOVA or mixed-effects analysis with Šídák multiple comparisons. Statistica annotations indicate significant comparisons as shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

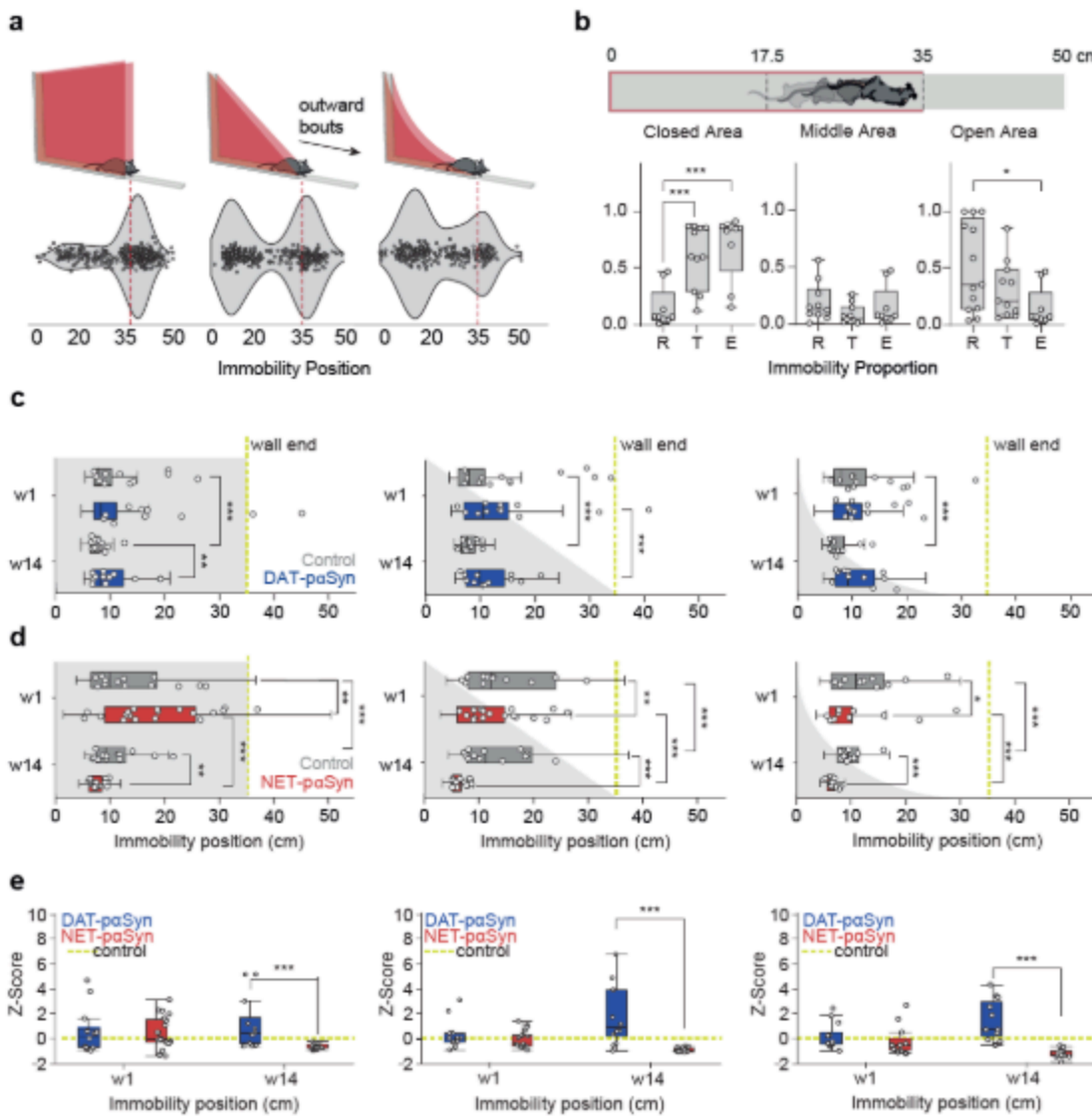


Figure 3

The Opening Track unmasks increased anxiety-like behavior induced by synucleinopathy in

noradrenergic, but not dopaminergic neurons. **a**, Schematic of the three wall geometries used in the OT (top; rectangular, triangular, and exponential) and distribution of outward-oriented immobility-bout positions in wild-type mice (bottom). Each point represents one immobility bout, and violin plots show the spatial density of bouts along the track. The red dashed line marks the wall end, corresponding to the transition between the enclosed and open segments. **b**, (top) Spatial segmentation of the track into Closed, Middle, and Open areas and (bottom) corresponding proportion of immobility in each zone across wall geometries in wild-type mice ($n = 13$). Immobility was redistributed as a function of wall geometry, indicating that the task is sensitive to graded environmental openness. Statistical analysis was performed using linear mixed-effects models with wall geometry as a fixed effect and mouse

identity as a random intercept, followed by Šidák-corrected multiple comparisons. Significant effects of wall geometry were detected in the Open area (OTR vs OTE, adjusted $p = 0.0204$) and Closed area (OTR vs OTTT, adjusted $p = 0.00018$; OTR vs OTE, adjusted $p = 0.000015$), but not in the Middle area.

c, Immobility position in DAT-paSyn mice (blue) and controls (grey) at week 1 and week 14, shown separately for the rectangular, triangular, and exponential wall configurations. Each point represents the mean immobility position per animal, calculated across all immobility bouts, whereas box plots show the distribution of all immobility bouts within each group. The dashed line marks the wall end. Statistical analysis was performed using linear mixed-effects models fitted separately for each wall geometry, with group, week and their interaction as fixed effects and animal identity as a random intercept. DAT-paSyn mice did not show a consistent proximal shift in immobility position. Instead, by week 14, immobility position tended to be displaced toward more distal regions relative to controls, reaching significance only in the rectangular configuration (OTR, $P = 0.00999$; OTT, $P = 0.07547$; OTE, $P = 0.07469$). These modest shifts were interpreted in the context of progressive motor impairment rather than a selective anxiety-like phenotype. **d**, Immobility position in NET-paSyn mice (red) and controls (grey) at week 1 and week 14, shown separately for the rectangular, triangular, and exponential wall configurations. Each point represents the mean immobility position per animal, calculated across all immobility bouts, whereas box plots show the distribution of all immobility bouts within each group. The dashed line marks the wall end. Statistical analysis was performed using linear mixed-effects models fitted separately for each wall geometry, with group, week and their interaction as fixed effects and animal identity as a random intercept. NET-paSyn mice showed a proximal displacement of immobility position relative to controls, which remained significant at week 14 across all wall geometries (OTR, $p = 0.00165$; OTT, $p = 9.27 \times 10^{-8}$; OTE, $p = 0.00072$). This proximal shift was consistent with reduced progression toward more exposed regions of the track. **e**, Direct comparison of DAT-paSyn and NET-paSyn immobility position expressed as Z-score relative to their respective control groups (control mean = 0; dashed line), shown at week 1 and week 14 for each wall geometry. Positive values indicate a shift toward more distal positions relative to controls, whereas negative values indicate more proximal immobility. NET-paSyn mice showed a stronger proximal bias than DAT-paSyn mice at week 14, particularly in the triangular and exponential configurations. Two-sided Mann–Whitney U tests showed no significant differences between DAT-paSyn and NET-paSyn mice at week 1 (OTR, $P = 0.6134$; OTT, $P = 0.6967$; OTE, $P = 0.4290$), but significant differences at week 14 across all wall geometries (OTR, $P = 0.0047$; OTT, $P = 0.00057$; OTE, $P = 0.0079$). Statistical annotations indicate significant comparisons as shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

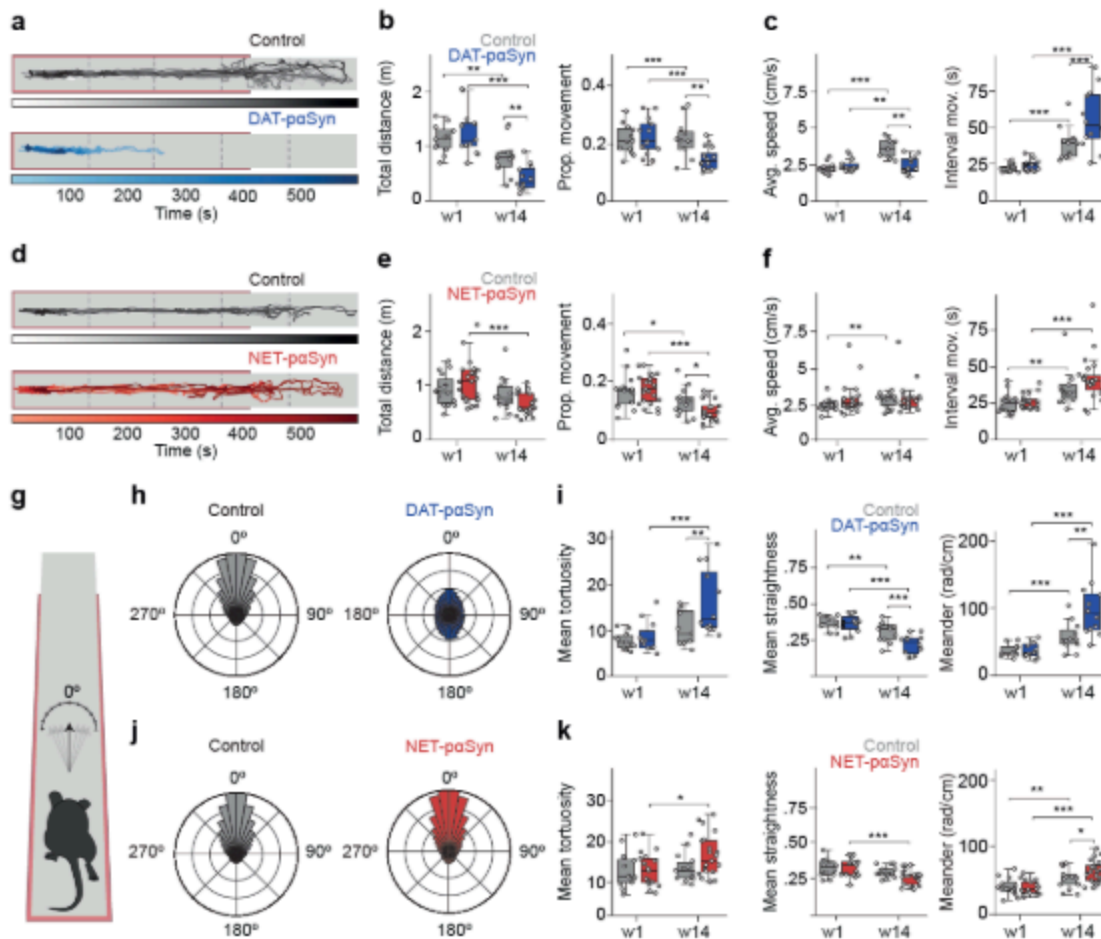


Figure 4

Motor dysfunction re-emerges in the Opening Track after SNpc, but not LC synucleinopathy.

a, Representative OT trajectories from DAT-control and DAT-paSyn mice recorded over the course of a session. Trajectories are color-coded by time and illustrate reduced track traversal in DAT-paSyn animals at late disease stages. **b,c**, Quantification of basic locomotor parameters in DAT-Cre mice at week 1 and week 14. **b**, Total distance travelled and proportion of movement. **c**, Average speed during movement and mean interbout interval. Box-and-whisker plots show median and interquartile range, with whiskers indicating 1.5× interquartile range. Individual points represent individual animals after averaging across OT wall configurations. Statistical comparisons were performed using linear mixed-effects models fitted to animal-level values averaged across wall configurations, with group, week and their interaction as fixed effects and animal identity as a random intercept. DAT-paSyn mice did not differ from DAT-controls at week 1, but showed reduced total distance travelled, reduced proportion of movement, reduced average speed during movement and increased mean interbout interval at week 14. Significant group × week interactions were detected for all four measures, indicating progressive motor deterioration in DAT-paSyn mice (total distance: week 14 group effect, $p = 0.00387$; group × week interaction, $p = 0.00138$; proportion movement: week 14 group effect, $p = 0.00206$; group × week interaction, $p = 0.00236$; average speed: week 14 group effect, $p = 5.35 \times 10^{-9}$; group × week interaction, $p = 2.67 \times 10^{-7}$; mean interbout interval: week 14 group effect, $p = 0.000605$; group × week interaction, $p = 0.0209$). **d**, Representative OT

trajectories from NET-control and NET-paSyn mice recorded over the course of a session.

e,f, Quantification of basic locomotor parameters in NET-Cre mice at week 1 and week 14. **e**, Total distance travelled and proportion of movement. **f**, Average speed during movement and mean interbout interval. NET-paSyn mice did not show the broad week-14 locomotor impairment observed in DAT-paSyn mice. Total distance travelled, average speed during movement and mean interbout interval did not differ significantly from controls at week 14, whereas the proportion of movement was modestly reduced. Significant group $\times$ week interactions were detected for total distance travelled, proportion of movement and average speed during movement, but not for mean interbout interval, indicating measurable but restricted changes in locomotor dynamics over time rather than a generalized progressive motor deficit (total distance: week 14 group effect, $p = 0.102$; group $\times$ week interaction, $p = 0.00873$; proportion movement: week 14 group effect, $p = 0.0463$; group $\times$ week interaction, $p = 0.0287$; average speed: week 14 group effect, $p = 0.269$; group $\times$ week interaction, $p = 0.0124$; mean interbout interval: week 14 group effect, $p = 0.0802$; group $\times$ week interaction, $p = 0.276$). **g**, Schematic of the OT geometry and angular coordinate frame used for trajectory-structure analyses. **h,j**, Rose plots illustrating angular movement directionality in control and paSyn animals in the DAT-Cre cohort and NET-Cre cohort. **i**, Quantification of trajectory-structure metrics at week 1 and week 14 in the DAT-Cre cohort. Metrics shown include straightness, tortuosity and meander. Statistical comparisons were performed using linear mixed-effects models fitted to animal-level values averaged across wall configurations, with group, week and their interaction as fixed effects and animal identity as a random intercept. DAT-paSyn mice developed progressively less straight, more tortuous and more meandering trajectories by week 14, with significant group $\times$ week interactions for all three measures (straightness: week 14 group effect, $p = 0.000869$; group $\times$ week interaction, $p = 0.00694$; tortuosity: week 14 group effect, $p = 0.00151$; group $\times$ week interaction, $p = 0.0271$; meander: week 14 group effect, $p = 5.23 \times 10^{-5}$; group $\times$ week interaction, $p = 0.00192$). **k**, Quantification of trajectory-structure metrics at week 1 and week 14 in the NET-Cre cohort. NET-paSyn mice showed a significant increase in meander at week 14, whereas straightness and tortuosity did not differ significantly from controls and no significant group $\times$ week interactions were detected for any of the three trajectory metrics (straightness: week 14 group effect, $p = 0.0512$; group $\times$ week interaction, $p = 0.161$; tortuosity: week 14 group effect, $p = 0.0554$; group $\times$ week interaction, $p = 0.210$; meander: week 14 group effect, $p = 0.0177$; group $\times$ week interaction, $p = 0.0562$). Statistical annotations indicate significant comparisons as shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Supplementary Files

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